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The Development of Quantitative Methods for Residues in Foods of Animal Origin with Validation According to Commission Decision 2002/657/EC

by

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Research Thesis submitted for the Degree of Masters of Science

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Declaration

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Statement 1

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Dedicated to my family

My parents Patrick and Kathleen whom have given me the gift of determination. Also their support and nurturing has allowed me to believe in myself and stand up and put up a good fight no matter what the cause or the task. My brother Paul and my sister's Lisa and Emma who accept me for who I am and their sense of humour has helped me tremendously throughout this journey. Mollie for always taking care of me.

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Abbreviations

EU	European Union
MRL	Maximum Residue Limit
CAM	Cambendazole
NETO	Netobimin
ABZ	Albendazole
ABZ-SO	Albendazole Sulphoxide
ABZ	Albendazole
ABZ-SO$_2$	Albendazole Sulphone
NH$_2$ABZ-SO$_2$	Amino Albendazole Sulphone
FEB	Febantel
OFZ/FBZ-SO	Oxfendazole/Fenbendazole Sulphoxide
FBZ-SO$_2$	Fenbendazole Sulphone
MBZ	Mebendazole
MBZ-OH	Hydroxy Mebendazole
MBZ	Mebendzole
TBZ	Thiabendazole
TBZ-OH	Hydroxy Thiabendazole
FLU	Flubendazole
NH$_2$FLU	Amino Flubendazole
TCB	Triclabendazole
TCB-SO	Triclabendazole Sulphoxide
TCB-SO$_2$	Triclabendazole Sulphone
OXI	Oxibendazole
SPE	Solid Phase Extraction
ASPEC XL4	Automated Solid Phase Extraction XL4
LC	Liquid Chromatography
I.D	Internal Diameter
CCα	Decision Limit
CCβ	Detection Capability
r^2	Regression Coefficient
LC-MS/MS	Liquid Chromatography Tandem Mass Spectroscopy
ES	Electrospray

ESP	Electrospray Positive
LC-UV	Liquid Chromatography Ultraviolet Detection
m/z	mass to charge ratio
GC	Gas chromatography
DCM	Dichloromethane
MG	Malachite Green
LMG	Leucomalachite green
CV	Crystal Violet
LCV	Leucocrystal Violet
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone

Abstract

Residue methods were developed for the determination of the coccidiostat robenidine in egg, the benzimidazoles (13) in liver (albendazole 2-amino albendazole sulphone, albendazole sulphoxide, albendazole sulphone, thiabendazole, oxfendazole or fenbendazole sulphoxide, hydroxy mebendazole, amino flubendazole, fenbendazole sulphone, oxibendazole, mebendazole, flubendazole and albendazole) and the triphenylmethane dyes (4) in salmon (malachite green, crystal violet, leucomalachite green and leucocrystal violet). The methods were validated according to the criteria defined in Commission Decision 2002/657/EC. Robenidine was extracted from egg with acetonitrile and the sample extracts analysed by liquid chromatography (LC) with ultraviolet (UV) spectrophotometric detection at 317 nm. The decision limit (CCα) and the detection capability (CCβ) were 10 $\mu g.kg^{-1}$ and 17 $\mu g.kg^{-1}$ respectively.

The benzimidazoles were extracted from liver samples with ethyl acetate, sample extracts were defatted with hexane and cleaned up by automated solid-phase extraction (SPE) on C_{18} cartridges. Aliquots of the extracts were analysed by LC with UV detection at 298 nm. The CCα values ranged between maximum residue limit (MRL) + 12% and MRL + 25% and the CCβ ranged between MRL + 25% and MRL + 45%.

The triphenylmethane dyes were extracted from salmon with acetonitrile and pH 3 buffer, extracts were cleaned up using cation-exchange SPE on sulphonic acid (SCX) cartridges and the sample extracts were analysed by liquid chromatography tandem mass spectroscopy (LC-MS/MS). CCα for malachite green, leucomalachite green, crystal violet and leucocrystal violet were 0.17, 0.15, 0.40 and 0.17 $\mu g.kg^{-1}$ respectively and CCβ were 0.30, 0.35, 0.80 and 0.32 $\mu g.kg^{-1}$ respectively.

All research undertaken in this thesis was published in peer reviewed journals. This work has made a significant contribution to residues science as more novel methods have become available for surveillance of these drugs at national and international level. The methods developed in this research also provide a legal basis for prosecuting individuals who use these veterinary products without adhering to EU legislation. Ultimately the work enhances food safety as methods developed help to eliminate the hazards associated with drug residues entering the food chain.

Chapter 1:

General Introduction

1

1.1 Residues in Food

1.1.1 Introduction

The administration of any pharmacologically active chemical to a food-producing animal can lead to the occurrence of residues in food. In recent years, the issue of veterinary drug residues in foods of animal origin has become increasingly important. Concerns over veterinary drug usage and residues are primarily related to food safety and the possibility of the development of antibiotic resistant pathogens. There is also an increased need to meet requirements relating to the levels of veterinary residues in food dictated by legislation. [1] Failure to meet legislative requirements in relation to levels of residues in food may have serious economic consequences for the country involved. There is also growing public concern regarding drug residues in food due to more intense methods of animal husbandry. There is increased consumption of meat and dairy products, especially poultry. To meet this demand, an increasing share of livestock production is by commercial enterprises and intensive or semi-intensive urban production systems rather than traditional mixed farming systems with a consequent increase in the use of veterinary compounds such as antibiotics, coccidiostats and growth promoting hormones. Aquaculture has also exhibited a rapid growth in recent years accounting for 26% of world fish production in 1999. About half of global aquaculture production is in developing countries. Contamination of aquaculture products from Asia and Latin America with residues has recently been the cause of international trade interventions leading to a loss of revenue for the exporting countries. [1]

Veterinary medicinal products are generally used in animal production for one of three purposes: [2]

1. Therapeutic treatment of active infection.
2. Vaccination or prophylactic medication to prevent or to minimise infection.

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3. Production enhancement-growth promotion and improvement of feed conversion efficiency with antimicrobial drugs and hormones.

1.1.2 Improper or illegal use of veterinary drugs

The occurrence of residues in food, in breach of the levels set down in legislation, can happen in a number of ways. These violations result from either the improper use of licensed products or the illegal use of unlicensed and prohibited substances. [3] Several factors contribute to the improper use of licensed products, including, poor treatment records or failure to identify treated animals and use of a drug other than as described on the product label. Mis-use occurs mainly by not observing adequate withdrawal periods before slaughter of animals.

Providing that a licensed drug is used in accordance with its product licence and providing that the drug withdrawal periods are respected by farmers, drug residues should not occur in human food at concentrations greater than the maximum residue limits (MRLs).

Intentional use of prohibited compounds occurs for a number of reasons. These include:- the use of growth promoting hormones in order to increase the weight of animals, as this leads to higher yields at market; the use of certain banned compounds that can be very effective at controlling particular infections eg nitrofurans or these banned compounds that maybe very cheap and readily available e.g. phenylbutazone.

Cross contamination in feed mills is also another way in which high levels of residues can occur in food.[3] Trace quantities of medicated feed may be retained at various points along the production line, contaminating subsequent batches of feed as they are processed. The electrostatic properties of some drugs, particularly those in powder form, aggravate the problem, making it more difficult to purge the equipment between batches. Manufacturers have responded to the problem by producing granular preparations with reduced electrostatic properties. The electrostatic properties of the drug nicarbazin caused problems during the manufacture of nicarbazin-free feeds, since nicarbazin (NCB) powder is strongly electrostatic and could not be removed from feed milling equipment. [4] NCB is a widely used coccidiostat drug, which is licensed as a feed additive for broiler

chickens, but not for laying hens. There is a problem in many countries world-wide with the occurrence of NCB residues in poultry tissues and eggs. Cannavan et al.[4] have reported a proportional relationship between the concentration of 4,4'-dinitrocarbanilide (DNC) and 4,6-dimethyl-3-hydroxypyrimidine (DHP) in eggs, the two marker residues for NCB, and the feed levels. Feed contaminated with NCB at concentrations higher than 2 mg kg $^{-1}$ gave rise to residue concentrations of DNC in eggs higher than 100 µg.kg^{-1}.

1.1.3 Environmental contamination

Potential environmental contamination may arise e.g in the case of commercial fish farming where veterinary drugs or other contaminants, which have been given as feed additives, accumulate in sediments and may become ingested by marine species in the vicinity of farms. [3] The presence of drug residues may pose a health risk, if these wild species are harvested for human consumption.

1.1.4 Animal-to-animal transfer

Recycling of drugs as a result of ingestion of faeces and/or urine significantly contributes to the concentration and persistence of residues in porcine tissues, poultry tissues and eggs. [3,5] Brief exposure of unmedicated animals to the excretions of medicated animals in improperly cleaned housing during transport or in the slaughter house can result in violative residues.

Canavan et al.[5] have observed a tenfold higher nicarbazin concentration in the liver of broiler chickens treated with this drug and housed on flooring where litter can accumulate compared with those housed on wire flooring. Complete exchange of the litter was the only way to prevent recycling.

1.2 The Risks to Human Health

1.2.1 Toxicity and allergenicity

Quite a large number of antibiotic and anthelmintic drugs administered in therapeutic and subtherapeutic forms to domestic animals are also approved for human use. These drugs have been shown to be relatively safe. Acute and chronic toxicities have been evaluated

and are well documented. [2] The likelihood of acute toxicity from veterinary drugs and their metabolites originating from animal tissues is extremely low. However the possibility of chronic toxicity expressed in longterm, cumulative allergenic, mutagenic, teratogenic or carcinogenic effects are difficult to assess.

Veterinary drugs with high potential to cause one or more of these toxic effects for human health were banned by the EC for veterinary use. Chloramphenicol is one such compound; it produces toxic aplastic anemia that is not related to dosage. Sulfonamides have been used widely at subtherapeutic and therapeutic concentrations in food-animal production, but increasing concern over their carcinogenic and mutagenic potential has led to decreased usage, longer withdrawal times and more intensive residue monitoring.

Tetracycline residues can reversibly slow down the growth of the skeleton and irreversibly discolour the teeth of children younger than 8 years since tetracyclines are deposited in bones and teeth. The residues of benzimidazole anthelmintics have a defined toxic potential. [6] Some benzimidazoles are mutagenic, but the effect is limited or low in mammals even at high doses. Teratogenicity may be considered as a general property of this drug group. Tetratogenic metabolites have been identified and quantified in animal products such as milk, eggs and meat. For levamisole, the most important of the observed adverse effects, which are rare, were agranulocytosis and neutropenia.[7]

To safeguard human health, the EU has established maximum residue limits (MRLs) for residues of licensed veterinary drugs in animal tissues entering the human foodchain. The term MRL may be defined as the maximum concentration of marker residue (e.g. parent compound, metabolites etc) resulting from the use of a veterinary drug, expressed as mg kg^{-1}, that is legally permitted or recognised as acceptable in or on a food.[1] The MRL is related to the acceptable daily intake (ADI). This is an estimate of the amount of a substance in food and/or drinking water, expressed on a body weight basis that can be ingested daily over a lifetime without appreciable health risk to the consumer on the basis of all the known facts at the time of the evaluation. It is usually expressed in milligrams of the chemical per kilogram of body weight. The ADI is determined from the no-observable-effect level (NOEL) taking into account a safety factor (usually 100).[8] The NOEL is the greatest concentration or amount of a substance, found by observation or experiment, which causes no detectable effect. The maximum acceptable total residue

level (TRL) is calculated from the ADI considering the consumption pattern. The MRL is the detectable proportion of the marker residue that corresponds to the TRL value. The MRL value refers to the permissible level for the marker residue.

In addition to conventional toxicological effects, other issues such as the effects of drugs on the immune system and pharmacological effects including specific effects of residues of veterinary antibiotics on the human gut flora, should be taken into account when considering safe residue levels.[9]

Besides toxicological problems, hypersensitive reactions in humans from ingesting antibiotic contaminated foods of animal origin can occur. Most reactions result from β-lactam (e.g penicillin) antibiotic residues in milk and meat [1,2] in sensitive individuals. Many people that went through prior medical treatment were hypersensitised to such a degree that following oral exposure a response was evoked.

Nevertheless, the risk to human health due to residues of veterinary drugs in foodstuffs have to be taken in context. Microbial contamination of food is a major health problem worldwide. Infections of poultry products with Salmonella and Campylobacter are greater risks to public health than are residues of veterinary drugs in food. Nevertheless, the public risks of antibiotics and their metabolites are difficult to define, and the presence of violative levels in food is illegal and subject to financial penalties in many countries.

1.2.2 Antibiotic resistance

Antibiotic resistance is a well documented major health threat around the world that has been given high priority by many health agencies [1,2] The potential of animal-to-human transfer of resistance exists. Clearly, the use of antibiotics in lifestock production has been associated with the development of human antibiotic resistance. Animals fed with low (prophylactic) levels of antibiotics may give rise to bacteria with evolving resistance to these/and other drugs. Humans may be exposed to these bacteria through consumption of food. It has been documented that humans developed drug resistant salmonellosis from food of animal origin. Examples of drugs which have been shown to cause the growth of resistant bacteria in food animals are fluoroquinolones and avoparcin. The resistance of microorganisms arising from subtherapeutic use of penicillin, tetracyclines and sulfonamides in aquaculture is suggested by WHO to be a high priority issue. [2]

1.2.3 Technological problems

The initial concerns with regard to antimicrobial residues in food were not expressed by consumers but by dairy processors who found that contaminated milk was inhibiting the starter cultures used in the production of fermented milk products as well as interfering with the results of dye reduction tests used for milk quality. [1] The sensitivity of thermophilic and mesophilic starter cultures for manufacturing high-value fermented products as yogurt, cheese, butter or raw sausages, requires biotechnologically safe raw materials.

1.2.4 Effect of processing on residues

Although most food of animal origin is cooked (except milk and honey) before consumption, none of the required studies for licensed drugs include the effect of processing on residues. Information about this influence is required to obtain more accurate estimates of consumer exposure to residues or possible breakdown products. The fate of several drug residues during normal cooking and processing procedures is described by Rose *et al.*, [10] Residues of levamisole were found to be stable to heating, but a fraction was lost from the meat into the juice. [10] Some evidence of oxfendazole instability in boiling water was found after 3 hours. Heating of samples with incurred residues of oxfendazole destroys the drug residues. Moats *et al.*, [11] reported that ordinary cooking procedures for meat, even to "well-done" can not be relied on to break down even the more heat sensitive compounds such as penicillins and tetracyclines. The relevance to food safety is uncertain since the nature of the degradation products is unknown in most cases. The identity of these degradation products should be established and their toxicity assessed.

The use of data generated from surveillance of raw tissue for dietary intake calculations and consumer exposure estimates has to be considered in the light of the effect of cooking on these residues.

1.3 Legislative Aspects

1.3.1 Overview of EC Decisions, Council regulations and guidelines

The occurrence of residues of veterinary drugs in food is a world-wide problem. Food-producing animals and animal products are transported within EU countries and between EU countries and the Third Countries. A lot of resources are expended by the EU to harmonise European legislation in the control of residues. The European Commission had regulated the inspection of animals and of fresh meat for the presence of residues of veterinary drugs and specific contaminants by Council Directive 86/469/EEC. [12] The current monitoring is governed by national surveillance schemes established under Council Directive 96/23/EC [14] on measures to monitor certain substances and residues thereof in animal products and under Commission Decision 97/747/EC [14] which lays down levels and frequencies of sampling in order to monitor some substances and residues thereof in certain animal products. The EU Decision establishes numbers of samples to be tested for each compound group.

Quality criteria for residue analyses are described in Commission Decision 93/256/EEC [15] which states the methods to be used for detecting residues of substances having a hormonal or thyreostatic action. Commission Decision 93/257/EEC [16] lays down the reference methods and the list of National Reference Laboratories for detecting residues. The two decisions are revised regularly in order to take into account the current scientific knowledge and the latest technological improvements. A revised version was submitted by the Directorate General for Agriculture as a draft Commission Decision SANCO/1805/2000 [17] laying down performance criteria for the analytical methods to be used for certain substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC. [13] This draft document was revised and published as Commission Decision 2002/657/EC [18] implementing Council Directive 96/23/EC [13] concerning the performance of analytical methods and the interpretation of results. The establishment of MRLs in the EU is governed by Council Regulation EEC/2377/90 amended by several EC Regulations. [19] This regulation establishes lists of compounds that have a fixed MRL (Annex 1), that need no MRL (Annex II), or that have a provisional MRL (Annex III). Annex IV of this regulation is a list of compounds that

are prohibited for use in livestock production. Annex V provides information and the data needed to determine the MRL values.

In addition to the protection of consumers from the potential risk of harmful residues, MRLs are also essential to facilitate international trade. However, the introduction of the MRL has a severe impact on the availability of veterinary medicinal products as the data required is very complex and the huge number of experiments and costs involved must be furnished by the pharmaceutical company. This is because pharmaceutical companies that want to market their products must submit comprehensive dossiers to regulatory authorities so MRLs can be set. These dossiers outline toxicological and pharmacological properties of their product. The financial implications of preparing and carrying out such trials makes most companies wary of carrying out this work so a lot of compounds that may have been used in the past are now no longer available. This maybe encouraging the use of unlicensed products. Since some drugs are licensed for use in particular species only, farmers may use the products in an off label manner by administering to a different species. The risk of the occurrence of residues in food when food producing animals are treated with un-licensed products or with products used in an off-label manner, is considerable.

1.4 Determination of Veterinary Drug Residues

1.4.1 Introduction

Analytical methods include screening, quantitative and confirmatory procedures. In the case of screening methods, they should ideally be rapid, easy to use and give a positive or negative result for the test compound at a specified level. Screening tools include rapid test kits such as microbial receptor, receptor binding, radio or enzyme immunoassays used to screen for veterinary residues. Screening tests can present some problems as they are not always sensitive at the required concentration (e.g MRL), are often drug class and not compound specific and do not give quantitative information. Therefore additional analytical tests are needed to determine if a sample is actually violative for an animal drug residue. Some tests such as immunoassays, provide semi-quantitiative results.

Quantitiative methods are designed to separate, quantify and provide some qualitiative information on the analyte of interest. Most gas and liquid chromatographic methods would fall into this category. Quantitative assays classify samples as positive or negative relative to specific drug concentration.

Confirmatory methods provide unambiguous identification of the drug residue in question. Due to its sensitivity and specificity, mass spectrometry is the preferred method for confirmation of drug residues.

1.4.2 Screening assays

A screening assay makes a distinction between compliant and suspect samples i.e samples containing residues over an allowed limit. The most important requirement for a screening assay is that they yield very few false negatives, in essence the β-error (possibility of false negative result or a risk for the consumer) should be lower than 5%; some false positives are acceptable as these will show a negative result when a confirmatory assay is performed. [18]

The earliest methods used for detection of antimicrobial residues in food were based on the detection of growth inhibition of various sensitive bacterial strains.[1] The major drawback of these assays are that they are not very specific for identification purposes, that they are not quantitative and they have a limited detection level for many antimicrobials. Moreover, they are time-consuming requiring several hours before results are obtained. However they are cheap, easy to carry out, adaptable for screening large numbers of samples and they have a reasonably broad antimicrobial detection spectrum.

The specificity of the immune system is demonstrated by its ability to distinguish subtle differences between antigens. Immunoassays exploit this and examples of such tests are enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA). Jackman *et al.,* [20] described class specific enzyme immunoassays for the detection of most benzimidazole drugs. For thiabendazole a compound specific ELISA had to be developed. Enzyme immunoassays are powerful tools for screening food products for the presence of veterinary drug residues. Benefits are high through-put of samples and low costs due to instrumental methods, however commercial kits can be expensive. Van der Made *et al,* [21] adjusted the sample preparation procedure of an enzyme immunoassay

(RIDASCREEN TM Acetylgestagene EIA of R-Biopharm) to create an easier and faster assay for the detection of medroxyprogesterone acetate (MPA) in kidney fat. A second commercial kit was tested (MPA ELISA of Euro-Diagnostica B.V) which showed poorer sensitivity for MPA. The results showed that screening using the former kit in combination with a simplified extraction procedure resulted in a reliable screening method to determine MPA in kidney fat of pigs. With this test it was shown that 30 samples could be analysed per day by one technician. Scortichini et al., [24] described a commercial enzyme linked immunosorbent assay (ELISA) test available from Euro-Diagnostica for the determination of chloramphenicol (CAP). Peippo et al., [23] describes a simple and rapid time-resolved fluoro immunoassay (TR-FIA) for the screening of narasin in poultry plasma. Noot et al., [22] described a surface plasmon resonance (SPR) based immunoassay using the Biacore Q [TM] SPR biosensor for multi-sulfonamide detection in porcine muscle. Following a simple preparation, results for 40 samples are available in just over five hours. After using the Qflex Kit Sulfonamides with Biacore Stead et al., [25] describes a rapid screening and subsequent confirmation of chloramphenicol using Q Flex [TM] Chloramphenicol kits employed on a Biacore [TM] Q system coupled with to LC-MS/MS technology.

1.4.3 Confirmatory methods

1.4.3.1 Introduction

Samples that screen positive need to be further analysed by a confirmatory analytical method in order to be unambiguously declared positive. Chromatographic techniques play an important role in the confirmatory tests for residues of veterinary drugs and contaminants. There are several types of chromatographic methods currently in use for residue analysis. These include thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). TLC has found some use, generally for screening or qualitative assays only. Most veterinary drugs are polar, non-volatile, heat sensitive and or difficult to derivatise for GC. Therefore HPLC is the most commonly used analytical technique for residue analysis. For confirmatory analysis

chromatography with selective detectors is used widely. Mass spectrometry (MS) is the preferred detection method for confirmation. Hyphenated techniques such as gas chromatography-tandem mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are the most powerful analytical tools in the analytical laboratory.

1.5 Validation Of Analytical Methods For Use In Veterinary Drug Residue Determination

1.5.1 Introduction

National surveillance laboratories within the member states are developing their own surveillance methods for veterinary drug residues. These methods have to be validated to demonstrate their reliability. Validation parameters to be evaluated for analytical methods are described in: Commission Decision 2002/657/EC [18] and they are: specificity, linearity, trueness, precision, recovery, analytical limits, stability and ruggedness. The criteria laid down in this decision were followed during the validation procedures carried out in this thesis.

1.5.2 Validation parameters

1.5.2.1 Specificity

The specificity describes the ability of the method to measure the analyte of interest in the presence of other components such as interfering analytes, metabolites and sample matrix.

1.5.2.2 Linearity

The mathematical relationship between the response and the concentration of the analyte in the matrix has to be established.

1.5.2.3 Trueness

This is a component of accuracy and is defined as the difference between the mean value measured for an analyte in a Certified Reference Material (CRM) and its certified value,

expressed as a percentage of its value. If no CRM is available, relevant parameters may be evaluated using fortified sample material.

1.5.2.4 Precision

The precision, another accuracy component, is the closeness of agreement between the results obtained by applying the experimental procedure several times under prescribed conditions and covers repeatability, within laboratory reproducibility and inter-laboratory reproducibility.

1.5.2.5 Recovery

Matrix effects in biological samples can cause loss of analyte during the analytical procedure and can affect the recovery or extraction efficency. Non matrix-related losses can occur due to the analytical procedure. If there is no CRM available to calculate trueness, the recovery has to be determined by experiments using fortified blank material. Whenever possible, incurred residue matrix material has to be used to optimise the method to recover as much incurred material as possible.

1.5.2.6 Analytical limits

Two analytical limits are defined in Commission Decision 2002/657/EC and these are the decision limit (CCα) and the detection capability (CCβ). The decision limit is the limit from which it can be decided that a sample is truly violative with an error probability of α. The α-error, which is the probability that the tested sample is not truly violative, even though a violative measurement has been obtained (false positive decision) shall be 1% or 5% or lower for banned and MRL compounds, respectively. In the case of banned substances, CCα is the lowest concentration level at which a method can discriminate with a statistical certainty of 1- α whether the identified compound is present. For substances with an established MRL, CCα is the concentration above which it can be decided with a statistical certainty of 1-α that the identified compound content is truly above the MRL.

The detection capability is the smallest content of a substance that may be detected, identified and quantified in a sample with an error probability of β. The β-error, defined

as the probability that the tested sample is truly violative, even though a non-violative measurement has been obtained (false negative decision), should be less than or equal to 5 %. In the case of banned substances, $CC\beta$ is the lowest concentration at which a method is able to detect truly residues in samples with a statistical certainty of $1-\beta$. For substances with an established MRL, $CC\beta$ is the concentration at which the method is able to detect MRL concentrations with a statistical certainty of $1-\beta$.

1.5.2.7 Stability

The validation protocol should always include an investigation of the stability of the analyte or matrix constituents in the sample matrix as well as the stability of the standard analyte in solution. Whenever possible, incurred samples should be used to check the stability in the matrix material.

1.5.2.8 Ruggedness

Ruggedness is tested by a separate systematic experimental procedure. Pre-investigation studies have to be carried out by selecting factors of the sample pretreatment, clean-up, instrument and analysis, which may influence the measured results. Such factors may include the operator, the reagents and the laboratory circumstances. The ruggedness of the method can be tested during determination of the within-laboratory reproducibility as part of the method validation.

1.5.3 Residue monitoring

Residue monitoring or surveillance programmes require the analysis of a large number of samples. Traditionally screening techniques, such as inhibitory substance tests and immunoassays, are used in residue laboratories. Multiresidue screening methods are needed. Screening methods reduce the number of samples requiring more detailed analysis. Screening assays are an important part of the integrated approach to residue monitoring. It is necessary to combine different methods in an integrated system in which a number of different tests are applied consecutively depending on the targets or objectives of the analysis. For regulatory purposes such a strategy should include at least two or more independent methods. [1]

- Screening with a method optimised to prevent false negative results and with an acceptable number of false positive results at a low cost (e.g microbial growth inhibition tests).
- Intermediate tests to identify the residue type.
- Quantitative confirmation with an independent method optimised to prevent false positive results.

1.6 Research Objectives and Relevance of the Presented Research Work

1.6.1 Introduction

The two main objectives of the research work described in three separate chapters in this Masters Thesis are:

1 The development of chromatographic screening methods for quantitative determination of anticoccidial (robenidine) and antiparasitic (benzimidazoles) substances in eggs and liver, respectively, and adoption of criteria governing method validation for these substances according to Commission Decision 2002/657/EC. [18]

2 The development of a multi-residue LC-MS/MS confirmatory method for the determination of the banned substance malachite green (MG), its major metabolite leucomalachite green (LMG), another related dye called crystal violet (CV) and its major metabolite leucocrystal violet (LCV) in farmed salmon and adoption of the rules governing confirmatory method validation for these banned substances according to Commission Decision 2002/657/EC. [18]

It is the also the objective to apply these methodologies to routine analysis, in which simple, reliable, accurate, sensitive methods need to be used due to the high number of samples that must be tested for under current legislation. Throughout this research, there has been a progression in the analytical technology used. In the first two chapters, methods for robenidine and benzimidazoles using HPLC with UV detection are described. In the final chapter(s) a LC-MS/MS confirmatory method is described.

1.6.2 Background to developed methodology in thesis

The work completed on the anticoccidial, robenidine, was carried out at The National Food Centre, Teagasc, Dublin. This laboratory is the National Reference Laboratory for a range of drugs including antiparasitics and anticoccidials. No method was available for determination of the feed additive robenidine hydrochloride in eggs by LC-UV. Although there were some methods using LC-MS/MS, the laboratory did not have LC-MS/MS technology available for this application. In chapter 3, a HPLC with UV detection method for robenidine in eggs is described. This method may be implemented into the Irish monitoring programme. No such method for the determination of robenidine in eggs by LC-UV existed prior to this study.

The work completed on the benzimidazoles which are anthelmintic veterinary drugs was also completed at The National Food Centre, Teagasc, Dublin. This laboratory is the National Reference Laboratory for benzimidazole drugs also. There are seven licensed groups of benzimidazoles (MRL compounds) of which there are 19 possible metabolites. Due to the wide variation in the chemical properties of these compounds it is very difficult to develop a multi-residue method for all of the licensed groups. In chapter 4, a HPLC-UV detection method for the determination of benzimidazoles using bovine liver as a model matrix is described. This method may also be implememted into the Irish monitoring programme. Compared to available published methods, the advantage of the developed method is the ability to simultaneously determine six of the seven licensed groups of these compounds.

The second part of the thesis deals with confirmatory methods. More sensitivity, specificity, selectivity and confidence for surveillance purposes is obtained with mass spectrometry, and especially so when an LC-MS/MS system is operated in the MRM mode. The work completed on MG,LMG, CV and LCV which are anti-fungal dyes that are prohibited for use in aquaculture, was carried out at the Marine Institute and the State Laboratory in Dublin. The Marine Institute Laboratory is the National Reference Laboratory for MG. In 2003, Ireland was audited by the EU Food and Veterinary Office (FVO) and a non-conformance regarding the illegal use of this compound was raised. In response to this audit, a research project was set up to develop an in-house LC-MS/MS method for the determination of MG and LMG in farmed salmon at the Marine Institute.

As a result of this study, chapter 5 describes a confirmatory method for the determination of MG, LMG, CV and LCV in salmon by LC-MS/MS. The residue method will be incorporated from 2006 onwards into the Irish monitoring programme. No such validated method according to 2002/657/EC for the determination of MG,LMG,CV and LCV in salmon by LC-MS/MS existed prior to this study.

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Chapter 2: Section A

Review of current methodology for the determination of robenidine residues

2.1 Introduction

Coccidiostats are substances that are used to kill or inhibit protozoa that cause coccidiosis. The coccidiostat, robenidine hydrochloride is therapeutically used to treat coccidiosis. Coccidiosis, is a contagious condition affecting lifestock, especially poultry, throughout the world. The disease is carried by unicellular organisms belonging to the genus *Eimeria* in the class Sporozoa.

In the poultry industry, coccidiostats are administered as feed additives. The European Commission regulates the inspection of animals and of fresh meat for the presence of residues of veterinary drugs and specific contaminants by Council Directive 86/469/EEC.[1] But since coccidiostat drugs are actually feed additives, legislative criteria for these feed additives is laid down in Regulation 1831/2003/EC.[10] This legislation states that coccidiostats can be used at a prescribed concentration during a certain time interval for broilers and young chickens, but these compounds cannot be used for laying hens. Currently monitoring of these feed additives is governed by national surveillance schemes within EU member states established under Council Directive 96/23/EC[2] on measures to monitor certain substances and residues thereof in certain animal products.

Even when withdrawal times have been established for these licensed feed additives, residues are frequently found in products derived from poultry, eggs and meat. One of the main reasons for this is cross contamination of non-medicated feed with medicated feed at time of manufacture. Subsequently another contributory factor to prolonged residues in farmed poultry is due to birds recycling their own faeces. This extends the exposure of poultry to medicated compounds which are contained in the ingested faecal material. Due to the presence of coccidiostat residues, phasing out of the use of coccidiostats as feed additives is being planned by 31 December 2012. The EC plan to submit a report to the European Parliament and the Council on the use of these substances as feed additives and recommend available alternatives before 1 Jan 2008. This report where appropriate will recommend legislative proposals. Until then, there are no maximum residue limits (MRL) set for these compounds in eggs and hence no residues of these compounds are permitted. Robenidine hydrochloride is a coccidiostat drug that has a structure that is unique among the anti-coccidials. It is the only anti-coccidial of the guanidine group that is unrelated chemically to any other coccidiostat in use and as a result of this attribute minimises the

risk of cross contamination. Cycostat 66 G (Cycostat) contains 6.6% of the active substance, the synthetic chemical robenidine hydrochloride and is marketed commercially as the feed additive.

Since it is licensed as a feed additive there is a need to evaluate potential problems related to the use of this compound in the poultry industry. Of all the licensed coccidiostats robenidine hydrochloride has been poorly studied.

This review focuses on the main stages of a residue detection method; namely,

Sample extraction procedures

Clean-up techniques

Methods for determination

Each of these stages will be discussed with specific reference to the determination of robenidine residues in biological fluids and tissues.

2.2 Sample Extraction Procedures

There are a limited number of methodologies available in the literature on the coccidiostat robenidine. Typically, methods developed in the 1970's and early 1980's used a variety of extraction methods requiring the use of, amongst other things, separatory funnels, volumetric flasks, glass pipettes, columns and evaporation flasks. Large sample sizes were used together with consequently large volumes of organic solvents for residue extraction, normally multiple extractions. Such large volumes of solvent were evaporated by rotary evaporation and the extracts reconstituted in volumetric flasks for further clean-up by liquid/liquid partitioning in separatory funnels or by open column chromatography. While such methodologies continue to be used in certain areas of residue analysis, notably for some applications in pesticide analysis, in most areas of residue analysis scaling down has occurred. Rather than flask-based extractions, test-tube extraction and liquid/liquid partitioning are common. Much smaller solvent volumes are used, and this use of smaller volumes is compatible with batch - based evaporations in heating blocks under nitrogen or in special evaporating apparatus such as centrifugal evaporators. Today most column clean-up technologies have been scaled down to solid phase extraction (SPE) cartridges. The above description is a

generalised view of changes in residue analysis through the decades. These trends are exemplified in robenidine analysis. In the 1970's, methods [3,7,8,9] that were developed for the quantitation of robenidine residues used large volumes of solvent. The tissues are generally first homogenised and following this they were extracted by liquid extraction with organic solvents alone or modified with the addition of an acid or a base. Smith *et al.,* [3] added heparin to blood samples and refrigerated for approximately 8 hr prior to extraction. With chicken skin, muscle, fat and liver tissues, ethyl acetate was usually the solvent of choice for extraction. The addition of the heparin to blood samples prevents them from clotting so that the blood can be later centrifuged and the plasma removed. This group extracted kidney, liver, plant tissue using acidic acetone and blood and soil using acetone and basic methanol. The extraction of chicken kidney with acidic acetone gave rise to a higher background level of interference so further clean-up was necessary. Exhaustive extraction studies of various sample matrices indicated that one extraction removed > 90% of the total robendine meaning repeat extractions may not be necessary. Zager *et al.,* [9] extracted feed premix samples with methanol using either manual or automated extraction procedures. These authors used a totally automated sample handling system called the Solidprep sampler [TM] which extracts weighed samples, dilutes and filters them onto the HPLC column. This group also used a liquid sampler to automatically inject onto the column manually prepared samples for separation and quantitation. Dubois *et al.,* [4] added anhydrous sodium sulphate to egg and muscle samples before extraction of robenidine with acetonitrile. Mortier *et al.,* developed simple direct methods to extract egg [5,6] with acetonitrile and feed pre-mixes [6] were extracted with methanol.

2.3   Sample Clean-Up Techniques for Different Matrices

Chicken Tissue

Smith et al., [3] showed that clean-up of chicken skin, muscle, fat and liver tissues extracted after evaporation could be re-dissolved in petroleum ether and successively partitioned into acetic acid (1+1) and washed with chloroform. It was found that these partitioning steps did not reduce interferences sufficiently for easy determination. Upon evaluation of

various column packings, including silica gel, alumina (acidic, basic and neutral), florisil, activated charcoal aswell as weak and strong cation exchange resins. It was found that the weak acid macroreticular cation exchange resin CG-50 separated robenidine from interfering co-extractives. Robendine could be extracted from chloroform layer in liquid/liquid partitioning with methanol, transferred to the column, and retained while interferences were washed from column with additional methanol (90:10, v/v). Robenidine was then eluted from the column with acidic hydrochloride acid in aqueous methanol. The eluate was deemed sufficiently clean for direct polarographic determination of robenidine. This group found acidic acetone to be suitable for extraction of robenidine from chicken kidney but it gave rise to huge intereferences so further clean-up was deemed necessary. The acidic acetone extract was cooled to precipitate high molecular weight co-extractives and filtered while cool, an aliquot of the filtrate was evaporated to remove the acetone. Sodium hydroxide was added to make the solution basic and partitioning against ethyl acetate was carried out. The ethyl acetate layer was dried and residue was dissolved in hexane and a further partitioning was carried out against acetic acid (50:50,v/v). This reduced background interferences sufficiently for direct polarographic determination of robenidine in acetic acid extraction without further clean-up on CG-50 chromtographic column.

Chicken Eggs

Smith et al.,[3] cleaned up egg samples evaporated to dryness by dissolving residue in petroleum ether by adding saturated sodium chloride solution to petroleum extract. The petroleum extract was partitioned against acetic acid (50:50, v/v) and then washed with chloroform. The clean-up on CG-50 column was same as described by this group in the clean up of chicken tissue.

Dubois et al.,[4] cleaned up acetonitrile extracts of egg by passing through a silica solid phase extraction cartridge. Mortier et al.,[5, 6] eliminated the need for clean-up of eggs samples by direct analysis of the extract by LC-MS/MS.

Plant Tissue

Smith et al., [3] cleaned up plant tissue acidic acetone extracts by rinsing with petroleum and successively partitioned against acetic acid (50:50, v/v) and washed with chloroform. The same clean-up procedure was utilised as described for animal tissue by this group except acetic acid (50:50, v/v) was added to the acidic hydrochloric acid in aqueous methanol prior to analysis. The majority of plant samples could be polarographed directly after clean-up on the CG-50 column. However in a small number of samples, an interfering polarographic wave was obtained which partially distorted the peak shape of the robenidine wave to make accurate measurements difficult. If a portion of the column effluent was concentrated down to < 1 ml and the residue re-dissolved in acetic acid (50:50, v/v). The robenidine wave was sufficiently separated for accurate quantitation.

Chicken Feed

Bories et al., [7] purified feed extracts using thin layer chromatography. Clean-up of acidified extracts of animal feed was accomplished on a column of aluminium oxide. The robenidine is eluted with acetonitrile containing 2-methoxyethanol and ammonia. In the determination of robenidine in premixes, no clean-up was required. In the preliminary study of animal feeds, it was found that acetonitrile with a low water content must be used. The recovery of robenidine was reduced if there was more than 3% water present in the feed extract. It was also found that different sources of equivalent grades of aluminium oxide did not perform equally as well. Results stated there was a tendency for some of the robenidine to be eluted from the column with wash liquor during the clean-up stage. It was found that drying of the extract with molecular sieves was satisfactory whereas drying with anhydrous sodium sulphate resulted in low recoveries so the former was utilised in the method. In the clean-up of turkey feeds containing grassmeal, the use of acidic aluminium oxide was found to be more useful when grassmeal was present in turkey feed. But in the absence of grassmeal, basic aluminium oxide was sufficient. Also nitrofurazone, acinitrazole and enheptin were found to interfere in the determination of robenidine by exhibiting a small apparent robenidine content. Gross interference was caused by the presence of dinitolmide in feed. However on using acidic aluminium oxide

in place of basic aluminium oxide, this interference could be overcome. [8] Mortier et al., [5] did not clean-up methanol feed/extracts as extracts were suitable for direct analysis by LC-MS/MS.

2.4 Methods for the Determination of Robenidine Residues

Few methods have been developed to analyse for robenidine and the ones that do exist are based on spectrometry,[8] chromatographic separations,[9] thin layer chromatography,[7] polarography [3] and liquid chromatography coupled to mass spectrometry detectors. [4,5,6]

Spectrometric Methods

Robenidine was determined in feeds after the feed extract was treated with ethanolic potassium hydroxide. [8] The absorbances were read at 440 nm and 550 nm. The absorbances were corrected by subtracting the absorbances at 440 nm and 550 nm of the treated eluate after acidification, and the difference between the corrected absorbances is directly proportional to the robenidine concentration. For the determination of robenidine in a pre-mix, the drug is extracted into methanolic sodium hydroxide and the extract's absorbance measured without the need for clean-up at 352 nm. A correction for background interference was made by subtracting the absorbance at 352 nm of the extract after acidification. The method was applicable to the determination of robenidine in poultry feeds down to 3 mg kg^{-1}.

Electrochemical Methods

Smith *et al.,* [3] analysed robenidine by cathode ray polarography. This technique provided the necessary sensitivity for determining robenidine at trace residue levels. Robenidine possesses an electrochemical azomethine group in its structure that is electrochemically reducible. This group evaluated polarographic waves in acidic, neutral and basic supporting electrolytes. The wave obtained in acidic supporting electrolytes proved to be the most useful in respect to stability, sensitivity and linearity. Acetic acid and acidic aqueous methanol (50:50, v/v) proved to be suitable supporting electrolytes. The differential mode of cathode ray polarography although, four times less sensitive than the

direct mode, produced a wave height that was linear and could be accurately quantitated with robenidine concentration over the range of 50 to 500 x 10^{-9} g ml^{-1}. The linear relationship between robenidine concentration and wave height was found to hold in the presence of the various types of sample background examined. Due to variability of sample background within a given sample type (e.g kidney) and the varying degrees of suppression of the polarographic response as a result of such a variation, a general linearity curve could not be applied to a given sample type. The standard addition technique was used during sample analysis. [14] C-Robenidine hydrochloride was used throughout the development of the polarographic procedure. The use of this radiolabeled compound greatly facilitated the method development process. Since this technique allowed for the investigation of (a) extractability of robenidine from a given sample matrix by a given solvent (b) partitioning of robenidine between two solvents, (c) the effect of variables such as pH and salt content on this partitioning, and (d) elution of robenidine from various chromatographic supports in the presence of sample background which otherwise would have prevented direct polarographic determination of the compound. Recoveries of robenidine ranged from 64 to 125% with an average overall recovery of 90%. The validated sensitivity was 0.1 mg kg^{-1} for chicken tissues, soil and plants, 0.01 mg^{-1} kg for eggs and 1 mg kg^{-1} for litter.

Liquid Chromatographic Separations

Four liquid chromatographic separations for the determination of robenidine residues have been developed. Three of these have been developed using C_{18} reverse phase systems. [4,5,6] The fourth method evaluated high pressure ion–exchange, reverse phase and absorption chromatography with appropriate supports and solvents mixtures. [9] In this method a mixture of methanol, acetic acid and methylene chloride were used as the mobile phase on a CPG-240 controlled pore glass stationary phase. The method provides rapid and specific method for the analysis of robenidine and is capable of distinguishing the intact drug from its degradation products. A precision study of the chromatography method was carried out and results indicated that the procedure was capable of giving quantitative results at a rate of 10 samples hr^{-1} with a coefficient of variation of 1.13%.

These results were achieved when replicate aliquots of the same sample solution were carried out to eliminate precision effects due to sample variation. A recovery of 101% was obtained for robenidine using this method.

Mortier *et al.*,[6] analysed robenidine, diclazuril, dimetridazole, halofuginone and nicarbazin by LC-MS/MS separating these compounds using reverse phase chromatography comprising of a Waters Symmetry C_{18} column (150 mm × 2.1 mm, 5 µm) and a gradient with water (A) and acetonitrile (B) each containing 0.1% formic acid. The mobile phase flow rate was 0.25 ml min^{-1} with a total runtime of 17 min. These conditions eluted robenidine at a retention time of 8.85 min. Dubois *et al.*,[4] separated halofuginone, diclazuril, dinitrocarbanilide, robenidine, monesin, lasalocid, narasin, salinomycin and maduramicin on a Merck Purospher C_{18} (125 mm × 3 mm, 5 µm) column. The flow rate was 1ml min^{-1} and a gradient of water (A) and acetonitrile (B) was applied, each containing 0.1% formic acid with a total run time of 14 .5 min. Zager *et al.*,[7] determined the operating conditions for the resolution of robenidine hydrochloride from 1,2,3- tris(p-chlorobenzylideneamino) guanidine hydrochloride and bis (4-chlorobenzal) azine two of its chemical impurities. The column used for the LC system was a 600 mm x 2.1 mm i.d stainless steel tube with end fittings containing 2 µm bed supports. The column was dry packed with 200 -400 mesh controlled pore glass CPG-10-240A packing. Mortier *et al.*,[5] separated halofuginone, robenidine, diclazuril, nicarbazin, dimetridazole and 2-hydroxydimetridazole on a Waters Symmetry C_{18} column (150 mm x 2.1 µm, 5 µm). The column temperature was maintained at 35 °C. The mobile phase flow rate was 0.25 ml min^{-1} and a gradient of eluent A (acetonitrile:water (95:5, v/v) containing 0.1 % formic acid) and eluent B (acetonitrile) was used to separate robenidine from the other coccidiostats investigated.

Thin Layer Chromatography (TLC) Determination

Bories *et al.*,[5] analysed robenidine in poultry feed by TLC using a plate coated with a thick layer of silica gel. The plate was developed with chloroform:methanol (95:5, v/v) as the developing solvent. After development the spots were made visible by spraying with Rhodamine B solution and a UV lamp (wavelength 265 nm) was used to detect

robenidine. The area on the plate containing the coccidiostat was scraped and the powder dissolved in dimethylformamide after addition of sodium hydroxide. The yellow coloration on development was measured at 464 nm. Robenidine contains a number of conjugated double bonds that undergo electronic reorganisation in an alkaline medium. This re-organisation results in a shift in the absorption maximum to a longer wavelength, so on the addition of potassium hydroxide solution this value shifts to 464 nm and at the same time adsorption by interfering substances is minimised. The efficiency of the chromatographic purification was tested by spotting with pure robenidine onto the plates, either on its own or in the presence of extra fatty or pigmented poultry feed. Extracts of the feed with no robenidine were measured under the same conditions, and results showed negligible blank values. The recovery of robenidine from poultry feed ranged between 96 and 100%.

Liquid chromatography detection systems

Only a small number of groups have developed methods for robenidine and there are no methods developed using UV or fluorescence detection. A limited number of methods have been developed for the determination of robenidine in tissue, egg, feed and feed pre-mixes using mass spectrometric detection. [4,5,6] Dubois *et al.,* [4] used positive and negative ion electrospray modes (ESP) in the separation of 9 coccidiostats. The first 7.4 min of the runtime were in positive ESP mode for robenidine and halofuginone, then switching to negative electrospray occurred until 9 min for dinitrocabanilide, DNC-d_8, diclazuril, diclazuril bis and then back to ESP [+] mode for the polyethers (monesin, lasalocid, salinomycin, narasin, maduramycin). For the unequivocal identification of each analyte, two ions were detected and chosen for multiple reaction monitoring (MRM). The parent ion monitored was 334.1 m/z at a cone voltage of 30-35 V. Daughter ions of 138.2 and 111.2 m/z were obtained with collision energies of (eV) 23 and 41. The recovery of robenidine from muscle samples fortified at 2 μg kg^{-1} level was 56 %. The coefficient of variation was not reported in this paper. The CCα and CCβ values obtained for robenidine were 0.2 and 0.5 μg kg^{-1}.

Mortier *et al.,* [5] used LC-MS/MS with electrospray ionisation interface (ESI) for the detection of robenidine and 4 other coccidiostats in poultry eggs and feed. Detection was performed in the selected reaction monitoring mode after ionisation in the positive or negative electrospray ionisation mode. The presence of the $[M+M]^+$ ion for dimetridazole, 2-hydrodimetridazole, dimitridazole-d_3, halofuginone and robenidine. The $[M-M]^-$ ion were monitored for diclazuril, diclazuril-bis and dinitrocarbamilide.

The parent ion monitored was 334.1 m/z at a cone voltage of 50 V. Daughter ions of 138.1 and 155.1 m/z were obtained with collision energies of (eV) 24 and 18. The recovery of robenidine from fortified egg and feed samples were between 101 -105 % and 96 – 102%. The coefficient of variation was between 4-7% and 5-6% in fortified egg and feed samples The CCα and CCβ values obtained for robenidine in fortified egg samples were 1.0 and 1.2 μg kg^{-1}. The CCα and CCβ values obtained for robenidine in fortified feed samples were 8.8 and 12.5 μg. kg^{-1}.

The use of LC-MS/MS with electrospray ionisation interface (ESI) was used by Mortier et al., [6] and robenidine was determined with ESI in the positive mode with $[M+H]^+$ ions. The parent ion monitored was 334.5 m/z using a cone voltage of 25 V. The most abundant daughter ion was 137.8 m/z which was obtained using a collision energy (eV) of 25. Daughter ions of 129 and 110.9 m/z were obtained using collision energies (eV) of 40 and 45. The recovery of robenidine from eggs fortified at 5 μg kg^{-1} level was 42 %. The coefficient of variation was 19 %. The CCα and CCβ values obtained for robenidine in eggs were 3.0 and 4.0 μg kg^{-1}.

2.5 Conclusions

The determination of the coccidiostat robenidine involves liquid partitioning and/or solid phase extraction in conjunction with liquid chromatography tandem mass spectrometry. This methodology allows for the determination of robenidine in multi-residue methods with other coccidiostats. Dubois *et al.,* [4] has developed the most complete method, allowing the determination of robenidine and 8 other coccidiostat drug residues in chicken muscle and eggs with detection by LC-MS/MS. The methods developed by

Mortier *et al.*, for the determination of robenidine and 4 other coccidiostats in eggs [5, 6] and feed [5] are also noteworthy.

However difficulties such as the high costs involved in performing analysis by LC-MS/MS mean that a variety of alternative techniques need to be made available so that robenidine determination is possible for all designated laboratories in the EU.

To date, however, no method exists that can screen for robenidine by LC-UV, this technique being readily available and of low cost. The development of such a method would allow for ease of determination of this compound using very accessible technology that is available in most basic analytical laboratories.

Robenidine, as mentioned before, has not been studied extensively hence there is also no information regarding the distribution of robenidine residues within tissues such as between egg yolk and egg white.

There is also little information on the depletion of robenidine in different species of poultry; different species effects may be important in determining withdrawal periods.

There is also no data on the stability of this compound upon storage. A study of this would give rise to vital information which would allow experiments to assess if harmful breakdown products may result from storage conditions.

Another factor to consider is that feed additives eventually end up in manure. Manure is dispersed in fields and the mobility of drugs in the soil system may pose a threat to ground water and soil dwelling organisms and ecosystems.

Further study is required to determine the environmental effects of administration of all feed additives in animal husbandry.

Since the licensed coccidiostat robenidine may be widely used, it is important to continue with the development of screening, validation or confirmatory methods in the areas where shortfalls have been discussed above.

Fundamental to this work is the development of a simple residue method capable of screening for the coccidiostat robenidine in eggs and subsequent availability of this method for implementation into The National Monitoring Plan for Ireland. Egg was selected as the matrix since this compound is not licensed for use in laying hens so any levels found in egg would be in breach of regulations. LC-UV methodology was chosen over LC-MS/MS technology since this technique is more available in residue

laboratories. The development of such methodology will provide a legal basis for prosecuting individuals who use this feed additive in breach of EU legislation. Acetonitrile was chosen as the extraction solvent since it has been widely used in the extraction of robenidine from a variety of matrices. There is an obligation on each member state in the EU to put methods in place to monitor for coccidiostat residues. For these reasons, the development of a residue method capable of monitoring for robenidine is extremely important.

2.6 References

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Chapter 2: Section B

Review of current methodology for the determination of benzimidazole residues

2.7   Introduction

Benzimidazoles are a group of drugs that have a fixed MRL and are therefore in Annex I of Council Regulation EEC/2377/90. Benzimidazoles are broad spectrum anthelmintics and have been used for about 40 years in animal husbandry and horticulture to control anthelmintic infections. Metabolism of these drugs is extensive. When the pro-drugs and active drugs and metabolites are counted, some 19 possible residues may occur in animal tissues.

Table 2.1 presents the pharmacologically active substances involved in the benzimidazole work for this thesis, for food producing animals including the established MRL values. The current status (fixed or provisional) of the MRL values are indicated. There are seven groups of benzimidazoles licensed for use and the MRL values range from 10 – 1000 µg kg^{-1}, depending on the compound and matrix. The development of a single multi-residue method capable of testing for all the residues in the MRL listings is very difficult since there is huge variability in terms of polarity and pKa within a group of these drugs and between the different groups. It is necessary for each member state to carry out a national surveillance scheme, established under Council Directive 96/23/EC [22] for all these benzimidazoles drugs. In Ireland, national surveillance of only 5 of these drugs was being carried out and work was necessary to improve the number of these residues detected in animals and animal products. This work was necessary not only to protect Irish consumers from the potential risk of harmful residues but also essential to facilitate international trade as other countries may be exporting animals treated with unlicensed products or concentration of residues above the maximum residue level into Ireland. Therefore a review of the literature concerning the types of residue methodologies employed for the screening and confirmation of these compounds was carried out. This evaluation would form a foundation for the approach that must be undertaken to incorporate a satisfactory multi-residue method into the National Monitoring Plan in Ireland to determine all 7 licensed groups.

Table 2.1: MRL listings for benzimidazole anthelmintic drugs

Drug	Marker Residue	Animal Species	MRL (µg/kg)	Target Tissue
NETO, ABZ and ABZ-SO	Sum of ABZ-SO, ABZ-SO$_2$, and ABZ-NH$_2$-SO$_2$ expressed as ABZ	Bovine, ovine	100	Milk
			100	Muscle
			100	Fat
			1000	Liver
			500	Kidney
FEB, FBZ and OFZ	Sum of extractable residues which may be oxidised to FBZ-SO$_2$	Bovine, ovine	10	Milk
		Bovine, ovine, porcine and equidae	50	Muscle
			50	Fat
			500	Liver
			50	Kidney
FLU	Sum of FLU and FLU-HMET	Porcine, poultry and game birds	50	Muscle
			50	Skin and Fat
			400	Liver
			300	Kidney
		Chicken	400	Eggs
TBZ	Sum of TBZ and 5-OH-TBZ	Bovine	100	Muscle
			100	Fat
			100	Liver
			100	Kidney
			100	Milk
OXI	OXI	Porcine	100	Muscle
			500	Skin and fat
			200	Liver
			100	Kidney
TCB	Sum of extractable residues that may be oxidised to ketotriclabendazole	Bovine and ovine	100	Muscle
			100	Liver
			100	Kidney
MBZ	Sum of MBZ, MBZ-OH and MBZ-NH$_2$ expressed as MBZ	Ovine, caprine and equidae	60	Muscle
			60	Fat
			400	Liver
			60	Kidney

This review focuses on the main stages of a residue detection method; namely,

Sample extraction procedures

Clean-up techniques

Methods for determination

Each of these stages will be discussed with specific reference to the determination of benzimidazole residues in biological fluids and tissues.

2.8   Sample Extraction Procedures

The presentation of biological matrices in a suitable format for residue extraction involves a sample pre-treatment step. The techniques used in sample pretreatment depend on the matrix under consideration, liquids requiring generally less pretreatment than tissues. The tissues are first homogenised, and after this step the tissues may be either hydrolysed for the release of conjugates or bound residues or liquid extraction of the tissue at high pH into immiscible organic solvent. Many groups investigated the effect of deconjugation of residues using enzymatic or acid hydrolysis since many benzimidazoles metabolites exist as conjugates. Tocco *et al.*,[1] investigated the urinary metabolites of TBZ and found that the glucuronide and sulfate conjugates of 5-OH-TBZ were the major metabolites. Conjugates were enzymatically hydrolysed using β-glucoranidase or glucosulase or by refluxing in 6N HCL (1 h). Similar procedures were later used by Tocco *et al.*,[2] to deconjugate 5-OH-TBZ residues present in cows' and goats' milk after treatment with TBZ. A more extensive study was completed by Chukwudebe *et al.*, [3] into the metabolism of TBZ in laying hens and lactating species using enzymatic and acid hydrolysis and it was found that conjugates were mainly present as sulfates. In a study of bluegill sunfish Van den Heuvel *et al.*,[4] also deconjugated 5-OH-TBZ by enzymatic and acid hydrolysis. Kriedel *et al.*,[5] deconjugated the glucuronide of MBZ-OH after digestion with β-glucuronidase. Glucosulase was used to enzymatically hydrolyse ABZ metabolites in urine by Gyurik *et al.*,[6] before detection by TLC. The incubation of urine by Short *et al.*,[7] showed that the majority of residues

present in urine were conjugated when tested on animals treated with FBZ and enzymes β-glucuronidase and sulfatase before extraction and determination by mass spectrometry. As a result of this other authors have included, hydrolysis steps in some residue methods. Markus et al.,[8] subjected liver samples to acid hydrolysis before extracting $ABZ-NH_2-SO_2$ residues. Arenas et al.,[9] used enzymatic hydrolysis to deconjugate 5-OH-TBZ sulphate. Coulet et al.,[10] used basic hydrolysis to release protein bound residues of TBZ from tissue.

The polarity and pKa values of benzimidazoles can differ to a huge extent between metabolites and parents. This not only makes it difficult to develop multi-residue methods for different benzimidazole drugs but also a single drug and its metabolites. The usual approach to extract benzimidazoles is liquid extraction. Commonly this is achieved using aqueous extraction at high pH with partitioning into immiscible organic solvents. [11,12,13] Many groups developing methodology for the determination of FBZ and its two metabolites OFZ and $FBZ-SO_2$ [14-19] have had difficulty in development due to FBZ and its metabolites having difficult physical properties, thus making it difficult to extract and determine by LC [15,16,20] Similar observations have been made with MBZ and its two metabolites MBZ-OH and $MBZ-NH_2$.[13] MBZ and $MBZ-NH_2$ are basic in nature with pKa values of 3.5 and 5.5, respectively. Therefore, only at pH levels >7.5 are both metabolites non-ionised. MBZ-OH possesses an acidic OH group with a pKa of 9.8 which is non-ionised below pH 7.8. This indicates that only in the pH range 7.5-7.8, are all three residues non-ionised. Alternatively, extraction may be carried out using a more polar organic solvent without pH manipulation. Some groups have extracted these drug residues using a single solvent system, such as acetonitrile [21,22],or binary water/organic solvent mixtures which would be around neutral pH.[28,20,24,25] Extraction with acidified extractants has only been used by a small number of groups for methods requiring hydrolysis or digestion steps [3,8,26,27] or methods including only a limited number of residues.[28]

The use of medium polarity water immiscible solvents has been used in the extraction of benzimidazoles from muscle, fat, liver and kidney at alkaline pH. A carbonate buffer/ethyl acetate system by Wilson et al., [11] to extract 8 benzimidazoles from muscle and liver has been used by a variety of groups [29,30-36].

The extraction of TBZ and 5-OH –TBZ from different tissues was achieved using a phosphate buffer (pH 7) from different tissues by Cannavan et al [37].

It was found that the ethyl acetate extraction gave rise to extracts that had more interferences when used as extraction solvent for cheese samples than acetonitrile, while dichloromethane gave low recovery. Takeba et al.,[49] used acetonitrile to extract TCB, TCB-SO and TCB-SO$_2$ from milk and obtained recovery of 89-95%. Sorensen et al.,[48] used a buffer at pH 6.8 to extract levamisole and TBZ from milk. An analogous extraction procedure was developed by the same group for eight other benzimidazoles using a buffer at pH 11.0.[50] Recovery in both methods was $\geq$ 81%. Brandon et al.,[51] found that milk samples needed only to be diluted in PBS-tween-BSA before detection of FBZ residues by ELISA.

The adjustment of plasma to pH 7.4 by Bogan et al., [52] in the extraction of FBZ, OFZ and ABZ residues with ether has been utilised by a number of groups extracting ABZ and FBZ residues [53, 54]. The substitution of ether with ethyl acetate was carried out by Lehr and Damn [55] to extract TBZ and TCB and their major metabolites.

Hoaksey et al.,[56] extracted ABZ and ABZ-SO from plasma samples with dichloromethane after protein precipitation with acetonitrile. Recovery of ABZ and ABZ-SO$_2$ was 97 and 75%, respectively. The extraction of TCB, TCB-SO and TCB-SO$_2$ from plasma by Alvinerie et al [57] used a simple ethyl acetate extraction procedure and gave mean recoveries of TCB and its two metabolites between 98 and 104%. Other groups [17,58] have mixed plasma with ammonium hydroxide or buffered solutions before extraction of residues by SPE.

The extraction of TBZ residues in fruit and vegetable matrices has been achieved using ethyl acetate [59-61], methanol [62] or acetone [63,64] and extending the methods to include benomyl, thiophanate-methyl and carbendazim. Extraction using binary mixtures usually a aqueous alkaline extraction with a less polar immiscible solvent such as dichloromethane [65], ethyl acetate [23,61,66] or ethyl acetate-hexane [67] have also been utilised.

Aqueous extraction using acetate buffer has also been applied to the extraction of TBZ residues with [68] and without [69] further solvent partitioning. Brandon et al.,[70] extracted TBZ from fruit and vegetables with tris-buffer or water, finding that the extracts needed only dilution with buffer before determination by ELISA. Shah et al.,[71] extracted OFZ

from feed using 10% glacial acetic acid in methanol (45 °C, 30 min), while Botsoglou *et al.*,[72] extracted FBZ from feed using acetonitrile.

Depending on the sample matrix, the presence of water, lipids, proteins or co-extractives can be a source of problems at the extraction stage or at subsequent stages. Therefore sample clean-up becomes important. Sample clean-up methodologies used to minimise problems and improve analysis of the benzimidazoles were subsequently evaluated.

2.9 Sample Clean-up Techniques for Different Matrices

Sample clean-up is dependent on a number of factors such as on the number of residues to be screened, the extraction procedure used, sensitivity requirements and the determination step. Extensive clean-up procedures are necessary if including a wide range of residues and require high sensitivity when extracting from complicated tissue matrices. Including sample pre-treatment steps and by using more selective detection systems such as fluorescence or mass spectrometry, the extent of clean-up needed can be reduced and sample throughput can be increased. Bushway *et al.*,[65] diluted fruit juice samples in mobile phase prior to determination by LC with fluorescence detection. A number of groups have analysed benzimidazole fungicides by GC with more selective detectors such as ECD [23,67,68], NPD [98,136,137] and MS [11,68,98,130,133,134,135,136,137,139,140] and found that residues may be determined without extensive clean-up. Kitada *et al.*[60] extracted citrus fruit with ethyl acetate and the extracts were sufficiently clean for analysis without further clean-up. Brandon *et al.* [24,70] found that sample extracts only needed dilution in buffer prior to determination by ELISA.

