Transfer of cross-contamination levels of coccidiostats, antibiotics and anthelmintics from feed to poultry matrices

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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADI</td>
<td>Acceptable Daily Intake</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, Distribution, Metabolism and Excretion</td>
</tr>
<tr>
<td>ALARA</td>
<td>As Low As Reasonable Achievabe</td>
</tr>
<tr>
<td>BCFI</td>
<td>Belgisch Centrum voor Farmacotherapeutische Informatie</td>
</tr>
<tr>
<td>Car</td>
<td>Carophyll</td>
</tr>
<tr>
<td>conc</td>
<td>concentration</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
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<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
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<tr>
<td>FA</td>
<td>Formic Acid</td>
</tr>
<tr>
<td>FCEC</td>
<td>Food Chain Evaluation Consortium</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>HACCP</td>
<td>Hazard Analysis and Critical Control Points</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophilic Lipophilic Balance</td>
</tr>
<tr>
<td>IB</td>
<td>Infectious Bronchitis</td>
</tr>
<tr>
<td>Koc</td>
<td>Organic Adsorption Coefficient</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid-Chromatography Tandem Mass Spectrometry</td>
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<tr>
<td>Log D</td>
<td>Distribution coefficient</td>
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<tr>
<td>Log P</td>
<td>Partition coefficient</td>
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<tr>
<td>LOD</td>
<td>Limit of Detection</td>
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<tr>
<td>LOQ</td>
<td>Limit of Quantification</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MRL</td>
<td>Maximum Residue Limit</td>
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<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Member state</td>
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<tr>
<td>Nc</td>
<td>Non-compliant</td>
</tr>
<tr>
<td>NCD</td>
<td>Newcastle Disease</td>
</tr>
<tr>
<td>NOEL</td>
<td>No Observed Effect Level</td>
</tr>
<tr>
<td>NSP</td>
<td>Non-Starch Polysaccharide-degrading</td>
</tr>
<tr>
<td>PBPK</td>
<td>Physiologically Based Pharmacokinetics</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated Biphenyl</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PK/PD</td>
<td>Pharmacokinetic-Pharmacodynamic</td>
</tr>
<tr>
<td>pKa</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>RASFF</td>
<td>Rapid Alert System for Food and Feed</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>SF</td>
<td>Safety Factor</td>
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<tr>
<td>SPE</td>
<td>Solid-Phase Extraction</td>
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<tr>
<td>TF</td>
<td>Transfer factor</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>VICH</td>
<td>International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products</td>
</tr>
<tr>
<td>Vit</td>
<td>Vitamin</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoproteins</td>
</tr>
<tr>
<td>VMP</td>
<td>Veterinary Medicinal Product</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Chapter 1: Literature review
1.1. Carry-over, Cross-contamination and Residue Monitoring

During the past decades, European citizens have become increasingly aware of food safety, of food production processes and of the protection of their health by national governments. Some food safety scandals have forced the European Commission into a fundamental rethink about the integrity of the food chain and how it should be regulated, which has manifested itself in the creation of food safety agencies in many member states, the reorganization of the directorates general into the Commission, subsequently the formation of the health and consumer protection directorate, and the setting up of the European Food Safety Authority (EFSA). An integrated “farm to fork” approach is required to ensure the safety of agri-food including the control of microbial contamination of food and the presence of potentially harmful residues of veterinary drugs and other contaminants (McEvoy 2002).

Two main actions should be taken to prevent residues in animal matrices. Firstly, an adequate administration, taking into account the European legislation, and Good Veterinary Practices (GVP) such as respecting the set withdrawal times (Kan and Meijer 2007). Attention needs to be focused on eggs which are produced on a daily base and withdrawal times most often do not apply since only a limited number of veterinary drugs are authorized for use in laying hens compared to other food-producing species (Kan and Meijer 2007). Furthermore, some veterinary drugs may be used off-label under certain conditions following the cascade system (The European Parliament and the Council of the European Union 2001; The European Parliament and the Council of the European Union 2004; BCFI 2011). Secondly, attention is needed to prevent carry-over in the feed mill (production of medicated feed) and at the farm (use of medicated feed) (Kan and Meijer 2007).

1.1.1. Medicated/supplemented feed

A feed or feeding stuff is legally defined as any substance or product, whether processed, partially processed or unprocessed, intended to be used for oral feeding to animals. Feed additives are substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform various functions such as having a coccidiostatic or histomonostatic effect (Borras et al. 2011). A Veterinary Medicinal Product (VMP) is any substance or combination of substances
presented for treating or preventing disease in animals (Food Chain Evaluation Consortium 2010). In Directive 2001/82/EC, a premix for medicated feed is defined as any veterinary medicinal product prepared in advance with a view to the subsequent manufacture of medicated feed (The European Parliament and the Council of the European Union 2001). According to Directive 2001/82/EC, a medicated feed is any mixture of a veterinary medicinal product or products and feed or feeds that is ready prepared for marketing and intended to be fed to animals without further processing, because of its curative, preventive or other properties (The European Parliament and the Council of the European Union 2001). Medicated feeds are a particular kind of feed since they contain relatively high concentrations of pharmacologically active substances, added by veterinary prescription for therapeutic purposes (Borras et al. 2011).

![Production of medicated feed (1000 tonnes) and the percentage of production of medicated feed to compound feed in some EU Member States in 2008](Data from Food Chain Evaluation Consortium or FCEC).

The production of medicated feed varies drastically amongst EU members, with a decrease of production in Germany and the UK, an increase in Belgium while production in France and Spain remained stable over 5 years (Food Chain Evaluation Consortium 2010). In Figure 1.1
the production of medicated feed and the percentage of production of medicated feed to compound feed in 2008 are presented.

The most important VMPs authorized as medicated premixes are antimicrobials with tetracyclines being by far the most commonly used VMP. Other frequently mentioned VMPs are sulfonamides/trimethoprim, macrolides and other antimicrobials. In Belgium, the group of endoparasiticides (including anthelmintics) is the most commonly used VMP for the production of medicated feed (Food Chain Evaluation Consortium 2010). In the European Union, several coccidiostats are authorized as feed additives for the prevention of coccidiosis in various animal species but mainly in chicken. Approximately 18.3 million tonnes of the 40.7 million tonnes produced annually for chickens for fattening, turkeys and rabbits is manufactured with the addition of a coccidiostat (Dorne et al. 2011). The coccidiostats and histomonostats are one of the five categories in which feed additives may be classified. The other categories are technological additives, sensory additives, nutritional additives and zootechnical additives. According to Regulation (EC) No 1831/2003, only additives that have been through an authorisation procedure may be placed on the market (The European Parliament and the Council of the European Union 2003). Authorisations are granted for specific animal species and specific conditions of use. In accordance with article 17 of Regulation (EC) No 1831/2003 on additives for use in animal nutrition, the Commission has established a Community Register of feed additives (The European Commission 2012).

In modern agricultural practice, veterinary drugs and feed additives are generally intentionally administered to animals and an adequate withdrawal time is prescribed (Kan and Meijer 2007; Stolker et al. 2007). Respiratory and enteric diseases in poultry, pigs and calves and mastitis in dairy cattle are the animal diseases requiring the most extensive use of therapeutic and prophylactic drugs (Anadon and Martinez-Larranaga 1999). Since individual animal treatment is from a practical point of view not feasible for poultry, veterinary drugs and feed additives for prevention, therapy and control are mainly administered in the feed or drinking water (Phillips et al. 2004). Therapy is the administration to a group of animals, which exhibit clear symptoms of clinical disease, whereas control refers to the administration to animals in a herd or a flock in which morbidity and/or mortality has exceeded baseline norms. The administration to exposed healthy animals considered to be at risk, but before expected onset of the disease, is called prevention or prophylaxis.
Metaphylaxis is used when there is clinical disease in some animals but all animals are treated (Phillips et al. 2004).

Controls on the use of veterinary drugs, including growth-promoting agents, particularly in food producing animal species has strictly been regulated by the European Union by issuing several Regulations and Directives (Stolker et al. 2007). Since 1998, the EU has prohibited antibiotics used in human medicine from being added to feed (Stolker et al. 2007). Concerns about the development of antimicrobial resistance and about the transfer of antibiotic resistance genes from animals to human microbiota, led to the withdrawal of antibiotics used as growth promoters in the European Union since January 1, 2006 (Castanon 2007). The use of veterinary drugs is regulated through Commission Regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding Maximum Residue Limits in foodstuffs of animal origin (The Commission of the European Communities 2010a). In this Regulation, all pharmacologically active substances are listed in one Annex in alphabetical order. Two separate tables have been established (The Commission of the European Communities 2010a):

Table 1: allowed substances (former annexes I, II, III of Regulation (EEC) No 2377/90)

Table 2: prohibited substances (former annex IV of Regulation (EEC) No 2377/90)

The CVMP applied Council Regulation 1055/2006/EC amending the Annexes I and III of Council Regulation 2377/90/EC to propose MRLs for a number of coccidiostats (EFSA 2007). In Commission Regulation (EC) No 124/2009, the maximum levels are set for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed.

1.1.2. Carry-over and cross-contamination

Almost all mixed feed formulations are prepared in multi product plants in the feed industry. Because different medicated/supplemented feeds have to be manufactured one after the other in the production line, traces of an active ingredient may remain in the production line at the switch-over from one product to the next and get mixed with the first batches of the next product (Segato et al. 2011). Cross-contamination occurs when trace amounts of drugs
or chemicals are incorporated into feeds that should not contain them and feeding these contaminated feed may lead to the presence of residues in the animals consuming them (Borras et al. 2011). It is currently accepted that carry-over seems to be hardly unavoidable under standard production, storage and manipulation conditions (Segato et al. 2011).

The residual amount remaining in the circuit and the degree of cross-contamination depends on technical facilities and procedures (various process parameters) as well as on product characteristics (physicochemical parameters) (EFSA 2007). The technological equipment in the feed mill can influence the level of cross-contamination, while the pharmaceutical properties also affect the degree of cross-contamination (Borras et al. 2011). Different sites in the technological equipment have been identified as places where fractions of the feed may be retained, such as areas with reduced flow, oversized and long conveying systems, silos or containers, mixing equipment and conveying systems, and filters (EFSA 2007). The parameters; high dusting potential, low product moisture, adherence due to electrostatic interactions and environmental conditions influence cross-contamination (EFSA 2007). Since cross-contamination can also occur during the production of the premix, the purchased premix itself can contain traces of contamination of other substances (EFSA 2007).

Carry-over of veterinary drugs or additives from one medicated/supplemented feed batch to the next non-medicated/non-supplemented one can occur during either manufacturing (critical contamination of production lines and storage at feed mills), or during transport to farms or even at the farm (storage, manipulation and mixing operations) (Kan and Meijer 2007; Segato et al. 2011). Critical control points are both in the feed mill and on the farm. Good Manufacturing Practices for feed production as well as Good Agricultural Practices at the farm should ensure that adequate precautions are taken (Kan and Meijer 2007). Prevention of exposure is the preferred risk management tool, several technical steps must be taken by feed operators according to Annex II of Regulation (EC) No 183/2005 and further to the separation of the medicated/supplemented feedstuff production line from the non-medicated/non-supplemented one (Kan and Meijer 2007; Segato et al. 2011). In Annex II of Regulation (EC) No 183/2005, requirements for feed businesses concerning facilities and equipment are laid down. The design, construction and size of the facilities and equipment shall permit adequate cleaning. Furthermore the facilities must be constructed in such a way
that the risk of error can be minimized and contamination, cross-contamination and any adverse effects on the safety and quality of the products can be avoided. Machinery coming into contact with feed shall be dried following any wet cleaning (The European Parliament and the Council of the European Union 2005). Sequential batching (manufacturing the feeds with higher drug levels first and ending with those containing lower levels), flushing and equipment clean-out are three methods commonly used to minimize cross-contamination of feeds (Borras et al. 2011). Sequencing or scheduling of production enables to manage carry-over. For each substance, the company must define the number of batches to be produced between a batch containing a given active substance and a batch for non-target species, for withdrawal feed or for continuous food producing animals. Flushing involves taking a known feed or feed material and moving a quantity through the system to purge feed from a previous batch that remains (FEFAC 2009). Before feed change-over, the feeding equipment needs to be cleaned and inspected (e.g. emptying and visual inspection of feed bins, hoppers and feeding lines) (O’Keeffe et al. 2007). Costs of measures related to management of risk of drug carry-over (cleaning, flush batches, tests for cross-contamination, additional equipment such as conveyors and elevators to reduce the risk of carry-over) are additional costs for manufacturing medicated feed compared to manufacturing compound feed (Food Chain Evaluation Consortium 2010).

1.1.2.1. Consequences of cross-contamination

The accidental administration of feed which has been contaminated with pharmacologically active substances may result in the marketing of products of animal origin, which may contain residues of substances for which a zero level is legally applicable or at concentrations higher than the authorized MRL. The residual concentrations of these substances may have pharmacological and/or microbiological effects (McEvoy 2002; Segato et al. 2011). Residue concentrations in food of animal origin depend on contamination levels in the feed, the chemical substance, the animal species, and the period of feed administration (Segato et al. 2011)

Antibiotic resistance is a microbiological phenomenon, which may or may not have clinical implications which has lead to the banning of several growth promoters as a precaution. In the case of multi-antibiotic resistant salmonellae, macrolide or fluoroquinolone-resistant
campylobacters, glycopeptides- or streptogramin-resistant enterococci and multi-antibiotic resistant *Escherichia coli*, the hypothesis is that the food chain is an important route of transmission (Phillips *et al.* 2004). As all antibacterial substances, coccidiostats may be an indirect risk for human health because their widespread usage could be responsible for the promotion of resistant strains of bacteria (Vincent *et al.* 2011).

Too high concentrations of coccidiostats in non-target feed could harm non-target animal species. These substances may also be of risk for human health since the presence of their residues in food products could cause directly toxic effects in sensitive individuals (Vincent *et al.* 2011). Harmful effects in animals may be seen when compounds have a low margin of safety in the animal species or when an adverse interaction of the contaminant and other medicines occurs (e.g. tiamulin and ionophore toxicity) (McEvoy 2002). Each coccidiostat (ionophoric carbocyclic polyethers and non-ionophoric compounds) has an individual toxicological profile. In general, ionophores (lasalocid, maduramicin, monensin, narasin, salinomycin, semduramicin) are lipophilic chelating agents that transport cations across cell membranes, including the plasma cell membrane and subcellular structures. Major clinical signs in acute intoxications with monensin and other ionophoric compounds are muscle weakness and myocard insufficiency. These symptoms have been observed in animals, but also in humans following accidental ingestion of high doses of monensin (Caldeira *et al.* 2001; Dorne *et al.* 2011). As the non-ionophoric coccidiostats (decoquinate, diclazuril, halofuginone, nicarbazin, robenidine) belong to different chemical classes, their toxicology varies considerably in comparison with ionophoric coccidiostats. The intensive use of most coccidiostats is accompanied by the development of tolerance and reduction in susceptibility of the target population (Anadon *et al.* 2009).

### 1.1.2.2. Legislation

Taking into account the application of Good Manufacturing Practices, the maximum levels of unavoidable carry-over of coccidiostats and histomonostats in non-target feed should be established following the As Low As Reasonably Achievable (ALARA) principle (The Commission of the European Communities 2009a). The EU Legislation established maximum levels for the unavoidable carry-over of coccidiostats and histomonostats (Vincent *et al.* 2011). According to Commission Directive 2009/8/EC, a carry-over rate of approximately 3%
compared to the authorized maximum content should be considered as regards feed for less sensitive non-target animal species. A carry-over rate of approximately 1% compared to the authorized maximum content should be retained for (The Commission of the European Communities 2009a):

- feed intended to sensitive non-target animal species;
- withdrawal feed which is feed used for the period before slaughter;
- cross-contamination of other feed for target species to which no coccidiostats or histomonostats are added;
- non-target feed for continuous food producing animals such as dairy cows or laying hens, where there is evidence of transfer from feed to food of animal origin.

Current feed production practices lead inevitably to carry-over between consecutive production batches. Cross-contamination of the batch following the production of a medicated/supplemented feed is practically unavoidable. Directive 2009/8/EC reflects the situation to some extent and adopts a pragmatic solution. A certain level of contamination is allowed for the coccidiostats and histomonostats, provided that their concentrations remain below an established limit but other veterinary medicinal products such as antibiotics are not yet considered. Therefore, the zero tolerance principle should be applied to these compounds in non-medicated feeds, and this causes major difficulties to the feed industry (Borras et al. 2011).

### 1.1.3. Residue monitoring

Council Directive 96/23/EC contains guidelines for controlling veterinary drug residues in animals and their products with detailed procedures for EU member states, which are required to adopt and implement a national residue monitoring plan for specific groups of residues (Stolker et al. 2007; EFSA 2011). This Directive lays down sampling levels and frequency for bovine, ovine, caprine, porcine, equine animals, poultry, aquaculture animals, milk, eggs, rabbit meat and the meat of wild game and farmed game, and honey as well as the groups of substances to be monitored for each food commodity (The Commission of the European Communities 1996). Additional rules for levels and frequencies of sampling for
milk, eggs, honey, rabbit meat and game are laid down in Commission Decision 97/747/EC (EFSA 2011).

For any type of animal or food, there are 2 main groups that need to be monitored according to Council Directive 96/23/EC: group A, substances having anabolic effect and unauthorized substances (e.g. stilbenes, antithyroid agents, steroids, beta-agonists, ... ) and group B, veterinary drugs and contaminants (Stolker et al. 2007; EFSA 2011). The group of antibacterials (B1) includes antibiotics such as beta-lactams, tetracyclines, macrolides, aminoglycosides but also sulphonamides and quinolones. The group of “other veterinary drugs” (B2) includes a variety of VMPs classified according to their pharmacological action in anthelmintics (B2a), anticoccidials (B2b), car bamates and pyrethroids (B2c), sedatives (B2d), non-steroidal anti-inflammatory drugs (B2e), and other pharmacologically active substances (B2f). The group of “other substances and environmental contaminants” (B3) includes organochlorine compounds including PCBs (B3a), organophosphorous compounds (B3b), chemical elements (B3c), mycotoxins (B3d), dyes (B3e), and others (B3f) (Stolker et al. 2007; EFSA 2011).

1.1.3.1. Methods for residue monitoring

In residue monitoring, banned substances, group A of Council Directive 96/23/EC, should be distinguished from the group B substances of this Directive. For the banned substances, not only the identification of the substances in a large number of matrices, but also in a concentration as low as possible, is important. Thus, qualitative multi-residue methods have to be developed first, followed by quantitative methods. For the substances belonging to the group B of Council Directive 96/23/EC, the emphasis lays on quantitative determination of these substances in edible matrices. For substances belonging to group A and B of Council Directive 96/23/EC, the detection is often not limited to only the parent substance but also one or more metabolites are analyzed (De Brabander et al. 2009).

Methods for surveillance testing of VMPs, belonging to the group B substances, may be subdivided into screening methods and confirmatory methods. Screening methods are used to detect the presence of an analyte or a class of analytes at the level of interest and have the capacity for high sample throughput. These methods are used to sift large numbers of
samples for potential non-compliant results and are especially designed to avoid false compliant results. Confirmatory methods are methods that provide full or complementary information, enabling the analyte to be identified unequivocally. If necessary, the analyte can be quantified at the level of interest. Confirmatory methods must be based on molecular spectrometry, such as GC-MS and LC-MS and must fulfill the criteria listed in Commission Decision 2002/657/EC (De Brabander et al. 2009).

Methods of analysis of antimicrobials can be grouped in different categories: microbiological, immunochemical or physicochemical (De Brabander et al. 2009). Microbiological methods are fast screening methods, which allow a high sample throughput but limited information is obtained about the substance identification and its concentration in the sample. When residues are found in a screening test, a confirmatory test will be carried out. Some EU member states have specific control systems which use microbiological tests (EFSA 2011). Microbiological screening relies on the common property of all antibacterials, being the inhibition of growth of microorganisms (De Brabander et al. 2009). Immunochemical methods include immunoassays and immunoaffinity chromatography. Physico-chemical methods, being more sophisticated and allowing a more accurate identification and quantification of the substance, are based on chromatographic purification/separation of residues followed by spectroscopic quantification such as UV, fluorescence or MS detection (De Brabander et al. 2009; EFSA 2011). A LC-MS/MS instrument allows the user to perform, within the limits of the instruments’ sensitivity, five different kinds of experiments: full scan, product ion scan, precursor ion scan, neutral loss scan and Multiple Reaction Monitoring (MRM). Over 90% of LC-MS/MS methods in clinical use are using MRM mode (Grebe and Singh 2011). Full scan MS technologies offer the advantage of retrospective analysis without re-analysis and makes it possible for hundreds of different substances to be screened in a single analysis (Stolker et al. 2007). Liquid-chromatography, combined with tandem mass spectrometry (triple-quadrupole or ion-trap multi stage) is the preferred technique in a large majority of all VMP classes. Also ultra-performance liquid-chromatography combined with time-of-flight mass spectrometric detection (UPLC/TOF MS) is used (De Brabander et al. 2009). In general, there is increasing interest in methods for the simultaneous analysis of various classes of veterinary drugs. Such multi-residue analyses can deal with more than 100 substances using full-scan MS
techniques. The key of multi-residue methods include sensitive and selective detection (Stolker et al. 2007). Multi-class-methods using LC-MS/MS for the simultaneous determination of 130 veterinary drugs and their metabolites in bovine, porcine and chicken muscle, for antibiotics from different classes in meat, honey, milk, eggs and shrimp have been developed (De Brabander et al. 2009). Multiresidues methods using LC-MS/MS with identification based on MRM transitions are also used for pesticide and mycotoxins analysis (Berthiller et al. 2007; Zhang et al. 2011).

Next to the selection of the method, a selection of type of sample material needs to be made when a monitoring program is set up. For monitoring drugs having a MRL, animal tissues such as muscle, liver, kidney, fat, skin with adhering fat and milk are selected most frequently. Eggs and fish are a second group of matrices of interest for residue monitoring and also honey and animal feed are gaining interest in national monitoring programs. Animal feed is a very complex matrix. Not only the composition differs for each species but starting material also differs for each production batch. This means that each sample of feed has its own characteristics. Sensitive analytical methods to determine pharmacologically active substances in animal feed are needed (Stolker et al. 2007).

**1.1.3.2. Data of residue monitoring**

Although there is relatively little information in literature dealing with animal feed contamination as a specific source of residues in animal products, it is clear that cross-contamination of animal feed may be a problem. McEvoy (2002) mentioned the detection of antimicrobials in 44.1% of the feeds which were considered to be free of medication by the manufacturers. In a survey, 247 medicated feeds were tested and 35.2% contained undeclared antimicrobials of which 23.9% were quantifiable. Chlortetracycline (15.2%), sulphonamides (6.9%), penicillin (3.4%) and ionophores (3.4%) were the most frequently identified contaminating antimicrobials (McEvoy 2002). In the past decade, the Rapid Alert System for Food and Feed (RASSF) reported 43 notifications concerning residues of veterinary medicinal products in animal feeds, most of these referred to the presence of chloramphenicol and to a lesser extent, tetracyclines, sulfadiazine, colistin or tylosin. The RASSF reported 1560 notifications regarding food matrices (Borras et al. 2011).
In Table 1.1, the number of targeted samples analyzed and the percentage of non-compliant samples in the European Union (inhibitor test reported by Germany not included) in 2009 for the groups of antibacterials, anthelmintics and anticoccidials in animal/product categories are presented. Targeted samples are taken with the aim of detecting illegal treatment or controlling compliance with the maximum levels laid down in the relevant legislation. A non-compliant sample is a sample that has been analyzed for the presence of one or more substances and failed to comply with the legal provisions for at least one substance, thus a sample can be non-compliant for one or more results. The total number of analyses carried out in 2009 for antibacterials and other veterinary drugs in targeted samples was 155,432 and 86,466 of which 0.21% and 0.26% were non compliant, respectively. The frequency of analyses for substances in the B2 subgroups followed a different pattern in each species, depending on their animal specific therapeutic application. For poultry, anticoccidials were the largest subgroup (EFSA 2011).
Table 1.2. The number of targeted samples analysed, non-compliant samples and non-compliant results in poultry and in eggs in 2009 for the group group B1 (antibacterials), B2a (anthelmintics), B2b (anticoccidials) (data from EFSA).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Group of substance</th>
<th>n</th>
<th>nc samples</th>
<th>nc results</th>
<th>MS-reporting nc results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry</td>
<td>B1</td>
<td>17942</td>
<td>32</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>B2a</td>
<td>2989</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B2b</td>
<td>6390</td>
<td>131</td>
<td>131</td>
<td>11</td>
</tr>
<tr>
<td>Eggs</td>
<td>B1</td>
<td>4604</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B2a</td>
<td>441</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B2b</td>
<td>3530</td>
<td>43</td>
<td>43</td>
<td>13</td>
</tr>
</tbody>
</table>

n: number of samples analysed, nc: non-compliant, MS: member state

In Table 1.2, the number of targeted samples analysed, non-compliant samples and non-compliant results in poultry and in eggs in 2009 for the antibacterials (B1), anthelmintics (B2a) and anticoccidials (B2b) are presented (EFSA 2011). For poultry matrices, most non-compliant samples and results were noted for antibacterials and anticoccidials, while in eggs, most non-compliant samples and results were obtained for anticoccidials. Mortier et al. (2005) analysed 320 eggs from eight different European countries for the presence of residues of 8 different coccidiostats, namely diclazuril, halofuginone, robenidine, nicarbazin, narasin, salinomycin, lasalocid and monensin, and the nitroimidazole, dimetridazole. Residues of one or more of the nine anticoccidials were detected in 35.6% of the analysed eggs with concentrations ranging from 0.1-63 µg/kg. Salinomycin and lasalocid accounted for more than 60% of all positive samples. In almost 90% of all positive samples, residue concentrations below 2 µg/kg were detected.
1.2. Laying hens

1.2.1. Egg production and consumption

Global egg production has rapidly grown in recent years with an increase from about 20 million tons in 1970 to almost 60 million tons in 2007. Experts from the International Egg Commission expect that the production volume of eggs will be greater than that of beef and veal within a few years. It is estimated that there are 4.93 billion egg-laying hens in the world of which 800-1000 million in China, 279 million in the United States, 380 million in the European Union (27 countries), 133 million in India and 115 million in Mexico. In 2007, around 59 million tons of eggs were produced around the world. The top five egg producing countries are presented in Figure 1.2 and contribute almost 60% to the global volume of egg production. The United States produced 5.3 million tons while the EU produced just over 6.4 million tons with France (14% of output), Germany (12% of output) and Spain (11% of output) being the main producing countries in 2007 (Food and Agriculture Organization of the United Nations 2010).

![Figure 1.2. Share of the main egg-producing countries in global output, 2007 (adapted from Agribusiness Handbook 2010).](image)

From 2000 to 2009, impressive growth in global egg markets has been seen for India (66%), Indonesia (65%), Mexico (33%), Brazil (32%) with the largest volume growth seen in the two largest producers, China (over 4 million tons) and the US (1 million tons). No growth was seen in the EU, with Germany falling from the top 10 of global markets and production in...
France being stagnant (Dreyer and Windhorst 2011). In Figure 1.3 and 1.4, the total egg production (1000 ton) and laying hens population for the production of consumption eggs (1000 animals) in Belgium are presented.

![Graph showing total egg production and laying hens population in Belgium](image)

**Figure 1.3 and 1.4.** Total egg production (1000 ton) (Data from EuroStat) and laying hens population for the production of consumption eggs (1000 animals) in Belgium (Data from VLAM).

The egg industry continues to change and evolves quickly due to an increased demand, technology changes and pressures from consumers and governmental regulators and will continue to grow as the egg offers consumers a source of protein with the least environmental impact (Dreyer and Windhorst 2011). Total egg consumption and the uptake of eggs per capita continue to grow everywhere but this varies widely among countries. The gain reflects an increase in purchase of in-shell eggs in developing countries and an increase in purchase of egg products in developed economies. During recent years, there has been a significant growth in the use of egg products such as liquid/frozen yolk, egg white, and ready-made omelets. EU consumption in most of the EU member states has been fairly stable over the last years. In Belgium, a total of 200 eggs per capita was noted in 2006 and 2007, of which 108 were in-shell eggs and 92 were egg products (Food and Agriculture Organization of the United Nations 2010) and a total of 184 eggs (92 in-shell and 92 products) per capita in 2008 (Evans 2009).

**1.2.2. Formation of the egg**

While a chicken lays an egg roughly every 24 h, each egg takes several days to develop *in vivo*. Egg white, egg yolk and egg shell are the three main components of the egg (Goetting *et al.* 2011). The components of the egg are presented in Figure 1.5. An egg weighing 60
gram consists for 17.4 g (29%) of egg yolk, 36.9 g (61.5%) of egg white and 5.6 g (9.5%) of egg shell (Food and Agriculture Organization of the United Nations 2003). The egg contains proteins (egg white and egg yolk), lipids (egg yolk), vitamins and minerals (Tian et al. 2010).

**Figure 1.5.** Different components of the egg (adapted from Goetting et al. 2011).

Egg white formation occurs in 3 general phases: 1) synthesis and storage of the majority of egg white protein, prior to ovulation; 2) the preplumping phase with secretion of stored proteins and secretion of new proteins during the passage of the ovum (yolk) down to the reproductive tract; and 3) addition of water in the plumping phase (Donoghue and Hairston 2000). The majority of egg white proteins are synthesized and stored in the magnum (Donoghue and Hairston 2000), the majority of the egg white is deposited by secretory cells of epithelial cells and tubular gland cells of the magnum (Furusawa 2002). The stored proteins are secreted around the ovum after ovulation and additionally new egg white proteins are synthesized and secreted onto the ovum. In the isthmus, shell membranes are formed around the ovum. Water is added in the shell gland (or uterus) and egg white weight approximately doubles (Donoghue and Hairston 2000). A large amount of the plumping water is extracted into the egg albumen from the tubular glands of the isthmus and the shell gland (Furusawa 2002). The approximate times spent in the reproductive tract during the different phases of egg white formation are 0.5 h (infundibulum), 3 h (magnum), 1 h (isthmus) and 20 h (shell gland). Ovulation occurs approximately 30 min after oviposition (Donoghue and Hairston 2000). The different phases of egg white production are visualized in Figure 1.6. Formation of egg white proteins takes 1-2 days and deposition of egg white around the yolk some 2-3 hours (Kan and Petz 2000). The major protein of the egg white is ovalbumin (Tian
et al. 2010), which constitutes 54% of the egg white’s total protein. Ovotransferrin is a glycoprotein that represents 13% of the egg white. Ovomucoid is a glycoprotein and ovomucin is a glycosulphiprotein that presents from one to 2% of egg white’s total protein. Lysozyme is a glycoprotein, representing 3.5% of the egg white and ovoglobulins encompass altogether 0.4% of the total egg white’s proteins (Alleoni 2006).

![Figure 1.6. Phases of egg production in the reproductive tract (adapted from Donoghue and Hairston 2000).](image)

Yolk develops over an extended period of time before ovulation and yolks within follicles are in various stages of physiological maturity. The majority of yolk development occurs over a period of 2 weeks, during which the individual yolks increase in size from approximately 0.2 g to a final mature weight of 17 g (Donoghue et al. 1996). Yolk components (predominantly lipoproteins) are formed in the liver and transported through the circulation into yolk follicles in the ovary. The ovary of hens in active production contains several follicles at varying developmental stages (Kan and Petz 2000; Goetting et al. 2011). Follicles may be in the slow phase of development (very small, development may take months or even years), the intermediate phase of growth which may last for 60 days and the rapid growth phase which lasts approximately 10 days. Since one follicle ovulates approximately every 24 hours, roughly 10 follicles are present in different stages of the rapid growth (Kan and Petz 2000). Estrogen secretion at the onset of the egg production in laying hens drastically increases serum levels of protein bound calcium, as well as liver metabolism and its production of total serum neutral lipids, triglycerides, and phospholipids. Furthermore, in mature egg-laying hens, very low density lipoprotein (VLDL) particles are the most predominant lipoprotein, followed by high density lipoprotein (HDL), and then low density lipoprotein. The plasma concentrations of HDL in laying hens are depressed twofold or threefold as compared to
those reported for non-laying hen (Peebles et al. 2004). The yolk precursors, very low density lipoproteins and vitellogenin become massively upregulated at the onset of egg-laying in mature hens. These yolk precursors are synthesized in the liver of the laying hen, secreted in the plasma and transported into the oocyte via receptor-mediated endocytosis (Yamamura et al. 1995; Saarela et al. 2009). After delivery into the oocyte, these lipoproteins are proteolytically processed into several fragments (Yamamura et al. 1995). The chicken oocyte receptor is responsible for the rapid uptake of the major yolk precursors (VLDL and vitellogenin). These yolk precursors bind specifically and with high affinity to the oocyte receptor, localized in coated pits in the plasma membrane of growing oocytes (Hiesberger et al. 1995). Egg yolk is not a homogeneous substance but it is primarily formed by the daily deposition of yolk material in a ring pattern (Donoghue 2001). The major egg yolk proteins and lipoproteins are distributed among two compartments: the yolk plasma and the yolk granular fraction. The yolk plasma contains the water–soluble fraction, which mainly consists of α-livetin (serum albumin), β-livetin (a2-glycoprotein), and the γ-livetin (IgY). The granular (or globular) fraction contains the lipovitellins as components of HDL, phosvitin, and LDL apoproteins (Mann and Mann 2008).

1.2.3. Residues in egg matrices

In Belgium, almost all drugs allowed for use in poultry, have to be administered by the oral route. Most of these pharmacologically active substances are registered for use in broilers (BCFI 2011) and only relatively few drugs are allowed for use in laying hens (Goetting et al. 2011). In order to produce eggs, free from residues or eggs, in which drug residues are below the MRL, it is necessary to administer pharmacologically active substances according to prescribed practices. It is therefore essential to clarify the pharmacokinetics of the target substances in the laying hens and the residual characteristics of these target substances in eggs (Furusawa 2001; Furusawa and Kishida 2002).

Drug residues in eggs may arise when laying hens are erroneously given medicated feed, when feed is contaminated at the mill during mixing, when drugs are administered off-label or illegal or when mislabeled feed is used (Donoghue et al. 1997a; Goetting et al. 2011). Some of these drugs are designed to work systemically so they must cross the intestinal wall to be absorbed and exert their function. Once these substances reach the blood stream,
they can be distributed over the whole body, including the ovary (with the growing follicles) and the oviduct, where formation and secretion of egg white occurs (Kan and Petz 2000). Veterinary drugs from a wide variety of drug classes may be deposited in eggs and residues can persist for some time after treatment has ended (Goetting et al. 2011).

The processes of egg white and egg yolk formation and the disposition in the egg matrices govern the shape of residue curves in eggs (Kan 2003). Residues in egg white are considered to be a reflection of plasma levels. That is why the time needed to achieve a constant level in egg white is generally 2-3 days. Residual concentrations in egg white show a constant level over time when plasma levels do (Kan and Petz 2000). Furthermore, egg whites of the first egg, laid after exposure of the laying hens, may contain residues since pharmacologically active substances may transfer into egg whites during the latter phase of formation, prior to oviposition (Donoghue and Hairston 2000). Residues in yolk reflect the plasma levels during the 10 days of rapid growth and reach a constant level after approximately 8-10 days (Kan and Petz 2000). Drug residues may be incorporated into preovulatory yolk, even when the drug transfer is limited to only 24 h and can be contained in laid eggs for weeks after the withdrawal of the residue (Donoghue et al. 1997b; Donoghue 2001). Incorporation of residues into the egg yolk structure is temporally related to the time of dosing. An egg laid soon after dosing has residues in the outer rings of yolks, whereas an egg laid some time after dosing has residues in the inner rings formed earlier in yolk development (Donoghue 2001).

Disappearance of drugs from the different egg matrices depends heavily on the plasma levels of the involved drugs. Drugs disappear from egg white after 2 or 3 days if the drug clears rapidly from the body, while approximately 10 days are needed for a drug to disappear from the egg yolk (Kan and Petz 2000). Developing, preovulatory egg yolks are an important storage depot for drug residues and many weeks may be required following exposure before eggs are free of drug residues (Donoghue and Myers 2000; Goetting et al. 2011).

In routine analysis, whole eggs are used for residue control. Investigators evaluating residue concentrations in whole eggs may be mislead because of the dilution effect of one edible matrix on the other. Since the edible egg mass consists for approximately two-thirds of egg
albumen, a threefold dilution of drug concentrations in egg yolk by uncontaminated albumen is possible. The dilution effect is especially true for residues deposited in yolk and since many drugs preferentially transfer into egg yolk, this dilution is a real possibility (Donoghue and Hairston 1999).

1.2.3.1. Residues of veterinary medicinal products in whole egg

Numerous studies have been carried out in which the residues in eggs following administration of pharmacologically active substances to laying hens were measured (Yoshida et al. 1973a; Yoshida et al. 1973b; Tomassen et al. 1996a; Tomassen et al. 1996b; Roudaut and Garnier 2002; Kan 2003; Mortier 2005; Hamscher et al. 2006; Lewicki 2006; EFSA 2007; EFSA 2008a; EFSA 2008b; EFSA 2008c; EFSA 2008e; EFSA 2008f; EFSA 2008g; EFSA 2008h; EFSA 2008i). However, only a few authors describe studies in which levels of various pharmacologically active substances in eggs under fixed conditions in similar laying hens are determined (Furusawa 2001). Furusawa investigated the concentrations of twelve veterinary drugs in eggs after feeding these drugs at dietary concentrations of 500 mg/kg to laying hens for 14 days. For 10 drugs, transfer ratios varied from 0.005% to 1.540%. Differences in time required to reach constant values in whole eggs were noted between the different compounds (Furusawa 2001).

