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CONFIRMATORY METHOD OF RESORCYLIC ACID LACTONES IN URINE AND TISSUE BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Abstract

This paper presents a liquid-chromatography negative ion electrospray tandem mass-spectrometry (LC-MS/MS) method to determine resorcylic acid lactones (RALs) in urine, muscle, fish, liver and kidney samples. The method comprises an extraction step followed by the clean-up of the samples in two steps using SPE cartridges: C18 Chromabond and Strata Amino from Phenomenex. The RALs were separated on a Pursuit C18 column. in isocratic mode with acetonitril/ 0.1% formic acid aqueous solution. The time for analysis was 12 minutes. A triple quadrupole mass spectrometric from Varian equipped with a ESI source in the multiple reaction monitoring was used for detection. The method was developed according to EU legislation and the parameters assessed were: selectivity, specificity, linearity, recovery, repeatability, within-laboratory reproducibility, decision limit (CCα), and detection capability (CCβ), ruggedness, control chart, uncertainty of measurement. The validation was performed at 1µg/kg for each compound. CCα and CCβ values were 0.16-0.79 µg/kg and the range of mean recoveries were 72.2-119.7% depending of the compound. The relative standard deviation (RSD) of the measurements was below 20%.The method is used to analyze samples originating from the Romanian Residue Control Monitoring Program or private samples. The main purpose of this method is the detection, quantification and confirmation of 6 RAL's in urine and tissue.

Key words: *acid lactones, LC-MS/MS spectrometry.*

INTRODUCTION

The group of resorcylic acid lactones comprises α-zearalanol- zeranol (α ZAL) and its metabolite β-zearalanol- taleranol (βZAL), zearalanone (ZAN). zearalenone (ZON) α/β zearalenol (α/β ZOL), presented in Figure 1.

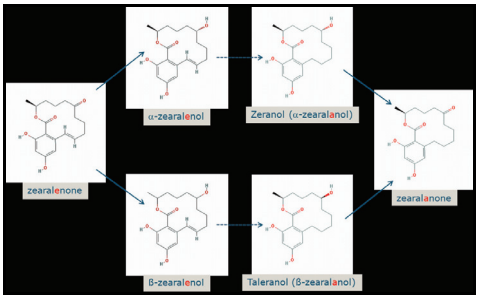


Figure 1 - Zearalenone and its metabolites

Substances with anabolic action are used to increase feed conversion, growth rate or muscle tissue. The EU banned the use of hormones as growth promoter or for fattening. The implantation of Zeranol in calves causes an improvement in mean live weight gain. The use of zeranol is allowed as a growth promoter in livestock in USA and Canada, but it is forbidden in Europe because of its non-steroidal estrogenic properties. RAL's were discovered when it noticed the pigs fed with moldy corn had a higher growth rate. Then, some fungal species were isolated in lab, included Ralgro, the commercial name for Zearanol. This is usually obtained from Zearalenone which is common produced by several species of Fusarium fungi grown in cereals like corn, wheat as Fusarium culmorum and Fusarium graminaerum. The both compounds, ZON and α ZAL give identical metabolites and they are often found in urine from bovine, pigs, ovine and horses. Zearalenone was reported at quantifiable levels in 15% of 20000 samples in grain in a recent EFSA (European Food Safety Authority) opinion. The chronic total dietary exposures to zearalenone ranges 2.4-29 ng/kg body weight/day.Metabolism produces α-

Zearalenol, a metabolite with greater affinity for estrogen receptors than the parent compound, and β- Zearalenol, a metabolite with lower affinity. A liquid chromatography negative ion electrospray tandem mass spectrometry was developed according to EU legislation in order to monitor the presence of RAL's and to know the cause of any illegal treatment with zeranol or natural environment contamination due to moldy feed.

