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Survey of the anticoccidial feed additive nicarbazin (as dinitrocarbanilide residues) in poultry and eggs

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Abstract

A survey was carried out on the occurrence of dinitrocarbanilide (DNC) the marker residue for nicarbazin, in poultry produced in Ireland during the years 2002 to 2004. Liver (n = 736) and breast muscle samples (n = 342) were tested. DNC residues were found in 40% and 26% of liver and breast muscle samples at levels greater than 12.5 and 5 μg kg^{-1}, respectively. DNC residues were found at >200 μg kg^{-1} in 12 and 0% of liver and muscle samples, respectively. Samples of breast muscle (n = 217) imported from 11 countries were also tested for DNC residues. A lower incidence of DNC residues (6%) was found in imported breast muscle. Egg samples (n = 546) were tested and DNC residues were found in nine samples, with levels ranging between 14 and 122 μg kg^{-1}. Analysis of poultry, carried out as part of official food inspection in the period 2004 to 2006, indicated a reduction in the number of broiler liver samples containing DNC at >200 μg kg^{-1}, to approximately 7%. Low levels of DNC residues continue to be found in <2% of egg samples.

Introduction

Nicarbazin is an equimolar mixture of 4,4´-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). It is administered to poultry in feed for the prophylactic treatment of coccidiosis. Coccidiosis is an infectious disease caused by a microscopic protozoan parasite, which damages the intestinal tract of the bird, causing illness and sometimes death. Intensively

reared broilers are particularly susceptible to the disease, owing to the warm and humid conditions of broiler houses. The disease is not as common when birds are raised under extensive conditions. The licence for feed premixes containing nicarbazin as a single active ingredient was withdrawn under Commission Regulation 2205/2001/EC [Anon. 2001]. Nicarbazin continues to be legally marketed at levels of 40-50 mg kg^{-1}, together with the feed additive ionophore narasin, as the combined product Maxiban® [Anon. 2007].

Nicarbazin is classified as a feed additive and not as a veterinary drug. No maximum residue limits (MRLs) have been set for nicarbazin in the European Union (EU). Nicarbazin depletion studies that have been carried out in broilers show that DNC is a more persistent residue than HDP in edible tissues (Porter and Gilfillan, 1955). The FAO/WHO Joint Expert Committee on Food Additives (JECFA) established an MRL of 200 µg kg^{-1} for DNC, as the marker residue, in edible tissue (liver and meat) [Wells 1999]. The JECFA MRL for DNC has been adopted as the action limit by regulatory authorities in the Republic of Ireland and in the UK. Liver samples are tested for DNC residues in monitoring programmes on the island of Ireland as this tissue tends to contain higher residue concentrations than muscle. No MRL has been defined for DNC in eggs because nicarbazin is not approved for use in laying hens. An action level of 100 µg kg^{-1} DNC in eggs has been adopted in the UK [Veterinary Residues Committee 2006].

The presence of DNC residues was first reported in poultry products nearly 20 years ago [De Giovanni et al. 1989, Oishi and Oda 1989]. Since then DNC residues have been reported in egg and liver samples tested in The Netherlands, UK, Italy and Ireland [Vertommen et al. 1989, Veterinary Residues Committee 2006, Gallo and Serpe 1997, O'Keeffe et al. 2005]. A number of studies have been carried out to identify the factors that may contribute to the presence of DNC residues in eggs and edible tissues, such as feed contamination [Cannavan and Kennedy 2000, Cannavan et al. 2000, McEvoy et al. 2003], withdrawal period [Cannavan and Kennedy 2000] and faecal recycling [Cannavan and Kennedy 2000, Penz et al. 1999]. The relationship between levels of nicarbazin in contaminated feed and corresponding levels of DNC residues in eggs and liver has proved useful in identifying trigger limits for nicarbazin as a contaminant in non-medicated feed. DNC residues in eggs can be attributed to poor practices at feedmills because nicarbazin is not licensed for use in laying hens. However, control of DNC residues in broiler tissues is more

complex than eggs because nicarbazin is widely applied to broilers and several on-farm factors that may contribute to residues. The contribution of on-farm factors to the DNC residues in broiler tissues has been described in more detail elsewhere [O'Keeffe et al 2006a].

In the study reported here, DNC contamination of tissues and eggs has been investigated in an integrated study across Ireland. Three surveys were organised to investigate the presence of DNC in eggs, liver and muscle between the years 2002 and 2004. In addition, a study on DNC residues in samples of imported poultry meat was undertaken.

