Analytical and Bioanalytical Chemistry
10.1007/s00216-005-0274-4

Determination of resorcylic acid lactones in biological samples by GC–MS. Discrimination between illegal use and contamination with fusarium toxins

M. H. Blokland1 , S. S. Sterk1, R. W. Stephany1, F. M. Launay2, D. G. Kennedy2 and L. A. van Ginkel1

(1) National Institute of Public Health and the Environment (RIVM), European Union Community Reference Laboratory for Residues, P.O. Box 1, Bilthoven, 3720 BA, The Netherlands
(2) Veterinary Sciences Division (VSD), Stoney Road, Stormont, Belfast, BT4 3SD, Northern Ireland

M. H. Blokland
Email: marco.blokland@rivm.nl
Phone: +31-302743966
Fax: +31-302744403

Abstract An EU project, FAIR5-CT-1997-3443, has been undertaken to distinguish illegal use of zeranol from consumption of food contaminated with Fusarium spp. toxin. One of the tasks was development of screening and confirmatory methods of analysis. This paper describes a new method based on two-step clean-up and GC–MS analysis. The first clean-up step is matrix-dependant; the second is applicable to both urine and meat. The MS is operated in negative chemical ionisation mode. The method is quantitative for zeranol and taleranol, α- and β-zearalenol, and zearalenone and qualitative for zearalanone. Validation was performed according to the latest EU performance criteria (Commission Decision 2002/657). For analysis of urine and for the method (μg L−1) were 0.06–0.11 for zeranol, 0.07–0.12 for taleranol, 0.07–0.11 for α-zearalenol, 0.21–0.36 for β-zearalenol, 0.35–0.60 for zearalenone, and 0.19–0.33 zearalanone. Within-laboratory reproducibility was 16.2, 11.2, 31.9, 30.1, 26.6, and 54.2% for zeranol, taleranol, α-zearalenol, β-zearalenol, zearalenone, and zearalanone, respectively. It was found that all the compounds are stable in urine at −20°C for at least a year. Part of the validation program was organisation of a small proficiency study (ringtest) and a correlation study with an LC–MS–MS method developed by the Veterinary Science Division (VSD; Belfast, UK-NI). This study showed there was good correlation between results from both laboratories. The method can be used for quantitative analysis discriminating illegal use of zeranol from consumption of zearalenone-contaminated food.
Keywords Zeranol - Taleranol - α/β-Zearalenol - Zearalanone - Zearalenone - GC–MS - Ringtest - Stability study

Introduction

Administration of zeranol, a non-steroidal oestrogenic growth-promoting compound, to animals raised as food is banned in the EU [1]. Zeranol (α-zearalanol) and its primary metabolite in bovine animals, taleranol (β-zearalanol), are resorcylic acid lactones (RALs), which also include α- and β-zearalenol, zearalanone, and zearalenone. Zearalenone is also known as the Fusarium spp. toxin (F2-toxin) and is commonly found in animal feed [2]. Zeranol and

zearalenone are known to give identical metabolites which explains why these metabolites, including zeranol itself, can also occur naturally in ovine urine and bovine bile after metabolism of Fusarium spp. toxin [3].

To distinguish illegal use of zeranol from consumption of food contaminated with Fusarium spp. toxin, an EU project, FAIR5-CT-1997-3443, was undertaken [4]. At the start it was hypothesized that testing for zeranol abuse would involve quantification of zeranol and its metabolite taleranol and of tearalenone and its two major metabolites α- and β-zearalenol. Validated quantitative methods were therefore necessary.

One of the tasks was development of screening and confirmatory methods of analysis suitable for all RALs. Recently published methods suitable for this purpose all are based on liquid chromatography–mass-spectrometry (LC–MS). Methods for urine have been published by van Bennekom et al. [5] and Launay et al. [6]. A procedure for bovine urine and tissue has been described by Jodlbauer et al. [7] and a procedure for rabbit and poultry liver by Fang et al. [8]. The major advantage of using LC–MS rather than gas chromatography–mass spectrometry (GC–MS) is that derivatisation can be avoided, although there are still concerns about the accuracy of quantification, because of ion-suppression phenomena [9], and although the published methods are validated according to commission decision 2002/657, they lack information about the stability of the analytes and results obtained by use of these methods were not compared with those obtained by use of other methods or by other laboratories.