MATERIALS AND METHOD

Reagents and samples

Zearalenol (αZOL), β Zearalenol (βZOL) and Zearalanone (ZAN) were purchased from Sigma-Aldrich, α Zearalanol (αZAL), β Zearalanol (βZAL) and Zearalenone (ZON) were purchased from NIM Australia. Zearalenone d6 (ZONd6) from TRC Canada and α/ β Zearalanol d4 (α/ β ZAL d4) from EURL (European Union Reference Laboratory) Rikilt, the Netherlands were used as internal standards. Stock solutions were prepared by dissolving 10 mg of standards into 10.0 mL of ethanol to obtain 1mg/mL concentration. For zearalenone d6, 1mg was dissolved into 1 ml etanol and it was obtained 1mg/mL concentration. For α/ β Zearalanol d4, 0.1 mg were dissolved into 1 ml etanol. The concentration obtained was 100µg/ml. These solutions were stored at −20 ºC. Intermediate solutions of 10 µg/ml concentration were prepared. For working standards, 250 µL of the intermediate solutions were diluted with ethanol to 25mL into volumetric flasks for a final concentration of 0.1µg/mL for each component. Working standard solutions were stored at 4 ºC. Methyl *tert*-butyl ether (TBME), ethanol, potassium dihydrogen phosphate and beta-glucuronidase from Helix Pomatia were obtained from Sigma-Aldrich, acetonitrile (CAN) and acetone from Merck, methanol and di-sodium hydrogen phosphate from Scharlau and formic acid from Lach:ner. For sample cleaning procedure there were used SPE cartridges: Chromabond C18ec 45 µm 500mg/6ml from Macherey-Nagel and Strata NH2 55 µm 1000mg/6ml from Phenomenex. The samples were collected started with 2011 up to present and were stored at −20 ºC until they were analyzed.

Samples preparation and clean-up Internal standards were added at urine samples and they were hydrolyzed by adding 1ml phosphate buffer 0.1M to 5.0 ml urine. The ph was adjusted at 7.0±0.3 using drops of NaOH 1M or HCl 1M, before adding 0.05 ml Helix Pomatia -β glucuronidase. The mixture was omogenizated by vortex mixing and then incubation at 37 ºC/2h. The samples had been cooled down at room temperature before performing the extraction with 10 ml TBME, vortex- mixing and centrifugation for 10 min/4000RPM. The upper layer was transferred in a glass tube and evaporated at dryness at 60 ºC under a gentle stream of nitrogen. For tissue, was weighted 5.0g well-homogenized sample and the internal standards were added. For extraction, 10 ml CAN were added in the tube, vortex- mixing and centrifugation for 10 min/4000RPM. The upper layer was transferred in a glass tube and evaporated at dryness at 60 ºC under a gentle stream of nitrogen. The residue from urine or tissue was redissolved in 5 ml methanol/water 50/50 v/v by carefully vortex mixing and added 2 ml of water to make the polarity of the solution suitable for the next step (SPE). The SPE column C18 was well-conditioned by passing 5 ml of methanol followed by rinsing with 5 ml water. The sample was passed through the column and washed with 5 ml 40/60 v/v-% methanol/water. The column was dried before the next step by applying a slight vacuum. The RAL's were eluted with 5 ml 80/20 v/v-% methanol/water. The eluate was collected in a test tube by applying a slight vacuum and evaporated at 50 ºC under a gentle stream of nitrogen. The dry residue was redissolved in 5 ml of 80/20 v/v-% acetone/methanol and passed through the SPE amino column which was conditioned before with 5 ml 80/20 v/v-% acetone/methanol. The sample was passed through the column and followed by the collection of the eluate. It was evaporated at 50 ºC under a gentle stream of nitrogen and the residue was redissolved in 100µl, 20% acetonitrile aqueous solution and injecting of 25 µl into LC-MS/MS. The

temperature for column thermostat was: 40 ºC, and the temperature at injection was 5 ºC.

Instrument conditions

The RAL's were separated using a column Varian Pursuit C18 (150mmx3mm, 3µm) equipped with a guard column Metaguard Pursuit 3u C18 4.6mm in isocratic mode 50%(v/v) B-CAN with 0.350 ml/min flow on the column. The mobile phase A was 0.1% formic acid aqueous solution. The analysis time was 12 min. LC–MS system was a Varian Triple Quad equipped with a detector 320MS, a Prostar 410 binary pump LC. Data analyses were performed using MS workstation version 6.9.3. The ion source was in negative ESI mode. Nitrogen gas was used for drying and argon for collision. The MS detector settings were as follows: housing temperature: 50ºC, shield voltage: - 500V, needle voltage: - 4000V, drying gas: 320 ºC, 18psi, nebulising gas : 40 psi

RESULTS AND DISCUSSIONS

According to CRL Guidance Paper 7december/2007 which is the Community Reference Laboratories (CRL's) view on state of the art analytical methods for national residue control plans established in accordance with Council Directive 96/23/EC, for A4 Resorcylic acid lactones and derivates **(**this document is to serve as technical guidance for analytical methods in residue control) the recommended concentration that are presented in Table 1.

