Narasin

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Addendum to the monograph prepared by the 70th meeting of the Committee
and published in FAO JECFA Monographs 6

Identity

International Non-proprietary names (INN): Narasin

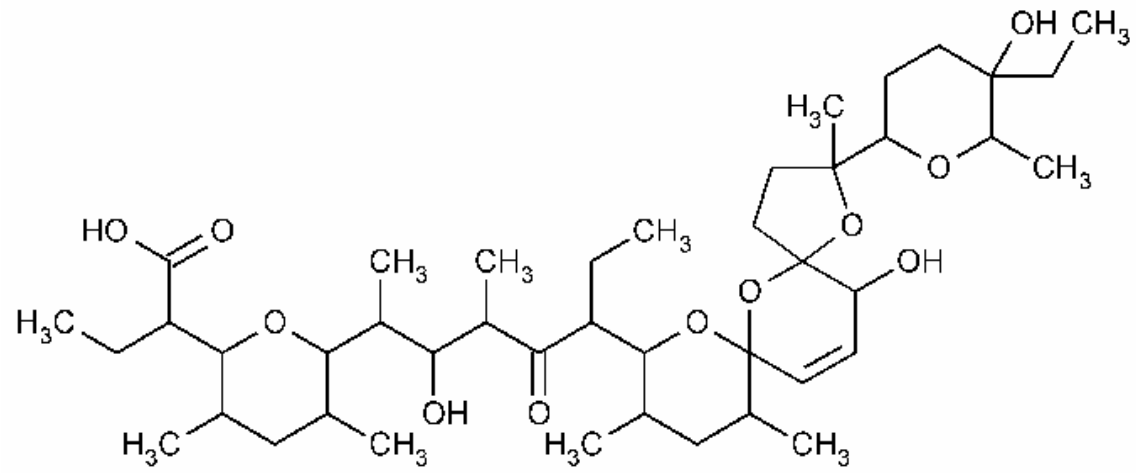
Synonyms: (4s)-4-methylsalinomycin, Narasin A, Monteban®, Naravin®

IUPAC Names: α-ethyl-6-[5-[2-(5 ethyltetrahydro-5-hydroxy-6-methyl-2H-pyran-2-yl)-15-hydroxy-2, 10, 12-trimethyl-1, 6, 8-trioxadispiro [4.1.5.3] pentadec-13-en-9-yl]-2-hydroxy-1, 3-dimethyl-4oxoheptyl] tetrahydro-3,5-dimethyl-2H-pyran-2-acetic acid.

Chemical Abstract Service Number: 55134-13-9

Molecular formula of Narasin A: $C_{43}H_{72}O_{11}$

Molecular mass: 765 g/mol



Background

The Committee evaluated the residue safety of narasin in multiple species of food animals at its 70th meeting (FAO, 2009). In the evaluation, the Committee considered narasin A (shown above) to be a suitable marker residue for narasin in animal tissues of cattle, pigs and chickens. At that time, the Committee recommended and CCRVDF concurred, that MRLs for narasin A in cattle tissues (15 µg/kg for muscle and kidney tissues and 50 µg/kg for liver and fat tissues) were temporary as the analytical method was not adequately validated. The Committee requested that before re-evaluation of narasin for consideration of permanent MRLs in tissues of cattle, a detailed description of a suitable regulatory method, including its

performance characteristics and validation data, be provided by the end of 2010. The sponsor has prepared and submitted detailed reports on a method validation for the determination of narasin A in cattle tissues.

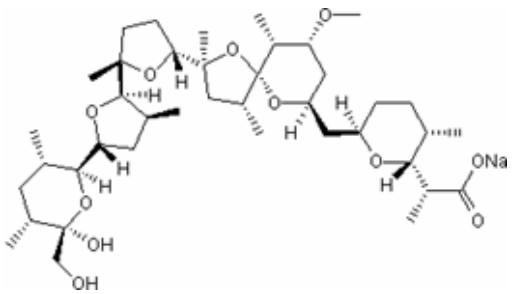
The sponsor submitted three documents in support of the method validation for narasin in cattle tissues. The first is a copy of the method formatted according to the ISO 78/2 format. The second document is a report for a GLP-compliant validation study conducted for monensin A and narasin A in cattle tissues. (MacDougall, 2011a). This study report includes data for monensin A and narasin A, however, only the narasin A data is reviewed here. The third document is a validation data summary for two additional fortification levels in muscle and liver (MacDougall, 2011b). This additional work was conducted to correct an error in the original validation protocol whereby the target concentrations for liver and muscle were transposed and did not correspond to the temporary MRLs. The full dataset is not available for these samples, but the data tables have been fully audited by a quality assurance unit for compliance with GLP. The cattle dataset is a subset of an extensive validation programme in conjunction with the AOAC International that will also include validation data for chicken and pig tissues.

Analytical method

A method for the determination and confirmation of monensin A and narasin A in cattle tissues and milk was validated (MacDougall, 2011a). The laboratory method (Charles River Method Number 1775 Version 1) was reported as written by Charles River Laboratory in Report Appendix 3 of that report. The method was formatted according to the ISO 78/2 format and assigned Version number 1A (Analytical Method Number 1775). Only the narasin data are relevant to this evaluation.

Sample preparation

Muscle, liver or kidney test samples are initially processed from sample material at approximately -20°C. Initial processing involves homogenizing each test sample with dry ice using a food grinder. Samples are allowed to freeze-dry for 24 h at -20°C before weighing 5 ± 0.1 g tissue samples. Test samples are mixed with iso-octane/ethyl acetate (90:10) followed by agitation using four 10 mm steel balls and mixed for 5 minutes in a high-speed tissue homogenizer (900 shakes/min). Samples are centrifuged for 5 minutes at 3000 rpm (*g* values not reported) at 4°C. Solvent is decanted and the procedure with the high-speed tissue homogenizer is repeated. Combined solvent extracts are mixed with anhydrous sodium sulphate. Dried samples are added to the silica solid phase extraction tubes and eluted with ethyl acetate/methanol (80/20) after pre-treatment of the silica SPE. Extracts are evaporated to dryness under nitrogen, and reconstituted in 1.0 ml methanol for LC-MS-MS analysis. Quantification is from a matrix-matched calibration line and is based on monensin A and narasin A. For fat samples, following treatment with the high-speed tissue homogenizer samples are centrifuged at 4000 rpm for 10 minutes at 20°C, refrigerated at 4°C for approximately 15 minutes, extraction solvent is decanted and the process repeated. The alternative is employed to minimize particulates forming a suspension. Nigercin ($C_{40}H_{67}NaO_{11}$, mol mass: 746.94) is used as an internal standard. The structure is reported below.



