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► **To cite this version:**

Gabrijela Tavcar-Kalcher, Katarina Vrtac, Anton Vengust. Validation of the procedure for the determination of maduramicin in concentrates, premixes, and feeds by liquid chromatography. *Food Additives and Contaminants*, 2009, 26 (01), pp.63-67. 10.1080/02652030802225973 . hal-00577404

HAL Id: hal-00577404

<https://hal.science/hal-00577404>

Submitted on 17 Mar 2011

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Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2008-052.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	15-May-2008
Complete List of Authors:	Tavcar-Kalcher, Gabrijela; University in Ljubljana, Veterinary Faculty-National Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene Vrtac, Katarina; University in Ljubljana, Veterinary Faculty-National Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene Vengust, Anton; University in Ljubljana, Veterinary Faculty-National Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene
Methods/Techniques:	Chromatography - HPLC, Extraction, Health significance, In-house validation
Additives/Contaminants:	Feeding, Veterinary drug residues
Food Types:	Animal feedingstuffs

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Validation of the procedure for the determination of maduramicin in concentrates, premixes, and feeds by liquid chromatography

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Abstract

A single laboratory validation was carried out for the determination of maduramicin in concentrates, premixes, and feed. The method comprised of sample extraction of maduramicin, derivatisation with dansylhydrazine and liquid chromatography with UV detection. The limit of detection (LOD) and limit of quantification (LOQ) were 0.4 mg kg⁻¹ and 1.0 mg kg⁻¹, respectively. The repeatability expressed as the average difference between results of duplicate measurements was 5.9 % at the concentration level of 1 % (concentrate), 7.1 % at the concentration level of 1 g kg⁻¹ (premix), and 11 % with the feed containing maduramicin with the nominal concentration of 5 mg kg⁻¹ and feed spiked at the concentration level of 1 mg kg⁻¹. The relative standard deviation for the within-laboratory reproducibility (RSD_w) was 9.2 %, 16 %, 18 %, and 17 % at the concentration levels of 1 %, 1 g kg⁻¹, 5 mg kg⁻¹, and 1 mg kg⁻¹, respectively. The measurement uncertainties were ±0.2 %, ±0.3 g kg⁻¹, ±1.9 mg kg⁻¹, and ±0.3 mg kg⁻¹ at the same concentration levels, respectively.

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4 **Keywords:** *Maduramicin; Concentrate; Premix; Feeds; Liquid chromatography; In-house*
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6 *validation*
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13 **Introduction**

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16 Maduramicin is a carboxylic acid ionophore for the treatment of chicken coccidiosis. It is
17 registered in EU as a feed additive for broiler and turkey feeds. It is contained in feeds at the
18 concentration level of 5 mg kg⁻¹. Premixes and concentrates marketed in Slovenia contain 1 g
19 kg⁻¹ and 1 % of maduramicin, respectively. To control the presence of maduramicin in
20 concentrates, premixes, and feeds, analytical methods with performance characteristics
21 complying with requirements of European regulation (European Parliament and Council,
22 2004) are needed. De Jong et al. (2004), described two possibilities for the determination of
23 maduramicin with liquid chromatography exist. The first method (Gliddon et al. 1988,
24 Markantonatos 1988) includes pre-column derivatisation with dansylhydrazine and
25 fluorescence detection and the second method (De Jong et al. 2004) includes post-column
26 derivatisation and the detection at 520 nm. While De Jong et al. (2004) carried out the
27 validation of the later one, in our laboratory, the procedure for the determination of
28 maduramicin in concentrates, premixes, and feeds based on the methods of Gliddon et al.
29 (1988) and Markantonatos (1988) was introduced and validated. An attempt was made to
30 obviate the drawbacks of the procedure indicated by De Jong et al. (2004) and the validation
31 of the procedure was performed in line with Decision 2002/657/EC (European Commission
32 2002). In view of the absence of suitable regulations, it seems to be a reasonable starting
33 point, although it provides rules for analytical methods used for the determination of residues
34 in products of animal origin. The purpose of the present work is to report the performance
35 characteristics of the procedure and to provide the comparison with data presented by De Jong
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4 et al. (2004). Nevertheless, the procedure might be an alternative for the determination of
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6 maduramicin avoiding the use of post-column derivatisation device.
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10 11 **Materials and methods**

