Projectnr.: 71.520.01
Nitroimidazole Interlaboratory Study 03/01

Projectmanager: Drs. J.A. Rhijn

Report 2001. 023 June 2001

Nitroimidazole Interlaboratory Study 03/01

B.J.A. Berendsen and J.A. van Rhijn

Department: Natural Constituents, Residues and Contaminants (NRC)

State Institute for Quality Control of Agricultural Products (RIKILT)
Bornsesteeg 45, 6708 PD Wageningen
P.O. Box 230, 6700 AE Wageningen
Telefoon +31 317 475400
Telefax +31 317 417717

MAILING LIST

INTERNAL:
director
program managers
authors
department marketing and communication
library (3x)
A.H. Roos
KVM

EXTERNAL:
Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BGVV) Berlin (1x)
Laboratorium van de Rijksdienst voor de keuring van Vee en Vlees (LRVV) (1x)

ABSTRACT

In the framework of the nitroimidazole interlaboratory study 03/01, organised by BGVV Berlin in its position as Community Reference Laboratory (CRL), four muscle samples were screened for the presence of dimetridazole (DMZ), ronidazole (RNZ), metronidazole (MNZ) and the hydroxy-metabolite of DMZ and RNZ: 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI).
The screening was carried out with HPLC-UV and resulted in a limit of detection of 0.5 µg/kg for all analytes. Three samples were suspected to contain HMMNI and/or MNZ and DMZ.
After screening, the results were confirmed by LC-MS/MS. The confirmatory analysis was also used for quantification of the amounts present. The results of the screening analyses could be confirmed with the exception of DMZ in sample 2001_521. The confirmatory analysis also indicated the presence of DMZ in a very small amount in this sample, but confirmation could not be achieved according to EU criteria.

SAMENVATTING

Interlaboratorium studie nitroimidazolen 03/01

Rapport 2001.023 Juni 2001

B.J.A. Berendsen en J.A. van Rhijn

Rijks-Kwaliteitsinstituut voor land- en tuinbouwprodukten (RIKILT)
Bornsesteeg 45, 6708 PD Wageningen
Postbus 230, 6700 AE Wageningen

6 bijlagen, 2 referenties

In het kader van de nitroimidazole interlaboratorium studie 03/01, georganiseerd door BGW Berlijn in haar bevoegdheid als Communautair Referentie Laboratorium (CRL), zijn vier vleesmonsters gescreend op de aanwezigheid van dimetridazole (DMZ), ronidazole (RNZ), metronidazole (MNZ) en het hydroxy-metaboliet van DMZ en RNZ: 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI).
De screening is uitgevoerd met behulp van HPLC-UV. Voor alle analyten werd een detectiegrens van 0,5 µg/kg verkregen. De screeningsresultaten wezen uit, dat drie monsters verdacht waren op HMMNI en/of MNZ en DMZ.
De screeningsresultaten zijn bevestigd met behulp van LC-MS/MS. De bevestigingsmethode is tevens gebruikt voor de kwantificering van de gehaltes. De screeningsresultaten konden bevestigd worden met uitzondering van DMZ in monster 2001-521. De bevestiging duidde net als de screening op de aanwezigheid van DMZ in een lage hoeveelheid, maar het signaal van het laagste diagnostische ion was onvoldoende voor bevestiging.

1 INTRODUCTION

In the framework of the nitroimidazole interlaboratory study 03/01, organised by BGVV Berlin in its position of Community Reference Laboratory (CRL), for participation of NRL's, four muscle samples were analysed for the presence of: dimetridazole (DMZ), ronidazole (RNZ), metronidazole (MNZ) and the hydroxy-metabolite of ronidazole and dimetridazole: 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI). Ipronidazole was not included in the method of analysis.
The nitroimidazoles are banned substances. The analytical approach for banned substances, in contrast to registered ones, has to focus on detecting and identifying the analyte at a level as low as possible.
The muscle samples were first screened for the presence of the above mentioned nitroimidazoles. The screening results were used to determine the amount present in the interlaboratory study samples. Subsequently, the suspect samples were re-analysed by LC-MS/MS for confirmation of identity of the analytes and for a more precise determination of the amount present. This report describes the analytical procedures for screening and confirmation and the results of the interlaboratory study are reported.

2 EXPERIMENTAL

2.1 Materials

All solvents and reagents used were of analytical grade or better. All chemicals were obtained from Merck (Darmstadt, Germany). Dimetridazole, ronidazole and metronidazole reference standards were obtained from Sigma (St. Louis, MO, USA). 2-hydroxymethyl-1-methyl-5-nitroimidazole, d_3-dimetridazole (d_3-DMZ) and d_3-ronidazole (d_3-RNZ) were obtained from RIVM (Bilthoven, The Netherlands). The ultrafilters used had a molecular weight cut-off (MWCO) of 30 kDa and were obtained from Millipore (Bedford, MA, USA). Oasis HLB® was used as adsorbens for the concentration column and was obtained from Waters (Milford, MA, USA). An Alltech (Deerfield, IL, USA) Alltima C18 column (L=15 cm, ID=3.2 mm) was used to establish the separation.
Lyophilised porcine muscle samples were received in a frozen condition from BGVV Berlin and were coded: 2001_164, 2001_346, 2001_416 and 2001_521.

2.2 Sample preparation

Blank porcine muscle for the preparation of QC samples, was thoroughly minced and homogenised. An aliquot of 5 g was taken and transferred to a stomacher bag.
An aliquot of 1.3 g of the lyophilised porcine muscle samples corresponding to 5 g of fresh tissue, was transferred to a stomacher bag and was first reconstituted with water, taking into account that the average loss of water was 73%, as specified by BGVV.
Internal standard was added to all samples and QC samples at a level of 1 µg/kg. The samples were thoroughly mixed. After 30 min, 10 ml water was added to the samples in the stomacher bag and the muscle homogenate was extracted in the stomacher apparatus during 3 minutes. The content of the stomacher bag was transferred to a centrifuge tube and centrifuged (15000 g, 15 min). The supernatant was filtered successively over a 5 µm and a 0.45 µm filter and a 30 kDa ultrafilter (UF). The ultrafiltrate was used for the analysis without further purification [1].

2.3 Screening analysis

The screening analysis was carried out with UV detection.
A Gilson (Villiers-le-Bel, France) 232 autosampler equipped with a 401 dilutor was used. A preconcentration column (L=1.0 cm, ID=2 mm) home-packed with Oasis HLB®-material replaced the sample loop. The precolumn was loaded by means of direct transfer of the sample by the dilutor unit. This permits loading of volumes of several millilitres. Exactly one ml of standard or ultrafiltrate was injected on the preconcentration column.
The chromatographic separation was established on an Alltech C18 (L=15 cm, ID=3,2 mm) column using isocratic elution with an eluent consisting of 15% acetonitril in ammonium acetate buffer (10 mM, pH=3,5) at a flow rate of 0,4 ml/min. Detection was carried out with an Applied Biosystems UV-detector, model 785 A (λ=320 nm).

Calibration was carried out using calibrants in matrix at a level of 2.0; 4.0; 8.0 and 15.0 µg/kg (referring to fresh tissue) for all analytes. Those calibrants were prepared by fortifying blank muscle before the clean-up procedure and were treated identical to the unknown samples.

2.4 Confirmatory analysis

The 232 autosampler used for the screening analysis was also used for the confirmatory analysis to facilitate the use of the same procedure of large volume injection and column switching. The chromatographic separation was established on the same Alltech Alltima C18 (L=15 cm, ID=3.2 mm) column, however, now gradient elution was used (Table 1).

Table 1: Gradient used for confirmatory analysis

Time (min)	% 5 mM formic acid	% methanol	Flow rate (ml/min)
0	100	0	0.4
5	10	90	0.4
8	10	90	0.4
9	100	0	0.4
15	100	0	0.4

The column was connected via a 1:1 splitting device to a Micromass (Manchester, UK) Quattro Ultima triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) interface.
The mass spectrometer was operated in multiple reaction monitoring mode (MRM) recording two product ions characteristic for the analytes (Table 2).

Table 2: Instrumental settings and diagnostic ions, the most abundant product ion is underlined

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Collision energy (eV)
DMZ	142	<u>95</u>	0.2	25
		96	0.2	25
d_3-DMZ	145	99	0.2	25
HMMNI	158	<u>140</u>	0.2	15
		55	0.2	15
MNZ	172	<u>128</u>	0.2	20
		82	0.2	20
RNZ	201	<u>140</u>	0.2	17
		55	0.2	17
d_3-RNZ	204	143	0.2	17

The signal of the most abundant product ion was used for quantitative determination, while the other product ion was included for confirmatory purposes. d_3-DMZ was used as an internal standard for the quantification of DMZ. d_3-RNZ was used as internal standard for the quantification of RNZ. For MNZ and HMMNI, d_3-RNZ was chosen as internal standard, because these analytes elute at about the same retention time.