Nigercin

Analytical measurement

HPLC separation employs a Phenomenex Aqua® 5 µm C_{18} 150 × 2 mm column with elution at 40°C using a gradient elution mixed phase of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The autosampler is maintained at 4°C and the injection volume is 2 µL. Run time is 10 minutes. The MS-MS analysis employs positive MRM using an electrospray ion source (5500 V). Transitions monitored for quantitative determination of narasin are 787.5 > 431.3 (CE = 67 eV) and confirmation 787.5 > 531.3 (CE = 60 eV) and 787.5 > 279.2 (CE = 73 eV). For the internal standard, nigercin, the transition monitored is 746.6 > 729.6 (CE = 55 eV). The latter is not highly specific (loss of –OH). A splitter system may be used as appropriate to ensure the detector source remains cleaner.

Method performance

System suitability was demonstrated based upon column efficiency, peak width at half height, tailing factor and system precision for each test item. System linearity was demonstrated over the range of 0.5 to 100 µg/kg (all tissues) for matrix-match calibration standards prepared in extracted control samples for each matrix. The accuracy (percent recovery) and precision (CV) determined for three sets of six replicate determinations at three levels are summarized in Table 6.1.

Table 6.1. Accuracy (percent recovery) and precision (CV) for determination of narasin A in cattle tissues at the LOQ (0.75 µg/kg in muscle, liver and kidney; 1.0 µg/kg fat)

Matrix	Narasin A Mean (n = 6)% Recovery (CV%)
Muscle	75.4 (2.88)
Liver	93.9 (6.23)
Kidney	96.4 (5.37)
Fat	88.2 (2.44)

The inter-day assay accuracy and precision was determined for each matrix at their respective ½MRL, MRL and 2MRL levels on three occasions. However, the fortification levels for liver and muscle were transposed during the validation. Additional fortification levels were subsequently validated in a separate study (See below, MacDougall, 2011b). The recoveries and precision (CV) are summarized in Table 6.2.

Table 6.2. Inter-day assay accuracy and precision for the determination of narasin A in cattle tissues on three occasions

Matrix	Temp MRL[2] (µg/kg)	½ × MRL[1]	MRL[1]	2 × MRL[1]
		Mean% Recovery (CV%)	Mean% Recovery (CV%)	Mean% Recovery (CV%)
Muscle	15 (50) [1]	97.8 (5.85)	96.2 (6.65)	99.7 (5.40)
Liver	50 (15) [1]	93.0 (9.55)	95.5 (7.70)	99.4 (5.85)
Kidney	15	87.8 (7.07)	87.2 (6.63)	86.6 (5.92)
Fat	50	81.6 (7.82)	82.0 (6.80)	84.9 (6.55)

NOTES: (1) Fortification levels for liver and muscle were transposed; parenthetical value was actually fortified instead of the MRL. (2) Recommended temporary MRLs at 70th meeting of the Committee.

The assay limit of detection (LOD) and limit of quantitation (LOQ) were determined for narasin A in each matrix, as summarized in Table 6.3.

Table 6.3. Assay limits of detection (LOD) and quantitation (LOQ) demonstrated in tissues fortified with narasin A

Matrix	Narasin A		Ratio LOQ/LOD
	LOD (µg/kg)	LOQ (µg/kg)	
Muscle	0.019	0.75	39.5
Liver	0.038	0.75	19.7
Kidney	0.026	0.75	28.8
Fat	0.151	1.00	6.6

NOTES: Values are rounded

Supplemental validation data for an analytical method for the determination of narasin A in bovine liver, and muscle by LC-MS/MS. Quality assurance verified

As noted above, during the original validation study for cattle tissues (MacDougall, 2011a), the tissue concentrations for the method validation for liver and muscle were transposed, resulting in the incorrect validation range for concentrations of narasin A (See Table 6.2). Additional liver and muscle control samples were fortified and analysed for narasin A using the same method reported in MacDougall, 2011a. The data in the Tables 6.4 and 6.5 summarize the individual data sets for narasin A employing the correct concentrations and provides the intra-day and inter-day accuracy and precision calculations (MacDougall, 2011b).

Table 6.4. Intra-day and inter-day assay accuracy and precision for the determination of narasin A in cattle liver on three independent replicates. (MacDougall, 2011b)