Experimental

Apparatus, chemicals and reagents

4,4′-dinitrocarbanilide (DNC) standard material was from Sigma-Aldrich (St. Louis, MO, USA). The deuterium-labelled internal standard (d8-DNC) was custom synthesised by Quchem (Queen's University, Belfast). Acetonitrile, methanol, dimethylformamide, water (all HiPerSolv grade), dimethylsulphoxide and *n*-hexane (Analar grade) were obtained from BDH (Merck, Poole, Dorset, UK). Cyclohexane (Pestican grade) was obtained from Labscan (Dublin, Ireland). DNC standard stock solution (1 mg ml^{-1}) was prepared in dimethylsulphoxide (Biacore and HPLC assays) and DNC and d8-DNC standard stock solutions (1 mg ml^{-1}) in dimethylacetamide (LC-MS/MS). The biosensor and HPLC standard solutions were prepared every 3 months and were stored in amber glass vials at room temperature in the dark preventing their solidification. LC-MS/MS standard solutions were stored at 4°C and were stable for at least one month. The optical SPR Biosensor system (Biacore™ Q) was obtained from Biacore™ (Uppsala, Sweden). Sensor chips (CM5), HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) buffer and an amine coupling kit were purchased from Biacore™. Biacore control software, version 3.1, was used for instrument operation and BIA evaluation software, version 3.0, for data handling. Chip surfaces were prepared by immobilisation of a DNC mimic, glutamic acid (1-glutamic acid-(*p*-nitroanilide)), which was obtained from Sigma (Poole, Dorset, UK). A DNC polyclonal antibody (R555) was used for the biosensor assay. Production of the DNC antibody are described in detail elsewhere

[Connolly et al. 2002]. Bond Elut™ cartridges (C_{18}, 500 mg, 3 ml) were from Varian (Harbor City, CA, USA).

Biosensor screening assays

Egg and liver samples were screened using a Biacore™ Biosensor assay [McCarney et al. 2003]. The assay was modified from the published assay to allow the sensitive detection of DNC residues in muscle tissue.

Egg and liver assays

Homogenised egg or liver test samples (1 g) were weighed into 30 ml polypropylene tubes. Acetonitrile (4 ml) was added to samples and they were vortexed (10 sec) and sonicated (2 min) prior to centrifugation (1200×*g*, 10 min). The supernatants were transferred into glass test tubes and evaporated to dryness under a stream of nitrogen at 60°C. Sample extracts were allowed to cool for 1 min. At this point liver sample extracts were further purified. Liver extracts were reconstituted in cyclohexane (1 ml) by vortexing (15 sec). An aliquot (250 µl) of methanol/water (75:25, v/v) was added, the tubes were vortexed (10 sec) and placed in a water bath (37°C, 10 min) prior to centrifugation (1200×*g*, 10 min). Aliquots (200 µl) of the aqueous layer were removed and evaporated to dryness under nitrogen.

Egg or liver sample extracts were subsequently reconstituted by sequentially adding methanol (200 µl) and HBS-EP buffer (800 µl), vortexing for 10 sec after each addition. Sample extracts were transferred to eppendorf tubes and centrifuged (14,000×*g*, 5 min). Sample extracts were further diluted 1:20 (v/v) in HBS-EP buffer prior to analysis on a Biacore™ Q system. Aliquots of the extracts (50 µl) were mixed with an equal volume of the antibody and injected for 2 min over the sensor chip surface at a flow rate of 25 µl min^{-1}. The response for the sample was determined as the difference in the signal (Response Units, RU) measured before and after injection. The surface was regenerated with a 1 min pulse of dimethylformamide/180 mM sodium hydroxide (20:80, v/v) at a flow rate of 25 µl min^{-1}.

Muscle assay

A number of modifications of the DNC liver assay were made to allow the detection of low levels of DNC in muscle tissue. Muscle samples (5 g) were weighed into 30 ml polypropylene tubes and extracted with acetonitrile (5 ml). The supernatants were transferred into glass test tubes and evaporated to dryness under a stream of nitrogen at 60°C. Sample extracts were allowed to cool for 1 min. Muscle extracts were reconstituted by sequentially adding methanol (200 µl) and HBS-EP buffer (400 µl) and vortexing for 10 sec after each addition. Reconstituted extracts were further diluted (1:1, v/v) with HBS-EP buffer prior to analysis. In a deviation from the liver and egg assays, three additional steps were required to regenerate the chip surface**.** It was found during the development of the assay to detect low levels of DNC in muscle that there was noticeable carry over of electrostatic nicarbazin residues between injections and a more intensive chip regeneration protocol was required. Chip surfaces were allowed to contact sequentially with (a) 180 mM NaOH/acetonitrile (80:20, v/v), (b) 100 mM HCl/acetonitrile (80:20, v/v) and (c) 180 mM NaOH/acetonitrile (80:20, v/v) at a flow rate of 50 µl min^{-1} for 60 sec.

Chemical assays

The presence of DNC residues were confirmed by LC-MS/MS [Yakkundi et al. 2001] or by HPLC-UV [Capurro et al. 2005].

LC-MS/MS assays

Homogenised liver samples (2.0 g) were weighed into 50 ml polypropylene centrifuge tubes. Internal standard (20 µl of the 10 µg ml^{-1} d8-DNC working standard) was added to samples, which were mixed and allowed to stand for 15 min prior to extraction. Samples were homogenised in acetonitrile (8 ml) using a Silverson homogeniser for 40 sec and centrifuged (600×*g*, 10 min at 4°C). An aliquot of the supernatant (2.5 ml) was transferred to glass tubes (10 ml) and evaporated to dryness under nitrogen at 60°C. After cooling, the residues were reconstituted in hexane (1 ml). This was extracted by vortexing (10 sec) with methanol/water (75:25 v/v, 250 µl). The tubes were centrifuged (600×*g*, 10 min at 4°C) and an aliquot (175 µl) of the aqueous layer was transferred to

microvials for analysis. Calibration curve standard solutions containing DNC and d8-DNC in methanol/water (75:25 v/v) were prepared at this time.