Because discrimination between zeranol abuse and the presence of Fusarium spp. toxins entails determination of five different analytes [10], we developed a method based on GC–MS as an alternative to the procedure of Launay et al. [6], developed within the same EU project. This paper describes the validation of a quantitative GC–MS method for the entire group of RALs, suitable for two of the most important matrices, urine and bovine tissue, enabling residue control on farms, at slaughter, and for import control.

Validation of the method for urine samples was performed according to the latest EU guidelines [11], including a study of analyte stability in the matrix, extension of the method to additional validation for muscle tissue, and a study of correlation of results from the method with those obtained by LC–MS–MS. For purposes of (long-term) quality control, QC materials were prepared. Both fortified materials and materials obtained by treatment of bovine tissue were prepared. Such materials are important because extensive phase II metabolism (conjugation) occurs [12, 13].

The results are discussed in relation to data currently available on discrimination of zeranol abuse from consumption of feed contaminated with Fusarium spp. toxins.

Experimental

Chemicals

Standard zeranol-d4, taleranol-d4, α-zearalenol-d4, β-zearalenol-d4, α-zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, zearalenone, and zearalanone were obtained from the bank of reference standards (RIVM), zearalenone-d6 was obtained from the Veterinary Sciences Division (VSD). Standard solutions of these analytes were prepared and stored at +4°C until analysis. Methanol, ethanol, acetone and iso-octane were of analytical grade and obtained from Baker. Heptafluorobutyric acid anhydride (HFAA) was obtained

from Pierce. Demineralised water was obtained by use of a Milli-Q purification system. Acetic acid, sodium-acetate, Tris(hydroxymethyl)aminomethane, and tert-butyl methyl ether (TBME) were obtained from Merck. Beta-glucuronidase/sulfatase containing 100,000 units β-glucuronidase and 1,000,000 units sulfatase per millilitre suc Helix pomatia was obtained from BioSepra. Subtilisin type VII from Bacillus licheniformis, 11.6 units mg−1, (protease) was obtained from Sigma.

Methods

Analytical procedure

Enzymatic deconjugation of urine samples

The internal standards (2 μg L−1 of each) were added to 5-mL urine samples. After adjusting the pH to 5.2 and buffering with 2 mL 2 mol L−1 acetate buffer, the urine samples were hydrolysed by addition of 40 μL suc Helix pomatia and incubation for 2 h at 37°C.

Enzymatic digestion of meat samples

Enzymatic digestion of meat was performed by adding 5 mL 0.1 mol L−1 Tris buffer, pH 9.5, and 5±1 mg protease to 5 g homogenized meat and incubation of the mixture for 2 h at 50°C.

Primary extraction of enzymatically treated samples

After enzymatic incubation the samples were cooled to room temperature and liquid–liquid extraction (LLE) was performed with 10 mL TBME. The mixture was centrifuged for 3 min at 2700g. The organic layer was collected in a glass tube and evaporated at 50°C under a gentle stream of nitrogen. To remove fat from the extract the dry residue was dissolved in 5 mL 50:50% v/v methanol–water and washed with 1 mL hexane. The mixture was centrifuged for 3 min at 2700g and the hexane layer was discarded. The washing procedure was repeated, after which 2 mL water was added. The methanol–water layer was passed through a disposable C18 SPE column (1000 mg; Alltech) preconditioned with 5 mL methanol and 5 mL Milli-Q water. The SPE C18 column was washed with 5 mL 40:60% v/v methanol–water after which the analytes were eluted with 5 mL 80:20% v/v methanol–water. The eluate was collected and evaporated to dryness at 50°C under a gentle stream of nitrogen and the residue was re-dissolved in 5 mL 80:20% v/v acetone–methanol. The dissolved extract was passed through an amino SPE column (1000 mg; Alltech) preconditioned with 5 mL 80:20% v/v acetone–methanol. The eluate was collected directly and evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was dissolved in 0.5 mL ethanol, transferred to a derivatisation vial, and evaporated to dryness at 50°C under a gentle stream of nitrogen. The dry residue was immediately derivatised with 10 μL HFAA and 30 μL iso-octane.