Tabel 1 - Recommended concentrations for Zeranol and Zearalanone

Substances	Marker residue-metabolite	Matrix	Recommended concentration
Zeranol	Taleranol	Urine	2 ppb
		Liver	2 ppb
		Muscle	1 ppb
Zearalanone		Urine	2 ppb
		Liver	2 ppb

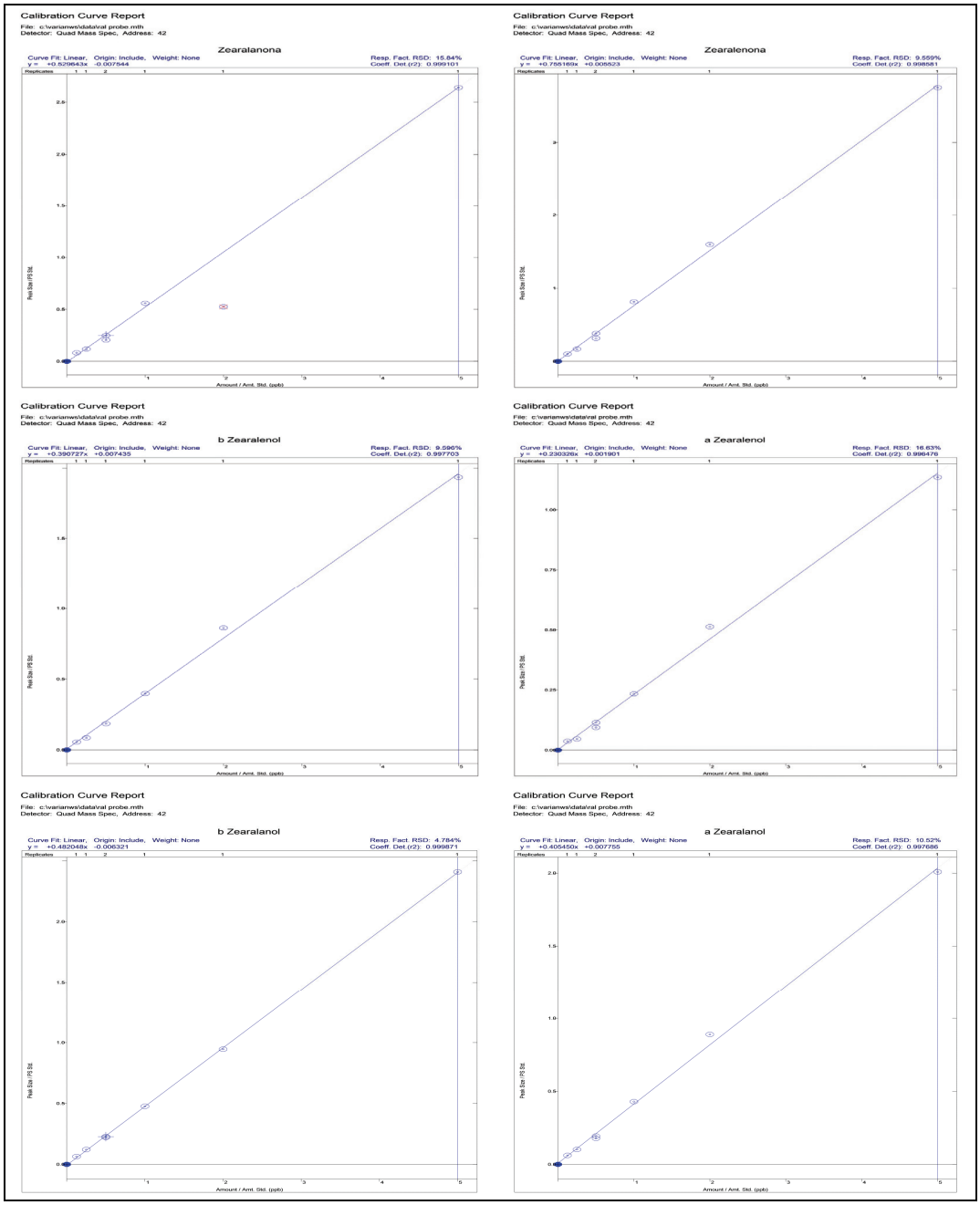
(In case both zeranol and zearalenone are present, the presence of zeranol is considered as the result of mycotoxin contamination). Therefore RAL's are banned substances beside to other anabolic compounds according to 96/22/EC, and the level of validation was chosen at 1ppb for every compound, each matrix. The seven points, including zero, were prepared for quantification of the blank and the spiked samples between 1.25 ng and 50 ng.The mixture of the three internal standards was added at the beginning of the sample preparation at 2ng/ml. The analytical method was validated according to the 2002/657 EC and the parameters assessed were: selectivity, specificity, linearity, recovery, repetability, within-laboratory reproducibility, decision limit (CCα), and detection capability (CCβ), rugedness, control chart, uncertainty of measurement. To demonstrate if the method is suitable, the validation was performed according to 657/2002/EC. It were analyzed many samples from different species to control the interferences and to check the compounds and the internal standards and their behavior in different matrix. The separation of RAL's could be observed without interference in Figure 3. Calibration was based on a standard solution curve. The calibration curves were formed with standard solutions of different concentrations. A seven-point calibration (including zero) was performed at levels 0.25-10ppb. Correlation coefficients were better than 0.98, as can be observed in Figure 2.