12 13 *Apparatus*

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17 Universal laboratory oven (Memmert, Schwabach, Germany) was used for drying samples
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19 and the linear shaker IKA HS 501 digital (IKA Labortechnik, Staufen, Germany) was used for
20
21 the extraction. Measurements were performed with an HPLC system Waters Alliance 2690
22
23 (Waters, Milford, MA, USA) equipped with a computer with a program Millennium for the
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25 system control and data processing, a column Luna C18, 5 μm , 150 x 4.6 (Phenomenex,
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27 Torrance, CA, USA), and UV/VIS detector Waters 2487. The mobile phase flow rate was 3
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29 mL min⁻¹, the injection volume 20 μL and the column was kept at ambient temperature. The
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31 detection was carried out at $\lambda = 254$ nm.
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39 40 *Samples*

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43 The validation of the procedure was performed with the samples of a concentrate, a premix
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45 and a feed containing 1 %, 1 g kg⁻¹, and 5 mg kg⁻¹ of maduramicin, respectively and samples
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47 of blank feeds. The concentrate was produced by Alpharma Animal Health (Fordingbridge,
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49 UK). The premix, intended for broilers and the feed for turkeys containing maduramicin were
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51 produced by Lek Veterina (Lipovci, Slovenia). As blank feeds, two commercially available
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53 finishers for broilers and two commercially available feedingstuffs for laying hens produced
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55 by Slovene feed plants were used. They were spiked with maduramicin prior to the extraction
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57 using a standard solution. The selected volume of a standard solution was applied to a
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4 weighed portion of a ground sample and the spiked sample was kept for half an hour prior to
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6 the addition of the extraction solvent.
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10 11 12 13 14 *Chemicals and reagents*

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17 A certified standard of maduramicin sodium was obtained from Alpharma Animal Health
18 (Willow Island, WV, USA). For the stock standard solution (1 mg mL^{-1}), 100 mg of
19 maduramicin standard was dissolved in 100 mL of acetonitrile. Working standard solutions
20 with the concentrations of 0.5, 1.0, and $1.5 \text{ } \mu\text{g mL}^{-1}$ were prepared diluting the stock standard
21 solution with acetonitrile. Acetonitrile, trichloroacetic acid (Merck, Darmstadt, Germany),
22 dansylhydrazine (1-dimethylaminonaphthalene-5-sulfonylhydrazine) (Fluka, Buchs,
23 Switzerland) and tetrabutyl hydrogensulfate (Aldrich, St. Louis, MO, USA) were of analytical
24 or chromatography grade purity. Dansylhydrazine solution and trichloroacetic acid solution
25 were prepared by dissolving 0.38 g of dansylhydrazine and 7.5 g of trichloroacetic acid,
26 respectively in 50 mL acetonitrile. The acetonitrile-water mixture was made of 50 mL of
27 deionised water made up to 500 mL with acetonitrile. For the mobile phase, 0.22 g of
28 tetrabutyl hydrogensulfate was dissolved in 150 mL of deionised water and made up to 1 L
29 with acetonitrile. For solid-phase extraction clean-up of samples, Florisil Sep-Pak columns, 3
30 mL, 500 mg (Waters, Milford, MA, USA) were used.
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51 52 53 *Analytical procedure*

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55 A modified procedure of Gliddon et al. (1988) and Markantonatos (1988) was used. A
56 weighed portion of a sample (20.0 g of a ground feed sample, 2.0 g of a premix or 2.0 g of a
57 concentrate) was extracted with 100 mL of acetonitrile for 1 hour using a linear shaker. Feed
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4 samples were dried prior to the extraction at 60°C in the oven overnight. 25.0 mL of a sample
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6 extract was transferred to a beaker (the extracts of a concentrate and a premix were diluted
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8 200-folds or 20-folds previously with acetonitrile). 600 µL dansylhydrazine solution and 1200
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10 µL trichloroacetic acid solution were added and the mixture was stirred for 10 seconds. The
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12 mixture was transferred promptly onto the SPE column. The column was washed twice with
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14 two 5.0 mL portions of acetonitrile. All the liquid from the column was discarded. The
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16 analyte was eluted from the column with 5.0 mL of acetonitrile-water mixture. Maduramicin
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18 was determined by an HPLC method with UV detection under conditions described above.
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20 The working standard solutions used for the preparation of the calibration curve were
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22 derivatised with dansylhydrazine and passed the SPE column in the same way as samples.
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31 *Validation procedure*