Calibration was carried out using calibrants in matrix at a level of 0.1; 0.5; 1.0; 2.0 and 5.0 µg/kg (referring to fresh tissue) or all analytes. Those calibrants were prepared by fortifying blank muscle and were treated identical to the unknown samples.

3 RESULTS AND DISCUSSION

3.1 Screening analysis

For the screening, a single analysis of the unknown muscle samples was carried out.
Figure 1 presents the relation between concentration and response for the matrix calibrants.
It is clear that there is an almost perfect linear relation (coefficient of correlation ranging from 0,996 to 0,999) between the response factor (Area) and the fortification level. This points out that the analytical procedure is performing satisfactory.

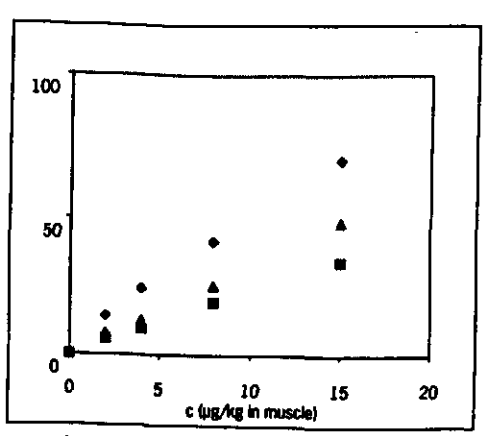


Figure 1: Plot of the response factor of HMMNI+MNZ (♦), RNZ (▼) and DMZ (▲) (UV 320 nm) versus fortification level of blank porcine muscle.

MNZ and HMMNI cannot be distinguished in the screening analysis, because these analytes co-elute under the above mentioned chromatographic conditions. No effort was made to achieve a better separation of these analytes, because, if the sample is suspected to contain HMMNI or MNZ, the identity of the analyte is always confirmed by LC-MS/MS. HMMNI and MNZ can readily be distinguished by LC-MS/MS. A typical chromatogram of the screening analysis of a blank muscle fortified at 2.0 µg/kg is presented in annex I.

The recovery was determined by comparing the fortified muscle samples to the standard aqueous solutions. The average recovery of the analytes in the range of 2 to 15 µg/kg varies from 86 to 108 %.
The limit of detection (LOD) and the limit of quantification (LOQ) can be estimated based on the chromatograms of the matrix-calibrants. The LOD is estimated at 0.5 µg/kg and the LOQ at 1.0 µg/kg for all analytes. The fresh muscle samples used for the preparation of the QC's showed a considerably lower background signal and less interfering peaks than the muscle lyophilates supplied in the framework of this interlaboratory study. Comparison of the chromatograms in annex I with those in annex II clearly illustrates this phenomenon. Consequently, actual detection limits in the lyophilates are slightly higher.

A chromatogram of each of the interlaboratory study samples is presented in annex II.

The results of the screening analysis are presented in Table 3.

Table 3: Screening results for the samples of the interlaboratory study (µg/kg)

Sample	DMZ	RNZ	MNZ / HMMNI
2001_164	2	-	16
2001_346	-	-	-
2001_416	2	-	15
2001_521	1	-	20

It was concluded that samples 2001_164, 2001_416 and 2001_521 are suspected to contain DMZ and either HMMNI or MNZ. RNZ was not found in the samples. Sample 346 was concluded to be negative for the targeted analytes.

3.2 Confirmatory analysis

LC-MS/MS was used for the confirmation of the identity of the analytes and for a more precise quantitative determination of the amounts present. A duplicate analysis was carried out for all the unknown muscle samples.

Figure 2 presents the relation between concentration and relative response for the matrix calibants.
There is a perfect linear relation (coefficient of correlation is 1,000) between the response factor and the fortification level for all analytes. This points out that the confirmatory LC-MS/MS analysis procedure is very suitable for the quantitative determination.

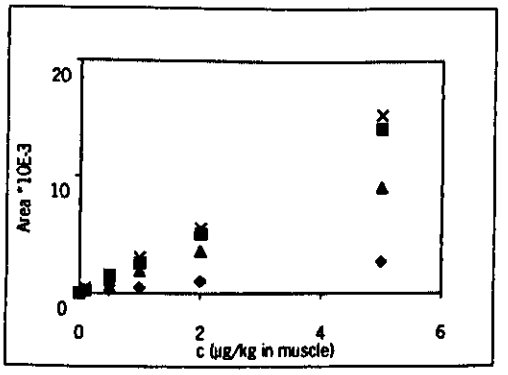


Figure 2: Plot of the response factors of HMMNI (♦), MNZ (v), RNZ (σ) and DMZ (s) (LC-MS/MS) versus fortification level of blank porcine muscle.

A chromatogram of a blank muscle fortified at 1.0 µg/kg with the targeted nitroimidazoles is presented in annex III. The recovery was determined by comparing the blank muscle samples fortified before the UF-clean-up to the blank muscle samples fortified after the UF-clean-up. The recovery of the analytes varied from 96 to 107 %.These results are in close agreement with the results of the screening analysis.
The limit of detection (LOD) and the limit of quantification (LOQ) were estimated from the chromatograms of the matrix calibrants and are presented in table 4.

Table 4: Limit of detection and limit of quantification using MS/MS detection (µg/kg in fresh muscle)

Analyte	LOD	LOQ
HMMNI	0.25	0.5
MNZ	0.1	0.2
RNZ	0.25	0.5
DMZ	0.1	0.2

For RNZ, DMZ and MNZ the most abundant product ion is used for the quantitative analysis to obtain a low LOD. RNZ has a higher LOD and LOQ because the intensity of the most abundant diagnostic ion is not as high as for DMZ and MNZ.
Confirmation was carried out using two product ions. MNZ and DMZ can be confirmed at very low levels (0.2 µg/kg in muscle). RNZ can be confirmed at levels slightly higher than for DMZ and MNZ, because the intensity of the second most abundant ion is less (lower relative abundance) (Annex IV).
For HMMNI, the blank showed an interference in the trace of the most abundant product ion.
This is not surprising since it represents the loss of water from the protonated molecule which is not a very characteristic transition. Unfortunately, however, these small molecules exhibit only few fragments upon CID and, hence, the choice of diagnostic ions is limited.

Therefore, quantification is carried out based on the second-most abundant product ion (m/z=55). A consequence is that confirmation cannot be carried out at the same low levels as for DMZ and MNZ. However, detection is readily achievable at 0.25 µg/kg.
The sensitivity of the confirmatory method (MS/MS) is better than the sensitivity of the screening method (UV) and due to its selectivity, provides cleaner chromatograms with fewer interfering peaks. Therefore and because of the availability of isotope-labelled analogues, the LC-MS/MS data are much better suited for quantification.

One chromatogram of each interlaboratory study sample is presented in annex V.
The results of the quantitative analysis using MS/MS detection are presented in table 5.
The presented values are the average of two separate analyses of the unknown samples. The separate results are quite consistent and are presented in the result forms (Annex VI).

Table 5: Quantitative results for nitroimidazole drugs obtained by LC-MS/MS

Sample	DMZ (µg/kg)	RNZ (µg/kg)	MNZ (µg/kg)	HMMNI (µg/kg)
2001_164	1.3	-	-	17.7
2001_346	-	-	-	-
2001_416	1.3	-	-	17.0
2001_521	0.1	-	28.7	-

The relative abundance of the diagnostic ions was found to be identical to the corresponding abundance in the standards. The average relative abundance of the diagnostic ions of the matrix calibrants is presented in table 6. The spectra of the four analytes, obtained in standard solutions, are presented in annex IV.

Table 6: Average (n=5) relative abundance of the diagnostic ions of the targeted analytes.

Analyte	Relative abundance (%)	RSD (%)
HMMNI	10.4	*
MNZ	68.2	9.8
RNZ	10.4	19.1
DMZ	63.7	9.6

* not determined because of interfering compound in one of the ion traces

Experiments indicate that the relative abundance of HMMNI depends on the amount present in the sample at low concentrations (<2.0 µg/kg). This is a result of the presence of the interference in the trace of the most abundant product ion. Confirmation below 2.0 µg/kg is therefore troublesome. The average relative abundance for amounts of HMMNI greater than 2 µg/kg in muscle is presented in table 6.
For RNZ a low abundance of one of the diagnostic ions was obtained and consequently a higher RSD was recorded. For confirmation of low amounts of RNZ, this may pose a problem.