A 25 µl portion of the final sample extract was injected onto the LC-MS/MS system. The LC-MS/MS system consisted of a Hewlett Packard (Stockport, Cheshire, UK) HPLC system, comprising an 1100 Series binary pump, autosampler and solvent degasser, was coupled *via* an electrospray interface to a Quattro LC (Micromass, Wythenshawe, UK), which operated in negative ion mode. The $[M-H]^-$ ion at *m/z* 301 was monitored along with two transition ions at *m/z* 137 and 107 for DNC and the $[M–H]^-$ ion at *m/z* 309 for the internal standard, d8-DNC. The LC column used was a Luna 5 µm C18 (2) 250 × 4.6 mm (Phenomenex, Macclesfield, Cheshire, UK). The mobile phase, which consisted of 0.05M ammonium acetate in acetonitrile/water (75:25 v/v), was pumped at a rate of 1.0 ml min^{-1}. The column effluent was split so that approximately 100 µl min^{-1} entered the mass spectrometer. The run time for each injection was 7 min.

HPLC assays

Homogenised egg, liver or muscle samples (2 g) were weighed into 30 ml polypropylene tubes. Fortified egg and liver samples were prepared at levels of 25 and 250 µg kg^{-1} by adding 50 µl portions of 1 and 10 µg ml^{-1} DNC standard solutions to negative control samples, respectively. Fortified muscle samples were prepared at levels of 10 and 100 µg kg^{-1} by adding 50 µl portions of 0.4 and 4 µg ml^{-1} DNC standard solutions to negative control samples, respectively. After fortification, samples were allowed stand for 15 min prior to extraction. Acetonitrile (10 ml) were added and samples were homogenised using a Polytron™. The homogeniser probe was washed with acetonitrile (5 ml), which was retained. Samples were vortexed (2 min), sonicated (3 min) and shaken (15 min), before centrifugation (1928×*g*, 10 min, 4°C). The supernatant was transferred to a clean polypropylene tube and the sample was re-extracted as before using the acetonitrile (5 ml) previously used to wash the homogeniser probe, plus water (1 ml). The supernatants were combined and defatted using hexane (2 × 10 ml) by vortex mixing, centrifugation and removal of the hexane layer. The acetonitrile layer was evaporated to dryness under nitrogen (60°C) and reconstituted in acetonitrile/water (70:30, v/v, 500 µl). Samples extracts were passed through C_{18}

SPE cartridges (preconditioned with 2.5 ml acetonitrile and 2.5 ml acetonitrile/water (70:30, v/v)) and eluted with 2.5 ml acetonitrile/water (70:30, v/v). The eluate from the cartridge was collected in a glass test-tube. Egg and liver extracts were evaporated to dryness under nitrogen (60°C) and reconstituted in acetonitrile/water (80:20, v/v, 500 µl). Muscle samples were reconstituted in acetonitrile/water (80:20, v/v, 200 µl). Extracts were allowed to sit for 15 min prior to transfer to HPLC vials; a phase separation may occur in some samples and care was taken not to transfer this lower oily layer into the HPLC vials.

A 25 µl portion of the final sample extract was injected onto the HPLC system. The HPLC system consisted of a model 600 HPLC pump with a model 717 autosampler and model 484 UV detector (set at 350 nm), all from Waters (Milford, MA, USA). The separation was carried out on a stainless-steel analytical column (250 × 4.6 mm i.d) equipped with a Securiguard™ pre-column, both packed with Hypersil BDS C_{18} material (Phenomenex, Cheshire, UK). The column temperature was maintained at 40°C. The mobile phase, consisting of water/acetonitrile (55:45, v/v), was pumped at 1 ml/min. Under these conditions the retention time of DNC was approximately 13 min. A Waters 746 data processing module was used for recording and processing chromatograms.

Calibration

Biacore

Standards were prepared by fortifying negative control egg and liver samples at concentrations of 0, 50, 100, 250, 500 and 1000 µg kg^{-1} and muscle at concentrations of 0, 1, 2.5, 5, 10, 25 and 50 µg kg^{-1} of DNC for calibration. Calibration curves were prepared by plotting response as a function of DNC concentration (µg kg^{-1}). The DNC concentration in test samples was read directly from the calibration curve prepared from fortified samples that were run with that particular batch.

LC-MS/MS

Calibration curve standard solutions containing DNC and d-8 DNC were prepared in methanol/water (75:25 v/v). The DNC standards were prepared at the concentration 0, 100, 200, 300, 400 and 500 µg kg^{-1} standard equivalent along with known equal amount of internal standard d-8 DNC. The calibration curve was obtained by plotting the standard concentrations against the peak area ratios of d-8 DNC and DNC. The DNC concentration in unknown samples was reported as internal standard corrected value.

