



HAL
open science

THE DETECTION, ACCUMULATION AND DISTRIBUTION OF NITROFURAN RESIDUES IN EGG YOLK, ALBUMEN AND SHELL.

David Glenn Kennedy, Robert J Mccracken

► **To cite this version:**

David Glenn Kennedy, Robert J Mccracken. THE DETECTION, ACCUMULATION AND DISTRIBUTION OF NITROFURAN RESIDUES IN EGG YOLK, ALBUMEN AND SHELL.. Food Additives and Contaminants, 2007, 24 (01), pp.26-33. 10.1080/02652030600967214 . hal-00577507

HAL Id: hal-00577507

<https://hal.science/hal-00577507>

Submitted on 17 Mar 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**THE DETECTION, ACCUMULATION AND DISTRIBUTION OF
NITROFURAN RESIDUES IN EGG YOLK, ALBUMEN AND
SHELL.**

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2006-055.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	14-Aug-2006
Complete List of Authors:	Kennedy, David; Chemical Surveillance Branch, VSD, DARD McCracken, Robert; Chemical Surveillance Branch, VSD, DARD
Methods/Techniques:	Chromatographic analysis, Extraction, LC/MS
Additives/Contaminants:	Veterinary drug residues
Food Types:	Eggs

SCHOLARONE™
Manuscripts

1
2
3
4 **The detection, accumulation and distribution of**
5
6
7 **nitrofurans residues in egg yolk, albumen and shell**
8
9

10
11 R.J. McCracken and D.G. Kennedy*.
12

13 Chemical Surveillance Branch, Veterinary Sciences Division, AFBI, Belfast,
14 Northern Ireland, UK.
15
16

17
18
19
20
21 *Corresponding author:
22

23 Dr Glenn Kennedy
24 Chemical Surveillance Department
25 Veterinary Sciences Division, AFBI
26 Stoney Road, Stormont, Belfast BT4 3SD
27 Northern Ireland, UK.
28
29

30 E-mail: glenn.kennedy@afbini.gov.uk

31 Telephone: 028 90525651

32 Facsimile: 028 90525626
33
34

35
36 Keywords: nitrofurans, eggs, LC-MSMS, eggshell, yolk, albumen.
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Abstract

Nitrofurans antibiotics have been banned for use in food producing animals in many countries including European Union, because of the threat they pose to human health. Research continues into the accumulation of these drugs in animal tissues and into the appropriate methods for their detection. In this study, an LC-MS/MS method is presented for the detection of the parent compounds, furazolidone, nitrofurantoin, furaltadone and nitrofurazone in eggs. The parent compounds are first extracted into ethyl acetate, fats are removed by partition between acetonitrile and hexane, and the concentrated sample is analysed by LC-MS/MS. Decision limits ($CC\alpha$) for the parents were $\leq 1 \mu\text{g kg}^{-1}$ for all four compounds. Within-day and between-day CVs are well within the limits stated in Commission Decision 2002/657/EC. The method provides an alternative to the testing of side-chain metabolites in eggs, which is particularly important in the case of nitrofurazone, where semicarbazide contamination of food has been attributed to sources other than nitrofurazone use. This method was used together with a method for the detection of the side-chain metabolite compounds, AOZ, AMOZ, AHD and SEM, to study the accumulation and distribution of the nitrofurans in eggs. Eggs were collected from 4 groups of hens that had been treated with one of the nitrofurans at a feed concentration of 300 mg kg^{-1} for 1 week. Parent compounds and metabolites were found in the yolk, albumen and shell. The albumen/yolk ratios for the parent compounds were 0.7, 0.82, 0.83 and 0.31 for furazolidone, furaltadone, nitrofurantoin and nitrofurazone, respectively. Ratios for the side-chain metabolites were 1.02, 1.06, 0.83 and 0.55 for AOZ, AMOZ, AHD and SEM, respectively. However, 50% of the total SEM residues were found in eggshell. This may be significant if eggshell products reach the consumer.

INTRODUCTION

Nitrofurans antibiotics were prohibited for use in food-producing animals in the European Union (EU) and other countries, in response to evidence that these compounds are both carcinogenic and mutagenic (van Koten-Vermeulen et al. 1993). In 2002 the EU had to impose restrictions on the import of poultry products from Thailand (Commission Decision 2002/251/EC) and Brazil (Commission Decision 2002/794/EC), countries whose food exports to the EU were found to contain nitrofurans residues. Since May 2003, more than 150 Rapid Alerts have been issued by the EU through its Rapid Alert System for Food and Feeds (http://europa.eu.int/comm/food/fs/sfp/ras_index_en.html) regarding the detection of nitrofurans in food from 29 countries. The presence of such residues in food, either through illegal use of the drugs or through inadvertent contamination, has made it essential for monitoring authorities to have appropriate detection methods in place to prevent such residues reaching the consumer.

An analytical method using LC-MS/MS was developed under the auspices of FoodBRAND (www.afsni.ac.uk/foodbrand), an EU funded project (Cooper et al. 2005a). This method and others, based on Solid-Phase Extraction (Leitner et al. 2001; Connely et al. 2003), are now used throughout the world to detect the marker residues of the nitrofurans drugs. Previous reports have shown that the tissue-bound side chain metabolites are the most suitable markers for nitrofurans detection in animal tissues, because they are more stable *in vivo* than the parent compounds and persist in tissues for many weeks (Cooper et al. 2005a). However, in the case of nitrofurazone (NFZ), whose side-chain metabolite is semicarbazide (SEM), this practice has proved unreliable, because SEM has been attributed to other sources of contamination in food

1
2
3 (Kennedy et al., 2004; Pereira et al. 2004). Following a spate of positive SEM
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(Kennedy et al., 2004; Pereira et al. 2004). Following a spate of positive SEM detection in coated chicken products, the source of SEM contamination was attributed to the presence of azodicarbonamide, a flour treatment agent, used in the coating material. It did not therefore indicate the use of nitrofurazone in chicken production. Consequently, attention has focused upon finding alternatives to SEM as a marker for the detection of nitrofurazone abuse. Recently, it was reported that intact nitrofurazone accumulates in whole eyes of chickens treated with the drug (Cooper et al. 2005b). The detection of the parent drug in retina is therefore a realistic alternative to SEM to monitor for abuse in primary production. This, of course, is not possible for the analysis of processed meat products. Arising from the crisis in 2002 of nitrofurans contamination in imported poultry and shellfish, and the subsequent doubts over the use of SEM as a marker residue is the need for further information on the accumulation of nitrofurans in food products. This requirement goes hand-in-hand with the need for sensitive confirmatory methods for detecting appropriate residues, as required under Commission Decision 2002/657/EC.