Confirmation was carried out in accordance with the EU criteria [2].
The results of the confirmatory analysis of the interlaboratory study samples are presented in table 7.

Table 7: Confirmatory results of nitroimidazole drugs in lyophilised porcine muscle

Sample	Analyte	Screening result (µg/kg)	MS/MS result (µg/kg)	Relative abundance (r.a.)	Deviation from average r.a.	Maximum allowed dif. of r.a. [2]	Confirmation
2001_164	HMMNI	16	17.7	12.1 %	16.3 %	30 %	POS
2001_164	DMZ	2	1.3	54.9 %	13.8 %	20 %	POS
2001_416	HMMNI	15	17.0	10.6 %	1.9 %	30 %	POS
2001_416	DMZ	2	1.2	52.2 %	18.1 %	20 %	POS
2001_521	MNZ	20	28.7	69.3 %	1.6 %	20 %	POS
2001_521	DMZ	1	0.1	0			NEG

Table 7 shows that the identity of all the analytes found in the screening could be confirmed, except for DMZ in sample 2001_521. The suspected presence of DMZ in this sample at LOD level was also demonstrated by LC-MS/MS, but the signal of the second-most abundant product ion (m/z=95) was lower than S/N=3. Therefore DMZ could not be confirmed in this sample.
In accordance with the results of the screening analysis, the confirmatory analysis indicated that sample 2001_346 was negative for the targeted nitroimidazoles.
The amount of the analytes present in the unknown samples determined by LC-UV compare quite well with the amounts determined by LC-MS/MS. Both are reported in the result forms (Annex VI).

4 CONCLUSION

Four samples of lyophilised muscle were analysed for the presence of nitroimidazoles in the framework of an interlaboratory study organised by the BGW, Berlin.
The screening indicated the presence of HMMNI and/or MNZ and DMZ in three of the four samples. The screening results were confirmed by LC-MS/MS according to EU criteria [2]. HMMNI and MNZ could be readily distinguished in the confirmatory analysis. The amounts present determined during the screening analysis compared well with the amount determined by the confirmatory analysis.

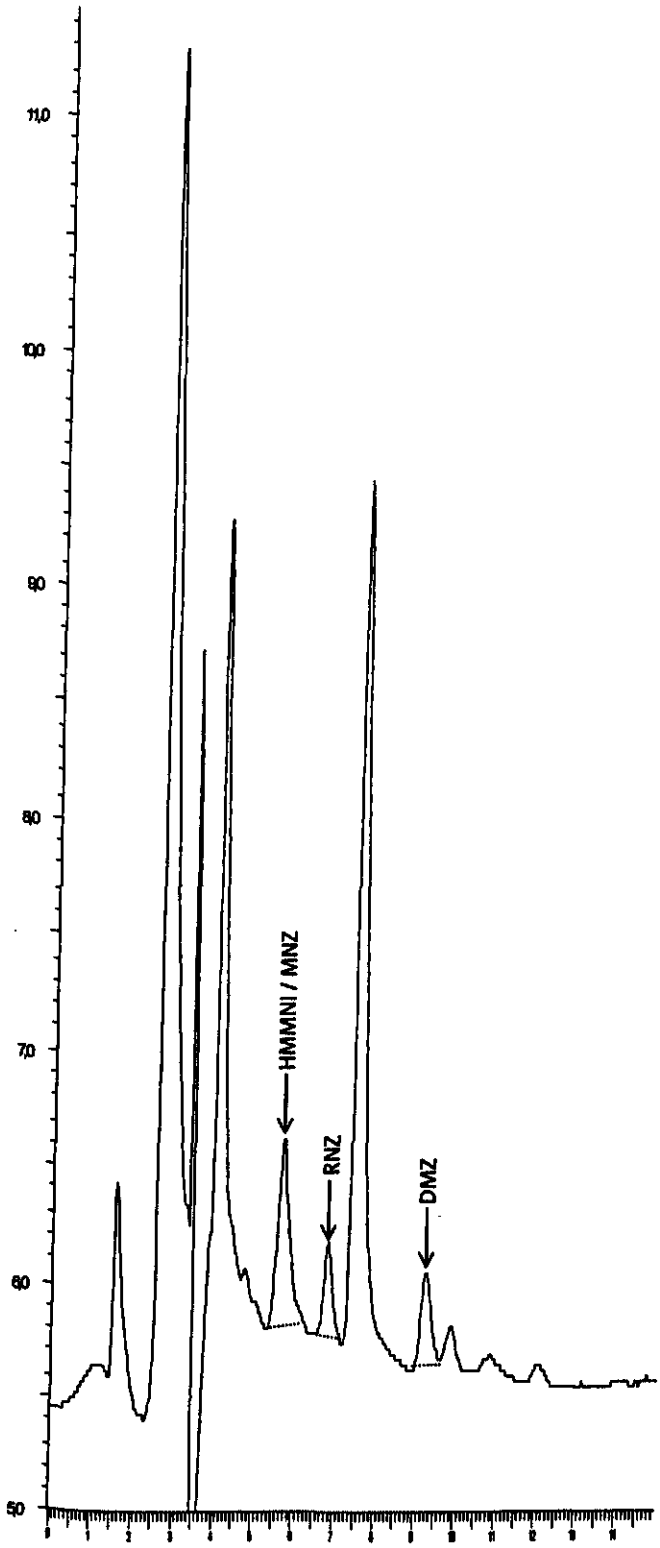
LITERATURE

[1] J.A. van Rhijn, B.J.A. Berendsen, J.J.P. Lasaroms and H.J. Keukens, *Confirmatory analysis of residues of dimetridazole in muscle by LC-MS. In:* Proceedings of the Euroresidue IV conference (Edited by: L.A> van Ginkel and A. Ruiter), **2**, (2000), 913-919.

[2] Final Draft Version of the Revision of EC Directive 93/256/EC, SANCO/1805/2000, version 1, December, 12, 2000.