Liquid-liquid partitioning is the most extensively used method for the elimination of polar and non-polar matrix interferences. A widespread approach is to modify the pH of extracts to alkaline conditions and partition residues into an immiscible organic solvent such as ethyl acetate to get rid of polar interference. Partitioning between an acidified extract (in dilute HCL or ethanol / HCL) and a non-polar solvent (ethyl acetate, heptane or hexane) can be used to remove non-polar interference, into the organic fraction. Solid phase extraction (SPE) is the most widely used procedure for the clean-up of sample

extracts. This procedure allows easier handling of a larger number of samples and can greatly reduce sample clean-up time and solvent usage. SPE can also offer greater selectivity. The availability of SPE sorbents in a wide variety of chemistries and differences between manufacturers of similar chemistries allow endless possibilities.

Animal Tissue

The use of liquid partitioning only in the clean-up of benzimidazoles from tissue [29,12,25,28, 38-41] have only been applied to a limited number of residues and generally residues from the same parent drugs. The most widely adopted procedure in liquid-liquid extraction (LLE) is an alkaline buffer-ethyl acetate extraction system for isolation of these drugs from tissue matrices while removing non-polar matrix interference. Samples can also be extracted using dilute HCL and non-polar interference removed by partitioning into non-polar solvent [28]. The acidified layer, neutralised and adjusted to alkaline pH before extraction with immiscible non-polar solvent. Blanchflower *et al.*,[25] extracted FBZ and OFZ from liver with methanol-water before defatting with petroleum spirit and extracting with diethyl ether and ethyl acetate. More simple defatting steps were used by Nafstad *et al.*,[41] and De Ruyck *et al.*,[39] by washing acetonitrile and methanol, respectively, with hexane. Mellergaard *et al.*,[28] extracted MBZ from eel muscle with 0.1N HCL, before defatting with heptane. The pH of the aqueous extracts was further adjusted to pH 9.6 before extraction into heptane/isoamyl alcohol.

The approach most commonly used in the extraction and clean-up of benzimidazole residues from animal tissue is a combination of liquid extraction (LLE), liquid-liquid partitoning and solid phase extraction (SPE). Wilson *et al.*,[11] purified extracts by partitioning between ethanol/0.2M HCL and hexane, before clean-up on C_2 SPE cartridges. The C_2 SPE step was necessary to remove polar compounds that interfered with ABZ-NH_2-SO_2 and TBZ-OH. This group evaluated a number of reverse phase and semi-polar sorbents and found C_2 to be most effective. This method was applied for the determination of 15 benzimidazoles in liver by Balizs *et al.*,[30] while Roudaut *et al.*,[33] modified the method by substituting Oasis HLB cartridges for C_2 cartridges and reported more reproducible results. Shaikh *et al.*,[29] applied the Wilson *et al.*,[11] method to the

determination of ABZ residues in fish muscle finding that the C_2 could be excluded by using more selective detection. Other groups have adopted similar LLE procedures and further purified tissue extracts by SPE on C_{18},[23] amino-propyl,[13] silica,[31] cyano,[36] neutral alumina.[73] using SPE cartridges Marti *et al.*,[23] and Farrington *et al.*,[74] developed multi-residue methods using dual SPE clean-up procedures based on normal phase and C_{18} sorbents. Rose *et al.*,[22] applied strong cation exchange (SCX) SPE to the isolation of OFZ and nine related residues in liver. Stubbings *et al.*,[75] developed an online SCX-SPE procedure for the purification of benzimidazole residues from tissue. Matrix Solid Phase Dispersion (MSPD) has been applied by a number of groups for the determination of benzimidazoles in liver.[29,76,16,18,77-81] This involves packing a tissue/C_{18} mixture into an empty SPE column, washing with hexane and eluting with acetonitrile or hexane.

Milk and milk products

The removal of polar interference from milk has been accomplished by liquid/liquid partitioning of ethyl acetate with water or partitioning of 0.2 N HCL with ethyl acetate by Fletouris *et al.*,[44] and Tsina *et al.*, [45]. Fletouris *et al.*,[15] found that acetonitrile was efficient at precipitating proteins, while non-polar interference could be removed by partitioning into hexane or iso-octane. The extraction of FBZ, ABZ, OXI and LEV from milk with acetonitrile; ethyl acetate was accomplished by De Ruyck et al., [122] The extract was washed with di-sodium hydrogen phosphate adjusted to pH 10.

A number of different SPE clean-up procedures have been developed using silica,[42] C_2,[82] C_{18},[21] cation exchange [9,81] and neutral alumina,[34] but liquid/liquid partitioning clean-up was still necessary prior to SPE.

Benzimidazoles were extracted using aqueous buffer pH 6.5 and pH 11 before purification by liquid/liquid partitioning and SPE on C_{18} by Sorenson et al. [48,50]

MSPD was applied to determine benzimidazoles in milk and cheese by Cinquina *et al.*, [82] and Longet *et al.*, [16] The former group found extracts needed further purification by C_2 SPE.

Plasma, Serum and other biological fluids

Plasma and other biological fluids are much cleaner than tissue and milk samples, requiring less clean-up. Bogan *et al.,*[52] developed a simple clean-up procedure for the determination of benzimidazoles in biological fluids. The pH of samples is adjusted to pH 7.4 and extracted with diethyl ether. This procedure has since been applied by other groups [53,54,83] for the determination of benzimidazole residues in plasma and blood. Similar extraction procedures have been developed by Zhangliu *et al.,*[84] and Alton *et al.,*[85], with partitioning between alkalised samples and non-polar solvents like chloroform[86,87] and ethyl acetate.[88] Allan *et al.,*[89] evaluated the clean-up procedures developed by Alton *et al.,*[84] and Karlaganis *et al.,*[87] and judged the latter method to be a much simpler extraction and clean-up procedure. Allan *et al.,*[89] applied this method to the determination of 4 MBZ related residues in plasma. Other groups [17, 58,90,91] have adjusted the pH of samples before purification on SPE cartridges, without prior liquid-liquid partitoning.

Rouan *et al.,*[92] developed an automated clean-up procedure for the determination of TCB and its metabolites in plasma using C_{18} SPE. Purified extracts were suitable for analysis without further clean-up and recovery was typically between 96 and 103%. Chiap *et al.,*[93] developed an automated clean-up procedure based on dialysis clean-up. Negro *et al.,*[94] isolated TCB-SO and $TCB-SO_2$ from serum and urine samples using a combination of protein precipitation and ultra-filtration through a 30,000 molecular mass cut-off filter. This group found that using this clean-up strategy greatly speeded up sample preparation, making the procedure faster than commonly used SPE clean-up procedures. Recovery of TCB-SO and $TCB-SO_2$ from serum was typically >90%.

Plant matrices

Farrow *et al.,*[95] simply adjusted the pH of hydrolysed extracts to pH 9.5 before partitioning into chloroform. Other groups have developed clean-up procedures based on a single LLE step between aqueous buffers and more non-polar solvents.[59,60,63,66] More

extensive LLE and liquid-liquid partitoning procedures have been adopted by other groups [67,68] Arenas *et al.*,[61] and Ito *et al.*,[96] applied cation exchange SPE to the determination of TBZ residues in fruit. Young *et al.*,[97] used Oasis® MCX SPE cartridges, finding that loading extracts in ammonium hydroxide produced better recovery than loading in acidic solution. Di Muccio *et al.*[63] used a combination of ExtreElut and SCX SPE for the isolation of TBZ, benomyl and carbendazim residues from fruit and vegetables. Oishi *et al.*,[98] isolated TBZ residues on an ExtreElut cartridge after extraction with an alkaline buffer. Zrostlikova *et al.*,[99] purified TBZ, carbendazim and other crop protection agents using Oasis MCX cartridges. Recovery of TBZ and carbendazim were typically greater than 88%.

Hiemstra *et al.*,[64] developed an on-line clean-up procedure for the isolation of TBZ and carbendazim residues from fruit and vegetable extracts. Samples were purified by liquid-liquid partitoning prior to automated off-line SPE clean-up on a diol SPE cartridge mounted on an ASPEC system. Shah *et al.*,[71] extracted OFZ from feeds, finding that plant pigments interfering with the determination could be removed by precipitation with zinc acetate before extracts were further purified by liquid-liquid partitoning. Botsoglou *et al.*,[72] extracted FBZ residues from feed, before removal of matrix interference by liquid-liquid partitoning against iso-octane.

The determination of the benzimidazoles in a variety of extracts was subsequently evaluated to identify which techniques have worked well for particular groups of bezimidazoles and to isolate a single technique that could be employed to develop the multi-residue national surveillance method for these licensed drugs.

Table 2.2: Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from animal tissues

Residues	Matrix[1]	Extraction	Clean-up	Recovery (%)	Ref
ABZ, CAM, MBC, MBZ, OFZ, FBZ, 5-OH-TBZ, TBZ	M, L	Ethyl acetate at alkaline pH	Acidified ethanol vs hexane C_2 SPE	81-100	11
ABZ, MBZ, OFZ, FBZ, OXI, FLU, TBZ, TCB	M, L, K	Acetonitrile-H_2O	LLP C_{18}, Florisil SPEs	65-87 39-80 (OFZ)	23
CAM, TBZ, 5-OH-TBZ, 5-NH_2-TBZ	L	DMSO-H_2O			24
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2 *(MBC, MBZ, FBZ-OH, FLU, 5-OH-TBZ, TBZ)	L	DMF-H_2O			116
ABZ, CAM, MBZ, OFZ, TBZ	L	Ethyl acetate	Alumina C_{18} SPE	67-90	74
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CAM, FBZ-SO_2, MBZ, OXI, 5-OH-TBZ, TBZ	Mk	Acetonitrile	LLP	62-108	35
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, OXI, 5-OH-TBZ, TBZ	M, L, K	Ethyl acetate at alkaline pH	Acidified ethanol vs hexane Oasis HLB SPE	44-87	33
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FEB, FBZ, OFZ, FBZ-SO_2, MBZ, OXI, FLU, CAM, TBZ, 5-OH-TBZ, TCB	M, L	Ethyl acetate at alkaline pH	Acidified ethanol vs hexane SDB SPE	36-117 8 (FEB)	30
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, FLU, MBZ, OXI, 5-OH-TBZ, TBZ, TCB, LEV	M, L, Fish	Ethyl acetate at alkaline pH	Methanol vs hexane	75-109	127
ABZ, FBZ, OFZ, FBZ-SO_2, FBZ-OH, MBZ, TBZ	M	MSPD	MSPD	63-86	77
ABZ, FBZ, OFZ, MBZ, TBZ	L	MSPD	MSPD	55-93	78
ABZ, FBZ, OFZ, FBZ-SO_2, FBZ-OH, TBZ	L	MSPD	MSPD	61-92	76
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, MBZ, MBZ-OH, FLU, OXI, TBZ		SF-CO_2	SCX	56-109	120
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, FLU, FLU-NH_2, MBZ, MBZ-OH, OXI, TBZ	L	Ethyl acetate at alkaline pH	C_{18} on ASPEC™	27-120	124

[1]Liver, fat, kidney, muscle and milk are abbreviated as L, F, K, M and Mk.

Table 2.3: Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from milk

Residues	Extraction	Clean-up	Recovery (%)	Ref
ABZ, ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$, FBZ, OFZ, FBZ-SO$_2$, FBZ-OH, MBZ, OXI	Ethyl acetate at pH 10		79-100	44
ABZ-NH$_2$-SO$_2$	Acid Hydrolysis (H$_3$PO$_4$) Acetonitrile (pH 5-7)	SCX SPE	>91	83
FBZ, OFZ, TBZ, 5-OH-TBZ	Ethyl acetate at alkaline pH	LLP Silica SPE	>80 56 (5-OH-TBZ)	48
ABZ, FBZ, OFZ, FBZ-SO$_2$, FBZ-OH, MBZ, TBZ	MSPD	MSPD	70-107	16
ABZ, ABZ-SO, ABZ-SO$_2$	Ethyl acetate at alkaline pH	LLP	78-100	14
ABZ, FBZ, TBZ, OXI, LEV	Acetonitrile-ethyl acetate	LLP	68-85	121
ABZ, FEB, FBZ, OFZ, OXI, TBZ, TCB, LEV	Ethyl acetate at alkaline pH		79-110	122
FBZ, OFZ	None	Diluted 1:10	95-122	51
ABZ, ABZ-SO, ABZ-SO$_2$, FEB, FBZ, OFZ, MBZ, OXI	Dilute buffer pH 11.0	C$_{18}$ SPE	81-96	50
TBZ, LEV	Defat Dilute buffer pH 6.5	C$_{18}$ SPE	81-89	48

Table 2.4: Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from crops

Residues	Matrix[1]	Extraction	Clean-up	Recovery (%)	Ref
BEN, MBC, 2-AB	F, V	Ethyl acetate	LLP	71-92	26
TBZ, MBC	F, V	LLE (Ethyl acetate vs 0.1N HCl)	LLP	91-97	138
BEN, TBZ	F, V	Reflux with Ethyl acetate at alkaline pH		73-109	115
BEN, MBC, 2-AB	F, V	Benzene at alkaline pH	LLP	70-90	136
BEN, MBC, TBZ	F, V	Reflux in 2N HCl Chloroform at pH 9.5			95
BEN, MBC, TBZ	F, V	Methanol	LLP	64-105	62
BEN, MBC, TBZ	F	LLE (Chloroform vs Aqueous NaCl)	LLP	>90	110
BEN, MBC, TBZ	F, V	Acetone	LLP, Normal Phase	48-86	104
BEN, MBC, TBZ	F, V	Acetone	Extrelut and SCX SPE	>80	63
MBC, TBZ	Fruit Juice	Dilution	Oasis® MCX	74, 97	108
BEN, MBC, T-M	F, V	SF-CO$_2$	C$_{18}$ Trap	85-93	141

[1]Fruit and vegetables are abbreviated as F and V, respectively.

2.10 Methods for the Determination of Benzimidazole Residues

Early work on benzimidazole analysis was carried out using spectrometric methods whereas today they are analysed by a variety of analytical techniques. Techniques such as mass spectroscopy, chromatographic separations, thin layer chromatography (TLC), bioassays in tandem with TLC and immunochemical methods have found widespread application in the screening of residues. Gas chromatography has also been used for the analysis of benzimidazole fungicides in plant based matrices but has not been used widely for the determination of veterinary drugs. The most widely used procedure for the determination of these veterinary drugs is liquid chromatography coupled to UV, fluorescence or mass spectrometry detectors.

Bioassays

There have been few applications of bioassays for the determination of benzimidazoles in a biological matrice. Erwin *et al.*[100] developed a bioassay system for the determination of benomyl. Later Petersen and Edginton [101] developed a more sensitive assay, allowing detection of benomyl and carbendazim in plant based materials. Benomyl and carbendazim residues were extracted and separated on a silica gel TLC plate, which was later sprayed with a combination of agar and *Penicillium* spores and incubated at room temperature for 20 h. Benomyl residues were indicated by 2 spots of inhibited fungal growth. The size of the zone of inhibition was related to the concentration of the fungicide. The method was 10 times more sensitive than UV detection. A multiwell screening assay for TBZ, ABZ, ABZ-SO, ABZ-SO$_2$ and MBZ using the larvae of Ascaris suum by Dew *et al.,* [102] showed the method could be used to screen for anthelmintics in edible tissue.

Spectrometric methods

Pease *et al.,* [103] determined benomyl, after conversion to 2-aminobenzimidazole, by fluorescent spectroscopy (λex 285 nm and λem 335 nm) or, after derivatisation with bromine, by colorimetric detection at 445 nm. The limit of detection was less than 100 μg kg^{-1}. The three benzimidazole fungicides, 2-aminobenzimidazole, TBZ and 2-(2-furyl)-benzimidazole all fluoresce naturally; however only 2-aminobenzimidazole reacts with bromine to produce coloured derivatives. As a result, colorimetric detection after bromination is a more selective method for the determination of 2-aminobenzimidazole. Aharonson *et al.,*[104] similarly determined benomyl and TBZ residues in crops by fluorescence spectroscopy after selective clean-up by column chromatography. MBZ and FLU were determined in pharmaceutical preparations by fluorescence (λex 300 nm and λem 400 nm) after reaction with 0.3% hydrogen peroxide in alkaline solution by Baeyens *et al.* [105] This group showed that the assay could be applied also to tetramisole, dexamisole and levamisole. A novel sorbent extraction procedure was developed by Capitan-Valvey *et al.,* [69] for the determination of TBZ in fruit. Samples were extracted using aqueous buffer before dilution of the extract in water and mixing with Sephadex G-15 gel beads. The Sephadex gel beads were isolated by filtration and packed into a glass cuvette, before determination using a luminescence spectrometer. The performance of the method developed by Barker *et al.,* [106] was similar to HPLC spectrometric detection systems.

Thin Layer Chromatography

TBZ in citrus fruits and carbendazim in crops were determined using a polyamide plastic TLC plate coated by a fluorescent indicator by Norman *et al.,* [107] and White *et al.,*[108] The plates were developed using a solvent of chloroform + ethyl acetate +acetic acid and visualised under UV light at 254 nm.

High Performance Thin Layer Chromatography (HPTLC) was investigated by

Corti *et al.*, [109] for the determination of TBZ and other residues in potatoes. C_8 plates were used with a mobile phase of methanol:water (10:3,v/v) and determination was achieved using a scanning densitometer at 248 nm. This technique was compared with HPLC but the latter was more sensitive. The same group later developed a method for the determination of benomyl, carbendazim and TBZ in fruit[110]. Residues were separated on NH_2 HPTLC plates (derivatised with polyamine) using a solvent of chloroform + cyclohexane + methanol (6 + 1 + 0.1, v/v/v) to elute carbendazim and TBZ. Residues were detected using a scanning densitometer at 285 nm. More recently, Abjean *et al.*,[73] developed a TLC screening method for the detection of ABZ-NH_2-SO_2 in liver. ABZ-NH_2-SO_2 was separated using a mobile phase of acetonitrile + ammonium hydroxide (10 + 0.6, v/v) on a silica TLC plate coated with a fluorescent indicator. The method was used for screening purposes only, based on a negative/positive result.

Immunochemical methods

A radioimmunoassay for the determination of OFZ in plasma where the polyclonal antibodies were raised in rabbits after immunisation with OFZ coupled to a polylysine carrier was developed by Nerenbery *et al.* [111]Brandon *et al.*,[24] developed a competitive ELISA for the determination of TBZ in liver, using a monoclonal antibody raised in mouse. Haptens of TBZ and 5-OH-TBZ were prepared and coupled to the carrier bovine serum albumin (BSA) for use as immunogens. It was found that antibodies raised after immunisation with the conjugate prepared from TBZ were more specific to TBZ and 5-OH-TBZ. While antibodies raised from the conjugates prepared with 5-OH-TBZ were found to be less specific and showed good cross-reactivity to CAM and TBZ-NH_2. Cross-reactivity to benzimidazoles not possessing a thiazolyl ring was also evaluated, but none of these residues (namely ABZ, MBZ and FBZ) showed significant cross-reactivity. Competitive ELISAs were developed based on the horseradish peroxidase (HRP) conjugate and were applied to liver samples extracted using water. This group later applied this ELISA for the determination of TBZ residues in fruit, vegetables and fruit juices.[113] It was found that fruit juice samples could simply be diluted in buffer prior to determination by ELISA.

Bushway et al., [114] evaluated a commercially available enzyme immunoassay tube kit for the determination of TBZ residues in processed/unprocessed fruit-vegetables. The polyclonal antibody used in the assay was raised against benomyl-carbendazim, but the antibody demonstrated sufficient cross-reactivity toward TBZ for its sensitive detection in food. Newsome et al., [115] developed ELISAs for the determination of benomyl and TBZ in crops, using polyclonal antibodies raised in rabbits. Immunogens were prepared by coupling the carrier human serum albumin (HSA) to the succinamide haptens of TBZ and carbendazim (the degradant of benomyl), and were used in rabbits for raising antisera. The cross-reactivity of the antibodies was evaluated for different benzimidazole residues but was shown only to inhibit the ELISAs at higher concentration levels.

Brandon et al., [116] produced a mouse monoclonal antibody that showed good cross-reactivity for 11 benzimidazole residues including ABZ, FBZ, OXI, MBZ, FLU, carbendazim and some metabolites. The antibody did not show cross reactivity to thiazolylbenzimidazoles (TBZ, CAM and 5-OH-TBZ). Antibodies were raised in mouse using conjugates prepared after coupling the succinamide hapten of ABZ to BSA carrier protein. This group developed a competitive ELISA HRP for the determination of benzimidazole residues in bovine liver samples after aqueous extraction. This group later developed a method for the determination of FBZ residues in milk using this ELISA step [51]. Milk samples were simply diluted in PBS-Tween-BSA before determination by ELISA.

Abad et al., [117] produced monoclonal antibodies raised against a TBZ-ovalalbumin conjugate. This group indicated that this was a very novel conjugate because it was prepared from a hapten functionalised at the nitrogen atom of the 1-position of the TBZ structure. This group developed an indirect ELISA based on HRP for the determination of TBZ residues in fruit juices. Moran et al., [118] produced mouse monoclonal antibodies raised against the novel immunogen 5-benzimidazole-carboxylic acid conjugated to the lipopeptide $Pam_3Cyst-T_H$. The antibodies were produced for the development of ELISAs

for the determination of protein bound residues of TBZ in tissue, although no applications were described.

Liquid Chromatographic separations

The development of chromatography methods for a range of benzimidazoles is difficult due to the wide range of polarities within the group and between groups of the benzimidazole drugs. The majority of LC methods for the determination of benzimidazoles have been developed using reverse phase systems and ion exchange systems. Other types of LC method columns that have been used but to a much lesser extent are silica or cation exchange. A normal phase separation was developed for separation of TCB, TCB-SO, TCB-SO$_2$ and OFZ on a silica column by Alvinere *et al.*, [57] The determination of MBZ in plasma using a silica column was used by Karlaganis *et al.* [87] Cation exchange columns were used by Kirkland *et al.*, [26] for the determination of benomyl residues in plants. Arenas *et al.*, [9] determined TBZ and 5-OH TBZ in milk. The use of polymeric columns crops when compared with silica by Hiemstra *et al.*, [64] for the analysis of TBZ and carbendazim residues in crops gave better peak shape and a longer column lifetime than the silica based columns.

Isocratic separations of benzimidazoles have given rise to long run times and only small numbers of residues can be determined due to the wide range of polarity of these drugs. An isocratic separation of eight benzimidazoles on a C$_{18}$ column in 14 min gave poor resolution for the substances. [52] The separation of FBZ and TCB metabolites was accomplished in 12 minutes but a runtime of 40 minutes was necessary if included TCB. [17] Wilson *et al.*. [11] and Long *et al.*, [18] developed a multi-residue LC methods for the determination of 7 and 8 benzimidazoles in less than 13 and 35 minutes. Gradient elution of the residues allows for the inclusion of more residues and improved resolution. Studies were carried out comparing isocratic and gradient systems for the determination of 5 benzimidazoles in plasma.[89] Sorenson *et al.*, [50] developed a gradient separation of 8 benzimidazoles in a 40 minute run with an added equilibration time. A C$_8$ column was used in the separation of OFZ and its 9 metabolites with a binary gradient in less that 35

minutes, [22] this column was also used in the separation of levamisole (LEV) and 8 benzimidazoles with gradient elution. [75]

A base deactivated C_{18} column was used to separate 10 benzimidazoles with a binary gradient; all the residues were separated in less than 16 min except OXI and FLU. [33] Rouan et al., [92] separated TCB, TCB-SO, TCB-SO_2 and 5,6-dihydro-11-oxo-11H-dibenz [b,e] aze-pine-5-carboxamide which was used as internal standard on a short C_{18} column (33 × 4.6 mm) in less than 10 min with detection by UV. Narrow bore columns (2.1 mm) have been evaluated (2.1 mm) for the determination of TBZ, MBZ, ABZ and FBZ residues. [119] Results showed that the peak heights for the analytes increased by a factor of 5 using narrow bore columns compared to columns of a standard diameter (4.6 mm). Isocratic separations were developed by Kan et al., [12] and Hajee et al., [13] on intermediate diameter (3 mm) columns for FLU and MBZ metabolites.

Domany et al.,[35] developed an isocratic separation of 10 benzimidazoles on a narrow bore (2.1 mm) C_{18} column. Shaikh et al., [29] determined ABZ and its 3 major metabolites using 2 separate isocratic systems and found that the metabolites could be eluted in less than 20 mins. An Xterra C_{18} column using gradient elution to separate 14 benzimidazole residues was carried out by Danaher et al. [120]

Ion pair chromatography was investigated for the determination of TCB-SO and TCB-SO_2 in plasma and urine. [94] The optimum mobile phase consisted of 0.05 M phosphate buffer, pH 7:acetonitrile (55:45,v/v) containing 0.001 M sodium decanesulphonate when the effects of pH, organic modifier content and chain length of sodium alkyl sulphonate ion pair reagent were evaluated.

The separation of FBZ and TBZ, ABZ and OXI by ion pairing chromatography was achieved using sodium 1- octane sulphonate with adjustment of mobile phase buffer to pH 3 and gradient elution using buffer, acetonitrile and water. [121]

The LC method was also used for LEV but with adjustment of mobile phase buffer solution to pH 3 and some ion pairing reagent. Gradient elution of LEV is accomplished using this buffer, methanol and water. [121] Other ion pairing studies investigated the effect of ion-pair reagents and pH on the retention time and peak height of FBZ and OFZ. [19] These studies showed that decreasing the pH to 2.2 shortened the retention time of FBZ

sharply and OFZ slightly. Over pH of 3.7 to 6.5 no change in retention time was observed with or without the addition of ion pair reagents. The addition of octane sulphonate anion increased retention time and tetrabutyl ammonium cation decreased retention time. The peak shape for FBZ was poor over the range of pH investigated in the absence of ion pair reagents, but addition of ion pair reagents greatly enhances peak shape at pH 2.2 even at the longer retention times. This group developed separations for FBZ residues using ion-pair chromatography. A multi-residue method was later developed for the separation of 10 benzimidazoles in milk in less than 30 min, using isocratic mobile phase containing 0.01 M pentane sulphonate and 0.5% triethylamine at pH 3.5.

In the area of LC-MS, most groups carry out separations using shorter columns (less than 150 mm in length). These columns require shorter equilibrium times during gradient chromatography because of the lower column volume, which increases LC-MS throughput. Blanchflower et al., [25] developed an isocratic separation of FBZ and OFZ, finding it gave more reproducible MS results than gradient separation. Cannavan et al., [37] developed a gradient method for the separation of TBZ and 5-OH-TBZ, using a mobile phase consisting of acetonitrile and 0.1M ammonium acetate. Balizs et al., [30] developed a method for the determination of 15 benzimidazole residues by LC-MS/MS with a gradient separation carried out on a narrow bore column (2.1 mm). The mobile phase flow rate ranged between 0.04 and 0.2 μl min^{-1} depending on the ionisation interface used. Substances were eluted from the column in less than 6 min but were not resolved. It was claimed that, because individual benzimidazoles have different molecular masses chromatographic separation was unnecessary. De Ruyck et al., [39] developed an LC-MS/MS method for the determination of benzimidazole residues using a column of similar dimensions to Balizs et al., [30] for the determination of FLU, FLU-HMET and FLU-RMET in eggs and muscle tissue. A method was later developed by the same group for the determination of levamisole and 7 benzimidazole residues in milk. [122]

A number of groups have developed LC methods for the determination of benzimidazoles in milk, tissue and crops using UV, fluorescence and mass spectrometric detection (Table 2.5). Benzimidazoles possess a strong UV chromophore and may be determined using LC-UV. However, it has been observed that certain benzimidazoles (namely ABZ, TBZ, CAM and their metabolites) possess naturally fluorescing chromophores, making them suitable for detection by LC fluorescence. Fluorescence is more sensitive and selective, but does not have the same range of applicability as UV detection. As a result, UV is the most widely used system for the detection of benzimidazole residues in extracts of biological matrices. LC with MS detection is becoming more widely used for the determination of benzimidazole residues in biological matrices, offering the capability of combined quantitative-confirmatory analysis.

Table 2.5:   Summary of detection methods used in LC for the determination of benzimidazoles

Detector	Derivitisation agent	Ref
UV	-	52,13,12,93,18,36,123,120,42,48,50
UV	Peracetic acid	40
UV, MS	P-nitrobenzyl bromide in presence of potassium carbonate	59
MS	-	25,37,30,21,126,39,122,39,127,128,99,78,129
FL	-	9,8,29
UV-FL	-	11,74,43,80,64,125
EC	-	86,126

Note: UV wavelengths ranged from 220 nm -318 nm

Detection by UV

Bogan *et al.*, [52] developed a method for the determination of eight benzimidazoles in body fluids. MBZ residues were determined in plasma at 313 nm [85] and eel muscle at 254 nm. [28] Karlanganis *et al.*, [87] determined MBZ in plasma demonstrating that there was less interference at a wavelength of 307 nm compared to 247 nm. Hajee *et al.*,[13] determined MBZ, MBZ-NH$_2$ and MBZ-OH in eel muscle at 289 nm. Kan *et al.*, [12] developed a

method for the determination of FLU and its metabolites in eggs using a wavelength of 250 nm. Chiap et al.,[93] determined ABZ, ABZ-SO and ABZ-SO$_2$ in plasma at 295 nm. UV photodiode array (PDA) detection has been used to determine 7 benzimidazole residues by Long et al.[18] This mode of detection was investigated as a confirmatory tool, but differences in spectra at lower and higher concentration levels were found and the PDA detector was not suitable for confirmatory analysis at concentrations lower that 5 ng on column. This group also developed methodology for the determination of these residues in milk and animal tissue. Neri et al.,[36] determined 8 benzimidazoles residues in tissue and De Buyanski et al.,[123] determined TBZ and LEV in tissue. Danaher et al.,[120] developed a method for the determination of 14 benzimidazole residues in liver tissue. FBZ, OFZ, TBZ and 5-OH TBZ were monitored similarly using the dual wavelength approach in milk.[42] TBZ and 5-OH TBZ were monitored at 318 nm whereas FBZ and OFZ residues were monitored at 298 nm. Similarly, Sorensen et al.,[48] determined TBZ and levamisole residues in milk. This group also developed a method for the determination of 8 other benzimidazoles in milk (ABZ, ABZ-SO, ABZ-SO$_2$, OFZ, OXI, MBZ, FBZ and FEB).[50]

Most derivatisation carried out in the determination of benzimidazoles has been for GC applications and only two papers on derivatisation for LC have been published. The conversion of FBZ and OFZ residues to FBZ-SO$_2$ was determined and accomplished by Capece et al. [40] Residues were derivatised by reaction with peracetic acid and purified by liquid partitioning prior to determination by LC-UV. The presence of TBZ residues was confirmed by derivatising with p-nitrobenzyl bromide in the presence of potassium carbonate (110 °C, 3 h) with LC-UV detection. [59] This derivatisation procedure was used to provide confirmatory analysis of TBZ residues in suspect samples and for quantitiation.

Fluorescence detection

The determination of TBZ and 5-OH TBZ in milk can be carried out using fluorescence detection with λ_{ex} 305 and 318 nm and λ_{em} 380 and 515 nm.[9] In order to achieve a more sensitive detection, two chromatographic runs were required. It was determined that in a

single run these residues could be analysed at their optimum wavelength using a dual wavelength monochromator. Markus *et al.,* [8] used fluorescence detection for the determination of ABZ-NH$_2$-SO$_2$ in bovine liver (λex 300 nm and λem 320 nm). Shaikh *et al.,*[29] determined ABZ, ABZ-SO, ABZ-SO2 and ABZ-NH2-SO2 and three metabolites in fish muscle using fluorescence detection with λex 290 nm and λem 330 nm.

UV and fluorescence detection in series

There are numerous advantages of coupling UV and fluorescent detectors in series such as, an improved sample throughput and reproducibility, increased recovery and provide selectivity. It was determined that extracts needed further purification on a C$_2$ SPE cartridge to remove matrix components that interfere in the UV detection of 2NH$_2$ABZSO$_2$ and 5-OH TBZ.[11] However, the influence of this matrix interference may be removed using fluorescence detection. The determination of ABZ-SO2, OFZ, CAM and MBZ using fluorescence and UV detectors in series was accomplished by Farrington *et al.*[74] Constantinou *et al.,*[43] determined 5 benzimidazole residues in milk using a similar approach. TBZ and ABZ residues were monitored using fluorescence (λex 312 nm, λem 355 nm) while other residues were detected at 290 nm.

Fluorescence detection and PDA detection was used to monitor for TBZ and MBZ in tissue by Le Boulaire *et al.,*[80] and it was found that fluorescence detection was 22 times more sensitive than PDA for TBZ. The determination of carbendazim residues in crops using UV (280 nm) and fluorescence (λex 235 nm and λem 280 nm) was performed and confirmation was made by comparison of relative UV spectra and fluorescent responses of standards.[64] TBZ, carbendazim and thiophanate-methyl were determined by UV at 285 nm while TBZ and carbendazim were determined also by LC with fluorescence detection (λex 305 nm, λem 345 and λex 282 nm, λem 307 nm respectively).[125] It was determined that matrix interferences were less with fluorescence detection and that TBZ detection was enhanced by this mode of detection. Detection of carbendazim was similar with both detectors.

Electrochemical detection

The determination of MBZ and MBZ-OH residues in biological fluids by electrochemical detection was accomplished by Oosterhuis *et al.*,[86] with a carbon paste electrode and a saturated calomel electrode as the working and reference electrode respectively. Flubendazole acted as internal standard for MBZ since has similar oxidative properties to MBZ. A method for the determination of a metabolite of carbendazim in urine by LC with electrochemical detection was developed by Leenheers *et al.*[126]

Mass spectrometry detection

Mass spectrometry detection has been widely used in the determination of benzimidazoles. LC-MS with a thermospray interface has been used for the determination of FBZ and OFZ residues [25] and TBZ and TBZ-OH residues.[37] FBZ, OFZ, TBZ and 5-OH TBZ were determined by selective ion monitoring as their [M+H+] ions. The presence of TBZ and 5-OH TBZ was confirmed using LC-MS with atmospheric chemical ionisation (APCI) interface. LC-MS/MS has been investigated using ion-spray and turbo-spray interfaces in the positive mode by Baliz *et al.*[30] The cross talk effects were evaluated and were approximately 10%; however, a 20% effect was observed for FBZ-SO_2 which was caused by OFZ.

The use of LC-MS/MS with electrospray ionisation interface (ESI) was evaluated by Takeba *et al.*,[21] monitoring the compounds as their [M-M]⁻ ions. Young *et al.*,[126] determined TBZ and carbendazim residues in fruit juices using LC-MS with ESI in the positive mode with [M+H]⁺ ions. Ruyck *et al.*,[39] developed an LC-MS/MS-ESI method for the determination of FLU residues. Residues were monitored as their [M+H]⁺ ions and confirmed using multiple reaction monitoring (MRM). The same group later developed a multi-residue method based on the LC-MS-MS-ESI for the determination of 8 anthelmintic drugs (LEV, TBZ, OFZ, FBZ, OXI, ABZ FEB and TCB) in milk.[122] Residues were monitored as their [M+H]⁺ ions and few additional daughter peaks were

generated using multiple reaction monitoring (MRM) for quantitative and confirmatory purposes. The method, however, was not applicable for netobimin and ABZ-SO. The same group also reported the optimisation of quantitative confirmatory assay by LC-MS/MS for the determination of MBZ, MBZ-OH and MBZ-NH$_2$ in sheep muscle, liver, kidney, liver and back fat. Atmospheric pressure electrospray ionisation in the positive ion mode (ESI$^+$) was applied.[39] Edder *et al.*,[127] analysed ABZ, ABZ-SO, ABZ-SO$_2$, TBZ, MBZ, FLU, OFZ, FBZ, LEV, TCB, OXI and 5-OH TBZ in meat, liver and fish matrices by LCMS/MS using positive electrospray and MRM mode.

The effect of different calibration procedures for the determination of benzimidazole fungicides in plant matrices was evaluated by Zrostlikova *et al.*[128] The calibrations were carried out using standards in solvent, spiked control samples and a technique called echo-peak internal calibration. The most accurate results were achieved using spiked controls. Curves that were produced from standards in organic solvent showed a tendency to over or under estimate the result due to either ion suppression or matrix enhancement. The echo peak method gave accurate results for 6 out of the 8 benzimidazoles. This group developed a method for the determination of fungicides in fruit juices using LC-MS systems with an ESI interface in the positive ion mode and an ion trap detector.[99] The recovery of FLU, FLU-HMET and FLU-RMET in eggs and poultry muscle using LC-MS/MS was also developed.[78] The compounds were measured by MS/MS transition of the molecular ion of the most abundant daughter ion. The same group also evaluated the disposition of residues of flubendazole in turkeys and guinea fowl. Atmospheric pressure electrospray ionisation in the positive mode (ES+) was applied. The compounds were detected by tandem mass spectroscopy using multiple reaction monitoring (MRM) functions of the two transitions.[129]

Gas chromatography

GC determination of benzimidazoles is difficult because of the basic nature and low volatility of these substances. However, TBZ and TCB are sufficiently volatile to allow their determination by GC without derivatisation. The main applications of GC methods have been in determining benzimidazole fungicides in crops. Some researchers have

found GC coupled to mass spectrometery useful for confirmation of the presence of benzimidazole residues. however, GC-MS procedures usually require derivatisation (see Table 2.6) of residues to induce volatility and allow the generation of suitable MS spectra for confirmatory analysis and, as a result, have been largely replaced by LC-MS/MS. There has been little work reported on the analysis of benzimidazoles by GC due to their low volatility. However, TBZ and TCB are sufficiently volatile to be determined by GC with nitrogen phosphorus detection (NPD) and electron capture detection (ECD) without derivatisation. NPD was found to be more sensitive than ECD.[67] The separation of TBZ was carried out on a fused silica capillary column (25 m x 0.2 mm) by Oishi *et al.*,[98] without derivatisation by GC-NPD. Polar amino or hydroxyl groups are reacted with alkylating/acylating reagents in the derivatisation of benzimidazoles for GC. Some groups also hydrolyse the carbamate functional group to form an amino functionality prior to reaction with alkyl/acylating reagent. Alternatively, residues may be derivatised directly without hydrolysis. Different functional groups form simple methyl esters to more complex pentafluorobenzyl groups. TBZ residues were derivatised with trimethylsilyl acetamide for analysis by GC-MS [130] and this method of derivatisation was also used in the determination of CAM and three urinary metabolites.[131,132]

Table 2.6: Summary of derivatisation reagents and detectors used in GC in the determination of benzimidazoles

Detector	Derivitisation agent	Ref
NPD, ECD	-	67
NPD	-	98
NPD	Acetic anhydride	136
FID	Dimethylformamide and dinethyl acetate	135
ECD, MS	Pentafluorobenzyl-bromide	68,137,138,139
ECD,NPD, MS	Pentafluorobenzyl-bromide and methyl iodide	
MS	Trimethylsilyl acetamide	130,131,132,133,134
MS, HRMS	Pentafluorobenzyl-bromide and triethylammonium hydroxide in methanol Hydrolysis with 2 N HCL followed by reaction with N-methyl-N- (t-butyldimethylsilyl) trifluoroacetamide (MTBSTFA)	140

An on-column methylation procedure for the confirmation of CAM residues by GC-MS-EI (electron impact mass spectrometry) in selective ion monitoring was developed by

VandenHeuvel et al.[133] This group later applied this method for the determination of TBZ and 5-OHTBZ.[134] A column with a polysulfone stationary phase was used because of background bleed and its adsorption characteristics were the most suitable of all columns evaluated. Methyl derivatives of TBZ were determined by GC with flame ionisation detection (FID) by reacting with dimethylformamide and dimethyl acetate in acetonitrile (120 °C, 40 min).[135]

TBZ was acylated by reaction with pentafluorobenzyl-bromide PFB-Br (120 °C, 30 min) before analysis by GC-ECD and confirmation by GC-MS-EI in full scan mode.[68] Benomyl residues were derivatised with acetic anhydride (100 °C, 30 min) by capillary GC-NPD by Physalo et al. [136] This technique was evaluated but found to be 10 times less sensitive. Bardalaye et al.,[137] later modified this procedure and applied it to the determination of TBZ residues. TBZ and cabendazim were determined in fruit using GC-ECD after derivatisation with PFB-BR.[138] This method was applied to the analysis of benomyl also, which is converted to carbendazim during the analysis. A confirmatory method was developed by this group using GC-MS-EI. This method was modified by Cline et al.,[139] for the determination of carbendazim in fruit treated with benomyl.

A range of derivatisation procedures were evaluated for GC determination of 8 benzimidazoles in animal tissue for substances with amino groups. It was discovered that acylation with PFB-BR and methylation with methyl iodide were suitable and were determined by GC-ECD and GC-NPD but derivatives were unstable due to the extreme temperatures used during injection. [23] GC-MS EI and GC-MSPICI (+ ion chemical ionisation) gave useful structural information and positive ion chemical ionisation provided a number of ions suitable for confirmatory analysis. Electron impact spectra of methyl derivatives produced fewer ions than the pentafluorobenzyl derivatives. Negative ion chemical ionisation was also evaluated and was shown to produce 1 or 2 ions for most residues. GC-MS EI in SIM mode was used for confirmation of benzimidazole residues in tissue and residues were derivatised firstly involving hydrolysis with 2 N HCL (110 °C, 1 h) to convert the carbamate group to an amino functionality. Hydrolysed residues were then reacted with N-methyl-N- (t-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), converting the primary and secondary amines to silyl amine. The phenol functional group of 5-OH TBZ was converted to a silyl ether and this method used a flash

alkylation procedure, in the GC injection port (260 °C), after injection for suitable applicability to CAM. This technique was used for the confirmation of ABZ-NH$_2$-SO$_2$ based on this derivatisation procedure with determination by GC-MS-EI.

The determination of TBZ and ABZ residues in tissue using capillary GC with high resolution mass spectrometry (HRMS) after derivatisation with PFB-BR was developed.[140] This procedure was later used by Anastassiades et al.,[141] for the confirmation of benzimidazole fungicides in crops after SFE. The determination of TBZ in fruit juices using capillary GC-NPD without derivatisation was carried out by Oishi et al.[98]

Jacob et al.,[130] determined TBZ residues by GC-MS after derivatisation with bis trimethylsilyl acetamide. This group later applied the same method to the determination of CAM and three urinary metabolites.[131,132] VandenHeuvel et al.,[133] developed an on-column methylation procedure for the confirmation of CAM residues by GC-MS-EI (electron impact mass spectrometry) in selective ion monitoring mode (SIM). Residues were dissolved in 0.125 M triethylammonium hydroxide in methanol just prior to injection into the GC-MS system. This method was later applied for the determination of TBZ and 5-OH-TBZ.[134] A column with a polysulfone stationary phase was employed because its background bleed and adsorption characteristics were more desirable than others tested. Tanaka and Fujimoto [135] determined the methyl derivative of TBZ by GC with flame ionisation detection (FID). Derivatives were prepared by reacting with dimethylformamide (DMF) and dimethyl acetate in acetonitrile (120°C, 40 min). The procedure was used to determine TBZ residues in fruit with a limit of detection of 100 µg kg^{-1}. Nose et al.,[68] acylated TBZ by reaction with pentafluorobenzyl-bromide PFB-Br (120°C, 30 min) before determination by GC-ECD and confirmation by GC-MS-EI in full scan mode. Physalo et al.,[136] derivatised benomyl residues with acetic anhydride (100°C, 30 min) before determination by capillary GC-NPD. GC-ECD was also evaluated for determining residues but was 10 times less sensitive. Bardalaye et al.,[137] later modified this procedure and applied it to the determination of TBZ in fruits. Tjan et al.,[138] determined TBZ and carbendazim in fruit using GC-ECD after derivatisation with PFB-Br. The procedure was also applicable to benomyl, which is converted to carbendazim

during the extraction and clean-up procedure. A confirmatory procedure was also developed by Tjan et al.,[138] using GC-MS-EI. Cline et al.,[139] later modified this procedure and applied it to the determination of carbendazim in fruit treated with benomyl.

Marti et al.,[23] developed GC methods for the determination of eight benzimidazoles in animal tissue, evaluating a range of derivatisation procedures used for substances with amino functional groups. They found that acylation with PFB-Br and methylation with methyl iodide were suitable. Derivatised extracts were determined by GC-NPD and GC-ECD; GC-NPD chromatograms were found to show less matrix interference. Derivatives were found to decompose during injection and chromatography because of the high temperature used, making quantitative determination impossible. However, GC-MS-EI and GC-MS-PICI (positive ion chemical ionisation) provided useful structural information. Electron impact spectra of the methyl derivatives produced fewer ions than the pentafluorobenzyl derivatives. Similarly, positive ion chemical ionisation provided a number of ions suitable for confirmatory analysis, while negative ion chemical ionisation produced only one or two ions for most residues.

Wilson et al.,[11] used capillary GC-MS-EI in SIM mode for the confirmation of benzimidazole residues in tissue. Residues were derivatised in a two-step procedure, firstly involving hydrolysis with 2N HCl (110°C, 1 h) to convert the carbamate group to an amino functionality. Hydrolysed residues were reacted with N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), converting the primary and secondary amines to a silyl amine. In the case of 5-OH-TBZ, the phenol functional group was converted to a silyl ether. The method was not applicable to CAM, which was alternatively derivatised using flash alkylation in the GC injection port (260°C) after injection in triethylammonium hydroxide in methanol. Markus et al.,[140] developed a method for the confirmation of ABZ-NH$_2$-SO$_2$ in tissue based on this derivatisation procedure, with determination by GC-MS-EI.

2.11 Conclusions

The benzimidazole drugs as a whole are a complicated group to develop a single surveillance method. The common methodology involves liquid extraction at a high pH followed by liquid partitioning alone or in tandem with solid phase extraction. This methodology allows for the broadest number of residues to be included in a multi-residue method when used in conjunction with LC-UV and LC-MS/MS. Balizs has developed the most complete method, allowing the determination of 15 benzimidazole residues in milk and liver with detection by LC-MS/MS. [30] The methods developed by Roudaut et al.,[33] Domany et al.,[35], Fletouris et al.,[44], Danaher et al.,[120] and Edder et al.,[127] allow 10 to 13 residues to be monitored.

To date, however, there is no method available that can screen for all the benzimidazole metabolites. The development of such a method could be simplified by a single step derivatisation technique. A method by Capece et al.,[40] used peracetic acid, which converted benzimidazoles in the sulphide/sulphoxide form to the sulphone. Extension of the number of benzimidazoles that could be derivatised should be investigated in future work.

Another approach would be to target the most persistant or prevalent members of each licensed group as shown in depletion tissue studies. More depletion work needs to be carried out in milk as work on this tissue is not as comprehensive as the other tissues. Also there appears to be no depletion data available in milk for OXI and CAM. Adopting either of these approaches reduces the number of residues hence simplifying analysis and could allow for the surveillance of all the groups licensed in the MRL listings.

There is also a lack of multi-residue methods, which target the TCB, FLU and MBZ licensed groups in conjunction with other residues. Since these drugs are widely used in veterinary medicine there is a need to develop such multi-residue methods.

There also appears to be limited depletion data on benzimidazoles in fish even though there are single residue methods for ABZ [29], MBZ [28,31,13], FBZ [20] and a multi-residue method developed by Edder et al.,[127] for the determination of ABZ, ABZ-SO2, ABZ-SO,

TBZ, MBZ, FLU, OFZ, FBZ, LEV, TCB and OXI by LC-MS/MS. Potential environmental contamination may arise, *e.g.*, from commercial fish farming where these compounds are given as feed additives, can accumulate in sediments and may be ingested by marine species in the vicinity of farms. Since substantial amounts of medicated feed is not eaten and can pass through the holding cages, it can be absorbed by marine flora and fauna.

Another consideration is, drugs used on farms for veterinary purposes will end up eventually in manure. Manure is eventually dispersed in fields and the mobility of drugs and drug metabolites in the soil system predicts if the drugs will be a threat to ground water.

The presence of drug residues therefore may pose a health risk, both directly and indirectly so further study is required on the environmental effects of administration of not only benzimidazole drugs but all veterinary drugs used in animal husbandry.

Since the benzimidazole class is one of the most widely used classes of anthelmintic drugs it is important to continue with the development of screening, validation or confirmatory methods in the areas where shortfalls have been discussed above.

Fundamental to this work is the development of a multi-residue method capable of screening for the seven benzimidazole licensed groups and subsequent availability of this method for implementation into the National Monitoring Plan in Ireland. Bovine liver was selected as a model matrix in which method development studies were based. LC-UV methodology was chosen over LC-MS/MS technology since this technique is more amenable to residues laboratories due to the high costs involved in LC-MS/MS analysis. A carbonate buffer/ethyl acetate extraction system employed by Wilson et al., [11] and generally used in the extraction of benzimidazoles from liver was used in method development. The introduction of automated solid phase extraction using the ASPEC XL4 for benzimidazole metabolites identified as the most likely to occur as a result of tissue depletion studies provides a faster more comprehensive screening method. This method has the potential to improve the analysis of the licensed benzimidazole drugs beyond current analysis.

The main aims of residue science are to develop methodology that is accurate, sensitive and has a high throughput. Therefore identification of consignments of contaminated animal products can be made quickly and decisions regarding isolation and disposal of these products can be made. This eliminates the hazards associated with drug residues entering the food chain and methods also provide a legal basis for prosecuting individuals who use veterinary products without adhering to EU legislation. There is also an obligation on each member state in the EU to put methods in place to monitor for benzimidazole residues. For these reasons, the development of a multi-residue method capable of monitoring for benzimidazole drugs is extremely important.

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Chapter 2: Section C

Review of current methodology for the determination
of malachite green and crystal violet residues

2.13   Introduction

Malachite green (MG) is a synthetic triphenylmethane dye. This compound has been used as a topical fungicide and antiprotozoal agent in salmon farming throughout the world for just over 60 years. Few known chemicals rival its effectiveness in treating external infections of fish and fish eggs by fungi of the genus *Saprolegnia* and by the protozoan parasite *Ichthyophthirius multifiliis.*[1] The occurrence of residues of MG is a universal problem. Currently monitoring is governed by national surveillance schemes established under Council Directive 96/23/EC. Since 1 st January 2000, the administration to food producing animals of veterinary medicinal products containing pharmacologically active substances which are not listed in Annex I or Annex II is prohibited. MG is not registered as a veterinary drug for use in food fish. Leucomalachite green (LMG) is the prevalent metabolite of MG and persists for a long time. LMG is stored in fat, and thus its elimination rate from fish muscle is strongly dependent on the fat content. Residues of MG and LMG have been reported in fish tissues, as well as in eggs and fry. Even though the use of MG in fish culture is not allowed under current European Union regulations, residues of MG and LMG in fish muscles are still being found in the residue monitoring schemes performed by EU Member States (EC Rapid Alerts 2003, 2004, 2005,2006). Studies carried out by Culp et al., [2] reports in general that LMG treatment resulted in a greater number of toxic effects and that the effects had greater severity. The presence of N-demethylated and N-oxidised MG and LMG metabolites including primary arylamines, was detected in the livers of treated rats. This suggested that both compounds were metabolised in a similar manner to carcinogenic aromatic amines. Data on the formation of DNA adducts showed that DNA adduct increased with increasing dose of each compound. As this process occurs in vivo, studies by this group found it was impossible to produce adducts in vitro. Therefore a more complete evaluation of the mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed MG was carried out. This study showed that the DNA adduct formed in the livers of rats fed LMG had little mutagenic or carcinogenic consequence. [3] Work carried out by Bose et al.,[4] studying the ability of MG to cause DNA damage, cell cycle arrest, apoptosis and possible roles of extracellular regulated kinases (ERKs), Jun-N-terminal kinases (JNK) and p38 kinases (α,β,γ). The

study reports that MG causes DNA damage, induces G2/M cell cycle arrest, apoptosis and causes elevated phosphorylation of ERK1 and JNK1 and no change in the phosphorylation of p38 kinase. A study by Stammati et al.,[5] was undertaken to ascertain the cytoxicity of MG and LMG using two distinct human cell lines. The first the HEp-2 cell line and the second is the Caco-2 cell line which are already used in food safety assessment. MG had a dose dependent toxic effect on Hep-2-cells. On the contary LMG gives only slight viability inhibition at the highest concentration tested. Increased signs of cytoxicity were found when Caco-2-cells were exposed to MG. By contrast no signs of cytotoxicity were ever noticed following cells exposure to LMG. LMG has also been shown to inhibit thyroid peroxidase, which is the enyme that affects the synthesis of thyroid hormone. [6] Other studies have shown that toxicity has been observed in some mammals, including organ damage, mutagenic, carcinogenic and developmental abnormalities. However, despite the large amount of data on its toxic effects residues are still being found worldwide. Toxicity is difficult to evaluate and to compare in different species of fish because they are influenced by various factors such as temperature, pH, water hardness and dissolved oxygen content of the water. Histopathological studies have revealed that MG causes detrimental effects in liver, gill, kidney, intestine, gonads and pituitary gonadotropic cells. [7]

MG has been used in aquaculture worldwide for the treatment of parasitic and fungal infections in fish and shellfish. Other dyes (such as crystal violet (CV)) exhibit similar antimicrobial properties and are widely marketed for the treatment of external infections in ornamental fish. None of these compounds are regulated for use in food fish production. MG is the primary focus of this review due to its toxicological properties and long history of use in aquaculture. However upon review of the literature for this contaminant it was noted that there is limited data available on the use of CV in fish. This is probably mainly due to the lack of analytical methodology available to determine this compound. There is a high probability that there is illegal use of this dye in domestic and foreign aquaculture and as a result of limited methodology there is not sufficient surveillance. Currently in Ireland there is no method implemented into the National Monitoring Plan for this compound and its metabolite leucocrystal violet (LCV). CV is a triphenylmethane dye but which has found use as a feed additive to inhibit mould and

fungal growth in poultry feed before 1990. [8] The metabolic profile for CV in mice, rat, hamster, guinea pig and chicken was the same in all animal species with the reduction of CV to LCV. [10, 11] Chicken detoxification of CV resulted in several demethylated metabolites in liver, thigh and breast muscle.[9] The fungicidal properties of this dye make it useful in treating infections in a variety of animal species. The therapeutic applications of dyes is well known but their use in aquaculture is highly restricted because of toxicological considerations. Therefore, the need arises to develop a multi-residue method to evaluate other dyes.

Each of these stages will be discussed with specific reference to the determination of MG and CV residues.

This review focuses on the main stages of a residue detection method; namely,

Sample extraction procedures

Clean-up techniques

Methods for determination

2.14   Sample Extraction Procedures

Allen *et al.,* [12] discovered that upon routine treatment of ethyl alcohol:formalin:acetic acid (85,10,5,v/v/v) to preserve fish, any fish exposed to MG residues would develop a bluish colour when this treatment was performed. This group exploited this sample pre-treatment step by measurement of this colour at the determination step. The determination was carried out using colorimetry with the measurement of absorbance of the sample extracts at 615 nm. The tissues are normally first homogenised, and then the residues are extracted using liquid extraction of the tissue at a low pH with organic solvent usually around pH 3-4 with acetonitrile. Scherpenisse *et al.,* [13] used McIlvaine buffer at pH 3:acetonitrile (1:6,v/v), para-toluenesulfonic acid, N,N,N',N'–tetramethyl-1,4-phenylenediamine dihydrochloride in the primary extraction of MG and LMG from pangasius, rainbow trout, salmon, tilapia and victoria perch tissue. The re-extraction of the sample pellet of each species was carried out with the following extraction solvent, McIllvain buffer at pH 6:acetonitrile (1:6, v/v). Valle *et al.,* [14] used this procedure to

extract these compounds from salmon muscle. Allen *et al.*, [15] used 1% acetic acid in acetonitrile to extract MG and LMG from the eggs and fry of rainbow trout. This group extracted rainbow trout muscle by firstly homogenising the tissue and mixing with sodium sulphate anhydrous. This extraction method was used by Mitrowska et al., [16] in the extraction of MG and LMG from carp except sodium acetate was used as the buffer instead of ammonium acetate. Doerge *et al.*, [17] used aqueous ammonium acetate containing hydroxylamine HCL and p-toluenesulfonic acid prior to the addition of acetonitrile to extract MG and LMG from catfish and trout. This group recognised that the demethylation of LMG can occur during sample preparation. Plakas *et al.*, [158] used acetonitrile to extract residues from catfish plasma. This group used an acetonitrile and acetate buffer mixture to extract residues from catfish muscle followed by re-extraction with acetonitrile. Under the low pH conditions of extraction, MG (pKa 6.9) exists primarily (> 99%) in its ionised, chromatic form. Alderman *et al.*, [19] extracted rainbow trout serum and bile tissues using a buffer at pH 4.0. Pentan-1-ol was added to the samples and the samples were subsequently extracted for 24 h. Liver, kidney, viscera and flank muscle tissues were blended with 2% pepsin and adjusted to pH 2 with HCl in distilled water. The blended samples were left for 18 h at room temperature (20 °C) and shaken at intervals, upon evaluation of the pH at extraction the pH of the mixture was at pH 4. This group found that re-adjustment to pH 4 was un-necessary after the 18 h extraction period. Maintenance of the low pH was to ensure that MG was in the form of the dye ion. Pentan-1-ol was added to samples and samples re-extracted and the samples were left to sit at room temperature for 24 h. The muscle samples were found to require more pentan-1-ol during extraction when compared with the other samples. Liver tissue was found to yield a possible haemolysis product and at low concentrations this could affect the determination of MG. Van Rhijn *et al.*, [20] used McIlvaine buffer pH 3 and acetonitrile (9:1, v/v) to extract MG and LMG from atlantic salmon. Van de Riet *et al.*, [21] used perchloric acid and acetonitrile to extract MG and LMG from salmon, rainbow trout, shrimp, tilapia and catfish tissue. Plakas et al., [22] used acetonitrile: acetate buffer pH 4.5, 1 % hydroxylamine in methanol and para-toluenesulfonic acid (TSA) to extract MG and LMG from catfish plasma. No interconversion of MG and LMG occurred during extraction and analysis of individually fortified plasma replicates. This group

homogenised the hydroxylamine solution, p-toluenesulfonic acid solution and acetate buffer with catfish tissue before addition of acetonitrile to the extract. Subsequently alumina was added and samples were centrifuged. The supernatant was removed and decanted into a water and diethylene glycol mixture. It was found that increasing the TSA concentration resulted in a 12% gain (from 68 to 80%) in the recovery of ^{14}C MG from the initial extract. The second extraction with acetonitrile resulted in an additional 16 % gain in recovery. This method of extraction was used by Turnipseed et al., [23] and Anderson et al., [24] in the extraction of MG and LMG from salmon. Tarbin *et al.*, [25] used pH 4 citrate buffer and acetonitrile to extract MG and LMG from trout muscle. It was found that recoveries were variable when using acetonitrile on its own as the extraction solvent. Roybal *et al.*, [26] used hydroxylamine hydrochloride, p-toluenesulfonic acid and acetate buffer at pH 4.5 with catfish tissue before addition of acetonitrile. Subsequently alumina was added to the extract and samples were centrifuged and the supernatant was decanted into water and diethylene glycol mixture. Hydroxylamine hydrochloride was added to fish samples to inhibit enzyme action on the analytes since Allen *et al.*, [15] reported the inter-conversion of MG to LMG after exposure to fish tissue. Turnipseed et al., [27] developed a method which was used by Roybal *et al.*, [26] to extract MG and LMG. Hajee *et al.*, [28] carried out a simple deproteinization of eel plasma with 2% trichloroacetic acid (50:50, v/v), 6% perchloric acid (50:50, v/v), 10% phosphoric acid (50:50, v/v) or acetonitrile (10:90,v/v) before the determination step which resulted in unsatisfactory recoveries. This group also found that alkalisation or acidification of the plasma prior to extraction with ethyl acetate also yielded poor results. It was concluded that a further clean-up stage in the method was necessary to improve results. Rushing *et al.*, [29] used hydroxylamine hydrochloride, aqueous P-TSA and aqueous ammonium acetate (adjusted to pH 4.5 with glacial acetic acid) blended with catfish or trout tissue, subsequently acetonitrile was added to extract MG and LMG from the tissues. Alumina was then added and a second acetonitrile extraction of sample was carried out. Bergwerff et al., [30] homogenised catfish, eel, trout, turbot and prawns with McIlvaine buffer pH 3.0, 1 M para-toluenesulphonic acid and 1 mg ml $^{-1}$ methanolic N,N,N',N' –tetramethyl 1, 4– phenylenediamine dihydrochloride and then acetonitrile was added to extract MG and LMG. A second extraction of the sample was carried out using McIlvaine buffer at pH

6.0 and acetonitrile. From correspondence with the author a higher extraction yield of LMG was accomplished when pH 6 was utilised in second extraction of the sample. The group found that on inspection of the liquid extraction process, homogenisation using an ultraturrax or household blender gave similar but better recoveries of the MG residues compared to ultrasonification. This group also found that the time interval between the moment of spiking and the moment of sample processing had a dramatic influence on the recovery of MG. It was shown that recoveries of 81, 63, 60, 54, and 45% were obtained for time intervals of 1, 15, 30 min and 1 h and 2 h respectively. Since no corresponding signal for LMG was observed, MG was apparently not reduced invitro into LMG during this incubation. In contrast, little effect was observed on the recovery of LMG since recoveries of 100, 96, 93, 93 and 95% were obtained for these time intervals respectively. Inspection of processed fish fillets, showed significant peaks, this suggested degradation of MG and especially LMG. Comparison of the analysis of spiked samples with standards indicated that this breakdown originated partly from the working up procedure. In this work it was found that the addition on TMPD and ascorbic acid to samples could largely reduce this degradation. Swarbrick *et al.*, [31] extracted MG and LMG with dichloromethane (2 ml), acetonitrile (10 ml) and 0.4 M perchloric acid in acetonitrile. This group evaluated a range of extraction systems, including several solvent/homogenisation and percolation techniques. Their effectiveness was limited by strong binding of the dyes to fish flesh and its oil content (10 − 15%) which causes emulsion formation. 3 hours was found to be effective time for the extraction of the analytes in the dark at 60 rpm on a rotary mixer. Acidification of the extraction solvent was shown to be vital for good yields.