Four identification points were obtained using the MRM (Multiple Reaction Monitoring) mode with one precursor ion and two product ions, in negative mode. First, the precursor ions were optimized in Scan mode. The mass spectra of the molecules were recorded and after choosing the optimized fragment there were determined the precursor ions. The collision energies of the ion transitions were optimized and presented in Table 2.

24 blank samples were spiked as follows: 6 at 0.5ppb, 6 at 1ppb, 6 at 1.5ppb, 6 at 2ppb. The data obtained were used to demonstrate the

recovery and the repeatability. Within-laboratory reproductibility was also evaluated by repeating the recovery test and employing the same method on two different days. Within-laboratory reproductibility was calculated for each level. According to the residue legislation, the recovery must be 50–120% for a concentration of 1ppb or lower and the RSD% lower than 20%. These conditions were observed for all RAL's.

Figure 2 - The separation of RAL's



Tabel 2 - The optimized collision energies of the ion transitions

Analyte	CAS	Retention time (min)	MRM I (quantification)	Collision energy (V)	MRM II (confirmation)	Collision energy (V)	Dwell time(S) MRM1/MRM2
ZON	17924-92-4	9.530	317>131	28.5	317>175	23	0.300/0.300
a ZOL	36455-72-8	6.067	319.1>275	18.5	319.1>301	19	0.400 /0.400
b ZOL	71030-11-0	5.025	319.1>275	18.5	319.1>301	19	0.400 /0.400
ZAN	5975-78-0	8.841	319.1>275	18.5	319.1>301	19	0.400 /0.400
a ZAL	26538-44-3	5.805	321.1>277	20.5	321.1>303.0	19	0.300/0.400
b ZAL	42422-68-4	4.878	321.1>277	20.5	321.1>303.0	19	0.300/0.400
ZON d6(SI)	1185236-04-7	9.126	323>131	28			0.100
a ZAL d4(SI)		5.760	325>281	19.5			0.200
b ZAL d4(SI)		4.878	325>281	19.5			0.200

To calculate the decision limit (CCα) and detection capability (CCβ), 8 blank samples were analyzed at: 2x0.5ppb, 2x1ppb, 2x1.5ppb, 2x2ppb. The calculation was done according to ISO 11843 using an EXCEL support. According to the 2002/657 EC, the decision limit of a substance should be lower than MRPL (maximum residue permitted limit) and the detection capability must be lower to MRPL. As shown in Table 3, the results of both the decision limit and the detection capability met the conditions of the 2002/657EC. The sources of uncertainty quantified were: within - laboratory reproductibility, relative standard deviation obtained from calibration curve.