GC–MS analysis

An Agilent HP5973 MSD mass spectrometer equipped with a 7683 autosampler and 6890 GC was used. The GC column used, a Zebron ZB-1 (L=30 mm, i.d.=0.25 mm, d f=0.25 μm), was obtained from Phenomenex. The volume of purified samples or standard solutions injected was 1 μL. The oven temperature was held constant at 80°C for 1 min and then increased at 25° min−1 to 300°C, which was held for 4 min. The helium carrier gas flow was kept constant at 1 mL min−1.

The GC–MS was operated in chemical ionisation negative ion mode; the ionisation gas was methane. The ions observed during single-ion monitoring were: zeranol and taleranol m/z 713, zeranol-d4 and taleranol-d4 m/z 717, α- and β-zearalenol

m/z 711, α- and β-zearalenol-d4 m/z 715, zearalenone m/z 513, zearalenone-D6 m/z 519 and zearalanone m/z 515.

Validation of the method for urine

The method was validated according to Commission Decision 2002/657/EC [11] which provides detailed guidance on validation of analytical methods used in residue control. The validation included determination of all the relevant performance characteristics of the method, a study of the stability of the analytes in the sample materials, and a correlation study in which the performance of the method in practice was compared with that of a different procedure (VSD).

The decision limit ($¡Emphasis Type="Italic"¿CC¡/Emphasis¿_\alpha$) and detection capability ($\left(¡Emphasis Type="Italic"¿CC¡/Emphasis¿_\beta\right)$) were determined by preparing a five-point standard plot for blank urine (urine with no detectable background levels of RALs) at the level of approximately 0.5 µg L^{-1}. From this calibration plot the y-intercept and slope were calculated ($y = ax + b$). $¡Emphasis Type="Italic"¿CC¡/Emphasis¿_\alpha$ is the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility (α=1%). The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability (β=5%).

To determine the absolute recovery a blank sample of bovine urine was spiked with a mixture of compounds at a concentration of 1 µg L^{-1} (n=3), the internal standard was added after clean-up, just before derivatisation.

The repeatability (within days) and within laboratory reproducibility (between days) were determined by spiking a blank urine sample at 1 µg L^{-1}. The urine sample was analysed, in triplicate, on five different days. Analysis of variance (ANOVA) was used to calculate the repeatability and the within-laboratory reproducibility.

The ruggedness was determined by spiking ten different samples of bovine urine at 1.0 µg L^{-1}. These analyses were performed exactly as routine analyses of unknown samples, with the addition on internal standards, but without subtracting background levels.

Validation of the method for meat

Changing to another matrix is regarded as a major change of a method and so supplementary validation is needed [11, 14]. During this procedure the performance characteristics obtained for the new matrix are compared directly with those for the original (validated) matrix. When the results are in agreement, the new matrix is added to the applicability range of the method. Supplementary validation entailed analysis of 10 urine samples and 10 samples of meat, the "new" matrix. All materials were fortified with 0.5 µg kg^{-1} of each compound. Background levels were determined for the materials and subtracted from the

results. A statistical t-test $\left(t = (\overline{x}_1 - \overline{x}_2)/s\sqrt{(1/n_1 + 1/n_2)}\right)$ was performed to compare the means and an F-test $F = s_1^2/s_2^2$ to compare the standard deviations [15]. According to these statistics there is a significant difference between the two matrices when the calculated value for the t-test exceeds 2.10 (eighteen degrees of freedom) and/or that for the F-test exceeds 4.026 (nine degrees of freedom).