2.15 Sample Clean-up Techniques for Different Matrices

The most widely adopted approach in liquid/liquid extraction of MG and LMG is a low pH buffer and acetonitrile extraction system. The clean-up of extracts is carried out by either liquid/liquid partitioning only or in combination with solid phase extraction. Allen et al., [15] carried out only a liquid/liquid partitioning clean-up of eggs, fry and muscle of rainbow trout tissues. This involved partitioning 1% acetic acid in acetonitrile extract after addition of anhydrous sodium bicarbonate with chloroform. The partitioning procedure was repeated twice and the combined chloroform extracts were made suitable for analysis directly by LC. This group extracted trout tissue by combining homogenised trout tissue with anhydrous sodium sulphate, packing into a column and eluting with acetic acid in methanol. The eluate was combined with anhydrous sodium bicarbonate and partitioned against chloroform. The chloroform fraction was removed and a second partitioning of the eluate with chloroform was performed. The combined chloroform fractions were concentrated and analysed. Valle et al., [14] and Bergwerff et al., [30] used a McIllvaine buffer: acetonitrile at pH 3 and pH 6 to extract MG and LMG from salmon muscle. The acetonitrile and McIlvaine buffer extracts were washed with dichloromethane (DCM). The DCM extracts were isolated from the aqueous extracts, combined and purified on a J.T Baker aromatic sulfonic acid cartridge. The analytes were eluted with a solution containing 25% ammonia, 1 mg ml^{-1} methanolic ascorbic acid and methanol. Elution studies of the MG and LMG from the SPE cartridges using between 1 to 15 ml of elution solvent showed that the volume of elution solvent should be over 6 ml. The cationic sulfonic acid cartridge was chosen since allows the separation of the analytes from low polarity compounds in the matrix. Due to the ionic character of MG, ion–exchange was the mechanism of choice. An ammonia gas pre-treatment of an aromatic sulphonic acid solid phase extraction cartridge with MG and LMG followed by elution with methanol showed only marginal losses of MG and LMG. This author suggests that ammonia gas treatment deprotonates MG and LMG, thus disrupting the electrostatic interation with the sulphonic acid solid phase extraction column which permits elution with a small volume of methanol. Scherpenisse et al., [13] purified

pangasius, rainbow trout, salmon, tilapia, and victoria perch extracts by combining the McIIIvaine buffer:acetonitrile extraction solvent at either pH 3 or pH 6 against chloroform. The organic phase was then passed through a J.T. Baker aromatic sulphonic acid cartridge. The addition of the chloroform was necessary to remove water. Plakas *et al.*, [18] partitioned catfish tissue extracts into methylene chloride with the final clean-up on alumina and propylsulfonic acid solid phase extraction column. Van de Riet *et al.*, [21] cleaned up a variety of aquaculture products including salmon, trout, catfish, tilapia and several shrimp species using Strata C_{18} solid phase extraction columns. The column was eluted with a mixture of 90 % methanol, 5 % of 1 mg ml^{-1} ascorbic acid and 5 % of 25 % aqueous ammonium hydroxide in methanol. The SPE clean-up was found to give a less colourless and viscous extract that enhanced MS response when compared with a hexane partitioning method investigated as an alternative clean-up method. The solid phase extraction clean-up appeared to remove a great deal of the non-polar coloured pigments and lipids that were possibly causing ion suppression in the MS interface. Plakas *et al.*,[22] partitioned catfish tissue extracts with two portions of methylene chloride and cleaned up using dual solid phase extraction (SPE) on an alumina column placed onto a propyl sulphonic acid (PRS) column. The analytes were eluted from the PRS columns using mobile phase. Similiarly Tarbin *et al.*, [25] washed acetonitrile-citric acid extracts using DCM with sodium chloride. The DCM layer after centrifugation was then passed through a J.T Baker sulphonic acid cartridge. Roybal *et al.*, [26] washed acetonitrile, acetate buffer pH 4.5 extracts with two portions of DCM. The combined extracts were modified with acetonitrile and passed through a dual SPE system using alumina and a propyl sulphonic acid solid phase extraction columns. The analytes were eluted with mobile phase, hydroxylamine hydrochloride in methanol and 0.005 M p-toluenesulfonic acid. Turnipseed *et al.*, [27] purified extracts using a J.T Baker cyano SPE cartridge. The elution solvent was an acetonitrile-sodium acetate buffer mixture that was designed to elute only the LMG from the cartridge even though MG and LMG are initially retained. Hajee *et al.*, [28] cleaned up extracts of protein denatured eel plasma using J.T Baker sulphonic acid SPE columns. Rushing *et al.*, [29] carried out a liquid/liquid partitioning step and solid phase extraction step in the clean-up of MG and LMG from catfish and trout tissue. The partitioning step involved combining distilled water, methylene chloride and diethylene

glycol with tissue extract of acetonitrile, ammonium acetate buffer and aqueous para-toluenesulphonic acid. This partitioning of the extract was carried out twice. The samples were then cleaned-up up using a dual SPE system which included an alumina cartridge and a PRS cartridge.

Bergwerff *et al.*, [30] cleaned up tissue extracts of McIlvaine buffer pH 3.0, 1 M para-toluenesulphonic acid and 1 mg ml $^{-1}$ methanolic N,N,N',N' –tetramethyl 1, 4 – phenylenediamine dihydrochloride and acetonitrile by washing extract with dichloromethane, removing the aqueous layer and passing through a Bakerbond aromatic sulphonic acid (SCX) solid phase extraction cartridge. The analytes were eluted with 25 % ammonium hydroxide, 1 mg ml $^{-1}$ ascorbic acid and methanol.

Swarbrick *et al.*, [31] used an alternative clean-up using activated charcoal instead of a C_{18} SPE column. This author cleaned up rainbow trout extracts by modifying the extractant with water before loading onto a C_{18} cartridge. The analytes were eluted with HPLC mobile phase. After centrifugation of the initial DCM /acetonitrile /perchloric acid extract of trout tissue, the extract was evaporated to approximately 0.5 mL at 60 °C and diluted with mobile phase. This extract was mixed with finely powdered charcoal and 5M nitric was added, the mixture shaken vigorously for 1 min and allowed to stand for 5 min. The acid was removed by centrifugation and the remaining paste was rinsed twice with water and then with HPLC mobile phase prior to dilution of sample with mobile phase for analysis. Sager et al., [32] analysed drinking and river water samples for MG residuals following adjustment of water samples to pH 5. The samples were passed through TechElut cyano solid phase extraction cartridges and the analytes were eluted with mobile phase. Mitrowska et al., [16] cleaned up tissue extracts of acetate buffer, hydroxylamine hydrochloride, para-toluenesulphonic acid and acetonitrile by partitioning extract with DCM. The organic phase was passed through a J.T Baker sulphonic acid cartridge. The analytes were eluted with 5 ml of a mixture containing acetonitrile and ammonium hydroxide (25%) (90:10,v/v).

Andersen et al., [24] cleaned up tissue extracts containing ammonium acetate buffer, hydroxylamine hydrochloride, para-toluenesulphonic acid and acetonitrile by the addition of water and diethylene glycol to the extract and partitioning twice against methylene chloride. The organic phase was then evaporated to dryness and the oily residue was re-

dissolved in acetonitrile followed by the addition of 0.001 M 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). Periodic mixing of the flask was carried out during the entire oxidation period of 30 min. The oxidised sample was transferred to an alumina solid phase extraction cartridge connected on top of a propylsulphonic acid (PRS) solid phase extraction cartridge. After loading of the sample the alumina solid phase extraction cartridge is discarded and the analytes are eluted from the PRS cartridge under gravity using mobile phase (ammonium acetate buffer:acetonitrile (50:50, v/v)). The conversion efficiency of DDQ to oxidise LMG to MG was investigated in this study. A 10-fold molar excess of DDQ added to a 20 ng ml $^{-1}$ standard solution of LMG and this was sufficient to completely oxidise LMG to MG. However a 10,000-fold excess of DDQ was required to completely oxidise 20 ng ml $^{-1}$ LMG to MG in salmon extract. This showed that one or more components of the salmon sample consume the majority of the DDQ. The quantity of alumina added to the sample at extraction was found to be critical to achieve a high MG recovery. Upon investigation it was recommended to use 6 g of alumina. Turnipseed et al., [23] used the extraction and clean-up method by Andersen et al., [24] in the determination of MG and LMG in salmon.

2.16 Methods for the Determination of Malachite Green and Crystal Violet Residues

MG analysis in early work was routinely carried out using spectrometric methods whereas today these compounds are analysed by a variety of analytical techniques. Initially, methods for determining MG residues in fish tissues were limited to measurements of the parent compound on the basis of its visible light absorption.

Spectrometric Methods

Alderman et al., [19] determined MG in pentan-1-ol extracts of tank water, serum, liver, bile, muscle, kidney and viscera of rainbow trout using a double beam spectrometer in concentration mode. Peak absorption for the MG dye ion in pentan-1-ol was 625 nm. The presence of a small amount of water in the extract of pentanol ensured that the MG remained in the dye ion. Therefore MG present as dye ion and as carbinol was

determined as the dye ion. The concentration in the dye extracts was determined by calibrating the spectrophotometer using extracts from the spiked control tissues against extracts from unspiked control tissues. Allen *et al.*, [12] determined residues of MG in muscle, eggs, and fry of Atlantic salmon and Chinook salmon by colorimetric analysis after fish had been routinely treated with the chemical at fish hatcheries. Ethyl alcohol, formalin and acetic acid (85:10:5, v/v/v) was used and the fish exposed to MG developed a blue colour. The absorbance of sample extracts at 615 nm were compared to the absorbance of MG standards in ethyl acohol, formalin and acetic acid, 85:10:5 v/v/v.

Chromatographic Methods

The majority of LC methods for the determination of MG have been developed using reverse phase systems. A reverse phase separation was developed for MG and LMG on a Phenomenex Luna C_{18} column (3 µm, 150 mm × 2mm) by Scherpenisse *et al.*, [13] The LC mobile phase consisted of 50 mM acetate buffer pH 4.5 and acetonitrile (25:75, v/v) at a flow rate of 200 µl min^{-1}. An in-line post-column reactor (20 mm × 20 mm) filled with a mixture of PbO_2 and celite in a weight ratio of 3:1 converted the lipophillic LMG into the cation MG. It was found that the oxidation capacity of the reactor did reduce noticeably over thousands of analytical runs. This LC method was suitable for the analysis of catfish, eel, rainbow trout, tropical prawns, salmon and turbot. Valle *et al.*, [14] used an isocratic mobile phase containing 60% acetonitrile in aqueous ammonium acetate (50 mM, pH 4.4) at a flow rate of 0.7 ml min^{-1} to detect MG using a Waters Symmetry C_{18} column (5 µm, 4.6 mm × 250 mm). An oxidative pre-column (4.6 mm × 20 mm) for the conversion of LMG into MG was hand packed with a mixture of 10% lead (IV) oxide and celite. The use of the oxidative reactor before the LC run allows both compounds, MG and LMG, to enter the LC column as MG. The advantage of merging both compounds into one signal is an increase in the signal-to- noise ratio.

A decomposition product of MG was formed and could be detected as a peak since the reactor was located before the LC column. The formation of the decomposition product desmethyl-MG depended on the amount of the PbO_2 in the reactor and the residence time of the sample inside the reactor.

Allen et al., [15] used an isocratic mobile phase containing methanol-water (81:19, v/v), buffered with 0.05 M sodium acetate and 0.05 M glacial acid with a Waters μBondapak C_{18} column (10 μm, 300 mm × 3.9 mm). The flow rate was 1.5 ml min $^{-1}$. MG and LMG were separated on column and LMG was oxidised to MG in a post-column reactor (stainless-steel tube, 32 mm long, 4 mm I.D.) packed with 10% PbO_2 suspended in Celite 545. The reactor was found to be efficient up to approximately 400 injections but it was recommended that the efficiency be checked periodically using standards. Doerge et al., [17] used a Phenomenex Prodigy ODS-3 column (5 μm, 250 mm × 4.6 mm) with a mobile phase gradient from 50% acetonitrile in aqueous ammonium acetate (50 mM, pH 4.5) to 100 % acetonitrile over a 10 min linear ramp period. The flow rate was 1.0 ml min $^{-1}$ and under these conditions MG and LMG eluted at approximately 5.3 and 12.9 min respectively.

Bergwerff et al., [30] used a Phenomenex Luna phenyl-hexyl column (3 μm, 50 mm × 4.6 mm) followed by a Phenomenex Luna C_8 column (3 μm, 50 mm × 4.6 mm) as the second phase HPLC column in series. The LC columns were eluted with a mixture of 60 % acetonitrile and 40% of 0.05 M ammonium acetate buffer, pH 4.5 at 0.6 ml min $^{-1}$.

Van Rhijn et al., [20] used a Waters Symmetry C_{18} column (150 mm × 3 mm) for the separation of MG and LMG in gradient mode from 90:10 10 mM formic acid:acetonitrile to 10:90, formic acid:acetonitrile using a linear gradient from 1 to 10 mins after injection. Under these conditions MG and LMG are eluted at 12.5 and 15.5 min respectively. Roybal et al., [26] used a Phenomenex Ultremex 5 Cyano column (5 μm, 150 mm × 4.6 mm) with a isocratic mobile phase of acetonitrile:acetate buffer (50:50, v/v). A PbO_2 post-column oxidation was performed with 10% PbO_2 suspended in Celite. The oxidation of LMG to MG [1] by the PbO_2 column occurred at 101% conversion. On evaluation of the performance of the PbO_2 column in maintaining optimum oxidation of LMG, it was found that after 67 injections no loss in peak response was noted. Plakas et al., [22] used a Phenomenex Ultremex 5 Cyano column (5 μm, 250 mm × 4.6 mm) with a isocratic mobile phase of acetonitrile:acetate buffer (50:50, v/v) at a flow rate of 1 mL min $^{-1}$. Under these conditions, retention times of LMG and MG were 6.3 and 16.2 min respectively. It was found that using the longer column in this study when compared with a study by Roybal et al., [26] improved the separation of LMG and other minor metabolites.

A PbO$_2$ post-column oxidation was performed with 25% PbO$_2$ suspended in Celite 545. Allen *et al.*, [15] used a Waters μBondapak C$_{18}$ column (10 μm, 300 mm × 3.9 mm) with a mobile phase of methanol:aqueous acetate buffer (85:15, v/v) at a flow rate of 1.5 ml min $^{-1}$. A reactor was placed after the column and was packed with 10% PbO$_2$ suspended in Celite 545.

Plakas et al., [18] used a Phenomenex Ultremex 5 Cyano column (5 μm, 250 mm × 4.6 mm) with a isocratic mobile phase of acetonitrile:acetate buffer (50:50, v/v) at a flow rate of 1 mL min $^{-1}$. A post-column oxidation chamber packed with 25 % lead oxide converted the colourless LMG to MG. Tarbin *et al.*, [25] used a Phenomenex Columbus C$_{18}$ column (5 μm, 250mm × 2 mm) with a binary gradient of aqueous ammonium acetate pH 4.5:acetonitrile (35:65, v/v) and aqueous ammonium acetate pH 4.5: acetonitrile (20:80, v/v) with a flow rate of 0.3 ml min $^{-1}$. A step gradient was used with mobile phase 1 being pumped for 10 min before switching to mobile phase 2 for 20 min. A 2 mm internal diameter analytical column was choosen since gave suitable chromatography for both analytes. Also the flow rate required by this column would be advantageous when moving the methodology over to mass spectrometric detection. Post-column oxidation was carried out using a column packed with PbO$_2$. It was found that if the pH of the mobile phase was kept at pH 7, then no oxidation occurred. Reduction of the pH to 4.5 gave good conversion of the LMG to MG.

Van de Riet *et al.*, [21] used a Phenomenex reverse phase Luna C$_{18}$ column (5 μm, 150 cm × 2 mm) held at a temperature of 35 °C with 30% of solvent B flowing at 0.25 ml min $^{-1}$. The system was held at 30% B for 1 min. and then a linear gradient was used ramping from 30% B to 100% B over 7 min; once at 100% B, the flow rate was ramped to 0.4 ml min $^{-1}$ over 3 min. The system was held at 100 % B for 10 min and returned to 30% B over a period of 1 min. MG and LMG were easily separated from one and other and completely eluted from the column in less than 25 min with the linear gradient from 30 to 100% solvent B at 0.25 ml min $^{-1}$. Hajee *et al.*, [28] analysed MG and LMG and their assumed demethylated degradation products on a variety of different analytical columns which included Chromopacks's Chromospher C$_8$ and C$_{18}$, Nucleosil 5 C$_{18}$, Hypersil ODS, Microspher C$_{18}$, Lichrosorb RP-8 and Chromspher 5B, Polymer Laboratories' PLRP-S and Hamilton's PRP-1 were tested for effective column efficiency and

resolution. Studies found that Nucleosil 5 C_{18}, LichroSorb C-8 and Chromspher 5B showed the best effective column efficiency and resolution of these compounds. The mobile phase was further optimised using the Chromopack Chromspher 5B column (11 × 3.0 mm). The optimum concentrations in the binary electrolyte system of the mobile phase appeared to be 25 mM sodium 1-pentane sulphonate and 50 mM sodium perchlorate. However, pH adjustment with dipotassiummonohydrogenphosphate (K_2HPO_4) crystals caused poor repeatability of the retention behaviour of MG and LMG. Also the required pH (3.5-4.5) of the mobile phase was outside the effective pH buffering range of phosphate. To overcome these problems, citric acid and acetate were tested as buffering agent in the aqueous ion-pair solution of the mobile phase. Citric acid caused clogging of the in-line post-column oxidation reactor. Sodium acetate was found to give no problems in this respect. The best resolution of MG and LMG and the demethylated degradation products was obtained when the pH of the aqueous 25 mM acetate-ion pair buffer is adjusted to pH 4.0 and an organic modifier content of 60% of acetonitrile is used. Also the size and PbO_2 content of the in-line post-column oxidation reactor were optimised regarding LMG and MG conversion performance. The large size of the post-column oxidation reactor (32 × 4 mm) used by Allen et al., [15] is possibly a cause of band broadening of the MG and LMG peaks. Hajee et al., [28] reduced the size of the reactor (10 × 2.1 mm) and this gave much sharper peaks for MG and LMG but the PbO_2 in the reactor was rapidly depleted. On increasing the PbO_2 content in the reactor from 10% to 25% this markedly prolonged the lifetime of the reactor. Also a guard oxidation reactor containing 50% PbO_2 in celite (50 × 4.6 mm) between HPLC pump and the auto-injector eliminated oxidisable interferences that occurred in the mobile phase and prolonged the lifetime of the post-column oxidation reactor to more than 800 injections. The stability of MG and LMG in mobile phase was investigated at room temperature since it is known that these compounds can be photo-oxidatively demethylated to N,N',N''-tri and/or N,N'-dimethylated compounds. MG was stable but LMG proved to be unstable. In a period of 13 hours the LMG decreased approximately 25% while the peak areas of the assumed demethylated degradation products of LMG increased. A standard solution of LMG in mobile phase stored at 4 °C did not show this photo-oxidative demethylation. A standard solution of LMG in anti-oxidative mobile phase was also stable at room

temperature. The anti-oxidative mobile phase contained 10 μg ml $^{-1}$ of ascorbic acid. The presence of ascorbic acid in standard solutions of MG and LMG influenced neither the stability of MG or the chromatography of MG and LMG. Rushing et al.,[29] investigated separation of MG,LMG,CV and LCV using a Supelco LC-CN column (250 × 4.6 mm) in-line with a post-column PbO$_2$ oxidation reactor (20 × 2.0 mm) using a mobile phase of acetonitrile-buffer (60:40, v/v) with a flow rate of 1 ml min $^{-1}$. The separation of these compounds was also investigated on a SynChropak SCD-100 column (150 × 4.6 mm) and a PbO$_2$ oxidative post-column (20 × 2.0 mm) with a mobile phase of acetonitrile-buffer (55:45, v/v). The isocratic elution of the analytes on a cyano column resulted in co-elution of LMG with LCV at a retention time of 5.8 min. This separation was inadequate. Complete separation of the mixture was achieved on a short-chained deactivated (SCD) reverse phase column in under 10 min. After separation on the column the LMG and LCV are oxidised by the PbO$_2$ reactor to their respective chromatic form. Bergwerff *et al.,*[30] used an Inertsil ODS-2 HPLC column (100 × 3.0 mm) with a mobile phase of 50 mM sodium perchlorate containing 25 mM sodium acetate and 25 mM 1-pentanesulfonic acid to pH 4.0 with acetic acid, and acetonitrile in the ratio of 2:3, v/v. A pre-column oxidation reactor (50 × 4.6 mm) filled with PbO$_2$ and celite in weight ratio of 50:50, v/v. The robustness of the LC separation was evaluated on three different HPLC columns and each different column was evaluated with three mobile phase batches. All chromatograms were similar.

Sager et al.,[32] used a Hichrom stainless steel cyano column (5 μm, 250× 4.6 mm) to chromatograph MG using a mobile phase of methanol:0.1 M sodium acetate pH 4.5 (70:30, v/v). Acetonitrile if used in the mobile phase was found to affect the longterm stability of the carbon fibre of the detector when using amperometric detection. Mitrowska et al.,[16] separated MG and LMG by isocratic elution on a phenomenex Luna-phenyl-hexyl (5μm, 150×4.6 mm) analytical column. A phenomenex phenyl-hexyl guard column (40 mm × 2 mm) was used prior to the analytical column. The mobile phase consisted of acetonitrile and acetate buffer ((0.05 M, pH 4.5) 60:40, v/v) and the separation was accomplished at a flow of 1 ml min $^{-1}$. Anderson et al.,[24] investigated the separation of MG and LMG on an Alltech Alltima C$_{18}$ (3 μm, 150×4.6 mm) with an Alltech C$_{18}$ guard column (5 μm, 7.5×4.6 mm) and a MacMod Analytical Inc Column

Saver column prefilter (0.5 μm). Mobile phase A consists of ammonium acetate buffer:acetonitrile (50:50,v/v) and mobile phase B consists of acetonitrile. The LC isocratic mobile phase elution profile is 95% A and 5% B. The mobile phase flow rate is 1 ml min $^{-1}$ and the column temperature is set at 35 °C. Turnipseed et al.,[23] used a Waters YMC phenyl 3-4-5 cartridge column (3 μm, 4.0 ×50 mm) with a guard cartridge insert (4.0 ×1mm) of the same composition. The column was maintained at 30 °C and the mobile phase flow rate was 700 μl min $^{-1}$.The analytes were eluted isocratically with 0.1% formic acid (63:37, v/v) for the first 10 min, followed by an increase to 100 % acetonitrile from 10 to 10.5 min, a column wash at 100% acetonitrile was carried out from 10.5 to 12 min, return to 0.1% formic acid:acetonitrile (63:37,v/v) from 12 to 12.5 min and the system was equilibrated at that concentration for a final 2.5 min. Roybal et al.,[33] used an Alltech Cyano (250 × 4.6 mm, 5 μm) for the determination of CV, its demethylated metabolites, LCV in chicken tissue using LC with electrochemical detection. The mobile phase was adjusted from acetate buffer:acetonitrile (2:3, v/v) to acetate buffer:acetonitrile (50:50,v/v). The flow rate was 1ml min $^{-1}$. Munns et al.,[34] used the method by Roybal et al., in the determination of LCV in chicken fat. Heller et al.,[35] determined LCV in chicken fat with baseline separation of LCV from matrix interferences using a DuPont Zorbax ODS column (150 × 4.6 mm, 5 μm) with a mobile phase of methanol:pH 4 ammonium acetate buffer (90:10,v/v). Thompson et al.,[36] used a supelco cyano (259 ×4.6 mm, 5 m) PbO_2 oxidative post-column (20 × 2.0 mm). The mobile phase was acetonitrile:ammonium acetate pH 4.5 (60:40, v/v) and the flow rate was 1 ml min $^{-1}$. The LCV was chromatographed on column as the leuco form but after separation on the column it was oxidised by PbO_2 post-column reactor to the chromatc form. Doerge et al.,[37] used a Synchrom Inc, SCD100 (250 × 4.6 mm, 5 μm) column using a mobile phase of acetonitrile:aqueous ammonium acetate pH 3.6 at a flow rate of 1.5 ml min $^{-1}$ for the separation of CV and LCV.

Liquid chromatography detection systems

A number of groups have developed methods that enable the simultaneous analysis of the chromatic parent compound and its colourless leuco metabolite. These methods

incorporate a post-column reactor containing PbO_2 in which the LMG after separation on an analytical column is oxidised back into the chromatic compound for visible light detection. Others have used electrochemical cells for detection.

Detection in the visible spectrum

Allen *et al.*, [15] developed a method for the simultaneous analysis of chromatic and leuco forms of MG using photometric detection. A reaction chamber containing 10 % PbO_2 in celite 545 was placed between the column and the spectrometric detector to oxidise the leuco form of MG to its chromatic form. Chromatic and LMG have been quantified by their absorbance at 618 nm. The detection limits was 0.12 $\mu g\ kg^{-1}$ for the leuco form of the dye and 0.28 $\mu g\ kg^{-1}$ for the chromatic MG oxalate. The detector response was lower for chromatic MG due to its different molecular weight. Plakas *et al.*, [18] determined residues of MG in plasma and tissue in channel catfish and holding tank water using a post-column oxidation chamber packed with 25% PbO_2 converting colourless LMG to MG for visible light detection at 618 nm and quantification. The limits of determination for MG and LMG were 0.023 $mg\ L^{-1}$ in plasma and 0.005 $mg\ L^{-1}$ in muscle. Bergwerff *et al.*, [30] determined the persistence of residues of MG in juvenile eels using post column reactor filled with a mixture of PbO_2 and celite in a weight ratio 3:1. The eluate was monitored at 620 nm after post column oxidation of LMG into chromic MG. The limit of detection for each MG and LMG was 1 $\mu g\ kg^{-1}$.

Plakas *et al.*, [22] using a LC cyano column with a PbO_2 post-column with visible detection at 618 nm. The limits of detection for MG amd LMG in plasma were approximately 10 $\mu g\ kg^{-1}$. The limits of detection of MG and LMG in muscle were approximately 2 $\mu g\ kg^{-1}$. The overall mean recoveries of parent MG and its major metabolite, LMG from plasma were 93 and 87% respectively. The overall mean recoveries of MG and LMG from muscle were 85 and 95% respectively. The relative standard deviations (RSDs) of recoveries at all fortification levels ranged from 4 to 7% for plasma and 2 to 5% for muscle. Roybal *et al.*, [26] determined MG and LMG by liquid chromatography with visible detection and post-column oxidation. The advantage of using the approach of post-column oxidation is that LMG is converted into MG and both analytes can be monitored by one procedure with a single LC determination. Recoveries of MG from fortified

catfish tissues were 73, 75 and 70 % for tissues spiked at 23 µg kg^{-1}, 11 µg kg^{-1} and 6 µg kg^{-1}. The relative standard deviations were 2, 7 and 7 % at each of these spiking levels respectively. Recoveries of LMG from fortified catfish tissues were 87, 3 and 88 % for tissues spiked at 21 µg g^{-1}, 10 µg kg^{-1} and 5 µg kg^{-1}. The relative standard deviations were 3, 6 and 12 % at each of these spiking levels. The limit of detection for MG and LMG were 4 and 5 µg kg^{-1}. Allen et al., [15] analysed MG and LMG by liquid chromatography with post-column oxidation of LMG to the chromatic form. Plakas et al., [18] determined MG using same procedure previously described. [8]

Tarbin et al., [25] determined MG and LMG in a screening method using HPLC with visible detection. Since MG has an absorption maxima in the visible range at 618 nm and its leuco metabolite has absorption maxima in the UV region at 265 nm post-column oxidation was used in order to see compounds at a single wavelength. The recovery was 71, 69 and 71% for MG when spiked at levels of 10 µg kg^{-1}, 5 µg kg^{-1} and 2 µg kg^{-1}. The recovery of LMG was 91, 97 and 97% when spiked at the same levels.

Hajee et al., [28] quantified MG and LMG in eel plasma after detection at 610 nm using post-column oxidation. The limits of detection and quantification were determined to be 3.5 and 5.0 µg kg^{-1}, respectively for MG and 0.6 and 0.9 µg kg^{-1} respectively for LMG. Recovery experiments were carried out in six replicates with blank eel plasma spiked at levels of 20, 50, 100, 200, 1000 and 2500 µg kg^{-1} for both MG and LMG. A good recovery at all levels investigated and a low standard deviation for repeatability was obtained.

Rushing et al., [29] determined MG and LMG with CV and LCV in catfish or trout muscle using in-line post column PbO_2 oxidation reactor permitting visible detection of all four compounds at 588 nm. Recoveries of LMG and MG from catfish tissues fortified at 10 µg kg^{-1} were 75 ± 3% and 61 ± 4% respectively while trout tissues fortified at the same level yielded recoveries of 83 ± 2% and 49 ± 2% (mean ± S.D., n=4), respectively. Visible maximum of 588 nm was chosen in this study rather than 618 nm as in other publications since the upper range of the detector in this study only reached 600 nm.

Bergwerff et al., [30] quantified MG and LMG by HPLC with pre-column oxidation and the effluent from the column was monitored at 620 nm with a tungsten light source. MG and its primary metabolite LMG were successfully determined at 2.5 – 2000 µg kg^{-1} in

catfish, eel, rainbow trout, salmon, tropical prawns and turbot with a limit of detection of 1 μg kg^{-1}. The recoveries for LMG were between 86 $\pm$ 15% (prawn) and 105 $\pm$ 14 % (eel). MG and LMG were determined by reverse phase HPLC with absorbance detection at 610 nm following post-column oxidation of the leuco form to MG using an electrochemical detector cell. The limits of detection for MG and LMG were 6 μg kg^{-1} and 3 μg kg^{-1} respectively. At concentrations in the range of 25 – 200 μg kg, recoveries in the range of 73-87% were achieved for MG and 89-98% for LMG.

Andersen et al., [24] analysed MG and LMG residues in salmon with insitu LMG oxidation upon reaction of MG with DDQ. The extracts were analysed for MG by LC with visible detection at 618 nm using isocratic elution and a C_{18} column. The recovery and coefficient of variation (CV %) of MG spiked into farm raised salmon was 95% and 11%. The recovery of MG and the CV % when MG was spiked into canned salmon was 90 and 3%. The detection limit of the method was 1 μg kg^{-1}.

Heller et al., [35] determined LCV in chicken fat by liquid chromatography with UV detection. Average recoveries of samples fortified at 5, 10 and 20 μg kg^{-1} were 76% with a CV % of 5%. The detection limit was 0.4 μg kg^{-1} and the limit of quantification was 2 μg kg^{-1}. Thompson et al., [36] described a method for the determination of CV and LCV in channel catfish were LCV was oxidised by PbO_2 post-column reactor to the chromatic form and both compounds were detected as CV. The limit of detection was 0.4 μg kg^{-1} and the limit of quantification was estimated to be 2 μg kg^{-1}.

Detection by visible-fluorescense detection

Mitrowska et al., [16] quantified MG and LMG in carp muscle using LC with visible (λ =620) and fluorescence detection (λ_{ex}=265 and λ_{em} 360). Both detectors were connected on-line which allowed direct analysis of the sample extract for MG and LMG without the need for post-column procedure. Average recoveries of MG and LMG from muscle fortified at three levels were 62% and 90% respectively. The relative standard deviations were less than 11 and 9% for MG and LMG. The CCα for MG and LMG were 0.15 and 0.13 μg kg^{-1} and CCβ were 0.37 and 0.32 μg kg^{-1}.

Electrochemical Detection

The determination of LMG and MG residues in rainbow trout flesh by post-column electrochemical oxidation was accomplished by Swarbrick *et al.,* [31] An electrochemical detector with ESA 5010 analytical cell operating at a potential of 0.45 V relative to the in-built proprietary reference electrode was coupled between the HPLC column and the visible detector. A selection of the operating potentials was achieved by performing repeated 10 µl injections of standard solutions of MG and LMG in the mobile phase at a flow rate of 0.1 mL min^{-1} after removal of the analytical column. The response of a visible detector was recorded at a range of potentials at the electrode of the electrochemical detector to determine the optimum voltage for analysis. The operating potential of 0.45 V was chosen since it gave maximal oxidation of LMG. The limits of detection for MG and LMG are 6 µg kg^{-1} and 3 µg kg^{-1}. At concentrations between 25-200 µg kg^{-1}, recoveries in the range of 73-87% were achieved for MG and 89-98% for LMG. Sager et al., [32] determined MG residues in water samples involving electrochemical detection using a carbon fibre electrode. The residues were detected amperometrically by employing a potential of +1.2 V at the working electrode. The limit of detection was 0.7 mg L^{-1}. A rapid method for the determination of LCV in chicken fat with electrochemical detection was described by Munns et al., [34] The electrochemical detector was set at a potential of 1.000V. Average recoveries of LCV from chicken fat were 84% with a coeffiecient of variation (CV%) of 13% for the 5 µg kg^{-1} level, 83 % with CV% of 14 % for the 10 µg kg^{-1} level and 78 % with CV % of 3 % for the 20 µg kg^{-1} level. Roybal et al., [33] developed a method for the determination of CV, its demethylated metabolites and LCV in chicken tissue. The analytes are detected by amperometric electrochemical detection at + 1.000 V. Average recoveries of CV and LCV from commercially purchased chicken liver fortified with 20 µg kg^{-1} were 92 %, CV % 7.6 and 86 %, CV % 8 % respectively. Average recoveries of CV and LCV from control chicken liver fortified with 20 µg kg^{-1} of each compound were 80 % with a CV % of 9 % and 70 % with a CV % of 4 % respectively. Compared to methanol, acetonitrile incorporated into the mobile phase improved the sensitivity of the LC/ED system and also the resolution for the analytes. Ethylenediaminetetraacetic acid (EDTA) was also added to the mobile phase as a masking agent to reduce background current and drift.

Detection by mass-spectrometry

Mass spectrometry has been widely used in the determination of MG and LMG residues. After conversion of LMG into MG by post-column oxidation with PbO_2, the effluent has been analysed by LC-ESI MS/MS in multiple reaction monitoring (MRM) mode.[13] The mobile phase consisted of 50 mM acetate buffer pH 4.5:acetonitrile (25:75, v/v) and was pumped at 200 µl min^{-1}. Species investigated in this study included salmon, pangasius, tilapia, trout and Victoria perch. Recoveries ranged from 66 % in trout spiked at a level of 4 µg kg^{-1} to 112 % in pangasius at 0.1 µg kg^{-1}. An average decision limit (CCα; α 1%) and detection capability (CCβ; β 5%) of 0.11 and 0.18 µg kg^{-1}, respectively.[1] was achieved. Valle *et al.,* [14] used pre-column oxidation which converts LMG into MG previous to liquid chromatography–atmospheric pressure chemical ionisation-mass spectroscopy (LC-APCI-MS). The determination of the combined signals provides a gain in sensitivity. The detection limit using the confirmatory ion m/z 313, was 0.15 µg kg^{-1}. The recoveries determined at a spiking level of 2 µg kg^{-1} were 85 and 70 % for LMG and MG respectively, with respective relative standard deviations of 1 and 3 %. Detection of a decomposition product of MG was identified as a peak which appeared about 1 min previous to the MG signal and was identified by LC-MS as desmethyl-MG (m/z 315). On using the APCI interface, the intensity of the signal obtained for MG was found to diminish when the mobile phase was enriched with acetonitrile. Van Rhijn *et al.,* [20] determined MG and LMG without oxidation of LMG to MG by LC-MS/MS. Doerge *et al.,* [17] determined MG and LMG using isotope dilution liquid chromatography atmosphere pressure chemical ionisation mass spectrometry. Tarbin et al., [25] confirmed the presence of MG in trout muscle using electrospray (ESP) MS. Post-column oxidation was used to convert LMG back to the parent MG after chromatographic separation and prior to detection. The procedure was validated down to 2 µg kg^{-1}. The recoveries were in the range of 61 to 94 % with RSD of 4-15 %. Investigation of ESP-MS method without post-column oxidation found that adequate sensitivity could be achieved but repeatability especially for the leuco compound was poor.

Van de Riet *et al.,*[21] developed a liquid chromatography tandem mass spectroscopy with electrospray method for residues of MG and LMG in a number of aquatic species.

Bergwerff et al., [30] determined these residues by LC-ESP-MS/MS with post column oxidation prior to the eluent being introduced into the ionisation chamber. Turnipseed et al., [33] confirmed the identify of MG and LMG residues in salmon using LCMS/MS with no-discharge atmospheric pressure chemical ionisation after their conversion to chromic MG in the extraction process. In this study, ESI was compared with no-discharge APCI for MS/MS determination of MG residues by ion-trap LC/MS. MG is a charged species (not protonated) in solution with a molecular ion at m/z 329. The product ion spectrum obtained by no-discharge APCI was virtually identical to spectrum obtained by ESI. The recoveries of LMG, measured as MG by the method at fortification levels of 1-10 µg kg^{-1} were high (86-109%), with low relative standard deviation (RSD) values (6-13%). The presence of LMG in salmon tissues fortified at 0.25 µg kg^{-1} was confirmed by this method with an average recovery of 70 % and an RSD of 12%. Doerge et al., [37] determined CV and LCV in catfish muscle by LC with atmospheric pressure chemical ionisation (APCI) mass spectroscopy. A full scan APCI mass spectra obtained at different cone voltages from 100 ng on-column injections of CV and LCV showed that at potentials less than 30 V, the spectra consisted predominantly of cationic portions of CV (m/z 372) and protonated molecule of LCV (m/z 374). As the voltage was raised, increasing numbers of fragment ions with different relative intensities due to CID reactions in the transport region of the ion source were observed. In the case of CV, the observed fragmentation is consistent with demethylation and dehydrogenation to form iminium ions. Fragment ions from LCV were consistent with losses of methyl and methyaniline and dimethylaniline moieties. The initial fragment ion of (m/z 359) formed from LCV is consistent with the loss of a methyl radical to yield a cation-radical species. This type of reaction is not a proton transfer typically seen for API-MS. Product ion spectra were obtained for APCI mass spectrometry analysis of LCV in order to determine the fragmentation reactions observed at various cone voltages that produced the ions used to monitor for LCV. As the collision energy was increased the same fragmentation ions as observed in the CID experiment.

Gas chromatography

There has been little work on the analysis of MG and LMG by GC due to their low volatility. A gas chromatographic/mass spectrometric (GC/MS) method was developed to confirm the presence of LMG in cat fish tissue. [27] MG was not sufficiently volatile and decomposes in the gas chromatograph so was removed from sample during clean-up. Both MG and LMG are initially retained on the cartridge, but only LMG is eluted with the acetonitrile buffer mixture prior to GC/MS analysis.

LMG was chromatographed on a cross-linked poly (dimethylsilicone) Hewlett Packard-1 12.5-M capillary column (0.2 mm i.d, 0.33 μm film thickness). LMG was found to fragment into several diagnostic ions. LMG was confirmed by SIM at m/z 330 $[M]^+$, 239 $[M-M]^+$, 253 $[M-Ph]^+$, 210 $[M-C_6H_4N(CH_3)_2]^+$ and 165 $[M]^{++}$. These ions correspond to molecular and fragment ions of relative high abundance and specificity for this compound. Wilson et al., [39] developed a procedure for confirming the identity of LCV in chicken fat by GC-MS/MS. Confirmatory identification of LCV was based on matching the retention times and relative abundances of 6 ions in the extract. Data acquisition consisted of selected ion monitoring at m/z 373, 372, 253, 252, 237 and 126. These m/z values represent the major ions in the EI mass spectrum of LCV. LCV was chromatographed on a DB-1 (100% methyl silicone) capillary column (30 × 0.25 mm, 0.25 μm film thickness). The column temperature was held at 150°C for 1 min, ramped at 16°C min $^{-1}$ to 300°C, held for 20 min. The linear velocity of the helium carrier gas at 300°C was measured at 25.5cm s^{-1}. LCV eluted at about 18 min. The procedure confirmed the identity of LGV in fat samples fortified at 5 μg kg $^{-1}$. All ions except that m/z 126 may be amenable to application below 5 μg kg $^{-1}$. An alternative approach would be to inject more extract so the m/z 126 can be used.

2.17   Conclusions

The determination of MG, LMG, CV and LCV involves liquid extraction using acetonitrile generally as the organic solvent and a buffer system at a low pH of between

3- 4. Sample clean-up is accomplished by washing extracts with dichloromethane alone or inconjunction with cation solid phase extraction. The determination of MG, LMG, CV and LCV is widely achieved using liquid chromatography separations with detection by UV-VIS spectrometry or tandem mass spectrometry.

A confirmatory method developed by Van Rhijn et al., [20] allows the determination of MG and LMG by LC-MS/MS without incorporating the oxidation of LMG to MG either pre-column or post-column. MG and LMG can easily be detected and some of the difficulties associated with post-column or pre-column oxidation are thus eliminated. Since MG is a banned substance, the development of confirmatory methods using tandem mass spectrometry in MRM mode is the most specific and widely used technique.

To date there is no LC-MS/MS method developed utilising a d_5 analogue of MG as internal standard for MG and a d_5 analogue of LMG. These internal standards are the preferred compounds in LC-MS/MS analysis as matrix suppression effects at the retention time of each compound may affect the ionisation efficacy differently. MG and LMG are well separated on reverse phase chromatography systems and ideally using deuterated standards of both substances would correctly evaluate any suppression effects at the retention time of each compound.

There are a number of single residue methods for MG in fish tissues but no methods on the effects that cooking of the fish tissue has on these residues and if there are differences between these cooking effects between different species.

There is also a lack of methods, which target CV and LCV as these are also classed in the same group of B3(e) dyes as MG. These compounds are banned also. It is very difficult to obtain certified reference materials for these compounds and their metabolites and impossible to obtain deuterated internal standards.

There is very limited data on the depletion of the other B3(e) contaminants in fish tissues

Therefore there is a need to develop a multi-residue LC-MS/MS method, which targets MG, LMG and the other Group B 3(e) dyes such as CV, LCV.

Environmental contamination may arise e.g from commercial fish farming where these compounds are given as feed additives, they can accumulate in sediments and may be injested by marine species in the vicinity of farms. Also substantial amounts of medicated feed is not eaten and can pass through the holding cages and be absorbed by marine flora and fauna. Freshwater fish farms are based inland and these compounds may pose a threat to groundwater and human water supplies.

There is no published confirmatory method for the simultaneous determination of MG, LMG, CV and LCV in salmon that is validated according to Commission Decision 2002/657/EC.

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Chapter 3

Development of a Screening Method for the Determination of Robenidine in Eggs by High Performance Liquid Chromatography with UltraViolet Detection

3.1 Introduction

Anticoccidials are compounds used extensively both prophylactically and therapeutically to treat coccidiosis in poultry. Coccidiosis is caused by unicellular organisms belonging to the genus *Eimeria* in the class *Sporozoa* and is the most important parasitic disease of poultry. [1] Robenidine (1,3-bis[(p-chlorobenzylidene) amino]- guanidine monohydrochloride is a common anticoccidial drug used in the poultry industry. The molecular structure of the compound is shown in Figure 3.1. It is authorised for use in poultry and other species under the Additives Directive 70/524/EC. [2] In the European Union according to Directive 70/524/EC, the use of robenidine is not authorised for laying hens. The widespread use of this anticoccidial drug may present a potential risk to the consumer if residues enter the food chain.

A range of methods have been described for the determination of robenidine in feed, including thin layer chromatography [3], a photometric method for feeds and premixes [4], and a LC-UV method for the determination of robenidine and its chemical precursors in feed. [5] A polarographic method for determination of robenidine in tissue, eggs, litter and soil [6], and methods for the determination of robenidine and other anticoccidials by LC-dual mass spectroscopy (MS/MS) in eggs [7,8] have been described. There are no methods available for determination of robenidine in egg by LC with UV detection.

In this study a fast, simple and reliable LC method is described for the detection of robenidine in eggs. The method was validated according to procedures described in Commission Decision 2002/657/EC. [9]
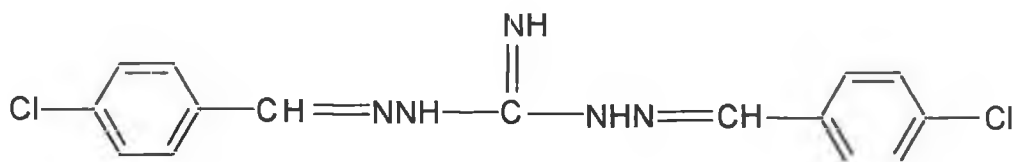
Figure 3.1: *Structure of robenidine (1,3-bis[(p-chlorobenzylidene) amino]- guanidine monohydrochloride*

3.2   Scope of the Research

The objective of this research is the development of a fast, simple and reliable method for the analysis of robenidine in eggs by LC-UV. The advantage of UV detection is that it is commonly found in use in analytical laboratories and more sophisticated techniques such as LC-MS/MS are not accessible to a number of laboratories due to excessive costs. Following the development, the method was validated according to the procedures described in Commission Decision 2002/657/EC. (See introduction). As robenidine is not authorised for use in laying hens, the decision limit (CCα) and detection capability (CCβ) were calculated according to the procedure outlined for banned substances.

3.3   Experimental

3.3.1   Materials and reagents

Water (HiPerSolv grade), acetonitrile (HiPerSolve grade), n-hexane, triethylamine and ammonium dihydrogen phosphate (Analytical grade) were obtained from BDH (Merck, Poole, Dorset, UK). N,N-dimethylformamide (Analytical grade) was obtained from Riedel-de-Haen (Seezle, Hannover, Germany). Robenidine hydrochloride was obtained from QMX (Essex CM6 2PY, UK). Ammonium phosphate buffer (0.05M, pH 6.8) was prepared by dissolving 5.75 g of ammonium dihydrogen phosphate in 800 ml LC grade water then adding triethylamine (BDH, Merck, Poole, Dorset, UK) to yield pH 6.8 and making up to 1 litre with water. A primary stock standard solution of robenidine was prepared in N,N-dimethylformamide at a concentration of 100 μg ml^{-1} and stored at 4 °C. Working standard solutions were prepared daily in acetonitrile:water (65:35, v/v) and stored at 4 °C.

3.3.2   LC conditions

The LC system consisted of a model 600 LC pump (Waters, Milford, USA) and a SIL-10I AVP autoinjector, a CTO-10 AVP column oven, a SPD-10 AVP UV detector operated at 317 nm and a SCL-10 AVP system controller linked to a PC with class-VP data handling software (Shimadzu Corporation, Japan). The mobile phase, consisting of acetonitrile:buffer pH 6.8 (65:35,v/v), was pumped at a flow of 1.5 ml min^{-1}. A stainless-steel Novapak C_{18} analytical column (250 × 4.6 mm I.D.), coupled to a Hypersil C_{18}, Securiguard ^{TM} guard column (3.0 × 2.0 mm. I.D), was used. The column temperature was maintained at 40 °C.

3.3.3   Procedure for egg samples

Egg samples (100 g approximately), prepared from pooling six individual eggs, were homogenised and stored at − 20 °C in 150 ml tubes. Egg samples, from commercial production units, previously analysed using method described in this chapter and found to contain no detectable residues of robenidine were used as negative controls.

3.3.4   Sample extraction and clean-up

Egg (2 g) was weighed into 50 ml polypropylene tubes. Acetonitrile (7.5 ml) was added and the test portion was vortexed (2 min), sonicated (3 min) and shaken on a mechanical shaker (15 min). The test portion was centrifuged (2400g, 10 min, 4 °C). The supernatant was transferred to a clean test-tube and the test portion was extracted with acetonitrile as before. The supernatants were combined and defatted with hexane (5 ml) by mixing on a vortex (30 s). The test portion was centrifuged (1250 x g, 5 min, 4 °C) and the hexane layer removed. The sample extract was evaporated to dryness under nitrogen (60 °C) before dissolving in 500 μl acetonitrile:water (65:35, v/v). An aliquot (100 μl) was injected onto the HPLC column.

3.3.5 Calibration

Standards were prepared in acetonitrile:water (65:35,v/v) at concentrations of 0, 50, 125, 250, 500, 1000 and 2000 ng ml^{-1}, equivalent to 0, 12.5, 31.25, 62.5, 125, 250 and 500 μg kg^{-1} egg, respectively. Calibration curves were prepared by plotting peak area as a function of analyte concentration (0 to 2000 ng ml^{-1}). Concentration of robenidine (ng ml^{-1}) was determined from the peak areas obtained for the test portion extracts, calculated from the calibration curve. The robenidine concentration in egg samples (μg kg^{-1}) was calculated, as follows:

Robenidine (μg kg^{-1} egg)

= robenidine (ng ml^{-1} extract) x 0.5 / 2

Recovery was measured from the peak areas obtained for fortified sample extracts, calculated from the calibration curve

3.3.6 Method validation

For estimation of recovery, blank egg samples were prepared and fortified with robenidine. Test portions (2 g) were fortified at 50, 75 and 100 μg kg^{-1} by adding 50, 75 and 100 μl portions of a 2000 ng ml^{-1} robenidine solution, respectively. After fortification, the test portions were held for 15 min prior to extraction with acetonitrile, as described above. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. For estimation of the precision of the method, within assay repeatability was calculated. The decision limit (CCα) and the detection capability (CCβ) of the method were calculated.

3.4 Results and Discussion

3.4.1 Preliminary experiments

An LC method, developed to analyse for robenidine in animal feed, was applied to the separation of robenidine on a C_{18} column with a mobile phase consisting of acetonitrile:water:potassium di-hydrogen phosphate:di-sodium hydrogen phosphate (65:25:5:5, v/v/v/v). [5] The peak shape was poor, with excessive tailing, using this mobile phase. Investigation of various LC conditions, including various percentages of organic content in the mobile phase, pH, injection volume and mobile phase flow rate, showed that a mobile phase consisting of 65% acetonitrile and 35% phosphate buffer, adjusted with triethylamine to pH 6.8, an injection volume of 100 μl and a flow rate of 1.5 ml min^{-1} gave good peak retention and shape. Following preliminary extraction and liquid partitioning between hexane and acetonitrile no further clean-up of the sample extracts using solid phase extraction was deemed necessary as the chromatograms were free of matrix interference. As no additional steps were necessary this satisfied the aim of developing a simple quantitative test method with a minimum of manipulations.

3.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [9] covering specificity, calibration curve linearity, recovery, repeatability, decision limit (CCα) and detection capability (CCβ).

3.4.2.1 Specificity

To establish the specificity of the method, egg samples fortified with robenidine and non-fortified samples were analysed. No interfering peaks were observed at the retention time for the robenidine peak (Figures 3.2 and 3.3).
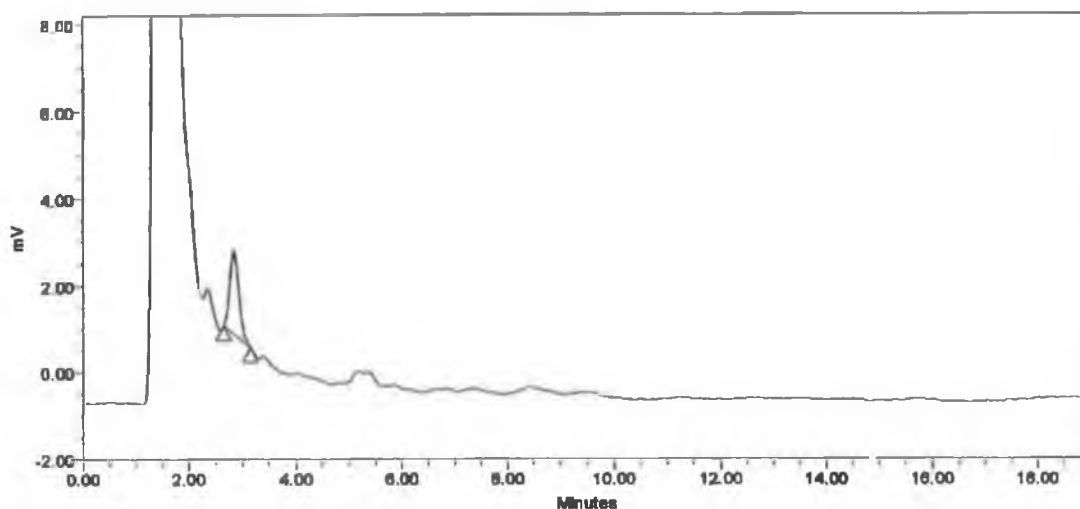
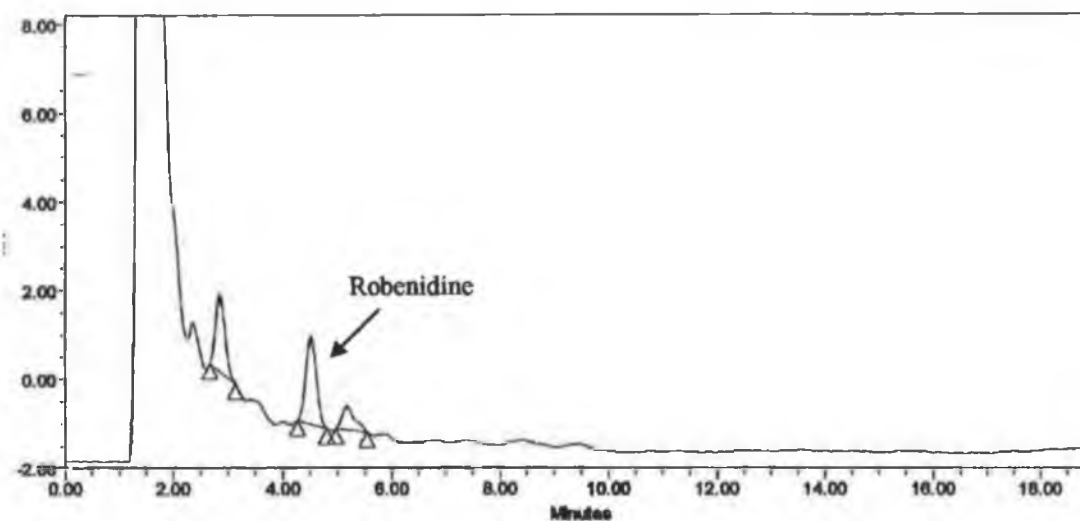**Figure 3.2:** Chromatogram of negative control egg sample

Figure 3.3: Chromatogram of negative control egg sample spiked at 50 μg kg^{-1}

3.4.2.2 Linearity of the response

The linearity of the LC-UV response was evaluated with a six-point calibration curve in the concentration range 0-2000 ng/ml (0-500 μg kg^{-1} egg). The regression coefficients (r^2) of the calibration curves were 0.98-0.99.

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3.4.2.3 Recovery

The recovery of the method was determined using egg samples fortified at 50, 75 and 100 $\mu g\ kg^{-1}$. Mean recovery (n=6) at each of the three levels of fortification and determined in three separate assays (n=54, total), was between 79 and 105 % (Table 3.1).

Table 3.1: Intra- and inter-assay variation for recovery of robenidine from egg

Analyte	Fortification level ($\mu g\ kg^{-1}$)	Recovery (%)							
		Assay 1		Assay 2		Assay 3		Inter-assay	
		Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
Robenidine	50	79.5	18.0	94.1	9.5	94.5	6.2	89	13.5
	75	95.8	6.7	105.0	8.9	102.8	7.0	101	8.2
	100	85.1	12.4	100.1	4.2	93.9	4.4	93	10.0

3.4.2.4 Repeatability

The inter-assay repeatability is shown in Table 3.1. At the three levels of fortification RSD values are $\leq$ 13.5 %. The repeatability of the method is satisfactory.

3.4.2.5 CCα and CCβ

The decision limit (CCα), in the case of substances for which no MRLs have been set, is the lowest concentration level at which it can be concluded that a sample is non-compliant, with an error probability of α=1%. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with an error of probability of β= 5%. Both of these parameters were determined using the calibration curve procedure in which blank egg samples were fortified at the minimum required performance level of the method (50 $\mu g\ kg^{-1}$) and at 1.5 and 2 times that level. CCα and CCβ values of 10 and 17 $\mu g\ kg^{-1}$, respectively, were determined.

3.5 Conclusions

This study reports the development and validation of a fast and simple LC method for the determination of robenidine in eggs. The method was validated according to the guidelines described in Commission Decision 2002/657/EC.

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Chapter 4

Development of a Multi-Residue Method for the Determination of Benzimidazoles in Animal Liver by High Performance Liquid Chromatography with UltraViolet Detection

Relevant publication: See Chapter 7

4.1 Introduction

Benzimidazoles are broad-spectrum anthelmintics and have been used for about 40 years in animal health and crop protection. Metabolism of the drugs is extensive. The metabolites found depend on the structure of the parent drug, the tissue and the animal species. One or more metabolites can be found in animal tissue for most drugs. [1,2] When the pro-drugs, active drugs and metabolites are counted, some 19 possible residues may occur in animal tissues. The EU has set maximum residue limits (MRLs) for benzimidazoles and their metabolites in animal products. The MRL values range from 10 to 1000 µg kg^{-1}, depending on the compound and matrix. Most benzimidazole drugs, with the exception of cambendazole, are licensed for use in food-producing animals. The development of a single multi-residue method capable of testing for all the residues in the MRL listings is very difficult.

Netobimin (NETO), albendazole (ABZ) and albendazole sulphoxide (ABZ-SO) are licensed for use in the treatment of cattle and sheep. ABZ-SO was used for treatment of pheasants, but because an analytical method was not satisfactorily validated for pheasant tissues, no final MRL values could be proposed for pheasants. [3] NETO is a prodrug, converted by the gut microflora to ABZ by splitting of the side chain and formation of the benzimidazole nucleus, and ABZ is converted further to ABZ-SO. In cattle, ABZ-SO, albendazole sulphone (ABZ-SO$_2$) and 2-amino albendazole sulphone (NH$_2$ABZ-SO$_2$) were found to be the major residues detected in liver. Febantel (FEB), fenbendazole (FBZ) and oxfendazole, or fenbendazole sulphoxide, (OFZ/FBZ-SO) are licensed for use in cattle, pigs, sheep and horses. FEB is a prodrug, converted to FBZ by cyclization to form the benzimidazole nucleus. Animal studies showed that FBZ, OFZ/FBZ-SO and fenbendazole sulphone (FBZ-SO$_2$) are the important residues. [4-8] However, depending on species, route of administration and time after treatment, the forms of residues may vary greatly. Mebendazole (MBZ) is approved for use in goats, horses and sheep. When sheep were treated with MBZ it was found that the major residues were hydroxy-mebendazole (MBZ-OH) and MBZ. [9] Thiabendazole (TBZ) is licensed for use in cattle. In studies on cattle [7] and sheep [10], residues of TBZ and hydroxy-thiabendazole (TBZ-OH) were found

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in liver. Flubendazole (FLU) is approved for use in pigs, chicken, turkeys and game birds. FLU and amino-flubendazole (NH_2FLU) are the important residues. In turkeys treated with FLU, residues were found in all tissues, with NH_2FLU accounting for 68 % of the total residues in liver. [11] Triclabendazole (TCB) is licensed for use in sheep and cattle. In cattle treated with radiolabelled TCB highest residue levels were detected in liver after a short withdrawal period. [12] In a study on lactating goats treated with TCB it was found that triclabendazole sulphoxide (TCB-SO) and triclabendazole sulphone (TCB-SO_2) residues could be detected in milk. [13] Oxibendazole (OXI) is licensed for use in pigs. The marker residue is the parent drug. In studies on cattle, sheep, pigs and horses, total OXI residues were found to be less than 100 μg kg^{-1} in muscle and fat.

There are many published methods for analysis of benzimidazoles in animal tissues, plants and milk products. A number of methods have been reported for the analysis of individual benzimidazoles and their metabolites in food products. [14-17] However, because of the large number of benzimidazoles licensed for use, multi-residue methods are more attractive. Multi-residue methods have been developed for the analysis of benzimidazole residues in milk [18-24] and tissues. [25] TBZ has been determined in fruit and vegetable matrices based on an ethyl acetate extraction. [26-28] Alternative solvents, such as methanol [29] and acetone [30, 31] have been used. Supercritical fluid extraction (SFE) has been used for extracting benzimidazole fungicides from fruit and vegetable matrices [32, 33] and from animal tissues. [34] Accelerated solvent extraction has been used to extract TBZ from fruit and vegetable samples and processed products. [35] Matrix solid phase extraction (MSPD) has been applied by a number of groups for the determination of benzimidazoles in liver. [6-45]

A combination of liquid-liquid partitioning and solid phase extraction is the most widely adopted approach for the clean-up of animal tissue extracts in benzimidazole residue analysis. Wilson et al., [7] purified tissue extracts by partitioning between ethanol: 0.2 N hydrochloric acid and hexane before clean-up on a C_2 solid phase extraction cartridge. Roudaut et al., [36] modified the method by substituting an Oasis HLB cartridge for the C_2 cartridge. Similar liquid-liquid partitioning approaches and further purification of tissue

extracts by SPE have used C_{18} [37], aminopropyl [38], cyano [39], neutral alumina [40] and silica [41] SPE cartridges. Rose *et al.,* [42] applied strong cation exchange (SCX) SPE to the isolation of OFZ and nine related residues in liver. Farrington *et al.,* [43] developed multi-residue methods using dual SPE clean-up procedures based on normal phase and C_{18} sorbents. Stubbings *et al.,* [44] developed an online SCX-SPE procedure for the purification of benzimidazole residues from tissue. Hiemstra *et al.,* [31] developed a clean-up procedure for the isolation of TBZ and carbendazim residues from fruit and vegetable extracts, using liquid-liquid partitioning prior to automated SPE clean-up on diol SPE cartridges mounted on an ASPEC system.