As part of a continuing investigation into the most appropriate markers for nitrofurans detection and the development of confirmatory methods, an evaluation of the residues for the four main nitrofurans in chicken eggs was undertaken. It has been known for some time that furazolidone (FZD) accumulates as intact parent compound in chicken eggs (Botsoglou 1988, McCracken et al. 2001). Levels of the drug reached a plateau of approximately $300 \mu\text{g kg}^{-1}$ after the fourth day of treatment, and parent compound could still be detected up to the ninth day after withdrawal from medication.

1
2
3 Two analytical methods were required to monitor the accumulation of residues in
4 eggs. Firstly, the existing LC-MSMS method for nitrofurans metabolites in tissues
5 was applied to the analysis of eggs. Secondly, a new LC-MSMS method for the
6 detection of the intact parent compounds was developed. This new method is
7 presented for the analysis of whole egg, with validation data obtained using the
8 guidelines contained in Commission Decision 2002/657/EC. The accumulation of
9 parent compound and side-chain metabolites in yolk, albumen and shell is presented
10 in order to illustrate, that for poultry eggs, the analysis of intact parent compound
11 residues is feasible as part of a residue monitoring programme. The procedure is less
12 complicated than the detection of metabolites, and samples are processed rapidly, thus
13 providing a satisfactory alternative to metabolite testing. This is particularly
14 important in the case of nitrofurazone, because the parent compound can be
15 determined instead of semicarbazide, which has been shown to be insufficiently
16 specific as an indicator for nitrofurazone use.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38

39 The study of nitrofurans accumulation in chicken eggs was extended to include the
40 analysis of the drugs and metabolites in eggshell. There are several varied
41 applications for the utilisation of eggshell waste from poultry production. In the US,
42 scientists have produced dietary phosphorous supplements for poultry, pigs and pets
43 (Miller 2001). Eggshell has been used to replace limestone as a source of calcium,
44 and has been combined with phosphoric acid to produce mono- and di-calcium
45 phosphate. The use of eggshell powder as a source of calcium has also been
46 evaluated for human nutrition (Schaafsma et al. 2000). It has been demonstrated that
47 eggshell matrix proteins enhance calcium transport in human intestinal epithelial cells
48 (Daengprok et al. 2002). Collagen, derived from eggshell membrane, is used in
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 cosmetics, wound dressings, surgical implants and intrusive device coatings. Gelatine
4 is used in the food and confectionery industries, and sialic acid is a precursor for use
5 in anti-inflammatory drugs. The utilisation of eggshell waste is therefore another
6 route potentially for nitrofurantoin contamination to reach the consumer.
7
8
9
10
11

12
13
14
15 The work presented therefore has three important implications. Firstly, that
16 confirmation of nitrofurantoin intact parent compounds in eggs is possible using
17 LC-MS/MS. Secondly, that intact parent compounds accumulate in eggs and are
18 appropriate markers for monitoring nitrofurantoin abuse in laying chickens. Thirdly, that
19 nitrofurans accumulate in eggshell and because of the utilisation of eggshell waste
20 there exists the potential for nitrofurans to reach the consumer by this route.
21
22
23
24
25
26
27
28
29
30
31
32
33

34 **EXPERIMENTAL**

35 **Materials**

36
37 Furazolidone (FZD), nitrofurazone (NFZ), furaltadone (FTD) and nitrofurantoin
38 (NFT) were obtained from Sigma-Aldrich (Poole, UK). The metabolite compounds:
39 3-amino-2-oxazolidinone (AOZ) and semicarbazide (SEM) hydrochloride were
40 obtained from Sigma-Aldrich; 1-amino-hydantoin (AHD) hydrochloride and 3-amino-
41 5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ) were obtained from Chemical
42 Synthesis Services (Craigavon, UK). D₄-Furazolidone, D₅-furaltadone,
43 ¹³C¹⁵N₂-nitrofurazone, D₄-AOZ, and D₅-AMOZ were also obtained from Chemical
44 Synthesis Services. ¹³C¹⁵N₂-SEM and ¹³C₃-AHD were obtained from Witega (Berlin,
45 Germany). All other reagents and solvents were analytical and HPLC grade,
46 respectively.
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Determination of parent nitrofurans residues in eggs.

Samples of whole egg, yolk, albumen (5 g) or shell (0.5 g) were placed in 30 ml glass centrifuge tubes. Internal standard solution (50 μl of 1 $\mu\text{g ml}^{-1}$ solutions in methanol) containing D⁴-FZD, D⁵-FTD and ¹³C ¹⁵N₂ -NFZ was added to each tube. Ethyl acetate (12 ml) was added, and samples were homogenised using a Silverson high-speed emulsifier for 1 min. Following centrifugation at 2200 rpm for 15 min., supernatants were transferred to clean tubes and evaporated to dryness under nitrogen at 50 °C. Acetonitrile (5 ml) was added to the dry residue, and hexane (3 ml) was added while vortexing. Following centrifugation, the hexane layer was discarded and the acetonitrile evaporated under nitrogen at 50 °C. The dry residue was redissolved in 500 μl of methanol-water (1:1, v/v), transferred to Eppendorf vials and centrifuged at 10 000 rpm for 10 min. Aliquots (140 μl) were transferred to autosampler vials prior to analysis by LC-MS/MS.

LC-MS/MS analysis of parent nitrofurans.