Narasin fortification	Analysis 1		Analysis 2		Analysis 3		Inter-day statistics			
	µg/kg	Recovery (%)	µg/kg	Recovery (%)	µg/kg	Recovery (%)	Mean µg/kg	Recovery (%)	s_r (µg/kg)	$RSD_{r\%}$
25 µg/kg	21.1	84.4	21.0	84.0	20.0	80.0	20.9	83.8	1.3	6.1
	18.0	72.0	22.2	88.8	20.6	82.4				
	19.6	78.4	22.5	90.0	19.0	76.0				
	22.0	88.0	21.2	84.8	21.5	86.0				
	20.9	83.6	21.3	85.2	22.8	91.2				
	22.6	90.4	20.8	83.2	19.9	79.6				
Mean	20.7	82.8	21.5	86.0	20.6	82.5				
s_r	1.67		0.687		1.35					
RSD_r	8.08		3.20		6.52					
50 µg/kg	37.5	75.0	42.8	85.6	43.1	86.2	42.4	84.7	3.4	8.1
	44.6	89.2	42.0	84.0	44.9	89.8				
	34.8	69.6	39.3	78.6	45.8	91.6				
	38.3	76.6	43.9	87.8	46.1	92.2				
	42.0	84.0	44.0	88.0	44.9	89.8				
	37.8	75.6	44.3	88.6	46.5	93.0				
Mean	39.2	78.3	42.7	85.4	45.2	90.4				
s_r	3.52		1.88		1.22					
RSD_r	8.99		4.41		2.70					
100 µg/kg	79.5	79.5	86.8	86.8	107	107.0	89.2	89.2	7.2	8.1
	90.1	90.1	88.4	88.4	97.7	97.7				
	85.5	85.5	82.2	82.2	91.5	91.5				
	94.7	94.7	91.7	91.7	87.6	87.6				
	85.9	85.9	83.0	83.0	99.9	99.9				
	78.3	78.3	86.5	86.5	88.9	88.9				
Mean	85.7	85.7	86.4	86.4	95.4	95.4				
s_r	6.22		3.51		7.46					
RSD_r	7.26		4.06		7.82					

NOTES: s_r = Standard Deviation. RSD = Reproducability standard deviation

Table 6.5. Intra-day and inter-day assay accuracy and precision for the determination of narasin A in cattle muscle on three independent replicates. (MacDougall, 2011b)

Narasin fortification (µg/kg)	Analysis 1		Analysis 2		Analysis 3		Inter-day statistics			
	µg/kg	Recovery (%)	µg/kg	Recovery (%)	µg/kg	Recovery (%)	Mean (µg/kg)	Recovery (%)	s_r (µg/kg)	$RSD_{r\%}$
7.5	7.15	95.3	7.18	95.7	6.82	90.9	7.2	96.4	0.4	5.0
	7.08	94.4	7.57	100.9	6.54	87.2				
	7.01	93.5	7.84	104.5	7.08	94.4				
	7.16	95.5	7.74	103.2	7.29	97.2				
	7.71	102.8	7.23	96.4	7.17	95.6				
	7.17	95.6	7.64	101.9	6.70	89.3				
Mean	7.21	96.2	7.53	100.4	6.93	92.4				
s_r	0.251		0.271		0.292					
RSD_r	3.48		3.59		4.21					
15	16.1	107.3	17.0	113.3	12.2	81.3	14.8	98.6	1.6	10.8
	13.8	92.0	16.9	112.7	13.2	88.0				
	15.4	102.7	17.0	113.3	12.5	83.3				
	14.4	96.0	16.1	107.3	13.1	87.3				
	14.3	95.3	15.3	102.0	13.1	87.3				
	14.7	98.0	16.6	110.7	14.6	97.3				
Mean	14.8	98.6	16.5	109.9	13.1	87.4				
s_r	0.833		0.674		0.828					
RSD_r	5.63		4.09		6.31					
30	30.2	100.7	33.2	110.7	24.2	80.7	28.7	95.7	2.5	8.8
	30.4	101.3	30.6	102.0	26.5	88.3				
	27.4	91.3	31.4	104.7	24.7	82.3				
	29.3	97.7	30.9	103.0	26.3	87.7				
	26.1	87.0	30.9	103.0	28.2	94.0				
	28.5	95.0	31.0	103.3	27.2	90.7				
Mean	28.7	95.5	31.3	104.4	26.2	87.3				
s_r	1.67		0.950		1.51					
RSD_r	5.84		3.03		5.75					

NOTES: s_r = Standard Deviation. RSD = Reproducibility standard deviation

System suitability

The column efficiency, peak width at half height and tailing factor for the test items and internal standards was established. The system precision for reproducibility of response and retention time was determined by replicate injections (n=10) of a standard solution of the test items and internal standard. The precision was defined as the coefficient of variation of the mean value for each parameter.

System linearity

The system linearity was determined by analysing non-extracted matrix-matched standard solutions of known amounts of each test item and the internal standard. Standards were prepared over a range of concentrations of each test item with a fixed concentration of internal standard. The detector response ratio for the test item/internal standard was plotted against the amount injected of the test item to generate a calibration curve. Calculated amounts of the injected standards were determined by using a least squares linear regression

analysis with weighting factor of 1/x. The origin was excluded from the regression analysis. The calculated amount injected for each prepared standard was required to be within ±15% of the actual amount injected (±20% at the lower limit of linearity) to define the linear range of the system.

System limit of detection

The limit of on-column detection (LOOCD) was determined by analysis of solutions of each test item with decreasing concentrations. The LOOCD was defined as the amount injected of each test item that gives a clearly discernible peak, and was at least 3 times greater than the background noise.

Assay limit of quantitation

The limit of quantitation (LOQ) for each test item was determined by the extraction and analysis of replicate aliquots (n=6) of control matrix fortified with decreasing concentrations of each test item, and assaying these samples with the standard method. The target intra-day assay accuracy at the LOQ (defined as the mean percentage measured concentration versus actual concentration) was 70–110%. The precision at each concentration (defined as the CV of the mean concentration) was ≤20%.

Intra-day assay accuracy and precision

The assay accuracy and precision was determined by the extraction and analysis of replicate aliquots (n=6) of each matrix fortified with each test item, together with a non-extracted matrix-matched calibration curve series of standard solutions. Samples were prepared at ½MRL, MRL and 2MRL for each matrix. The target intra-day assay accuracy at each concentration (defined as the mean percentage measured concentration versus actual concentration) was 70–110%. The target intra-day assay precision at each concentration (defined as the CV of the mean concentration) was ≤20%.

Inter-day assay accuracy and precision

The assay accuracy and precision was determined on three occasions, as detailed for the intra-day assay accuracy and precision. The target inter-day assay accuracy at each concentration (defined as the mean percentage determined concentration/actual concentration of all the replicate samples from all three occasions) was 70–110%. The target inter-day assay precision at each concentration (defined as the CV of the mean requirement concentration) was ≤20%. To demonstrate the ruggedness of the assay, at least one of the occasions was extracted by a second analyst.