4.2   Scope of the Research

The objective of this work was to develop an improved, automated, quantitative method for determination of an extended range of benzimidazoles in liver within a single multi-residue method by LC-UV. The advantage of UV detection is that it is commonly found in use in analytical laboratories and more sophisticated techniques such as LC-MS/MS are not accessible to a number of laboratories due to excessive costs.

Most of the published methods, such as that by Wilson *et al.,* [7], deal only with the determination of some of the benzimidazole drugs. Using the approach of identifying the most likely residues to occur in different animal species, a multi-residue method might be developed that would provide more complete surveillance for these compounds. Following the development, the method was validated according to the procedures described in Commission Decision 2002/657/EC. As the benzimidazoles are authorised for use, the decision limit (CCα) and detection capability (CCβ) were calculated according to the procedure outlined for authorised substances.

4.3   Experimental

4.3.1 Materials and reagents

Water, methanol, acetonitrile, ethyl acetate and dichloromethane (HiPerSolv grade) were obtained from BDH (Merck, Poole, Dorset, UK). Triethylamine, glacial acetic acid,

ammonia, hydrochloric acid, dimethyl sulphoxide, acetone, hexane, sodium chloride and ammonium dihydrogen phosphate (Analytical grade) were from BDH. Ammonium hydroxide (ACS grade) was obtained from Sigma (St. Louis, MO, USA). N,N-dimethylformamide and sodium bicarbonate (Analytical grade) were obtained from Riedel-de-Haën (Seezle, Hannover, Germany). Mebendazole (MBZ), thiabendazole (TBZ), albendazole (ABZ) and oxibendazole (OXI) were purchased from Sigma. Flubendazole (FLU), amino-flubendazole (NH_2FLU) and hydroxy-mebendazole (MBZ-OH) were donated by Janssen (Beerse, Belgium). Fenbendazole sulphone ($FBZ-SO_2$) and fenbendazole sulphoxide (OFZ/FBZ-SO) were donated by Intervet (Dublin, Ireland). Albendazole sulphoxide (ABZ-SO), albendazole sulphone ($ABZ-SO_2$) and 2-amino albendazole sulphone ($NH_2ABZ-SO_2$) were donated by GlaxoSmithKline (Harlow, UK). Primary stock standard solutions of TBZ in methanol; OXI, MBZ, OFZ/FBZ-SO and $FBZ-SO_2$ in dimethylsulphoxide; MBZ-OH in N,N-dimethylformamide; FLU and NH_2FLU in N,N-dimethylformamide:methanol (3:2, v/v); $NH_2ABZ-SO_2$, ABZ, ABZ-SO and $ABZ-SO_2$ in N,N-dimethylformamide:methanol (2:8, v/v), were prepared at a concentration of 2 mg ml^{-1}. Working standard solutions were prepared in methanol and stored at 4°C. Bond Elut™ C_{18} cartridges (3ml, 500 mg) were obtained from Varian (Harbor City, USA), Solid phase extraction was performed using an ASPEC XL4 instrument (Gilson, Middleton, USA).

4.3.2 LC conditions

The LC system consisted of a model 616 HPLC pump with a model 717 plus autosampler and a model 996 photodiode array detector operated at 298 nm (Waters, Milford, MA, USA). A gradient LC system (Table 4.1) using ammonium dihydrogen phosphate buffer (pH 6.8), methanol and acetonitrile at a flow of 0.5 ml min^{-1}, was used to separate the analytes on a stainless-steel analytical column (150 × 3.0 mm I.D.) equipped with a guard column (3.0 × 2.0 mm I.D.), both packed with Xterra™ (3.5 μm) C_{18} material (Waters). The column temperature was maintained at 40°C. Data acquisition and integration were performed using Millenium[32] chromatographic management software (Waters).

Table 4.1: HPLC gradient profile for benzimidazoles

Time (min)	Component A (%)	Component B (%)	Component C (%)
0	100	0	0
3	45	55	0
10.5	30	70	0
14	15	85	0
18	0	59	41
23	0	0	100
36	0	60	40
43	100	0	0

A: Ammonium dihydrogen buffer pH 6.8 + methanol + acetonitrile (76 + 15 + 9, v/v/v).

B: Ammonium dihydrogen buffer pH 6.8 + methanol + acetonitrile (52 + 30 +18, v/v/v).

C: Ammonium dihydrogen buffer pH 6.8 + methanol (18 + 82, v/v).

4.3.3 Procedure for liver samples

Liver samples (100 g approximately) were homogenised in a Robot Coupe™ bowl-blender (Montceau-en-Bourgogne, France) and stored at -20°C in 150 ml tubes. Liver samples previously analysed using method described in this chapter and found to contain no detectable residues of the analytes were used as negative controls. For the preparation of fortified liver samples, 3 g portions of negative control liver were weighed into 50 ml test tubes. Samples were fortified at levels corresponding to 0.5 × MRL, 1 × MRL and 1.5 × MRL by adding 50 µl portions of diluted stock solutions. After fortification, samples were held for 15 min prior to extraction.

4.3.4 Sample extraction and clean-up

Sodium sulphate (1.5 g), 4 M potassium carbonate (0.5 ml) and ethyl acetate (5 ml) were added and the samples were homogenised (30 sec). Samples were centrifuged (2500 rpm, 10 min, 4 °C). The supernatant was transferred to a clean test-tube and the sample was re-extracted with ethyl acetate as before. The supernatants were combined and evaporated to dryness under nitrogen (50°C). Ethanol: 0.2N hydrochloric acid (66:33, v/v) (1 ml) and n-hexane (5 ml) were added to the dry residue, vortexed (2 min) and centrifuged (1500 rpm, 5 min , 4°C). The upper hexane phase was removed. This clean-up step was repeated with a second portion of hexane (5 ml). Sample extracts were

further purified by automated SPE using C_{18} SPE cartridges. Sample extracts (0.6 ml) were modified by addition of 2% potassium bicarbonate solution (4 ml). Modified sample extracts (3.8 ml) were loaded onto C_{18} cartridges (preconditioned with 6 ml ethyl acetate, 3 ml ethanol and 3 ml distilled water). The cartridges were washed with distilled water (2 × 1 ml) and then dried. The C_{18} cartridges were eluted with ethyl acetate (3 × 1 ml). The eluates were reduced to dryness under nitrogen ($50^{\circ}C$) before dissolving in 400 µl methanol:water (50:50, v/v). An aliquot (50 µl) was injected onto the LC column.

4.3.5 Calibration

Standards were prepared at concentrations of 0, 0.25, 0.50, 0.75, 1.5, 3.75, 7.5 and 15 µg ml^{-1} in methanol:water (50:50, v/v). Calibration curves were prepared by plotting peak area as a function of analyte concentration (0 to 15 µg ml^{-1}). Recovery was determined using the peak area values obtained for fortified sample extracts, calculated from the calibration curves.

4.3.6 Method validation

For estimation of recovery, blank liver samples were fortified with benzimidazoles at 0.5, 1 and 1.5 times the MRLs. For estimation of the precision of the method, an intra-assay study was carried out by fortifying bovine liver samples at each level (n = 6) and extracting and analysing in a single run. The inter-assay study involved fortifying bovine liver samples as described and extracting and analysing in three separate assays. The decision limit (CCα) and the detection capability (CCβ) of the method were calculated using the intra-assay validation results. The selectivity/specificity and linearity of the method were also established.

4.4 Results and Discussion

4.4.1 Preliminary experiments

Previous work in this laboratory had developed a ternary gradient LC method for separation of 19 benzimidazole drugs, with detection at 298 nm. [34] This method was applied to the separation of benzimidazole drugs in this study. From an evaluation of different extraction solvents, ethyl acetate was found to give the best recovery for the compounds of interest. Ethyl acetate has good solvating power for weakly basic drugs because it can provide emulsion-free extraction from liver and muscle tissue samples. Multi-residue extraction of benzimidazoles at a set pH is difficult because of their varied, and in some cases multiple, pKa values. The effect of pH was studied over the range pH 5 to pH 10, based on a method described by Cannavan *et al.*, [39] for extraction of TBZ from tissue using buffering at pH 7 and extraction with ethyl acetate. The possibility of using the ChemElut™ diatomaceous earth buffered and unbuffered columns was also evaluated but low recoveries were obtained. Wilson *et al.*, [7] developed an extraction procedure based on carbonate buffer/ethyl acetate for ABZ, TBZ-OH, OFZ, TBZ, CAM, MBZ and FBZ. Similarly, carbonate buffer extraction and liquid partitioning steps were used by Roudaut *et al.*, [36] for TBZ-OH, TBZ, NH_2ABZSO_2, ABZ-SO, OXI, OFZ, $ABZ-SO_2$, ABZ and FBZ. After extraction, liquid partitioning between hexane and ethanol:hydrochloric acid, 0.2 N (33:66 v/v) was used in both these methods. Further clean-up of the tissue extracts was achieved by solid phase extraction on C_2 cartridges [7] or on Oasis HLB™ cartridges. [36]

A carbonate buffer/ethyl acetate extraction and liquid partitioning, followed by solid phase extraction using C_{18} cartridges was developed in this work and applied to a wider range of benzimidazoles to include MBZ-OH, NH_2FLU, $FBZ-SO_2$ and FLU. TBZ-OH, which is a marker residue for thiabendazole, was not included because of incomplete chromatographic separation of this analyte from NH_2ABZSO_2; preliminary experiments indicate that TBZ-OH is extracted with similar yield as TBZ using the method. In total, drugs from six of the seven classes of benzimidazoles listed are included; triclabendazole and its metabolites are not extractable using this method (Table 2:1).

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Hiemstra *et al.,* [31] developed an automated SPE clean-up on diol cartridges for determination of thiabendazole and carbendazim residues in fruit and vegetable extracts. The C_{18} solid phase extraction step in the current method was automated using an ASPEC XL4™ system. Compared with the method described by Wilson *et al.,* [7] the method developed here incorporates automated SPE on C_{18} cartridges and has been applied to an extended range of benzimidazoles, including most of the marker residues designated by EU MRL regulations.

4.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [46] covering specificity, calibration curve linearity, recovery, repeatability, decision limit (CCα) and detection capability (CCβ).

The method was validated using bovine liver, as a model matrix, and applying the MRL values specified for liver of each species. Preliminary experiments with liver samples of other species indicate that comparable extraction yield is obtainable and that the developed method may be applicable to liver samples of other species. While most MRL values are expressed as the sum of the marker residues, validation was undertaken applying the MRL value for each individual marker residue; this was necessary because of the variability in the proportions of marker residues which might occur in samples. For benzimidazoles for which MRL values have not been specified for a particular species, the method would require further validation according to the procedures described in Commission Decision 2002/657/EC for substances for which no permitted limit has been established.

4.4.2.1 Specificity

To establish the selectivity/specificity of the method, liver samples fortified with the benzimidazoles and non-fortified samples were analysed. No interfering peaks were observed at the retention time for the benzimidazoles (Figure 4.1) except at the retention time for ABZ where an interfering peak of negligible size, relative to the response for MRL concentration, was observed.
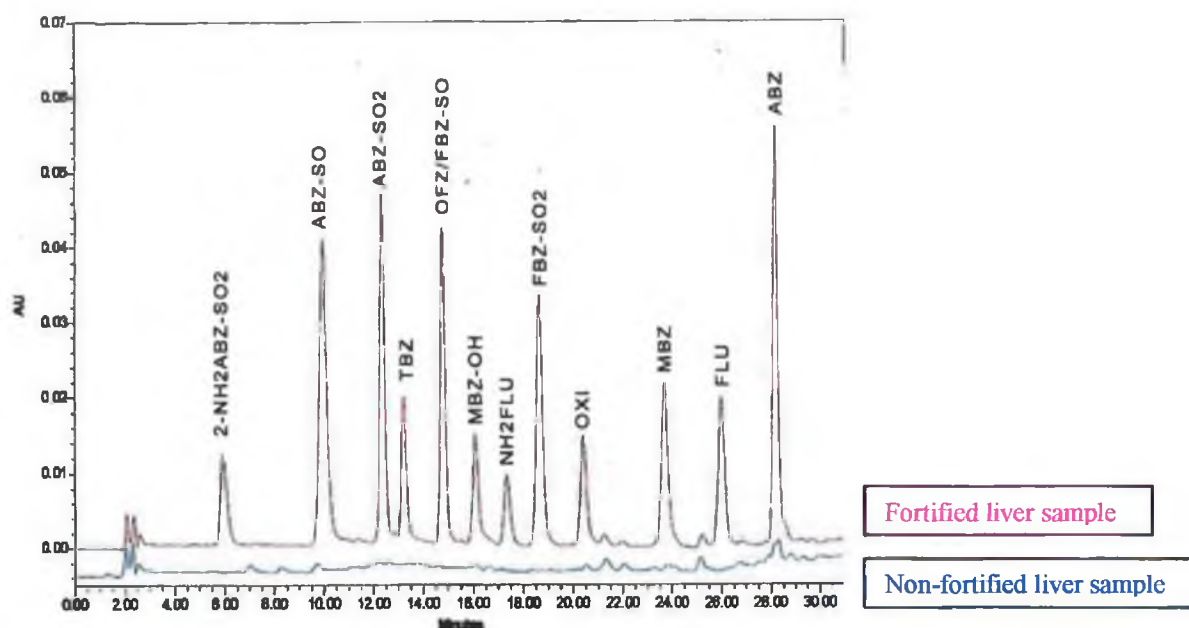

Figure 4.1 Chromatogram of blank liver sample and sample fortified at the MRLs

4.4.2.2 Linearity of the response

The linearity of the chromatographic response was tested with 7 calibration points in the concentration range of 0.25 to 15 μg ml^{-1}. The regression coefficients (r^2) for the calibration curves used in the study were $\geq$ 0.99.

4.4.2.3 Recovery

The accuracy of the method was determined using bovine liver samples fortified at $0.5 \times$ MRL, MRL and $1.5 \times$ MRL for each benzimidazole. Mean recovery (n = 6) of the analytes, determined in three separate assays, was between 60 and 100 % for ABZ-SO, ABZ-SO$_2$, TBZ, OFZ/FBZ-SO, MBZ-OH, FBZ-SO$_2$, OXI, MBZ and FLU (Table 4.2). Lower mean recoveries of approximately 50 % were obtained for NH$_2$FLU and ABZ. NH$_2$ABZ-SO$_2$ was recovered at approximately 25 %, probably due to the difficulty in extracting this relatively polar compound from tissue.

4.4.2.4 Repeatability

The inter-assay repeatability is shown in Table 4.2. For most analytes and at the three levels of fortification ($0.5 \times$ MRL, MRL and $1.5 \times$ MRL), RSD values were at less than 25 %. In some instances (NH$_2$FLU at $0.5 \times$ MRL, OXI at MRL, and ABZ at MRL), RSD values were in excess of 25 %. More work involving incorporation of an internal standard would be necessary for these compounds. Overall, the repeatability of the method for benzimidazoles is satisfactory.

4.4.2.5 CCα and CCβ

The decision limit (CCα) is, in the case of substances for which MRLs have been defined, the lowest concentration level at which it can be concluded that a sample is non-compliant, with an error probability of $\alpha = 5$ %. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with an error probability of $\beta = 5$ %. Both of these parameters were determined using the calibration curve procedure, in which blank bovine liver was fortified around the MRL values in equidistant steps ($0.5 \times$ MRL, MRL, $1.5 \times$ MRL). The concentration at the MRL plus 1.64 times the standard deviation at the MRL gives the CCα value for each benzimidazole. The CCβ value for each benzimidazole is determined as the CCα value + 1.64 times the standard deviation. Table 4.3 lists the CCα and CCβ values for the benzimadazoles, using the data from Assay 1. With the exception of NH$_2$ABZ-

SO2, CCα values ranged between MRL + 12 % and MRL + 25 % and CCβ values ranged between MRL + 25 % and MRL + 45 % for the benzimidazoles included in the study.

Table 4.2: Intra and inter-assay variability for the recovery of benzimidazoles from liver

Analyte	Fortification level ($\mu g\ kg^{-1}$)	Recovery (%) Assay 1 Mean	R.S.D	Assay 2 Mean	R.S.D	Assay 3 Mean	R.S.D	Inter-assay Mean	R.S.D
NH$_2$ABZ-SO$_2$	500	27	10.0	22	17.8	26	15.4	25	15.9
	1000	24	10.2	22	3.3	23	6.7	23	7.7
	1500	24	12.4	19	12.2	19	21.9	20	16.4
ABZ-SO	500	68	9.3	60	11.0	73	6.4	67	11.7
	1000	62	8.7	57	5.3	62	6.3	61	7.5
	1500	61	9.8	61	1.1	52	6.5	57	10.2
ABZ-SO$_2$	500	75	6.3	61	8.2	73	10.5	70	12.1
	1000	72	9.0	58	8.7	69	17.1	67	15.1
	1500	66	7.0	62	1.7	63	10.9	64	7.8
TBZ	50	74	8.6	63	24	96	4.6	78	21.8
	100	82	8.9	56	5.4	85	9.1	76	19.2
	150	83	5.5	66	8.7	78	7.2	76	11.4
OFZ/FBZ-SO	250	85	5.2	61	5.8	73	6.6	73	15.1
	500	83	7.3	57	9.3	67	18.5	69	19.6
	750	83	7.0	64	2.6	62	8.9	70	15.8
MBZ-OH	200	83	6.3	63	6.7	103	14.2	83	21.3
	400	89	8.4	60	10.9	77	20.5	76	20.9
	600	83	7.1	61	5.1	64	8.5	70	16.3
NH$_2$FLU	150	62	6.1	40	12.2	35	9.1	46	27.7
	300	68	10.5	39	5.7	54	16.7	54	24.3
	450	62	7.7	40	14	56	14.5	54	20.7
FBZ-SO$_2$	250	87	4.9	58	4.0	74	5.9	73	17.4
	500	86	9.2	55	7.8	71	24.1	71	23.5
	750	84	7.3	62	2.8	59	16.3	69	19.2
OXI	100	92	4.3	69	4.9	120	13.6	93	24.9
	200	90	10.1	61	7.3	119	22.1	94	32.5
	300	85	7.0	66	2.1	76	18.2	76	15.3
MBZ	200	94	3.6	67	6.5	100	5.2	87	17.6
	400	87	6.0	59	7.5	84	23.2	77	22.8
	600	79	8.1	61	3.4	65	21.7	69	17.2
FLU	200	78	3.0	54	7.7	85	8.2	72	19.9
	400	78	7.5	48	8.1	67	15.2	65	21.4
	600	69	8.9	53	5.2	54	20.8	59	17.8
ABZ	500	63	3.2	39	7.3	56	6.9	53	20.6
	1000	59	9.0	36	8.4	48	30.4	48	27.4
	1500	54	9.2	40	12.0	44	21.3	46	19.0

Table 4.3: Calculated CCα and CCβ values based on data from Assay 1 (Table 4.2)

Benzimidazole	MRL (μg kg^{-1})	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
NH$_2$ABZ-SO$_2$	1000	1303	1556
ABZ-SO	1000	1164	1343
ABZ-SO$_2$	1000	1225	1417
TBZ	100	116	132
OFZ/FBZ-SO	500	561	627
MBZ-OH	400	481	544
NH$_2$FLU	300	376	435
FBZ-SO$_2$	500	587	670
OXI	200	242	281
MBZ	400	467	520
FLU	400	493	558
ABZ	1000	1216	1397

4.5 Conclusions

A method was developed and validated for quantitative analysis of 11 benzimidazole veterinary drugs in animal liver; ABZ, ABZ-SO and ABZ-SO$_2$, TBZ, OFX/FBZ-SO and FBZ-SO$_2$, MBZ and MBZ-OH, FLU and NH$_2$FLU, and OXI. TCB and its metabolites, TCB-SO and TCB-SO$_2$, were not quantitatively determined using this method. This multi-residue method covers six of the seven benzimidazole groups that are licensed for use within the EU. Future work might concentrate on the development of a separate method for the TCB group to allow complete surveillance for the mis-use of the benzimidazole drugs in agriculture. To date however there is no method available that can screen for all the benzimidazole metabolites. The development of such a method could be accomplished using the technique of derivitisation.

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Chapter 5

Confirmatory Analysis of Malachite Green, Leucomalachite Green, Crystal Violet and Leucocrystal violet in Salmon by Liquid Chromatography Tandem Mass Spectrometry

Relevant Publication: See Chapter 7

Article in press

Chapter 5

Confirmatory Analysis of Malachite Green, Leucomalachite Green, Crystal Violet and Leucocrystal violet in Salmon by Liquid Chromatography Tandem Mass Spectrometry

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5.1 Introduction

Malachite green (MG) and crystal violet (CV) are triphenylmethane dyes. MG has been used as a topical fungicide and antiprotozoal agent in salmonid farming throughout the world for over 60 years. Few known chemicals are as effective in treating ectoparasites of fish and fish eggs by fungi of the genus *Saprolegnia* and by the protozoan parasite *Ichthyophthirius multifiliis.* [1] CV is also known to be effective in the treatment of fungal infections. It had found widespread use as a feed additive to inhibit mould and fungal growth in poultry feed before 1990. [2] According to EU law, all substances for veterinary use need to be included in annexes 1, 2 or 3 of Regulation 2377/90. MG or CV, however, are so-called "non-defended" compounds that have never been registered for veterinary use. As a result of these anti-fungal properties there is a serious potential for the mis-use of these drugs in aquaculture. Recently there has been a number of reports of these residues in aquaculture products (cf. EC Rapid Alert Reports for Food and Feed 2003/2004/2005/2006). Concerns regarding residues of MG in aquaculture products have prompted studies of the pharmacokinetics and metabolism of this compound. However there is limited data regarding the pharmacokinetics and metabolism of CV. Leucomalachite green (LMG) and leucocrystal violet (LCV) are formed by the metabolic reduction of MG and CV and in this form the drug persists in the tissues of exposed fish. [3,4]

The U.S Food and Drug Administration explicitly banned the use of MG in fish farming in 1991 due to its suspected carcinogenic properties. MG and CV are structurally related to other triphenylmethane dyes such as rosaniline which has been linked to increased risk of human bladder cancer. The leuco form of rosaniline induces renal, hepatic and lung tumors in mice. [5] CV has been implicated in the induction of thyroid and liver tumors. [6] Studies show that LMG is an *in-vivo* mutagen and the mutagenicity of MG and LMG correlates with their tumorgenicities in mice. [7] MG has also been shown to cause DNA damage. [8] CV was found to be cytotoxic to mammalian cells [9] as was MG. [10] Compared to LMG, MG is much less toxic to cells. [10] No safe levels for the presence of MG, LMG,

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CV and LCV in fish for human consumption could be established and therefore, detection of these compounds at sub μg kg^{-1} levels is required.

Within the EU, each member state is required to monitor for MG and LMG residues with analytical methodology that at least meets the Minimum Required Performance Limit (MRPL). The MRPL, a quality parameter for residue laboratories, is set as the sum of MG and LMG at 2 μg kg^{-1}.[11] There is no MRPL set for CV so developed methods must be capable of monitoring levels as low as is reasonably achievable.

Most methods currently used are based on the solvent extraction of MG and LMG from fish tissues using acetonitrile or methanol with aqueous buffer (pH 3-4.5). [12-25] In the clean-up of these samples a combination of liquid/liquid partitioning and solid phase extraction is used. Bergwerff *et al.,* [21] purified catfish, eel, trout, turbot and prawn samples by partitioning acetonitrile:McIIIvaine pH 3 buffer (90:10, v/v) against chloroform before passing the chloroform extract through a Bakerbond [TM] strong cation exchange solid phase extraction cartridge. This clean-up approach has been adopted by other groups using a single solid phase extraction cartridge [12,20] or using dual solid phase extraction with aluminia and propylsulphonic acid cartridges. [3,16,19] Van Rhijn *et al.,* [18] modified the method of Bergwerff *et al.,* [21] by eliminating the liquid/liquid partitioning step. Other methodologies eliminated the liquid/liquid partitioning steps and purified tissue extracts directly using C$_{18}$ [24] and cyano [22] SPE cartridges. Swarbrick *et al.,* used an alternative clean-up using activated charcoal instead of C$_{18}$ SPE. [26]

A number of methods have been reported for the analysis of MG,LMG,CV and LCV residues by visible light detection with oxidation of the leucometabolites carried out either pre-column or post-column. [3,14,16,19,20,21,23,27] Other methods developed for the detection of these residues are based on this oxidation but with detection by mass spectrometry. [12,13,20,21] Detection of these residues by mass spectrometry in the absence of this oxidation has also been accomplished. [15,18,24] An alternative oxidation approach using 2,3-dichloro-5,6-dicyano-1,4- benzoquinone (DDQ) before the determination step to

oxidise LMG to MG has also been utilised. [30,31] Determination based on electrochemical detection, [27] GC-MS, [23] and spectrophotometry [17] has also been achieved.

The method developed in this study was based on the method developed by Van Rhijn *et al.*, [18] but adapted to include CV, LCV, and deuterated internal standards for LMG and for MG. The method omits the often-proposed pre-column or post-column oxidation step used in the determination of MG or CV. The method also omits the liquid/liquid partitioning stage often used in the clean-up of extracts containing these residues. The method is a simple extraction with acetonitrile:McIlvaine buffer pH 3 (90:10, v/v), with clean-up using aromatic sulphonic acid solid phase extraction and LC-MS/MS analysis. The applicability of the method for regulatory control will be discussed.

5.2 Scope of the Research

The objective of this work was to develop a multi-residue confirmatory method for the determination of MG, LMG, CV and LCV by LC-MS/MS. The advantage of MS detection is that it provides unambiguous identification of unknowns and this technique is the method of analysis of choice in screening for banned substances.

To date there is no method that simultaneously determines MG, LMG, CV and LCV by LC-MS/MS that is validated according to the guidelines of 2002/657/EC. Following the development, the method was validated according to the procedures described in Commission Decision 2002/657/EC. As these dyes are not authorised for use, the decision limit (CCα) and detection capability (CCβ) were calculated according to the procedure outlined for unauthorised substances.

5.3 Experimental

5.3.1 Materials and reagents

Water, methanol, acetonitrile, ethyl acetate and hexane (HiPerSolv grade) were obtained from BDH (Merck, Poole, Dorset, UK). Ammonium hydroxide (ACS grade) was obtained from Sigma (St. Louis, MO, USA). Citric acid (ACS grade) was obtained from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate (ACS grade) was obtained from Sigma (St. Louis, MO, USA). Ascorbic acid (ACS grade) was obtained from Fluka

(CH-9470 Buchs, Switzerland). MG, LMG and CV were purchased from Sigma. d_5-MG and d_5-LMG were obtained from Witega (Berlin, Germany). LCV was purchased from Dr. Ehrenstorfer (QMX Laboratories, UK). Primary stock standard solutions of MG, LMG, d_5-MG, d_5-LMG, CV and LCV were prepared in acetonitrile at a concentration of 100 µg ml^{-1}. Intermediate single standards solutions of MG, LMG, d_5-MG, d_5-LMG, CV and LCV were prepared in acetonitrile:water (50:50, v/v) at a concentration of 1 µg ml^{-1}. Mixed standard fortification solutions of MG, LMG, CV and LCV were prepared in acetonitrile:water (50:50, v/v) at concentrations of 50 ng ml^{-1} and 200 ng ml^{-1}. Mixed standard fortification solution of d_5-MG, d_5-LMG was prepared in acetonitrile:water (50:50, v/v) at a concentration of 100 ng ml^{-1}. All standards were stored at 4 °C. Bakerbond ™ SCX cation exchange solid phase extraction cartridges (3 ml, 500 mg) were obtained from J.T Baker (Harbor City, USA). McIIIvaine pH 3 buffer was prepared by adding 0.1 M citric acid (250 ml) and adjusting to pH 3 with 0.2 M disodium hydrogen phosphate and diluting to 1000 ml with water. Extraction solvent was prepared by making a solution in the ratio of acetonitrile:McIIIvain pH 3 buffer (90:10, v/v). A 2 % ammonium hydroxide solution in methanol was used as the solid phase extraction elution solvent. Injection solvent consisted of 1% ascorbic acid in acetonitrile:water (50:50, v/v).

5.3.2 LC conditions

The LC system consisted of a model Finnigan TSQ Quantum Ultra (MS) coupled to a Finnigan Surveyor LC Pump and a Finnigan Surveyor Autosampler (Thermo Electron Corporation, CA, USA). A gradient LC system (Table 5.1) using ammonium formate :water (5:95, v/v Mobile phase A) and ammonium formate:acetonitrile (5:95, v/v Mobile phase B) at a flow of 0.350 µl min^{-1}, was used to separate the analytes on a Phenomenex 3 µm C$_{18}$ 100 A (100 x 2.0 mm) (Phenomenex, UK) column equipped with a guard column (10.0 × 2.0 mm I.D.) packed with Hypersil ™ (3.0 µm) C$_8$ material (Thermo Electron Corporation, UK). The column temperature was maintained at 45°C. Data acquisition and integration were performed using XCalibur version 1.4 chromatographic management software (Thermo Electron Corporation, CA, USA).The LC system was equilibrated for 30 min at a column oven temperature of 45 °C. A linear gradient (Table

5.1) was used ramping from 10 % B to 90 % B over 7.2 min: once at 90 % B, the system was held for 7.3 min and returned to 10 % B over a period of 2.0 min. The runtime was 21 min. The described gradient LC system was shown to be suitable for the analysis of MG, LMG, CV and LCV (Figures 5.1-5.4 (weak transitions)).

Table 5.1: LC gradient profile for determination of MG, LMG, CV and LCV.

Time (min)	Component A (%)	Component B (%)
0.0	90	10
0.5	90	10
3.0	50	50
7.2	10	90
14.5	10	90
16.5	90	10
21.0	90	10

Component A: Ammonium formate buffer + water (5 + 95, v/v) with 200 µl of formic acid

Component B: Ammonium formate buffer + acetonitrile (5 + 95, v/v) with 200 µl of formic acid

5.3.3 MS-MS parameters

The analysis was performed using positive-ion electrospray (ESI) interface with multiple reaction monitoring (MRM) mode. Two transitions per compound were used and the spray and collision voltages were optimised as shown (Table 5.2). The MS/MS detector conditions were as follows: Ion mode ESI+; gas sheath pressure 60 psi; capillary temperature 375 °C; source CID -10 V; collision pressure 1.5 torr; collision gas argon.

Table 5.2: MS/MS parameters for determination of MG, LMG, CV and LCV

Compound	Transition	Spray Voltage [V]	Collision [eV]	Rt [min]
MG	329>313 (strong)	4000	35	8.1
	329>208 (weak)	4000	37	8.1
LMG	331>239 (strong)	4000	35	13.2
	331>316 (weak)	4000	23	13.2
d_5-MG (IS)	334>318	4000	35	8.1
d_5-LMG(IS)	336>239	4000	35	13.2
CV	372>356 (strong)	4000	57	8.7
	372>340 (weak)	4000	40	8.7
LCV	374>358 (strong)	4000	35	13.5
	374>238 (weak)	4000	30	13.5

5.3.4 Procedure for Salmon Samples

Whole salmon obtained from a local supermarket were homogenised in a bowl-blender (Waring, UK) and stored at –20 °C in 150 ml glass jars. Laboratory samples of salmon tissue were previously analysed by method described in this chapter and those found to contain no detectable residues of the analytes were used as negative controls.

5.3.5 Sample extraction and clean-up

Salmon samples (2 g) were weighed into 50 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 2.5 μg kg^{-1} by adding 50 μl portion of 100 ng ml^{-1} mix solution of d$_5$-MG and d$_5$-LMG. Samples were fortified at levels corresponding to 1, 1.5 and 2 μg kg^{-1} by adding 40, 60 and 80 μl portions of 50 ng ml^{-1} mix solution of MG, LMG, CV and LCV. After fortification, samples were held for 15 min prior to extraction. Acetonitrile:McIIIvaine pH 3 buffer (90:10, v/v) (20 ml) was added and the samples were homogenised (30 sec). Samples were shaken (5 min) and sonicated (15 min). The samples were centrifuged (3000 rpm, 5 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The samples were re-extracted with acetonitrile:McIIIvaine pH 3 buffer (90:10, v/v) as before. The supernatants were combined and sample extracts were further purified by ion exchange solid phase extraction using Bakerbond SCX SPE cartridges. Sample extracts (40 ml) were loaded onto the cartridges (preconditioned with 2 ml methanol, 2 ml acetonitrile:McIIIvain buffer (90:10, v/v). The cartridges were washed with distilled water (1 ml), methanol (1 ml), ethyl acetate (1 ml), hexane (1 ml) and then dried using a vacuum pump (3 min). The cartridges were eluted with 2 % ammonium hydroxide in methanol (3 × 2 ml). The eluates were reduced to dryness under vacuum at 45°C before re-dissolving in 250 μl of 1% ascorbic acid in acetonitrile:water (50:50, v/v). An aliquot (20 μl) was injected onto the LC column.

5.3.6 Matrix-Matched Calibration curve

Matrix matched calibration curves were prepared and used for quantification. Control tissue previously tested and shown to contain no residues was prepared as above (5.3.4). One control tissue sample was used for each calibration standard level. Salmon samples

(2 g) were weighed into 50 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 2.5 µg kg^{-1} by adding 50 µl portion of 100 ng ml^{-1} mix solution of d$_5$-MG and d$_5$-LMG. Samples were fortified at levels corresponding to 0, 0.5, 1 and 2 µg kg^{-1} by adding 0, 20, 40 and 80 µl portions of a 50 ng ml^{-1} standard solution. Samples were fortified at the 5 and 10 µg kg^{-1} calibration levels by adding 50 and 100 µl portions of a 200 ng ml^{-1} standard solution. After fortification, samples were held for 15 min prior to extraction procedure as above (5.3.5).

Calibration curves were prepared by plotting the response factor as a function of analyte concentration (0 to 10 µg kg^{-1}).

5.3.7 Method validation

For estimation of accuracy, blank salmon tissue samples were fortified with MG, LMG, CV and LCV at 1, 1.5 and 2.0 µg kg^{-1}. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. For the estimation of the precision of the method, intra-assay and inter-assay repeatability was calculated. The decision limit (CCα) of the method were calculated according to the calibration curve procedure using the intercept (value of the signal, y, where the concentration, x is equal to zero) and 2.33 times the standard error of the intercept for a set of data with 6 replicates at 3 levels. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα.

5.4 Results and Discussion

5.4.1 Preliminary experiments

The LC-MS/MS method was developed to provide confirmatory data for the analysis of salmon for MG, LMG, CV and LCV. The MS/MS fragmentation conditions were investigated and collision energies were optimised for each individual compound. For a method to be deemed confirmatory one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion) must be monitored (Table 5.2) this yields 4 identification points, which is a suitable confirmatory method in accordance with CD 2002/657/EC.

MG and LMG are separated on a Phenomenex C_{18} column with a retention time of 8.1 and 13.2 min, respectively. Similarly CV and LCV are separated with a retention time of 8.7 and 13.5 min, respectively. d_5-MG was used as I.S for MG and similarly d_5-LMG was utilised as internal standard for LMG. As there are no deuterated standards of CV and LCV currently available, d_5-MG and d_5-LMG were used as internal standard for these analytes as well.

5.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [29] covering specificity, calibration curve linearity, recovery (accuracy), repeatability, decision limit (CCα) and detection capability (CCβ).

5.4.2.1 Specificity

The technique of LC-MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, salmon samples were fortified with the four analytes and the internal standards and non-fortified samples were also analysed. Interfering peaks were observed at the retention time for some transitions but upon quantification were so low as to be of little significance. (Figures 5.1-5.4)

Figure 5.1: Chromatogram of Negative Control Salmon (top) and Negative Control Salmon Fortified at 0.5 µg kg^{-1} with MG
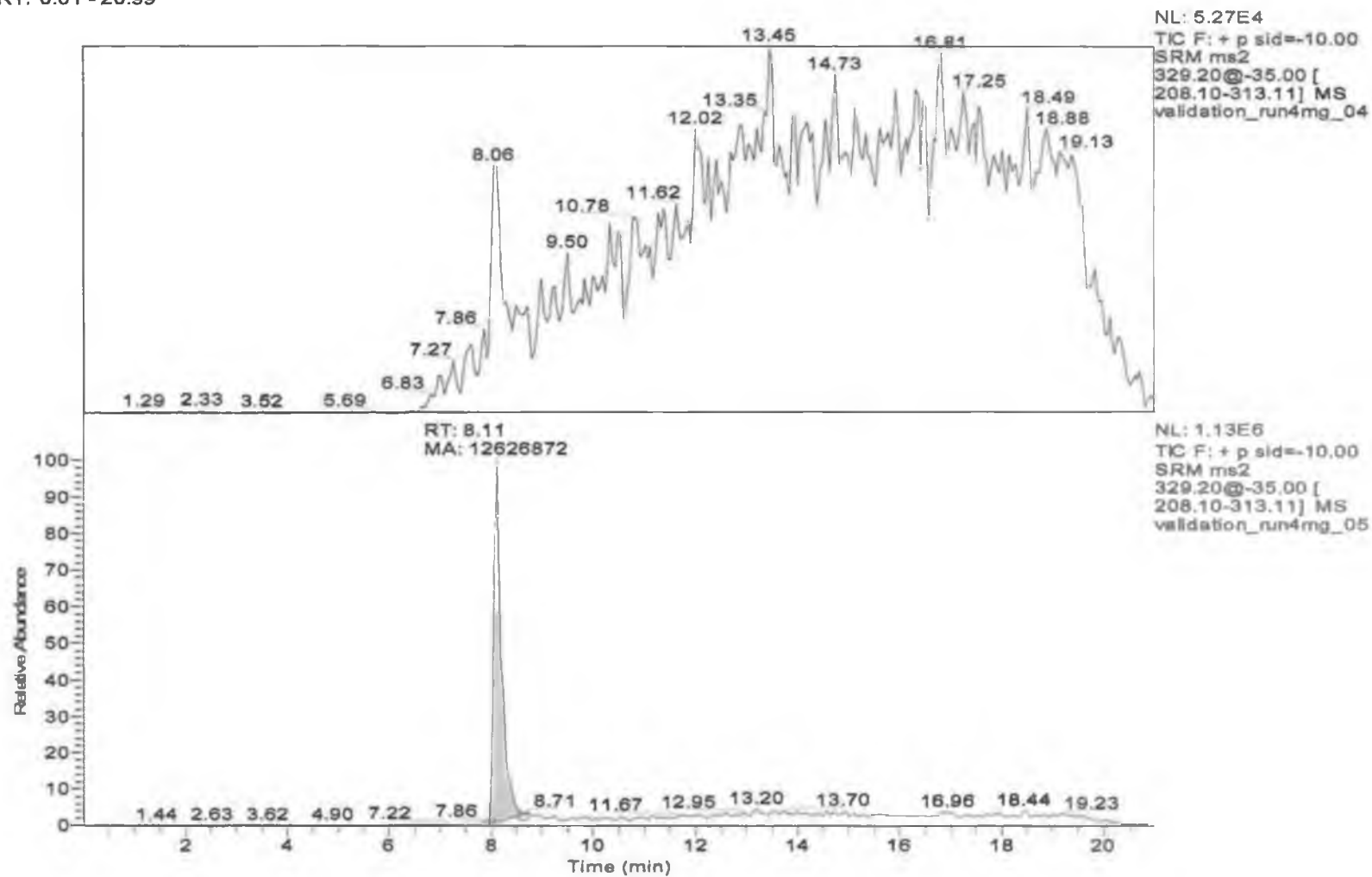
Figure 5.2: Chromatogram of Negative Control Salmon (top) and Negative Control Fortified at 0.5 µg kg^{-1} with LMG
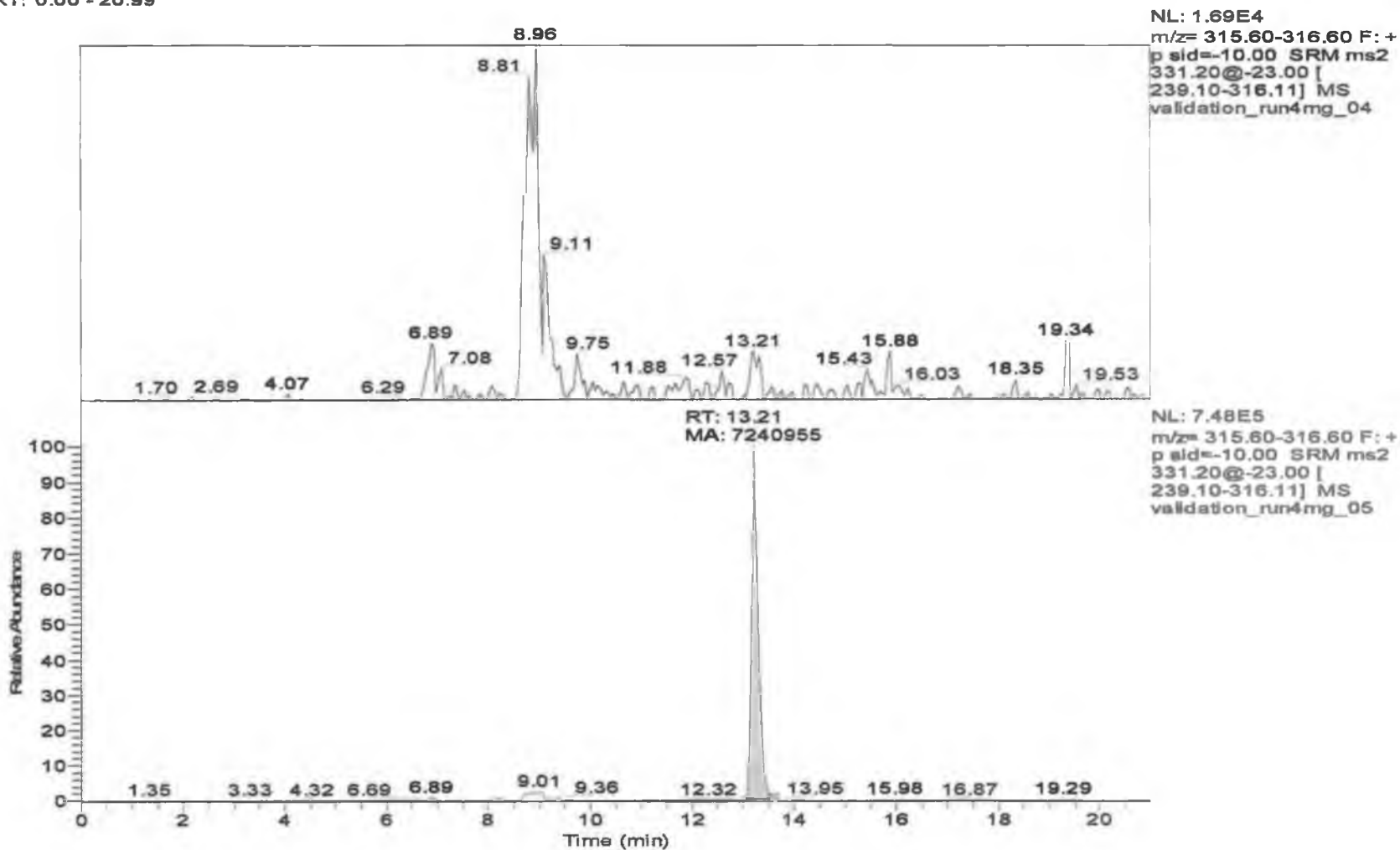
Figure 5.3: Chromatogram of Negative Control Salmon (top) and Negative Control Fortified at 0.5 µg kg^{-1} with CV
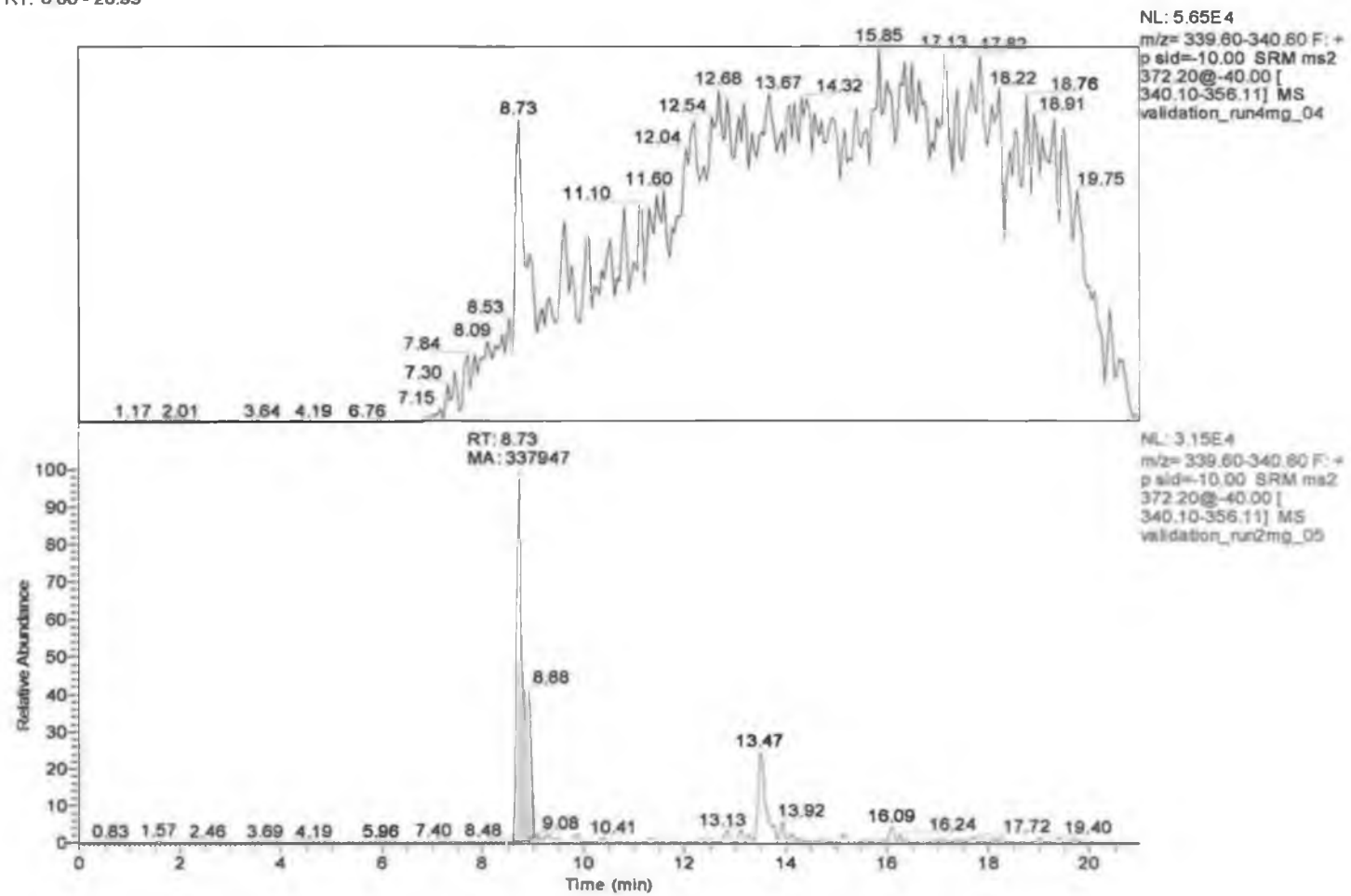
Figure 5.4: Chromatogram of Negative Control Salmon (top) and Negative Control Fortified at 0.5 µg kg $^{-1}$ with LCV
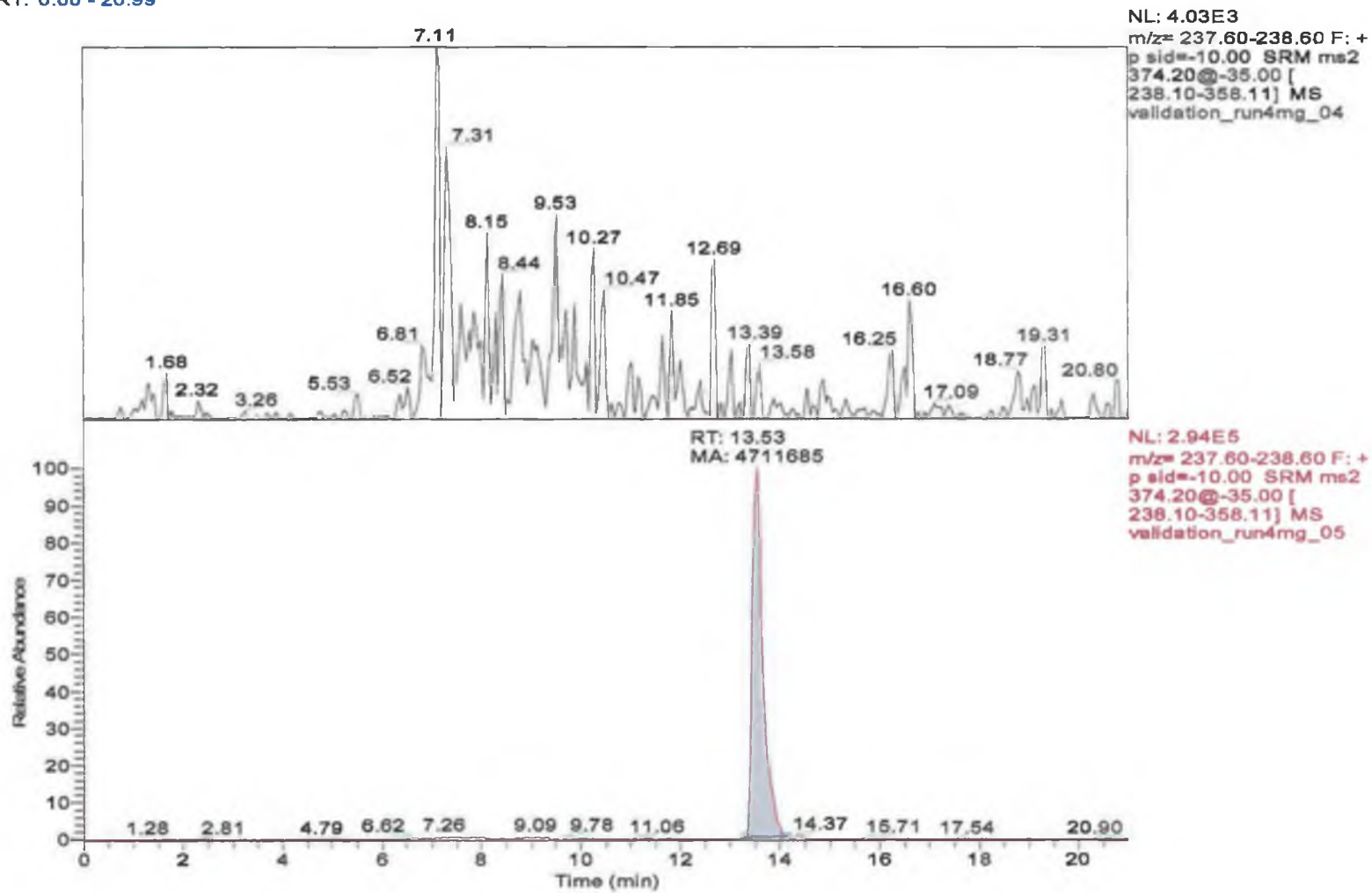
5.4.2.2 Linearity of the response

The linearity of the chromatographic response was tested with matrix matched curves using 6 calibration points in the concentration range of 0 to 10 μg kg^{-1}. The regression coefficients (r^2) for all the calibration curves used in this study were ≥ 0.990.

5.4.2.3 Recovery

The recovery of the method was determined using salmon samples fortified at 1.0, 1.5 and 2 μg kg^{-1} for each dye. Mean corrected recovery (n = 6) of the analytes, determined in three separate assays shown in Table 5.3 was between 77 and 113 % for MG, LMG, LCV and CV.

5.4.2.4 Repeatability

The usefulness of suitable deuterated standards is demonstrated in the excellent intra and inter repeatability obtained for MG and LMG (Table 5.3). Although no deuterated analogue is available for LCV, still a very acceptable repeatability is obtained by using the IS of LMG. Less favourable is the situation for CV. The rather high RSD values obtained for both the intra and inter repeatability of CV (between 20 to 25%), even by applying correction by means of the IS of MG indicates the necessity of the incorporation of a structurally identical isotopically-labelled IS in the method.

Table 5.3: Intra- and inter-assay variation for accuracy of malachite green, leucomalachite green, crystal violet and leucocrystal violet from salmon

Analyte	Fortification level (μg kg^{-1})	Accuracy							
		Assay 1		Assay 2		Assay 3		Inter-assay	
		Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
MG	1.0	104	1.9	96	6.9	107	2.5	102	6.2
	1.5	108	3.3	98	7.1	113	3.7	106	7.6
	2.0	104	2.3	96	2.9	109	4.5	103	6.1
LMG	1.0	96	10.9	99	3.0	102	0.1	100	6.3
	1.5	97	2.7	97	6.3	103	1.0	90	4.7
	2.0	97	1.4	96	4.3	98	3.5	97	3.2
CV	1.0	92	10.4	89	9.3	105	28.8	96	20.2
	1.5	102	24.3	81	14.0	94	29.0	92	24.7
	2.0	111	15.1	90	20.3	104	23.5	101	20.0
LCV	1.0	77	7.3	99	3.0	100	5.2	92	13.1
	1.5	89	6.0	102	9.0	97	2.3	96	8.4
	2.0	96	8.7	103	6.0	95	5.8	98	7.6

5.4.2.5 CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1 % is applied. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of 1-β, were β = 5 %. CCα and CCβ were calculated using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (1, 1.5 and 2 μg kg^{-1}). Blank salmon tissue was fortified at 1, 1.5 and 2 times the MRPL of 2 μg kg^{-1} set for MG; 1 μg kg^{-1} for each compound has been used for the method validation in this work as the MRPL is set for the sum of MG and LMG. There is no limit set for CV and LCV and the method was validated at the same levels as described in the study for MG. CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. CCα values of 0.17, 0.15, 0.35 and 0.17 μg kg^{-1} respectively were achieved for MG, LMG, CV and LCV. CCβ is the concentration

corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e the intercept + 3.97 times that standard error of the intercept). CCβ values of 0.30, 0.35, 0.80 and 0.32 μg kg^{-1} were achieved for MG, LMG, CV and LCV.

5.5   Conclusions

A relatively fast, simple and selective liquid chromatography-tandem mass spectrometric method for the simultaneous detection of MG, LMG, CV and LCV in salmon has been developed. There is no published confirmatory method for the simultaneous determination of MG, LMG, CV and LCV in salmon that is validated according to Commission Decision 2002/657/EC. The obtained data fulfils the requirements laid down in Commission Decision 2002/657/EC and allows the calculation of all relevant performance characteristics. This study shows that the required sensitivity for MG and LMG were obtained that easily meets the MRPL of 2 μg kg^{-1} and that the method is suitably sensitive for CV and LCV. The method performs very well in terms of accuracy and repeatability for MG, LMG and LCV. For CV improvements can be made if a suitable deuterated analogue becomes available.

The reduced number of analytical steps within the method makes it very amenable for high through-put regulatory monitoring of these compounds. There is no published confirmatory method for the simultaneous determination of MG, LMG, CV and LCV in salmon that is validated according to Commission Decision 2002/657/EC. The objective of the work to develop a method for these residues in salmon at sub μg kg^{-1} levels and validate according to the requirements in Commission Decision 2002/657/EC therefore has been achieved successfully. [29]

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Chapter 6

Conclusions

This thesis discusses the development and validation of methodology for determination of the coccidiostat robenidine, the benzimidazoles and the triphenylmethane dyes. A particular emphasis is placed on the development of methods that allow high through-put sample analysis. The thesis also applies newer applications in sample clean-up and detemination of these residues.

The main objectives of the research work described in this thesis were:

1   The development of chromatographic screening methods for quantitative determination of banned (robenidine) and MRL (benzimidazoles) substances in chicken eggs and bovine liver respectively and adoption of criteria governing method validation for banned and MRL substances according to Commission Decision 2002/657/EC.

2   The development of a confirmatory method for the quantitative determination of the banned substance MG and its major metabolite LMG, CV and LCV in farmed salmon and adoption of the rules governing confirmatory method validation for these banned substances according to Commission Decision 2002/657/EC.

3   As a byproduct of the research the implementation of the developed methods into national surveillance schemes in Ireland established under Council Directive 96/23/EC on measures to control certain substances and residues thereof in animal products.

These objectives have been accomplished and a number of practical developments have been made in the areas studied. In chapter 3, The first reported LC-UV method was developed for the analysis of robenidine in eggs. In the future the development of a confirmatory method for this compound by LC-MS/MS could be developed and work might also focus on the development of multi-residue methodology for the determination of other coccidiostats in egg, feed and animal tissues using this technique. Although there are a large number of methods for benzimidazoles, not all of these methods are multi-residue. To date, however there is no method available that can screen for all the benzimidzole metabolites. The development of such a method could be undertaken by using a derivitisation technique. Future investigations should concentrate on the development of this technique. Using the approach of identifying the most likely residues to occur in different animal tissues, a multi-residue method was developed that provides more practical surveillance of these compounds. The first

reported multi-residue automated SPE using this approach for the determination of benzimidazoles in liver was described in chapter 4. Further improvements in the analysis of these compounds in liver could be achieved by the implementation of an automated extraction step. This coupled with the automated SPE described in chapter 4 would improve sample handling. In future the possibility of developing on-line clean-up procedure using trace enrichment cartridges and column switching could be investigated. There is also a lack of multi-residue methods, which target TCB, FLU and MBZ licensed groups in conjunction with other residues. Since these drugs are used widely in veterinary medicine there is a need to develop such multi-residue methods. There is also a need to develop multi-residue methodology for the determination of benzimidazoles in milk.

Although there are a number of methods for MG and LMG, there is a lack of single and multi-residue methods that target other Group B3 (e) dyes such as CV, LCV, brilliant green, leucobrilliant green, ethyl violet, methyl violet and methylene blue. In chapter 5 the first reported multi-residue method for the determination of MG, LMG, CV and LCV in salmon by LC-MS/MS that is validated according to 2002/657/EC was described. Future work should concentrate on developing single residue methods for the determination of these dyes in fish. There is also limited data available on the depletion of these dyes from fish tissues. Toxicological studies on these dyes are also very limited. Future work should concentrate on expanding the method described in chapter 5 to include the other dyes. No such multi-residue method currently exists. A future application of the method would be to survey a variety of fish and aquaculture products to determine prevalence of these residues which is currently unknown. The dyes are possibly the least researched of the three topics investigated in this thesis in the world today. There are a number of methods available for coccidiostats and benzimidazoles but with the exception of malachite green and its metabolite limited information regarding the other dyes.

Chapter 7

Publications

1.   Conference Papers

G. Dowling, M. O'Keeffe and M.R. Smyth, Multi-residue method for the determination of benzimidazoles in bovine liver, In: *EuroResidue V*, 2004, Noordwijkerhout, The Netherlands, 10-12 May 2004, 435-440

2.   Conference Abstracts and Poster Presentations

G. Dowling, M. O'Keeffe and M.R. Smyth, Determination of robenidine in eggs by liquid chromatography with UV spectrometric detection, *In Fifth International Symposium on Hormone and Veterinary Drug Residue Analysis*, 2006, Antwerp, 16-19 June 2006

G. Dowling, M. O'Keeffe and M.R. Smyth, Confirmatory analysis of malachite green, leucomalachite green, crystal violet and leucocrystal violet in salmon by liquid chromatography tandem mass spectroscopy, *In Fifth International Symposium on Hormone and Veterinary Drug Residue Analysis*, 2006, Antwerp, 16-19 June 2006

G. Dowling, M. O'Keeffe and M.R. Smyth, Multi-residue method for the determination of benzimidazoles in bovine liver, In: *EuroResidue V*, 2004, Noordwijkerhout, The Netherlands, 10-12 May 2004

3.   Oral Presentations

Presented work on benzimidazoles at Teagasc Research Seminar to staff at Teagasc and international food safety colleagues.

Presented work on robenidine, benzimidazoles and triphenylmethane dyes to academic staff and students in DCU.

Presented work on triphenylmethane dyes to Food Safety Promotion Board Ireland and staff at the Marine Institute.

4.   Awards

Won 1 [st] prize at Teagasc Research Seminar for a power-point presentation describing the development of a multi-residue method for the determination of benzimidazoles in bovine liver.

Won 1 [st] prize at University College Cork Conference on Food Safety for poster on benzimidazoles.

Appendix 1

ELSEVIER

Determination of robenidine in eggs by liquid chromatography with UV spectrophotometric detection

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Abstract

A method has been developed to analyse for robenidine drug residues in eggs. Egg samples were extracted with acetonitrile and the sample extracts were defatted with hexane and aliquots of the extracts were analysed by liquid chromatography with UV spectrophotometric detection (317 nm). The method was validated according to the criteria defined in Commission Decision 2002/657/EC. The validation parameters linearity, precision, recovery, specificity, decision limit ($CC\alpha$) and detection capability ($CC\beta$) were determined. The decision limit was $10\,\mu g\,kg^{-1}$ and the detection capability was $17\,\mu g\,kg^{-1}$. The results of the inter-assay study, which was performed by fortifying egg samples ($n=6$) at three levels in three separate assays, show mean recoveries to be between 79 and 105%. The precision of the method, expressed as CV (%) values for the within-laboratory repeatability, was ≤13.5%.
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Keywords: Robenidine; Residue analysis; Egg; Method validation; Liquid chromatography with UV spectrophotometric detection

1. Introduction

Anticoccidials are compounds used extensively both prophylactically and therapeutically to treat coccidiosis in poultry. Coccidiosis is caused by unicellular organisms belonging to the genus *Eimeria* in the class *Sporozoa* and is the most important parasitic disease of poultry [1]. Robenidine (1,3-bis[(p-chlorobenzylidene) amino]-guanidine monohydrochloride) is a common anticoccidial drug used in the poultry industry. The molecular structure of the compound is shown in Fig. 1. It is authorised for use in poultry and other species under the Additives Directive 70/524/EEC [2]. In the European Union, according to Directive 70/524/EEC, the use of robenidine is not authorised for laying hens. The widespread use of this anticoccidial drug may present a potential risk to the consumer if residues enter the food chain. A range of methods have been described for determination of robenidine in feed, including thin layer chromatography [3],

a photometric method for feed and premixes [4], and a LC-UV method for determination of robenidine and its chemical precursors in feed [5]. A polarographic method for determination of robenidine in tissue, eggs, litter and soil [6], and methods for determination of robenidine and other anticoccidials by LC–dual mass spectrometry (MS/MS) in eggs [7,8] have been described. There are no methods available for determination of robenidine in egg by LC with UV detection.