An Agilent 1100 Series HPLC system (Agilent Technologies, USA) coupled to a Quattro Ultima[®] Platinum tandem mass detector (Micromass, UK) incorporating MassLynx[®] software were used for nitrofurans analysis. The mass spectrometer was operated in electrospray positive mode for the analysis of FZD and FTD, and in electrospray negative mode for NFT and NFZ. Data acquisition was performed in multiple reaction monitoring (MRM) mode. The time-sectored ion transitions, dwell times, cone voltages and collision energies are shown in Table 1. The source settings were as follows: source and desolvation temperatures were 120 and 350°C, respectively, cone and desolvation gas flows were 99 and 800 L hr⁻¹, respectively,

1
2
3 capillary 3.2 kV, RF lens 5.0. Argon was used as collision gas and the multiplier was
4
5 operated at 650 V.
6
7

8
9
10 The HPLC analytical column was a Columbus C18 (2) 5 μ m, 2.0 x 150 mm
11
12 (Phenomenex, UK). A binary gradient mobile phase was used at a flow rate of 0.2 ml
13
14 min⁻¹. Solvent A consisted of 0.5 mM ammonium acetate and methanol (80:20, v/v),
15
16 and solvent B was methanol. The gradient ran from 10 % solvent B initially to a
17
18 maximum of 80% after 8 min, was held until 15 min, returning to 10 % after 15.5
19
20 min. The sample injection volume was 5 μ l. Nitrofurans concentrations in samples
21
22 were calculated by comparing the ratio of the nitrofurans base peak to the internal
23
24 standard peak with the same ratio in the calibration curve standards.
25
26
27
28
29
30
31

32 **Validation of the method for parent nitrofurans in eggs.**

33
34 The method for the determination of parent nitrofurans compounds was validated for
35
36 whole egg homogenates in accordance with the guidelines stated in Commission
37
38 Decision 2002/657/EC. Whole egg homogenate, fortified with each of the nitrofurans
39
40 (FZD, FTD, NFT and NFZ) at concentrations of 2, 3 and 4 μ g kg⁻¹ were analysed on
41
42 three separate occasions (n=7). Samples were quantified against standard calibration
43
44 curves in the range, 0, 1, 2, 5, 10 and 20 μ g kg⁻¹.
45
46
47
48
49
50

51 **Determination of nitrofurans side-chain metabolites in eggs using LC-MS/MS.**

52
53 The protein-bound, side-chain metabolites for each of the nitrofurans were determined
54
55 using the extraction procedure reported previously for the determination of AOZ in
56
57 eggs (McCracken et al. 2001). Identification and quantification was performed using
58
59 liquid chromatography tandem mass spectrometry. The mass spectrometric
60

1
2
3 conditions used were developed under the auspices of EU project, QLK1-CT-1999-
4 00142 known as “FoodBRAND” (Cooper et al. 2005a). Briefly, egg homogenate,
5 albumen, yolk (1 g) or shell (0.5 g) was mixed in a 50 ml centrifuge tube with internal
6 standard solution containing D⁴-AOZ, D⁵-AMAZ, ¹³C ¹⁵N₂-SEM and ¹³C₃-AHD (50 μl
7 of 100 ng ml⁻¹), 4 ml of water, 0.5 ml of 1 M hydrochloric acid and 150 μl of
8 2-nitrobenzaldehyde (50 mM in dimethylsulphoxide). The tube was stoppered and
9 the mixture incubated in a water bath at 37 °C for 16 hours. Nitrofurans side-chain
10 metabolites (AOZ, AMAZ, SEM and AHD) present in the egg sample were
11 derivatised by the 2-nitrobenzaldehyde to produce the corresponding nitrophenyl
12 derivative. Following the incubation, the pH of the mixture was adjusted to
13 approximately 7.4 by the addition of 5 ml of 0.1 M dipotassium hydrogen
14 orthophosphate and 0.4 ml of 1M sodium hydroxide. The nitrophenyl derivatives
15 were extracted by shaking twice with 5 ml of ethyl acetate. The solvent was removed
16 under nitrogen at 50°C and the residue redissolved in 0.5 ml of methanol:water (1:1,
17 v/v). The sample was transferred to a centrifuge tube and centrifuged at 10 000 rpm
18 for 10 min. An aliquot of the supernatant was transferred to an autosampler vial for
19 LC-MS/MS analysis.

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 **Treatment of laying hens with nitrofurans.**

48 Four groups of six laying hens were held in wire-floored cages. They were allowed
49 *ad libitum* access to fresh water at all times. Each group was fed a standard layers
50 diet for three days prior to commencement of the experiment. After this period one
51 group was fed a layer feed medicated at 300 mg kg⁻¹ with furazolidone. In the same
52 way the other groups were fed with furalfadone, nitrofurantoin or nitrofurazone. After
53 feeding for 1 week and with no withdrawal from treatment, eggs were collected for 2
54
55
56
57
58
59
60

1
2
3 days from each cage. They were separated into yolk, albumen and shell, and analysed
4
5 for the presence of bound residues of AOZ, AMOZ, SEM and AHD, and for parent
6
7 compounds. The analysis for parent compounds was performed on the day of
8
9 sampling.
10
11

12 13 14 15 **RESULTS AND DISCUSSION**

16
17 Method validation results for the analysis of parent nitrofurans are shown in Tables 2
18
19 and 3. Calibration curves for standard solutions were linear in the range 1-20 $\mu\text{g kg}^{-1}$
20
21 (Table 2). Decision Limits ($CC\alpha$) for the parents were $<1 \mu\text{g kg}^{-1}$ for all four
22
23 compounds (Table 3). Detection Capabilities ($CC\beta$) were lower for furazolidone and
24
25 furaltadone by comparison with nitrofurazone and nitrofurantoin. Nevertheless,
26
27 confirmation of residues was possible at $2 \mu\text{g kg}^{-1}$ for all four compounds in all of the
28
29 replicates analysed. Intra and inter run CVs are well within the acceptable values
30
31 predicted by the Horwitz equation (Commission Decision 2002/657/EC).
32
33 Concentrations for furazolidone, furaltadone and nitrofurazone were calculated using
34
35 the appropriate internal standard. An internal standard was not available for
36
37 nitrofurantoin, however concentrations were calculated using the D⁴ furazolidone.
38
39 For this reason, recovery values for nitrofurantoin were lower than the other 3
40
41 nitrofurans.
42
43
44
45
46
47
48
49
50