HPLC

Standards were prepared at concentrations of 0, 50, 100, 250, 500, 1000 and 2000 ng ml^{-1} in acetonitrile/water (80:20, v/v). Calibration curves were prepared by plotting peak area as a function of DNC concentration (ng ml^{-1}). The DNC concentration in test samples was determined from the peak areas obtained for test sample extracts, as calculated from the calibration curve. The DNC concentration in test samples was reported corrected for recovery using recovery factors calculated from fortified samples that were run with that particular batch.

Validation of methods

The methods used in this comparison were validated according to current EU guidelines as described in Commission Decision 2002/657/EC. The assays were validated to allow detection or determination of DNC to an MRL of 200 µg kg^{-1}. The sensitivity of the Biacore™ biosensor, HPLC-UV and LC-MS/MS assays are much lower than the MRL. Limits of determination of DNC are 33.2, 12.5 and 10 µg kg^{-1} in liver; 34.8, 12.5 and 10 µg kg^{-1} in egg; and 5, 5 and 2 µg kg^{-1} in muscle, respectively.

Industry surveys

Broiler liver survey

Liver samples (n = 736) were taken from 16 poultry companies (coded A to P), which were representative of over 95% of domestic broiler production on the island of Ireland. In 2002, samples were collected from companies A to H between March and November; no samples were taken during May. The majority of the 238 samples were collected during the months of September (n = 52), October (n = 89) and November (n = 81). In 2003, samples were collected from companies A to H between February and November. The lowest and highest number of samples were taken during the months of June (n = 7) and April (n = 46), respectively. Samples were collected from companies I to P between May 2002 and April 2003. A total of 265 samples were collected and the monthly sampling numbers are described in Figure 2. Samples were screened using the immunobiosensor assay and samples found to contain DNC at levels greater than 33 µg kg^{-1} were selected for confirmatory analysis.

Broiler muscle survey

Breast meat samples (n = 342) were taken from 13 poultry companies. Three of the companies involved in the liver survey (A, G and H) did not participate in this study. Samples were collected from companies B to F (n = 127) between July and December 2003. A total of 215 samples were collected from companies I to P between May 2003 and April 2004 at a frequency of 16 to 21 samples per month. Samples were screened using the immunobiosensor assay and samples found to contain DNC at levels greater than 5 µg kg^{-1} were selected for confirmatory analysis.

Egg survey

Egg samples (n = 546) were taken by official agriculture inspectors from egg packers, representative of small, medium and large egg producers on the island of Ireland between 2003 and 2004. Samples were screened by the immunobiosensor assay and samples found to contain DNC at levels greater than 12.5 µg kg^{-1} were selected for confirmatory analysis. During sample screening, it was found that the immunobiosensor could detect DNC residues in egg samples at <12.5 µg kg^{-1}.

Imported breast muscle survey

A survey of imported broiler breast muscle was undertaken in the period 2003 to 2004. In total, 217 samples of imported breast muscle were obtained from retail outlets, from Environmental Health Officers carrying out inspections at food premises, by official agriculture inspectors at border inspection posts and meat processing companies. Samples were screened using the immunobiosensor assay and samples found to contain DNC at levels greater than 5 µg kg^{-1} were selected for confirmatory analysis.

Confirmatory analyses

Samples from all surveys screened as containing DNC residues were confirmed by an independent chemical assay (HPLC-UV or LC-MS/MS). HPLC-UV has been described as suitable for confirming the presence of Group B substances, when used in conjunction with a second independent detection assay (Anon. 2002). In this survey, an immunobiosensor was used as the second independent detection assay.

Results and discussion

Liver survey

An overall summary of the poultry liver survey is presented in Figure 1. The survey showed that 12 and 3% of samples contained DNC residues at >200 and >1000 µg kg^{-1}, respectively. DNC residues were found at <200 µg kg^{-1} in 28% of samples and no residues were detectable (i.e. not detected above 33 µg kg^{-1} by immunobiosensor and/or confirmed above 12.5 µg kg^{-1} using chemical assays) in 60% of samples.

Seasonal variation in levels of DNC in liver

The seasonal variation in DNC levels in broiler liver samples from eight companies (I to P) is shown in Figure 2. In general, there was a similarity in the proportion of samples containing DNC and of samples containing DNC at >200 µg kg^{-1} over time. In the period June to August the incidence of DNC containing liver samples was lower by a factor of at least twofold compared to

other months. A number of hypotheses may be proposed for the lower incidence of DNC residues in poultry during the June to August period. One hypothesis is that reduced usage of nicarbazin may have occurred during the summer months; nicarbazin can cause heat stress, resulting in increased mortality in broilers during warm weather. A second hypothesis is that the lower incidence of DNC residues may be related to the removal of licensing for products containing nicarbazin alone that occurred in May 2002. A third hypothesis is that the higher incidence of DNC residues in broiler liver from September onwards may have been due to changes in the feeding programmes used on poultry farms, with nicarbazin-containing feed being given to birds closer to slaughter time.

