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Liquid chromatography–electrospray tandem mass spectrometric method for quantification of monensin in plasma and edible tissues of chicken used in pharmacokinetic studies: Applying a total error approach

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Abstract

A liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed and validated for use in pharmacokinetic studies in order to determine the concentrations of monensin in plasma and edible tissues of chicken. Two sample preparations were performed, one for determining monensin concentrations in plasma using acetonitrile for protein precipitation and another one for determining monensin concentrations in muscle, liver, and fat using methanol–water followed by a clean up on a solid-phase extraction cartridge. Sample extracts were injected into the LC–MS/MS system, and a gradient elution was performed on a C_{18} column. Narasin was used as internal standard. The LC–MS/MS method was validated using an approach based on accuracy profiles, and applicability of the method was demonstrated for the determination of monensin in chicken plasma, muscle, liver, and fat in a pharmacokinetic study.

Keywords: Coccidiostats; Monensin; Polyether ionophores; Chicken; Plasma; Muscle; Liver; Fat; Validation; Pharmacokinetics; Total error

1. Introduction

Coccidiosis is a parasitic disease caused by unicellular organisms such as those belonging to the genus Eimeria, class Sporozoa. Economic losses related to coccidiosis are significant in avian industry. For this reason, coccidiostats in the form of feed additives are used on a large scale to prevent and treat the disease. For the moment, coccidiostats are licensed for use in Europe as feed additives by Regulation 1831/2003/EC [1], but no MRLs have yet been set, except those for lasalocid. Thus, to comply with EU requirements, the European Food Safety Authority (EFSA) council on efficacy and safety will establish classification of additives into two categories: authorized additive with MRL or banned additive included in Annex IV of Council Regulation 2377/90 [2]. Monensin, a known coccidiostat used worldwide and marketed under the name Elancoban, can be added to broiler feed at concentrations ranging from 100 to 125 mg/kg for treatment until 3 days before slaughtering according to the Council Directive 70/524/EC [3]. Currently, EFSA has proposed a MRL of 50 μg/kg for all tissues [4].

Monensin is a coccidiostat produced by certain strains of Streptomyces belonging to the group of polyether ionophore antibiotics. To determine monensin in different biological matrices, several analytical methods have been used. These methods are based on colorimetry [5], high performance liquid chromatography with UV [6,7] or fluorescence detection [8,9] and liquid chromatography linked with mass spectrometry [10–14].

Monensin depletes rapidly from chicken tissues. After roughly 8 h, monensin is detected at only very low concentrations in tissues [15]. Although LC/MS–MS methods for polyether ionophore detection in tissues have been published, no specific method for monensin determination in poultry tissues for use in pharmacokinetic applications exists to the best of our knowledge. Therefore, our first objective was to develop

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a LC/MS–MS method for the quantification of monensin in chicken plasma, liver, muscle and fat since tandem mass spectrometry offers the possibility to detect residues at very low levels. Detecting residues at very low levels is essential for running a correct depletion study where monensin is tested. Our second objective was to use the total error approach described in harmonization guidelines of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) [16–18] in order to check if our method is applicable in the field of residues.

The total error approach has proven itself to be efficacious in allowing evaluation of a number of methods such as UV [19], NIR [19], HPTLC [20], HPLC [21], GC [22], and LC–MS/MS [23] methods of quantification. We propose to illustrate its usefulness with a LC–MS/MS method intended to quantify residual concentrations of monensin not only in plasma, but also in complex matrices such as muscle, fat and liver. The decision tools that are proposed by the European Commission permit to conclude that the performance of the analytical method is fit for its purpose with respect to the defined acceptance limits. The total error approach is based on the total error measurement, *i.e.* systematic and random errors, which is easily visualized by the accuracy profile. The accuracy profile is characterized by the use of two-sided β-expectation tolerance intervals calculated at each concentration level. In fact, the $1-\beta$ proportion of measurements falling outside the acceptance limits allows to evaluate the risk of the procedure and was chosen initially equal at 5%. A number of accuracy profiles were determined per matrix and the best accuracy profile was selected considering the objective of the method.

For plasma, the selected acceptance limit was higher than that proposed by the FDA [24]. In pharmacokinetic studies, it is very important to determine the plasma/tissue ratio, in particular for low concentrations, in order to describe the distribution in tissues. For tissues, the acceptance limits were dependant on concentration level [25], and we retained an acceptance limit of 30 or 50% depending on the matrix. Consequently, we applied an acceptance limit of 30% for plasma. In a pilot pharmacokinetic study in chicken, the concentration profile was established in plasma and other tissues. From these results, the calibration and validation standards were selected.

2. Experimental

2.1. Chemical and reagents

Acetonitrile, methanol, and ammonium acetate used were of HPLC or analytical grade and purchased from Fisher Scientific (Leicestershire, UK). Formic acid was purchased from Merck (Darmstadt, Germany). Water was HPLC grade generated by a Milli-Q (Millipore, Molsheim, France) purification system. Filters for filtration of the extract were from Millipore (Millex GV, 0.45 μm).