Stability study

Bovine urine was collected from animals that had not been intentionally exposed to the Fusarium spp. toxins. The urine samples were pooled, sub-sampled, and frozen at −20°C. Urine samples were subsequently filtered through two glass-fibre filters then through 0.45 μm and 0.2 μm pore size membrane filters. The urine was fortified with 3 ppb α-zearalanol and 30 ppb α- and β-zearalenol to furnish typical "natural" levels or with 30 ppb α-zearalanol and 30 ppb α- and β-zearalenol to furnish typical "abuse" levels. Samples (10±0.1 mL) were transferred to individual 20-mL amber vials using an automatic filling system (ASPEC). One set of vials was stored at −20°C. All the remaining vials were freeze-dried (Christ Delta II) and filled with nitrogen before closing and sealing.

A 16-week stability study was conducted on the stored materials. Samples were taken in duplicate at zero time and after 1, 2, 4, 8, and 16 weeks of storage, reconstituted by adding 10 mL water, and analysed immediately.

After analysis of the samples, the mass concentration (expressed as a percentage of the spiked value) for each compound was plotted against time. To determine trends over time the slope and y-intercept were calculated for each batch of samples. From the standard deviation of the slope the 95% confidence interval was calculated. The stability period is determined as the point at which the slope plus the 95% confidence limit crosses the −20% line of the y-intercept. If this line never crosses the −20% line the samples are stable under the described conditions. These materials were used for evaluation of screening procedures developed within the project.

Ringtest

A ringtest among five laboratories, each using its own analytical method based on MS, was included in the validation procedure. Bovine urine was collected from animals that had not been intentionally exposed to Fusarium spp. toxins (materials were obtained from the VSD). The urine samples were pooled, sub-sampled, spiked at the concentrations given in Table 1, and frozen at −20°C. All vials were freeze-dried (Christ Delta II) and filled with nitrogen before closing and sealing. Five lyophilised samples (in duplicate) were sent to each participant.

Table 1 Amount of analyte added to the urine samples (n=5)

ID	Zeranol	Taleranol	α-Zearalenol	β-Zearalenol	Zearalenone	Zearalanone
A	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	Blank	10 ng mL^{-1}	10 ng mL^{-1}	Blank	Blank
C	2 ng mL^{-1}	2 ng mL^{-1}	Blank	Blank	Blank	Blank
D	2 ng mL^{-1}	2 ng mL^{-1}	10 ng mL^{-1}	10 ng mL^{-1}	2 ng mL^{-1}	10 ng mL^{-1}
E	5 ng mL^{-1}	5 ng mL^{-1}	5 ng mL^{-1}	5 ng mL^{-1}	5 ng mL^{-1}	5 ng mL^{-1}

Participants analysed the samples with their own in-house GC–MS procedure.

Correlation study
To evaluate quantification by GC–MS, 20 randomly selected real-life samples were analysed by VSD both by this procedure and by LC–MS–MS [16].

Results and discussion
The described method was validated according to Commission Decision 2002/657/EC [11]. Several performance characteristics were determined. The important data are the decision limit (CC_α), the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant, and the detection capability $\left(\text{¡Emphasis Type="Italic"¿CC¡/Emphasis¿}_\beta\right)$, the smallest amount of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β. For substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1−β. For substances with an established permitted limit, the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1−β.

The results, of the validation study are summarized in Table 2.

Table 2 Overview of results from validation of the method for urine

Analyte	CC_α	CC_β	Recovery (%)		Repeatability	Reproducibility	Robustness (%)	
	(µg L^{-1})	(µg L^{-1})	Average	*CV*	*CV* (%)	*CV* (%)	Average	*CV*
Zeranol	0.06	0.11	71.7	1.8	4.1	16.2	119.6	7.4
Taleranol	0.07	0.12	67.8	3.3	3.2	11.2	126.4	11.1
α-Zearalanol	0.07	0.11	65.5	0.4	5.9	31.9	126.3	8.2
β-Zearalanol	0.21	0.36	71.5	3.6	3.3	30.1	239.5	48.6
Zearalenone	0.35	0.60	37.5	5.5	6.1	26.6	132.3	35.5
Zearalanone	0.19	0.33	86.9	11.9	6.9	54.2	227.5	31.6