51
52 There are numerous methods reported for the determination of nitrofuran residues in
53
54 eggs. Kumar et al. (1994) reported an LC method with UV detection. The limits of
55
56 detection were $1 \mu\text{g kg}^{-1}$ for nitrofurazone and furazolidone; and $2 \mu\text{g kg}^{-1}$ for
57
58 furaltadone. This procedure, however, would not meet present EU criteria as a
59
60 confirmatory method. Draisci et al. (1997) confirmed nitrofuran residues in eggs

1
2
3 using LC-ion spray mass spectrometry with an atmospheric pressure ionisation
4 interface. Limits of detection were 3.2, 1.6 and 1.0 $\mu\text{g kg}^{-1}$ for nitrofurazone,
5
6
7
8 furazolidone and furaltadone, respectively. Only single protonated-molecular ions
9
10 were monitored, and again this would not meet EU criteria as a confirmatory method.
11
12 Since 2002, and the start of the crisis related to nitrofurantoin contamination in poultry
13 and shellfish, most residues monitoring programmes have focussed upon the detection
14
15 of the side-chain metabolites. This was justified on the basis of the stability *in vivo* of
16
17 metabolite residues compared to the parent compounds. However, this is not
18
19 necessarily the case when considering other matrices such as eggs. Botsoglou (1988)
20
21 and McCracken et al. (2001) demonstrated that intact furazolidone residues
22
23 accumulate in eggs and may still be detected as such up to 9 days after withdrawal of
24
25 medicated feed. A Minimum Required Performance Limit (MRPL) of 1.0 $\mu\text{g kg}^{-1}$ has
26
27 been set for all four side-chain metabolites in poultry meat (Commission Decision
28
29 2003/181/EC). Subsequently, Commission Decision 2005/34/EC extended the MRPL
30
31 definition to cover all matrices. No MRPL has been established for the nitrofurantoin
32
33 parent drugs in eggs, or in any other tissue. The developed method may find two
34
35 potential applications. Firstly, it may be used as the primary test used to detect
36
37 nitrofurantoin residues in eggs. Secondly, it is quicker and simpler than methods for the
38
39 determination of the side chain metabolites. It may also be used to back up the results
40
41 obtained using the more widely used method for the detection of the side chain
42
43 metabolites. Specifically, it might be used to confirm the presence of nitrofurazone
44
45 following the finding of semicarbazide in eggs. This would provide unambiguous
46
47 evidence for the administration of nitrofurazone to the chickens and would remove
48
49 any possible ambiguity associated with the use of semicarbazide as a marker. Stability
50
51 experiments performed at this laboratory and reported elsewhere indicate that while
52
53
54
55
56
57
58
59
60

1
2
3 SEM has a depletion half-life in eggs of 2.4 days, the half-life for parent nitrofurazone
4 is 1.1 days (Cooper et al., 2006). Parent nitrofurazone was detected in eggs up to 3
5 days post-withdrawal when chickens were fed at only 1% of the therapeutic dose (3
6 mg kg⁻¹). In eggs pasteurised for 4 min. at 64-66 °C, followed by spray drying at 180-
7 230 °C, parent nitrofurazone depleted by 65%. It is possible, however that albumen
8 powder containing over 5 µg kg⁻¹, SEM may contain detectable quantities of parent
9 nitrofurazone.
10
11
12
13
14
15
16
17
18
19
20
21

22 The distributions of parent compound and side chain metabolite residues in yolk,
23 albumen and shell for each nitrofurazone are shown in Table 4. For all four drugs,
24 residues of parent compounds accumulated in yolk, albumen and shell. The chemical
25 basis for the detection of AOZ, AMOZ, AHD and SEM means that any parent
26 compound present in the sample will also be measured as its corresponding metabolite
27 together with any other moiety containing acid-releasable AOZ, AMOZ, AHD or
28 SEM. In this study, where there was no withdrawal from treatment before eggs were
29 collected, it was expected that most of the residues occurring in the yolk and albumen
30 might be present as intact parent compound. This was not the case. In terms of the
31 number of moles detected, the concentrations of side-chain metabolites greatly
32 exceeded that of the parent compound for all four nitrofurans. This suggests that over
33 the 6-day treatment period, and despite zero withdrawal, most of the drug was present
34 not as the parent – but as other moieties which contain the intact side-chain.
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

1
2
3 degradation therefore, occurred during the period of treatment, and much of it prior to
4
5 deposition in the egg.
6

7
8 For furazolidone the albumen/yolk ratio of parent residues was 0.7 compared to 1.02
9
10 of AOZ. Similar values were found for furaltadone: 0.82 of parent and 1.06 for
11
12 AMOZ; and for nitrofurantoin: 0.83 parent compared to 1.01 for AHD. Residue
13
14 concentrations of nitrofurantoin were lower than the other nitrofurans, once again
15
16 reflecting the poor absorption of this drug in chickens (McCracken et al. 2005). The
17
18 concentration of residues and their distribution in yolk and albumen was quite
19
20 different in the case of nitrofurazone. Higher concentrations of parent compound
21
22 were detected in yolk (73%) compared to albumen (23%), giving an albumen/yolk
23
24 ratio of 0.31. The albumen/yolk ratio for SEM was 0.55. Reasons for the difference
25
26 in distribution between compounds may be attributable to the physiochemical
27
28 characteristics of the drugs and to the physiology of the hen and the formation of the
29
30 egg. Kan and Petz (2000) compiled a database from the literature on drug residues in
31
32 egg yolk and white. White/yolk ratio values for the four nitrofurans compare
33
34 favourably with those reported here, but the authors concluded that there was no
35
36 single factor apparent that determines the distribution of drug between yolk and
37
38 albumen. The difference between the yolk/albumen ratio for nitrofurazone and the
39
40 other nitrofurans therefore may not be significant. Contrary to this, Kan and Petz also
41
42 discussed the striking difference in distribution between yolk and white of narasin and
43
44 salinomycin (white/yolk ratios of 0.31 and <0.007, respectively). These large
45
46 molecules (molecular weights: 765 and 751 for narasin and salinomycin, respectively)
47
48 differ only by one methyl group. Similarly, the differences in distribution of the
49
50 nitrofurans may in part be due to the molecular structure of the compounds. The most
51
52 striking difference in residue distribution of the nitrofurans however, was that 50% of
53
54
55
56
57
58
59
60