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34 For the linearity test as well as for the determination of LOD and LOQ, working standard
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36 solutions of maduramicin with concentrations from 0.1 µg mL⁻¹ to 2 µg mL⁻¹, which
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38 corresponds to the maduramicin content in feeds from 0.5 mg kg⁻¹ to 10 mg kg⁻¹, were
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40 prepared and HPLC measurements were performed. The identity of the analyte was examined
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42 by co-chromatography. Chromatographic measurements of a sample extract, obtained from a
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44 feed sample containing maduramicin and the sample extract with an addition of a standard
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46 solution of maduramicin were performed. For the within-laboratory reproducibility test, the
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48 concentrate, the premix, and the feed containing maduramicin were analysed by three analysts
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50 in three consecutive days. Each analyst prepared each sample in duplicates every day. Blank
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52 feeds were spiked with maduramicin at the concentration level of 1 mg kg⁻¹ and prepared in
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54 duplicates as well (each of two finishers for broilers and one of two feedingstuffs for laying
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56 hens were prepared twice, and one feedingstuffs for laying hens was prepared three times).
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4 The design of experiments is presented in Table 1. The results of these experiments were used
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6 also for the repeatability test, for the evaluation of measurement uncertainty and for the
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8 determination of the recovery. For the determination of the recovery, blank samples were
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10 spiked also at the concentration level of 5 mg kg^{-1} .
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13 14 15 16 *Statistical procedures* 17

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19 In the present study, LOD was defined as a concentration giving a signal $b_0 + 3 \times s(b_0)$, where
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21 b_0 and $s(b_0)$ were the intercept of a calibration curve and the standard deviation of the
22
23 intercept, respectively. As LOQ, a value of 2.5 times LOD was selected. The within-
24
25 laboratory reproducibility was expressed with the standard deviation s_w and the relative
26
27 standard deviation RSD_w . RSD_w values were compared to the values derived from the
28
29 Horwitz equation, given as the reference value in Decision 2002/657/EC (European
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31 Commission 2002). Prior to the calculation of listed parameters, results of experiments were
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33 examined by the Cochran test and the Grubbs test to eliminate results with too high difference
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35 between duplicate measurements and outliers (Miller and Miller 2000). The repeatability was
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37 expressed with the average differences between results of duplicate measurements obtained
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39 within the within-laboratory reproducibility test. It was compared to the values given as the
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41 maximum permitted difference between parallel determinations at similar concentration levels
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43 of other substances (European Commission 1999). To check the stability of test results, the
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45 stability of the recovery was used. It was followed over the period of one year at the spiking
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47 level of 5 mg kg^{-1} .
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55 The expanded measurement uncertainties U (EA 2003) at the tested concentration levels
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57 were calculated from the within-laboratory reproducibility s_w using coverage factor 2
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59 ($U = \pm 2 \times s_w$).
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Results and discussion

The equation describing the calibration curve, constructed from six points in the range from 0.1 $\mu\text{g mL}^{-1}$ to 2 $\mu\text{g mL}^{-1}$, which corresponds to the maduramicin content in feeds from 0.5 mg kg^{-1} to 10 mg kg^{-1} , was $y = 6917x - 2.4$, where y is the peak area expressed in μVs and x is the concentration of maduramicin expressed in $\mu\text{g mL}^{-1}$. The correlation coefficient was 0.9987 and it was considered acceptable. LOD and LOQ calculated as mentioned above were 0.4 mg kg^{-1} and 1.0 mg kg^{-1} of maduramicin in feed, respectively. They are comparable to the values reported by De Jong et al. (2004).

The identity of a peak obtained with the analysis of a feed containing maduramicin was confirmed with co-chromatography. From the chromatograms of an extract of a feed containing maduramicin and of the extract spiked by a standard solution of maduramicin presented in Figure 1, it is evident that only the height of the maduramicin peak was enhanced by the addition of the maduramicin standard, hence the peak can be assigned to maduramicin.