For zeranol and its metabolite taleranol the analytical performance data are well within the criteria for methods used for residue analysis [11] with absolute recoveries of approximately 70%, a value for $\text{¡Emphasis Type="Italic"¿CC¡/Emphasis¿}_\beta$ <0.2 µg L^{-1} and good repeatability and reproducibility. For α-zearalenol and β-zearalenol very similar values were

obtained, with slightly higher values for the reproducibility, but still within the limits [11]. For the other analytes higher values were observed, with relative poor recovery for zearalenone, resulting in a significantly higher value for ¡Emphasis Type="Italic"¿CC¡/Emphasis¿$_\beta$. From the data for ¡Emphasis Type="Italic"¿CC¡/Emphasis¿$_\alpha$ and ¡Emphasis Type="Italic"¿CC¡/Emphasis¿$_\beta$ it is concluded that analytes at a concentration of 0.5 μg L^{-1} can be detected reliably. To confirm this for true samples representative blank urine samples (n=9) were spiked with the analytes at 0.5 μg L^{-1}. Signals with S/N ratios exceeding 6 were always clearly observed. No false compliant (negative) results were obtained. Robustness data, based on different samples, can be evaluated only for compounds for which there is no significant background value. Some of these samples contained α/β-zearalenol, zearalenone, and zearalanone with mass concentrations in the range 0.5–5 μg L^{-1}.

Supplementary validation

The performance characteristics of the method for urine and meat arecompared in Table 3.

Table 3 Overview of supplementary validation data, comparison for urine (n=10) and meat (n=10); the values given are the accuracy and *CV* (%) at 0.5 μg L^{-1} or 0.5 μg kg^{-1}

	Zeranol		**Taleranol**		**α-Zearalenol**		**β-Zearalenol**		**Zearalenone**		**Zearalanone**	
	Urine	**Meat**	**Urine**	**Meat**	**Urine**	**Meat**	**Urine**	**Meat**	**Urine**	**Meat**	**Urine**	**Meat**
Average	99.0	100.1	105.2	98.6	97.0	91.1	101.9	97.7	184.6	124.6	173.7	173.5
CV (%)	3.9	3.2	7.3	4.5	12.3	13.4	5.5	3.4	27.4	29.9	12.5	29.7
t-test	0.1		0.4		0.2		0.4		0.6		0	
F-test	1.5		3.0		1.1		2.9		2.9		0.2	

The different samples of urine used in this experiment contained concentrations of α- and β-zearalenol and zearalenone in the range 0.5–5 μg L−1. The concentrations of these compounds found in unspiked urine were subtracted from the results. For zeranol, taleranol, and α- and β-zearalenol the results of the procedure were indicative of high accuracy and robustness. Values for zearalenone and zearalanone remain biassed. Very important, however, is the conclusion that differences between performance data for urine and meat for all the compounds is not significant. Based on the criteria for supplementary validation, the applicability of the method can be extended to include meat as a sample material [11].

Stability study

The stability of the compounds in urine under different storage conditions is given in Table 4.

Table 4 Stability of the different compounds over time (weeks) under different storage conditions

ID	Condition	Zeranol	α-Zearalenol	β-Zearalenol
Cattle urine	−20°C Frozen	Stable	Stable	Stable
	−20°C Lyophilised	Stable	Stable	52
	+4°C Lyophilised	176	20	18

Zeranol, taleranol, and β-zearalenol are stable at −20°C in frozen or lyophilized urine. In lyophilized urine the stability of β-zearalenol is limited to 52 weeks. When urine is stored at +4°C, none of the compounds is stable, however, with significant changes within a few months of storage.

Ringtest
The aim of the ringtest was to compare the results obtained by use of GC–MS for the five laboratories participating. The highest and lowest concentrations found are shown in Fig. 1.