1
2
3 the total SEM residues found in the egg were detected in the shell. A mean
4
5 concentration of 24.2 nmol g⁻¹ of SEM was found in shell compared to only 0.24 nmol
6
7 g⁻¹ of parent nitrofurazone. Eggshells used as negative controls were free of SEM,
8
9
10 which eliminates the possibility of SEM occurring by any means other than
11
12 nitrofurazone treatment of the laying birds. This concentration is significantly higher
13
14 than for the side-chain metabolites of the other nitrofurans and illustrates the potential
15
16 for SEM contamination to occur in eggshell products. The concentration of residues
17
18 in shell were slightly higher for furazolidone compared to furaltadone but did not
19
20 amount to more than 10% of the total residues in the egg. The result reported for NFT
21
22 in shell represents detection of the drug in only 1 of the six samples tested.
23
24
25
26
27
28

29 The utilisation of eggshell waste is a growing industry. Eggshell is mainly composed
30
31 of calcium carbonate (94%), but also contains small amounts of magnesium
32
33 carbonate, calcium phosphate and organic matter including protein. AOZ, AMOZ,
34
35 AHD and SEM have been considered as part of the side-chain metabolites of the
36
37 nitrofurans that are bound to protein in the tissues of the animal. It is not known
38
39 whether all of the metabolites detected in the eggshell are bound to the eggshell
40
41 proteins, but it remains a possibility. However, it is clear that nitrofurans residues
42
43 accumulate in eggshell and therefore pose a threat to health, where eggshell products
44
45 are consumed.
46
47
48
49
50
51
52

53 In conclusion, residues of nitrofurans are deposited throughout the stages of egg
54
55 production: in the development of yolk, in the albumen and during the shelling
56
57 process. These residues, at least with zero withdrawal from treatment, can be detected
58
59 as intact parent compound. The method presented is suitable as a confirmatory
60

1
2
3 procedure and can be used together with the established method for the metabolites to
4
5
6 form an effective residue control programme.
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review Only

REFERENCES

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Botsoglou NA. 1988. Determination of furazolidone in eggs by high-performance liquid chromatography. *Journal of Agricultural Food Chemistry*, 36, pp1224-1227.
- Commission Decision 2002/251/EC, of 27 March 2002 concerning certain protective measures with regard to poultry meat and certain fishery and aquaculture products intended for human consumption and imported from Thailand. *Official Journal of the European Communities*, L84, pp 77-78.
- Commission Decision 2002/657/EC, of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Communities*, L221, pp 8-36.
- Commission Decision 2002/794/EC, of 11 October 2002 concerning certain protective measures with regard to poultry meat, poultry meat products and poultry meat preparations intended for human consumption and imported from Brazil. *Official Journal of the European Communities*, L276 pp 66-67.
- Commission Decision 2003/181/EC, of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Official Journal of the European Union*, L71 pp 17-18.
- Commission Decision 2005/34/EC, of 11 January 2005 laying down harmonised standards for the testing for certain residues in products of animal origin imported from third countries. *Official Journal of the European Union*, L61 pp 61-63.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Conneely A, Nugent A, O’Keeffe M, Mulder PPJ, Van Rhijn JA, Kovacsics L, Fodor A, McCracken RJ, Kennedy DG. 2003. Isolation of bound residues of nitrofurans from tissue by solid-phase extraction with determination by liquid chromatography with UV and tandem mass spectrometric detection. *Analytica Chimica Acta*, 483, pp 91-98.
- Cooper KM, Mulder PPJ, Van Rhijn JA, Kovacsics L, McCracken RJ, Young PB, Kennedy DG. 2005a. Depletion of four nitrofurans and their tissue-bound metabolites in porcine tissues and determination using LC-MS/MS and HPLC-UV. *Food Additives and Contaminants*, 22, pp 406-414.
- Cooper KM, McCracken RJ, Kennedy DG. 2005b. Nitrofurazone accumulates in avian eyes—a replacement for semicarbazide as a marker of abuse. *Analyst*, 130, pp 824-827.
- Cooper KM, Jian L, Kane C, Kennedy DG. 2006. A study of the kinetics of semicarbazide and nitrofurazone in chicken eggs and egg powders. Presented at the Fifth International Symposium on Hormone and Veterinary Drug Residues Analysis, 16-19 May, Antwerp, Belgium.
- Daengprok W, Rupa P, Mine Y. 2002. Hen eggshell matrix proteins enhance calcium transport in the human intestinal epithelial cells. Annual meeting and food expo-Anaheim, California 17th June, session 61D, Nutraceuticals and Functional Foods II. Institute of Food Technologists, The society for Food Science and Technology.
- Draisci R, Giannetti L, Lucentini L, Palleschi L, Brambilla G, Serpe L, Gallo P. 1997. Determination of nitrofurans in avian eggs by liquid chromatography-UV photodiode array detection and confirmation by liquid chromatography-ion spray mass spectrometry. *Journal of Chromatography A* 777, pp 201-211.
- European Commission Rapid Alert System for Food and Feed web site, http://europa.eu.int/comm/food/fs/sfp/ras_index_en.html.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Kan CA, Petz M. 2000. Residues of veterinary drugs in eggs and their distribution between yolk and white. *Journal of Agricultural Food Chemistry*, 48, pp 6397-6403.
- Kennedy, DG, van Rhijn, J.A., & Kanarat, S. 2004. Azodicarbonamide, a flour treatment agent, produces semicarbazide residues in bread. In: *EuroResidue V Conference on Residues of Veterinary Drugs in Food*. The Netherlands FECS Event No. 277 10-12 May, 2004 (Editors: van Ginkel, L. A. and Bergwerff, A. A.) pp 612-617.
- Kumar L, Toothill JR, Koon Bay Ho. 1994. Determination of nitrofurans residues in poultry muscle tissues and eggs by liquid chromatography. *Journal of AOAC International*, 77, pp 591-595.
- Leitner A, Zollner P, Lindner W. 2001. Determination of the metabolites of nitrofurans antibiotics in animal tissue by high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 939, pp 49-58.
- McCracken RJ, Spence DE, Floyd SD, Kennedy DG. 2001. Evaluation of the residues of furazolidone and its metabolite, 3-amino-2-oxazolidinone (AOZ), in eggs. *Food Additives and Contaminants*, 18, pp 954-959.
- McCracken RJ, Van Rhijn JA, Kennedy DG. 2005. Transfer of nitrofurans residues from parent broiler breeder chickens to broiler progeny. *British Poultry Science*, 46, pp 287-292.
- Miller V. 2001. Using the old egg (shell). Research Nebraska, University of Nebraska-Lincoln Agricultural Research Division web site, <http://ard.unl.edu/rn/0901/egg.html>.
- Pereira AS, Donato JL, De Nucci G. 2004. Implications of the use of semicarbazide as a metabolic target of nitrofurans contamination in coated products. *Food Additives and Contaminants*, 21, pp 63-69.