Variation in DNC levels in liver sampled from different companies

The breakdown by company of samples containing DNC residues and containing DNC residues >200 µg kg^{-1} is shown in Table I. For two companies (F and G) no samples containing DNC residues were found; however, few samples were sourced from these companies during the survey. The percentages of samples containing DNC residues range between 15 and 84% for the other companies. The mean value for 16 companies was 42% of samples contained DNC residues. The mean result for companies containing DNC residues >200 µg kg^{-1} was 14%, with the lowest at 0% and the highest at 42%. Three companies, C, E and L had 10, 22 and 33% of samples containing DNC residues but 0, 0 and 1% of samples from these companies contained DNC residues >200 µg kg^{-1}, respectively. Companies M and N had less than 10% of samples containing DNC >200 µg kg^{-1}.

Survey of breast meat

A total of 342 samples of broiler breast meat (muscle) were tested for DNC residues. The overall breakdown of results is shown in Figure 3. DNC residues were determined in 26% of samples at levels ranging between 5 and 183 µg kg^{-1}. No residues were detected in 74% of muscle samples. No muscle sample contained DNC residues above 200 µg kg^{-1}. A lower percentage of muscle samples contained measurable DNC residues compared to liver samples. A lower incidence of

DNC-positive muscle, compared with liver, is supported by the pharmacokinetic profiles, which show that DNC residues occur at higher concentrations in liver compared to muscle [Anon. 1999]. As a result, DNC residues may be detected for a longer period of time in liver and the probability of finding DNC residues in tissue is lower for muscle than for liver. The results for the muscle and liver surveys indicate that liver is the most appropriate matrix to use for monitoring DNC residues because it is the matrix in which residues persist at highest concentrations.

Seasonal variation in levels of DNC in muscle

The seasonal variation for DNC residues in breast meat is shown in Figure 4. The average monthly incidence of muscle samples containing DNC is 20%. The highest incidence occurred in the months of May, November and March with 28, 28 and 42% of muscle samples containing DNC residues, respectively. The proportion of liver samples likely to contain DNC at levels >200 μg kg^{-1} was estimated from the muscle survey results. A level of approximately 7 μg kg^{-1} DNC in muscle has been estimated as equivalent to approximately 200 μg kg^{-1} in liver tissue. This approximation is derived from the mean values obtained for a comparison of DNC residues in liver and muscle for birds raised in deep litter (9.0 μg kg^{-1} muscle ≈ 200 μg kg^{-1} liver) and those on wire flooring (5.5 μg kg^{-1} muscle ≈200 μg kg^{-1} liver) [Cannavan and Kennedy 2000]. Using this estimation, the percentage of liver samples likely to contain DNC at >200 μg kg^{-1} was calculated (Figure 4); the estimated incidence of liver samples containing >200 μg kg^{-1} DNC was similar to that observed for the liver survey, with a mean value of 14% (Table I).

Variation in DNC levels in muscle sampled from different companies

The breakdown by company of muscle samples containing DNC residues and corresponding liver samples (estimated as containing DNC residues >200 μg kg^{-1}) is shown in Table I. No samples from companies E and F contained measurable DNC residues and company C had only 3% of samples containing measurable DNC residues. For the other companies, the proportion of muscle samples containing measurable DNC ranged between 20 and 50%. A comparison between the liver survey (2002/03) and estimated liver survey (2003/04) shows that for some companies (B, I,

J, L and M) there was a higher incidence of DNC residues at >200 µg kg^{-1} in the second survey, while for companies K, N, O and P however a lower incidence in liver may be concluded from the muscle survey (Table I). Companies C and D showed no appreciable change in incidence between the two surveys.

The proportion of liver samples containing DNC residues >200 µg kg^{-1} (determined for 2002/03 and estimated for 2003/04) may vary between companies and within companies from year to year. The results of the surveys show that DNC residues in broilers occur throughout the year and that flocks require frequent sampling (such as each month), proportional to scale of production, to identify the situation.

Imported breast muscle

A total of 217 samples of imported breast meat were sampled over the period May 2003 to September 2004. The objective of the survey was to make a comparison between DNC residues in domestic and imported poultry meat consumed on the island of Ireland. The results of the survey are shown in Table II. Samples were sourced from wholesalers, meat processing companies, border inspection posts and retail outlets. The imported breast meat samples originated from 11 different countries. DNC residues were determined in 13 samples at levels ranging from 2.7 to 18.7 µg kg^{-1}. The results of the survey indicate that DNC residues were at a lower level in imported meat (6%) compared to domestic meat samples (26%). Samples from only three countries had measurable DNC residues (France, Thailand and The Netherlands). The relatively low number of positives for DNC in imported meat suggests (a) that alternative anticoccidial agents may be used in other countries and/or (b) practices are adopted at feed mills and on farms that allow better control of DNC residues in poultry production.

