

Determination of nicarbazin in feeds using liquid chromatography–electrospray mass spectrometry

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A method is presented for the determination of the 4,4'-dinitrocarbanilide component of the coccidiostat nicarbazin in animal feeds. Samples are extracted by shaking with methanol and analysed, without further clean-up, using liquid chromatography–electrospray mass spectrometry. A deuterated form of the analyte is employed as internal standard to improve the repeatability of the method. The method has been validated at levels between 0.1 and 100 mg kg⁻¹ with internal standard corrected recoveries between 88 and 101% and RSD values <8%.

Introduction

Nicarbazin, a mixture of 4,6-dimethyl-2-hydroxypyrimidine (DMHP) and 4,4'-dinitrocarbanilide (DNC) in a 1:1 molar ratio, is a drug that is used globally in the prevention of coccidiosis in broiler chickens. In the UK, nicarbazin is licensed for use as a feed additive, at concentrations of 100–125 mg kg⁻¹, in broiler chickens up to a maximum age of 28 d. The licence requires that treatment must be withdrawn for at least 9 d prior to slaughter. Nicarbazin is not licensed for use in commercial egg-laying chickens in the UK, and consequently eggs should be free from nicarbazin residues. A joint FAO/WHO Expert Committee recommended the use of DNC alone as the marker residue for nicarbazin, and fixed a maximum residue limit (MRL) of 200 µg kg⁻¹ in broiler chicken tissues. However, no MRL has yet been fixed by the EU.

The preparation of drug-free withdrawal diets can be difficult because nicarbazin powder is strongly electrostatic. This property can lead to contamination of feed mill production lines after milling a medicated feed, and hence to contamination of supposedly nicarbazin-free feeds with the drug. Feeding of diets containing contamination-level concentrations of nicarbazin can cause unwanted residues in eggs^{1,2} and broiler chickens.³ Although drug manufacturers have responded to this challenge by the introduction of granular preparations of the drug that are less prone to contaminate feed milling equipment, there are still persistent reports of the occurrence of nicarbazin in eggs and poultry tissues.⁴ It is therefore important that methods capable of the determination of nicarbazin in feeds at low levels are available to aid the animal feed industry in identification and elimination of contaminating processes.

Several groups have reported methods for nicarbazin in feeds using high performance liquid chromatography (HPLC) with UV detection. The DNC and DMHP components were extracted and chromatographed separately by Macy and Loh,⁵ with quantification down to 25 mg kg⁻¹. Extraction and quantification of the DNC component at 0.1 mg kg⁻¹ was reported by Hurlbut *et al.*⁶ Micro HPLC was used by Draisci *et al.*⁷ to measure DNC down to 4 mg kg⁻¹. Liquid chromatography–thermospray mass spectrometry has been applied as a confirmatory method for DNC in chicken tissues after determination by HPLC–UV.^{8,9} A method has also been reported¹ for the simultaneous determination of both DNC and DMHP in eggs by liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry (LC–APCI–MS).

The present study describes a method for the determination of nicarbazin in feeds using liquid chromatography–electrospray mass spectrometry (LC–ESI–MS). Nicarbazin is extracted with methanol and an aliquot of the extract is evaporated to dryness and redissolved in methanol + water (75 + 25, v/v). DNC is analysed, without any further clean-up, by reversed phase chromatography with detection by ESI–MS. Deuterated (d8) DNC is employed as an internal standard. The method was validated for DNC at concentrations between 0.1 and 100 mg kg⁻¹.

Experimental

Materials

All solvents were of HPLC grade and other chemicals were of analytical reagent grade. Distilled or de-ionised water was used throughout. DNC was obtained from Sigma-Aldrich Co Ltd (Gillingham, Dorset, UK). The deuterium-labelled internal standard (d8-DNC) was custom-synthesised by Quchem (The Queen's University of Belfast, UK). Stock standard solutions (1 mg ml⁻¹) of DNC and d8-DNC were prepared by sonication in dimethylacetamide. Dilute standards (10 µg ml⁻¹) were prepared by dilution of the stock standards in methanol. All stock and dilute standard solutions were stable for at least 1 month when stored in a refrigerator. Working standards (0.2 µg ml⁻¹) were prepared weekly by mixing aliquots of each of the dilute standards and diluting with methanol + water (75 + 25, v/v) and were stored in a refrigerator.