Monensin sodium and narasin were provided by Sigma–Aldrich Co. (St Quentin Fallavier, France).

Stock standards (1 mg/ml) of monensin and narasin (Fig. 1) were prepared in methanol. They were stored at 4 °C until further

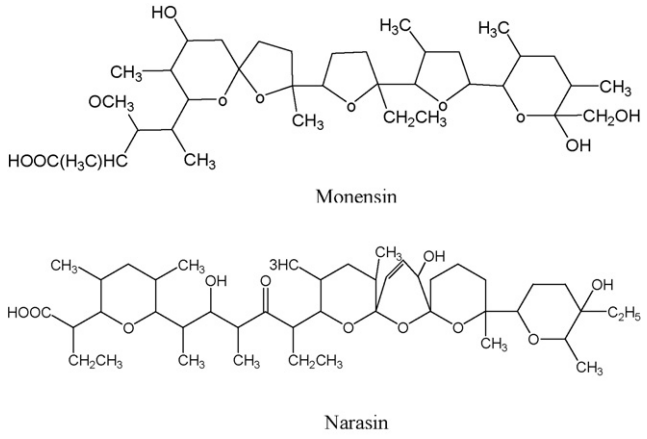

Fig. 1. Chemical structures of the analyte and its internal standard.

dilutions were made in methanol for calibration and validation standards.

2.2. Spiking of samples

Samples (blank and naturally incurred from the studies) of chicken plasma, muscle, liver and abdominal fat were collected at the AFSSA Ploufragan laboratory facilities and stored at −20 °C until analysis was performed. Stock standards were diluted daily in the same solvent to obtain five working solutions of concentrations ranging from 5 to 200 μg/l. As the concentration levels for calibration and validation standards were the same, the spiking was reproduced in the same way for these two kinds of standards. However, working standards were different. Calibration and validation samples were obtained by adding 100 μl for plasma and 200 μl for the other matrices of the appropriate working solutions to thawed, homogenized blank samples. Blank samples were previously shown to contain no detectable polyether ionophore antibiotic. Spiking of analyte followed spiking of the internal standard (IS).

2.3. Instrumentation

The SPE column used was Bond Elut C_{18}, 200 mg, 3 ml (Varian, Les Ulis, France). Chromatography was performed on a Hewlett Packard 1100 system (Waldbronn, Germany) fitted with a Luna C_{18} (2) (3 μm, 150 mm × 2.1 mm) column (Phenomenex, Ashaffenburg, Germany) and protected with a C_{18} guard column containing the same material. The MS equipment consisted of a PE Sciex API 2000 (Foster City, CA, USA) controlled by Version 1.4.1 of the Analyst software. The validation data were processed by e.noval® software Version 1.1a (Arlenda, Liège, Belgium).

2.4. Sample preparation

2.4.1. Plasma

One millilitre of plasma was placed in a 50 ml centrifuge tube, and the internal standard was added at a concentration of 50 μg/kg. The sample was vortex-mixed and allowed to stand

for 10 min. Six millilitres of acetonitrile were added and the sample was again vortex-mixed to homogenize the material and solvent. The sample was then placed on a mechanical shaker for 10 min at 100 rpm and centrifuged for 10 min at 4000 × *g* at 5 °C. An aliquot of the supernatant of 5 ml was transferred to 15-ml plastic tubes and evaporated to dryness under nitrogen at 45 °C. The sample was redissolved in 300 μl of a 50 mM acetonitrile–ammonium acetate (80/20, v/v) mixture. The concentrated extract was vortex-mixed briefly, filtered through a 0.45 μm syringe filter, and transferred to HPLC autosampler vials, and 50 μl of the extract were injected into the LC–MS/MS system.

2.4.2. Chicken muscle, liver, and fat

The method was based on the one described by Rosén [26]. It is used for the determination of selected polyether ionophore antibiotics in liver and eggs.

Two grams of sample were weighed in a 50 ml centrifuge tube, and narasin, the internal standard, was added at a concentration level of 50 μg/kg. The sample was vortex-mixed and allowed to stand for 10 min. Six millilitres of a mixture of methanol–water (87/13, v/v) were added, and the sample tube was again vortex-mixed in order to homogenize the material and solvent. The sample was then placed in an ultrasonic bath for 10 min and centrifuged for 10 min at 4000 × *g* at 5 °C. A 5 ml aliquot was purified on the SPE cartridge and preconditioned with 4 ml of methanol and 2 ml of water. Once the extract had passed through the cartridge, the cartridge was rinsed with 2 ml of a mixture of methanol–water (80/20, v/v) and the extract was collected in a 15-ml plastic tube with 4 ml of methanol. Then the extract was evaporated to dryness under nitrogen at 45 °C. The sample was redissolved in 300 μl of a mixture of acetonitrile–ammonium acetate 50 mM (80/20, v/v), vortex-mixed briefly, and transferred to HPLC autosampler vials, and 50 μl of the sample (20 μl for fat sample) were injected into the LC–MS/MS system.