Assay specificity

The specificity of the assay for each test item and the internal standard was examined by extraction and analysis of aliquots of each matrix with and without the addition of the test item. The assay requirement for each test item was "no significant interfering substances >20%" (a very permissive value) of peak area at LOQ level eluting at the same retention times as the test items or the internal standard. The following analytes were also used to evaluate the potential for interference: penicillin, tylosin, tilmicosin, tetracycline, lasalocid, ceftiofur, ractopamine and ketoprofen. This study was limited to injection of solution standards.

Assay limit of detection

The assay limit of detection (LOD) was determined by extraction and analysis of 20 aliquots (4 extractions from each of 5 different animals) of the matrix to determine the mean background noise. The LOD was defined as the concentration of each test item equivalent to the mean background noise plus 3 times the standard deviation.

Storage stability at room temperature

Storage stability was demonstrated to be acceptable for each matrix at room temperature (about 4 h) and for extended frozen storage (approximately -20°C) up to 2 months. The stability of samples after three freeze-thaw cycles was demonstrated for each matrix. The stability of extracts stored at about 4°C was shown to be approximately 72 h for each matrix.

The effects of storing samples at room temperature was investigated by preparing replicate matrix samples fortified with each test item at the relative MRL concentrations, followed by storage at room temperature for about 4 h prior to extraction (representative of the actual times necessary to prepare and analyse a batch of samples prior to extraction). To define the reference (initial) concentrations, replicate aliquots (n=6) of the matrix was prepared (fortified with solutions prepared independently from the calibration standard solutions) and assayed. The mean calculated concentration of the replicate samples was determined and was defined as the reference (i.e. 100%) concentration. Acceptance criteria for reference concentration samples were the same as for assay accuracy and precision analysis. The stability of the test items was defined as the mean 4 h post-storage concentration/reference concentration (expressed as a percentage) for that level. The test items were considered to be stable in the matrix at room temperature if the stability was 100 ± 20%.

Freeze-thaw stability

The effects of repeatedly freezing and thawing samples were investigated by preparing replicate matrix samples fortified with each test item at the relative MRL, and repeatedly freezing and thawing prior to extraction. To define the reference (initial) concentrations, replicate aliquots (n=6) of the matrix were prepared (fortified with solutions prepared independently from the calibration standard solutions) and assayed. The mean calculated concentration of the replicate samples was determined and defined the initial concentration. Acceptance criteria for reference concentration samples were the same as for assay accuracy and precision analysis.

To determine the freeze-thaw stability of the test item in the matrix, 3 sets of replicate aliquots (n=6) of the matrix were prepared (fortified with solutions prepared independently from the calibration standard solutions). Following fortification of the tissues the samples were stored frozen for a minimum of 24 h. The samples were then thawed until they reach room temperature, and then re-frozen for a minimum of 24 h. Samples were subjected to 1, 2 or 3 freeze-thaw cycles. The samples were assayed together with a non-extracted matrix-match calibration curve and freshly prepared samples at the same fortified fortification level after the appropriate number of cycles.

The stability of each test item for each freeze-thaw cycle was defined as the mean post freeze-thaw concentration/concentration of the fresh extracts expressed as a percentage for that number of cycles. The test items were deemed to be stable in the matrix if the stability was 100 ± 20%.

Autosampler stability

The effect of storing extracts of samples was investigated by extracting replicate matrix samples fortified with each test item at the MRL and 2MRL and storing at +4°C for about 72 h prior to analysis. Replicate aliquots (n=6) of the matrix were prepared at each concentration (fortified with solutions prepared independently from the calibration standard solutions) and extracted, together with a non-extracted matrix-matched calibration curve series of standard solutions. The calibration samples and the replicates at each concentration were analysed before being stored at about +4°C for about 72 h prior to analysis. This was representative of the actual times necessary to prepare and analyse a batch of samples and to permit a repeat analysis (without re-extraction), if required, and then assayed. The mean

post-storage calculated concentrations of the replicate samples at each level were determined. The stability was defined with respect to the recovery data prior to the circa 72 h storage period. The autosampler storage was considered to be acceptable if the recoveries were 100 ± 20% of the samples analysed immediately.

Solution stability

The stability of selected calibration solutions of each test item was investigated by periodically re-preparing standard and QC solutions and analysing them together with a non-extracted calibration series of standards previously prepared. The solutions were deemed to be stable for a period corresponding to difference in time between the preparations of the two sets of standard solutions if the mean accuracy of freshly prepared solutions (defined as the mean percentage determined concentration/actual concentration) was 100 ± 20%.

Extended frozen storage stability

The effects of storing samples frozen at about -20°C were investigated by preparing samples fortified with each test item at the MRL in each matrix. Sufficient samples were prepared in each matrix to permit replicate samples (n=6), which were had not been thawed since preparation, to be taken at each time point. To define the reference (initial) concentrations, replicate aliquots (n=6) of the matrix were prepared (fortified with solutions prepared independently from the calibration standard solutions) and assayed. The mean calculated concentration of the replicate samples was determined and was defined as the initial concentration. Acceptance criteria for reference concentration samples were the same as for assay accuracy and precision analysis.

To determine the storage stability of the test item in the matrix at about -20°C after approximately 1 and 2 month frozen storage, replicate samples (n=6) were thawed and assayed together with a non-extracted matrix-match calibration curve and freshly prepared fortified samples. The stability of the test item was defined as the percentage difference between the freshly fortified extracts on each occasion and the time points T=1 and 2 months. The test item was deemed to be stable in the matrix under frozen conditions if the stability was 100 ± 20% of the fresh extracts.

Assay acceptance criteria

For the analysis of the test items in bovine tissues described above, the following additional criteria were met. The determined concentration for each prepared non-extracted matrix-match (injected at the front and the end of any batch) standard used to construct the calibration curve was within 100 ± 15% of the actual concentration (100 ± 20% at the lower limit of quantitation). At least 75% of the calibration standards met the above criteria.