In this paper a fast, simple and reliable LC method is described for the detection of robenidine in eggs. The method was validated according to procedures described in Commission Decision 2002/657/EC [9].

2. Experimental

2.1. Materials and reagents

Water (HiPerSolv grade), acetonitrile (HiPerSolv grade), *n*-hexane, triethylamine and ammonium dihydrogen phosphate (Analytical grade) were obtained from BDH (Poole,

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* Corresponding author.
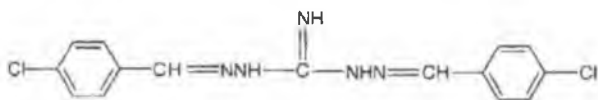*E-mail address:* mokeeffe@nfc.teagasc.ie (M. O'Keeffe).

Fig. 1. Structure of robenidine (1,3-bis[(p-chlorobenzylidene) amino] – guanidine monohydrochloride).

Dorset, UK). N,N-dimethylformamide (Analytical grade) was obtained from Riedel-de-Haen (Seezle, Hannover, Germany). Robenidine hydrochloride was obtained from QMX (Essex, UK). Ammonium phosphate buffer (0.05 M, pH 6.8) was prepared by dissolving 5.75 g of ammonium dihydrogen phosphate in 800 ml LC grade water then adding triethylamine to yield pH 6.8 and making up to 1 l with water. A primary stock standard solution of robenidine was prepared in N,N-dimethylformamide at a concentration of 100 μg ml^{-1} and stored at 4 °C. Working standard solutions were prepared daily in acetonitrile:water (65:35, v/v) and stored at 4 °C.

2.2. LC conditions

The LC system consisted of a model 600 LC pump (Waters, Milford, USA) and a SIL-101 Avp autoinjector, a CTO-10 Avp column oven, a SPD-10Avp UV detector operated at 317 nm and a SCL-10 Avp system controller linked to a PC with class-VP data handling software (Shimadzu Corporation, Japan). The mobile phase, consisting of acetonitrile:buffer (65:35, v/v) pH 6.8, was pumped at a flow of 1.5 ml min^{-1}. A stainless-steel Novapak C$_{18}$ analytical column (250 mm × 4.6 mm ID), coupled to a Hypersil C$_{18}$, Securiguard^{TM} guard column (3.0 mm × 2.0 mm ID), was used. The column temperature was maintained at 40 °C.

2.3. Egg samples

Egg samples (100 g approximately), prepared from pooling six individual eggs, were homogenised and stored at −20 °C in 150 ml tubes. Egg samples, from commercial production units, analysed and found to contain no detectable residues of robenidine were used as negative controls.

2.4. Sample extraction and clean-up

Egg (2 g) was weighed into 50 ml polypropylene tubes. Acetonitrile (7.5 ml) was added and the test portion was vortexed (2 min), sonicated (3 min) and shaken on a mechanical shaker (15 min). The test portion was centrifuged (2400 g, 10 min, 4 °C). The supernatant was transferred to a clean testtube and the test portion was extracted with acetonitrile as before. The supernatants were combined and defatted with hexane (5 ml) by mixing on a vortex (30 s). The test portion was centrifuged (1250 × g, 5 min, 4 °C) and the hexane layer removed. The sample extract was evaporated to dryness under nitrogen (60 °C) before dissolving in 500 μl acetoni-

trile:water (65:35, v/v). An aliquot (100 μl) was injected onto the HPLC column.

2.5. Calibration

Standards were prepared in acetonitrile:water (65:35, v/v) at concentrations of 0, 50, 125, 250, 500, 1000 and 2000 ng ml^{-1}, equivalent to 0, 12.5, 31.25, 62.5, 125, 250 and 500 μg kg^{-1} egg, respectively. Calibration curves were prepared by plotting peak area as a function of analyte concentration (0–2000 ng ml^{-1}). Concentration of robenidine (ng ml^{-1}) was determined from the peak areas obtained for test portion extracts, calculated from the calibration curve. The robenidine concentration in egg samples (μg kg^{-1}) was calculated, as follows:

robenidine (μg kg^{-1} egg)

   = robenidine (ng ml^{-1} extract) × 0.5 ÷ 2

2.6. Method validation

For estimation of recovery, blank egg samples were prepared and fortified with robenidine. Test portions (2 g) were fortified at 50, 75 and 100 μg kg^{-1} by adding 50, 75 and 100 μl portions of a 2000 ng ml^{-1} robenidine solution, respectively. After fortification, the test portions were held for 15 min prior to extraction with acetonitrile, as described above. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. For estimation of the precision of the method, within assay repeatability was calculated. The decision limit (CCα) and the detection capability (CCβ) of the method were calculated.

3. Results and discussion

3.1. Preliminary experiments

An LC method, developed to analyse for robenidine in animal feed, was applied to the separation of robenidine on a C$_{18}$ column with a mobile phase consisting of acetonitrile:water:potassium di-hydrogen phosphate:di-sodium hydrogen phosphate (65:25:5:5) [5]. The peak shape was poor, with excessive tailing, using this mobile phase. Investigation of various LC conditions, including various percentages of organic content in the mobile phase, pH, injection volume and mobile phase flow rate, showed that a mobile phase consisting of 65% acetonitrile and 35% phosphate buffer, adjusted with triethylamine to pH 6.8, an injection volume of 100 μl and a flow rate of 1.5 ml min^{-1} gave good peak retention and shape. Following preliminary extraction with acetonitrile, liquid partitioning between hexane and acetonitrile was carried out. Further clean-up of the sample extracts was not necessary; the chromatograms were free of matrix interference and satisfied the aim of developing a sim-

Table 1
Intra- and inter-assay variation for recovery of robenidine from egg

Fortification level (μg kg^{-1})	Recovery (%)											
	Assay 1			Assay 2			Assay 3			Inter-assay		
	n	Mean	R.S.D.	n	Mean	R.S.D.	n	Mean	R.S.D.	n	Mean	R.S.D.
50	6	79.5	18.0	6	94.1	9.5	6	94.5	6.2	18	89	13.5
75	6	95.8	6.7	6	105.0	8.9	6	102.8	7.0	18	101	8.2
100	6	85.1	12.4	6	100.1	4.2	6	93.9	4.4	18	93	10.0
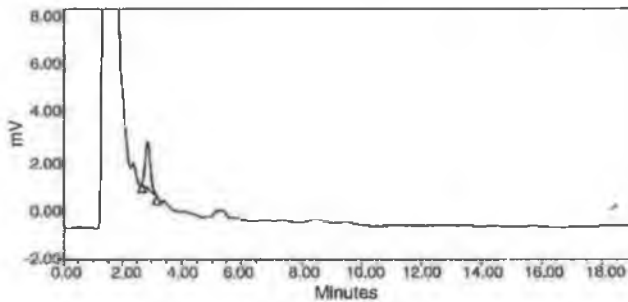
Fig. 2. Chromatogram of negative control egg sample.

ple quantitative test method with a minimum of manipulations.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [9], covering specificity, calibration curve linearity, recovery, repeatability, decision limit (CCα) and detection capability (CCβ).

3.2.1. Specificity

To establish the specificity of the method, negative control egg samples ($n = 3$) and egg samples fortified with robenidine were analysed. No interfering peaks were observed at the retention time for the robenidine peak (Figs. 2 and 3).

3.2.2. Linearity of the response

The linearity of the LC-UV response was evaluated with a six-point calibration curve in the concentration range 0–2000 ng/ml (0–500 μg kg^{-1} egg). The regression coefficients (r^2) of the calibration curves were 0.98–0.99.
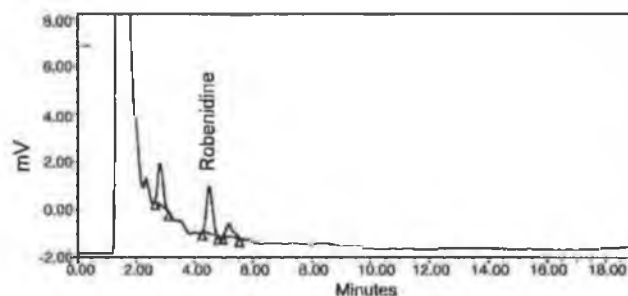
Fig. 3. Chromatogram of negative control egg sample spiked at 50 μg kg^{-1}.

3.2.3. Recovery

The accuracy of the method was determined using egg samples fortified at 50, 75 and 100 μg kg^{-1}. Mean recovery ($n = 6$), at each of the three levels of fortification and determined in three separate assays ($n = 54$, total), was between 79 and 105% (Table 1).

3.2.4. Repeatability

The inter-assay repeatability is shown in Table 1. At the three levels of fortification residual standard deviation (RSD) values are <13.5%. The repeatability of the method is satisfactory.

3.2.5. CCα and CCβ

The decision limit (CCα), in the case of substances for which no MRLs have been set, is the lowest concentration level at which it can be concluded that a sample is non-compliant, with an error probability of $\alpha = 1\%$. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with an error probability of $\beta = 5\%$. Both of these parameters were determined using the calibration curve procedure in which blank egg samples were fortified at the minimum required performance level of the method (50 μg kg^{-1}) and at 1.5 and 2 times that level. CCα and CCβ values of 10 and 17 μg kg^{-1}, respectively, were determined for the method.

4. Conclusions

This paper reports the development and validation of a fast and simple LC method for the determination of robenidine in eggs. The method was validated according to the guidelines described in Commission Decision 2002/657/EC.

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Appendix 2

ELSEVIER

Multi-residue method for the determination
of benzimidazoles in bovine liver

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Abstract

A method has been developed to analyse for 12 benzimidazole drug residues in bovine liver. Liver samples were extracted with ethyl acetate, sample extracts were defatted with hexane and cleaned-up by automated SPE on C_{18} solid phase extraction cartridges. Aliquots of the extracts were analysed by LC with UV detection (298 nm). The method was validated in bovine liver, according to the criteria defined in Commission Decision 2002/657/EC. The decision limit (CC_{α}) was between MRL + 12% and MRL + 25% and the detection capability (CC_{β}) was between MRL + 25% and MRL + 45% for the range of benzimidazoles investigated. The results of the inter-assay study, which was performed by fortifying bovine liver samples ($n = 6$) in three separate assays, show the mean recovery to be between 60% and 100% for albendazole sulphoxide, albendazole sulphone, thiabendazole, oxfendazole/fenbendazole sulphoxide, hydroxy-mebendazole, fenbendazole sulphone, oxibendazole, mebendazole and flubendazole. Lower mean recovery was obtained for amino-flubendazole and albendazole (approximately 50%) and amino–albendazole sulphone (approximately 25%). The precision of the method, expressed as R.S.D. values for the within-laboratory repeatability, was generally below 25%.
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Keywords: Benzimidazoles; Automated solid phase extraction; Residue analysis; Liver; Method validation

1. Introduction

Benzimidazoles are broad-spectrum anthelmintics and have been used for about 40 years in animal health and crop protection. Metabolism of the drugs is extensive. The metabolites found depend on the structure of the parent drug, the tissue and the animal species. One or more metabolites can be found in animal tissue for most drugs [1,2]. When the pro-drugs, active drugs and metabolites are counted, some 19 possible residues may occur in animal tissues. The EU has set maximum residue limits (MRLs) for benzimidazoles and their metabolites in animal products. The MRL values range from 10–1000 $\mu g \, kg^{-1}$, depending on the compound and matrix (Table 1). Most benzimidazole

drugs, with the exception of cambendazole, are licensed for use in food-producing animals. The development of a single multi-residue method capable of testing for all the residues in the MRL listings is very difficult.

Netobimin (NETO) albendazole (ABZ) and albendazole sulphoxide (ABZ-SO) are licensed for use in the treatment of cattle and sheep. ABZ-SO was used for treatment of pheasants, but because an analytical method was not satisfactorily validated for pheasant tissues, no final MRL values could be proposed for pheasants [3]. NETO is a pro-drug, converted by the gut microflora to ABZ by splitting of the side chain and formation of the benzimidazole nucleus, and ABZ is converted further to ABZ-SO. In cattle, ABZ-SO, albendazole sulphone (ABZ-SO_2) and 2-amino albendazole sulphone (NH_2ABZ-SO_2) were found to be the major residues detected in liver. Febantel (FEB), fenbendazole (FBZ) and oxfendazole, or fenbendazole sulphoxide,

Table 1
EU MRL values for benzimidazole anthelmintic drugs (based on Council Regulation No. 2377/90 and Commission Regulation Nos. 508/1999, 2385/1999, 2393/1999 and 807/2001)

Benzimidazole	Marker residue	Animal species	MRL (μg kg^{-1})	Target tissue
Netobimin (NETO), albendazole (ABZ), albendazole sulphoxide(ABZ-SO)	Sum of ABZ-SO, albendazole sulphone (ABZ-SO$_2$) and amino-albendazole sulphone (NH$_2$ABZ-SO$_2$), expressed as albendazole	Bovine, ovine	100 100 100 1000 500	Milk Muscle Fat Liver Kidney
Febantel (FEB), fenbendazole (FBZ) and oxfendazole (OFZ)	Sum of extractable residues that may be oxidised to fenbendazole sulphone (FBZ-SO$_2$)	Bovine, ovine	10	Milk
		Bovine, ovine, porcine, equidae	50 50 500 50	Muscle Fat[a] Liver Kidney
Flubendazole (FLU)	Sum of FLU and amino-flubendazole (NH$_2$FLU)	Porcine, game birds, chicken, turkey	50 50 400 300	Muscle Fat[a] Liver Kidney
		Chicken	400	Eggs
Thiabendazole (TBZ)	Sum of TBZ and hydroxy-thiabendazole(TBZ-OH)	Bovine	100 100 100 100 100	Muscle Fat Liver Kidney Milk
Oxibendazole (OXI)	OXI	Porcine	100 500 200 100	Muscle Fat[a] Liver Kidney
Triclabendazole (TCB)[b]	Sum of extractable residues that may be oxidised to keto-triclabendazole	Bovine, ovine	100 100 100	Muscle Liver Kidney
Mebendazole (MBZ)[b]	Sum of hydroxy-mebendazole (MBZ-OH) and amino-mebendazole (NH$_2$-MBZ), expressed as mebendazole equivalents	Ovine, caprine, equidae	60 60 400 60	Muscle Fat Liver Kidney

[a] For porcine, and/or poultry, species this MRL relates to "skin and fat in natural proportions".

[b] Not for use in animals from which milk is produced for human consumption.

(OFZ/FBZ-SO) are licensed for use in cattle, pigs, sheep and horses. FEB is a prodrug, converted to FBZ by cyclization to form the benzimidazole nucleus. Animal studies showed that FBZ, OFZ/FBZ-SO and fenbendazole sulphone (FBZ-SO$_2$) were the important residues [4–8]. However, depending on species, route of administration and time after treatment, the forms of residues could vary greatly. Mebendazole (MBZ) is approved for use in goats, horses and sheep. When sheep were treated with MBZ it was found that the major residues were hydroxy-mebendazole (MBZ-OH) and MBZ [9]. Thiabendazole (TBZ) is licensed for use in cattle. In studies on cattle [7] and sheep [10], residues of TBZ and hydroxy-thiabendazole (TBZ-OH) were found in liver. Flubendazole (FLU) is approved for use in pigs, chicken, turkeys and game birds. FLU and amino-flubendazole (NH$_2$FLU) are the important residues. In turkeys treated with FLU, residues were found in all tissues, with NH$_2$FLU accounting for 68% of the total residues in liver [11]. Triclabendazole (TCB) is licensed for use in sheep and cattle. In cattle treated with radiolabelled TCB highest residue levels were detected in

liver at early withdrawal period [12]. In a study of lactating goats treated with TCB it was found that triclabendazole sulphoxide (TCB-SO) and triclabendazole sulphone (TCB-SO$_2$) residues could be detected in milk [13]. Oxibendazole (OXI) is licensed for use in pigs. The marker residue is the parent drug. In studies on cattle, sheep, pigs and horses total OXI residues were found to be less than 100 μg kg^{-1} in muscle and fat.

There are many published methods for analysis of benzimidazoles in animal tissues, plant tissues and milk products. A number of methods have been reported for the analysis of individual benzimidazoles and their metabolites in food products [14–17]. However, because of the large number of benzimidazoles licensed for use, multi-residue methods are more attractive. Multi-residue methods have been developed for the analysis of benzimidazole residues in milk [18–24] and tissues [25]. TBZ has been determined in fruit and vegetable matrices based on an ethyl acetate extraction [26–28]. Alternative solvents, such as methanol [29] and acetone [30,31] have been used. Supercritical fluid extraction (SFE) has been

used for extracting benzimidazole fungicides from fruit and vegetable matrices [32,33] and from animal tissues [34]. Accelerated solvent extraction has been used to extract TBZ from fruit and vegetable samples and processed products [35]. Matrix solid phase dispersion (MSPD) has been applied by a number of groups for the determination of benzimidazoles in liver [6,45].

A combination of liquid–liquid partitioning and solid phase extraction is the most widely adopted approach for the clean-up of animal tissue extracts in benzimidazole residue analysis. Wilson et al. [7] purified tissue extracts by partitioning between ethanol:0.2 N hydrochloric acid and hexane before clean-up on a C_2 solid phase extraction cartridge. Roudaut and Garnier [36] modified the method by substituting an Oasis HLB cartridge for the C_2 cartridge. Similar liquid–liquid partitioning approaches and further purification of tissue extracts by SPE have used C_{18} [37], aminopropyl [38], cyano [39], neutral alumina [40] and silica [41] SPE cartridges. Rose [42] applied strong cation exchange (SCX) SPE to the isolation of OFZ and nine related residues in liver. Farrington et al [43] developed multi-residue methods using dual SPE clean-up procedures based on normal phase and C_{18} sorbents. Stubbings et al. [44] developed an online SCX-SPE procedure for the purification of benzimidazole residues from tissue. Hiemstra et al. [31] developed a clean-up procedure for the isolation of TBZ and carbendazim residues from fruit and vegetable extracts, using liquid–liquid partitioning prior to automated off-line SPE clean-up on diol SPE cartridges mounted on an ASPEC system.

Most of the published methods, such as that by Wilson et al. [7], deal only with the determination of some of the benzimidazole drugs. Using the approach of identifying the most likely residues to occur in different animal species, a multi-residue method might be developed that would provide more complete surveillance for these compounds. The objective of this work was to develop an improved, automated, quantitative method for determination of an extended range of benzimidazoles within a single multi-residue method.

2. Experimental

2.1. Materials and reagents

Water, methanol, acetonitrile, ethyl acetate and dichloromethane (HiPerSolv grade) were obtained from BDH (Merck, Poole, Dorset, UK). Triethylamine, glacial acetic acid, ammonia, hydrochloric acid, dimethyl sulphoxide, acetone, hexane, sodium chloride and ammonium dihydrogen phosphate (Analytical grade) were from BDH. Ammonium hydroxide (ACS grade) was obtained from Sigma (St. Louis, MO, USA). N,N-dimethylformamide and sodium bicarbonate (Analytical grade) were obtained from Riedel-de-Haën (Seezle, Hannover, Germany). Mebendazole (MBZ), thiabendazole (TBZ), albendazole (ABZ)

Table 2
LC gradient profile for determination of benzimidazoles

Time (min)	Component A (%)	Component B (%)	Component C (%)
0	100	0	0
3	45	55	0
10.5	30	70	0
14	15	85	0
18	0	59	41
19	0	60	40
23	0	0	100
36	0	60	40
43	100	0	0

Component A: ammonium dihydrogen phosphate buffer pH 6.8 + methanol + acetonitrile (76 + 15 + 9, v/v/v); component B: ammonium dihydrogen phosphate buffer pH 6.8 + methanol + acetonitrile (52 + 30 + 18, v/v/v); component C: ammonium dihydrogen phosphate buffer pH 6.8 + methanol (18 + 82, v/v).

and oxibendazole (OXI) were purchased from Sigma. Flubendazole (FLU), amino-flubendazole (NH₂FLU) and hydroxy-mebendazole (MBZ-OH) were donated by Janssen (Beerse, Belgium). Fenbendazole sulphone (FBZ-SO₂) and fenbendazole sulphoxide (OFZ/FBZ-SO) were donated by Intervet (Dublin, Ireland). Albendazole sulphoxide (ABZ-SO), sulphone (ABZ-SO₂) and 2-amino albendazole sulphone (NH₂ABZ-SO₂) were donated by GlaxoSmithKline (Harlow, UK). Primary stock standard solutions of TBZ in methanol; OXI, MBZ, OFZ/FBZ-SO and FBZ-SO₂ in dimethylsulphoxide; MBZ-OH in N,N-dimethylformamide; FLU and NH₂FLU in N,N-dimethylformamide:methanol (3:2, v/v); NH₂ABZ-SO₂, ABZ, ABZ-SO and ABZ-SO₂ in N,N-dimethylformamide:methanol (2:8, v/v), were prepared at a concentration of $2\,mg\,ml^{-1}$. Working standard solutions were prepared in methanol and stored at 4 °C. Bond Elut™ C_{18} cartridges (3 ml, 500 mg) were obtained from Varian (Harbor City, USA). Solid phase extraction was performed using an ASPEC XL4 instrument (Gilson, Middleton, USA).

2.2. LC conditions

The LC system consisted of a model 616 HPLC pump with a model 717 plus autosampler and a model 996 photodiode array detector operated at 298 nm (Waters, Milford, MA, USA). A gradient LC system (Table 2) using ammonium dihydrogen phosphate buffer (pH 6.8), methanol and acetonitrile at a flow of $0.5\,ml\,min^{-1}$, was used to separate the analytes on a stainless-steel analytical column (150 mm × 3.0 mm i.d.) equipped with a guard column (3.0 mm × 2.0 mm i.d.), both packed with Xterra™ (3.5 μm) C_{18} material (Waters). The column temperature was maintained at 40 °C. Data acquisition and integration were performed using Millenium^{32} chromatographic management software (Waters).

2.3. Liver samples

Liver samples (100 g approximately) were homogenised in a Robot Coupe™ bowl-blender (Montceau-en-Bour-

gogne, France) and stored at -20 °C in 150 ml tubes. Liver samples analysed and found to contain no detectable residues of the analytes were used as negative controls.

2.4. Sample extraction and clean-up

Liver (3 g) was weighed into 50 ml polypropylene tubes. Samples were fortified at levels corresponding to 0.5 × MRL, 1 × MRL and 1.5 × MRL by adding 50 μl portions of diluted stock solutions. After fortification, samples were held for 15 min prior to extraction. Sodium sulphate (1.5 g), 4 M potassium carbonate (0.5 ml) and ethyl acetate (5 ml) were added and the samples were homogenised (30 s). Samples were centrifuged (2500 rpm, 10 min, 4 °C). The supernatant was transferred to a clean test-tube and the sample was re-extracted with ethyl acetate as before. The supernatants were combined and evaporated to dryness under nitrogen (50 °C). Ethanol: 0.2N hydrochloric acid (66:33, v/v) (1 ml) and n-hexane (5 ml) were added to the dry residue, vortexed (2 min) and centrifuged (1500 rpm, 5 min, 4 °C). The upper hexane phase was removed. This clean-up step was repeated with a second portion of hexane (5 ml). Sample extracts were further purified by automated SPE using C_{18} SPE cartridges. Sample extracts (0.6 ml) were modified by addition of 2% potassium bicarbonate solution (4 ml). Modified sample extracts (3.8 ml) were loaded onto C_{18} cartridges (preconditioned with 6 ml ethyl acetate, 3 ml ethanol and 3 ml distilled water). The cartridges were washed with distilled water (2 × 1 ml) and then dried. The C_{18} cartridges were eluted with ethyl acetate (3 × 1 ml). The eluates were reduced to dryness under nitrogen (50 °C) before dissolving in 400 μl methanol:water (50:50, v/v). An aliquot (50 μl) was injected onto the LC column.

2.5. Calibration

Standards were prepared at concentrations of 0, 0.25, 0.50, 0.75, 1.5, 3.75, 7.5 and 15 μg ml^{-1} in methanol:water (50:50, v/v). Calibration curves were prepared by plotting peak area as a function of analyte concentration (0–15 μg ml^{-1}). Recovery was determined using the peak area values obtained for fortified sample extracts, calculated from the calibration curves.

2.6. Method validation

For estimation of recovery, blank liver samples were fortified with benzimidazoles at 0.5, 1 and 1.5 times the MRLs. For estimation of the precision of the method, an intra-assay study was carried out by fortifying bovine liver samples at each level ($n = 6$) and extracting and analysing in a single run. The inter-assay study involved fortifying bovine liver samples as described and extracting and analysing in three separate assays. The decision limit (CC_α) and the detection capability (CC_β) of the method were calculated using the intra-assay validation results. The selectivity/specificity and linearity of the method were also established.

3. Results and discussion

3.1. Preliminary experiments

Previous work in this laboratory had developed a ternary gradient LC method for separation of 19 benzimidazole drugs, with detection at 298 nm [34]. That method was applied to separation of benzimidazole drugs in this study. From an evaluation of different extraction solvents, ethyl acetate was found to give the best recovery for the compounds of interest. Ethyl acetate has good solvating power for weakly basic drugs because it can provide emulsion-free extraction from liver and muscle tissue samples. Multi-residue extraction of benzimidazoles at a set pH is difficult because of their varied, and in some cases multiple, pK_a values. The effect of pH was studied over the range 5–10, based on a method described by Cannavan et al. [39] for extraction of TBZ from tissue using buffering at pH 7 and extraction with ethyl acetate. The possibility of using the ChemElut^{TM} diatomaceous earth buffered and unbuffered columns was also evaluated but low recoveries were obtained. Wilson et al. [7] developed an extraction procedure based on carbonate buffer/ethyl acetate for ABZ, TBZ-OH, OFZ, TBZ, CAM, MBZ and FBZ. Similarly, carbonate buffer extraction and liquid partitioning steps were used by Roudaut and Garnier [36] for TBZ-OH, TBZ, NH_2ABZSO_2, ABZ-SO, OXI, OFZ, ABZ-SO$_2$, ABZ and FBZ. After extraction, liquid partitioning between hexane and ethanol:hydrochloric acid, 0.2N (33:66, v/v) was used in both these methods. Further clean-up of the tissue extracts was achieved by solid phase extraction on C_2 cartridges [7] or on Oasis HLB^{TM} cartridges [36].

A carbonate buffer/ethyl acetate extraction and liquid partitioning, followed by solid phase extraction using C_{18} cartridges was developed in this work and applied to a wider range of benzimidazoles to include MBZ-OH, NH_2FLU, FBZ-SO$_2$ and FLU. TBZ-OH, which is a marker residue for thiabendazole, was not included because of incomplete chromatographic separation of this analyte from NH_2ABZSO_2; preliminary experiments indicate that TBZ-OH is extracted with similar yield as TBZ using the method. In total, drugs from six of the seven classes of benzimidazoles listed in Table 1 are included; triclabendazole and its metabolites are not extractable using this method.

Hiemstra et al. [31] developed an automated SPE clean-up on diol cartridges for determination of thiabendazole and carbendazim residues in fruit and vegetable extracts. The C_{18} solid phase extraction step in the current method was automated using an ASPEC XL4^{TM} system. Compared with the method described by Wilson et al. [7], the method developed here incorporates automated SPE on C_{18} cartridges and has been applied to an extended range of benzimidazoles,

including most of the marker residues designated by EU MRL regulations.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [46] covering specificity, calibration curve linearity, recovery, repeatability, decision limit (CC_α) and detection capability (CC_β).

The method was validated using bovine liver, as a model matrix, and applying the MRL values specified for liver of each species. Preliminary experiments with liver samples of other species indicate that comparable extraction yield is obtainable and that the developed method may be applicable to liver samples of other species. While most MRL values are expressed as the sum of the marker residues (Table 1), validation was undertaken applying the MRL value for each individual marker residue; this was necessary because of the variability in the proportions of marker residues which might occur in samples. For benzimidazoles for which MRL values have not been specified for a particular species, the method would require further validation according to the procedures described in Commission Decision 2002/657/EC for substances for which no permitted limit has been established.

3.2.1. Specificity

To establish the selectivity/specificity of the method, liver samples fortified with the benzimidazoles and non-fortified samples were analysed. No interfering peaks were observed at the retention time for the benzimidazoles (Fig. 1) except at the retention time for ABZ where an interfering peak of negligible size, relative to the response for MRL concentration, was observed.

3.2.2. Linearity of the response

The linearity of the chromatographic response was tested with seven calibration points in the concentration range of 0.25–15 µg ml^{-1}. The regression coefficients (r^2) for the calibration curves used in the study were ≥ 0.99.

3.2.3. Recovery

The accuracy of the method was determined using bovine liver samples fortified at 0.5 × MRL, MRL and 1.5 × MRL for each benzimidazole. Mean recovery ($n = 6$) of the analytes, determined in three separate assays, was between 60% and 100% for ABZ-SO, ABZ-SO$_2$, TBZ, OFZ/FBZ-SO, MBZ-OH, FBZ-SO$_2$, OXI, MBZ and FLU (Table 3). Lower mean recoveries of approximately 50% were obtained for NH$_2$FLU and ABZ. NH$_2$ABZ-SO$_2$ was recovered at approximately 25%, probably due to the difficulty in extracting this relatively polar compound from tissue.

3.2.4. Repeatability

The inter-assay repeatability is shown in Table 3. For most analytes and at the three levels of fortification (0.5 × MRL, MRL and 1.5 × MRL), R.S.D. values were at less than 25%. In some instances (NH$_2$FLU at 0.5 × MRL, OXI at MRL, and ABZ at MRL), R.S.D. values were in excess of 25%.
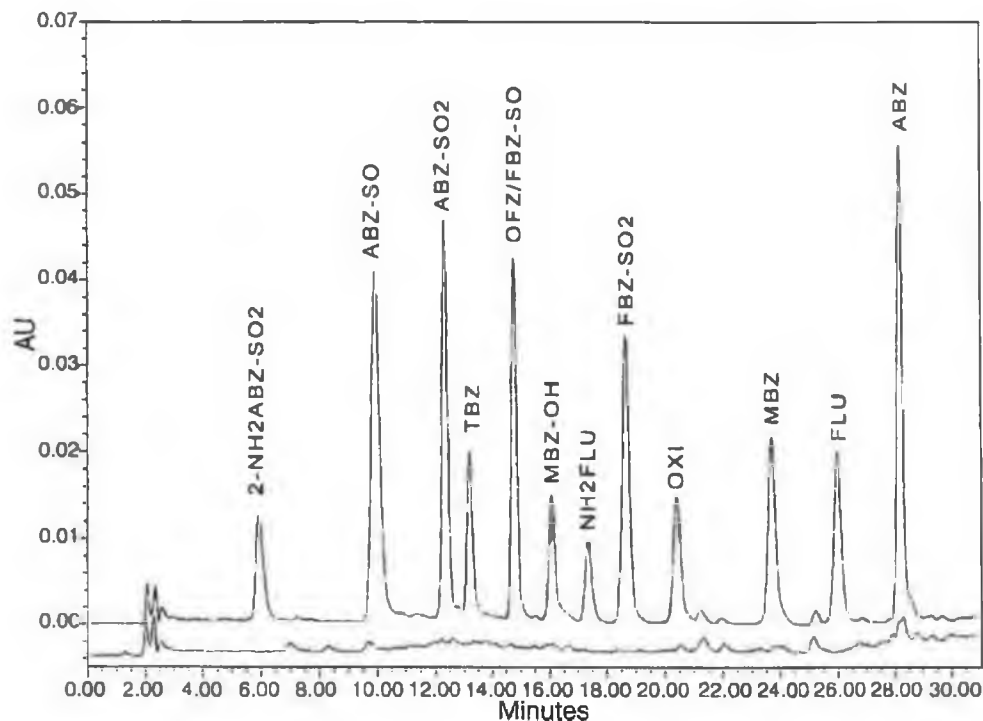

Fig. 1. Chromatogram of blank liver sample and liver sample fortified at MRLs.

Table 3
Intra- and inter-assay variation for recovery of benzimidazoles from liver

Analyte	Fortification level ($\mu g\,kg^{-1}$)	Recovery (%)							
		Assay 1		Assay 2		Assay 3		Inter-assay	
		Mean	R.S.D.	Mean	R.S.D.	Mean	R.S.D.	Mean	R.S.D.
$NH_2ABZ-SO_2$	500	27	10.0	22	17.8	26	15.4	25	15.9
	1000	24	10.2	22	3.3	23	6.7	23	7.7
	1500	24	12.4	19	12.2	19	21.9	20	16.4
ABZ-SO	500	68	9.3	60	11.0	73	6.4	67	11.7
	1000	62	8.7	57	5.3	62	6.3	61	7.5
	1500	61	9.8	61	1.1	52	6.5	57	10.2
$ABZ-SO_2$	500	75	6.3	61	8.2	73	10.5	70	12.1
	1000	72	9.0	58	8.7	69	17.1	67	15.1
	1500	66	7.0	62	1.7	63	10.9	64	7.8
TBZ	50	74	8.6	63	24.0	96	4.6	78	21.8
	100	82	8.9	56	5.4	85	9.1	76	19.2
	150	83	5.5	66	8.7	78	7.2	76	11.4
OFZ/FBZ-SO	250	85	5.2	61	5.8	73	6.6	73	15.1
	500	83	7.3	57	9.3	67	18.5	69	19.6
	750	83	7.0	64	2.6	62	8.9	70	15.8
MBZ-OH	200	83	6.3	63	6.7	103	14.2	83	21.3
	400	89	8.4	60	10.9	77	20.5	76	20.9
	600	83	7.1	61	5.1	64	8.5	70	16.3
NH_2FLU	150	62	6.1	40	12.2	35	9.1	46	27.7
	300	68	10.5	39	5.7	54	16.7	54	24.3
	450	62	7.7	40	14.0	56	14.5	54	20.7
$FBZ-SO_2$	250	87	4.9	58	4.0	74	5.9	73	17.4
	500	86	9.2	55	7.8	71	24.1	71	23.5
	750	84	7.3	62	2.8	59	16.3	69	19.2
OXI	100	92	4.3	69	4.9	120	13.6	93	24.9
	200	90	10.1	61	7.3	119	22.1	94	32.5
	300	85	7.0	66	2.1	76	18.2	76	15.3
MBZ	200	94	3.6	67	6.5	100	5.2	87	17.6
	400	87	6.0	59	7.5	84	23.2	77	22.8
	600	79	8.1	61	3.4	65	21.7	69	17.2
FLU	200	78	3.0	54	7.7	85	8.2	72	19.9
	400	78	7.5	48	8.1	67	15.2	65	21.4
	600	69	8.9	53	5.2	54	20.8	59	17.8
ABZ	500	63	3.2	39	7.3	56	6.9	53	20.6
	1000	59	9.0	36	8.4	48	30.4	48	27.4
	1500	54	9.2	40	12.0	44	21.3	46	19.0

Overall, the repeatability of the method for benzimidazoles is satisfactory.

3.2.5. CC_α and CC_β

The decision limit (CC_α) is, in the case of substances for which MRLs have been defined, the lowest concentration level at which it can be concluded that a sample is non-compliant, with an error probability of $\alpha = 5\%$. The detection capability (CC_β) is the smallest content of the substance that may be detected, identified and quantified in a sample, with an error probability of $\beta = 5\%$. Both of these parameters were determined using the calibration curve procedure, in which blank bovine liver was fortified around the MRL values in equidistant steps ($0.5 \times$ MRL, MRL and $1.5 \times$ MRL). The concentration at the MRL plus 1.64 times the standard deviation at the MRL gives the CC_α value for each benzimidazole. The CC_β value for each benzimidazole is determined as the CC_α value + 1.64 times the standard deviation. Table 4 lists the CC_α and CC_β values for the benzimadazoles, using the data from Assay 1. With the exception of $NH_2ABZ-SO_2$, CC_α values ranged between MRL + 12% and MRL + 25% and CC_β values ranged between MRL + 25% and MRL + 45% for the benzimidazoles included in the study.

Table 4
Calculated CC_α and CC_β values based on data from Assay 1 (Table 3)

Benzimidazole	MRL ($\mu g\,kg^{-1}$)	CC_α ($\mu g\,kg^{-1}$)	CC_β ($\mu g\,kg^{-1}$)
NH_2ABZ-SO_2	1000	1303	1556
ABZ-SO	1000	1164	1343
ABZ-SO_2	1000	1225	1417
TBZ	100	116	132
OFZ/FBZ-SO	500	561	627
MBZ-OH	400	481	544
NH_2FLU	300	376	435
FBZ-SO_2	500	587	670
OXI	200	242	281
MBZ	400	467	520
FLU	400	493	558
ABZ	1000	1216	1397

4. Conclusions

This paper reports the development and validation of a relatively fast and simple LC method for the simultaneous determination of albendazole, albendazole sulphoxide and albendazole sulphone, thiabendazole, oxfendazole and fenbendazole sulphone, mebendazole and hydroxy-mebendazole, flubendazole and amino-flubendazole, and oxibendazole. Triclabendazole and its metabolites, triclabendazole sulphoxide and triclabendazole sulphone, were not quantitatively determined using this method. This multi-residue method covers six of the seven benzimidazole groups that are licensed for use within the EU.

Acknowledgements

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Appendix 3

ELSEVIER

Review

Review of methodology for the determination of benzimidazole residues in biological matrices

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Abstract

Benzimidazoles are anthelmintic agents widely used in the treatment of parasitic infections in a range of species and as fungicidal agents in the control of spoilage of crops during storage and transport. In this paper, the more important benzimidazoles are introduced and their pharmacological effects and physiochemical properties discussed. The metabolism of these drugs is described relating to the occurrence and persistence of residues in biological matrices, providing information for selection of suitable matrices and target residues for testing. Methods for determination of benzimidazoles are reviewed for a range of biological matrices. The importance of selecting suitable extraction and clean-up procedures is discussed, along with the difficulties encountered in adapting single residue methods to multi-residue methods. The importance of suitable detection systems for determination of benzimidazoles, namely, screening, HPLC, GC and confirmatory methods is described in detail. The future for benzimidazole residue analysis is discussed, focusing on selection of appropriate residues for screening methods and protocols for confirmation of benzimidazole residues.
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Contents

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1. Introduction

Benzimidazoles are veterinary drugs widely used for prevention and treatment of parasitic infections in agriculture and aquaculture. Some benzimidazoles have also found applications as pre- or post-harvest fungicides for control of a wide range of fungi affecting field crops, stored fruit and vegetables. Thiabendazole (TBZ) was the first benzimidazole to be marketed over 40 years ago [1]. It has been used widely for control of gastrointestinal nematodes, lungworms and as a fungicidal agent. After

its introduction, a number of alternative benzimidazoles offering similar activity came on the market, such as parbendazole (PAR) [2] cambendazole (CAM) [3] mebendazole (MBZ) [4] and oxibendazole (OXI) [5].

Benzimidazoles possessing sulphide and sulphoxide functional groups were subsequently introduced, offering a wider spectrum of activity and improved efficacy. Albendazole (ABZ) [6], fenbendazole (FBZ) [7] and oxfendazole (OFZ) [8] were the first such benzimidazoles to be successfully used in the treatment of all growth stages of gastrointestinal nematodes. They may be

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used also in the treatment of lungworms, tapeworms and adult stages of liver fluke. The benzimidazole, triclabendazole (TCB) was later introduced as an anthelmintic agent for treatment of all stages of liver fluke, but it is ineffective against nematodes [9]. Luxabendazole (LUX) is another benzimidazole-sulphide used in the treatment of food-producing animals but is not licensed for use in the EU [10]. The low solubility of benzimidazole sulphides and sulphoxides leads to their low absorption from the gut, resulting in low bioavailability [11,12]. Netobimin (NETO) [13] and febantel (FEB) [14], which are the pro-drugs of ABZ and FBZ, respectively, have greater water solubility resulting in improved absorption and increased bioavailability. Similar pro-benzimidazoles have found widespread use as fungicidal agents, including benomyl (BEN) and thiophanate-methyl (TM), which are precursors of carbendazim (MBC).

In this paper, a comprehensive review of methodologies for the determination of benzimidazole residues in biological matrices is presented. Firstly, an introduction is given on benzimidazole residues in relation to their mode of action, activity and toxicity. The metabolism and pharmacokinetics of these substances is then presented and discussed in relation to MRLs, and the selection of appropiate residues to monitor the presence of residues in food. Methodology for determination of benzimidazole residues in food is subsequently discussed in terms of sample handling, analysis, residues included and sensitivity. The paper concludes with comments on method validation, a general summary and notes on future perspectives.

2. Mode of action, biological activity and toxicity

2.1. Mode of action

A number of different modes of action have been proposed for benzimidazole drugs. ABZ has been shown to block glucose uptake in the larval and adult stages of susceptible parasites, by depleting their glycogen reserves and thereby decreasing ATP formation [15]. Blocking of glucose uptake was shown to be a mechanism of action for MBZ and TBZ [16]. A number of researchers have shown that benzimidazoles inhibit the enzyme fumaric acid reductase in the parasite, thus blocking the formation of succinic acid [16–18]. Consequently, this disturbs the normal operation of the dicarboxylic acid cycle, which replaces the Krebs cycle in anaerobic parasites [19,20]. The dicarboxylic acid cycle starts by fixing CO_2 on pyruvate, or preferably on phosphoenolpyruvate, to form oxaloacetate, and proceeds via malate and fumarate to form succinate as an end-product. Therefore, this cycle runs in anaerobic parasites in an opposite direction to the Krebs cycle, and its final electron receiver is fumarate. It is also coupled with a special electron transport system particular to anaerobic parasites for production of ATP. By blocking the cycle at the fumarate level, TBZ deprives the parasites of a significant source of energy, thus ultimately causing their paralysis and death [21,22].

Inhibition of microtubule formation has been identified as the primary mode of action of benzimidazole drugs by a number of researchers [15,23,24]. Many of the processes proposed as targets for benzimidazoles are dependent on the integrity of the microtubule matrix. Microtubules are associated with the following cellullar functions—formation of the mitotic spindle in cell division, maintenance of cell shape, cell motility, cellular secretion, nutrient absorption and intracellular transport. In view of the crucial roles microtubules play in many cellular processes, their drug-induced destruction eventually leads to the death of the organism. The dynamics of microtubule formation has been described in a number of papers, which should be consulted for more detail [25–28]. Inhibition of microtubule formation has been investigated in detail with the classical antimitotic agent colchicine showing that this compound binds to tubulin prior to its polymerisation [29]. Inhibition of microtubule assembly appears to be achieved through addition of colchicine-bearing tubulin to the end of a growing microtubule and loss of the ability of these subunits to accept other tubulin molecules for further microtubule growth. Data obtained from fluorescence spectroscopy and labelling experiments have indicated that the colchicine binding site is on the β-tubulin monomer, obviously close to the α,β subunit interface [30,31]. Other spectroscopic analyses suggest that inhibition of tubulin polymerisation is due to a drug-induced local unfolding of a small region within the β-tubulin monomer [29]. A similar mechanism may be responsible for inhibition of tubulin polymerisation by benzimidazoles, as the binding site of these compounds appears also to be located on the β-tubulin monomer [32–34].

The reason for selective effect of benzimidazoles towards parasites and high safety in host species is not well understood. Friedman and Platzer [35] demonstrated that the binding of FBZ and MBZ was 250- to 400-fold greater in parasite tubulin than mammalian tubulin. This provided the first evidence that the species selectivity of the benzimidazoles may derive from differences in their affinities for host and parasite tubulin. Kohler and Bachmann [36] showed in subsequent work that selectivity was more likely achieved by differential drug pharmacokinetics in the host and parasite. Russell et al. [37] found that the rate of dissociation of MBZ from parasite tubulin was much lower than the rate for dissociation of MBZ from mammalian tubulin. Therefore, it would appear that the selective toxicity of benzimidazoles may be due to differences in the strength of binding in parasite and host tubulin.

2.2. Activity

A summary of the activity of different benzimidazole drugs in the major food-producing animals is presented in this section. More detailed reports of drug activity are described elsewhere [11,38]. At the recommended dosage rates in cattle, TBZ (66 mg/kg bw) and OXI (10 mg/kg bw) are effective only against adult and developing larval stages of the common gastrointestinal (G.I.) roundworms. FEB shows additional activity against lungworms. ABZ-SO_2, FBZ and OFZ have broader spectra of activity which include adult larvae. ABZ-SO and OFZ are also effective against tapeworms. The spectra of activity are extended further to include adult fluke for NETO and ABZ although the dosages have to be increased to 20 and 10 mg/kg, respectively, for efficacy against this parasitic disease. TCB is ineffective

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against nematodes and cestodes but has excellent activity against all stages of the fluke (a trematode).

2.2.1. Sheep

OX1 is effective against adult and developing larval stages of the common G.I. roundworms. TBZ and MBZ have additional activity against lungworms, although TBZ has to be given at a higher dose rate for treatment of G.I. roundworms and lungworm. At a higher dose rate, TBZ is also effective against developing G.I. roundworms. MBZ is also active against tapeworms. ABZ-SO, FEB, FBZ and OFZ are effective against all stages of G.I. roundworms, they are also effective against lungworms and tapeworms. NETO and ABZ have similar activity to this group but are also effective against adult stages of fluke if given at an increased dose rate. TCB is effective against all stages of fluke.

2.2.2. Goats

MBZ and FBZ are effective against all the major G.I. roundworms, lungworms and tapeworms in goats.

2.2.3. Horses

All benzimidazoles and probenzimidazoles licensed for use in horses are effective against adult G.I. roundworms, although TBZ needs to be given at an increased dose rate for efficacy against *P. equorum*. TBZ, OX1 and FBZ are effective against G.I. roundworms if given at an increased dose. FBZ and OFZ are effective against migrating G.I. roundworm larva, although FBZ must be given at a much higher dose rate.

2.2.4. Pigs

OX1, flubendazole (FLU) and FEB are effective against G.I. roundworms in pigs. FLU, FEB and FBZ are also effective against lungworm. FBZ should be given at a higher dose rate for lungworms if treatment is being carried out on a single occasion.

2.2.5. Poultry

CAM is effective against larval and adult stages of G.I. roundworms in pigeons at a dose rate of 75 mg/kg given on two consecutive days. In pheasants, partridges and waterfowl, MBZ is effective against gapeworm and G.I. roundworms. FLU offers the broadest spectrum of activity of benzimidazoles in poultry and game birds, showing activity against G.I. roundworms, gapeworms and tapeworms.

2.3. Toxicity

McKellar and Scott [11] have reviewed the toxicity of benzimidazole veterinary drugs in detail. Excerpt from this review are summarised in this section. Benzimidazoles are regarded as safe up to 20 to 30 times the recommended dose. Acute toxicity is difficult to induce with these drugs and LD_{50} values are almost impossible to define for drugs such as TBZ and FBZ. Reports of acute toxicity of benzimidazoles in animals are very limited. The main toxic effect of the benzimidazole compounds involves their teratogenic effect, which was first reported for

PAR [39]. Congenital malformations resulting from administration of benzimidazole anthelmintics during gestation in ewes have been observed with CAM [40], OFZ [41] and ABZ [42]. However, FEB [43], FBZ [44], MBZ [45] and OX1 [46,47] do not appear to exert a teratogenic effect in sheep when administered in early gestation. Polyploidy was observed in cultured hamster ovary cells incubated with OX1 and, as a result, OX1 has been restricted to use in weaning piglets or at batch formation [48]. In breeding poultry, treatment with FLU in feed caused transient diarrhoea but there was no effect on egg production, fertility and hatchability [49]. In general, if these drugs are to be used in early pregnancy, it must be for good reason and at the lowest recommended doses. Seiler observed anaemia in dogs after prolonged treatment with TBZ [50]. In calves, treatment with cambendazole caused pulmonary oedema and necrotic lymphadenopathy, which was fatal in some cases [51].

Of particular note is the fact that some benzimidazole metabolites are more toxic than the parent drug; for example, hydroxymebendazole (MBZ-OH) has been found to be more embryotoxic than MBZ, in rat [52]. In rat, OFZ is teratogenic at about one-half the highest reported no-effect level for FBZ [52].

3. Important physical and chemical properties of benzimidazoles

The benzimidazoles are the largest chemical family used to treat endoparasitic disease in domestic animals. This group includes thiabendazole analogues and benzimidazole carbamates; substitution of various side chain and radicals on the parent benzimidazole nucleus produces the individual members. Newer benzimidazole carbamates are characterised by novel substituents on the benzimidazole nucleus and replacement of the thiazole ring by methylcarbamate. Such modifications have given rise to a new generation of benzimidazoles with much slower rates of elimination, higher potencies and broader activity spectra.

Much information about the physical and chemical properties of benzimidazoles can be found in reference books, from chemical suppliers and by simple calculations. However, information relating to important properties such as octanol–water partition coefficients and pK_a values are not readily available. Some researchers have referenced pK_a values in published papers for determination of benzimidazole residues in biological matrices, but information regarding determination of pK_a values (either by experimentation or computer software) is generally not quoted [12,53–56]. These properties, pK_a values in particular, provide important information regarding solubility and ion exchange properties of residues. It is known that benzimidazoles possess an imidazole ring containing both acidic and basic nitrogen atoms. Under suitable conditions, the molecule may be protonated ($pK_a \sim 5$–6) or deprotonated ($pK_a \sim 12$), as shown in Fig. 1 [57].

The pK_a values of a limited number of benzimidazoles have been quoted in the literature but mostly only one pK_a value, is listed when two or more usually exist. A summary of the pK_a values and octanol–water partition coefficients (K_{ow}) are listed in Table 1 (N = acidic or basic nitrogen groups and
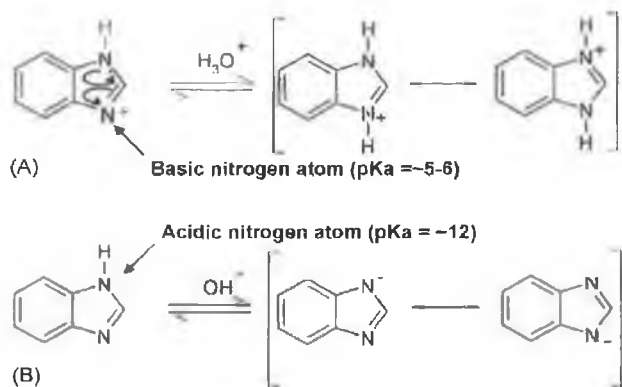
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Fig. 1. Ionisation of the benzimidazole molecule under acidic (A) and basic (B) conditions.

OH = hydroxyl groups) [57]. Experimental (from literature) and calculated (using computer software) values are quoted for each benzimidazole listed, where available. Each benzimidazole in Table 1 has pK_a values listed relating to acidic and basic nitrogen atoms on the imidazole ring. Other benzimidazoles, namely CAM, TBZ, TCB, FLU reduced metabolite (FLU-RMET) and hydroxy mebendazole (MBZ-OH), are more complex molecules possessing more than two ionisable groups. FLU-RMET and MBZ-OH possess acidic hydroxyl groups, with pK_a values of 13.1 and 13.8, respectively. This has important consequences for extraction and clean-up of 5-hydroxy thiabendazole (5-OH-TBZ) residues from biological matrices. A common approach is to carry out aqueous extraction of residues under acidic or, more commonly, alkaline conditions, before adjusting the pH and partitioning into ethyl acetate. Unfortunately, because 5-OH-TBZ is ionised over the complete pH range, it is readily soluble in most acidic or basic solutions and, therefore, difficult to extract into ethyl acetate. In the case of CAM, TCB and TCB metabolites, two or more pK_a values are indicated for each substance. Again, for these molecules it can be seen that the pH of the extraction solvent needs careful manipulation. Triclabendazole (TCB), triclabendazole sulphoxide (TCB-SO), triclabendazole sulphone (TCB-SO$_2$) and CAM are neutral in the ranges of pH 7.3–10.9, 5.0–9.0, 3.8–8.0 and 7.4–11.1, respectively. Therefore, the only pH range that would give non-ionised forms of these molecules is 7.4–8.0. The problem gets more complicated when the other

Table 1
Listing of important physical and chemical properties of benzimidazoles

Benzimidazole	CAS number	Octanol–water partition coefficient		pK_a values[a]			pH range at which substance is in neutral state
		Experimental	Calculated[b]	Experimental pK_a	Calculated		
					Basic groups	Acidic groups	
ABZ	54965-21-8		2.2–2.92		N: 5.54	N: 13.11	7.7–11.2
ABZ-SO	54029-12-8		0.82–0.94	7.8	N: 5.69	N: 13.25	7.6–11.2
ABZ-SO$_2$			0.9–1.01		N: 3.50	N: 11.20	5.5–9.2
ABZ-NH$_2$-SO$_2$	80983-34-2		0.69–0.75		N: 5.98	N: 13.30	7.9–11.3
FBZ	43210-67-9	1.95	3.07–4.01		N: 5.12	N: 12.72	7.1–9.8
OFZ	53716-50-0		1.88–2.13		N: 4.13	N: 11.79	6.1–9.8
FBZ-SO$_2$			2.13–3.30		N: 3.41	N: 11.12	5.4–9.2
FLU	31430-15-6		1.98–2.41		N: 4.10	N: 11.76	6.1–9.8
FLU-HMET			1.72		N: 6.58	N: 13.87	6.5–11.9
FLU-RMET			1.94		N: 5.57	N: 13.14 OH: 13.68	7.6–11.2
MBZ	31431-39-7		2.44–2.52	3.5	N: 4.13	N: 11.79	6.1–9.8
MBZ-OH			2.22–2.61	9.8	N: 5.60	N: 13.16 OH: 13.77	7.6–11.2
MBZ-NH$_2$			1.84–2.27	5.5	N: 6.61	N: 13.89	8.6–11.9
OXI	20559-55-1		1.86–2.63		N: 6.26	N: 13.78	8.2–11.8
TBZ	148-79-8	5.3–6.2	1.58–1.76	2.5,4.7	N: 5.22	N: 12.83	7.2–10.9
5-OH-TBZ			1.29–1.37	4.5	N: 7.65	Phenol: 5.46 N: 15.10	Always in ionised state
TCB	68786-66-3		4.90–6.66	10.5, 2.5	N: 5.31 N: 1.82	N: 12.91	7.3–11.0
TCB-SO			3.39–3.66		N: 3.00 N: 1.82	N: 10.95	5.0–9.0
TCB-SO$_2$			3.58–5.14		N: 1.86 N: 1.82	N: 9.99	3.8–8.0
CAM	26097-80-3		1.81–1.91		N: 5.44 N: −4.62	N: 13.04 N: 15.97	7.4–13.1
MBC	10605-21-7		1.29–1.69	9.52	N: 5.52	N: 13.09	7.5–11.1
Benzimidazole		1.5	0.8–0.95	4.36, 16.4	N: 5.30	N: 12.90	7.3–10.9
PAR	14255-87-9		1.86–2.63		N: 5.99	N: 13.53	7.9–11.6

N, ionisable nitrogen atom. OH, ionisable hydroxyl group.
[a] pK_a values calculated using Pallas Software Version 3.0 (CompuDrug International Inc., Grandview Drive, South San Francisco, CA 94080, USA).
[b] Octanol–water partition coefficient calculated using ChemDraw Ultra Software Version 6.0.1 (CambridgeSoft, 100 Cambridge Park Drive, MA 02140, USA).

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benzimidazoles are included; predicted pK_a values indicate that at a pH value of 8.0 most molecules are in their non-ionised state. However, a few exceptions exist, such as amino mebendazole (MBZ-NH$_2$) and OX1 which exist in a non-ionised form at pH values of >8.6 and 8.3, respectively.

The octanol–water partition coefficient, K_{ow}, although not generally quoted in the scientific literature, is also a very important property. It can provide an indication of the solubility of these residues in different solvents and give an indication of the elution conditions necessary for liquid chromatography. Most of these molecules have similar K_{ow} values, which are generally in the range 0.8–3.3. However, FBZ and the TCBs residues appear to be much more lipophilic in nature than the other benzimidazoles; these properties appear to be in good agreement with the polarity of the molecules, as indicated by chromatographic elution order in reversed phase LC systems.

4. Factors affecting the occurence of benzimidazoles in tissues of animal origin

4.1. Route of administration

Benzimidazoles are typically administered as an oral or intraruminal dose or in feed. The bioavailability of benzimidazoles depends greatly upon the route of adminstration and the drug in question. After administration of intraruminal doses of FBZ and ABZ to sheep, improved bioavailability of the drug was observed compared to oral doses [58]. Earlier work proposed that a portion of the oral dose can on occasion bypass by the rumen and rapidly enter the abomasum by closure of the oesophageal groove, reducing bioavailability [59]. In the case of NETO, the drug rapidly cleared from the plasma 12 h post-treatment, when administered parentally in sheep and cattle [60–62]. It was found that over 94% of the dose was excreted in urine as parent drug, with ABZ metabolites making up the remainder of residues [60]. This metabolism profile was reversed after intra-ruminal administration at the same dose [60]. In contrast to the other pro-benzimidazole drugs, FEB and NETO are not transformed by liver microsomal enzymes. NETO is converted in the gastro-intestinal tract, leading to greater bioavailability of the active form of the drug after intraruminal administration [63]. Group administration entails the inclusion of drugs in feed supplements and drinking water. Results show that there is great variation between farms in rate of consumption of feed blocks and between sheep on the same farm in their plasma levels of the drug [64,65].

4.2. Physiochemical properties of the drug

Drug particles must dissolve in the enteric fluids to facilitate absorption of the benzimidazole molecule through the GI mucosa. The dissolution rate influences the rate and extent of its absorption (systemic bioavailability), its maximal plasma concentration and its subsequent distribution and disposition kinetics. Drugs such as TBZ and CAM, the most hydrosoluble moeities among the benzimidazole anthelmintics, are extensively dissolved in aqueous ruminal fluid and rapidly absorbed

reaching maximal plasma concentrations as early as 4 h post administration to cattle [11]. Newer benzimidazole compounds show limited GI absorption due to their poor solubility in water, resulting in extended residence times for active metabolites of the substituted benzimidazole compound compared with those of TBZ or CAM [66,67]. Prichard et al. [67] showed that maximal plasma concentrations of TBZ in cattle occurs 4 h post treatment, compared with 24 h for FBZ, and that FBZ persists much longer than does TBZ in plasma and the GI tract. Consistently, TBZ and its metabolites are recovered in urine much more rapidly than are FBZ and its metabolites. This difference in pharmacokinetics in many ways accounts for the greater anthelmintic potency of FBZ compared with TBZ. Similar slow absorption of MBZ has been observed and residues can be found for 15 and 30 days post-treatment [68]. Different distribution patterns among benzimidazole sulphides (FBZ, ABZ), sulphoxides (OFZ, ABZ-SO) and sulphone (FBZ-SO$_2$, ABZ-SO$_2$) metabolites, based on their differential lipophilicities, may be expected. The distribution rate depends on molecular weight, lipid solubility, and plasma protein binding of each drug metabolite. The majority of benzimidazole compounds show a binding of less than 50% to plasma protein, relatively high volume of distribution, and a relatively fast elimination rate [11]. In contrast, TCB is strongly bound to plasma proteins, especially albumin which reduces its distribution in the body and increases its elimination half-life [69,70].

4.3. Animal species

On administration of either NETO prodrug or ABZ, ABZ-SO and ABZ-SO$_2$ are the major metabolites recovered in plasma of both sheep and cattle. However, pronounced differences in the plasma disposition and in the bioavailability of these metabolites between sheep and cattle have been shown [60–62,71,72]. Studies have shown the ratio of plasma area under curve (AUC) for ABZ-SO$_2$/ABZ-SO obtained in cattle to be higher in comparison to sheep, after treatment with NETO [60–62]. In addition, the elimination half-life and mean residence of the ABZ-SO metabolite were significantly longer in sheep compared to cattle [73]. The bioavailability of OFZ and its metabolites after oral administration was significantly lower in goats than in sheep [74,75]. However, the intravenous administration of OFZ in both species resulted in similar AUC values. In addition, FBZ was more rapidly cleared from plasma in goats than in cattle, and the detection of the sulphone metabolite [FBZ-SO$_2$] in plasma was delayed in cattle compared to goats [76,77].