1
2
3 Schaafsma A, Pakan I, Hofstede GJH, Muskiet FAJ, Van Der Deer E, De Vries PJF.
4
5 2000. Mineral, amino acid and hormonal composition of chicken eggshell
6
7 powder and the evaluation of its use in human nutrition. Poultry science 79, pp
8
9 1833-1838.

10
11
12 Van Koten-Vermeulen, JEM, Wouters, MFA and Van Leeuwen, FXR 1993,
13
14 Toxicological evaluation of certain veterinary drug residues in food. Report of
15
16 the 40th Meeting of the Joint FAO/WHO Expert Committee on Food
17
18 Additives (JECFA). World Health Organisation, Geneva, pp 85-123.
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Data acquisition parameters for the Multiple Reaction Monitoring (MRM) of parent nitrofurans.

Compound	Ion transition	Dwell time (sec)	Cone voltage (V)	Collision energy (eV)
Function: 1: Electrospray positive mode (0 – 20 min)				
Furazolidone	226.2 > 139.2	0.40	35	13
	226.2 > 209.3	0.60	35	13
D ₄ FZD	230.2 > 139.2	0.30	35	13
Function 2: Electrospray negative mode (0 – 20 min)				
Nitrofurazone	197.2 > 124.2	0.40	35	7
	197.2 > 150.2	0.70	35	7
¹³ C ¹⁵ N ₂ NFZ	200.2 > 153.2	0.70	35	7
Nitrofurantoin	237.1 > 152.2	0.40	35	12
	237.1 > 194.2	0.70	35	8
Function 3: Electrospray positive mode (5 – 20 min)				
Furaltadone	325.3 > 252.4	0.20	35	15
	325.3 > 281.3	0.20	35	9
D ₅ -FTD	330.3 > 286.3	0.20	35	10

Table 2. Typical values for calibration of parent nitrofurantoin standard solutions in the range 1-20 $\mu\text{g kg}^{-1}$

	Typical equation of curve	Correlation coefficient (r)
Nitrofurazone	$y = 0.7872x - 0.033$	0.9989
Furazolidone	$y = 0.8322x + 0.194$	0.9971
Furaltadone	$y = 0.9698x + 0.206$	0.9993
Nitrofurantoin	$y = 0.0781x + 0.011$	0.9977

Table 3. Summary of validation results for the determination of parent nitrofurans in whole egg homogenate.

	Fortification Level ($\mu\text{g kg}^{-1}$)	Overall Mean ($\mu\text{g kg}^{-1}$) n=21	Overall recovery (%)	Within Day CV	Between Day CV
Furazolidone	2.0	2.2	110	6.4	7.4
	3.0	3.3	110	7.0	6.8
	4.0	4.5	113	5.7	9.0
	CCα	0.7 $\mu\text{g kg}^{-1}$			
	CCβ	1.2 $\mu\text{g kg}^{-1}$			
Furaltadone	2.0	2.3	115	6.7	6.2
	3.0	3.3	110	4.8	4.9
	4.0	4.6	115	4.5	7.6
	CCα	0.5 $\mu\text{g kg}^{-1}$			
	CCβ	0.8 $\mu\text{g kg}^{-1}$			
Nitrofurazone	2.0	2.3	115	9.6	11.8
	3.0	3.3	110	10.1	12.9
	4.0	4.6	115	7.0	12.1
	CCα	1.0 $\mu\text{g kg}^{-1}$			
	CCβ	1.7 $\mu\text{g kg}^{-1}$			
Nitrofurantoin	2.0	1.5	75	10.0	23.5
	3.0	2.3	77	7.3	6.9
	4.0	2.8	70	7.3	8.4
	CCα	1.0 $\mu\text{g kg}^{-1}$			
	CCβ	1.7 $\mu\text{g kg}^{-1}$			

Table 4. The distribution of nitrofuran parent compound and side chain metabolite residues in yolk, albumen and shell.

	Parent compounds (Mean \pm SE, n=6)			Side-chain metabolites (Mean \pm SE, n=6)		
	Yolk	Albumen	Shell	Yolk	Albumen	Shell
Furazolidone						
nmol g ⁻¹	1.82 \pm 0.30	1.29 \pm 0.16	0.23 \pm 0.03	4.68 \pm 0.57	4.81 \pm 0.66	1.12 \pm 0.38
Furaltadone						
nmol g ⁻¹	1.83 \pm 0.08	1.50 \pm 0.10	0.11 \pm 0.03	2.93 \pm 0.23	3.10 \pm 0.14	0.28 \pm 0.07
Nitrofurantoin						
nmol g ⁻¹	0.28 \pm 0.02	0.23 \pm 0.03	0.15	1.47 \pm 0.12	1.49 \pm 0.11	0.17 \pm 0.03
Nitrofurazone						
nmol g ⁻¹	4.18 \pm 0.59	1.30 \pm 0.13	0.24 \pm 0.04	15.13 \pm 0.81	8.44 \pm 1.19	24.2 \pm 2.97