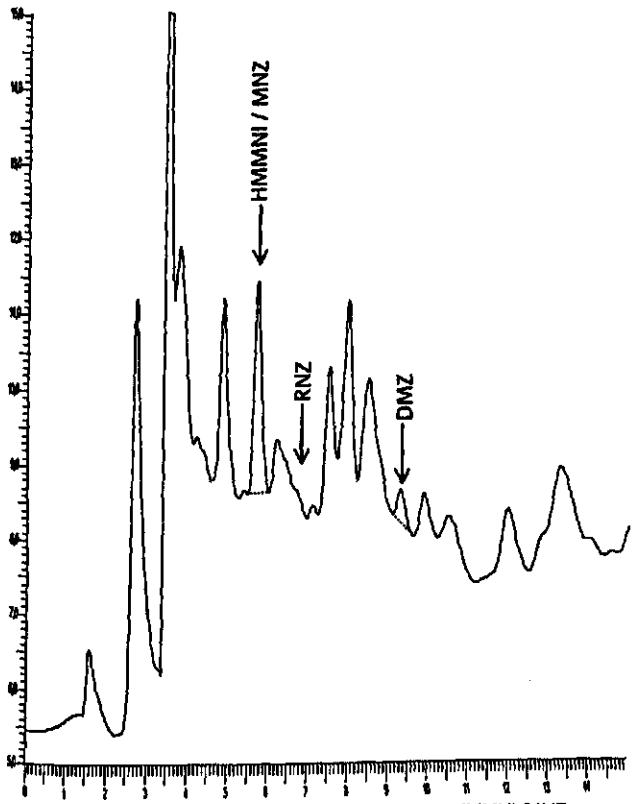
Annex I: Screening analysis

Chromatogram of a blank muscle fortified at 2.0 µg/kg

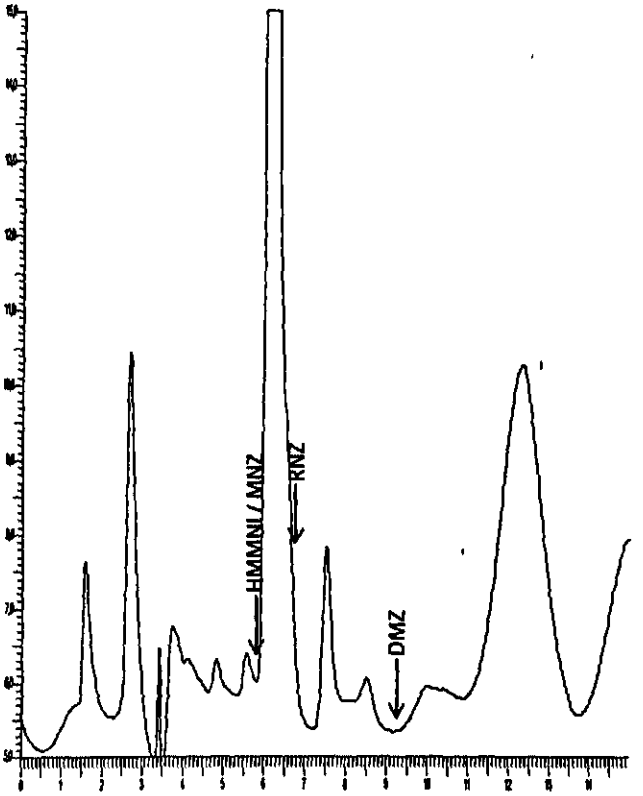


Annex II: Screening analysis

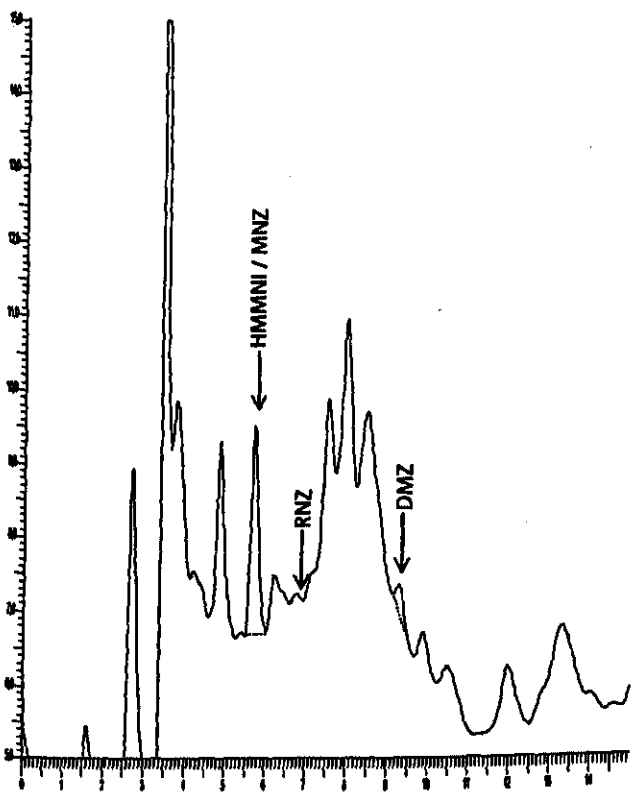
Chromatograms of interlaboratory study samples



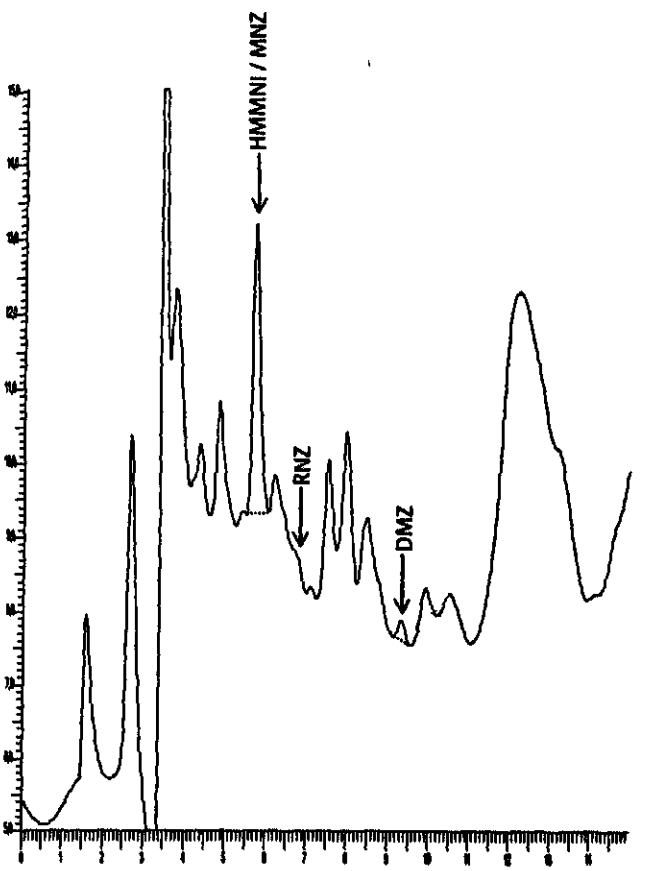
Sample 2001_164, 2 µg/kg DMZ, 16 µg/kg HMMNI/MNZ



