SESSION E: FOOD SAFETY AND RESIDUES

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Food Safety and Residues

INTRODUCTION

Traditionally, genotoxic compounds are considered as potential carcinogens and not to have a threshold for their effects, while for nearly all other biological endpoints a threshold is assumed. Genotoxicity tests are generally not designed to establish a NOAEL but only to give a qualitative answer, in a series of appropriate tests, whether a substance is genotoxic or clearly not. Therefore, results of genotoxicity testing normally do not affect the value of the ADI but influence whether an ADI can be established or not. As a consequence genotoxic substances, such as nitrofurans, nitroimidazoles or carbadox, are commonly banned for use in farm animals in the EU. The validity of this approach is questionable since genotoxic substances, such as nitrofurans, nitroimidazoles or carbadox, are commonly banned for use in farm animals in the EU. The validity of this approach is questionable since genotoxic substances, such as nitrofurans, nitroimidazoles or carbadox, are commonly banned for use in farm animals in the EU.

KN09 Risk assessment of substances which are both genotoxic and carcinogenic
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MODELS OF LINEARISED DOSE-RESPONSE EXTRAPOLATION TO A ‘VIRTUAL SAFE DOSE’

Quantitative risk assessment has been used for non-threshold effects, such as cancer, by applying linearised dose-response extrapolation to a virtually safe dose or to a safe exposure/“no residue” level [2,3]. The differently sophisticated mathematical models are the (i) Mantel-Bryan probit model; (ii) one-hit linear extrapolation model; (iii) multistage model and (iv) tolerance distribution models, such as Weibull distribution for extrapolation of the region of experimental data to a region of acceptable risk, which may be defined as the level of a compound of carcinogenic concern in the diet of test animals that corresponds to a maximum lifetime risk of cancer in the test animals of 1 in 1 million. Those models are, however, heavily dependent on the mathematical model used and on the assumption that at least one event, in the sequence of events leading to cancer, is linearly related to dose. Because of the high doses used to generate a reproducible incidence of adverse effects in experimental studies of subchronic or chronic toxicity, extrapolation over three or more orders of magnitude beyond the range of experimental data to very low exposure levels may be necessary.

This extrapolation has to make assumptions relating to the (i) choice of starting point taken; (ii) slope of the curve used and (iii) mechanisms of interspecies differences and interindividual variability taken into account. The slope of the dose-response and the mathematical model used will have a profound influence on the final risk characterisation. It will be the major
variable in the numerical estimate derived by quantitative risk assessment with extrapolation to low exposure for genotoxic and non-genotoxic compounds. Although linear low-dose extrapolation is based on the worst case assumption, it largely ignores certain aspects, such as human variability, of the biology of the hazard or toxicokinetic influences. The common use of 95th percentile to produce a dose-response relationship, which allows for variability in the test animals, may not be appropriate to represent the slope arising from human variability and will not necessarily represent the dose-response relationship in the human population or various subpopulations. The models are inappropriate if hormesis, which has to be considered for genotoxic carcinogenesis, is demonstrated for a compound. A suitable database is mostly lacking for developing more predictive biologically based dose-response models (e.g. the Molgakar-Venzon-Knudsen model). These models require extensive knowledge of toxicokinetics, -dynamics and rate-limiting stages to transform the external dose into an internal dose and target organ dose and to predict the target organ response and toxic response. To fill the gaps in the knowledge of mode of action (MOA) for chemical carcinogenesis, the International Programme on Chemical Safety has initiated a conceptual framework for evaluating the key pathophysiological events and their association with tumour response, their dose-response relationships and temporal association [4]. Potential errors arise from the extent of unquantified assumptions being made, such as the arbitrary selection of the slope. Thus, the actual numerical values of the dose levels of risk-specific exposure derived by simple mathematical models, such as the one-hit or linearised multistage models, are largely determined by the model than by the experimental data.

THE ‘CARBADOX STORY’

The spurious precision with such ‘safe’ risk estimates and the considerable diversity of the prediction of mathematical models for extrapolation with variations by a factor of orders of magnitude are highlighted by the results of the safety evaluation of the residues of the antimicrobial growth promoter carbadox by the US-FDA and FAO/WHO-JECFA [3,5]. On its first evaluation JECFA could not establish an ADI because of evidence for carcinogenicity of the parent substance and its metabolite desoxycarbadox, which act by a genotoxic mechanism. At that time, no exposure to carcinogenic carbadox residues was assumed when the concentrations of the non-carcinogenic quinoxaline-2-carboxylic acid (QCA) metabolite were at or below 0.03 mg kg⁻¹ in liver and 0.005 mg kg⁻¹ in muscle, established as MRL. The US-FDA codified a revised tolerance for carbadox residues and their risk to the consumer based on determination of ‘no residue levels’ of carcinogenic carbadox residues corresponding to a maximum lifetime risk of cancer in the test animals of 1 in 1 million. The calculation using low dose linear extrapolation models, however, revealed largely divergent ‘virtual safe doses’ of desoxycarbadox: 680 ng kg⁻¹ was calculated using the Mantel-Bryan model and 61 ng kg⁻¹, respectively, with one-hit linear extrapolation. It was noted, at the reevaluation by JECFA, that residues of carcinogenic concern were detectable by a more sensitive analytical method at the MRL of QCA at five times the ‘no residue level’. JECFA rejected the approach of US-FDA. No ADI for carbadox was allocated and the MRL for the metabolite QCA was withdrawn because of exposure to carcinogenic carbadox residues or at below the MRL and no amounts of residues in food could be determined that represent an acceptable risk to the consumers. JECFA has not to date used the approach of extrapolating irreversible effects of chemicals at low doses for which thresholds have not been identified to a ‘virtual safe dose’ using mathematical models for evaluating residues of veterinary drugs in food. In summary, the risk estimates resulting from mathematical models of linear low-dose extrapolation are considered poorly or not to be scientifically credible.

MARGIN OF EXPOSURE

Recently a different approach to the low exposure risk assessment of substances that are both genotoxic and carcinogenic has been proposed by the Scientific Committee of the European Food Safety Agency, known as the margin of exposure (MOE) approach [1]. This model is designated to provide advice from risk assessors to risk managers to reduce the unavoidable contamination of food with genotoxic carcinogens to a level that is as low as reasonably achievable (ALARA). The principle of the MOE approach differs from the mathematical models in that it uses a dose that induces low measurable effects in a repeated dose toxicity study in animals as the reference point in the range of experimental data, instead of extrapolating to a virtual safe dose far beyond the experimental dose level. The Committee recommends the lower limits of a one-sided 95% confidence interval of the benchmark dose (BMDL) as an appropriate reference point on the dose-response curve. The BMDL is a more quantitative alternative to the NOAEL/LOAEL concept and is based on a mathematical model being fitted to the experimental data within the observed range of a dose-response curve. It estimates the dose that causes a low but measurable effect, chosen at no more than a 10% incidence of cancer above the control (BMD10). In cases where data are inadequate for deriving a BMDL, the less conservative T25 dose, corresponding to a 25% tumour incidence, should be used. The BMDL is related to human dietary exposure to residues of the compound and the MOE is calculated by dividing the BMDL by the estimated long-term average human intake. It is supposed that in the case of an MOE of a magnitude of ≥10 000 (indicating a minimum of 10 000-fold difference in BMDL between an animal study and human exposure) residues of substances that are both genotoxic and carcinogenic would be considered of low concern for public health. The 10 000-fold difference consists of the usual default safety factor of 100 for non-genotoxic compounds to compensate for interspecies differences and human variability in toxicokinetics and –dynamics and an additional default uncertainty factor of 100 for genotoxic carcinogens, to allow for additional uncertainties, such as human variability in cell cycle control and DNA-repair, and for unknown dose effects below the BMDL. The advantage of the MOE concept is the use of the BMDL as reference point within the region of measurable effects of the dose-response curve and would not normally require extrapolation outside the experimental observed data. However, this approach of risk assessment has to make assumptions related to different inherent uncertainties with respect to the selection of human intake estimates, the nature of the carcinogenic process, the type of reference point selected (BMDL or T25) and the use of default uncertainty factors of 10 000 (100 × 100) to allow for interspecies and human variability. In the opinion of the Scientific Committee of EFSA, the MOE approach should apply to unavoidable contaminants of food only and not to substances.
deliberately used in the food chain, such as veterinary drugs if they leave residues that are both genotoxic and carcinogenic.

EXPOSURE ASSESSMENT – THE ‘ESTRADIOL STORY’

For veterinary drugs that are potential genotoxic carcinogens the different approaches of linear dose extrapolation or the MOE concept are considered unsuitable for risk assessment. In the absence of an ADI, exposure assessment has to demonstrate either no detectable residues of genotoxic concern or no excessive exposure to endogenous levels of the contaminant or no bioavailability of the residues. This approach failed in the case of genotoxic and carcinogenic carbadox residues but was successfully used for the risk assessment of endogenous compounds such as estradiol-17β [6]. In vitro and in vivo genotoxicity testing, with and without metabolic activation, revealed genotoxic effects of estradiol-17β at concentrations ≥10^-6 mol L^-1. At physiological concentrations of 10^-12 to 10^-10 mol L^-1 this hormone induced tumours in estrogen-sensitive tissues by non-genotoxic mechanisms subsequent to increased cell proliferation. It was concluded that the carcinogenicity of estradiol-17β is most probably a result of its interaction with estrogen receptors. Residue data in meat indicate that treatment with anabolic or therapeutic doses of estradiol-17β does not lead to tissue concentrations of residues exceeding the physiological range in untreated animals, with the only possible exception being injection sites. The estimated daily human excess intake from meat of hormone-treated animals is several orders of magnitude lower than the daily endogenous production of the identical hormone in the most sensitive human subpopulations, prepubertal boys or postmenopausal women, with the lowest daily production rate. It is concluded that the potential hazard of genotoxic and carcinogenic effects of estradiol-17β is not of concern for public health because exposure of consumers to a biologically insignificant amount of exogenous hormone concentrations through ingestion of tissue from treated animals will be incapable of exerting a hormonal effect. Therefore, JECFA and CVMP concluded that ADI and MRL for estradiol-17β need not to be established. A common and harmonised approach to hazard and risk characterisation is deemed necessary to ensure a rational framework for the development and use of more predictive models for dose-response extrapolation purposes and for defining safe levels of residues of substances that are both genotoxic and carcinogenic. An agreement on what constitutes an acceptable risk is required.

REFERENCES


US, contaminated ball clay had caused increased dioxin levels in chickens and cat fish [5,6]. The contamination of these clay minerals is thought to be derived from ancient forest fires. The use of kaolinic clay was subsequently abandoned in the feed area. Surprisingly it returned at the end of 2004. Routine monitoring of pooled milk samples revealed a sample containing 1.5 pg TEQ dioxins/g fat, being clearly above the normal background [3]. Analysis of samples from the different farms revealed a sample with one of the highest levels in milk ever observed in the Netherlands, 20 pg TEQ g⁻¹ fat [7]. The congener pattern was compared with those from other sources and pointed to the use of kaolinic clay. At the same time it was shown that the source of the dioxins was potato peels, a waste product obtained from a neighbouring French fries producer. It turned out that kaolinic clay was used for the selection of poor quality potatoes with a low density. Kaolinic clay replaced salt that had previously been used for this process. This application was not limited to The Netherlands but apparently the problem was aggravated by the very high dioxin levels in this particular batch of clay and the fact that large amounts of the contaminated material was fed to the cows. Another incident in 2000 with feed was the use of choline chloride produced in Belgium and distributed through Spain, which was mixed with pine saw dust that was contaminated with the wood preserving agent pentachlorophenol (PCP), another known source of higher chlorinated dioxins [8]. A similar incident occurred in Italy, where wood chips derived from the production of coffins were used as beddings in a chicken farm [9]. Again the wood turned out to be contaminated with PCP. Contaminated wood actually ruined a carry-over study by the USDA [10]. Calves were fed with clean or dioxin contaminated feed, but it turned out that the wood in the stable contained PCP, thus providing an uncontrollable source of higher chlorinated dioxins. In 2003, bakery waste from Germany, used for the production of animal feed, had been dried on an open fire for which painted wood was used [11]. This again resulted in a very typical congener pattern and levels in the bakery waste up to 12 ng TEQ kg⁻¹. The feed prepared from this ingredient was primarily fed to pigs and resulted in levels around the EU limit of 1 pg TEQ g⁻¹ fat. Improper drying of feed ingredients is certainly a hazard that requires proper control. In January 2006, a survey was performed by RIKILT focusing on recycled ingredients for feed production. Pig fat turned out to contain dioxins at a level of 50 pg TEQ g⁻¹: 25 times the EU limit. Other batches were tested and shown to contain levels up to 440 pg TEQ g⁻¹ fat. The feed was primarily used for pig feed with a highest observed level of 8.4 ng TEQ kg⁻¹. Levels in fat from exposed pigs amounted to 3 pg TEQ g⁻¹ fat. The fat resulted from the production of gelatin and was traced back to the use of contaminated hydrochloric acid. The HCl was normally filtered but a failure of this process resulted in the contamination. The actual production process leading to the formation of these dioxins remains to be identified.

**DIOXINS IN EGGS FROM FREE-RANGE CHICKENS**

Following the increased monitoring, it was shown that eggs from free-ranging chickens contain elevated levels of dioxins and dl-PCB, and often exceed the limit for eggs of 3 pg TEQ g⁻¹ fat. Levels in eggs amount to 10–15 pg TEQ g⁻¹ fat; up to five times the EU limit. The major source appeared to be the soil and possible organisms living in the soil. Studies at farms pointed to a relation between the number of chickens and the dioxin levels. Surprisingly, the relation between soil and egg levels was poor. Follow-up studies indicate that the major factor is the extent to which the chickens actually forage outside. This seems to be less at large commercial farms than at small farms and for private owners with just a few hens [12].

**HEALTH RISK**

In most of these incidents, the levels in the products for human consumption are slightly elevated and impose no direct risk to the consumer. However, the EU strategy, aiming at a reduction of the long-term exposure of the population, requires the elimination of all major sources. Only in some cases, did the contamination result in clear and observable effects in the animals exposed to the contaminated feed. This involved only chickens and other birds, which seem very sensitive to the effects. Pigs contain large quantities of fat and thus appear to deal more easily with these contaminants.

**PHARMACOKINETIC MODELS**

In order to estimate the consequences of elevated levels in feed it is essential to have pharmacokinetic models that can be used to predict the levels in edible products. During the incident in 2004, such a model was used by RIVM to predict the period required for reducing the levels in milk to below the EU limit [7]. This was important for the farmer to decide on the measures to be taken with respect to his herd. Models can also be used for harmonizing...

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**Table 1. Incidents with dioxins and PCB in animals**

<table>
<thead>
<tr>
<th>Year</th>
<th>Incident</th>
<th>Source</th>
<th>Animal species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1957</td>
<td>Chicken edema disease</td>
<td>Fat from cow hides treated with pentachlorophenols</td>
<td>Chickens</td>
</tr>
<tr>
<td>1969</td>
<td>Chicken edema disease</td>
<td>Vegetable oil mixed with pesticide waste water</td>
<td>Chickens</td>
</tr>
<tr>
<td>1996</td>
<td>Ball clay</td>
<td>Contaminated ball clay, ancient forest fires</td>
<td>Chickens, cat fish</td>
</tr>
<tr>
<td>1998</td>
<td>Citrus pulp</td>
<td>Contaminated lime from PVC production plant</td>
<td>Cows</td>
</tr>
<tr>
<td>1999</td>
<td>Belgium PCB incident</td>
<td>Fat with PCB oil containing dioxins</td>
<td>Chickens, pigs</td>
</tr>
<tr>
<td>1999</td>
<td>Kaolinic clay</td>
<td>Kaolinic clay, ancient forest fires</td>
<td>Pigs</td>
</tr>
<tr>
<td>2000</td>
<td>Choline chloride</td>
<td>Pine saw dust treated with pentachlorophenol</td>
<td>Chickens, pigs</td>
</tr>
<tr>
<td>2003</td>
<td>Bakery waste</td>
<td>Drying on fire from waste wood</td>
<td>Pigs</td>
</tr>
<tr>
<td>2003</td>
<td>SQM minerals</td>
<td>Minerals mixed with kelp in novel process</td>
<td>Cows, pigs</td>
</tr>
<tr>
<td>2004</td>
<td>Potato peels</td>
<td>Kaolinic clay, ancient forest fires</td>
<td>Pigs, chickens</td>
</tr>
<tr>
<td>2006</td>
<td>Gelatin</td>
<td>Hydrochloric acid, process leading to dioxin production unknown</td>
<td>Pigs, chickens</td>
</tr>
</tbody>
</table>
the feed and food levels. In cooperation with RIKILT and ASG, RIVM has developed such models for lactating cows, laying hens and growing pigs.

REFERENCES

INTRODUCTION
The effect of gastrointestinal parasitism on the patterns of edible tissue depletion of doramectin (DRM) was studied in two groups of lambs. The aim of the study was to test the hypothesis that parasitic disease can have significant influences on the patterns of tissue depletion of DRM through the changes induced on the host’s gastrointestinal tract and nutritional condition in comparison to those observed in healthy non-parasitized lambs.

MATERIALS AND METHODS
Twenty-four parasitized Suffolk Down lambs (23.3 ± 0.7 kg body weight) were selected. The lambs were ranked in descending order of body weight and sequentially paired from heaviest to lightest. Faecal examinations were performed on all lambs to determine faecal egg counts (FEC). Twelve pairs of lambs were allocated into two groups equally balanced for body weight. Once the pairs of animals were established, their distribution to the experimental groups was performed according to values of nematode egg counts in order to obtain the higher values in the parasitized group. Group I (non-parasitized) was pre-treated with three repeated administrations of 5 mg kg⁻¹ of fenbendazole (FBZ) to maintain a parasite free condition. While in Group II (parasitized), the lambs did not receive any anthelmintic treatment. After 70 days, both groups were treated with a s.c. dose of 0.2 mg kg⁻¹ body weight of DRM. The animals were slaughtered in groups of three at the following times after DRM administration: 7, 14, 21 and 28 days. Samples of the target tissues (liver, kidney, muscle and fat) were obtained from each animal and stored frozen at −18°C until analysis. Tissue samples were analysed by HPLC with fluorescence detection after solid phase extraction as previously described [1]. The concentrations obtained for each tissue were processed statistically using a computer program based on the EMEA guidelines for estimation of withdrawal periods [2]. Results were compared by the unpaired Student t-test.

RESULTS
Pre-treatment in the healthy non-parasitized animals with FBZ reduced the FEC and increased the body weight of the lambs, changes that were significantly different from those observed in parasitized lambs. The analytical method was validated appropriately. The percentage of DRM recovery from liver, muscle, kidney and adipose tissue ranged from 76.8 to 85.7%. The limits of quantitation obtained for the different tissues ranged from 0.2 ng g⁻¹ (kidney) to 2.5 ng g⁻¹ (fat). The mean DRM concentrations measured at 7, 14, 21 and 28 days are shown in Table 1. DRM was detected in all of the tissues analysed up to 28 days after s.c. administration. For both groups, higher concentrations of DRM were obtained in adipose tissue than the other tissues analysed (Table 1). When compared between the two groups of animals, higher and more persistent concentrations of DRM were observed in the non-parasitized group, differences that were significant in comparison to those obtained in parasitized lambs for kidney, muscle and fat at 7 days after treatment. Taking into consideration the MRL established for DRM in the liver (50 µg kg⁻¹) and fat (100 µg kg⁻¹) by the EMEA [3], the calculated withdrawal times for the group of healthy lambs were higher than for the parasitized animals: 43 vs. 26 days in fat and 32 vs. 22 days in liver.

DISCUSSION
The results obtained in the healthy lambs are similar to those previously described in sheep [4]. According to the concentration values observed in the current study it is evident that liver and fat are the most suitable tissues for monitoring DRM residues, and that the unchanged drug is the most appropriate compound for use as a marker residue. The differences in body weight observed between the two groups resulted in differences in tissue distribution of DRM, where the higher and most persistent drug concentrations are observed in the non-parasitized group. In conclusion, gastrointestinal parasitism, through the reduction in body weight gain of lambs significantly reduced the tissue depletion of doramectin (DRM) was studied in two groups of lambs. The aim of the study was to test the hypothesis that parasitic disease can have significant influences on the patterns of lambs. The aim of the study was to test the hypothesis that parasitic disease can have significant influences on the patterns of tissue depletion of DRM through the changes induced on the host’s gastrointestinal tract and nutritional condition in comparison to those observed in healthy non-parasitized lambs.

Table 1. Tissue residue concentrations of DRM (Mean ± SEM) in parasitized and non-parasitized lambs (n = 3)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time (days)</th>
<th>Parasitized (ng/g)</th>
<th>Non-parasitized (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>7</td>
<td>118.6 ± 49.6</td>
<td>204.8 ± 12.2</td>
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<tr>
<td></td>
<td>14</td>
<td>26.9 ± 7.1</td>
<td>47.8 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>10.6 ± 2.6</td>
<td>12.2 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>2.2 ± 1.5</td>
<td>10.1 ± 3.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>7</td>
<td>37.1 ± 12.0</td>
<td>64.8 ± 2.0*</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>10.8 ± 4.1</td>
<td>127 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>3.4 ± 1.1</td>
<td>5.1 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>1.5 ± 0.7</td>
<td>2.4 ± 1.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>7</td>
<td>23.7 ± 7.2</td>
<td>72.7 ± 22.7*</td>
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<tr>
<td></td>
<td>14</td>
<td>8.3 ± 3.8</td>
<td>11.3 ± 2.0</td>
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<tr>
<td></td>
<td>21</td>
<td>4.2 ± 1.4</td>
<td>13.7 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.4 ± 0.2</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>Fat</td>
<td>7</td>
<td>199.1 ± 62.7</td>
<td>530.0 ± 48.7*</td>
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<tr>
<td></td>
<td>14</td>
<td>65.1 ± 20.9</td>
<td>155.1 ± 33.7*</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>23.9 ± 8.9</td>
<td>40.5 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>13.7 ± 5.1</td>
<td>23.4 ± 12.8</td>
</tr>
</tbody>
</table>

*P < 0.05
distribution and persistence of DRM. Consequently, the calculated withdrawal times of DRM were shorter in parasitized than in healthy lambs.

ACKNOWLEDGEMENTS
This study was supported by research Grant FONDECYT 1030609.

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E02
Does anatomical location affect the pattern of drug residues in muscle? Evaluation of ivermectin (sheep) and doramectin (cattle) residues
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Laboratorio de Farmacología, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro, Tandil, Argentina

INTRODUCTION
Veterinary drugs are widely used to protect animal health, prevent production loss and ensure a safe food supply. The fate of chemical residues from veterinary drugs in animal tissues destined for human consumption is a pivotal issue for food safety. To reach the required food protection level, reliable data must be available to permit adequate risk evaluation and subsequent action. Ivermectin (IVM) and doramectin (DRM) are lipophilic avermectin compounds extensively used for broad-spectrum parasite control in food-producing animals. Both molecules are extensively distributed from the bloodstream to different body tissues, including fat, muscle and liver [1]. Adipose and other edible fat-containing tissues are important tissue reservoir for the avermectins and related compounds in ruminants, which accounts for their extended persistence of antiparasitic activity. Muscular tissue in food-producing animals may have different fat infiltration grades, with variable blood supply according to its anatomical location and physiological role. Thus, the pattern of residues distribution for highly lipophilic drugs could be variable in muscular tissue from different anatomical sites. Considering that meat is the main edible tissue destined for human consumption, the current work provides some tissue residue kinetic data for compounds widely used in livestock, which may be useful in assuring food safety. The trial presented here characterized the residual drug concentrations in muscular tissue obtained from different anatomical locations after s.c. administration of IVM (sheep) and topical treatment with DRM (calves) at recommended therapeutic dose rates.

MATERIALS AND METHODS
Two parallel experiments were carried out. Animals received food and water ad libitum during the whole experimental period. Residues in sheep Twelve adult male Corriedale sheep (49.4 ± 7.93 kg) were treated with a commercial formulation of IVM at 200 μg kg⁻¹ s.c. Residues in Cattle Twelve Holstein calves (180 ± 30.9 kg) were treated with a commercial formulation of DRM by topical (pour-on) administration at the recommended dose rate (500 μg kg⁻¹). In both experiments, four animals were randomly assigned for sacrifice at: 15, 20 and 30 days post-treatment (Exp 1) and at 2, 5 and 10 days post-treatment (Exp 2). Animals were sacrificed according to the American Veterinary Medical Association animal euthanasia ethical guidelines [2]. Muscle samples from the following anatomical regions were collected at the above indicated sampling times: neck, shoulder, thigh (semitendinosus), rump, loin, inter-costal and diaphragm. They were collected and processed (1 g) as previously reported [1]. DRM and IVM were determined by HPLC using fully validated analytical techniques [1]. ANOVA plus Tuckey test was used for statistical comparisons.

RESULTS
The highest residual concentrations of IVM in all the muscle locations were found at 15 days post-treatment in sheep. Although the highest mean concentrations of IVM were measured at 15 (16.8 ± 10.3 ng g⁻¹) and 20 (10.5 ± 7.0 ng g⁻¹) days post-administration in the intercostals muscles, the highest IVM residues (4.91 ± 5.2 ng g⁻¹) at 30 days post-administration were measured in the diaphragm. DRM residual concentrations were quantified in muscular tissue from all the anatomical locations after the topical administration to calves (Table 1). The maximum residue level was determined at 10 days post-treatment at all the anatomical sites. The diaphragm had the highest levels of DRM residues at 2, 5 and 10 days post-treatment in calves.

Table 1. Mean±SD (n = 4) doramectin (DRM) concentrations (ng g⁻¹) measured in muscular tissue from different anatomical regions after its topical administration to calves (500 μg kg⁻¹)

<table>
<thead>
<tr>
<th>Time post-administration</th>
<th>DRM concentration (ng g⁻¹) in muscle from different anatomical regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diaphragm</td>
</tr>
<tr>
<td>2 days</td>
<td>22.08±8.7</td>
</tr>
<tr>
<td>5 days</td>
<td>26.12±2.4</td>
</tr>
<tr>
<td>10 days</td>
<td>27.92±19.1</td>
</tr>
</tbody>
</table>

DISCUSSION
While the highest residual levels of IVM were measured in the inter-costal muscles in sheep, the diaphragm was the muscle where the highest DRM residues were recovered from cattle treated topically. The observed tendency for differential residue patterns between anatomical sites for both molecules did not reach statistical significance (P > 0.05) due to large individual variability. However, the pattern of IVM and DRM residues depletion seems to differ according to the anatomical location and/or physiological role. Regardless the preliminary data shown here, this work is part of a broader study in which several conflictive issues related to chemical residues in edible tissues are under evaluation: (a) assessment of tissue residues at the site of injection, including criteria for sample collection and drug diffusion into the muscular tissue surrounding the injection site, and (b) the use of tissue biopsy as a technique to obtain valid information on residue profiles in treated animals.

REFERENCES
**INTRODUCTION**

The recent adoption of the guideline on injection site residues in food producing animals by the CVMP (Committee for Medicinal Products for Veterinary Use) raised the question of the risk specifically related to injection site consumption [1]. The aim of this study was to assess the risk of human exposure to consumption of injection site residues and to review the recommended statistical approach for withdrawal period calculation.