Tabel 3- Asessed performance parameters

No.	Compound/matrix	ccα ug/kg	Ccß ug/kg	r ug/kg	R ug/kg	RSD_r %	RSD_R %	U %	Rec %
1.	Zearalenone/urine	0.29	0.36	0.228	0.287	7.95	10.39	20.83	83.8-114.3
2.	b Zearalenol/urine	0.28	0.35	0.176	0.217	7.11	8.47	17.02	81.5-109.4
3.	a Zearalenol/urine	0.33	0. 40	0.096	0.207	3.24	7.02	16.68	91.5-116.6
4.	Zearalanone/urine	0.24	0.30	0.161	0.385	5.20	13.74	29.74	75.5-119.7
5.	b Zearalanol/urine	0. 16	0. 19	0.181	0.190	5.82	6..32	15.73	95.9-119.7
6.	a Zearalanol/urine	0.21	0.26	0.099	0.190	3.40	6.74	19.98	88.7-111.3
7.	Zearalenone/liver	0.30	0.37	0.239	0.340	9.45	12.24	24.51	72.2-116.8
8.	b Zearalenol/liver	0.37	0. 46	0.147	0.348	6.03	12.90	25.85	80.8-116.6
9.	a Zearalenol/ liver	0.27	0.33	0.079	0.368	3.40	13.86	29.15	81-116.8
10.	Zearalanone/ liver	0. 49	0. 61	0.083	0.471	3.88	18.52	38.75	73.1-115
11.	b Zearalanol/ liver	0. 25	0.31	0.242	0.266	8.84	9.46	21.10	81.7-119.7
12.	a Zearalanol/ liver	0. 24	0. 30	0.306	0.333	11.09	11.55	27.42	80.6-116
13.	Zearalenone/fish	0. 54	0. 67	0.142	0.233	5.47	8.78	17.62	83.4-110.4
14.	b Zearalenol/ fish	0. 64	0. 79	0.169	0.204	7.28	8.43	16.94	76.5-97.6
15.	a Zearalenol/fish	0. 61	0. 76	0.154	0.179	6.13	6..96	16.57	85.9-105.9
16.	Zearalanone/ fish	0. 35	0. 43	0.181	0.208	7.07	8.06	19.73	83.1-110.5
17.	b Zearalanol/ fish	0. 38	0. 48	0.105	0.184	4.20	7.10	17.00	83.9-105.5
18.	a Zearalanol/ fish	0. 33	0. 40	0.122	0.157	4.64	5.78	18.73	86.8-110,6
19.	Zearalenona/kidney	0. 22	0. 27	0.239	0.340	9.45	12.24	24.51	72.2-116.8
20.	b Zearalenol/ kidney	0. 52	0. 65	0.147	0.348	6.03	12.90	25.85	80.8-116.6
21.	a Zearalenol/ kidney	0. 27	0. 33	0.079	0.368	3.40	13.86	29.15	81-116.8
22.	Zeralanone/ kidney	0. 51	0. 63	0.083	0.471	3.88	18.52	38.75	73.1-115
23.	b Zearalanol/ kidney	0. 42	0. 52	0.242	0.266	8.84	9.46	21.10	81.7-119.7
24.	a Zearalanol/ kidney	0. 34	0. 52	0.306	0.333	11.09	11.55	27.42	80.6-116

There were analyzed 5 liver blank samples spiked with the RALs at 1ppb to check if the metod is suitable and robust toward some minor changes. One sample was worked folowing the all steps of the procedure, for 2 samples it changed the methanol percent with

+/-10% at the washing phase at C18 column, and for 2 samples it changed the acetone percent with +/-10% at the washing phase at amino column. It noticed that the deviation standard obtained was lower than the reproductibility standard deviation. In conclusion the method is enough robust to the chosen modifications.

Detection/Identification

The presence of RALs in a sample is confirmed if there are fulfilled the next criteria:

- the ratio of the retention time of the analyte to that of the internal standard, the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ±2.5% for LC;
- the both transitions MRM I and MRM II must be presented;
- for screening (quantification) it uses MRM I.

For confirmation measurements is performed on both MRM I and MRM II.