Survey of eggs

A survey of table eggs was carried out during 2003 to 2004. A total of 546 egg samples were collected and DNC residues were determined in nine samples. No residues were detectable (i.e. not detected above 34.8 µg kg^{-1} by immunobiosensor and/or confirmed above 12.5 µg kg^{-1} using

chemical assays) in 98% of egg samples. The levels of DNC residues present ranged between 14 and 122 µg kg^{-1}. DNC residues occurred in egg samples throughout the year. The contaminated egg samples were sourced from four companies and one of these companies had five positive samples. Nicarbazin is not approved for the treatment of layer hens and residues in eggs may be attributed to contamination of layer feed with low levels of nicarbazin [Cannavan et al. 2000]. DNC residues may be eliminated from eggs through adequate quality control of feed samples shipped to farms.

Performance of the biosensor screening assay for detection of nicarbazin residues

Connolly et al. (2003) characterised the antibody applied in this assay. The antibody was shown to be specific towards DNC and did not cross-react to other potential interfering substances namely, the anti-coccidials, toltrazuril, halofuginone and ronidazole. In routine application, it has been found that the antibody does not cross-react to lasalocid and other ionophore (monensin, salinomycin and narasin) residues detected in egg and liver samples. The cut-offs for sending samples for confirmatory analysis was determined through the analysis of 20 negative liver and 20 known negative egg samples. Briefly, analysis of 20 known negative livers yielded values of 1.19 ± 5.32 µg kg^{-1}. The limits of detection (mean + 3 S.D.) and determination (mean + 6 S.D.) were calculated as 17.1 and 33.2 µg kg^{-1}, respectively. A second validation study was carried out in eggs and analysis of 20 known negative eggs yielded values of 3.07 ± 5.29 µg kg^{-1}. The limits of detection and determination were calculated as 18.9 and 34.8 µg kg^{-1}, respectively. A more comprehensive evaluation of the suitability of the limits of determination was carried out during the egg and liver surveys. A total of 145 samples confirmed to contain DNC residues by HPLC were also screened by immunobiosensor. Evaluation of the results indicated that false negatives rates for the assay are generally <5%. In the concentration ranges >33 - 100 (n = 42 samples), >100 – 200 (n = 62), and >200 µg kg^{-1} (n = 41); 2, 1 and 0 false negative results were observed, respectively. This corresponded to false negative rates of <5, <2 and 0%, respectively. It is highlighted that no false negative results were observed at or around the MRL.

Comparison of results from official food inspection

The results for DNC residues in poultry liver and eggs samples between 2002 and 2005 indicate that the percentage of liver samples containing DNC at levels ≥200 µg/kg decreased to 7% in 2005 (O'Keeffe et al. 2005; O'Keeffe et al. 2006). This was a 50% reduction in positives from the previous three years even though the useage of Maxiban throughout Ireland had increased. The results of official food inspection for 2006 will provide a clearer indication if this reduction in DNC positives is sustainable or just a brief improvement. The reduction in DNC positives can be largely attributed to on-farm investigations, which were carried out with the aim of identifying the cause of DNC residues during 2004 to 2005 (O'Keeffe et al. 2007). In addition, there has been increased scrutiny placed on the control of DNC residues by regulatory agencies. As a result, a number of companies have instigated their own testing programs, which are aimed at fulfilling self-monitoring criteria. The results for egg testing between 2002 and 2005 indicate that the percentage of DNC positives in eggs remains static at approximately 1.5%.

Conclusions

DNC is a frequent contaminant of poultry products on the island of Ireland and has been shown to be a contaminant both of liver and breast meat. Residues may also occur in eggs but are typically at less than 2% of samples. Residues in eggs may be largely attributed to contaminated feed because administration of nicarbazin to layers is not approved because it affects bird fertility and egg shells. DNC residues occur in poultry samples throughout the year, indicating that this anticoccidial needs to be monitored continuously in poultry. Residues in liver and meat can be caused by a number of different factors, but may be attributed mainly as due to improper feed management on farms and at feed mills (O'Keeffe et al. 2007). The number of liver samples exceeding the MRL of 200 µg kg^{-1} DNC may vary considerably within individual companies between years. In 2005, there was a reduction in the number of broiler liver samples containing DNC at >200 µg kg^{-1}, to approximately 7% of samples tested in the monitoring programmes (O'Keeffe et al. 2006, Shortt 2006). The available results from official food inspections for 2006 indicate a similar lower proportion of samples containing DNC residues at levels >200 µg kg^{-1}. The reduction in DNC positives may be attributed partly to on-farm investigations, which were carried

out during 2004/05 with the aim of identifying the causes for DNC residues in poultry (O'Keeffe et al. 2007). In addition, there has been increased scrutiny on the control of DNC residues in poultry by regulatory authorities and a number of companies have instigated there own testing programmes to fulfil self-monitoring as required by EU food law (96/23/EC).