Equipment

The HPLC system consisted of an L6200A intelligent pump and an AS2000 autosampler (Merck, Poole, Dorset, UK). The LC column was a Luna 5µ C18 (2), 250 × 4.6 mm (Phenomenex, Macclesfield, Cheshire, UK). The mobile phase was acetonitrile + water (75 + 25, v/v) containing ammonium acetate (0.05 M). The LC was coupled *via* an ESI probe to a Platform LC–MS system (Micromass, Altrincham, Cheshire, UK). The source was maintained at 125 °C. Nitrogen was used as the drying gas and ESI nebulising gas at flow rates of 300 and 15 l h⁻¹, respectively. Spectra were obtained in negative mode over the range *m/z* 50–400 with the cone set to 15 V, by flow injection of

standard solutions (10 µg ml⁻¹) with no column installed. Selected ion monitoring (SIM) was used for sample analysis. The negative ions at *m/z* 301 and 309 were monitored for DNC and d8-DNC, respectively, with dwell times of 0.5 s for each ion. The mobile phase flow rate was 1 ml min⁻¹, with the column effluent split so that approximately 100 µl min⁻¹ was introduced into the MS. The run time was 7 min.

Sample extraction

Meal samples were pulverised, if necessary, in a Knifetec 1095 sample mill (Tecator, Hoganas, Sweden) and aliquots (10 g) weighed into 125 ml polyethylene bottles. Aliquots of known negative meal were weighed out for spiking and to provide blank chromatograms with each batch of samples.

Medicated feeds (100–125 mg kg⁻¹)

Spiked negatives were prepared by adding DNC (1 mg ml⁻¹, 1 ml). Internal standard (d8-DNC, 1 mg ml⁻¹, 1 ml) was added to all samples, negatives and spiked negatives. The bottles were allowed to stand for approximately 15 min before proceeding with the extraction. Methanol (50 ml) was added and the bottles were capped and shaken on a mechanical shaker for 30 min. The extracts were allowed to settle and aliquots (10 ml) transferred to centrifuge tubes and centrifuged (600g, 4 °C, 10 min). Aliquots of the supernatants (100 µl) were transferred to volumetric flasks and diluted to 10 ml with methanol + water (75 + 25, v/v). Aliquots (200 µl) of these dilute solutions were transferred to microvials for analysis.

Contamination levels (0.1, 1.0 and 10.0 mg kg⁻¹)

Spiked negatives were prepared by adding DNC (10 µg ml⁻¹, 1 ml). Internal standard (d8-DNC, 10 µg ml⁻¹, 1 ml) was added to all samples, negatives and spiked negatives. The samples were shaken with methanol and centrifuged as described above. Aliquots (1 ml) of the supernatants were evaporated to dryness under nitrogen at 60 °C using a Techne Sample Concentrator (Jencons Scientific), allowed to cool and re-dissolved in methanol + water (75 + 25, v/v, 1 ml). Aliquots (200 µl) were transferred to microvials for analysis.

LC-MS analysis

The system was equilibrated by pumping mobile phase for 15 min. Peak reproducibility was checked by injecting, typically, three aliquots (25 µl) of the working standard before beginning an analytical sequence. Samples were then injected, with a standard injection after every 3–4 samples. Peak area data were collected for the ions at *m/z* 301 (DNC) and 309 (d8-DNC). Results were calculated by comparing the peak area ratio of analyte to internal standard in a sample with the ratio in the mean of the standards bracketing the sample.

Results and discussion

The structures and LC-ESI-MS spectra of DNC and d8-DNC are shown in Fig. 1. With the sample cone voltage set to 15 V, both DNC and d8-DNC gave a strong response in negative mode, with prominent peaks for the [M – H]⁻ ions at *m/z* 301 and 309, respectively. Peaks were also present at *m/z* 361 and 369 for DNC and d8-DNC, respectively, probably corresponding to acetate adducts. Our previously published method¹ used LC-APCI-MS to determine both the DNC and the DMHP

components of nicarbazin in eggs. In the present study, which focussed specifically on the determination of DNC alone in animal feedingstuffs, we chose to use electrospray ionisation because this ionisation mode is more sensitive for this compound. In contrast to the LC-APCI-MS spectra produced by Blanchflower *et al.*,¹ in which the [M – H]⁻ ion was the only prominent ion formed regardless of sample cone voltage, it was possible, using ESI, to initiate collision induced dissociation of the DNC molecule by increasing the sample cone voltage. This apparently anomalous result (more fragments formed by a softer ionisation technique) may be explained by the fact that the earlier study¹ was performed on an earlier version of the VG Platform than that used in the current study. We have previously noticed differences in the extent of fragmentation induced by the two machines. Fragment ions were formed at *m/z* 137 and 107. The ion at *m/z* 137 results from cleavage of the molecule between the carbonyl and either of the adjoining secondary amino groups. The fragment at *m/z* 107 may result from the neutral loss of NO[•]. These ions could be useful for the unequivocal identification of the compound, if required.