2.5. LC/MS–MS conditions

2.5.1. Chromatography

Water (A) and acetonitrile (B) were used to apply a gradient. Each contained 0.1% formic acid. The gradient conditions were as follows: from 0 to 6 min ramp from 80 to 100% B; hold for 4 min; ramp over 2 min to 80% B; hold for 6 min to re-equilibrate the system. The flow rate was 0.30 ml/min, and no split was necessary to introduce the LC effluent in the mass spectrometer.

2.5.2. Mass spectrometry

Tuning was performed in electrospray positive ionisation mode with solutions of 5 ng/μl in acetonitrile/ammonium acetate 0.2 M (50/50, v/v) at a flow rate of 10 μl/min. The most prominent ions were ammonium adducts [M + 18] at *m*/*z* 688.4 for monensin and 782.6 for narasin. For both full scan MS and SRM MS/MS, the settings of the mass spectrometer were as follows: capillary voltage 5.5 kV; source temperature 100 °C and desolvatation temperature 450 °C. The nebulisation and desolvatation gas (air) pressure were 40 and 50 psi, respectively. Cone voltage and collision energy were 13 and 23 V for monensin, and 10 and 30 V for narasin, respectively. The curtain gas pressure was 20 psi. The collision gas (nitrogen) pressure was 2 psi. The dwell time was 0.25 s and the interscan delay was 5 ms. The most favourable SRM transition monitored for monensin was 688.4 > 635.3 and 782.6 > 747.5 for narasin.

2.6. Preparation of standards

In order to validate the analytical method, we prepared two kinds of samples for calibration and validation in an independent way. The independence of the samples was obtained by different working solutions prepared daily from a stock solution. Subsequent validation allowed the analysts to obtain estimates of bias and variance.

2.6.1. Calibration standards

The calibration standards consist of samples with matrix, containing known concentrations of the analyte of interest. The samples are only used for calibration. They must be prepared according to the protocol that will be applied routinely. Two calibration standards' series of five concentration levels replicated on 3 different days were performed for each matrix. The concentration levels were chosen according to a pharmacokinetic study that served as a pilot experiment (unpublished data) for the current study. The concentration levels by matrix were: 2.5, 5, 10, 25, and 100 μg/kg for plasma; 1, 2.5, 5, 10, and 100 μg/kg for liver; 0.5, 2.5, 5, 10, and 100 μg/kg for muscle; 2.5, 5, 10, 100, and 200 μg/kg for fat. This protocol was chosen in order to select the most appropriate response function. The best model of the response function is a guarantee of a reliable quantification, namely at the limit of quantification (LOQ).

2.6.2. Validation standards

The validation standards are reconstituted samples with matrix containing known concentrations of the analyte of interest. In the validation phase, the validation standards represent the futures samples that the analytical procedure will have to quantify. The concentration levels selected for the validation standards were the same as the levels of the calibration standards, corresponding to low (estimated limit of quantification), three intermediate, and high concentrations levels. Three repetitions were used at each concentration level for 3 days.

3. Results and discussion

3.1. LC–MS/MS optimization

A simple and sensitive method for the detection of monensin in poultry plasma, muscle, liver, and fat was developed. To enhance the ruggedness and quantification of the method, the use of an internal standard was investigated. In fact, no stable isotope labelled molecule exists for use as internal standard since polyether ionophores are natural products. Therefore, several polyether ionophores antibiotics having similar properties

were tested. Narasin was found to be suitable as internal standard because its behaviour was closest to that of monensin in the Electrospray (ESI) interface. Thus, variations were reduced and response was more stable with narasin allowing optimization of the method. Under the analytical conditions of our protocol, monensin is chromatographically separated from the IS, but not completely. However, complete separation was not our priority since tandem mass spectrometry is very selective. The method was developed to convert the two ionophores into ammonium adduct species prior to electrospray ionization (ESI) in positive ion and selected reaction monitoring mode (SRM) analysis by adding sufficient ammonium acetate to the extracts. It did not require ammonium buffer to be added to the mobile phase. Actually, previous assays showed problems of reproducibility of the method when sodium adduct ions were usually used. So we decided to compare the formation of these adducts to that of ammonium adduct ions. In fact, it is possible to change the cation which forms an adduct with the ionophore. Usually, sodium complexes occur easily. The ammonium adducts were increased by adding ammonium acetate into the vials.

Precision of the method was improved and our results proved that adding ammonium acetate could have a beneficial effect.