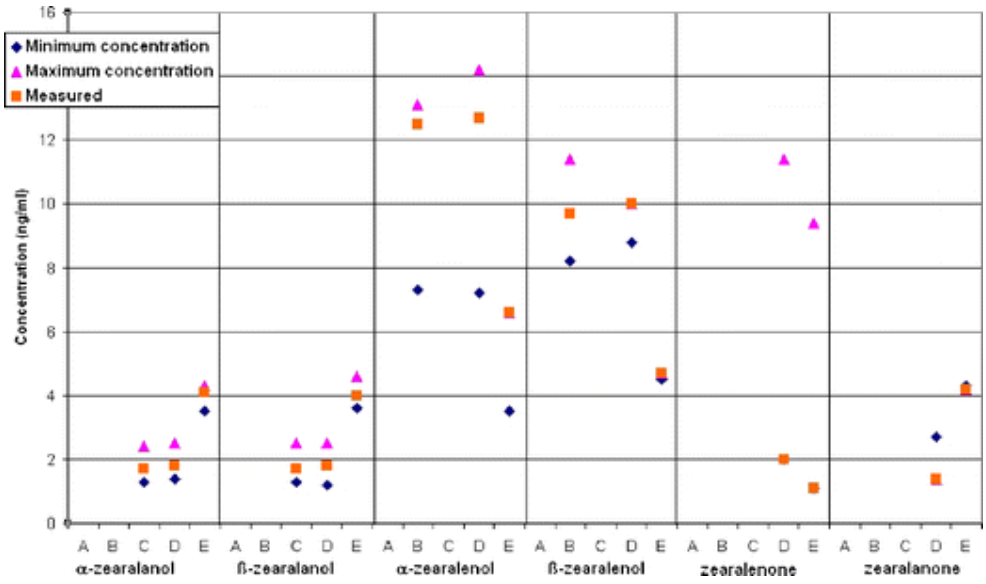

Fig. 1 Highest (triangles) and lowest (diamonds) concentrations found in this ringtest and the concentrations found by use of the method presented (squares)

Results from GC–MS (NCI) analysis of the urine are in agreement with the concentrations expected for all analytes except zearalanone. Overall variability for zeranol and taleranol, α- and β-zearalenol, and zearalenone was 10–35% for all the concentrations tested. These values are in agreement with general performance during international ringtests (proficiency tests) conducted within the EU. The variability for zearalanone was higher.

Correlation study
The applicability of the method to real-life samples, and the specificity of detection, is demonstrated by comparing the results obtained by the VSD using LC–MS–MS [16] and by the Community Reference Laboratory (CRL) using GC–MS. An overview of the results is shown in Fig. 2.

