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Note

High-performance liquid chromatographic screening method for low levels of nicarbazin in eggs with off-line cartridge sample clean-up

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Nicarbazin, a molecular complex of 4,4'-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine, has been in worldwide use from 1955 to control coccidiosis in poultry. In the Netherlands, nicarbazin is used as feed additive at a maximum level of 125 mg/kg for the prophylactic treatment of *ca.* 200 million broilers, representing 70% of the total annual production. A residual level of 4 mg/kg in uncooked chicken muscle, liver, skin and kidney is allowed by the U.S. Food and Drug Administration. The Dutch broiler industry attempts to eliminate nicarbazin residues by a compulsory withdrawal period of 9 days before slaughter.

Because of adverse effects on shell colour, yolk pigmentation and hatchability[1,2] the product is not used in layers and breeders. Because feed for both broilers and layers is produced in the same factory it is unavoidable that cross-contamination between different batches occurs and low levels of nicarbazin may be detected in feed for layers and breeders. This problem of carry-over is confirmed by the investigations of Friedrich *et al.*[3], among others. Investigations on the kinetics of nicarbazin in layers by Friedrich *et al.*[4] showed that even very low levels of nicarbazin in the feed result in detectable residues of nicarbazin in eggs.

In these studies the dinitrocarbanilide moiety of nicarbazin was analysed by the high-performance liquid chromatographic (HPLC) method of Malisch[5]. The Malisch procedure was developed for the determination of some 54 different substances in milk, eggs and meat, and has a detection limit for nicarbazin of *ca.* 5 μg/kg, with recoveries of 30–40% in 50-g test portions.

A practical disadvantage of this method is the low number of samples that can be analysed per day due to the time-consuming cleanup procedure. The need to analyse a larger number of samples at lower cost was the reason for developing a rapid screening method for nicarbazin in eggs.

MATERIALS AND METHODS

Reagents and standards

Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The following reagents and solvents were used: acetic acid glacial (p.a. quality, Merck, Darmstadt, F.R.G.); acetonitrile (HPLC grade, Fisons, Loughborough, U.K.); sodium sulphate anhydrous (p.a. quality, Merck); *n*-hexane (Baker Grade,

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Baker, Deventer, The Netherlands); chloroform ("Baker Analysed", Baker); sodium acetate anhydrous (p.a. quality, Merck).

Nicarbazin standard (97.4%) was supplied by Merck, Sharp & Dohme (Haarlem, The Netherlands). For HPLC, a standard solution of nicarbazin in eluent (0.020 μg/ml) was prepared from a 50 μg/ml stock solution of nicarbazin in acetonitrile. For spiking, a standard solution of nicarbazin in water (1.25 μg/ml) was prepared from the same stock solution.

Spiking of egg material

To 49 g of blank homogenized whole egg, 1.0 ml of an aqueous standard solution of nicarbazin (1.25 μg/ml) was added by mixing on a magnetic stirrer for 10 min. From this material, containing a concentration of nicarbazin of 25 μg/kg, dilutions were prepared in blank whole egg material to obtain samples containing 2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 μg of nicarbazin, respectively. To determine the recovery these materials were treated in the same way as the egg samples.

Sample preparation

Before analysis the total egg content was homogenized by shaking. The homogenate can be stored at 4°C for up to 4 days without measurable loss of nicarbazin content. For longer storage the samples can be kept at −20°C for several weeks.

For the extraction, 50 μl of 10% acetic acid solution was pipetted into a polystyrol tube. Using a 1-ml pipettor with a disposable tip, 1.0 g of sample was weighed into the tube and a magnetic stirring rod was added. While this was stirred on the magnetic stirrer (Model IKA, Combimag RCT) at maximum speed, 2.5 ml of acetonitrile were slowly added. The tube was closed with a stopper and stirring continued. After 4 min, 0.25 g of anhydrous sodium sulphate was added and stirring was continued for 2 min. The tube was centrifuged for 2 min at 3000 rpm (1400 *g*), on a Heraeus Minifuge. The supernatant was decanted into a glass centrifuge tube through a disposable funnel with a silanized glasswool plug (Baker). The sediment in the polystyrol tube was re-extracted with 2.5 ml of acetonitrile by stirring for 3 min at maximum speed. After centrifugation for 2 min at 3000 rpm, the supernatant was combined with the first extract through the funnel. The funnel was rinsed with 1 ml of acetonitrile. The combined extracts were evaporated to dryness under nitrogen, using a water-bath at 60°C.