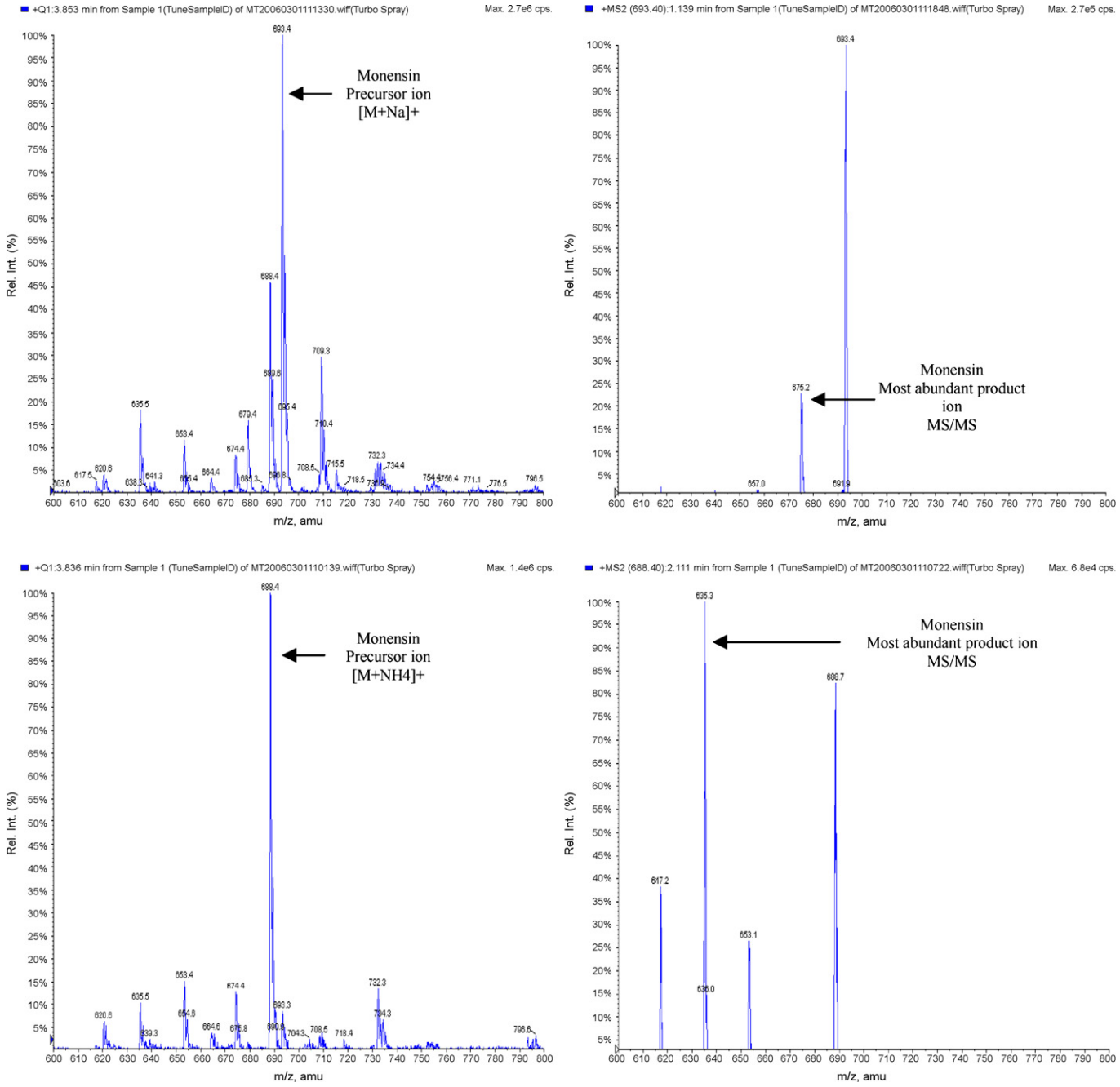

Fig. 2. ESI–MS (left) and ESI–MS/MS (right) spectra of monensin in sodium adduct specie (top) and ammonium adduct specie (bottom).

Signal was found to be increased in these conditions especially for narasin and salinomycin (another polyether ionophore) in NH_4^+ form species, in comparison with signal of Na^+ form species detected in their usual conditions (acetonitrile as frequent injection solvent).

Fig. 2 shows the full spectra of the sodium adduct, used beforehand, at *m/z* 693.4 and the ammonium adduct at *m/z* 688.4. Below each spectra, their prevailing product ion spectra in MS/MS, each under their optimal conditions, are presented. The spectra show the good fragmentation of the product ion from the ammonium precursor at *m/z* 635.3 compared to that at 675.2 from the sodium precursor. Moreover, no protonated molecules and few sodium adducts were present in the full scan spectrum of the ammonium species. This finding illustrates the good conversion of majority sodium adducts into ammonium adducts that was usually observed.

Quantification of monensin was then performed in the SRM mode using the ammonium adduct as precursor ion for both monensin and narasin as IS.