In practice, the pattern of residue transfer in eggs will vary depending upon the drug dosing, route of administration, age of hens, rate of egg production, and health of the hens (Donoghue et al. 1996). The quantity of drug transfer and the duration of time that eggs, containing residues are laid, is influenced by the drug’s elimination half-life, the propensity of the drug to transfer into either egg white and egg yolk (physicochemical properties of a drug), and the dynamic nature of egg white and egg yolk formation (Donoghue et al. 1997a). Difference in body accumulation is also an important variable; drugs that are stored in body tissues (fat and liver) and released back into the blood long after the drug withdrawal will enhance daily carry-over accumulation and produce residues in eggs for a longer period than drugs without extended body tissue storage (Donoghue et al. 1996). Some veterinary medicinal products such as macrocyclic lactones are stored in fat, from which they are slowly released, metabolized and excreted (Merck Veterinary Manual 2012).
1.2.3.2. Residues of veterinary medicinal products in egg white and egg yolk

Many drugs deposit preferentially in the yolk or the albumen (Goetting et al. 2011). Appreciable levels in egg white and egg yolk are noted for sulphonamides. For most molecules of this group, levels in egg white were at least equal to those in egg yolk, but often they were much higher. Quinolones showed much higher residues in egg yolk than in egg white. Macrolides and nitrofurans showed a more divergent distribution but levels in egg white were substantial. Within the group of the tetracyclines, higher levels in egg white compared to egg yolk were noted for the very lipophilic ones such as doxycycline and minocycline. Other substances such as trimethoprim, decoquinate and ivermectine showed very low levels in egg white. Residue concentrations of trimethoprim, decoquinate and ivermectine were higher in yolk compared to residue concentrations in egg white (Kan and Petz 2000). In Table 1.3, the distribution preference of various substances available as premix in Belgium are demonstrated. Different studies have been carried out to investigate the distribution in egg white or egg yolk (Donoghue et al. 1996; Donoghue et al. 1997a; Donoghue et al. 1997b; Donoghue and Hairston 2000; Donoghue and Myers 2000). The concentration of substances in the different matrices depends on their physicochemical characteristics, the physiological processes in the hen and the egg formation (Kan and Petz 2000). Kan (2003) investigated the distribution of 11 sulphonamides with variable physicochemical parameters (e.g. pKa range from 4.7 - 11.3) between egg white and egg yolk. These physicochemical parameters included lipid solubility, distribution between the aqueous and organic phase, pKa value and in vivo protein binding in egg white. None of these parameters could predict or explain the distribution between egg white and egg yolk (Kan 2003). Levels in yolk depend on yolk solubility as well as on the affinity of the drug for the lipid phase. Levels in egg white probably depend on circulating plasma levels, on specific binding to proteins in plasma and egg white and the affinity constants of that binding (Kan 2003).
Table 1.3. The distribution preference for egg white and egg yolk of various pharmacologically active substances available as premix in Belgium.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Egg white</th>
<th>Egg yolk</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>chlortetracycline</td>
<td>≈</td>
<td>≈</td>
<td>(Yoshida et al. 1973a)</td>
</tr>
<tr>
<td>decoquinate</td>
<td>x</td>
<td></td>
<td>(EFSA 2008a)</td>
</tr>
<tr>
<td>diclazuril</td>
<td>x</td>
<td></td>
<td>(EFSA 2008b)</td>
</tr>
<tr>
<td>doxycycline</td>
<td>x</td>
<td></td>
<td>(Tomassen et al. 1996a)</td>
</tr>
<tr>
<td>flubendazole</td>
<td>x</td>
<td></td>
<td>(Kan et al. 1998)</td>
</tr>
<tr>
<td>halofuginone</td>
<td>x</td>
<td></td>
<td>(EFSA 2008c)</td>
</tr>
<tr>
<td>ivermectin</td>
<td>x</td>
<td></td>
<td>(Kan and Petz 2000)</td>
</tr>
<tr>
<td>lincomycin</td>
<td>x</td>
<td></td>
<td>(EMEA 1998)</td>
</tr>
<tr>
<td>monensin</td>
<td>≈</td>
<td>≈</td>
<td>(Kan 2003)</td>
</tr>
<tr>
<td>narasin</td>
<td>x</td>
<td></td>
<td>(EFSA 2008f)</td>
</tr>
<tr>
<td>nicarbazin</td>
<td>x</td>
<td></td>
<td>(Mortier 2005)</td>
</tr>
<tr>
<td>oxytetracycline</td>
<td>≈</td>
<td>≈</td>
<td>(Yoshida et al. 1973b)</td>
</tr>
<tr>
<td>robenidine</td>
<td>x</td>
<td></td>
<td>(Mortier 2005)</td>
</tr>
<tr>
<td>salinomycin</td>
<td>x</td>
<td></td>
<td>(EFSA 2008i)</td>
</tr>
<tr>
<td>sulfadiazine</td>
<td>x</td>
<td></td>
<td>(Tomassen et al. 1996b; Atta and El-zeini 2001)</td>
</tr>
<tr>
<td>tiamulin</td>
<td>x</td>
<td></td>
<td>(EMEA 1999c)</td>
</tr>
<tr>
<td>trimethoprim</td>
<td>x</td>
<td></td>
<td>(Nagata et al. 1991; Atta and El-zeini 2001)</td>
</tr>
<tr>
<td>tylosin</td>
<td>≈</td>
<td>≈</td>
<td>(Yoshida et al. 1973c)</td>
</tr>
</tbody>
</table>

1.2.4. Legislation and control systems

According to European legislation (Commission Decision 97/747/EC), each official sample must be taken by official authorities in such a way that it is always possible to trace it back to the origin of the eggs. The samples can be taken either at farm level or at the level of the packing center and the sample size is at least 12 eggs or more, according to the analytical methods used. The number of samples to be taken each year must be at least equal to 1 per 1000 tonnes of the annual production of consumption eggs with a minimum of 200 samples. Each country may decide the breakdown of samples according to the structure of its industry. Packing centers represent the most significant proportion of eggs supplied for
human consumption so at least 30% of the samples must be collected from packing centers. Seventy percent of the samples must be tested for at least one compound from each of the following groups: A6 (compounds included in table 2 of Commission Regulation (EU) No 37/2010), B1 (antibacterial substances including sulphonamides, quinolones) and B2(b) (anticoccidials) mentioned in annex II to Directive 96/23/EC. Thirty percent of the samples must be allocated according to the situation in the individual Member State, but must include some analyses for substances in group B3(a) of Annex I (organochlorine compounds including PCBs) (The Commission of the European Communities 1996; The Commission of the European Communities 1997). Since MRL values are not set for non-target animals (thus a zero tolerance applies) the detection in eggs of any pharmacologically active substance, not licensed for use in laying hens will constitute a violation of legal limits (Kan and Meijer 2007).

In Belgium, the Federal Agency for the Safety of the Food Chain performs controls of eggs and egg products on residues of drugs (prohibited substances, antibiotics, sulphonamides, coccidiostats,...) and contaminants (heavy metals, dioxins, dioxin-like PCBs (polychlorinated biphenyls), PCBs, pesticides, ...) on the layer farms. Eggs from caged hens, barn eggs, free range eggs and organic eggs are analyzed. All of the 495 analyses (2009) and all of the 529 analyses (2010) were compliant. Control was also performed in the processing and distribution units. In 2009, all of the 20 analyses were compliant for dioxins, dioxin-like PCB’s and PCB-markers. In 2010, all of the 106 analyses were compliant for residues of drugs (prohibited substances, antibiotics, sulphonamides, coccidiostats,...) and contaminants (heavy metals, dioxins, dioxin-like PCBs, PCBs, pesticides,...) (Federal Agency for the Safety of the Food Chain 2009; Federal Agency for the Safety of the Food Chain 2010).
1.3. Broilers

1.3.1. Broiler production and consumption

Of all meat sectors, the poultry sector has shown the greatest growth as reflected in world consumption. It is expected that the meat sector as a whole will continue the upward production in the next ten years, driven by world population growth. Poultry meat represented 33% of the global meat production of 282.1 million tons in 2008. Bovine meat and pig meat represented 23% and 37% of the global meat production, respectively. The poultry sector was the most dynamic meat sector during the last decade, which has been supported by a strong growth in demand. The five major poultry meat producers are the United States, China, the EU-27, Brazil and Mexico (Food and Agriculture Organization of the United Nations 2010). These producers represent more than two-third of global poultry meat output and their share in global output is presented in Figure 1.7.

Figure 1.7. Share of the main poultry meat producers in global output (2008) (Adapted from Agribusiness book).

Total meat production increased with 35% from 2000 (69 million tons) to 2008 (94 million tons). The United States are the world’s biggest meat producers. In Figure 1.8, the evolution of poultry meat production by the main producing countries is presented. In Figure 1.9, the evolution of the netto poultry meat production in Belgium is shown. In the EU, little change...
in poultry meat production is anticipated (Food and Agriculture Organization of the United Nations 2010).

Figure 1.8. Evolution of poultry meat production by the main producing countries from 2004-2008 (Data from Agribusiness book).

Figure 1.9. Evolution of the netto poultry meat production (ton carcass weight) in Belgium from 1999-2009 (Data from VLAM).
In 2008, global poultry consumption increased by 4% and China became with 19% of the global consumption the world’s leading poultry meat consuming country. The United States and the EU represented 17% and 12% of the global consumption, respectively. In developing countries, continued economic growth resulted in an increase in protein demand, especially for low-priced food such as poultry. The demand for low-calorie food and changes in lifestyle in developed countries resulted in an increased consumer demand for lean and easy-to-cook meat and thus an increase in poultry meat consumption (Food and Agriculture Organization of the United Nations 2010). In the EU, poultry meat consumption per capita has been stable with a consumption of 24 kg/year. A slight increase in consumption of 2% to 11.5 million tons of poultry meat in 2008 was noted, due to population growth (Food and Agriculture Organization of the United Nations 2010). In Belgium, consumption of poultry meat (kg/capita) increased from 18 in 1991 over 19.3 in 2000 to 22.6 in 2005 (Eurostat).

1.3.2. Residues in broiler matrices

The administration of pharmacologically active substances to food-producing animals may lead to residues in the edible tissues of the treated animals (Goudah 2009). Knowledge about the persistence of a drug as well as its active metabolite(s) in edible tissues is important from a public health viewpoint (Anadon et al. 2008). Tissue distribution studies need to be supplemented with pharmacokinetic studies in order to determine the persistence of residues in animal products (Goudah 2009). The time needed for a pharmacologically active substance to disappear from animal tissue needs to be defined. Thus depletion of drugs from food-producing animals must be assessed to determine when the treated animal can be safely consumed (Anadon et al. 2008).

1.3.2.1. Absorption, distribution, biotransformation and excretion

The disposition of a drug includes the processes of absorption, distribution, metabolism and excretion. Pharmacokinetics describes the time course of the blood and tissue concentration profiles, while pharmacodynamics refers to the relationship between dose and the intended pharmacological response (Drummer 2004). An overview of the pharmacokinetic and pharmacodynamic processes of a drug is presented in Figure 1.10.
Figure 1.10. Overview of the pharmacokinetic and pharmacodynamic processes.

The pharmacokinetic behavior of a substance is mainly determined by its clearance and volume of distribution (Martinez 1998a). The four key pharmacokinetic parameters are bioavailability, clearance, volume of distribution and elimination half-life (Lees 2004).

Absorption of a drug by a living organism is a complex process (Rescigno 1997). Drug absorption is an important process of drug pharmacokinetics and the administration route and pharmaceutical formulation are important factors in determining the absorption rate and extent (Drummer 2004). During the absorption phase, drugs are distributed by the blood to all parts of the body (Drummer 2004). The orally administered drug passes through the intestinal wall and to the liver before it is transported via the blood stream to its target site. The intestinal wall and liver alter (metabolize) many drugs, decreasing the amount of drug that reaches the blood stream (Kopacek 2007). Since drug administration is generally performed by the oral route in poultry, oral bioavailability and absorption are important factors (De Backer 2006). Passive diffusion is by far the most common mechanism for absorption from the gastro-intestinal tract (Drummer 2004). Absorption is possible throughout the whole gastro-intestinal tract, from the stomach to the rectum. As in mammals, drugs are mainly absorbed in the duodenum after oral administration in birds (Vermeulen et al. 2002; De Backer 2006). Bioavailability is defined as the rate and extent to which the active drug ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action (Rescigno 1997). The oral bioavailability of a
drug is expressed as $F$ and represents the fraction of an oral dose which is effectively absorbed into the circulatory system (Daniel 2009).

The uptake of a drug into tissues is a time dependent process and differs between tissues and from drug to drug (Drummer 2004). The distribution of a drug can be described in terms of several discrete body compartments, including the central (or blood) compartment, a peripheral (or systemic) compartment and sometimes a third (or deep) compartment, which is a body space where a drug resides for a prolonged time (Martinez 1998a). The volume of distribution is used as a measure of the extent to which a pharmaceutical compound distributes around the body (Yates and Arundel 2008). The volume of distribution of a compartment is the ratio between the amount of a drug present in the body and its concentration in the compartment (Rescigno 1997). A drug enters the body through the blood compartment after which it is either distributed to the other tissues or is cleared from the system. Shortly after entry into tissues, drugs can be subject to biotransformation as well as excretion (unchanged or as metabolite) from the body (Drummer 2004).

Metabolism is a major determinant of pharmacokinetics and plays a major role in the mode of action of many drugs due to production and removal of metabolites (Clark et al. 2004). Drugs are mostly eliminated by a combination of biotransformation (mainly hepatic) and renal excretion of the parent drug and metabolites (Drummer 2004; De Backer 2006). Drug metabolism is an integral part of elimination, the metabolites of most drugs are more polar (and water soluble) and are therefore more likely to be excreted from the body. Drugs and metabolites are mainly excreted by the kidneys into the urine. However, drugs and metabolites present in the liver are often also excreted into bile, pass into the duodenum and may be reabsorbed or passed into the feces (Drummer 2004). Clearance, the total of elimination processes such as metabolism, renal excretion, and other processes, can be defined as the amount of drug eliminated per unit of time and has the dimension of a flow (Rescigno 1997; Drummer 2004). Clearance is expressed in terms of the volume of blood totally cleared of a substance per unit of time and can be determined in terms of total systemic clearance or in terms of clearance associated with a particular eliminating organ such as the kidney or the liver (Martinez 1998a). Elimination half-life is defined as the interval or time for the concentration of the drug in a compartment to decrease with 50%
Elimination half-life is a function of volume of distribution and clearance (Drummer 2004).

Drugs and metabolites may bind to plasma proteins found in the blood. Albumine and $\alpha_1$-acid glycoprotein are the two major drug-binding proteins but lipoproteins can also have a role, particularly for highly lipophilic drugs. Protein binding is reversible with an equilibrium established between bound and unbound drug but only free drug is available for entering into tissues (Pellegatti et al. 2011). The extent of binding to plasma proteins has a large influence on the pharmacokinetics, pharmacodynamics, and toxicology of a drug in vivo (Wenlock et al. 2011). Variations in binding capacity can affect clearance and volume of distribution (De Backer 2006).

1.3.2.2. Residues of veterinary medicinal products

In Commission Regulation No 37/2010, the marker residue, animal species, MRL and target tissues are described for various pharmacologically active substances. The target tissue, the most appropriate tissue for residue monitoring, is defined as the edible tissue in which the concentration of residues remains above the MRL for the longest period of time compared with other edible tissues (Reyes-Herrera et al. 2008). The target tissues for which an MRL has been set in poultry are muscle, liver, kidney and fat or skin and fat (The Commission of the European Communities 2010b). In Table 1.4, the preferential disposition site of various substances, available as premix in Belgium is noted, with “1” being the tissue with the highest concentrations measured in animal studies and “4” the lowest. The preferential site of disposition for a substance may vary between different animal studies. Fat constitutes a rather stable reservoir because of the relatively low blood flow but drug distribution into fat tissue may be rapid. Lipophilic compound residues preferentially deposit in adipose tissues (MacLachlan 2010).
Table 1.4. Preferential tissue for deposit of various pharmacologically active substances, available as premix in Belgium

<table>
<thead>
<tr>
<th>Substance</th>
<th>Liver</th>
<th>Kidney</th>
<th>Muscle</th>
<th>Skin+fat</th>
<th>Fat</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>apramycin</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td>(Afifi and Ramadan 1997)</td>
</tr>
<tr>
<td>apramycin</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td>(EMEA 1999a)</td>
</tr>
<tr>
<td>apramycin</td>
<td>1</td>
<td>3</td>
<td></td>
<td>2</td>
<td></td>
<td>(EMEA 1999a)</td>
</tr>
<tr>
<td>decoquinate</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td></td>
<td>(EFSA 2008a)</td>
</tr>
<tr>
<td>decoquinate</td>
<td>2</td>
<td>3</td>
<td></td>
<td>1</td>
<td></td>
<td>(EFSA 2008a)</td>
</tr>
<tr>
<td>decoquinate</td>
<td>1</td>
<td>2</td>
<td></td>
<td>3</td>
<td></td>
<td>(EFSA 2008a)</td>
</tr>
<tr>
<td>diclazuril</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td></td>
<td>(EFSA 2008b)</td>
</tr>
<tr>
<td>diclazuril</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>(Mortier et al. 2005)</td>
</tr>
<tr>
<td>doxycycline</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td>(Atif et al. 2002; Ismail and El-Kattan 2009)</td>
</tr>
<tr>
<td>florfenicol</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
<td>(Afifi and ElSooud 1997)</td>
</tr>
<tr>
<td>florfenicol</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td></td>
<td>(EMEA 1999b)</td>
</tr>
<tr>
<td>halofuginone</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td></td>
<td>(EFSA 2008c)</td>
</tr>
<tr>
<td>lasalocid</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td></td>
<td>(EMEA 2004)</td>
</tr>
<tr>
<td>lasalocid</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td></td>
<td>(EMEA 2004)</td>
</tr>
<tr>
<td>lincomycin</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td>(EMEA 1998)</td>
</tr>
<tr>
<td>narasin</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>(EFSA 2008f)</td>
</tr>
<tr>
<td>narasin</td>
<td>2</td>
<td>3</td>
<td></td>
<td>1</td>
<td></td>
<td>(EFSA 2008f)</td>
</tr>
<tr>
<td>maduramycin</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>(EFSA 2008d)</td>
</tr>
<tr>
<td>monensin</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td></td>
<td>(EFSA 2008e)</td>
</tr>
<tr>
<td>monensin</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td></td>
<td>(WHO 2009)</td>
</tr>
<tr>
<td>monensin</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td></td>
<td>(Henri et al. 2009)</td>
</tr>
<tr>
<td>robenidine</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td></td>
<td>(EFSA 2008h)</td>
</tr>
<tr>
<td>salinomycin</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td></td>
<td>(EFSA 2008i)</td>
</tr>
<tr>
<td>salinomycin</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td></td>
<td>(EFSA 2008i)</td>
</tr>
<tr>
<td>semduramicin</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>(EFSA 2008j)</td>
</tr>
<tr>
<td>spectinomycin</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td></td>
<td>(EMEA 2000)</td>
</tr>
<tr>
<td>spectinomycin</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td></td>
<td>(EMEA 2000)</td>
</tr>
<tr>
<td>tiamulin</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
<td>(EMEA 1999c)</td>
</tr>
<tr>
<td>tiamulin</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>(EMEA 1999c)</td>
</tr>
<tr>
<td>tilmicosin</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>(EMEA 1997a)</td>
</tr>
<tr>
<td>trimethoprim</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td></td>
<td>(EMEA 1997b)</td>
</tr>
</tbody>
</table>

1, 2, 3 and 4: residue concentrations in decreasing order. 1: highest residue concentrations are measured in this tissue, 4: lowest residue concentrations are measured in this tissue.

1.3.2.3. Difference between thigh muscle and breast muscle tissue residues

MRLs are the reference points for the control of residues in food of animal origin (Kozarova et al. 2011). However, the type of muscle that should be tested for residue monitoring is not mentioned. A specific edible muscle tissue may be a better indicator of the highest residue.
concentrations that may exceed established MRLs. The preferential muscle disposition site could vary between different molecules (Reyes-Herrera et al. 2005). Higher concentrations in thigh muscle compared to breast muscle were noted for monensin, diclazuril and polychlorinated biphenyl residues, while enrofloxacin concentrations were higher in breast muscle compared to thigh muscle. Possible explanations are the higher blood flow in the thigh or the greater intramuscular fat content of the thigh (Mortier et al. 2005; Reyes-Herrera et al. 2005; Henri et al. 2009).

1.3.3. Legislation and control systems

The European Union has set MRLs to ensure human food safety (Anadon et al. 2008). A MRL is the maximum concentration of residue resulting from the use of a veterinary medicinal product which is permitted in a food product and is expressed in µg/kg. MRLs are the reference points for establishing the withdrawal periods in marketing authorizations for veterinary medicinal products to be used in food-producing animals (Kozarova et al. 2011). The withdrawal period, the period between treatment or last treatment in a multidose regimen and when the animal may be slaughtered for human consumption, is the time necessary to ensure that the residues monitored will fall below the established MRL (Woodward 2005a). MRL violations may arise from failure to observe withdrawal time but there is also a significant contribution from contamination of unmedicated feed with components of medicated feed at feed mills. However in the US, the major factor in the origin of violative residues is the failure to observe withdrawal periods (Woodward 2005b).

In Council Directive 93/23/EC, the minimum number of animals from which samples must be taken are laid down. For broilers, a sample consists of one or more animals depending on the requirements of the analytical methods. The minimum number of samples to be taken each year must at least equal one per 200 tonnes of annual production (dead weight), with a minimum of 100 samples for each group of substances if the annual production is over 5000 tonnes. The following breakdown must be respected: group A (substances having an anabolic effect and unauthorized substances) and group B (veterinary drugs and contaminants): 50% of the total samples. In group B, 30%, 30% and 10% must be checked for group B1 (antibacterial substances, including sulphonamides, quinolones), B2 (other veterinary drugs) and B3 (other substances and environmental contaminants) substances,
respectively (The Commission of the European Communities 1996). In Belgium, samples in the slaughter house are taken from the carcasses for control of residues and contaminants. Poultry samples were analysed for the presence of anabolic substances, forbidden substances, antibiotics, anthelmintics, coccidiostats, pyrethroids, non-steroidal anti-inflammatory drugs, dioxins and PCBs, organochloropesticides, lead and cadmium. For antibiotics, anthelmintics and coccidiostats respectively, 607, 114 and 223 analyses were performed in 2010. For antibiotics and coccidiostats, 99.7% and 97.8% of the analyses resulted in compliant results, respectively (Federal Agency for the Safety of the Food Chain 2010). These results were comparable to those of 2009 (Federal Agency for the Safety of the Food Chain 2009).

1.3.4. Interspecies variation

The drug bioavailability, clearance, and volume of distribution may be influenced by factors such as genetic variability (breed effects), disease/stress, specific physiological conditions (body composition), hepatic and renal function, environment, food, gender and age. Body condition has been found to influence the pharmacokinetic behavior of various drugs, especially the highly lipophilic drugs (Martinez and Modric 2010). Species differences in growth patterns and fat deposition may result in different patterns of residue accumulation. The interplay between elimination half-life and growth rate of the fat depot determines whether or not the concentration of residues continues to increase with age. An increase of the pool of body fat is correlated with a dilution of residues if the source of exposure has been removed (MacLachlan and Bhula 2009). Poultry production has evolved into a separate egg and meat production system with the development of specialized breeds and strains for maximum production efficiency (MacLachlan 2010). The rapid growth of broilers potentially allows dilution of residues after exposure as the lipid component of body weight increases with age (MacLachlan 2008). The transformation from non-laying hens to laying hens results in a dramatic shift in lipid metabolism. In laying hens, egg production is associated with an increased liver activity with approximately 50% of the liver’s daily protein synthesis attributed to vitellogenin production, potentially tripling the amount of protein in circulation (Vezina et al. 2003).
Aims of the study
In modern agricultural practice, veterinary drugs and feed additives are being used on a large scale to prevent or control the outbreak of diseases. In poultry industry, veterinary drugs and feed additives are generally given by mass application through water or in-feed administration. As feed companies may produce a broad range of feeds in the same production line, carry-over of residues of active ingredients from one batch to the following may occur. Consequently, the administration of cross-contaminated feed may result in the presence of residues in food products of poultry origin, including egg (laying hen), muscle and liver (broiler). Although cross-contamination is considered to be unavoidable, little is known about the impact of feeding cross–contaminated feed on residue concentrations in edible poultry matrices. Especially for laying hens, Maximum Residue Limits are only available for a minority of active ingredients.

The general aim of this thesis was to investigate the transfer of representative veterinary drugs and feed additives, belonging to different classes of compounds, from the feed mixed at cross-contamination levels to various poultry matrices. Furthermore, several factors such as various physicochemical parameters and plasma protein binding that may influence the distribution of veterinary drugs and feed additives to matrices of laying hens and broilers were studied.

Therefore, the following specific aims were proposed:

- To determine the residue concentrations of representative veterinary drugs and feed additives in egg matrices (whole egg, egg white and egg yolk) following in-feed administration of these compounds at cross-contamination levels.

- To determine the residue concentrations of representative veterinary drugs and feed additives in broiler matrices (liver, breast muscle and thigh muscle) following in-feed administration of these compounds at cross-contamination levels.

- To develop a predictive mathematical model based on physicochemical, pharmacokinetic and physiological parameters for residues in broiler tissues and egg matrices, applicable to various veterinary drugs and feed additives.
Chapter 2: Animal experiments and LC/MS-MS
2.1. Choice of substances

In this study, six pharmacologically active substances were investigated. The choice to include the ionophoric coccidiostats, lasalocid and monensin, was already made at the start of the project since these compounds are frequently used. For the selection of the other substances, various criteria were used:

- the substances should belong to various pharmacotherapeutic classes, and with different physiochemical parameters and pharmacokinetic properties;
- the frequent use of the substance by in-feed administration in animal husbandry;
- the occurrence of residues of veterinary medicinal products in animal feeds, based on notifications of the Rapid Alert System for Food and Feed;
- the occurrence of non-compliant results of targeted samples in poultry meat and eggs, based on the supporting publications of EFSA;
- the possibility to analyse the substances in one of the cooperating laboratories.

Based on these criteria, the choice was made to investigate also substances belonging to the group of antiparasitic agents and antibacterials. Flubendazole, belonging to the class of benzimidazoles, was the substance of choice in the group of the antiparasitic agents. In the group of the antibacterials, the classes of the sulphonamides, tetracyclines and macrolides were selected. For the sulphonamides, sulfadiazine was chosen as representative pharmacologically active substance. In the class of the tetracyclines; chlortetracycline, oxytetracycline and doxycycline were mentioned as possible candidates. For the macrolides, tylosin and tilmicosin were two possible substances. During the annual meetings of the user committee of the project, these possibilities were discussed and the choice was made to investigate doxycycline and tylosin as representative substances of respectively tetracyclines and macrolides. The chemical structure of the six substances are presented in Figure 2.1. In Table 2.1, the Maximum Residue Limit or maximum level, marker residue and target tissue of the six pharmacologically active substances in poultry matrices are presented.
<table>
<thead>
<tr>
<th>Substance</th>
<th>Marker residue</th>
<th>MRL or max. level (µg/kg)</th>
<th>Target tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>doxycycline</td>
<td>100 muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 skin and fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>600 kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flubendazole</td>
<td>Sum of flubendazole and (2-amino 1H-benzimidazol-5-yl) (4fluorophenyl) methanone flubendazole</td>
<td>50 muscle</td>
<td></td>
<td>Commission Regulation No 37/2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 skin and fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>400 liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>400 eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lasalocid</td>
<td>lasalocid A</td>
<td>20 muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 skin and fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>150 eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monensin</td>
<td>monensin A</td>
<td>8 muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 skin and fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 other food</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>parent drug</td>
<td>100 muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 skin and fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tylosin</td>
<td>tylosin A</td>
<td>100 muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 skin and fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 eggs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1. Chemical structure of the six selected pharmacologically active substances
2.2. Preparation of the experimental feed

The preparation of the experimental feeds was performed at the Animal Sciences Unit of ILVO. For laying hens, 50 kg of each experimental feed was prepared. For broilers, the preparation was done in 2 steps of each 125 kg (sulfadiazine, doxycycline, flubendazole and tylosin) or in 3 steps of each 90 kg (lasalocid and monensin). The animals received a standard feed according to the National Research Council (NRC) recommendations. The feed composition and the nutrient composition of the laying hen feed is described in Table 2.2. The feed and nutrient composition of the broiler feed is described in Table 2.3 and Table 2.4.

Table 2.2. Feed and nutrient composition of laying hen feed

<table>
<thead>
<tr>
<th>Feedstuffs laying hen</th>
<th>%</th>
<th>Nutrient composition laying hen</th>
<th>MJ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow corn</td>
<td>45.00</td>
<td>MEn-layers, MJ/kg</td>
<td>11.88</td>
</tr>
<tr>
<td>Wheat</td>
<td>20.74</td>
<td>C-protein, %</td>
<td>15.5</td>
</tr>
<tr>
<td>Soybean meal (44)</td>
<td>15.79</td>
<td>d-Lys, %</td>
<td>0.71</td>
</tr>
<tr>
<td>Soybeans (toasted and full fat)</td>
<td>6.30</td>
<td>d-S-amino acids, %</td>
<td>0.57</td>
</tr>
<tr>
<td>CaCO₃ pellet form</td>
<td>4.50</td>
<td>d-Thr, %</td>
<td>0.53</td>
</tr>
<tr>
<td>CaCO₃ powder form</td>
<td>3.38</td>
<td>Ca, %</td>
<td>3.65</td>
</tr>
<tr>
<td>Rendered animal fat</td>
<td>1.33</td>
<td>Pav.⁴, %</td>
<td>0.33</td>
</tr>
<tr>
<td>di-Ca phosphate.2H₂O</td>
<td>1.31</td>
<td>Fat, %</td>
<td>4.82</td>
</tr>
<tr>
<td>Vit and trace elements¹ premix</td>
<td>1.00</td>
<td>C 18:2, %</td>
<td>1.79</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.32</td>
<td>Na+K-Cl, meq</td>
<td>177</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.15</td>
<td>Na, %</td>
<td>0.14</td>
</tr>
<tr>
<td>L-lysine.HCl</td>
<td>0.10</td>
<td>Cl, %</td>
<td>0.25</td>
</tr>
<tr>
<td>NSP-enzyme²</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>² (g/100 kg): Carrot, 2.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>³ (g/100 kg): Carrot, 1.80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ (mg/kg diet): vitamin A, 12000 (IE); cholecalciferol, 3000 (IE); vitamin E, 50; vitamin K, 2.5; thiamin, 2.2; choline-Cl, 650; riboflavin, 7.5; pantothenic acid, 38; pyridoxin, 5.5, cyanocobalamin, 0.035; nicotinic acid, 13; biotin, .20; folic acid, 1; ethoxyquin, 35; BHT, 25; I, 2; Co, 1; Se, .4; Cu, 25; Mn, 60; Zn, 70; Fe, 45.

⁴ NSP enz = non-starch polysaccharide-degrading enzyme

⁵ carophyll

⁶ phosphorus available
Table 2.3. Feed composition of broiler feed

<table>
<thead>
<tr>
<th>Feedstuffs broiler</th>
<th>% 1-12 d</th>
<th>% 13-42 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>36.42</td>
<td>47.82</td>
</tr>
<tr>
<td>Yellow corn</td>
<td>20.00</td>
<td>10.50</td>
</tr>
<tr>
<td>Soybean meal (48)</td>
<td>24.06</td>
<td>24.72</td>
</tr>
<tr>
<td>Soybeans (toasted and full fat)</td>
<td>10.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Rendered animal fat</td>
<td>3.83</td>
<td>6.23</td>
</tr>
<tr>
<td>Soy oil</td>
<td>1.50</td>
<td>1.00</td>
</tr>
<tr>
<td>CaCO₃ powder form</td>
<td>0.51</td>
<td>0.70</td>
</tr>
<tr>
<td>di-Ca phosphate.2H₂O</td>
<td>1.41</td>
<td>1.04</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.28</td>
<td>0.29</td>
</tr>
<tr>
<td>R phytase</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>L lysine.HCl</td>
<td>0.33</td>
<td>0.24</td>
</tr>
<tr>
<td>DL methionine</td>
<td>0.27</td>
<td>0.23</td>
</tr>
<tr>
<td>L threonine</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>Nsp enz²</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Vit and trace elements premix¹</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.00</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

¹(mg/kg diet): vitamin A, 12000 (IE); cholecalciferol, 3000 (IE); vitamin E, 50; vitamin K, 2.5; thiamin, 2.2; choline-Cl, 650; riboflavin, 7.5; panthothenic acid, 38; pyridoxin, 5.5, cyanocobalamin, 0.035; nicotinic acid, 13; biotin, .20; folic acid, 1; ethoxyquin, 35; BHT, 25; I, 2; Co, 1; Se, .4; Cu, 25; Mn, 60; Zn, 70; Fe, 45.

²NSP enz = non-starch polysaccharide-degrading enzyme

Table 2.4. Nutrient composition of broiler feed

<table>
<thead>
<tr>
<th>Nutrient composition broiler</th>
<th>MJ % 1-12 d</th>
<th>MJ % 13-42 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEn-broilers, MJ/kg</td>
<td>12.3</td>
<td>12.6</td>
</tr>
<tr>
<td>C protein, %</td>
<td>21</td>
<td>20.4</td>
</tr>
<tr>
<td>d-Lys, %</td>
<td>1.15</td>
<td>1.07</td>
</tr>
<tr>
<td>d-S-amino acids, %</td>
<td>0.8</td>
<td>0.76</td>
</tr>
<tr>
<td>d-Thr, %</td>
<td>0.75</td>
<td>0.7</td>
</tr>
<tr>
<td>Ca, %</td>
<td>0.95</td>
<td>0.88</td>
</tr>
<tr>
<td>Pav., %</td>
<td>0.45</td>
<td>0.39</td>
</tr>
<tr>
<td>C 18:2, %</td>
<td>2.99</td>
<td>2.48</td>
</tr>
</tbody>
</table>

The premixes of lasalocid (Avatec 150G), monensin (Elancoban G200), sulfadiazine (Tucoprim powder), doxycycline (Doxyprex 75% Kela), flubendazole (Flubenol 5% premix) and tylosin (Tylan 250) were kindly provided by Alpharma (Antwerp, Belgium), Elanco Animal Health (Brussels, Belgium), Pfizer (Brussels, Belgium), Kela Veterinaria (Sint-Niklaas,
Belgium), Janssen Pharmaceutica (Beerse, Belgium) and Elanco Animal Health, respectively. The amount of premix needed to reach an experimental feed at 2.5%, 5% and 10% of the therapeutic dose was weighed for each of the active substances. Each experimental feed was prepared in 2 phases. First, the premix was added to a small amount of blank feed in the Varmixer Bear (Figure 2.2A, Dehaeck Construct, Ghent, Belgium). Then, this small mixed amount was added to the remaining blank feed in a custom-made mixer (Figure 2.2B, Silobouw, Zulte, Belgium). This final experimental feed was mixed during 30 min at a speed of 35 rpm with a change of direction every 5 minutes. Three rinsing batches were carried out before the preparation of each experimental feed. Ten dynamic samples of the experimental feed were taken from a moving stream at different stages: 3 samples at the top, 4 samples at the middle and 3 samples at the bottom of each experimental feed. These were analysed to determine the achieved concentration. Furthermore, 2 samples were taken from each of the rinsing batches to exclude cross-contamination during the preparation of the experimental feed.

![Figure 2.2A](image1.png)  ![Figure 2.2B](image2.png)

**Figure 2.2A.** Small mixer, Varmixer Bear and **B:** Large mixer, custom-made mixer

### 2.3. Animal experiments: laying hens

The animal experiment with monensin was performed at CER, in which the laying hens were housed individually. The animal experiments with lasalocid, sulfadiazine, doxycycline, flubendazole and tylosin were performed at the Animal Sciences Unit of ILVO. The laying hens (medium weight ISA-Brown) were vaccinated and arrived at ILVO at the age of 18
weeks. The animals were housed under conventional conditions of ventilation, temperature and lighting (16 hours light/day). The hens had free access to water and feed. The laying hens, in stable egg production, were randomly divided in 3 (lasalocid, monensin and sulfadiazine) or 4 (doxycycline, flubendazole and tylosin) concentration groups. Each group counted 18 laying hens. The animals of the fourth group served as a control group for zootechnical parameters and received blank feed during the whole period (pre-treatment, treatment and depletion period). The animals were housed in 6 tier battery pens of 3 laying hens each (Figure 2.3A, lasalocid and sulfadiazine) or in 2 enriched cages of 9 laying hens each (Figure 2.3B, doxycycline, flubendazole and tylosin). Daily inspections were carried out to monitor general and individual health, feed and water supply, ventilation and a temperature of 20°C. After an adaptation period of 10 days, the laying hens received the experimental feed for 15 days (sulfadiazine) or 14 days (lasalocid, monensin, doxycycline, flubendazole and tylosin), followed by a 17-day depletion period. Eggs were collected daily and egg weight and number of eggs per group were noted. Per group, 10 whole eggs were mixed; the remaining eggs were separated into egg white and egg yolk and these matrices were mixed separately. All samples were stored at –18°C until analysis.

Figure 2.3A. Tier battery pens (3 hens/cage) and B: Enriched cages (9 hens/cage)
Analysis of the whole egg was performed for all concentration groups every 2-days between day 1 to day 13 of treatment period, and daily from day 1 to the last day of depletion period. Residue concentrations have also been measured in both egg white and egg yolk but only for the 10% cross-contamination group and only during expected plateau phase.

Animal experiments and sample analysis are schematically presented in Figure 2.4A and B.

![Diagram](image)

**Figure 2.4A.** Scheme and B. Time line of the animal experiment and sample analysis

### 2.4. Animal experiments: broilers

The monensin experiment was performed at CER, while the animal experiments for lasalsocid, sulfadiazine, doxycycline, flubendazole and tylosin were performed at the Animal Sciences Unit of ILVO.

A growth trial was set-up with 576 1-day-old, male Ross 308 broilers, which were housed in a concrete floor pen under conventional conditions of ventilation, temperature and lighting (16 hours light/day). The animals, randomly assigned to 4 treatments, were distributed among 4 pens and separated from each other by a narrow walkway (Figure 2.5A and 2.5B).
Each group that received 1 of the 3 experimental feeds included 182 chickens, of which 102 were slaughtered. The control group consisted of 30 broilers. A standard 2-phase feeding including a starter diet and grower+finisher diet was used in each study. After an adaptation period of 12 days (pre-treatment), experimental feed was provided to the animals during 14 days, followed by a 17-days depletion period, during which the animals received blank feed. The animals had free access to water and feed. They were vaccinated for Infectious Bronchitis (IB), Newcastle Disease (NCD) and were treated for coccidiosis (by vaccination for the lasalocid and monensin experiments and by in-feed administration of a coccidiostat for the sulfadiazine, doxycycline, flubendazole and tylosin experiments). Each day, the status of the animals and their housing were inspected to determine the animals’ health, feed and water supply, temperature and ventilation, and occurrence of dead animals.

Figure 2.5A and B. Housing of broilers

Starting on day 11 of the pre-treatment period, every 2 days 6 animals per concentration group were weighed and then slaughtered by cervical dislocation. Chickens were weighed individually and samples were taken at day 11 of the pre-treatment period, days 1, 3, 5, 7, 9, 11, 13 of the treatment period, and days 1, 3, 5, 7, 9, 11, 13, 15, 17 of the depletion period. Breast feathers were removed and the complete breast muscles, the upper thigh muscles, and the liver (without the gall-bladder) were sampled. All samples were minced and ground using a Robot Coupe 2 and stored at -18°C until analysis. The samples of breast and thigh muscle and liver were taken for each chicken individually but a pooled sample was used for analysis. The pooled sample was prepared for every tissue and for each sampling day by
mixing 5-10 g of the tissue from each of the 6 chickens. Besides the analyses of the pooled samples, extra analyses were performed. The individual samples collected on day 13 of treatment period were also analysed for each matrix of the 10% concentration group.

Analysis of the breast muscle was performed every 2 days starting from day 1 of the treatment period. Because of the large number of samples, the upper thigh muscles and the liver were only analyzed for the 10% cross-contamination group and only during the expected plateau phase.

Animal experiments and sample analysis are schematically presented in Figure 2.6A and B.

![Scheme and Time line of the animal experiment and sample analysis](image)

**Figure 2.6A.** Scheme and B. Time line of the animal experiment and sample analysis
2.5. Liquid Chromatography-Tandem Mass Spectrometry or LC-MS/MS

2.5.1. Principles of LC-MS/MS

Chromatography is a physical separation method in which the components to be separated are selectively distributed between two immiscible phases: a mobile phase and a stationary phase. The chromatographic process occurs as a result of repeated sorption/desorption steps during the movement of the analytes along the stationary phase. The separation is due to differences in distribution of the analyte between the stationary phase and the mobile phase. Differences in distribution coefficients and retention of the individual analytes in the sample result in separated peaks on the chromatogram. Column chromatography, in which the stationary phase is solid, granular, packed in a column, can be divided according to the nature of the mobile phase. The most important mobile phases are gas (gas chromatography or GC) or liquid (liquid chromatography or LC). Ultra Performance LC (UPLC) makes use of very short columns, with smaller particle diameter, compared to LC. Several chromatographic techniques can be distinguished: adsorption (normal phase and reverse phase), ion exchange and size-exclusion chromatography. In reversed phase chromatography, the mobile phase is a mixture of polar and non-polar solvents, while the stationary phase is apolar. Typical mobile phases are a mixture of water or aqueous buffer with methanol, acetonitrile or tetrahydrofuran (Baars and Crombeen 1995; Mortier 2005; Reyns 2008).

Mass spectrometry is based on the production of ions, which are subsequently separated and filtered according to their mass-to-charge (m/z)-ratio and the detection of these ions. Mass spectrometry, used for quantitative and qualitative analysis is characterized by selectivity, sensitivity and specificity. Different ionisation techniques can be used to generate ions: electron ionisation, chemical ionisation, desorption/ionisation methods (e.g. fast atom bombardment or FAB, matrix assisted laser desorption/ionisation or MALDI) and nebulizing techniques such as thermospray and electrospray ionization. The generated ions can be separated according to their m/z ratio in space or time by different types of analysers: sector mass analyser, quadrupole mass analyser, ion-trap mass analyser, time-of-flight mass analyser and Fourier-transform ion cyclotron resonance mass analyser. A detector is used to transform ions coming from the mass analyser into an appropriate signal, which is recorded
by a computer system and displayed as a mass spectrum. The mass spectrum represents the (relative) intensity of the produced ions as a function of the m/z ratios (Niessen 1997; Mortier 2005; Reyns 2008). The mass spectrometer basically exists of five parts as presented in Figure 2.7.