Both methods can be combined. If a screening result looks non-compliant, MRM II is used for confirmation. The ratio MRM I/II should fulfill 2002/657/EC, described in Table 4.

Table 4 – MRM I/II ratio according to 2002/657/EC

Relative intensity(%)	LC-MS
>50 %	± 20%
from >20 % to 50 %	± 25%
from >10 % to 20 %	± 30%
≤10 %	± 50%

When the two ion traces appeared with the same defined ion ratios and expected retention times, the compound is positively identified. Confirmation of the substance was conducted using a minimum of four identification points. According to 657/2002/EC one precursor ion earned 1 point and each of the both 2 product ions earned 1.5 points which satisfy the condition of the substance identification.

Tabel 5 - The ion ratios in the standard solution and in the samples

COMPOUND	Ion ratios of standard solutions	Criteria 657/2002/EC	Maximum permitted tolerances	Ion ratios of spiked urine samples
ZON	97	± 20 %	77.6-116.4	91.2-103.8
α ZOL	26.8	± 25 %	20.10-33.5	23.3-32.7
β ZOL	27.3	± 25 %	20.48-34.13	23.4-32.7
ZAN	27.4	± 25 %	20.55-34.25	24.4-29.2
α ZAL	26.7	± 25 %	20.03-33.38	23.2-31.8
β ZAL	27.4	± 25 %	20.55-34.25	24.1-32.9

The ion ratios in the standard solution and in the samples during the validation were applied. As shown in Table 5, the ion ratios of each spiked sample fell within the maximum permitted tolerances for positive identifications. MRM chromatograms are shown in Figure 3. There are 2 transitions for compounds and one transition for internal standard.

The internal control of the results

- The suitability of the LC-MS-MS system is checked by autotune conform the manual of the instrument.
- Injection of a standard at 1.25ng. S/N must be >6 for all the compounds. In that case the system is considered as suitable for analysis of all samples
- Injection of a standard at 5ng
- a blank sample spiked at 1ng/ml; the results were introduced in the control chart.

The injection of the extracts into the anlytical instrument were made in the following order: standard blank, standards for calibration curve including a standard at 5ng, reagent blank with internal standard, compliant control sample with internal standard, samples to be quantified including a sample in duplicat, compliant control sample with internal standard, non-compliant control sample spiked at 1ng/ml, standard at 5ng.