Sample 2001_346, no nitroimidazoles detected



Sample 2001_416, 2 µg/kg DMZ, 16 µg/kg HMMNI/MNZ

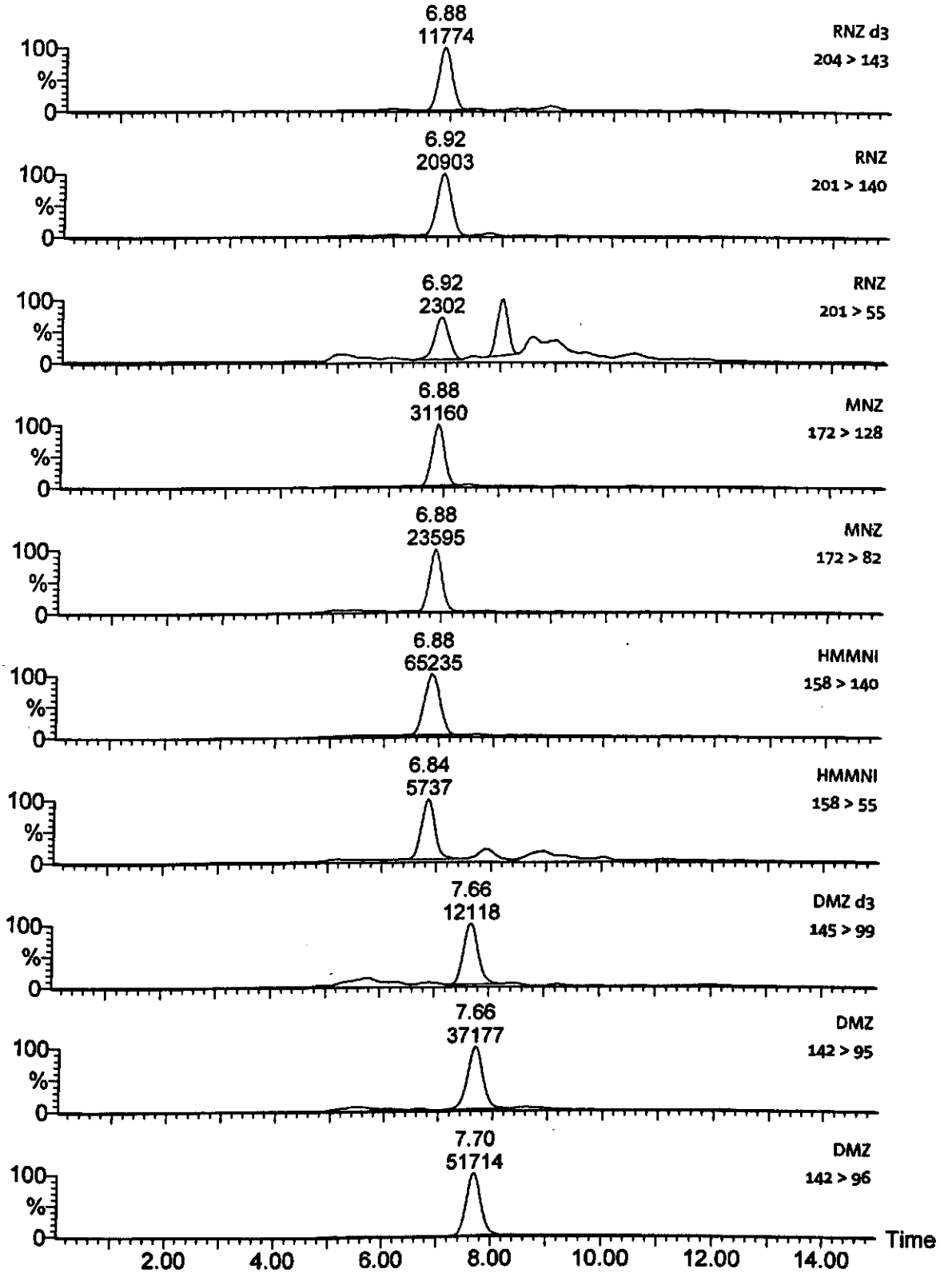


Sample 2001_521, 1 µg/kg DMZ, 20 µg/kg HMMNI/MNZ

Annex III: Confirmatory analysis

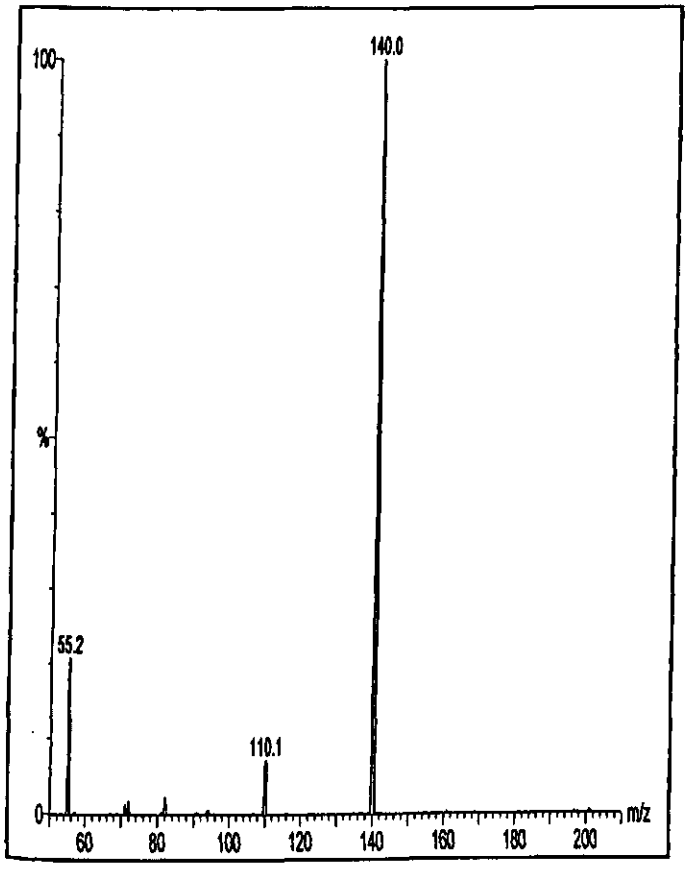
Chromatogram blank muscle fortified at 1.0 µg/kg