4.4. Animal husbandry

Animal husbandry factors can have major effects on the disposition of benzimidazole drugs. Prichard et al. [78] found that the passage of digesta could influence the kinetics of orally administered benzimidazole drugs via binding of benzimidazoles to dietary fibre, modifiying bioavailability. Hennessy et al. [79] demonstrated that an increased rate of digesta passage through increased feed intake reduced bioavailability of OFZ and its metabolites in sheep. Lanusse et al. [73] proposed that
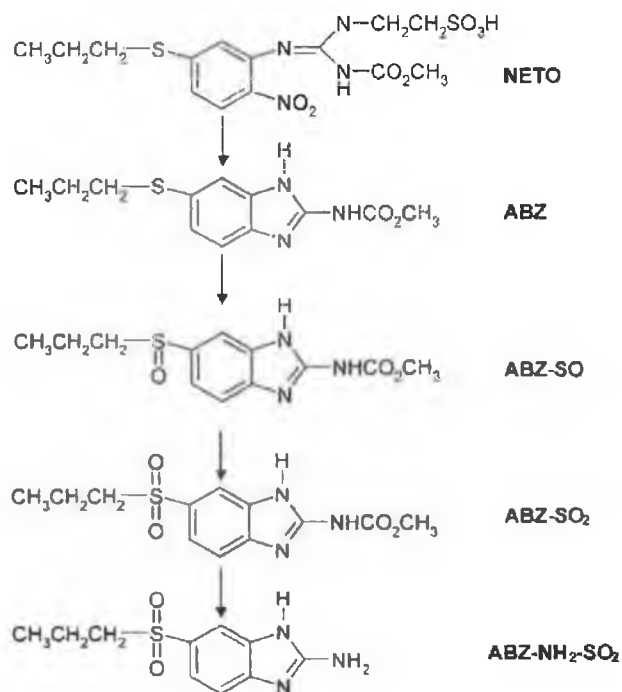
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Fig. 2. Major metabolites of netobimin and albendazole related drugs in tissue and milk.

Fig. 3. Major metabolites of febantel and fenbendazole related drugs in tissue and milk.

bacterial population in microflora can play an important role on biotransformation of benzimidazole drugs in the gasrointestinal tract. In particular, bacteria-mediated reduction of benzimidazole sulphoxides (OFZ and ABZ-SO) back to the parent drug (FBZ and ABZ), extending residence time of the active drug in the animal's body. Therefore, changes in microflora populations and variations in ruminal pH, based on dietary modifications, may affect the reductive activity of the GI fluids. This could lead to differences in pharmacokinetic behaviour and clinical efficacy between animals fed on grain-based diets and those reared exclusively on pasture.

5. Metabolism and distribution of benzimidazole residues in tissues

5.1. Metabolism of benzimidazoles

It has been found in animal studies that metabolism of these drugs is extensive (Figs. 2–7), with one or more major metabolites found in animal tissue or milk for each drug [11,52,80–82]. The metabolites found depend on the structure of the parent drug, the tissue and animal species. The drugs ABZ, FBZ, and TCB possess a sulphide linkage, which is susceptible to oxidation. As a result, two metabolites of the parent sulphide are possible, a sulphoxide (SO) and a sulphone (SO₂). MBZ and FLU possess a carbonyl group that may be reduced to form a hydroxy group. ABZ, FBZ, MBZ and FLU possess carbamate groups that may be hydrolysed to form an amino-benzimidazole. TBZ may be oxidised, resulting in the formation of 5-OH-TBZ. A 4-hydroxy metabolite of TCB and a keto metabolite have also been observed in rat, goat and sheep [18]. For other drugs, such as CAM, LUX,
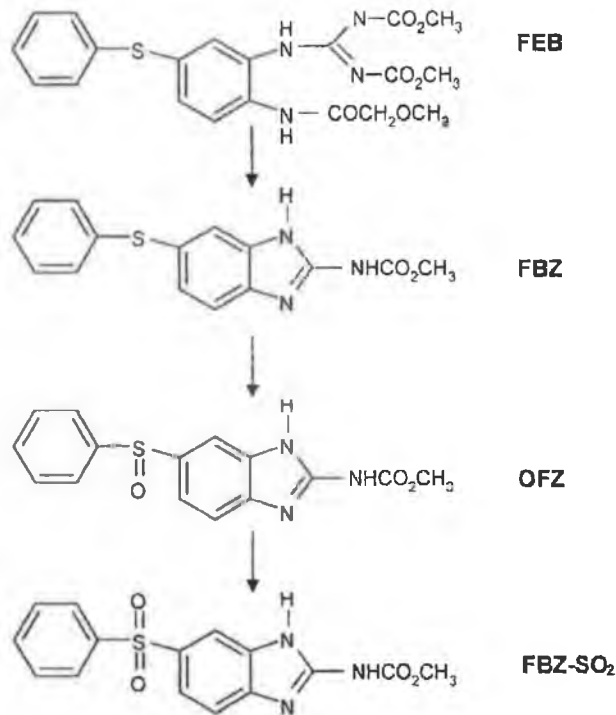
OX1 and PAR, little information has been reported. The structures of these drugs, along with some benzimidazole fungicides, are shown in Figs. 8 and 9. In the case of the pro-drugs FEB and NETO, they are converted into their benzimidazole form after dosing. FEB is rapidly converted into FBZ and does not persist as a major residue. NETO is rapidly converted into ABZ with only low levels of NETO residues occurring in tissue.

5.2. Albendazole, albendazole sulphoxide and netobimin

In cattle treated with single oral doses of radiolabelled ABZ, it was shown that residues mainly occur in the liver followed by the kidney with lower levels of residues detected in other tissues [83]. In calves, 90% of residues were extractable at 1 day after treatment with an oral dose of radiolabelled ABZ [83]. However, 4–10 days post-treatment only 20–30% of residues were extractable. The parent drug and its marker metabolites (sulphoxide, sulphone and amino-sulphone) accounted for 27
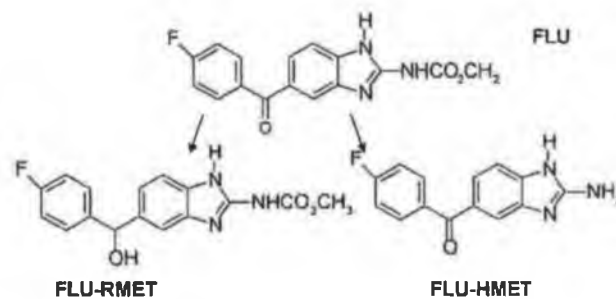

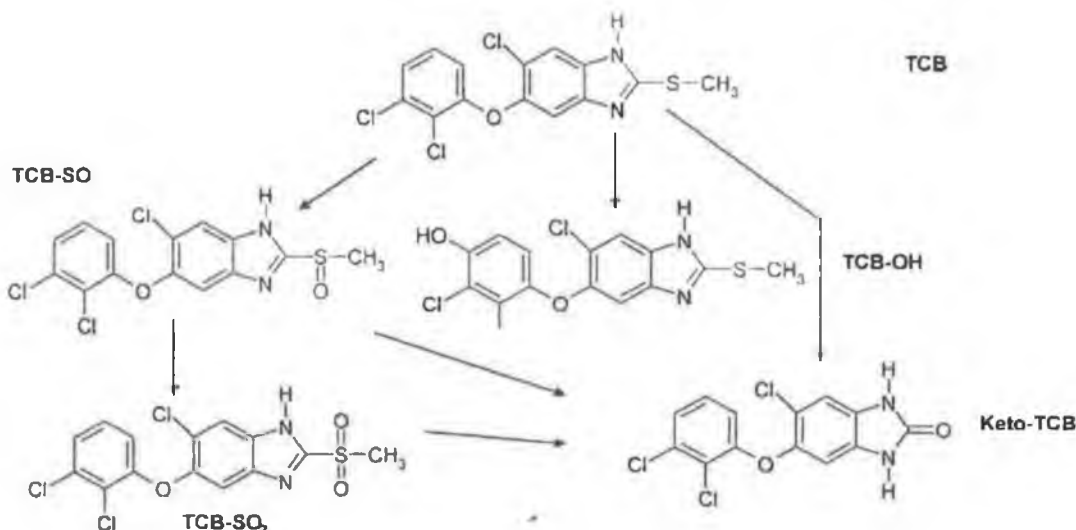Fig. 4. Major metabolites of flubendazole in tissue and eggs.

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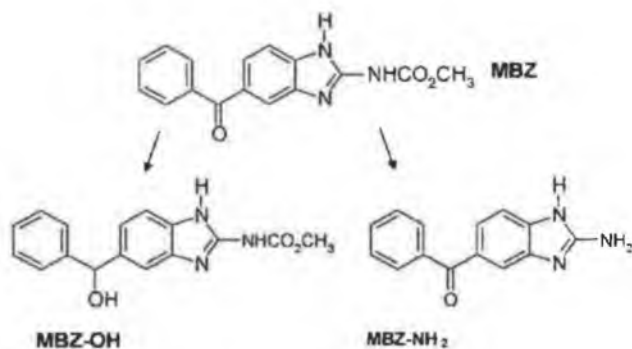Fig. 5. Major metabolites of triclabendazole in rats, goats and sheep.

Fig. 6. Major metabolites of mebendazole in tissue.

SO_2 was the major metabolite detected at 1 day post-treatment in liver and kidney [84]. A similar residue depletion profile was observed in poultry and pheasant [84]. In cattle and sheep treated with oral doses of NETO, ABZ was the major residue detected at 18 h post-treatment [85]. ABZ-SO and ABZ-NH_2-SO_2 were the major residues found at 3 days post-treatment [85]. Depletion studies of ABZ in trout, tilapia and salmon up to 120 h after treatment showed that ABZ-SO and ABZ-NH_2-SO_2 were the major residues found in trout and tilapia muscle and skin tissue. In salmon ABZ and ABZ-SO were the major residues found [86].

and 52% of total extractable residues at 1 day post-treatment, respectively. At 4 days post-treatment, ABZ was not detected in tissues and marker metabolites accounted for 40–50% of the total extractable residues. In sheep treated with a single oral dose of ABZ, 100% of residues were extractable at 1 day post-treatment. The distribution of residues in tissues was similar to that in cattle. However, only 37 and 13% of residues were extractable at 4 and 8 days post-treatment, respectively. In sheep treated with an intra-ruminal dose of ABZ for 7 and 14 days, it was shown that the marker residues accounted for 80–100, 52–58 and 47–74% of total extractable residues in muscle, liver and kidney at zero days post-treatment, respectively [83]. In sheep treated with an oral dose of ABZ-SO, it was shown that ABZ-

Fig. 7. Major metabolites of thiabendazole in tissue and milk.

Fig. 8. Structures of oxibendazole, cambendazole, parabendazole and luxbendazole.

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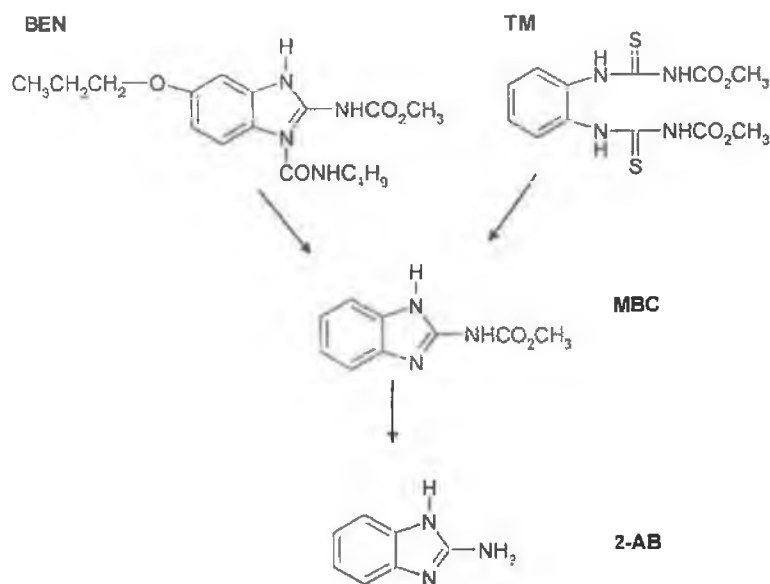*M. Danaher et al. / J. Chromatogr. B xxx (2006) xxx–xxx*

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Fig. 9. Structures of some benzimidazole fungicides and their breakdown products.

5.3. Febantel, fenbendazole and oxfendazole

Studies on animals treated with FEB, FBZ and OFZ have shown that FBZ, OFZ and FBZ-SO$_2$ were the major residues [25–29]. Similarly to ABZ, FBZ and related drug residues are mainly found in the liver and kidney, with lower levels found in the muscle and fat tissues. In cattle treated with an oral dose of FEB, 90% of residues were readily extractable 18 h post treatment [87]. At this time-point, FBZ, OFZ, FBZ-SO$_2$ and FEB accounted for 30–41, 4–19, 14–15 and 3–6% of total extractable residues. At 10 days post-treatment, <25% of residues were readily extractable from liver. The major residue found at 10 days post-treatment was the FBZ amino-sulphone (FBZ-SO$_2$-NH$_2$), which accounted for 12–35% of total residues. In sheep treated with an oral dose of FEB, residues were shown to occur mainly in liver, with much lower levels of residues being found in other tissues [88]. Separate studies in cattle and sheep treated with oral doses of FBZ reported approximately 20 and 50 times higher residue levels, respectively in liver than in the next highest residue containing tissue [89]. Blanchflower et al. [90] found that OFZ was the major residue found in the liver of sheep after administration of an oral dose of FBZ (5 mg/kg bw); FBZ was also detected but at lower levels. In pigs treated with an oral dose of FEB (5 mg/kg bw), residue levels in liver were 10 times higher in liver than in other edible tissues [88]. Capece et al. [91] investigated the distribution of oxidised FBZ residues in tissues after treatment of pigs with FBZ in medicated feed (5 mg/kg bw per day for 5 days). The study showed that highest residue levels were detected in the liver with lower levels found in kidney, followed by fat and muscle [91]. Sorensen and Hansen [92] investigated the distribution of FBZ and metabolites in the muscle and skin of trout after administration in medicated feed (1 g/kg feed for 1 week). The study showed that FBZ and OFZ residues were slightly higher in skin compared to muscle tissue, with lower levels of FBZ-SO$_2$ found.

5.4. Flubendazole

In pigs treated with FLU in medicated feed (1.2 mg/kg bw per day for 5 days), 71, 80, 90 and 89% of residues were shown to be readily extracted from liver, kidney, muscle and fat tissues, respectively [93]. At 5–30 days post-treatment, approximately 50% of residues were readily extractable from liver and kidney. At 6 h post-treatment, the hydrolysed metabolite of FLU (FLU-HMET) was the major residue found and accounted for 47, 94, 94 and 31% of total extracted residues in liver, kidney, muscle and fat tissues, respectively. At 10 days post-treatment, FLU-HMET accounted for 18 and 23% of residues in liver and kidney. In laying hens treated with FLU (2.7 mg/kg bw per day for 7 days), it was shown that 76–79% of residues were extractable at 24 h post-treatment from muscle, skin and fat tissues [93]. At this time-point, 49 and 61% of residues were extractable from kidney and liver tissues, respectively. At later time-points, 30–35% of residues were extractable from liver and kidney. Residues were distributed mainly in the liver and kidney with much lower levels being found in muscle, skin and fat. It was reported that the parent drug accounted for <3% of total extracted residues in liver and kidney tissues at 24 h post-treatment. The majority of residues comprised of FLU-HMET and FLU-RMET (the reduced metabolite of FLU). De Ruyck et al. [94] investigated the fate of FLU residues in turkey after administration of FLU in medicated feed (1.2 mg/kg bw for 7 days). They found that FLU-HMET was the major residue present in turkey breast muscle with lower levels of the parent drug being present. De Ruyck et al. investigated the fate of FLU residues in the tissues of guinea fowl in a separate study, finding that FLU-RMET was the major residue present in liver and muscle tissues [95]. FLU was also detected with lower levels of FLU-HMET present. It was shown that 80% of FLU are readily extractable from the eggs of laying hens treated with this drug. FLU was the major residue found in eggs, accounting for 40% of total residues at 1 and 9 days post-

treatment [93]. A study carried out by Kan et al. [96] supported the findings of this study.

5.5. Mebendazole

In sheep treated with an oral dose of mebendazole (15 mg/kg bw), 74, 95, 92 and 99% of residues were extractable from liver, muscle, kidney and fat tissues at 1 day post-treatment, respectively [97]. At 7 and 14 days post-treatment, 87 and 74–78% of residues were extractable from liver and kidney, respectively. The parent drug, accounted for 8, 3, 20 and 30% of total residues in liver, muscle, kidney and fat, at 1 day post-treatment. At the same time-point, MBZ-OH accounted for 47, 90, 14 and 67% of total residues in liver, muscle, kidney and fat, respectively. At 3 days post-treatment, MBZ and MBZ-OH accounted for 2 and 3.5% of total residues. De Ruyck et al. [98] found a similar distribution pattern of residues in the tissues of sheep treated with an oral dose of MBZ (20 mg/kg bw). The fate of MBZ residues in goat was found to be similar to that of sheep [97]. In horse, it was shown that the major residue found in edible tissues is $MBZ-NH_2$, with much lower levels of MBZ and MBZ-OH detected [97]. Residues were found at highest levels in the liver and kidney, with lower levels found in muscle and fat. Iosifidou et al. [99] investigated the fate of MBZ in farmed eel, finding that residues mainly occurred in the liver and kidney with lower levels found in skin and muscle. $MBZ-NH_2$ was the major residue found with lower levels of MBZ and MBZ-OH present.

5.6. Thiabendazole

In calves treated with an oral dose of TBZ, it was found that residues mainly occurred in the liver and kidney [100]. At 1 day post-treatment, residues were found to occur mainly in the kidney with lower levels found in liver. At later withdrawal periods (>2 days), residues were mainly found in the liver [100]. Chukwudebe et al. [101] investigated the fate of TBZ residues in goats treated with oral doses (120 mg per animal daily for 7 days). Residues were shown to occur mainly in the liver and kidney. The major residue found in tissue at 1 day post-treatment was TBZ, with lower levels of 5-OH-TBZ present. The same study showed that at 1 day post-treatment, 5-OH-TBZ was the major residue detected in the tissues of laying hen, with lower levels of TBZ found [101]. Residues occurred mainly in the kidney with lower levels found in the liver. Wilson et al. [102] investigated the fate of TBZ in cattle, sheep and pig liver. In sheep treated with an oral dose of TBZ (50 mg/kg bw), animals were sacrificed at 7 and 10 days post-treatment. TBZ was the only residue found and in one animal at 10 days post-treatment. In pigs, treated with an oral dose (50 mg/kg bw), residues were not detected in liver at 7 and 10 days post-treatment. In cattle treated with an oral dose (50 mg/kg bw), TBZ was the major residue found with lower levels of 5-OH-TBZ. Coulet et al. [103] carried out an in vitro study of the metabolism of TBZ in the cultured hepatocytes of rats, rabbits, sheep, calves and pigs, finding 5-OH-TBZ to be the major metabolite in calf and pig hepatocytes. Several metabolites were found in sheep, consisting mainly of 5-OH-TBZ (40%) and three unknown metabolites representing 26, 25 and 15% of residues found.

5.7. Triclabendazole

In cattle and sheep treated with oral doses of TCB it was shown at early withdrawal periods (2 days) that residues occurred at higher levels in the liver and kidney, with lower levels found in muscle and fat [104]. At longer withdrawal periods (>28 days), it was found that similar levels of residues could be found in muscle, liver and kidney. The contribution of individual TCB metabolites to the marker residues in animal tissues has not been reported. Most studies report the depletion of total marker residues and not individual components.

5.8. Oxibendazole

There is very little information available on the metabolism of OXI, but studies indicate that the drug is significantly metabolised in the liver and kidney [47,105]. Two metabolites 5-OH-OXI and 6-OH-OXI have been identified in urine and tissues, but only accounted for 15–20% of the total residues (the remainder are unidentified) [105].

5.9. Cambendazole

Early studies on the drug found that it was rapidly metabolised producing at least 13 urinary metabolites [106,107]. The identification of marker residues in animal tissues is not well described because the drug is not marketed for use in food producing animals.

5.10. Benomyl

Gardiner et al. [108] investigated the elimination of benomyl (BEN) residues in animals, finding 5-OH benzimidazole carbamate to be the major metabolite, with lower levels of the 4-OH metabolite present.

5.11. Summary

In cattle and sheep treated with ABZ, ABZ-SO or NETO, it has been shown that ABZ-SO or $ABZ-SO_2$ are the most likely residues to be found in the case of an MRL violation. $ABZ-NH_2-SO_2$ is perhaps the most persistent residue in tissue but generally occurs at levels below the MRL. There is very little information available on the presence of individual FBZ residues in animal tissues, although Blanchflower et al. [90] found that OFZ was the major residue present in the tissues of sheep treated with FBZ. In cattle treated with FEB, FBZ was the major residue found at early withdrawal periods, with lower levels of OFZ and $FBZ-SO_2$ found.

FLU parent drug is the major residue found in the eggs of laying hens, while FLU-HMET is the major metabolite present in pig and turkey tissues. A recent study has shown that FLU-RMET is the major residue found in the tissue of guinea fowl, indicating that it should be listed as a marker residue. MBZ-OH

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and MBZ-NH_2 are the major MBZ residues found in sheep and horse liver, respectively. In cattle, goats and sheep treated with TBZ, the parent drug was shown to be the major residue found in liver after treatment. The 5-OH-TBZ metabolite occurs only at low levels in these species, but was shown to be the major metabolite in poultry at early withdrawal periods. There is very little information available on the occurrence of individual TCB residues in tissues. However, pharmacokinetic data indicates that TCB-SO and TCB-SO_2 would be the most likely residues to be found. Very little data has been published on the metabolism and distribution of CAM and OXI residues in tissues.

6. Depletion of benzimidazole residues in tissues

6.1. Netobimin, albendazole and albendazole sulphoxide

In cattle treated with an oral dose of ABZ-SO (12 mg/kg bw), ABZ-SO marker residues were below the MRLs in liver and kidney at 2 days post-treatment [84]. At 1 day post-treatment, ABZ-SO_2 was detected in liver and kidney at levels of 2953 and 1355 μg/kg, respectively. ABZ-SO was detected at 1 day post-treatment in liver and kidney at levels of 294 and 233 μg/kg. In cattle treated with an oral dose of netobimin (20 mg/kg bw), marker residues were below the MRL at 3 days post-treatment [85]. At 10 h post-treatment, ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2, were found at levels of 7354, 771, 5289 and 0 μg/kg in liver, respectively. These parent/metabolite residues were at levels of 3285, 467, 1879 and 0 μg/kg in kidney, 792, 1909, 1740 and 0 μg/kg in muscle; and 556, 195, 474 and 0 μg/kg in fat, respectively. ABZ residues were detectable at 1 day post-treatment with NETO but were not detected at 3 days. At 3 days post-treatment and thereafter, the only residue present was ABZ-NH_2-SO_2, which could be found at levels of 243, 213 and 81 μg/kg at 3, 7 and 14 days post-treatment. In sheep treated with an oral dose of NETO (20 mg/kg bw), marker residues were below the MRL at 3 days post-treatment [85]. At 18 h post-treatment, ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2, were found at levels of 18,000; 3100; 4200 and 78 μg/kg in liver, respectively. At 18 h post-treatment, these residues were at levels of 7400, 2400, 110 and 110 μg/kg in kidney, 2200, 3400, 1100 and 0 μg/kg in muscle, and 303, 983, 555 and 0 μg/kg in fat, respectively. At 3 days post-treatment, ABZ-SO and ABZ-NH_2-SO_2 were found at levels of 150 and 770 μg/kg in liver tissues, respectively. At 3 days post-treatment, ABZ-NH_2-SO_2 was found in kidney at a level of 226 μg/kg. At 10 days post-treatment, ABZ-NH_2-SO_2 was the major residue found in liver and kidney at levels of 150 and 31 μg/kg, respectively.

In pheasant treated with ABZ-SO in medicated feed (17 mg/kg bw per day for 3 days), residues were found to be below the MRL at 1 day post-treatment [84]. At this time-point, combined ABZ-SO and ABZ-SO_2 residues were found at levels of 168, 479 and 366 μg/kg in muscle, liver and kidney tissues, respectively. Combined ABZ-SO and ABZ-SO_2 residues were more persistent in liver than other tissues and were found at levels of 90 and 45 μg/kg at 3 and 7 days post-treatment, respectively. ABZ-NH_2-SO_2 was only found in liver tissue and at levels of 24, 168 and 99 μg/kg at 1, 3 and 7 days post-treatment, respectively.

Shaikh et al. investigated the depletion of ABZ and its metabolites in salmon, tilapia, trout after treatment with ABZ (oral dose 10 mg/kg bw) [86]. In salmon, ABZ and ABZ-SO were found in muscle tissue at levels of 22 and 39 μg/kg, respectively at 24 h post-treatment. ABZ-SO_2 and ABZ-NH_2-SO_2 were also present but at lower levels, typically less than 5 and 10 μg/kg, respectively. ABZ was found in tilapia at a level of 678 μg/kg at 8 h post-treatment but was not present at later time-points. ABZ-SO was found at levels of 39 and 31 μg/kg at 8 and 12 h post-treatment, respectively. ABZ-SO was generally at 7 μg/kg or less at 24 h. ABZ-SO_2 was at or below 7 μg/kg at all time-points. ABZ-NH_2-SO_2 was the more persistent residue, present at levels of 18, 100 and 53 μg/kg at 8, 72, and 120 h post-treatment, respectively. ABZ parent drug was not found in trout muscle. The major residues found were ABZ-SO (18–47 μg/kg) followed by ABZ-SO_2 (5–14 μg/kg) and ABZ-NH_2-SO_2 (3–10 μg/kg).

6.2. Febantel, fenbendazole and oxfendazole

In cattle treated with FBZ (oral dose 7.5 mg/kg bw), animals were sacrificed at 7 and 21 days post-treatment. Marker residues (FBZ, OFZ and FBZ-SO_2) were below the MRL in all tissues at 7 days post-treatment at levels of 5, 7, 8 and 194 μg/kg in fat, kidney, muscle and liver, respectively [89]. In sheep treated with FBZ (oral dose 10 mg/kg bw), animals were sacrificed at 5 and 9 days post-treatment [89]. At 9 days post-treatment, marker residues were below the MRL in all tissues, with the exception of liver. At this time, combined marker residues were present at levels of <5, <6, 6 and 745 μg/kg in kidney, fat, muscle and liver, respectively. At 5 days post-treatment, marker residues were found to be at levels of 79 and 3659 μg/kg in kidney and liver. In sheep treated with an oral dose of OFZ (5 mg/kg bw), animals were sacrificed at 10 and 24 days post-treatment [109]. Marker residues were below the MRLs at 10 days post-treatment. Highest residue levels (at 10 days post-treatment) were found in the liver at a level of 476 μg/kg. In sheep treated with an oral dose of FEB (5 mg/kg bw), animals were sacrificed at 3 and 7 days post-treatment [88]. Marker residues were above the MRL at 7 days post-treatment. At this time-point, marker residues were found at a level of 942 μg/kg in liver (nearly twice the MRL). At 3 days, marker residues were found at levels of 40, 4617, 199 and 133 μg/kg in muscle, liver, kidney and fat, respectively.

In pigs treated with an oral dose of FEB (5 mg/kg bw), animals were sacrificed at 12, 20 and 34 days post-treatment [88]. Marker residues were below the MRL at 34 days post-treatment. At 24 days post-treatment, marker residues were found at levels of 402, 6, 12 and 100 μg/kg in liver, muscle, kidney and fat, respectively. The marker residues were above the MRL in fat, which is 50 μg/kg. Blanchflower et al. [90] investigated the depletion of FBZ and OFZ in sheep treated with an oral dose of FBZ (5 mg/kg bw). Animals were sacrificed at 1, 2, 4, 7, 10 and 15 days post-treatment. It was shown in this study that marker residues were typically below the MRL of 500 μg/kg at 14 days post-treatment in liver. At 2 days post-treatment, OFZ and FBZ were both found at levels of approximately 3000 μg/kg. OFZ was shown to be a much more persistent residue in liver tissue than FBZ. Capece et al. [91] monitored the depletion of oxi-

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dised FBZ residues in pigs after treatment with an oral dose of FBZ (5 mg/kg bw per day for 5 days). Marker residues were below the MRLs in all tissues at 2 days post-treatment. At 1 day post-treatment, marker residues were found in liver, kidney, muscle and fat, at levels of 5552, 917, 623 and 1423 μg/kg, respectively. Sorensen and Hansen [92] investigated the depletion of FBZ, OFZ and FBZ-SO$_2$ in trout after treatment with an oral dose of FBZ in medicated feed (1 g/kg feed for 1 week). At 1 day post-treatment, FBZ, OFZ and FBZ-SO$_2$ were found at levels of 56–389, <2–252 and <4–53 μg/kg, respectively. At 6 weeks post-treatment, only low concentrations of residues were reported.

6.3. Flubendazole

Pigs treated with FLU in medicated feed (1.2 mg/kg bw per day for 7 days), animals were sacrificed at 6 h and 10 days post-treatment [93]. Residue depletion data showed that FLU marker residues (FLU and FLU-HMET) were below the MRL at 10 days post-treatment. At 6 h post-treatment, FLU-HMET was found at levels of 1817, 2517, 246 and 66 μg/kg in liver, kidney, muscle and fat, respectively. FLU was found at levels of 39, 51, 30 and 62 μg/kg in liver, kidney, muscle and skin-fat, respectively. In turkeys treated with FLU in medicated feed (30 mg/kg feed per day for 7 days), birds were sacrificed at 6 h, 1 day, 3 days, 7 days and 9 days post-treatment [93]. Marker residues were found to be below the MRL at 1 day post-treatment. At 6 h post-treatment, FLU was found at levels of 64, 67, 18 and 60 μg/kg in liver, kidney, muscle and skin-fat, respectively. At 6 h post-treatment, FLU-HMET was found at levels of 29, 11, 0 and 0 μg/kg in liver, kidney, muscle and skin-fat, respectively. At 6 h post-treatment, FLU-RMET was found at levels of 200, 80, 42 and 32 μg/kg in liver, kidney, muscle and skin-fat, respectively. FLU and FLU-HMET are the marker residues for FLU in all species. De Ruyck et al. [94] monitored the depletion of FLU marker residues in turkeys (1.2 mg/kg bw per day for 7 days). At 1 day post-treatment, combined FLU and FLU-HMET residues were at levels of 102 and 120 μg/kg in thigh muscle, respectively. In liver, combined FLU and FLU-HMET residues were found at a level of 2334 μg/kg. At 2 days post-treatment, marker residues were below the MRL in all tissues.

In laying hens treated with FLU in medicated feed (3.6 mg/kg bw per day for 7 days) eggs were collected at 7 and 11 days post-treatment [93]. FLU levels in egg ranged between 118 and 230 μg/kg at 7 days post-treatment to 13 μg/kg at 11 days post-treatment. Kan et al. [96] investigated the depletion of FLU residues in eggs collected from laying hens treated with rations of medicated feed containing different concentrations of FLU (2.6, 9.4 and 27 mg/kg feed) for 21 days. The study showed that FLU residues in egg reached a maximum level of 150 μg/kg in whole egg during the treatment period after consuming the ration containing the highest levels of FLU. In pheasant treated with FLU in medicated feed (3.6 mg/kg bw per day for 7 days), birds were sacrificed at 6 h, 1 and 10 days post-treatment [93]. This group monitored the depletion of FLU only and not metabolites. At 6 h post-treatment, FLU was found at levels of 35, 58, 18.5 and 76 μg/kg in liver, kidney, muscle, and skin-fat, respec-

tively. FLU was more persistent in skin-fat and could be found at levels of 29 and 12 μg/kg at 1 and 7 days post-treatment, respectively. De Ruyck et al. [95] investigated the depletion of FLU and it's metabolites in guinea fowl after administration in medicated feed (56 mg/kg feed for 7 days). The highest concentration in thigh muscle during the treatment period for FLU-RMET (Day 3) and FLU plus FLU-HMET (Day 4) was 312 and 114 μg/kg, respectively. At 1 day post-treatment, FLU-RMET and combined FLU plus FLU-HMET residues were detected in thigh muscle at levels of 81 and 50 μg/kg, respectively. In liver, highest levels of FLU, FLU-HMET and FLU-RMET during treatment were 108, 23 and 1043 μg/kg, respectively. At 1 day post-treatment, FLU, FLU-HMET and FLU-RMET were 17, 11 and 283 μg/kg in liver, respectively.

6.4. Mebendazole

In horse treated with an oral dose of MBZ (8.8 mg/kg bw), animals were sacrificed at 1 and 28 days post-treatment [97]. At 1 day post-treatment, MBZ-NH$_2$ was the major residue, which was found at concentrations of 5047, 5851, 497 and 156 μg/kg in liver, kidney, muscle and fat, respectively. At the same time-point, MBZ was found at levels of 728, 29, 16 and 57 μg/kg in liver, kidney, muscle and fat, respectively. MBZ-OH was found at levels of 293, 85, 84 and 60 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, MBZ-NH$_2$ was the only marker residue detected in liver and kidney, at levels of 182 and 23 μg/kg, respectively. In sheep treated with an oral dose of MBZ (20 mg/kg bw), animals were sacrificed at 1, 7, 14, 21 and 28 days post-treatment [97]. At 28 days post-treatment, marker residues (MBZ, MBZ-OH and MBZ-NH$_2$) were below the MRL in all tissues. At 1 day post-treatment, MBZ was at 1016, 1460, 21 and 343 μg/kg; and MBZ-OH was at 7582, 1531, 1783 and 758 μg/kg; and MBZ-NH$_2$ was at 18, 147, 0 and 0 μg/kg in liver, kidney, muscle and fat, respectively. At 7 days post-treatment, residues were only detectable in liver and kidney. MBZ, MBZ-OH and MBZ-NH$_2$ were determined in liver at levels of 56, 272 and 27 μg/kg, respectively. In kidney, MBZ, MBZ-OH and MBZ-NH$_2$ were determined at 27, 38 and 44 μg/kg, respectively. De Ruyck et al. [98] carried out a similar study in sheep at the same dosage. However, in this study residues were shown to be below the MRL in all tissues at 7 days post-treatment. In goats treated with an oral dose of MBZ (20 mg/kg bw), animals were sacrificed at 1 and 28 days post-treatment [97]. At 28 days post-treatment, marker residues were below the MRL in all tissues. In liver, MBZ-OH and MBZ-NH$_2$ were found at levels of 308 and 19 μg/kg, respectively. At 1 day post-treatment, highest residues levels were found in the liver, where MBZ, MBZ-OH and MBZ-NH$_2$ were present at levels of 1020, 7502 and 151 μg/kg, respectively.

6.5. Thiabendazole

A number of studies reporting the depletion of combined marker residues (TBZ and 5-OH-TBZ) have been described in different species after treatment with TBZ [100]. In sheep treated with an oral dose of TBZ (44 mg/kg bw), marker residues were

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found in liver at levels of 4680, 326, 825 and <50 μg/kg at 1, 2, 3 and 4 days post-treatment, respectively [100]. In calves treated with an oral dose of TBZ (75 mg/kg bw), combined marker residues at 1, 2 and 6 days post-treatment were 472, 215 and 63 μg/kg in liver; 638, 97 and <50 μg/kg in kidney; 75, 50 and <28 μg/kg in muscle and 89, <50 and <50 μg/kg in fat [100]. Wilson et al. [102] reported the depletion data for individual TBZ marker residues in a study in sheep and cattle. In sheep treated with an oral dose of TBZ (50 mg/kg bw), animals were sacrificed at 7 and 10 days post-treatment. TBZ was found in one animal at a level of 72 μg/kg at 10 days post-treatment. In cattle treated with an oral dose of TBZ (50 mg/kg bw), animals were sacrificed at 1 and 2 days post-treatment. Marker residues were below the MRL in the liver of cattle at 2 days post-treatment. TBZ was the major residue found at this withdrawal period at a level of 71 μg/kg. At 1 day post-treatment, TBZ and 5-OH-TBZ were found in one animal at levels of 677 and 259 μg/kg, respectively.

6.6. Triclabendazole

In cattle treated with an oral dose of TCB (12 mg/kg bw), animals were sacrificed at 2 and 42 days post-treatment [104]. At 2 days post-treatment, combined marker residues (TCB, TCB-SO) and TCB-SO$_2$) were found at levels of 5870, 4300, 1420 and 2470 μg/kg in liver, kidney, muscle and fat, respectively. At 42 days post-treatment, combined marker residues were found at levels of 80, 75, 100 and <60 μg/kg in liver, kidney, muscle and fat, respectively. In a second study, cattle treated with an oral dose of TCB (12 mg/kg bw) showed, at 2 days post-treatment, combined marker residues at levels of 3250, 3050, 875 and 1800 μg/kg in liver, kidney, muscle and fat, respectively [104]. At 28 days post-treatment, combined marker residues were found at levels of 52, 47, 23 and <3 μg/kg in liver, kidney, muscle and fat, respectively. In a study on cattle treated with an oral dose of a combination product (12 mg/kg bw (TCB) and 7.5 mg/kg bw (levamisole)), the cattle were slaughtered at 1, 21 and 28 days post-treatment [104]. At 1 day post-treatment, marker residues were found at levels of 7050, 5800, 1400 and 5800 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, TCB marker residues were found at levels of 144, 102, 142 and <40 μg/kg in liver, liver, kidney, muscle and fat, respectively. In a similar study on sheep treated with an oral dose of a combination product (12 mg/kg bw (TCB) and 7.5 mg/kg bw (levamisole)), the animals were sacrificed at 2 and 28 days post-treatment [104]. At 2 days post-treatment, TCB marker residues were found at levels of 3500, 3100, 1420 and 1350 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, TCB marker residues were found at levels of 127, 119, 95 and <29 μg/kg in liver, kidney, muscle and fat, respectively. In a study on sheep treated with an oral dose of a combination product (10 mg/kg bw (TCB) and 7.5 mg/kg bw (levamisole)), the animals were slaughtered at 1, 21 and 28 days post-treatment [104]. At 1 day post-treatment, TCB marker residues were found at levels of 7400, 6800, 2100 and 10,100 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, TCB marker residues were found at

levels of 238, 198, 321 and 23 μg/kg in liver, kidney, muscle and fat, respectively.

6.7. Summary

The marker residues of ABZ, ABZ-SO and netobimin are rapidly cleared from the edible tissues of cattle and sheep; and are typically below the MRL at 4 days post-treatment. Results of studies verify the suitability of the withdrawal periods that are specified in cattle (14 days) and sheep (3–10 days) [110]. FLU marker residues were shown to be below the MRLs in turkey and pigs at 1 and 10 days post treatment, respectively. The withdrawal period specificed for FLU marker residues in turkey and pig is 7 days. Two separate studies have been carried out in eggs, showing that FLU residues do not occur at levels above the MRL during or after treatment. As a result, a zero withdrawal period has been specified for FLU in eggs. Marker residues for FBZ and related drugs (OFZ and FEB) were shown to be below the MRL in the edible tissues of cattle, sheep and pig at 10, 7 and 2 days post-treatment, respectively. The withdrawal periods for FBZ related drugs in cattle, sheep and pigs range between 14–35, 7–21 and 3–35 days, respectively. MBZ and TCB have been identified as the most persistent benzimidazole drug in animal tissues. In horse and sheep, MBZ marker residues were below the MRL at 28 days post-treatment. TCB residues were shown to be below the MRL in cattle at 28 to 42 days (two separate studies) and above the MRL in sheep at 28 days post-treatment. As a result, withdrawal periods for MBZ and TCB are typically >14 and 28 days, respectively. TBZ is rapidly cleared from the edible tissues of cattle and sheep, to below the listed MRLs, at 6 and 4 days post-treatment, respectively. No withdrawal period has been specified for TBZ, which may be an indication that this drug has been withdrawn from the market. The depletion of others benzimidazole drugs, namely OXI and CAM, are not well described for the edible tissues of food-producing animals. The withdrawal period for OXI is 7 days in pig, indicating that it is one of the least persistent benzimidazole residues. CAM is not approved for treatment of food-producing species and detailed residue depletion studies have not been conducted.

7. Depletion of benzimidazole residues in milk

7.1. Netobimin, albendazole and albendazole sulphoxide

In dairy cows treated with NETO (oral dose 20 mg/kg bw), ABZ-SO residues were found at levels of 7374, 2278 and 210 μg/kg at 7.5, 22 and 31.5 h post-treatment, respectively. ABZ-SO$_2$ residues were found at levels of 2336, 3410 and 81 μg/kg at 7.5, 22 and 31.5 h post-treatment, respectively. Residues of NETO, ABZ and ABZ-NH$_2$-SO$_2$ were not detectable at any timepoint [85]. Fletouris et al. [111] investigated the depletion of ABZ, ABZ-SO and ABZ-SO$_2$ in the milk of dairy cows treated with ABZ (oral dose 15 mg/kg bw). At 12 h post-treatment, ABZ-SO and ABZ-SO$_2$ were found at levels of 163 and 931 μg/kg, respectively. At 24 h post-treatment, ABZ-SO and ABZ-SO$_2$ residues were found at levels of 800 and 853 μg/kg, respectively. ABZ-SO and ABZ-SO$_2$ residues were

below the MRL at 36 h post-treatment. Cinquina et al. [112] investigated the depletion of ABZ residues in the milk of sheep and goats treated with ABZ (oral dose 3.75 mg/kg bw). At 24 h post-treatment, ABZ-SO residues were found approximately at 1100 and 1700 μg/kg in goat and sheep milk, respectively. At 24 h post-treatment, ABZ-SO$_2$ residues was found at an approximate level of 500 μg/kg in goat and sheep milk. At 48 h post-treatment, ABZ-SO and ABZ-SO$_2$ were the major residues found in sheep milk. At 48 h post-treatment, ABZ-NH$_2$-SO$_2$ was the major residue found in goat milk, with lower levels of ABZ-SO$_2$ present. At 72 h post-treatment, residues were typically at less than 100 μg/kg in goat and sheep milk. De Liguoro et al. [113] investigated the depletion of ABZ marker residues in milk from sheep treated with ABZ (oral dose 12.5 mg/kg). ABZ residues were not detected in milk at any time-point. At 12 h post-treatment, ABZ-SO and ABZ-SO$_2$ were found at levels of 3896 and 902 μg/kg, respectively. At 48 h post-treatment, ABZ-SO and ABZ-SO$_2$ were found at levels of 62 and 106 μg/kg, respectively. ABZ-NH$_2$-SO$_2$ was only found at 36 h post-treatment and at a level of 89 μg/kg. Chu et al. [114] investigated the depletion of ABZ marker residues in cows milk after treatment with ABZ (oral dose 10 mg/kg bw). At 24 h post-treatment, combined marker residues were found at a level of 576 μg/kg. At 48 h post-treatment, combined marker residues were found at a level of 67 μg/kg.

Moreno et al. [115] compared the milk residue profiles of ABZ marker residues in dairy cows treated with ABZ oral (oral dose 5 mg/kg bw) and ABZ-SO aqueous injectable (3 mg/kg bw) formulations. At 12 h post-treatment, ABZ-SO and ABZ-SO$_2$ residues were found at levels of 280 and 860 μg/kg after oral administration, respectively. After administration by sub-cutaneous injection ABZ-SO and ABZ-SO$_2$ were at levels of 180 and 80 μg/kg, respectively. In both studies, residues were not detectable at 36 h post-treatment.

7.2. Febantel, fenbendazole and oxfendazole

Fletouris et al. [111] investigated the depletion of FBZ marker residues in the milk of a dairy cow treated with FBZ (oral dose 10 mg/kg bw). At 12 h post-treatment, FBZ, OFZ and FBZ-SO$_2$ residues were found at levels of 85, 196 and 48 μg/kg, respectively. Marker residues were shown to be below the MRL at 84 h post-treatment. In dairy cows treated with FEB (unde-scribed dosage), marker residues depleted from 268 to <5 μg/kg at 24–130 h post-treatment, respectively [89]. Residues were below the MRL at 106 h post-treatment. In sheep treated with FEB (undescribed dosage), marker residues depleted from 357 to <5 μg/kg at 10–130 h post-treatment, respectively [88]. Residues were below the MRL at 120 h post-treatment. Tai et al. [116] investigated the depletion of FBZ and OFZ residues in the milk of dairy cows treated with FBZ (oral dose 10 mg/kg bw). At 28 h post-treatment, FBZ and OFZ residues were found at levels of 105 and 441 μg/kg, respectively. OFZ residues were found to be more persistent in milk than FBZ residues and OFZ could be found at a level of 29 μg/kg at 76 h post-treatment. Moreno et al. [115] compared the milk residue profiles of OFZ marker residues in dairy cows treated with oral (5 mg/kg bw)

and aqueous injectable (3 mg/kg bw) formulations. At 12 h post-treatment, OFZ, FBZ and FBZ-SO$_2$ residues were found at levels of 390, 90 and 70 μg/kg after oral administration, respectively. Residues were not detectable after 72 h post-treatment. After administration by subcutaneous injection OFZ and FBZ-SO$_2$ were the only residues detected. OFZ and FBZ-SO$_2$, reached maximum milk levels at 12 h (30 μg/kg) and 36 h (42 μg/kg), respectively. In both studies, residues were not detectable after 72 h post-treatment.

7.3. Thiabendazole

In cows treated with TBZ (oral dose 66 mg/kg bw), marker residues (TBZ and 5-OH-TBZ) were found at a level of 5175 μg/kg at 12 h post-treatment [100]. Residues were found to be present at a level of 45 μg/kg at 84 h post-treatment (below the MRL of 100 μg/kg). Tocco et al. [66] investigated the depletion of TBZ residues in the milk of dairy cows and goats treated with TBZ (oral dose 66 and 150 mg/kg bw, respectively). The sulfate conjugate of 5-OH-TBZ was found to be the major and most persistent residue found in milk. At 6 and 72 h post-treatment, 5-OH-TBZ was found in goat milk at levels of 39,000 and and 800 μg/kg, respectively. In cow's milk, TBZ was only found to be present at a level of 60 μg/kg at 12 h post-treatment. At 12 and 48 h post-treatment, 5-OH-TBZ was found at a level of 1860 and 80 μg/kg, respectively. Tai et al. [116] found that the sum of TBZ and 5-OH-TBZ residues were below the MRL of 100 mg/kg in cows milk at 48 h post-treatment (oral dose of 100 μg/kg bw).

7.4. Triclabendazole

Kinabo and Bogan [117] showed that TCB-SO and TCB-SO$_2$ could be detected up to 3 and 6 days in the milk of goats treated with an oral dose of TCB (12 mg/kg bw). TCB parent drug was not detectable at any timepoint. Highest residue levels of TCB-SO and TCB-SO$_2$ were found at 1 (160 μg/kg) and 3 (640 μg/kg) days post-treatment, respectively. Counotte et al. [118] investigated the depletion of TCB residues in the milk of cows treated with an oral dose of TCB (12 mg/kg bw). TCB-SO$_2$ was the major residue present with lower levels of TCB-SO determined. At 50 h post-treatment, TCB-SO$_2$ and TCB-SO were found at levels of 1415 and 100 μg/kg. Residues of TCB-SO and TCB-SO$_2$ could be detected up to 45 and 240 h post-treatment. The study showed that 1.5% of the administered dose was excreted in milk. Takeba et al. [119] carried out a similar study in cows treated with an oral dose of TCB (12 mg/kg bw). This group only reported residue data for the first 48 h post-treatment. At 48 h post-treatment, TCB, TCB-SO and TCB-SO$_2$ were found at levels of 6–35, 17–114 and 51–595 μg/kg, respectively.

7.5. Summary

In dairy cows, sheep and goats treated with ABZ, levels of marker residues in milk were typically below the MRL at 72 h post-treatment [83,111–113]. At early withdrawal periods, ABZ-SO was found to be the major residue, ABZ-SO$_2$ was

Table 2
MRL listings for benzimidazole anthelmintic drugs

Drug	Marker residue	Animal species	MRL (μg/kg)	Target tissue
NETO, ABZ and ABZ-SO	Sum of ABZ-SO, ABZ-SO$_2$, and ABZ-NH$_2$-SO$_2$, expressed as ABZ	Bovine, ovine	100	Milk
			100	Muscle
			100	Fat
			1000	Liver
			500	Kidney
FEB, FBZ and OFZ	Sum of extractable residues which may be oxidised to FBZ-SO$_2$	Bovine, ovine	10	Milk
			50	Muscle
		Bovine, ovine, porcine and equidae	50	Fat
			500	Liver
			50	Kidney
FLU	Sum of FLU and FLU-HMET	Porcine, poultry and game birds	50	Muscle
			50	Skin and fat
			400	Liver
			300	Kidney
		Chicken	400	Eggs
TBZ	Sum of TBZ and 5-OH-TBZ	Bovine	100	Muscle
			100	Fat
			100	Liver
			100	Kidney
			100	Milk
OXI	OXI	Porcine	100	Muscle
			500	Skin and fat
			200	Liver
			100	Kidney
TCB	Sum of extractable residues that may be oxidised to ketotriclabendazole	Bovine and ovine	100	Muscle
			100	Liver
			100	Kidney
MBZ	Sum of MBZ, MBZ-OH and MBZ-NH$_2$ expressed as MBZ	Ovine, caprine and equidae	60	Muscle
			60	Fat
			400	Liver
			60	Kidney

found to be the pre-dominant residue at later timepoints. The withdrawal periods for ABZ and related drugs in species producing milk for human consumption range between 36 and 72 h. In dairy cows treated with FBZ, OFZ and FEB, marker residues were shown to be below the MRL at 84, 72 and 120 h post-treatment, respectively [89,115,116]. The withdrawal periods for FBZ and related drugs in milk range between 2 and 7 days. In dairy cows treated with TBZ, marker residues were typically below the MRL at 84 h post-treatment. 5-OH-TBZ was found to be the major residue in cow and goat milk [66,100,116]. TCB is not licensed for use in lactating species. Kinabo and Bogan [117] found that TCB residues could be detected in goat milk for up to 6 days post-treatment. Counotte et al. [118] found that TCB residues could be detected in cow milk up to 10 days post-treatment. TCB-SO$_2$ was shown to be the most persistent residue in the milk of cows and goats.

8. Monitoring for benzimidazole residues in food

It has been shown that some twenty or so different benzimidazole residues could occur in animal tissue. However, it would be difficult to develop and maintain a robust residue method that would cover all of these residues. Such a method would probably require the application of expensive LC–MS/MS technology. An alternative approach would be to select the most likely residues of a particular benzimidazole drug that would give rise to a MRL violation or that would be most persistent in tissue (to identify unapproved use). It is important to be aware of the depletion pattern of benzimidazole residues in tissues before developing a residue method. Non-compliant benzimidazole residues may occur in tissues because products are administered to the wrong species, are administered at more than the recommended dose, withdrawal periods are not observed or because animals consume contaminated feed.

For most benzimidazoles, the marker residue is defined as the sum of the parent drug or/and its' major or most persistent metabolites. After administration of ABZ related drugs, the residues found depend on the drug used (ABZ, ABZ-SO or NETO), route of administration, target tissue or time that has elapsed since treatment. At early withdrawal periods, the most likely marker residues to be found are ABZ-SO and ABZ-SO$_2$, while at longer withdrawal periods the most prevalent residue is ABZ-NH$_2$-SO$_2$. As a result, the marker residue is defined as the sum of ABZ-SO, ABZ-SO$_2$ and ABZ-NH$_2$-SO$_2$, expressed

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Table 3
Identification of target analytes for monitoring residues of benzimidazoles in liver and milk

Drug	Target analytes	
	Liver	Milk
NETO, ABZ or ABZ-SO	$ABZ\text{-}SO_2$, and $ABZ\text{-}NH_2\text{-}SO_2$	$ABZ\text{-}SO_2$, and $ABZ\text{-}NH_2\text{-}SO_2$
FEB, FBZ or OFZ	FBZ, OFZ and $FBZ\text{-}SO_2$	OFZ and $FBZ\text{-}SO_2$
FLU	FLU and FLU-HMET	Not defined
TBZ	TBZ	5-OH-TBZ
OXI	OXI	Not defined
TCB	TCB-SO and $TCB\text{-}SO_2$	$TCB\text{-}SO_2$
MBZ	$MBZ\text{-}OH$, $MBZ\text{-}NH_2$	Not defined

as ABZ. The MRLs for ABZ related drugs have been set at 100 μg/kg for milk, muscle and fat, 500 μg/kg for kidney, and 1000 μg/kg for liver in bovine and ovine species (Table 2) [120]. Similarly, for FEB, FBZ and OFZ the marker residue is defined as the sum of FBZ, OFZ and FBZ SO_2, expressed as $FBZ\text{-}SO_2$. Data from residue depletion studies indicate that OFZ and FBZ-SO_2 are the most suitable residues to test for in tissues. The MRLs range between 10 μg/kg for milk and 500 μg/kg for liver, in the listed species.

The marker residue for FLU is defined as FLU parent drug in eggs and as the sum of FLU and FLU-HMET in other edible tissues of avian and porcine species. The MRLs range between 50 μg/kg for muscle and 400 μg/kg for eggs and liver. The marker residue for MBZ is defined at the sum of MBZ, $MBZ\text{-}OH$ and $MBZ\text{-}NH_2$, expressed as MBZ. Residue depletion data indicate that $MBZ\text{-}NH_2$ or $MBZ\text{-}OH$ are the most persistent residues and most likely contribute to an MRL violation. The MRLs in ovine, caprine and equine species are set at 60 μg/kg for muscle, fat and kidney and 400 μg/kg for liver. The marker residue for TBZ is defined as the sum of TBZ and 5-OH-TBZ. In edible tissues, the parent drug is the most likely residue to be found. While the sulfate conjugate of 5-OH-TBZ is the predominant metabolite in milk. The MRL for bovine tissues and milk is 100 μg/kg. The marker residue for TCB is defined as the sum of extractable residues (TCB, TCB-SO and $TCB\text{-}SO_2$) that may be derivatised to form ketotriclabendazole. Residue depletion studies in tissue have been published reporting the depletion of the sum of extractable residues derivatised to form ketotriclabendazole, but do not indicate the contribution of individual residues. Studies indicate that because TCB is not licensed for use in lactating species and it should be best monitored as its' most persistent residue, $TCB\text{-}SO_2$. The MRL for TCB is 100 μg/kg for muscle, liver and kidney. The marker residue for OXI is the parent drug and MRLs are set at 100 μg/kg for muscle and kidney, 200 μg/kg for liver, and 500 μg/kg for skin and fat. Other benzimidazoles such as CAM, LUX, PAR and BEN have no EU MRL listings because they are not licensed as veterinary drugs in food producing animals. It is recommended that target certain target residues (as indicated in Table 3) should be selected in the event that all marker residues of benzimidazole are not included in a method. In the event that a positive residue is detected, a method capable of detecting the complete range of marker residues of a drug should be applied.

9. Sample extraction and clean-up procedures

9.1. Sample pre-treatment

9.1.1. Incorporation of hydrolysis steps

Hydrolysis may be used as a pre-treatment step to release protein bound residues, drug conjugates or simply convert residues to a common structure. However, in the area of benzimidazole analysis most hydrolysis steps have been used to release conjugates. Tocco et al. [121] found that urinary metabolites of TBZ mainly occurred as glucuronide and sulfate conjugates of 5-OH-TBZ. Conjugates were released using enzymatic (β-glucoranidase or glucosulase) or acid hydrolysis (refluxing in 6N HCl (1 h)). Tocco et al. [66] later used similar procedures to deconjugate 5-OH-TBZ residues present in the milk of cows and goats treated with TBZ. Chukwudebe et al. [101] completed a more detailed study into the metabolism of TBZ in laying hens and lactating species, using enzymatic and acid hydrolysis, and found that conjugates were mainly present as sulfates. Vanden-Heuvel et al. [122] also deconjugated 5-OH-TBZ by enzymatic and acid hydrolysis in a study in bluegill sunfish. Kriedel et al. [123] deconjugated the glucuronide of MBZ-OH after digestion with β-glucuronidase. Gyurik et al. [124] used glucosulase to enzymatically hydrolyse ABZ metabolites in urine before detection by TLC. Short et al. [76] incubated urine samples from animals treated with FBZ with β-glucuronidase and sulfatase before extraction and determination by mass spectrometry, showing that the majority of residues present in urine were conjugated.

As a result of these studies, hydrolysis steps have been included in some residue methods. Markus and Sherma [125] subjected liver sample extracts to acid hydrolysis to allow conversion of ABZ metabolite residues to a common $ABZ\text{-}NH_2\text{-}SO_2$ residue. Arenas and Johnson [126] used enzymatic hydrolysis to deconjugate 5-OH-TBZ sulfate, in a method for determination of TBZ residues in milk. Coulet et al. [103] used basic hydrolysis to release protein bound residues of TBZ from tissue.

9.2. Extraction and clean-up of benzimidazole residues

Liquid extraction is a common approach used to extract benzimidazoles, usually with aqueous extraction at high pH with partitioning into an immiscible organic solvent [53,96,102].

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However, polarity and pK_a can differ greatly between parent benzimidazoles and metabolites, not only making it difficult to develop a multi-residue method for different benzimidazole drugs, but also for individual drugs and their metabolites. This has been observed by many researchers developing methodology for determination of FBZ and its two metabolites OFZ and FBZ-SO_2 [127–131]. FBZ has differing physical properties to its two metabolites, making it difficult to extract and determine by LC [128,129,132,133]. Similar difficulties have been encountered with MBZ and its two metabolites MBZ-OH and MBZ-NH_2 [53]. MBZ and MBZ-NH_2 are basic in nature with pK_a values of 3.5 and 5.5, respectively. Therefore, only at pH levels >7.5 are both metabolites non-ionised. MBZ-OH possesses an acidic OH group with a pK_a of 9.8 which is non-ionised below pH 7.8. This indicates that only in the pH range 7.5–7.8, are all three residues non-ionised. Alternatively, extraction may be carried out using a more polar organic solvent without pH manipulation. Some researchers have extracted these drug residues using a single solvent system, such as acetonitrile [119,134], or binary water/organic solvent mixtures which would be around neutral pH [131,135–137]. Extraction with acidified extractants has only been used by a small number of researchers for methods requiring hydrolysis or digestion steps [101,125,136,137] or methods including only a limited number of residues [138].

9.2.1. Plasma, serum or other biological fluids

Benzimidazole residues may be extracted from biological fluids using water immiscible organic solvents with or without pH adjustment and analysed without further purification. Bogan and Marriner [139] adjusted the pH of plasma to pH 7.4 before extracting FBZ, OFZ and ABZ residues with diethyl ether. Bogan's procedure has been applied for isolation of benzimidazole residues from plasma and blood [140–142]. Other groups have developed alternative procedures, while substituting solvents such as chloroform [143,144] and ethyl acetate [145–147] for diethyl ether. Hoaksey et al. [148] extracted ABZ and ABZ-SO from plasma samples with dichloromethane after protein precipitation with acetonitrile. Alvinerie and Galtier [149] extracted TCB, TCB-SO and TCB-SO_2 directly from plasma without pH adjustment using a simple ethyl acetate extraction procedure. Galtier et al. [150] later applied this method for determination of TBZ and 5-OH-TBZ in plasma.

Allan et al. [151] simply diluted plasma samples in acid prior to application onto a C_{18} SPE cartridge. The SPE cartridge was washed with water (20 ml), methanol + water (40 + 50, 0.5 ml) and methanol (0.4 ml) prior to elution with 1.6 ml of methanol. Recovery of mebendazole and its metabolites was typically greater than 80%. The procedure was subsequently applied by Galtier et al. [150] and Behm et al. [152] to determine mebendazole in plasma. Hennessy et al. [70] subsequently modified this procedure and applied it to investigate the pharmacokinetics of TCB. Hennessy et al. also found the procedure to be suitable for determination of TCB in bile (after pH adjustment) but samples required liquid–liquid extraction (LLE) clean-up into ethyl acetate before SPE. This procedure was later applied by other researchers to determine ABZ related residues in plasma [60,153].

The use of automated purification systems has been shown to enhance reproducibility, improve throughput of samples and reduce manual handling. Rouan et al. [154] described an automated C_{18} clean-up procedure for isolation of TCB residues on an ASPEC system. They demonstrated, with a limited number of samples, that recovery values greater than 90% could be obtained under optimal conditions. Chiap et al. [155] developed an automated procedure based on dialysis clean-up for isolation of albendazole and its metabolites from plasma with on-line determination by HPLC through the use of switching valve technology. Recovery of ABZ residues from plasma was typically greater than 65%. Negro et al. [56] isolated TCB-SO and TCB-SO_2 from serum and urine samples using a combination of protein precipitation and ultra-filtration through a 30,000 molecular mass cut-off filter. This approach was shown to greatly reduce sample preparation time in comparison to conventional off-line clean-up procedures. Recovery of TCB marker residues from serum and urine were typically greater than 82%.

9.2.2. Tissue

Benzimidazole residues are typically extracted from animal tissues using organic solvent and purified by LLP and/or SPE. Some researchers have opted to carry out comprehensive purification of residues through LLP and SPE, usually with determination by HPLC-UV. Other groups have moved towards the development of simple clean-up procedures based on LLP only, with determination by more selective fluorescence and LC–MS/MS systems. These simpler clean-up procedures (although less selective) may be more suitable to allow for isolation of a broad range of benzimidazoles, which are difficult to recover using more comprehensive clean-up procedures. Brandon et al. [135] extracted TBZ residues from liver tissues using DMSO-H_2O (10:90, v/v) and diluted extracts in PBS Tween prior to determination by ELISA. Brandon et al. [156] developed a similar type of extraction procedure for determination of some 11 benzimidazole residues in liver tissues. To allow recovery of a range of benzimidazole residues including the poorly water soluble FBZ, samples had to be extracted in DMF-H_2O (10:90, v/v).