3.2. Validation

Several criteria were studied in order to ensure the reliability of the developed method:

- Selectivity;
- Response function;
- LOQ;
- Trueness;
- Precision (repeatability and intermediate precision);
- Accuracy;
- Extraction efficiency.

3.2.1. Selectivity

The selectivity of the method was tested by comparing chromatograms of different blank plasma samples, supplemented matrices and standard solutions. No interferences were observed at the retention times of monensin and narasin.

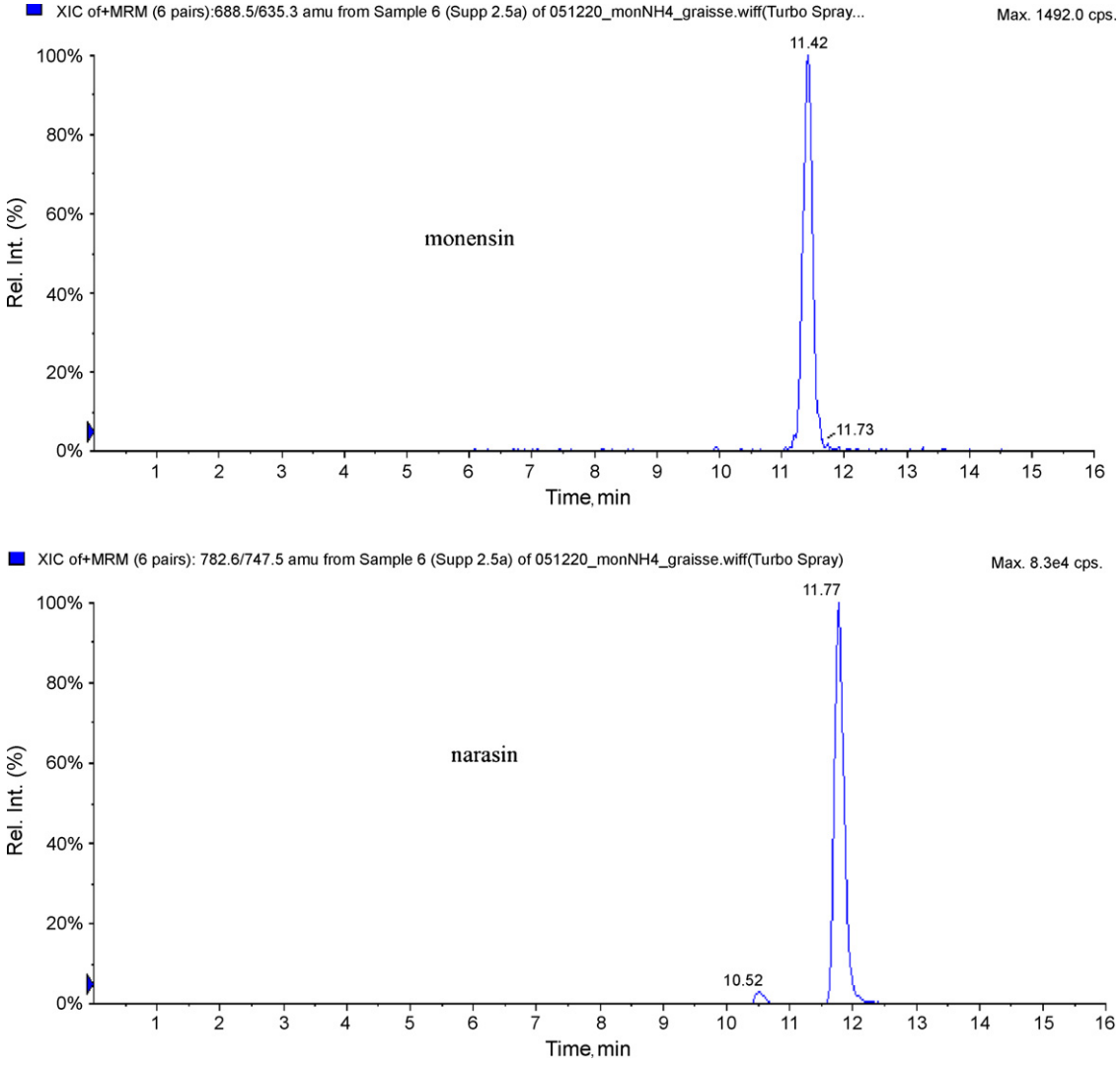

Fig. 3. LC–ESI–MS/MS chromatogram of fat spiked with monensin at 2.5 μg/kg and narasin (IS).