Solid phase extraction (SPE) columns packed with 100 mg of Bond-Elut No. 601101 from Analytichem were washed twice with 1 ml of chloroform–acetonitrile (80:20) and once with 1 ml of chloroform, and finally air-dried before use. After evaporation the residue was redissolved in 1 ml of hexane-chloroform (50:50) and transferred to a pre-treated SPE column on a vacuum manifold (Alltech). The tube was rinsed with 1 ml of hexane-chloroform (50:50). The rinsing fluid was transferred to the SPE column without allowing it to run dry. After the liquid had flowed through, the column was dried by vacuum for 1 min. The column was then eluted with chloroform–acetonitrile (80:20), and the first 1 ml of the eluate was collected in a 1.5-ml glass autosampler vial. After evaporation to dryness under nitrogen at room temperature, 1.0 ml of the HPLC eluent was added. The vial was closed with a cap and the residue was dissolved by shaking on a Vortex mixer for 4 min.

HPLC conditions

The HPLC conditions were derived from those described by Malisch[5], Hoshino *et al.*[6] and Petz[7].

An HPLC system with an autosample (Promis, Spark Holland), a solvent-delivery pump (SF 400, Applied Biosystems), an UV–VIS detector (SF 783, Applied Biosystems) and an integrator (4290, Spectra Physics) was used. Sample volumes of 50 μl were injected.

The analytical column consisted of two cartridges (100 × 3.0 mm I.D.) packed with ChromSpher C_{18} (5 μm particle size) and was obtained from Chrompack. The guard column cartridge (10 × 2.1 mm I.D.) packed with pellicular RP (30–40 μm particle size) was also from Chrompack. The mobile phase was acetonitrile–acetate buffer (0.02 *M*, pH 4.8)–water (54:10:36). A flow-rate of 0.60 ml/min was used and nicarbazine was detected at 360 nm using a Kratos Model SF 783 UV detector.

The amount of nicarbazin in the samples was calculated by comparison of the peak height with that of the standard solution. The result was corrected for the recovery found in the spiked egg material.

RESULTS AND DISCUSSION

Chromatography

Chromatrograms from spiked and control samples and the standard are shown in Fig. 1.

Recovery

Samples spiked at 2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 μg/kg were used in recovery experiments and in studies on the linearity of the photometric detector response. The results of the spiking experiments are shown in Table I. The linearity of the photometric detector response in presented in Fig. 2.

A good recovery for all the levels investigated and a low standard deviation for

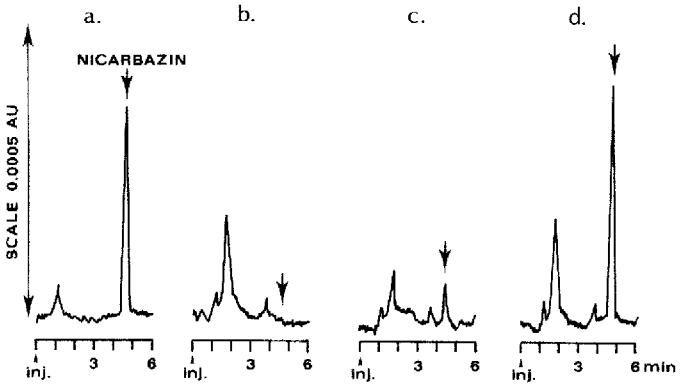

Fig. 1. Representative chromatogram from HPLC injections of (a) nicarbazin standard solution; (b) blank egg sample; (c) egg sample, spiked with 5 μg of nicarbazin per kg; (d) egg sample, spiked with 25 μg of nicarbazin per kg.

TABLE I

STATISTICAL SUMMARY OF ANALYSIS OF EGG MATERIAL SPIKED WITH SEVERAL LEVELS OF NICARBAZIN

Correlation coefficient for average content and spiking level: 0.99915.

Spiking level (μg/kg)	*n*	*Average content*	*Standard deviation*	*Coefficient of variation (%)*	*Mean recovery (%)*
2.5	4	2.91	0.65	22.41	116.4
5.0	5	4.41	0.67	15.11	88.2
10.0	5	9.21	0.38	4.08	92.1
15.0	5	14.03	0.28	1.96	93.5
20.0	5	18.40	0.37	2.00	92.0
25.0	8	22.31	0.53	2.39	89.0

the repeatability were obtained. The relationship between peak height and concentration of the analyte was linear in the range 2.5–25.0 μg of nicarbazin per kilogram of egg material.