Originally procedures for tissue samples were based on multiple LLP and/or SPE clean-ups. Marti et al. [134] extracted eight benzimidazole residues from muscle, liver and kidney tissues using acetonitrile. Extracts were subsequently purified using a series of LLP steps which included washing with hexane, saturated NaCl and dichloromethane. Extracts were concentrated and further purified using a combination of C_{18} and Florisil SPE prior to analysis by HPLC-UV or confirmation by GC-MS. Recovery of benzimidazole residues was typically >65% for all residues with the exception of OFZ, which gave a lower recovery of 45 and 39% for liver and kidney, respectively. Farrington et al. [157] extracted five benzimidazole residues from liver tissue using ethyl acetate and purification on acid alumina and C_{18} SPE before determination by HPLC-UV. Recovery of benzimidazoles ranged between 73 and 90%. Blanchflower et al. [90] extracted FBZ and OFZ from liver and muscle tissues using methanol–water (20:7, v/v). Extracts were washed with light petroleum, diluted with phosphate buffer and extracted with

diethyl ether–ethyl acetate (60:40, v/v). Recovery of FBZ and OFZ was typically greater than 80 and 70%, respectively. de Buyanski et al. [158] developed a simple procedure for extraction of TBZ and levamisole from muscle tissue at alkaline pH using ethyl acetate with subsequent partitioning into 0.5 M HCl. The ethyl acetate layer was discarded before alkalisation of the aqueous phase and extraction with chloroform. Levan and Barnes [159] developed a labour intensive procedure for extraction of five benzimidazole residues from liver tissue using large volumes of organic solvent. Samples were purified using LLP steps prior to purification on silica SPE cartridges.

Wilson et al. [102] developed a simpler procedure based on ethyl acetate extraction for isolation of eight benzimidazole residues from muscle and liver tissues. Ethyl acetate was chosen as the extraction solvent because of its good solvating power for weakly basic drugs and its ability to form emulsion-free interfaces with muscle and liver tissues. Samples were subsequently purified using LLP (acidified ethanol versus hexane wash) and C_2 SPE clean-up prior to determination by HPLC-UV or GC–MS. This group concluded that C_2 was the most effective sorbent material for removing polar matrix interference while giving good recovery of benzimidazole residues in comparison to other SPE sorbent materials evaluated. Wilson's method is the most widely used method for isolation of benzimidazole residues from animal tissues. In some cases, researchers have reported modifications of this method, such as size of sample used, extraction pH and SPE cartridge clean-up.

A number of researchers have applied the Wilson method, modified to exclude the SPE clean-up. Domany and Kovacsics [160] found that the method could be extended to 10 benzimidazole residues in liver and muscle tissues, while excluding SPE clean-up. Shaikh et al. [86] found that ABZ marker residues could be determined in salmon, tilapia and trout without the need for SPE clean-up through the application of a more selective HPLC fluorescence detection system. They found that ABZ recovery increased after adding dimethyl sulphoxide (0.5 ml) to the extraction solvent (ethyl acetate, 5 ml), while extraction efficiency for ABZ marker residues did not vary greatly over the pH range from 9 to 11. It was also found that addition of sodium metabisulfite to the extraction medium inhibited the oxidation of ABZ-SO to ABZ-SO$_2$, resulting in increased recovery for ABZ-SO. Recovery of ABZ, ABZ-SO, ABZ-SO$_2$ and ABZ-NH$_2$-SO$_2$ was typically greater than 82, 78, 75 and 63%, respectively.

Hajee and Haagsma [53] extracted MBZ, MBZ-OH and MBZ-NH$_2$ from eel muscle with ethyl acetate after adjusting the pH to 7.5 with phosphate buffer. They concluded that only in the narrow pH range between 7.5 and 7.8 were all MBZ marker residues in a non-ionised state thereby facilitating their extraction using ethyl acetate. Ethyl acetate extracts were modified with hexane prior to application onto a preconditioned aminopropyl SPE cartridge. The authors evaluated a range of normal phase and reverse phase SPE systems but found aminopropyl to be most suitable. After loading of sample extract, the aminopropyl cartridge was washed with iso-octane and MBZ marker residues were eluted with methanol. Mean recovery of MBZ, MBZ-NH$_2$ and MBZ-OH was typically greater than 80, 60 and 85%, respectively. Cannavan et al. [161] developed a simple procedure for isolation of TBZ and 5-OH-TBZ from liver, kidney and muscle tissues based on an ethyl acetate extraction at pH 7.0, and SPE clean-up on cyanopropyl (CN) SPE cartridges. Deuterated TBZ was used as an internal standard to improve reproducibility. Recovery of TBZ and 5-OH-TBZ ranged between 96–103 and 70–85%, respectively. It was concluded that reproducibility of 5-OH-TBZ recovery could be improved through the introduction of a deuterated internal standard for 5-OH-TBZ.

Acetonitrile was found to be an effective solvent for extraction of benzimidazoles from animal tissues by some researchers. Sorensen and Hansen [92] extracted the marker residues of FBZ from the muscle and skin tissues of trout using acetonitrile. Extracts were washed with hexane prior to purification on C_{18} and CN SPE cartridges. Under acidic conditions benzimidazole residues may become ionised making them amenable for more selective ion exchange SPE clean-up. Rose extracted nine metabolites of OFZ from liver using acetonitrile prior to purification on strong cation exchange (SCX) SPE cartridges [133]. Acetonitrile extracts were modified using acetic acid and applied onto the SCX cartridge, which was subsequently washed with acetone, methanol and acetonitrile. The cartridge was eluted with acetonitrile containing 5% ammonia (5 ml). Recovery of benzimidazoles varied between 28 and 117%. Su et al. [162] extracted six benzimidazole residues from muscle and liver tissues using acetonitrile after adjusting the pH to 10. Dibutyl hydroxytoluene (BHT) was added to prevent oxidation of 5-OH-TBZ. Extracts were further purified on C_{18} SPE prior to determination by HPLC. Recovery ranged between 71 and 105%.

Polymeric sorbents have been successfully applied to isolate benzimidazole residues from animal tissue in some multiresidue methods. Roudaut and Garnier [163] extracted 10 benzimidazole residues from liver using the Wilson procedure, while substituting Oasis HLB for C_2 SPE cartridges. Sample extracts were loaded onto HLB cartridges in methanol–0.1 M ammonium acetate (50:50, v/v, 1.0 ml) and eluted with methanol (1 ml) before determination by HPLC-UV. They compared a number of other polymeric SPE cartridges including Isolute 101 and Nexus but found that Oasis HLB gave the cleanest sample extracts and most satisfactory recovery. Balizs loaded muscle extracts onto styrol-divinyl-benzene SPE cartridges in methanol–0.1 M ammonium acetate (50:50, v/v, 1.0 ml) and eluted them with methanol–ethyl acetate (1:4, v/v, 3 ml) before analysis by LC–MS/MS [164]. Balizs applied the method to isolate 15 benzimidazole residues from muscle tissues. Recovery of benzimidazole residues was typically in the range of 36–117%. A low recovery of 8% was obtained for FEB.

Capece et al. [91] extracted FBZ marker residues from muscle, fat and kidney samples using ethyl acetate after adjusting the pH to 8.0. Extracts were analysed by HPLC without further purification. De Ruyck et al. [94] extracted FLU, FLU-HMET and FLU-RMET from egg and muscle tissues with ethyl acetate at alkaline pH. Extracts were subsequently evaporated to dryness and purified by LLP (methanol versus hexane) before analysis by LC–MS/MS. Recovery of FLU marker residues was typically greater than 70% (eggs) and 87% (muscle). De Ruyck et al. [98] subsequently applied this method to the determina-

tion of MBZ marker residues in sheep liver. Mean recovery of MBZ marker residues was typically greater than 85%. Edder et al. [165] extracted 11 benzimidazoles and levamisole (LEV) from liver and muscle tissues at alkaline pH while using MBC as an internal standard. Extracts were further purified by LLP (methanol versus hexane), before determination by LC–MS/MS. Recovery of residues was typically greater than 70%.

Some novel procedures have been developed for isolation of benzimidazole residues from animal tissues. Fletouris et al. [166] isolated ABZ-SO, ABZ-SO$_2$ and ABZ-NH$_2$-SO$_2$ residues from muscle, liver, kidney and fat tissues using a LLP procedure based on ion-pair extraction with octane sulphonate under acidic conditions. Extracts were further purified by partitioning between phosphate buffer pH 8.5 and ethyl acetate. However, this group concluded that the ion pair extraction was critical for purification of tissue extracts. Recovery of ABZ-SO, ABZ-SO$_2$ and ABZ-NH$_2$-SO$_2$ was greater than 81, 88 and 76%, respectively. Techniques such as matrix solid phase dispersion (MSPD) [77,129,131,167–171] and supercritical fluid extraction [172] have been applied for isolation of benzimidazoles residues from liver. Long et al. [168] developed an MSPD method for isolation of five benzimidazole residues from liver tissue. Liver samples (0.5 g) were blended with C$_{18}$ material and packed into a SPE column between two frits. The column was washed with hexane and eluted with acetonitrile prior to determination by HPLC-UV. Recovery of the benzimidazole residues ranged between 55 and 93%. Danaher et al. extracted 13 benzimidazoles from fortified liver samples using unmodified supercritical CO$_2$ (601, 690 bar and 80 °C) [172]. Benzimidazole residues were trapped off-line on a neutral alumina SPE cartridge and further purified on a SCX cartridge prior to determination by HPLC-UV. Mean recovery for 10 benzimidazoles ranged between 51 and 113%. TBZ, CAM and TCB-SO$_2$ residues were also extracted but results were not quantitative.

Some groups have implemented automated SPE clean-up procedures. Stubbings et al. [173] developed an automated on-line clean-up system for isolation of benzimidazole residues from liver and tissue samples. Benzimidazole sample extracts were purified on SCX cartridges prior to on-line clean-up on cation exchange trace enrichment cartridges. Dowling et al. [174] modified the method developed by Wilson and extended it to isolate 12 benzimidazole residues from liver tissue. Purification of samples was carried out on C$_{18}$ SPE cartridges using an automated SPE system (ASPEC^{TM}). They found that this automated system allowed unattended clean-up of samples and enhanced reproducibility of assay results. Mean recovery of residues was typically in the range of 60–100%. Lower recovery of 25% was observed for ABZ-NH$_2$-SO$_2$. HPLC-UV chromatograms of negative control and fortified liver samples are presented in Fig. 10.

9.2.3. Milk and milk products

Milk samples are generally easier to manipulate than corresponding solid tissue samples. In addition, milk contains less pigments and matrix interence components that typically occur in tissue. Important purification issues that need to be overcome with milk samples include removal of proteins and fat through
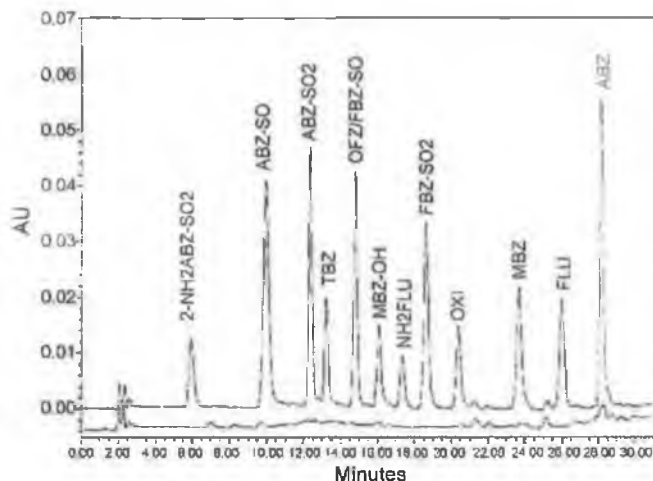

Fig. 10. Chromatogram of blank liver sample and liver sample fortified at MRLs (Reprinted from reference [174], with permission from Elsevier, Copyright 2005).

introduction of precipitation and defatting steps. A number of groups have simply adjusted the pH of samples prior to extraction with an immiscible solvent such as ethyl acetate, chloroform or dichloromethane. The required pH adjustment may depend on the type or number of drug residues included in a particular method. Tocco et al. [66] found that TBZ and 5-OH-TBZ could be successfully extracted from milk samples at pH 6.0 with ethyl acetate. Fletouris et al. [175] showed that ABZ, ABZ-SO and ABZ-SO$_2$ were best extracted at pH 9.8 with ethyl acetate, giving recovery values of 81, 78 and 100%, respectively. Fletouris et al. [111] later extracted 10 benzimidazole residues from milk samples at pH 10 with ethyl acetate. They evaluated extraction efficiency over the range pH 3–11.5, finding pH 10 gave best recovery for the majority of benzimidazole residues selected. Mean recovery for benzimidazole residues was typically in the range of 79–100%. A lower recovery in the range of 56–66% was obtained for FBZ. Fletouris et al. [128] developed an alternative acetonitrile based extraction procedure that gave recovery near to 100% for FBZ over the pH range 2–9, indicating pH adjustment of milk samples was unnecessary with acetonitrile. They later used a similar extraction procedure for determination of ABZ residues in cheese [176], and FBZ metabolites in milk [128]. It was found that solvent extraction with ethyl acetate extraction gave dirtier extracts for cheese samples than acetonitrile, while dichloromethane gave low recovery. Samples (0.5 g) were extracted with acetonitrile (3 ml), defatted with hexane, evaporated to dryness and further purified by LLP (ethyl acetate versus phosphate buffer pH 8.5). Recovery of ABZ-SO, ABZ-SO$_2$ and ABZ-NH$_2$-SO$_2$ were typically greater than 83, 80 and 74%, respectively. De Ruyck et al. [177] later scaled this method up (larger volmes of solvent and sample) and applied it for extraction of four benzimidazole residues from milk. Mean recovery of FBZ, TBZ, ABZ and OXI was typically between 68 and 77%. De Ruyck et al. [178] subsequently developed a procedure to isolate eight benzimidazoles and levamisole from milk samples at alkaline pH using ethyl acetate with determination by LC–MS/MS. Recov-

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ery of benzimidazole residues was typically in the range of 79–110%.

The amount of purification required to successfully isolate benzimidazole residues from milk samples may depend on the number of residues included and the detection system being used. Brandon et al. [179] simply diluted milk samples in phosphate buffer saline prior to direct determination by ELISA. They found that milk samples had to be diluted 10-fold to eliminate matrix effects and ensure good agreement between standards prepared in buffer and fortified or incurred samples. Fletouris et al. [111] extracted 10 benzimidazoles from milk samples (1 ml) at pH 10 with ethyl acetate (6 ml). Samples extracts were subsequently evaporated to dryness and reconstituted in mobile phase before determination by HPLC. Other researchers have developed more labour intensive sample preparation procedures using multiple LLE/LLP/SPE steps that require larger quantities of organic solvent, glassware and cumbersome rotary evaporation steps [116,180]. Tai et al. [116] extracted four benzimidazole residues from milk at alkaline pH using large volumes of ethyl acetate (150 ml). Sample extracts were purified by LLP (phosphoric acid versus hexane wash) and back extracted into ethyl acetate after alkalisation. Sample extracts were further purified on silica SPE prior to determination of residues by HPLC-UV. Recovery of FBZ, OFZ, TBZ and 5-OH-TBZ was greater than 78, 86, 81 and 25%, respectively. They proposed that the poor recovery of 5-OH-TBZ might be due to degradation during the long extraction procedure. Constantinou et al. [180] developed a similarly labour intensive procedure for isolation of OFZ, FLU, ABZ and FBZ from milk based on LLE, with further purification on C_{18} SPE. Recovery of TBZ, OFZ, FLU, ABZ and FBZ were greater than 68, 63, 57, 24 and 29%, respectively.

Other researchers have developed simpler sample preparation procedures based on solvent extraction and SPE. Moreno et al. [115] deproteinised milk samples (0.5 ml) with acetonitrile (0.5 ml) and applied the supernatant layer onto a preconditioned C_{18} SPE cartridge, which was washed with water (0.5 ml) and eluted with methanol (2 ml). The method was suitable for determination of ABZ, FBZ and their metabolites. Mean recovery ranged between 77 and 97%. Sorensen and Petersen [181] mixed milk samples (5 ml) with phosphate buffer pH 11 (5 ml) and incubated the mixture in a water bath (45 °C, 20–30 min) prior to application onto a C_{18} SPE cartridge. The cartridge was washed with water (5 ml) and eluted with acetonitrile (6 ml). Samples were evaporated to dryness and further purified by LLP (phosphate buffer pH 9 versus dichloromethane extraction) before determination by HPLC-UV. Mean recovery of ABZ-SO, ABZ-SO₂, OFZ, OXI, ABZ, MBZ, FBZ and FEB was in the range 81–96%. Sorensen and Petersen [182] developed an alternative procedure for determination of TBZ and LEV in milk. Defatted milk samples (10 ml) were mixed with phosphate buffer pH 6.5 (10 ml) and incubated in a water bath (45 °C, 20–30 min.) prior to passing through a C_{18} SPE cartridge. The cartridge was washed with phosphate buffer (5 ml) and eluted with methanol (2 ml). Sample extracts were evaporated to dryness and further purified by LLP (phophate buffer pH 10 versus dichloromethane extraction) before determination by HPLC-UV. Mean recovery of TBZ and LEV was 81–89%, respectively.

A number of novel methods, including use of MSPD, have been developed to allow determination of benzimidazole residues in milk and milk products. Long et al. [129] mixed milk samples (0.5 ml) with C_{18} material (2 g) and packed the mixture between two filter paper discs in a 10 ml syringe barrel and compressed to a volume of 4.5 ml. The column was washed with hexane (8 ml), air-dried and eluted with dichloromethane–ethyl acetate (1:2, v/v, 8 ml). Recovery of the benzimidazole residues was typically in the range 81–108%. A slightly lower recovery of 69% was obtained for FBZ. De Liguoro et al. [113] extracted ABZ residues from milk, whey and whey solubles using acetonitrile, prior to defatting with hexane, LLP clean-up and C_{18} SPE. Recovery of ABZ, ABZ-SO, ABZ-SO₂ and ABZ-NH₂-SO₂ were typically greater than 78, 82, 79 and 81%, respectively. De Liguoro et al. [113] developed an MSPD procedure for determination of ABZ residues in cheese. Cheese samples (1 g) were mixed with C_{18} material and packed between two filter paper discs in a 10 ml syringe barrel and compressed to a volume of 7 ml. The column was washed with hexane (10 ml), air-dried and eluted with methanol–acetic acid (97:3, v/v). The eluate was defatted with hexane and further purified by C_{18} SPE prior to determination by HPLC. Recovery of ABZ metabolites were similar to those achieved by the procedure developed for milk (77–86%).

Residue scientists face similar problems when trying to determine benzimidazole residues in milk as in animal tissue. Development of methods that will cover the complete number of marker residues or most persistent or predominant metabolites is most difficult. As a result, a number of important benzimidazole residues are not included in multiresidue methods. TCB and it's metabolites have been included in few milk residue methods. De Ruyck et al. [178] developed a method that included TCB parent drug but not the more important TCB-SO₂ metabolite, which is the most persistent residue of TCB in milk. Some researchers have developed methods to isolate TCB and its metabolites from milk but methods do not include other benzimidazole drugs. Takeba et al. [119] extracted TCB, TCB-SO and TCB-SO₂ from milk using acetonitrile with subsequent defatting with hexane. The acetonitrile fraction was modified with carbonate buffer and extracted with dichloromethane prior to further purification on C_{18} SPE and determination by HPLC-UV. Mean recovery was in the range of 89–95%. Kinabo and Bogan [117] extracted TCB and it's metabolites from milk (4 ml) with acetone (4 ml). Acetone extracts were simply diluted with water and applied onto C_{18} SPE cartridges, which were washed with water (5 ml) and eluted with methanol (3 ml). Purified extracts were subsequently determined by HPLC fluoresence. Su et al. [162] demonstrated that their earlier method for the determination of benzimidazole residues in animal tissues was also applicable to milk samples with minor modification.

Furthermore, few multi-residue methods for determination of benzimidazole residues in milk include a deconjugation step. Inclusion of such a step is particularly important when testing for residues of TBZ in milk, which mainly occur as the sulfate conjugate of 5-OH-TBZ. Arenas and Johnson [126] subjected milk samples (5 ml) to acid hydrolysis in the presence of concentrated HCl (85–90 °C, 4 h) to free the sulphate conjugate of 5-OH-TBZ.

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Hydrolysed extracts were neutralised with NaOH and adjusted to pH 8.0 before extraction with ethyl acetate. Ethyl acetate extracts were subsequently purified on PRS cation exchange cartridges prior to determination by HPLC fluorescence. Recovery of TBZ, 5-OH-TBZ and the sulphate conjugate of 5-OH-TBZ were typically greater than 87, 98 and 96%, respectively. Arenas and Johnson proposed enzymatic hydrolysis as an alternative to acid hydrolysis but required an overnight incubation. One factor to be considered when including an acid hydrolysis step is that benzimidazole carbamate residues (ABZs, FBZs, MBZs and FLUs) may be hydrolysed to amino benzimidazoles. Chu et al. [114] included an acid hydrolysis in a method for determination of ABZ metabolites in milk samples. Milk samples were subjected to acid hydrolysis (1 h, 100 °C), followed by purification on SCX SPE cartridges. In this procedure, acid hydrolysis was used to convert ABZ metabolites to a common residue (ABZ-NH_2-SO_2). The procedure should be suitable for determination of ABZ-SO_2, ABZ-NH_2-SO_2 and their conjugates as ABZ-NH_2-SO_2. However, the procedure was not demonstrated to be suitable to allow determination of ABZ, netobimin or ABZ-SO as ABZ-NH_2-SO_2. Chu et al. demonstrated that the method was suitable for determination of more than 40% of ABZ residues in cow's milk 36–120 h post-treatment. Recovery by the method was typically in the range of 91–105%.

9.2.4. Plant matrices

There are fewer benzimidazole compounds used in the treatment of crops compared to animal health. TBZ and thiophanate-methyl (TM) are used in crop protection and animal health, while BEN is used in crop protection only. The purpose of this section is to give an overview of methods for determination of BEN, TBZ, TM and their breakdown products in crops. Those interested in reading in more detail should consult the reviews by Gorbach [183] and Singh and Chiba [184]. It will be found throughout this section that the techniques used to isolate benzimidazole fungicides from crops are typical of those applied in a pesticide laboratory. In general, larger weights of sample and volumes of solvent are used in procedures for isolation of benzimidazoles from crops. Samples are typically purified by LLE/LLP steps, which require relatively large volume glassware and concentration of samples one at a time using rotary evaporation. In recent years, researchers in this area of testing have moved towards scaling down methods to reduce solvent usage and increase sample throughput.

The control of sample pH is critical to ensure satisfactory recovery of benzimidazole residues from crops. Benzimidazole residues have been extracted from certain crops with [185–187] or without [188–190] adjustment of sample pH. However, when extracting acidic samples such as citrus fruits, sample pH should be controlled to ensure a robust method [188–190]. A principle applied in most LLE/LLP procedures for isolation of benzimidazole residues is that they are highly soluble in dilute acid and less soluble in aqueous solutions at higher pH values (>7.0). This allows the washing or extraction of water immiscible organic extracts with basic or acidic aqueous solutions, respectively. In a typical ethyl acetate extraction, extracts may be washed with water or alkaline buffers (to remove polar inter-

ference) and extracted with 0.1N HCl (while non-polar matrix components remain in the ethyl acetate). The acidified extracts are subsequently adjusted to alkaline pH with NaOH and re-extracted with ethyl acetate, which is evaporated to dryness or reduced to a small volume prior to analysis. Alternative extractions have been developed by other researchers using solvents such as ethanol–dichloromethane [191] or hexane–ethyl acetate [192]. Corti et al. [193] extracted TBZ from samples using a saturated sodium chloride-dichloromethane system. Other researchers have extracted samples with more polar solvents such as methanol [191] or water [194], prior to dilution of sample extracts and determination by ELISA.

Extraction and clean-up procedures that include BEN, TM and their marker residue (MBC) are more complex than those used to isolate TBZ. The approach adopted in most methods is to convert the parent fungicides to MBC or 2-aminobenzimidazole (2-AB) through acid or basic hydrolysis prior to analysis. Acid hydrolysis converts BEN to MBC, while basic hydrolysis converts MBC to amino-benzimidazole. Pease and Gardiner [195] extracted BEN residues from fruit and vegetable matrices using ethyl acetate. Samples were subjected to acid and basic hydrolysis prior to LLP and determination by fluorescence spectrophotometry. Recovery of BEN from fortified fruit, vegetable, animal tissue and soil was typically greater than 76%. Pease and Holt [196] later improved this clean-up procedure through the introduction of alkaline wash steps to remove matrix components that interfered with fluorescent quenching, allowing the more effective determination of BEN in oranges.

Kirkland et al. [136] extracted BEN from soil and crops by refluxing in the presence of methanol and 1N HCl. Hydrolysed extracts were purified by a series of LLP steps prior to HPLC determination. The method allowed the determination of BEN and MBC (both as MBC), and 2-aminobenzimidazole. Recovery of the respective residues was 92, 88 and 71%, respectively. Physalo [197] later modified this clean-up procedure and used it to isolate MBC and 2-AB from crops, prior to determination by GC. Recovery ranged between 70 and 90%. Tjan and Jansen [186] extracted BEN, MBC and TBZ from crops with ethyl acetate–0.1N HCl. After adjustment of the acidified fraction to pH 7.5, residues were extracted with ethyl acetate, concentrated, derivatised and determined by GC. Recovery of BEN, MBC and TBZ were typically greater than 91, 93 and 95%, respectively. Farrow et al. [198] extracted BEN, MBC, TM, TBZ and some non-benzimidazole fungicides from citrus fruit by refluxing in 2N HCl. Sample extracts were washed with chloroform and adjusted to a high pH prior to extraction with chloroform. Recovery of BEN, MBC, TM and TBZ were typically greater than 70, 66, 32 and 84%, respectively.

Gilvydis and Walters [199] extracted five benzimidazole residues from crops using acidified methanol and partitioning into dichloromethane after dilution in NaCl solution, prior to HPLC determination. This fraction contained TM. The acidified fraction (containing the other benzimidazoles) was adjusted to pH 7.7–8.0 and extracted with dichloromethane. Recovery of BEN, MBC, TBZ and TM were typically greater than 78, 87, 81 and 63%, respectively. Corti et al. [200] extracted BEN, MBC and TBZ from fruit and vegetable samples using satu-

rated NaCl solution–chloroform. The chloroform extract was re-extracted with 0.1N HCl and incubated at 50 °C (30 min.). Acidified extracts were alkalised and extracted with chloroform prior to determination by HPLTC or HPLC. Recovery of BEN, MBC and TBZ was typically greater than 91, 93 and 94%, respectively.

The application of sorbent based clean-up for isolation of benzimidazole residues has become more popular in recent years. However, there was some application of sorbent clean-up in early methods, using either column chromatography or preparative TLC. Aharonson and Ben-Aziz [201] extracted BEN, MBC and TBZ from crops using acetone, with subsequent purification on magnesium oxide-celite-alumina columns prior to determination by TLC or fluorescence spectrophotometry. Recovery of BEN, MBC and TBZ were typically greater than 68, 60 and 70%, respectively. White and Kilgore [202] extracted BEN from different fruits with benzene and partitioned residues into 0.1N HCl, before converting the BEN to MBC at room temperature. Sample extracts were purified by LLP and preparative TLC prior to determination by UV spectrophotometry. Recovery of BEN was >87%. More recently, groups have developed procedures on commercial SPE materials. Oishi et al. [203] adjusted fruit juice samples to pH 8–9 before application onto an Extrelut™ cartridge (supported LLE), which they subsequently eluted with hexane–ethyl acetate (3:1, v/v). This group found that the highest recovery was achieved at pH values >4. Recovery of TBZ ranged between 90 and 96%.

The development of chemically more sophisticated cation exchange and polymeric sorbent materials has resulted in improved procedures for isolation of benzimidazole fungicide residues from crops. Di Muccio et al. [204] extracted MBC and TBZ from crops using an aqueous-acetone mixture. A portion of the aqueous-acetone extract (20 ml) was dispersed over the diatomaceous earth material (Extrelut-20) packed into a SPE cartridge. Acetone was removed by passing nitrogen through the cartridge from bottom to top. Residues were subsequently eluted with 0.1 M phosphoric acid and isolated on a phenyl-SCX cartridge attached to the end of the Extrelut™ cartridge, while leaving material poorly soluble in dilute phosphoric acid retained on the Extrelut™ cartridge. The Extrelut™ cartridge was disconnected and the SCX cartridge was washed with water, methanol and eluted with acetonitrile–methanol–ammonium formate buffer pH 6.8 (50:25:25, v/v/v). Arenas and Johnson [205] extracted TBZ from banana with ethyl acetate and applied extracts onto PRS-SCX cartridges. TBZ was eluted with 0.1 M KH_2PO_4 in acetonitrile and purified extracts were determined by HPLC with fluorescence detection. Recovery of TBZ ranged between 87 and 101%. Arenas et al. [206] later modified this procedure through the inclusion of sample pH control and applied it to determine TBZ in citrus fruits. Young et al. [207] developed two procedures for the isolation of TBZ and MBC residues from fruit juice samples using Oasis® mixed mode cation exchange (MCX) SPE cartridges. They found that isolation of TBZ and MBC residues from orange juice could be effectively achieved using MCX operating sequentially in SCX and reversed phase retention modes. Orange juice samples were loaded at pH 2 and catridges were washed with 0.1N HCl,

methanol and high aqueous basic solutions, prior to elution with NH_4OH in methanol. In contrast, MCX operating sequentially in reversed phase and SCX modes provided a more effective clean-up for apple and grape juice samples. It was proposed that the apple and grape juice samples contained polyphenolic (acidic) interferences which were strongly retained after loading under acidic conditions. Young et al. [208] later applied this clean-up in an LC–MS assay, showing that the matrix effect from purified extracts had little or no ion suppression effects. Recovery of MBC and TBZ in both methods was typically greater than 74 and 90%, respectively. Zrostlíková et al. [209] isolated 17 crop protection agents including TBZ and MBC from fruit samples using Oasis® MCX cartridges. Capitan-Valvey et al. [210] developed a novel sorbent extraction procedure for the determination of TBZ in fruit. Samples were extracted using aqueous buffer before dilution of the extract in water and mixing with Sephadex G-15 gel beads. The Sephadex gel beads were isolated by filtration and packed into a glass cuvette, before determination using a luminescence spectrometer.

There have been some applications of automated extraction and clean-up procedures for isolation of benzimidazole residues from crops in recent years. Hiemstra et al. [54] developed an automated on-line clean-up procedure to isolate MBC and TBZ residues from crops. Samples were extracted using acetone and partitioned into dichloromethane–petroleum ether. Sample extracts were evaporated to dryness and reconstituted in methanol (2 ml) and an aliquot (1 ml) was applied onto a Diol SPE cartridge using an ASPEC™ system. The cartridge was washed with methanol (1 ml) and eluted with methanol–0.1 M H_3PO_4 (2 ml) into a vial containing 1 M NaOH (100 μl). Recovery of MBC and TBZ ranged between 97 and 108%. Supercritical fluid extraction (SFE) has been used by some researchers to isolate benzimidazole residues from crops. Aharonson et al. [211] mixed samples (2 g) with hydromatrix (HMX) prior to extracting BEN, MBC, TM and TBZ residues using supercritical carbon dioxide modified with 3% methanol. Optimum extraction was achieved using 90 g of SF-CO_2 (400 atm, 60 °C). Residues were trapped off-line on a C_{18} trap maintained at 25 °C and desorbed with methanol (10 ml). It was found that recovery of residues was affected by the water content in samples but this variable could be controlled using a HMX to sample ratio of 1:1. At higher ratios of HMX recovery of residues was reduced and at ratios greater than 3:1 residues were not recovered. Mock and Scherbaum [212] extracted TBZ and other non-benzimidazole fungicides from fruit using unmodified SF-CO_2 (350 bar, 50 °C), with off-line trapping on a C_{18} trap maintained at 20 °C. Recovery of TBZ ranged between 63 and 70%. Anastassiades and Schwack [213] extracted MBC and TM from crops using unmodified SF-CO_2 (329 atm, 55 °C). Extracts were trapped off-line on a C_{18} trap maintained at 10 °C before desorption with acetonitrile (1.3 ml, 50 °C). This group investigated the influence of pH on the extraction of residues from fortified fruit juice samples, finding that sample pH had to be adjusted to pH 7 to ensure quantitative extraction of MBC. Mean recovery of MBC and TM ranged between 85 and 93%. Kakimoto et al. [214] extracted TBZ and other fungicides from fruit and vegetable samples and processed products using accelerated sol-

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Table 4
Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from animal tissues

Residues	Matrix[a]	Extraction	Clean-up	Recovery (%)	Ref.
ABZ, CAM, MBC, MBZ, OFZ, FBZ, 5-OH-TBZ, TBZ	M, L	Ethyl acetate at alkaline pH	Acidified ethanol vs. hexane C_2 SPE	81–100	[102]
ABZ, MBZ, OFZ, FBZ, OXI, FLU, TBZ, TCB	M, L, K	Acetonitrile–H_2O	LLP C_{18}, Florisil SPEs	65–87 39–80 (OFZ)	[134]
CAM, TBZ, 5-OH-TBZ, 5-NH_2-TBZ	L	DMSO–H_2O			[135]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2 *(MBC, MBZ, FBZ-OH, FLU, 5-OH-TBZ, TBZ)	L	DMF–H_2O			[156]
ABZ, CAM, MBZ, OFZ, TBZ	L	Ethyl acetate	Alumina C_{18} SPE	67–90	[157]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CAM, FBZ-SO_2, MBZ, OXI, 5-OH-TBZ, TBZ	Mk	Acetonitrile	LLP	62–108	[160]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, OXI, 5-OH-TBZ, TBZ	M, L, K	Ethyl acetate at alkaline pH	Acidified ethanol vs. hexane Oasis HLB SPE	44–87	[163]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FEB, FBZ, OFZ, FBZ-SO_2, MBZ, OXI, FLU, CAM, TBZ, 5-OH-TBZ, TCB	M, L	Ethyl acetate at alkaline pH	Acidified ethanol vs. hexane SDB SPE	36–117 8 (FEB)	[164]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, FLU, MBZ, OXI, 5-OH-TBZ, TBZ, TCB, LEV	M, L, fish	Ethyl acetate at alkaline pH	Methanol vs. hexane	75–109	[165]
ABZ, FBZ, OFZ, FBZ-SO_2, FBZ-OH, MBZ, TBZ	M	MSPD	MSPD	63–86	[167]
ABZ, FBZ, OFZ, MBZ, TBZ	L	MSPD	MSPD	55–93	[168]
ABZ, FBZ, OFZ, FBZ-SO_2, FBZ-OH, TBZ	L	MSPD	MSPD	61–92	[170]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, MBZ, MBZ-OH, FLU, OXI, TBZ		SF-CO_2	SCX	56–109	[172]
ABZ, ABZ-SO, ABZ-SO_2, CAM, FBZ, OFZ, FBZ-SO_2, MBZ, LEV	L, M, Mk, Fish	Acetonitrile	SCX SPE On-line TEC	57–89	[173]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, FLU, FLU-NH_2, MBZ, MBZ-OH, OXI, TBZ	L	Ethyl acetate at alkaline pH	C_{18} on ASPEC^{TM}	27–120	[174]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, MBZ, OXI, FLU	L	Dilution juice			[230]

[a] Liver, fat, kidney, muscle and milk are abbreviated as L, F, K, M and Mk.

vent extraction (ASE). Samples were mixed with diatomaceous earth prior to extraction with acetonitrile or methanol (100 °C, 2000 psi) over a 20 min period. Mean recovery of TBZ ranged between 90 and 110%. Pawlowski and Poole [215] extracted MBC and TBZ from crops using subcritical water (40 ml, 75 °C, 50 bar). The pH of aqueous extracts was adjusted to pH 8–9 with 1 M NaOH before partitioning into ethyl acetate. Mean recovery of MBC and TBZ ranged between 80 and 101%.

Some methods have been described for isolation of benzimidazole veterinary residues from medicated feed. Shah et al. [216] extracted OFZ from medicated feeds using methanol–acetic acid (90:10, v/v) at 45 °C. Plant pigments and other feed excipients were removed using zinc acetate treatment and LLP prior to determination by HPLC-UV. Mean recovery of OFZ from fortified feed samples were at 98%. Botsoglou et al. [217] extracted ABZ residues from medicated feed using acetonitrile and washing with hexane. Recovery of ABZ was typically greater than 98%. This group later developed a method for the isolation of FBZ from medicated feed using acetonitrile before washing with iso-octane and determination by HPLC-UV [218]. Mean recov-

ery of FBZ from fortified feed samples ranged between 95 and 100%.

9.3. Summary on sample preparation

The extraction and purification of benzimidazole residues from biological tissues offers a difficult challenge. However, some of these molecules possess common functional groups, which in some cases allows the selective isolation of a range of residues through extraction at a particular pH or through ion exchange SPE clean-up. However, to extract a wide range of residues a more generic acetonitrile based extraction procedure might be required, with hexane defatting and washing with saturated sodium chloride to remove polar components. Extracts may invariably require further purification by SPE prior to determination of residues.

At present, the majority of methods used for isolation of benzimidazole residues from animal tissues and milk may be described as screening methods. This is because the majority do not generally include the complete range of marker residues

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Table 5
Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from milk

Residues	Extraction	Clean-up	Recovery (%)	Ref.
ABZ, ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$, FBZ, OFZ, FBZ-SO$_2$, FBZ-OH, MBZ, OXI	Ethyl acetate at pH 10		79–100	[111]
ABZ-NH$_2$-SO$_2$	Acid hydrolysis (H$_3$PO$_4$) Acetonitrile (pH 5–7)	SCX SPE	>91	[114]
ABZ-SO, ABZ-SO$_2$, FBZ, OFZ, FBZ-SO$_2$	Acetonitrile	C$_{18}$	77–97	[115]
FBZ, OFZ, TBZ, 5-OH-TBZ	Ethyl acetate at alkaline pH	LLP Silica SPE	>80 56 (5-OH-TBZ)	[116]
ABZ, FBZ, OFZ, FBZ-SO$_2$, FBZ-OH, MBZ, TBZ	MSPD	MSPD	70–107	[129]
ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$, 5-OH-TBZ, TBZ, MBZ	Acetonitrile at pH 10 in presence of BHT	C$_{18}$	71–105%	[162]
ABZ, ABZ-SO, ABZ-SO$_2$	Ethyl acetate at alkaline pH	LLP	78–100	[175]
ABZ, FBZ, TBZ, OXI, LEV	Acetonitrile-ethyl acetate	LLP	68–85	[177]
ABZ, FEB, FBZ, OFZ, OXI, TBZ, TCB, LEV	Ethyl acetate at alkaline pH		79–110	[178]
FBZ, OFZ	None	Diluted 1:10	95–122	[179]
ABZ, ABZ-SO, ABZ-SO$_2$, FEB, FBZ, OFZ, MBZ, OXI	Dilute buffer pH 11.0	C$_{18}$ SPE	81–96	[181]
TBZ, LEV	Defat Dilute buffer pH 6.5	C$_{18}$ SPE	81–89	[182]

[916] for each substance. Some researchers have developed methods
[917] for a wide range of substances using LC–MS/MS but these
[918] methods do not include the complete range of marker residues
[919] [164,165,178]. These multi-residue screening methods are sum-

marised in Tables 4 and 5. In some cases, it can be seen from the [1920]
methods that they are suitable for determination of the marker [1921]
residues of certain substances such as ABZ, FBZ, OXI and TBZ. [1922]
However, the majority of these methods fall short when it comes [1923]

Table 6
Summary of methods suitable for the isolation of marker residues of individual benzimidazole drugs from food of animal origin

Residues	Matrix[a]	Extraction	Clean-up	Recovery (%)	Ref.
MBZ, MBZ-OH, MBZ-NH$_2$	Eel	Ethyl acetate at pH 7.8	NH$_2$ SPE	>83, >83, >59	[53]
ABZ, ABZ-SO, ABZ-SO$_2$ ABZ-NH$_2$-SO$_2$	Fish	Ethyl acetate-DMSO at pH 11		82, 78, 75 and 63	[86]
FBZ, OFZ	L	Methanol–H$_2$O	LLP	80, 70	[90]
FBZ, OFZ, FBZ-SO$_2$	M, F, K	Ethyl acetate at pH 8.0			[91]
FBZ, OFZ, FBZ-SO$_2$	Fish, M, Sk	Acetonitrile and hexane wash	C$_{18}$ and CN SPE		[92]
FLU, FLU-HMET, FLU-RMET	E, M	Ethyl acetate at pH 10	Methanol vs. hexane	>70 (E) and >87 (M)	[94]
MBZ, MBZ-NH$_2$, MBZ-OH	L	Ethyl acetate at pH 10	Methanol vs. hexane	>85	[98]
ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$	Mk	Acetonitrile	LLP	74–90	[112]
ABZ, ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$	Dairy products	Acetonitrile	LLP	78–86	[113]
TCB, TCB-SO, TCB-SO$_2$	Mk	Acetone	C$_{18}$	76–92	[117]
TCB, TCB-SO, TCB-SO$_2$	Mk	Acetonitrile and hexane wash	C$_{18}$	89–95	[119]
ABZ marker residues	L	Acid hydrolysis Ethyl acetate at pH 8–9.5	C$_{18}$ SPE	>75	[125]
TBZ, 5-OH-TBZ, 5-HSO$_4$-TBZ	Mk	Acid hydrolysis (HCl) Ethyl acetate at pH 8.0	PRS SPE	87–115	[126]
Nine metabolites of OFZ	L	Acetonitrile	SCX SPE	28–118	[133]
TBZ, 5-OH-TBZ	M, L, K	Ethyl acetate at pH 7.0	CN SPE	>96, >70	[161]
ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$	M, L, F, K	Acetonitrile	Ion-pair extraction LLP	>81, >88, >76	[166]
ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$	Mk	Acetonitrile	LLP	73–85	[175]

[a] Egg, fat, kidney, liver, muscle, milk and skin, are abbreviated as E, F, K, L, M, Mk and Sk.

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Table 7
Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from crops

Residues	Matrix[a]	Extraction	Clean-up	Recovery (%)	Ref.
MBC, TBZ	F, V	Acetone	LLE Diol SPE on ASPEC	97–108	[54]
BEN, MBC, 2-AB	F, V	Ethyl acetate	LLP	71–92	[136]
TBZ, MBC	F, V	LLE (ethyl acetate vs. 0.1N HCl)	LLP	91–97	[186]
BEN, TBZ	F, V	Reflux with ethyl acetate at alkaline pH		73–109	[227]
BEN, MBC, 2-AB	F, V	Benzene at alkaline pH	LLP	70–90	[197]
BEN, MBC, TBZ	F, V	Reflux in 2N HCl Chloroform at pH 9.5			[198]
BEN, MBC, TBZ	F, V	Methanol	LLP	64–105	[199]
BEN, MBC, TBZ	F	LLE (chloroform vs. aqueous NaCl)	LLP	>90	[200]
BEN, MBC, TBZ	F, V	Acetone	LLP, normal phase column chromatography	48–86	[201]
BEN, MBC, TBZ	F, V	Acetone	Extrelut and SCX SPE	>80	[204]
MBC, TBZ	Fruit juice	Dilution	Oasis® MCX	74, 97	[207,208]
MBC, TBZ	F	Acetonitrile	Oasis® MCX	>88	[209]
BEN, MBC, TBZ, T-M	F, V	SF-CO_2	C_{18} Trap	78–102	[211]
BEN, MBC, T-M	F, V	SF-CO_2	C_{18} Trap	85–93	[213]
TBZ, MBC	F, V	H_2O (100 °C, 50 bar)	LLP	80–101	[215]

[a] Fruit and vegetables are abbreviated as F and V, respectively.

to the inclusion of the marker residues of FLU, MBZ and TCB. It may be proposed that these methods are suitable for residue monitoring purposes. However, in some cases, if the most persistent and/or predominantly occurring residues such as MBZ-OH in sheep, MBZ-NH_2 in horse, and the sulphate conjugate of TBZ and TCB-SO_2 in milk are not included in methods then this is not strictly true. It may be concluded that for more effective determination of benzimidazole residues in food of animal origin, more residues need to be included in methods. Alternatively, laboratories should apply methods that are suitable for the determination of individual marker residues such as those outlined in Table 6, in combination with multi-residue methods.

The methodology developed for the isolation of benzimidazole fungicides from crops is generally fit for purpose. In general, there are only a few benzimidazole fungicides used in crop protection, requiring methods for the isolation of four residues BEN, MBC, TBZ and TM. A summary of these methods are outlined in Table 7. It may be concluded that methods that have been developed for the determination of MBC and TBZ are suitable for the determination of benzimidazole fungicides in crops. Some have looked at the researcher's applicability of these extraction and purification procedures also for recovery of the pre-benzimidazoles (BEN and TM).

10. Methods for measurement of benzimidazole residues

10.1. Bioassays

Bioassays have been used to detect benzimidazole residues in food but are more routinely used to evaluate the potency of anthelmintic substances. A common approach adopted in most bioassays is to first separate residues on a TLC plate and subsequently spray the plate sequentially with nutrient agar and a solution containing an indicator organism. The presence of benzimidazole residues is indicated by a zone of inhibited growth on the TLC plate. The size of the zone of inhibition is related to the concentration of the benzimidazole residue. Erwin et al. [219] developed a bioassay method for determination of BEN. Later Petersen and Edginton [220] developed an assay to detect BEN and MBC in crops. BEN and MBC residues were extracted and separated on a silica gel TLC plate, which was subsequently sprayed with a mixture of agar and *Penicillium* spores prior to incubation (ambient, 20 h). The authors concluded that the bioassay was 10 times more sensitive than detection by UV spectrometry. The procedure offered a number of advantages over an alternative bioassay procedure developed by Erwin et al. [219]. These advantages included the possibility to allow separation of different metabolites and improved sensitivity. Dew et al. [221] developed a multiwell screening assay for TBZ, ABZ, ABZ-SO, ABZ-SO_2 and MBZ using larvae of *Ascaris suum*. The effect of different drugs and concentrations was evaluated on the different larval development stages (L2–L4). L2 is defined as larvae hatched from eggs, L3 the first moult and L4 the second moult. All of the benzimidazole residues selected were shown to inhibit development of the L2–L3 stage at a concentration of 10 ng/ml. The L3–L4 stage was also inhibited by each of the benzimidazole residues. However, inhibition of this larval stage was strongly influenced by the concentration and benzimidazole type. ABZ, MBZ, TBZ, ABZ-SO and ABZ-SO_2 inhibited larval development at concentrations of 10, 10, 100, 100 and 1000 ng/ml, respectively. The bioassay was proposed as a low cost screening system for detection of benzimidazole residues

in food. However, it can be concluded that the variable sensitivity of test organisms to benzimidazole metabolites or marker residues is a key issue that needs to be evaluated more closely.

10.2. Direct spectrometric detection

In early work, a number of groups developed spectrometric methods for determination of benzimidazole residues in crops. Pease and Gardiner [195] determined BEN, after conversion to 2-aminobenzimidazole (2-AB), by fluorescence spectrophotometry (λex 285 nm and λem 335 nm) or by colorimetric detection at 445 nm after derivatisation with bromine. The limit of detection was less than 100 μg/kg. The three benzimidazole fungicides, 2-AB, TBZ and 2-(2-furyl)-benzimidazole all fluoresce naturally; however, only 2-AB reacts with bromine to produce coloured derivatives. As a result, colorimetric detection after bromination is a means of discriminating between 2-AB and other benzimidazole fungicide residues. Aharonson and Ben-Aziz [201] similarly determined BEN and TBZ residues in crops by fluorescence spectroscopy after selective clean-up by column chromatography. Baeyens et al. [222] determined MBZ and FLU in pharmaceutical preparations by fluorescence (λex 300 nm and λem 400 nm) after reaction with 0.3% hydrogen peroxide in alkaline solution. This group indicated that the assay could be applied also to tetramisole, dexamisole and levamisole. In summary, spectrometric methods may be suitable for quantitation of high levels of benzimidazole residues in food. However, when quantitation of benzimidazoles in the low μg/kg range and greater selectivity is required, chromatographic separation of residues prior to spectrometric detection is generally required.

10.3. Thin layer chromatography

TLC is a suitable technique to detect benzimidazole residues in food, while providing semi-quantitative analysis. The technique has been applied to allow cost-effective detection of benzimidazole residues in crops and animal tissues. Norman et al. [185] determined TBZ in citrus fruits by TLC with fluorescence detection (λex 302 nm and λem 360 nm). White and Kilgore [202] determined MBC in crops at a concentration lower than 50 μg/kg on polyamide plastic TLC plates coated with a fluorescent indicator. Plates were developed using a solvent of chloroform–ethyl acetate–acetic acid (190:10:4, v/v/v) and visualised under UV light at 254 nm. Corti et al. investigated the use of high performance TLC (HPTLC) for determination of TBZ and other residues in potatoes [193]. The residues were separated on C_8 plates using a mobile phase of methanol–water (10:3, v/v) and quantitation was completed using a scanning densitometer at 248 nm. The sensitivity of the method was compared with HPLC, but HPLC was found to be much more sensitive. The same researchers later developed a method for determination of BEN, MBC and TBZ in fruit [200]. Residues were separated on NH_2 HPTLC plates (derivatised with polyamine) using a solvent of chloroform–cyclohexane–methanol (6:1:0.1, v/v/v) for MBC and TBZ. Residues were detected using a scanning densitometer at 285 nm. More recently, Abjean [223] developed a TLC screening method for detection of ABZ-NH_2-SO_2 in

liver. ABZ-NH_2-SO_2 was separated using a mobile phase of acetonitrile–ammonium hydroxide (10:0.6, v/v) on a silica TLC plate coated with a fluorescent indicator. The limit of detection of the method was less than 500 μg/kg.

10.4. Immunochemical methods

Immunoassays can provide simple, sensitive and selective detection of benzimidazole residues in biological matrices. In some cases, samples such as plasma, serum and milk may be analysed directly or diluted prior to analysis. In early work, radioimmunoassays were applied to detect benzimidazole residues in animal tissue and crops. Nerenberg et al. [224] developed a radioimmunoassay for determination of OFZ in plasma. Polyclonal antibodies were raised in rabbits after immunisation with OFZ coupled to a polylysine carrier. In subsequent work, the assay was applied to OFZ residues in sheep fat tissue [137]. Newsome and Shields [225] developed a radioimmunoassay using a polyclonal antibody for determination of BEN in crops, as it's major residue MBC. Polyclonal antibodies were raised in rabbits after immunisation with the hapten 2-amino benzimidazole coupled to a human serum albumin (HSA) carrier.

In recent years, enzyme linked immunoassays (ELISAs) have found widespread application and have largely taken over from radioimmunoassays. There have been a number of significant applications of ELISA technology in the area of benzimidazole analysis. Brandon et al. [135] developed a competitive ELISA for determination of TBZ in liver, using a monoclonal antibody raised in mouse. Haptens of TBZ and 5-OH-TBZ were prepared and coupled to the carrier bovine serum albumin (BSA) for use as immunogens. It was found that antibodies raised after immunisation with the conjugate prepared from TBZ were more specific for TBZ and 5-OH-TBZ, while antibodies raised from the conjugates prepared with 5-OH-TBZ were found to be less specific and also showed good cross-reactivity to CAM and TBZ-NH_2. Cross-reactivity to benzimidazoles not possessing a thiazolyl ring was also evaluated, but none of these residues (namely ABZ, MBZ and FBZ) showed significant cross-reactivity. Competitive ELISAs were developed based on the horseradish peroxidase (HRP) conjugate and were applied to liver samples extracted using water. They later applied this ELISA for determination of TBZ residues in fruit, vegetables and fruit juices [226]. It was found that simple dilution of fruit juice samples in buffer was sufficient prior to determination by ELISA. Brandon et al. [156] later produced a mouse monoclonal antibody that showed good cross-reactivity to 11 benzimidazole carbamate residues including ABZ, FBZ, OXI, MBZ, FLU, MBC and some metabolites. The antibody did not show cross reactivity to thiazolylbenzimidazoles (TBZ, CAM and 5-OH-TBZ). Antibodies were raised in mice using conjugates prepared after coupling the succinamide hapten of ABZ to BSA carrier protein. They developed a competitive ELISA based on HRP for determination of benzimidazole residues in bovine liver samples after aqueous extraction and later developed a method for determination of FBZ residues in milk using this ELISA [179]. Milk samples were simply diluted in PBS-Tween-BSA before determination by ELISA.

Bushway et al. [191] evaluated a commercially available enzyme immunoassay tube kit for determination of TBZ residues in processed/unprocessed fruit and vegetables. The polyclonal antibody used in the assay was raised against BEN-MBC, but the antibody demonstrated sufficient cross-reactivity towards TBZ to allow its sensitive detection in food. Newsome and Collins [227] developed ELISAs for determination of BEN and TBZ in crops, using polyclonal antibodies raised in rabbits. Immunogens were prepared by coupling the carrier human serum albumin (HSA) to the succinamide haptens of TBZ and MBC (the breakdown product of BEN), and were used in rabbits for raising antisera. Abad et al. [228] produced monoclonal antibodies raised against a TBZ-ovalbumin conjugate. They indicated that this was a very novel conjugate because it was prepared from a hapten functionalised at the nitrogen atom of the 1-position of the TBZ structure. They developed an indirect ELISA based on HRP for determination of TBZ residues in fruit juices. Moran et al. [229] produced mouse monoclonal antibodies raised against the novel immunogen 5-benzimidazole–carboxylic acid conjugated to the lipopeptide $Pam_3Cyst-T_H$. The antibodies were produced for development of ELISAs for determination of protein-bound residues of TBZ in tissue, although no applications were described.

Crooks [230] later produced a polyclonal antibody that showed good cross-reactivity to eight benzimidazole carbamate residues. Antibodies were raised in sheep using conjugates prepared after coupling the succinamide hapten of ABZ to HSA carrier protein. This group developed a competitive ELISA based on HRP for determination of benzimidazole residues in the tissue juice produced from bovine liver. Johnsson et al. [231] later applied the antibody produced by Crooks in a Biacore® biosensor assay for detection of benzimidazole residues in bovine serum. They evaluated the effect of different sample processing conditions on the biosensor assay response, finding that benzimidazole standard curves prepared in serum, without protein precipitation did not agree with standards in buffered solutions. The assay was shown to give good cross-reactivity (>74%) to five benzimidazoles. The results also indicated an improved cross-reactivity to FLU (75%) compared to the original ELISA developed by Crooks (23%). The LOD and LOQ of the method were determined using 20 blank serum samples to be 2.6 and 4.8 ng/ml, respectively.

10.5. Liquid chromatography based separations

Benzimidazole drugs and their metabolites may differ greatly in terms of physical and chemical properties. As a result, development of a chromatographic method for a range of benzimidazole residues is a challenging process. A number of problems need to be overcome including resolution, peak shape, peak sharpness and reasonable run time. The majority of LC methods for determination of benzimidazoles have been developed using reversed phase columns (C_8 or C_{18}) and ion suppression based mobile phase systems. In early work, some methods were developed using alternative column types such as silica or cation exchange. Karlaganis et al. [144] determined MBZ in plasma using a silica column with a mobile phase of acetonitrile–water saturated chloroform–formic acid (75:92.5:0.25, v/v/v). Alvinerie and Galtier [149] developed a normal phase separation of TCB, TCB-SO, TCB-SO$_2$ and OFZ on a silica column achieving good separation in a 15 min run. Kirkland et al. [136] used a cation exchange column for determination of BEN residues in plants and soil. Arenas and Johnson [126] determined TBZ and 5-OH-TBZ in milk using a strong cation exchange column. Hiemstra et al. [54] used a polymeric column for analysis of TBZ and MBC residues in crops giving better peak shape, separation and longer column lifetime than silica-based columns.

In early work, Bogan and Marriner [139] developed an isocratic separation for eight benzimidazole residues on a C_{18} column in 14 min, but the substances were poorly resolved. Bull and Shume [130] later separated metabolites of FBZ and TCB in a 40 min run time. Long et al. [131] and Wilson et al. [102] developed multi-residue LC methods for determination of 7 and 8 benzimidazoles in less than 13 and 35 min, respectively. Other researchers have developed gradient separations achieving improved separation or inclusion of more residues. Allan et al. [151] compared isocratic and gradient systems for determination of five benzimidazoles in plasma. Sorensen and Petersen [181] developed a gradient separation of eight benzimidazoles in a 40 min run with additional time needed for equilibration. Rose [133] separated OFZ and nine metabolites in less than 35 min using a binary gradient on a C_8 column. Similarly, Stubbings et al. developed a gradient separation for levamisole and eight benzimidazoles on a C_8 column [173]. More recently, Roudaut and Garnier [163] separated 10 benzimidazoles using a binary gradient on a base deactivated C_{18} column, all residues with the exception of OX1 and OFZ were separated in less than 16 min.

Rouan et al. separated TCB, TCB-SO, TCB-SO$_2$ and an internal standard on a short C_{18} column (33 mm × 4.6 mm) in less than 10 min [154]. Porter and Johnston [232] evaluated narrow bore columns (2.1 mm) for determination of TBZ, MBZ, ABZ and FBZ residues. This group found that the peak heights for analytes increased by a factor of five using narrow bore columns compared to a column of standard diameter (4.6 mm). This group concluded that use of narrow bore columns resulted in a reduction in solvent usage, analysis time and pump wear. Kan et al. [96] and Hajee and Haagsma [53] developed isocratic separations on columns of intermediate diameter (3 mm) for FLU and MBZ metabolites, respectively. Likewise, Domany and Kovacsics [160] developed an isocratic separation of 10 benzimidazoles on a narrow bore (2.1 mm) C_{18} column. Shaikh et al. found that ABZ and its three major metabolites could not be eluted in less than 20 min without the use of gradient elution [86]. This group chose to determine ABZ and its three metabolites using two separate isocratic systems. Danaher et al. [172] separated 14 benzimidazole residues on an Xterra® C_{18} column using gradient elution in a run time of 60 min.

Negro et al. [56] evaluated the use of ion-pair chromatography for determination of TCB-SO and TCB-SO$_2$ in plasma and urine. They investigated the effect of pH, organic modifier content and the chain length of the sodium alkylsulphonate ion-pair reagent on chromatography. The optimum mobile phase consisted of 0.05 M phosphate buffer pH 7.0–acetonitrile (55:45, v/v) con-

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taining 0.001 M sodium decanesulphonate. De Ruyck et al. [177] developed an ion pair gradient separation for FBZ, TBZ, ABZ, OXI and LEV. Satisfactory separation conditions were achieved with sodium 1-octanesulphonate as the ion-pairing agent and adjustment of the mobile phase buffer solution to pH 3.5 with ortho-phosphoric acid. Botsoglou et al. [132] investigated the effect of ion-pair reagents and pH on the retention time and peak height of FBZ and OFZ, and showed that decreasing the pH to 2.2 shortened the retention time of FBZ markedly and OFZ slightly. Addition of octane-sulphonate anion increased retention time and tetrabutyl ammonium cation decreased retention time. Over the range of pH 3.7 to 6.5 no change in retention time was observed with or without the addition of ion-pair reagents. It was found that the peak shape of FBZ was poor over a range of pH values in the absence of ion-pair reagents, but addition of ion-pair reagents greatly enhanced peak shape at pH 2.2, even at longer retention times. The same group developed a range of separations for FBZ residues using ion-pair chromatography. A multi-residue method was later developed for separation of 10 benzimidazoles in milk in less than 30 min, using an isocratic mobile phase. Macri et al. [233] developed an ion-pair separation of eight benzimidazoles using a mobile phase containing 0.01 M pentane-sulphonate and 0.5% triethylamine at pH 3.5.

In the area of LC–MS, most groups carry out separations using shorter columns (less than 150 mm in length). These columns require shorter equilibration times during gradient chromatography because of the lower column volume, which increases LC–MS throughput. Blanchflower et al. [90] developed an isocratic separation of FBZ and OFZ, finding it gave more reproducible MS results compared to gradient separation. Cannavan et al. [161] developed a gradient method for separation of TBZ and 5-OH-TBZ, using a mobile phase consisting of acetonitrile and 0.1 M ammonium acetate. Balizs [164] developed a method for determination of 15 benzimidazole residues by LC–MS/MS with a gradient separation carried out on a narrow bore column (2.1 mm). The mobile phase flow rate ranged between 0.04 and 0.2 ml/min depending on the ionisation interface used. The benzimidazoles were eluted from the column in less than 6 min but were not resolved. It was claimed that, because individual benzimidazoles have different mass-to-charge values, chromatographic separation was unnecessary. De Ruyck et al. [94] developed an LC–MS/MS method for determination of benzimidazole residues using a column of similar dimensions for determination of FLU, FLU-HMET and FLU-RMET in eggs and muscle tissue. Later, they developed a method for determination of levamisole and seven benzimidazole residues in milk [178].

10.5.1. Liquid chromatography detection systems

The determination of benzimidazoles in milk, tissue and crops is routinely carried out using UV, fluorescence and mass spectrometric (MS) detection systems coupled to LC. Benzimidazoles possess a strong UV chromophore and may be determined using LC–UV. However, it has been found that certain benzimidazoles (namely ABZ, CAM, FLU, TCB, TBZ, and their metabolites) possess naturally fluorescing chromophores, making them suitable for detection by LC fluorescence. Fluorescence is more sensitive and selective, but does not have the same range of applicability as UV detection. As a result, UV is the most widely applied system to detect benzimidazole residues in biological extracts. MS detection is becoming more widely used for determination of benzimidazole residues in biological matrices, offering the capability for combined quantitative-confirmatory analysis.

10.5.1.1. Detection by UV.
Alton et al. [146] determined MBZ residues in plasma by UV detection at 313 nm. Karlaganis et al. [144] determined MBZ in plasma, finding less interference at a wavelength of 307 nm compared to 247 nm. Bogan and Marriner [139] developed a method for determination of eight benzimidazoles in body fluids, using UV detection at 292 nm. Mellergaard et al. [138] determined MBZ residues in eel muscle at 254 nm. Hajee and Haagsma [53] determined MBZ, MBZ-NH$_2$ and MBZ-OH in eel muscle at 289 nm. Kan et al. [96] developed a method for analysis of FLU and its metabolites in eggs using UV detection at 250 nm. Chiap et al. [155] determined ABZ, ABZ-SO and ABZ-SO$_2$ in plasma at 295 nm.