**Figure 2.7.** Mass spectrometer: sample introduction, ion source, mass analyser, detector, data analysis (adapted from Mortier, 2005).

In this study, the quadrupole mass analyser was used. In a quadrupole instrument, electric fields are used to separate the ions, according to their m/z ratio. A quadrupole mass analyser consists of four hyperbolic or circular rods, placed parallel, equidistant and with electronic connections between them as presented in Figure 2.8. By applying specific voltages to opposing set of poles, a certain mass filter is created (Niessen 1997; Mortier 2005; Reyns 2008).

**Figure 2.8.** Scheme of a quadrupole mass analyser
Tandem mass spectrometry or MS/MS brings in higher specificity and selectivity compared to single MS and requires two mass analysers connected in tandem. The basic approach of tandem MS/MS is the measurement of m/z ratios of ions before and after an induced fragmentation within the mass spectrometer. Fragmentation reactions are induced by energetic collision of the ions, with a gas (e.g. argon or xenon). This process is referred to as collision induced dissociation or CID. The tandem MS/MS instrument, used in this study is the triple quadrupole mass spectrometer, which consists of two scanning quadrupole mass analysers separated by a collision cell. Tandem MS/MS can be used to perform a variety of experiments: a product ion scan, precursor ion scan or neutral loss scan can be recorded or multiple reaction monitoring (MRM) (Figure 2.9) can be performed (Niessen 1997; Mortier 2005; Reyns 2008).

- Multiple reaction monitoring

![Diagram of tandem MS/MS](image)

**Figure 2.9.** Scheme of the multiple reaction monitoring mode (adapted from Mortier, 2005)

To perform quantitative analysis, the tandem MS/MS is used in the selected reaction monitoring mode (SRM). Both mass analysers are set at a selected m/z ratio. The first mass analyser is set to transmit the precursor ion into the collision cell, the second mass analyser monitors its product ions. The transition of several precursor ions each into its product ions can be monitored. SRM plots usually contain a single peak. Sensitivity is increased compared to scanning methods because the mass analysers can focus longer on a specific ion. High specificity is guaranteed since both mass analysers only pass the mass of interest (Niessen 1997; Mortier 2005; Reyns 2008).
2.5.2. Validation of the methods.

The methods, used for the qualitative and quantitative detection of the analyte of interest were making use of LC-MS/MS. Performance criteria for analytical methods are described in Commission Decision No 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

- Linearity

The linearity is determined on matrix-matched calibration curves which are created using at least five (including zero) concentration levels. The coefficient of correlation ($R^2$) is determined.

- Accuracy

Accuracy means the closeness of agreement between a test result and the accepted reference value. It is determined by trueness and precision. Minimum trueness criteria for quantitative methods are presented in Table 2.5.

Table 2.5. Minimum trueness criteria for quantitative methods

<table>
<thead>
<tr>
<th>Mass fraction</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 1 µg/kg</td>
<td>-50% to +20%</td>
</tr>
<tr>
<td>&gt; 1 µg/kg to 10 µg/kg</td>
<td>-30% to +10%</td>
</tr>
<tr>
<td>≥ 10 µg/kg</td>
<td>-20% to +10%</td>
</tr>
</tbody>
</table>

The precision of a method, expressed as the inter-laboratory coefficient of variation (CV) for repeated analysis of a reference or fortified material, under reproducibility conditions, shall not exceed the level calculated by the Horwitz equation.

This equation is $CV = 2^{(1-0.5 \log C)}$ where $C= $ mass fraction expressed as a power (exponent) of 10.

For analyses carried out under repeatability conditions, the intra-laboratory CV would typically be between one half and two third of the above values.
- Decision limit (CCα)

Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant.

- Detection capability (CCβ)

Detection capability (CCβ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β.

- Limit of Quantification (LOQ)

The Limit of Quantification is determined as the lowest concentration for which the method is validated with a trueness and precision that fall within specified ranges.

- Limit of Detection (LOD)

The Limit of Detection is determined as the lowest concentration from which it is possible to deduce the presence of the analyte with statistical certainty, using the criterion of the signal-to-noise ratio (s/n) of 3/1.

In our study, we used fully validated methods for the analysis of monensin (muscle and egg), lasalocid (muscle and egg), doxycycline (muscle), flubendazole (muscle and egg) and tylosin (muscle). For sulfadiazine (muscle and egg), doxycycline (egg) and tylosin (egg), limited validations were performed as the validated methods did not cover these matrices. This limited validation needed to respond to the performance characteristics of linearity and trueness and precision. In Tables 2.6, 2.7, 2.8 and 2.9, various performance parameters of the validated methods for lasalocid, monensin and flubendazole are demonstrated.

**Table 2.6.** CCα (µg/kg) and CCβ (µg/kg) for lasalocid and flubendazole determination in egg and muscle tissue (validated as MRL compound)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Matrix</th>
<th>CCα (µg/kg)</th>
<th>CCβ (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lasalocid</td>
<td>egg</td>
<td>175</td>
<td>200</td>
</tr>
<tr>
<td>Lasalocid</td>
<td>muscle</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>Flubendazole</td>
<td>muscle</td>
<td>54</td>
<td>56</td>
</tr>
<tr>
<td>H-flubendazole</td>
<td>muscle</td>
<td>58</td>
<td>59</td>
</tr>
</tbody>
</table>
Table 2.7. Various performance parameters for lasalocid and flubendazole determination in egg and muscle tissue

<table>
<thead>
<tr>
<th>Substance</th>
<th>Matrix</th>
<th>Performance parameter</th>
<th>0.5MRL</th>
<th>MRL</th>
<th>1.5MRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lasalocid</td>
<td>egg</td>
<td>CV (%) repeatability conditions</td>
<td>3</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV (%) reproducibility conditions</td>
<td>13</td>
<td>22</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trueness (%)</td>
<td>102</td>
<td>101</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>muscle</td>
<td>CV (%) repeatability conditions</td>
<td>12</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV (%) reproducibility conditions</td>
<td>16</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trueness (%)</td>
<td>117</td>
<td>113</td>
<td>117</td>
</tr>
<tr>
<td>Flubendazole</td>
<td>muscle</td>
<td>CV (%) repeatability conditions</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV (%) reproducibility conditions</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trueness (%)</td>
<td>98</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>H-flubendazole</td>
<td>muscle</td>
<td>CV (%) repeatability conditions</td>
<td>7</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV (%) reproducibility conditions</td>
<td>8</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trueness (%)</td>
<td>102</td>
<td>95</td>
<td>105</td>
</tr>
</tbody>
</table>

Table 2.8. CCα (µg/kg) and CCβ (µg/kg) for monensin and flubendazole determination in egg and muscle tissue (validated as forbidden compound)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Matrix</th>
<th>CCα (µg/kg)</th>
<th>CCβ (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin</td>
<td>egg</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Monensin</td>
<td>muscle</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Flubendazole</td>
<td>egg</td>
<td>0.34</td>
<td>0.5</td>
</tr>
<tr>
<td>H-flubendazole</td>
<td>egg</td>
<td>0.30</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2.9. Various performance parameters of monensin and flubendazole determination in egg and muscle tissue

<table>
<thead>
<tr>
<th>Substance</th>
<th>Matrix</th>
<th>Performance parameter</th>
<th>0.5 µg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin</td>
<td>egg</td>
<td>CV (%) repeatability conditions</td>
<td>28</td>
</tr>
<tr>
<td>Monensin</td>
<td>muscle</td>
<td>CV (%) repeatability conditions</td>
<td>13</td>
</tr>
<tr>
<td>Flubendazole</td>
<td>egg</td>
<td>CV (%) repeatability conditions</td>
<td>20</td>
</tr>
<tr>
<td>H-flubendazole</td>
<td>egg</td>
<td>CV (%) repeatability conditions</td>
<td>24</td>
</tr>
</tbody>
</table>
The methods used for detection of tylosin and doxycycline in poultry muscle and liver tissue were based on validated and published methods (Cherlet et al. 2002, 2003). The trueness and precision at the Limit of Quantification for the limited validations are presented in Table 2.10.

Table 2.10. The trueness and repeatability (CV) at the Limit of Quantification (LOQ) for the limited validations

<table>
<thead>
<tr>
<th>Substance</th>
<th>Matrix</th>
<th>LOQ (µg/kg)</th>
<th>Trueness (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>egg</td>
<td>10</td>
<td>86</td>
<td>12</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>egg</td>
<td>2</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>muscle</td>
<td>2</td>
<td>110</td>
<td>11</td>
</tr>
<tr>
<td>Tylosin A</td>
<td>egg</td>
<td>2.5</td>
<td>108</td>
<td>5</td>
</tr>
</tbody>
</table>

The trueness and repeatability (CV) at expected (based on literature data) concentration levels for the limited validations are presented in Table 2.11.

Table 2.11. The trueness and repeatability (CV) at expected concentration levels for the limited validations

<table>
<thead>
<tr>
<th>Substance</th>
<th>Matrix</th>
<th>Expected concentration (µg/kg)</th>
<th>Trueness (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>egg</td>
<td>100</td>
<td>106</td>
<td>13</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>muscle</td>
<td>100</td>
<td>94</td>
<td>9</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>egg</td>
<td>200</td>
<td>110</td>
<td>7</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>muscle</td>
<td>250</td>
<td>104</td>
<td>6</td>
</tr>
<tr>
<td>Tylosin A</td>
<td>egg</td>
<td>50</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>Tylosin A</td>
<td>muscle</td>
<td>5</td>
<td>106</td>
<td>3</td>
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</tbody>
</table>
Chapter 3: Laying hens
3.1. Residues of sulfadiazine and doxycycline in egg matrices due to cross-contamination in the feed of laying hens and the possible correlation with physicochemical, pharmacokinetic and physiological parameters
Based on:

Abstract

In the poultry industry, the widespread use of veterinary drugs such as antimicrobial compounds may lead to the presence of residues in whole eggs, egg white and egg yolk. During this study, laying hens received experimental feed containing sulfadiazine or doxycycline at cross-contamination levels of 2.5%, 5% and 10% of the therapeutic concentration. Since the therapeutic dose is 250 mg/kg for both molecules, cross-contamination concentrations in the feed of 6.25, 12.5 and 25 mg/kg were expected. Whole egg, egg white and egg yolk samples were collected during the treatment and depletion period and were analysed via liquid chromatography-tandem mass spectrometry. For both drugs, a plateau phase was reached within 3-5 days and residue concentrations were detected in all egg matrices. For the 10 % cross-contamination group, residual sulfadiazine concentrations of 208, 299 and 60 µg/kg and residual doxycycline concentrations of 455, 332, 206 µg/kg were detected in whole egg, egg white and egg yolk on day 13 of the treatment period, respectively. Both sulfadiazine and doxycycline had higher concentrations in egg white than in egg yolk but the egg white-egg yolk ratio was higher for sulfadiazine than for doxycycline. As neither drug is allowed in Belgium for use in laying hens, residues may pose food safety concerns.

3.1.1. Introduction

Tetracyclines and sulphonamides have been widely used in poultry industry to control poultry diseases, to treat coccidiosis and various bacterial infections (Yoshimura et al. 1991;
Roudaut and Garnier 2002; Shaik et al. 2004). In Belgium, neither sulfadiazine nor doxycycline are registered for use in laying hens whose eggs are used for human consumption (BCFI 2011). Laying hens may be exposed to veterinary drugs by illegal or extra-label use of drugs, use of feed unintentionally cross-contaminated during feed mixing and the use of mislabeled feed (Donoghue et al. 1997). Although feed containing veterinary drugs is prepared following good manufacturing practices’ guidelines, carry-over of active ingredients from previous formulations cannot be ruled out because almost all mixed feed formulations are prepared in multiproducts plants. Cross-contamination may occur during the whole production process, but also during storage at feed mills, transport to farms or at the farm (Segato et al. 2011).

Most veterinary drugs are designed to work systemically, meaning that they must cross the intestinal wall to exert their function (Kan and Petz 2000). After absorption from the gastrointestinal tract, the drug reaches the blood-stream and is distributed over the whole body, including the ovary with growing follicles and the oviduct where the egg white is formed and secreted (Kan and Petz 2000). This distribution can be quantified by some pharmacokinetic parameters such as volume of distribution, which is largely determined by the physicochemical parameters of the compounds such as plasma protein binding, molecular weight, lipid solubility and pKa (Kan and Petz 2000). Moreover, the presence of residues of veterinary drugs in the egg is dependent on general factors. These factors include the regulation of the drug uptake by the ovary; the formation of the egg, including the dynamic nature of yolk and albumen; pharmacokinetic parameters such as the drug’s elimination half-life and physicochemical parameters such as the propensity of the drug to transfer into either albumen or yolk (Donoghue et al. 1997). Egg white formation occurs in 3 phases: (1) a pre-ovulation phase, where albumen proteins are synthesized and stored; (2) a preplumping phase, where stored proteins are secreted and new proteins are synthesized and secreted during the passage of the ovum down the reproductive tract; and (3) plumping phase, where water is added to the egg (Donoghue and Hairston 2000). The formation of proteins takes 1-2 days and the deposition of egg white around the yolk occurs at 2-3 h after ovulation (Roberts and Brackpool 1994). Yolk components are formed in the liver and transported by the blood. Very low-density lipoproteins and vitellogenin are quantitatively the most important yolk precursors (Stifani et al. 1990; Kan and Petz 2000). The yolk is then
deposited in the ovary, which contains 3 types of follicles. Follicles may be in the slow growth phase (these very small follicles take months or even years to develop), the intermediate growth phase (requiring approximately 60 days to develop) or the rapid growth phase (requiring only 10 days to develop) (Kan and Petz 2000). Donoghue et al. (1997) concluded that preovulatory yolks that incorporate drugs during dosing may be an important storage depot for residues in eggs laid later.

When antimicrobial agents are used in laying hens, eggs laid during and after treatment may contain drug residues (Roudaut and Garnier 2002). Since no Maximum Residue Limits for eggs have been set for most of these drugs, these substances are not allowed to be used for animals producing eggs for human consumption (EMEA1995a; EMEA 1997; BCFI 2011). Some drugs such as chlortetracycline are allowed for use in laying hens (BCFI 2011). Residues in eggs may pose food safety concerns and may affect consumers’ confidence in these matrices (Donoghue et al. 1997). Therefore the aim of this study was to investigate the transfer ratios of sulfadiazine and doxycycline from cross-contamination in the feed to poultry egg matrices including whole egg, egg white and egg yolk.

3.1.2. Material and methods

3.1.2.1. Premix, oral powder, reagents and standards

The premix of sulfadiazine in combination with trimethoprim (Tucoprim powder®) and the doxycycline oral powder (Doxycycline 75 % Kela®) were kindly provided by Pfizer (Brussels, Belgium) and Kela Veterinaria (Sint-Niklaas, Belgium), respectively. Analytical standards of sulfadiazine, sulfachloropyridazine (internal standard) and demethylchlortetracycline (internal standard) were purchased from Sigma (Bornem, Belgium), doxycycline hyclate came from Acros (Geel, Belgium). Acetonitrile (ACN) and methanol originated from Biosolve (Valkenswaard, the Netherlands). The water, HPLC grade, was generated by a Milli-Q Gard 2 system (Millipore, Billerica, MA, USA). The sulfadiazine extract was filtered with a 0.22 µm Millex® GV from Millipore. The solid phase extraction (SPE) columns used for the doxycycline clean-up were Oasis® columns (HLB solid-phase extraction column 60 mg/3 ml, Waters, MA, USA).The egg matrices were homogenized with an Ultra-turrax at 13,500 - 20,500 rpm (Ika-Werke, Yellow line DI25, Staufen, Germany). Amicon filters (30,000 MWCO)
used to determine the protein binding came from Millipore. Trifluoroacetic acid used in plasma protein determination was from Sigma.

3.1.2.2. Preparation of the experimental feed and animal experiments

The feed preparation and the animal experiments were carried out at the Animal Sciences Unit of the Institute for Agricultural and Fisheries Research under approval of the ILVO’s ethical committee (EC no. 2008/87, 2009/109).

The preparation of the experimental feed and the animal experiments are described in chapter 2. Animal experiments and sample analysis are schematically presented in Figure 3.1.

Figure 3.1. Scheme of the animal experiment and sample analysis

Analysis of the whole egg (sulfadiazine and doxycycline) and analysis of egg white and egg yolk (sulfadiazine) was performed for all concentration groups every 2-days between day 1 to day 13 of treatment period, and daily from day 14 of treatment period to the last day of treatment.
depletion period. Doxycycline has also been measured in both egg white and egg yolk but only for the highest cross-contamination group. This analysis was performed from day 5 of the treatment period to day 3 of the depletion period.

3.1.2.3. Extraction and clean-up of feed samples

For sulfadiazine and doxycycline analysis, 5 grams of feed was weighed. For the sulfadiazine analysis, 100 µl of the internal standard sulfachloropyridazine (1 mg/ml) was added to the feed sample and the sample was allowed to stand for 10 minutes. A total of 25 ml of methanol was added, the tube was vortexed, placed on the horizontal shaker for 30 minutes, and centrifuged during 10 minutes at 3000 rpm. A total of 5 ml of the supernatant was transferred into a tube, evaporated under nitrogen to dryness in a water bath at 45°C and redissolved in 10 mL acetonitrile:water (ACN:H₂O, 50:50, v:v). A further dilution of 1:15 was performed in ACN:H₂O (50:50, v:v), followed by filtration through a 0.22 µm Millex®GV. For the analysis of doxycycline, 1 ml of the internal standard demethylchlortetracycline (20 µg/ml) and 25 ml of methanol were added to the feed samples. The samples were vortexed, placed on a rotary shaker for 20 minutes and centrifuged for 10 min at 4000 rpm. A total of 200 µl of the supernatant was transferred in a vial, 800 µl of HPLC water was added and the vial was vortexed.

3.1.2.4. Extraction and clean-up of egg samples

The method of Mortier (2005) was used as basis for the sample preparation for analysis of the sulfadiazine content in the eggs. An egg consists for 65% - 70% of egg white and 30% - 35% of egg yolk. A total of 10 grams of homogenized egg (whole egg, egg white and egg yolk) were weighed in a 50 ml centrifuge tube. A total of 100 µl of the internal standard sulfachloropyridazine (10 µg/ml) was added, the sample was briefly vortexed and was then allowed to stand for 10 min. A total of 10 ml of acetonitrile (ACN) was added, the sample was vortexed for 1 min and then placed in an ultrasonic bath for 5 min. After centrifuging the sample for 10 min at 3000 rpm, the supernatant was transferred into a graduated tube. The eluent was evaporated under nitrogen in a water bath at 45°C until about it reached a volume of 4 ml and filtered through a 0.22 µm Millex®GV. For doxycycline analysis in eggs, the method described by Cherlet et al. (2003) was used. Two grams of homogenized egg were weighed and 50 µl of the internal standard demethylchlortetracycline (10 µg/ml) was
added. The samples were vortexed for 15 s and 10 ml of a 0.1M sodium succinate solution were added. The samples were vortexed during 15 s, rotated for 20 min on a home made apparatus and centrifuged at 4000 rpm for 10 min. The supernatant was collected in a new centrifuge tube and 1 ml of a 20% (w/v) trichloroacetic acid (TCA) solution was added. After homogenization on a vortex mixer and centrifugation (4000 rpm, 10 min), the supernatant was filtered through a Whatman® filter paper (90 mm) followed by a solid-phase clean-up. An Oasis® Hydrophilic Lipophilic Balance column (HLB, 60 mg/3ml, Waters, Brussels, Belgium) was installed on a vacuum-manifold. The columns were preconditioned consecutively with 3 ml of methanol, 3 ml of a 1N HCl solution and 3 ml of water. The egg extracts were allowed to pass slowly through the HLB column, which was washed with 1 ml of water. After drying, the analytes were eluted with 3 ml of methanol and the eluate was evaporated to dryness at 50°C, under a gender stream of nitrogen gas. The dry residue was re-dissolved in 250 µl of a 0.5% (v/v) formic acid solution and vortexed during 15 s. The re-dissolved sample was transferred in an Eppendorf cup, centrifuged at 3000 rpm for 10 min and the supernatant was transferred into a HPLC vial.

3.1.2.5. Plasma protein binding

Blood samples from laying hens that received no sulfadiazine nor doxycycline, were collected in lithium heparin vacuum blood collection tubes, then centrifuged after which the plasma was collected. The determination of the protein binding was performed by calculating the ratio of the free or unbound drug concentration and total drug concentration. Internal standards sulfachloropyridazine and demethylchlortetracycline were used. Samples were spiked at a low and high level (25 µg and 250 µg/ml for sulfadiazine, 400 and 800 µg/ml for doxycycline, 2 samples/level) for the determination of the unbound and the total concentration. The free drug concentration was determined by incubation of the spiked samples and the calibration samples during 1 h at body temperature (41 °C) in a warm water bath to mimic protein binding. After ultracentrifugation using Amicon centrifugal device filters during 2 h at 13,000 rpm, the free fraction (250 µl) was collected. For sulfadiazine total plasma concentration, 500 µl ACN and 200 µl phosphate buffer (pH = 6.8) were added to 500 µl plasma samples, spiked with the internal standard and the calibration standards. The samples were vortexed and ultracentrifuged at 13,000 rpm, the extract was added to a tube.
containing 4 ml of ethyl acetate and was then placed on a rocking table for 20 minutes. After centrifugation at 3000 rpm for 20 minutes, the organic phase was transferred into a tube and was evaporated to dryness. This was re-dissolved in 0.1% acetic acid in HPLC H2O and transferred to a vial. The free plasma concentration was determined using the same method used for the total concentration but with only half of the volume of the reagents.

To determine the free and total plasma concentration of doxycycline, 75 µl (total plasma concentration) or 37.5 µL (free plasma concentration) of trifluoracetic acid (TFA) was added to the samples. After centrifugation during 10 minutes at 13,000 rpm, the supernatant was transferred into a HPLC vial.

The determination of sulfadiazine and doxycycline plasma protein binding was determined by LC-MS/MS. Sulfadiazine analysis was performed on a Zorbax Eclipse plus column (C18, 3.5 µm, 3.0*100 mm, Agilent) and a gradient of HPLC H2O + 0.1% acetic acid and ACN. For sulfadiazine analysis, a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro Ultima® MS instrument was used (m/z 250.9 > 155.8) The Limit of Quantification (LOQ) of the sulfadiazine analysis was 10 µg/ml. For doxycycline, the same conditions were used as for the feed and egg samples with an LOQ of 200 µg/ml.

The final plasma protein binding was calculated in different steps. For each concentration level, the mean of the 2 samples was calculated. The final plasma protein binding was then calculated by the mean of the values for both the low and high concentration level.

3.1.2.6. LC-MS/MS analysis

LC-MS/MS analysis was performed in the same manner as described in Vandenberge et al. (2012). The analysis of sulfadiazine was performed using a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro LC® MS instrument. The analysis was performed on an X-terra® column (C18 5µm, 2.1*150 mm) and a gradient of 0.1% formic acid (FA) in H2O (solvent A) and 0.1% FA in ACN. For doxycycline analysis, a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro Ultima® MS instrument was used. A PRLP-S polymer column (5 µm, 2.1*150 mm) was used and the analysis was performed with an isocratic mobile phase of 70% solvent A (HPLC H2O + 0.5% FA, 0.001 M oxalic acid, 0.5% tetrahydrofuran) and 30% of solvent B (ACN). The sulfadiazine and doxycycline analysis was performed in MRM mode and electrospray positive ion mode.
(sulfadiazine: m/z 250.9 > 107.9, 250.9 > 92.2, 250.9 > 156.1; doxycycline: m/z 462.5 < 428.1). In this study, the LOQ of the method for the feed analysis was 5 mg/kg and 2.5 mg/kg for sulfadiazine and doxycycline, respectively. For the egg matrices, the method LOQ was 2 and 10 µg/kg for sulfadiazine and doxycycline, respectively. The method for sulfadiazine and doxycycline had been fully validated before for honey, and muscle and liver tissue, respectively. For the egg matrices, limited validations were carried out in which the LOQ met the requirements for repeatability and accuracy, as well as the requirements for the linearity ($R^2 > 0.99$ and goodness of fit of the calibrators < 20%).

3.1.2.7. Pharmacokinetic and statistical analysis
Since this study has not been repeated, no statistically obvious conclusions about the influence of dosing regimen on weight and number of eggs can be made. Because of the limited feed dataset, no statistics could be performed to determine the homogeneity of the experimental feed. The terminal elimination half-lives in egg matrices were calculated using pharmacokinetic (PK) functions for Excel and were based on the terminal slope of the tissue concentration-time curve after withdrawal of the experimental feed (Usansky et al., 2011).

3.1.3. Results

3.1.3.1. Zootechnical parameters and experimental feed
No large differences in mean egg weight and mean laying percentage were noted between the different concentration groups for the same period and between the different periods for the same group. In general, performance in this study was high for both molecules as demonstrated by the mean laying rate and relatively favourable feed conversion ratios. A mean laying percentage of 85.6 ± 1.58 % and 88.9 ± 0.88 % and a mean feed conversion of 1.98 ± 0.05 g and 2.03 ± 0.07 g were noted for the sulfadiazine and doxycycline experiment, respectively. For both sulfadiazine and doxycycline the maximum authorised dose is 250 mg/kg, therefore a concentration of 25, 12.5 and 6.25 mg/kg in the feed was expected for respectively the 10%, 5% and 2.5% carry-over groups. A value of 26.8 ± 1.1 (107%), 13.6 ± 0.6 (109%) and 7.4 ± 0.4 (118%) mg/kg was reached for the 10%, 5% and 2.5% group receiving sulfadiazine. For the group receiving doxycycline, values of 25.3 ± 6.2 (101%), 14.8 ± 3.1
(118%) and 5.8 ± 1.3 (93%) mg/kg were observed for respectively the 10%, 5% and 2.5% groups.

3.1.3.2. Residues in egg matrices

Both drugs quickly reached a plateau phase but both showed a rather slow depletion. The sulfadiazine residue concentrations measured in whole egg, egg white, and egg yolk are presented in respectively Figures 3.2 and 3.3.

![Figure 3.2](image-url)

**Figure 3.2.** Residue concentrations (µg/kg) in whole egg for the 3 concentration groups of sulfadiazine during treatment period (day 1 to day 15 included) and depletion period (day 16 to day 32 included).

For whole eggs, the three concentrations reached a plateau phase at day 3 of treatment period. The residue concentrations were below LOQ at day 3 of the depletion period for the 2.5% group and at day 8 for the 5% and 10% group. Linearity between the different groups was noted. Residue concentrations reached a plateau phase in egg white on day 3, although the 10% concentration did not reach a distinct plateau phase. Values below LOQ were noted at day 6, 7 and 9 of the depletion period for respectively the 2.5%, 5% and 10% concentration group. In egg yolk, residue concentrations reached a plateau phase at day 3 of treatment period (2.5% and 5% group) or at day 5 (10% group) and residue concentrations
dropped below the LOQ at day 6, 7 and 8 in depletion period for respectively the 2.5%, 5% and 10% concentration group. The highest residue concentrations were measured in the egg white, being approximately 5 times higher than the residue concentrations in egg yolk.

Figure 3.3. Residue concentrations (µg/kg) in whole egg, egg white and egg yolk for the 10% concentration group of sulfadiazine during the treatment (day 1 to day 15 included) and depletion period (day 16 to day 32 included).

Transfer ratios were calculated as the ratio of the concentration of the veterinary drug in the egg to the concentration in the diet. This study determined the transfer ratio for each of the egg matrices for the 10% concentration group by dividing the measured residue concentration on day 13 of the treatment period by the measured concentration in the feed. For sulfadiazine, transfer ratios were 0.0077, 0.011, and 0.0022 for respectively whole egg, egg white and egg yolk. Elimination half-lives were calculated for the various egg matrices. For whole egg, elimination half-lives were 0.95 and 1.24 days for respectively the 10% and 5% concentration groups. As the residue concentrations dropped quickly below LOQ, no elimination half-life could be calculated for the 2.5% concentration group. Elimination half-lives in egg white for the 2.5%, 5% and 10% concentration groups were respectively 0.90, 0.93 and 1.15 days, these values in egg yolk were respectively 1.38, 1.67 and 1.77 days.
The doxycycline residue concentrations in whole egg for the 3 concentration groups are presented in Figure 3.4. The concentrations measured in the different egg matrices for the 10% concentration group during the plateau phase are presented in Figure 3.5. Although no clear plateau phase was reached for the 10% concentration group, the 2.5% and 5% concentration group reached a plateau phase at day 3 of treatment period. Residue concentrations in whole egg dropped below the LOQ at day 10 of the depletion period for the 2.5% concentration group and at day 11 for the 5% and 10% concentration group.

![Figure 3.4](image)

**Figure 3.4.** Residue concentrations (µg/kg) in whole egg for the 3 concentration groups of doxycycline during the treatment (day 1 to day 14 included) and depletion period (day 15 to day 31 included).

The highest concentrations were measured in the whole egg. Concentrations in egg white were approximately 1.5 times higher in comparison to the egg yolk concentrations. Doxycycline transfer ratios for respectively whole egg, egg white and egg yolk were 0.018, 0.013 and 0.008. Elimination half-lives were only calculated for the whole egg since not enough data were available for the other egg matrices. Elimination half-lives in whole egg were 3.05, 2.07 and 1.8 days for respectively the 2.5%, 5% and 10% doxycycline concentration groups.
Figure 3.5. Residue concentrations (µg/kg) in whole egg, egg yolk and egg white for the 10% concentration group of doxycycline during the treatment (day 9 to day 14 included) and depletion period (day 15 to day 17).

Plasma protein binding in laying hens was determined for sulfadiazine and doxycycline. A plasma protein binding of 57% and 89% was found for respectively sulfadiazine and doxycycline. Some physicochemical and pharmacokinetic parameters, as well as the residue concentrations on day 13 in different egg matrices for both molecules are presented in Table 3.1.

Table 3.1. Various physicochemical, and pharmacokinetic parameters and residue concentrations of sulfadiazine and doxycycline.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sulfadiazine</th>
<th>Doxycycline</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>250.25</td>
<td>444.43</td>
<td>SciFinder</td>
</tr>
<tr>
<td>pKa (most acidic)</td>
<td>6.81</td>
<td>4.50</td>
<td>SciFinder</td>
</tr>
<tr>
<td>pKa (most basic)</td>
<td>1.64</td>
<td>10.84</td>
<td>SciFinder</td>
</tr>
<tr>
<td>pKa</td>
<td>6.36</td>
<td></td>
<td>PHYSPROP</td>
</tr>
<tr>
<td>Log D (pH = 7)</td>
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<td>-0.92</td>
<td>SciFinder</td>
</tr>
<tr>
<td>Log P</td>
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<td>1.777</td>
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</tr>
<tr>
<td></td>
<td>-0.09</td>
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<td>PHYSPROP</td>
</tr>
<tr>
<td></td>
<td>-0.09</td>
<td>-0.02</td>
<td>ALOGPS</td>
</tr>
<tr>
<td></td>
<td>-0.34</td>
<td>-1.36</td>
<td>EPI Suite</td>
</tr>
<tr>
<td>Plasma elimination half-life (h)</td>
<td>3.71</td>
<td>3.64</td>
<td>(Atef et al. 2002; Baert et al. 2003)</td>
</tr>
<tr>
<td>Volume of distribution (L/kg)</td>
<td>0.50</td>
<td>2.77</td>
<td></td>
</tr>
<tr>
<td>Clearance (mL/min per kg)</td>
<td>1.5</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Plasma protein binding (%)</td>
<td>57</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/kg) in egg white at day 13 of treatment period</td>
<td>298.88</td>
<td>332.33</td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/kg) in egg yolk at day 13 of treatment period</td>
<td>60.21</td>
<td>206.05</td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/kg) whole egg at day 13 of treatment period</td>
<td>208.40</td>
<td>455.43</td>
<td></td>
</tr>
</tbody>
</table>

Note: n.d., not determined

Plasma elimination half-life, volume of distribution, and clearance, were pharmacokinetic values, reported in literature for broilers after oral administration. No specific data for laying hens were found. A literature search and consultation of different databases resulted in various experimental and predicted values for several physicochemical parameters, especially for the lipophilicity parameters (ALOGPS, EPI Suite, SciFinder, PHYSPROP).

3.1.4. Discussion

Both molecules reached a plateau phase after 3 days of treatment, except for doxycycline at the 10% cross-contamination level. This may be explained by the affinity of both drugs for egg white. Drug residues appear first in egg white (Donoghue and Hairston 2000), reflect drug plasma levels, and generally reach a constant level in 2-3 days (Kan 2003). In the present experiment, sulfadiazine residue concentrations in egg white reached a plateau phase within 3 days. Generally, an exposure for about 8-10 days is required to reach constant levels of residues in egg yolk (Kan 2003). However, in our study, sulfadiazine reached a plateau phase in yolk within 3-5 days. The highest doxycycline concentrations in egg yolk were found at the last days of treatment, although Yoshimura et al. (1991)
measured maximum concentrations of doxycycline in egg yolk 2 days after withdrawal of the medicated drinking water. The difference in time, needed to reach a plateau phase in either egg white or egg yolk may be explained by the difference in development of egg white and egg yolk at different stages of the egg production. Egg yolk formation takes 8 to 11 days, whereas the formation of egg white takes only approximately 10 h (Hekman and Schefferlie 2011). Egg white is formed in three phases. Although the majority of protein deposited as egg white is probably formed the day prior to secretion, drugs may transfer into egg whites during the latter phase of formation prior to oviposition. This may lead to the accumulation of drug residues in egg white of the first egg laid after drug intake (Donoghue and Hairston 2000). Yolk formation is a dynamic process. Yolks develop in the ovary over a period of months with the latter period of rapid yolk accumulation occurring approximately 2 weeks before ovulation. The transfer of drugs into developing yolk is an important storage reservoir and may lead to the production of contaminated eggs, even if the drug is no longer provided (Donoghue et al. 1997a).

Residue concentrations of sulfadiazine as well as doxycycline were higher in egg white compared to egg yolk. The egg white:egg yolk ratio was highest for sulfadiazine and was approximately 5 compared to 1.5 for doxycycline. Similar ratios have also been found by other researchers (Yoshimura et al. 1991; Tomassen et al. 1996a; Tomassen M.J.H. et al. 1996a; Tomassen et al. 1996b; Tomassen M.J.H. et al. 1996b; Kan 2003). Residue concentrations of doxycycline were highest in whole egg compared to the separate egg matrices. No clear explanation for this unexpected finding can be given. Several hypotheses such as animal experimental set-up, the use of mixtures as sample material and analytical methods (different matrix effects) can be formulated. Several authors have investigated the distributions of different veterinary drugs such as ampicillin, oxytetracycline and gentamicine in either egg white or egg yolk (Yoshimura et al. 1991; Donoghue et al. 1997a; Donoghue and Hairston 1999; Donoghue and Hairston 2000; Roudaut and Garnier 2002; Kan 2003; Filazi et al. 2005). Sulphonamides have appreciable levels in egg white and yolk, egg white levels are at least equal to yolk levels but often they are much higher (Roudaut and Garnier 2002; Kan 2003). The literature mentions that some more lipophilic tetracyclines such as doxycycline show higher levels in egg white than in egg yolk, whereas the more
water soluble oxytetracycline and chlortetracycline show similar levels in egg white and egg yolk (Yoshimura et al. 1991; Kan 2003).

Physicochemical parameters such as molecular weight, lipid solubility, pKa and plasma protein binding determine the drug pharmacokinetics (Martinez 1998b) and are suggested as possible explanations for the distribution between the egg white-egg yolk. Kan (2003) could not explain the distribution of 11 sulphonamides between egg white and egg yolk by some of their physicochemical characteristics. Important physicochemical parameters are molecular weight, lipophilicity, and acid dissociation constant. Since the molecular weight is correlated with the diffusion coefficient, an increase of molecular weight will give rise to a decrease of the compound’s ability to diffuse through any liquid medium (Martinez 1998b).

Log P is a parameter which determines the lipophilicity of the drug (Martinez 1998b) and the lipophilicity influences the deposition of the drug in the fat-rich yolk (Blom 1975). Log P is defined as the logarithm of the partition coefficient between octanol and water for the neutral form of the compound, whereas log D is known as the logarithm of the partition coefficient between octanol and water at a given pH for the mixture of the neutral and ionized forms of a compound. The lipophilicity scale, expressed by the log P value, defines drugs as lipophilic (>1) or hydrophilic (<1) (Grabowski and Jaroszewski 2009). Log P is positively correlated with the volume of distribution and the ability to cross biological membranes (Martinez 1998b). The acid dissociation constant (pKa) of a compound is described as the pH at which equal proportion of the drug exist in its ionized and unionized form (Martinez 1998b). It’s generally accepted that mainly unionized compounds would be able to penetrate biological membranes by passive diffusion (Martinez 1998b; Kan 2003). Plasma protein binding determines the drug’s availability to other compartments as only the free (unbound) drug distributes to the tissues (Martinez 1998; Kan 2003). Since the ratio egg white:egg yolk is 5 for sulfadiazine and 1.5 for doxycycline, we may conclude that doxycycline has a higher affinity for egg yolk compared to sulfadiazine. Doxycycline has a higher molecular weight (444.43) compared to sulfadiazine (250.25) and this would suggest a decreased ability to diffuse through any liquid medium and would result in less affinity for egg yolk, which is opposite to our findings. However, since log P of doxycycline (log P: 1.777) is higher compared to sulfadiazine (log P: -0.074) and doxycycline (log P > 1) is thus defined as being lipophilic; it is expected to have a higher affinity for the fat-rich yolk. Since plasma
protein binding of doxycycline (89%) is higher compared to that of sulfadiazine (57%), there is less free doxycycline in the plasma to distribute to the tissues. Residues in egg white are regarded to be a reflection of the drug plasma level thus a higher affinity of sulfadiazine for the egg white is expected, which is confirmed by our results. Given the large differences noted between the found values of some physicochemical parameters and because only 2 molecules have been investigated in this study, correlations are difficult to find between any of these parameters and the measured residue concentrations. Further research is needed to determine the characteristics (physicochemical and/or pharmacokinetic) that may explain the distribution of veterinary drugs to egg white or yolk. Metabolisation and excretion are important drug disposition processes that may influence the residue concentrations in various matrices. Doxycycline is eliminated by the bile and thus the intestine, while sulphonamides are partly metabolized in the liver and excreted mainly by the kidneys (BCFI, 2011). A possible hypothesis for the relative higher preference of doxycycline in egg yolk compared to sulfadiazine could be that the longer persistence of doxycycline in the liver might be responsible for the occurrence of residues in egg yolk, since the main yolk proteins are already synthesized in the liver.

The slope of the depletion curve in whole egg is different for both molecules as the whole egg elimination half-life of doxycycline is twice that of sulfadiazine. One hypothesis is the relatively higher affinity of doxycycline for egg yolk, compared to that of sulfadiazine. This could be due to higher lipid solubility and/or specific yolk precursor protein binding. For sulfadiazine, the elimination half-life in egg yolk is longer than the elimination half-life in egg white. Disappearance from the different egg matrices of a drug is correlated with the drug plasma levels. Unfortunately, in this study no blood samples were collected to determine plasma concentration. If the drug is cleared rapidly from the body, disappearance from the egg white is noted within 2-3 days after cessation of the drug exposure. Residues disappear from egg yolk in about 10 days (Kan 2003).