Blank muscle fortified at 1.0 µg/kg

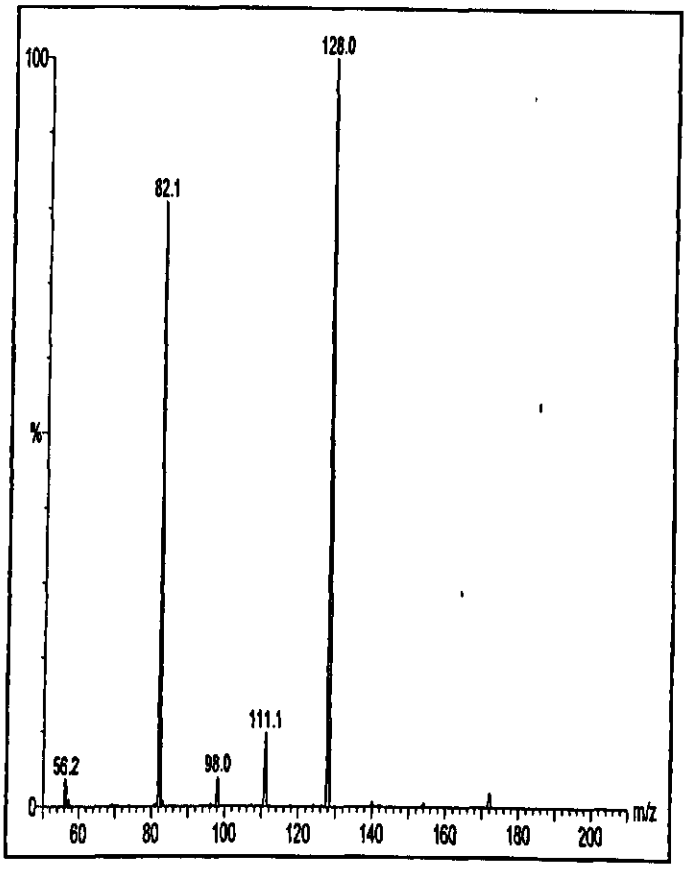


Annex IV: Confirmatory analysis

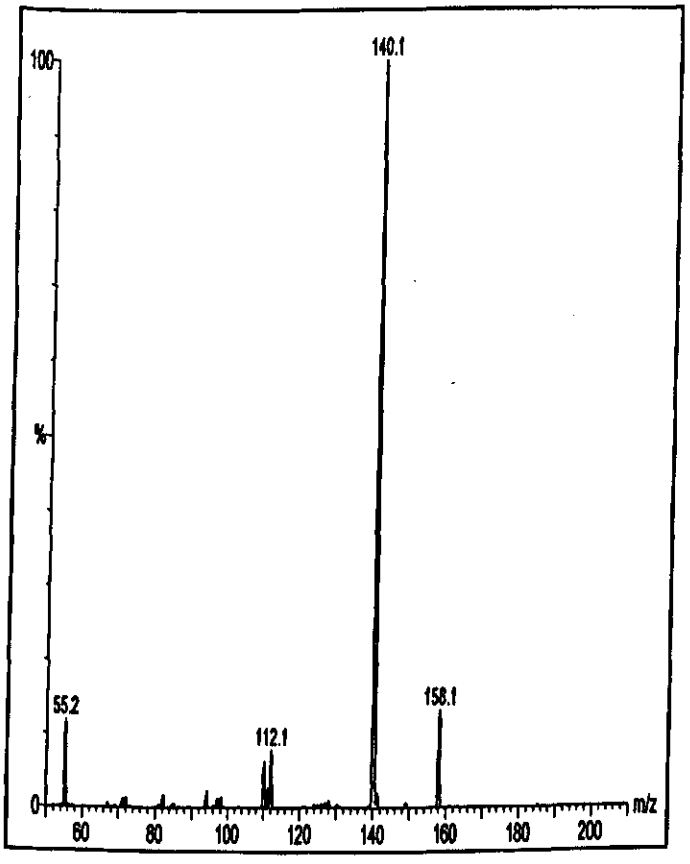
CID Spectra of targeted nitroimidazoles



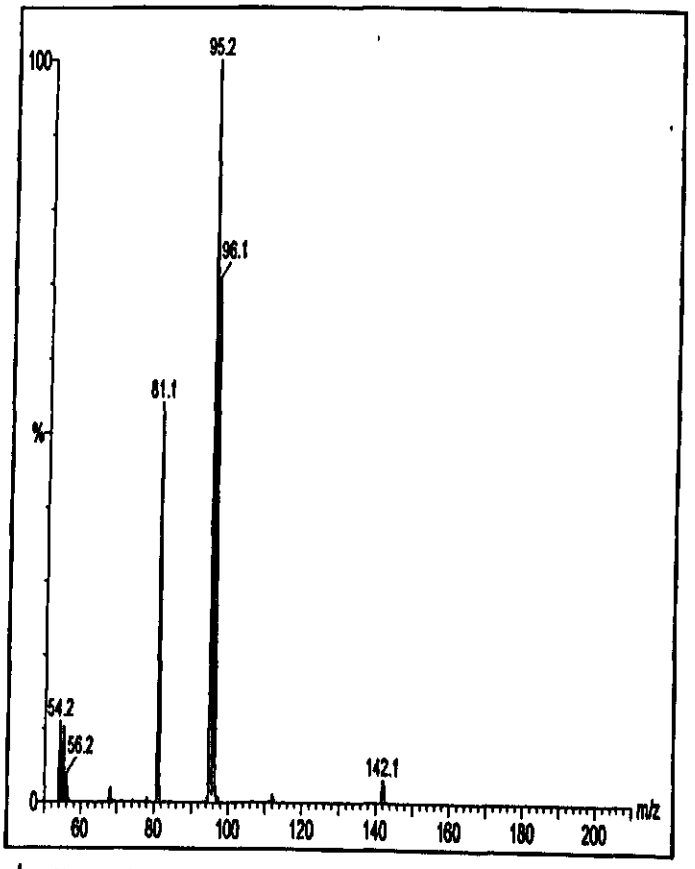
a: ronidazole, precursor: m/z=201



b: metronidazole, precursor: m/z=172



c: 2-hydroxymethyl-1-methyl-5-nitroimidazole, precursor: m/z=158

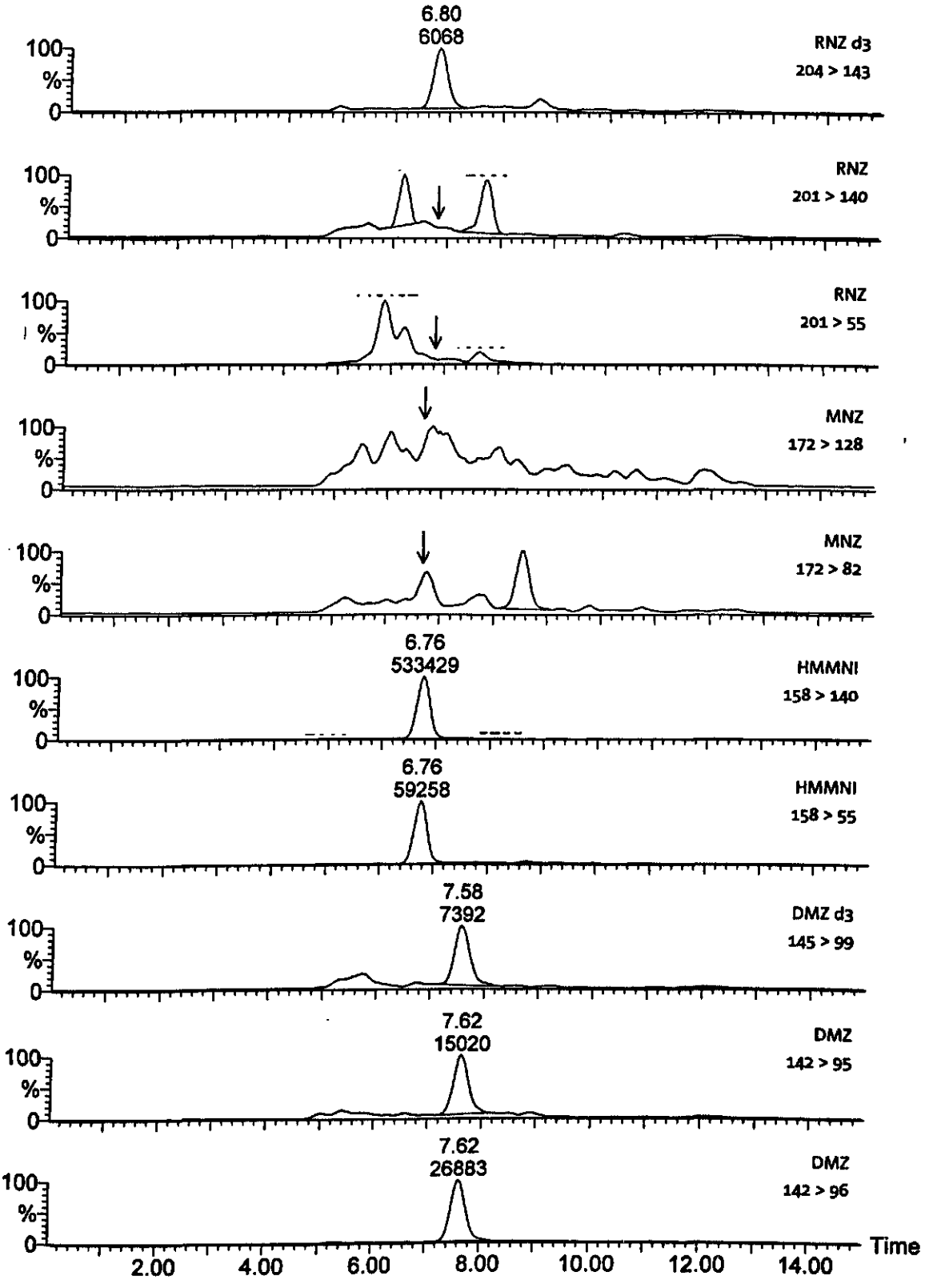


d: **dimetridazole**, precursor: m/z=142

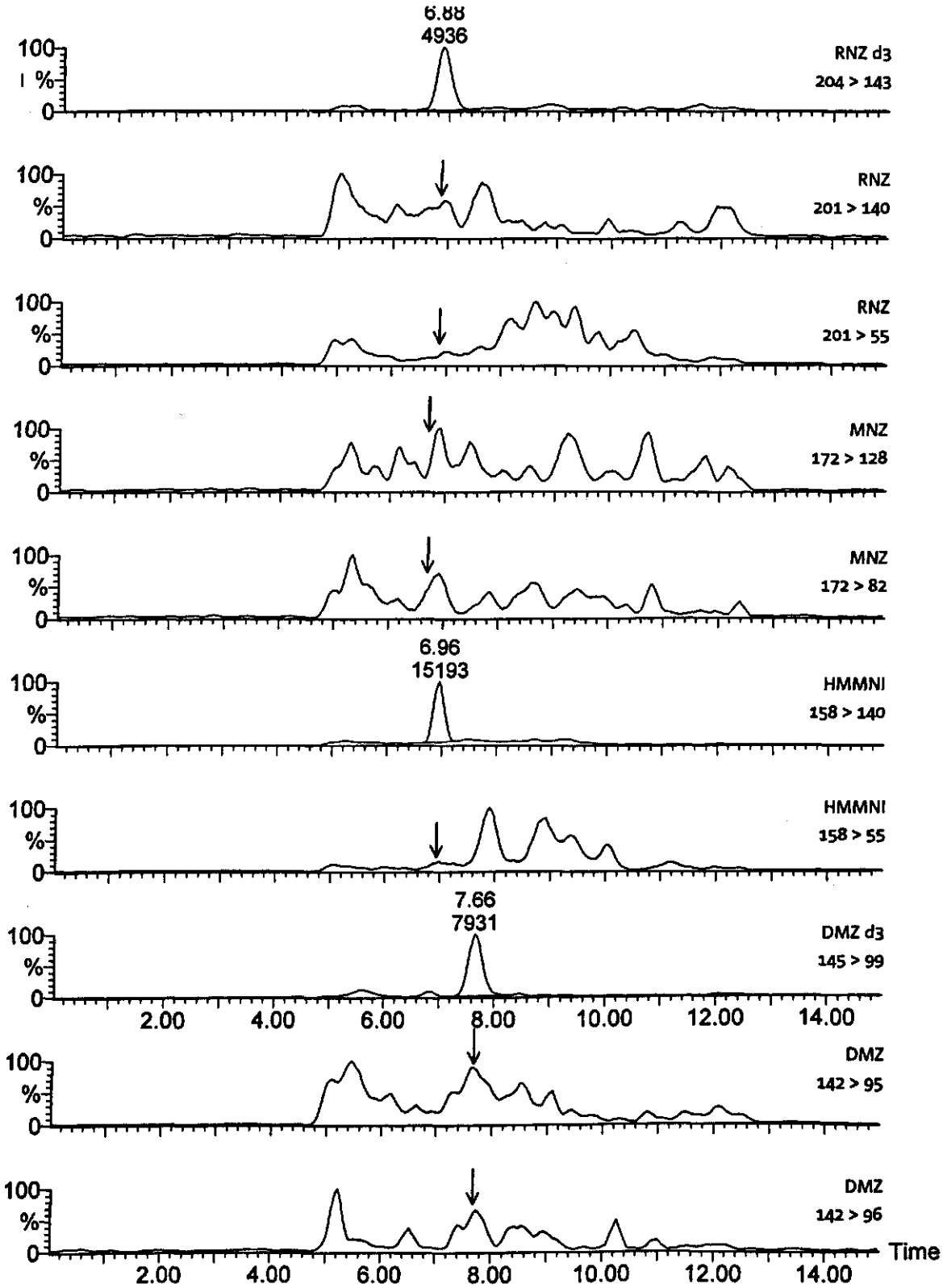
Annex V: Confirmatory analysis

Chromatograms of interlaboratory study samples

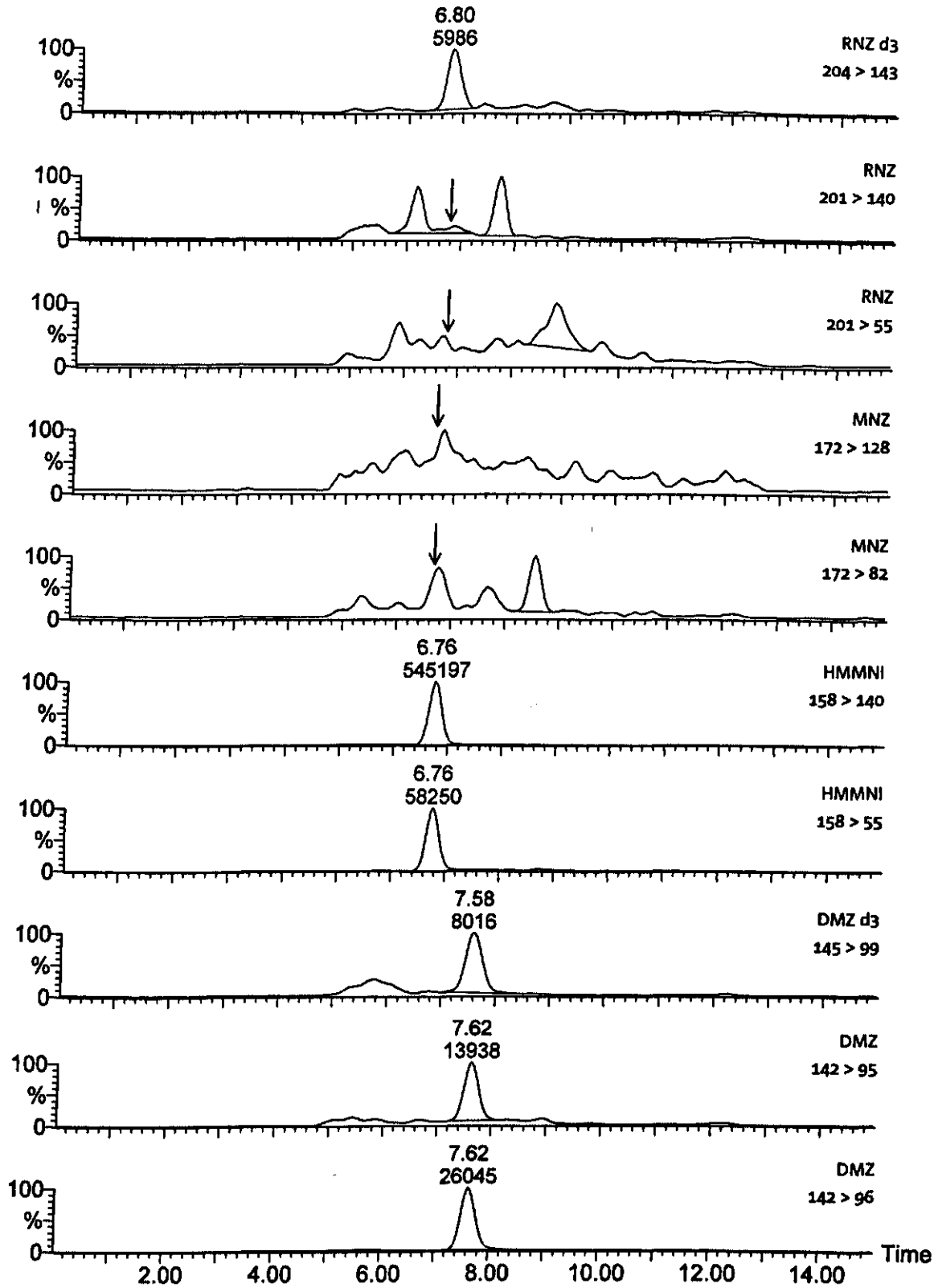
a. sample 2001_164, 1.3 µg/kg DMZ, 17.7 µg/kg HMMNI



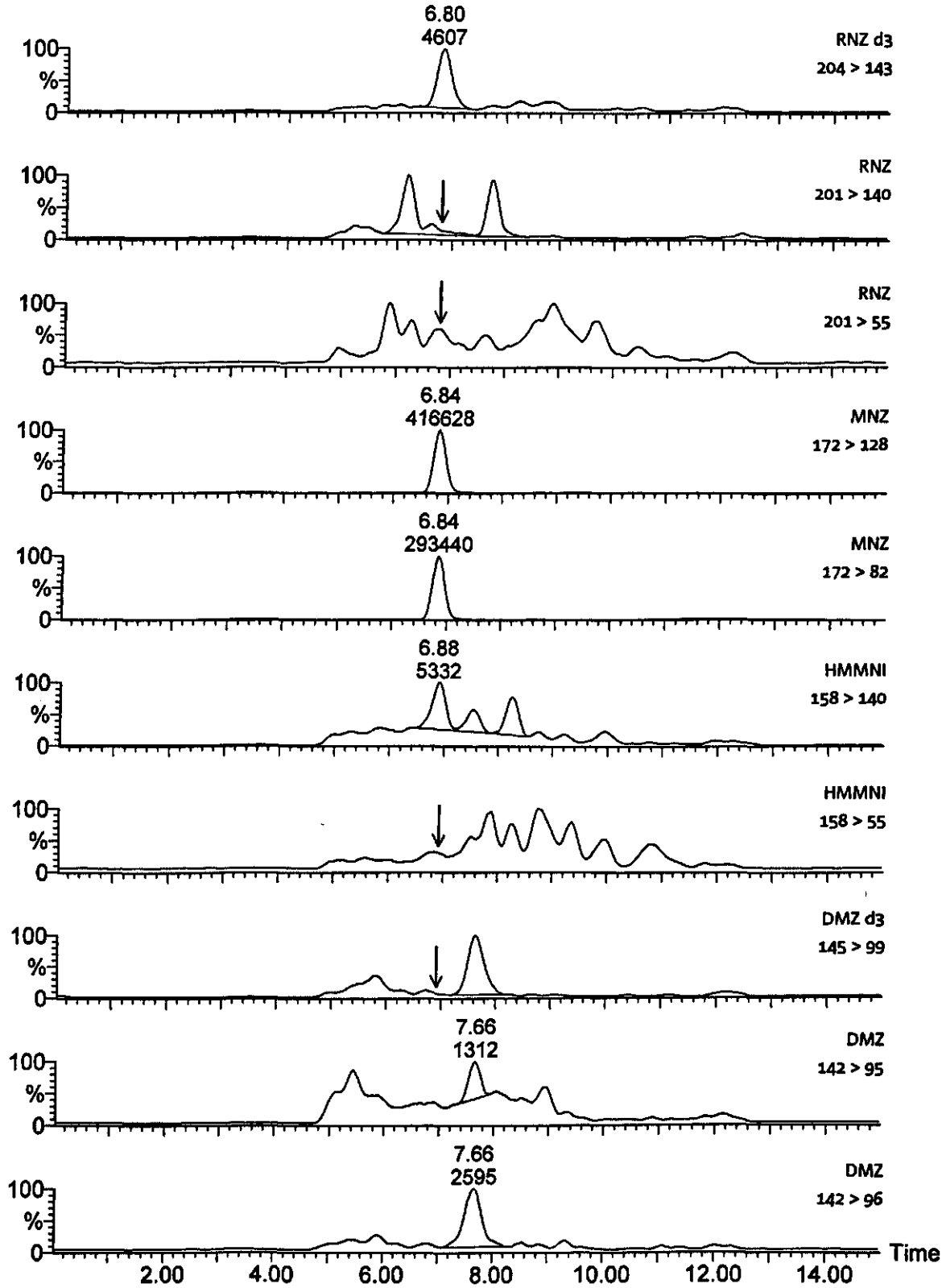
b. sample 2001_346, no nitroimidazoles detected



c. sample 2001_416, 1.2 µg/kg DMZ, 17.0 µg/kg HMMNI



d. sample 2001_521, 0.1 µg/kg DMZ (not confirmed), 28.7 µg/kg MNZ



Annex VI: result forms BGVV

1 METHOD DESCRIPTION	SCREENING ANALYSIS	