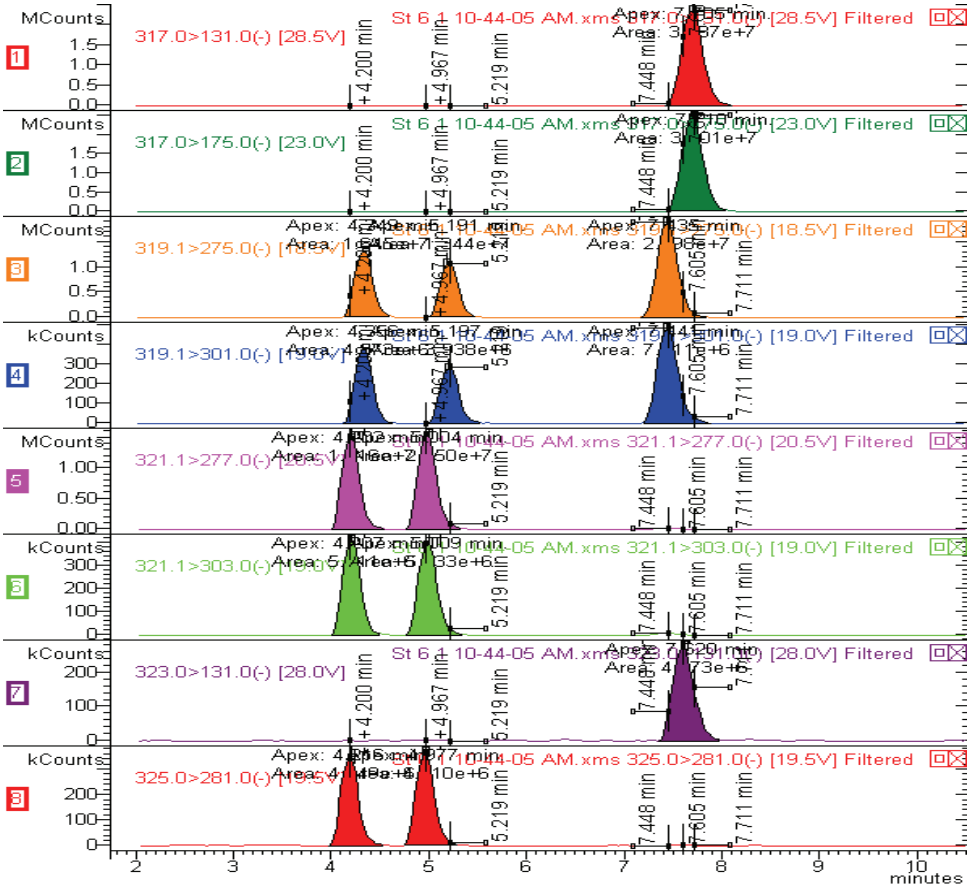


Figure 3 – MRM chromatograms for each compound

Calculation

Areas of the selected ion of the standard and of the internal standard were calculated by the software.

The ratio is a response variable.

Quantitative results are obtained by constructing linear curve fitting using least squares linear regression calculation of the response variable versus the concentration. Unknown concentrations are calculated by interpolation.

According to 2002/657 EC quantification is valid only if:

- In the blank control sample all the internal standards are present (S/N ratio >3 for internal standards).
- In the spiked control sample all components are present (S/N ratio >3 for internal standards and for the non-deuterated compounds).
- In samples all the internal standards are present; for the positive samples, the S/N ratio>3 for the identified analytes

Interpretation

Considering the results interpretation it has been made different approaches by authorities and can be reminded two different opinions made by EFSA and SENASA (National Service for Food Safety and Quality of the Argentine Republic).

To distinguish illegal use of Zeranol from the consumption of *Fusarium spp.* toxin contaminated 8000 samples were analysed by different countries within Europe and there were made different interpretations.

In EFSA's opinion, samples were classified as follows:

- False-positive: Fusarium spp. toxins present, zeranol (or taleranol) absent.

- Equivocal: Fusarium spp. toxins present, zeranol (or taleranol) present.
- True-positive: Fusarium spp. toxins absent, zeranol (or taleranol) present.

In Argentina's case, samples were classified as follows,:

- False-positive: Fusarium spp. toxins present, zeranol (or taleranol) absent.
- Equivocal: Fusarium spp. toxins present, zeranol (or taleranol) present.
  - Ratio (a/ß-zearalanol)/(a/ß-zearalenol +zearalenone)>10 positive
  - Ratio (a/ß-zearalanol)/(a/ß-zearalenol +zearalenone)>1-10 farm
  - Ratio (a/ß-zearalanol)/(a/ß-zearalenol +zearalenone) <1 negative.
- True-positive: Fusarium spp. toxins absent, zeranol (or taleranol) present.

The both criteria give the same results, but at the moment, there is no legal basis for these tools, just screening. It is recommended to do the investigation in farms, also.

Application of the method

About 700 samples, originating from the romanian Residue Control Monitoring Program of 2012 and 2013 or private samples were subjected to the analysis of RAL's using the developed method.

4. CONCLUSIONS

Urine samples were analyzed and RAL's were detected in twenty-three urine samples from horse, pig and bovine.

Zeralenone and its metabolites were detected and confirmated according to the both criteria presented upper, the zeranol abuse was not confirmated and the presence of RAL's in urine sample had a natural environment contamination cause. Also liver samples were investigated and neither zeranol nor taleranol were presented.

The method was developed for the determination of RAL's in urine and tissue. The method was validated successfully, based on the 2002/657 EC. Selectivity, specificity, linearity, recovery, repeatability, within-laboratory reproductibility, decision limit (CCα), and detection capability (CCβ), ruggedness, control chart, uncertainty of measurement were the requirements evaluated. The values obtained during the validation were also in agreement with limits set by the EU.

The method was successfully applied to the proficiency test and to the intercomparison test and it has been accredited by the Romanian National Accreditation Body.

This method was also applied to screening and confirmation of resorcylic acid lactones in urine and tissue.

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