Based on the results of this study, we may conclude that the presence of sulfadiazine and doxycycline in feed at cross-contamination levels results in important residue concentrations in the whole egg but also in both egg white and egg yolk. No MRL’s have been set for either molecules in eggs but a MRL of 100 µg/kg was set for all sulphonamides in muscle, fat, liver
and kidney for all food-producing animals. MRL’s of 100 µg/kg, 300 µg/kg and 600 µg/kg have been set in muscle, liver and kidney for chlortetracycline (all food-producing animals), doxycycline (poultry) and oxytetracycline (all food producing animals) (The Commission of the European Communities 2010b), but for chlortetracycline and oxytetracycline, a MRL of 200 µg/kg has also been set in eggs (EMEA 1995a; EMEA 1995b; EMEA 1997). If for sulfadiazine, the MRL of 100 µg/kg should also be applied for egg matrices, a cross-contamination in the feed of 10% would results in exceeding this MRL in the whole egg and the egg white, while 5% cross-contamination would result in residue concentrations exceeding the MRL in the egg white and residue concentrations around the MRL in the whole egg. No residue concentrations of any cross contamination group would exceed the MRL in egg yolk. If for doxycycline a MRL of 200 µg/kg was set in accordance with the other tetracyclines, 10% cross-contamination in the feed would result in exceeding the MRL for whole egg and egg white while yolk residue concentrations would be around the MRL. As neither sulfadiazine, nor doxycycline have been approved for use in laying hens, cross-contamination may result in potential food safety problems. Because of the physiology of the hen and the complexity of the egg formation, residues may appear very quickly and eggs may remain positive for a long time. The egg white may contain residues starting from the first day of drug exposure. The yolk may be responsible for the persistence of residues during several days. This is determined by the affinity of the drug for the egg yolk or egg white. However, no clear explanations for the distribution between the egg matrices can be given. When positive samples are identified, not only the whole egg but also the separate egg matrices need to be analysed, as concentrations between the egg matrices may vary substantially. In order to guarantee consumers safety, this implies that not only the whole egg but also the different egg matrices or at least the preferential egg matrix should be included in routine analysis.
3.2 Transfer of flubendazole and tylosin at cross-contamination levels in the feed to egg matrices and distribution between egg yolk and egg white

Based on:


Abstract

Chemical residues may be present in eggs from laying hens’ exposure to drugs or contaminants. These residues may pose risks to human health. In this study, laying hens received experimental feed containing flubendazole or tylosin at cross-contamination levels of 2.5%, 5% and 10% of the therapeutic dose. Eggs were collected daily and analysis of the whole egg, egg white and egg yolk was performed using liquid chromatography tandem mass spectrometry. Highest concentrations of the parent molecule flubendazole (128.5 µg/kg) as well as the hydrolyzed (33.3 µg/kg) and the reduced metabolite (34.9 µg/kg) were detected in egg yolk. Residue concentrations of the parent molecule were higher compared to those of the metabolites in all egg matrices. No tylosin residue concentrations were detected above the limit of quantification for all concentration groups and in all egg matrices. Neither molecule exceeded the set Maximum Residue Limits.
3.2.1. Introduction

Therapeutic doses of some veterinary drugs given to laying hens may lead to the deposition of drugs and metabolites in the eggs laid. To ensure consumer safety, withdrawal periods have been set and only eggs laid after the withdrawal period may be used for consumption (Hekman and Schefferlie 2011). Residues in eggs may occur from illegal or extra-label use of drugs, the use of unintentionally cross-contaminated feed or the use of mislabeled feed (Donoghue et al. 1997). Regardless of adherence to good manufacturing practice guidelines, contamination of non-medicated feed by residues of antimicrobials or coccidiostats may occur during several steps of the production process. Besides the production lines in the multi-product plants of the feed industry, contamination may also occur during storage at the feed mills, transport to farms and at the farm (Segato et al. 2011).

In Belgium, flubendazole is registered as a premix, oral powder in feed or emulsion in drinking water for use in poultry with a withdrawal time of 0 days in eggs. Tylosin is available as an oral powder administered in drinking water or feed or as premix. Only the formulation in the drinking water is registered for use in laying hens with an egg withdrawal time of 0 days. The other formulations are not allowed for use in laying hens that produce eggs for human consumption (BCFI 2011). Flubendazole, a benzimidazole anthelmintic and tylosin, a macrolide antibiotic have flubendazole and tylosin A as marker residue and a Maximum Residue Limit (MRL) of 400 µg/kg and 200 µg/kg in eggs, respectively (EMEA 2000; EMEA 2006c).

Egg white and egg yolk are formed at different stages in egg production. The development of egg white takes approximately 10 h while 2-3 h is needed for the deposition of egg white around the yolk. The formation of egg yolk takes 8-11 days; yolk components (predominantly lipoproteins) are formed in the liver and transported via the blood to the ovary containing follicles in which the yolk is deposited. The majority of yolk formation occurs during the last 2 weeks prior to ovulation (Hekman and Schefferlie 2011; Donoghue et al. 1997; Kan and Petz 2000). Residue levels in egg white reflect the plasma levels, while residues in yolk reflect the plasma levels during the 10 days of the rapid growth phase of the follicles (Kan and Petz 2000). The concentration of a drug is determined by the compounds pharmacokinetic behavior, which in turn is a reflection of animal physiology (organ
perfusion, renal and hepatic function, membrane permeability, tissue composition and tissue pH) and the drug’s physicochemical properties (Martinez 1998a) such as molecular weight, lipid solubility, and acid dissociation constant, as well as binding to plasma proteins. These physicochemical properties largely determine a drug’s pharmacokinetics. For example, as molecular weight increases, the compound’s ability to diffuse through any liquid medium decreases. The lipophilicity of a drug, expressed as the octanol/water partition coefficient (log P), is positively correlated with the ability to cross biological membranes. The acid dissociation constant (pKa) represents the pH at which an equal proportion of the drug exists in its ionized and unionized forms and theoretically only unionized, non-polar drugs penetrate biological membranes mainly by passive diffusion. Plasma protein binding determines the drug’s availability to other compartments as only the unbound drug molecules are distributed to the tissues (Martinez 1998b; Kan and Petz 2000; Kan 2003).

In this study, we have determined 1) whether providing feed containing flubendazole or tylosin at cross-contamination levels to laying hens leads to the presence of residues in egg matrices thus presenting a risk for food safety, 2) the preference of both molecules for either egg white or egg yolk and the recommended matrix for sampling to ensure food safety and 3) whether testing for metabolites should be included in routine analysis.

### 3.2.2. Material and methods

#### 3.2.2.1. Premix, reagents and standards

The premixes containing flubendazole or tylosin were Flubenol 5% premix® and Tylan 250 vet premix®. These premixes were kindly provided by Janssen Pharmaceutica (Beerse, Belgium) and Elanco Animal Health (Hampshire, England), respectively. Analytical standards of flubendazole, H-flubendazole, R-flubendazole and tylosin A were obtained from Sigma (Bornem, Belgium), Witega (Berlin, Germany), Janssen (Beerse, Belgium), and Biovet (Peshtera, Bulgaria), respectively. The internal standards, D₃ flubendazole and spiramycine, were purchased from Witega and Sigma. K₂CO₃ and HCl 37% for flubendazole analysis and acetic acid 100% for tylosin analysis came from Merck (Darmstadt, Germany). An ultra-turrax at 13,500-20,500 rpm (Ika-werke, Yellow-line DI25, Staufen, Germany) was used to homogenize the egg matrices.
3.2.2.2. Preparation of the experimental feed and animal experiments

The feed was prepared at ILVO’s Animal Sciences Unit. Animal experiments were performed at ILVO’s Animal Sciences Unit following the recommendations for euthanasia of experimental animals and under supervision of ILVO’s ethical committee (EC. No 2010/133 and 2010/139).

The preparation of the experimental feed and the animal experiments are described in chapter 2. Animal experiments and sample analysis are schematically presented in Figure 3.6.

**Figure 3.6.** Scheme of the animal experiment and sample analysis
3.2.2.3. Sample preparation for the experimental feed

Flubendazole and its metabolites and tylosin A were analyzed respectively by the Department Santé of the Centre d’Economie Rurale and at Ghent University’s Department of Pharmacology, Toxicology and Biochemistry of the Faculty of Veterinary Medicine.

For flubendazole analysis, 3 grams of the feed was weighed and the internal standard D₃-flubendazole (30 µl of 100 µg/ml) was added. After the addition of 500 µl of K₂CO₃ 4M in water and 5 ml ethyl acetate, the samples were vortexed during 1 min and placed on a shaker during 30 min. Samples were centrifuged at 4000 rpm during 5 min, the supernatant was transferred into a 15 ml tube, re-extraction was performed with 5 ml ethyl acetate and both supernatants were pooled. One ml was evaporated to dryness at 50°C under nitrogen, 5 ml of hexane was added and samples were vortexed. One ml of an ethanol/HCl 0.2 M solution was added, samples were vortexed during 1 min, which was followed by shaking during 30 min and centrifugation during 5 min at 3000 rpm. The hexane was removed and samples were evaporated to dryness at 50°C under nitrogen. After redissolution with 4 ml HPLC H₂O + 0.1% formic acid, the samples were diluted by adding 100 µl of the extract to a sample vial, containing 900 µl HPLC H₂O + 0.1% formic acid. For the analysis of tylosin A, 20 g of feed was weighed and 1 ml of the internal standard spiramycine (50 µg/ml) was added. The sample was allowed to stand for 10 min, 100 ml of methanol was added, the tube was vortexed and subsequently placed on a horizontal shaker for 20 min. Six ml of the supernatant was transferred in a tube, centrifuged during 10 min at 4000 rpm and 200 µl of the supernatant was transferred in a vial, containing 800 µl of HPLC H₂O.

3.2.2.4. Sample preparation for the egg matrices

For flubendazole analysis, 3 grams of homogenized egg matrices were weighed and internal standard (30 µl of 1 µg/ml) was added. A nearly identical procedure as described for the feed sample preparation was used. The supernatants were pooled and the complete sample instead of 1 ml was evaporated to dryness. After the last evaporation to dryness, extracts were redissolved with 4 ml HPLC H₂O + 0.1% formic acid and placed in a HPLC vial. For tylosin analysis, one gram of whole egg, egg white and egg yolk was weighed. The internal standard spiramycine (100 µl of 10 µg/ml) was added, samples were vortexed and allowed to stand for five minutes. Five ml of methanol was added; samples were vortexed and placed
on a rotary shaker for 20 minutes. After centrifugation (10 minutes, 4000 rpm), the supernatant was transferred into a new tube then 500 µl of a 10% acetic acid solution in HPLC water was added and samples were vortexed. Ten ml of hexane was added, samples were vortexed, placed on a rotary shaker for 10 minutes and then centrifuged for 10 minutes at 4000 rpm. The hexane was removed and the extract was transferred into a new tube, evaporated to dryness and redissolved in 250 µl HPLC H2O. The samples were vortexed and transferred into a cup, which was ultra-centrifuged during 10 minutes at 13,000 rpm. The supernatant was transferred into an HPLC vial.

### 3.2.2.5. Liquid chromatography tandem mass spectrometry analysis

The analysis of flubendazole was performed on a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro Ultima® MS instrument. A Symmetry® column (C18 5 µm, 150 x 2.1 mm) was used. Analysis was performed with a gradient of 0.1% formic acid (FA) in HPLC H2O and 0.1% FA in acetonitrile (ACN). The tylosin A analysis was performed on a similar LC-MS system as for flubendazole analysis. Separation was performed on a PLRP-S polymer column (5 µm, 150 x 2.1 mm). A gradient mobile phase, HPLC H2O + 0.01 M ammonium acetate (pH 3.5) and ACN, was used. The analysis of both molecules was performed in MRM mode and electrospray positive ionization mode. For flubendazole, the parent molecule and the 2 major metabolites, i.e. the reduced and the hydrolyzed forms were detected (flubendazole: m/z 314.4 > 123.4, 314.4 > 282.3; H-flubendazole: m/z 256.4 > 123.4, 256.4 - 95.4; R-flubendazole: m/z 316.2 > 284.2, 316.2 > 97.2). Tylosin A was the marker residue of tylosin (tylosin A: m/z 916.4 > 174.1). In this study, the Limit of Quantification (LOQ) of the analytical method for feed was 250 µg/kg and 1.25 mg/kg for flubendazole and tylosin, respectively. For egg matrices, the method LOQ was 1 and 2.5 µg/kg for flubendazole and tylosin, respectively.

### 3.2.2.6. Pharmacokinetic and statistical analysis

Because the treatments of this study were not repeated, no statistically obvious conclusions about the influence of dosing regimen on weight and number of eggs can be made. Due to the limited dataset, no statistical analysis (correlation factors) could be performed on the residue concentrations and physicochemical and pharmacokinetic parameters. Pharmacokinetic (PK) functions for Excel were used to calculate the terminal elimination
half-life in egg matrices and calculation was based on the terminal slope of the tissue concentration-time curve after withdrawal of the experimental feed (Usansky et al., 2011).

3.2.3. Results

3.2.3.1. Zootechnical parameters

Zootechnical parameters such as egg weight and number of eggs were noted daily. This study occurred only once for each treatment group, therefore no statistical analysis on the available data can be performed. Nevertheless, no large differences in mean egg weight and mean laying percentage were observed between the different concentration groups for the same period and between the different periods for the same group. During this study, performance in general was high for both molecules, which is demonstrated by the mean laying rate and relatively favourable feed conversion ratios. A mean laying percentage of 92.43 ± 1.84% and 89.33 ± 0.56% and a mean feed conversion of 1.97 ± 0.05 g and 1.94 ± 0.06 g were calculated for respectively the flubendazole and tylosin experiment.

3.2.3.2. Experimental feed

The maximum allowed doses for flubendazole and tylosin in feed are 30 mg/kg and 100 mg/kg respectively, thus values of 750, 1,500 and 3,000 μg/kg and 2.5, 5 and values of 10 mg/kg are expected for respectively the flubendazole and tylosine 2.5%, 5% and 10% groups. Since tylosin A counted for 90.9% of the tylosin in the used premix, values of 2.3, 4.5 and 9.09 mg/kg for tylosin A were expected for the 2.5%, 5% and 10% concentration group, respectively. Concentrations of 790 ± 54 (105%), 1504 ± 160 (100%), 2707 ± 126 (90%) μg/kg and 2.04 ± 0.83 (90%), 4.58 ± 2.17 (100%), 9.42 ± 2.63 (103%) mg/kg were reached for respectively flubendazole and tylosin A.

3.2.3.3. Residue concentrations in egg matrices

For tylosin, no residue concentrations were measured above LOQ for all concentration groups in all egg matrices. Because of this, no transfer factors and no elimination half-lives could be determined.

Flubendazole and its metabolites reached a plateau phase rather slowly and also showed a slow depletion. The residue concentrations of flubendazole and both metabolites in whole
egg are presented in respectively Figures 3.7 and 3.8. For flubendazole, the plateau phase was reached at day 9 of treatment period for all concentration groups. Residue concentrations of flubendazole below LOQ were observed at day 8, 9 and 10 of the depletion period for respectively the 2.5%, 5% and 10% concentration groups. For both metabolites, the plateau phase was reached at day 7 for the 10% concentration group and at day 9 for the 2.5% and 5% concentration groups. The residue concentrations of the metabolites dropped below LOQ at day 6 of the depletion period for the 2.5% and 5% concentration group and at day 9 for the 10% concentration group. The 3 groups showed linearity in concentrations reached for the parent molecule as well as for the metabolites in whole eggs. The highest concentrations were measured for the parent molecule and residue concentrations of the hydrolyzed and the reduced metabolites were in the same range, i.e. 20%-25% of the parent molecule.

![Graph](image-url)

**Figure 3.7.** Residue concentrations (µg/kg) in whole egg of flubendazole for 3 concentration groups (2.5%, 5% and 10% concentration group) during the treatment period (day 1 to day 14 included) and the depletion period (day 15 to day 31 included).
Figure 3.8. Residue concentrations (µg/kg) in whole egg of the hydrolyzed metabolite (H-flu) and the reduced metabolite (R-flu) for 3 concentration groups (2.5%, 5% and 10% concentration group) during the treatment period (day 1 to day 14 included) and the depletion period (day 15 to day 31 included).

Transfer factors, calculated by dividing the measured concentration in the 10% group on day 13 of treatment period by the reached feed concentration is determined for the parent molecule as well as for both metabolites for all matrices. Residue concentrations are expected to reach a plateau level on day 13 of treatment period for the different egg matrices. Transfer factors in whole egg of 0.017, 0.0037 and 0.0048 are calculated for respectively the parent molecule, the hydrolyzed metabolite and the reduced metabolite, Elimination half-lives were calculated for the parent molecule and both metabolites. For the parent molecule elimination half-lives of 2.06, 1.58 and 1.44 days were calculated for respectively the 2.5%, 5% and 10% groups. The 2.5%, 5% and 10% group showed elimination half-lives of respectively 3.4, 1.49 and 2.07 days for the hydrolyzed metabolite and respectively 2.77, 1.88 and 2.12 days for the reduced metabolite.

The highest concentrations of flubendazole and its metabolites were detected in egg yolk. The residue concentrations of flubendazole and both metabolites for the 10% concentration
group in the different egg matrices are presented in respectively Figures 3.9 and 3.10. For the parent molecule and the metabolites, residue concentrations were highest in egg yolk followed by the whole egg, with the lowest concentrations measured in the egg white. The residue concentrations of metabolites in egg white of the 2.5% and 5% concentrations groups were below LOQ, while both metabolites in the 10% concentration group and the parent molecule in the 2.5% concentration group were around LOQ. Egg white transfer factors of 0.0018, 0.00037 and 0.00079 and egg yolk transfer factors of 0.047, 0.012 and 0.011 were determined for respectively flubendazole, the hydrolyzed metabolite and the reduced metabolite. On day 14 of the treatment period, the ratios egg yolk/egg white were 25, 30 and 14 for respectively the parent molecule, the hydrolyzed and the reduced metabolite. Flubendazole residue concentrations were higher compared to the residues of the metabolites in both matrices while residues of the metabolites were in the same range.

Figure 3.9. Residue concentrations (µg/kg) in whole egg, egg white and egg yolk of flubendazole for the 10% concentration group during the treatment period (day 5 to day 14 included) and the depletion period (day 15 and day 16).
Figure 3.10. Residue concentrations (µg/kg) in whole egg, egg white and egg yolk of the hydrolyzed metabolite (H-flu) and the reduced metabolite (R-flu) for the 10% concentration group during the treatment period (day 5 to day 14 included) and the depletion period (day 15 and day 16).

3.2.3.4. Physicochemical and pharmacokinetic parameters

In Table 3.2, physicochemical parameters such as molecular weight, lipophilicity parameters and acid dissociation constant (pKa), the pharmacokinetic parameters plasma protein binding and volume of distribution (Lewicki 2006) and the measured concentrations in the different egg matrices for the parent molecule are listed. Different databases have been used which resulted in various (predicted as well as experimental) values for the physicochemical parameters partition coefficient (log P) and pKa (ALOGPS; EPI Suite; PHYSPROP; SciFinder).
Table 3.2. Various physicochemical, pharmacokinetic parameters and residue concentrations of flubendazole and tylosin.

<table>
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<th>Flubendazole</th>
<th>Tylosin</th>
<th>Reference</th>
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<td>SciFinder</td>
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<tr>
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<td>/</td>
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</tr>
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<td></td>
<td>2.91</td>
<td>1.63</td>
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<td></td>
<td>2.91</td>
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<td>/</td>
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<td>&lt; LOQ</td>
<td></td>
</tr>
</tbody>
</table>

3.2.4. Discussion

Flubendazole was the major component in all egg matrices. The plateau phase was reached within 9 days and values dropped below LOQ at 8-10 days once the experimental feed was no longer provided. These findings have been confirmed by Kan et al. (1998), however a transfer ratio of 0.0038 (transfer factor in our study: 0.017) was mentioned in a study in which the feed was only provided for 7 days at a concentration of 60 mg flubendazole/kg feed (EMEA 2006c). Although large differences were observed between residue concentrations of both metabolites in different egg matrices (egg yolk > whole egg > egg white), no large difference in residue concentration was observed between the hydrolyzed and reduced metabolite in each of the egg matrices. This was not confirmed by Kan et al. (1998), who found higher concentrations of the hydrolyzed metabolite compared to the reduced metabolite. Flubendazole is rapidly absorbed in laying hens and there is no evidence of bioaccumulation, but a keto reduction in the liver occurs (EMEA 2006c). Despite of its metabolization in the liver, no differences in disappearance rate were observed between
flubendazole and its metabolites (Kan et al. 1998) and the percentage of residues present as flubendazole remained constant (EMEA 2006) after withdrawal of the feed. No tylosin A concentrations above LOQ could be detected in the whole egg for the 3 concentration groups. Other researchers have also detected very low transfer ratios from the feed to the whole egg (EMEA 2000; Furusawa 2001; Hamscher et al. 2006; Lewicki 2006). Tylosin reached constant values 4 days after the start of experimental feeding (Furusawa 2001), but variations in residue concentrations in individual hen’s eggs have been noticed (Hamscher et al. 2006).

Flubendazole as well as its metabolites are mostly present in the egg yolk, which Kan et al. (1998) also observed. No tylosin A residues above LOQ were measured in egg yolk and in egg white, therefore no conclusions about difference in distribution can be made. Other researchers also determined tylosin residue concentrations in whole egg, egg white and egg yolk (Yoshida et al. 1973; Lewicki 2006). When laying hens received feed containing tylosin at a dose of 8000 g/ton for 7 days, mean residue concentrations in egg white and egg yolk were 2818 and 1820 µg/kg on day 3 of treatment period, 5160 and 5150 µg/kg on day 7 of treatment period and 3930 and 4840 µg/kg on day 1 of depletion period. Residue concentrations were higher in egg yolk than in egg white in 20% of the eggs on day 3 of treatment period and in 60% of the eggs on day 7 of treatment period and on day 1 of depletion period. Residue concentrations in decreasing order were found in egg yolk, whole egg and egg white starting on day 3 of the treatment period when laying hens received tylosin via their drinking water for 5 days (Yoshida et al. 1973; Lewicki 2006). Several authors mentioned differences in distribution of veterinary drugs between egg white and egg yolk and various hypotheses have been suggested (Kan 2003; Lewicki 2006). According to Furusawa, drugs that are lipid soluble are found in a higher concentration in egg yolk than in albumen (Furusawa 2001). A log P value > 1 defines a drug as lipophilic (Grabowski and Jaroszewski 2009). Due to the variation on values for log P provided by different databases, it is difficult to find correlations between the physicochemical parameters of the molecule and the preference of a molecule for egg white or egg yolk. Besides the physicochemical and pharmacokinetic parameters, metabolism and elimination processes may also influence drug residue concentrations. Flubendazole is highly metabolized in the liver and primary metabolism of tylosin also occurs in the liver (Lewicki 2006; EMEA 2006c). Yolk components,
very low density lipoproteins, vitellogenin, vitamin-binding proteins and immunoglobulins are formed in the liver, transported via the blood and are actively and specifically taken up by the growing oocytes (Nimpf and Schneider 1991; Kan 2003). De novo lipogenesis does not occur in the ovary therefore triglyceride storage depends on the availability of plasma lipid substrate. The lipid substrate in the plasma originates from either the diet or lipogenesis in the liver. Commercial poultry diets are usually lipid-poor (less than 10%) therefore the liver plays an important role in lipid provision. Hepatic lipogenesis is dramatically enhanced in laying hens due to the demand for vitellogenesis. Triglycerides are the main products of de novo lipogenesis. However, the liver is also the major site of cholesterol and phospholipids synthesis, which are the main components of lipoproteins in combination with proteins. Lipoproteins are generally classified as either very low density (VLDL) and high density (HDL) lipoproteins. Due to the very limited catabolisation of VLDL in plasma of laying hens, lipids are transported to the oocytes, where VLDL are endocytosed, rather than to other tissues (Hermier 1997). Since flubendazole is highly metabolized in the liver, and the liver is the main source of e.g. VLDL, we hypothesize that this might contribute to the deposition of the drug in the egg yolk.

Some veterinary drugs used in laying hens may result in residues being retained in the eggs. This study demonstrates that the presence of flubendazole in feed of laying hens caused by cross-contamination may lead to detectable levels in egg although no MRL is exceeded. Transfer of tylosin from the feed to the egg matrices was very low. MRLs are set for whole egg, although highest flubendazole as well as tylosin concentrations were measured in the egg yolk. Sampling of the different egg matrices is recommended to ensure food safety. Flubendazole and tylosin A are retained as marker residues and MRLs are set for the whole egg. Given that the parent molecule flubendazole was present in higher concentrations compared to the metabolites, flubendazole must indeed be assigned as the marker residue.
3.3. Transfer of the coccidiostats monensin and lasalocid from feed at cross-contamination levels to whole egg, egg white and egg yolk

Based on:


Abstract

Recent legislation has addressed the unavoidable carry-over of coccidiostats and histomonostats in feed which may lead to the presence of residues of these compounds in eggs. In this study, laying hens received cross-contaminated feed at a ratio of 2.5%, 5% and 10% of the therapeutic dose of monensin and lasalocid for broilers. The eggs were collected during the treatment and depletion period and were analysed using liquid chromatography tandem-mass spectrometry. The different egg matrices were separated and analysed during the plateau phase. High lasalocid concentrations, which exceeded the maximum residue level, and low monensin concentrations were found in whole egg. Plateau levels were reached at day 7-9 for lasalocid and at day 3-5 for monensin. For lasalocid, the highest concentrations were measured in egg yolk while residue concentrations in egg white were very low.
3.3.1. Introduction

Coccidiosis, an important parasitic disease in poultry, is caused by protozoa, more in particular by *Eimeria* spp. It causes high mortality rates and symptoms such as bloody diarrhea, high mortality, reduced feed and water intake, emaciation and loss of egg production. Prevention is more important than treatment as much of the economic loss arises prior to diagnosis (Elliott *et al*. 1998). Coccidiostats are given as the main preventive measure to combat this disease. Only coccidiostats and histomonostats can be used as feed additives within the European Union as a number of antibiotics have been banned as feed additives (Vincent *et al*. 2008). Lasalocid as well as monensin are both ionophoric coccidiostats. They are authorized for use in chickens for fattening, chickens reared for laying and turkeys (EFSA 2007; The Commission of the European Communities 2008b; EFSA 2008e).

Feed manufacturing companies commonly produce a broad range of compound feedingstuffs, many of which are made on the same production line. These practices may result in cross-contamination of feed batches. This product or establishment-related cross-contamination can occur when traces of the first product remain in the production line and contaminate the following batch. However, cross-contamination of residues is regarded to be unavoidable (EFSA 2007). Contamination of animal feedingstuffs may result in animals and their products (meat, milk and eggs) being contaminated with residues of veterinary drugs and coccidiostats (McEvoy 2002). Many veterinary drugs have been shown to transfer into egg, thus the drug residue concentration in the food supply must be constrained (Donoghue and Hairston 2000). In European legislation, 1.25 mg/kg is given as the maximum content of lasalocid sodium and monensin sodium in feed materials due to the unavoidable carry-over in non-target feed (The Commission of the European Communities 2009a). A maximum monensin sodium content of 2 µg/kg wet weight in food of animal origin from animal species other than chickens for fattening, turkeys and bovine (including dairy cattle) has been set (The Commission of the European Communities 2009b) and a Maximum Residue Level (MRL) of 150 µg/kg in eggs is set for lasalocid. Lasalocid A and monensin A have been set as marker residues (EMEA 2006a; The Commission of the European Communities 2007).
Most orally administered veterinary drugs are designed to act systemically, which means they must cross the intestinal wall. Once these compounds reach the blood stream, they are distributed over the whole body, including the ovary with growing follicles and the oviduct. The follicles grow on the ovary and after ovulation, the free ovum is picked by the infundibulum of the oviduct, where the egg white is formed and secreted. Yolk components are formed in the liver, and then transported via the blood to the ovary. The ovary contains 3 types of follicles; very small follicles, follicles in the intermediate growth phase and those in the rapid growth phase (Kan 2003). Egg white formation occurs in 3 general phases. Prior to ovulation, albumen proteins are synthesized and stored. During the preplumping phase, stored proteins are secreted and new proteins are synthesized and secreted during the passage of the ovum in the reproductive tract. Water is then added in the plumping phase (Donoghue and Hairston 2000). Yolk protein precursors, very low density lipoprotein and vitellogenin, are synthesized in the liver, secreted in the plasma and delivered into the oocyte by receptor-mediated endocytosis (Yamamura et al. 1995). Ovalbumin, the major protein in avian egg-white is synthesized by the hen’s oviduct (Huntington and Stein 2001). These ovalbumin molecules might partly be secreted into the blood circulatory system, may be taken up by the ovary and may accumulate in the yolk sphere (Sugimoto et al. 2001).

In this study, we investigated the transfer of 2 coccidiostats (i.e. lasalocid and monensin) to eggs as well as the distribution of both molecules in the different egg matrices. Based on these results, we determined the transfer ratio from feed to eggs and concluded whether this transfer to the egg poses food safety concerns for both molecules. We have also determined the preferential site of deposition of both molecules and evaluated the importance of each matrix.

3.3.2. Material and methods

3.3.2.1. Premixes, reagents and standards

Premixes for lasalocid and monensin are registered for use in chickens for fattening, chickens reared for laying, and turkeys. The authorized maximum lasalocid content in complete feed is 125 mg/kg for all target animals, while a maximum monensin content of 125 mg/kg, 120 mg/kg and 100 mg/kg in the complete feed is authorized for chickens for fattening, chickens
reared for laying, and turkeys, respectively (EFSA 2007; EFSA 2008e). Premixes of lasalocid (Avatec 150 G) and monensin (Elancoban G200) were kindly provided by Alpharma and Elanco, respectively. Analytical standards of lasalocid and monensin were obtained from respectively Alpharma and Sigma (Bornem, Belgium). The internal standard nigericine was obtained from Sigma. Acetonitrile (ACN) originated from Biosolve (Valkenswaard, the Netherlands). HPLC-grade water was generated by a Milli-Q Gard 2 system. The 0.22 µm Millex® GV filters used to filtrate the lasalocid extract were obtained from Millipore (Billerica, MA, USA). HPLC water and Na₂CO₃ for monensin extraction came from Acros (Geel, Belgium) and VWR (Leuven, Belgium), respectively. Homogenization of the egg matrices was performed with an Ultra-turrax operating at 13,500-20,500 rpm (Ika-Werke, Yellow line DI25, Staufen, Germany).

3.3.2.2. Preparation of the experimental feed and animal experiments

The preparation of the experimental feed and the animal experiments are described in chapter 2. Animal experiments and sample analysis are schematically presented in Figure 3.11.

![Figure 3.11. Scheme of the animal experiment and sample analysis](image-url)
3.3.2.3. Extraction and clean-up of feed samples

Centre d’Economie Rurale performed the analysis of lasalocid and monensin in feed samples. Ten grams of feed were weighed and, the internal standard nigericine (50 µl of 1 mg/ml) was added. The samples were allowed to stand for 10 min to equilibrate. Fifteen milliliters of Na₂CO₃ (10% w/v) was added and the samples were shaken manually, followed by addition of 20 ml of acetonitrile. The samples were placed on a horizontal shaker for 20 min, followed by sonication for 10 min. After centrifugation (5 min, 2000 rpm), the supernatant was transferred into a new tube and re-extraction was performed 4 times. This resulted in 100 ml supernatant. One milliliter of the supernatant was added to 3 ml of acetonitrile and 1 ml H₂O + 0.1% formic acid (FA). One milliliter was vortexed and placed in a HPLC vial.

3.3.2.4. Extraction and clean-up of egg samples

The lasalocid analysis was performed at the ILVO’s Technology and Food Science Unit. Ten grams of each egg matrix was weighed. The internal standard nigericine (100 µl of 10 µg/ml, 150 µl of 1 µg/ml, and 50 µl of 100 µg/ml for whole egg, egg white and egg yolk, respectively) was added, the samples were briefly vortexed and they were allowed to stand for 10 min. Ten milliliters of acetonitrile was added, the samples were vortexed for 1 min and were placed in an ultrasonic bath during 5 min. After centrifugation (10 min, 3000 rpm), the supernatant was transferred into a graduated tube, evaporated under nitrogen to 4 ml in a warm water bath of 60°C and filtered through a 0.22 µm Millex® GV. The egg yolk samples were diluted by adding 100 µl of the extract to 900 µl ACN:H₂O (90:10,v:v) in a HPLC vial.

The monensin analysis was performed at the Centre d’Economie Rurale, 5 grams of egg matrices were weighed, the internal standard nigericine (50 µl of 1 µg/ml) was added and the samples were allowed to stand for 10 min. Ten milliliters of acetonitrile were added and the samples were placed on the horizontal shaker for 30 min. After centrifugation (5 min, 2000 rpm), the supernatant was transferred and re-extraction was performed. The extracts were pooled and were evaporated to complete dryness under nitrogen in a warm water bath of 50°C. The samples were redissolved in 1 ml H₂O: ACN (80:20, v:v) + 0.1% FA.
3.3.2.5. LC-MS/MS analysis

The analysis of lasalocid was performed on a Waters Acquity Ultra Performance LC combined with a Waters Micromass Quattro Ultima Pt® MS instrument. An C₁₈ Symmetry column (5 µm, 150 x 2.1 mm) was used. An isocratic mobile phase consisting of HPLC H₂O:ACN (95:5, v:v) + 0.1% FA (10%) and ACN + 0.1% FA (90%) was used. The monensin analysis was performed on a Waters 2690 Separation Module combined with a Waters Micromass Quattro Ultima Pt® MS instrument. A Merck Lichrocart Purospher® column (5 µm, 125 x 3.0 mm) was used and samples were analyzed using a gradient of H₂O +0.1% FA and ACN + 0.1% FA. The analysis of monensin A sodium (m/z 693.6 > 461.5, 693.6 > 479.5) and lasalocid A sodium (m/z 613.5 > 577.5, 613.5 > 377.4, 613.5 > 359.5) was performed in MRM mode and electrospray positive ionization mode. In this study, the Limit of Quantification (LOQ) of the method in feed was 500 µg/kg for lasalocid and monensin, respectively. For all egg matrices, the method LOQ was 2 and 0.5 µg/kg for respectively lasalocid and monensin.

3.3.2.6. Pharmacokinetic and statistical analysis

As this study has been carried out only once and since the number of animals is limited, no statistically obvious conclusions about the influence of dosing regimen on weight and number of eggs can be made. Since the feed samples were not analysed in duplicate, which is needed to determine sufficient homogeneity, no statistics could be performed for the determination of the homogeneity of the experimental feed. The terminal elimination half-life in egg matrices was calculated using pharmacokinetic (PK) functions for Excel which were based on the terminal slope of the tissue concentration-time curve after withdrawal of the experimental feed (Usansky et al., 2011).

3.3.3. Results

3.3.3.1. Zootechnical parameters and experimental feed

The number of eggs and mean egg weight were noted every day during treatment and depletion period. As these experiments have not been repeated, no statistically-based conclusions of effects of the experimental feed on zootechnical parameters can be made. A maximum allowed concentration of 125 mg/kg in the feed has been set for both molecules in broilers, therefore we expected values of 3.125, 6.25, and 12.5 mg/kg. Lasalocid
concentrations of $3.35 \pm 1.34$ (107%), $6.55 \pm 2.04$ (105%) and $13.45 \pm 6.74$ (108%) mg/kg and monensin concentrations of $3.09 \pm 0.78$ (99%), $5.22 \pm 1.21$ (84%) and $11.69 \pm 1.41$ (94%) mg/kg were achieved in the experimental feed, indicating that the measured levels correspond to the expected values.

### 3.3.3.2. Residue concentrations in egg matrices

Lasalocid residue concentrations of the 3 concentration groups, measured in whole egg are presented in Figure 3.12. A plateau level was reached at day 7 for the 2.5% and the 5% group and at day 9 for the 10% concentration group, although the 5 and 10% group did not reach a distinct plateau phase. The residue concentrations were below Limit of Quantification (LOQ) at day 13 of the depletion period for the 2.5% concentration group and at day 17 of depletion period for the 5% and the 10% concentration group.

![Figure 3.12. Residue concentrations (µg/kg) of lasalocid in whole egg for 3 concentration groups (2.5%, 5% and 10% concentration group) during the treatment period (day 1 to day 14 included) and the depletion period (day 15 to day 31 included).](image)

Monensin residue concentrations in whole egg are shown in Figure 3.13. For monensin, the 2.5% concentration group did not reach values above the LOQ. The 5% and the 10% concentration group reached plateau levels at day 3 and day 5, respectively. Residue
concentrations were below LOQ at day 3 of depletion period for the 5% and the 10% concentration groups. The elimination half-life of lasalocid in whole egg was 1.23, 1.66 and 1.58 days for respectively the 2.5%, 5% and the 10% concentration group. As the measured concentrations of monensin in the whole egg were below LOQ within 3 days, no elimination half-life could be calculated. Transfer ratios were calculated by dividing the concentrations in the matrix on day 11 for the 10% group by the achieved feed concentration. Transfer ratios of the whole egg were 0.076 and 0.00012 for respectively lasalocid and monensin.

![Figure 3.13. Residue concentrations (µg/kg) of monensin in whole egg for 3 concentration groups (2.5%, 5% and 10% concentration group) during the treatment period (day 1 to day 14 included) and the depletion period (day 15 to day 30 included).](image)

Residue concentrations in the different egg matrices (whole egg, egg white and egg yolk) are presented in Figures 3.14 and 3.15 for respectively lasalocid and monensin. Highest lasalocid residue concentrations were measured in egg yolk, followed by the whole egg. Very low lasalocid concentrations were measured in egg white compared to the egg yolk. Residue concentrations measured in egg yolk are approximately 3 times higher than the residue concentrations in whole egg, while lasalocid residue concentrations were more than 200 times higher in egg yolk compared to egg white. No large differences were noted between
the monensin concentrations in the different egg matrices. Transfer ratios were calculated for both molecules for egg white as well as for egg yolk. Transfer ratios of lasalocid in egg white and egg yolk were respectively 0.0076 and 0.28.

**Figure 3.14.** Residue concentrations (µg/kg) of lasalocid in whole egg, egg white and egg yolk for the 10% concentration group during the treatment period (day 5 to day 13).

**Figure 3.15.** Residue concentrations (µg/kg) of monensin in whole egg, egg white and egg yolk for the 10% concentration group during the treatment period (day 6 to day 12).
For monensin, a transfer ratio of 0.00014 and 0.000098 was noted for respectively egg white and egg yolk. Since the residue concentrations were only measured during the plateau phase for both molecules, no elimination half-lives could be calculated.

### 3.3.3.3 Physicochemical parameters

Various physicochemical parameters and the residue concentrations in the different egg matrices on day 11 of the treatment period for the 10% concentration group of both molecules are summarized in Table 3.3. In the different on-line databases consulted, the values varied for the following parameters: octanol/water partition coefficient or log P, and the acid dissociation constant, pKa (EPI Suite, SciFinder, PHYSPROP). In the European Food Safety Authority (EFSA) report (EFSA 2007), the lasalocid log octanol/water partition coefficient varies between 1.4 and 2.3.

**Table 3.3.** Various physicochemical, pharmacokinetic parameters and residue concentrations of lasalocid and monensin in various egg matrices.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lasalocid</th>
<th>Monensin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>590.79</td>
<td>670.87</td>
<td>SciFinder</td>
</tr>
<tr>
<td>Log P</td>
<td>6.446</td>
<td>4.011</td>
<td>SciFinder</td>
</tr>
<tr>
<td></td>
<td>6.74</td>
<td>5.43</td>
<td>EPI Suite</td>
</tr>
<tr>
<td></td>
<td>6.74</td>
<td>5.43</td>
<td>PHYSPROP</td>
</tr>
<tr>
<td>Log D (pH = 7)</td>
<td>3.31</td>
<td>1.3</td>
<td>SciFinder</td>
</tr>
<tr>
<td>pKa (most acidic)</td>
<td>3.15</td>
<td>4.26</td>
<td>SciFinder</td>
</tr>
<tr>
<td>pKa</td>
<td>/</td>
<td>4.3</td>
<td>PHYSPROP</td>
</tr>
<tr>
<td>Bioconcentration factor (pH = 7)</td>
<td>33.9</td>
<td>1.29</td>
<td>SciFinder</td>
</tr>
<tr>
<td>Concentration on day 11 in whole egg (µg/kg)</td>
<td>1029.4</td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td>Concentration on day 11 in egg white (µg/kg)</td>
<td>18.9</td>
<td>1.63</td>
<td></td>
</tr>
<tr>
<td>Concentration on day 11 in egg yolk (µg/kg)</td>
<td>3728.6</td>
<td>1.15</td>
<td></td>
</tr>
</tbody>
</table>

### 3.3.4. Discussion

No effects on the zootechnical parameters, egg weight or number of eggs was noted although no statistically-based conclusions can be made from this experiment. Rokka et al. (Rokka et al. 2005) have found, however, that egg production was not (monensin) or significantly (lasalocid) decreased in case laying hens received feed containing respectively monensin at 100 mg/kg or lasalocid at 115-150 mg/kg.
Lasalocid is transferred to the whole egg in high amounts. This has also been demonstrated by other researchers (Rokka et al. 2005; EFSA 2007). Feed containing 0.1–5 mg/kg lasalocid resulted in whole egg concentrations of 6–8 µg/kg and 300 µg/kg lasalocid, respectively (EFSA 2007). Lasalocid reached a plateau level at day 7-9 and after more than 10 days of depletion, eggs still had positive values above LOQ for lasalocid. This has been confirmed by other researchers (Rokka et al. 2005). Lasalocid showed a rapid absorption with maximum plasma levels within two hours (EFSA 2007). Lasalocid is highly distributed to the liver (EMEA 2004). Yolk precursors are formed in the liver and transported via the blood to the oocyte (Yamamura et al. 1995; Saarela et al. 2009). So high residue concentrations in the liver may be correlated with high residue concentrations in the whole egg, existing for 29% of yolk.