2	RESULT FORM	Sample number 2001_164
3	RESULT FORM	Sample number 2001_346
4	RESULT FORM	Sample number 2001_416
5	RESULT FORM	Sample number 2001_521
6 METHOD DESCRIPTION	CONFIRMATORY ANALYSIS	
7	RESULT FORM	Sample number 2001_164
8	RESULT FORM	Sample number 2001_346
9	RESULT FORM	Sample number 2001_416
10	RESULT FORM	Sample number 2001_521
11 QUALITY ASSURANCE MEASURES	CONFIRMATORY ANALYSIS	

Participant : RIKILT

Lab.-Code : 27

METHOD DESCRIPTION

SCREENING ANALYSIS

Short description of the screening method / equipment

Pre-treatment of the sample: Hydrolysis yes ☐ no ☒
how ?

Clean-up: liquid/liquid ☐ solvent:
SPE ☐ phase: solvent:
other: Extraction (water), ultrafiltration, in-line SPE (Oasis HLB®)

Derivatisation: yes ☐ no ☒
if yes, reagent:

Measurement method: GC ☐
HPLC ☒
TLC ☐
other:

Detection method: MS ☐
DAD ☐
UV ☒
other:

Reference:

Which nitroimidazoles can be detected with this screening method? What are the limits of detection for these nitroimidazoles in this matrix ?