Table 1
Results of the regression analysis of the data of the standard calibration graphs

Matrix	Curve	Intercept	Slope	Quadratic term	Weight	R^2
Plasma	1	0	9.160E−05	–	1	–
	2	0	9.475E−05	–	1	–
	3	0	9.125E−05	–	1	–
			RSD: 2.1%			
Muscle	1	0	8.785E−05	–	1	–
	2	0	8.205E−05	–	1	–
	3	0	7.975E−05	–	1	–
			RSD: 5.0%			
Fat	1	0	3.058E−05	–	1	–
	2	0	2.955E−05	–	1	–
	3	0	2.893E−05	–	1	–
			RSD: 2.8%			
Liver	1	4.813E−04	1.168E−04	−2.457E−09	$1/X^2$	0.9987
	2	−6.231E−04	1.338E−04	−2.073E−09	$1/X^2$	0.9997
	3	5.383E−04	1.246E−04	−1.784E−09	$1/X^2$	0.9978
			RSD: 6.8%			

A representative chromatogram from blank fat sample spiked with monensin at 2.5 μg/kg and IS is given in Fig. 3.

Moreover, the effect of interferences or matrix effect of plasmas from different origins was tested. In fact, this effect can be important where both an ESI interface is utilized and the internal standard has to be an analogue. As no stable isotope labelled molecule standard is available on the market for polyether ionophore antibiotics, the decision was made to replace it by an analogue. The 5th simplified approach described in a recent paper of Matuszewski et al. [27] for assessing the matrix effect in plasma was retained. The experiment was performed only in plasma because a clean-up is applied for the other matrices and we considered one experiment to be sufficient. Thus, five standards lines were constructed in five different matrices from different origins, and the CV on slopes was equal to 4.1%. So no significant matrix effect on quantification is present.

3.2.2. Response function

For each matrix, the following models of regression were tested: linear, linear through zero, weighted linear, linear after logarithm transformation of both concentration and response, linear after square root transformation of both concentration and response, weighted linear after square root transformation of both concentration and response, quadratic and weighted quadratic regressions. The regression models were chosen parsimoniously according to the best accuracies covering the entire dosing ranges.

The best models for calibration are linear regressions passing through 0 fitted with the level 5 only, except for liver. The calibration model for the liver is a weighted quadratic model. Table 1 summarizes the results of the regression analysis.

3.2.3. Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [17,28]. Trueness values are shown, for each matrix, in Table 2. As can be seen in Table 2, trueness is expressed in term of absolute bias (μg/l or μg/kg) and recoveries (%). The overall recoveries do not exceed the threshold of 15%. The trueness of the first level of calibration in muscle (0.5 μg/kg) is not satisfying.

3.2.4. Precision

By studying the criteria for determining precision, it is possible to estimate the dispersion of the results around their mean value. As can be seen in Table 2, precision is evaluated by repeatability (RSD%) and intermediate precision (RSD%) at each concentration level. The relative standard deviation values are calculated from the estimated amounts of the validation standards. Repeatability and intermediate precision values are acceptable for the field of pharmacokinetic studies since they are distributed between 1.9 and 13.3%.

3.2.5. Limit of quantification

Usually only the lower limit of quantification is defined. The concept of total error also introduces the upper limit of quantification given by the intersection between the accuracy profile and the acceptance limits. The other intersection defines the lower limit. Thus, the measurement interval was chosen according to the results of the validation results. According to these results, the upper limit was chosen to be the highest concentration investigated and the lower level to be the lowest concentration investigated, except for muscle. Indeed, the unacceptable recovery of 58.65% in muscle for the first level of quantitation (0.5 μg/kg) led us to take 2.5 μg/kg as low LOQ for that matrix.

3.2.6. Extraction efficiency

The extraction rate was not determined because we considered that validation results gave an indication about the extraction efficiency. No significant problem of accuracy occurred during the development and the validation between the different calibration levels.

Table 2
Detailed results of validation for all matrices (plasma, muscle, fat, and liver)

Mean introduced concentration (μg/l (plasma) or μg/kg)	Trueness		Precision		Accuracy	
	Absolute bias (μg/l or μg/kg)	Recovery (%)	Repeatability (RSD%)	Intermediate precision (RSD%)	β-Expectation tolerance limit (μg/l or μg/kg)	Risk (%)
Plasma						
2.5	−0.02	99.2	8.5	13.3	[1.85, 3.11]	18.3
5	−0.07	98.5	8.8	8.8	[4.24, 5.62]	2.1
10	−0.30	97.0	3.7	3.7	[9.12, 10.29]	<0.1
25	−0.40	98.4	3.4	3.4	[23.27, 25.93]	<0.1
100	1.91	101.9	4.5	4.5	[94.87, 109.00]	<0.1
Muscle						
0.5	−0.21	58.7	10.9	10.9	[0.16, 0.43]	94.1
2.5	0.05	102.2	5.2	5.7	[2.34, 2.77]	0.1
5	−0.59	88.2	4.6	4.6	[4.07, 4.75]	0.6
10	−0.99	90.1	3.2	6.5	[7.78, 10.24]	12.1
100	−1.72	98.3	2.8	7.7	[82.87, 113.70]	10.1
Fat						
2.5	0.11	104.3	3.3	4.0	[2.34, 2.88]	<0.1
5	0.35	107.0	6.5	8.1	[4.23, 6.47]	6.4
10	0.05	105.4	3.0	4.3	[9.24, 11.85]	0.2
100	0.07	100.7	4.8	4.8	[88.73, 112.60)	<0.1
200	−14.00	93.0	1.9	2.8	[168.90, 203.10]	<0.1
Liver						
1	−0.02	97.9	6.4	6.4	[0.82, 1.14]	<0.1
2.5	0.12	105.0	8.3	8.3	[2.12, 3.13]	<0.1
5	0.23	104.5	8.9	9.4	[4.05, 6.41]	0.1
10	−0.80	92.0	11.8	11.8	[6.32, 12.09]	1.2
100	0.69	100.7	3.5	3.5	[92.18, 109.20]	<0.1

