Research Article

An integrated sample preparation to determine coccidiostats and emerging *Fusarium*-mycotoxins in various poultry tissues with LC-MS/MS

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The usefulness of an existing sample preparation technique used for ionophoric coccidiostats (lasalocid, monensin, salinomycin and narasin) was applied in the analysis of emerging *Fusarium*-mycotoxins beauvericin (BEA) and enniatins (ENNs) in poultry tissues (liver and meat). Also, maduramicin and liver as a new sample matrix was introduced. The developed methods were validated and applied for the determination of coccidiostats and BEA/ENNs in Finnish poultry tissues in 2004 – 2005. The validation parameters demonstrated that the integrated sample preparation technique is applicable to the parallel determination of these contaminants in poultry tissues. Of the samples analysed (276 meat and 43 liver), only trace levels of LAS, MON, SAL, NAR and MAD were detected in 7, 3, 5, 6 and 4% of the samples, respectively. Interestingly, for the first time, traces of BEA and ENNs could also be detected in animal tissues. BEA and ENNs A, A1, B and B1 were found in 2, 0.3, 0.6, 4 and 3% of the samples, respectively. The simultaneous presence of coccidiostats and mycotoxins was detected in three turkey samples in 2004.

Keywords: Beauvericin / Coccidiostat / Enniatins / Poultry / Residue

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1 Introduction

The ionophoric coccidiostats lasalocid (LAS), monensin (MON), salinomycin (SAL), narasin (NAR) and maduramicin (MAD) (Fig. 1) are widely used feed additives in the poultry industry to prevent and control coccidiosis caused by the genus *Eimeria*. In Finland, they are added into feeds as their sodium salts at concentration levels of 10 – 100 mg/kg. Anticoccidial drugs are also given to other farm animals to improve feed efficacy, *i.e.* to increase the body mass. Broiler chickens receive coccidiostats in the feed during their entire lifespan. In order to avoid coccidiostat residues in edible tissues a withdrawal period of 5 days for LAS, SAL, NAR and MAD and 3 days for MON is used. The European Commission has established official maximum residual levels (MRLs) for LAS in poultry tissue (muscle 20 μg/kg, liver 100 μg/kg) [1]. For other ionophoric compounds MRLs have not been set.

Coccidiostats are not used in human medicine because of their cardiovascular effects [2]. For example, LAS has been found to cause contraction of human heart in test systems [3]. In an accidental feeding, LAS caused leg weakness, ataxia and microscopic muscular damage in broiler breeder chickens [4]. SAL caused weight loss, cannibalism, necrotic hepatitis with infarcts for breeders, which were fed with feed contaminated accidentally with SAL [5]. SAL toxicity seems to be mainly neurological in nature in mice, rats and rabbits. However, in pigs, bulls and horses SAL damaged both liver and myocardium [6]. Even low concentrations of MON and NAR can increase coronary blood flow in dogs [7]. MON has also been found to interfere with feather growth in young chicks, and to evoke hyperexcitability [8], whereas NAR has caused anorexia, hypoactivity, leg weakness, ataxia, depression and diarrhea in animals of various species [7]. The residues of coccidiostats have not induced any short-term clinical symptoms in humans [9].

Beauvericin (BEA) and enniatins A, A1, B and B1 (ENNs) are mycotoxins produced by different *Fusarium*

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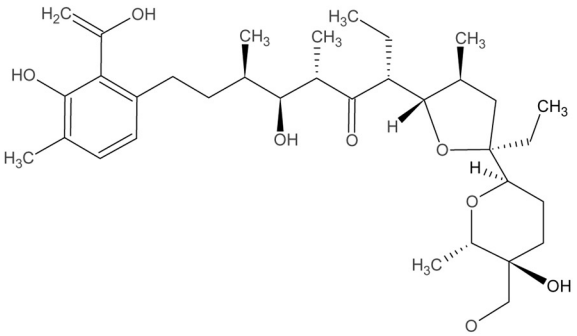
Abbreviations: BEA, beauvericin; **CCα,** decision limit; **CCβ,** detection limit; **ENNs,** enniatins; **LAS,** lasalocid; **MAD,** maduramicin; **MON,** monensin; **NAR,** narasin; **SAL,** salinomycin



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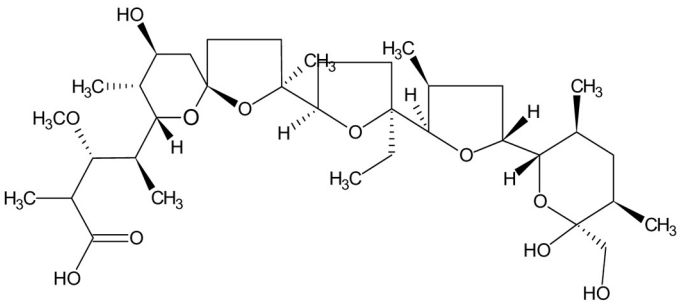

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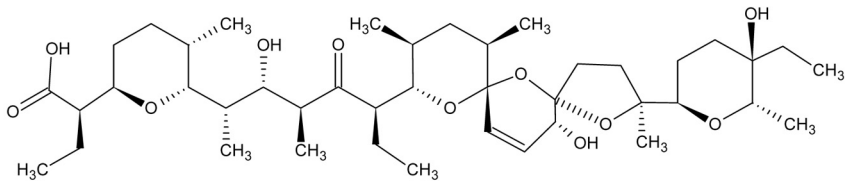

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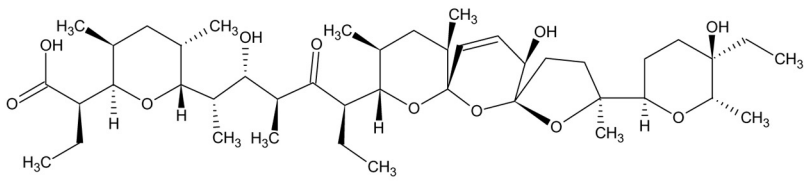
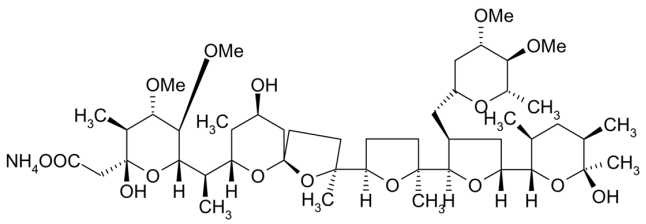
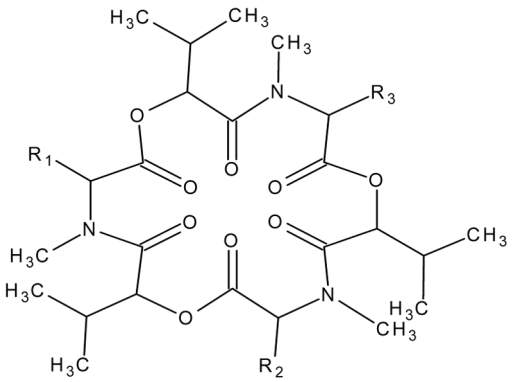


narasin



maduramicin

Figure 1. The structures of ionophoric coccidiostats.



	R1	R2	R3
BEA	Phenylmethyl	Phenylmethyl	Phenylmethyl
A	*sec*-butyl	*sec*-butyl	*sec*-butyl
A1	*sec*-butyl	*sec*-butyl	*iso*-propyl
B	*iso*-propyl	*iso*-propyl	*iso*-propyl
B1	*iso*-propyl	*iso*-propyl	*sec*-butyl

Figure 2. The structures of BEA and ENN.

species such as *F. avenaceum*, *F. poae* and *F. tricinctum* (*e.g.* [10]). These specific mycotoxins are cyclic hexadepsipeptides consisting of alternating amino acid residues. BEA has phenyl substituents on the *N*-methylamino acid residue as ENNs have aliphatic substitutions on the same positions (Fig. 2). BEA and ENNs are common contaminants of grains in Finland. However, the contamination levels are usually low, particularly in the case of BEA, which has been detected only in trace amounts in Finnish barley and wheat. ENNs, instead, may occur in mg/kg levels, especially with the samples harvested late in the autumn [11].

The primary toxic action of BEA and ENNs is considered to be related to their ionophoric properties. They affect to electrochemical gradient in membranes resulting to toxic actions *via* disturbances in the normal physiological concentrations of cations in the cell [12, 13]. The toxic actions of BEA and ENNs also includes the induction of apoptosis by triggering an increase in the cytoplasmic calcium concentration [14]. BEA is known to form noncovalent products, *i.e.* interchelate with DNA [15]. As BEA and ENNs are structurally related, the same toxicodynamic action can be suggested to be relevant also to ENNs. Until now, the toxic outcomes observed for BEA and ENNs are mostly from *in vitro* tests [16]. This can be seen as a shortcoming because it is possible that BEA and ENNs may have toxic effects on different tissues and organs, although no recognised mycotoxicoses of these particular mycotoxins have been reported. In the few conducted *in vivo* studies the toxicities of BEA and ENNs are reported to be low at the concentration levels tested, except for the study of McKee *et al.* [17]. Leitgeb *et al.* [18, 19] did not observe any toxic interactions between different mycotoxins (BEA, moniliformin, deoxynivalenol, fumonisin B_1) in a feeding study using broilers and turkeys. However, due to their lipophilic nature, BEA and ENNs may bioaccumulate [20], and therefore the possible residues in animal tissues should also be studied.

Poultry are one of the most sensitive farm animals to the toxic effects of low amounts of some mycotoxins, whereas they are very tolerable for some other mycotoxins. Although poultry do not live long enough to develop tumours, mycotoxin-related diseases may adversely affect their health and productivity [21]. Several mycotoxicoses have been reported on poultry. These include especially the well-known turkey-X disease caused by aflatoxins and related with acute hepatic necrosis and even death [22], as well as the mixture of aflatoxins, ochratoxins, sterigmatocystine and citrinin that resulted in different diseases such as hepatitis, salmonellosis, coccidiosis and infectious bursal disease [23]. In addition, moniliformin, has been suspected to promote ascites, a poultry cardiomyopathy, although the etiology of the disease is not clear [24]. Mycotoxicoses caused by BEA or ENNs has not been reported.

As the chemical structures and particularly the mode of action of BEA/ENNs and coccidiostats are closely related, the question raised whether these lipophilic mycotoxins could accumulate in the same animal tissues as coccidiostats [2, 25]. The aims of the present study were (i) to study the suitability of the sample preparation technique of ionophoric coccidiostats for BEA and ENNs, (ii) to validate the method for the analysis of BEA and ENNs from poultry tissues (meat and liver), (iii) to add ionophoric coccidiostat MAD to the existing method for ionophoric coccidiostats, (iv) to validate the coccidiostat method for liver samples, (v) to study the simultaneous presence of coccidiostats as well as BEA and ENNs in Finnish poultry tissues.

2 Materials and methods

2.1 Samples

Poultry meat and liver samples originated from the national residue monitoring programme conducted in Finland. Meat samples (2004: 40 broiler and 114 turkey samples, 2005: 88 broiler and 34 turkey samples) and liver samples (2004: 18

broiler and 9 turkey samples, 2005: 6 broiler and 10 turkey samples) were collected from slaughterhouses from different locations in Finland.

2.2 Chemicals and reagents

Standards MON (sodium salt, ~90–95%), SAL (~96%), NAR (~97%), BEA (≤97%) and ENNs (A + A1 + B + B1, ≤97%) were purchased from Sigma (St. Louis, MO, USA), LAS (sodium salt) from Fluka Chemie (Buchs, Switzerland) and MAD (ammonium, ~96.5%) from Alpharma (Willow Island, NE, USA). Standard stock solutions of coccidiostats (1 mg/mL) and working standard solution (a mixture of standard stock solutions, 0.1 μg/mL) were prepared in methanol. The standard solutions of BEA (0.01–0.1 μg/mL) and ENNs (0.02–0.2 μg/mL) were prepared in ACN. ACN, methanol, acetic acid (analytical grade), anhydrous sodium sulphate and ammonium acetate were purchased from J. T. Baker (Deventer, Holland). The water used was milli-Q water purified with a Millipore Milli-Q Plus System (Millipore, Espoo, Finland). Silica SPE columns (Bond elut, 500 mg, 3 mL) were purchased from Varian (Lake Forest, CA, USA). Argon (AGA, Finland) was used as a collision gas in the MS/MS.

2.3 Sample preparation

Sample preparation was performed as described by Rokka and Peltonen [26]. Homogenised material (5 g) was dried with anhydrous sodium sulphate and extracted with 20 mL of ACN. Samples were shaken using a horizontal shaker for 30 min and centrifuged at 4000 rpm for 15 min. Five millilitres of crude extract was cleaned with SPE using a silica column. Before loading the sample onto the column, the column was conditioned with ACN (2 mL). A sample was passed through the conditioned column and the eluate was collected into a test tube. The column was further washed with ACN (2 mL), which was also collected. The combined eluate was concentrated under the steam of nitrogen, dissolved into the mobile phase of coccidiostats separation (95% ACN: 5% 2 mM ammonium acetate containing 2% acetic acid) and injected into an LC – tandem mass spectrometer (Waters Alliance 2695 liquid chromatograph; Waters, Milford, MA, USA and MicroMass Quattro Micro triple-quadrupole mass spectrometer; MicroMass, Manchester, UK).

2.4 LC-MS/MS analysis

Ionophoric coccidiostats were analysed with Luna $C_{18}(2)$ (5 μm), 3.0 × 150 mm analytical column (Phenomenex, Cheshire, UK). The flow rate of the mobile phase was 0.5 mL/min and the injection volume was 10 μL. The instrument was operated in the positive ion electrospray mode using the following parameters: capillary voltage 3.75 kV, cone voltage 34 kV, source temperature 130°C, desolvation temperature 250°C and collision gas energy 38 eV. Coccidiostats were detected as their sodium adducts $[M + Na]^+$ (LAS *m/z* 613, MON *m/z* 693, SAL *m/z* 773, NAR *m/z* 787, MAD *m/z* 939) and the adducts were fragmented to corresponding product-ions (LAS *m/z* 359 and 377, MON *m/z* 461 and 675, SAL *m/z* 431 and 531, NAR *m/z* 431 and 531, MAD *m/z* 877, 895). For the quantification, the following product-ions were used: *m/z* 377 for LAS, *m/z* 675 for MON, *m/z* 431 for SAL, *m/z* 431 for NAR and *m/z* 877 for MAD.

The separation of BEA and ENNs was conducted as described by Jestoi *et al.* [27]. Ten microlitres of the sample was injected into Symmetry C_{18} (3.5 μm), 2.1 × 150 mm analytical column (Waters). An isocratic elution was used in the HPLC analysis in which the mobile phase used was ACN/methanol/ammonium acetate (10 mM, pH 7) in a ratio of 45:45:10 v/v. The flow rate of the mobile phase was 0.2 mL/min. The instrument was operated in the positive ion electrospray mode using the following parameters: cone voltage 45 V, capillary voltage 3.80 kV, source temperature 150°C and desolvation temperature 270°C and collision gas energy 25 eV. MRM-technique was used for identification and quantification, in which protonated molecules $[M + H]^+$ of the analytes (BEA *m/z* 784, ENN A *m/z* 682, ENN A1 *m/z* 668, ENN B *m/z* 640, ENN B1 *m/z* 654) were fragmented in the collision cell to the product ions (BEA *m/z* 244 and 262, ENN A *m/z* 210 and 228, ENN A1 *m/z* 210 and 228, ENN B *m/z* 196 and 214, ENN B1 *m/z* 196 and 214). For the quantification were used the following product-ions *m/z* 244 for BEA, *m/z* 210 for ENN A, *m/z* 210 for ENN A1, *m/z* 196 for ENN B and *m/z* 196 for ENN B1.

2.5 Method validation

The following validation parameters were determined for all compounds analysed: selectivity, linearity, specificity, recovery and repeatability. Based on a recommendation of Commission Decision 2002/657/EC [28] decision limit (CCα) and detection limit (CCβ) were determined for coccidiostats. For BEA and ENNs the LOD and the LOQ were determined. For ionophoric coccidiostats six replicates of spiked samples, at three concentration levels (Table 1) were analysed on three different days for both matrices. The calibration curves with and without matrix were prepared at five different concentration levels (1.0–5.0 μg/kg). For BEA and ENNs, 12 replicates of spiked samples, at three concentrations levels (Table 1) and calibration curves with and without matrix (BEA 0.2–20 μg/kg; ENN A 0.01–1.2 μg/kg; ENN A1 0.03–8.4 μg/kg; ENN B 0.03–8 μg/kg; ENN B1 0.09–22.4 μg/kg) were analysed for both matrices.

Table 1. The mean recoveries and the CVs for the ionophoric coccidiostats and mycotoxins analysed at three spiking levels in poultry meat and poultry liver

	Spiking level 1 (μg/kg)	Recovery% (spiking level 1; *n* = 12)	Spiking level 2 (μg/kg)	Recovery% (spiking level 2; *n* = 12)	Spiking level 3 (μg/kg)	Recovery% (spiking level 3; *n* = 12)
Poultry meat						
LAS	2.0	80 ± 12	3.0	67 ± 6.2	4.0	69 ± 11
MON	2.0	94 ± 8.2	3.0	83 ± 13	4.0	85 ± 12
SAL	2.0	84 ± 12	3.0	81 ± 12	4.0	83 ± 6.8
NAR	2.0	84 ± 6.2	3.0	76 ± 5.0	4.0	82 ± 6.7
MAD	2.0	78 ± 7.9	3.0	78 ± 8.1	4.0	82 ± 7.6
BEA	2.0	95 ± 4.6	8.4	91 ± 6.6	17	78 ± 9.1
ENN A	0.12	76 ± 18	0.5	60 ± 5.7	1.0	74 ± 7.9
ENN A1	0.84	62 ± 12	3.5	71 ± 8.8	6.7	54 ± 8.1
ENN B	0.8	97 ± 9.4	3.2	98 ± 4.8	6.3	98 ± 3.8
ENN B1	2.24	81 ± 7.0	9.0	97 ± 4.6	18	97 ± 4.7
Poultry liver						
LAS	2.0	71 ± 9.1	3.0	65 ± 5.5	4.0	73 ± 4.8
MON	2.0	78 ± 4.4	3.0	77 ± 4.6	4.0	79 ± 4.5
SAL	2.0	76 ± 19	3.0	77 ± 15	4.0	78 ± 12
NAR	2.0	76 ± 4.6	3.0	76 ± 4.2	4.0	83 ± 4.4
MAD	2.0	77 ± 15	3.0	80 ± 17	4.0	88 ± 7.7
BEA	2.0	48 ± 13	8.4	62 ± 12	17	58 ± 7.8
ENN A	0.12	38 ± 12	0.5	54 ± 20	1.0	62 ± 11
ENN A1	0.84	45 ± 14	3.5	69 ± 3.1	6.7	68 ± 7.1
ENN B	0.8	70 ± 20	3.2	70 ± 9.1	6.3	88 ± 8.5
ENN B1	2.24	78 ± 18	9.0	61 ± 5.6	18	94 ± 8.3

3 Results

3.1 Method validations

Selectivity of the analytical methods was tested by comparing (two-sided *t*-test) the slopes of the calibration curves obtained with and without matrix. Due to the matrix effects observed (data not shown), the calibration curves for all compounds analysed were prepared in poultry meat or in liver. The acceptable linearity of each point of the matrix-assisted calibration curves was tested with the method of van Trijp and Roos [29]. A tolerance of 100 ± 10% was accepted for the separate calibration points for good linearity (data not shown). On this basis, the method for coccidiostats as well as for BEA and ENNs can be considered linear over the ranges studied. The specificity of the methods was tested by analysing 20 blank meat and liver samples, and no interference signals close to the retention times of the analytes were detected in any blank samples analysed. The chromatograms of poultry meat spiked with coccidiostats or BEA/ENNs are shown in Figs. 3 and 4, respectively. A positive broiler meat sample for coccidiostats and a positive turkey meat sample for BEA and ENNs are shown in Figs. 5 and 6, respectively. The CV% of the repeatability for coccidiostats measured in poultry meat and liver were 5.0–13 and 4.2–19, respectively. The recoveries of coccidiostats in meat and liver varied between 67–94% and 65–88%, respectively (Table 1). The CV% of the repeatability of the method for BEA and ENNs in meat and liver were 3.8–18% and 3.1–20%, respectively. The recoveries of BEA and ENNs in meat and liver varied between 54–98% and 38–94%, respectively (Table 1). CCα varied from 2.0 to 2.4 μg/kg in meat and from 1.7 to 2.2 μg/kg in liver (Table 2). CCβ varied from 2.2 to 3.0 μg/kg in meat and from 1.9 to 2.6 μg/kg in liver (Table 2). LODs were between 0.015 and 0.56 μg/kg and LOQs 0.03 and 1.12 μg/kg in both matrices (Table 3).

Table 2. CCα and CCβ for coccidiostats analysed

	Broiler meat		Broiler liver	
	CCα (μg/kg)	CCβ (μg/kg)	CCα (μg/kg)	CCβ (μg/kg)
LAS	2.4	3.0	1.7	1.9
MON	2.2	2.5	1.8	1.9
SAL	2.3	2.7	2.2	2.6
NAR	2.0	2.2	1.8	1.9
MAD	2.0	2.3	2.1	2.5

3.2 Sample analyses

Coccidiostats were detected in a few broiler meat, turkey meat and liver samples in 2004. The levels were lower than the CCβs, except for SAL for which the highest measured level was 4.2 μg/kg in one broiler meat sample. In three turkey liver samples two coccidiostats (LAS and MON in one

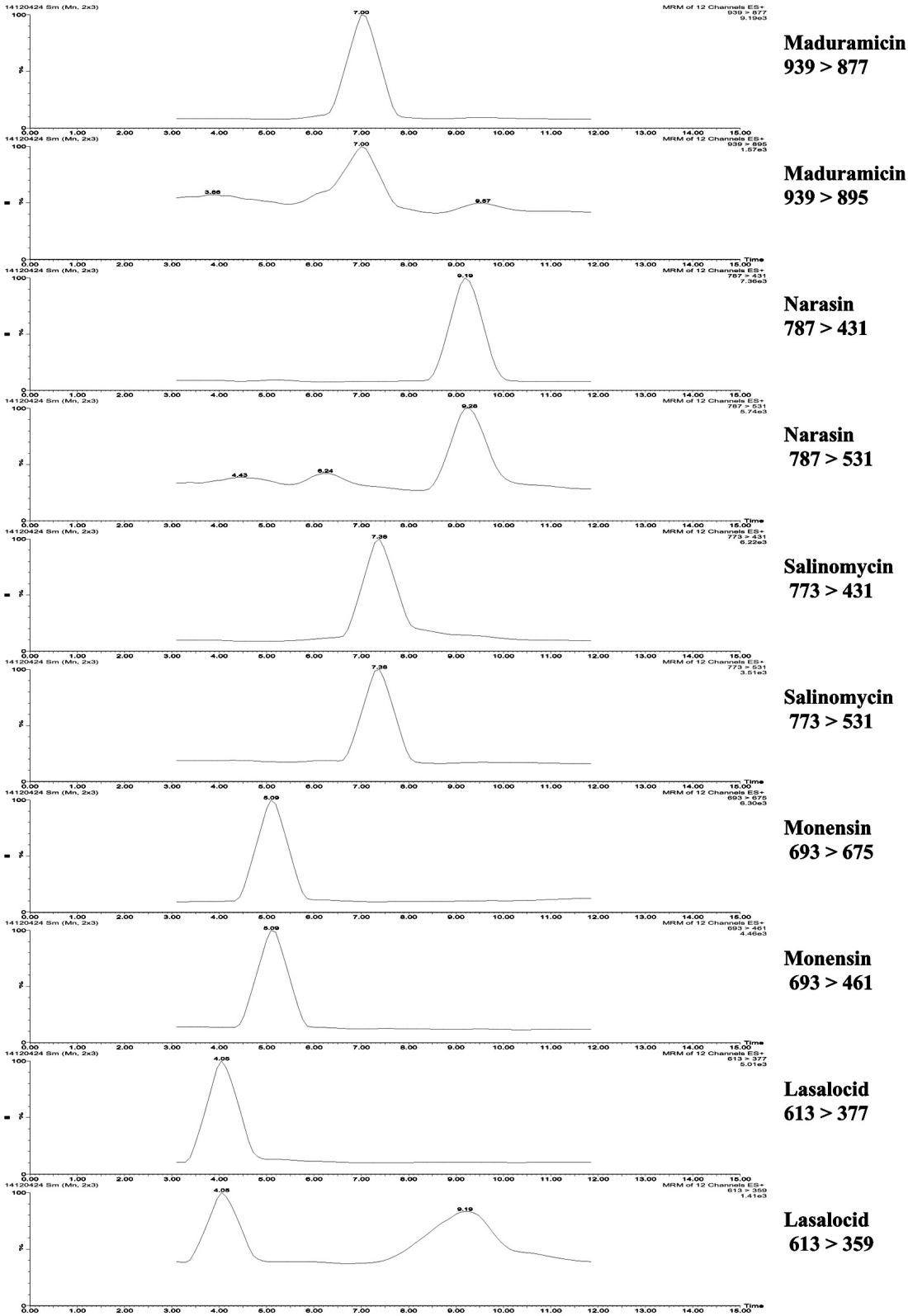


Figure 3. The extracted ion chromatograms of coccidiostats of a spiked broiler meat sample (spiking level 3.0 μg/kg).

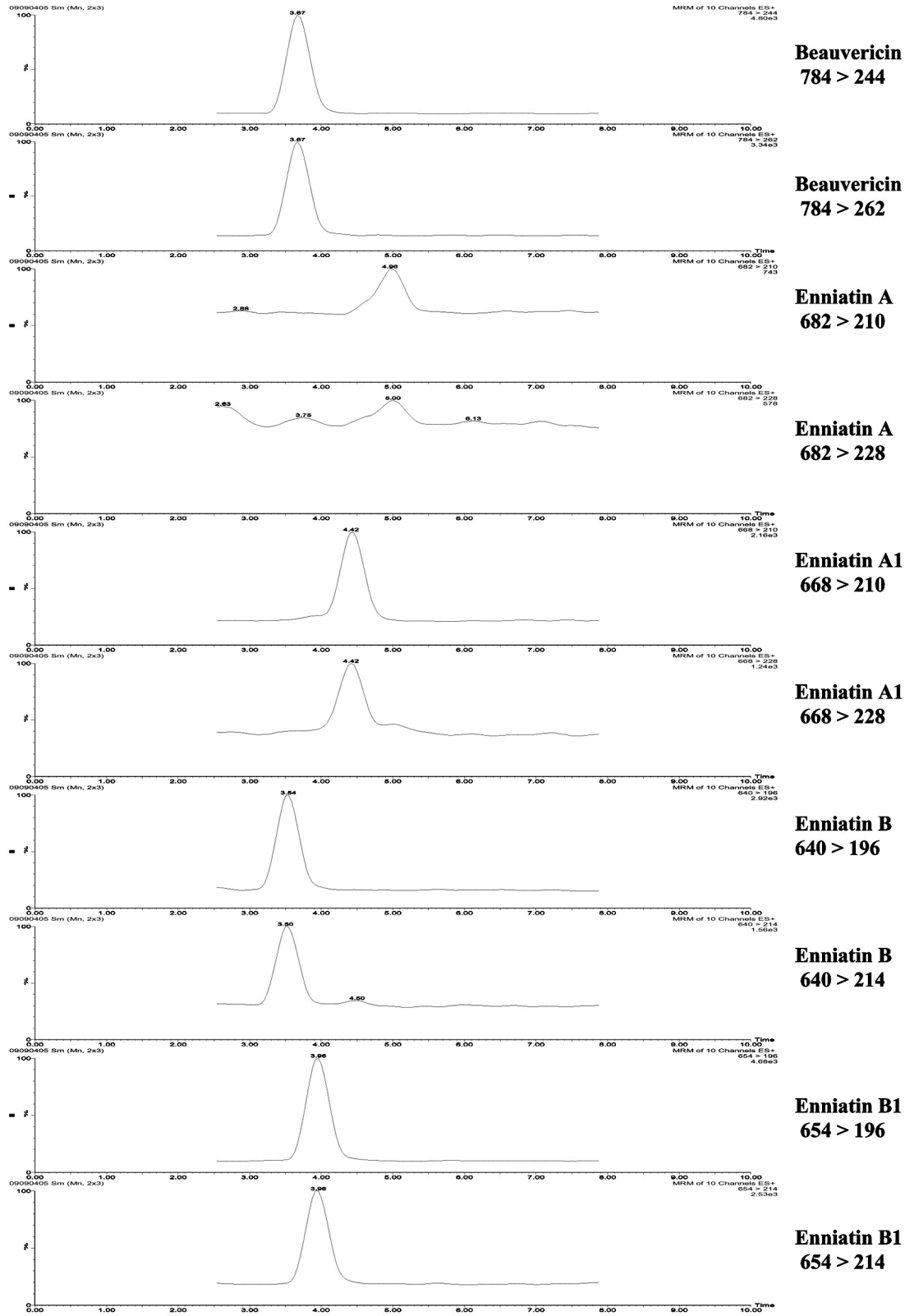


Figure 4. The extracted ion chromatograms of BEA and ENN of a spiked poultry meat sample. Spiking were for BEA 2.0 μg/kg, enniatin A 0.12 μg/kg, enniatin A1 0.84 μg/kg, enniatin B 0.8 μg/kg and enniatin B1 2.24 μg/kg.

Table 3. The LOD and LOQ for mycotoxins analysed

	Broiler meat		Broiler liver	
	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
BEA	0.50	1.0	0.50	1.0
ENN A	0.015	0.03	0.015	0.03
ENN A1	0.21	0.42	0.21	0.42
ENN B	0.20	0.40	0.20	0.40
ENN B1	0.56	1.12	0.56	1.12

sample; MON and NAR in two samples) were detected. In 2005, traces of coccidiostats were found in both sample groups (meat and liver). LAS was detected in one turkey meat sample at the concentration level of 3.1 μg/kg. More than three coccidiostats were detected in three broiler liver (LAS + MON + SAL + NAR + MAD in three samples), two turkey meat (LAS + SAL + NAR + MAD in one samples; SAL + NAR + MAD in one sample) and five turkey liver samples (LAS + MON + SAL + NAR + MAD in three samples; LAS + SAL + NAR + MAD in two samples). The results for coccidiostats in Finnish poultry samples in 2004 and 2005 are presented in Tables 4 and 5, respectively.

BEA and ENNs were detected in turkey meat samples in 2004 (1 – 11% of the samples were positive for various mycotoxins). The levels were below the LOQs, except for ENN B for which the highest measured level was 2 μg/kg. Only one type of mycotoxin was found in five samples as two different mycotoxins were present in seven samples. More than two different mycotoxins were detected in three individual turkey meat samples (Table 6). In 2005, only ENN B was detected in one broiler liver sample (<0.4 μg/kg). Mycotoxins and coccidiostats together were found in three turkey meat samples in 2004 (NAR + ENN B in one sample; LAS + BEA + ENN B + ENN B1 in one sample; LAS + BEA + B in one sample).

4 Discussion

A method for the quantitative determination of LAS, MON, SAL and NAR in broiler meat was already available [26]. Based on the European Union's recommendation [28] MAD was included into the method. The revalidation procedure of the method demonstrated that MAD can be analysed with the existing method. In addition, to that the method proved to be applicable to poultry liver as well.

As the structures and especially the mode of action of BEA/ENNs and coccidiostats are closely related, the sample preparation method used for coccidiostats was tested for BEA and ENNs in poultry meat and liver matrices. Although the recovery was low for some of the mycotoxins, the repeatability was good (Table 1). The use of the same sample preparation method for mycotoxins and coccidiostats is convenient as the LC-MS/MS analyses can be performed from the same samples. The simultaneous analyses of various analytes should be the aim of the future method development analysis, whenever possible, as many of the present (analytical) methods only described the determination of a single compound or compound groups The lack of resources is a ubiquitous problem of analytical laboratories.

Physicochemical properties (octanol/water partition coefficient K_{ow}, pK_a-value, binding to plasma proteins, M_r) of exogenous substances determine the toxicodynamic

Table 4. The maximum concentration levels (μg/kg) of ionophoric coccidiostats in Finnish poultry tissues in 2004. The proportion of positive samples are shown in parenthesis

	LAS	MON	SAL	NAR	MAD
Broiler meat (n = 38)	<3.0 (2.6%)	<2.5 (5.3%)	4.2 (7.9%)	n.d.	n.d.
Broiler liver (n = 18)	n.d.	n.d.	n.d.	n.d.	n.d.
Turkey meat (n = 114)	<3.0 (5%)	n.d.	n.d.	<2.2 (3%)	<2.2 (1%)
Turkey liver (n = 9)	<1.9 (11%)	<1.9 (33%)	n.d.	<1.9 (33%)	<2.5 (11%)

n.d., Not detected.

Table 5. The maximum concentration levels (μg/kg) of ionophoric coccidiostats in Finnish poultry tissues in 2005. The proportion of positive samples are shown in parenthesis

	LAS	MON	SAL	NAR	MAD
Broiler meat (n = 88)	<3.0 (2.2%)	n.d.	n.d.	<2.2 (1.1%)	n.d.
Broiler liver (n = 6)	<1.9 (67%)	<1.9 (50%)	<2.6 (50%)	<1.9 (67%)	<2.5 (50%)
Turkey meat (n = 34)	3.1 (6%)	n.d.	<2.7 (6%)	<2.2 (6%)	<2.3 (6%)
Turkey liver (n = 10)	<1.9 (50%)	<1.9 (30%)	<2.6 (50%)	<1.9 (50%)	<2.5 (50%)

n.d., Not detected.

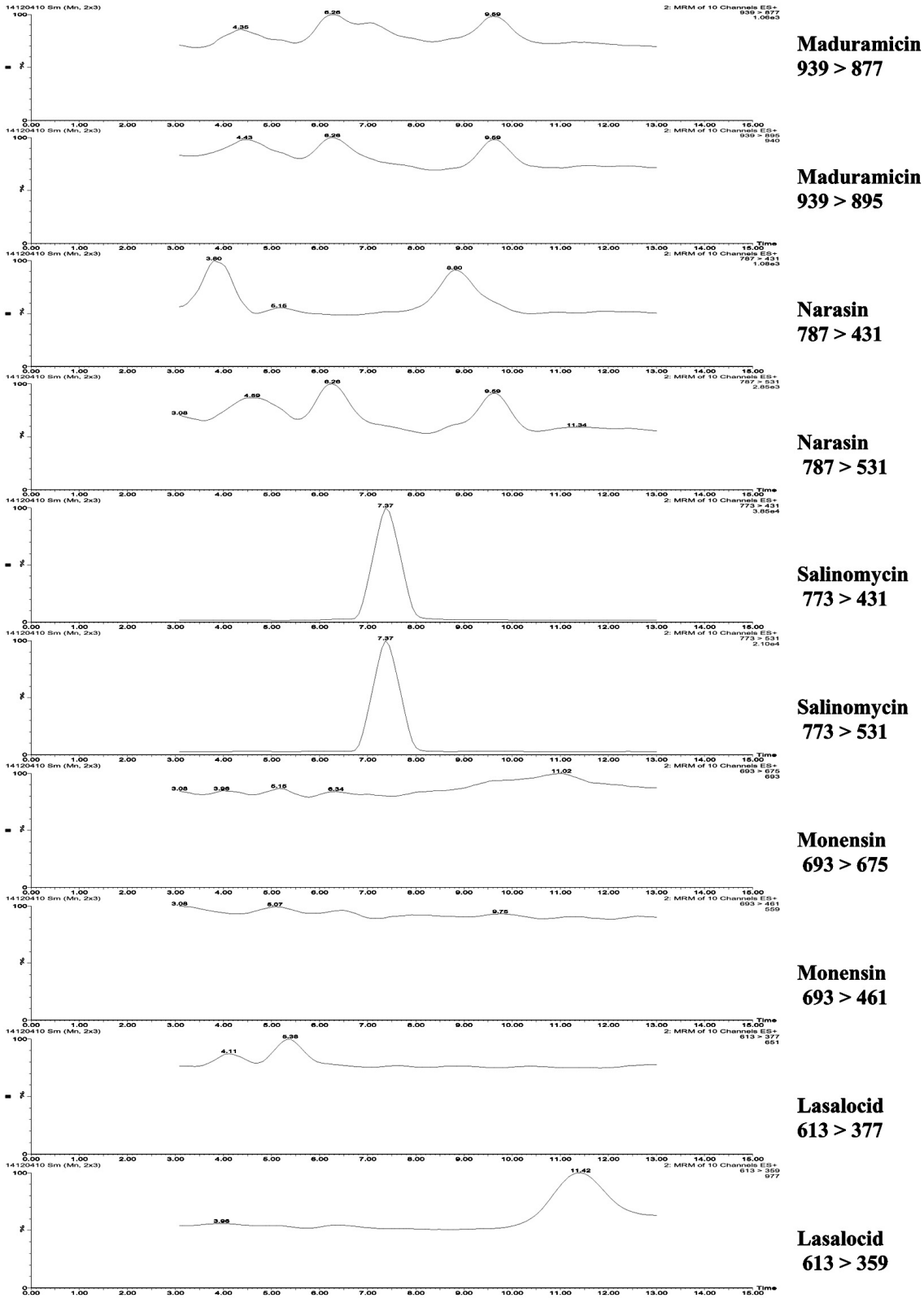


Figure 5. The extracted ion chromatograms of coccidiostats of a positive (SAL 4.2 μg/kg) broiler meat sample.

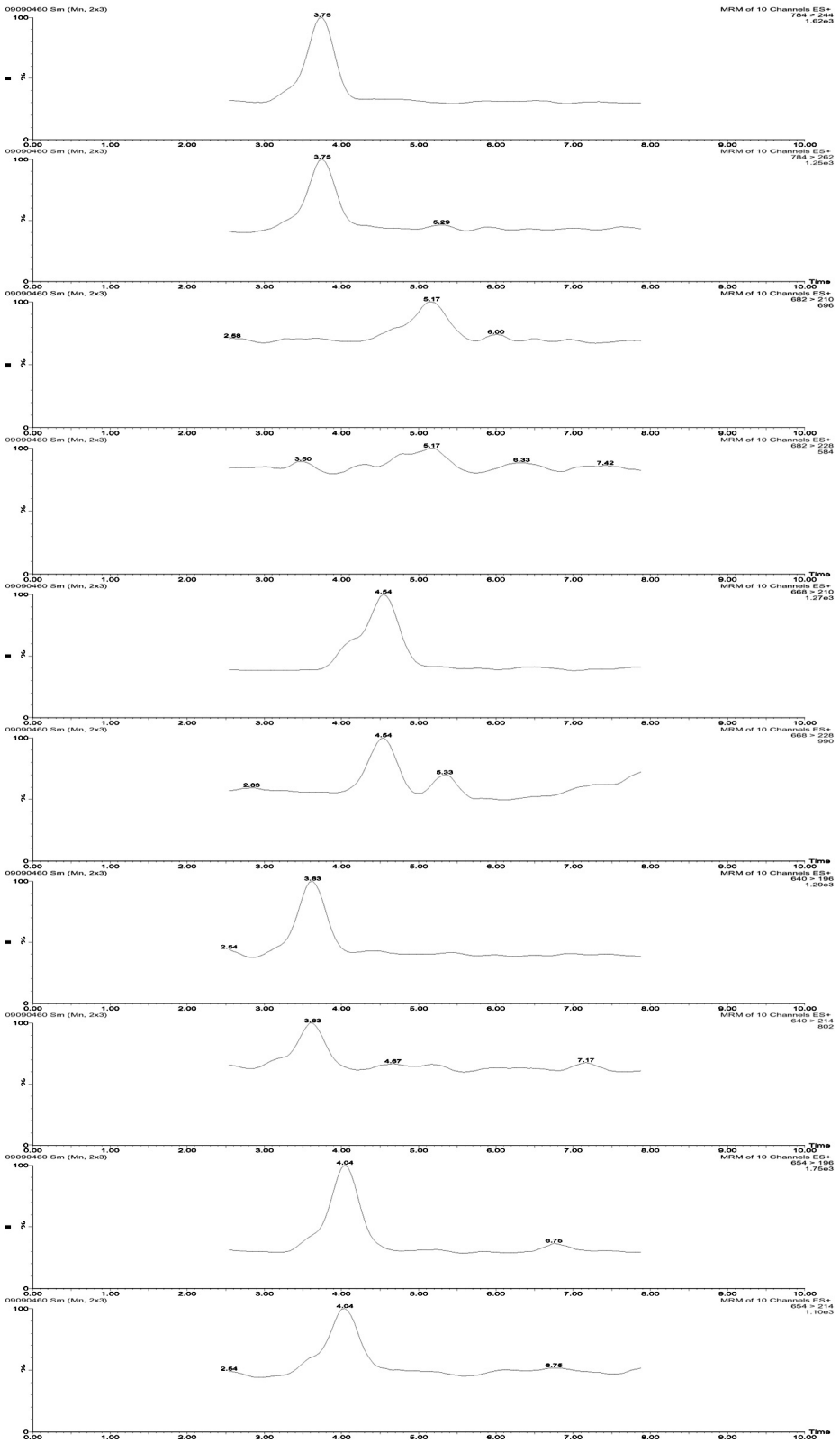


Figure 6. The extracted ion chromatograms of BEA and ENN of a positive turkey meat sample (BEA < 1 μg/kg, ENN A not detected, ENN A1 < 0.42 μg/kg, ENN B < 0.4 μg/kg, ENN B1 < 1.12 μg/kg).

Table 6. The number of turkey meat samples positive for one or more mycotoxins in 2004. The number in parenthesis describes in how many samples the defined mycotoxin(s) were detected

Number of analytes	Number of samples	Compounds detected
1	5	BEA (1), B (4)
2	7	BEA + B (1), B + B1 (4), BEA + B1 (2)
3	1	BEA + B + B1 (1)
4	1	BEA + A1 + B + B1 (1)
5	1	BEA + A + A1 + B + B1 (1)

behaviour and distribution to tissues small change in the structure may lead to a remarkable difference in the adsorption/distribution [30]. The biotransformation reactions for xenobiotics include well-established enzymatic phase I (hydrolysis, reduction, oxidation) and phase II (glucuronidation, sulphonation, acetylation, methylation, conjugation with glutathione) reactions which modify the substrates to more water-soluble form to improve elimination. The metabolism takes place especially in the liver and the excretion occurs mostly through kidneys and/or faeces. It must be noted, however, that large species differences in the metabolism of xenobiotics exists.

The main metabolic pathway of NAR and LAS in the chicken involves an oxidative process leading to the formation of di-, tri- and tetra-hydroxy compounds as well as keto compounds [31]. For example, unchanged NAR is a minor component (up to 5% of chicken excreta), whereas a great number of metabolites have been identified, some of them representing more than 20% of the whole NAR-related excreted compounds. Generally speaking, metabolites in tissues and excreta are qualitatively similar. However, in the case of NAR and LAS, each metabolite represents less than 10% of the whole tissue residues.

The residues of unmetabolised NAR detected particularly in the samples taken from the leg muscle (*e.g.* [32]) may indicate the low level of the expression of the P450 enzymes involved with the oxidative metabolism of the ionophores. Interestingly in this study, mycotoxins were not detected in leg muscle samples which may indicate the fact that the two coccidiostat, *i.e.* LAS and NAR and mycotoxins are oxidised by the different isoenzymes of P450. From the food safety perspective, it seems important to know the isoenzymes involved in the metabolism of the mycotoxins and coccidiostats. This would facilitate the identification of the key metabolites in laboratory for further actions.

In addition to the enzymes involved in the metabolism of xenobiotics, other protective systems have been identified. These include membrane-bound efflux transproteins, namely P-glycoprotein and multidrug resistance protein (MRP) that effectively lower the intracellular concentrations of toxins [33, 34]. Their role as a barrier to toxin absorption is supported, in part, by their specific expression in tissues (*e.g.* intestine, where the activity of metabolising enzymes is usually lower than in the liver) responsible for the detoxification/metabolism of toxicants. The level of toxicants in the diet might influence to the levels of membrane-bound efflux proteins in an adaptation manner [35].

Walker [36] postulated that cytochrome P450 expression involved in the metabolism of xenobiotics corresponds to their evolutionary exposure to substrates as the expression of P-glycoprotein is strongly regulated by the dietary constituents. Therefore, it could be possible that as the poultry in Finland have presumably been exposed to *Fusarium avenaceum*-mycotoxins interminably, they have adopted effective metabolising/elimination systems for these compounds, as for coccidiostats the exposure has not been continuous. This could, in part, explain the differences in the presence of these compounds in poultry tissues observed in this study.

Residues of ionophoric coccidiostats have been detected in poultry meat and liver in several studies [2, 25, 32, 37]. For example, MAD was found in liver samples at the concentration level of 160 μg/kg, with no withdrawal period [37]. After different withdrawal times, the residue levels were drop close to the LOQs or were not detected, except LAS, which was detected in liver samples (10 μg/kg), after a seven-day withdrawal period [2].

Traces of several *Aspergillus*-, *Penicillium*- and *Fusarium*-mycotoxins, including aflatoxins, zearalenone, ochratoxin A [38 – 41] have been detected in the different tissues (kidney, liver, muscle) of poultry species (hens, broilers, ducks). The transmission rates, *i.e.* the average residue to intake ratios, have varied greatly but been very low in average (<0.01) suggesting the existence of effective metabolising system and that only a small part of the administered toxin does bioaccumulate. The residue levels have usually declined soon after the removal of contaminated feed from the diet. It has also been proposed, that hens are more susceptible to mycotoxin accumulation than broilers because of the metabolic differences and/or the prolonged treatment [39].

Unfortunately, the levels of mycotoxins in feed consumed by animals tested in this study were not available. The average feed consumption by different avian species is about 100 – 150 g per day and the combined BEA/ENNs contamination levels of Finnish raw grains (harvested in 2001 – 2002) ranging from trace-levels up to 25 mg/kg [11]. Based on these facts, it can be postulated that the exposure of poultry to these natural contaminants may vary between negligible trace-levels and about 3.75 mg/day. It is worth to notice that the climatic conditions can have a significant effect on the levels of mycotoxins produced. The mycotoxin levels in the years 2004 – 2005 may be markedly different from the mycotoxins levels in 2001 – 2002. For instance, the summer of 2004 was remarkably rainier than the average seasons in Finland (www.fmi.fi/news/index.html?Id=1111661315.html, accessed on 2.11.2006), which led

to heavy contamination of Finnish grains with *Fusarium* spp. (Parikka *et al.*, unpublished data). The contamination levels of BEA and ENNs were, however, not determined from the harvest of this rainy season. In the rainy season of 1998, high levels of BEA and ENNs were detected in Finnish grains [42], so it may be possible that the levels of the emerging mycotoxins have been high in 2004, as well.

It is interesting that BEA could be detected more often than ENNs in Finnish poultry tissues, although ENNs are found in much higher concentrations in Finnish grains samples [14]. This could be due to the possible higher transmission rate of BEA as compared to ENNs or to increased hydrophobicity relating to the phenylmethyl-side chain (Fig. 2). The decreased polarity leads to higher K_{ow} value, which in turn leads to the increased adsorption. Leitgeb *et al.* [18, 19] analysed the residues of BEA in turkey and broiler meat after exposure in combination with other mycotoxins. No traces of BEA were detected in the tissues. However, the analytical technique used (HPLC combined with UV-detection) can, in most cases, be considered as a nonsensitive detection technique, which can partly explain the negative findings of the researchers.

Despite the fact that trace amounts of both coccidiostats and selected *Fusarium*-mycotoxins could be detected in Finnish poultry tissues, the levels were negligible if the acute risks on human or animal health are concerned. The observation that these compounds can accumulate in animal tissues is, however, interesting. MON, NAR and LAS are reported to go through extensive metabolic transformation reactions [31], but this is an unknown fact of mycotoxins. Further investigations are needed to clarify the main metabolites of these compounds using metabolomic studies and to determine the possible bioaccumulation of these metabolites in tissues, as it is strongly possible that the accumulation of molecule(s) unmodified by the biotransformation reactions in organisms is less susceptible than that of metabolites. In the light of this, we suggest that a metabolite rather than a parent molecule should be used for food safety purposes.

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