In Table 2, the results for the within-laboratory reproducibility expressed with the standard deviation (s_w) and the relative standard deviation (RSD_w), as well as the reference values are given. s_w and RSD_w values were calculated after eliminating results with too high difference between duplicates and outliers as mentioned above. In the case of the premix and the feed containing maduramicin, no measurement was eliminated either by the Cochran test or by the Grubbs test. With the concentrate, a result of one experiment done in duplicates was eliminated by the Grubbs test and with the feed spiked with maduramicin at the concentration

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4 level of 1 mg kg^{-1} , results of two experiments were eliminated by the Cochran test and a result
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6 of one experiment done in duplicates was eliminated by the Grubbs test. Therefore, the results
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8 for s_w and RSD_w were calculated from nine experiments done in duplicates with the premix
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10 and the feed containing maduramicin, from eight experiments done in duplicates with the
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12 concentrate, and from six experiments done in duplicates with the feed spiked with
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14 maduramicin at the concentration level of 1 mg kg^{-1} . The reason for a high rejection rate at the
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16 concentration level of 1 mg kg^{-1} was a small volume of a spike applied to a sample ($20 \mu\text{L}$).
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18 Unfortunately, it was found out that the pipetting of such a small volume was not accurate
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20 enough. A bigger volume of a diluted standard solution was proved to be favourable. As it is
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22 obvious from Table 2, the experimental RSD_w values are higher than RSD_w values derived
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24 from Horwitz equation, used as references, but only in the case of the concentrate and the
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26 premix, HORRAT values (the ratio between the obtained RSD_w and Horwitz value) were
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28 higher than 2, which is considered unacceptable (Horwitz and Albert, 1991). The reason for a
29
30 poor reproducibility was supposed to be the off-line pre-column derivatisation of sample
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32 solutions as already mentioned by De Jong et al. (2004). Beside the within-laboratory
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34 reproducibility test no collaborative trial was performed.
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45 The repeatability of the procedure was evaluated by means of differences between results of
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47 duplicate measurements. The average differences were 5.9 % at the concentration level of 1 %
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49 (concentrate), 7.1 % at the concentration level of 1 g kg^{-1} (premix), and 11 % with the feed
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51 containing maduramicin with the nominal concentration of 5 mg kg^{-1} and feed spiked at the
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53 concentration level of 1 mg kg^{-1} . They are lower than 15 % relative to the higher value, taken
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55 as the maximum permitted difference as mentioned above, thus they are deemed acceptable.
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4 The recoveries at the concentration levels of 1 and 5 mg kg⁻¹ were calculated from six
5 experiments carried out in duplicate at the concentration level of 1 mg kg⁻¹ and from twelve
6 experiments done in duplicate at the concentration level of 5 mg kg⁻¹. The recoveries were
7 93.4 % (ranging between 76 % and 118 %) and 99.7 % (ranging between 82 % and 118 %) at
8 the concentration levels of 1 mg kg⁻¹ and 5 mg kg⁻¹, respectively and the mean recovery was
9 96.6 %. To achieve acceptable recoveries, drying of feed samples at 60°C prior to the
10 extraction was introduced as mentioned above. It was found out in previous experiments that
11 the presence of water in the extraction solvent affects the recovery crucially, what is in
12 accordance with the finding of De Jong et al. (2004). The use of molecular sieve suggested
13 by Gliddon et al. (1988) was not found effective enough, so it was omitted and replaced by
14 sample drying.
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33 The stability of test results was tested following the recovery over the period of one year and
34 presented by means of the Shewhart control chart (Figure 2). The oscillation of the recovery
35 between 55 % and 135 % in the first part of the control chart might indicate a poor robustness
36 of the method. However, after certain time, the recovery settled between 81 % and 119 %. In
37 the study reported by De Jong et al. (2004), the recovery fluctuated between 96 % and 105 %.
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47 The measurement uncertainties determined at the concentration levels of 1 % (concentrate),
48 1 g kg⁻¹ (premix), 5 mg kg⁻¹ (feed) and 1 mg kg⁻¹ (blank feed spiked with maduramicin) were
49 ±0.2 %, ±0.3 g kg⁻¹, ±1.9 mg kg⁻¹, and ±0.3 mg kg⁻¹, respectively. They will be reported along
50 the analytical results and taken into account for checking the compliance of samples. As
51 mentioned above, the measurement uncertainties were calculated from the within-laboratory
52 reproducibility. Since the mean recovery was close to 100 % and results of routine analysis
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4 are going to be reported uncorrected for recovery, the contribution of recovery was not
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6 included in the measurement uncertainty.
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10 11 **Conclusions**