3.2.7. Accuracy

The accuracy of the method takes into account the total error, *i.e.* systematic and random errors, related to the test result [16,17,24,28]. An important decision tool, the accuracy profile, has been introduced by the SFSTP in order to evaluate easily the capability of the method to quantify samples with a known accuracy and a risk fixed according to the requirements of the food safety analysis. The accuracy profile is constructed from the tolerance interval based on total error that allows us to evaluate the proportion of expected measures inside the acceptance limits (±λ).

The accuracy profiles for plasma, muscle, liver, and fat are illustrated in Fig. 4. The plain lines are the relative bias, the dashed lines are the β-expectation tolerance limits, and the dotted curves represent the acceptance limits. The values of the β-expectation tolerance limits and of the associated risk are detailed in Table 2.

As shown in Fig. 4, most important variations of trueness and precision are found in the lower concentration levels. However, as these levels are fundamental for the depletion study, we decided to increase the acceptance limits and take a slightly more important risk (initially taken at 5%) (*cf.* Table 2) at certain concentrations considering the complexity of matrices and the residual values of concentration. In these conditions, the method of determination of monensin in the four matrices is accurate over the whole concentration range.

Validation in plasma is characterized by a good accuracy. Upper and lower β-expectation tolerance limits at the lowest concentration level do not exceed the threshold of 30% selected for the acceptance limits. As shown in Table 2, the associated risk at this concentration level is about 18%, but for the next concentration levels, the tolerance interval does not exceed ±15% with a risk falling under 2%. Therefore, the risk of 18% is kept, for plasma values, around 2.5 μg/l, value which is retained for the depletion study.

For muscle, the accuracy profile of the method presents acceptable results, even if a more important bias is visible for the 10 μg/kg level of concentration. Moreover, the risk at the first quantification level is very low (<1%), but increases to about 10% for the higher concentration levels. This highlights that risk can be greater for upper than for lower concentrations. The risks were accepted because we knew that very few concentrations would be found at high levels in muscle, and targeted tissues are preferentially fat and liver.

As can be seen on the accuracy profile of fat, β-expectation tolerance limits are also higher for the second level of calibration. The reason for this could be a lack of replicates or series to evaluate the variability of the method and subsequently the corresponding tolerance interval. However, relative bias still ranged from −7 to 7% and risk values are close to 5%.

Contrary to the other matrices, the acceptance limits were not set at 30% for liver. The 10 μg/kg level obliged us to increase the limits to 50% to validate this method. However, the bias of 8% at this level is still reasonable.