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Table I *Variation in the percentage of liver and muscle samples containing DNC residues that were surveyed from companies A to P*

Company	Liver Survey (n = 736)		Domestic Muscle Survey (n = 342)	
	Samples containing DNC ≥12.5 μg kg^{-1} (%)	Samples containing DNC ≥200 μg kg^{-1} (%)	Samples containing DNC ≥5 μg kg^{-1} (%)	Estimate of liver samples containing DNC ≥200 μg kg^{-1} (%)
A	84	26	-	-
B	47	16	38	29
C	15	1	3	0
D	41	13	36	14
E	22	0	0	0
F	0	0	0	0
G	0	0	-	-
H	40	33	-	-
I	46	11	36	17
J	67	25	30	33
K	83	25	22	9
L	33	0	27	25
M	25	6	20	17
N	44	8	20	3
O	63	42	50	29
P	59	16	20	8
Mean	42	14	23	14

Table II *The incidence of DNC residues in breast muscle samples imported into the island of Ireland*

Country	Number of samples tested	Number of samples containing DNC at >5 µg/kg
The Netherlands	68	4
Denmark	26	0
Belgium	7	0
France	10	8
Brazil	58	0
Chile	2	0
Germany	14	0
Thailand	22	1
Romania	5	0
New Zealand	4	0
UK	1	0

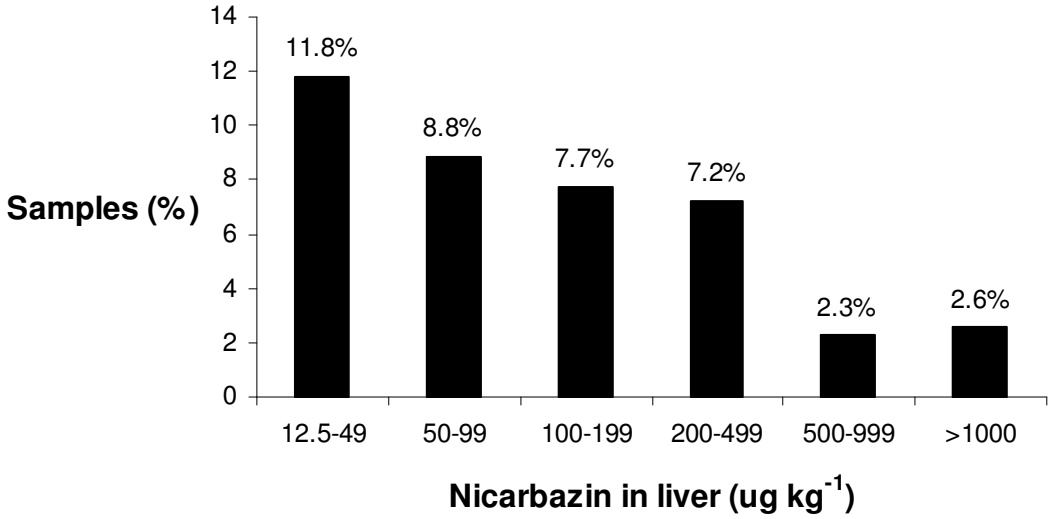

Figure 1 *Levels of DNC residues in broiler liver samples (n = 736)*

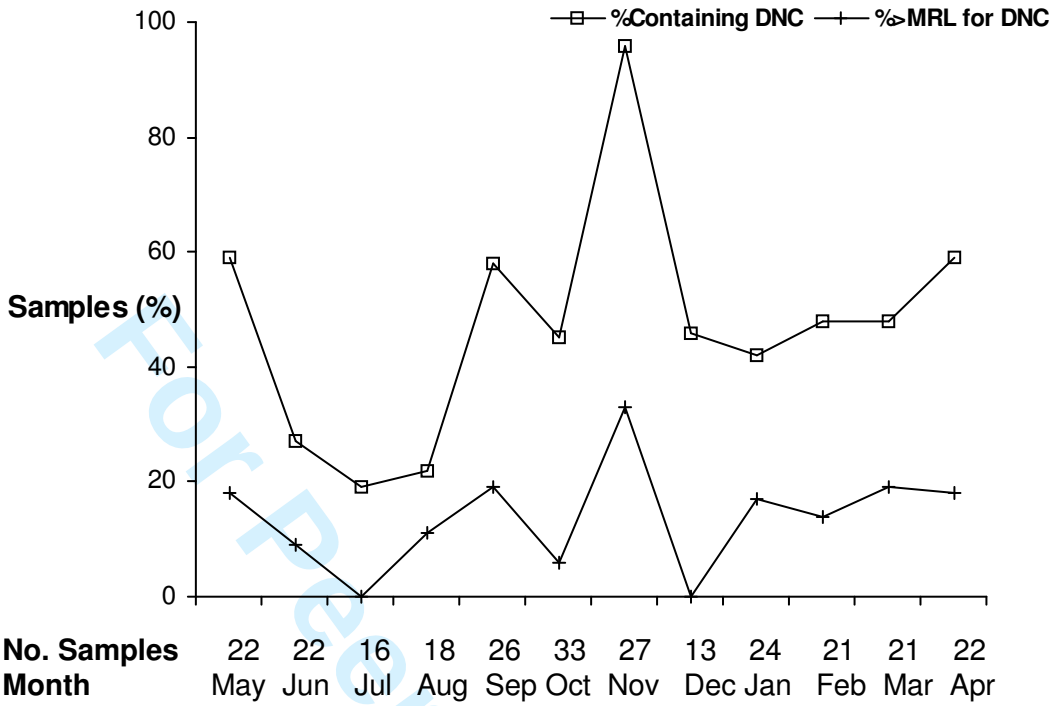

Figure 2 *Seasonal variation of DNC residues in broiler liver samples, May 2002 to April 2003 (companies I to P)*