Fig. 2 shows SIM chromatograms for a DNC standard (equivalent to 1 mg kg⁻¹ in meal), a negative meal and a meal fortified at 1 mg kg⁻¹ with DNC. Peak symmetry is good and the chromatograms are free from interference at the retention time of DNC.

Inter- and intra-assay precision and recovery were assessed by extracting and analysing five replicates of known nicarbazin-negative meal spiked at 0.1, 1, 10 and 100 mg kg⁻¹ with DNC on three separate occasions. The results are presented in Table 1. The absolute recovery achieved by the method was approximately 65%. Internal standard corrected recoveries ranged from 88–101% and RSDs were <8%.

The limit of determination, defined as the lowest level at which the method was validated, is 0.1 mg kg⁻¹. Quantification to this level should be adequate to identify any significant contamination of meals produced by the animal feed industry. However, the method is sufficiently sensitive to achieve quantitative results at levels at least a factor of 10 lower than this if required. The LC-UV method described by Hurlbut *et al.*⁶ is also capable of measuring DNC at 0.1 mg kg⁻¹ levels, and provides a possible alternative in laboratories where LC-ESI-MS instrumentation is not available. However, that method involves the use of hot solvents and requires an alumina clean-up step, which makes the procedure more time consuming than the method described in this paper. Mass spectrometric detection provides a greater degree of specificity than does UV

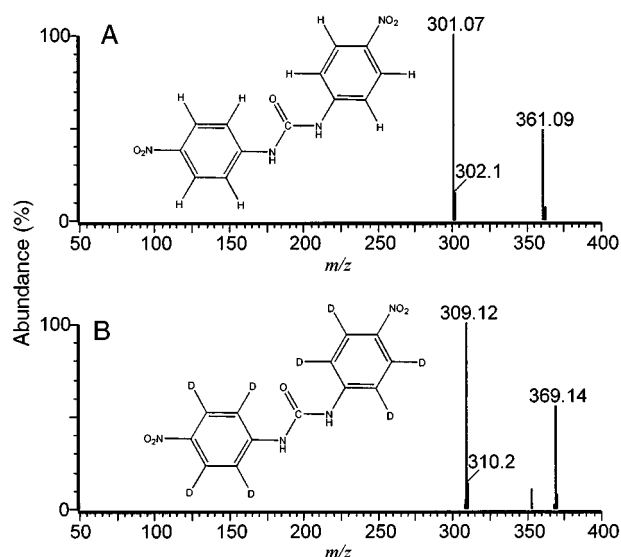


Fig. 1 Structures and LC-ESI-MS spectra of (A) DNC and (B) d8-DNC. The sample cone voltage was 15 V.