Statistical analysis

Statistical analysis was limited to derivation of means, standard deviations, coefficient of variation and regression parameters.

Means, standard deviations and precisions (CV%).

Table 6.6 provides assay limits of quantitation; Tables 6.7–6.10 provide accuracy and precision data in fortified tissues; and Figures 6.1–6.4 describe matrix match calibration lines in control tissue.

Table 6.6. Narasin A assay limits of quantitation

Analyte	Fortification level (µg/kg)	Recovery (%)	Mean recovery (%)	CV (%)
In Muscle				
Narasin	0.75	71.9 76.6 76.5 77.0 77.0 73.5	75.4	2.88
In Liver				
Narasin	0.75	88.0 86.7 94.5 101 99.7 93.7	93.9	6.23
In Kidney				
Narasin	0.75	101 98.9 96.7 98.4 86.3 96.8	96.4	5.37
In Fat				
Narasin	1.00	86.1 91.5 87.6 89.0 85.8 89.3	88.2	2.44

Table 6.7. Assay accuracy and precision in muscle tissue fortified at 25 µg/kg with Narasin A

			Intraday		Interday	
Fortification Level (µg/kg)	Occasion	Recovery (%)	Mean Recovery (%)	CV (%)	Overall Mean Recovery (%)	Overall CV (%)
25	1	102	99.9	6.36	97.8	5.85
		105				
		94.4				
		98.6				
		108				
		91.3				
	2	97.3	97.6	3.74		
		90.6				
		98.1				
		98.7				
		101				
		99.7				
	3	86.1	96.1	7.27		
		93.9				
		103				
		102				
		101				
		90.4				

Table 6.8. Assay accuracy and precision in liver tissue fortified at 30 µg/kg with Narasin A

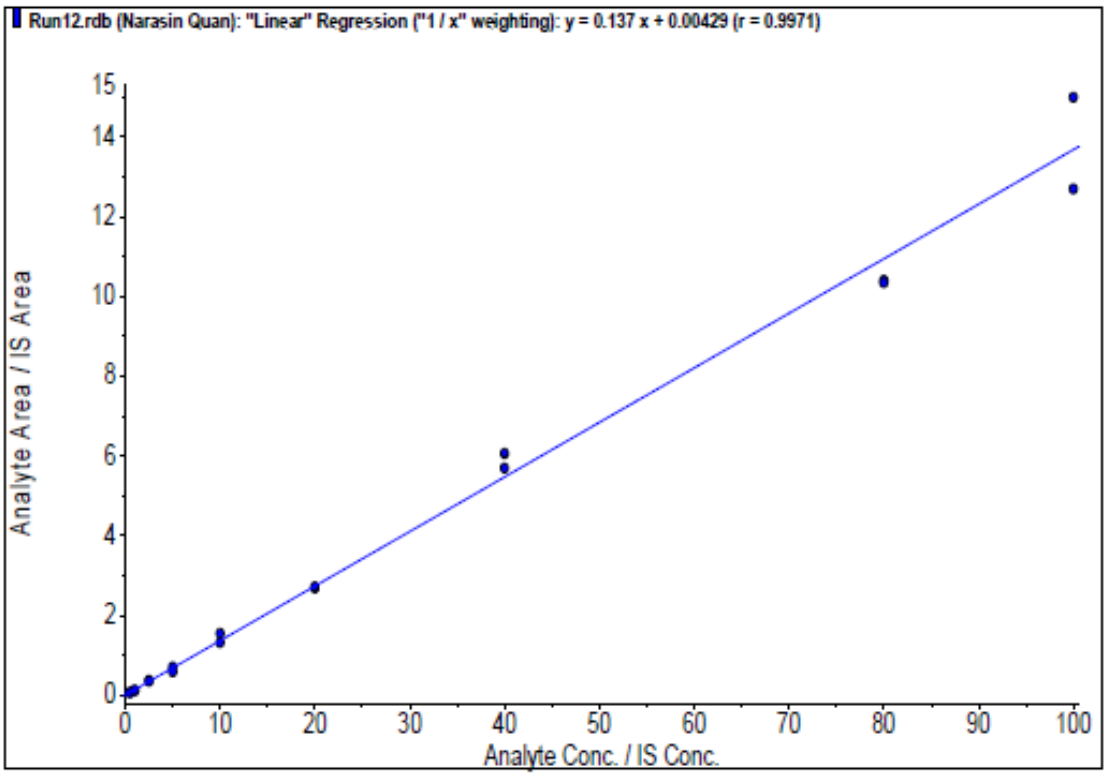
			Intraday		Interday	
Fortification Level (µg/kg)	Occasion	Recovery (%)	Mean Recovery (%)	CV (%)	Overall Mean Recovery (%)	Overall CV (%)
30	1	98.0	104	5.17	99.4	5.85
		105				
		98.8				
		108				
		112[A]				
		103				
	2	98.0	99.6	4.54		
		94.7				
		101				
		103				
		95.0				
		106				
	3	92.8	94.3	2.91		
		93.1				
		97.2				
		98.2				
		91.2				
		93.4				

Table 6.9. Assay accuracy and precision in kidney tissue fortified at 15 µg/kg with Narasin A

			Intraday		Interday	
Fortification Level (µg/kg)	Occasion	Recovery (%)	Mean Recovery (%)	CV (%)	Overall Mean Recovery (%)	Overall CV (%)
15	1	93.0	92.1	4.98	87.2	6.63
		88.8				
		93.4				
		98.7				
		93.5				
		85.3				
	2	83.1	87.0	4.67		
		90.8				
		86.5				
		92.9				
		85.9				
		82.9				
	3	77.1	82.5	5.44		
		83.0				
		84.1				
		79.9				
		90.1				
		80.5				