Nitroimidazoles	limit of detection* [µg/kg]
2-Hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI)	0.5
Metronidazole (MNZ)	0.5
Ronidazole (RNZ)	0.5
Dimetridazole (DMZ)	0.5

* fresh tissue

Remarks: HMMNI and MNZ are co-eluting analytes. Therefore, the screening analysis does not distinguish MNZ and HMMNI.

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_164**

RESULT FORM

SCREENING ANALYSIS

The screening results of the above mentioned sample were positive for the following nitroimidazoles:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values***):

Detected Nitroimidazoles	**Nature of the screening results**			**Concentration*** [µg/kg]
	estimated	semi-quantitative	quantitative	
HMMNI / MNZ	☐	☐	X ☐	16 [1]
DMZ	☐	☐	X ☐	2
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	

* fresh tissue

☐ No nitroimidazoles were detected in the above mentioned sample.

What quality assurance measures were taken to avoid false negative screening results?

A blank muscle sample was extracted and analysed under similar conditions to the unknown samples. Blank muscle samples were fortified at four different levels in the range of 2 to 15 µg/kg.

Remarks:
1) The screening method cannot distinguish between HMMNI and MNZ.

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_346**

RESULT FORM

SCREENING ANALYSIS

The screening results of the above mentioned sample were positive for the following nitroimidazoles:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values***):

Detected Nitroimidazoles	**Nature of the screening results**			**Concentration*** [µg/kg]
	estimated	Semi-quantitative	quantitative	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	

* fresh tissue

☒ No nitroimidazoles were detected in the above mentioned sample.

What quality assurance measures were taken to avoid false negative screening results?

A blank muscle sample was extracted and analysed under similar conditions to the unknown samples. Blank muscle samples were fortified at four different levels in the range of 2 to 15 µg/kg.

Remarks:

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_416**

RESULT FORM

SCREENING ANALYSIS

The screening results of the above mentioned sample were positive for the following nitroimidazoles:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values***):

Detected Nitroimidazoles	**Nature of the screening results**			**Concentration*** (µg/kg)
	estimated	semi-quantitative	quantitative	
HMMNI / MNZ	☐	☐	X	15[1)]
DMZ	☐	☐	X	2
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	

* fresh tissue

☐ No nitroimidazoles were detected in the above mentioned sample.

What quality assurance measures were taken to avoid false negative screening results?

A blank muscle sample was extracted and analysed under similar conditions to the unknown samples. Blank muscle samples were fortified at four different levels in the range of 2 to 15 µg/kg.

Remarks:
1) The screening method cannot distinguish between HMMNI and MNZ.

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_521**

RESULT FORM

SCREENING ANALYSIS

The screening results of the above mentioned sample were positive for the following nitroimidazoles:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values***!):

Detected Nitroimidazoles	**Nature of the screening results**			**Concentration*** [µg/kg]
	estimated	semi-quantitative	quantitative	
HMMNI / MNZ	☐	☐	X ☐	20[1]
DMZ	☐	☐	X ☐	1
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	
	☐	☐	☐	

* fresh tissue

☐ No nitroimidazoles were detected in the above mentioned sample.

What quality assurance measures were taken to avoid false negative screening results?

A blank muscle sample was extracted and analysed under similar conditions to the unknown samples. Blank muscle samples were fortified at four different levels in the range of 2 to 15 µg/kg.

Remarks:
1) The screening method cannot distinguish between HMMNI and MNZ.

Participant	**: RIKILT**
Lab.-Code	**: 27**

METHOD DESCRIPTION

CONFIRMATORY ANALYSIS

Short description of the confirmatory method / equipment

Pre-treatment of the sample: Hydrolysis yes ☐ no ☒
how ?

Clean-up: liquid/liquid ☐ solvent:
SPE ☐ phase: solvent:
other: Extraction (water), ultrafiltration, in-line SPE (Oasis HLB®)

Derivatisation: yes ☐ no ☒
if yes, reagent:

Measurement method: GC ☐
HPLC ☒
TLC ☐
other:

Detection method: MS ☒
DAD ☐
UV ☐
other:

Reference:

Which nitroimidazoles are detected by this confirmatory method ? What are the limits of detection, limits of determination and recovery rates for these nitroimidazoles in this matrix?

Nitroimidazoles	limit of detection* (µg/kg)	limit of determination* (µg/kg)	recovery rate (%)
HMMNI	0.25	0.5	95
MNZ	0.1	0.2	104
RNZ	0.25	0.5	107
DMZ	0.1	0.2	95

*fresh tissue

Remarks:

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_164**

RESULT FORM

CONFIRMATORY ANALYSIS

The following nitroimidazoles were confirmed by the described method in the above mentioned sample:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values***):

Confirmed nitroimidazoles	Number of parallel analyses	Concentration* [µg/kg]
HMMNI	2	17.7
		17.7
DMZ	2	1.3
		1.3

* fresh tissue

Recovery correction was carried out: [1] yes ☒ no ☐
Note: The results are taken over into the evaluation without any further correction !

☐ The presence of nitroimidazoles could not be confirmed in the above mentioned sample.

Remarks:
[1] Recovery correction was intrinsically made by applying isotope dilution. No further correction was made.

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_346**

RESULT FORM

CONFIRMATORY ANALYSIS

The following nitroimidazoles were confirmed by the described method in the above mentioned sample:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values***):

Confirmed nitroimidazoles	Number of parallel analyses	Concentration* [µg/kg]

* fresh tissue

Recovery correction was carried out: yes ☐ no ☐
Note: The results are taken over into the evaluation without any further correction !

☒ The presence of nitroimidazoles could not be confirmed in the above mentioned sample.

Remarks:

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_416**

RESULT FORM

CONFIRMATORY ANALYSIS

The following nitroimidazoles were confirmed by the described method in the above mentioned sample:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values!***):

Confirmed nitroimidazoles	Number of parallel analyses	Concentration* [µg/kg]
HMMNI	2	17.2
		15.7
DMZ	2	1.1
		1.2

* fresh tissue

Recovery correction was carried out: [1] yes ☒ no ☐
Note: The results are taken over into the evaluation without any further correction!

☐ The presence of nitroimidazoles could not be confirmed in the above mentioned sample.

Remarks:
[1] Recovery correction was intrinsically made by applying isotope dilution. No further correction was made.

Participant	**: RIKILT**
Lab.-Code	**: 27**
Sample number	**: 2001_521**

RESULT FORM

CONFIRMATORY ANALYSIS

The following nitroimidazoles were confirmed by the described method in the above mentioned sample:
(*To remind you again, only one sample preparation per sample was requested. If parallel analyses were carried out, please report* ***every individual result*** *and* ***no mean values***!):

Confirmed nitroimidazoles	Number of parallel analyses	Concentration* [µg/kg]
MNZ	2	26.0
		31.4

* fresh tissue

Recovery correction was carried out: [1] yes ☒ no ☐
Note: The results are taken over into the evaluation without any further correction !

☐ The presence of nitroimidazoles could not be confirmed in the above mentioned sample.

Remarks: DMZ was detected during the confirmatory analysis. The amount of DMZ was quantified at 0,1 µg/kg in muscle. The presence of DMZ could not be confirmed in accordance with EU regulations.

[1] Recovery correction was intrinsically made by applying isotope dilution. No further correction was made.

Participant	**: RIKILT**
Lab.-Code	**: 27**

QUALITY ASSURANCE MEASURES

CONFIRMATORY ANALYSIS

Which criteria were applied to identify the nitroimidazoles?
The used method complies with EU regulations concerning confirmatory analysis of banned substances: Draft revision of Commission Decision 93/256/EC, May 1999.

Which quality assurance measures were taken to avoid false positive and false negative results?
A blank muscle sample was extracted and analysed under similar conditions to the unknown samples. Blank muscle samples were fortified at five different levels in the range 1 to 5 µg/kg.

Which quality assurance measures were taken to ensure that the analytical system was under statistical control?
A simple test is performed every week, to make sure that the analytical system is working properly.

Please fill in the following table and indicate all nitroimidazoles confirmed and quantified in the samples:

confirmed nitroimidazoles	Number of Calibration points	concentration range of the calibration [µg/kg]	correlation coefficient
DMZ	5	0.1-5	0.999
MNZ	5	0.1-5	0.999
HMMNI	4	0.5-5	0.999

Remarks: