FULL PAPER
THE ANALYST
www.rsc.org/analyst

Efficient and sensitive screening and confirmation of residues of selected polyether ionophore antibiotics in liver and eggs by liquid chromatography-electrospray tandem mass spectrometry

Johan Rosén

National Food Administration, Box 622, SE-751 26, Uppsala, Sweden.
E-mail: johan.rosen@slv.se

Received 12th June 2001, Accepted 23rd July 2001
First published as an Advance Article on the web 2nd October

A method using liquid chromatography tandem mass spectrometry (LC-MS-MS) with electrospray (ES) for the determination of traces of narasin, monensin and salinomycin in chicken liver and eggs was developed, validated and used for routine surveillance. The essence of this paper is to demonstrate that one single method can serve very well for two entirely different purposes, *i.e.*, screening and confirmation. Highly reliable confirmation of the identity at low concentrations was demonstrated when residues of narasin were detected and quantified (0.2 to 11 ng g^{-1}) in 50% of the Swedish eggs analysed in 1999. Four daughter ions were detected with ion ratios meeting suggested confirmation criteria for the European Union, even at 0.2 ng g^{-1}. The method was found to be highly cost-effective since both screening and confirmation of 98 liver samples were performed in only two analytical runs (the Swedish national surveillance scheme of 1999, report level 5 ng g^{-1}). The high performance of the method for the different applications was possible due to a combination of the power of ES-LC-MS-MS, a procedure involving screening of pooled samples, and method optimisation of the work-up (automated solid phase extraction), LC and MS parameters. Validation data for narasin (0.5 to 20 ng g^{-1}) in eggs are presented (accuracy 94 to 108%, relative standard deviation 4 to 10%, limit of detection 0.026 ng g^{-1}). The time for an LC-MS-MS run was 4 min, corresponding to 48 s per sample in a pool.

Introduction

The substances narasin, salinomycin, monensin and lasalocid belong to a group of polyether ionophore antibiotics. They are carboxylic acids and form cyclic complexes with cations, *e.g.*, sodium complexes. They are commonly used as coccidiostats, *i.e.*, to prevent and treat coccidiosis in poultry. In Sweden narasin in particular is used as a feed additive for most broiler chickens. Prior to slaughter a withdrawal period is required to minimise residues in edible tissues. Although no maximum residue limit (MRL) has yet been fixed by the European Union (EU), narasin is included in the Swedish national surveillance scheme for drug residues in chicken liver, and residues exceeding 5 ng g^{-1} are reported. The feed for egg-laying hens, however, should be free from coccidiostats. Hence, eggs included in the scheme are expected to test negative.

The tests are performed at the Swedish National Food Administration (NFA). A previous method used at the NFA for chicken liver was based on high performance liquid chromatography (HPLC) with post column derivatisation,[1] a method that was too slow for screening and not reliable enough for confirmation. An alternative strategy based on screening with a non-commercial ELISA kit (enzyme-linked immunosorbent assay)[2] and confirmation with single quadrupole LC-MS[3] did not prove to be efficient at our laboratory. The ELISA was not specific enough, giving too many false positives, and the LC-MS method was not quick enough to confirm all the samples that tested positive in the ELISA.

Therefore a simpler work-up (an automated solid phase extraction (SPE) step only) was developed for narasin, salinomycin and monensin, but this has not yet been done for lasalocid. A more powerful detection method with LC-MS-MS for all four substances was also developed.

Among other published methods carefully reviewed by Elliot *et al.* in 1998,[4] no method for narasin, salinomycin and/or monensin was as efficient as the present method. Presented HPLC methods for these substances all required derivatisation to introduce a chromophore. Thin-layer chromatography (TLC)-bioautographic procedures used inhibition of bacterial growth, and relied on lengthy solvent-based extraction systems. According to the review ELISA methods normally detected only one compound and the cross-reactivity with metabolites was poorly investigated. Clean up for the reviewed ELISA methods almost exclusively relied on solvent partitioning, and is therefore not regarded as simpler than the present method. If ELISA kits are commercially available they are often rather expensive. If not available, method development can be expected to be costly. Such costs should be considered when comparing an ELISA method with the analysis using expensive MS-MS equipment. The best mass spectrometric methods at the time of the review were described by Blanchflower and Kennedy.[3,5] However, their use of single quadrupole detection gave no fragmentation and the limit of quantification was 5 ng g^{-1}.

Regarding the most recent LC-MS(-MS) methods for polyether ionophores published after the review, some authors have had a similar approach as in the present paper, *i.e.*, to develop a high throughput confirmatory method.[6–9] However, either application results were not presented,[6,7] or an extensive work-up was still required.[8] A promising method was presented by Matabudul *et al.* using no clean up at all. The only drawback was that it was only applied to lasalocid, and there was higher

 DOI: 10.1039/b105133b

relative standard deviations (RSD) at the lowest validated levels (RSD 14 to 28% at 1 to 6 ng g^{-1}).[9]

Experimental

Materials

Narasin, salinomycin, monensin and lasalocid were supplied by Sigma (St. Louis, MO, USA). Methanol ("LiChrosolv") and ammonium acetate (p.a.) were both obtained from Merck (Darmstadt, Germany), and HPLC-grade acetonitrile from Lab-Scan (Dublin, Ireland). The SPE-column used was Isolute MF C18, 100 mg (IST, Hengoed, Mid Glamorgan, UK). Water was Milli-Q water from a Millipore purification system (Bedford, MA, USA).

Stock solutions of standards 1 mg ml^{-1}were prepared in methanol and stored at −20 °C. Working standard solutions, for spiking samples as well as for the standard curve, were obtained by dilutions in methanol. The HPLC mobile phase was obtained by mixing 800 ml of acetonitrile with 200 ml of an aqueous solution of ammonium acetate (0.050 mol l^{-1}). Different methanol solutions denoted as, *e.g.*, "80% MeOH" are all % v/v methanol in water.

Equipment

Mass spectrometry was performed using a triple quadrupole: Micromass Quattro LC with Z-spray and the standard electrospray probe (Micromass UK Ltd, Altrincham, Cheshire, UK). The HPLC system was a Waters Alliance 2690 (Waters Ltd, Watford, Hertfordshire, UK). The HPLC column was a Genesis C18, 4 μm, 50 × 2.1 mm, with a guard column 10 × 2.1 mm (Jones Chromatography, Hengoed, Mid Glamorgan, UK). Automated work-up with SPE columns was performed using Gilson ASPEC XL (Villers-le-Bel, France). The homogeniser was a DISP 25 (InterMed, Roskilde, Denmark) operated at 13 500 min^{-1}.

Samples

Samples of chicken liver were collected at slaughterhouses, homogenised and stored at −20 °C until analysis. Eggs were collected from packing houses, and each egg sample was obtained by mixing 12 eggs (yolk and white) from the same packing house. Egg samples were stored in the same way as the liver samples. However, eggs were also analysed fresh, which gave no significant difference regarding recovery or precision. Check samples and calibration samples (0.5 to 20 ng g^{-1}) were obtained by adding the analyte (50 to 250 μl of a standard solution 50 to 400 ng ml^{-1}) to thawed or fresh samples (5 g) earlier proved to be free from ionophores. After mixing, these samples were allowed to stand for 10 min and then treated in the same way as the normal samples. For screening of liver samples five samples of 1 g each were mixed, and the resulting pool (5 g) was screened by the present method.

Sample extraction

Samples (5 g) were extracted with 87% MeOH (15 ml) by means of a homogeniser (30 s), and the extracts were centrifuged (3600*g*, 5 min). The rest of the work-up was performed automatically. SPE-columns were pre-treated with methanol (4 ml) and water (2 ml) before the supernatant (9 ml) was loaded. After washing with 80% MeOH (800 μl), elution was performed with methanol (600 μl), and the extracts were analysed by LC-MS-MS.

LC-MS-MS analysis

The HPLC flow was maintained at 500 μl min^{-1}, and the injection volume was 10 μl. Injections were made every fourth minute. The LC-MS-MS was operated in the positive electrospray mode. Nitrogen was used as drying gas (670 l h^{-1}, 500 °C) and nebulising gas (80 l h^{-1}), and argon as collision gas (2.3 10^{-3} mbar). The source temperature was maintained at 135 °C, the capillary voltage at 4.0 kV, and the extractor voltage at 5 V. Cone voltage and collision energy were optimised for each transition for multiple reaction monitoring (MRM) as shown in Table 1. The dwell time for each MRM transition was 0.2 s, and the interchannel delay was 0.03 s. The instrument was operated at unit resolution.

Results and discussion

Optimisation of the extraction procedure

The first aim of this work was to develop a simple work-up procedure for narasin, monensin and salinomycin. With standard solutions and SPE columns (Varian C18, 500 mg) different conditions (type of solvent, pH, volumes, *etc.*) were tested for loading, washing and elution of the three substances. It was found that optimal conditions were obtained with methanol-water solutions. Results from one of the elution profile experiments are presented in Table 2. The three compounds are effectively retained at the column even at 80% MeOH. The slight differences in hydrophilicity between the compounds resulted in a difference in elution at 91% MeOH. When the two steps with 91% MeOH were omitted and the columns were

Table 1 The MRM transitions and instrument settings used in (A) screening of four substances, (B) confirmation of narasin

Substance	Mother ion *m/z*	Daughter ion *m/z*	Cone voltage/V	Collision energy/eV
A Screening of four substances				
Narasin	787	431	60	50
Monensin	693	479	70	50
Salinomycin	773	431	60	50
Lasalocid	613	377	50	35
B Confirmation of narasin				
Narasin	787	431	60	50
Narasin	787	265	60	50
Narasin	787	279	60	50
Narasin	787	531	60	40

Table 2 Optimisation of the SPE step. Columns (Varian C18, 500 mg) were loaded with 900 ng of each substance and eluted successively by increasing concentrations of methanol in water. The loss or yield in each step was measured by analysing the eluate with LC-MS as described by Blanchflower *et al.*[3]

	Ionophore found in each step compared to the total amount loaded (%)		
Step	Monensin	Salinomycin	Narasin
9 ml 80% MeOH, 100 ng ml^{-1} of each ionophore	2	2	3
4 ml Water	< 1	< 1	< 1
2 ml 80% MeOH	< 1	< 1	< 1
2 ml 91% MeOH (first portion)	41	6	2
2 ml 91% MeOH (second portion)	37	57	36
2 ml MeOH	6	26	53
Total	86	91	94

eluted with 4 ml of MeOH only, the yield was 89–92% for all three compounds. When a smaller SPE column was used (Varian C18, 100 mg) 9 ml of a liver extract (approx. 80% MeOH) could still be loaded without significant losses of analytes. After having scaled down optimal volumes for solvents, *i.e.,* to 800 μl 80% MeOH for washing, and 600 μl methanol for elution, these smaller columns performed well in a method using the LC-MS-detection as described by Blanchflower *et al*.[3]

However, after having used this work-up method with stable recoveries for a long period of time, the SPE columns stopped working properly: the recovery of narasin sometimes dropped dramatically. It was found that narasin sometimes bound harder to the column, probably due to batch variations, and that a larger volume of methanol (1–2 ml) was needed for efficient elution. A larger elution volume would, however, dilute the analytes and give a lower detection capability. Solving this by concentrating the extract would slow down the procedure. The original elution volume could be kept though by using a column with slightly less retention. Among several columns tested, Isolute C18 MF (mono functional) 100 mg was found to solve the problem, and was used throughout this work.

As concluded elsewhere (*e.g.* Elliot *et al*.[4]) narasin, monensin and salinomycin can readily be combined in a single analytical procedure, while lasalocid requires alterations to the extraction procedure. Thus, the work-up was not developed to obtain a quantitative method for lasalocid; only the HPLC and MS-MS parameters were optimised for this substance (see below).

Optimisation of the LC-MS-MS procedure

The second aim of this work was to develop a quick, selective and sensitive LC-MS-MS procedure. Identification of mother and daughter ions in positive and negative electrospray, and preliminary optimisation of instrument settings were performed by continuous infusion of the individual compounds at 1 μg ml^{-1}in 50% MeOH. The sodium adducts were found to give the best response as reported elsewhere,[3,5] and the mass spectrometer was optimised to obtain MRM transitions for each sodium adduct (see Table 1A). A standard column for LC-MS-MS (see materials) and different combinations of acetonitrile, methanol, formic acid, and ammonium acetate solutions with different pH, were used to find the optimal LC-MS-MS conditions. These were defined as retention times within minutes, as high signal to noise ratios as possible, and stable response. The shapes of the peaks and the separation of the compounds were of less interest. Source temperatures and gas and LC flows were optimised at the same time. Finally all parameters were fine-tuned for optimal performance and a high sensitivity was demonstrated for standard solutions (see Fig. 1 and Table 3). For all parameter settings refer to Experimental. However, no guard column was used at this stage.

High selectivity, robustness and sensitivity

To test the selectivity and robustness of the LC-MS-MS part of the method, a liver sample was fortified with narasin (40 ng g^{-1}) and a raw extract thereof (5 μl supernatant, see sample extraction) injected into the LC-MS-MS. Single ion monitoring (SIM) and MRM were compared (see Fig. 2). It was found that the signal to noise ratio was approximately a factor of 3000 higher for MRM, which demonstrates the high selectivity of MS-MS. Repeated injections (n = 12) of this "extremely dirty" extract, mixed with numerous injections of blank extracts (equally dirty) (n = 36) as well as standard solutions (n = 48), gave an RSD of 3% for the spiked extracts, which shows the high robustness of the interface. However, to use such raw extracts for routine analysis would probably shorten the lifetime of the column and lead to frequent cleaning of the interface.

Validation

The results from the validation of the method for narasin in eggs are presented below. At this stage an HPLC guard column was introduced (see Materials). All quantitative results have been based on the transition m/z 787 > 431 even though four transitions were always acquired as described in Table 1B. On two occasions blank egg samples (in total 18) were spiked with narasin at levels ranging from 0.5 to 20 ng g^{-1} and analysed by LC-MS-MS (see Fig. 3A). On each occasion three additional samples were spiked (2 to 20 ng g^{-1}) to obtain calibration samples. The average recovery from the latter samples was used to correct the results for losses during the work-up and for any ion suppression in the LC-MS-MS. However, the absolute recovery of the calibration samples averaged 93%, indicating

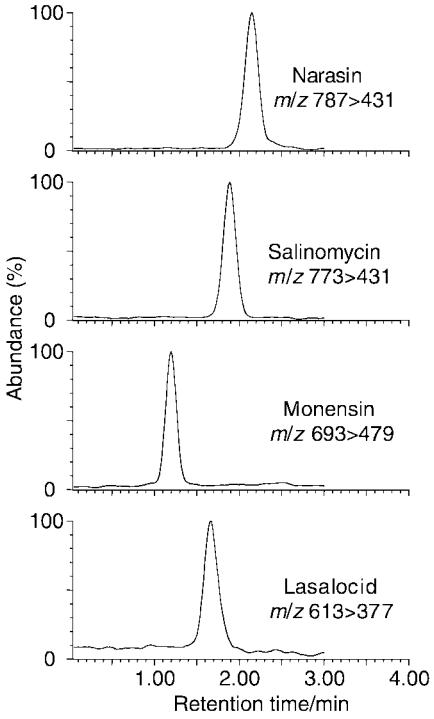

Fig. 1 MRM chromatograms from a 10 μl injection of a mixed standard containing 1.0 ng ml^{-1} narasin, salinomycin, monensin and lasalocid. Chromatograms were normalised to 100% = 1330, 1560, 684 and 335 respectively.

Table 3 Calculated limit of detection (LOD) from repeated (n = 3) injections of standard solutions using the described method including HPLC separation

Substance	S/N ratio[a] (2 pg)	LOD[b]/ ng ml^{-1}	LOD[c]/pg	LOD[c]/ fmol
Narasin	15	0.04	0.4	0.5
Salinomycin	15	0.04	0.4	0.5
Monensin	7	0.09	0.9	1.3
Lasalocid	5	0.12	1.2	2.0

[a] Average signal to noise ratio at 10 μl injections of 0.2 ng ml^{-1}, *i.e.* 2 pg on column. [b] LOD as the calculated concentration giving a signal to noise ratio of 3 at 10 μl injections. [c] LOD as the calculated amount of analyte giving a signal to noise ratio of 3.

that losses and ion suppression were of minor importance. The corrected results are presented in Table 4, where trueness is expressed as bias%. According to EU-regulations[10] the criterion for trueness at levels over 10 ng g^{-1} is that bias should be in the interval −20% to +10%, and for imprecision that RSD should be less than 20% at 10 ng g^{-1}(under repeatability conditions). These criteria were fulfilled even at 0.5 ng g^{-1}. The limit of detection (LOD) was estimated to be 26 pg $g^{-1}$by analysing 12 different blank samples (see Fig. 3C), quantifying the largest peak in a retention time window of 0.85 min centred on the retention time of narasin, and calculating mean + 3*s*. The limit of quantification, defined as the lowest validated level fulfilling trueness and precision criteria and "mean − 3.2*s* > LOD", was fulfilled at 0.5 ng g^{-1} (mean − 3.2*s* = 0.35). Standard curves (0.2 to 50 ng ml^{-1}) typically produced correlation coefficients of 0.99.

For confirmation of identity with low resolution MS-MS it has been suggested that a minimum of two daughter ions must be present with a relative intensity that do not exceed certain tolerances when compared with a standard analyte.[11] In this validation four daughter ions were measured and three ratios calculated: *m/z* 265/431, 279/431 and 531/431. These ratios averaged 0.17, 0.30 and 0.33 respectively for the standard solutions. The suggested maximum permitted tolerances for these three ratios were ±30%, ±25% and ±25% respectively.[11] Using any of these ratios, and the suggested criteria, all spiked samples were confirmed positive.

The method was also validated for narasin in chicken liver. Since it was difficult to find narasin-free samples the results at low levels were highly influenced by incurred residues. However, at higher levels the results were similar: On four occasions two liver samples were spiked with narasin at both 5 and 10 ng g^{-1}. All samples (eight) originated from different animals. The analyses were carried out as described above including calibration samples. Biases were −2% and 5% respectively, and RSD 8% and 9% respectively (n = 8). The

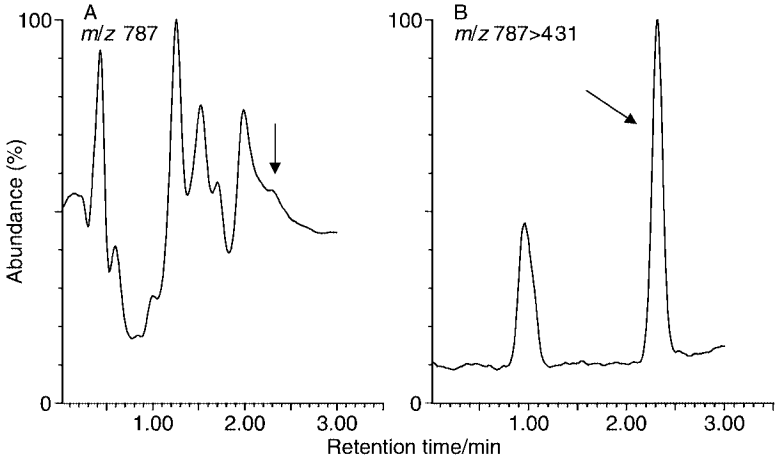

Fig. 2 Chromatograms for a crude extract of liver fortified with narasin for comparison of (A) SIM (single ion monitoring) and (B) MRM (multiple reaction monitoring). Homogenised liver (5 g) was fortified with narasin (40 ng ml^{-1}), extracted with 87% MeOH (15 ml) and centrifuged (3600*g*, 5 min). The supernatant (5 μl) was injected into the described LC-MS-MS system. Chromatograms were normalised to (A) 100% = 65000 and (B) 100% = 2000.

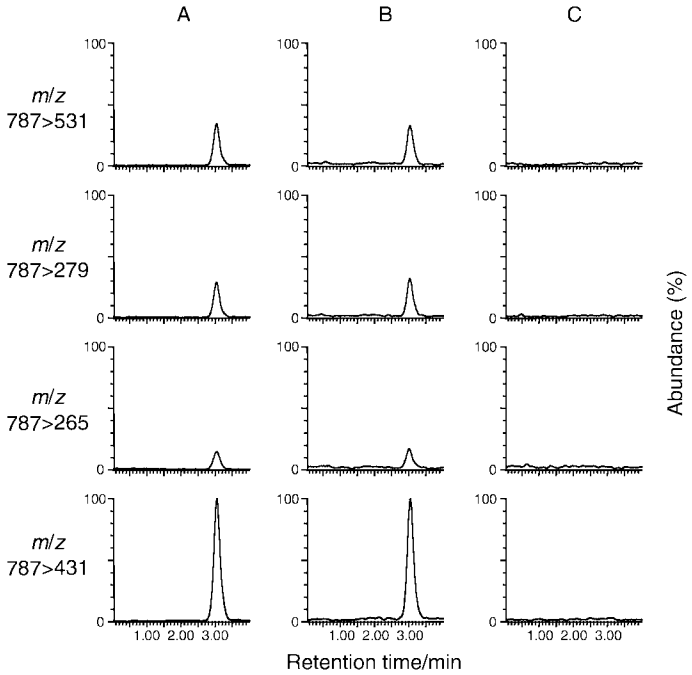

Fig. 3 Chromatograms of four daughter ions for narasin from samples cleaned up with the present method (including SPE). (A) Egg fortified with 0.5 ng ml^{-1} narasin, (B) an incurred sample (from the survey of Swedish eggs) with narasin residue corresponding to ≈ 0.2 ng g^{-1}, and (C) blank egg. MRM chromatograms were normalised to (A) 100% = 4100, (B) and (C) 100% = 1460.

Table 4 Validation data for narasin in eggs

Spike level/ng g^{-1}	Single determinations/ng g^{-1}				n	Mean/ng g^{-1}	Bias (%)	RSD (%)
0.5	0.57	0.52	0.47		3	0.52	4	10
1	1.09	1.10	1.06		3	1.08	8	2
2	1.96	2.19	2.05	1.97	4	2.04	2	5
5	4.94	5.08	5.07	4.60	4	4.92	−2	5
10	9.88	9.33			2	9.61	−4	4
20	19.32	18.36			2	18.84	−6	4
					Total 18			

validation of monensin and salinomycin in tissues from cattle and pig will appear elsewhere.

Narasin residues in Swedish eggs

From 1999 the determination of narasin in eggs has been included in the Swedish national surveillance scheme. The eggs were expected to test negative since the feed for egg-laying hens should be free from coccidiostats. However, when 24 egg samples were analysed in 1999, 12 of them tested positive in the range 0.2 to 11 ng g^{-1}. Confirmation of the identity of narasin was performed as described above (see validation) and showed positive results for all three ion ratios even at 0.2 ng g^{-1} (see Fig. 3B). Cross-contamination of unmedicated feeds with narasin during feed manufacture is the most probable cause of residues in eggs. Similar findings have been reported from Northern Ireland where the incidence of detectable lasalocid residues in eggs was 66% in a survey performed in 1994.[12] Residues of monensin, salinomycin and narasin were also detected in a few cases in the same survey.[13] Due to the present findings Swedish manufacturers of poultry feed are at present taking measures to reduce cross-contamination of feeds to reduce the incidence of narasin in eggs. A study in progress at this laboratory will reveal whether it has been successful.

Effective screening of polyether ionophores in liver by pooling samples

The power of LC-MS-MS is generally associated with high throughput analysis. This is normally achieved by minimising the work-up. However, in cases where the detection capability or limit of quantification is lower than necessary and when most of the samples are expected to be negative, screening can be performed on pooled samples. The latter can be exemplified by the determination of polyether ionophores in liver at this laboratory. Five samples of 1 g each were mixed, and the resulting pool was screened by the present method, detecting one daughter ion per substance (see Table 1A). Since only residues over 5 ng g^{-1} were reported (see the introduction) the limit for the pool of five samples was 1 ng g^{-1}, which is well above the limit of quantification. For confirmation all samples from any pool exceeding this limit were analysed individually. The same method was used. However, only the substance that gave a positive result during screening was detected (by monitoring at least two daughter ions of that substance, *e.g.* see Table 1B). Using this procedure the 98 samples scheduled for 1999 were screened and confirmed in only two analytical runs (see Table 5). Fewer results to evaluate, less documentation (only one method description and one validation for both screening and confirmation, and fewer control charts), easier planning and fewer persons involved were all factors leading to cost-effective activities. Expensive instrument time was minimised since extracts were injected into the LC-MS-MS at intervals of four minutes, which at screening of pooled samples resulted in an instrument time of less than one minute for each individual sample.

Table 5 The arrangements of, and the results from, the screening and confirmation of narasin in 98 chicken liver samples in 1999. All detectable residues over the limit of detection are presented even though the method was not validated below 0.5 ng g^{-1}. No sample contained detectable residues of monensin or salinomycin

Series number	Purpose	Sample number	Number of samples	Found concentration of narasin/ ng g^{-1}
1	Screening	1–5 (pooled)	5	0.14
1	Screening	6–10 (pooled)	5	ND[a]
1	Screening	11–15 (pooled)	5	0.67[b]
1	Screening	16–20 (pooled)	5	ND
1	Screening	21–25 (pooled)	5	0.22
1	Screening	26–30 (pooled)	5	0.13
1	Screening	31–35 (pooled)	5	ND
1	Screening	36–40 (pooled)	5	ND
1	Screening	41–45 (pooled)	5	0.19
1	Screening	46–50 (pooled)	5	ND
2	Confirmation	11	1	2.3
2	Confirmation	12	1	1.3
2	Confirmation	13	1	0.29
2	Confirmation	14	1	ND
2	Confirmation	15	1	0.04
2	Screening	51–55 (pooled)	5	ND
2	Screening	56–60 (pooled)	5	0.06
2	Screening	61–65 (pooled)	5	0.10
2	Screening	66–70 (pooled)	5	ND
2	Screening	71–75 (pooled)	5	0.11
2	Screening	76–80 (pooled)	5	0.09
2	Screening	81–85 (pooled)	5	0.27
2	Screening	86–90 (pooled)	5	ND
2	Screening	91–95 (pooled)	5	0.04
2	Screening	96–98 (pooled)	3	ND

[a] ND = Not detected, *i.e.* below the detection limit of 0.03 ng g^{-1}. [b] The report level for liver was 5 ng g^{-1}. Therefore a pool of five samples should not contain more than 1 ng g^{-1}. Even though this result was below 1 ng g^{-1} samples 11–15 were confirmed in series number 2. The results from the confirmation averaged 0.79 ng g^{-1}, which should be compared to 0.67 ng g^{-1} found in the pool.

Conclusion

The present paper presents an example of how the power of an analytical method based on LC-MS-MS can be utilised for different purposes. The method was not just found to be suitable for reliable confirmation of identity and quantification at low concentration levels, but also excellent for cost-effective screening. Even though the results presented above do not just depend on a powerful technique, but also on well optimised parameters for work-up and detection, the idea of multipurpose utilisation could probably be applied more generally.

Acknowledgements

The author wishes to thank Dr Karl-Erik Hellenäs and Dr Kristina Granelli for their valuable advice in the preparation of this manuscript.

References

1 J. W. Moran, J. M. Rodewald, A. L. Donoho and M. R. Coleman, *J. Assoc. Off. Anal. Chem.*, 1994, **77**, 885.
2 D. G. Kennedy, W. J. Blanchflower and B. C. O'Dornan, *Food Addit. Contam.*, 1995, **12**, 93.
3 W. J. Blanchflower and D. G. Kennedy, *J. Chromatogr.*, 1996, **675**, 225.
4 C. T. Elliott, D. G. Kennedy and W. J. McCaughey, *Analyst*, 1998, **123**, 45R, and references cited therein.
5 W. J. Blanchflower and D. G. Kennedy, *J. Chromatogr.*, 1995, **120**, 1129.
6 D. A. Volmer and C. M. Lock, *Rapid Commun. Mass Spectrom.*, 1998, **12**, 157.
7 J. A. Harris, C. A. L. Russell and J. P. G. Wilkins, *Analyst*, 1998, **123**, 2625.
8 V. Hormazábal and M. Yndestad, *J. Liq. Chromatogr. Rel. Technol.*, 2000, **23(10)**, 1585.
9 D. K. Matabudul, B. Conway and I. D. Lumley, *Analyst*, 2000, **125**, 2196.
10 Commission Decision 93/256/EC, *Off. J. Eur. Communities,* 1993, **L118**, 64.
11 Draft Revision of Commission Decision 93/256/EC, final version 1999, approved by EU Community Reference Laboratories for residues.
12 D. G. Kennedy, W. J. Blanchflower, P. J. Hughes and W. J. McCaughey, *Food Addit. Contam.*, 1996, **13**, 787.
13 D. G. Kennedy, P. J. Hughes and W. J. Blanchflower, *Food Addit. Contam.*, 1998, **15**, 535.