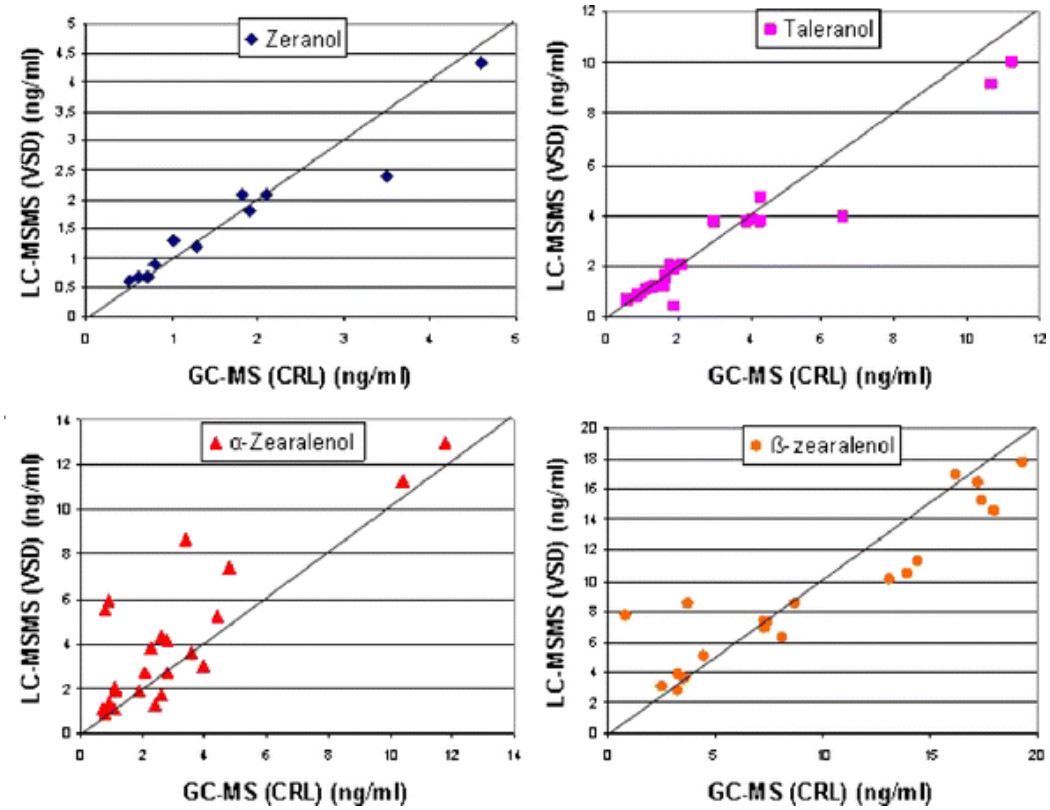

Fig. 2 Graphical representation of results obtained by GC–MS and LC–MS–MS

From Fig. 2 it is apparent there is good correlation between quantitative results for zeranol, taleranol and β-zearalanol. For α-zearalanol LC–MS–MS results tend to be slightly higher. No false positives or negatives were obtained.

Discussion and conclusions

Reliable methods for detection and quantification of zeranol and taleranol residues are necessary within the framework of modern residue-control programmes.

The possible presence of Fusarium spp. toxins as a consequence of contamination of feed makes detection and quantification of other, related, compounds necessary, however. The objective of the "Natural Zeranol" project FAIR5-CT-1997-3443, during which the method described was used, was to study and to establish criteria for discriminating between illegal treatment with zeranol-containing preparations and the presence of Fusarium spp. toxins because of contamination of feed. A statistical model was developed after screening and confirmation of 8008 samples collected from different parts of Europe [10]. The model developed is a tool which could help laboratories involved in zeranol testing to distinguish between illegal use of zeranol and natural contamination. It is based on comparing the sum of the zeranol and taleranol concentrations with the sum for zearalenone and its two major metabolites, α- and β-zearalenol. Validated quantitative methods are necessary for practical applications.

From the results obtained during this study it is clear that the performance is not identical for all compounds. Extensive validation reveals differences in performance for different (groups of) RALs. The best results, in terms of CC values, recovery, repeatability and reproducibility, and ruggedness, were obtained for zeranol and taleranol, the two compounds primarily indicative of illegal treatment. For α- and β-zearalenol also the results were good, with only a higher value for the within-laboratory reproducibility. The most critical compound

is zearalenone itself for which recovery was relative poor. Despite this, however, the other results were acceptable. On the basis of these results it can be concluded that the three RALs most indicative of consumption of zearalenone-contaminated feed can also be determined quantitatively. For zearalanone, results from the four-laboratory ringtests indicated there is no correlation between the expected and observed concentrations. It is, therefore, concluded that quantitative analysis is not currently possible. For the current purpose of the test this is not relevant.

From supplementary validation it was concluded that the method can be applied to urine and meat. The stability study showed that storage of bovine urine at +4°C, even when lyophilized, will result in reduction of the concentrations of all analytes.

After lyophilization and storage at −20°C the concentration of β-zearalenol decreases; the other compounds are stable under these conditions. A very important conclusion for practical residue control is that when urine samples are stored at −20°C all the analytes are stable for at least a year.

The performance of the method is further demonstrated by the results obtained from a ringtest and the comparison of results obtained by analysis of real-life samples by the VSD and the CRL. This method is a practical analytical tool for quantitative analysis of urine and muscle tissue to detect the presence of RALs and can be used, with the statistical tool described, to discriminate treatment with a zeranol-containing preparation from consumption of zearalenone-contaminated feed [10].