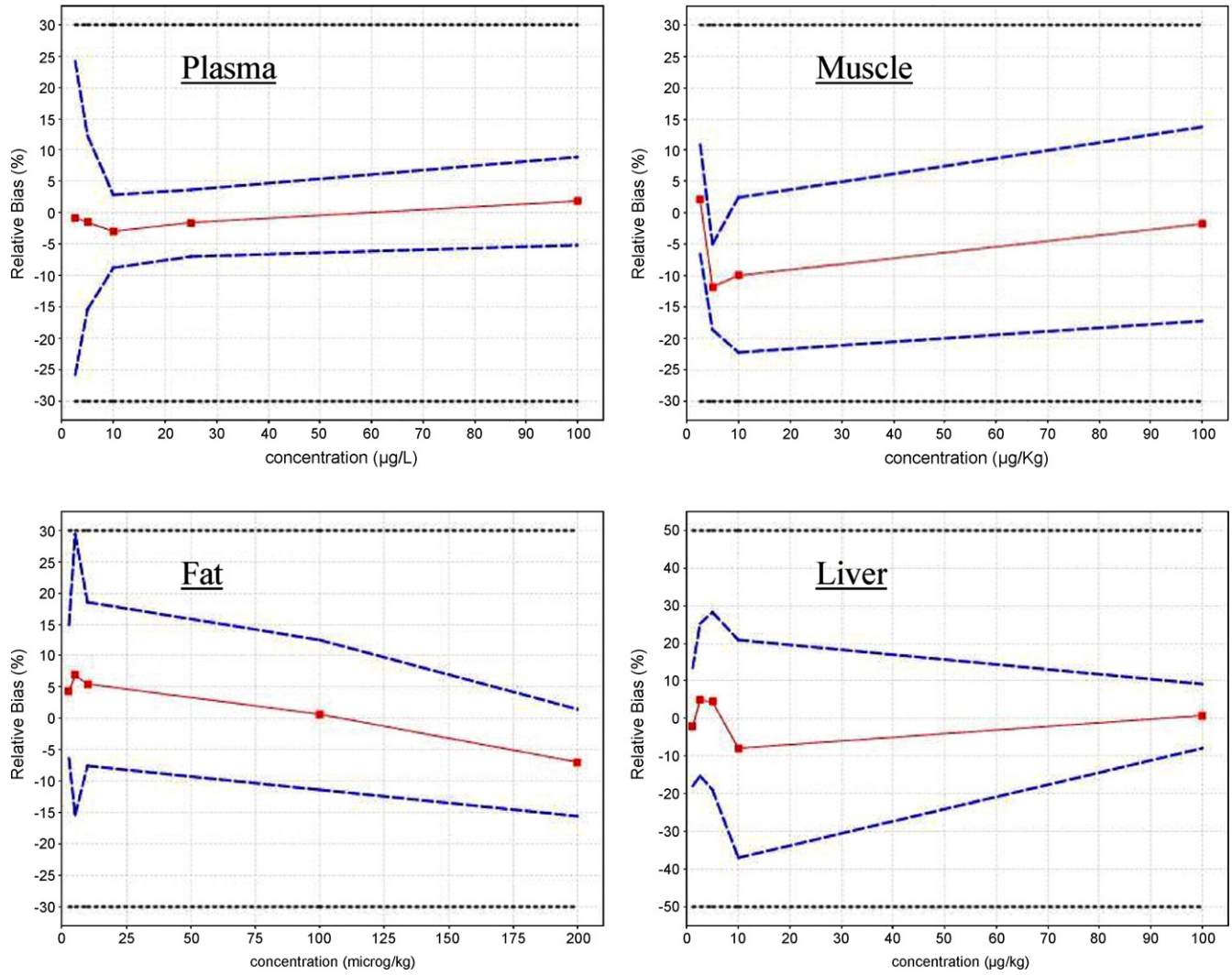

Fig. 4. Accuracy profiles from the validation of plasma, muscle, fat, and liver.

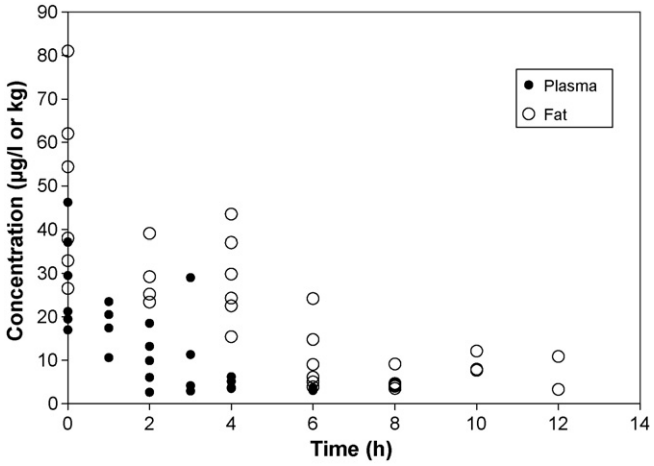

Fig. 5. Depletion of residues of monensin in chicken plasma and fat after withdrawal of medicated feed at a concentration of 121 mg/kg per feed.

3.2.8. Demonstration of applicability to biological samples

In order to demonstrate the applicability of the LC–MS/MS method to samples of a pharmacokinetic study, the method was used to quantify concentrations of monensin in plasma, fat, muscle and liver of chickens. In the depletion study, 68 chickens were treated orally with monensin from first day until day 33, via medicated feed at a concentration of 121 mg/kg per feed. During the depletion phase until day 36, 6–10 animals were slaughtered at each sample time. Residues declined rapidly and were only observed during 8–10 h in plasma, liver and thigh muscle whereas monensin was still present 18 h in fat. Detailed pharmacokinetic data will be reported in a separate article. Fig. 5 illustrates the kinetic of monensin in plasma and fat during the depletion.

4. Conclusion

An appropriate method was developed in LC–MS/MS for the quantitative determination of monensin in plasma and the edible tissues of chicken for pharmacokinetic applications. The

mass spectrometric parameters and the detection conditions were optimized in order to improve sensitivity and precision of the method. The method was then successfully validated using a new approach based on the accuracy profile as described in guidelines of SFSTP. For the validation, the conventionally defined acceptance limits were re-examined. Indeed, the total error showed that for the method variability of results could be greater in complex matrices than in plasma. It is particularly the case in electrospray ionisation mass spectrometry for residual concentrations. The method proved to be applicable in a pharmacokinetic study we conducted.

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