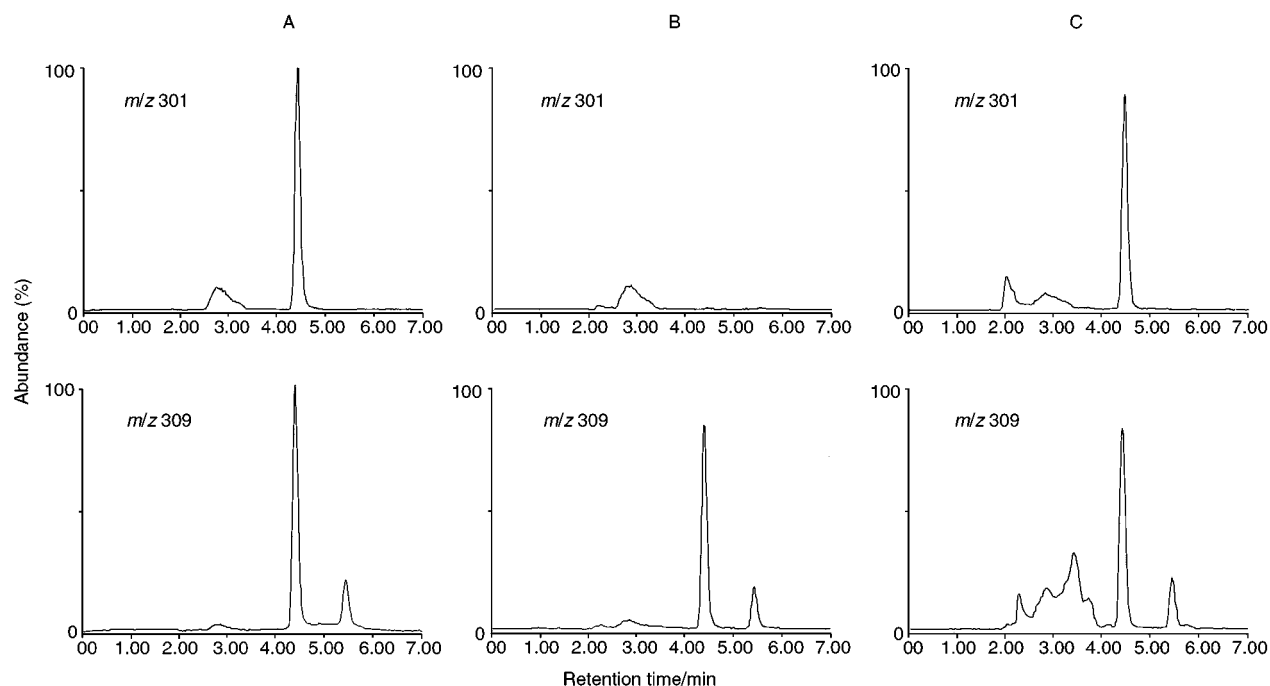


Fig. 2 SIM chromatograms for (A) a DNC standard at a concentration of 1.0 mg kg^{-1} , (B) a negative meal sample and (C) a negative meal sample fortified with DNC at 1.0 mg kg^{-1} . The top row shows traces at m/z 301, normalised to $100\% = 5.5 e,^5$ for the $[\text{M} - \text{H}]^-$ ion of DNC. The bottom row shows traces at m/z 309, normalised to $100\% = 3.8 e,^5$ for the $[\text{M} - \text{H}]^-$ ion of d8-DNC.

Table 1 Inter- and intra-assay reproducibility and recovery for meal spiked with DNC at 0.1, 1, 10 and 100 mg kg^{-1} . Results are calculated using the internal standard

	Day 1	Day 2	Day 3	Overall
<i>0.1 mg kg⁻¹—</i>				
Mean/ mg kg^{-1}	0.10	0.09	0.09	0.09
<i>s</i> / mg kg^{-1}	0.005	0.004	0.005	0.006
RSD (%)	5.7	5.1	5.8	6.4
Mean recovery (%)	96.0	88.0	94.0	92.7
<i>n</i>	5	5	5	15
<i>1.0 mg kg⁻¹—</i>				
Mean/ mg kg^{-1}	0.99	0.93	1.01	0.98
<i>s</i> / mg kg^{-1}	0.031	0.014	0.021	0.043
RSD (%)	3.2	1.5	2.1	4.4
Mean recovery (%)	99.3	92.7	101.0	97.7
<i>n</i>	5	5	5	15
<i>10.0 mg kg⁻¹—</i>				
Mean/ mg kg^{-1}	8.83	9.44	10.06	9.44
<i>s</i> / mg kg^{-1}	0.323	0.718	0.472	0.714
RSD (%)	3.7	7.6	4.7	7.6
Mean recovery (%)	88.3	94.4	100.6	94.4
<i>n</i>	5	5	5	15
<i>100 mg kg⁻¹—</i>				
Mean/ mg kg^{-1}	91.1	93.4	95.4	93.3
<i>s</i> / mg kg^{-1}	0.792	1.199	1.178	2.043
RSD (%)	0.9	1.3	1.2	2.2
Mean recovery (%)	91.1	93.4	95.4	93.3
<i>n</i>	5	5	5	15

detection, and minimizes the requirement for sample clean-up before analysis.

The LC-ESI-MS response was shown to be linear by preparing and analysing a series of eight standard solutions containing DNC over the range $0\text{--}2 \mu\text{g ml}^{-1}$ (equivalent to $0\text{--}1000 \text{ mg kg}^{-1}$ in meal). Each standard solution also contained d8-DNC at a concentration of $0.2 \mu\text{g ml}^{-1}$. A standard curve was prepared by plotting the peak area ratio (m/z 301/309) against DNC concentration. The correlation coefficient (r) was 0.9999.

The inclusion of a deuterated internal standard contributes significantly to the repeatability of the method since it corrects

not only for any minor losses of the analyte during the extraction, but also for changes in the response of the MS during an analytical sequence. Over a period of hours a gradual decrease in abundance is frequently observed, possibly due to contamination of the ESI source or the sample cone. The sample matrix may also suppress, or in some instances enhance, the ionisation of the analyte in the ESI source. These effects can make calculation of results, including recovery values, using absolute areas difficult. Calculations based on the comparison of the ratios of analyte to internal standard in unknown samples with the ratios found in standards overcome these difficulties.

In conclusion, the method presented is simple and rapid, and has been used in this laboratory to measure nicarbazin in commercially produced meals, and in meals formulated for experimental purposes, at medicated and contamination levels. Approximately 15 samples, plus controls and negatives, can be extracted in duplicate in one working day. The inclusion of an autosampler in the LC-ESI-MS system facilitates overnight analysis of the extracts.

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