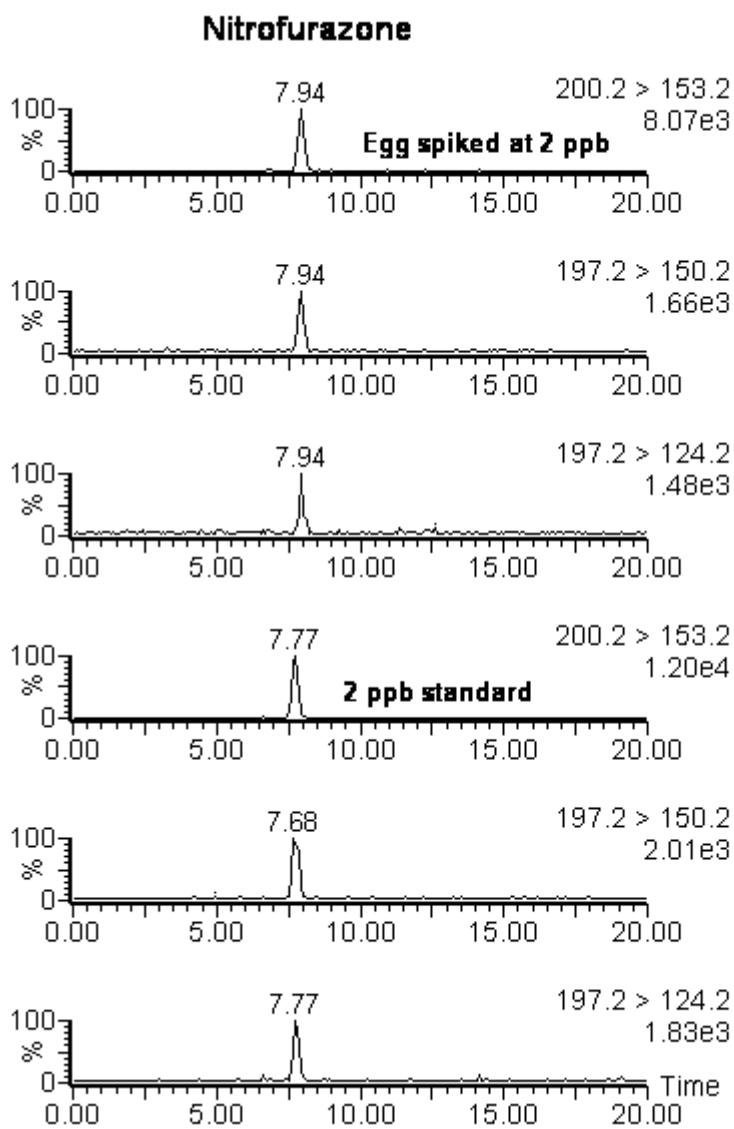


Figure 1a.