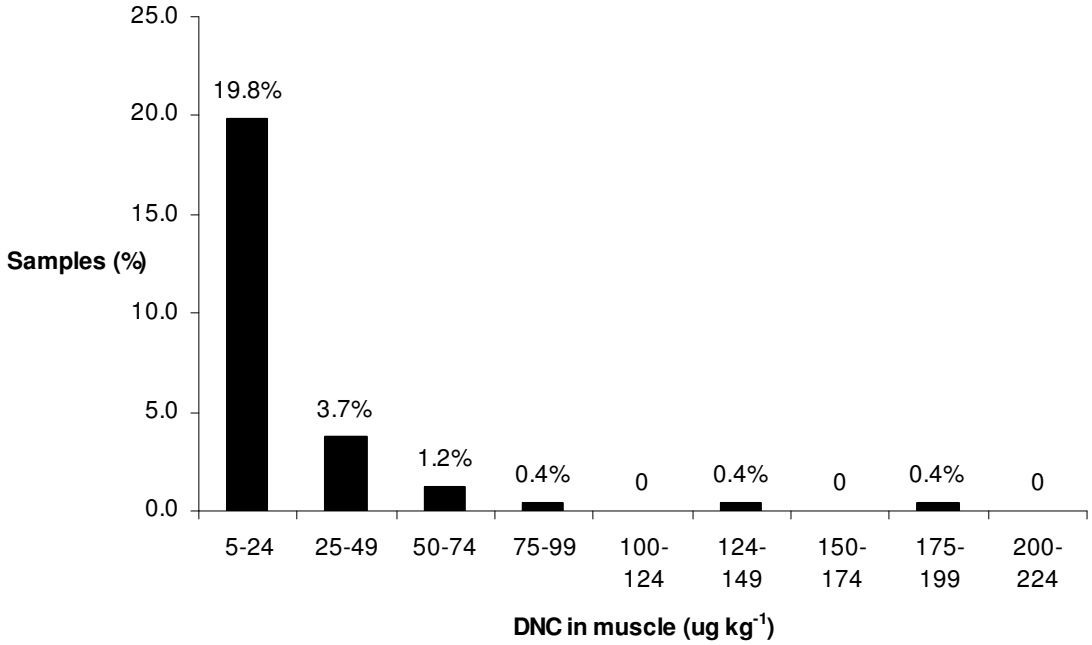

Figure 3 *Distribution of DNC residues in broiler breast samples (n = 342)*

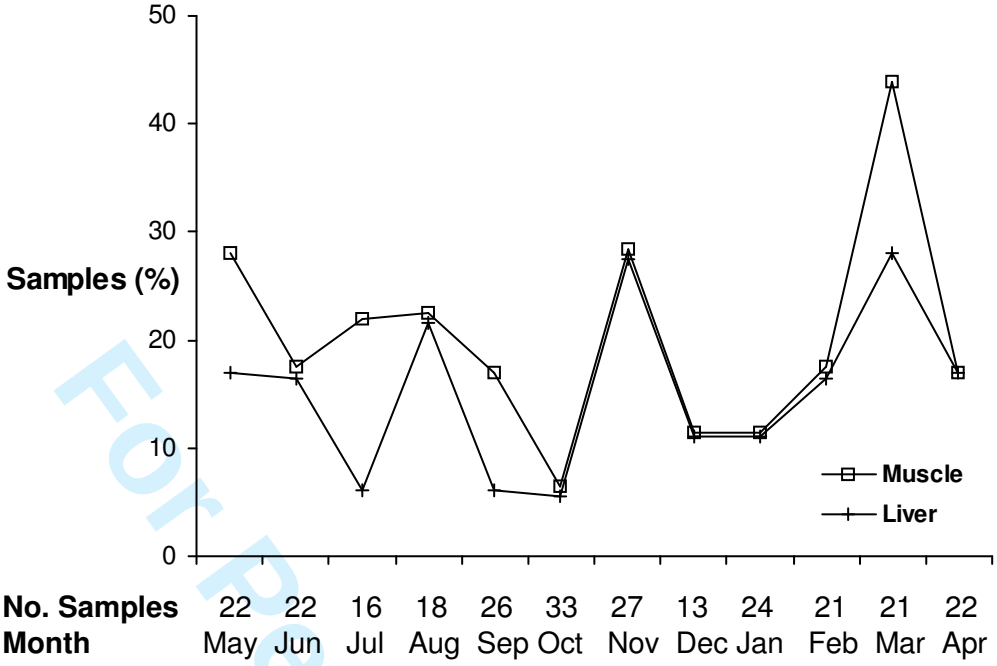

Figure 4 *Seasonal variation of DNC residues in poultry breast muscle and estimation of percentage of liver samples containing >200 µg/kg DNC, May 2003 to April 2004 (companies I to P)*