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13 The method performance characteristics of LOD (0.4 mg kg⁻¹), LOQ (1 mg kg⁻¹), and
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15 repeatability (the average differences between results of duplicate measurements of 5.9 % at
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17 the concentration level of 1 %, 7.1 % at the concentration level of 1 g kg⁻¹, and 11 % with the
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19 feed containing maduramicin with the nominal concentration of 5 mg kg⁻¹ and with the feed
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21 spiked at the concentration level of 1 mg kg⁻¹) were comparable to those reported by De Jong
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23 et al. (2004). The correlation coefficient of the calibration curve (0.9987) and the within-
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25 laboratory reproducibility expressed by RSD_w of 9.2 %, 16 %, 18 %, and 17 % at the
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27 concentration levels of 1 %, 1 g kg⁻¹, 5 mg kg⁻¹, and 1 mg kg⁻¹, respectively indicate that the
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29 performance of the analytical procedure is not as good as the performance of the method
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31 reported by De Jong et al. (2004). Except RSD_w of 9.2 % and 16 % in the case of the
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33 concentrate and the premix (HORRAT >2), the determined parameters correspond to adopted
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35 reference values, hence the procedure might nevertheless provide an alternative for the
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37 determination of maduramicin in concentrates, premixes and feeds avoiding the use of post-
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39 column derivatisation device.
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Figure captions

Figure 1. Chromatograms of (a) a feed sample containing 5 mg kg^{-1} of maduramicin and (b) a feed sample containing 5 mg kg^{-1} of maduramicin and a standard addition of maduramicin (5 mg kg^{-1}).

Figure 2. Shewhart control chart for the recovery of maduramicin. (a) mean recovery, (b) lower action limit ($\text{mean}-3s_w$), (c) lower warning limit ($\text{mean}-2s_w$), (d) upper warning limit ($\text{mean}+2s_w$), (e) upper action limit ($\text{mean}+3s_w$). The spiking level: 5 mg kg^{-1} .

Table 1. The design of experiments for the within-laboratory reproducibility test.

	Concentrate	Premix	Feed containing 5 mg kg ⁻¹	Feeds spiked at the level 1 mg kg ⁻¹
Day 1	Analyst 1,2,3	Analyst 1,2,3	Analyst 1,2,3	Analyst 1,2,3
Day 2	Analyst 1,2,3	Analyst 1,2,3	Analyst 1,2,3	Analyst 1,2,3
Day 3	Analyst 1,2,3	Analyst 1,2,3	Analyst 1,2,3	Analyst 1,2,3

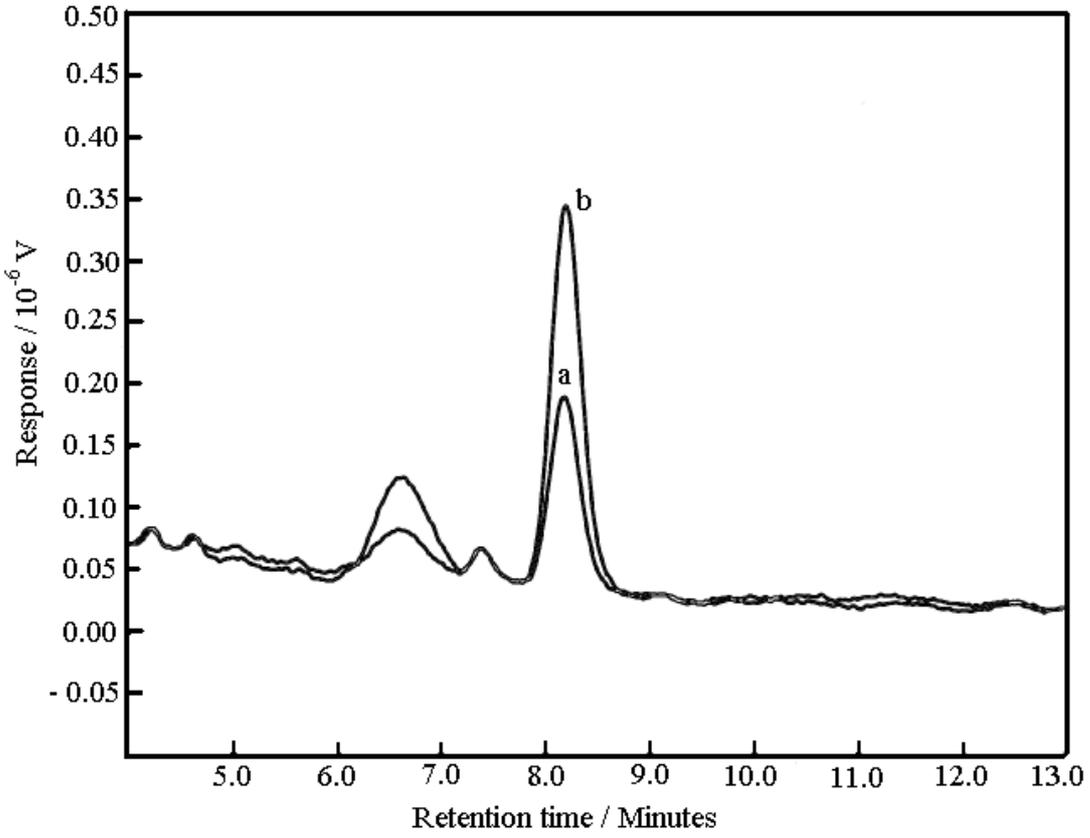
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Table 2. The within-laboratory reproducibility of measurements, expressed with the standard deviation (s_w) and relative standard deviation (RSD_w).