Only

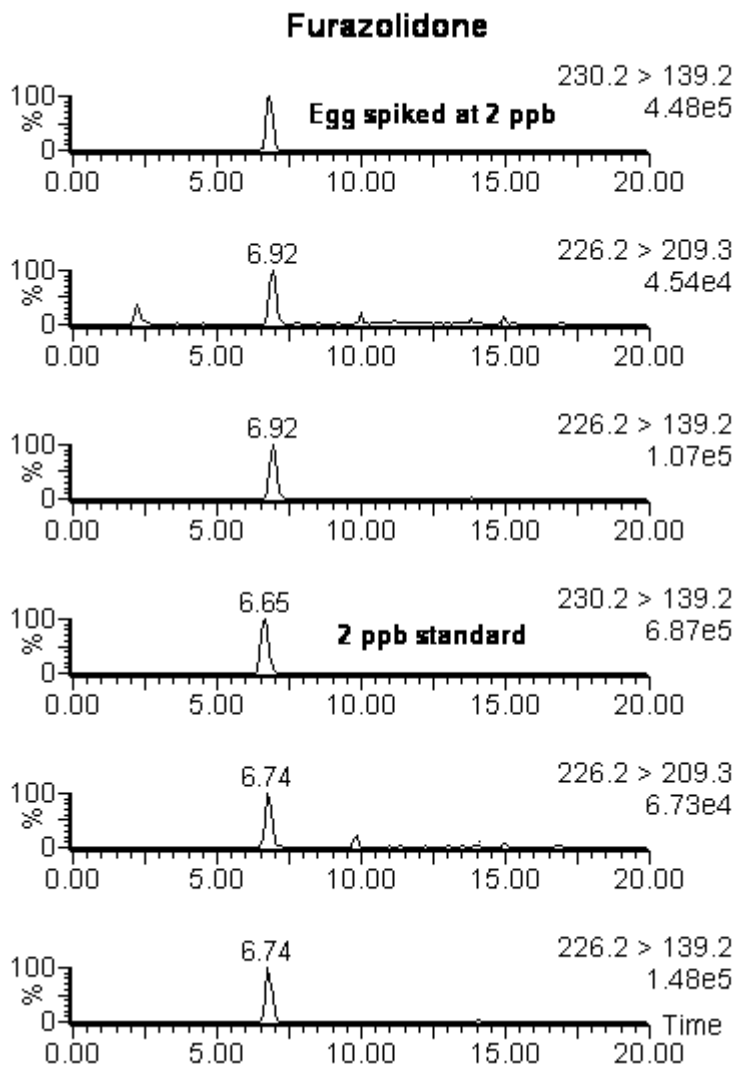


Figure 1b

Manuscript Only

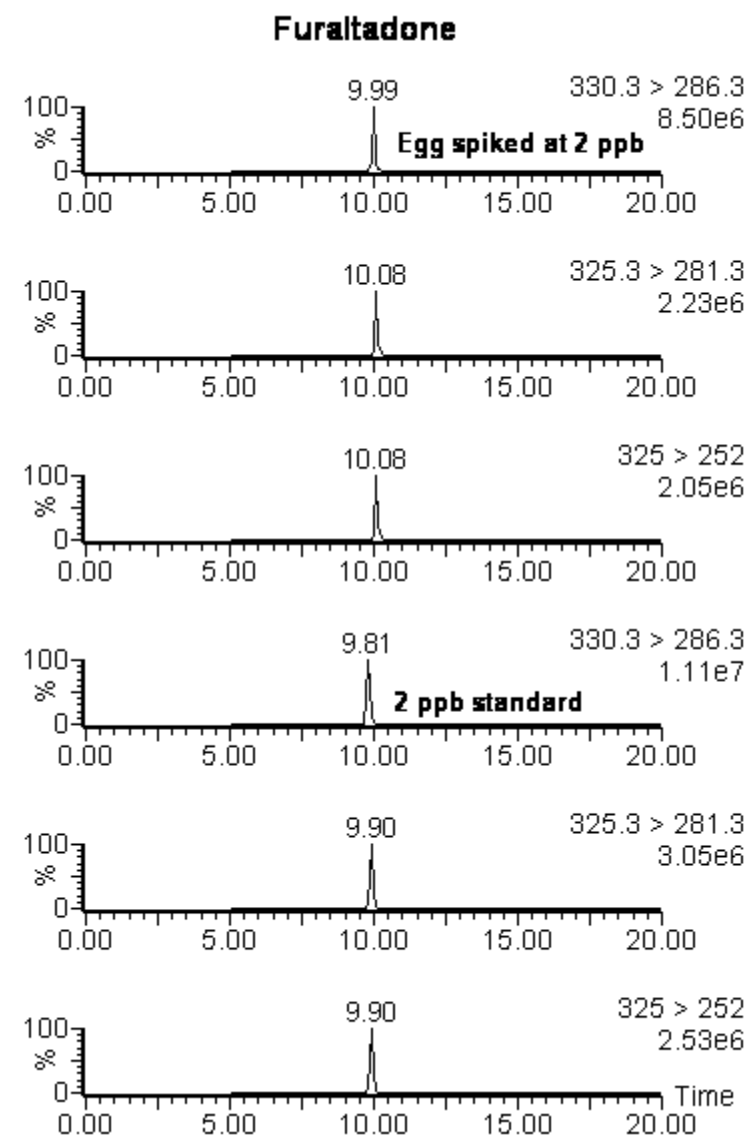


Figure 1c

View Only

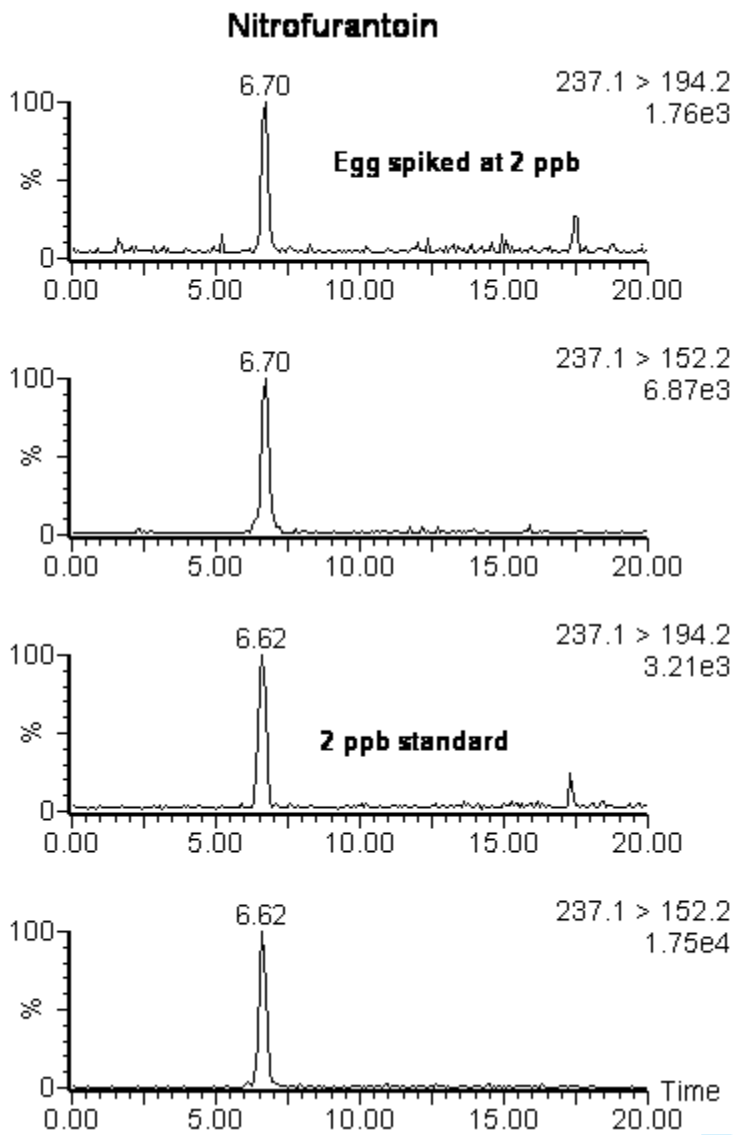


Figure 1d.

Only

Legends to figures.

Figure 1a. Chromatograms (un-smoothed data) of MRM in electrospray negative mode of a blank egg extract spiked with nitrofurazone at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for nitrofurazone: $197.2>150.2$ and $197.2>124.2$; and $200.2>153.2$ corresponding to the internal standard $^{13}\text{C}^{15}\text{N}_2$ -nitrofurazone.

Figure 1b. Chromatograms (un-smoothed data) of MRM in electrospray positive mode of a blank egg extract spiked with furazolidone at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for furazolidone: $226.2>209.3$ and $226.2>139.2$; and $230.2>139.2$ corresponding to the internal standard D_4 -furazolidone.

Figure 1c. Chromatograms (un-smoothed data) of MRM in electrospray positive mode of a blank egg extract spiked with furaltadone at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for furaltadone: $325.3>281.3$ and $325>252$; and $330.3>286.3$ corresponding to the internal standard D_5 -furaltadone.

Figure 1d. Chromatograms (un-smoothed data) of MRM in electrospray negative mode of a blank egg extract spiked with nitrofurantoin at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for nitrofurantoin: $237.1>194.2$ and $237.1>152.2$.