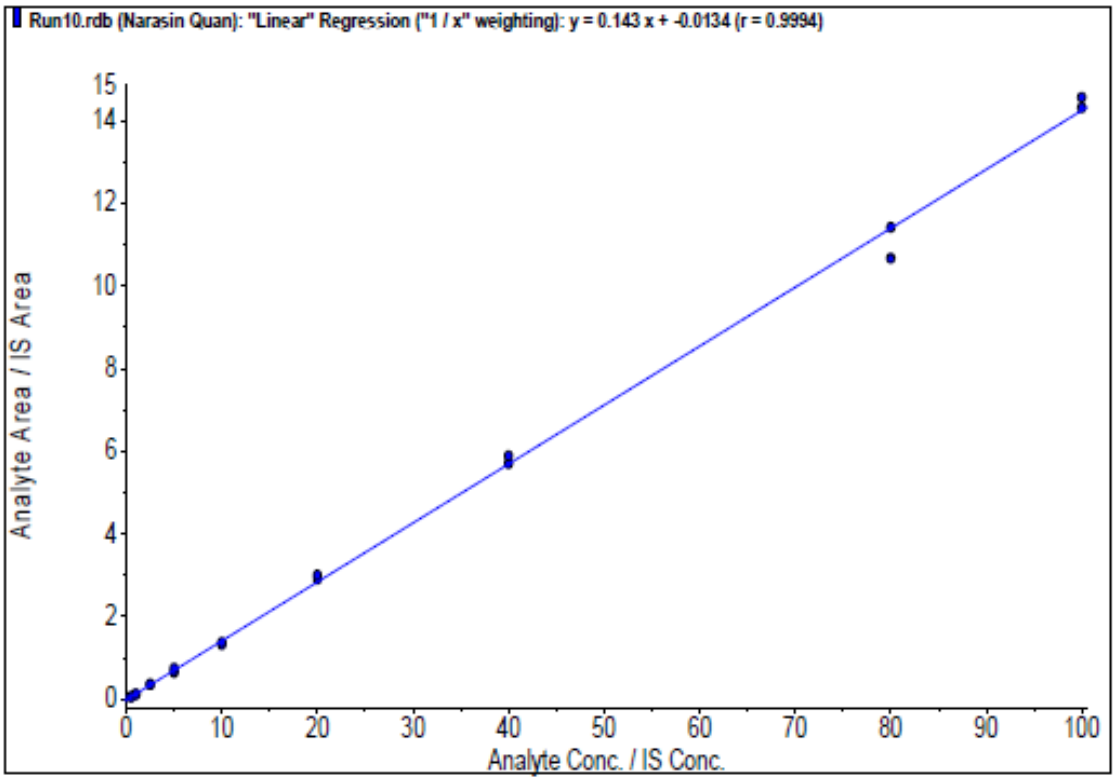
Table 6.10. Assay accuracy and precision in fat tissue fortified at 50 µg/kg with Narasin A

			Intraday		Interday	
Fortification Level (µg/kg)	Occasion	Recovery (%)	Mean Recovery (%)	CV (%)	Overall Mean Recovery (%)	Overall CV (%)
50	1	81.4	83.8	3.41	82.0	6.80
		81.7				
		83.4				
		88.8				
		82.0				
		85.3				
	2	83.0	85.7	3.69		
		84.7				
		88.4				
		83.4				
		83.7				
		90.7				
	3	73.6	76.7	7.63		
		85.3				
		72.7				
		80.5				
		69.4^				
		78.7				



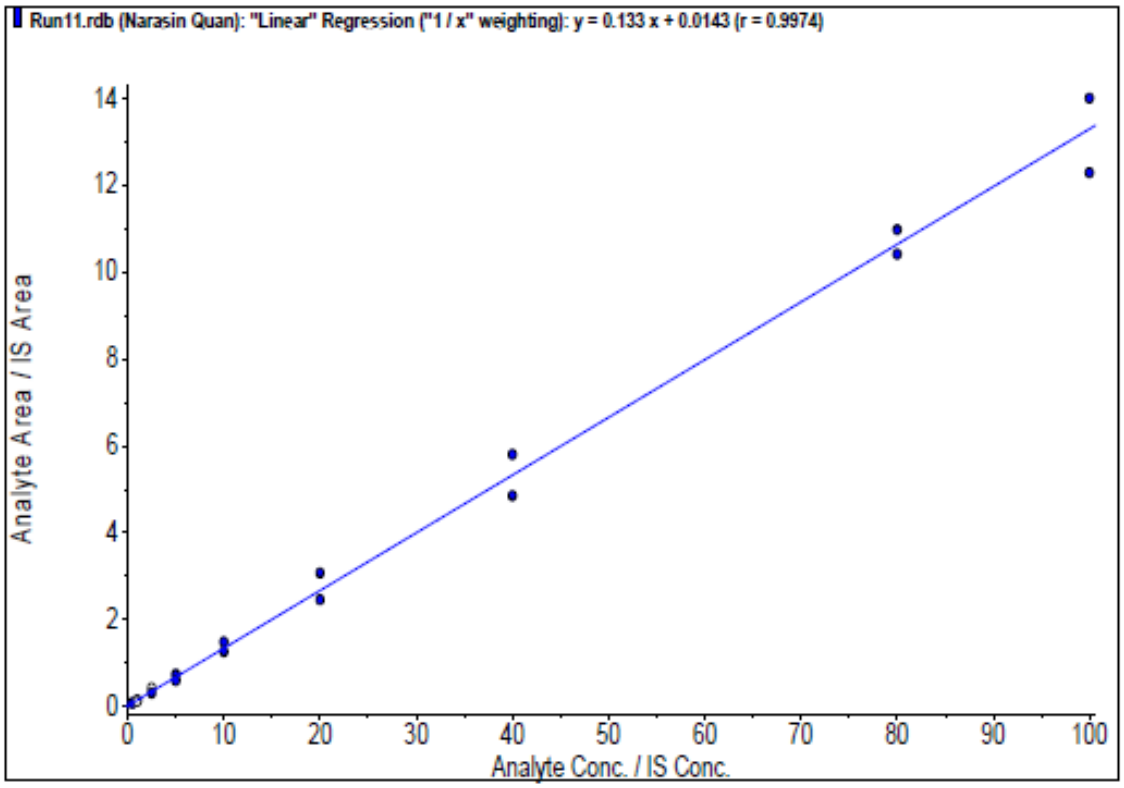
Standard ID	Actual Concentration (ng/mL)	Front Calibration Line		Back Calibration Line	
		Found Concentration (ng/mL)	Accuracy (%)	Found Concentration (ng/mL)	Accuracy (%)
MM L	0.5000	0.454	90.8	0.509	102
MM K	1.000	0.765	76.5 [A]	0.937	93.7
MM J	2.500	2.73	109	2.56	103
MM I	5.000	5.16	103	4.27	85.5
MM H	10.00	11.3	113	9.63	96.3
MM G	20.00	19.6	98.0	19.9	99.4
MM F	40.00	41.6	104	44.3	111
MM E	80.01	76.0	95.0	75.6	94.5
MM D	100.0	110	110	92.8	92.8