Monensin reached a plateau phase at day 3-5 which differs from the day 7 found by other researchers. This may be explained by the very low concentration levels in our study (Rokka et al. 2005). Rokka et al. (2005) found that feeding monensin at a rate of 1.1-12.9 mg/kg feed resulted in monensin whole egg concentrations between 0.1 to 4.0 µg/kg, which is comparable to our findings. The low residue concentrations of monensin may be explained by the limited absorption (11-31%) and the rapid elimination, mainly through the faeces (EFSA 2008e).

Transfer of other coccidiostats from feed to eggs has also been investigated for ionophoric as well as for non-ionophoric coccidiostats (Mortier 2005; Mulder et al. 2005; EFSA 2008a; EFSA 2008c; EFSA 2008g). Mortier (2005) found narasin plateau concentrations of 6 and 90 µg/kg in whole eggs when laying hens received feed containing 2144 µg/kg and 40.9 mg/kg narasin. Salinomycin ranged from 1-60 µg/kg on a diet containing 0.9 to 13.9 mg/kg salinomycin (Rokka et al. 2005). A significant difference in ability to accumulate in eggs is observed for coccidiostats. Lasalocid (63 ng/g per mg/kg feed) is much more readily transferable than salinomycine (3.3 ng/g per mg/kg feed). Monensin is less transferable into eggs (0.12 ng/g per mg/kg feed) (Kennedy et al. 1998a). Large differences in the degree of transfer to the whole egg were noted between the different molecules, which belong to the same class of compounds. This resulted in different equations to express the relationship between residue concentrations in eggs and concentration in feed for each individual coccidiostat. These are (EFSA 2007; EFSA 2008c; EFSA 2008g; EFSA 2008i):
Lasalocid concentration in eggs (µg/kg) = 63.6 * concentration in feed (mg/kg);

Salinomycin concentration in eggs (µg/kg) = 3.33 * concentration in feed (mg/kg);

Feed nicarbazin (mg/kg) = 0.0195 * whole egg residue dinitrocarbanilide (µg/kg) + 0.05 and feed nicarbazin (mg/kg) = 0.230 * 2-hydroxy-4.6-dimethylpyrimidine (µg/kg) + 0.737; and

Halofuginone in egg (µg/kg) = 77.2 * concentration in feed (mg/kg) – 0.002.

These results demonstrate that each molecule needs to be regarded as an individual compound and extrapolations or generalization must be avoided.

Lasalocid has a great affinity for egg yolk and a very low affinity for egg white, which has also been noted by other researchers. If laying hens received $^{14}$C labelled lasalocid, similar to the administration of 125 mg/kg lasalocid in feed, peak total residue concentrations of 291 µg/kg and 32,500 µg/kg were detected in albumen and egg yolk, respectively. The same study reported mean steady state concentrations of 11,000-12,000 µg/kg in whole eggs (EFSA 2007). For monensin, no large differences between egg white and egg yolk residue concentrations have been observed (Kan and Petz 2000). Differences between distribution in egg white and egg yolk have also been noted for other coccidiostats. Narasin, halofuginone, diclazuril, dinitrocarbanilide and salinomycin have mainly been found in egg yolk (Mulder et al. 2005; Rokka et al. 2005; EFSA 2008a; EFSA 2008b; EFSA 2008c; EFSA 2008f; EFSA 2008i). Dimetridazole and its metabolite are mostly present in egg white (Mortier 2005). Several researchers have tried to explain the distribution between egg white and egg yolk. Some authors investigated possible explanations such as plasma protein binding, lipid solubility, partition coefficient and molecular weight, but no clear correlation between any of these parameters and the preference for one of the egg matrices could be demonstrated (Kan and Petz 2000; Kan 2003; Hekman and Schefferlie 2011). No clear conclusions could be made in our study either. If veterinary drugs are administered to laying hens, residues may appear in egg white and egg yolk. After intestinal absorption, veterinary drugs are transported via the bloodstream, followed by deposition in the yolk in the ovary or egg white in the oviduct. The amount of the drug, deposited in the egg matrices is determined by the physicochemical parameters of the drug but also by the animal’s physiology and egg formation (Kan and Petz...
The lipophilicity scale, expressed by the log P value defines substances with log P > 1 as lipophilic and substances with log P < 1 as hydrophilic (Grabowski and Jaroszewski 2009). Based on log P values for lasalocid and monensin, both coccidiostats can be defined as lipophilic. Log P of lasalocid (6.446) is slightly higher compared to the log P of monensin (4.011) so a higher preference for egg yolk would be expected for lasalocid compared to monensin (SciFinder), which was confirmed by our results. Furthermore, marked differences between lasalocid and monensin have been seen for the physicochemical properties log D and bioconcentration factor. These parameters might show some correlation with egg yolk concentrations, as seen in our study. Donoghue at al. (1997) demonstrated that various chemical compounds may be incorporated into preovulatory yolks in a similar pattern. Donoghue and Hairston investigated the possibility of incorporation into egg white during the latter phases of formation (Donoghue and Hairston 1999). Hekman and Schefferlie (Hekman and Schefferlie 2011) recently presented a physiologically-based pharmacokinetic model that describes and predicts drug residue profiles of certain veterinary drugs in eggs based on their plasma kinetics. No plasma concentrations were measured in our study, as a result this model could not be tested using our results.

This study demonstrates that lasalocid is transferred at a high degree to eggs; all the 3 concentration groups exceeded the MRL of 150 µg/kg. If a cross-contamination level of 1% of the maximum authorized dose and a transfer ratio of 0.076 is considered, lasalocid concentrations in whole egg would be 95 µg/kg, which would be below the set MRL of 150 µg/kg. Monensin on the other hand showed a very low transfer ratio from feed to egg; all concentration groups reached values below or just above the set maximum level. Even molecules belonging to the same group result in different transfer ratios, highlighting the uniqueness of each molecule. Lasalocid and most other coccidiostats had the highest affinity for egg yolk although the MRL for lasalocid has been set for whole egg and no separate egg matrices are included in official control procedures. In this study, we only investigated 2 molecules and therefore, no final conclusions about possible correlations between any of the physicochemical parameters and the preferable deposition site could be gathered.
Chapter 4: Broilers
4.1. Residues of sulfadiazine and doxycycline in broiler liver and muscle tissues due to cross-contamination of feed

Based on:


Abstract

Veterinary drugs, such as antimicrobial compounds, are widely used in poultry and may lead to the presence of residues in matrices of animal origin such as muscle and liver tissue. In this study, broilers received experimental feed, containing sulfadiazine or doxycycline at cross-contamination levels of 2.5%, 5% and 10% of the therapeutic dose in feed. Breast- and thigh muscle and liver samples were collected during treatment and depletion period and analyzed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Concentrations reached a plateau phase 3-5 days after starting the experimental feed. A rapid depletion of residues was noted after withdrawal of the experimental feed. No significant differences in measured concentrations were observed between the different muscle types. The residue concentrations for some experimental groups, the 10% group of sulfadiazine and the 5 and 10% group of doxycycline, however exceeded the corresponding Maximum Residue Limits (MRLs).
4.1.1. Introduction

The veterinary drugs used therapeutically in poultry industry are generally given via mass application in feed or drinking water (Kan and Petz 2000). Antibiotics are used to raise production efficiency, as they enhance growth and feed efficiency and reduce disease (Donoghue 2003). Antibiotics have been used as growth promoters in the member states of the European Union but concerns about development of antibacterial resistance led to the withdrawal of these agents as growth promoters in the European Union since January 1, 2006 (Castanon 2007). Some antimicrobial drugs, such as sulphonamides are used to treat coccidiosis (Roudaut and Garnier 2002; Sentepe and Eraslan 2010). In veterinary medicine the sulphonamides are effective chemotherapeutics for bacterial or protozoal diseases (EMEA 1995; BCFI 2010). Doxycycline belongs to the group of tetracycline antibiotics, which have a broad spectrum activity (EMEA 1996). These drugs are widely used as mass medication in both pig and poultry industry. Concerning feed medication, in Belgium, sulfadiazine in combination with trimethoprim is available as premix for pigs and non-laying hens and as oral powder to be mixed in the feed of pigs. Doxycycline is registered as a premix for pigs only and is also available as an oral powder to be mixed in the feed of pigs (BCFI 2010). These drugs work systemically meaning that they must cross the intestinal wall and distribute in the body to exert their function (Kan and Petz 2000). After absorption from the gastrointestinal tract, the drug reaches the blood stream and is distributed throughout the whole body. This distribution can be quantified by some pharmacokinetic characteristics such as volume of distribution, which is largely determined by the physicochemical parameters of the compounds (Kan and Petz 2000), plasma and tissue protein binding and animal physiological status (Toutain and Lees 2004).

Residues exceeding established Maximum Residue Limits may be the result of misuse of antibiotics, due to misreading of the product label or not respecting the withdrawal time or the accidental administration of feed contaminated with pharmacologically active residues (Reyes-Herrera et al. 2005; Segato et al. 2011). According to Regulation 178/2002, a feed or feeding stuff is any substance or product, including additives, whether processed, partially processed or unprocessed, intended to be used for oral feeding to animals. In Directive 2001/82/EC, a medicated feeding stuff is described as any mixture of a veterinary medicinal
product or products and feed or feeds that is ready prepared for marketing and intended to be fed to animals without further processing, because of its curative, preventive or other properties. Medicated feeding stuffs are prepared in multi-product plants following good manufacturing practice guidelines (Segato et al. 2011). Carry-over, transfer from one production batch to the following batch may result in cross-contamination and may occur during feed processing for many reasons (Borras et al. 2011). In the EFSA reports, cross-contamination is defined as: “contamination of feeds that are produced after the production of a mixed feed, containing additives with residual amounts of the previous feed batch”. Cross-contamination may be product- or establishment- related. Some feed additives and premixes properties, such as adhesive strength-adhesion to walls, particle size and density (carrier, substance) and electrostatic properties influence cross-contamination behavior and affect how cross-contamination occurs. The technological equipment in the feed mill, such as the design of dosage and grinding and mixing equipment can influence the level of cross-contamination (EFSA 2007; Borras et al. 2011). Under practical conditions, cross-contamination of residues is unavoidable, even when preventive measures are taken (EFSA 2007). A totally risk/residue free food production system does not exist. Consequently, active surveillance and compliance programs to ensure the proper use of antibiotics and the safety of the food supply are needed (Sasanya et al. 2005). Many governments have established antibiotic residue tolerances in edible animal tissues and have determined the target tissues for residue monitoring (Reyes-Herrera et al. 2005). In the European Union, these Maximum Residue Limits (MRLs) for doxycycline in pigs and poultry are set at 100 µg/kg (muscle), 300 µg/kg (skin and fat, liver) and 600 µg/kg (kidneys) (EMEA 1996; BCFI 2010). For all sulphonamides, the MRL is 100 µg/kg in muscle, fat, liver and kidneys for all food-producing animals (AFSCA 2004; BCFI 2010). Taking into account the unavoidable cross-contamination in practical field conditions, the aims of this study were to investigate the transfer ratios of two frequently used drugs from the feed to poultry edible tissues when provided at cross-contamination levels and to evaluate this transfer in relation to current MRLs.
4.1.2. Material and methods

4.1.2.1. Premix and oral powders, reagents and standards

The premix containing sulfadiazine and thrimetoprim was Tucoprim® powder, kindly provided by Pfizer (Brussels, Belgium). Doxycycline oral powder to be mixed in the feed was Doxycycline 75% Kela®, kindly provided by Kela Veterinaria (Sint-Niklaas, Belgium). Analytical standards of sulfadiazine and doxycycline hyclate were from Sigma (Bornem, Belgium) and Acros (Geel, Belgium), respectively. The internal standards, namely sulfachloropyridazine and demethylchlortetracycline were purchased from Sigma. Acetonitrile, distilled water (for doxycycline analysis) and methanol came from Biosolve (Valkenswaard, The Netherlands). Water for sulfadiazine analysis was HPLC grade and generated by a Milli-Q Gard 2 system (Millipore, Billerica, MA, USA). The anhydrous sodium sulphate came from Merck (Darmstadt, Germany). Filters for filtration of the sulfadiazine extract, i.e. 0.22 µm Millex® GV were from Millipore (Billerica, MA, USA). The solid–phase extraction (SPE) columns used for the clean-up of doxycycline-containing samples were Oasis® columns (HLB SPE column 60 mg/3 ml, Waters, Milford, MA, USA). The Robot Coupe 2, used to mince muscle and liver samples, came from C-Tech Systems (Lille, Belgium).

4.1.2.2. Preparation of the experimental feed and animal experiments

Feed preparation and the animal experiments were carried out at ILVO’s Animals Sciences Unit with approval from the ILVO ethical committee (EC no. 2008/89, 2009/108). The preparation of the experimental feed and the animal experiments are described in chapter 2. Animal experiments and sample analysis are schematically presented in Figure 4.1. Analysis of the breast muscle was performed every 2 days starting from day 11 of the pre-treatment period. Samples from the upper thigh muscles and the liver were only analyzed for the 10% carry-over concentration and only during plateau phase.
Figure 4.1. Scheme of the animal experiment and sample analysis

4.1.2.3. Extraction and clean-up of feed samples

For the analysis of sulfadiazine, 5 grams of feed was weighed, 100 µl of the internal standard sulfachloropyridazine (1 mg/ml) was added and the sample was allowed to stand for 10 minutes. After addition of 25 ml of methanol, the tube was vortexed and placed on the horizontal shaker for 30 minutes. After centrifugation for 10 min at 3000 rpm, 5 ml of the supernatant was transferred into a tube and evaporated under nitrogen to dryness in a water bath at 45°C. After redissolving the residues in 10 ml acetonitrile:water (ACN:H₂O, 50:50,v:v), a dilution of 1:15 was performed in ACN:H₂O followed by filtration using a 0.22 µm Millex®Gv. For analysis of doxycycline, 5 grams of feed was weighed and 1 ml of the internal standard demethylchlortetracycline (20 µg/ml) was added. After the addition of 25 mL of methanol, the samples were placed on a rotary shaker for 20 minutes and centrifuged for 10 min at 4000 rpm. Then, 200 µl of the supernatant was transferred in a vial, 800 µl of HPLC water was added and the vial was vortexed.
4.1.2.4. Extraction and clean-up of broiler samples

For sulfadiazine analysis in muscle and liver, we used the method described by Mortier (2005). In brief, 2 grams of minced muscle or liver were weighed and the internal standard (20 µl of 10 µg/ml) was added. The samples were vortexed, allowed to stand for 10 min and 6 grams (muscle) or 8 grams (liver) of anhydrous sodium sulphate was added. The tissue was carefully mixed with a spatula until a powdery mixture was obtained and 10 ml (muscle) or 20 ml (liver) of acetonitrile was added. After vortexing, the tubes were placed on a horizontal shaker for 30 min. The tubes were centrifuged (15 min, 4000 rpm), 5 ml (muscle) or 10 ml (liver) of the supernatant was transferred into a tube and was evaporated to dryness under nitrogen in a water bath of 45°C. Then, the sample was redissolved in 1 ml of an acetonitrile:water mixture (50:50,v:v) containing 0.1 % formic acid, vortexed, sonicated for 5 min and filtered through a 0.22 µm filter into a HPLC vial.

For doxycycline analysis in tissues, 2 grams of minced tissue were weighed and extracted as described by Cherlet et al. (2003). After the addition of the internal standard demethylchlortetracycline (50 µl of 10 µg/ml), the samples were vortexed and allowed to stand for 5 min. Then, 10 ml of a 0.1 M sodium succinate buffer was added, the samples were placed on a rotary shaker for 20 minutes and centrifuged at 4000 rpm for 10 min. The supernatant was added to a centrifuge tube containing 1 ml of a 20 % trichloroacetic acid, vortex mixed and centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a Whatman® filter paper and was ready for a further solid-phase clean-up step. In the solid-phase clean-up, the Oasis® HLB (Hydrophilic Lipophilic Balance) column was preconditioned consecutively with 3 ml methanol, 3 ml of a 1N HCl solution and 3 ml of water, and the extract was allowed to pass slowly through the HLB column. The HLB column was washed with 1 ml of water and dried. The analytes were eluted with 3 ml of methanol and the eluate was evaporated to dryness at 40°C under nitrogen. After redissolution with 250 µl of a 0.5% formic acid solution and vortexing, the sample was transferred to an autosampler vial.
4.1.2.5. LC-MS/MS analysis

Sulfadiazine analysis was performed on a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro® MS instrument. An X-terra® column (C_{18} 5 µm, 150 x 2.1 mm) was used and the analysis was performed with a gradient of 0.1 % formic acid (FA) in H_{2}O (solvent A) and 0.1 % FA in ACN (solvent B). The analysis of doxycycline was performed on a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro Ultima® MS instrument. Separation was performed on a PLRP-S polymer column (5 µm, 150 x 2.1 mm). An isocratic mobile phase consisting of 70% solvent A (HPLC H_{2}O+0.5% formic acid and 0.001 M oxalic acid; 0.5 % tetrahydrofuran) and 30% of solvent B (ACN) was used. The analysis of sulfadiazine and doxycycline was performed in MRM mode and electrospray positive ion mode (sulfadiazine: m/z 250.9 > 107.9, 250.9 > 92.2, 250.9 > 156.1; doxycycline: m/z 462.4 > 428.1). In this study, the Limit of Quantification (LOQ) of the method was 5 mg/kg and 2.5 mg/kg for respectively sulfadiazine and doxycycline for feed. For muscle, the method LOQ was 2 and 10 µg/kg for respectively sulfadiazine and doxycycline.

4.1.2.6. Pharmacokinetic and statistical analysis

These studies were not repeated, therefore, no statistical analysis of the effect of the experimental feed on the zootechnical parameters could be performed. Due to the limited dataset of the experimental feed, no statistical analysis to determine the homogeneity of the experimental feeds could be carried out. The terminal elimination half-life and elimination rate constant in tissues were calculated using pharmacokinetic (PK) functions for Excel and were based on the terminal slope of the tissue concentration-time curve after withdrawal of the experimental feed (Usansky et al. 2011). Statistical analysis was performed with Statistica 9.0 (StatSoft. Inc., Tulsa, OK, USA). Significance level α was set at 0.05. Statistical analysis was performed on the mean of the residue concentrations of the individual chickens and the pooled sample for each of the different matrices. The comparison between measured residue concentrations in thigh and breast muscle was performed with t-test for dependant samples. The pooled sample on day 13 of the treatment period was analysed six times. The mean of these results was compared with the mean of the individual chickens by an independent t-test.
4.1.3. Results

4.1.3.1. Zootechnical parameters and experimental feed

The effects of experimental feed on the performance parameter, broiler weight, were studied. The mean broiler weights are presented for sulfadiazine and doxycycline in Figure 4.2.

Figure 4.2. Mean broiler weight of the 3 concentration groups of sulfadiazine and doxycycline during the pre-treatment period (day 1 to day 12 included), treatment period (day 13 to day 26 included), and depletion period (day 27 to day 43 included).

For both sulfadiazine and doxycycline the maximum authorized dose is 250 mg/kg (instruction leaflets). Therefore a value of 25, 12.5 and 6.25 mg/kg was calculated for respectively the 10%, 5% and 2.5% carry-over groups. Mean concentrations ± standard deviation and recovery for the 10%, 5% and 2.5 % groups were 21.2 ± 1.8 (79 %), 12.7 ± 0.9 (93 %) and 5.5 ± 0.4 (88 %)mg/kg for sulfadiazine and 24.7 ± 4.6 (99 %), 13.2 ± 4.1 (106 %), 5.5 ± 1.2 (88 %) mg/kg for doxycycline.
4.1.3.2. Residue concentrations in breast muscle, thigh muscle and liver

The residue concentrations for respectively sulfadiazine and doxycycline for all groups in breast muscle are shown in Figure 4.3. The plateau phase was reached at day 3 for 2.5% and 5% carry-over group of sulfadiazine and the 2.5% carry-over group of doxycycline. For the 5 and 10% carry-over groups of doxycycline and the 10% group of sulfadiazine, a plateau phase was reached at day 5. A rapid depletion phase was noted after withdrawal of the experimental feed. Breast muscle tissue elimination half-life was calculated for both molecules but due to the rather fast decline below the LOQ, elimination half-life could only be calculated for respectively the 5 and 10% group of doxycycline, namely 3.1 and 3.5 days.

![Graph showing residue concentrations in breast muscle](image)

**Figure 4.3.** Residue concentrations of the 3 sulfadiazine and doxycycline concentration groups (2.5%, 5% and 10%) in breast muscle during treatment period (day 1 to day 14 included) and depletion period (day 15 to day 31 included).

The concentrations in the three tissue matrices for the 10% group during the plateau phase are presented in Figure 4.4. Small differences in concentrations were noted between the different types of muscles for sulfadiazine as well as for doxycycline but these were not statistically significant (P>0.05). Measured residue concentrations in liver were similar to the concentrations in muscle for sulfadiazine, although higher concentrations were measured in
liver compared to muscle for doxycycline. The liver/breast muscle ratio was 1.2 and 1.8; the liver/thigh muscle ratio was 1.3 and 1.9 for respectively sulfadiazine and doxycycline. Transfer factors, i.e (concentration in the matrix on day 13 of the treatment period for the 10% group/measured concentration in the feed) x 100 for breast muscle, thigh muscle and liver were respectively 1.04, 0.95 and 1.37 % for sulfadiazine and 1.27, 1.62 and 2.90 % for doxycycline.

Figure 4.4. Residue concentrations in breast muscle, thigh muscle and liver for sulfadiazine 10% and doxycycline 10% during the treatment period (day 5 to day 13 included) and depletion period (day 15).
Table 4.1. Concentration of sulfadiazine and doxycycline (mean ± standard deviation; µg/kg) in the pooled sample and individual chickens on day 13 of the treatment period.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Sample type</th>
<th>Matrix</th>
<th>Mean and standard deviation (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfadiazine</td>
<td>Six analyses of the pooled sample</td>
<td>Breast muscle</td>
<td>201.6 ± 15.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thigh muscle</td>
<td>172.2 ± 7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>358.0 ± 20.4</td>
</tr>
<tr>
<td></td>
<td>Analysis of six individual chickens</td>
<td>Breast muscle</td>
<td>202.6 ± 49.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thigh muscle</td>
<td>173.1 ± 30.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>355.8 ± 109.0</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Six analyses of the pooled sample</td>
<td>Breast muscle</td>
<td>485.7 ± 88.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thigh muscle</td>
<td>344.4 ± 13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>728.4 ± 74.4</td>
</tr>
<tr>
<td></td>
<td>Analysis of six individual chickens</td>
<td>Breast muscle</td>
<td>474.8 ± 107.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thigh muscle</td>
<td>327.7 ± 32.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>726.2 ± 74.7</td>
</tr>
</tbody>
</table>

The measured residue concentrations of the tissues from the 6 individual chickens and the six repetitive analyses’ of the pooled sample were compared. The calculated means and standard deviations are presented in Table 4.1. For both molecules no significant difference between the two means was noted for all matrices (P>0.05).

4.1.4. Discussion

For the experimental feed of sulfadiazine and doxycycline, the target concentrations of 2.5%, 5% and 10% of the maximum allowed dose were reached (79 – 106 %) but no statements can be made about the homogeneity of the experimental feeds. The achievement of adequate mixing of an active ingredient in final feed depends on the composition of the final feed, the precision and the size of samples taken for analysis and other factors such as particle size, electrostatic properties, types of mixing machinery and mixing in stages or trituration (EMEA 1996a). The ideal way to mix a drug is to add it at the beginning of the premix process just prior to pelleting (Daniel 2009). Good sampling requires sufficient samples of adequate size and sampling from a moving stream is better than static sampling (Borras et al. 2011). Since very small volumes of premixes were added to large amounts blank finished feed and because the experimental feed differs in composition from the feed used for target species for which the medicated premix is intended, it is not easy to guarantee a homogeneous feed. For each experimental feed, 10 dynamic samples were
taken after 30 min of mixing, at the top (n = 3), the middle (n= 4) and the bottom (n=3) of the final feed in order to provide the best possible sampling procedure.

For both compounds studied, no significant differences were found between the concentrations in breast and thigh muscle. On most sampling occasions, the residue concentrations in breast muscle were slightly higher than the concentrations in thigh muscle. For doxycycline, this was confirmed by Atef et al. (2002) while Reyes-Herrera and co-workers found significantly higher concentrations of enrofloxacin in breast muscle compared to thigh muscle but no difference in concentrations between different breast sections (Reyes-Herrera et al., 2005; Reyes-Herrera et al., 2008). Other authors reported differences between residual concentrations in thigh and breast muscle, which may be explained by a variation in intramuscular fat content. According to Reyes-Herrera et al. (2005), different antibiotics may have different sites of preferential deposition and therefore it may be interesting to determine the edible tissue with the highest concentration if the target tissue is muscle. Elimination half-lives were calculated for breast muscle. Atef et al. (2002) found an elimination half-life of 1.8 days for breast muscle in contrast to our calculated value of 3.1 to 3.5 days. This difference may be due to the different ways of oral administration and the duration of the administration, a dose of 15 mg/kg was provided orally twice daily during 5 successive days. Doxycycline had higher residue concentrations in the liver compared to muscle as reflected by the feed/tissue transfer ratios, which has also been described previously (Atef et al. 2002; Ismail and El-Kattan 2004). Doxycycline is known to be well absorbed from the gastro-intestinal tract (Yoshimura et al. 1991), to have a high volume of distribution with the highest detected levels in liver and kidney (EMEA 1996) and a high tissue binding (Santos et al. 1997). Doxycycline has an absolute oral bioavailability of 73.4% and may be metabolised up to 40% by the liver and is largely excreted in faeces, via bile (EMEA 1996; Laczay et al. 2001). After oral dosing, a good bioavailability (approximately 80%) of sulfadiazine is obtained in non-fasted chickens (Baert et al. 2003). Sulphonamides may be metabolized in various tissues, mainly the liver, at varying degrees and through various mechanisms such as acetylation and oxidation and are mainly excreted via the kidneys (Sentepe and Eraslan 2010).
In addition to individual tissue analysis, we also analyzed a pooled sample. It was concluded that pooled tissue samples are a good reference for the mean of the 6 individual chickens for the studied matrices. It is important to note animal-dependent variability for each of the different matrices, which needs to be considered while sampling and interpreting results when a cross-contamination problem occurs on a farm.

For muscle, MRLs have been set at 100 µg/kg for sulfadiazine as well as for doxycycline (BCFI 2010). The 10% group of sulfadiazine and the 5% and 10% group of doxycycline generated concentrations above the MRL, whereas intake of 5% carry-over of sulfadiazine and 2.5% of doxycycline resulted in concentrations around the MRL. Only the 2.5% group of sulfadiazine generated concentrations below the MRL. For liver, MRLs have been set at 100 µg/kg and 300 µg/kg for respectively sulphonamides and doxycycline (BCFI 2010). Only liver tissue from the animals receiving the 10% concentration in feed was analyzed and resulted in concentrations exceeding the MRLs. It may be concluded that cross-contamination may lead to the presence of residual concentrations above MRL. However, once the “cross-contaminated feed” was no longer administered, a rapid decline in measured concentrations was observed.

The use of cross-contaminated feed may have several consequences for animal as well as human health. Besides the possible adverse effect on the health of non-target species and the indirect toxic effects by the promotion of resistant strains of bacteria, the MRL may also be exceeded in food matrices of animal origin, and consequently food safety may not be guaranteed (Donoghue et al. 1997a; Segato et al. 2011; Vincent et al. 2011). These experiments indicate that important residual levels of both molecules occur in both muscle and liver after feeding experimental cross-contamination levels. An unaware farmer, who provides accidentally feed contaminated with pharmacologically active residues to his animals, may also face legal repercussions because of exceeding the MRL in matrices of animal origin. However, the problem of residual concentrations due to the use of cross-contaminated feed can be resolved within days after switching to blank feed since both molecules showed quick residue depletion once the experimental feed is withdrawn.
4.2. Transfer of flubendazole and tylosin from feed at cross-contamination levels to various poultry matrices

Based on:


Abstract

Residues of veterinary drugs and feed additives used extensively in animal husbandry are sometimes found in edible matrices. In this study, broilers received experimental feed, containing either flubendazole or tylosin at cross-contamination levels of 2.5%, 5% and 10% of the therapeutic dose in order to determine the transfer ratio of these molecules from feed to poultry matrices. Breast and thigh muscle and liver samples were collected during the treatment and depletion periods, and then analyzed using liquid chromatography-tandem mass spectrometry. The parent molecule flubendazole and its 2 major metabolites were quantified. After 3 to 5 days, a plateau phase was reached and a few days after withdrawal of the experimental feed a depletion of residues was noted. A significant difference between both muscle types was observed for flubendazole. Strong metabolization of flubendazole in the liver was seen. For tylosin, no residue concentrations above the limit of quantification could be detected in muscle. None of the residue concentrations for either molecules exceeded the corresponding Maximum Residue Limits.
4.2.1. Introduction

In-feed administration of veterinary drugs and feed additives is an essential treatment/prophylactic route for intensively reared species (McEvoy 2002; Kan and Meijer 2007). Since different compounds are manufactured in the same production line, traces of the first product may remain in this line and get mixed with the first batches of the next product. Transfer from one production batch to the following is called carry-over, cross-contamination is the transfer of undesirable materials (Borras et al. 2011). Carry-over during feed production can lead to contamination of unmedicated feed with zootechnical feed additives and veterinary drugs. Cross-contamination can occur during production and handling in the feed mill, during transport, and on the farm (McEvoy 2002). McEvoy (2002) reports that antimicrobials were detected in 44.1% of feeds declared by manufacturers to be free of medication. Cross-contamination of feed can be prevented by using Good Manufacturing Practice (GMP) for feed production and Good Agricultural Practice (GAP) on the farm (Kan and Meijer 2007).

Flubendazole, one of the benzimidazole anthelmintics, is widely used for treatment and prevention of endoparasitic diseases in poultry and swine and is used in human medicine as well (Moreno et al. 2004; Baliharova et al. 2004). In Belgium, flubendazole is available as a premix to be mixed in the feed, a powder to add to feed, or an emulsion to add to the drinking water. It is allowed for use in pigs, chickens, geese, turkeys and pheasants (BCFI 2011). Tylosin, a macrolide antibiotic, is registered exclusively for veterinary use and is effective against Gram-positive bacteria, Mycoplasma and Chlamydia spp. (Prats et al. 2002; Kim et al. 2011). In Belgium, tylosin is available as a premix, a powder to add to feed, or to the drinking water. It is registered for use in pigs, chickens, turkeys and cattle (BCFI 2011)

Livestock animals are exposed to a variety of veterinary drugs and feed additives. These compounds may be metabolized by different enzymatic systems of the hepatic and extra-hepatic tissues (Mate et al. 2008). Biotransformation of benzimidazoles has been shown to be catalyzed by hepatic microsomal mixed-function oxidases; metabolic reactions are sulfoxidation, demethylation and hydroxylation (Moreno et al. 2004). Flubendazole, which has a low oral bioavailability, is rapidly metabolized so that concentrations of the parent drug in the blood are very low (EMEA 2002). Various species metabolize flubendazole mainly
through keto-reduction and carbamate hydrolysis (EMEA 2006b). The main metabolite of flubendazole is the reduced metabolite in chickens, turkeys (EMEA 2006b) and sheep (Moreno et al. 2004), and the hydrolyzed metabolite in pigs (Moreno et al. 2004). Macrolides are well and quickly absorbed from the intestine and have a good tissue distribution, with highest concentrations in tissues with a pH higher than that of plasma (BCFI 2011). In broilers, tylosin has an oral bioavailability of 30-34%, a high apparent volume of distribution and an elimination half-life of approximately 2 h after oral administration (Lilia et al. 2008). A circadian pattern of serum concentrations of tylosin in broiler has been noted after feed and water medication (Lilia et al. 2008). The biotransformation of tylosin was qualitatively similar for rats, chickens, pigs and cattle. Tylosin is principally metabolized in the liver, resulting in various metabolites. In cattle, pigs, chickens and rats, tylosin A is the most abundant residue (WHO 2009). The use of veterinary drugs may result in residue concentrations in edible tissues that exceed the corresponding Maximum Residue Limits (MRLs) (Prats et al. 2002; Phillips et al. 2004). In poultry, MRLs were set for the sum of flubendazole and (2-amino-1H-benzimidazole-5-yl)(4-fluorophenyl)-methanone, at 50 µg/kg in muscle and skin with adhering fat, 400 µg/kg in liver and 300 µg/kg in kidney (EMEA 2006b). An MRL of 200 µg/kg for muscle and 500 µg/kg for liver is mentioned in the information document in support to the discussion on the Maximum Residue Limits for veterinary drugs (Codex Alimentarius Commission 2012). In poultry, the MRL of tylosin A was set at 100 µg/kg for muscle, skin + fat, liver and kidney (EMEA 2002).

In this study, we have determined the transfer factors of flubendazole and its metabolites and tylosine from the feed at cross-contamination levels to various poultry matrices such as breast and thigh muscle and liver. Based on these results, we can determine 1) whether cross-contamination of both molecules in the feed leads to the presence of residues in edible tissues and presents a risk for food safety, 2) which tissues should be considered as target sampling tissue, and 3) whether testing for metabolites should be included in routine analysis.
4.2.2. Material and methods

4.2.2.1. Premixes, reagents and standards

The premix containing flubendazole was Flubenol 5% premix®, kindly provided by Janssen Pharmaceutica (Beerse, Belgium). Tylosin premix was Tylan 250 VET premix®, kindly provided by Elanco Animal Health (Hampshire, England). Analytical standards of flubendazole, H-flubendazole, R-flubendazole and tylosin A were obtained from Sigma (Bornem, Belgium), Witega (Berlin, Germany), Janssen (Beerse, Belgium) and Biovet (Peshtera, Bulgaria), respectively. The internal standards, D₃-flubendazole and spiramycin, were purchased from Witega and Sigma, respectively. Other reagents such as K₂CO₃, HCl 37%, KH₂PO₄ and NH₃ 25%, came from Merck (Darmstadt, Germany). A KH₂PO₄ buffer (pH = 5) was prepared by dissolving 13.6 g KH₂PO₄ in 1 l HPLC H₂O. A solution of concentrated ammonia in methanol was prepared by dissolving 5 ml of ammonia 25% in 95 ml of methanol. The SCX Isolute columns (500 mg/10 ml) were from Sopachem (Brussels, Belgium). The Robot Coupe 2, used to mince muscle and liver samples, came from C-Tech Systems (Lille, Belgium).

4.2.2.2. Preparation of the experimental feed and animal experiments

The feed preparation and the animal experiments were carried out at ILVO’s Animals Sciences Unit. Animal experiments were performed following the recommendations for euthanasia of experimental animals and under supervision of ILVO’s ethics committee (EC no. 2009/117, 2010/127).

The preparation of the experimental feed and the animal experiments are described in chapter 2. Animal experiments and sample analysis are schematically presented in Figure 4.5. The experimental feed was prepared by incorporation of flubendazole or tylosin at cross-contamination levels of 2.5%, 5% and 10% of the maximum allowed dose of respectively 30 mg/kg and 100 mg/kg in the feed.
For flubendazole, analysis of the breast muscle was performed every 2 days starting from day 1 of the treatment period. Because of the large sample quantity, samples from the upper thigh muscles and the liver were only analyzed for the 10% cross-contamination group and only during the plateau phase. No residue concentrations of tylosin A could be measured above the Limit of Quantification (LOQ) in the muscle tissues, therefore the liver of the 3 concentration groups was analyzed every 2 days during treatment and depletion period.

**4.2.2.3. Sample preparation for the experimental feed**

Analysis of the samples for flubendazole and tylosin was performed by the Department Santé of the Centre d’Economie Rurale and at the department of Pharmacology, Toxicology and Biochemistry of the Faculty of Veterinary Medicine (Ghent University), respectively.

For flubendazole analysis, 3 grams of the feed was weighed and the internal standard D$_3$-flubendazole (30 µl of 100 µg/ml) was added. Five hundred µl of K$_2$CO$_3$ 4M and 5 ml ethyl acetate were added, the samples were vortexed during 1 min and placed on a shaker during 30 min. After centrifugation at 4000 rpm during 5 min, the supernatant was transferred into a 15 ml tube and re-extraction was performed with 5 ml ethyl acetate. Both supernatants were pooled and 1 ml was evaporated to dryness at 50 °C under nitrogen. Five ml of hexane
was added, samples were vortexed and 1 ml of an ethanol/HCl 0.2 M solution was added. Vortexing during 1 min was followed by shaking during 30 min and centrifugation during 5 min at 3000 rpm. The hexane was removed and samples were evaporated to dryness at 50°C under nitrogen. Samples were redissolved with 4 ml HPLC H₂O + 0.1% formic acid (FA) and diluted by adding 100 µl of the extract to a sample vial, containing 900 µl HPLC H₂O + 0.1% FA. For the analysis of tylosin A, 20 grams of feed were weighed. One ml of the internal standard spiramycine (50 µg/ml) was added and the sample was allowed to stand for 10 min. After addition of 100 ml of methanol, the tube was vortex mixed and subsequently placed on a horizontal shaker for 20 min. Six ml of the supernatant was transferred in a tube, centrifuged during 10 min at 4000 rpm. Two hundred µl of the supernatant was transferred in a vial, containing 800 µl of HPLC H₂O.

4.2.2.4. Sample preparation for poultry muscle and liver

For flubendazole analysis, 3 grams of minced tissue were weighed, internal standard (30 µl of 1 µg/ml) was added, and a nearly identical procedure as described for the feed sample preparation was used. The supernatants were pooled and the complete sample instead of 1 ml was evaporated to dryness. After the last evaporation to dryness, samples were redissolved with 4 ml HPLC H₂O + 0.1% formic acid and placed in a HPLC vial. The method described by Cherlet et al. (2002) was used for analysis of tylosin A in poultry liver and muscle.

4.2.2.5. LC-MS/MS analysis

The flubendazole analysis was performed on a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro Ultima® MS instrument. A Symmetry® column (C₁₈ 5 µm, 150 x 2.1 mm) was used and the analysis was performed with a gradient of 0.1% FA in HPLC H₂O and 0.1% FA in acetonitrile (ACN). The analysis of tylosin A was performed on a Waters Alliance 2695 Separation Module combined with a Waters Micromass Quattro Ultima MS® instrument. Separation was performed on a PLRP-S polymer column (5 µm, 150 x 2.1 mm). A gradient mobile phase consisting of HPLC H₂O + 0.01 M ammonium acetate (pH 3.5) and ACN was used. The analysis of flubendazole and tylosin was performed in MRM mode and electrospray positive ionization mode. For flubendazole, the parent molecule and the 2 major metabolites, the reduced and the hydrolyzed forms were detected.
(flubendazole: m/z 314.4 > 123.4, 314.4 > 282.3; H-flubendazole: m/z 256.4 > 123.4, 256.4 > 95.4; R-flubendazole: m/z 316.2 > 284.2, 316.2 > 97.2). Tylosin A was the marker residue of tylosin (tylosin A: m/z 916.4 > 174.1). In this study, the Limit of Quantification (LOQ) of the method in feed was 250 µg/kg and 1.25 mg/kg for flubendazole and tylosin A, respectively. For muscle, the method LOQ was 1 and 2.5 µg/kg for flubendazole including parent molecule and metabolites and for tylosin A, respectively. For liver, the method LOQ was 2 and 2.5 µg/kg for respectively flubendazole including parent molecule and metabolites and for tylosin A.

4.2.2.6. Statistical analysis

Because no repetitive studies were carried out, no statistical analysis of the effect of the experimental feed on the zootechnical parameter, chicken weight could be performed.