Long et al. [131] determined seven benzimidazole residues in tissue using UV photodiode array (PDA) detection. They also investigated the use of PDA as a confirmatory tool, but found differences between spectra at lower and higher concentration levels. The PDA detector was not suitable for confirmatory analysis at lower concentrations (5 ng on-column). They later developed methodology for determination of these residues in milk and animal tissues. De Buyanski et al. [158] determined TBZ and levamisole in tissue using PDA detection at 300 and 240 nm, respectively. Danaher et al. [172] determined 14 benzimidazole residues in liver tissue using UV detection at 298 nm.

Tai et al. [116] adopted a dual wavelength approach for determination of FBZ, OFZ, TBZ and 5-OH-TBZ residues in milk. FBZ and OFZ residues were monitored at 298 nm, while TBZ and 5-OH-TBZ were monitored at 318 nm. Similarly, Sorensen and Petersen [182] determined TBZ and levamisole residues in milk using a PDA detector at wavelengths of 300 and 220 nm, respectively. This group also developed a method for determination of eight other benzimidazoles in milk (ABZ, ABZ-SO, ABZ-SO$_2$, OFZ, OXI, MBZ, FBZ and FEB) [181].

A range of derivatisation methods can be used for determination of benzimidazoles. Most of these have been used for GC applications and only two papers on derivatives for LC have been published. Capece et al. [91] converted FBZ and OFZ residues to FBZ-SO$_2$ and measured the sum of total residues as this peak. Residues were derivatised by reaction with percetic acid and purified by liquid–liquid partitioning prior to determination by LC–UV. Tafuri et al. [234] confirmed the presence of TBZ residues, by derivatising with p-nitrobenzyl bromide in the presence of potassium carbonate (110 °C, 3 h) with detection by LC–UV. The derivatisation procedure was used to provide quantitative and confirmatory analysis of TBZ residues in suspect samples.

10.5.1.2. Detection by fluorescence.
A number of researchers have developed methods for determination of benzimidazole residues using HPLC coupled to fluorescence detection. Wilson

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et al. [102] found that sample extracts needed further purification on C_2 SPE cartridges to remove matrix components that interfered with UV detection of ABZ-NH$_2$-SO$_2$ and 5-OH-TBZ. However, the influence of this matrix interference could be greatly reduced using fluorescence detection. It was proposed because less clean-up is required with fluorescence that recovery and reproducibility of assays should improve. Le Boulaire et al. [169] determined TBZ and MBZ in tissue, finding fluorescence to be 20 times more sensitive than UV detection for TBZ. Kinabo and Bogan [117] developed a method for determination of TCB and its metabolites in milk using fluorescence detection (λex 300 and λem 676 nm). The limit of detection of the method was 20, 40 and 40 μg/l for TCB-SO$_2$, TCB-SO and TCB, respectively. Arenas and Johnson determined TBZ and 5-OH-TBZ in milk using fluorescence detection with λex 305 and 318 nm and λem 380 and 515 nm, respectively [126]. Two separate chromatographic runs were required to achieve more sensitive detection, but it was concluded that these residues could be determined at their optimum wavelengths in a single run using a dual grating monochromator. The LOD and LOQ were 5 and 50 μg/l, respectively. Markus and Sherma [125] used fluorescence detection for determination of ABZ-NH$_2$-SO$_2$ in bovine liver (λex 300 nm and λem 320 nm). Shaikh et al. [86] determined ABZ, ABZ-SO, ABZ-SO$_2$ and ABZ-NH$_2$-SO$_2$ and three metabolites in fish muscle using fluorescence detection with λex 290 nm and λem 330 nm. LOQs for the method were 20, 1.5, 0.5 and 5 μg/kg for ABZ, ABZ-SO, ABZ-SO$_2$ and ABZ-NH$_2$-SO$_2$, respectively. Aharouson et al. [211] determined benzimidazole fungicides in fruit and vegetable matrices using UV and fluorescence detection. TBZ, MBC and TM were determined by UV at 285 nm while TBZ and MBC were determined also by fluorescence (λex 305 nm, λem 345 nm and λex 282 nm, λem 307 nm, respectively). It was found that detection of TBZ residues could be enhanced using fluorescence instead of UV detection, but detectability of MBC was similar using both detectors. However, matrix interference was found to be less significant with fluorescence detection.

10.5.1.3. Detection by UV and fluorescence in series. The use of fluorescence detection can offer added sensitivity and selectivity to methods, potentially reducing the need for extensive sample clean-up. These added advantages may allow the development of multiresidue methods that would allow the determination of all major benzimidazole residues, a task that has not yet been completed by any researcher. However, since some benzimidazoles do not fluoresce naturally (namely FBZ, OFZ, FBZ-SO$_2$, MBZ, FLU, MBZ-NH$_2$ and FLU-NH$_2$), UV is still required as it applicable to all benzimidazole residues.

Some researchers have developed methods for the determination of benzimidazole residues using UV and fluorescence detection in series, these methods are described in this section. Farrington et al. [157] determined benzimidazole residues (ABZ-SO$_2$, OFZ, CAM and MBZ) using UV and fluorescence detectors in series. ABZ-SO$_2$, TBZ and CAM were monitored using fluorescence detection (λex 312 nm, λem 355 nm), while OFZ and MBZ were monitored using UV detection at 290 nm. Su et al. [162] developed a method for determination of benzimi-dazole residues using UV and fluorescence detection. A generic wavelength of 290 nm was found to be suitable for detection of ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$ and TBZ. This group found that more selective detection of 5-OH-TBZ and MBZ could be achieved using UV at 320 nm. Fluorescence detection (λex 290 nm, λem 320 nm) was found to be suitable for the determination of ABZ-SO, ABZ-SO$_2$, ABZ-NH$_2$-SO$_2$ and TBZ. Fluorescence detection was found to be unsuitable for the detection of 5-OH-TBZ and MBZ because of weak signal and lack of fluorescent characteristics, respectively. Constantinou et al. [180] determined five benzimidazole residues in milk using a similar approach. TBZ and ABZ residues were monitored using fluorescence (λex 312 nm, λem 355 nm) while other residues were detected by UV at 290 nm. Hiemstra et al. [54] determined MBC residues in crops using UV (280 nm) and fluorescence (λex 235 nm, λem 280 nm) detectors in series. Residues were confirmed by comparing the relative UV and fluorescence responses for positive samples with standards.

10.5.1.4. Detection by electrochemical potential. There are few reported applications of electrochemical detection for benzimidazole residues. Oosterhuis et al. [143] determined MBZ and MBZ-OH residues in biological fluids by electrochemical detection. The electrochemical detector consisted of a carbon paste electrode and a saturated calomel electrode; MBZ residues were determined at 950 mV versus the reference electrode. FLU, which possesses similar oxidative properties to MBZ, was used as internal standard. Leenheers et al. [235] similarly developed a method for determination of a metabolite of MBC in urine by HPLC with electrochemical detection.

10.5.1.5. Detection by mass-spectrometry. It will be seen from the applications reviewed in this section that LC–MS is the technique of choice for sensitive and selective detection of a wide range of benzimidazole residues in complex biological matrices. In contrast to traditional LC–UV methods, LC–MS can be used to allow stand-alone determination and confirmation of benzimidazole residues. LC-fluorescence could be applied as an alternative technique to allow determination and confirmation of selected benzimidazole residues. However, not all benzimidazole residues fluoresce naturally. In this section, the suitability of LC–MS methods for determination and confirmation of benzimidazole residues will be reviewed.

Some early methods were reported for determination of benzimidazole residues in animal tissues using single quadrupole MS instruments equipped with thermospray ionisation interfaces, while operating in selected ion monitoring (SIM) mode. The disadvantage with such systems is that insufficient fragmentation of molecules is achieved and resulting mass spectra do contain a suitable number of diagnostic ions for confirmatory purposes. Blanchflower et al. [90] used LC–MS with a thermospray interface for determination of FBZ and OFZ residues in tissue. FBZ and OFZ were determined in SIM mode as their [M + H]$^+$ ions. Cannavan et al. [161] used LC–MS with a thermospray interface for determination of TBZ and 5-OH-TBZ residues in bovine tissue, while using a deuterated TBZ internal standard to improve reproducibility. TBZ and 5-OH-TBZ were

monitored in SIM mode as their $[M+H]^+$ ions. Cannavan et al. confirmed the presence of residues on a single quadrupole instrument equipped with an atmospheric pressure chemical ionisation (APCI) interface. They demonstrated that suitable fragmentation could be induced with an APCI interface to produce four diagnostic ions, which were suitable for confirmation of residues. Other researchers have developed methods for determination or confirmation of benzimidazole residues using single quadrupole instruments equipped with electrospray ionisation (ESI) interfaces. Takeba et al. briefly described a method for confirmation of TCB marker residues, which were monitored as their $[M-H]^-$ ions [119]. The LOD of the method ranged between 4 and 6 μg/kg in bovine milk. The protocol used for confirmation of residues was poorly described and it was not clear if residues could be confirmed at these low levels. Young et al. [208] determined TBZ and MBC residues in fruit juices using a single quadrupole LC–MS instrument equipped with an ESI interface in positive ion mode. TBZ and MBC were monitored as their $[M+H]^+$ ions. The LOQ of the method was 2 μg/kg in fruit juice. TBZ and MBC residues were fragmented through in-source collision induced dissociation at different cone voltages to produce five and six diagnostic ions for confirmation of MBC and TBZ, respectively.

In the last decade, the application of tandem mass spectrometry (MS/MS) has resulted in improved determination and confirmation of benzimidazole residues in biological matrices. Most of these methods have been reported on triple quadrupole instruments operating in multiple reaction monitoring (MRM) mode or using the MS/MS capabilities of ion trap instruments. Balizs [164] developed a method for determination of 15 benzimidazoles using a triple quadrupole instrument equipped with ionspray and turbospray interfaces while operating in positive ion mode. $[M+H]^+$ ions were fragmented through CID and monitored as a single daughter transition through MRM. The LOQ ranged between 5 and 30 μg/kg in meat. De Ruyck et al. [94] developed an LC–MS/MS–ESI method for determination of FLU and metabolites in muscle, liver and eggs. Residues were monitored as their $[M+H]^+$ ions and confirmed using MRM of two daughter ion transitions. Quantitation was carried using the most abundant daughter ion while using chlorobenzimidazole as an internal standard and matrix matched calibration. The LOQ was approximately 1 μg/kg in egg and meat tissues. They also reported an LC–MS/MS–ESI for determination of MBZ, MBZ-OH and MBZ-NH$_2$ in sheep muscle, liver, kidney, liver and back fat [98]. The limit of detection of the method was less than 1 μg/kg. De Ruyck et al. [178] later developed a multi-residue method based on the LC-MS/MS-ESI for determination of eight anthelmintic drugs (LEV, TBZ, OFZ, FBZ, OXI, ABZ FEB and TCB) in milk (Fig. 11). Residues were monitored as their $[M+H]^+$ ions and confirmed using MRM. The decision limit (CCα) and the detection capability (CCβ) were calculated by analysing samples fortified at 1 μg/kg (for non-permitted substances) and at the MRL (for permitted substances). Edder et al. [165] determined levamisole and 11 benzimidazole residues in meat and liver by LC–MS/MS using positive ESI and MRM mode. They similarly confirmed the presence of residues based on two daughter transitions. The LOD and LOQ of the method

were 5 and 10 μg/kg (except for ABZ, which were 10 and 20 μg/kg), respectively.

A limited number of LC–MS methods have been developed for determination of benzimidazole residues on ion trap instruments. Zrostlíková et al. [209] reported a method for determination of TBZ, MBC and other crop protection agents in fruit using an LC–MS system equipped with an ESI interface in positive ion mode and an ion trap detector. Benzimidazole parent $[M+H]^+$ ions were fragmented using collision induced dissociation through the MS/MS capability of the ion trap and monitored as a single product ion transition. The LOQ of the method for MBC and TBZ was 2 and 4.8 μg/kg, respectively.

10.6. Gas chromatography

GC determination of benzimidazoles is difficult because of the basic nature and low volatility of these substances. However, TBZ and TCB are sufficiently volatile to allow their determination by GC without derivatisation. The main applications of GC methods have been in determining benzimidazole fungicides in crops. Some researchers have found GC coupled to mass spectrometry useful for confirmation of the presence of benzimidazole residues. However, GC–MS procedures usually require derivatisation of residues to induce volatility and allow the generation of suitable MS spectra for confirmatory analysis and, as a result, have been largely been replaced by LC–MS/MS. Quantitative GC methods have been developed by some researchers to determine the presence of benzimidazole fungicides in crops. Lafuente et al. [192] determined TBZ residues in fruit by direct GC using nitrogen–phosphorous detection (NPD) and electron capture detection (ECD). Separation was carried out on a wide-bore capillary column (10 m × 0.53 mm) with a silicone-bonded phase. NPD was found to be more sensitive than ECD. The LOD of the method was 10 μg/kg. Oishi et al. [203] similarly determined TBZ (without derivatisation) by GC–NPD. The separation was carried out on a fused silica capillary column (25 m × 0.2 mm), giving a LOD of 10 μg/kg. However, most GC methods for determination of benzimidazoles include derivatisation steps, where more polar amino or hydroxyl groups are reacted with suitable alkylating/acylating reagents. An overview of these derivatisation procedures is outlined in Fig. 12 and Table 8. Some researchers hydrolysed the carbamate functional group to form an amino functionality prior to reaction with the alkylating/acylating reagent. Alternatively, residues may be derivatised directly without hydrolysis. A wide range of alkylating and acylating reagents have been used to derivatise benzimidazoles, these are summarised in Table 8. These allow the introduction of functional groups ranging from simple methyl esters to more complex pentafluorobenzyl groups.

Jacob et al. [236] confirmed the photolysis products of TBZ residues by GC–MS after derivatisation with bis(trimethylsilyl)acetamide. They later applied the same method to confirm the structures of urinary metabolites of CAM [106,237]. VandenHeuvel et al. [238] developed an on-column methylation procedure for confirmation of CAM residues by GC–MS–EI (electron impact mass spectrometry) in SIM mode. Residues were dissolved in 0.125 M triethylammonium hydrox-
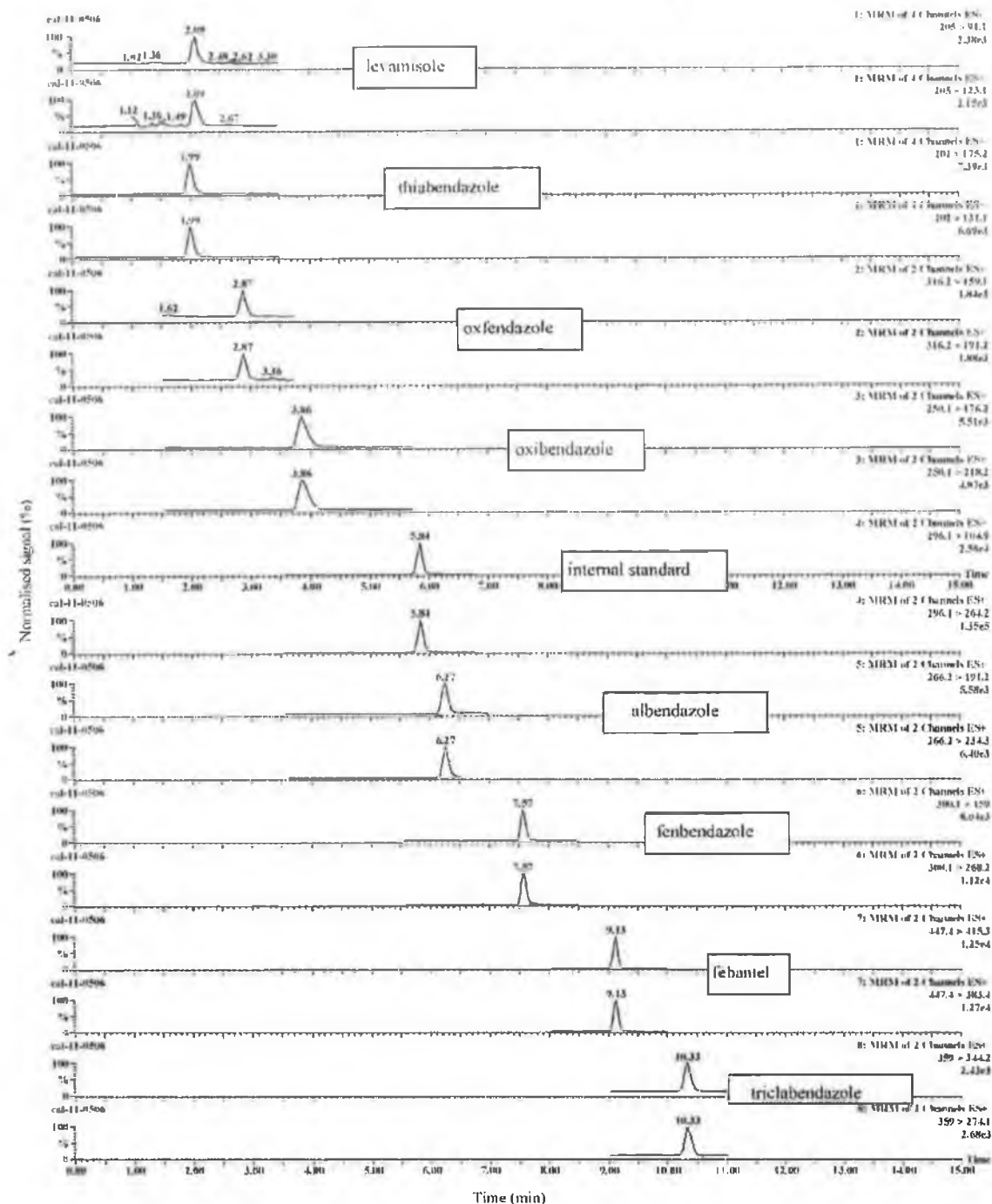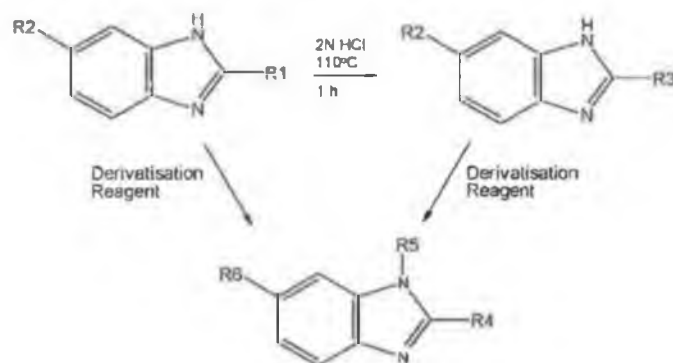
Fig. 11. Chromatogram of a blank milk sample spiked with a mixture of the anthelmintics at 1 μg/l and with the internal standard mebendazole at 10 μg/l (reprinted from reference [178], with permission from Elsevier, Copyright (2002)).

ide in methanol just prior to injecting into the GC–MS system. They later applied this method for TBZ and 5-OH-TBZ [239]. They used a column with a polysulfone stationary phase because its background bleed and adsorption characteristics were more desirable than others tested. Tanaka and Fujimoto [188] determined the methyl derivative of TBZ by GC with flame ionisation detection (FID). Derivatives were prepared by reacting with dimethylformamide and dimethyl acetal in acetonitrile (120 °C, 40 min). The procedure was used to determine TBZ residues

in fruit with a limit of detection of 100 μg/kg. Nose et al. [190] acylated TBZ by reaction with pentafluorobenzyl-bromide PFB-Br (120 °C, 30 min) before determination by GC–ECD and confirmation by GC–MS–EI in full scan mode. Physalo derivatised BEN residues with acetic anhydride (100 °C, 30 min) before determination by capillary GC-NPD [197]. The LOD was 10 μg/kg. GC–ECD was also evaluated for determining residues but was 10 times less sensitive. Bardalaye and Wheeler later modified this procedure and applied it to the determination of

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Fig. 12. Overview of derivatisation procedures used in the preparation of benzimidazole derivatives for gas chromatography. The R1 functional group remains unchanged if residues are not hydrolysed prior to alkylation/acylation. The structures for the R5 functional group are listed in Table 8 (reprinted from reference [102] with permission from AOAC International, Copyright (1991)).

Table 8
Summary of derivatisation reagents, reaction conditions and functional group modifications

Derivatisation reagent	Residues	Conditions	R5	Ref.
MTBSTFA	MBC, TBZ, 5-OH-TBZ, ABZ-NH2-SO2, FBZ, MBZ, OFZ	110 °C, 2 h	—Si(CH3)2 C(CH3)3	[102,213,241]
BSA in pyridine	CAM, TBZ	60 °C, 30 min	—Si(CH3)2 C(CH3)3	[106,236,237]
PFB-Br + K2CO3	TBZ, TCB, ABZ, FBZ, OFZ, MBZ, FLU, OXI, MBC	100 °C, 3 h or 60 °C, 3 h	—C-H2 (pentafluorobenzyl)	[134,186,240]
PFB-CO-Cl + Na2CO3 (TBZ)	TBZ	120 °C, 30 min or 100 °C, 45 min	—CO (pentafluorobenzoyl)	[134,187,190]
PFB-CO-Cl + Na2CO3 (other benzimidazoles)			No reaction	[134]
CH3-I	TBZ, TCB, ABZ, FBZ, OFZ, MBZ, FLU, OXI	60 °C, 30 min	—CH3	[134]
Trimethylphenylammonium hydroxide in methanol	CAM, TBZ, 5-OH-TBZ	On-column methylation	—CH3	[102,238,239]
DMA-DMF	TBZ	120 °C, 40 min	—CH3	[188]

TBZ in fruits [187]. Tjan and Janssen [186] determined TBZ and MBC in fruit using GC–ECD after derivatisation with PFB-Br. The procedure was also applicable to BEN, which was converted to MBC during the extraction and clean-up procedure. The LOQ of the method was 50 μg/kg. Tjan and Janssen also developed a confirmatory procedure using GC–MS–EI. Cline et al. [240] later modified this procedure and applied it to determine MBC in fruit treated with BEN. The LOD of the method was 10 μg/kg.

Marti et al. [134] developed GC methods to confirm the presence of eight benzimidazole residues in animal tissue, while evaluating a range of derivatisation procedures used for substances with amino functional groups. They found that acylation with PFB-Br and methylation with methyl iodide gave satisfactory results. Derivatised extracts were determined by GC–NPD and GC–ECD; GC–NPD chromatograms were found to contain less matrix interference than GC–ECD chromatograms. However, derivatives were found to decompose during injection and chromatography because of the high temperature used, making quantitative determination difficult. The main advantages of these derivatives could be seen with GC–MS–EI and GC–MS–positive ion chemical ionisation (PICI) determination where particularly useful structural information was provided for confirmatory analysis. Electron impact spectra of the methyl derivatives produced fewer ions than the pentafluorobenzyl derivatives. Positive ion chemical ionisation provided a similar number of ions suitable for confirmatory analysis, while negative ion chemical ionisation produced only one or two ions for most residues. Wilson et al. [102] used capillary GC–MS–EI in SIM mode for confirmation of benzimidazole residues in tissue. Residues were derivatised in a two-step procedure, firstly involving hydrolysis with 2N HCl (110 °C, 1 h) to convert the carbamate group to an amino functionality. Hydrolysed residues were reacted with N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), converting the primary and secondary amines to a silyl amine. In the case of 5-OH-TBZ, the phenol functional group was converted to a silyl ether. The method was not applicable to CAM, which was alternatively derivatised using flash alkylation in the GC injection port (260 °C) after injection in triethylammonium hydroxide in methanol. Markus and Sherma [241] developed a method for confirmation of ABZ-NH$_2$-SO$_2$ in tissue based on this derivatisation procedure, with determination by GC–MS–EI. Baliza and Erbach [242] determined TBZ and ABZ residues in tissue using capillary GC with high-resolution mass spectrometry (HRMS), after derivatisation with PFB-Br. Anastassiades and Schwack later applied a similar GC–MS procedure for confirmation of benzimidazole fungicides in crops after SFE [213].

10.7. Summary on detection systems

At present most methods for the determination of benzimidazole residues in biological matrices using HPLC with UV and/or fluorescence detection. HPLC–UV is a more universal detection system but fluorescence is generally more selective and in some cases more sensitive. In the last 10 years, LC–MS(–MS) has found more widespread application in benzimidazole residue analysis offering more sensitive detection and increased confidence in reporting results. In the future, with an increasing demand to include additional residues in methods there should be an increase in the number of LC–MS/MS methods. Such methods can offer the ability to include additional residues in methods and offer improved limits of detection. In particular, when testing for low levels of residues, such as those that may occur in milk due to unapproved used of licensed veterinary drugs, LC–MS/MS is the technique of choice. Alternatively, HPLC coupled to UV and fluorescence detection in series may offer a low cost alternative to LC–MS/MS, and may be particularly effective for independently confirming the presence of MRL substances in food.

It is important to emphasise the need for rapid methods to detect the presence of residues in food prior to its entry into the marketplace. There have been considerable developments in the area of immunochemical screening methods. No antibody has been developed yet that shows cross-reactivity to the complete range of benzimidazole residues. Future work in this area should concentrate on exploring the cross-reactivity of antibodies to important residues and improvement of cross-reactivity through genetic engineering of antibodies. The application immuno-biosensor assays has been explored in a limited study in serum. However, future work should investigate the application of this technique to allow more sensitive detection of residues and reduce non-specific binding effects.

11. Conclusions

There has been intensive research in the last decade into developing methods for the determination of benzimidazole residues in food. At present, methodology for the determination of benzimidazoles in crops would appear to be fit for purpose. However, methodology for determination of benzimidazole residues in foods of animal origin needs improvement. This is not a reflection on the quality of the research that has been published but of the difficult challenge faced, owing to the extensive metabolism of these molecules.

The authors of this paper have carefully considered these problems and have come up with the following recommendations:

- There is a need to develop multi-residue methodology that will allow the determination of the complete range of benzimidazole veterinary drug residues in foods of animal origin.
- Sample preparation techniques need to be developed to allow simultaneous extraction of polar residues such as TBZ and non-polar residues such as TCB.
- These methods may require the application of more selective detection such as LC–MS/MS or a combination of HPLC-UV-fluorescence or through the application of a combination of more specific analytical methods.
- An alternative approach might be to include a rational scientific selection of benzimidazole residues according to their occurrence/persistence in the tissues of treated animals. However, in the event that a drug residue is found the presence of other marker residues of the drug should be checked. The disadvantage of such an approach is that it would necessitate the

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validation of alternative residue methods for confirming the marker residues of each drug.

- The need for rational selection of residues particularly applies when testing for benzimidazole residues in milk. In the case of TBZ and TCB the most prevalent and persistent residues in milk are the sulfate conjugate of 5-OH-TBZ and TCB-SO_2, respectively. This would indicate that hydrolysis steps should be included to allow the effective determination of TBZ residues in milk. To monitor for the unapproved use of TCB in dairy animals, TCB-SO_2 in milk would be the most appropriate residue to select.

- One problem encountered when selecting residues for a method is that the most prevalent/persistent residue is not always clearly identifiable. This is the case with benzimidazole veterinary drugs, where the residue is often expressed as the sum of marker residues. The marker residues of ABZ may be expressed as the hydrolysed/oxidised residues and FBZ as the sum of oxidised residues. In the case of TCB, residues are expressed as the sum of residues, which may be derivatised to form keto-TCB. It is not clear whether keto-TCB is a metabolite or an artifact of derivatisation. In the interest of food safety, it would be more appropriate if residues were expressed as the individual metabolites to allow rational selection of marker residue(s) for analysis.

- Another problem faced, is that current validation criteria for testing of foods of animal origin, as outlined in Commission Decision 2002/657/EC, are not appropriate for all approved benzimidazoles because MRLs are often expressed as the sum of marker residues. This indicates that determination of CCα and CCβ is not always appropriate. This is a particular problem for MBZ and FLU because no method is available that allows the determination of total marker residues.

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Appendix 4

Confirmatory analysis of malachite green, leucomalachite green, crystal violet and leucocrystal violet in salmon by liquid chromatography–tandem mass spectrometry

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Abstract

A method has been developed to analyse for malachite green (MG), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet (LCV) residues in salmon. Salmon samples were extracted with acetonitrile:McIIIvain pH 3 buffer (90:10 v/v), sample extracts were purified on a Bakerbond strong cation exchange solid phase extraction cartridge. Aliquots of the extracts were analysed by LC–MS/MS. The method was validated in salmon, according to the criteria defined in Commission Decision 2002/657/EC. The decision limit (CC_α) was 0.17, 0.15, 0.35 and 0.17 µg kg^{-1}, respectively, for MG, LMG, CV and LCV and for the detection capability (CC_β) values of 0.30, 0.35, 0.80 and 0.32 µg kg^{-1}, respectively, were obtained. Fortifying salmon samples ($n = 6$) in three separate assays, show the accuracy to be between 77 and 113% for MG, LMG, LCV and CV. The precision of the method, expressed as RSD values for the within-laboratory reproducibility, for MG, LMG and LCV at the three levels of fortification (1, 1.5 and 2.0 µg kg^{-1}), was less than 13%. For CV a more variable precision was obtained, with RSD values ranging between 20 and 25%.
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Keywords: Malachite green; Leucomalachite green; Crystal violet; Leucocrystal violet; Salmon; Method validation

1. Introduction

Malachite green (MG) and crystal violet (CV) are triphenylmethane dyes. MG has been used as a topical fungicide and antiprotozoal agent in salmonid farming throughout the world for over 60 years. Few known chemicals are as effective in treating ectoparasites of fish and fish eggs by fungi of the genus *Saprolegnia* and by the protozoan parasite *Ichthyophthirius multifiliis* [1]. CV is also known to be effective in the treatment of fungal infections. It had found widespread use as a feed additive to inhibit mould and fungal growth in poultry feed before 1990 [2]. According to EU law, all substances for veterinary use need to be included in annexes 1–3 of Regulation 2377/90. MG

or CV, however, are so-called "non-defended" compounds that have never been registered for veterinary use. As a result of these anti-fungal properties there is a serious potential for the miss-use of these drugs in aquaculture. Recently there has been a number of reports of these residues in aquaculture products (cf. EC Rapid Alert Reports for Food and Feed 2003–2006). Concerns regarding residues of MG in aquaculture products have prompted studies of the pharmacokinetics and metabolism of this compound. However there is limited data regarding the pharmacokinetics and metabolism of CV. Leucomalachite green (LMG) and leucocrystal violet (LCV) are formed by the metabolic reduction of MG and CV and in this form the drug persists in the tissues of exposed fish [3,4].

The US Food and Drug Administration explicitly banned the use of MG in fish farming in 1991 due to its suspected carcinogenic properties. MG and CV are structurally related to other triphenylmethane dyes such as rosaniline which has been linked

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to increased risk of human bladder cancer. The leuco form of rosaniline induces renal, hepatic and lung tumors in mice [5]. CV has been implicated in the induction of thyroid and liver tumors [6]. Studies show that LMG is an *in vivo* mutagen and the mutagenicity of MG and LMG correlates with their tumorgenicities in mice [7]. MG has also been shown to cause DNA damage [8]. CV was found to be cytotoxic to mammalian cells [9] as was MG [10]. Compared to LMG, MG is much less toxic to cells [10]. No safe levels for the presence of MG, LMG, CV and LCV in fish for human consumption could be established and therefore, detection of these compounds at sub $\mu g\,kg^{-1}$ levels is required.

Within the EU, each member state is required to monitor for MG and LMG residues with analytical methodology that at least meets the MRPL. The European minimum required performance limit (MRPL), a quality parameter for residue laboratories, is set as the sum of MG and LMG at 2 $\mu g\,kg^{-1}$ [11]. There is no MRPL set for CV so developed methods must be capable of monitoring levels as low as is reasonably achievable.

Most methods currently used are based on the solvent extraction of MG and LMG from fish tissues using acetonitrile or methanol with aqueous buffer (pH 3–4.5) [12–25]. In the clean-up of these samples a combination of liquid/liquid partitioning and solid phase extraction is used. Bergwerff et al. [21] purified catfish, eel, trout, turbot and prawn samples by partitioning acetonitrile:McIllvain pH 3 buffer (90:10 v/v) against chloroform before passing the chloroform extract through a Bakerbond™ strong cation exchange solid phase extraction cartridge. This clean-up approach has been adopted by other groups using a single solid phase extraction cartridge [12,20] or using dual solid phase extraction with aluminia and propylsulphonic acid cartridges [3,16,19]. Van Rhijn et al. [18] modified the method of Bergwerff et al. [21] by eliminating the liquid/liquid partitioning step. Other methodologies eliminated the liquid/liquid partitioning steps and purified tissue extracts directly using C_{18} [24] and cyano [22] SPE cartridges. Swarbrick et al [26] used an alternative clean-up using activated charcoal instead of C_{18} SPE.

A number of methods have been reported for the analysis of MG, LMG, CV and LCV residues by visible light detection with oxidation of the leucometabolites carried out either pre-column or post-column [3,14,16,19–21,23,27]. Other methods developed for the detection of these residues are based on this oxidation but with detection by mass spectrometry [12,13,20,21]. Detection of these residues by mass spectrometry in the absence of this oxidation has also been accomplished [15,18,24]. An alternative oxidation approach using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) before the determination step to oxidise LMG to MG has also been utilised [30,31]. Determination based on electrochemical detection [27], GC–MS [23] and spectrophotometry [17] has also been achieved.

The method developed in this study was based on the method developed by Van Rhijn et al. [18] but adapted to include CV, LCV, and deuterated internal standards for LMG and for MG. The method omits the often-proposed pre-column or post-column oxidation step used in the determination of MG or CV. The method also omits the liquid/liquid partitioning stage often used in the clean-up of extracts containing these residues. The method is a simple extraction with acetonitrile:McIlvain buffer

Table 1
LC gradient profile for determination of MG, LMG, CV and LCV

Time (min)	Component A (%)	Component B (%)
0.0	90	10
0.5	90	10
3.0	50	50
7.2	10	90
14.5	10	90
16.5	90	10
21.0	90	10

Component A: ammonium formate buffer + water (5 + 95 v/v) with 200 μL of formic acid; component B: ammonium formate buffer + acetonitrile (5 + 95 v/v) with 200 μL of formic acid.

pH 3 (90:10 v/v), with clean-up using aromatic sulphonic acid solid phase extraction and LC–MS/MS analysis. The applicability of the method for regulatory control will be discussed.

2. Experimental

2.1. Materials and reagents

Water, methanol, acetonitrile, ethyl acetate and hexane (HiPerSolv grade) were obtained from BDH (Merck, Poole, Dorset, UK). Ammonium hydroxide (ACS grade) was obtained from Sigma (St. Louis, MO, USA). Citric acid (ACS grade) was obtained from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate (ACS grade) was obtained from Sigma (St. Louis, MO, USA). Ascorbic acid (ACS grade) was obtained from Fluka (CH-9470 Buchs, Switzerland). MG, LMG and CV were purchased from Sigma. d_5-MG and d_5-LMG were obtained from Witega (Berlin, Germany). LCV was purchased from Dr. Ehrenstorfer (QMX Laboratories, UK). Primary stock standard solutions of MG, LMG, d_5-MG, d_5-LMG, CV and LCV (stable for 3 months) were prepared in acetonitrile at a concentration of 100 $\mu g\,mL^{-1}$. Intermediate single standards solutions of MG, LMG, d_5-MG, d_5-LMG, CV and LCV (stable for 1 month) were prepared in acetonitrile:water (50:50 v/v) at a concentration of 1 $\mu g\,mL^{-1}$. Mixed standard fortification solutions of MG, LMG, CV and LCV (stable for 1 month) were prepared in acetonitrile:water (50:50 v/v) at concentrations of 50 and 200 $ng\,mL^{-1}$.

Table 2
MS/MS parameters for determination of MG, LMG, CV and LCV

Compound	Transition	Spray voltage (V)	Collision (eV)	R_t (min)
MG	329 > 313 (strong)	4000	35	8.1
	329 > 208 (weak)	4000	37	8.1
LMG	331 > 239 (strong)	4000	35	13.2
	331 > 316 (weak)	4000	23	13.2
d_5-MG (IS)	334 > 318	4000	35	8.1
d_5-LMG (IS)	336 > 239	4000	35	13.2
CV	372 > 356 (strong)	4000	57	8.7
	372 > 340 (weak)	4000	40	8.7
LCV	374 > 358 (strong)	4000	35	13.5
	374 > 238 (weak)	4000	30	13.5

Mixed standard fortification solution of d_5-MG and d_5-LMG (stable for 1 month) was prepared in acetonitrile:water (50:50 v/v) at a concentration of 100 ng mL^{-1}. All standards were stored at 4 °C. Bakerbond^{TM} SCX cation exchange solid phase extraction cartridges (3 mL, 500 mg) were obtained from J.T. Baker (Harbor City, USA). McIllvain pH 3 buffer was prepared by adding 0.1 M citric acid (250 mL) and adjusting to pH 3 with 0.2 M disodium hydrogen phosphate and diluting to 1000 mL with water. Extraction solvent was prepared by making a solution in the ratio of acetonitrile:McIllvain pH 3 buffer (90:10 v/v). A 2% ammonium hydroxide solution in methanol was used as the solid phase extraction elution solvent. Injection solvent consisted of 1% ascorbic acid in acetonitrile:water (50:50 v/v).

2.2. LC conditions

The LC system consisted of a model Finnigan TSQ Quantum Ultra (MS) coupled to a Finnigan Surveyor LC Pump and a Finnigan Surveyor Autosampler (Thermo Electron Corporation, CA, USA). A gradient LC system (Table 1) using ammonium formate:water (5:95 v/v mobile phase A) and ammonium formate:acetonitrile (5:95 v/v mobile phase B) at a flow of 0.350 μL min^{-1}, was used to separate the analytes on a Phenomenex 3 μm C_{18} 100 Å (100 mm × 2.0 mm) (Phenomenex, UK) column equipped with a guard column (10.0 mm × 2.0 mm i.d.) packed with Hypersil^{TM} (3.0 μm) C_8 material (Thermo Electron Corporation, UK). The column temperature was maintained at 45 °C. Data acquisition and integration were performed using XCalibur version 1.4 chromatographic management software (Thermo Electron Corporation, CA, USA). The LC system was equilibrated for 30 min at a column oven temperature of 45 °C. A linear gradient (Table 1) was used ramping from 10% B to 90% B over 7.2 min: once at 90% B, the system was held for 7.3 min and returned to 10% B over a period of 2.0 min. The runtime was 21 min. The described gradient LC system was shown to be suitable for the analysis of MG, LMG, CV and LCV (Figs. 1–4 (weak/strong transitions)).

2.3. MS/MS parameters

The analysis was performed using positive-ion electrospray (ESI) interface with multiple reaction monitoring (MRM) mode. Two transitions per compound were used and the spray and collision voltages were optimised as shown (Table 2). The MS/MS detector conditions were as follows: ion mode ESI+; gas sheath pressure 60 psi; capillary temperature 375 °C; source CID 10 V; collision pressure 1.5 Torr; collision gas argon.

2.4. Salmon samples

Whole salmon obtained from a local supermarket were homogenised with bones in a bowl-blender (Waring, UK) and stored at −20 °C in 150 mL glass jars. Laboratory samples of salmon tissue were analysed and those found to contain no detectable residues of the analytes were used as negative controls.

2.5. Sample extraction and clean-up

Salmon samples (2 g) were weighed into 50 mL polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 2.5 μg kg^{-1} by adding 50 μL portion of 100 ng mL^{-1} mix solution of d_5-MG and d_5-LMG. Samples were fortified at levels corresponding to 1, 1.5 and 2 μg kg^{-1} by adding 40, 60 and 80 μL portions of 50 ng mL^{-1} mix solution of MG, LMG, CV and LCV. After fortification, samples were held for 15 min prior to extraction. Acetonitrile:McIllvain pH 3 buffer (90:10 v/v) (20 mL) was added and the samples were homogenised (30 s). Samples were shaken (5 min) and sonicated (15 min). The samples were centrifuged (3000 rpm, 5 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The samples were re-extracted with acetonitrile:McIllvain pH 3 buffer (90:10 v/v) as before. The supernatants were combined and sample extracts were further purified by ion exchange solid phase extraction using Bakerbond SCX SPE cartridges. Sample extracts (40 mL) were loaded onto the cartridges (preconditioned with 2 mL methanol, 2 mL acetonitrile:McIllvain buffer 90:10 v/v). The cartridges were washed with distilled water (1 mL), methanol (1 mL), ethyl acetate (1 mL), hexane (1 mL) and then dried using a vacuum pump (3 min). The cartridges were eluted with 2% ammonium hydroxide in methanol (3 × 2 mL). The eluates were reduced to dryness under vacuum at 45 °C before re-dissolving in 250 μL of 1% ascorbic acid in acetonitrile:water (50:50 v/v). An aliquot (20 μL) was injected onto the LC column.

2.6. Matrix matched calibration

Matrix matched calibration curves were prepared and used for quantification. Control tissue previously tested and shown to contain no residues was prepared as above (Section 2.4). One control tissue sample was used for each calibration standard level. Salmon samples (2 g) were weighed into 50 mL polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 2.5 μg kg^{-1} by adding 50 μL portion of 100 ng mL^{-1} mix solution of d_5-MG and d_5-LMG. Samples were fortified at levels corresponding to 0, 0.5, 1 and 2 μg kg^{-1} by adding 0, 20, 40 and 80 μL portions of a 50 ng mL^{-1} standard solution. Samples were fortified at the 5 and 10 μg kg^{-1} calibration levels by adding 50 and 100 μL portions of a 200 ng mL^{-1} standard solution. After fortification, samples were held for 15 min prior to extraction procedure as above (Section 2.5).

Calibration curves were prepared by plotting the response factor of the strong transitions as a function of analyte concentration (0–10 μg kg^{-1}) to quantify samples.

2.7. Method validation

For estimation of accuracy, blank salmon tissue samples were fortified with MG, LMG, CV and LCV at 1, 1.5 and 2.0 μg kg^{-1}. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. For the estimation of the precision
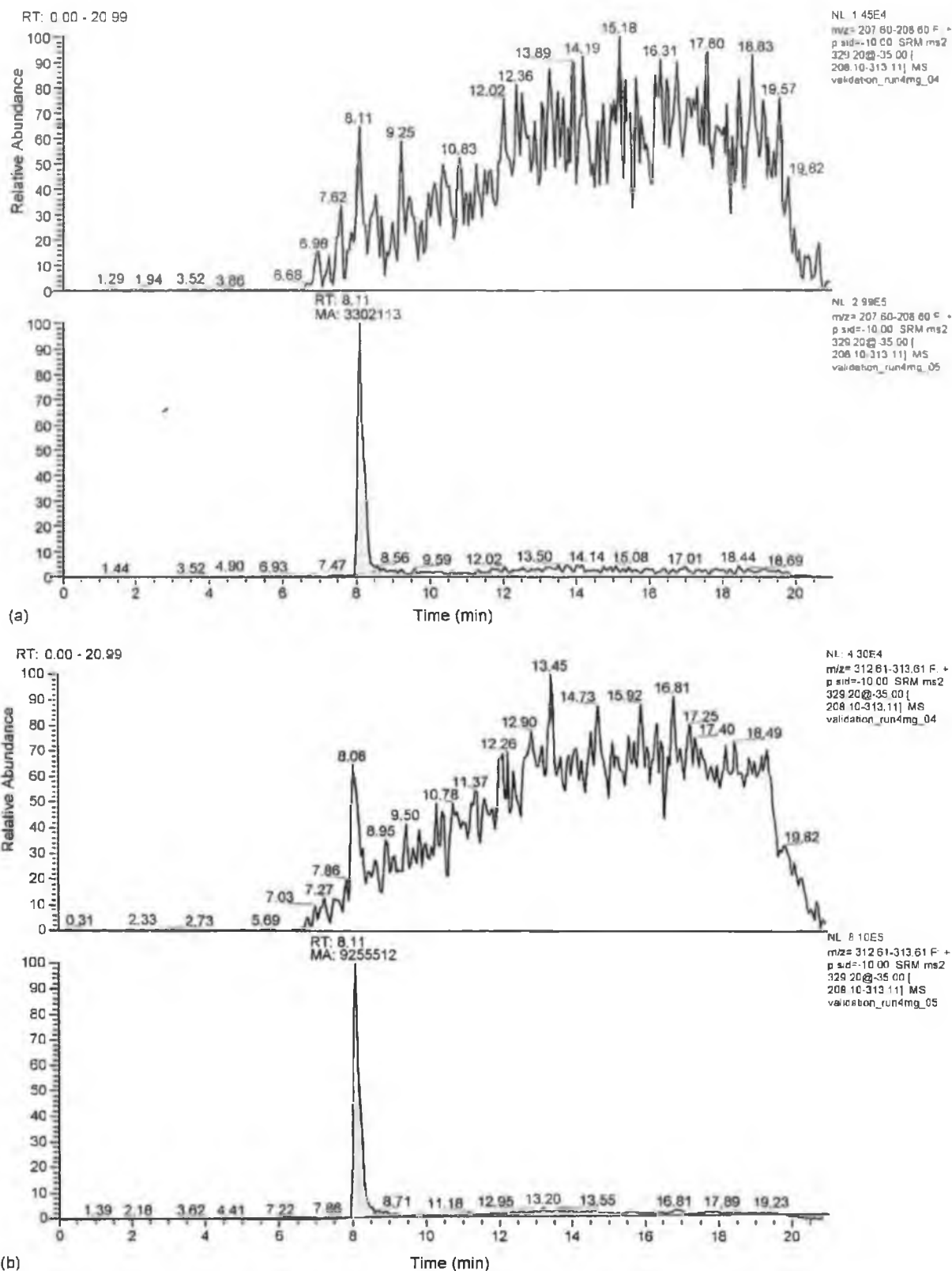
Fig. 1. Chromatogram of negative control salmon (top) and negative control salmon fortified at 0.5 μg kg⁻¹ with: (a) MG (weak transition) and (b) MG (strong transition).

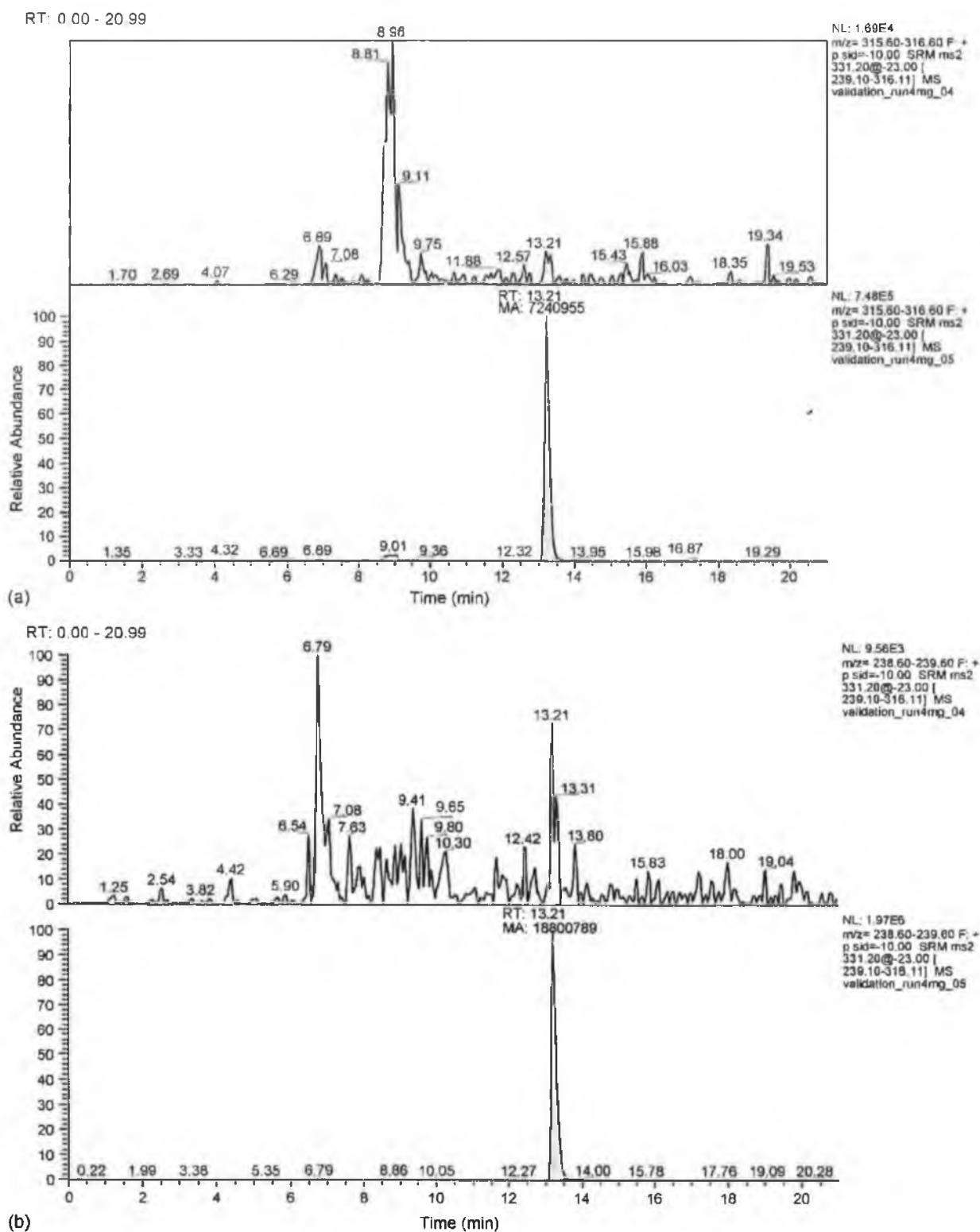Fig. 2. Chromatogram of negative control salmon (top) and negative control fortified at 0.5 µg kg^{-1} with: (a) LMG (weak transition) and (b) LMG (strong transition).

of the method, repeatability and within-laboratory reproducibility was calculated. The decision limit (CC$_\alpha$) of the method were calculated according to the calibration curve procedure using the intercept (value of the signal, y, where the concentration, x is equal to zero) and 2.33 times the standard error of the intercept for a set of data with six replicates at three levels. The detection capability (CC$_\beta$) was calculated by adding 1.64 times the standard error to the CC$_\alpha$.
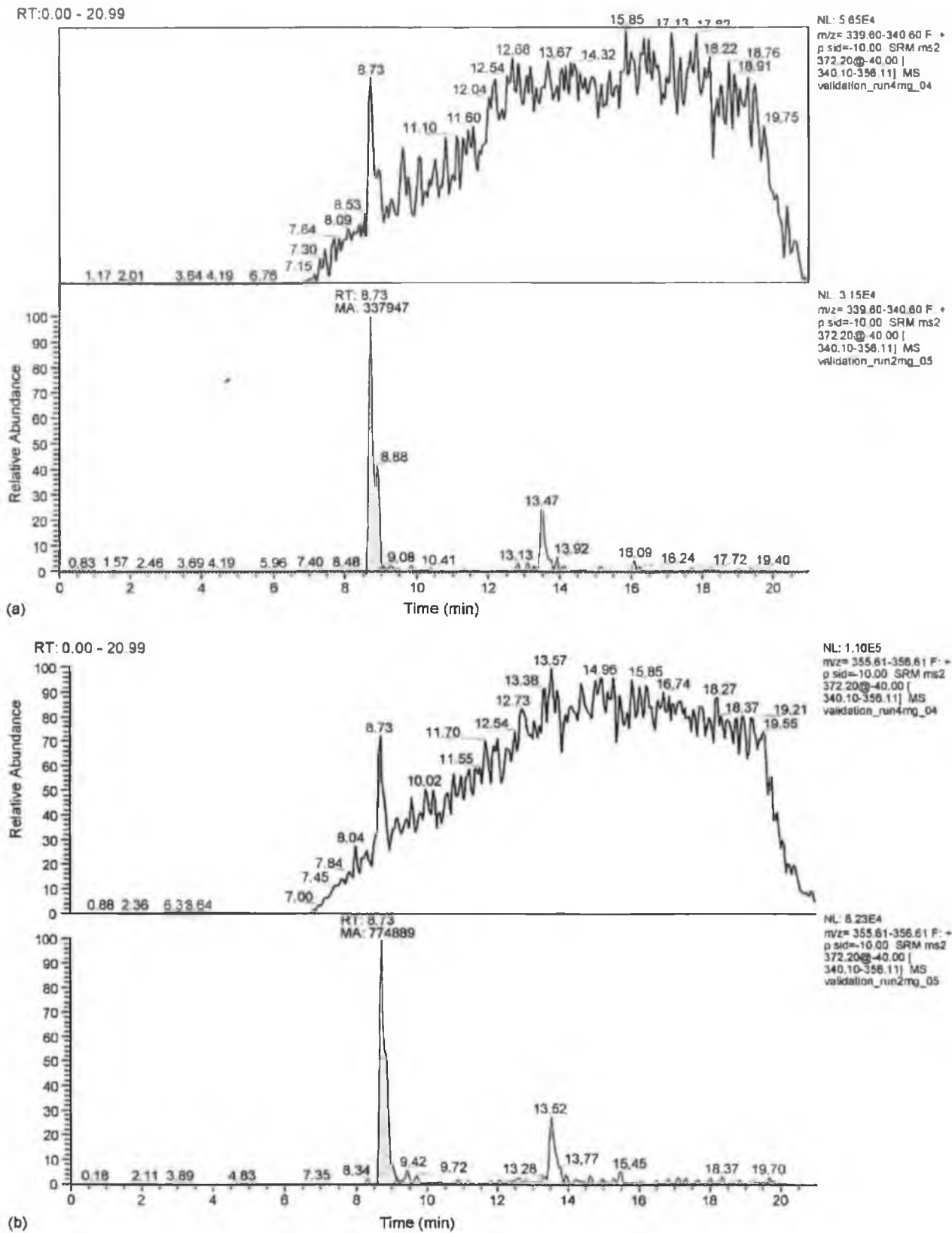
Fig. 3. Chromatogram of negative control salmon (top) and negative control fortified at 0.5 μg kg^{-1} with: (a) CV (weak transition) and (b) CV (strong transition).

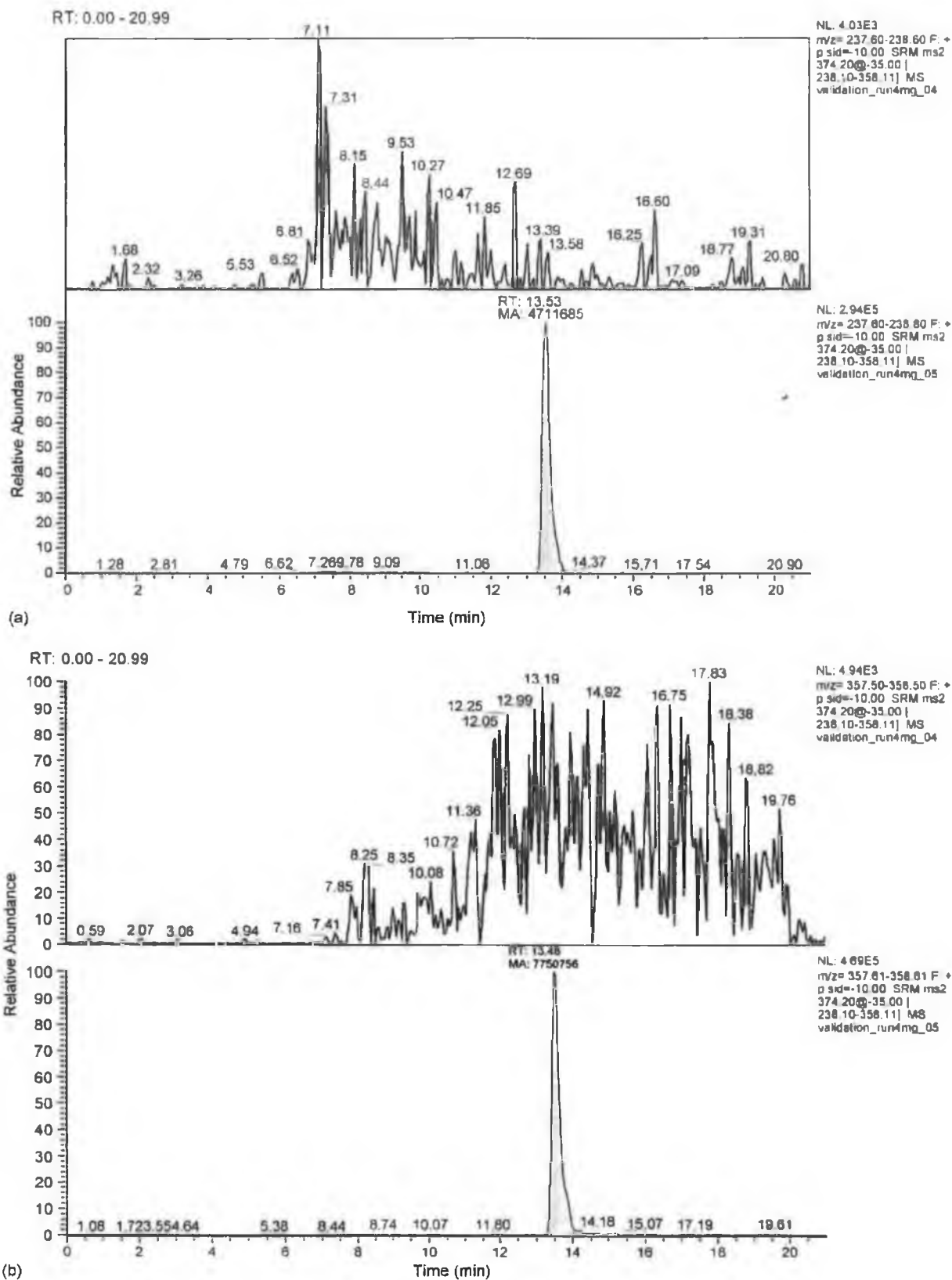Fig. 4. Chromatogram of negative control salmon (top) and negative control fortified at 0.5 µg kg⁻¹ with: (a) LCV (weak transition) and (b) LCV (strong transition).

Table 3
Results for repeatability and within-laboratory reproducibility of malachite green, leucomalachite green, crystal violet and leucocrystal violet in salmon

Analyte	Fortification level (μg kg^{-1})	Accuracy							
		Assay 1 repeatability		Assay 2 repeatability		Assay 3 repeatability		Within-laboratory reproducibility	
		Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
MG	1.0	104	1.9	96	6.9	107	2.5	102	6.2
	1.5	108	3.3	98	7.1	113	3.7	106	7.6
	2.0	104	2.3	96	2.9	109	4.5	103	6.1
LMG	1.0	96	10.9	99	3.0	102	0.1	100	6.3
	1.5	97	2.7	97	6.3	103	1.0	90	4.7
	2.0	97	1.4	96	4.3	98	3.5	97	3.2
CV	1.0	92	10.4	89	9.3	105	28.8	96	20.2
	1.5	102	24.3	81	14.0	94	29.0	92	24.7
	2.0	111	15.1	90	20.3	104	23.5	101	20.0
LCV	1.0	77	7.3	99	3.0	100	5.2	92	13.1
	1.5	89	6.0	102	9.0	97	2.3	96	8.4
	2.0	96	8.7	103	6.0	95	5.8	98	7.6

3. Results and discussion

3.1. Preliminary experiments

The LC–MS/MS method was developed to provide confirmatory data for the analysis of salmon for MG, LMG, CV and LCV. The MS/MS fragmentation conditions were investigated and collision energies were optimised for each individual compound. For a method to be deemed confirmatory one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion) must be monitored (Table 2) this yields four identification points, which is a suitable confirmatory method in accordance with CD 2002/657/EC.

MG and LMG are separated on a Phenomenex C$_{18}$ column with a retention time of 8.1 and 13.2 min, respectively. Similarly CV and LCV are separated with a retention time of 8.7 and 13.5 min, respectively. d_5-MG was used as IS for MG and similarly d_5-LMG was utilised as internal standard for LMG. As there are no deuterated standards of CV and LCV currently available, d_5-MG and d_5-LMG were used as internal standard for these analytes as well.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [29] covering specificity, calibration curve linearity, recovery (accuracy), precision, decision limit (CC$_\alpha$) and detection capability (CC$_\beta$).

3.2.1. Specificity

The technique of LC–MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, salmon samples were fortified with the four analytes and the internal standards and non-fortified samples were also analysed. Interfering peaks were observed at the retention time for some transitions but upon quantification were so low as to be of little significance (Figs. 1–4).

3.2.2. Linearity of the response

The linearity of the chromatographic response was tested with matrix matched curves using six calibration points in the concentration range of 0–10 μg kg^{-1}. The regression coefficients (r^2) for all the calibration curves used in this study were ≥ 0.990.

3.2.3. Accuracy

The recovery of the method was determined using salmon samples fortified at 1.0, 1.5 and 2 μg kg^{-1} for each dye. Mean corrected recovery ($n = 6$) of the analytes, determined in three separate assays shown in Table 3 was between 77 and 113% for MG, LMG, LCV and CV.

3.2.4. Precision

The usefulness of suitable deuterated standards is demonstrated in the excellent repeatability and within-laboratory reproducibility obtained for MG and LMG (Table 3). Although no deuterated analogue is available for LCV, still a very acceptable repeatability and within-laboratory reproducibility is obtained by using the IS of LMG. Less favourable is the situation for CV. The rather high RSD values obtained for both the repeatability and within-laboratory reproducibility of CV (between 20 and 25%), even by applying correction by means of the IS of MG indicates the necessity of the incorporation of a structurally identical isotopically-labelled IS in the method (Table 4).

3.2.5. CC$_\alpha$ and CC$_\beta$

The decision limit (CC$_\alpha$) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an

Table 4
Calculated CC$_\alpha$ and CC$_\beta$ values

	CC$_\alpha$ (μg kg^{-1})	CC$_\beta$ (μg kg^{-1})
MG	0.17	0.30
LMG	0.15	0.35
CV	0.35	0.80
LCV	0.17	0.32