Limit of detection

The use of the 5-μm ChromSpher C_{18} column and an UV detector such as the Kratos Model SF 783 was essential for sensitive detection of nicarbazin. With this combination of column and detector the detection limit, with the signal three times the noise level, was achieved with 125 pg of nicarbazin. This amount represents 2.5 μg of nicarbazin per kilogram of egg material.

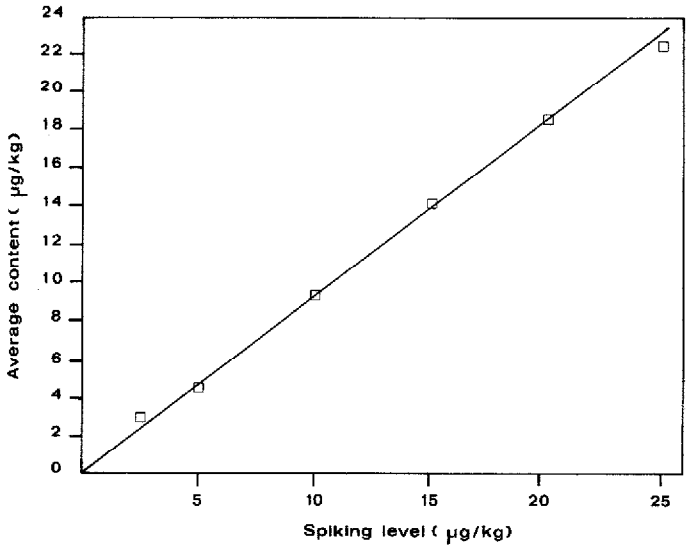

Fig. 2. Linearity of the photometric detector response to nicarbazin in spiked egg materials.

Extraction and cleanup

Working with the method of Haagsma and Van de Water[8] for the determination of sulphonamides in egg material, it was found that adding acetic acid to the sample, to adjust the pH, reduced the viscosity of the sample. This reduced viscosity made the sample more suitable for homogenizing with acetonitrile and improved the recovery.

Adding 0.25 g of anhydrous sodium sulphate to the homogenate is essential to remove part of the water, which makes evaporation to total dryness with nitrogen possible. Moreover, the sodium sulphate has a grinding effect, which possibly enhances partition of the egg material in the extraction fluid. Evaporation of the extract proved to be unavoidable, since only a part of the water from the sample could be removed by sodium sulphate during extraction. Moreover, evaporation of the extract to total dryness proved to be essential to obtain the proper polarity of the solution of the extracted material for further cleanup.

In experiments with different mixtures of chloroform and hexane, it was established that a 50:50 ratio of chloroform–hexane was optimal for dissolving the extracted material and prevented adsorption of egg matrix material on the SPE column as selectively as possible. Chloroform–acetonitrile (80:20) proved to be optimal for quantitative elution of nicarbazin in a small volume, minimizing co-elution of matrix material from the SPE column.

The need to evaporate twice during cleanup has little influence on the analysis time because of the small volumes involved. With simple equipment it is possible to evaporate several extracts simultaneously. The described cleanup procedure, in combination with a relatively short HPLC run time of 6.0 min, makes it possible for two technicians to analyse 30 samples per day.

During development of this method chromatograms of samples showed impurities from the SPE column material, but chromatograms were improved by washing as described. In present batches of SPE columns pretreatment has been proven to be less essential because of improvements in manufacture.

Applications

The procedure described has been used to determine the nicarbazin content of eggs obtained from hens fed with different levels of the anticoccidial drug in the feed. In accordance with the studies of Polin *et al.*[9] and Friedrich *et al.*[4] a maximum level of nicarbazin was detected *ca.* 8 days after first administration of the medicated ration. This finding was also in accordance with the results of the investigations of Anhalt[10] for other drugs.

The method was also used to screen several thousand samples from egg producers and exporters during a period of 2 years. In every case of positive findings a relation was found with carry-over of low levels of nicarbazin in the feedmill. Further research is being conducted to automate the method and to make it applicable to the analysis of nicarbazin in meat, feed and other matrices.

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