Acknowledgements The financial contribution of the European Commission through the project "Natural Zeranol" (FAIR4-CT-1997-3443) and DG SANCO (CRL for residues) is gratefully acknowledged.

References

1. European Community (1996) Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having hormonal or thyrostatic action and of beta-agonists and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EC. Off J Eur Communities L125:3

2. Jonker M, Egmond H, Stephany R (1999) Mycotoxins in food of animal origin, CRL-document 389002 096. pp 39, December 1999

3. Kleinova M, Zöllner P, Kahlbacher H, Hochsteiner W, Lindner W (2002) Metabolic profiles of the mycotoxin zearalenone and the growth promoter zeranol in urine, liver and muscle of heifers. J Agric Food Chem 50:4769–4776

4. FAIR-CT97-3443. An improved immunoassay for zeranol to distinguish zeranol abuse from natural contamination with Fusarium spp. toxins in food animals

5. van Bennekom EO, Brouwer L, Laurant EHM, Hooijering H, Nielen MWF (2002) Confirmatory analysis method for zeranol, its metabolites and related mycotoxins in urine by liquid chromatography-negative ion electrospray tandem mass spectrometry. Anal Chim Acta 473:151–160

6. Launay FM, Young PB, Sterk SS, Blokland MH, Kennedy DG (2004) Confirmatory assay for zeranol, taleranol and the Fusarium spp. toxins in bovine urine using liquid chromatography–tandem mass spectrometry. Food Addit Contam 21(1):52–62

7. Jodlbauer J, Zöllner J, Lindner W (2000) Determination of zeranol, taleranol, zearalenone, α- and β-zearalenol in urine and tissue by high-performance liquid chromatography–tandem mass spectrometry. Chromatographia 51:681–687

8. Fang X, Chen J, Gua D, Wang G (2002) Detection and Identification of zeranol in chicken or rabbit liver by liquid chromatography–electrospray tandem mass spectrometry. J AOAC Int 85(4):841–847

9. Fiori M, Civitareale C, Mirante C, Magaro S, Brambilla G (2005) Evaluation of two different clean-up steps, to minimise ion suppression phenomena in ion trap liquid chromatography–tandem mass spectrometry for the multi-residue analysis of beta agonists in calves urine. Anal Chim Acta 529:207

10. Launay FM, Ribeiro L, Alves P, Vozikis V, Tsitsamis S, Alfredsson G, Sterk SS, Blokland M, Iitia A, Lovgren T, Tuomola M, Gordon A, Kennedy DG (2004) Prevalence of zeranol, taleranol and Fusarium spp. toxins in urine: implications for the control of zeranol abuse in the European Union. Food Addit Contam 21(9):833–839

11. European Comunity (2002) Commission Decision No. 2002/657/EC of 12 August 2002 implementing Council Directive 96/23 concerning the performance of analytical methods and the interpretation of results. Off J Eur Comm L221:8

12. Miehl ML, Prelusky DB, Koritz GD, Hartin KE, Buck WB, Trenholm HL. Biliary excretion and enterohepatic cycling of zearlenone in immature pigs. Toxicol Appl Pharmacol 121:152–159

13. Meyer K, Usleber E, Martlbauer E, Bauer J (1996) Untersuchungen zum nachweis und vorkommen von zearalenon und alpha-zearalenol in gallen von zuchtsauen mit fertilitatsstorungen, proceedings 18-mykotoxin workshop 960610–960612 kulmbach DE, pp 180–184

14. Stolker AAM, van Ginkel LA. Procedure for the validation of analytical methods, ARO-SOP425

15. Miller JC, Miller JN. Statistics for analytical chemistry (2nd edn) ISBN 0-13-845439-6

16. Launay FM, Young PB, Sterk SS, Blokland MH, Kennedy DG (2004) Confirmatory assay for zeranol, taleranol and the Fusarium spp. toxins in bovine urine using liquid chromatography–tandem mass spectrometry. Food Addit Contam 21(1):52–62