On day 13 of the treatment period, 6 different samples of the pooled sample material of each matrix (breast muscle, thigh muscle and liver) of the 10 % concentration group were taken and analyzed on residue concentrations of flubendazole and both metabolites. The coefficient of variation (CV) of these pooled samples was calculated in order to determine the variation due to the sampling method. Next, the residue concentrations of flubendazole and both metabolites in each matrix were determined for the 6 individual chickens of the 10% concentration group on day 13 of treatment period. A single-sample t-test was performed to test wether the pooled sample is a good representation of the mean concentration of the individual chickens. In addition, the comparison between measured residue concentrations in thigh muscle and breast muscle was performed using a t-test for dependent samples. This test was performed for flubendazole and both metabolites for the pooled samples (day 5 of treatment period to day 1 of depletion period) for all concentration groups and for the 6 individual chickens of the 10% concentration group on day 13 of treatment period. Significance level α was set at 0.05 (Statistica 9.0, StatSoft. Inc.,Tulsa, OK, USA).
4.2.3. Results

4.2.3.1. Body weight and experimental feed

At arrival, 1-day-old Ross 308 broilers were healthy and had an average body weight of 41.0 grams. Since this study has not been repeated, no statistical analysis could be performed but no distinct effect of the administration of the experimental feed on the broiler weight was observed. The maximum allowed concentration in feed is 30 mg/kg and 100 mg/kg for respectively fluben
dazole and tylosin. Therefore, concentrations of 750, 1500, and 3000 µg/kg and 2.5, 5 and 10 mg/kg were expected for the 2.5%, 5% and 10% concentrations of respectively fluben
dazole and tylosin. Since tylosin A counted for 90.9% of the tylosin concentration in the used premix, a tylosin A concentration of 2.27, 4.55, and 9.09 mg/kg is expected in the final feed. When feed was analyzed, flubendazole concentrations of 591 ± 26 (79%), 1270 ± 92 (85%) and 2822 ± 120 (94%) µg/kg and tylosin A concentrations of 2.1 ± 1.5 (95%), 3.6 ± 2.2 (78 %) and 9.9 ± 3.8 (109 %) mg/kg were determined in the feed for respectively the 2.5%, 5% and 10% concentration groups. Analysis of the experimental feed samples indicated that the measured levels of flubendazole and tylosin correspond to the expected values.

4.2.3.2. Residue concentrations in various poultry matrices

4.2.3.2.1. Flubendazole and metabolites

![Chemical structures of flubendazole and its metabolites: the hydrolyzed and the reduced flubendazole metabolite.](image)

Figure 4.6. Chemical structures of flubendazole and its metabolites: the hydrolyzed and the reduced flubendazole metabolite.
The chemical structures of the parent molecule flubendazole and its metabolites are presented in Figure 4.6. Residue concentrations in breast muscle for the 3 concentration groups of the parent molecule and the metabolites are presented in Figures 4.7 and 4.8, respectively. Residue concentrations of the reduced metabolite (R-flubendazole) were in the same range as the parent compound and were clearly higher than the hydrolyzed metabolite (H-flubendazole). For the hydrolyzed metabolite, no residues above the LOQ (1 µg/kg) could be measured for the 2.5% and the 5% group.

![Graph showing residue concentration of flubendazole (µg/kg) in breast muscle for the 3 carry-over concentration groups during the treatment period (day 1 to day 14 included) and depletion period (day 15 to day 31 included).](image)

**Figure 4.7.** Residue concentration of flubendazole (µg/kg) in breast muscle for the 3 carry-over concentration groups during the treatment period (day 1 to day 14 included) and depletion period (day 15 to day 31 included).

Linearity in residue concentrations in breast muscle was seen between the different concentration groups. The hydrolyzed metabolite concentrations in the 10% group reached values similar to those of the reduced metabolite concentrations in the 2.5% group. For the 2.5% and the 5% concentration group of the parent molecule as well as for the 10% of the hydrolyzed metabolite and the 5% and the 10% of the reduced metabolite, a plateau phase was reached at day 3 of the treatment period. The 2.5% concentration group of the reduced metabolite reached a plateau phase on day 5 of the treatment period. The 10% group of flubendazole never reached a distinct plateau phase, highest residue concentrations were
measured on day 13 of treatment period. Once the experimental feed was no longer provided, the residue concentrations of the parent molecule and the metabolites dropped below the LOQ for all concentration groups starting from day 3 of the depletion period. Transfer factors were calculated for the 10% group by dividing the residue concentration in the matrix at day 13 of treatment by the measured concentration in the feed. The transfer factors for flubendazole, the hydrolyzed metabolite and the reduced metabolite in breast muscle were respectively 0.006, 0.0007 and 0.005.

![Graph](image)

**Figure 4.8.** Residue concentration of the reduced metabolite (µg/kg) in breast muscle for the 3 carry-over concentration groups and the hydrolyzed metabolite for the 10% group during the treatment period (day 1 to day 14 included) and depletion period (day 15 to day 31 included).

In thigh muscle, flubendazole was present in higher concentrations than the reduced metabolite which was in turn present in higher concentrations than the hydrolyzed metabolite. In the liver, the parent molecule was highly metabolized, mostly to the reduced metabolite. Residue concentrations in the different broiler matrices (breast muscle, thigh muscle, and liver) are presented for the 10% group during plateau phase for the parent molecule, the hydrolyzed metabolite and the reduced metabolite (Figures 4.9 and 4.10.
respectively). The parent molecule as well as its metabolites reached a plateau phase in thigh muscle and liver. Residues in liver and thigh muscle dropped below the LOQ at day 5 of depletion for flubendazole and the reduced metabolite in all concentration groups and for the hydrolyzed metabolite in the 10% concentration group. In thigh muscle, the transfer factors for flubendazole, the hydrolyzed metabolite and the reduced metabolite were respectively 0.009, 0.001 and 0.003. For the parent molecule, residue concentrations in the liver were in the same range as the concentration in thigh muscle. For the metabolites, the concentrations in the liver were clearly higher than the residue concentrations in the muscles. Liver residue concentrations were the highest for the reduced metabolite. The transfer factors for flubendazole, the hydrolyzed metabolite and the reduced metabolite in liver were respectively 0.005, 0.01 and 0.05.

![Figure 4.9](image-url). Residue concentration of flubendazole (µg/kg) for the different poultry matrices (breast muscle, thigh muscle and liver) for the 10% concentration group during the treatment period (day 5 to day 13 included) and depletion period (day 15 and day 17).
Figure 4.10. Residue concentration of the reduced and the hydrolyzed metabolite of flubendazole (µg/kg) for the different poultry matrices (breast muscle, thigh muscle and liver) for the 10% concentration group during the treatment period (day 5 to day 13 included) and depletion period (day 15 and day 17).

A significant difference was noted between the residue concentrations in thigh muscle and in breast muscle for flubendazole and in most cases for the hydrolyzed and the reduced metabolite. No significant difference between residue concentrations in breast and thigh muscle was noted for the pooled samples of the 2.5% hydrolyzed metabolite and the 5% reduced metabolite concentration groups and for the individual samples of the reduced metabolite 10% concentration group. Concentrations in thigh muscle were higher compared to those in the breast muscle (P < 0.05) for the pooled samples of all concentration groups of flubendazole and the hydrolyzed metabolite and the 2.5% concentration group of the reduced metabolite. Higher residue concentrations in thigh muscle compared to breast muscle were noted for the pooled samples of the 5% and 10% concentration of the reduced metabolite. The mean residue concentration and the standard deviation of the six individual chickens and the six samples of the pooled sample material of the 10% concentration group on day 13 of treatment period are shown for flubendazole, the hydrolyzed metabolite and the reduced metabolite in Table 4.2. The CV for flubendazole was 5.3%, 3.3% and 7.1% for
breast muscle, thigh muscle and liver indicating a limited variation within the pooled sample material. For the hydrolyzed and the reduced metabolite, CV of 7.0%, 6.2%, and 12.5% and 6.6%, 5.3%, 11.4% were determined for respectively the breast muscle, thigh muscle, and liver. No significant difference was found between the residue concentrations in individual chicken samples and the mean of the repetitive samples of the pooled sample for flubendazole and both metabolites for each matrix (P> 0.05), except for the hydrolyzed metabolite in thigh muscle (P< 0.05).

Table 4.2. Mean and standard deviation of flubendazole and metabolites for the individual samples and the pooled samples in the different poultry matrices. Significance (P) between residue concentrations in breast and thigh muscle is indicated with P<0.05.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Sample type</th>
<th>Matrix</th>
<th>Mean and standard deviation (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flubendazole</td>
<td>6 analyses of the</td>
<td>Breast muscle</td>
<td>19.33 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>pooled sample</td>
<td>Thigh muscle</td>
<td>31.72 ± 1.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>27.31 ± 1.94</td>
</tr>
<tr>
<td></td>
<td>Analysis of 6</td>
<td>Breast muscle</td>
<td>16.28 ± 7.28 (P &lt; 0.05)</td>
</tr>
<tr>
<td></td>
<td>individual chickens</td>
<td>Thigh muscle</td>
<td>30.83 ± 15.9 (P &lt; 0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>30.04 ± 13.23</td>
</tr>
<tr>
<td>Hydrolyzed</td>
<td>6 analyses of the</td>
<td>Breast muscle</td>
<td>2.26 ± 0.16</td>
</tr>
<tr>
<td>flubendazole</td>
<td>pooled sample</td>
<td>Thigh muscle</td>
<td>1.90 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>31.29 ± 3.92</td>
</tr>
<tr>
<td></td>
<td>Analysis of 6</td>
<td>Breast muscle</td>
<td>2.05 ± 0.40 (P &lt; 0.05)</td>
</tr>
<tr>
<td></td>
<td>individual chickens</td>
<td>Thigh muscle</td>
<td>1.54 ± 0.32 (P &lt; 0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>30.47 ± 5.81</td>
</tr>
<tr>
<td>Reduced</td>
<td>6 analyses of the</td>
<td>Breast muscle</td>
<td>7.57 ± 0.50</td>
</tr>
<tr>
<td>flubendazole</td>
<td>pooled sample</td>
<td>Thigh muscle</td>
<td>7.60 ± 0.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>104.95 ± 11.95</td>
</tr>
<tr>
<td></td>
<td>Analysis of 6</td>
<td>Breast muscle</td>
<td>6.97 ± 3.55 (P &gt; 0.05)</td>
</tr>
<tr>
<td></td>
<td>individual chickens</td>
<td>Thigh muscle</td>
<td>7.97 ± 4.69 (P &gt; 0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>121.77 ± 69.89</td>
</tr>
</tbody>
</table>

4.2.3.2.2. Tylosin

For tylosin, none of the 3 concentrations groups reached concentrations above the LOQ in the breast muscle; neither did the 10% group in the thigh muscle. The measured residue concentrations in the liver for the 3 concentration groups are presented in Figure 4.11. Some
residue concentrations of the 2.5% concentration group were below the LOQ. Tylosin did not reach a distinct plateau phase in the liver for any of the concentration groups. Highest concentrations were reached on day 7 of the treatment period for the 5% and 10% concentration groups and at day 11 for the 2.5% concentration group. The transfer factor for tylosin in liver is 0.0006. Once the experimental feed was no longer provided, the measured residue concentrations dropped below the LOQ on day 3 of the depletion period.

![Figure 4.11](image_url)

**Figure 4.11.** Residue concentration of tylosine A (µg/kg) in liver for the 3 carry-over concentration groups during the treatment period (day 1 to day 14 included) and depletion period (day 15 to day 31 included).

4.2.4. Discussion

Upon starting the treatment with the experimental feed, flubendazole and its metabolites were rapidly detected and a very rapid decline in residue concentration was noted after cessation of the experimental feed. These results are similar to those found in guinea fowl (De Ruyck *et al.* 2004). The reduced metabolite was the major component in the different matrices. In breast muscle, residue concentrations of flubendazole were similar to the concentrations of the reduced metabolite but concentrations of the parent molecule in the thigh muscle were higher compared to the concentrations of the reduced metabolite. This is
different from the findings in guinea fowl, in which the reduced metabolite was the most important residue in breast muscle and thigh muscle (De Ruyck et al. 2004). In turkeys, residue concentrations of the reduced metabolite were higher compared to the parent molecule but the muscle type was not specified (EMEA 2006b). In our experiment, the hydrolyzed metabolite was the minor compound, which was also observed in different other bird species (De Ruyck et al. 2004; EMEA 2006b). In the broiler liver, the highest residue concentrations were measured for the reduced metabolite, followed by the hydrolyzed metabolite which was slightly higher than the residue concentrations of the parent molecule. The reduced metabolite was present in the highest concentrations in the liver of guinea fowl, followed by the parent molecule, which in turn had higher residue concentrations than the hydrolyzed metabolite (De Ruyck et al. 2004). In turkeys, residue concentrations were highest for the reduced metabolite, followed by the parent molecule with the hydrolyzed metabolite having the lowest concentrations (EMEA 2006b).

For flubendazole and in most cases for both metabolites, a significant difference in residue concentrations was determined between breast and thigh muscle for all concentration groups. Limited information is available on residue disposition between different muscle types. Differences between thigh and breast muscle have been mentioned for percentage recovery of the coccidiostat zoalene, polychlorinated biphenyl residue amount, and diclazuril residue concentrations (Parks and Doerr 1986; De Vos et al. 2003; Mortier et al. 2005). Possible explanations suggested were the lipophilic nature of the molecules, the greater intramuscular fat content in thigh muscle or the higher thigh blood flow. Reyes-Herrera et al. found higher enrofloxacine concentrations in breast muscle versus thigh muscle (Reyes-Herrera et al. 2005) but no difference was noted within the different regions of the same muscle type (Reyes-Herrera et al. 2008).

The pooled sample was analyzed several times and the coefficient of variation (CV) was determined. The CV was lowest for flubendazole in all matrices, the CV was highest for the liver, especially for metabolites. We may conclude that the repeatability of the pooled sample is rather good for flubendazole in all matrices and for both flubendazole metabolites in muscle. The residue concentrations of the individual chickens were compared to the mean of the repetitive analyses of the pooled sample. In general, we might conclude that pooled
tissue samples are a good reference for the mean of the 6 individual chickens for the parent molecule and in most cases for the metabolites. However, the dataset used for statistical analysis is rather limited. The variability between the different animals of the same group as indicated by the standard deviation needs to be kept in mind if a cross-contamination problem has occurred.

No residues were detected above the LOQ for tylosin A in both muscle types and small amounts of tylosin A could be detected in the liver. This has also been demonstrated by other researchers. Tylosin was administered in the drinking water at a dose of 500 mg/l for 5 days. At 0 h after oral administration via the drinking water, residues of tylosin A were less than 100 µg/kg in liver and muscle (WHO 2009). Residues have only been found in liver (180-270 µg/kg) at 24 hours withdrawal of feeding growing chickens tylosin phosphate at a dose of 2000 - 8000 g/ton feed for 8 weeks (Lewicki 2006). Various tylosin studies have been carried out in pigs and turkeys and similar findings were noted (Montesissa et al. 1999; Prats et al. 2002). In laying hens, residues were detected in the muscle and higher residue concentrations were found in thigh muscle compared to breast muscle (Lewicki 2006).

The administration of flubendazole at cross-contamination levels to broilers leads to detectable residues. In poultry, MRLs were set for the sum of flubendazole and (2-amino-1H-benzimidazole-5-yl)(4-fluorophenyl)-methanone at 50 µg/kg in muscle and 400 µg/kg in liver (EMEA 2006b). None of the concentration groups of the parent molecules as well as both metabolites exceeded the MRL in each matrix. Transfer of tylosine from feed to broiler matrices was very low in poultry and did not exceed the set MRL of 100 µg/kg for muscle and liver (EMEA 2002). Although differences in transfer factors were noted between the 2 studied molecules, cross-contamination of each of the studied molecules would not result in residue concentrations above MRL in edible broiler tissues. Veterinary drugs such as antibiotics are used to treat diseases and improve animal production. In order to ensure the safety of the food supply and the consumer’s confidence, the type of tissue that should be monitored for residues (the target tissue) has been determined (Reyes-Herrera et al. 2005). The target tissue is the edible tissue in which the concentration of residues remains above the tolerance level for the longest period of time compared with the other edible tissues and is important during the post-marketing monitoring of veterinary drug usage (Reyes-Herrera
et al. 2008). Food safety authorities do not delineate what type of edible muscle type should be tested for residue determination (Reyes-Herrera et al. 2005). However, significant differences in residue concentrations between breast and thigh muscle for different molecules have been noted in the current study and in literature. Several food safety authorities have established tolerance levels or safe concentrations of residues in edible tissues. For flubendazole and its metabolites and for tylosine, the MRLs were not exceeded in any of the investigated poultry matrices. The MRL for poultry has been set for the sum of the parent molecules flubendazole and the hydrolyzed metabolite, (2-amino-1H-benzimidazole-5-yl)(4-fluorophenyl)methanone, although the main residue is the reduced metabolite in poultry, particularly (EMEA 2006b). To ensure food safety, the parent molecule and its metabolites should be determined and the muscle type, used for monitoring programs should be specified.
4.3. Residue concentrations of lasalocid and monensin in broiler matrices due to cross-contamination in the feed

Based on:


Abstract

European legislation has set maximum levels of unavoidable carry-over of coccidiostats or histomonostats in non-target feed and maximum levels for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed. In this study, broilers received experimental feed that contained the coccidiostats lasalocid or monensin at cross-contamination levels of 2.5%, 5% and 10% of the maximum therapeutic dose. Samples from the breast muscle (lasalocid + monensin), the upper thigh muscle (monensin) and the liver (monensin) were taken during the treatment and depletion periods and were analyzed using liquid-chromatography tandem mass spectrometry (LC-MS/MS). Residues above Maximum Residue Limit (MRL) were found for lasalocid in breast muscle. Very low concentrations of monensin (below the MRL) were detected in both muscle types and in the liver. Transfer factors in breast muscle were determined for both molecules. Considering these transfer factors, a carry-over rate of 1% would result in residue concentrations below MRL values.
4.3.1. Introduction

Coccidiosis is a highly contagious parasitic disease of the intestinal tract. Minor coccidiosis infections can cause poor feed conversion and weight loss, whereas major infections cause significant mortality (Delahaut et al. 2010). Under common farm conditions, herd health management alone is insufficient to mitigate coccidial infections in large poultry units, therefore coccidiostats must be administered. Several coccidiostatic substances have been authorised in the EU for the prevention of coccidiosis in one or more animal species (EFSA 2007). Because of the economic impact of coccidiosis, at least 45% of the feed is manufactured with an added coccidiostat, corresponding to 18.33 million tonnes of the 40.65 million tonnes produced annually for chickens for fattening, turkeys and rabbits (Delahaut et al. 2010; Dorne et al. 2011). In feed companies, a broad range of feeds may be produced in the same production line and carry-over of coccidiostats may occur, leading to cross-contamination (Delahaut et al. 2010). Cross-contamination can be defined as “contamination of feeds that are produced after the production of a mixed feed containing additives with residual amounts of the previous feed batch” and can be divided into product-related cross-contamination (properties of the feed additives and premixes such as adhesive strength-adhesion to walls, particle size and density, electrostatic properties) or establishment-related cross-contamination (design of the dosage, grinding and mixing equipment). Cross-contamination of residues is practically unavoidable under current conditions, even when all prevention measures such as rinsing batches are taken (EFSA 2007). Unavoidable cross-contamination may occur at any stage of production, processing, storage or transport of the feed and could lead to the presence of residues of coccidiostats in edible products (Delahaut et al. 2010). European legislation has set maximum levels of unavoidable carry-over of coccidiostats or histomonostats in non-target feed and maximum levels for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed (The Commission of the European Communities 2009a; The Commission of the European Communities 2009b). A carry-over rate of approximately 3% compared to the authorized maximum content should be retained for the feed given to less sensitive, non-target animals; a carry-over rate of 1% compared to the authorised maximum content should be retained for feed intended for
sensitive non-target animal species, and for withdrawal feed. The 1% carry-over rate should also be considered for non-target feed for continuous food-producing animals such as dairy cows and laying hens (The Commission of the European Communities 2009a). A maximum content of 1.25 mg/kg is set for chickens for fattening, chickens reared for laying and turkeys for lasalocid sodium and monensin sodium for the period before slaughter in which the use of lasalocid or monensin sodium is prohibited (The Commission of the European Communities 2009a). Coccidiostats, present in excessive concentrations in non-target feed could be harmful to non-target animal species but they may also pose a risk to human health. The human health risk could be direct, as in the toxic effects of residues in foodstuffs in sensitive individuals, and/or indirect, such as promotion of bacterial resistance (true for all antibacterial substances) (Vincent et al. 2011).

In this study, we determined 1) the transfer ratios of two coccidiostats (i.e lasalocid and monensin) from feed to broiler matrices with feed containing cross-contamination levels, and 2) whether this transfer poses human food safety risks.

4.3.2. Material and methods

4.3.2.1. Premixes, reagents and standards

Lasalocid and monensin premixes are registered for use in chickens for fattening, chickens reared for laying, and turkeys. For all target animals, the authorised maximum lasalocid content in complete feed is 125 mg/kg, while the maximum authorised monensin contents are 125 mg/kg, 120 mg/kg and 100 mg/kg in the complete feed for chickens for fattening, chickens reared for laying and turkeys, respectively (EFSA 2007; EFSA 2008e). Lasalocid (Avatec 150 G) and monensin (Elancoban G200) premixes were kindly provided by Alpharma and Elanco, respectively. Internal standard nigericin came from Sigma (Bornem, Belgium), and the analytical standards lasalocid and monensin were obtained from Alpharma and Sigma, respectively. Acetonitrile (ACN) originated from Biosolve (Valkenswaard, the Netherlands). HPLC grade water was generated by a Milli-Q Gard 2 system and 0.22 µm filters, used for filtration of the lasalocid extracts, came from Millipore (Billerica, MA, USA). Na₂CO₃ and HPLC H₂O for monensin extraction came from VWR (Leuven, Belgium) and Acros
(Geel, Belgium), respectively. Muscle and liver samples were minced with the Robot coupe 2 (C-Tech Systems, Lille, Belgium).

### 4.3.2.2. Preparation of the experimental feed and animal experiments

The Animal Sciences Unit of the Institute for Agricultural and Fisheries Research (ILVO) prepared both experimental feeds. The broiler lasalocid experiment was carried out at the Animal Sciences Unit of ILVO and the monensin experiment was carried out at the Centre d’ Economie Rurale (EC 2008/91).

The preparation of the experimental feed and the animal experiments are described in chapter 2. Animal experiments and sample analysis are schematically presented in Figure 4.12.

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**Figure 4.12.** Scheme of the animal experiment and sample analysis

Since residue concentrations for monensin in breast muscle were very low, residue concentrations were also determined in liver and thigh muscle at expected plateau phase. All samples were minced and grounded with a Robot Coupe 2 and stored at -18 °C until analysis. A pooled sample was prepared by mixing 5 to 10 g (depending on the available amount) of each type of tissue sampled from each of the six chickens. This pooled sample was used for analysis.
4.3.2.3. Extraction and clean-up of feed samples

Centre d’Economie Rurale performed the analysis of lasalocid and monensin in feed samples. Ten grams of feed were weighed and, the internal standard nigericine (50 µl of 1 mg/ml) was added. The samples were allowed to stand for 10 min to equilibrate. Fifteen milliliters of Na₂CO₃ (10% w/v) was added and the samples were shaken manually, followed by addition of 20 ml of acetonitrile. The samples were placed on a horizontal shaker for 20 min, followed by sonication for 10 min. After centrifugation (5 min, 2000 rpm), the supernatant was transferred into a new tube and re-extraction was performed 4 times. This resulted in 100 ml supernatant. One milliliter of the supernatant was added to 3 ml of acetonitrile and 1 ml H₂O + 0.1% formic acid (FA). One milliliter was vortexed and placed in a HPLC vial.

4.3.2.4. Extraction and clean-up of poultry samples

The extraction of the lasalocid muscle is the same as described by Mortier et al. (2005). Two grammes of muscle tissue were weighed, internal standard (20 µl of 10 µg/ml) was added and the samples were briefly vortexed and allowed to stand for 10 min. Next, six grammes of anhydrous sodium sulphate were added to the muscle. The sample was well-mixed using a spatula until powdery. Ten milliliters of acetonitrile (ACN) were added to the muscle sample. The samples were vortexed and placed on a horizontal shaker for 30 min, followed by centrifugation for 15 min at 4000 rpm. Five milliliters of supernatans were transferred into a tube and the samples were evaporated to dryness in a warm water bath of 60 °C. The samples were redissolved in 1 ml ACN:H₂O (50:50,v:v) + 0.1% formic acid (FA), vortexed for 30 sec, and placed in an ultrasonic bath for 5 min. The extract was then filtered and poured into a HPLC vial. The monensin analysis was performed at the Centre d’Economie Rurale, 5 grams of muscle matrices were weighed, the internal standard nigericine (50 µl of 1 µg/ml) was added and the samples were allowed to stand for 10 min. Ten milliliters of acetonitrile were added and the samples were placed on the horizontal shaker for 30 min. After centrifugation (5 min, 2000 rpm), the supernatant was transferred and re-extraction was performed. The extracts were pooled and were evaporated to complete dryness under nitrogen in a warm water bath of 50° C. The samples were redissolved in 1 ml H₂O:ACN (80:20, v:v) + 0.1% FA .
4.3.2.5. LC-MS/MS analysis

The analysis of lasalocid was performed on a Waters Alliance 2695 Separation Module combined with a Waters Micromass LC Quattro® MS instrument. A C18 Symmetry column (5 µm, 150 x 2.1 mm) was used for the separation. An isocratic mobile phase consisting of 10% HPLC H2O:ACN (95:5, v:v) + 0.1% FA and 90% ACN + 0.1% FA was used. Monensin analysis was performed on a Waters 2690 Separation Module combined with a Waters Micromass Quattro Ultima® MS instrument. A Merck Licrochart Purospher® column (5 µm, 125 x 3.0 mm) was used and the analysis was performed with a gradient of H2O +0.1% FA and ACN + 0.1% FA. The analysis of monensin A sodium (m/z 693.6 > 461.5, 693.6 > 479.5) and lasalocid A sodium (m/z 613.5 > 577.5, 613.5 >377.4, 613.5 >359.5) was performed in multi-reaction monitoring mode and electrospray positive ionization mode. In this study, the Limit of Quantification (LOQ) of the method in feed was 500 µg/kg for lasalocid and monensin. For all broiler samples, the method LOQ was 2 µg/kg and 1 µg/kg for lasalocid and monensin, respectively.

4.3.3. Results

4.3.3.1. Zootechnical parameters and experimental feed

At arrival, 1-day-old Ross 308 broilers were healthy and had an average body weight of 41.0 g. Since this study has not been repeated, no statistical analysis could be performed but no distinct effect of the administration of the experimental feed on the broiler weight was observed. For both lasalocid and monensin, the maximum allowed dose is 125 mg/kg feed; therefore values of 3.125, 6.25 and 12.5 mg/kg were expected. For the lasalocid experiment mean values and standard deviations of 2.78 ± 1.56 (89%), 6.86 ± 2.08 (109%) and 13.13 ± 2.79 (105%) mg/kg were reached for respectively the 2.5%, 5% and 10% group. Concentrations of 3.09 ± 1.08 (99%), 6.41 ± 2.42 (103%) and 13.49 ± 2.27 (108%) were measured for respectively the monensin 2.5%, 5% and 10% concentrations groups.

4.3.3.2. Residue concentrations in breast muscle, thigh muscle and liver

The lasalocid concentrations (µg/kg) in breast muscle are presented in Figure 4.13. The plateau level was reached at day 5 of the treatment period for all concentration groups, although no stable plateau phase was reached. The values dropped below LOQ at day 5 of
depletion period for all concentration groups. A decline in residue concentrations was already noted at the end of the treatment period, for which no clear explanation could be given. The transfer factors were calculated by dividing the residue concentrations of the 10% concentration group at day 13 of the treatment period by the measured concentration in the feed. Transfer factor in breast muscle was 0.0065 for the 10% concentration group.

![Graph showing residue concentrations of lasalocid (µg/kg) in breast muscle of the 3 concentrations groups (2.5%, 5% and 10%) during the treatment period (day 1 to day 14 included) and the depletion period (day 15 to day 31 included).](image)

**Figure 4.13.** Residue concentrations of lasalocid (µg/kg) in breast muscle of the 3 concentrations groups (2.5%, 5% and 10%) during the treatment period (day 1 to day 14 included) and the depletion period (day 15 to day 31 included).

As the monensin residue concentrations in breast muscle of the 2.5% and the 5% concentrations groups were below the LOQ, also residue concentrations in thigh muscle and liver were determined. The values of the breast muscle, thigh muscle and liver of the 10% group during treatment and depletion period were close to the LOQ and are presented in Figure 4.14. For the three matrices, highest concentrations were reached at day 7 of the treatment period but in general very low concentrations were measured. No large differences were noted between the measured concentrations of the three matrices. Transfer ratios of the 10% concentration group were 0.000092; 0.000069 and 0.000043 for respectively breast muscle, thigh muscle and liver.
Figure 4.14. Residue concentrations (µg/kg) of monensin in breast muscle, thigh muscle and liver of the 10% concentration group during the treatment period (day 1 to day 13 included) and the depletion period (day 15).

4.3.4. Discussion

A number of pharmacokinetic studies were carried out for lasalocid to determine the organ distribution in broilers. Lasalocid showed a rapid absorption with maximal plasma levels within two hours and an elimination half-life of approximately three hours (EFSA 2007). The treatment of chickens with 125 mg/kg feed of unlabelled lasalocid for 34 days, followed by 132 mg/kg feed of radiolabelled lasalocid for 21 days resulted in radioactive residue concentrations in liver and muscle of 11,930 and 610 µg/kg, 2,630 and 60 µg/kg, 1,720 and 30 µg/kg at 0 day, 24 h and 48 h after treatment, respectively (EMEA 2004). High residue concentrations were noted but also a quick decline in residual concentrations once the animals were no longer exposed to the drug. This has also been seen in our study. If 25-day-old broiler chickens received capsules containing the equivalent of 125 mg/kg of $^{14}$C lasalocid for seven days in feed, total residue concentrations of 1,220 and 80 µg/kg were found in liver and muscle at 0 h withdrawal time (EMEA 2004). Differences in transfer factors are noted between different studies.
Monensin is absorbed to a limited extent (11-31%) and is eliminated rapidly mainly through faeces but also by metabolisation and by biliary excretion (EFSA 2008e). Several monensin kinetic studies have been carried out. For chickens receiving feed containing 110-125 mg/kg $^{14}$C monensin, residue concentrations were highest (434-935 µg/kg) in the liver and lowest (6-59 µg/kg) in muscle. Chickens treated with feed containing 80, 100 or 120 mg monensin/kg, showed residue concentrations from undetectable to 35 µg/kg and undetectable to 39 µg/kg in respectively muscle and liver at zero withdrawal time. Treatment of chickens with 125 mg/kg monensin/kg feed for 42 days resulted in residue concentrations at zero withdrawal time of 14.8 µg/kg in liver and no residues in muscle (WHO 2009). Treatment of broilers with feed containing monensin at therapeutic level (100 mg/kg) resulted in mean liver residue concentrations of 90 µg/kg (Kennedy et al. 1998b). These studies demonstrate variable results in monensin transfer but in general very low transfer ratios were obtained which has also been shown in our study. No large differences between residue concentrations in liver and muscle were noted for monensin. Other researchers found higher or similar residue concentrations in liver, as compared to muscle (WHO 2009).

For lasalocid and monensin, lasalocid A and monensin A were retained as marker residue. A Maximum Residue Limit (MRL) of 20 µg/kg (muscle) and 100 µg/kg (liver), and of 8 µg/kg (muscle and liver) was set for respectively lasalocid and monensin (The Commission of the European Communities 2008a; The Commission of the European Communities 2010a). These studies demonstrate that treatment of chickens with feed containing lasalocid or monensin at cross-contamination levels results in lasalocid residue concentrations above MRL and monensin residue concentrations below MRL. Both compounds, belonging to the group of ionophore coccidiostats, showed a different transfer rate. Although a high transfer of lasalocid was noted, a quick decline in residue concentrations was seen once the drug was no longer provided.

In recent legislation, maximum carry-over rates of 1% and 3% are described. The 1% carry-over rate should be retained for withdrawal feed or for feed intended for target species to which no coccidiostats or histomonostats are added (The Commission of the European Communities 2009a). The therapeutic dose for lasalocid and monensin is 125 mg/kg so a 1%
carry-over rate would result in a feed containing 1.25 mg/kg lasalocid or monensin. For the 10% group, transfer ratio’s in breast muscle were 0.0065 and 0.000092 for lasalocid and monensin, respectively. Using this transfer rate, providing feed containing lasalocid at a carry-over rate of 1% would result in concentrations in breast muscle of 8.1 µg/kg and 0.115 µg/kg for lasalocid and monensin, respectively. These values are below the set MRL.
A supplementary animal study was carried out with lasalocid (described in section 4.4) to

- check whether a depletion in lasalocid concentration in breast muscle could already be determined at the end of treatment as seen in the previous lasalocid experiment (cfr 4.3);

- determine lasalocid residue concentration in breast muscle but also in thigh muscle and liver;

- compare residue concentrations between different muscle types, more in particular breast muscle versus thigh muscle;

- check whether the method of euthanasia influences the transfer factor. In all performed experiments, animals were euthanized by cervical dislocation. However, in poultry production practice, animals are euthanized in the slaughterhouse. The most common slaughter method includes neck-cutting with a minimum bleed time. The animal experiment was carried out to determine whether the bleeding influences the rate of transfer. In this way, it can be checked whether birds killed by cervical dislocation are a good representative for birds killed by conventional slaughter methods.
4.4. Residue concentrations in various poultry matrices after in-feed administration of lasalocid at cross-contamination levels

Based on:


**Abstract**

Maximum levels of coccidiostats and histomonostats in feed and food have been set in European legislation, resulting from the unavoidable carry-over of these additives in non-target feed. In this study, broilers received experimental feed that contained the coccidiostat lasalocid at cross-contamination levels of 2.5%, 5% and 10% of the maximum authorized content. Samples from the breast muscle, the upper thigh muscle and the liver were taken during the treatment and depletion periods and analyzed using liquid-chromatography tandem mass spectrometry (LC-MS/MS). Residues above Maximum Residue Level (MRL) were found in both muscle types and in the liver. Significant differences in residue concentrations were noted between the thigh muscle and the breast muscle. Different methods of euthanasia were used but no differences in measured residue concentrations were observed. Transfer factors were determined for each matrix. Using these transfer factors, a carry-over rate of 1% would result in residue concentrations below or just above the MRL.
4.4.1. Introduction

In modern agricultural practice, veterinary medicinal products are used on a large scale and may be administered in order to prevent the outbreak of diseases (Kan and Meijer 2007). In the European Union, coccidiostats are incorporated in the feed as feed additives and compounds used in poultry are the polyether ionophores and various substances obtained by chemical synthesis. Residues of polyether ionophores in foods of animal origin could cause pharmacologically adverse effects on human health since these substances possess potent cardiovascular properties, but there is no information in literature describing the association of ionophore toxicity and meat consumption (Anadon and Martinez-Larranaga 1999). Lasalocid sodium is authorized for the control of coccidiosis in the European Union at a minimum-maximum concentration of 75-125 mg/kg feed in chickens for fattening and chickens reared for laying (up to 16 weeks of age) and at a minimum-maximum concentration of 75-125 mg/kg in turkeys up to the age of 16 weeks (EFSA 2007; The European Commission 2010).

As feed manufacturing companies produce a broad range of compound feedingstuffs, different feeds have to be manufactured after each other. Traces of a feed additive may remain in the production line and end up in the beginning of the production of the following batch (EFSA 2007). Cross-contamination of additives from one supplemented feed batch to the next non-supplemented one can occur during either manufacturing, transport or even at the farm so the critical control points are in the feed mill and during transport. Feed, supplemented with feed additives is generally administered to animals on purpose and an adequate withdrawal time is prescribed so the farmer is also a critical control point. Systems such as good manufacturing practices for feed production and good agricultural practices at the farm should ensure that adequate precautions are taken (Kan and Meijer 2007). The ability of the poultry farmers to produce meat with coccidiostat residues below the MRL depends on several factors such as the ability of feed manufacturers to produce drug-free withdrawal rations and the tissue pharmacokinetics of the individual drug (Kennedy et al. 1998b). Withdrawal times should be followed but prevention of exposure is by far the preferred risk management tool (Kan and Meijer 2007). The application of good manufacturing practices by the feed operator to avoid the cross-contamination of residues
of the coccidiostat in subsequent batches of compound feedingstuffs to the largest extent possible is of major importance. However, cross-contamination of residues is unavoidable under practical conditions even if all preventive measures are applied. In recent legislation, maximum levels of 1% and 3% are set for the presence of coccidiostats or histomonostats in feed resulting from the unavoidable carry-over of these substances in non-target feed (The Commission of the European Communities 2009b).

In this study, we determined 1) the transfer of lasalocid from feed to various poultry matrices 2) whether this transfer poses human food safety risks, 3) if residue concentrations varied within muscle type, 4) whether the method of euthanasia influences the residue concentrations.

4.4.2. Material and methods

4.4.2.1. Premixes, reagents and standards

Lasalocid premix is registered for use in chickens for fattening, chickens reared for laying, and turkeys. For all target animals, the authorized maximum lasalocid content in complete feed is 125 mg/kg (EFSA 2007; The European Commission 2010). Lasalocid (Avatec 150 G) premix was kindly provided by Alpharma. Internal standard nigericin came from Sigma (Bornem, Belgium), and the analytical standard lasalocid was obtained from Alpharma (Antwerp, Belgium). Acetonitrile (ACN) originated from Biosolve (Valkenswaard, the Netherlands). HPLC grade water was generated by a Milli-Q Gard 2 system and 0.22 µm filters used for filtration of the lasalocid extracts came from Millipore (Billerica, MA, USA). Muscle and liver samples were minced with the Robot coupe 2 (C-Tech Systems, Lille, Belgium).

4.4.2.2. Preparation of the experimental feed and animal experiments

The production of the experimental feed and the animal experiment was performed at the Animal Sciences Unit of the ILVO (EC 2010/143). Animal experiments were performed following the recommendations for euthanasia of experimental animals and under supervision of ILVOs ethics committee (EC 2010/143).
The experimental feed is prepared as described in chapter 2. The animal experiment was carried out with four groups (2.5%, 5%, 2 x 10%) of 204 animals of which 192 animals were euthanized. The animals were randomly assigned to 4 treatments. Per concentration group, six animals were euthanized at day 7, 9, 11 and 13 of the treatment period and at day 1, 3, 5 and 7 of depletion period. In this experiment, 2 groups received experimental feed containing lasalocid at 10% contamination level and the birds of each group were killed in a different way to determine whether the method of euthanasia influenced the transfer ratios. The birds of one of the 10% concentration groups were killed in the same manner as in the slaughterhouse (conventional slaughterhouse method, including neck-cutting with bleeding). On the other hand, the 2.5%, 5% and the other 10% concentration groups were euthanized by cervical dislocation. The samples from the breast muscle, the upper thigh muscle and liver (without the gallbladder) were analyzed for all concentration groups. All samples were minced and grounded with a Robot Coupe 2 and stored at -18°C until analysis. A pooled sample was prepared by mixing 5 to 10 g (depending on the available amount) of each type of tissue sampled from each of the six chickens. This pooled sample was used for analysis.

The result obtained for the pooled sample of the 10% group on day 13 of the treatment period was compared to the mean result of the six individual samples to determine the reliability of the pooled samples as a reference.

4.4.2.3. Extraction and clean-up of feed samples

Centre d’Economie Rurale performed the analysis of lasalocid in feed samples. Ten grams of feed were weighed and, the internal standard nigericine (50 µl of 1 mg/ml) was added. The samples were allowed to stand for 10 min to equilibrate. Fifteen milliliters of Na₂CO₃ (10% w/v) was added and the samples were shaken manually, followed by addition of 20 ml of acetonitrile. The samples were placed on a horizontal shaker for 20 min, followed by sonication for 10 min. After centrifugation (5 min, 2000 rpm), the supernatant was transferred into a new tube and re-extraction was performed 4 times. This resulted in 100 ml supernatant. One milliliter of the supernatant was added to 3 ml of acetonitrile and 1 ml H₂O + 0.1% formic acid (FA). One milliliter was vortexed and placed in a HPLC vial.
4.4.2.4. Extraction and clean-up of poultry samples

The extraction of the lasalocid muscle and liver samples is the same as described by Mortier et al (2005). Two grammes of muscle and liver tissue were weighed. The internal standard nigericin (20 µl of 10 µg/ml) was added, the samples were briefly vortexed and allowed to stand for 10 min. Six grammes of anhydrous sodium sulphate were added to the muscle preparation and 8 g to liver. The sample was well-mixed using a spatula until a powdery mixture was obtained. Ten milliliters of acetonitrile were added to the muscle and 20 ml to the liver. The samples were vortexed, placed on a horizontal shaker for 30 min, and centrifuged for 15 min at 4000 rpm. Five milliliters of the supernatants from the muscle extract and 10 ml from the liver extract was transferred into a tube. The samples were evaporated to dryness in a warm water bath of 60 °C. The samples were redissolved in 1 ml ACN:H₂O (50:50,v:v) + 0.1% formic acid (FA) (muscle) or 2 ml ACN:H₂O (50:50,v:v) + 0.1% formic acid (liver) and vortexed for 30 sec. The samples were placed in an ultrasonic bath for 5 min and the extract was then filtered and poured into a HPLC vial.

4.4.2.5. LC-MS/MS analysis

The analysis was performed on a Waters Acquity Ultra Performance LC combined with a Waters Micromass Quattro Ultima Pt® MS instrument. A C₁₈ Symmetry® column (5 µm, 150 x 2.1 mm) was used for the separation. An isocratic mobile phase consisting of 10% HPLC H₂O:ACN (95:5,v:v)+ 0.1% FA and 90% ACN + 0.1% FA was used. The analysis of lasalocid A sodium (m/z 613.5 > 577.5, 613.5 >377.4, 613.5 >359.5) was performed in multi-reaction monitoring mode and electrospray positive ionisation mode. In this study, the Limit of Quantification (LOQ) of the method in feed was 500 µg/kg, the method LOQ was 2 µg/kg for all muscle and liver samples.

4.4.2.6. Pharmacokinetic and statistical analysis

Statistical analysis was performed with Statistica 9.0 (StatSoft. Inc., Tulsa, OK, USA) with a significance level α, set at 0.05. Statistical analysis was performed on the residue concentrations (breast muscle, thigh muscle and liver) of the individual chickens and the pooled sample collected on day 13 of the treatment period for the 10% concentration groups. The result of the pooled sample collected on day 13 was compared with the results
of the individual chickens by a single-sample t-test. Measured residue concentrations in thigh muscle and in breast muscle of the 6 individual chickens of the 10% concentration groups on day 13 of treatment period were compared with a t-test for dependent samples.

4.4.3. Results

4.4.3.1. Experimental feed

For lasalocid, the maximum allowed dose is 125 mg/kg feed; therefore values of 3.125, 6.25 and 12.5 mg/kg were expected. For 2.5%, 5% and 10% concentration groups, mean values of respectively 2.21 ± 1.06 (71%), 6.74 ± 2.76 (108%), 10.16 ± 1.71 (81%) mg/kg were reached. For the lasalocid 10 % slaughter method group, a mean value of 12.12 ± 2.77 (97%) mg/kg was reached.

4.4.3.2. Residue concentrations in breast muscle, thigh muscle and liver

Residue concentrations in various broiler matrices (i.e. breast muscle, thigh muscle and liver) during plateau phase for the 3 concentration groups (2.5%, 5%, 10%), euthanized by cervical dislocation are presented in respectively Figure 4.15. and 4.16. A plateau phase was reached at day 7 of treatment period. Residue concentrations in thigh muscle were higher compared to breast muscle for the 5% and the 10% concentration group, whereas residue concentrations in breast muscle were higher compared to thigh muscle for the 2.5% concentration group. The ratio breast muscle/liver and thigh muscle/liver was 0.16, 0.10 and 0.13, and 0.16, 0.15 and 0.22 for respectively the 2.5%, 5% and 10% concentration group. The residue concentrations in various matrices for the two 10% concentration groups, euthanized in different ways is presented in Figure 4.17.
Figure 4.15. Residue concentrations of lasalocid (µg/kg) in breast muscle and thigh muscle of the 2.5, 5 and 10% concentration group, euthanized by cervical dislocation during the treatment period (day 7 to day 13 included) and the first day of the depletion period (day 15).

Figure 4.16. Residue concentrations of lasalocid (µg/kg) in the liver of the 2.5, 5 and 10% concentration group, euthanized by cervical dislocation during the treatment period (day 7 to day 13 included) and the first day of the depletion period (day 15).
A significant difference between the residue concentrations in breast and thigh muscle of the individual samples of the 10% concentration groups was noted. Concentrations in thigh muscle were higher compared to concentrations in breast muscle. The highest concentrations were measured in the liver, which were approximately 9 and 3-5 times the concentrations in respectively breast muscle and thigh muscle.

![Figure 4.17](image_url)

**Figure 4.17.** Residue concentrations of lasalocid (µg/kg) of the 10% concentration group in breast muscle, thigh muscle and liver for the two methods of euthanasia, i.e. cervical dislocation (CD) and slaughterhouse method (Sl) during the treatment period (day 7 to day 13 included) and the first day of the depletion period (day 15).

The transfer factors were calculated by dividing the residue concentrations of the 10% concentration group at day 13 of the treatment period by the measured concentration in the feed. Transfer factors of the cervical dislocation group and the slaughterhouse method group were 0.015, 0.020, 0.095 and 0.011, 0.024 and 0.073 for respectively the breast muscle, thigh muscle, and liver. No large difference between transfer factors was seen between the different methods of euthanasia. The results of the six individual chickens was compared with a pooled sample (Table 4.3). In general, no significant difference was noted between the results of the 6 chickens and the value of the pooled sample indicating that a
pooled sample is a good representative of the six individual chickens. The difference between the mean of the six individual and the pooled samples seems larger for the slaughterhouse method group compared to the cervical dislocation group but no clear explanations can be given for this.

**Table 4.3.** Mean residue concentration and standard deviation of lasalocid in individual chickens and concentration of the pooled sample for the 10% concentration groups.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Method of euthanasia</th>
<th>Mean and standard deviation of the 6 individual chickens on day 13 of the treatment period (µg/kg)</th>
<th>Concentration of the pooled sample of 6 chickens on day 13 of the treatment period (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast muscle</td>
<td>Cervical dislocation</td>
<td>116 ± 81</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Slaughterhouse method</td>
<td>123 ± 47</td>
<td>107</td>
</tr>
<tr>
<td>Thigh muscle</td>
<td>Cervical dislocation</td>
<td>266 ± 124</td>
<td>266</td>
</tr>
<tr>
<td></td>
<td>Slaughterhouse method</td>
<td>235 ± 97</td>
<td>338</td>
</tr>
<tr>
<td>Liver</td>
<td>Cervical dislocation</td>
<td>906 ± 350</td>
<td>929</td>
</tr>
<tr>
<td></td>
<td>Slaughterhouse method</td>
<td>875 ± 244</td>
<td>1004</td>
</tr>
</tbody>
</table>

**4.4.4. Discussion**

A number of pharmacokinetic studies were carried out for lasalocid to determine the organ distribution in broilers. The treatment of chickens with 125 mg/kg feed of unlabelled lasalocid for 34 days, followed by 132 mg/kg feed of radiolabelled lasalocid for 21 days resulted in radioactive residue concentrations in liver and muscle of 11,930 and 610 µg/kg, 2,630 and 60 µg/kg, 1,720 and 30 µg/kg at 0 day, 24 h and 48 h after treatment, respectively. If 25-day-old broiler chickens received capsules containing the equivalent of 125 mg/kg of $^{14}$C lasalocid for seven days in feed, total residue concentrations of 1220 and 80 µg/kg were found in liver and muscle at 0 h withdrawal time (EMEA 2004). Mean residue concentrations of 500 µg/kg were measured in the liver if broilers received feed containing lasalocid at therapeutic dose (100 mg/kg) (Kennedy *et al*. 1998b). These studies indicated differences in transfer factors.

In the present study, residue concentrations in muscle were compared to the liver as well as between muscle types. The highest concentrations were measured in the liver compared to muscle. This has also been confirmed by other researchers who found residue
concentrations (in decreasing order) in liver, skin + fat, kidney and muscle. In our study, liver residue concentrations were 3 to 9 times higher compared to muscle. In other studies, concentrations measured in liver were 6, 15 or 20 times higher compared with the residue concentrations measured in muscle (EMEA 2004; EFSA 2007).

Significant differences were observed between the thigh and the breast muscle. This has also been seen for other molecules such as flubendazole, zoalene, polychlorinated biphenyls, diclazuril and enrofloxacine. Explanations given include the lipophilic nature of the molecule, greater intramuscular fat content in thigh muscle and the higher blood flow in the thigh (Parks and Doerr 1986; De Vos et al. 2003; Mortier et al. 2005; Reyes-Herrera et al. 2005). To our knowledge, no studies have been carried out that compared the residue concentrations from chickens, euthanised in different ways. We have demonstrated that the method of euthanasia did not influence the transfer factors from the feed to the different matrices, thus birds euthanized by cervical dislocation are representative for birds euthanised in the slaughter house.

A Maximum Residue Limit (MRL) of 20 µg/kg (muscle) and 100 µg/kg (liver) was set for lasalocid (The Commission of the European Communities 2010a). This study demonstrates that treatment of chickens with feed containing lasalocid at cross-contamination level results in lasalocid residue concentrations above MRL and may pose food safety problems. If a cross-contamination level of 1% and the transfer factors of the euthanasia method by cervical dislocation method are considered, lasalocid concentrations in breast muscle, thigh muscle and liver would be 18.75 µg/kg, 25 µg/kg and 118.75 µg/kg. Residue concentrations in thigh muscle and liver still exceed the set MRL. If a cross-contamination level of 1% and the transfer factors of the euthanasia method by the slaughter house procedure is considered, lasalocid concentrations in breast muscle, thigh muscle and liver would be 13.75 µg/kg, 30 µg/kg and 91.25 µg/kg. In that case, only residue concentrations in thigh muscle exceed the set MRL. Although significant differences were noted between the different muscle types, no specification of muscle type as target tissue for analysis has been stipulated in legislation. A pooled sample may be used for the analysis of various broiler matrices and no great influence of method of euthanasia was noted.
Chapter 5: Prediction models
5.1. An attempt to determine correlation factors between physicochemical parameters and the residues in various poultry matrices of veterinary medicinal products and to create a prediction model

Abstract

Use of veterinary drugs and coccidiostats by the poultry industry has the potential to give rise to the presence of residues in edible tissues and products of the treated birds. The transfer of veterinary drugs and coccidiostats from the feed to egg and broiler tissues is substance dependent. In this research, we tried to define possible correlations between the physicochemical parameters and the residue concentrations in the egg and the muscle of various substances. Furthermore, we tried to determine a prediction model for various poultry matrices. Literature has been searched for animal experiments with in-feed administration of veterinary drugs and coccidiostats and the residual concentrations in egg and poultry tissues were collected in a database. Equations have been proposed for the egg matrix whole egg and for the poultry matrix muscle. Where applicable, literature data has been used to assess the validity of the prediction models. At the moment, the predictive ability of the proposed models is considered to be insufficient to be used in practice.
5.1.1. Introduction

The use of veterinary drugs and coccidiostats, usually via feed or drinking water, for the prevention and treatment of disease may lead to the disposition of these drugs and their metabolites in edible tissues (Furusawa 2001; Reyes-Herrera et al. 2008; Hekman and Schefferlie 2011). Maximum residue limits have been established and withdrawal times are set to ensure consumer safety (Furusawa 2001; Hekman and Schefferlie 2011). Modern food systems for food production require the identification and the management of risks to food safety, however the usefulness of any risk assessment depends on the quality of data used as input to develop management strategies. In poultry industry, specialized breeds and strains have been developed to maximize egg and meat production efficiency (MacLachlan 2010).

Pharmacodynamics is the science of drug action on the body, on organisms or parasites within or on the body. Drugs may act on many target molecules in many tissues but for a great majority of drugs, the action on the body is crucially dependent on the chemical structure (Lees et al. 2004a). Pharmacokinetics on the other hand is the science of drug disposition: absorption, distribution, metabolism and excretion. Pharmacokinetics is generally characterized on the basis of a drug's absorption rate, volume of distribution and total body clearance; the latter two parameters are determining the terminal elimination half-life (Martinez 1998b). The pharmacokinetic behavior of a drug reflects animal physiology and the physicochemical properties of the drug (Martinez 1998b). Drug action depends on the concentration-time at the site of action, which is generally not identical but proportionally related to the plasma concentration-time profile (Toutain and Lees 2004). Clearance, total systemic clearance or clearance associated with a particular eliminating organ system is expressed in terms of volume of blood totally cleared of a substance per unit time. Total systemic clearance is the sum of all elimination processes. The rate of systemic drug clearance may be dependent on free drug concentrations, intrinsic clearance or organ blood flow. The distribution of a drug can be described by several body compartments, including the central (or blood) compartment, a peripheral (or tissue) compartment and in some instances a third or deep compartment. The third compartment describes a body space where a drug resides for a prolonged period (Martinez 1998b).
In veterinary pharmacology, different types of (kinetic) drug modeling are used. Physiologically based pharmacokinetic (PBPK) models provide a framework for incorporating information on the various production-specific factors that can have an impact on residues into the risk assessment process (MacLachlan 2010). Pharmacokinetic-pharmacodynamic (PK-PD) modeling is used to compute in vivo key pharmacodynamic parameters and enables documentation of the pharmacological profile of a drug (Lees et al. 2004b). MacLachlan (2010) describes a generic PBPK model that can be used to estimate tissue residues for different chicken production types following exposure to lipophilic chemicals dependent only on the availability of adequate chemical specific partitioning and metabolism information. Hekman and Schefferlie (2011) presented a PBPK model in order to describe or predict the residue profiles of certain veterinary drugs in eggs based on their plasma kinetics. Since the formation of both egg matrices are two distinct and totally different physiological processes, the disposition of a veterinary drug into the egg white or egg yolk compartment may be described independently from the other (Hekman and Schefferlie 2011).

In this research, possible correlations between physicochemical parameters and residue concentrations of various pharmacologically active substances in different poultry matrices after in-feed administration were investigated. Furthermore, we tried to determine prediction models based on the physicochemical parameters of different substances. Prediction models are proposed by Crescendo Stat Gent (Ghent University) for the poultry matrices whole egg and broiler muscle. Moreover, these prediction models have been evaluated by the incorporation of substances which had not been used for the development of the model. Residual concentrations provided by the prediction model were compared with residual concentrations found in the literature. The predictive value of the proposed models for egg white and egg yolk was insufficient and no prediction model could be made for the liver due to the limited data set available. Although prediction models for whole egg and broiler muscle tissue are proposed, none of these are ready to be used in practice.
5.1.2. Material and methods

5.1.2.1. Databases

Databases with selected substances, used for modeling in both laying hens and broilers were created, and were slightly different for both poultry species. The final list for both laying hens and broilers was the result of a step by step elimination process. The first list (Table 5.1) consisted of 31 substances, i.e. veterinary drugs that are available in Belgium as premix for different species (BCFI 2011) and the coccidiostats allowed for use in the European Union.

Table 5.1. Premixes of veterinary drugs and coccidiostats available in Belgium (BCFI 2011)

<table>
<thead>
<tr>
<th>Premix</th>
<th>Active ingredient</th>
<th>Class of substance</th>
<th>Type of substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aivlosin 42.5 mg/g premix pigs</td>
<td>tylosin</td>
<td>macrolides</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Apralan 100 vet premix</td>
<td>apramycin</td>
<td>aminosides</td>
<td>anti-infectives</td>
</tr>
<tr>
<td>Aurofac 100 mg/g premix</td>
<td>chlortetracycline</td>
<td>tetracyclines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Doxyprex 100mg/g premix</td>
<td>doxycycline</td>
<td>tetracyclines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Econor 10%</td>
<td>valnemulin</td>
<td>pleuromutulines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Feedmix Oxy 10%</td>
<td>oxytetracycline</td>
<td>tetracyclines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Flubenol 5% premix</td>
<td>flubendazol</td>
<td>benzimidazoles</td>
<td>antiparasitic agents</td>
</tr>
<tr>
<td>Ivomec premix 0.6%</td>
<td>ivermectin</td>
<td>macrocyclic lactones</td>
<td>antiparasitic agents</td>
</tr>
<tr>
<td>Lincomix 110 premix</td>
<td>lincomycin</td>
<td>lincomycines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Lincomix 600 premix</td>
<td>lincomycin</td>
<td>lincomycines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Linco-spectin 22+22</td>
<td>lincomycin+ spectinomycin</td>
<td>combined antibiotics</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Noramectin premix</td>
<td>ivermectin</td>
<td>macrocyclic lactones</td>
<td>antiparasitic agents</td>
</tr>
<tr>
<td>Nuflor premix 40mg/g</td>
<td>florfenicol</td>
<td>florfenicol</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Panacur 4% premix</td>
<td>fenbendazol</td>
<td>benzimidazoles</td>
<td>antiparasitic agents</td>
</tr>
<tr>
<td>Pharmasin 250mg/g premix</td>
<td>tylosin</td>
<td>macrolides</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Pracetam 10% premix for pigs</td>
<td>paracetamol</td>
<td>analgesics + antipyretics</td>
<td>NSAIDs</td>
</tr>
<tr>
<td>Promomycine 400 IU/mg premix</td>
<td>colistin</td>
<td>polymyxines</td>
<td>anti-infectives</td>
</tr>
<tr>
<td>Pulmotil 200 Vet premix</td>
<td>tilmicosin</td>
<td>macrolides</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Rheinox premix</td>
<td>amoxicillin</td>
<td>betalactam antibiotics</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Suramox 5% premix</td>
<td>amoxicillin</td>
<td>betalactam antibiotics</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Tetramin 20%</td>
<td>oxytetracycline</td>
<td>tetracyclines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Tiamutin 10% premix</td>
<td>tiamulin</td>
<td>pleuromutulines</td>
<td>antimicrobials</td>
</tr>
<tr>
<td>Premix</td>
<td>Active ingredient</td>
<td>Class of substance</td>
<td>Type of substance</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------</td>
<td>--------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Tilmovet 200g/kg premix</td>
<td>tilmicosin</td>
<td>macrolides</td>
<td>Antimicrobials</td>
</tr>
<tr>
<td>Tucoprim powder</td>
<td>sulfadiazine +</td>
<td>sulfonamides +</td>
<td>antimicrobials</td>
</tr>
<tr>
<td></td>
<td>trimethoprim</td>
<td>trimethoprim</td>
<td></td>
</tr>
<tr>
<td>Tylan 250 VET premix</td>
<td>tylosin</td>
<td>macrolides</td>
<td>Antimicrobials</td>
</tr>
<tr>
<td>Vetmulin 100g/kg premix</td>
<td>tiamulin</td>
<td>pleuromutulines</td>
<td>Antimicrobials</td>
</tr>
<tr>
<td>Maxiban</td>
<td>nicarbazin + narasin</td>
<td>non-ionophoric +</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ionophoric</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Clinacox</td>
<td>diclazuril</td>
<td>non-ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Cycostat</td>
<td>robenidine</td>
<td>non-ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Deccoxx</td>
<td>decoquinate</td>
<td>non-ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Koffogran</td>
<td>nicarbazin</td>
<td>non-ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Stenorol</td>
<td>halofuginone</td>
<td>non-ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Sacox</td>
<td>salinomycin</td>
<td>ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Salinomax</td>
<td>salinomycin</td>
<td>ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Avatec</td>
<td>lasalocid</td>
<td>ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Coxidin</td>
<td>monensin</td>
<td>ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Cygro</td>
<td>maduramicin</td>
<td>ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Aviax</td>
<td>semduramicin</td>
<td>ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
<tr>
<td>Monteban</td>
<td>narasin</td>
<td>ionophoric</td>
<td>coccidiostats</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coccidiostats</td>
<td></td>
</tr>
</tbody>
</table>

For all these substances, the Chemical-Abstract-Service (CAS) number (Martindale) was put in different computer programs or on-line applications, providing information on various physicochemical parameters. The sources that were consulted were the computer program SciFinder, the on-line applications ALOGPS, PHYS PROP and EPI Suite. Remarkably, this search resulted frequently in different values for the physicochemical parameters of the same substance. Some databases reported predicted values, others experimental values. SciFinder was the most complete database and provided information on bioconcentration.
factor (different pHs), Koc (different pHs), log D (different pHs), log P, mass intrinsic solubility, mass solubility (different pHs), molar intrinsic solubility, molar solubility (different pHs), molecular weight and pKa (most acidic and most basic). Only predictive values were available and these parameters are defined as follows by the CAS, a division of the American Chemical Society (www.cas.org).

- **Bioconcentration factor**: ratio of the concentration of a substance in an aquatic organism to the average concentration of the substance in the surrounding water

- **Koc**: organic carbon adsorption coefficient: for a substance added to a mixture of soil and water, Koc is the ratio of the amount of the substance adsorbed per unit weight of organic carbon in the soil to the concentration of the substance remaining in the water at equilibrium

- **Log D**: logarithm of the partition coefficient between octanol and water at a given pH for the mixture of the neutral and ionic forms of a compound

- **Log P**: logarithm of the partition coefficient between octanol and water for the neutral form of a compound

- **Mass intrinsic solubility**: the solubility of the neutral form of a compound expressed in grams of solute per liter of solution

- **Mass solubility**: the number of grams of a compound that dissolve in pure water at 25°C to produce a liter of saturated solution

- **Molar intrinsic solubility**: the solubility of the neutral form of a compound expressed in moles of solute per liter of solution

- **Molar solubility**: the number of moles of a compound that dissolve in pure water at 25°C to produce a liter of saturated solution

- **pKa**: negative logarithm of the acid-base dissociation constant (in the range of 0 to 14) at 25 degrees C and zero ionic strength in aqueous solutions for the most acidic and/or most basic sites in a molecule. The pKa for the most basic site is the pKa of the molecule after the most basic site has been protonated.
Only the substances with values in SciFinder were retained. Subsequently, literature search was performed on animal experiments during which the animals received these substances via in-feed administration and residue concentrations reached a plateau level. For laying hens and broilers, residue concentrations in respectively whole egg, egg white and egg yolk, and muscle and liver were recorded. In most experiments, no specification of muscle type is given but in case a distinction between thigh and breast muscle was made, only the breast muscle values were incorporated. Breast muscle is regarded as the edible tissue type in the marker residue depletion studies (VICH 2009). The final list of pharmacologically active substances for broilers and laying hens each consisted of a list of compounds of which the physicochemical parameters were known and residue concentrations in broiler or egg matrices after in-feed administration were available.

For laying hens, residue information in egg matrices following in-feed treatment was found for 15 substances, i.e. chlortetracycline, decoquinate, diclazuril, doxycycline, flubendazole, halofuginone, lasalocid, monensin, narasin, oxytetracycline, robenidine, salinomycin, sulfadiazine, trimethoprim, and tylosin (Yoshida et al. 1973a; Yoshida et al. 1973b; Yoshida et al. 1973c; Tomassen et al. 1996a; Tomassen et al. 1996b; Kan et al. 1998; Kan and Petz 2000; Furusawa 2001; Furusawa and Kishida 2002; Mortier et al. 2005; Mulder et al. 2005; Rokka et al. 2005; Hamscher et al. 2006; Lewicki 2006; EFSA 2007; EFSA 2008a; EFSA 2008b; EFSA 2008f; EFSA 2008i).

For broilers, the final list also composed of 15 substances, i.e. chlortetracycline, decoquinate, diclazuril, doxycycline, flubendazole, halofuginone, lasalocid, monensin, narasin, oxytetracycline, robenidine, salinomycin, semduramicin, sulfadiazine, and tylosin (Meredith et al. 1965; EMEA 2004; Mortier et al. 2005; EFSA 2007; EFSA 2008a; EFSA 2008c; EFSA 2008e; EFSA 2008f; EFSA 2008h; EFSA 2008i; EFSA 2008j; WHO 2009).

5.1.2.2. Determination of correlation factors and prediction model building

Prediction models were built by Crescendo Stat Gent (Ghent University) and were based on multiple linear regression models. Two model building strategies were followed. First a backward elimination procedure on a model including physicochemical variables and their interactions with the feed concentration was performed. Second, the selection of the
physicochemical variables was determined by means of a backward elimination procedure starting from a model containing all main effects (i.e. the physicochemical variables and feed concentration), and subsequently interactions of the remaining variables were added in a forward selection fashion. Experimental data obtained by ILVO (experiments by V. Vandenberge or L. Mortier (2005)) were used to build the prediction models. These substances were diclazuril, dimetridazole, doxycycline, flubendazole, halofuginone, lasalocid, monensin, narasin, nicarbazin, robenidine, sulfadiazine and tylosin for laying hens and diclazuril, doxycycline, flubendazole, lasalocid, monensin, sulfadiazine and tylosin for broilers. The Akaike’s Information Criterion (AIC) was used as selection criterion, because it is considered as an approximation of the prediction error. In this way, overfitting was avoided.

The correlations between the different physicochemical parameters are demonstrated in Table 5.2. When two physicochemical variables were highly correlated, only one variable of that pair was included in the model building process, to avoid the problem of multicolinearity. In this respect, the variables Koc and bioconcentration factor were observed to be highly correlated ($\rho = 0.996$); bioconcentration factor was selected for the model building. High correlations were also present between mass solubility and molar solubility ($\rho = 0.957$) and between mass intrinsic solubility and molar intrinsic solubility ($\rho = 0.993$). The molar solubility variables were selected for the model building.

Table 5.2. Correlations between the various physicochemical parameters

<table>
<thead>
<tr>
<th></th>
<th>bioconc</th>
<th>Koc</th>
<th>log D</th>
<th>log P</th>
<th>mass intr sol</th>
<th>mass sol</th>
<th>mol intr sol</th>
<th>mol sol</th>
<th>mol w</th>
<th>pKa acid</th>
<th>pKa basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>bioconc</td>
<td>1</td>
<td>0.996</td>
<td>0.392</td>
<td>0.305</td>
<td>-0.065</td>
<td>-0.114</td>
<td>-0.069</td>
<td>-0.118</td>
<td>0.351</td>
<td>0.193</td>
<td>-0.183</td>
</tr>
<tr>
<td>Koc</td>
<td>0.996</td>
<td>1</td>
<td>0.412</td>
<td>0.309</td>
<td>-0.071</td>
<td>-0.125</td>
<td>-0.075</td>
<td>-0.129</td>
<td>0.339</td>
<td>0.2</td>
<td>-0.221</td>
</tr>
<tr>
<td>log D</td>
<td>0.392</td>
<td>0.412</td>
<td>1</td>
<td>0.884</td>
<td>-0.686</td>
<td>-0.509</td>
<td>-0.691</td>
<td>-0.613</td>
<td>0.226</td>
<td>-0.004</td>
<td>-0.223</td>
</tr>
<tr>
<td>log P</td>
<td>0.305</td>
<td>0.309</td>
<td>0.884</td>
<td>1</td>
<td>-0.536</td>
<td>-0.33</td>
<td>-0.546</td>
<td>-0.436</td>
<td>0.398</td>
<td>-0.18</td>
<td>0.121</td>
</tr>
<tr>
<td>mass intr sol</td>
<td>-0.065</td>
<td>-0.071</td>
<td>-0.686</td>
<td>-0.536</td>
<td>1</td>
<td>0.541</td>
<td>0.993</td>
<td>0.699</td>
<td>0.013</td>
<td>0.233</td>
<td>0.136</td>
</tr>
<tr>
<td>mass sol</td>
<td>-0.114</td>
<td>-0.125</td>
<td>-0.509</td>
<td>-0.33</td>
<td>0.541</td>
<td>1</td>
<td>0.514</td>
<td>0.957</td>
<td>0.387</td>
<td>0.22</td>
<td>0.194</td>
</tr>
<tr>
<td>mol intr sol</td>
<td>-0.069</td>
<td>-0.075</td>
<td>-0.691</td>
<td>-0.546</td>
<td>0.993</td>
<td>0.514</td>
<td>1</td>
<td>0.678</td>
<td>-0.031</td>
<td>0.026</td>
<td>0.13</td>
</tr>
<tr>
<td>mol sol</td>
<td>-0.118</td>
<td>-0.129</td>
<td>-0.613</td>
<td>-0.436</td>
<td>0.699</td>
<td>0.957</td>
<td>0.677</td>
<td>1</td>
<td>0.27</td>
<td>0.279</td>
<td>0.202</td>
</tr>
<tr>
<td>mol w</td>
<td>0.351</td>
<td>0.339</td>
<td>0.226</td>
<td>0.398</td>
<td>0.013</td>
<td>0.387</td>
<td>-0.031</td>
<td>0.27</td>
<td>1</td>
<td>0.129</td>
<td>0.356</td>
</tr>
<tr>
<td>pKa acid</td>
<td>0.193</td>
<td>0.2</td>
<td>-0.004</td>
<td>-0.18</td>
<td>0.233</td>
<td>0.22</td>
<td>0.026</td>
<td>0.279</td>
<td>0.129</td>
<td>1</td>
<td>-0.107</td>
</tr>
<tr>
<td>pKa basic</td>
<td>-0.183</td>
<td>-0.221</td>
<td>-0.223</td>
<td>0.121</td>
<td>0.136</td>
<td>0.194</td>
<td>0.13</td>
<td>0.202</td>
<td>0.356</td>
<td>-0.107</td>
<td>1</td>
</tr>
</tbody>
</table>

bioconc: bioconcentration factor, Koc: organic adsorption coefficient, log D: distribution coefficient, log P: partition coefficient, mass intr sol: mass intrinsic solubility, mass sol: mass solubility, mol intr sol: molar intrinsic solubility, mol sol: molar solubility, mol w: molecular weight, pKa: acid dissociation constant (acid and basic)
The missing values for pKa (most acidic) and pKa (most basic) were imputed via a multivariate model on the basis of the other physicochemical variables. This procedure is known as single imputation. It is not the best method of dealing with missing data, but given the practical constraints it is considered as the best available way forward. A ranking of ‘importance’ of a variable in the prediction model was obtained by removing that variable from the final model and using the increase in the AIC value as a measure of importance.

Observations resulting from the highest feed concentrations in the ILVO data (>18,000 µg/kg) were not taken into account for the model building, as it was found that these observations appeared to be too influential. However, for the broilers muscle tissue, all ILVO observations could be used. The prediction models are only valid within the feed concentration range observed in the ILVO data used for the model building, so no extrapolations could be done.

The statistical package R (version 2.13.1) was used for the model building.

The developed prediction models have been validated with substances which have not been used for the model building. The predicted values were compared with the residual concentrations mentioned in the literature. Furthermore, a Jackknife validation was performed on the ILVO data. In a Jackknife validation, the prediction for an observation is based on a model that does not include that observation. This means that the model is refitted excluding one observation and that this observation is predicted on the basis of the new model. The procedure was repeated for each observation in the ILVO dataset.

5.1.3. Results

The final models and their results and restrictions are described one by one in the next sections. The physicochemical variable names were abbreviated as follows: Feed = concentration in the feed (µg/kg); BioConcFactor = the bioconcentration factor at pH 7; logD = log D at pH 7; logP = logP; MolWeight = molecular weight; Mol_intr_sol = molar intrinsic solubility; Mol_sol = molar solubility at pH 7; pKa_acid = pKa most acidic; pKa_basic = pKa most basic.
5.1.3.1. Laying hens

The best model found for the residue concentration in the whole egg was found to be:

\[
\text{Whole Egg} = -18.612 - (0.197 \times \text{Feed}) + (0.082 \times \text{BioConcFactor}) - (0.147 \times \text{Feed} \times \log D) + (0.166 \times \text{Feed} \times \log P) - (0.000287 \times \text{Feed} \times \text{MolWeight}) + (0.033 \times \text{Feed} \times \text{pKa}_\text{acid}) - (0.022 \times \text{Feed} \times \text{pKa}_\text{basic})
\]

The model building was based on 19 ILVO observations, with a feed concentration range of 47 to 14,790 µg/kg. Of the literature data, 11 observations were within this feed range, for which a prediction can be obtained without extrapolation. The variables in the model were ranked in decreasing order of importance (these values give the increase in AIC value when the variable is removed from the presented model): MolWeight (70.2), log P (62.2), log D (56.0), pKa_acid (54.3), pKa_basic (49.2) and bioconcentration factor (8.5). The significance P-values for each of the terms (physicochemical parameter or feed x physicochemical parameter) present in the final model are given in Table 5.3, together with the correlation coefficients of these terms with the residue concentrations in whole egg.

Table 5.3. P-value (p) and correlation coefficient (ρ) with the residue concentration for the various terms in the final whole egg model

<table>
<thead>
<tr>
<th>Term in final model</th>
<th>P</th>
<th>ρ</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.122</td>
<td></td>
</tr>
<tr>
<td>feed</td>
<td>&lt; 0.0001</td>
<td>0.427</td>
</tr>
<tr>
<td>BioConcFactor</td>
<td>0.016</td>
<td>-0.112</td>
</tr>
<tr>
<td>feed x log P</td>
<td>&lt; 0.0001</td>
<td>0.722</td>
</tr>
<tr>
<td>feed x log D</td>
<td>&lt; 0.0001</td>
<td>0.787</td>
</tr>
<tr>
<td>feed x MolWeight</td>
<td>&lt; 0.0001</td>
<td>0.519</td>
</tr>
<tr>
<td>feed x pKa_acid</td>
<td>&lt; 0.0001</td>
<td>0.179</td>
</tr>
<tr>
<td>feed x pKa_basic</td>
<td>&lt; 0.0001</td>
<td>0.542</td>
</tr>
</tbody>
</table>

The predicted versus the observed values, with 95% prediction interval (PI) for the ILVO observations, used to build the model, and the 11 literature validation observations are presented in Figure 5.1. The half-widths of the 95% PI on the ILVO data is between 58 and 78 µg/kg and on the literature data between 59 and 126 µg/kg. For 8 out of the 11 literature values, the 95% PI contains the observed value. The 3 observations that lie outside the 95% PI were for the molecules halofuginone, lasalocid and salinomycin. Egg concentrations of 873.7 µg/kg (halofuginone), 428.9 µg/kg (lasalocid) and -125.8 µg/kg (salinomycin) were
predicted. These were higher than the results mentioned in literature being 450 µg/kg (halofuginone), 200 µg/kg (lasalocid) or lower than the results mentioned in literature being 60 µg/kg (salinomycin). The Jackknife procedure on the basis of the ILVO data resulted in a mean absolute difference between the observed concentrations and the Jackknife predictions of 59.7 µg/kg (with a range of 2.6 to 320.2 µg/kg)

Figure 5.1. Predicted versus observed residue concentrations in the whole egg for the ILVO data used to build the prediction model (left) and for the ILVO data and literature validation data (right). The vertical bars represent the 95% prediction interval.

For the egg weight and egg yolk, predictive value of the proposed prediction models was insufficient. For egg white, the parameters Feed, MolWeight, pK$_{acid}$ and pK$_{basic}$ were found to be the best correlation parameters for residue concentrations. For egg yolk, the parameters Feed, BioconcFactor, Molar$_{intr\_sol}$, Molar$_{sol}$ and MolWeight were found to be the best correlation parameters for residue concentrations.

5.1.3.2. Broilers

The best model found for the residue concentration in the broilers muscle tissue was found to be:

\[
\text{Muscle} = -2.85 + (0.0171 \times \text{Feed}) - (25107.2 \times \text{Molar}_{intr\_sol}) - (0.165 \times \text{Feed} \times \text{Molar}_{sol}) - (0.00150 \times \text{Feed} \times \text{LogD})
\]

The model building was based on 15 ILVO observations, with a feed concentration range of 591 to 24,700 µg/kg. Of the literature data, only 2 observations were within this feed range,
for which a prediction can be obtained without extrapolation. The predicted versus the observed values, with 95% PI for the ILVO observations, used to build the model, and the 2 literature validation observations are given in Figure 5.2. The half-widths of the 95% PI on the ILVO data is between 35 and 44 µg/kg and for the 2 literature observations between 35 and 36 µg/kg. For one of the literature observations, i.e. halofuginone, the 95% PI contains the observed value, but the predicted value of 28.9 µg/kg was lower than the measured concentration (60 µg/kg). For the other observation of diclazuril the prediction is too low, the predicted value of -4.4 µg/kg is lower than the measured concentration of 90 µg/kg. The 95% PI of the diclazuril observation does not cover the observed residue concentrations.

Following variables in the model were ranked in decreasing order of importance (these values give the increase in AIC value when the variable is removed from the presented model): Molar_sol (39.2), Molar_Intr_sol (26.2) and Log D (13.8). The significance P-values for each of the terms (physicochemical parameter or feed x physicochemical parameter) present in the final model are given in Table 5.4, together with the correlation coefficients of these terms with the residue concentrations in broiler muscle.

**Table 5.4.** P-value (p) and correlation coefficient (ρ) with the residueconcentration for the various terms in the final broiler muscle model

<table>
<thead>
<tr>
<th>Term in final model</th>
<th>p</th>
<th>ρ</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.6980</td>
<td></td>
</tr>
<tr>
<td>feed</td>
<td>&lt; 0.0001</td>
<td>0.859</td>
</tr>
<tr>
<td>Molar_intr_sol</td>
<td>&lt; 0.0001</td>
<td>0.106</td>
</tr>
<tr>
<td>feed x molar_sol</td>
<td>&lt; 0.0001</td>
<td>-0.256</td>
</tr>
<tr>
<td>feed x log D</td>
<td>0.0015</td>
<td>-0.606</td>
</tr>
</tbody>
</table>

A Jackknife validation was performed on the ILVO data. The mean absolute difference between the observed concentrations and Jackknife predictions is 14.4 µg/kg with a range of 2 to 42 µg/kg for the absolute difference. This points towards a stable prediction model.
5.1.4. Discussion

In order to develop the prediction models, we combined physicochemical parameters of substances with their residue concentrations in edible tissues after in-feed administration. However, no plasma concentration versus time results and pharmacokinetic data were included. Information on plasma levels and pharmacokinetic data for broilers and especially for laying hens is scarce. Animal studies which combine information on plasma profiles and residue profiles are exceptional (Hekman and Schefferlie 2011). Different computer programs and on-line applications have been consulted for physicochemical parameters but depending on the consulted source, information on physicochemical parameters could be collected for different numbers of substances (less to 10 in one database to more than 20 in another database). Some of these databases provided information on only predictive parameters, others on experimental determined parameters. Remarkably, for some substances, large differences were noted between the predicted and the experimental values (ALOGPS, EPI Suite, PHYS PROP, SciFinder). When using the prediction models, it is important to use the same source as was used for the development of the model since values for the same parameter may vary between different sources.
Different databases with selected substances were used for laying hens and for broilers since literature information on residue concentrations after in-feed administration was different for laying hens and broilers as the physiological processes of both bird types are different. The extent of systemic drug exposure and the pharmacokinetic behavior can be affected by variation in parameters such as animal species, gender, age or disease (Martinez 1998a). Even the transition of pullets to laying hens may influence certain parameters such as the dramatically increase of serum triglycerides (MacLachlan 2010). Pharmacokinetic parameters might not be amenable to interspecies extrapolation because of interspecies differences in biotransformation, protein binding, saturation of elimination mechanisms, active processes, glycoprotein systems, drug induced alterations in physiology and differences in entero-hepatic circulation (Riviere et al. 1997). Only studies in which the substance of interest was provided with in-feed administration were considered. Veterinary drugs may be administered by different ways, such as through the intramuscular, intravenous or oral route. Oral administration in poultry experimental studies includes drinking water administration, administration by gagave or in-feed administration (Lilia et al. 2008; EFSA 2008h; Ismail and El-Kattan 2009).

The results in residual concentration in edible poultry tissues were gathered from different studies, which were carried out under different circumstances. Most residue studies which are carried out are parallel titration studies in which different doses of drugs were administered to separate groups of animals. It is important that a steady-state level in tissues is reached. The time needed to reach a steady-state level in plasma depends on the elimination half-life but also on the distribution and accumulation into tissues. The latter is important to reach plateau levels in the egg. Blood flow is considered to be the rate-limiting step for most lipophilic drugs since partitioning of lipophilic compounds from blood to tissues is assumed to be instantaneous and takes place in a homogeneous manner within the tissue compartment. Hekman and Schefferlie (2011) presented a PBPK model used to describe the relationship between the plasma concentration of a drug and its disposition in the egg yolk and the egg white compartment. However, according to Donoghue (1997), decisions based solely on the kinetics of drugs in plasma could significantly underestimate the extent and duration of contaminated eggs due to the development stages of an egg into the laying hen. Furusawa (2001) described the drug concentration in whole egg ($C_w$) as $C_w$
\[(\text{mg/kg}) = (C_a \cdot W_a + C_y \cdot W_y)/(W_a + W_y)\] with \(C_a\) and \(C_y\) being drug concentrations (mg/kg) in the albumen and yolk and \(W_a\) and \(W_y\) the weights (g) of the albumen and yolk, respectively. For lipophilic compounds, MacLachlan (2010) considered residues partition from blood into yolk and the laid egg is modeled as the sum of yolk deposited on the preceding 10 days. Since muscle contains low amounts of lipids and high concentrations of proteins, muscle can be regarded as a hydrophilic compartment and diffusion of lipophilic drugs to the muscle tissue should be lower than that of hydrophlic compounds (Grabowski et al. 2012). However, Grabowski et al. (2012) could not observe a direct correlation between physicochemical parameters of compounds and their MRL values determined for muscle tissues.

Many equations have been formulated for the determination of the transfer of residues from the feed to egg matrices and are described as concentration in egg (µg/kg): constant * concentration in feed (mg/kg) (+ constant or - constant). However most of these equations are only applicable for a certain substance (EFSA 2007; EFSA 2008c; EFSA 2008g; EFSA 2008i).

In this research we collected data from molecules from different classes of antibiotics, antiparasitic agents and coccidiostats and we tried to find a correlation between the found residue concentrations in various poultry matrices and one or more of the physicochemical parameters. The research resulted in different equations for the egg matrix whole egg and the broiler matrix muscle. In this study, the best prediction model for egg matrices was found for the whole egg, predictive value of the prediction models for egg white and egg yolk was very limited. In broiler different prediction models for muscle and liver have been built, although no satisfactory model for liver was found in our study. In most studies, residue concentrations are mentioned in muscle but no specification about the term muscle has been given. If residue concentrations were determined in breast muscle as well as in thigh muscle, only the residue concentrations measured in breast muscle were considered.

In conclusion, it can be stated that the proposed prediction models are certainly not applicable for use in practice. Prediction models are only proposed for the whole egg and broiler muscle. The prediction models for egg white and egg yolk resulted in insufficient validation results and it was not possible to build a prediction model for the liver based on the current information. Further research on different aspects of the prediction models are needed. In our opinion, pharmacokinetic parameters specific for laying hens and broilers are

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absolutely necessary to build reliable prediction models. A further limitation of the proposed prediction models is the use a specific database for physicochemical parameters. The same database, used for the development of the prediction models must be consulted in order to predict residual concentrations. Furthermore, the prediction results should only be applied for the feed concentration ranges used to set up the prediction models (50-18,000 µg/kg for laying hens and 600-25,000 µg/kg for broilers). Considering the interaction between physiochemical parameters and pharmacokinetic properties of a substance, combined with the complex species specific physiology and the between animal variability, it is however uncertain that a reliable mathematical model for prediction of residues in poultry matrices can be developed.
Chapter 6: General discussion
6.1. Carry-over and cross-contamination

Medicated/supplemented feed contains relatively high concentrations of a pharmacologically-active substance, added by veterinary prescription (medicated feed) for therapeutic purposes. Veterinary medicinal products can be present in feed by three different ways: (1) authorized, (2) unauthorized, or (3) unintentional, as a result of carry-over during production resulting in cross-contamination. Practices in current feed production lead inevitably to carry-over between consecutive production batches. Production of premixes and compound feeds, free of contamination is very difficult in practice. However, cross transference of veterinary medicinal products could be reduced with an appropriate Hazard Analysis and Critical Control Points (HACCP) system (Borras et al. 2011).

Control of carry-over must always be considered within the HACCP system of the industrial compound feed and premixes manufacturing sector, delivering feed for food producing animals in the European Union. Carry-over has to be measured with an appropriate method at least once a year or after adaptation of the facilities. Several methods, making use of a tracer, exist to measure the plant bound carry-over. Each method must follow general principles, concerning aspects of the tracer, sampling procedure, analysis methods and the interpretation of results. The carry-over is calculated as a percentage of the concentration in the first batch manufactured without tracer divided by the concentrations of the tracer in the last preceding batch containing the tracer (FEFAC 2009). In recent European legislation, maximum levels are set for the presence of coccidostats and histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed (The Commission of the European Communities 2009b). However, other veterinary medicinal products are not contemplated in this Directive so the zero-tolerance principle should be applied to these compounds in non-medicated feed which causes difficulties to the feed industry (Borras et al. 2011). The detection of cross-contamination concerns the analysis of feed, produced after a medicated/supplemented feed and deals with the detection of authorized veterinary medicinal products. The main difficulty of detecting cross-contamination is the quite low concentrations of the active ingredients (Borras et al. 2011) and the non-homogeneous distribution. In our studies, rinsing batches were carried out between the preparations of each experimental feed by mixing 10 kg of blank feed in the
custom-made mixer. These rinsing batches were analysed for the presence of the active ingredient of interest. None of the rinsing batches however contained detectable levels of the active ingredient.

6.2. Production of medicated feed and experimental feed

Directive 90/167/EEC stipulates that “the manufacturing process (of medicated feeding stuffs) must comply to the rules of good manufacturing practices”. Rules on good manufacturing practice with regard to production of medicated/supplemented feed are in place in a large majority of the member states of the EU (Food Chain Evaluation Consortium 2010). The person responsible for the production of medicated feed ascertains, by use of regular controls of the content of active ingredients, the full homogeneity and the stability of produced medicated feed (Die Keure NV warenwetgeving 2006). Premix for medicated feed must be mixed in an appropriate quantity and in a homogeneous and stable way following the manufacturer’s instructions of use, to ensure that medicated feed contains the quantity as specified. The composition of a batch of animal feed to which additives are added must respect the fixed tolerances set in the production specifications. The inclusion rate of the premix into the compounds feed should be predefined on the basis of the assessment of the efficiency of each production line, taking into account the specifications of the equipment, the accuracy of calibration and the results of homogeneity tests. A minimum of 8 samples need to be taken as close to the mixer discharge as possible, at predetermined intervals throughout the batch and put into sequentially numbered containers. When interpreting the data, one must look at variation between samples and at average recovery. A target maximum percent coefficient of variation (CV) and mean percent recovery must be established taking into account the analyte, the target levels and background values. A CV is expressed as the ratio of standard deviation (SD)/mean. The CV is expressed as a percentage and a target CV of less than 10% should be achieved in most cases (FEFAC 2009). In our study, experimental feed was prepared by mixing a premix, containing an active ingredient in blank feed at the cross-contamination levels of 2.5, 5 and 10%, respectively. For some substances of the broiler experiments and almost all substances of the laying hens experiments, the composition of the experimental feed was different from the feed for the target species, which makes it more difficult to guarantee homogeneity. Interpretations and
conclusions on the reached concentrations and homogeneity of the experimental feeds are very difficult. Sampling was carried out in the best possible way for the determination of homogeneity. Ten samples were taken as close to the mixer discharge as possible and were taken from a moving stream at the top, middle and bottom of the experimental feed. However, no duplicate analysis of the material has been performed as needed for the use of a formal test (Fearn and Thompson 2001). Since we are dealing with cross-contamination levels it is hard to determine the target maximum coefficient of variation. The acceptable higher and lower levels of active ingredients in the usual final feed are determined with regard to safety and efficacy (EMEA 1996b). Since the active ingredient was incorporated at cross-contamination levels, no higher and lower levels could be determined. For laying hens and broilers, experimental feeds (containing the active ingredient at cross-contamination levels of 2.5%, 5% and 10% of the therapeutic dose) reached concentrations within a range of respectively 84% - 118% and 71% -109% of the expected values.

6.3. Residues of veterinary medicinal products in poultry matrices

Poultry products are an important source of proteins and the demand for poultry meat is increasing throughout the world. In poultry industry, coccidiosis is the most important parasitic infection in terms of distribution, frequency and economic losses. Furthermore, field trials show the widespread presence of helminthic infections. Parasitic infections may result in production losses due to reduced appetite, lower body weights, reduced egg production and death. Veterinary medicinal products are essential in poultry production but the potential to generate drug residues in poultry products is always present (Bistoletti et al. 2011). For all licensed veterinary medicinal products, the EU has established MRLs in the major edible tissues, which are presented per tissue and per animal species. These MRL values take into account the total consumption of animal-derived foods as well as potential species differences and are established in order to minimize the risk of undesirable amounts of residues to consumers (Haritova and Fink-Gremmels 2010). Transfer factors are often used for the description of the potential for substances to transfer from feed to tissues or eggs. A transfer factor is defined as the ratio of the residue concentration in tissue or egg to the concentration of the substance in the diet. Values of transfer factors are obtained from
dosing experiments with a sufficient duration of the study to reach plateau levels of residues (MacLachlan 2008).

6.3.1. Laying hens

The administration of pharmacologically active substances to laying hen results in the appearance of residues in the whole egg. From literature and from the results obtained in our studies, it can be concluded that large differences between the different substances exist in transfer factor to the whole egg, egg white and egg yolk. Halofuginone showed the highest transfer factor, while transfer factors of monensin, tylosin and oxytetracycline were very low and this for whole egg as well as for egg white and egg yolk. Even substances belonging to the same class of molecules such as lasalocid and monensin (both ionophoric coccidiostats) or chlortetracycline and doxycycline (both tetracyclines) have different transfer factors to egg matrices (Yoshida et al. 1973a; Yoshida et al. 1973b; Yoshida et al. 1973c; Nagata et al. 1991; Tomassen et al. 1996a; Tomassen et al. 1996b; Kan et al. 1998; Furusawa 2001; Mortier et al. 2005; Mulder et al. 2005; Rokka et al. 2005; Hamscher et al. 2006; Lewicki 2006; EFSA 2007; EFSA 2008a; EFSA 2008b; EFSA 2008f; EFSA 2008i). This highlights the uniqueness of each molecule in the possibility to give rise to the presence of residues in the whole egg and the different egg matrices. For some substances, a MRL in the whole egg is set. For the substance lasalocid, the MRL in whole egg may be exceeded after exposure of laying hens to feed containing the pharmacologically active ingredient at the cross-contamination levels used in our study. For other substances (flubendazole, monensin and tylosin), the set MRL was not exceeded. No MRL was set for sulfadiazine or doxycycline but the exposure of laying hens to feed containing these substances resulted in rather high residue levels in the whole egg.

The main components of an egg are water (74%), proteins (12%) and lipids (11%) and there are 3 main egg parts, being egg yolk, egg white and egg shell (Belitz et al. 2009). Egg white and egg yolk develop at different stages of egg production (Hekman and Schefferlie 2011). The egg white proteins are formed and secreted in the magnum. Formation of the proteins takes 1-2 days and deposition of egg white around the yolk about 2-3 hours. Donoghue and Hairston (2000) demonstrated the possibility that residues may accumulate in the first eggs produced after drug exposure. Yolk components are formed in the liver, transported via the
blood to the ovary, where the yolk can be deposited in three types of follicles (Kan and Petz 2000). The formation of yolk takes 8-11 days while egg white is formed in approximately 10 h which explains the delayed peak of residue concentrations in egg yolk compared to egg albumen (Hekman and Schefferlie 2011). Residues in egg white are a reflection of plasma levels which means that 2-3 days are generally needed to achieve a constant residue level in egg white. Residues in egg yolk reflect the plasma levels during the 10 days of the rapid growth of the follicles and generally an exposure of 9-10 days is required to reach a constant residue level (Kan and Petz 2000). The difference in formation time between egg white and egg yolk influences the time needed to reach constant residue levels in the whole egg. Substances with higher residue concentrations in egg white compared to egg yolk (sulfadiazine and doxycycline) will reach constant levels in the whole egg within 3-5 days. However, for substances with yolk as preferential site such as lasalocid and flubendazole, 9 days after exposure are needed to reach constant levels. Donoghue et al. (1997) emphasized the importance of transfer and storage of drugs in pre-ovulatory yolks as a significant contributing mechanism to the production of incurred drug residues in eggs. This has also been demonstrated in our study. The time needed for residues to disappear from the whole egg is longer for substances with the yolk as preferential egg disposition site such as lasalocid and flubendazole.

Although plasma profiles are recognized to have an important influence on drug disposition in eggs, the current models of residues in eggs do not include plasma drug concentration profiles. Very few authors presented residue concentration data in plasma along with residue data in eggs (Hekman and Schefferlie 2011). Also, in our study, no plasma samples were taken and no information on the concentration ratio plasma:whole egg, plasma:egg white or plasma:egg yolk was available. Since plasma levels influence residue concentrations in egg white and egg yolk, studies during which residue concentrations are determined in both egg white/egg yolk and plasma are needed. Moreover, determination of the plasma protein binding would be interesting since this parameter determines the availability to other compartments (Kan and Petz 2000).

The administration of veterinary medicinal products to laying hens results in the appearance of residues in both egg white and egg yolk. Intestinal absorption is a first requirement, and
transport via blood/plasma is responsible for the deposition of drugs in the egg white (oviduct) and the egg yolk (ovary with follicles). Physicochemical properties of the drug and the physiology of the hen and egg formation will determine how much drug will be deposited and where (Kan and Petz 2000). Kan (2003) investigated the possible correlation between some physicochemical parameters such as lipid solubility, partitioning coefficient, pKa value and \textit{in vivo} protein binding in egg white and distribution between egg white and egg yolk. However, it was not possible to explain or predict what will happen \textit{in vivo} from variables measured \textit{in vitro}. Moreover, recent results obtained by Grabowski et al. (2012) suggest that there is no direct correlation between individual physicochemical parameters and MRL values in muscle tissues of various compounds. Also from our results, no clear correlations between one of the physicochemical parameters and the measured residue concentrations or the distribution between the different egg matrices could be defined. Highest residue concentrations were measured in egg yolk for lasalocid and flubendazole and in egg white for sulfadiazine and doxycycline, whereas no preferential disposition egg matrix could be determined for monensin. The preference of disposition in the egg yolk for the investigated substances, based on the egg yolk/egg white ratio in decreasing order is lasalocid, flubendazole, monensin, doxycycline and sulfadiazine. This decreasing order could not be observed for the values of log $P$, log $D$ and pKa acid. Log $D$ of sulfadiazine is higher compared to log $D$ of doxycycline. Log $P$ of monensin is higher compared to log $P$ of flubendazole and log $P$ of doxycycline is $> 1$. The acid dissociation constant (pKa acid) was lowest for lasalocid but highest for flubendazole. Although residue concentrations in one of the egg matrices may be higher compared to those in the whole egg, MRL legislation only considers residue concentration in the whole egg.

The egg white is a 10% aqueous solution of various proteins and has a negligible lipid content (0.03%). Yolk is a fat in water emulsion that contains 32.6% of lipids, of which the fatty acid composition is correlated with that of the feed. The main components of egg yolk are low density lipoproteins (68%), high density lipoproteins (16%), livetins (10%) and phosvitins (4%) (Belitz et al. 2009). These yolk LDLs are synthesized in the liver of the laying hen and are similar to the very low density lipoproteins present in chicken blood. The water-soluble globular protein fraction of livetin (egg yolk) can be separated electrophoretically into $\alpha$-, $\beta$- and $\gamma$- livetins, which have been proven to correspond to chicken blood serum
proteins, i.e. serum albumin, α₂-glycoprotein and γ-globulin (Belitz et al. 2009). The process of yolk formation in rapidly growing oocytes is achieved by receptor-mediated endocytosis of the hepatically synthesized circulating yolk protein precursors, very low density lipoproteins and vitellogenin, which both bind to the oocyte vitellogenesis receptor (Elkin et al. 1995; Elkin et al. 1999). Almost 12% of the egg white are proteins. The main proteins are the glycoproteins; ovalbumin (54%), ovotransferrin (13%), ovomucoid (11%) and α- and β-ovomucin (1.5- 3.0%); and lysozyme (3.5%) (Roberts 2004). Multiple serum proteins, including very low density lipoprotein, vitellogenin and some egg white precursors (such as ovotransferrin and lysozyme) are present in large quantities in the blood stream of chickens during the laying period (Liou et al. 2007). Ovotransferrin (egg white protein) is identical to serum transferrin in chicken (Belitz et al. 2009). Further investigation of binding of molecules to different types of proteins (plasma protein, yolk lipoprotein precursors, yolk proteins and egg white proteins) is recommended in order to understand possible processes governing the disposition of veterinary medicinal products in eggs and thus the transfer ratio to the egg and the distribution into egg white and yolk, respectively.

6.3.2. Broilers

The exposure of animals to undesirable substances in feed materials and drinking water as well as the therapeutic use or abuse of veterinary medicinal products may result in drug residues in tissues (Haritova and Fink-Gremmels 2010). For the substances, we investigated, an MRL is set in muscle and liver. The exposure of broilers to feed containing lasalocid, sulfadiazine or doxycycline at the investigated cross-contamination levels may result in some cases to residue concentrations above the MRL. Various transfer factors were observed between substances, belonging to different classes of molecules. Based on literature data, diclazuril was the substance with the highest transfer rate to muscle, whereas monensin showed the lowest transfer factor. Differences in transfer factor were also seen between substances belonging to the same class, such as monensin ad lasalocid (both ionophoric coccidiostats). The transfer from feed to liver was the highest for diclazuril, followed by halofuginone and the lowest for monensin (Mortier et al. 2005; EFSA 2008e; WHO 2009). In our study, residue concentrations in liver compared to breast muscle were higher (lasalocid,
doxycycline, flubendazole metabolites and tylosin) or in the same range (sulfadiazine, flubendazole and tylosin).

According to MacLachlan et al. (2010) the partition of lipophilic compounds from blood to tissues is assumed to be instantaneous and takes place in a homogeneous manner within the tissue compartment. Several researchers have proposed the use of body fluids such as blood to determine and monitor drug concentrations in live animals and carcasses of poultry (Haasnoot et al. 2005; Schneider et al. 2007; Reyes-Herrera et al. 2011). Highest correlations were found between serum-liver samples, followed by serum–muscle samples, lowest correlations were obtained for serum-skin+fat samples. However, higher concentrations of both sulphonamides were found in serum compared to tissues during withdrawal time, whereas enrofloxacine concentrations were 2-3 times higher in muscle compared to blood and serum concentrations (Haasnoot et al. 2005; Schneider et al. 2007; Reyes-Herrera et al. 2011). Similarities between the enrofloxacin incorporation patterns for blood versus muscle residue concentrations support the ability to use blood to estimate muscle residue concentrations (Reyes-Herrera et al. 2011). Further research determining the relationship between blood/serum and target tissue is however needed.

The use of blood samples as an initial screening method to detect residues of veterinary medicinal products could represent a simple and inexpensive option (Reyes-Herrera et al. 2011). Only a small portion of carcasses is tested for the presence of violative residues because of the loss of valuable edible products and the cost of sampling all carcasses. Unlike tissue, blood is readily available from all birds at the beginning of processing. Blood samples are easy to collect, would not interfere with the processing procedure and have no economic value. Furthermore this matrix does not require special, complicated, or expensive preparations before analysis (Haasnoot et al. 2005; Schneider et al. 2007; Reyes-Herrera et al. 2011).

Muscle tissues are considered to be the major consumable product of slaughter animals (Haritova and Fink-Gremmels 2010). Broiler meat contains high protein and low fat content (Yang et al. 2010). Animal tissues can be classified as hydrophilic (more or less) and lipophilic based on physicochemical structure only, muscles can be classified as a hydrophilic compartment. Thus water-soluble drugs should show high affinity and distribution to the
muscle tissues (Grabowski et al. 2012). For some compounds such as enrofloxacin, lasalocid, diclazuril, zoalene, polychlorinated biphenyls and flubendazole investigated in our studies and by other researchers, significant differences were noted between breast muscle and thigh muscle (Mortier et al. 2005; Reyes-Herrera et al. 2005). For other molecules such as sulfadiazine and doxycycline, investigated in our studies, no significant differences between the different muscle types were observed (Vandenberge et al. 2012). Most of the compounds with marked differences in residue concentrations between the different muscle types, showed higher residue concentrations in thigh muscle compared to breast muscle. However, enrofloxacin residue concentrations were higher in breast muscle compared to thigh muscle (Reyes-Herrera et al. 2005). Explanations such as a greater intramuscular fat content in thigh muscle and a higher blood flow are proposed (Mortier et al. 2005; Reyes-Herrera et al. 2005). Muscle tissue consists of muscle fibers, connective tissues and fat, but in poultry, thigh muscle and back muscle contain more connective tissue and fat than breast muscle. Breast muscle consists only of white muscle fibers, whereas thigh muscle contains both red and white fibers (Grashorn 2010). Although differences in residue concentrations between different muscle types were found, no specification of muscle type is mentioned in MRL legislation. The MRL, the maximum concentration of residues in a food product accepted, is the derivative of the no observed effect level value (NOEL), related safety factor (SF) and consequently the acceptable daily intake (ADI) (Grabowski et al. 2012). In the guideline of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products considering the sample collection from animals in marker residue depletion studies, breast muscle is described as edible muscle type in poultry (VICH 2009).

### 6.4. Prediction model for residue transfer in eggs and poultry tissues

The prediction models have been based on 2 different datasets, which have been combined and different models are presented for laying hens and for broilers. The first dataset is the dataset with physiochemical parameters. Literature has been searched for physicochemical parameters, but remarkably information was very limited. Different databases (computer programs and on-line databases) have been consulted and resulted in predicted or experimentally determined values ( ALOGPS, EPI Suite, PHYS PROP, SciFinder). Interestingly,
for some compounds the values of certain physicochemical parameters varied substantially, depending on the consulted sources. Finally, it was decided to use the computer program SciFinder as this provided the largest dataset of information. This implies that only data from SciFinder may be entered in the proposed mathematical models, which restricts the amount of molecules that can be investigated using the proposed formulas. The other database consisted of residue concentrations for different compounds based on literature. Physicochemical properties of drugs, physiological parameters of birds and pharmacokinetics but also analytical methods are the 3 main factors that must be considered when evaluating drug residues. The wide variation in experimental design concerning medication route, duration of treatment, dose and analytical methods for residue monitoring makes it difficult to compare the results (Hafez 1991). This has also been noticed during the literature search e.g. residue concentrations of salinomycin varied substantially between studies. In 2 different studies, chickens received feed, containing 60 mg/kg salinomycin for 14 days, resulting in residue concentrations in liver and muscle varying from 14 to 1,100 µg/kg in liver and from 2.5 to 670 µg/kg in muscle (EFSA 2008i). Transfer studies following a strict protocol concerning dose regimen, route of administration, type and age of bird, treatment duration, analytical methods and analytical sample types are needed.

Since only limited public information on pharmacokinetic data is available in broiler chickens and pharmacokinetic data are totally lacking in laying hens, data on this particular field of interest could not be included in the predictive mathematical model. However, the input of both physicochemical and pharmacokinetic data would improve the predictive mathematical model drastically since physicochemical parameters influence the pharmacokinetic behavior and distribution of a compound (Kan and Petz 2000). The volume of distribution, clearance, mean residence time and elimination half-life influence not only the amount but also the persistence of residue concentrations in poultry matrices. Animal studies investigating pharmacokinetic data by measuring blood or serum levels in laying hens and broilers combined with residue concentration studies in the respective bird types would provide very useful information. Studies in laying hens, measuring residue levels in blood, liver and the separate egg matrices could provide insight in the possible correlation between liver and yolk residual concentrations. Furthermore, specific protein binding of active ingredients
needs to be investigated including binding to plasma proteins, yolk precursor proteins, and the proteins of egg white and egg yolk.

6.5. Future perspectives

The presence of residues in edible poultry matrices depends on various factors during the total food chain. This includes the production of medicated/supplemented feed, consumption of this feed by non-target animals and the possibility of each substance to transfer from feed to food. More study on different stages of the “farm to fork principle” is necessary. Further research is needed at the level of (1) occurrence and importance of cross-contamination, (2) the processes governing the distribution towards edible tissues of poultry, especially the egg, (3) the unambiguous determination of the compound dependent physicochemical parameters and the species specific pharmacokinetic behavior, (4) the identification of variables that can be used as predictive tool for the determination of residue concentrations in edible poultry matrices.

Firstly, investigation on the risk of carry-over of active ingredients from medicated/supplemented feed to non-medicated/non-supplemented feed is needed. In current medicated/supplemented feed production, cross-contamination of active ingredients from one batch to the following batch during production is considered to be unavoidable. Veterinary drugs as well as feed additives may be transferred into poultry matrices at varying degrees but legislation with regard to maximum carry-over levels is only set for coccidiostats and histomonostats. An amplification of this legislation setting maximum levels for the presence of veterinary drugs in non-target feed, due to carry-over of these substances is recommended. The technological equipment of the feed mill influences the level of cross-contamination and HACCP studies are essential. However, cross-contamination may also occur during storage and transport of the feed and at the farm. Identification of the critical point at each of these different stages needs to be determined. The cross-contamination behavior and the way cross-contamination can occur depends on the properties of the active ingredient and the used formulation. Further research of these properties is needed, taking into account the possible differences in composition/structure between target feed and non-target feed, which may be cross-contaminated.
Secondly, the processes governing the distribution towards edible tissues of poultry need to be studied. In literature and in our research, differences between substances in transfer factors from feed to poultry matrices were noticed for broilers as well as for laying hens. Since the number of veterinary medicinal products registered for use in laying hens is limited, no MRL values are set for a lot of compounds in eggs. However, some coccidiostats (e.g. halofuginone, lasalocid, diclazuril, nicarbazin) show a high transfer ratio towards egg and may pose problems for food safety. Eggs are produced on a daily base and are frequently consumed thus special attention needs to be paid for laying hens. In laying hens, some substantial differences between residue concentrations in egg white and egg yolk were noticed for some substances. Further research on the egg matrix of preference is needed to evaluate the use of a certain egg matrix as a target tissue for routine analysis. For broilers, differences in residue concentrations between different muscle types were observed for some substances. Furthermore, in legislation, muscle is defined as one of the tissues to be analysed during monitoring plans but the target muscle type is not specified.

Thirdly, the substance dependent physicochemical parameters and the species specific pharmacokinetic behavior must be investigated. For some substances, large differences in values for some physicochemical parameters are noticed depending on the consulted source. A determination of some physicochemical parameters such as log P, log D, bioconcentration factor, pKa (at different pHs: plasma pH, egg white pH and egg yolk pH) using standardized protocols would be interesting. Limited species specific pharmacokinetic data based on blood levels are available in literature, especially for laying hens. Also, determination of various pharmacokinetic parameters such as volume of distribution, clearance, and consequently the elimination half-life is interesting for broilers as well as for laying hens. Besides these, determination of the oral bioavailability and plasma protein binding is mandatory as well.

Finally, variables influencing the residual concentrations in edible poultry matrices needs to be investigated. In this way, blood/matrix partition coefficient could be determined and the use of blood/plasma as a predictive tool, especially for broilers may be evaluated. Further research on possible correlations between physicochemical/pharmacokinetic parameters and blood or plasma concentrations/matrix concentrations is needed in order to develop
reliable predictive models. Special attention needs to be paid to the egg. Due to specific physiological aspects of the hen (changes in physiology, more specific in liver metabolism and lipid metabolism by the onset of egg production) and the different processes governing the development of egg white and egg yolk, residual concentrations in the egg are not only and mainly determined by the physicochemical properties of the active substance and the pharmacokinetic characteristics of the active substance in the laying hen. As egg yolk precursors are formed in the liver and transported by the blood to the oocyte, research on binding of active ingredients (parent molecule and/or metabolites) to the yolk precursors, i.e. vitellogenine and VLDL is necessary. These yolk precursors are transported into the oocyte by receptor-mediated endocytosis. Knowledge on this receptor mediated uptake of yolk precursors combined with active substance or the active substance itself would be very valuable. Once the yolk precursors are delivered into the oocyte, they are proteolitically processed into several fragments. Research on binding of the active ingredient to the yolk proteins and lipoproteins as well as on binding to egg white proteins such as ovalbumin (major egg white protein) is recommended. Since residue concentrations in egg white are suggested to be correlated with plasma concentrations, knowledge on binding of the active ingredient to plasma proteins such as albumin but also ovalbumin is needed.


Die Keure NV warenwetgeving. 2006 Koninklijk besluit van 21 februari 2006 tot vaststelling van de voorwaarden inzake erkenning en toelating van inrichtingen in de diervoedersector


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In poultry industry, the treatment of animals with pharmacologically active substances by mass-application, via drinking water or in-feed administration is generally accepted for the control and treatment of diseases. Cross-contamination, resulting from the carry-over of undesirable trace amounts of pharmacologically active ingredients from a medicated/supplemented feed to a non-medicated/non-supplemented feed, may occur at different stages of the production process, during the storage and transport but also on the farm. Although preventive actions are taken by the feed industry, cross-contamination is considered to be unavoidable. The consumption of a feed, containing a pharmacologically active ingredient due to cross-contamination, by a non-target species such as the laying hen may generate unwanted residual concentrations of these substances in edible matrices.

In Chapter 1, a general introduction on the feed industry as well as the broiler industry and laying hen industry, is presented. Attention is paid to the production of medicated/supplemented feed and to the consumption and production of broiler meat and eggs, respectively. Furthermore, information on residue issues and the related legislation and residue monitoring is provided.

The general aim of this thesis was to determine the transfer factors from feed to poultry matrices for various veterinary drugs and feed additives, belonging to different classes of pharmacologically active substances. The animal experiments (broiler and laying hen) and the preparation of the experimental feed are described in Chapter 2. The investigated substances were sulfadiazine (sulphonamides), doxycycline (tetracyclines), flubendazole (benzimidazoles), tylosin (macrolides), lasalocid and monensin (both ionophoric coccidiostats). The transfer of these veterinary drugs and feed additives from feed to food was investigated in broilers as well as in laying hens. Furthermore, various parameters which may influence the distribution of a substance to a specific poultry matrix, were studied. For this reason, animal experiments during which the animals received an experimental feed for a 14-day period were carried out. This experimental feed contained a pharmacologically active substance at cross-contamination levels of 2.5%, 5% and 10% of the therapeutic dose. Residual concentrations in various matrices of broilers (breast muscle, thigh muscle and liver) and laying hens (whole egg, egg white and egg yolk) were determined at different time points during the experiment.
In **Chapter 3**, the results of the laying hen cross-contamination experiments are presented. In these experiments analyses were performed on the whole egg as well as on egg white and egg yolk. In general, it can be concluded that large differences in transfer factors between the investigated substances were observed. Lasalocid, sulfadiazine, doxycycline and flubendazole showed a higher transfer compared to monensin and tylosin, which showed a negligible transfer. For the substances with no set Maximum Residue Limit (MRL) in eggs such as sulfadiazine and doxycycline, residual amounts were detected in the whole egg as well as in each of the egg matrices. In case a MRL in whole egg was set, residue concentrations may exceed this MRL (lasalocid) or were below MRL (flubendazole, monensin and tylosin). A large variation in preference for either egg white or egg yolk was noticed. Residue concentrations of lasalocid and flubendazole were highest in egg yolk, whereas sulfadiazine and doxycycline had a preference for the egg white. No clear conclusions could be made concerning the possible correlation between some physicochemical parameters such as lipophilicity, acid dissociation constant and distribution coefficient and the preference for one of the egg matrices: egg white or egg yolk.

In **Chapter 4**, the results of the broiler cross-contamination experiments are presented. In these studies, residue concentrations were measured in breast muscle, upper thigh muscle and liver. Generally, large differences in transfer factors for the six investigated substances were observed. Transfer factors of lasalocid, sulfadiazine and doxycycline were higher compared to flubendazole. Low transfer factors were determined for monensin and tylosin. MRLs are set for all substances in muscle and liver. Residue concentrations of some cross-contamination groups of doxycycline, sulfadiazine and lasalocid exceeded this MRL. Liver concentrations were higher compared to muscle concentrations for lasalocid, doxycycline and tylosin. A significant difference in residue concentrations between the two different muscle types (breast muscle versus thigh muscle) was obtained for flubendazole and lasalocid.

An attempt to design a predictive mathematical model for broilers and laying hens is presented in **Chapter 5**. A mathematical formula was presented for broiler muscle and the whole egg. However, the practical relevance of these formulas for use in the field is very limited for several reasons. The presented formulas are based on physicochemical
parameters and residual concentrations in edible poultry matrices after in-feed administration of veterinary medicinal products. Very limited information considering the plasma concentration-time course and the possible correlated pharmacokinetic parameters is available. Therefore, such data could not be used for the development of the models and no appropriate prediction models could be established.

In Chapter 6, general conclusions are presented and discussed. Topics such as cross-contamination, residues in poultry matrices and legislation are highlighted. In addition, some critical factors and some hiatus in literature as well in the performed research are highlighted. The absence of standardized animal experiments and analytical methods, the limited information on pharmacokinetics as well as on species specific physiology are a few examples. This results in some suggestions on further research concerning different steps in the “farm to fork” principle.
Samenvatting
In de pluimveehouderij wordt vaak geopteerd voor een groepsbehandeling van de dieren via drinkwatermedicatie of via gemedicineerd/gesupplementeerd voeder, en dit voor de controle en de behandeling van ziektes. Kruiscontaminatie, voortvloeiend uit de overdracht van ongewenste stoffen van een gemedicineerd of gesupplementeerd voeder naar een niet-gemedicineerd of niet-gesupplementeerd voeder kan gebeuren gedurende de aanmaak, de opslag en het transport van het gemedicineerd of gesupplementeerd voeder, maar kan ook optreden op het pluimveebedrijf zelf. Kruiscontaminatie wordt ondanks talrijke maatregelen in de veevoederindustrie als onvermijdelijk beschouwd. De toediening van een voeder, dat een farmacologisch actieve substantie bevat door kruiscontaminatie, aan niet-doeldieren kan aanleiding geven tot de aanwezigheid van residuen in eetbare producten.

In Hoofdstuk 1 wordt een algemeen overzicht gegeven van de veevoederindustrie en de vleeskuiken- en leghennenindustrie. In dit hoofdstuk komt onder meer de productie van gemedicineerd/gesupplementeerd voeder, en de productie en consumptie van kippenvlees en eieren aan bod. Tevens wordt informatie omtrent de residu problematiek en de hiermee verbonden wetgeving en controles verschaft.

De algemene doelstelling van deze thesis was het bepalen van de mate van overdracht van voeder naar pluimveematrices voor diverse diergeneesmiddelen en voederadditieven, behorende tot verschillende klassen van farmacologisch actieve bestanddelen. De dierexperimenten (vleeskuikens en leghennen) en de aanmaak van het experimenteel voeder worden beschreven in Hoofdstuk 2. De onderzochte substanties waren sulfadiazine (sulfonamiden), doxycycline (tetracyclines), flubendazole (benzimidazoles), tylosine (macroliden), lasalocid en monensin (beide ionofore coccidiostatica) en de overdracht werd onderzocht bij zowel vleeskuikens als bij leghennen. Bovendien werden diverse factoren, die een rol kunnen spelen bij de distributie van een substantie naar een bepaalde matrix onderzocht. Hiertoe werden dierproeven uitgevoerd waarbij de dieren gedurende 14 dagen een experimenteel voeder toegediend kregen. Dit voeder bevatte een farmacologisch actieve substantie aan kruiscontaminatie niveaus van 2.5%, 5% and 10% van de therapeutische concentratie. Residuconcentraties in diverse matrices van vleeskuikens en leghennen werden op regelmatige tijdstippen bepaald.
In Hoofdstuk 3 worden de resultaten van de overdrachtstudies uitgevoerd bij leghennen besproken. Bij de leghennenstudies werden enerzijds residuconcentraties gemeten in volledig ei en anderzijds in eiwit en dooier afzonderlijk. Algemeen kan men besluiten dat er een grote variatie werd waargenomen in overdrachtsfactor van de zes onderzochte substanties. Lasalocid, doxycycline, sulfadiazine en flubendazole werden in hogere mate overgedragen in vergelijking tot monensin en tylosine, die een te verwaarlozen overdracht vertoonden. Voor sulfadiazine en doxycycline, waarvoor geen Maximale ResiduLimiet (MRL) in ei beschikbaar is, werden residuconcentraties in volledig ei en in eiwit en dooier waargenomen. Voor substanties, waarvoor een MRL in ei vastgelegd is, werden residuconcentraties boven MRL (lasalocid) of onder MRL (flubendazole, monensin en tylosine) genoteerd. De hoogste residu concentraties van lasalocid en flubendazole werden waargenomen in de dooier. Sulfadiazine en doxycycline vertoonden een duidelijke voorkeur voor eiwit. Daarenboven werd een grote variatie waargenomen tussen de diverse substanties betreffende hun voorkeur voor eiwit of eidooier. Er kon geen duidelijke correlatie opgemerkt worden tussen enerzijds de voorkeur voor eiwit of eidooier en anderzijds de fysicochemische eigenschappen zoals vetoplosbaarheid, dissociatieconstante en distributiecoëfficiënt.

In Hoofdstuk 4 worden de resultaten van de overdrachtstudies, uitgevoerd bij vleeskuikens, besproken. Bij de vleeskuiken studies werden niet alleen residuconcentraties gemeten in borstspier maar ook in bilspier en lever. Algemeen kan men besluiten dat er opnieuw duidelijke verschillen werden waargenomen in overdrachtsfactoren van de zes onderzochte substanties. Lasalocid, doxycycline en sulfadiazine werden in hogere mate overgedragen dan flubendazole. Monensin en tylosine werden slechts in beperkte mate overgedragen. Voor elk van de onderzochte substanties zijn er MRLs in spier en lever vastgelegd. Deze MRL waarden werden overschreven voor enkele kruiscontaminatie groepen van lasalocid, sulfadiazine en doxycycline. Voor lasalocid, doxycycline en tylosine werd een hogere residuconcentratie waargenomen in de lever in vergelijking met de spier. Daarenboven werd een significant verschil in residuconcentraties in borstspier in vergelijking met bilspier voor flubendazole en lasalocid waargenomen.
In **Hoofdstuk 5** wordt een poging ondernomen om een voorspellend model voor te stellen voor de overdracht van substanties vanuit voeder naar pluimveematrices. Voor volledig ei en spier werd een afzonderlijke formule voorgesteld. De relevantie van deze formules voor gebruik in de praktijk is echter zeer beperkt omwille van diverse redenen. Deze formules zijn gebaseerd op enerzijds fysicochemische parameters en anderzijds op residuconcentraties in matrices van dierlijke oorsprong tengevolge van een in-voeder medicatie. Gezien de informatie met betrekking tot het plasmaconcentratie-tijdsverloop en relevante farmacokinetische parameters beperkt is, konden dergelijke data niet worden aangewend bij de ontwikkeling van het model, waardoor geen geschikte modellen konden worden tot stand gebracht.

Curriculum Vitae


Valerie Vandenberge is auteur van meerdere wetenschappelijke publicaties. Ze gaf lezingen op internationale congressen en begeleidde ook een eindwerk.

Publicaties in internationale tijdschriften met peer-review


Abstracts, proceedings, posters en lezingen op internationale congres sen.


Begeleiding van eindverhandelingen

Dankwoord
Het ei is gelegd. Na 4 jaren van bloed, zweet en tranen, kan ik met enige trots mijn doctoraat voorstellen. Een doctoraat tot een goed einde brengen, kan vergeleken worden met een “start-to-run”. Al lijkt het einddoel in het begin onmogelijk, training na training, kom je dichter tot het vooropgestelde einddoel: de wedstrijd en natuurlijk de finish. Dit kan echter niet zonder de deskundige begeleiding van de coaches. Ik heb het geluk gehad om 2 “Evy’s” te hebben: Prof. Dr. S Croubels en Dr. Apr E. Daeseleire. Dus Els en Siska, dank voor de begeleiding gedurende de voorbije jaren in een toch niet altijd even gemakkelijke materie van transferfactoren, residuen, physicochemische parameters, farmacokinetiek en fysiologie. Een ei zal nooit meer zomaar “een ei” zijn.

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