Figure 6.1. Narasin matrix-match calibration line in control muscle extracts (0.5–100 ng/ml)



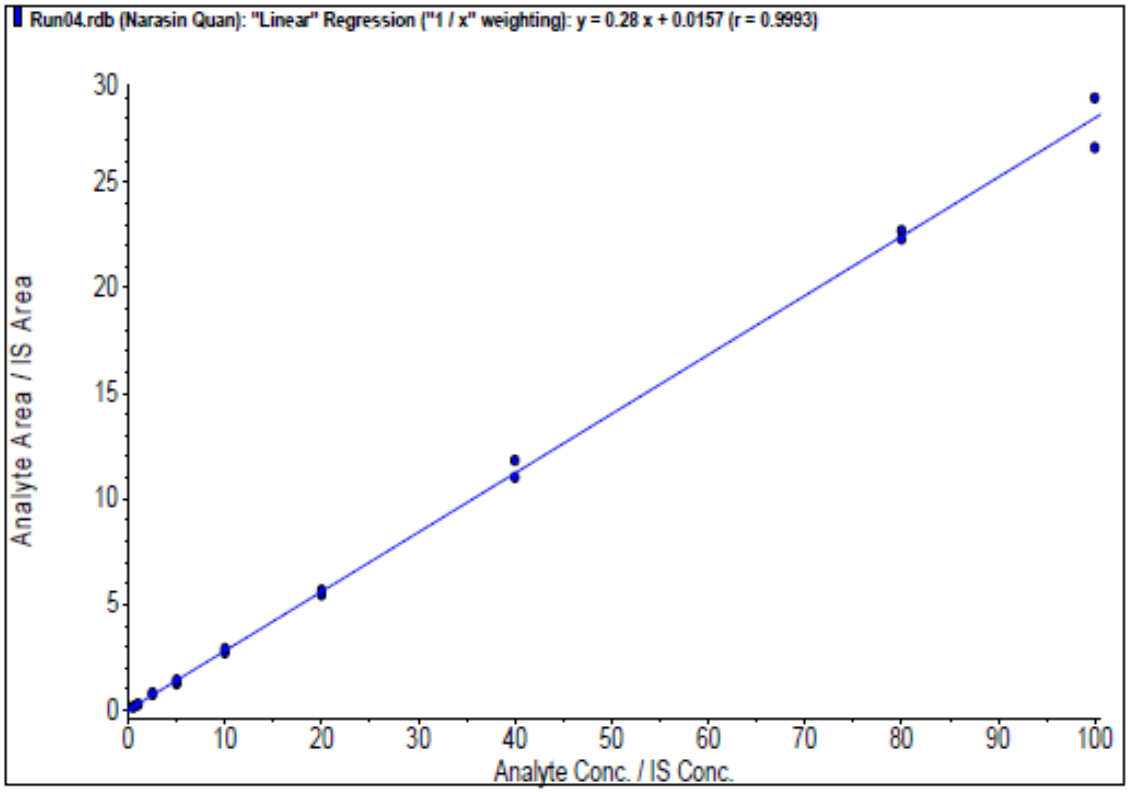
Standard ID	Actual Concentration (ng/mL)	Front Calibration Line		Back Calibration Line	
		Found Concentration (ng/mL)	Accuracy (%)	Found Concentration (ng/mL)	Accuracy (%)
MM L	0.5000	0.512	102	0.507	101
MM K	1.000	0.911	91.1	0.968	96.8
MM J	2.500	2.62	105	2.62	105
MM I	5.000	4.65	93.0	5.35	107
MM H	10.00	9.36	93.6	9.73	97.3
MM G	20.00	20.5	102	21.1	106
MM F	40.00	40.1	100	41.4	103
MM E	80.01	74.9	93.6	80.2	100
MM D	100.0	100	100	102	102

Figure 6.2. Narasin matrix-match calibration line in control liver extracts (0.5–100 ng/ml)



		Front Calibration Line		Back Calibration Line	
Standard ID	Actual Concentration (ng/mL)	Found Concentration (ng/mL)	Accuracy (%)	Found Concentration (ng/mL)	Accuracy (%)
MM L	0.5000	0.496	99.3	0.509	102
MM K	1.000	1.03	103	0.794	79.4 [A]
MM J	2.500	3.07	123 [A]	2.23	89.1
MM I	5.000	5.46	109	4.38	87.5
MM H	10.00	11.1	111	9.39	93.9
MM G	20.00	23.0	115	18.4	91.9
MM F	40.00	43.6	109	36.4	91.1
MM E	80.01	78.3	97.8	82.5	103
MM D	100.0	92.4	92.4	105	105

Figure 6.3. Narasin matrix-match calibration line in control kidney extracts (0.5–100 ng/ml)