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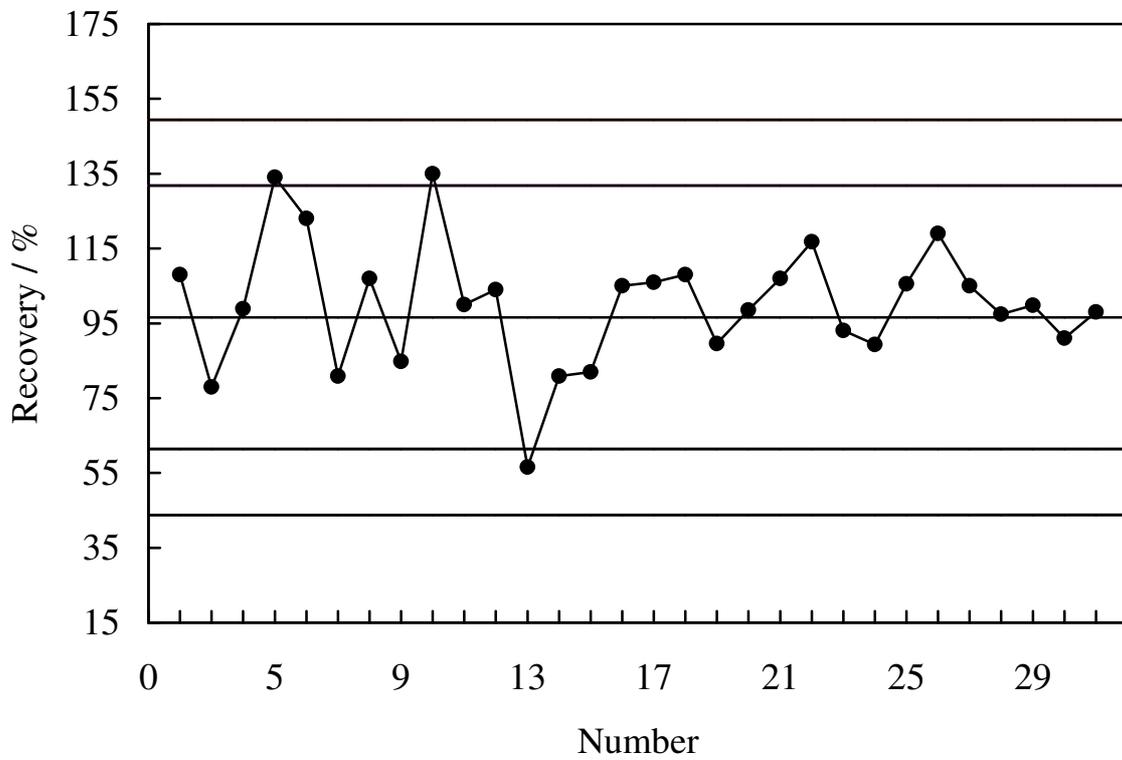
Nominal concentration	Determined conc. level	Number of repetitions	s_w	RSD_w (%)	Horwitz value (%)
1 % (concentrate)	1.18 %	16	0.11 %	9.2	4.0
1 g kg ⁻¹ (premix)	0.93 g kg ⁻¹	18	0.15 g kg ⁻¹	16	5.7
5 mg kg ⁻¹ (feed)	5.14 mg kg ⁻¹	18	0.94 mg kg ⁻¹	18	13
1 mg kg ⁻¹ (feed)	0.95 mg kg ⁻¹	12	0.16 mg kg ⁻¹	17	16

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