**MATERIALS AND METHODS**

The risk assessment of human exposure to consumption of injection site residues was performed using a probabilistic approach. The calculations were based on meat consumption figures in the EU, taking into account consumption of each species, in each Member State. The assessment was applied to injectable antibiotics. It evaluated the maximal number of times, in a year, with consumption of a whole or part of an injection site for a European consumer. The guideline on injection site residues recommends the same method be applied for the calculation of withdrawal period for injection site residues as for other edible tissues [2]. Two alternative approaches were presented here. Firstly, a simple pragmatic approach with the last slaughter time with all data below the reference threshold combined with a safety span, and secondly a non-parametric approach proposed by Concordet & Toutain [3]. Examples derived from industry were used to illustrate the different methods. The reference threshold commonly used in the withdrawal period calculation is the MRL and the ADI (Acceptable Daily Intake). The replacement of these thresholds, to deal with the acute risk exposure of injection site consumption, by the ASDI (Acceptable Single Dose Intake) was thus considered for the withdrawal period calculation.

**RESULTS**

The probabilistic approach led to a maximal risk of 4 days of injection site consumption during a year in the EU. It also indicated that 37% of consumers will never eat an injection site in the same period. Three datasets were used as examples to calculate the withdrawal periods for the different methods proposed (Table 1).

**DISCUSSION**

The probabilistic approach permitted to conclude to an acute exposure risk of consuming injection site residues, rather than a chronic one, as for other edible tissues. This led to the recommendation to use the ASDI or the ARD (Acute Reference Dose) for withdrawal period calculation, instead of the MRL and ADI. The specificity of injection site residue (erratic and slower depletion) was deemed incompatible with the usual statistical assumptions of the recommended approach. Indeed, the homoscedasticity and the linearity were often violated (even if it was not statistically detected), resulting in unreliable withdrawal periods with too high extrapolation. The pragmatic and non-parametric methods were considered as sound alternatives to these problems. Alternative methods and reference thresholds (e.g. ADI, MRL, ASDI or ARD) should be scientifically discussed for the withdrawal period calculation for injection site residues.

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---

**Table 1. Results of withdrawal period calculations with the recommended, alternative and non-parametric approaches, using the MRL and the ASDI**

<table>
<thead>
<tr>
<th>Assessment based on MRL</th>
<th>Example 1</th>
<th>Example 2</th>
<th>Example 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time when all data &lt;MRL</td>
<td>56 days</td>
<td>28 days</td>
<td>20 days</td>
</tr>
<tr>
<td>Recommended time</td>
<td>95 days</td>
<td>43 days</td>
<td>188 days</td>
</tr>
<tr>
<td>Statistical approach</td>
<td>70%</td>
<td>20%</td>
<td>282%</td>
</tr>
<tr>
<td>Extrapolation from last slaughtered time</td>
<td>56 days + 30% = 73 days</td>
<td>28 days + 30% = 37 days</td>
<td>20 days + 30% = 26 days</td>
</tr>
<tr>
<td>Alternative approach</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Non-parametric approach</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

**Drug withdrawal from farmed fish: depletion of oxytetracycline in gilthead sea bream (Spauros aurata, Limnaeus 1758)**


Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Complutense University, Madrid, Spain

**INTRODUCTION**

The widespread use of antibiotics and chemotherapeutic drugs in modern intensive aquaculture is well recognized, but inherent to this application of drugs to food-producing species is the public health aspect associated with tissue residue levels. Thus, drug withdrawal periods are required. Since oxytetracycline (OTC), a broad-spectrum antibiotic, is now used extensively in farmed fish, the present study was designed to apply a statistical approach [1] to the setting of withdrawal times based on quantitative analysis of OTC residues in muscle and skin samples from gilthead sea bream.

**MATERIALS AND METHODS**

Gilthead sea bream (Spauros aurata, Limnaeus 1758) that each weighed 200 ± 50 g were used to investigate the tissue depletion of OTC after oral administration (20 and 30 mg kg⁻¹ body weight day⁻¹, for five consecutive days). Fish treated at a dose rate of 20 mg kg⁻¹ body weight, received OTC at a concentration of 2.25 g kg⁻¹ feed. Fish treated at a dose rate of 30 mg kg⁻¹ body weight, received OTC at a concentration of...
3.2 g kg\(^{-1}\) feed. The dose was administered during 20 min per day. The fish were kept in 6000 L tanks with an aerated continuous flow of sea water. Water temperature was 20–20.5°C. Fish, previously kept alive on ice for 10 min, were euthanized by decapitation at intervals (seven fish per time) after treatment and samples of muscle + skin in natural proportions were taken. Tissue samples were stored frozen at −45°C until analysed. Tissue concentrations of OTC and its 4-epimer were measured by HPLC using solid-phase extraction as described previously [2]. Recovery rate for the various OTC concentrations was 80–92%. The limit of quantitation was 50 ng g\(^{-1}\) for muscle + skin. The withdrawal period was estimated by linear regression analysis of the log-transformed tissue concentrations determined at the time when the upper one-sided tolerance limit, with a confidence of 95%, was below the MRL [1, 3].

RESULTS
OTC plus 4-epi-OTC concentrations obtained after administration of 20 or 30 mg kg\(^{-1}\) body weight of OTC as medicated feed demonstrated that OTC plus 4-epi-OTC concentrations increased with the dose administered; concentrations measured 3 h after treatment (day 0), were 189.257 ± 33.30 ng g\(^{-1}\) (20 mg kg\(^{-1}\)) or 260.575 ± 63.843 ng g\(^{-1}\) (30 mg kg\(^{-1}\)). After a withdrawal period of 1 day, OTC plus 4-epi-OTC concentrations were below the MRL. Residual levels of OTC and its 4-epimer in muscle plus skin samples were estimated (95% tolerance limit 95%) to fall below the MRL after a withdrawal period of 4.73 days (rounded up to 5 days) and 4.84 days (also rounded up to 5 days) for the 20 and 30 mg kg\(^{-1}\) day\(^{-1}\) dose, respectively.

DISCUSSION
The sampling protocol adopted in the present study, which was intended to evaluate the depletion of OTC residues, does not make it possible to describe the plasma kinetic profile of OTC and therefore it is not possible to draw any conclusion about the clinical efficacy of the proposed dosage regimens. However, it is possible to speculate that at least the dosage regimen of 30 mg kg\(^{-1}\) for five consecutive days could be therapeutically useful in the treatment of bacterial diseases in fish. In the present study, OTC was cleared slowly. The elimination rate results in a withdrawal period of 5 days; the withdrawal period was estimated by the statistical method [3]. This withdrawal period of 5 days is proposed to avoid, with the assayed formulation, the presence of violative residues in edible tissues of gilt-head sea bream.

ACKNOWLEDGEMENTS
This was supported in part by the Spanish Ministry of Agriculture, Fish and Food (Secretaria General de Pesca Maritima). The authors thank to CIFPA “El Toruño”, Puerto de Santa María, Cadiz, for help in this work (Dr. M. Manchado).

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INTRODUCTION
Tyrosine aminotransferase (TAT, EC 2.6.1.5), a pyridoxal phosphate dependent cytosolic enzyme mostly expressed in mammalian liver, is the rate-limiting enzyme in tyrosine catabolism. It is considered one of the most sensitive biological markers of the gluconeogenic effects of glucocorticoids (GC), inasmuch as it has been selected for the establishment of the Acceptable Daily Intake for this class of drugs [1]. Dexamethasone (DEX) and other synthetic GC are increasingly used in bovines at very low dosages for performance enhancing purposes [2]. The aim of this study was to characterize the modulation of TAT activity by a number of natural and synthetic GC in a well established in vitro model, to assess whether TAT could be proposed as a biomarker for GC exposure.

MATERIALS AND METHODS
Hepatocytes were isolated from male Fischer rats by the collagenase perfusion method [3] and plated on culture dishes coated with collagen at 7 × 10^4 viable cells/cm\(^2\) in M199 medium supplemented with BSA, HEPEs, antibiotics, horse serum and insulin. After allowing 4 h for cell attachment, the medium was changed and the plates were incubated for 24 h with concentrations of cortisol or dexamethasone (DEX) ranging from 10^{-6} M to 10^{-8} M. To compare the inducing capacity of different GC, other plates were incubated with cortisol, flumethasone, beclomethasone, prednisone, prednisolone, methylprednisolone or DEX for 24 h, all at a final concentration of 10^{-7} M. The same concentration was used to assess the time course of DEX-mediated TAT induction and the effects of the repeated exposure to this compound. To this end, culture medium was removed at 24 h intervals and replaced with fresh medium containing 10^{-7} M DEX. After appropriate incubation times, dishes were washed twice in PBS and cells were removed using potassium phosphate buffer and lysed through homogenization with Dounce. After centrifugation, TAT activity was assayed in supernatants by a modification of the method of Diamondstone [4] and expressed as nmol/min/mg protein (mean ± SEM).

RESULTS
After 24 h incubation, the natural hormone cortisol had a less effective inducing effect than DEX, although the highest tested concentration (10^{-6} M) did not result in the maximal rate of induction (Fig. 1). A 10^{-7} M concentration was therefore used in all the subsequent experiments. At this concentration of DEX, TAT activity increased proportionally to the incubation time up to 24 h, when it displayed a more than six-fold increase over controls (21.3 ± 1.8 vs. 3.6 ± 0.4), and thereafter exhibited a progressive decrease reaching control values at 72 h. Repeated
A diclazuril oral suspension (Vecoxan®) is the first anticoccidial formulation available for ruminants. The recommended dose is 1 mg diclazuril/kg body weight, given metaphylactically as a single oral treatment. Considering consumer safety, diclazuril has been included in Annex II of Council Regulation (EEC) No 2377/90 for oral use in all ruminant and porcine species.

**INTRODUCTION**

A diclazuril oral suspension (Vecoxan®) is the first anticoccidial formulation available for ruminants. The recommended dose is 1 mg diclazuril/kg body weight, given metaphylactically as a single oral treatment. Considering consumer safety, diclazuril has been included in Annex II of Council Regulation (EEC) No 2377/90 for oral use in all ruminant and porcine species, indicating that no numerical MRL (maximum residue limits) are required because use cannot result in residues of consumer concern. Results from residue studies show that at 1 day after dosing, at a dose of 5 mg kg$^{-1}$ body weight, residues represented only 2.2% of the ADI, while the recommended dose is 1 mg kg$^{-1}$ body weight [1,2]. Since diclazuril included in Annex II for calves and since only very low residue concentrations are measured in edible tissues, a ‘zero’ withdrawal time has been set. With respect to environmental safety, only small quantities of diclazuril are released into the environment after administration to calves. Predicted environmental concentrations (PEC) values range from 1 to 6 µg/litre whereas PEC values are even in the ppt range. Diclazuril is immobile and degradable in soil. It is devoid of insecticidal properties and exerts no or very low toxicity to earthworms, plants, soil micro-organisms and aquatic organisms. The use of diclazuril oral suspension in calves has no environmental impact and in this respect no specific precautions should be taken. Next to consumer safety and environmental safety, target animal safety should be considered.

**MATERIALS AND METHODS**

A Target Animal Safety (TAS) study was performed in Friesian calves aged between 5 and 10 weeks, which represent the target age class for the administration of an anticoccidial used as metaphylactic treatment. Four groups of eight animals each were included and administered 1x, 3x and 5x the recommended dose of 1 mg kg$^{-1}$ b.w. The calves were dosed orally for three consecutive days although the dose regime foresees a single application. The frequency, timing and selected parameters for evaluating safety: i.e. complete clinical veterinary examination, extensive haematological and blood chemistry, feed and water intake, body weight, gross examination at necropsy completed with histological examination of the target organs for this type of drug, such as liver, rumen, abomasum, jejunum, ileum and colon, allows an objective assessment on the safety and the tolerance of the product to bemade.

**RESULTS**

The outcome of the study did not reveal abnormalities, directly correlated with the dose and/or the duration of the administration of the drug. None of the dose regimes had any noteworthy detrimental effect on the observed clinical parameters, or in general behaviour. The haematological and biochemical analyses did not reveal any group, sex nor time related abnormalities. The high concentration of alanine aminotransferase (ALT), in some cases outside the reference range, noted throughout the administration period, could be considered as an indicator of hepatocellular changes. However, the statistical analysis showed no evolution throughout the study or differences between the treatment groups and the control group, except for times prior to the administration of the test product. In the aspartate transaminase (AST) values differences were noticed only between the group treated at 3x and the control group on the last day of administration and the following day. Since no differences were observed for the other doses, one can conclude that there is no direct relationship with the administration of diclazuril oral suspension. On the contrary, the differences found in the alkaline phosphatase values appear to be due to the improvement of this parameter in the course of the study. In general, the differences in the haematological and biochemical parameters did not follow a specific pattern. The pasty faeces and...
diarrhoea, mainly in the overdosed groups, did not indicate an important physiological or behavioural change. The main parameters sensitive to this effect, i.e. hematocrit and electrolytes, were not altered. Throughout the study the animals gained considerable weight, without statistical difference between the various groups, which supports indirectly that the administered drug, even at five times the recommended dose for three consecutive days, had no detrimental effects on health. Abomasal ulceration was noted on histopathology in all groups but without related clinical signs. This was probably caused by the change in diet from milk to dry feed.

DISCUSSION
As a general conclusion, the diclazuril oral suspension can be considered as safe, even when overdosed and administered for three consecutive days. This conclusion is correlated with the high safety profile of the compound noticed during the toxicity studies in laboratory species. Moreover, the pharmacokinetic data in the target species elaborated in combination with this TAS study, indicate that the bioavailability of diclazuril, administered orally as a 0.25% suspension, is not higher than 1%. This extremely low absorption contributes to the safe profile of the product. Next to its extremely high consumer and environmental safety, diclazuril oral suspension was also well tolerated in the target species, even at 15 times the recommended dose.

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INTRODUCTION
Albendazole (ABZ) is a potent broad-spectrum benzimidazole anthelmintic that is widely used in veterinary medicine for the treatment of intestinal helminth infections. Following administration, ABZ is extensively metabolised by progressive oxidation of its sulphide moiety and further cleavage of its carbamate group to albendazole sulphoxide (ABZ-SO), albendazole sulphone (ABZ-SO2) and albendazole 2-aminosulphone (ABZ-2-NH2SO2). In order to protect human health, the EU has established a maximum residue limit (MRL) for ABZ at a level of 1000 µg kg⁻¹ in liver, 500 µg kg⁻¹ in kidney and 100 µg kg⁻¹ in muscle and fat. The marker residue is the sum of ABZ-SO, ABZ-SO2 and ABZ-2-NH2SO2, expressed as ABZ [1]. For the determination of ABZ and its metabolites in edible tissues, high-performance liquid chromatographic methods with ultraviolet and fluorescence detection and tandem mass spectrometric detection (LC-MS/MS) have been reported. Many of these methods do not quantify all of the target compounds (i.e. parent ABZ and ABZ-SO, ABZ-SO2 and ABZ-2-NH2SO2) in one analytical run. This was one of the aims of the present study. The second objective was to use the developed method for study of residue depletion of ABZ, ABZ-SO, ABZ-SO2 and ABZ-2NH2SO2 and calculation of a withdrawal time in sheep after oral administration of a commercial formulation of ABZ [2].

MATERIALS AND METHODS
Sample preparation One g of tissue was extracted twice with ethylacetate in alkaline medium. The combined ethylacetate fractions were further purified using an Oasis<sup>®</sup> MCX solid-phase extraction column (60 mg/3 mL, Waters). After evaporation of the eluate, the dry residue was reconstituted in an ethanolic HCl solution and defatted using hexane. The lower layer was evaporated and an aliquot of the reconstituted residue was injected onto the LC-MS/MS system. Chromatographic analysis: the LC-MS/MS system comprised a Waters Acquity UPLC sample and solvent manager in combination with a Quattro Premier XE mass spectrometer (Micromass, Waters) operating in the positive ionisation mode. Chromatographic separation was achieved on an Acquity C18 UPLC column (2.1 × 100 mm, dp: 1.7 µm), using gradient elution at a flow-rate of 1 mL min⁻¹. Multiple reaction monitoring (MRM) transitions for all compounds are shown in Table 1. Method validation: The method was validated according to the recommendations of the EC [3]. Residue study: Twenty sheep (males and females equally represented) were treated once with an oral suspension of ABZ at a dose rate of 10 mg kg⁻¹ body weight. Animals were slaughtered at 1, 3, 5, 7 and 9 days after drug administration and relevant edible tissues (liver, kidney, muscle and fat) were sampled. The samples were stored at ≤ −15°C until analysis.

Table 1. MRM transitions for ABZ and metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM transition</th>
<th>Collision (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABZ</td>
<td>265.9&gt;234.0</td>
<td>20</td>
</tr>
<tr>
<td>ABZ-SO</td>
<td>282.1&gt;191.1</td>
<td>23</td>
</tr>
<tr>
<td>ABZ-SO2</td>
<td>298.0&gt;265.9</td>
<td>20</td>
</tr>
<tr>
<td>ABZ-2-NH2SO2</td>
<td>240.0&gt;133.0</td>
<td>25</td>
</tr>
<tr>
<td>MEB (IS)</td>
<td>296.0&gt;264.0</td>
<td>25</td>
</tr>
</tbody>
</table>

Figure 1. LC-MS/MS chromatogram of a liver sample, spiked with ABZ and its metabolites at the MRL level (1000 µg kg⁻¹)
RESULTS AND DISCUSSION
Method optimization and validation: Chromatographic separation of all target compounds could be accomplished within 2.5 min, as is shown in Fig. 1. This is at least four times faster than any method reported previously in the literature. The validation results fell within the accepted ranges: linearity: \( r \geq 0.99\) and \( g \leq 10\%\), trueness: \(-20\%\) to \(+10\%\) of the target concentration, between-run and within-run precision: \( \text{RSD} \leq \text{RSD}_{\text{max}}\), LOQ \( \leq \frac{1}{5} \text{MRL}\), LOD: \( S/N=3\) and no interference of endogenous and analogous compounds. We succeeded in the development and validation of a quantitative LC-MS/MS method for the simultaneous determination of ABZ and its major metabolites in sheep edible tissues. In addition, by using the UPLC technique, the LC-MS/MS analysis could be performed within 2.5 min, which is attractive for application in routine analysis. The method was fully validated and will be used for the analysis of real samples. This will prove its validity for implementation in the field of residue analysis.

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E08
Development of an HPLC-UV method for simultaneous quantification of quinoxaline-2-carboxylic acid and 3-methyl-quinoxaline-2-carboxylic acid in muscle and liver of swine, chicken and fish
Z. H. YUAN, Y. J. WU, Y. L. WANG, L. L. HUANG, D. M. CHEN & Y. F. TAO
National Reference Laboratory of Veterinary Drug Residues/MOA Key Laboratory of Food Safety Evaluation, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China, E-mail: iovp@mail.hzau.edu.cn

INTRODUCTION
Carbadox (CBX) and olaquindox (OLA) were banned by the EU for use in food-producing animals as antimicrobial and growth promoting agents due to their toxicities [1]. Residue monitoring is a key to the control of the use of these drugs in animals. Quinoxaline-2-carboxylic acid (QCA) and 3-methyl-quinoxaline-2-carboxylic acid (MQCA), respectively, are considered as the marker residues in animal tissues [2,3]. Most of the methods adopted for the analysis of QCA and MQCA such as HPLC [4], GC-MS [5] and LC-MS [6] are not only time consuming and labour intensive but also matrix limiting; suitable for one or two tissues from one or two animal species. The method reported here is a simple and sensitive HPLC-UV method for simultaneous determination of QCA and MQCA in liver (as target tissue) and muscle (as the most consumed tissue) from swine, chicken and fish, the target species of CBX and OLA used legally and illegally in some countries.

MATERIALS AND METHODS
5.0 g of samples were extracted with \( 2 \times 8 \) mL of 5\% (w/v) metaphosphoric acid in methanol:water(10:90, v/v), the supernatant extracted with \( 2 \times 8 \) mL of ethyl acetate, and the pooled upper layer partitioned with \( 2 \times 5 \) mL of 0.01 M phosphate buffer (pH 7.0). The buffer extract was cleaned-up using a

### Table 1. Recovery and precision for QCA and MQCA in spiked pork (\( n = 5 \) at each concentration, on five separation days)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Spiked level (µg kg(^{-1}))</th>
<th>Recovery (%)</th>
<th>Intra-day CV (%)</th>
<th>Inter-day CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QCA</td>
<td>2</td>
<td>101</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>87</td>
<td>12</td>
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<td></td>
<td>8</td>
<td>75</td>
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<td></td>
<td>8</td>
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</table>

Figure 1. The HPLC chromatograph of blank porcine sample spiked with 4.0 µg kg\(^{-1}\) of QCA and 4.0 µg kg\(^{-1}\) of MQCA

DISCUSSION
The chromatogram of pork spiked with 4.0 µg kg\(^{-1}\) of mixed QCA and MQCA is shown as Figure 1. Decision limits (CC\(_{\alpha}\)) are 0.7–2.6 µg kg\(^{-1}\), and detection capabilities (CC\(_{\beta}\)) 1.3–4.4 µg kg\(^{-1}\) for both analytes in three types of muscle. Recoveries of QCA and MQCA from muscle are 69%–102% with intra- and inter-day variation coefficient (CVs) <16% (Table 1). The calibration curves for both show good linearity (\( s > 0.999\)) within 20–1000 µg kg\(^{-1}\).

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RESIDUES OF SEVERAL NITROFURANS IN EGG

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INTRODUCTION
Furazolidone, furaltadone, nitrofurantoin and nitrofurazone are veterinary drugs belonging to the group of nitrofurans. Nitrofurans were used for the treatment of gastrointestinal infections in cows and pigs and especially for the treatment of coccidiosis in poultry. Due to their carcinogenic and mutagenic effects, the use of nitrofurans as veterinary drug for food-producing animals is banned completely in the EU since 1995 [1]. To ensure food safety, food has to be controlled efficiently for residues of these drugs. In muscle, nitrofurans metabolise very rapidly, escaping detection within hours after administration. Nevertheless, their metabolites are partially stored for weeks or even months as protein-bound residues. Whether the same is true for nitrofurans and their metabolites in eggs was examined in this study.

MATERIALS AND METHODS
Laying hens were fed with complete feed for laying hens with furazolidone, furaltadone nitrofurantoin or nitrofurazone added to result in an uptake of about 7.5 mg of the respective nitrofuran/kg body weight per day. The eggs from one day were pooled (whole egg). The method used for analysis is an adaptation of a method developed for muscle tissue [unpublished data]. The LC-MS/MS method was validated for egg in our laboratory according to Commission Decision 2002/657/EC [2].

RESULTS
The residues are detectable in eggs as early as after the first day of treatment. The concentration of the marker residues rises to a plateau value within four to five days. For AOZ a plateau concentration of about 70 µg kg⁻¹ whole egg was measured. After withdrawal of the drug, the residue concentrations declined rapidly but with different slopes for the different nitrofurans. In the case of furazolidone, AOZ was no longer detectable five days after treatment withdrawal, while the AMOZ concentration was still around 10 µg kg⁻¹.

REFERENCES

BIOMARKERS REVEALING ABUSE OF STEROID HORMONES IN CALVES

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INTRODUCTION
Combinations of anabolic androgenic steroids and estrogens and/or glucocorticoids are used to increase muscle protein accretion. In the EU, the use of such hormonal growth promoters is illegal in production animals. State of the art to prove the illegal administration of drugs with anabolic effect is the detection of residues of the administered drugs, including their metabolites, in faeces, hair and urine before animals are slaughtered or in fat, kidney, liver, meat or retina after slaughter. However, the detection of endogenous hormones and hormone cocktails is an analytical challenge and difficult to achieve [1]. The 6th FP integrated project BioCop scouts innovative technologies for alternatives for the detection of foreign compounds in the food chain. Such a new analytical approach could rely on the analysis of biomarkers indicating illegal exposure of the inspected animal to anabolic compounds.

MATERIALS AND METHODS
Six female and six male calves (Holstein-Friesian x Friesian-Holland, 10 weeks of age) were treated three times with both 17β-estradiol benzoate (25 mg i.m.) and 19-nortestosterone decanoate (nandrolone; 150 mg i.m.) on days 0, 14 and 28. The same group was treated once with dexamethasone on day 35 (4 mg s.c.). Six female calves and six male calves served as controls and were treated with a placebo. At regular intervals urine, hair and blood were sampled for incurred residue and biomarker analysis. From day 42 onwards, animals were slaughtered and tissues were sampled for histological and pathological inspection. Plasma samples from all animals were...
analysed for over a dozen potential biomarkers using affinity assays (western blotting, surface plasmon resonance biosensor, RIA and ELISA), and simultaneously protein profiles of treated calves were compared with those of untreated animals using two-dimensional (2DGE) and differential (DIGE) gel electrophoresis.

RESULTS
Treated animals gained more weight than untreated animals. Publications were mined for potential protein biomarkers. While 2DGE and DIGE experiments were developed for plasma, these candidates were immunochemically screened in the plasma samples acquired from the (un)treated animals. Most potential biomarkers belonging to e.g. the group of growth factors/ regulators and bone metabolism, did not differ by more than a factor of two in concentration and were, in particular, reflecting effects in male animals and dexamethasone treatment.

DISCUSSION
The extra weight gain of treated animals was on average 10 kg compared to the untreated group. The applied analytical strategy, namely electrophoresis/mass spectrometry and affinity assays, was not without challenges. The abundant proteins in plasma obscured biomarker discovery by 2DGE and DIGE, whereas affinity assays failed especially to acquire good-performing anti-bovine protein antibodies. Unexpectedly, most candidate biomarkers, including insulin-like growth factor I (IGF-I), as extracted from published data, were not found valuable in the treated juvenile calves. In the ongoing project, once identified, potential biomarkers will be validated and then used in a biosensor enabling the screening of plasma to support inspection actions.

ACKNOWLEDGEMENTS
This project is funded by the European Commission under the Food Quality & Safety Priority Thematic Area (Food-CT-2004-06988).

REFERENCE

E11
Identification of hydroxyl metabolites by liquid chromatography tandem mass spectrometry in urine of veal calves administered boldenone and boldione
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INTRODUCTION
Preliminary data obtained in vitro by incubating subcellular fraction from calf liver with 17β-boldenone (androsta-1, 4-diene-17β-ol-3-one (17β-BOL)) or androsta-1,4-diene-3,17-dione (ADD or boldione) showed that some polar metabolites were produced from both compounds at detectable quantities. Indeed when ADD was incubated, HPLC analysis of the extracts showed 17β-BOL as metabolite, but another polar compound occurred in detectable amounts (Fig. 1); the putative identity as a hydroxyl derivative was tentatively assessed by means of LC/MS/MS fragmentation. When 17β-BOL was added to the incubation mixture two polar compounds were recovered in addition to ADD, and their nature as hydroxyl derivatives was confirmed with pure standard comparison [1]. Because urinary hydroxyl derivatives can help confirm illegal treatment and anabolic steroids are usually completely metabolized, the main aim was the in vivo investigation of the urinary excretion profile of 17β-BOL and its metabolites, in an attempt to identify possible new in vivo markers of illicit administration.

MATERIALS AND METHODS
Animal husbandry and treatment The experiment involved, according to a 2 × 2 design, 28 German Brown male calves (10 days old. about 68.7 ± 0.14 kg body weight). Calves were kept in separate boxes for 3 months and fed a commercial milk replacer. At the end of this period, the diet was changed: 14 animals received a milk replacer with a higher percentage of plant sterols added whereas the other 14 were given the milk replacer. At the end of this period, the diet was changed: 14 animals received a milk replacer with a higher percentage of plant sterols added whereas the other 14 were given the milk replacer.

Table 1. Precursor, most abundant fragments and method performances of the boldenone hydroxyl metabolites.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor m/z</th>
<th>Product m/z</th>
<th>CE (eV)</th>
<th>tR (min)</th>
<th>CCββ (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6β,17β-BOL</td>
<td>303 (M + H)²</td>
<td>121, 171, 147</td>
<td>30</td>
<td>6.0</td>
<td>0.6</td>
</tr>
<tr>
<td>6β,17α-BOL</td>
<td>303 (M + H)²</td>
<td>121, 171, 147</td>
<td>30</td>
<td>7.0</td>
<td>0.3</td>
</tr>
<tr>
<td>16β,17β-BOL</td>
<td>303 (M + H)²</td>
<td>121, 147, 171</td>
<td>30</td>
<td>7.8</td>
<td>0.6</td>
</tr>
<tr>
<td>16α,17β-BOL</td>
<td>303 (M + H)²</td>
<td>121, 147, 171</td>
<td>30</td>
<td>9.1</td>
<td>0.6</td>
</tr>
</tbody>
</table>
with sterols of animal origin. At the age of about 5 months, seven out of the 14 calves from each group received a combination of 17β-BOL and ADD (0.9 mg and 0.1 mg, respectively) daily, *per os* and *pro capite*, for 40 days. *Sample collection and extraction* Urine samples were collected at least once a week within 2–3 h of treatment for two months into a clean container: about 400–500 mL was collected after cleaning of the area of interest and divided into 100 mL aliquots that were immediately stored in the dark at –20°C. Extraction was performed as previously described [2] only on urine from treated animals, with some modifications. *LC/MS/MS analysis* Analyses were carried out in positive ion mode on a API 3000 triple quadrupole Mass Spectrometer, equipped with a Turbo Ion Spray source by gradient elution using a mobile phase acetonitrile-formic acid 0.3%, flow-rate of 200 μL min⁻¹ (Table 1).

RESULTS

Conjugated 17α-BOL and 17β-BOL were present at concentrations in the range 1.0–36.3 and 0.2–1.0 ng mL⁻¹, respectively. Detectable amounts of hydroxyl metabolites, conjugated 17α-BOL and 17β-BOL were measured in the urine samples from treated animals, while ADD was mainly below CCβ.

DISCUSSION

17β-BOL was confirmed as a marker of illegal use of anabolic agents. Work is ongoing however the hydroxyl metabolites 6βOH-BOL and 6β, 17αOH-BOL present after 3 days of treatment are potential new markers of anabolic abuse.

ACKNOWLEDGEMENT

The project was funded by Servizi Veterinari Regione del Veneto 2005 (Del 3655 29.11.05).

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E12 Impact of ampicillin administration on excretion of blaTEM genes in swine feces

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INTRODUCTION

Comparable to the effects of their use in human medicine, the utilisation of β-lactam antibiotics in animals contributes to selective pressure on intestinal bacteria leading to the emergence and the distribution of strains carrying resistance genes [1]. The plasmid-mediated blaTEM genes code for β-lactamases, enzymes responsible for most of the plasmid-mediated resistance to β-lactamases [2]. The development of relevant markers for the detection and quantification of these genes is the cornerstone for establishing strategies aimed at minimizing the impact of antibiotic therapy on the antibiotic resistance of animal microlora [3]. In the present study we developed a PCR based method for the quantification of blaTEM genes in faeces and applied it to evaluate the impact of three modes of administration of ampicillin to swine on the faecal excretion of resistance to β-lactams using both genotypic and phenotypic methods.

MATERIALS AND METHODS

To quantify blaTEM genes in swine feces a quantitative PCR assay, using SYBR®Green, was developed. The specificity of amplification, the quality of the standard curve and the absence of inhibitors were confirmed. Ampicillin was administered for 7 days at 20 mg kg⁻¹ to three groups of four pigs: oral route fed, oral route fasted, and an intramuscular route group. Four pigs were used as controls. The study was carried out in accordance with applicable animal welfare laws. Bacterial ampicillin resistance in faeces was measured both phenotypically and genotypically: percentage of enterobacteria resistant to ampicillin and MIC of *E. coli* isolates (phenotype); quantification of faecal blaTEM by real-time PCR (genotype). Changes in antibiotic resistance were statistically analysed using a generalized linear mixed-effects model.

RESULTS

The quantitative PCR assay of blaTEM genes was validated for quantities between 10 and 10⁶ copies μL⁻¹, corresponding to 10⁴ to 10⁹ copies g⁻¹ faeces. *Phenotype* Before treatment, mean percentages of enterobacteria resistant to ampicillin (MIC >16 μg mL⁻¹) ranged from 2.5% to 12.0%. From day 4 to day 7, for all administration routes, fecal enterobacterial flora was dominated by highly resistant strains (MIC >256 μg mL⁻¹). Statistical analysis indicated that ampicillin treatment led to an increase in faecal resistance to ampicillin, relative to the control group. These phenotypic evaluations did not however discriminate between the three dosage regimens. *Genotype* The following figure shows the means of blaTEM quantities excreted during the 7 days of treatment per treatment group; prior to treatment, the quantities of blaTEM excreted were below 10⁻⁷ copies g⁻¹ of faeces. During treatment, these quantities fluctuated between 10⁻⁴ and 10⁻⁶ for the control group and 10⁻⁷–10⁻⁹ and 10⁻⁶–10⁻⁸ copies g⁻¹ faeces for the oral routes and the intramuscular route, respectively. Fecal excretion of blaTEM genes was significantly higher amongst treated animals relative to the control group. Fecal excretion of blaTEM genes was significantly higher for the oral route in fed animals than for the intramuscular route.

DISCUSSION

These results indicate that the quantification of blaTEM genes by real-time PCR, applied concomitantly with a phenotypic evaluation of ampicillin resistance, is a useful tool for comparing the effect of different β-lactam dosage regimens on faecal excretion of resistant bacteria. Moreover, successful development
of this real-time PCR assay will facilitate the exploration of the dynamics of resistance genes in the digestive tract of swine.

REFERENCES

E13
A comparative study of the withdrawal period in milk of two antimicrobials administered by the intrauterine route to healthy cows
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INTRODUCTION
Oxytetracycline and the combination of sulphadiazine-trimethoprim are two commonly used formulations for the prevention and treatment of post-partum uterine infections in cows [1,2]. The aim of this study was to detect and quantify oxytetracycline and its 4-epimer (epi-oxytetracycline) and sulphadiazine-trimethoprim in bovine milk after post-partum intrauterine administration of two commercial formulations. The results were used for the determination of the withdrawal time of each formulation [3].

MATERIALS AND METHODS
Thirty-eight Holstein Friesian cows in the age range 2–9 years were used. All animals were randomly allocated to two groups (A and B). After parturition, animals from group A were administered Oxyvet® twice with at a dose of four pessaries per animal (2 g oxytetracycline hydrochloride/animal) per administration. There was an interval of approximately 12 h between the two administrations and the second administration was at about the time of milking. After parturition, each cow from Group B was administered Optiprime® once at a dose of four boluses per animal (4 g sulphadiazine plus 0.8 g trimethoprim/animal). There was an interval of approximately 12 h between the administration of the test item and the first milking. Milk was collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 days after the last administration. Concentrations of oxytetracycline and its 4-epimer were determined in milk using a validated HPLC method with UV photometric detection. Determination of sulphadiazine and trimethoprim in milk was performed GLP-accredited laboratory (Veterin) using HPLC coupled with a Mass Spectrometer. Throughout the study the following examinations were performed daily in order to identify any evidence of infection which could affect the test product absorption: body temperature, complete blood count, fibrinogen concentration and a general clinical evaluation with closer inspection of the udder and the reproductive system. Any abnormalities were recorded, including lesions in teats, presence of clots in milk or abnormal vaginal discharge.

RESULTS
The sum of oxytetracycline and its 4-epimer were below the MRL in all of the milk samples on day 3 and was no longer detected 3.5 days after the last administration of the pessaries. Sulphadiazine could not be quantified or detected in any of the milk samples taken after 2.5 days of administration of the boluses. Trimethoprim could only be found in the samples collected during the first milking and was not detected at subsequent milkings. No abnormalities indicative of infection were recorded in any cows throughout the study and therefore it can be assumed that the concentrations detected in milk are the maximum that could be achieved after administration of the two products by the intrauterine route.

DISCUSSION
Based on these results and the absence of any clinical or clinicopathological abnormalities that could reduce drug absorption, a withdrawal period of 3 days is justified for colostrum, whereas, in milk a zero withdrawal period is justified, following intrauterine administration of Oxyvet® pessaries or Optiprime® bolus at the maximum recommended dosage post partum to healthy cows.

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E14
Macro cyclic lactone residues in milk: chemical stability and pattern of residual concentrations during milk processing
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INTRODUCTION
The presence of drug residues in milk is a problem that has focused mainly on antimicrobial agents due to their impact on industrial milk processing. Nevertheless, it is necessary to know the consequences that the residues of other substances, used extensively in dairy animals, could have on the processes of elaboration of milk products. The presence of antiparasitic drug residues usually refers to drug concentrations in raw tissues (liver, muscle, fat) but the effect of different food processing methods (pasteurization, cooking, ripening, etc.) on the chemical stability and persistence of drug residues in milk and dairy products is unknown. Ivermectin (IVM), moxidectin (MXD) and eprinomectin (EPM) are broad-spectrum macrocyclic lactone (ML) antiparasitic drugs extensively used in food-producing animals [1]. The pattern of milk residue excretion for different ML compounds in dairy sheep has been recently determined in our laboratory [2]. As a follow up, the current trial addressed the evaluation of the chemical stability of residual concentrations of IVM, MXD and EPM in milk and to determine the comparative pattern of residues in whey, curd and cheese prepared from milk from dairy sheep treated with those ML compounds.
MATERIALS AND METHODS
IVM (0.25–10 ng mL\(^{-1}\)), EPM (0.1–5 ng mL\(^{-1}\)) and MXD (10–200 ng mL\(^{-1}\)) was added to drug-free milk samples collected from untreated lactating ewes. Milk samples with drug added were heated at 65°C for 30 min (pasteurization) or at 75°C for 15 s (high temperature short time pasteurization). IVM, MXD and EPM concentrations were measured prior to and after the heating process as described below. Semi-hard cheeses were elaborated with pooled milk collected daily (up to 25 days post-treatment) from ewes treated with either IVM, MXD or EPM. Drug residues in curd and whey collected during cheese-making were measured. IVM, MXD and EPM concentrations were measured in raw milk, heated milk and in dairy products (whey, curd and cheese) using an HPLC methodology previously reported [3]. Additionally, the acidity, dry matter, fat content and total nitrogen in milk collected from untreated and ML-treated-dairy sheep before milk processing were determined.

RESULTS
No significant changes in the IVM, MXD and EPM residue profiles were observed after either thermal treatment (65°C, 30 min or 75°C, 15 s), the variation observed in heated milk residual concentration being within the range of the analytical method. The presence of ML residues affected neither the acidity, nor the dry matter, fat or total nitrogen content of raw milk. High residual concentrations of the parent compounds were found in curd. The ML residue profiles measured in curd were significantly higher than those detected in milk collected from treated sheep. The ratios between drug residue concentrations measured in curd and milk were: 2.8 ± 0.23 (IVM), 2.4 ± 0.17 (MXD) and 3.4 ± 0.24 (EPM). A lower proportion of these lipophilic analytes ended in whey due to the high water content of this dairy product. The highest residual concentrations of these ML were detected in ripened cheese. The ratios between drug concentrations in semi-hard cheese and milk were: 3.3 ± 0.45 (IVM), 3.4 ± 0.38 (MXD) and 5.1 ± 0.65 (EPM). Weight reduction in the cheese due to water loss during ripening was observed. This water loss and the subsequent increment of total solid and fat content in ripened cheese may have accounted for the high residual concentrations of ML found after 40 days of maturation. Linear correlations between percentages of water loss, total solid, fat contents and drug residual concentration during cheese maturation were observed (\(r > 0.90\)).

DISCUSSION
IVM, MXD and EPM residues in sheep milk were chemically stable after exposure to pasteurization temperatures, which agrees with results previously reported for IVM in heated milk [4]. The total amount of drug residues recovered from milk was markedly lower for EPM compared to both IVM and MXD. However, the concentration ratios cheese/milk were similar for the three ML compounds. The highest residual concentrations were consistently recovered from curd and cheese, in line with their higher fat and total solid contents compared to the milk. The outcome of the work presented here is complementary to the available information [2,5,6] and demonstrates that the presence of ML residual concentrations does not affect the physicochemical features of milk. IVM, MXD and EPM residual concentrations persist unchanged during milk processing reaching concentration levels three to five-fold higher in ripened cheese than in milk.

REFERENCES

E15
Local tolerance and milk withdrawal period of a veterinary medicinal product containing ceftiofur
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INTRODUCTION
This study aimed to assess the local tolerance and to determine the withdrawal period in cows milk for ceftiofur sodium according to the current European guideline [1].

MATERIALS AND METHODS
Ceftiofur sodium sterile powder for injection (Virbacet\(^\text{®}\)) was administered intramuscularly according to the recommended treatment regime to 20 healthy dairy cows (mixed breeds, 2.5 to 9 years old), weighing 607 ± 53 kg and with an average daily milk production of 29.2 ± 10.0 L (milk yielding: 13.4 to 47.8 L per day). The daily administered doses were 1.00 ± 0.03 mg ceftiofur kg\(^{-1}\) body weight (one daily injection of 1 mL per 50 kg body weight in the neck) for five consecutive days. Milk samples (pooled milk from the four quarters) were collected at 0 h (just before the 5th dosing), then at 6, 8, 12 and 24 h. The samples were analysed for ceftiofur and metabolites (desfuroylceftiofur equivalents) using a HPLC with UV detection based on published results [2,3] and fully validated according to the European regulations [4]. The LOQ was set to 50 \(\mu\)g kg\(^{-1}\), i.e. half the ceftiofur maximal residue limit (MRL) in milk. Both the in-life and the analytical phases were performed according to Good Laboratory Practices. The withdrawal period was determined using the EMEA software MELK14 [5] allowing the time when milk concentrations are below the MRL for the 95th percentile of the population with a 95% confidence level to be determined. For this purpose, values not detectable were entered as half the LOD (10 \(\mu\)g kg\(^{-1}\)), and values <LOQ at half the LOQ, according to EMEA/CVMP/473/98-Final. Zero h was entered as 0.001 h because MELK14 software does not allow input values of zero for sampling time.

RESULTS
Whatever the animal, no swelling, induration or warmth was detected at the injections sites during the study. All ceftiofur concentrations in milk were below the MRL, irrespective of sampling time and animal (Table 1). Only safe concentration per milking (SCPM) was useful and the withdrawal period was determined to be nil.
DISCUSSION
Because all concentrations were below the MRL, the time-to-safe-concentration (TTSC) method cannot be used. The other statistical method safe concentration based on linear-regression (SCLR) was also not useful because of the lack of linear relation between log concentration and time. Thus, the safe concentration per milking (SCPM) was the only method allowing the determination of a withdrawal period. Using the MELK14, the withdrawal period was statistically calculated to be nil, confirming the intuitive assumption that there is no risk for the consumer since all concentrations cefquinome and its metabolites were below the MRL of 100 \( \mu \text{g kg}^{-1} \) for, whatever the animal (even low yielding animals) or sampling time. Thus the withdrawal period for Virbacet in milk is nil.

RESULTS
Cefquinome concentrations were already below the maximum residue limit (MRL) at the first milking post-calving for all cows with a DP of 31 days or more. For shorter DP, a WHP of 35 days from the time of treatment is determined statistically using 19 cows with a DP of 20–31 days or 23 cows with a DP 16–31 days. The time to safe concentration post-calving shows a strong negative correlation with the length of the dry period (Pearson’s coefficient of correlation: \( R = -0.78 \), Fig. 1).

DISCUSSION
The results clearly indicate that the depletion of cefquinome residues in milk after calving mainly depends on the length of the dry period. This is further supported by a study where Cobactan DC was administered to lactating cows [1]. A 35-day WHP was also calculated although the cows were milked out twice daily.

REFERENCE

INTRODUCTION
The determination of the withholding period (WHP) in milk after calving for dry cow products is difficult because different management practices and individual variations lead to a high variability in the length of the dry period (DP). Two residue studies were conducted with Cobactan DC, an intramammary cefquinome formulation to be administered at drying-off. The aim of the studies was to determine a WHP in the post-calving milk following DPs of variable durations.

MATERIALS AND METHODS
Fifty-seven cows were selected, 31 cows at the end of the normal lactation period (Study 1) and, in order to shorten the DP, 26 cows after a prolonged lactation (Study 2). All of the cows were treated with 150 mg cefquinome/quarter (Cobactan DC) at drying-off. At calving, after a dry period ranging from 16 to 59 days, milk/colostrum was taken twice a day from all four quarters of 54 cows (three exclusions). The samples were analysed for cefquinome content using a validated HPLC/MS method. Samples were processed until at least four consecutive samples were below the LOQ (10 ng mL\(^{-1}\)). The time to safe concentration (<MRL = 20 ng mL\(^{-1}\)) post-treatment was determined for each individual animal as described by the guideline (EMEA/CVMP/473/98-Final) and a withdrawal period was calculated. Additionally, times to safe concentrations post-calving were determined and compared with the length of the DP.
MEGLUMINE PREPARATIONS FOR THE TREATMENT OF mastitis IN CATTLE IS APPROVED IN SEVERAL EU Member States. Therefore EMEA/CVMP has established a MRL of 40 µg kg⁻¹ for flunixin, as measured by the marker residue 5-hydroxy flunixin (5-OH FLU), in bovine milk. Several authors, however, have reported that in milk obtained from cows treated i.v. with FLU not only 5-OH FLU is present, but approximately 50% of the total residues (¹⁴C) were residues other than the 5-hydroxylated derivative [1,2]. A compound different from FLU and 5-OH FLU was further identified [3]. Moreover, it was observed that up to 75% of the total residue level in milk. The aim of this study was to determine the concentrations of FLU and 5-OH FLU in the milk from cows dosed with flunixin meglumine and to compare the results with those obtained after treating the same milk samples with β-glucuronidase.

MATERIALS AND METHODS

Six Holstein-Friesian dairy cows, with a mean body weight of 498 kg and a range of 13.1–15.8 kg daily milk yield were used. Analysis of milk collected before the beginning of the experiment proved that subclinical mastitis was present in the cows. The range of concentrations of FLU and 5-OH FLU measured in milk samples with or without prior deconjugation from cows treated i.v. are presented in Table 1.

RESULTS

The range of concentrations of FLU and 5-OH FLU measured in milk samples collected after the last i.v. administration of FLU and 5-OH FLU was present as a β-glucuronide derivative, which is not extractable during conventional analysis [4]. All these observations suggest that the marker residue 5-OH FLU may not represent the total residue level in milk. The aim of this study was to determine the concentrations of FLU and 5-OH FLU in the milk from cows dosed with flunixin meglumine and to compare the results with those obtained after treating the same milk samples with β-glucuronidase.

DISCUSSION

The results from this study are in agreement with the findings of Rupp et al. [4] and showed that a high percentage of FLU in cows is present as β-glucuronide conjugates and is therefore not extracted by routine analytical methods, where enzymatic deconjugation is not performed. This may explain the choice of 5-OH FLU as marker residue of flunixin in milk. From a toxicological point of view, it would be therefore reasonable to recognize the sum of the parent compound and the main metabolite as the marker residue of FLU.

REFERENCES


E18

EXTRACTION METHODOLOGY FOR ANTIBIOTICS DETECTION IN MILK

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INTRODUCTION

Antibiotics are widely used in veterinary medicine and animal nutrition for disease treatment and as dietary supplements. Their use may result in drug residues being present in milk, especially if they are not employed according to label indications. Microbiological assays have been most commonly used for milk residue detection, but they are non-specific [1]; therefore, a method combining simple, rapid and precise chromatographic separation, such as TLC and HPTLC, offers significant advantages. This work describes extraction procedures for penicillin G, amoxycillin ( β-lactams), tetracycline (tetracyclines), spiramycin (macrolides), spectinomycin (aminoglycosides), and lincomycin in the same milk sample. The different chemical structures of such groups provide difficulties in the recovery of compounds in extraction method. Antibiotic identification was done using TLC and HPTLC [2] methods where compound discrimination could be obtained. For spot visualization on TLC and HPTLC plates, two techniques were used: examination under UV light (365/254 nm) and/or plate spraying with chemical reagents that react with compounds to produce a colored product.

MATERIAL AND METHODS

Four extraction procedures were made in 10 mL aliquots of fortified fat milk. These procedures were: (a) a liquid-liquid extraction with acetonitrile and chloroform; (b) a solid phase extraction (SPE), with acetonitrile protein precipitation and cartridge wash with n-hexane and centrifugation at 3400 rpm before elution; (c) a SPE, with acetonitrile protein precipitation and cartridge wash with acetonitrile without centrifugation before elution; (d) a SPE without protein precipitation with cartridge wash with n-hexane, followed by elution and n-hexane purification. All SPE elutions were performed with methanol. The SPE extractions were performed in HLB Plus (225 mg...
Waters Oasis) cartridges [3]. For both TLC and HPTLC procedures silica gel G 60 F254 Merck plates were used. The HPTLC was developed in a CAMAG system (Nanomat 4), with horizontal development.

RESULTS
With the liquid-liquid extraction, only tetracycline, amoxicillin and spiramycin were extracted; with the first SPE extraction procedure (b), penicillin G and lincomycin were extracted; with the second SPE extraction procedure (c) penicillin G and tetracycline were extracted. With the last SPE extraction procedure (d), tetracycline and spectinomycin were extracted at 0.2 µg mL⁻¹, amoxicillin, penicillin G and lincomycin were extracted at 0.5 µg mL⁻¹, and spiramycin was extracted at 1 µg mL⁻¹. The elution systems for efficient antibiotic separation in TLC and HPTLC were: chloroform/methanol (4: 1, v/v) (2: 3, v/v) and butanol/acetic acid/water (3: 1: 1, v/v/v). Antibiotic spot visualization was optimized with the following reaction spraying reagents: p-anisaldehyde for tetracycline, spectinomycin and spiramycin and iodine for penicillin G, lincomycin, amoxicillin and spiramycin. The detection limits in TLC for spiramycin, spectinomycin, tetracycline, penicillin G, lincomycin and amoxicillin were 0.05, 0.005, 0.008, 0.007, 0.08 and 0.08 µg, respectively. The detection limits in HPTLC for spiramycin, spectinomycin, tetracycline, penicillin G, lincomycin and amoxicillin were 0.02, 0.1, 0.05, 1, 0.05 and 1 µg, respectively.

DISCUSSION
Liquid-liquid extraction only resulted in three antibiotics being detected because of their high solubility in milk aqueous layer. Some compounds could not be extracted, and consequently not visualized, in procedures with protein precipitation, cartridge centrifugation before elution and subsequent wash with acetonitrile. With SPE extraction, without protein precipitation and with n-hexane elution, it was possible to extract all of the antibiotics selected in the same aliquot irrespective of their different physico-chemical properties. Using the TLC and HPTLC methods proposed, it was possible to identify, for each antibiotic studied, eluents, spraying reagents, retention factor values and detection limits. However detection limits of some compounds were lower in TLC than HPTLC. Although the spiked concentrations were greater than the established MRL for the examined compounds, work is in progress to further validate the described methodology for lower drug concentrations.

ACKNOWLEDGEMENTS
In memory of the friend and colleague E. Marques Fontes. This work was supported by CIISA/FMV.

REFERENCES
INTRODUCTION
The potentiated sulfonamides have been used as chemotherapeutic agents against bacterial diseases in fish for decades. Several studies indicated that sulfadimethoxine and ormetoprim (SDM-OMP) has potential as shrimp aquaculture antibacterial medicated feed treatment [1,2]. This study was conducted to evaluate the bioavailability and tissue depletion of SDM-OMP 5:1 following medicated feed treatment of tiger shrimp.

MATERIALS AND METHODS
The SDM-OMP in a 5:1 ratio was added to the feed to achieve active ingredient concentrations of 30 and 50 mg kg\(^{-1}\) body weight and given ad libitum to shrimps, 25–35 g, for five consecutive days. During the treatment, nine shrimp were sampled twice daily and residual concentrations were followed for 50 days. Samples of muscle were analysed by HPLC, with a limit of detection of 0.01 mg kg\(^{-1}\) for SDM and OMP.

RESULTS
Drug concentrations found during 4 and 24 h were 0.5–1.46 mg kg\(^{-1}\) and 0.65–2.45 mg kg\(^{-1}\) for SDM and 0.16–0.88 mg kg\(^{-1}\) and 0.25–2.40 mg kg\(^{-1}\) for OMP, following 30 and 50 mg kg\(^{-1}\) body weight medicated feed treatments, respectively. Significant SDM residues in muscle were below 0.01 mg kg\(^{-1}\) by 28–35 days after the withdrawal of the treatment, while OMP residues were <0.01 mg kg\(^{-1}\) by 72 h in muscle. The corresponding ratio in shrimp muscle at 4 h after administration of SDM-OMP at low and high dose were 2.5:1 and 1:1, respectively. Mean drug concentrations of SDM and OMP during 5 days of medicated feed treatment are summarized in the table below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean concentration (mg kg(^{-1})) at 4 h and 24 h after feeding (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDM 4 h</td>
<td>2.45 ± 0.01 0.45 ± 0.00 1.56 ± 0.07 0.54 ± 0.02</td>
</tr>
<tr>
<td>24 h</td>
<td>1.46 ± 0.12 0.50 ± 0.01 0.88 ± 0.01 0.16 ± 0.01</td>
</tr>
<tr>
<td>OMP 4 h</td>
<td>2.45 ± 0.07 0.65 ± 0.01 2.4 ± 0.10 0.25 ± 0.01</td>
</tr>
<tr>
<td>24 h</td>
<td>1.46 ± 0.12 0.50 ± 0.01 0.88 ± 0.01 0.16 ± 0.01</td>
</tr>
</tbody>
</table>

DISCUSSION
Ours results suggest that both the dihydrostreptomycin ELISA and the Charm Test II may be successfully applied to detect aminoglycoside residues in milk samples at the established MRL. The agreement between results of the two different post-screening methods on treated milk samples was quite good.

ACKNOWLEDGEMENT
This work was supported by National Bureau of Agricultural Commodity and Food Standards, MOAC, Thailand.

REFERENCES


### Table 1. Results of tests performed on samples collected from treated animals

<table>
<thead>
<tr>
<th>Animal No./Sample</th>
<th>Time from the last treatment (days)</th>
<th>Delvotest®Sp</th>
<th>Elisa Dihydrostreptomycin</th>
<th>Charm Test II Dihydrostreptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/A</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1/B</td>
<td>6</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2/A</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2/B</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/C</td>
<td>7</td>
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<td>+</td>
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<td>5/A</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5/B</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>6/A</td>
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<td>-</td>
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<tr>
<td>6/B</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7/A</td>
<td>not declared</td>
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<td>-</td>
</tr>
<tr>
<td>7/B</td>
<td>not declared</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>From 8 to 19</td>
<td>not declared</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
INTRODUCTION

According to earlier studies of i.m. and oral administration of a 5:1 sulfadimethoxine (SDM) and trimethoprim (TMP) combination to tiger shrimp, TMP has low bioavailability after oral administration. The aim of this study was to evaluate the bioavailability and tissue depletion of an SDM-TMP 5:1 combination following medicated feed treatment in tiger shrimp.

MATERIALS AND METHODS

SDM-TMP in combination at a 5:1 ratio was added to feed and given ad lib to shrimp, 25–35 g body weight, to achieve 30, 50 and 100 mg kg$^{-1}$ body weight, for five consecutive days. During the treatment, nine shrimps were sampled twice daily and tissue residual concentrations were followed for 50 days. Samples of muscle tissue were analysed by HPLC, with a limit of detection of 0.01 mg kg$^{-1}$ for SDM and 0.05 mg kg$^{-1}$ for TMP [1].

RESULTS

Drug concentrations after 4 and 24 h were 0.5–1.9 mg kg$^{-1}$, 0.6–1.9 mg kg$^{-1}$ and 0.8–2.0 mg kg$^{-1}$ for SDM and 0.04 mg kg$^{-1}$ to undetectable, 0.04 mg kg$^{-1}$ to undetectable and 0.07–0.19 mg kg$^{-1}$ for TMP, following 30, 50 and 100 mg kg$^{-1}$ body weight in medicated feed, respectively. Significant SDM residues in muscle tissues were below 0.01 mg kg$^{-1}$ by 28–36 days after withdrawal of medicated feed, while TMP concentrations were below 0.05 mg kg$^{-1}$ (LOQ) at all sampling times in all groups. With the increase of the dose from 50 to 100 mg kg$^{-1}$ body weight, the shrimp feeding rates decreased after 2 days of medication. As a result, the drug concentrations found in muscle did not increase as expected. Mean drug concentrations of SDM and TMP during 5 days of medicated feed treatment are summarized in the table below.

DISCUSSION

Results of the present study indicate that increasing SDM-TMP concentrations in feed to obtain a dose of 100 mg kg$^{-1}$ body weight in shrimp caused feed rejection after only two days of the required 5-day feeding regimen. As a result, the drugs remained unconsumed and the shrimp unmedicated. The low bioavailability at dose rates higher than 50 mg kg$^{-1}$ body weight probably resulted from poor absorption due to the low solubility of SDM-TMP in the gastrointestinal tract of the shrimp [2]. These results clearly show that it is difficult to calculate pharmacokinetic values in shrimp on which to base dosage recommendations. A reasonable suggestion is to reduce the dose and extend the treatment period.

ACKNOWLEDGEMENT

This work was supported by National Bureau of Agricultural Commodity and Food Standards (ACFS), Ministry of Agriculture and Cooperatives (MOAC), Thailand.

REFERENCES


E22 Residue depletion of flumequine in gilthead sea bream (Spauros aurata, Limnaeus 1758)


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INTRODUCTION

Aquaculture is a rapidly growing industry. Farmers frequently increase stocking density to enhance production. This results in increased disease prevalence and the use of antibiotic-containing feed. Increasing use of antibiotics in modern aquaculture to increase production may result in a significant economic advantage for farmers, but the concern for consumer safety associated with drug residues and development of pathogen resistance is on the rise. Flumequine is a broad spectrum synthetic antimicrobial agent belonging to the 4-quinolones, often used in livestock and fish farm industries in cases of pulmonary, urinary and gastrointestinal infections, which act by inhibiting bacterial DNA-gyrase. There is now a strict legislative framework controlling the use of such substances, with the aim of minimising the risk to human health associated with consumption of their residues. The pharmacokinetic properties of flumequine have been evaluated in fish [1,2], but there is no published information on residues of flumequine in edible tissues. The aim of this study was to evaluate the residue depletion of flumequine in the gilthead sea bream.

MATERIALS AND METHODS
Gilthead sea bream (Sparus aurata, Limnaeus 1758), ranging in weight from 200 to 250 g, were used to investigate the tissue depletion of flumequine after oral administration (12 mg kg⁻¹ body weight day⁻¹, for five consecutive days). Fish which are treated at the dose of 12 mg kg⁻¹ body weight, receive the feed mixed with flumequine at a concentration of 1.75 g kg⁻¹ feed. The dose was administered during 20 min per day. Fish were kept in 6000 L tanks with an aerated continuous flow of sea water. Water temperature was 20–20.5°C. After treatment, the fish were sacrificed on ice during 10 min. Once confirmed dead, fish were decapitated and samples of muscle plus skin in natural proportions were taken at intervals (seven fish/time point). Tissue samples were stored frozen at −45°C until analysis. Tissue concentrations of flumequine were measured using the HPLC method reported by Yorke & Froc [3]. Homogenized tissue samples were extracted in three aliquots of ethyl acetate. After shaking and centrifugation, Yorke & Froc [3]. Recovery rate for the various flumequine concentrations was 81–88%. The LOQ was 4 ng g⁻¹. The withdrawal period was estimated by linear regression analysis of the log-transformed tissue concentrations determined at the time when the upper one-sided tolerance limit, with a confidence of 95%, was below the MRL [4,5].

RESULTS
After a withdrawal of 3 h (0 days) 1, 2 and 3 days, tissue concentrations were 1564.979 ± 242.636, 131.107 ± 46.644 ng g⁻¹, 13.528 ± 4.379 ng g⁻¹ and 7.520 ± 2.426 ng g⁻¹. After a withdrawal of 8, 10 and 14 days, flumequine concentrations were below the LOQ in tissue samples (muscle plus skin). Residual levels were estimated to fall below the MRL after a withdrawal period of 1.37 days.

DISCUSSION
Flumequine appears to be a promising candidate for use as an antimicrobial in fish. In the present study, treatment of gilthead sea bream with flumequine at a dose of 12 mg kg⁻¹ body weight for 5 days by means of medicated feed resulted in residue levels that were cleared quite rapidly. A withdrawal period of 2 days would ensure that concentrations in muscle plus skin would be less than MRL (EMEA/MRL/823/02).

ACKNOWLEDGEMENTS
Supported in part by the Spanish Ministry of Agriculture, Fish and Food (Secretaría General de Pesca Marítima). The authors would like to thank CIFPA “El Toruño”, Puerto de Santa Maria, Cadiz for help in this work (Dr. M. Manchado).

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4. EMEA/CVMP/036/95.
5. EMEA/MRL/823/02, 2002.

INTRODUCTION
Diclazuril (Clinacox®) is a chemical anticoccidial from the benzeneacetonitrile group authorised for use in chickens, turkeys and rabbits. Diclazuril is also granted an Annex II listing (no MRL required) in EC Regulation 2377/90 for oral use in ruminants and pigs. The maximal inclusion rate for diclazuril in target animal feedstuffs is 1 mg diclazuril/kg feed, or 1 ppm. Carry-over in feed-mills could lead to exposure of non-target species. The feed industry considers that an As Low As Reasonably Achievable (ALARA) level of 5% feedstuff cross-contamination is an achievable limit using current feed-mill practices. The non-target species that could potentially be exposed to diclazuril in feed include cattle, sheep, horses, goats and poultry.

MATERIALS AND METHODS
Based on the available data on the tolerance of diclazuril in non-target species, an assessment was made to determine the potential risk posed to non-target species following ingestion of feedstuffs containing Clinacox® at rates of 1 ppm and at 50 ppb. This corresponds to a level of carry-over of 100% and 5%, respectively. Assessment of the risk to consumers following ingestion of the EU standard food basket from non-target species that have been exposed to feedstuffs containing 1 ppm and 50 ppb diclazuril was conducted.

RESULTS
Non-Target Species Safety Assessment Diclazuril is an authorised veterinary medicinal product for cattle and sheep [1,2]. Doses of 5 mg kg⁻¹ body weight administered for 3 days have been shown to be safe in calves. Assuming a possible exposure to diclazuril at 1 ppm in feed gives a safety margin of about 250. Acute toxicity studies where diclazuril was administered as an oral suspension to sheep and goats have shown that diclazuril was safe at doses up to 60 and 80 mg diclazuril kg⁻¹ body weight, respectively. Assuming a possible exposure to diclazuril at 1 ppm in feed gives a safety margin of more than 4000. In pigs, doses of 5 mg diclazuril kg⁻¹ body weight administered for 3 days have shown to be safe. This gives a safety margin of about 75 assuming possible exposure to diclazuril at 1 ppm in feed. Although no long time exposure was investigated in cattle, sheep, goats or pigs, with the wide safety margin demonstrated, no concerns related to toxicity are anticipated after exposure to complete feed. Diclazuril has been used therapeutically in the horse at a dose of 5 mg kg⁻¹ body weight for 21 consecutive days, with no reported adverse events [3,4]. Also doses up to 20 mg diclazuril kg⁻¹ body weight administered for 21 days have been shown to be safe in horses. In relation to medicated feed containing diclazuril levels of 1 ppm, this leaves a safety margin of 2000. The safety of diclazuril at levels of up to 1000 ppm in feed administered to mallard ducks for 28 days was demonstrated. Diclazuril levels of up to 25 ppm in feed
administered to quail and guinea fowl for 31 and 85 days and up to 50 ppm for 14 days in quail proved to be safe. **Consumer Safety Assessment** The margin of consumer safety following consumption of the EU standard food basket of 0.5 kg from non-target species that have been exposed to feedstuffs containing diclazuril at 1 ppm was estimated. For this, the Acceptable Daily Intake (ADI) of 1800 µg/person, as set by the Committee for Veterinary Medicinal Products (CVMP), was taken into account. Most typical body weights and daily feed consumption were obtained from literature. As diclazuril appears to be poorly absorbed from the intestinal lumen of all species, a worse-case estimate assumed that 10% of the ingested diclazuril is absorbed and distributed evenly over the tissues of the animal (see table). The margin of consumer safety after ingestion of a non-target species exposed to feed containing diclazuril levels of 1 ppm remains always well above 200 times.

**DISCUSSION**

Based on the available data, it can be concluded that: there are no safety concerns in non-target species (cattle, sheep, goats, pigs, horses, ducks, guinea-fowl and quail) when exposed to diclazuril at 1 ppm in feed; there is no risk to the consumer from ingestion of non-target species exposed to diclazuril at 1 ppm in feed (100% carry over); for cross contamination, acceptable concentrations of diclazuril of 1 ppm (100% carry over) could be considered. Even at a 100% carry over, diclazuril is safe for non-target species and for the consumer ingesting these non-target species.

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1. EMEA/MRL/89/5/04-Final-rev.
2. EMEA/MRL/086/96-Final.

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**E24**

**Tissue residue depletion after multiple subcutaneous administration of florfenicol to sheep**

S. E. WETZLICH1, V. M. LANE2 & A. L. CRAIGMILL1

1Western Region Minor Use Animal Drug Program (NRSP-7), Environmental Toxicology Extension, University of California, Davis, CA, USA; 2Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA, USA

**INTRODUCTION**

Florfenicol is a broad-spectrum antibiotic that is a structural analogue of thiamphenicol. It is approved for the treatment of bovine respiratory disease in beef cattle. Respiratory disease in sheep is often caused by the same pathogens as those causing disease in cattle, therefore florfenicol may have clinical applications in sheep. The objective of this study is to provide tissue residue depletion data necessary to fulfill the human food safety requirements of the United States Food and Drug Administration, Center for Veterinary Medicine (FDA-CVM) for florfenicol in sheep.

**MATERIALS AND METHODS**

Twenty-six mixed breed, Polypay type sheep (13 wethers and 13 ewes) weighing between 40 and 65 kg were randomized into five treatment groups by weight and sex. The extra animal was used as a control. Animals were dosed once a day for three days at a dose of 40 mg kg⁻¹ by s.c. injection in the neck. Serum samples were collected prior to each dose, at 24 h intervals for 4 days following the last dose, and prior to euthanasia. Treatment groups were euthanatized at 5, 10, 20, 30, and 40 days following the final dose. At necropsy, tissue samples from the second and third injection sites, kidney, liver, semimembranosus/semitendinosus muscle, diaphragm muscle, renal fat, and carcass fat were collected for residue analysis. Samples were assayed for florfenicol levels using an HPLC method [1]. Tissue samples were assayed for the marker residue, florfenicol amine, using an HPLC method [2]. This method converts all florfenicol residues and related metabolites to the marker residue. The University of California, Davis Animal Use and Care Administrative Advisory Committee approved the protocol for this study.

**RESULTS**

The mean concentration ± SD of the marker residue, florfenicol amine, in the sampled tissues is shown in Table 1. The highest levels, discounting the injection sites, were found in the liver. Injection site levels rapidly decreased to levels similar to those found in the other muscle tissues by 40 days. Serum florfenicol levels were detectable at 10 days post dosing.

**Table 1. Mean tissue concentrations of florfenicol amine (µg g⁻¹) in sheep**

<table>
<thead>
<tr>
<th>Day</th>
<th>Liver</th>
<th>Kidney</th>
<th>Muscle</th>
<th>Diaphragm</th>
<th>Inj Site 2</th>
<th>Inj Site 3</th>
<th>Fat</th>
<th>Renal Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>18.27±1.25</td>
<td>4.12±0.62</td>
<td>0.59±0.12</td>
<td>0.56±0.18</td>
<td>2.14±0.12</td>
<td>2.14±0.12</td>
<td>0.03±0.03</td>
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<tr>
<td>10</td>
<td>11.45±0.69</td>
<td>1.66±0.38</td>
<td>0.22±0.05</td>
<td>0.16±0.15</td>
<td>0.14±0.14</td>
<td>0.15±0.15</td>
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<tr>
<td>20</td>
<td>8.59±0.12</td>
<td>0.92±0.30</td>
<td>0.16±0.04</td>
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</tr>
<tr>
<td>30</td>
<td>1.27±0.06</td>
<td>0.11±0.07</td>
<td>0.08±0.06</td>
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</tr>
<tr>
<td>40</td>
<td>1.99±0.05</td>
<td>0.17±0.05</td>
<td>0.08±0.03</td>
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<td>0.04±0.04</td>
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</tr>
</tbody>
</table>

**DISCUSSION**

Cattle given a single dose s.c. at 40 mg kg⁻¹ had florfenicol amine levels in muscle, liver, and injection site of 0.08 ± 0.01, 2.12 ± 0.78, and 0.16 ± 0.06 µg g⁻¹ respectively at 35 days and 0.14 ± 0.41, and 0.14 ± 0.06 at 42 days [3]. This study found that sheep given three doses s.c. at 40 mg kg⁻¹ had similar levels to the cattle. It was established that the liver is the edible tissue with the highest and most persistent total residues and should be the target tissue for determining the withdrawal time in sheep. It has been shown in cattle that the majority of the total residue in the liver is bound and that the regulatory assay will release florfenicol amine in high yield from the bound and unbound residues. Thus, florfenicol amine has been set as the
marker residue [3]. The FDA-CVM has set the tolerance for florfenicol amine in cattle liver at 3.7 ppm based on a comparison of total residues of florfenicol to measured levels of the marker residue and a safe concentration of florfenicol of 6.0 ppm [3]. A USA withdrawal period of 38 days has been set for cattle after s.c. dosing. The data from this study in sheep suggests a USA withdrawal period of 42 days using the individual animal liver data. It is expected that the withdrawal period would be longer in most other countries based on their longer withdrawal periods for the s.c. route in cattle (UK, Ireland, Switzerland – 44 days, Canada – 55 days).

ACKNOWLEDGEMENTS
This work was financially supported by the Western Region Minor-Use Animal Drug Program, United State Department of Agriculture National Research Support Project (NRSP) No. 7. The analytical methods, analytical standards, and drug were generously supplied by Schering Plough Animal Health.

REFERENCES

INTRODUCTION
Florfenicol (FF), a broad-spectrum antibiotic, is a fluorinated structural analogue of thiamphenicol and chloramphenicol, which lacks the human health risk associated with chloramphenicol. It is bacteriostatic and its mechanism of action is similar to that chloramphenicol [1]. Moreover, FF is unionized in a pH range from 3 to 9 [2] and also poorly soluble in aqueous solution. Due to its lipophilicity, FF shows a good tissue penetration and, when used to control systemic infections in food producing species, undergoes biotransformation into the main metabolite florfenicol amine (FFA). This study provides pharmacokinetic properties necessary to fulfill the safety requirements for FF in chickens. In poultry, oral doses of 20 mg kg\(^{-1}\) body weight are suggested [3]. The aim of this study was to define FF (and FFA) tissue distribution and depletion in chickens.

MATERIALS AND METHODS
Eighteen healthy Ross male broiler chickens weighing 1.5 kg were used to investigate the tissue distribution and depletion of FF. The animals were treated orally at doses of 20 mg kg\(^{-1}\) body weight/day for 3 days and killed at 1, 5, and 7 days post treatment. Tissue (muscle, kidney, liver, fat and skin) samples were analyzed separately for FF and FFA using a HPLC method [4]. Tissue was homogenized with 0.1 M phosphate buffer, pH 7, then shaken and centrifuged. The supernatant was extracted in methylene chloride and then evaporated at 30°C under nitrogen. Mean recovery was 100% for FF and 73% for FFA. The limit of quantitation for FF and FFA in muscle, liver, kidney and fat and skin was 40 μg kg\(^{-1}\).

RESULTS
Table 1 shows the FF tissue distribution and residues, respectively (expressed also as a sum of FF and FFA).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 day</th>
<th>5 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>FF</td>
<td>FFA</td>
<td>FF</td>
</tr>
<tr>
<td></td>
<td>142</td>
<td>142</td>
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</tr>
<tr>
<td></td>
<td>95</td>
<td>95</td>
<td>86</td>
</tr>
<tr>
<td>Kidney</td>
<td>FF</td>
<td>FFA</td>
<td>FF</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>56</td>
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</tr>
<tr>
<td></td>
<td>267</td>
<td>267</td>
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</tr>
<tr>
<td>Muscle</td>
<td>FF</td>
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<td>FF</td>
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<td></td>
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<td></td>
<td>140</td>
<td>140</td>
<td>248</td>
</tr>
<tr>
<td>Fat + skin</td>
<td>FF</td>
<td>FFA</td>
<td>FF</td>
</tr>
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<td></td>
<td>46</td>
<td>46</td>
<td>34</td>
</tr>
</tbody>
</table>

DISCUSSION
As observed in other species (cattle, sheep and goat), FF showed a wide distribution in chickens. A 9-day withdrawal time can be considered adequate for the respect of established MRL [5]. The withdrawal period was estimated by the statistical method proposed by the EMEA [6].

REFERENCES

INTRODUCTION
Monensin is a polyether ionophore antibiotic used as feed additive to control coccidiosis in chickens and turkeys. The European Food Safety Authority (EFSA) have proposed that monensin and three other growth promoting antibiotics [1] be phased out by 2006. The most recent data on monensin residue distribution in chickens were obtained in the 1990s [2]. The design of residue studies implies that only one sample per animal is available: the pharmacokinetic analysis of these data is then based on a population approach [3]. To develop a PB-PK model the knowledge of bioavailability is also necessary. The aim of the study is to use actual data on residues and bioavailability of
monensin in chickens, as a basis for further investigations on the PB-PK of coccidiostats in chickens and turkeys.

MATERIALS AND METHODS
A pilot study was conducted to assess tissue and plasma depletion. Forty birds were fed with monensin (Elnancoban® 125 mg kg⁻¹ of feed) for 32 days. Birds (n = 6) were sacrificed at 20, 25, 32, 32.25, and 33 days. Plasma, thigh muscle, breast muscle, fat, and liver were collected. From this study, experimental design for the pivotal residues study was built, in which 138 birds were fed a monensin sodium premix at 20, 25, 32, 32.25, and 33 days. Plasma, thigh muscle, breast muscle, fat, and liver were collected. From this study, experimental design for the pivotal residues study was built, in which 138 birds were fed a monensin sodium premix (125 mg kg⁻¹ of feed) ad libitum: 70 birds were involved in a 24-h steady state study and 68 birds in a 4-day depletion study. The third study assessed bioavailability: the drug was administered, at a dose rate of 0.4 mg kg⁻¹ of body weight, by the i.v. route to 78 birds or into the crop with or without feed (66 birds per group). These studies were carried out according to national and international guidelines and laws on animal welfare. Quantification of monensin was performed with a validated HPLC-MS-MS method, with LOQ, depending on the matrix, ranging from 1 to 2.5 µg kg⁻¹. Population PK analysis was performed with WinNonMix 2.0 (Phartsicht, Mountain View, CA, USA).

RESULTS
The pilot study showed low concentrations in all tissues, and short half-lives in liver (2.69 h) and in fat (3.14 h). Six hours after the end of the treatment detectable plasma levels (>LOQ) were found in only one bird, while at steady state 31.56 ± 16.95 µg mL⁻¹ was measured. This value had marked inter-individual variability with a coefficient of variation of 53.71%. This was confirmed in the pivotal depletion study (Table 1). Monensin distributed to a higher extent to fat followed by liver and muscle. Furthermore, higher concentrations of monensin were detected in thigh muscle than in breast muscle.

<table>
<thead>
<tr>
<th>Table 1. Pivotal depletion study</th>
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<tr>
<td>Concentration in µg L⁻¹ or kg⁻¹</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Plasma</td>
</tr>
<tr>
<td>[n = 6]</td>
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<tr>
<td>28.42 ± 11.44</td>
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<tr>
<td>10.06 ± 6.17</td>
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<tr>
<td>4.66 ± 1.26</td>
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<tr>
<td>(n = 5)</td>
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<tr>
<td>Thigh muscle</td>
</tr>
<tr>
<td>[n = 6]</td>
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<tr>
<td>5.77 ± 2.01</td>
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<tr>
<td>6.15</td>
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<tr>
<td>Fat</td>
</tr>
<tr>
<td>[n = 6]</td>
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<tr>
<td>49.13 ± 20.54</td>
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<tr>
<td>29.23 ± 7.02</td>
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<tr>
<td>28.75 ± 10.23</td>
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<tr>
<td>(n = 4)</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>[n = 6]</td>
</tr>
<tr>
<td>16.98 ± 6.44</td>
</tr>
<tr>
<td>4.94 ± 1.90</td>
</tr>
<tr>
<td>3.82 ± 1.83</td>
</tr>
<tr>
<td>(n = 6)</td>
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</tbody>
</table>

DISCUSSION
It is surprising that data from the depletion studies reveal lower tissues concentrations than those found by Atef et al. [2] using a similar supplemented monensin premix (120 mg kg⁻¹ of feed). This could be explained by the analytical method used by those authors, i.e. TLC coupled with bioautography where the metabolites of monensin could have been quantified with the monensin leading to an overestimation of monensin concentration. Since thigh muscle contains more fat than breast muscle, the lower concentrations of monensin found may be due to the lipophilic nature of the ionophore: similar behaviour has been described for other lipophilic molecules like polychlorinated biphenyls [4]. The present results also suggest that the data should be analysed using appropriate fitting techniques, such as the population approach. The final results of the pivotal study should confirm the pilot study results. These in vivo data will help to validate a descriptive PB-PK model of monensin in chickens that could then be extrapolated to other avian species, and especially to turkeys. It will be important to incorporate variability and uncertainty into the PB-PK model because of the variability of in vivo data. Further investigations will be performed to adapt it to all polyether ionophores family when validating the model for two species.

ACKNOWLEDGEMENTS
C. Burel, R. Maurice and their teams at AFSSA’s laboratory in Ploufragan.

REFERENCES

E27
Safety profile an oral emulsion formulation of flubendazole
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INTRODUCTION
Flubendazole is an anthelmintic of the benzimidazole carbamate group. For many years, medicated feed premix formulations have been marketed for treatment of porcine and avian food producing species. Recently, a water emulsifiable formulation of flubendazole was developed to facilitate treatments in modern farming: an oral emulsion containing 100 mg flubendazole/g (Solubenol®). The recommended dose rate is 5 mg kg⁻¹ body weight for 5 days in pigs and 10 mg kg⁻¹ body weight for 7 days in hens. The present contribution addresses some salient features of the pharmacokinetics, consumer safety and environmental safety of flubendazole oral emulsion.

MATERIALS AND METHODS
For the quantification of flubendazole and its principle metabolites in plasma and edible tissues LC-MS/MS bioanalytical methods were developed and validated in compliance with regulatory guidance in EU. The methods achieved LOQ of 0.01 mg kg⁻¹. Pharmacokinetic studies were performed in pigs and hens at the recommended dose as well as at doses exaggerated in magnitude and duration. Residue studies were conducted at the recommended dose in pigs, hens and eggs. Environmental risk assessments (ERA) were made in accordance with regulatory guidance in the EU.

RESULTS
A prominent characteristic of flubendazole is its low oral bioavailability. This is due to limited absorption and a strong first pass metabolism in the liver. The result is a limited
distribution into the tissues. Flubendazole is metabolised by hydrolysis of the carbamate moiety and reduction of the carbonyl group. It is excreted, mainly unchanged, in faeces. Hydrolysed flubendazole is the dominant flubendazole-related substance in the plasma of pigs. At the recommended dose, it occurs at concentrations of 0.05–0.50 mg L\(^{-1}\) and depletes with a half-life of 15 h. Flubendazole and reduced flubendazole are measurable at exaggerated doses only. In hens, parent flubendazole and the reduced metabolite are measurable. At the recommended dose they attain concentrations in the range of 0.01–0.53 mg L\(^{-1}\) and 0.01–0.17 mg L\(^{-1}\), respectively, and deplete with a half-life of 13 h and 31 h, respectively. The hydrolysed metabolite is observed in hens only at exaggerated doses. For flubendazole, a no observed effect level (NOEL) of 2.5 mg kg\(^{-1}\) body weight was derived from the toxicology studies. With a Safety Factor of 200, this results in an Acceptable Daily Intake (ADI) of 0.750 mg per consumer day\(^{-1}\). The EU has established MRL for flubendazole. The description of the target species has been subject to change over the years, but in the most recent CVMP opinion it is expressed as pigs and poultry. The marker residue for meat and organs is the sum of flubendazole and its hydrolysed metabolite: in eggs the marker residue is parent flubendazole only. The MRL are 0.400 mg kg\(^{-1}\) in liver, 0.300 mg kg\(^{-1}\) in kidneys, 0.050 mg kg\(^{-1}\) in muscle and skin/fat, and 0.400 mg kg\(^{-1}\) in eggs [1]. Hydrolysed flubendazole represents the marker residue in the tissues of pigs whereas the parent drug is not quantifiable. Except for the first day after treatment, residues are present in pig liver and kidney only. The MRL is exceeded only in kidney on the first day post treatment. Both flubendazole and hydrolysed flubendazole are quantifiable in hen tissues. Except from the first to third day post treatment, residues are present in liver and kidneys only. Again, the MRL is exceeded only in kidney on the 1st day post treatment. A 4-day withdrawal is recommended for the edible tissues of both pigs and hens [2]. Residue concentrations in eggs do not even reach half the MRL at, during and post treatment. This means that anthelmintic treatment can be carried out without need to discard eggs. A number of release scenarios were considered to evaluate the safety of the use in fattening pigs, sows, pullets and hens for the environment. None of the resulting predicted environmental concentration (PEC)\(_{soil}\) indicated an environmental exposure requiring the study of environmental fate, behaviour or effects of flubendazole.

**DISCUSSION**

The pharmacokinetics, consumer safety and environmental safety of the use of flubendazole oral emulsion in pigs and hens is adequately documented. Suitable bioanalysis methods for flubendazole and its relevant metabolites in plasma, edible tissues and eggs have been developed and validated. The proposed withdrawal time of 4 days after treatment in tissues of pigs and hens, and the absence of the need to discard eggs during and after treatment of laying hens, are compatible with the patterns of use of flubendazole oral emulsion. Finally, the use of flubendazole oral emulsion in pigs and hens is safe for the environment. Solubenol® oral emulsion is an appropriate alternative to the premix formulations for the deworming of pigs and hens.

**REFERENCES**

1. EMEA/MRL/267/97-FINAL.

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**E28**

**An overview of antimicrobial resistance in bacteria of food animal origin in Kenya**

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**INTRODUCTION**

Antimicrobial resistance of bacteria has become a worldwide problem [1]. The emergence and spread of resistant bacteria is unavoidable unless antimicrobial agents are not used at all. The prevalence of resistant bacteria can lead to erroneous empirical selection of either non-effective or expensive drugs, increased morbidity or mortality [2]. The intent of this review is to describe the situation of antimicrobial resistance in bacteria of food animal origin in Kenya.

**MATERIAL AND METHODS**

Literature on antimicrobial resistance in *Escherichia coli* [2,3] and non-typhi *Salmonella* spp [4] from apparently healthy cattle, pigs and poultry and *Staphylococcus aureus* from milk and meat [5] for years between 1994 and 2002 was reviewed and evaluated. These data were for ampicillin, cotrimoxazole, streptomycin, tetracycline, kanamycin, gentamicin and chloramphenicol.

**RESULTS**

Available data suggest that the resistance problem in bacteria of animal origin is comparatively high in Kenya. Resistance to cotrimoxazole ranged from 51% in pathogenic bacteria to 100% in *Escherichia coli* [2]. Resistance to ampicillin, streptomycin and tetracycline ranged between 42.5 and 62.2% in both pathogenic and commensal (*E. coli*) bacteria. Resistance to the other antimicrobials remained low (≤ 38.5%). *Commensal* (*E. coli*) [2, 3] and pathogenic [4] *E. coli* bacteria of animal origin were often resistant to various antimicrobial agents. The high prevalence of resistant bacteria of animal origin in Kenya seems to be related to antibiotic usage; easy availability without prescription at drug stores and injudicious use in farms [6].

**DISCUSSION**

Antimicrobial resistance problems in bacteria of animal origin should be regarded as a major public health concern in Kenya. Enforcement of the laws that prohibit the sale of veterinary antimicrobial agents without prescriptions and the establishment of control measures of bacterial infections in food animals is urgently needed. These issues are not easy to address and require the collective action of governments, the pharmaceutical industry, animal health providers and the consumers.

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E29
High liver zinc concentrations associated with the use of oral zinc-oxide in pigs. Implications arising from routine diagnostic post mortem examination

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INTRODUCTION
Zinc is widely distributed throughout the body and plays an essential role in many body processes. A supplement of 2500 ppm zinc for 2 weeks post-weaning in pigs has been shown to reduce the incidence of diarrhoea by up to 50% and improve weight gain and food conversion efficiency [1]. It is believed that zinc inhibits the growth of pathogenic bacteria that might otherwise adversely affect the newly weaned pig. For this reason, in Great Britain weaner diets are frequently supplemented with zinc oxide (ZnO). Standard inclusion rates are 3.1 kg per tonne contributing 3100 mg kg\(^{-1}\) ZnO, which is 2500 ppm zinc.

MATERIALS AND METHODS
Index case: two eight-week-old pigs were submitted live for post mortem examination with a history of anaemia and scouring. Pigs had been weaned at 3–4 weeks and had recently received feed medicated with 3100 ppm ZnO for four to five weeks. Post Weaning Multi Systemic Wasting Syndrome (PMWS) had been present in growing pigs on the farm of origin for two years. The clinical signs shown by the pigs examined had been recognised by the farmer to be distinct from the usual presentation of PMWS on his farm, the clinical signs being anaemia and scour (diarrhoea). For the index case a diagnostic post mortem was carried out following standard laboratory procedures. Pilot study 1 was conducted to investigate the relationship between PMWS, zinc supplementation and accumulation of liver zinc was due entirely to zinc supplementation or if intestinal lesions associated with PMWS might have predisposed to zinc accumulation by affecting absorption or excretion. Alternatively excessive zinc accumulation of liver zinc was due entirely to zinc supplementation or if intestinal lesions associated with PMWS might have predisposed to zinc accumulation by affecting absorption or excretion.

RESULTS
PMWS was confirmed in the index case by histopathological examination of tissues. Liver zinc concentrations of 26, 441 and 47, 498 \(\mu\)mol kg\(^{-1}\) DM were measured (laboratory reference range 1820–4211 \(\mu\)mol kg\(^{-1}\) DM). Pilot study 1: Liver zinc concentrations from age-targeted pigs, some of which had been exposed to 3100 ppm in feed ZnO.

In pilot study 2, the results of monitoring slaughter weight pigs revealed that liver zinc residues were within the expected reference range when supplementation had been withdrawn 14 to 20 weeks prior to slaughter.

DISCUSSION
In the index incident it was uncertain if the apparently excessive ZnO supplementation and accumulation of liver zinc was due entirely to zinc supplementation or if intestinal lesions associated with PMWS might have predisposed to zinc accumulation by affecting absorption or excretion. Alternatively excessive zinc supplementation may have directly caused zinc toxicity and the clinical signs observed. Results from the first pilot study suggest that ZnO supplemented pigs tend to accumulate higher liver zinc concentrations. The causes and mechanisms are not apparent from this study but further work in this area is probably justifiable. ZnO is insoluble and very poorly absorbed following oral administration. Our results in Pilot study 1 suggest that the presence of PMWS in a herd may increase ZnO absorption (see table). The results of pilot study 2 confirm that zinc tissue residues arising from approved use of ZnO are unlikely to undermine food safety. High zinc concentrations may also affect other aspects of veterinary husbandry and therapeutics by interacting with other nutrients, antibiotics and by potentially facilitating the selection of resistant bacteria.

REFERENCE

E30
Validation of methodology for the analysis of tetracycline, chlortetracycline, 4-epichlortetracycline and oxytetracycline in porcine tissues using LC-MS/MS

S. BREWIN, D. AIRS, M. TODD & M. COLEMAN
Huntingdon Life Sciences, Eye, Suffolk, UK

INTRODUCTION
Tetracyclines are antibacterials which are used to treat a wide range of infections caused by Gram-positive and Gram-negative bacteria. Tetracyclines are also commonly used in veterinary medicine usually in combination with sulphonamides (e.g. sulfadimethoxine). There are a variety of instrumental techniques available to enable the analysis of tetracyclines, such as liquid chromatography with ultra-violet absorbance [1,2], fluorescence or with mass spectrometric detection [3,4]. Liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) was selected as the quantification technique for this work, due its high selectivity and sensitivity. Methodology was developed and validated for the determination of tetracycline, chlortetracycline, 4-epichlortetracycline and oxytetracycline in porcine tissues (muscle, liver and kidney).

MATERIALS AND METHODS
The methodology developed and validated involves extraction into a methanol/oxalic acid mixture by maceration, clean-up by liquid-liquid partition with hexane and quantification by LC-MS/MS using positive electrospray, monitoring characteristic MS/MS ion transitions for each analyte. These were \(m/z\) 445>410 for tetracycline, \(m/z\) 461>426 for oxytetracycline, and \(m/z\) 479>444 for both chlortetracycline and 4-epichlortetracycline.
The chromatography conditions used involved the use of a C₈ column with a gradient elution system and mobile phases containing methanol, water, and formic acid. Quantification was by the comparison of peak areas of extracted samples against a series of matrix matched calibration solutions using least squares regression analysis.

RESULTS
The detection limit for the methodology was 1 ng mL⁻¹ (equivalent to 25 µg kg⁻¹ in muscle, 75 µg kg⁻¹ in liver and 150 µg kg⁻¹ in kidney for all four tetracyclines). The response of all four tetracyclines using the LC-MS/MS system was shown to be linear over the range 1–50 ng mL⁻¹ in all matrices. The methodology was validated over the range to 50–200 µg kg⁻¹ in muscle, 150–600 µg kg⁻¹ in liver and 300–600 µg kg⁻¹ in kidney for all four tetracyclines. These levels were selected based on the MRL allowed in porcine tissues. Lower levels could have been achieved if required due to the sensitivity of the LC-MS/MS system. All the recoveries for all four tetracyclines were in the range 62–120% with mean recoveries on each validation occasion in the range 73–104%. All overall relative standard deviations (RSD) were between 4 and 16%. The recovery data was obtained for six replicates, at three concentrations on three separate occasions for each tissue type for all four tetracyclines. In addition, stability tests also carried out demonstrate that all the tetracyclines were stable in matrix for a period of one month when stored at approximately −20°C and that all the tetracyclines were stable in the final extract for one week when stored at approximately +4°C.

DISCUSSION
This methodology selected enables sample extracts to be quantified following only limited sample clean-up procedures. In addition, using this technique the four tetracyclines could be quantified together in a single analytical session, resulting in relatively short sample analysis times. For this reason, large numbers of samples may be analysed with a high degree of confidence in the data produced. The analytical method has been fully validated in porcine muscle, liver and kidney, with the accuracy and precision data demonstrating that this methodology is acceptable for routine sample analysis. Porcine tissues can be stored for periods of up to one month prior to analysis if required and tissues that have already been extracted using this methodology can be stored for up to one week prior to quantification.

REFERENCES

E31 Validation of methodology for the analysis of maduramicin in bovine tissues and milk, and avian tissues and eggs using LC-MS/MS
S. BREWIN, D. BURTON, D. AIRS & M. TODD
Huntingdon Life Sciences, Eye, Suffolk, UK

INTRODUCTION
Maduramicin is used in veterinary medicine principally to prevent or control coccidiosis (mainly in chickens). It allows the development of immunity in the chicken and prevents further proliferation of various strains of coccidia. An analytical method was developed and validated for the determination of maduramicin in bovine tissues (muscle, liver, kidney and fat) and milk, and avian tissues (muscle, liver, kidney and fat) and eggs in order to update previous methodology. A few instrumental techniques exist for the analysis of maduramicin, such as liquid chromatography with atomic absorption spectroscopy [1] or with mass spectrometry [2] detection. For this project, liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) was selected as the quantification technique.

MATERIALS AND METHODS
The developed and validated methodology involves extraction into acetonitrile by maceration, clean-up by liquid-liquid partition with hexane and quantification by LC-MS/MS using positive electrospray, monitoring the MS/MS ion transition m/z 934.5>629.5. The chromatography conditions used involved the use of a C₈ column and a mobile phase containing acetonitrile, water, ammonium acetate and acetic acid. Quantification was by the comparison of peak areas of extracted samples against a series of solvent based calibration solutions using least squares regression analysis.

RESULTS
A detection limit of 0.2 ng mL⁻¹ was easily obtained (equivalent to 2 µg kg⁻¹ in avian fat, bovine fat and milk and 4 µg kg⁻¹ in the remaining matrices). The response of maduramicin using the LC-MS/MS system was shown to be linear over the range 0.2–20.0 ng mL⁻¹. The methodology was validated over the range 10–1000 µg kg⁻¹ in all matrices. Recovery data was obtained for five replicates, at three different concentrations for all matrices. Individual recoveries were all in the range 72–120% with mean recoveries on each validation occasion in the range 85–106% and overall relative standard deviations all less than 13%. In addition, stability tests carried out demonstrated that maduramicin is stable in all the matrices when stored at approximately −20°C for 87 days and that maduramicin is also stable in the final extract when stored at approximately −20°C for 6 days.

DISCUSSION
This technique is highly selective and enables sample extracts to be quantified following only limited sample clean-up. For this reason, large numbers of samples may be analysed with a high degree of confidence in the data produced. LC-MS/MS also enables low detection limits to be achieved. The analytical method has been fully validated in these matrices and precision/accuracy data confirm its suitability for routine sample analysis. Samples may be stored for up to 87 days prior to analysis if required and sample extracts may be stored for up to 6 days prior to quantification.

REFERENCES
E32
The analysis of amoxicillin in bovine liver and fat by LC-MS/MS
D. BURTON, D. AIRS, S. BREWIN & M. TODD
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INTRODUCTION
Amoxicillin is a β-lactam antibiotic used to treat bacterial infections caused by susceptible micro-organisms. Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, and so may be given with clavulanic acid to increase its spectrum. Amoxicillin and clavulanic acid combinations have been used in veterinary medicine since 1980 for treating livestock and companion animals for a wide range of Gram-positive and a limited range of Gram-negative organisms. The analysis of amoxicillin has been performed previously, utilising various instrument techniques such as liquid chromatography with ultra-violet absorbance [1], fluorescence [2] or mass spectrometric (MS) detection. As this analyte responds well by MS, liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) was selected as the most appropriate quantitation technique for this work. The aim of this work was to develop and validate an analytical method for the determination of amoxicillin in bovine tissues.

MATERIALS AND METHODS
The analytical method for fat involved extraction of the analyte from the sample with aqueous 0.01 M KH$_2$PO$_4$/acetonitrile before clean-up using liquid: liquid partition with hexane. The method for liver involved extraction of the analyte from the sample with aqueous 0.01 M KH$_2$PO$_4$. The extract was cleaned-up using a C$_{18}$ solid phase extraction cartridge. Quantitation in both cases was by reverse phase liquid chromatography using tandem mass spectrometric detection (LC-MS/MS). Quantitation was with reference to external matrix matched standard solutions of amoxicillin. Electrospray ionisation was used and the mass fragment transition m/z 366.4>114.2 monitored. The method for each matrix was evaluated in terms of linearity of detector response, specificity, limit of detection, limit of quantitation, analytical accuracy and analytical precision. The stability of amoxicillin in bovine liver during freezer storage at approximately −20°C and −80°C was also assessed. Amoxicillin (50 μg kg$^{-1}$) was added to samples of tissue from untreated control animals. These were subjected to the analytical procedure immediately (Day 0), after 14 days storage at approximately −80°C and after 21 days and 35 days storage at approximately −20°C.

RESULTS
The response of the LC-MS/MS system to amoxicillin was found to be linear (coefficient of determination >0.9993) over the range 1−50 ng mL$^{-1}$ in both matrices. The use of LC-MS/MS incurred high specificity and enabled low detection limits to be achieved. The LOD was equivalent to 5 μg kg$^{-1}$ in matrix. No matrix interference (<LOD) was found in any batch of matrix. The analytical method was fully validated for amoxicillin in bovine liver and fat, with a LOQ of 25 μg kg$^{-1}$. Recovery data was obtained for six replicates at three concentration levels on three separate occasions. For bovine fat, the recoveries ranged from 85−108%, with a mean recovery of 94% and a coefficient of variation (CV) of 6.8%. For liver, the recovery data ranged from 71−100% with a mean recovery of 87% and a CV of 9.0%. Following storage, the recovery of amoxicillin was still >70%, showing it to be stable under the conditions tested.

DISCUSSION
As LC-MS/MS is a highly selective technique, it enables sample extracts to be quantified following only limited clean-up procedures. The use of LC-MS/MS can also lead to relatively short sample analysis times. For this reason, large numbers of samples may be analysed with a high degree of confidence in the data produced. The validation data demonstrates that this methodology is acceptable to be used for the determination of amoxicillin in both matrix types. Amoxicillin is stable in bovine liver for 14 days when stored at approximately −80°C and for 35 days when stored at approximately −20°C. This work demonstrates that this analytical method is suitable for the analysis of tissue samples.

REFERENCES

E33
The analysis of tiamulin and 8x-hydroxy mutilin in various animal tissues and fluids by LC-MS
D. BURTON, D. AIRS, S. BREWIN & M. TODD
Huntingdon Life Sciences, Eye, Suffolk, UK

INTRODUCTION
Tiamulin is an antimicrobial with its main activity largely confined to Gram-positive micro-organisms and mycoplasma. In veterinary medicine, tiamulin is used for treatment and prophylaxis of dysentery, pneumonia and mycoplasma infections in pigs and poultry. In animal tissues, tiamulin is metabolised to form 8x-OH mutilin, which is defined as the marker residue. A validated analytical method was required for the determination of both compounds in a variety of porcine, rabbit and chicken matrices. There are only a limited number of instrument techniques available to enable the analysis of tiamulin and 8x-OH mutilin due to the low level of ultra-violet or fluorescence activity. There are also published methods for the analysis of 8x-OH mutilin by gas chromatography with mass spectrometric detection (GC-MS), where all of the relevant tiamulin metabolites are derivatised to form this compound. However, as both analytes respond to electrospray ionisation, liquid chromatography with mass spectrometric detection (LC-MS) was selected as the quantification technique for this work.

MATERIALS AND METHODS
The method for the analysis of 8x-OH mutilin involved extraction of tiamulin metabolites into a hydrochloric acid/acetone mixture by maceration, followed by hydrolysis of these compounds to 8x-OH mutilin. The extract then underwent liquid/liquid partition into dichloromethane followed by silica solid phase extraction cartridge clean-up. Quantification was by reverse phase LC-MS, with reference to external matrix matched standard solutions of 8x-OH mutilin. Electrospray ionisation was used and the mass fragment m/z 359.4 monitored. Tiamulin residues in tissue, egg or plasma were determined by extraction/
dilution by maceration with 0.1% aqueous tartaric acid, precipitation of proteins by addition of acetonitrile (plasma only), followed by clean-up using liquid/liquid partition with hexane. Quantification was performed by reverse phase liquid chromatography using tandem mass spectrometric detection (LC-MS/MS). Quantification was with reference to external standard solutions of tiamulin. Electrospray ionisation was used and the mass fragment transition m/z 495.5>192.2 monitored. In addition, a freezer storage stability test was conducted at approximately −20°C.

RESULTS

The analytical method was fully validated for 8α-OH mutilin in porcine muscle and liver, rabbit muscle and liver and chicken muscle, liver and skin/fat over the range 50–1000 μg kg⁻¹. These levels were selected based on the MRL allowed in tissues. Individual recovery values were in the range 63–126% with mean recoveries in the range 89–100% and coefficient of variation (CV) values less than 17% for all matrices. The tiamulin analytical method was validated in porcine plasma, muscle and chicken eggs. Individual recovery values were in the range 69–112%, with mean recoveries in the range 90–92% and CV values less than 11%. For both methods, recovery data was obtained for six replicates, at three concentration levels on three separate occasions. Detection limits equivalent to was obtained for six replicates, at three concentration levels on and CV values less than 11%. For both methods, recovery data range 69–112%, with mean recoveries in the range 90–92% only if the method is robust, quick, cheap and simple. Hence, we propose a new method for the control of antimicrobials in meat. The methods use on six plates for the simultaneous screening and post screening and is a modification of a method described previously [5].

MATERIAL AND METHODS

Assay plates All test micro-organisms are inoculated at the final concentration of 5 × 10⁶ cfu mL⁻¹ in the agar media (6 mL of seeded medium/plate). Plate 1 (β-lactams - tetracyclines): B. subtilis strain BGA on T. agar pH 6; Plate 2 (sulfonamides): B. subtilis strain BGA on DST plus TMP [0.12 μg mL⁻¹ agar]; Plate 3 (aminoglycosides): B. subtilis strain BGA on A. medium n. 5 TMP [0.12 μg mL⁻¹ agar]; Plate 4 (macrolides - β-lactams): M. luteus ATCC 9341 on T. agar pH8; Plate 5 (quinolones): E. coli ATCC 11303 on T. agar pH 6; Plate 6 (quinolones): E. coli ATCC 11303 on T. agar pH 8. Confirmatory solutions Penicillinase [10⁷ IU mL⁻¹] on plates 1 and 4. PABA [80 μg mL⁻¹] on plate 2, and MgSO₄ [20% v/v water] on plates 1, 3, 5 and 6. Sample assay In the same plate, slices of tissue samples are put on the agar surface with and without a 20 μL drop of confirmatory solutions. In the analysis of kidney samples, squares of the dialysis membranes were placed between the tissue samples and the agar surface. All samples were tested in duplicate. Plates 1–3 were incubated at 30°C and plates 4–6 at 37°C for 18 h and thereafter the width of each inhibition zone was recorded. When testing a large number of samples, it can be advantageous to screen and then to re-test only the positive samples.

RESULTS

Detection levels were obtained with ten replicates of standard solutions all less than or equal to the corresponding MRL [1]. Reversible concentrations were defined as the highest concentration of an antibiotic producing no halo when the corresponding confirmatory solution was present. When testing a sample, the simultaneous presence of inhibition around the first tissue slice and its disappearance around the others with confirmatory solution added, can be interpreted as presence of the family of antibacterial agents whose activity can be detected and neutralized in that plate of the system.

DISCUSSION

Penicillinase and PABA are respectively specific for β-lactams and sulfonamides, while MgSO₄ acts on tetracyclines,
aminoglycosides and quinolones. The presumptive identification by MgSO$_4$ must therefore take into account the profiles of inhibition obtained on the different plates. Dialysis membranes for kidney samples prove useful to prevent the action of natural inhibitors and reduce false positive results, frequently associated with this matrix. Validation of the method was made with standard solutions, but the detection of concentrations of antibacterials well below their respective MRL makes us confident that the method is suitable for the control of the listed antibacterials. For this, the Decision 2002/657/EC [2] seems to be only partially applicable in the requirement of false compliant rate below 5% at the level of interest, while it is fully satisfied for the two other parameters, specificity and applicability. In conclusion, our method proves useful as a routine screening method for very large sample throughput, cost-effectiveness, rapidity, and reduced proportion of false positive results.

REFERENCES

**E35**

**Fully validated analytical method for the determination of toltrazuril sulfone in edible tissues of food producing animals by HPLC with fluorescence detection**

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**INTRODUCTION**

Toltrazuril (Baycox®) is an anticoccidial widely used in poultry and swine and approved recently in Denmark for the use in cattle. In edible tissues the marker residue is toltrazuril sulfone. MRL are set between 100 and 600 μg kg$^{-1}$ for all mammalian food producing animals and poultry. For the surveillance of possible residues an analytical method was developed and validated according to the requirements of the EU [1].

**MATERIALS AND METHODS**

*Sample extraction* Five grams of minced liver, kidney or muscle were weighed into a centrifuge vial. After addition of 40 mL acetonitrile the sample was homogenised using a high speed blender. After centrifugation the supernatant was transferred into a separatory funnel. This extraction step was repeated and the combined extracts were partitioned against 150 mL acetonitrile-saturated n-heptane. After evaporation to dryness the residue was dissolved in 20 mL of a potassium carbonate solution. Fat samples (5 g) were first extracted with 50 mL n-heptane and then with 40 mL acetonitrile. Partitioning of the combined extracts was performed against 40 mL acetonitrile and 150 mL acetonitrile-saturated n-heptane. After evaporation to dryness the residue was dissolved in 20 mL of a potassium carbonate solution. *Clean-up of the extract* Potassium carbonate solution was poured onto an Extrelut disposable column. After a waiting period of 15 min the elution was performed twice with 30 mL of a dichloromethane/ethyl acetate mixture (1/1, v/v). The residue was evaporated to dryness and then dissolved in 10 mL of an acetonitrile/water mixture (57/43, v/v). An aliquot of 25 μL from this solution was injected into the HPLC system.

*HPLC determination* The chromatography was performed using a reversed phase analytical column by isocratic elution with a mixture of acetonitrile (570 mL L$^{-1}$) and a diluted buffer solution (mixture of citric acid/sodium hydroxide-buffer pH 5 and water 1/1, v/v). Detection was performed by a fluorescence detector after post column derivatisation with UV-light using a photochemical reaction unit with UV-lamp 254 nm and reaction coil 20 m (e.g. Beam Boost, i.e.t) [2].

**RESULTS**

For validation tissue recovery samples of cattle, swine, poultry and sheep were analysed. The mean accuracy for these species ranged between −5% and −2% and the precision was 7.5% to 10.6%. The limit of quantification was 30 μg kg$^{-1}$ for kidney, muscle and fat and 40 μg kg$^{-1}$ for liver. Linearity could be demonstrated for toltrazuril sulfone concentrations of 0.015 to 2.5 mg L$^{-1}$.

**DISCUSSION**

This method allows a reliable determination of toltrazuril sulfone, the marker residue for toltrazuril, in edible tissues of food producing animals. The recovery rates are neither dependent on the concentration nor on the species or matrix. This robust method was fully validated for cattle and pig tissues and has also been tested for the analysis of tissues samples from poultry and sheep tissue.

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**E36**

**Mass Spectrometry Protein Profiling in bovine sera: a potential proteomic approach to detecting illicit treatment of calves with growth promoters**

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**INTRODUCTION**

At present, control programs by EU Member States to detect illicit treatment of cattle with growth promoters (GP) consists of the analytical identification of banned molecules by immunochemical and physico-chemical methods, which are not always suited to the detection of compounds present in very low concentrations or of unknown chemical structure [1]. Among current biological methodologies, the proteomic approach has already been successfully applied to finding biomarkers in liver subfractions from veal calves exposed to a combination of 17β-estradiol (17βE), clenbuterol (CLEN) and dexamethasone (DEX) [2]. According to the National Residues Monitoring Plans, samples should be taken not only from animal products but also from live animals. Among the body fluids that may be
RESULTS
A typical MALDI-TOF-MS spectrum of bovine serum, showing about 30 protein signals in the low molecular weight region investigated, is depicted in Fig. 1. Some of the signals highlighted in the spectra were identified by LC-MS/MS fragmentation analysis of the purified fraction, for example, apolipoprotein C-III (m/z = 8582 Th), β2-microglobulin (m/z = 11628 Th) and haemoglobin alpha chain (m/z = 15148 Th). Other signals, like fibrinogen alpha chain (m/z = 8806 Th), were attributed to fragments of higher molecular weight proteins not visible in the mass range investigated. Our preliminary results did not show any protein signal capable of discriminating between control and treated animals. The intra-day and inter-day variability were lower than 15% (n = 6) and lower than 16% (n = 18), respectively.

DISCUSSION
In this study, the protein profiles of serum samples from untreated or GP-treated calves were characterized. The developed method performed well in the relative-quantitative application of linear MALDI-TOF MS. Although this preliminary study did not disclose statistically significant differences in the protein pattern between control and treated calves, possibly because of the limited number of cases investigated, this innovative approach could be used to detect the exposure to many other hormonal compounds or β-adrenergic drugs illegally employed as GP. Overall, Mass Spectrometry Protein Profiling in bovine sera may allow the identification of proteins that may not be characterized by other classical methodologies like 2D electrophoresis. To confirm these preliminary data, which represent one of the first attempts to identify bovine proteins by MS, further studies are currently in progress.

REFERENCES

E37
Application of gas chromatography – ion trap mass spectrometry for the determination of anabolic substances in biological matrices
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INTRODUCTION
Gas chromatography combined with high-resolution mass spectrometry (GC-HRMS) or low-resolution tandem mass spectrometry (GC-MS-MS) has been widely used to quantify trace amounts of anabolic substances in biological matrices. Because GC-HRMS is a very expensive technology, low-resolution mass spectrometry remains essential for the development of a residues laboratory. In recent years, gas chromatography coupled with ion trap mass spectrometry (GC-ITMS) became an alternative technique to GC-HRMS for residues analysis [1]. The aim of the present study is to demonstrate the ability of the GC-ITMS to analyse anabolic substances in biological matrices.

MATERIALS AND METHODS
Samples of meat, liver, kidney, urine, bile, retina, hair and reference material BCR-474/475, BCR-411, BCR-389, BCR-648/649, BCR-504 (IRMM) were analyzed using GC-ITMS techniques. The sample preparation procedures were optimized for GC-ITMS using standard methods. Analysis of anabolic substances was carried out on a SATURN 2000 ion trap mass spectrometer (Varian) coupled to a 3380 gas chromatograph equipped with a Varian 8200 autosampler. The Saturn 5.52 software version of the workstation was used. The chromatographic separation was performed using a CP-Sil 24 CB low bleed MS (Varian, USA) fused-silica capillary column (30 m x 0.25 mm I.D. x 0.15 μm). The 1079 injector temperature was 280°C. Column temperature programs were as follows:
100°C for 0.5 min, 8°C min to 290°C and hold time was up to 45 min. Helium flow was 0.8 mL min⁻¹ and 1–3 µL sample injected in splitless mode. The operation conditions for the ion trap mass spectrometer working at MS-MS mode were the following: positive electron ionization mode, ion trap temperature 220°C, transfer line temperature 250°C, emission current 30–50 µA, AGC target 5000, axial modulation 4.0 V. For the best sensitivity and selectivity, collision induced dissociation (CID) was performed on precursor ions isolated with a 3 m/z window. The non-resonant or resonant CID parameters were optimized for each derivative. The samples were analyzed by GC-ITMS using deuterated internal standard anabolic substances (RIVM). Quantification was performed on the sum of the major product ions. EC concept of identification points was used to set up quality criteria for qualitative methods [2]. To check trueness, the bias of the analytical results was controlled using reference material.

RESULTS
GC-ITMS is an efficient method for a wide range of anabolic substances. Optimization of sample preparation by using consecutive cleaning by solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) yielded the most reproducible results. Optimum results for MS-MS analysis were gained with trimethylsilyl, pentafluoropropionic and mixed methyloxime-trimethylsilyl derivatives. As a result tandem mass-spectrometry parameters for anabolic steroids, stilbenes and beta-agonists were optimized. For example, the analysis of MO-TMS derivatives of trenbolone, nortestosterone and testosterone can be carried out on ion trap in two modes. Isolation of ions with m/z 371, 340, 266 and 240 was carried out for trenbolone in µSIS. In MS-MS mode a molecular ion with m/z 371 was used as the precursor ion. As a result of non-resonant dissociation, product ions with m/z 340 (100), 281 (50), 240 (28), 249 (19), 266 (14) were received. The excitation amplitude of non-resonant activation in 70–75 V is effective for the majority of MO-TMS derivatives. For the analysis beta-agonists, MS-MS or MS-MS-MS with non-resonant activation were used. For MS-MS of di-S derivatives, clenbuterol was used an ion cluster with m/z 334–337 at excitation amplitude 85 volt and for tris-TMS salbutamol was used a precursor ion with m/z 369 at excitation amplitude 63 Volt. As a result of non-resonant dissociation, product ions for clenbuterol with m/z 336 (50), 334 (75), 300 (100), 227 (25) and for salbutamol with m/z 369 (40), 207 (100), 191 (35) were received. For MS-MS-MS of S derivatives of clenbuterol a precursor ion with m/z 300 was used, and for salbutamol a precursor ion with m/z 207 was used. As a result of non-resonant dissociation, product ions for clenbuterol with m/z 284 (100), 210 (65), 226 (10) and for salbutamol with m/z 191 (60), 189 (40), 163 (100), 161 (45) were received. For GC-ITMS the limit of detection of anabolic substances in biological matrices ranged from 1.0 to 20.0 pg on-column.

DISCUSSION
Application of GC-ITMS requires intensive sample preparation. The most accurate results followed the use of HPLC cleaning using C18 reversed phase analytical columns. Adjustment of CID parameters in Automated Method Development (AMD) mode delivered optimum values for activation energy of the precursor ions for confirmatory analyses. Among the anabolic substances used for the growth promotion in animals, anabolic steroids and beta-agonists are the most widely used. For the control of these substances effective mass spectrometry techniques are offered, however application of GC-ITMS can be an effective and economic method of the analysis of prohibited substances.

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E38
Diclazuril: 20 years of safety research in support of residue surveillance
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INTRODUCTION
Diclazuril is an anticoccidial from the benzeneacetonitrile group. Extensive research has demonstrated the efficacy of diclazuril in various food producing species, e.g. broiler chickens, turkeys, rabbits, lambs, calves and piglets. Simultaneously, a vast amount of residue safety data has been gathered. This contribution attempts to comprehensively review these data with particular emphasis on consumer safety.

MATERIALS AND METHODS
Safety data were gathered from toxicology studies, including carcinogenicity, and studies on the magnitude, nature and depletion of residues in animals. The studies were conducted compliant with regulatory guidance from EU and USA and OECD Guidelines where appropriate. For in vivo and in vitro metabolism studies, 14C-diclazuril was available. Quantification of residues was done with GC-ECD (electron capture detection), HPLC-UV and LC-MS/(MS). Residue depletion studies were conducted with premix formulations in broiler chickens, turkeys and rabbits and with oral suspension formulations in lambs, calves and piglets.

RESULTS
There are no indications of significant metabolism of diclazuril in animals. Diclazuril-related substances are eliminated mainly as unchanged drug in the excreta of avian species and in the faeces of mammals. The lack of metabolism in tissues is a dominant feature in all species and, as the tissue metabolites are quantitatively extractable, there is no substantial formation of bound residues. Parent diclazuril is indicated as the marker residue substance for surveillance purposes [1]. A no observed effect level (NOEL) of 3 mg kg⁻¹ body weight and per day can be derived from a carcinogenicity study in mice. With a Safety Factor of 100, this results in an Acceptable Daily Intake (ADI) of 1.8 mg/consumer and /day [2,3]. Alternatively, a NOEL of 2.5 mg kg⁻¹ body weight and per day can be selected on the basis of reproduction studies in the rat. With a safety factor of 100, this results in an ADI of 1.5 mg per consumer and per day [4]. The bioanalysis methods for diclazuril in edible products of animal origin, validated on behalf of the drug sponsor, attain LOQ in the range 0.05–0.10 mg kg⁻¹ for HPLC, 0.01–0.05 mg kg⁻¹ for GC-ECD, 0.025 mg kg⁻¹ for LC-MS and 0.005 mg kg⁻¹ for LC-MS/MS. Bioanalysis methods available in open literature also attain LOQ in the ppb range [5,6]. Studies

on the depletion of residues after the recommended continuous treatment at 1 mg diclazuril/kg feed with the premix formulations revealed the highest concentrations in the tissues of rabbits; the concentrations in tissues of broilers and turkeys were substantially lower, but of equal magnitude in both avian species. Lambs display the highest tissue concentrations after single treatment with the oral suspension formulation at 1 mg kg\(^{-1}\) body weight, although the concentrations are much lower than in rabbits, broilers and turkeys. In calves and piglets, the tissue concentrations are virtually insignificant even at an exaggerated dose of 5 mg kg\(^{-1}\) body weight. The depletion half-life is in the range of 2–2.5 days in broilers, turkeys and rabbits and about 1 day in lambs; in calves and piglets the bioavailability is too low for accurate determination of the depletion rate, but the half-life is shorter than 1 day. The available safety and residue data allow establishment of MRL for diclazuril in edible products. JECFA Codex was the first to propose 3 mg kg\(^{-1}\) for liver, 2 mg kg\(^{-1}\) for kidney, 1 mg kg\(^{-1}\) for (skin)/fat and 0.5 mg kg\(^{-1}\) for muscle for poultry, sheep and rabbits. Residue intake at these MRL would represent 33% of the ADI in the mammalian species and 31% in the avian species. The same values were accepted in the USA as Food Tolerances, although FDA/CVM had calculated substantially higher Safe Concentrations. The USA accordingly granted the ‘Zero Withdrawal’ Period for the premix formulation of diclazuril in broilers and turkeys. In the EU, diclazuril as veterinary medicinal product has been included in Annex II of Council Regulation (EEC) No 2377/90 for oral use in all ruminants and in porcine species, indicating that no numerical MRL are required because use cannot result in residues of consumer concern. No numerical MRL have been established yet for diclazuril in the regulatory framework of feed additives. The relevance of the residues of diclazuril in the edible products of its target species can be evaluated most appropriately by comparing the potential residue intake at the practical ‘zero withdrawal’ (6 h in broilers and turkeys, 24 h in rabbits, lambs, calves and piglets) with the ADI of 1.8 mg per consumer per day mentioned above. The % ADI consumed is 16.5 in rabbits, 6.6 in turkeys, 5.9 in broilers, 4.4 in lambs, 2.2 in calves and 1.4 in piglets. Although laying hens are not a target species for diclazuril, studies have indicated that at continuous feeding at 1 mg kg\(^{-1}\) feed, hens would lay eggs containing residues representing not more than 0.8% of the ADI.

DISCUSSION
The residue safety of diclazuril has been extensively documented in various target species. Based on its ADI, it can be concluded that diclazuril is an intrinsically safe substance. Moreover, the residue concentrations are very low in comparison with the ADI, even at steady-state during continuous administration as feed additive to broilers, turkeys and rabbits and virtually insignificant after single administration as veterinary medicine in lambs, calves and piglets. Furthermore, residues deplete rapidly from the edible tissues. The use of diclazuril in the target species is very safe from a food safety standpoint. Unintended use, for example in laying hens, would result in quantifiable residues, but these would not constitute any threat to consumer safety. Other data demonstrate that unintended use in non-target species is unlikely to provoke an indirect risk to public health due to residues. Therefore, the question can be raised whether there is a justified cost-benefit to target diclazuril in residue surveillance programs.

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E39
A sensitive LC-MS/MS method for the quantitative determination of dexamethasone in porcine plasma, muscle, liver and kidney
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INTRODUCTION
Dexamethasone is a synthetic glucocorticoid, which has been used for many years in the treatment of metabolic and inflammatory diseases in human and veterinary medicine. Within the EU, the use of dexamethasone is approved in cattle only for therapeutic indications, and tissues and milk intended for human consumption have to be analysed for their MRL. The following MRL have been established by the Committee for Veterinary Medicinal Products (EMEA) for dexamethasone in cattle: 2 μg kg\(^{-1}\) in liver, 0.75 μg kg\(^{-1}\) in kidney and muscle, and 0.3 μg kg\(^{-1}\) in milk. As a result, sensitive analytical methods are required to determine MRL in edible tissues derived from cattle. The ultimate goal of our work was to develop and validate a sensitive and reliable bioanalytical LC-MS/MS method suitable for the determination of dexamethasone in porcine plasma, muscle, liver and kidney.

MATERIALS AND METHODS
After homogenising tissue samples in saline solution, an internal standard (prednisolone) was added to plasma and tissue samples and the samples then subjected to liquid-liquid extraction using acetonitrile. After dilution with water and washing with pentane, the samples were extracted using TBME. The evaporated residues were dissolved in injection solvent and the samples then subjected to liquid-liquid extraction using pentane, the samples were extracted using TBME. The evaporated residues were dissolved in injection solvent and injected into the LC-MS/MS system for quantification. The samples were chromatographed on a LUNA C18 LC column (3 μm, 150 × 3.0 mm). The mass-spectrometer consisted of a

<table>
<thead>
<tr>
<th>Medium</th>
<th>Porcine muscle</th>
<th>Porcine liver</th>
<th>Porcine kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC level</td>
<td>Bias (%)</td>
<td>CV (%)</td>
<td>Bias (%)</td>
</tr>
<tr>
<td>LLOQ (0.10 MRL)</td>
<td>3.7</td>
<td>13.4</td>
<td>20.2</td>
</tr>
<tr>
<td>Low (1.00 MRL)</td>
<td>0.3</td>
<td>6.4</td>
<td>14.3</td>
</tr>
<tr>
<td>Medium (2.00 MRL)</td>
<td>6.5</td>
<td>4.9</td>
<td>6.8</td>
</tr>
<tr>
<td>High (5.00 MRL)</td>
<td>4.3</td>
<td>7.0</td>
<td>11.8</td>
</tr>
</tbody>
</table>
Sciex API 4000 equipped with a Turbo Ion Spray interface and was operated in the positive ion mode. A full validation of the method was performed according to Volume 8 of The Rules governing Medicinal Products in the EU. The calibration range for dexamethasone was 0.100–25.0 ng mL\(^{-1}\) for porcine plasma and 0.10 10.0 MRL for porcine tissue. The validation included the determination of the parameters: calibration, accuracy and precision, recovery, specificity, dilution, and stability.

RESULTS
The assay for dexamethasone was validated for the concentration range of 0.100–25.0 ng mL\(^{-1}\) for plasma, 0.0750–7.50 µg kg\(^{-1}\) for kidney and muscle, and 0.200–20.0 µg kg\(^{-1}\) for liver. The method showed acceptable accuracy (expressed as bias) and precision (expressed as CV) for all tissues (Table 1). In porcine plasma, accuracies of –5.8, –6.4, –10.7 and –8.7% and precision of 11.9, 12.6, 9.1 and 6.5% were obtained at the QC levels LLOQ (0.100 ng mL\(^{-1}\)), Low (0.500 ng mL\(^{-1}\)), Medium (5.00 ng mL\(^{-1}\)) and High (20.0 ng mL\(^{-1}\)), respectively. A lower limit of quantitation (LLOQ) of 0.10 MRL and 0.100 ng mL\(^{-1}\) was achieved in porcine tissue and plasma, respectively. In addition, the method was valid with respect to recovery (>55% in tissue), specificity, the 10-fold dilution of samples with blank matrix, and stability (24 h storage of samples on the autosampler and one additional freeze/thaw cycle) for all matrices.

DISCUSSION
At ABL, a sensitive bioanalytical LC-MS/MS method for the determination of dexamethasone in porcine plasma, muscle, liver and kidney tissue has been developed and validated successfully. The method produces accurate and precise results, and very low LLOQ were achieved (up to 0.10 MRL in porcine tissue) in comparison to other available methods for the determination of dexamethasone (usually 0.50 MRL). Due to the low LLOQ, the assay is extremely suitable for the quantitative determination of dexamethasone in porcine plasma, muscle, liver and kidney and, as such, offers an alternative to the methods available for the investigation of the pharmaco- and depletion kinetics of dexamethasone in porcine plasma, muscle, liver and kidney. Moreover, this method can be used as a lead for the validation of dexamethasone in other species (bovine).

**RESULTS**

The method can detect the four metabolites simultaneously at very low levels (CC\(\beta\) ≤ MRPL). The method was fully validated according to the EU requirements for the detection of veterinary drug residues in products of animal origin, and has the major advantage of yielding high recoveries compared to the methods reported in literature [4,5].

**MATERIALS AND METHODS**

**Sample extraction** After acidic hydrolysis from the protein bound form, and derivatization with o-nitrobenzaldehyde, the samples were brought to pH 7.2, then extracted (2 × 5 mL) with ethyl acetate. The organic phase was collected and evaporated to dryness. The samples were reconstituted with 2 mL of acetonitrile and extracted with 2 × 2 mL of n-hexane (defatting). The n-hexane layer was discarded and the acetonitrile was evaporated to dryness under a gentle stream of nitrogen and reconstituted in methanol and 5 µL was injected into the LC/MS/MS system. LC-MS/MS analysis was carried out in positive ion mode on a API 3000 triple quadrupole mass spectrometer equipped with a Turbo Ion Spray source by gradient elution with 1% acetic acid (A) ACN/1% acetic acid 90/10 v/v (B), flow rate 0.4 mL min\(^{-1}\).

**DISCUSSION**

The method can detect the four metabolites simultaneously at very low levels (CC\(\beta\) ≤ MRPL). The method was fully validated according to the EU requirements for the detection of veterinary drug residues in products of animal origin, and has the major advantage of yielding high recoveries compared to the methods reported in literature [4,5].

**E40**

Multi residue method for the determination of nitrofuran metabolites in egg using liquid chromatography coupled to tandem mass spectrometry

G. FERRETTI, C. CIVITAREALE, L. PALLESCHI, L. FANTOZZI & M. FIORI

Istituto Superiore di Sanità, Department of Food Safety and Veterinary Public Health, Viale Regina Elena 299, Roma, Italy

**INTRODUCTION**

Furazolidone, furaltadone, nitrofurantoin and nitrofurazone are veterinary drugs belonging to the class of nitrofurans. Nitrofurans were banned for food producing animals by the EU in 1995 due to their potentially carcinogenic and mutagenic effects in humans. Nitrofurans are very rapidly metabolized, as reported in various studies and their metabolites remain for a long period as protein bound residues. The marker residues identified are 3-amino-2-oxalidinone (AOZ) for furazolidone, 5-methylmorpholino-3-amino-2-oxalidinone (AMOZ) for furaltadone, 1-amino-hydantoin (AHD) for nitrofurantoin and semicarbazide (SEM) for nitrofurazone. Those marker residues are detected by LC-MS/MS as their nitrophenyl (NP) derivates and confirmation is possible at the MRPL (Minimum Required Performance Limit) concentration of 1 µg kg\(^{-1}\) or lower [1]. Even although a MRPL has not yet established for the matrix egg, it is suggested that this can be extrapolated from all the other matrices [2]. The present method has the advantage of high recoveries, and is applicable to the detection and identification of nitrofuran metabolites according to EU requirements for confirmatory analysis in fresh whole eggs.

**DISCUSSION**

At ABL, a sensitive bioanalytical LC-MS/MS method for the determination of dexamethasone in porcine plasma, muscle, liver and kidney tissue has been developed and validated successfully. The method produces accurate and precise results, and very low LLOQ were achieved (up to 0.10 MRL in porcine tissue) in comparison to other available methods for the determination of dexamethasone (usually 0.50 MRL). Due to the low LLOQ, the assay is extremely suitable for the quantitative determination of dexamethasone in porcine plasma, muscle, liver and kidney and, as such, offers an alternative to the methods available for the investigation of the pharmaco- and depletion kinetics of dexamethasone in porcine plasma, muscle, liver and kidney.

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**E40**

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**MATERIALS AND METHODS**

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**RESULTS**

Full validation was achieved, according to the criteria of the Commission Decision 2002/657/EC [3]. Table 1 shows the validation parameters calculated for the four metabolites considered as NP derivatives (CC\(z\) and CC\(\beta\), CV and recovery).

**Table 1 Validation parameters.**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CC(z) (ng g(^{-1}))</th>
<th>CC(\beta) (ng g(^{-1}))</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP-AOZ</td>
<td>0.4</td>
<td>0.5</td>
<td>Lev 1 (1 ppb)</td>
<td>96.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Lev 2 (1.5 ppb)</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lev 3 (2 ppb)</td>
<td>100.9</td>
</tr>
<tr>
<td>NP-AMOZ</td>
<td>0.3</td>
<td>0.4</td>
<td>Lev 1 (1 ppb)</td>
<td>96.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lev 2 (1.5 ppb)</td>
<td>99.7</td>
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<td></td>
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<td>Lev 3 (2 ppb)</td>
<td>100.9</td>
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<tr>
<td>NP-SEM</td>
<td>0.5</td>
<td>0.7</td>
<td>Lev 1 (1 ppb)</td>
<td>96.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lev 2 (1.5 ppb)</td>
<td>103.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lev 3 (2 ppb)</td>
<td>105.7</td>
</tr>
</tbody>
</table>


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**DISCUSSION**

The method can detect the four metabolites simultaneously at very low levels (CC\(\beta\) ≤ MRPL). The method was fully validated according to the EU requirements for the detection of veterinary drug residues in products of animal origin, and has the major advantage of yielding high recoveries compared to the methods reported in literature [4,5].
E41
Oral administration of boldenone and boldione to veal calves: disposition and elimination rate of boldenone metabolites

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INTRODUCTION

Boldenone (1,4-androstadiene-3-one, 17-ol) (BOL) and boldione (1, 4-androstadiene-3,17-dione) (ADD) are chemical derivatives of testosterone, known as strong anabolics and low to moderate androgenic agents. ADD, the dione form of BOL, is a direct precursor of that anabolic steroid, and it is activated by the same widely distributed 17beta-hydroxysteroid dehydrogenase enzyme that converts androstenedione to testosterone [1]. Poor oral bioavailability is one of the most fundamental problems with pro-hormones, because the liver processes natural steroid hormones so efficiently that following oral administration only a small fraction of the active form reaches the blood stream. ADD is one of the most orally active pro-hormones, and displays a level of oral bioavailability far superior to any other compound. The structure of ADD is intrinsically resistant to 17-ketosteroid deactivation during this first pass through the liver: as the presence of its two double bond (1, 4-diene) shifts its hepatic metabolism (the 17-keto redox potential) in favour of activation (17β-ol). ADD is converted to estrogen at approximately half the rate of androstenedione and testosterone but at a rate high enough to potentially support its effectiveness, and low enough to avoid unwanted side effects [2]. To optimize anabolic surveillance strategies, ADD and BOL were administered to veal calves, at a similar dosage and the same ratio present in ‘ready to use’ cocktails; their disposition in plasma and their elimination rate in urine were followed for 24 h.

MATERIALS AND METHODS

Animal treatment Three veal calves aged 30 days were kept under controlled conditions and were fed a commercial milk replacer until the age of four months (150 ± 15 kg body weight), when the experiment started. Each calf was then fed 200 mL of reconstituted milk containing a combination of BOL (9 mg) and ADD (1 mg) dissolved in ethanol (5 mL). The remainder of the daily milk was fed immediately thereafter. Sampling During the 8-h post-administration, blood samples were collected from the jugular vein into vacutainer tubes. Plasma was separated and stored at –20°C until analysis. Within 4 h after feeding, spontaneous urination occurred and urine samples were collected using a clean container. The urine was sampled again at about 9 and 24 h after treatment.

RESULTS

ADD was not detectable in any of the urine or plasma samples at any time. The concentrations of β-BOL and α-BOL, measured after deconjugation, are reported in Table 1. For both compounds the highest concentrations were detected in the first plasma samples (15 min after administration), their concentrations then decreased at similar rates and both were undetectable within 4 h after administration. In urine samples collected within 4 h after administration, concentrations of α-BOL were considerably higher than those of β-BOL, and 36 h after administration α-BOL was the only detectable metabolite. β-BOL was detectable over a period of 24 h only.

Table 1. Plasma and urine concentrations of β-BOL and α-BOL

<table>
<thead>
<tr>
<th></th>
<th>Plasma (ng mL⁻¹)</th>
<th>Urine (ng mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>β-BOL</td>
<td>nd</td>
<td>0.94</td>
</tr>
<tr>
<td>α-BOL</td>
<td>149</td>
<td>5.1</td>
</tr>
</tbody>
</table>

DISCUSSION

The absence of ADD confirmed its double identity, i.e. not only as a metabolite but also a precursor of β-BOL, as already shown in vitro after incubation of ADD or β-BOL with calf liver microsomes [4]. The in vivo results confirm that both ADD and β-BOL were promptly absorbed when administered to veal calves before feeding, and that their disappearance from plasma was even more rapid. About 3 h after administration a significant quantity of α-BOL was recovered in urine, while β-BOL attained concentrations only slightly higher than the EU action limit. Surprisingly, after 9 h α-BOL concentrations were already less than 2% of those detected at the previous sampling time and β-BOL concentrations were closed to the limit of quantitation. One day after treatment only α-BOL gave a feeble indication of the ‘illegal’ treatment. These findings indicate that sampling time is crucial when urine samples are collected from farmed calves for surveillance purposes.

ACKNOWLEDGEMENT

This study was supported by a grant from Regione del Veneto (Delibera n. 2411 del 08.08.2003).

REFERENCES

E42
Biotransformation pattern of 17β-boldenone and of its precursor/metabolite androsta-1,4-diene-3,17-dione in bovine hepatic subfractions
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INTRODUCTION
The androgenic steroid 17β-boldenone (BOL) may be illegally used for cattle fattening, but there is evidence indicating its natural occurrence under certain circumstances [1]. A thorough knowledge of its biotransformation pathways may help in discriminating the exogenous exposure from the ‘endogenous’ production. When incubating BOL with bovine liver microsomes, the oxidation to androsta-1,4-diene-3,17-dione (ADD) appeared as the major biotransformation pathway, while in isolated hepatocytes the hydroxylated metabolites of both BOL and ADD were further identified. Nevertheless, neither preparation was able to produce the 17α-epimer (17α-BOL) to any significant extent, which is always found in urine samples from cattle treated with BOL or ADD [2]. To our knowledge, however, no in vitro studies have been conducted in cattle to elucidate the biotransformation pattern of ADD and to identify the sub-cellular localization of the enzymes involved in the generation of the above metabolites. Therefore, the main objectives of this in vitro study, which is still in progress, were to: (i) investigate the biotransformation profile of BOL and ADD; (ii) examine the inter-conversion between BOL and ADD and characterize the nature of the cofactor(s) involved; (iii) assess if the use of liver preparations from phenobarbital (PB) pre-treated cattle could result in a qualitative and quantitative modulation of the mentioned biotransformation pathways.

MATERIALS AND METHODS
Liver subcellular fractions from untreated (UT, n = 3) or PB-induced male Friesian cattle (n = 4) were isolated by differential ultracentrifugation, pooled and stored in liquid nitrogen. The metabolic fate of BOL or ADD was studied at pH 7.4 using post-mitochondrial (S9)- (2 mg protein, 1 mM substrate) or microsomal fractions (0.2–0.5 mg protein, 0.25 mM substrate) in the presence of an NADPH–generating system or of NADPH, NADH, NADP+ or NAD+ (each at 1 mM concentration), respectively. After 5–30 min incubation at 37°C, the reactions were stopped on ice and the samples were snap-frozen in liquid nitrogen. The steroids and metabolites (1 mL) were extracted twice with tert-butylmethylether, centrifuged and evaporated to dryness under vacuum. The residue was dissolved in 200 μL of mobile phase (CH3CN/H2O-CH3COONH4 2 mM, 30/70) and analyzed by a LC/MS (LTQ, Thermo Electron Corporation) equipped with an atmospheric pressure chemical ionization (APCI) interface.

RESULTS
Incubations of S9 with BOL and an NADPH–generating system revealed ADD as the most prominent product in both UT- and PB preparations. Moreover, three hydroxylated metabolites were identified, and the amount of one of them was 4 times higher in PB than in UT fractions. Incubations with ADD yielded both 17α- and 17β-BOL; irrespective of the fraction source, the β-epimer was produced to a many fold greater extent than the α epimer. In addition, in this case, PB pre-treatment resulted in the generation of a much higher amount of one of the OH-derivatives formed (about six-fold). The oxidation (dehydrogenation) of 17β-BOL to ADD in microsomes occurred at a slightly higher rate with NAD+ as the cofactor. Reductive reactions of ADD to both 17α- and 17β-BOL utilised NADH as the preferred cofactor and, as previously observed in S9, the β-epimer was the major product with either cofactor (Table 1). The oxidation rate was not significantly affected by PB-pretreatment, whereas the production of 17β-Bol from ADD was about four times higher in PB vs. UT samples using NADH as the cofactor.

DISCUSSION
Taken together, the above preliminary results confirm that ADD is both a precursor and a metabolite of BOL [1] and strongly suggest that ADD and not BOL is the source of the 17α-epimer of BOL, as previously reported in cattle for the reduction of androstenedione to epitestosterone [3]. Although NAD(H) would appear to be more involved than NADP(H) either in the oxidation of BOL to ADD or in the reverse (reductive) pathway, the formation of either androgen seems to occur with either cofactor. This points to the presence of several 17-hydroxysteroid dehydrogenases, with different substrate affinities, subcellular localization and cofactor requirements [4]. Interestingly, upon incubation of ADD, the production of the β-epimer largely predominated over that of the α- one, while upon the administration of either BOL or ADD to cattle the opposite holds true [1,2]. This would indicate that in cattle 17(2)-hydroxysteroid dehydrogenase (also referred to as epimerase) or other enzyme(s) involved in the biosynthesis of 17α-BOL are more expressed in other tissues (kidney, testes) than in liver. The previously mentioned similar pathway (androstenedione → epitestosterone) has been reported to occur extensively in bovine blood [5]. Finally, both the generation of the OH-derivatives of BOL or ADD and of 17β-BOL seem to be PB-inducible pathways.

ACKNOWLEDGEMENT
This study was supported by a grant of the Italian Ministry of Health ISZLER 02/02 “Ricerca e determinazione delle possibili cause della produzione, endogena e esogena, del boldenone in bovini da carne”.

REFERENCES
INTRODUCTION
The presence of boldenone (α- and β-BOL) in bovine urine has given rise to a long debate in the EU about the possibility of a ‘natural’ origin of these steroids [1]. It has been shown that contamination with faecal matter may result in the detection of BOL in bovine urine [2] and that neoformation of high concentrations of α-BOL, androstenedione (AED), androstadienedione (ADD) and epitestosterone (ET) occurs in faeces after excretion [3]. To examine this in further depth, the degradation and conversion of these steroids and testosterone (T) in faeces of veal calves was tested using a simple in vitro model.

MATERIALS AND METHODS
Pooled rectal faeces from 5 veal calves, were divided in 0.5 g samples, suspended in 50 mL of sodium chloride 0.9% and kept gently shaken at 25°C. Six anabolic steroids (T, ET, α-BOL, β-BOL, AED and ADD) were added separately to each sample (200 ng mL⁻¹ equal to 20 µg g⁻¹ faeces) and 1 ml aliquots was collected at 0, 0.5, 1, 2, 4, 8 h and 1, 2 and 3 days. After thermal treatment (10 min at 80°C) and centrifugation (1400 g for 10 min), the supernatants were diluted 1:2 with methanol/water (50/50) and analysed by HPLC/MS-MS as reported elsewhere (Pompa et al. Food Addit Contam 2006; 23: 126–32). A blank sample (suspected faeces without steroid addition) and six control samples (suspected faeces thermally treated and separately spiked with each steroid) were also prepared. Two replicates were carried out in two different weeks.

RESULTS
No degradation of the steroids was observed in control samples and none of the six steroids were detected in the blank samples after 3 days. T and β-BOL readily transformed and vanished between 2 and 24 h (Fig. 1). Few conversions were observed for AED, ET and ADD until day 1, and their concentrations tended to 0 on day 3 (Fig. 1). α-BOL concentration remained stable from day 2 and fell to approximately 30% on day 3 (Fig. 1). The maximum conversion percentages relatives to added steroids are reported in Tab. 1. T and β-BOL were largely converted into AED (about 80%) and ADD (about 85%) within 2 h. ET caused the production on day 2 of low percentages of ADD (about 10%), AED (about 4%) and α-BOL (about 4%). Two days after addition of α-BOL, ET (about 65%) and ADD (about 25%) were detected. AED produced ADD (about 11%) and ET (about 3%) at day 1, while ADD produced β-BOL 1 h (about 4%) and induced formation of ET (about 3%) and AED (about 7%) at day 1.

DISCUSSION
These preliminary results show the greater stability of the α-17OH steroids compared with their β-forms in faeces (aerobic conditions). T and β-BOL were readily transformed into their oxidized forms, probably by a 17-hydroxysteroid dehydrogenase. The rapid transformation of T may account for its usual absence

REFERENCES

E44
Effects of dietary phytosterols on the presence of boldenone in faeces of veal calves
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INTRODUCTION
The in vitro conversion of phytosterols to androstenedione (AED) [1], progesterone, 17α-hydroxyprogesterone, androstadienedione...
(ADD) and likely boldenone (α- and/or β-BOL) [2] by Mycobacterium strains is well documented. In vivo it has been demonstrated that ADD (precursor of 17β-BOL) can be detected in the faeces of rats fed phytosterols [3]. Other authors have proposed that the presence of BOL and/or ADD in calf faeces is correlated to the use of vegetable fat in veal calf diets [4]. The aim of this study was to estimate the influence of dietary phytosterols on the presence of ADD, α-BOL, β-BOL and AED in the faeces of veal calves fed skimmed milk with added vegetable or animal fat.

MATERIALS AND METHODS
A total of thirty Friesian calves (45 ± 10 kg at the beginning of the experiment) were used. After a 15-day adaptation period in which they were fed with ‘milk no milk’, calves were divided into two groups. Fifteen were fed corn silage and skimmed milk with added vegetable fat (coconut oil) (Group A), while the others were fed corn silage and skimmed milk with added animal fat (tallow) (Group B). Rectal faeces were collected from each calf the day before the start of the experiment (T0) and once monthly for 3 months and analysed by HPLC MS-MS using methods already described [5]. All animals were under control by Istituto Zooprofilattico Sperimentale Lombardia Emilia Romagna and received no anabolic treatment. ANOVA followed by Student-Newman-Keuls was performed to compare mean steroid concentrations at T0 with the mean of the three months of the two diets, while t-test was performed to compare the mean concentrations of each steroid at each time (Group A vs. Group B); significance was set at P < 0.05.

RESULTS
The results of samples positive for one or more detected steroids are reported in Table 1. A quite random distribution of positive samples was observed (i.e. one animal was not always positive or negative to the same steroid at different times). There were only statistically significant differences between control animals (T0) and groups A and B for ADD and between groups A and B for β-BOL at 2 months and for AED at 3 months.

DISCUSSION
The results of this experiment showed that α-BOL and β-BOL can be present in rectal faeces of untreated calves along with the precursor ADD. These results are in contrast with those of other authors that did not find α-BOL and β-BOL in rectal faeces of veal calves [6]. However the most frequent steroid found in faeces at the highest concentration was AED. The diet with vegetable fat did not seem to influence the frequency or concentration of the four steroids studied. These data do not confirm the hypothesis on the origin of boldenone and ADD from dietary phytosterols. Justification of the few statistical differences found in this study is not possible at present. It is also impossible to correlate the increase of the concentrations of steroids observed in faeces with time in the dietary study or with the age of animals.

ACKNOWLEDGEMENT
Project financially supported by the Italian Ministero della Salute (IZLER PRC–02–2002).

REFERENCES

E45 Which ratio should be adopted to evaluate the urinary excretion of testosterone and epitestosterone in veal calves?

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INTRODUCTION
Control of the illicit use of endogenous hormones in meat producing animals requires a detailed knowledge of both the metabolism of endogenous hormones and their physiological profile in each species. Current approaches in cattle do not involve the determination of the absolute urinary concentration of endogenous hormones because no validated physiological levels are recognised; the same is true when the ratio between metabolites is considered, as applied for testosterone (T) and epitestosterone (E) in human athletes [1]. In man, urinary E has attracted the attention as a reference substance in the doping control of T abuse. The nearly constant urinary ratio of T to E (T/E) in adults became the basis of the method of detection of exogenously administered T, since in humans ET does not originate from exogenous T in significant amounts [2]. In cattle ET is a metabolite of T probably produced in liver and blood by hydroxysteroid-oxidoreductase enzymes via androstenedione [2–4]. The aim of this study was to assess physiological concentrations of T and E in urine of veal calves, and to evaluate which urinary ratio could be useful to indicate hormone treatment of calves.

MATERIAL AND METHODS
Thirty-one Friesian male calves were housed for 6 months and fed a liquid diet based on a milk replacer. At the age of 6 months, the animals were divided into four groups: K group (n = 10, control), A group (n = 9; 17β-oestradiol benzoate and T enanthate 10 + 200 mg per head), B group (n = 9;
17β-estradiol benzoate and boldenone undecylenate 10 and 200 mg per head) and C group (n = 3; boldenone undecylenate 200 mg per head) by s.c. administration. Groups A and B were treated every 15 days on four occasions (t1, t2, t3, t5); the C group every 7 days for three doses (t3, t4, t5). Urine samples were collected, early in the morning, before beginning the treatment and twice, after 1 and 7 days, following treatment. After enzymatic hydrolysis, urine samples were extracted using a C18 cartridge coupled to an amino-cartridge. Analysis was performed by a triple-quadrupole mass-spectrometer (LC-MS-MS). Mass detection was set at m/z 289 and main fragments were set at m/z 109 and m/z 97. The data were analysed using ANOVA (Mann–Whitney Test; Kruskal–Wallis Test followed by Dunn’s Multiple Comparisons Test).

RESULTS

Between the 6 and 7 months the mean E/T ratio of control calves showed a significant reduction (13.9 ± 8.8 vs. 9.5 ± 4.0; P < 0.005). At 6 months of age (t1), the E/T ratio (mean values at each time of sampling) of all treated groups (A, B, C) compared with K group (overall mean values) was not significantly different. At 7 months of age, there was a significant reduction of E/T ratio in group A group compared with group K lasting for the three further samplings. There was no significant difference between groups B and C compared with group K (Fig. 1).

DISCUSSION

Despite the large variations of T and E concentrations in urine collected from veal calves, the physiological excretion of E was about ten times greater than T, and E excretion tended to decrease with increasing age, as reported in young and adult men [2]. In men urine concentrations of E and T are quite similar, and the excretion of E remain rather constant when exogenous T is administered; a cut-off value for T/E in urine was established for doping control [5]. In a previous paper we applied the T/E ratio approach to urine of veal calves, but the influence of treatment was masked and there were no significant differences between treated and control calves [6]. Our recent data confirm that repeated treatment with natural hormone could cause a reduction in urinary E as already reported [3,7]; applying the E/T ratio, the reduction of E urinary excretion was confirmed in T treated (A) but not in boldenone treated (B and C) calves.

ACKNOWLEDGEMENT

This study was supported by a grant from Regione del Veneto (Delibera n. 8 - 7. 12. 2001).

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viable, apoptotic and necrotic thymocytes, including nuclear fragments.

RESULTS
At slaughter the weight of the thymus was severely reduced in group A at day 14 and in group B at day 30 compared to control animals. The trend of relative weight reduction was statistically significant in both treated groups compared to controls (P = 0.022). Histological lesions included moderate lymphoid depletion and hyperplasia of adipose tissue at day 7 and day 14 in all treated animals. In situ evaluation of apoptosis in the thymus showed an increase in the percentage of positive nuclear areas from day 3 until day 14 in anabolic and therapeutic groups. The percentage of Annexin V-positive apoptotic cells increased at day 14, more in per os treatment group (36.2%) than in the i.m. group (27.1%). High percentages were present until day 52.

DISCUSSION
In our study lymphoid depletion and fatty infiltration of the thymus were evident in animals treated with both anabolic and therapeutic dosages of dexamethasone. A clear correlation of these phenomena with thymocyte apoptosis was detected: an increase of apoptotic was already revealed after 3 days of treatment with anabolic dosages of dexamethasone, and persisted until 7 days, followed by a progressive reduction, while the increase of Annexin V-positive cells was present by T14 till T52. The differences may be due to the different apoptotic modifications recognized by the two methods: the DNA fragmentation (ApopTag®) and phosphatidylserine expression on cell membrane (Annexin V). The trend of apoptosis was similar in animals treated with anabolic and therapeutic dosages of dexamethasone, although a higher percentage of apoptosis was induced by anabolic treatment. In conclusion, our investigation indicates that thymus weight and percentage of apoptosis was induced by anabolic treatment. In therapeutic dosages of dexamethasone, although a higher apoptosis was similar in animals treated with anabolic and anabolic dosages of dexamethasone, and persisted until 7 days, followed by a progressive reduction, while the increase of Annexin V-positive cells was present by T14 till T52. The differences may be due to the different apoptotic modifications recognized by the two methods: the DNA fragmentation (ApopTag®) and phosphatidylserine expression on cell membrane (Annexin V). The trend of apoptosis was similar in animals treated with anabolic and therapeutic dosages of dexamethasone, although a higher percentage of apoptosis was induced by anabolic treatment. In conclusion, our investigation indicates that thymus weight and percentage of apoptosis was induced by anabolic treatment.

ACKNOWLEDGEMENT
This work was supported by a Grant from the Direzione di Sanità Pubblica, Assessorato alla Sanità, Regione Piemonte, Italy.

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E47
Genetically modified feeding and food safety: evaluation of cell metabolism by enzymatic analysis in rabbits
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INTRODUCTION
The insertion of new genes can be a useful tool for obtaining specific characteristic which can lead to an improvement of agronomical relevant traits or food quality [1]. Potential toxicological risks of genetically modified (GM) plant as whole food are evaluated in animal studies according to the classical methods used for drugs, including blood and urine chemistry, organ weight and histopathological examination [2]. It has also been suggested to find and apply specific biomarkers of early effects, in order to increase the diagnostic value and the sensitivity of toxicity tests [3]. With the present studies we aimed to evaluate possible adverse health effects of a GM diet by measuring the activity of organ specific enzymes in rabbits.

MATERIALS AND METHODS
Twenty weaned New Zealand rabbits were equally assigned to control and treated groups. Animals were fed a diet of pelleted concentrate (80%) and soybean meal (20%), from conventional or genetically modified beans, for controls and treated groups, respectively. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactic dehydrogenase (LDH), gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP) were assayed in serum and in homogenates from liver, kidney, heart and skeletal muscle. Enzyme activity was determined spectrophotometrically using reagents from Spinreact SA, Spain. Since significant differences were found for LDH between control and treated groups, in order to assess the isoenzymatic distribution of the enzyme, electrophoretic separation was performed on each sample.

RESULTS
The levels of the enzymes in serum did not show significant differences between controls and treated groups, thus suggesting that no adverse effects were induced by GM soybean meal. In contrast, the analysis of the relative enzyme activities in tissues showed significant increases in enzyme levels, particularly of LDH in the treated animals. As depicted in the table, the distribution of LDH isoenzymes showed significant differences between the groups for heart LDH1 and LDH2, kidney LDH1 and liver LDH1 and LDH4.

DISCUSSION
LDH1 was the dominant isoenzyme in heart and kidney and a significant increase in this isoenzyme and a decrease of LDH4 were shown in the liver, although no significant differences were found for total LDH activity in this organ. The reason for this shift remains unclear, but this finding supports the hypothesis that some metabolic changes also occurred in the liver following ingestion of GMO soy bean meal. Since LDH1 is known to be involved in cell metabolism, by favouring the reaction of lactate to pyruvate [4], our results indicate a general increase in cell metabolism. This hypothesis is in agreement with other studies.
that showed significant modifications in GM-fed mice, suggesting a higher metabolic rate and intense molecular trafficking [5]. Since no disease was detected in treated animals and serum activities of all the enzymes showed similar levels in both groups, these findings should be taken into account for future research activities.

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1. EFSA J (2004); 99: 1–19.

E48
Tissue distribution and persistence of malachite green and leucomalachite green in common carp (Cyprinus carpio L.)
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INTRODUCTION
Malachite green (MG) is a cationic triphenylmethane dye which has been used worldwide as fungicide and ectoparasicide in cultured fish eggs, fingerlings and adult fish since 1930s [1]. Due to its potential carcinogenicity, mutagenicity and teratogenicity [2,3], MG has never been registered as a veterinary medicine in the EU [4]. Despite this, it has a high probability of abuse due to its high efficacy and the lack of equally effective alternatives. In fish, including in carp, MG is easily absorbed during waterborne exposure and extensively metabolized to the reduced, colourless compound, leucomalachite green (LMG). The aim of this study was to investigate the tissue distribution and persistence of MG and its main metabolite in carp after a therapeutic bath.

MATERIALS AND METHODS
One hundred carp (average weight 100 ± 11 g) were placed in a tank containing aerated water. After an acclimatization period of 7 days fish were exposed to a bath of 2 mg L\(^{-1}\) MG for 3 h, and then transferred to fresh water. During the experiment, temperature varied between 14°C and 17°C and pH between 7.5 and 8.1. Carp were randomly sampled (n = 6) and euthanized at 0, 1, 3, 7, 14, 21, 28, 42, 56, 84, 112, 140, 196, 252 and 308 days after the end of the treatment. Six individuals were killed before the treatment and kept as controls. Gill, kidney, liver, spleen and muscle samples were collected and stored at −20°C until the analysis, which was performed by liquid chromatography with visible and fluorescence detection [5]. The LOQ for all tissues was 0.5 μg kg\(^{-1}\).

RESULTS AND DISCUSSION
Data shown in Table 1 indicate a wide distribution of MG and LMG in all analysed carp tissues. CVs of the concentrations detected in the samples were ≤ 35%. On day 0, MG concentration in all tissues exceeded the initial concentration in therapeutic water; the dye was thereafter rapidly and extensively metabolized to LMG, which was slowly eliminated from the tissues. Higher concentrations of MG and LMG were detected in gill, liver and kidney than in the spleen and muscle. The parent compound was more persistent in kidney, liver and spleen (up to 112 days) than in gill and muscle (up to 56 days). LMG concentrations declined more slowly in all tissues being still detectable in kidney and muscle 252 days after treatment. At that time the concentration of LMG in muscle was below the Minimum Required Performance Limit (MRPL, 2 μg kg\(^{-1}\)). The elevated persistence of LMG is in accordance with earlier findings and partly arises from the non-ionic, lipophilic character of this metabolite [6].

ACKNOWLEDGEMENT
This study was supported by grant no. 2P06K00729 from the State Committee for Scientific Research, Warsaw, Poland.

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E49
Chloramphenicol residues in commercial milk produced for human consumption in Venezuela
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INTRODUCTION
Dairy cattle and the milk production industry play an important socioeconomic role in Venezuela not only as a source of income for a significant number of families in certain regions of the country but also because milk is one of the most important sources of high quality protein for the population. Antibiotics have been traditionally used as a tool to improve the productivity of dairy cattle. Chloramphenicol is an antibiotic obtained from

Table 1. Mean concentrations (μg kg\(^{-1}\)) of MG and its reduced metabolite LMG in carp tissues after exposure to MG

<table>
<thead>
<tr>
<th>Days after exposure</th>
<th>Gill</th>
<th>Kidney</th>
<th>Liver</th>
<th>Spleen</th>
<th>Muscle</th>
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<td>0</td>
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<td>3</td>
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<td>1781.3</td>
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<td>7</td>
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<td>1773.3</td>
<td>155.4</td>
<td>3380.9</td>
<td>113.4</td>
</tr>
<tr>
<td>14</td>
<td>35.9</td>
<td>1214.6</td>
<td>181.8</td>
<td>1662.4</td>
<td>113.6</td>
</tr>
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<td>21</td>
<td>36.5</td>
<td>1308.4</td>
<td>75.6</td>
<td>1647.4</td>
<td>108.6</td>
</tr>
<tr>
<td>28</td>
<td>13.4</td>
<td>703.6</td>
<td>32.8</td>
<td>707.3</td>
<td>24.8</td>
</tr>
<tr>
<td>42</td>
<td>6.0</td>
<td>92.4</td>
<td>41.2</td>
<td>134.4</td>
<td>14.5</td>
</tr>
<tr>
<td>56</td>
<td>4.8</td>
<td>74.3</td>
<td>18.0</td>
<td>123.1</td>
<td>17.5</td>
</tr>
<tr>
<td>84</td>
<td>&lt;LOQ</td>
<td>2.1</td>
<td>4.0</td>
<td>11.0</td>
<td>5.0</td>
</tr>
<tr>
<td>112</td>
<td>&lt;LOQ</td>
<td>3.1</td>
<td>2.1</td>
<td>5.6</td>
<td>1.7</td>
</tr>
<tr>
<td>140</td>
<td>&lt;LOQ</td>
<td>1.9</td>
<td>&lt;LOQ</td>
<td>3.8</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>196</td>
<td>&lt;LOQ</td>
<td>0.7</td>
<td>&lt;LOQ</td>
<td>2.8</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>252</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>0.6</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>308</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
</tbody>
</table>

Streptomyces venezuelae, with bacteriostatic effect and a broad spectrum of action against Gram-positive and Gram-negative microorganisms, including H. influenzae, S. pneumoniae and N. meningitidis, as well as against Chlamydia and Rickettsia [1]. At present this antibiotic is produced in a synthetic form as esters (succinate and palmitate) which are hydrolyzed to their active forms. The mechanism of action is based on the inhibition of synthesis of bacterial proteins. Several years ago, chloramphenicol used to be extensively used in dairy herds; the adverse effects associated with the use of this antibiotic, however, have led to a ban on a worldwide basis. Nevertheless, its illegal introduction in Venezuela has been matter of discussion and led to a ban on a worldwide basis. Nevertheless, its illegal introduction in Venezuela has been matter of discussion and research. The present study investigated the presence of chloramphenicol in bovine long-life milk in Venezuela.

MATERIAL AND METHODS
Milk samples were collected from twelve different brands of milk in Venezuela. A total of 120 samples (ten per brand) were obtained and screened for the presence of the antibiotic using an ELISA test (detection limit: 50 ng kg\(^{-1}\)) [2].

RESULTS AND DISCUSSION
Residues of chloramphenicol, ranging from 22.7 to 932.7 µg kg\(^{-1}\), were detected in all of the examined samples. These results showed evidence of violation of national and international rules prohibiting the use of this antibiotic, which could be the cause of serious public health problems in Venezuela [3].

Table 1. Chloramphenicol levels in different brands of milk intended for human consumption in Venezuela

<table>
<thead>
<tr>
<th>Brand</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>386.4 ± 0.01</td>
<td>1200 ± 1.2</td>
<td>586.0 ± 1.3</td>
<td>886.8 ± 1.3</td>
<td>1413.3 ± 2.0</td>
<td>912.7 ± 2.0</td>
<td>286.4 ± 2.0</td>
<td>1099.4 ± 3.0</td>
<td>528.7 ± 4.0</td>
<td>87.9 ± 5.0</td>
<td>99.7 ± 6.0</td>
<td>22.7 ± 6.0</td>
</tr>
<tr>
<td>(µg kg(^{-1}))</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

REFERENCES

INTRODUCTION
Ochratoxin A (OTA) is produced principally by the moulds Penicillium verrucosum and Aspergillus ochraceus [1]. It is a potent renal toxin in almost all animal species; in laboratory animals its nephrotoxicity is associated with renal tumours. OTA is also immunotoxic and teratogenic but at higher dosages [2,3]. Pigs are particularly sensitive to the nephrotoxicity, a LOEL (8 µg kg\(^{-1}\) body weight day\(^{-1}\) 90 days feeding study) and a NOEL (40 µg kg\(^{-1}\) body weight day\(^{-1}\) in a 2-year feeding study) have been established [4]. A special problem is the presence of OTA in meat and meat products, because carry over from feed to meat has been shown. In the exposed organism, the kidneys contain the highest toxin concentrations, followed by the liver, muscle and fat; also in blood considerable amounts of the toxin have been found [5,6]. The aim of the present study was to evaluate the in vivo adsorption efficacy of autoclaved Saccharomyces cerevisiae (SC) and the carry-over of OTA alone or in combination with aflatoxin (AF) B\(_1\).

MATERIALS AND METHODS
Sixty pigs (120 kg mean body weight) were randomly divided into six experimental groups. Six different diets were administered for 5 weeks: 0; 0; diet supplement; 0% SC; 0% OTA; diet supplemented with 200 ppb OTA; 0% OTA+AFB\(_1\); diet supplemented with 200 ppb OTA+280 ppb AFB\(_1\); SC-OTA: diet supplemented with 0% SC and 200 ppb OTA; SC-OTA+AFB\(_1\); diet supplemented with 0% SC and 200 ppb OTA+280 ppb AFB\(_1\). The diets were artificially contaminated with the toxins adding pure OTA and AFB\(_1\) to the basal diet. At the beginning of the trial and immediately before slaughter, blood samples were collected. At the end of the experiment, all the animals were slaughtered and liver, kidney, muscle and adipose tissue were sampled. HPLC analysis with fluorometric detection as proposed by Cirilli et al. [7] and by Simonella et al. [8] were slightly modified and were used to measure OTA, AFB\(_1\) and its main polar metabolite aflatoxin M1 (AFM\(_1\)) in the biological samples. Animal care and experimental procedures were conducted according to Directive 86/609/EEC (1986). The study was performed according to ISO 9001: 2000 requirements. Differences between treatments were compared using the Student’s t-test (paired data); a difference with P < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION
During the trial all the pigs remained clinically healthy, and the mycotoxins and Saccharomyces had no apparent effects on feed intake and weight gain. These results are in contrast to the observations in pigs fed 0.2 mg kg\(^{-1}\) of OTA in the diet for 90 days [9], but the shorter feeding period most likely accounts for these differences. For the three toxins, the HPLC methods were characterized by accuracy, good specificity and linearity in the considered ranges of concentrations. The levels of OTA, AFB\(_1\) and AFM\(_1\) found in the considered biological matrices are summarized in Table 1. The levels of AF are always lower than OTA, confirming the records of Stubblefield et al. [10]. The concentrations of OTA are, in descending order: blood>>kidney>>liver>muscle and adipose tissue, our results sufficiently agree with data reported by different authors [11,12]. Our data (Table 1) underline differences and opposing results in the in vivo efficacy of autoclaved SC to adsorb OTA and AFB\(_1\).

Our results confirm, also in the swine, the limited ability of AFB\(_1\) and AFM\(_1\) to accumulate in tissues, so contaminated food of animal origin can be usually considered safe to humans as long as dietary concentrations comply with the limits set by the EU. On the contrary, OTA is characterized by a long persistence and accumulation in the kidneys, resulting in a contamination of serum and kidney. In contrast, toxin concentrations in meat (muscle tissue) and fat are rather low. Hence the contribution of
meat products to overall human exposure remains low, with exception of young children and those individuals that consume larger amounts of local specialities. Finally, our in vivo study does not confirm the demonstrated in vitro ability of Saccharomyces cerevisiae to adsorb OTA and AFB1 administered orally.

ACKNOWLEDGEMENT
This study was supported by a grant from Regione Emilia-Romagna (LR28, 2002).

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11. EFSA J (2004); 101: 1–36.

E51
Preliminary evaluation of aflatoxin M1 absorption/secretion profile on a Caco-2/TC7 clone, a human intestinal in vitro model, cultured on semi-permeable inserts
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1Department of Veterinary Sciences and Technologies for Food Safety, University of Milan, Milan, Italy; 2Department of Environmental and Primary Prevention, Istituto Superiore di Sanità, Rome, Italy

INTRODUCTION
Aflatoxin M1 (AFM1), the principal hydroxylated aflatoxin (AF) B1 metabolite, which is present in the milk of all mammals after consumption of feed contaminated with AFB1, is classified as a probable human carcinogen (group 2B of the IARC). Most countries have regulated its maximum permissible levels in milk in order to reduce the risks of AFM1 exposure (50 ppt EU and 500 ppt USA). In a recent study [1] it was demonstrated that AFM1 was highly absorbed in differentiated Caco-2/TC7 cells, which represent a very well characterised model of human intestinal enterocytes, and is not cytotoxic to these cells, in the range of possible milk contamination level. The present study with AFM1 was carried out on Caco-2/TC7 cells, cultured on a microporous filter support, to evaluate the absorption/secretion profile [2].

MATERIALS AND METHODS
Caco-2/TC7 cells have been cultured on microporous filter supports, which separate the apical (Ap) side (corresponding to the in vivo intestinal lumen) from the basolateral (Bl) compartment that faces the interstitial space and the vascular systems in vivo, allowing to evaluate the absorptive component (Ap to Bl) separately from the excretory pathway (Bl to Ap). Cells were cultured for 21 days, to allow the complete differentiation process, and were then exposed to several concentrations of AFM1 (1,000–10,000 ppt) either on the Ap or the Bl surface. After 48 h, AFM1 concentrations were determined by HPLC-fluorimetry either in culture media or in cell pellets (1). In addition, barrier integrity of the cellular layer was observed by TEER (Trans-epithelial Electrical Resistance) measurement, during the entire treatment.

RESULTS
AFM1 passage through the Caco-2/TC7 layer, was mainly observed after Bl exposure at all concentrations tested. In both treatments, the same low concentration of mycotoxin has been detected in the cells, indicating that no significant absorption occurs into this cell line (Table I). A slight but significant decrease of TEER values was reported after 6 h and 24 h of treatment at all concentrations tested. This decrease is partially reversed at the end of the exposure period (48 h), indicating a reversible opening of the tight junctions.

Table 1. AFM1 (ppt) detection in Caco-2 absorption model

<table>
<thead>
<tr>
<th>AFM1 ppt</th>
<th>Donor Medium</th>
<th>Acceptor Medium</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>4.09± 0.2</td>
<td>20.57± 0.1</td>
<td>71.7± 3.5</td>
</tr>
<tr>
<td>5.000</td>
<td>427.1± 0.2</td>
<td>20.70± 7.4</td>
<td>63.4± 10.4</td>
</tr>
<tr>
<td>10.000</td>
<td>714.2± 2.2</td>
<td>216.2± 7.1</td>
<td>59.7± 6.1</td>
</tr>
</tbody>
</table>

DISCUSSION
Our preliminary results show that AFM1 is able to cross the intestinal barrier, without impairment of its integrity (as demonstrated by TEER measurement results), particularly after Bl treatment, and that it is poorly absorbed by intestinal cells. Considering that other mycotoxins, like ochratoxin A and patulin [3], strongly impair intestinal barrier functions, the absence of barrier damage and the dose dependent AFM1 BL-AP passage observed here, could point to a mechanism comparable to the transport of aminopentol [4], the fumonisin B1 metabolite, where it was suggested that P-glycoprotein was involved in the influx/exflux across the intestinal mucosal barrier. Further studies are needed to clarify the kinetic of absorption/secretion profile of AFM1 in order to give a realistic estimate of the potential rate of exposure (internal dose) following the consumption of contaminated food.

REFERENCES
E52 Distribution of high molecular weight PAH in Atlantic mackerel (Scomber scombrus L, 1758) and European hake (Merluccius merluccius L, 1758)
M. PERUGINI, P. VISCIANO, M. MANERA, A. GIAMMARINO & M. AMORENA
Department of Food Science, University of Teramo (TE), Italy

INTRODUCTION
Polycyclic aromatic hydrocarbons (PAH), above all those characterized by high molecular weight, play a prominent role as they interact with the aryl hydrocarbon receptor, producing, dioxin-like responses in vitro [1,2]. Adsorption of PAH from water by fish may be regarded as a simple partition process between water and the lipids within the organism. Consequently PAH with a higher molecular weight and more hydrophobic nature are expected to be more efficiently adsorbed than the compounds with lower molecular weight that are less hydrophobic [3]. Biotransformation of PAH in tissues is controlled by simple diffusion in and out of lipid pools and the rate of incorporation into storage lipids slows down their loss by diffusion or metabolism. The aim of this study was to investigate the distribution of benzo(b)fluoranthene (BbF), benzo(k)fluoran-thene (BkF), benzo(ghi)perylene (BghiP), benzo(a)pyrene (BaP), fluoranthene (F) and indeno(1,2,3-cd)pyrene (IP) in a species of fish with a high lipid content and to compare these data with the results from a species with a low lipid content.

MATERIALS AND METHODS
Fourteen samples of Atlantic mackerel and 14 of European hake were caught in the Central Adriatic Sea. The lipid content of each species was gravimetrically determined using Accelerated Solvent Extraction. PAH extraction was carried out as described by Dafflon et al. [2]. Quantitative analysis was performed by an external standard method (PAH mix 38, Dr. Ehrenstorfer Laboratories) using a Varian HPLC equipped with a fluorescence detector. PAH were separated using a C18 Enviroweep-pg column (Phenomenex) and a gradient elution programme with a flow rate of 1 mL min⁻¹. The recoveries were 70–110% with relative standard deviations of 5–15%. The external standard multipoint calibration technique was used. The detection limit of this method ranged from 0.05 to 0.25 μg kg⁻¹. Statistical significance was assessed by SPSS 13.0.1. Normality of data of PAH was assessed by means of Kolmogorov–Smirnov test. A Kruskal–Wallis H test was performed since the data was not distributed normally.

RESULTS
The results are reported in Fig. 1. BaP, BghiP and IP were below the limit of detection in all samples.

DISCUSSION
Disposition of PAH in fish is often influenced by lipid content and metabolic ability of individual species. Our results showed a significant difference (Kruskal-Wallis H test, P < 0.01) in PAH concentrations between European hake and Atlantic mackerel. It can be assumed that the low PAH levels in European hake are due to a low muscle lipid content (0.39%) compared to that of Atlantic mackerel (1.79%). As reported by Meador et al. [4], species with a high hepatic lipid content accumulate higher levels of PAH in the liver than in muscle. The distribution of PAH in tissues may be of considerable interest to consumers of seafood, especially if it is considered that muscle is the main edible part. Further investigations will help to estimate the contribution of fish to the dietary intake of PAH in individual geographic regions.

REFERENCES

Figure 1. Concentrations (μg kg⁻¹) of BbF, BkF, F and PAH Sum expressed on fresh weight basis in two marine species (mean ± SEM) selected were Norwegian lobster (Nephrops norvegicus), red mullet (Mullus barbatus), European flying squid (Todarodes sagittatus), Atlantic mackerel (Scomber scombrus), blue whiting (Micromesistius poutassou) and European hake (Merluccius merluccius). For

E53 Total mercury in fish from the Central Adriatic Sea in relation to levels found in the hair of fishermen
M. PERUGINI¹, N. DORAZIO², M. MANERA¹, B. GIANNELLA¹, A. ZACCARONI³, M. ZUCCHINI¹, A. GIAMMARINO, G. RICCIONI², C. FICONERI² & M. AMORENA¹
¹Department of Food Science, University of Teramo, Italy;
²Department of Human Nutrition, University of Chieti, Italy;
³Department of Veterinary Public Health and Animal Pathology, University of Bologna, Ozzano Emilia (BO), Italy

INTRODUCTION
Mercury (Hg) is a global pollutant, without any environmental boundaries. Environmental exposure to Hg occurs primarily via the food chain due to accumulation of methylmercury (MeHg) in fish. Epidemiological studies underline the strong correlation between chronic exposure to MeHg and teratogenic and neurotoxic effects. In facts this metal readily crosses the blood-brain barrier and the placenta accumulating in the foetus and exerting toxic effects [1]. Furthermore MeHg is excreted through breast milk exposing newborns to mercury toxicity. Scalp hair represents another important excretion route in which organic mercury accounts for more than 70–80% of total mercury [2]. The aim of the present study was to document the total Hg content in different commonly eaten fish species from Adriatic Sea and to correlate these levels with the concentration of Hg in scalp hair of habitual fish consumers (fishermen), to assess possible exposure scenarios.

MATERIALS AND METHODS
Fish, crustacean and cephalopods were caught in the Central Adriatic Sea (Italy), within 40 miles of Pescara (42°-40’-67’’ North and 14°-38’-05’’ East) by fishing boats equipped with bottom trawls. Samples were collected in 2004. The species selected were Norwegian lobster (Nephrops norvegicus), red mullet (Mullus barbatus), European flying squid (Todarodes sagittatus), Atlantic mackerel (Scomber scombrus), blue whiting (Micromesistius poutassou) and European hake (Merluccius merluccius). For

the determination of total mercury, a wet digestion method was used. The quantitative analysis was performed using a hydride generator spectrophotometer (Varian). Scalp hair samples of fishermen living in Abruzzo were also collected during 2004. Quantitative analysis of approximatively 0.204 g of hair was performed by Doctor’s Data Inc, using ICP-MS. The obtained results were subjected to a Kruskal–Wallis test (z = 0.05) and ANOVA (General Linear Model) were applied on the obtained log-transformed values to evaluate differences between species, sampling seasons (summer and winter) and analysed matrix (muscle and bone). Correlation between Hg content of scalp hair and the amount of fish eaten (on average) by fishermen was assessed by means of the partial correlation method.

RESULTS
Benthic fish (Norwegian lobster, red mullet) displayed higher values (599.48 ± 98.07 ng g⁻¹ wet weight) of Hg than pelagic fish (340.73 ± 33.03 ng g⁻¹ wet weight) (European flying squid, Atlantic mackerel, blue whiting and European hake (P < 0.01), with Norway lobster showing the highest values (969.13 ± 237.90 ng g⁻¹ wet weight) (P < 0.01). Season affected Hg values only in pelagic fish with highest values during winter (495.33 ± 60.23 ng g⁻¹ wet weight) (P < 0.01). With regard to the matrix, muscle (473.95 ± 54.80 ng g⁻¹ wet weight) contained higher values than bone (260.38 ± 26.71 ng g⁻¹ wet weight) (P < 0.01). Analysis of scalp hair from fishermen indicated that the Hg content of hair correlated with the amount of fish consumed, excluding interfering sources like dental amalgams (partial correlation coefficient, 0.58; P < 0.05). Moreover Hg content in human tissue appeared to be the highest of the species studied (7815.76 ± 1887.86 ng g⁻¹ wet weight; P < 0.05).

DISCUSSION
The statistical analysis provided convincing evidence of significant differences in total Hg load between benthic and pelagic fish, muscle and bone and between winter and summer. This variegated distribution of Hg among the different species analysed is in accordance with the process of Hg uptake by fish, and with the interaction of biotic parameters such as size, sex, longevity, growth rate, feeding habits, trophic level, and habitat [3]. Benthic fish may potentially accumulate higher amounts of Hg because they spend considerable time searching for food on the bottom in the sediment [4]. The concentrations of mercury in these species are not acceptable levels for human consumption according to the standards for mercury concentration set by the European Commission Decision 93/351 of 19 May 1993 (0.5 μg g⁻¹ wet and 1 μg g⁻¹ wet weight in species with high potential accumulation). The content of total Hg in scalp hair from fishermen indirectly confirmed the elevated levels detected in fish because of the good correlation with fish consumption. Considering the observed average Hg content and the daily fish intake estimated for Italian population, the provisional tolerable weekly intake, recommended by the Joint FAO/WHO Expert Committee on Food Additives, is largely exceeded.

ACKNOWLEDGEMENT
The study was financed by MIUR ex 40% 2002.

REFERENCES


E54
Arsenic and mercury in fish from the Northern Adriatic and Tyrrhenian Seas in relation to sampling period and trophic level
A. ZACCARONI¹, M. ZUCCHINI¹, M. PERUGINI², M. MANERA², A. LUCISANO³ & M. AMORENA²
¹Department of Veterinary Public Health and Animal Pathology, University of Bologna, Ozzano Emilia (BO), Italy; ²Department of Food Science, University of Teramo, Teramo, Italy; ³Department Animal Pathology and Health, University of Napoli, Napoli, Italy

INTRODUCTION
Arsenic (As) and mercury (Hg) are amongst the most important pollutants in aquatic environments [1]. Arsenic can be present in animals both in its organic and inorganic forms. Even although it can be present in terrestrial animals at tissue levels lower than 1.0 mg kg⁻¹ wet weight, in marine environments mean levels of As are dramatically higher. Marine organisms, especially crustaceans, can present As levels as high as 100 mg kg⁻¹ dry weight in the form of arsenobetaine, with is virtually non toxic. Some variations are observed with age, habitat and to proximity of the habitat of the fish to human activity. Although it can be concentrated in animal tissues, it is not biomagnified along the food chain. Environmental exposure of humans to Hg occurs primarily via the food chain due to accumulation of methylmercury (MeHg) in fish. Epidemiological studies underline a strong correlation between chronic MeHg exposure and teratogenic and neurotoxic effects; in fact this metal readily crosses the blood–brain barrier and the placenta, accumulating in the foetus and exerting its toxic effects [2,3]. The aim of the present study was to evaluate total As and Hg content in muscle and bone present in different commonly eaten fish species from the Adriatic Sea and Tyrrhenian Sea and to correlate it with sampling period and trophic level.

MATERIALS AND METHODS
Norwegian lobster (Nephrops norvegicus), red mullet (Mullus barbatus), European flying squid (Todarodes sagittatus), Atlantic mackerel (Scomber scombrus), Blue whiting (Micromesistius poutassou), European Hake (Merluccius merluccius) and mussels (Mytilus galloprovincialis) were caught in two different areas: the Northern Adriatic Sea (Italy) and the Tyrrhenian Seas in 2004. For the determination of As and Hg, a wet digestion method was used and quantitative analysis of metals was performed using an hydride generator spectrophotometer (Varian) for Hg and a ICP-MS (Agilent HP) for As. No speciation of As was performed, this allows us to include also toxic effects.
heavy metal contamination. Metal concentrations were also analysed considering the trophic level of each species and the food chain they belong to, in order to identify if any magnification occurred.

RESULTS

The concentrations (mean ± SEM wet weight) of Hg and As found in each species sampled stratified by sampling area are reported in the table below, where statistical differences (P < 0.01) between seas are reported for comparison of the same species.

<table>
<thead>
<tr>
<th>Species</th>
<th>As (ng g⁻¹ wet weight)</th>
<th>Hg (ng g⁻¹ wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adriatic Sea</td>
<td>Tyrrenian Sea</td>
</tr>
<tr>
<td>Nephrops norvegicus</td>
<td>5761 ± 0.76</td>
<td>93864 ± 0.54*</td>
</tr>
<tr>
<td>Mullus barbatus</td>
<td>41533 ± 0.38*</td>
<td>11374 ± 0.52</td>
</tr>
<tr>
<td>Scomber scombrus</td>
<td>3299 ± 0.27</td>
<td>7894 ± 0.52*</td>
</tr>
<tr>
<td>Micromesistius poutassou</td>
<td>-</td>
<td>3793 ± 0.39</td>
</tr>
<tr>
<td>Merluccius merlucius</td>
<td>11817 ± 0.35*</td>
<td>6669 ± 0.27</td>
</tr>
<tr>
<td>Mytilus galloprovincialis</td>
<td>13283 ± 0.64</td>
<td>196.1 ± 0.47</td>
</tr>
<tr>
<td>Todarodes sagittatus</td>
<td>5761 ± 0.78</td>
<td>172.6 ± 0.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>As (ng g⁻¹ wet weight)</th>
<th>Hg (ng g⁻¹ wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adriatic Sea</td>
<td>Tyrrenian Sea</td>
</tr>
<tr>
<td>Nephrops norvegicus</td>
<td>172.6 ± 0.56</td>
<td>60.4 ± 0.59*</td>
</tr>
<tr>
<td>Mullus barbatus</td>
<td>298.1 ± 0.39*</td>
<td>173.3 ± 0.38</td>
</tr>
<tr>
<td>Scomber scombrus</td>
<td>129.6 ± 0.31</td>
<td>218.3 ± 0.51*</td>
</tr>
<tr>
<td>Micromesistius poutassou</td>
<td>-</td>
<td>203.2 ± 0.38</td>
</tr>
<tr>
<td>Merluccius merlucius</td>
<td>367.9 ± 0.30*</td>
<td>186.6 ± 0.29</td>
</tr>
<tr>
<td>Mytilus galloprovincialis</td>
<td>196.1 ± 0.47</td>
<td>-</td>
</tr>
<tr>
<td>Todarodes sagittatus</td>
<td>172.6 ± 0.56</td>
<td>-</td>
</tr>
</tbody>
</table>

ACKNOWLEDGEMENT

The study was financed by MIUR ex 40% 2002.

REFERENCES


INTRODUCTION

Arsenic (As) is a global pollutant and is found as a naturally occurring element in soil, food and water [1]. As toxicity depends on its oxidation states and molecular form; inorganic As compounds are generally considered more toxic than organic (arsenobetaine and arsenocholine) [2]. Most environmental inorganic As is present as arsenate (As⁵⁺), but arsenite (As³⁺), which is more toxic than arsenate, is also found [3]. Environmental exposure to As in humans occurs primarily via the food, due to the accumulation of As in fish. Indeed, in marine environments, methylation or demethylation is influenced by different factors, such as pH, water temperature and the composition of sediments. Since most of the As in marine organisms is in organic form (methylated), it is readily excreted by humans and of low toxicity [4]. Populations eating contaminated fish display increased incidence of cancer of the skin, urinary bladder, liver and kidney [1]. It is possible to estimate human exposure using a trichologial survey because As, as other metals, is also eliminated in hair [5]. The aim of the present study was to document the total As content in different commonly eaten fish species from the Adriatic Sea and to correlate this with the As concentration of scalp hair of habitual fish consumers (fishermen) to assess possible exposure scenarios.

MATERIALS AND METHODS

Fish, crustaceans and cephalopods were caught in the Central Adriatic Sea (Italy), within 40 miles of Pescara (42°40’–67” North and 14°38’–05” East) by fishing boats equipped with bottom trawls. Samples were collected in the year 2004. The species selected were Norwegian lobster (Nephrops norvegicus), red mullet (Mullus barbatus), European flying squid (Todarodes sagittatus), Atlantic mackerel (Scomber scombrus), blue whiting (Micromesistius poutassou) and European hake (Merluccius merlucius). For the determination of total As, a wet digestion process of metal uptake by fish and with the interaction of biotic organisms is in organic form (methylated), it is readily excreted by humans and of low toxicity [4]. Populations eating contaminated fish display increased incidence of cancer of the skin, urinary bladder, liver and kidney [1]. It is possible to estimate human exposure using a trichologial survey because As, as other metals, is also eliminated in hair [5]. The aim of the present study was to document the total As content in different commonly eaten fish species from the Adriatic Sea and to correlate this with the As concentration of scalp hair of habitual fish consumers (fishermen) to assess possible exposure scenarios.
method was used and an ICP-MS (Agilent HP) technique was applied for quantitative analysis. No speciation of As was performed, this study being focused on a comparison of species and not on human health risk assessment. Additionally, MRI and acceptable levels for human consumption refer to total Hg and As load, respectively. Scalp hair samples from fishermen living in Abruzzo were collected during 2004. Metal quantitative analysis of close to 0.204 g hair was performed by Doctor’s Data Inc. using ICP-MS. A Kruskal Wallis test (x = 0.05) and an ANOVA (General Linear Model) were, respectively, applied to the values obtained and log-transformed values to evaluate differences between species, sampling season (summer and winter) and tissue analysed (muscle and bone). Correlation between Hg content of the fishermen’s scalp hair and the amount of eaten fish (on average basis) was also assessed by mean of partial correlation method.

RESULTS
Invertebrates (Norwegian lobster, European flying squid) displayed higher values (66048.38 ± 8258.04 ng g⁻¹ wet weight) of As than finfish (34820.95 ± 2581.18 ng g⁻¹ wet weight) (red mullet, Atlantic mackerel, blue whiting and European hake) (P < 0.01). With regard to habits, benthic animals (Norwegian lobster, red mullet) displayed higher values (57448.90 ± 5515.77 ng g⁻¹ wet weight) than pelagic fish (34282.20 ± 3088.61 ng g⁻¹ wet weight) (Atlantic mackerel, blue whiting and European hake) (P < 0.01) though European flying squid showed values (67377.88 ± 12065.51 ng g⁻¹ wet weight) comparable with those of Norwegian lobster (64718.89 ± 11759.05 ng g⁻¹ wet weight). Season affected As values in some pelagic fish only, namely in Atlantic mackerel (summer: 4231.39 ± 1025.11 ng g⁻¹ wet weight; winter: 36018.23 ± 8230.96 ng g⁻¹ wet weight) and European hake (summer: 17689.58 ± 2462.61; winter: 44759.92 ± 36018.23 ng g⁻¹ wet weight), with highest values during winter (P < 0.01). With regard to matrix, muscle (42452.24 ± 4349.30 ng g⁻¹ wet weight) contained higher values than bone (27339.30 ± 2439.82 ng/g wet weight) (P < 0.01). As content in hair correlated with the amount of fish consumed, excluding interfering sources like dental amalgams, smoking cigarettes and drinking coffee (partial correlation coefficient, 0.70; P < 0.01). Moreover, As content in human hair appeared to be the lowest of the species studied (75.37 ± 11.90 ng g⁻¹ wet weight; P < 0.01).

DISCUSSION
The total amount of As found in our sample fish was higher than in previous studies [4,6]. Even although no As speciation was performed, it can be assumed that more than 90–95% of the total As load is in the organic forms [7–9], which are virtually non-toxic and do not accumulate. This is confirmed by analysis of close to 0.204 g hair was performed by Doctor’s Data Inc. using ICP-MS. A Kruskal Wallis test (x = 0.05) and an ANOVA (General Linear Model) were, respectively, applied to the values obtained and log-transformed values to evaluate differences between species, sampling season (summer and winter) and tissue analysed (muscle and bone). Correlation between Hg content of the fishermen’s scalp hair and the amount of eaten fish (on average basis) was also assessed by mean of partial correlation method.

ACKNOWLEDGEMENT
The study was financed by MIUR ex 40% 2002.

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detected during the study. The systemic tolerance of the cows was normal. There was no significant change in daily milk production following the GENTA i.m. injections. The milk samples resulted collected from all four udder quarters were as follows presented (mean ± SD) in the Table that follows.

CONCLUSION
The results indicated that the time required for the milk residue concentration to fall below the maximal residue level (MRL 100 µg kg⁻¹) when lactating cows have been given five, twice daily consecutive i.m. injections of GENTA was no less than 48 h after the last dose.

REFERENCES

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Evaluation of a multi-component feed additive for the safe use in poultry production
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INTRODUCTION
Assessment of the main criteria for evaluation of probiotics in terms of identity, efficacy and safety is an essential part of the development of zootechnical feed additives for use in European broiler production. In the course of an EU-promoted project (C-EX QLK-CT-2002-71662) a well-defined multi-component feed additive was developed that combines various effective strains originally isolated from the gastrointestinal tract of healthy broilers. A complex risk assessment had to be established to carefully evaluate each strain with regard to the main risk factors related to the use of microorganisms as a feed additive. Considering the risks for antimicrobial resistance associated with agricultural sources, the Scientific Committee on Animal Nutrition (SCAN) adopted an Opinion in 2001 (updated in 2003) that provides guidance for the assessment of feed additives, demanding that bacterial strains used as probiotics should not carry resistance genes and should not be able to transfer resistance genes to other microorganisms.

MATERIAL AND METHODS
Bacterial resistance to clinically relevant antimicrobials was examined intensively on the basis of the pheno- and genotype of each strain, as recommended by the SCAN [1] and by the FEEDAP panel [2]. In a first step, the susceptibility to therapeutic antibiotics was quantitatively assessed by determining the MIC of the panel of antibiotics defined by the SCAN using the microdilution technique. The risk of possible horizontal antibiotic resistance gene transfer by mobile genetic elements was studied by direct plate colony mating under pre-antibiotic pressure using appropriate recipients. Plasmid DNA was isolated with the Nucleo Spin Plasmid DNA Purification Kit (Machery-Nagel) according to the manufacturer’s instructions, and additionally on a large scale using the alkaline lysis method followed by the separation in a caesium chloride gradient. The presence of added genes coding for antibiotic resistance, particularly associated with mobile genetic elements, was examined using molecular methods specifically targeting transmissible antibiotic resistance genes (e.g. vanA, tet genes). The incidence of enterococcal virulence factors was examined as described previously [3].

RESULTS
The probiotic candidate strains were sensitive to the majority of clinically effective antibiotics, although some of them showed single agent resistance (e.g. vancomycin, tetracycline, enrofloxacin). None of the vancomycin resistant strains carried the enterococcal vanA gene. The strains contained no extrachromosomal DNA and were not able to transfer single agent resistance (e.g. to vancomycin, tetracycline) by means of conjugation. The enterococcal strain was demonstrated to lack the most concerning virulence markers, specific for the surface protein gene esp, the cytolysin activator cylA and the gelatinase gelE gene, the cylB gene involved in transport of cytolysin, the cell wall adhesion-encoding efaAs and efaAfms as well as sex pheromones (cpd, ccf).

DISCUSSION AND CONCLUSION
Five well-studied chicken strains (Pediococcus acidilactici, Enterococcus faecium, Bifidobacterium animalis ssp. animalis, Lactobacillus reuteri, L. salivarius subsp salivarius) showing antagonistic activity against common poultry pathogens in vitro were carefully evaluated with regard to the safety of their combined use as natural feed additive in young chickens. Easy transferable resistances or potential virulence traits were excluded, mainly on basis of the absence of plasmids and non-transferability by conjugation. Anyhow horizontal dissemination of single agent resistance in some resistant strains could not be excluded per se. According to the actual guidelines [1,2] acquired antibiotic resistance traits are not acceptable unless it can be shown that the genetic basis of the resistance is due to chromosomal mutation. Therefore, work is in progress to localize the resistance gene in the chromosome of the resistant strains. This will fill the gaps relating to the potential for transmission of resistance via these resistance genes.

REFERENCES
1. SCAN 2001.