		Front Calibration Line		Back Calibration Line	
Standard ID	Actual Concentration (ng/mL)	Found Concentration (ng/mL)	Accuracy (%)	Found Concentration (ng/mL)	Accuracy (%)
MM L	0.5000	0.486	97.1	0.512	102
MM K	1.000	0.896	89.6	0.995	99.5
MM J	2.500	2.62	105	2.88	115
MM I	5.000	4.43	88.6	5.08	102
MM H	10.00	9.66	96.6	10.3	103
MM G	20.00	19.5	97.3	20.2	101
MM F	40.00	39.3	98.3	42.2	105
MM E	80.01	81.0	101	79.6	99.4
MM D	100.0	95.0	95.0	103	103

Figure 6.4. Narasin matrix-match calibration line in control fat extracts (0.5–100 ng/ml)

Appraisal

The 70th meeting of the Committee reported on the availability of screening, quantitative and confirmatory methods for narasin in chicken, pig and cattle tissues that may be appropriate for regulatory control programmes (FAO/WHO, 2009). GLP-compliant screening methods were reported based on extraction followed by thin layer chromatography-bioautography methods (Maruyama and Sugimoto, 2000). The bioautography was performed by melting agar over the surface of the TLC plate seeded with *Bacillus stearothermophilus* var. *calidolactis* C-953 inoculum. The limit of quantitation (LOQ) was estimated to be 25 µg/kg. Calibration curves showed good linearity within the tested concentrations of 0.1–3.2 mg/kg. However, the accuracy, precision and the limit of detection (LOD) of the assay were not given. In another GLP-compliant screening study (Handy, Thomson and Tamura, 1985), a TLC-bioautographic method, using *Bacillus subtilis* as the indicator organism, was described. The limit of detection was 5 µg/kg.

A Time-Resolved Fluorescence Immunoassay (TR-FIA) screening method for the detection of narasin was developed in a non-GLP compliant study (Peippo *et al.,* 2004). Muscle tissue extracts were applied to a microtitre well containing an antibody (goat anti-sheep IgG), and an aliquot of unlabelled narasin-transferrin conjugate in a reconstitution buffer. The time resolved fluorescence was measured by a multi-label counter. The LOD of this method was 560 µg/kg, the LOQ was 800 µg/kg. The results of the precision intra-assay and inter-assay were 3.5 and 3.6% (CV) respectively.

Confirmatory methods for narasin were also reported using HPLC methods with UV_{vis} detection using chromatographic analysis post-column derivatization with vanillin reagent that produces a product that absorbs at 520 nm. (Ward *et al.*, 2005; Lacoste and Larvor, 2003). Different authors have described the use of LC coupled to mass spectrometry to determine narasin in edible chicken tissues. The analyses are performed in the positive ion electrospray modes. The mass spectrometric methods are suitable and provide better specificity and sensitivity than do the HPLC-UV methods. Because the methods require only a simple extraction with a short run time (about 12 minutes), large-sample batches (more than 20 samples) can be processed daily.

However, the 70th meeting of the Committee noted that suitable analytical methods have been described for the determination and confirmation of narasin only in edible tissues of chickens and pigs. Residues in cattle could only be determined using a TLC-bioautographic method. This method, while having a reported test sensitivity of 5 µg/kg, had results of residue values reported only as a range (e.g. 10–20; 5–10). As a result, only temporary MRLs for cattle were recommended by the Committee using the LOQ values for the HPLC-UV methods.

The sponsor has provided a new GLP-compliant HPLC-MS/MS method. Three documents were submitted in support of the method validation for narasin in cattle tissues: a copy of the method formatted according to the ISO 78/2 format; a report for a GLP-compliant validation conducted for monensin A and narasin A in cattle tissues; and a validation data summary for two additional fortification levels in muscle and liver. This additional work was conducted because the original validation protocol transposed concentrations for liver and muscle temporary MRLs. The full dataset was not available for these samples, but the data tables have been fully audited by a quality assurance unit for compliance with GLP. The cattle dataset is a subset of an extensive validation programme in conjunction with the AOAC International that will include validation data for chicken and pig tissues as well. While not noted in the report, the method has been developed for narasin and monensin in tissues of cattle, chickens and pigs.

The reports document acceptable system suitability, system linearity, accuracy and precision, limits of detection and quantitation, but do not specifically specify the limit of identification, although expected to be consistent with the LOQ. The LOD was determined to be 0.026–0.151 µg/kg for the four primary tissues and the LOQ was determined to be 0.75 µg/kg for muscle, liver and kidney tissues and 1.0 µg/kg for fat tissue. All values are well below the recommended temporary MRLs from the 70th meeting of the Committee. Other performance factors demonstrating method performance and method validation include intra- and inter-day accuracy and precision performance, analytical specificity with a number of veterinary antibiotic drugs, analyte-fortified storage stability, freeze-thaw and extended frozen storage stability, autosampler stability and solution stability. The method description, reagents, equipment, mass spectrometry settings and conditions are adequately described. Data provided should enable a regulatory laboratory to develop specific quality control and quality assurance documents to support laboratory and regulatory control use.

Maximum residue limits

In recommending MRLs for narasin in cattle, the Committee considered the following factors:

- A new GLP-compliant validated HPLC-MS/MS complete with adequate performance factors and method validation was provided that was considered suitable for routine monitoring for narasin A as marker residue.
- The analytical method has been validated for use in cattle tissues and is also appropriate for chicken and pig tissues.

The 70th meeting of the Committee recommended temporary MRLs of 50 µg/kg for cattle liver and fat, and 15 µg/kg for cattle muscle and kidney, determined as narasin A. The LOQs for the new analytical method for cattle tissues are more than adequate to accommodate the MRLs recommended at the 70th meeting of the Committee for other animal species and tissues.

The Committee recommended full MRLs for narasin of 15 µg/kg for cattle muscle and kidney, and 50 µg/kg for liver and fat tissues, determined as narasin A.

The 70th meeting of the Committee decided not to calculate the Estimated Daily Intake because there were insufficient data points in the residue depletion studies to calculate the median values for residues. Using the model diet and a marker:total residue ratio of 5%, the MRLs recommended above would result in a theoretical maximum daily intake of 255 µg per person per day, which represents approximately 85% of the upper bound of the ADI.

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