Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 860–868

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Determination of semduramicin in poultry feed at authorized level by liquid chromatography single quadrupole mass spectrometry

María José González de la Huebra*, Ursula Vincent, Christoph von Holst

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (EC-JRC-IRMM), Retieseweg 111, B-2440 Geel, Belgium

ARTICLE INFO

Article history:
Received 26 February 2010
Received in revised form 10 June 2010
Accepted 14 June 2010
Available online 23 June 2010

Keywords:
Coccidiostats
Semduramicin
Feedingstuffs
LC–MS
Validation

ABSTRACT

A novel liquid chromatography single quadrupole mass spectrometry (LC–MS) method for the determination of the feed additive semduramicin, in poultry feed, was developed and single-laboratory validated. This work was selected as a real case scenario to outline the different steps that may be needed whenever the standardisation of an analytical method in the field of methods of analysis for animal feedingstuffs is attempted. In this manuscript the main achievements reached within the development and the single-laboratory validation of an analytical method for the determination of semduramicin in feedingstuffs are detailed.

Semduramicin is extracted from the feedingstuffs with acetonitrile. The obtained extracts are then filtered and diluted appropriately. The separation has been carried out in a reverse phase C18 column using isocratic elution with a mixture of methanol and 20 mM ammonium formate solution as mobile phase. The ammonium adducts have been selected for monitoring the coccidiostats signals in the mass spectrometry detector.

The method has been successfully validated for the determination of semduramicin concentrations ranging between half of the minimum authorized concentration (10 mg kg^{-1}) to twice of the maximum authorized concentration (50 mg kg^{-1}). A good relative standard deviation for repeatability (RSD_r) varying from 2.8 to 3.2% has been obtained whereas the relative standard deviation for intermediate precision ($RSD_{Int.}$) ranged from 3.7 to 7.3%.

The obtained analytical performance characteristics of the method demonstrated its fitness for the purpose, making thus the proposed method suitable to be submitted for the last phase within the standardisation procedure, i.e. the inter-laboratory study.

1. Introduction

During the last years scientists and legislators have acknowledged the impact of using antibiotics as feed additives for human health. Resistant microbes may be transferred from animals to humans via the food chain or through direct contact. Taking into account that the feedingstuffs are the first link within the food chain, their appropriate control is thus a key issue in order to prevent the spread of resistant microbes through the whole food chain.

Consequently, to help to decrease resistance to antibiotics used in animal nutrition, five feed additives namely avoparcin [1], bacitracin zinc, spiramycin, virginiamycin and tylosin phosphate [2] were prohibited and their authorisations withdrawn in the late 90s. Subsequently, a Commission Regulation [3] implementing a new system regarding the authorisation of feed additives limited the use of antibiotics as feed additives to those belonging to the groups of coccidiostats and histomonostats.

Coccidiostats are compounds that have been widely used to inhibit parasites which cause coccidiosis in farmed animals [4]. This disease is caused by highly host-specific protozoan parasites belonging to the genera *Eimeria* in the class Sporozoa and it is considered the most important parasitic disease in poultry [5]. The main successful strategy of controlling this disease is the combination of a good implementation of the prescribed hygiene requirements [3] together with the addition of coccidiostats to the feed at the authorized level [6].

Semduramicin presents a broad spectrum of anticoccidial activity against *Eimeria* spp. at dietary inclusion levels within the authorized concentration range (20–25 mg kg^{-1} complete feedingstuffs) [7,8]. The conditions of use of this feed additive including the authorized concentration range are specified in the corresponding Commission Regulation [9].

Semduramicin is produced by a strain of *Actinomadura roseorufa* [10] and belong to the group of ionophore coccidiostats which are

* Corresponding author.
E-mail address: maria-jose.gonzalez-de-la-huebra@ec.europa.eu (M.J.G. de la Huebra).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.06.011

characterized by having within their chemical structure a polyether group. Another five compounds of the same family namely monensin, lasalocid, maduramicin, narasin and salinomycin complete the list of ionophore coccidiostats currently authorized as feed additives in poultry feed.

Enforcing the target levels of semduramicin in feedingstuffs by Member States' official feed laboratories requires the availability of appropriate analytical methods that comply with the criteria established by Regulation (EC) No. 882/2004 [11].

In the light of the enforcement of this legislation the use of standardized methods of analysis is of utmost importance to ensure the control in a harmonized way of the compliance with the European legislation and thus to enforce the regulatory requirements. However opposite to its equivalent in food, the existing methods currently used to carry out the control of feed additives in feedingstuffs are in many cases based on obsolete methodologies and often lack an appropriately documented standardisation procedure that ensured the establishment of its performance characteristics under recognized international criteria.

Aimed to close this gap between the characterization of the existing analytical methods for food and feed, the European Commission requested the standardization of different analytical methods in the field of animal feedingstuffs via a mandate to the European Committee for Standardisation (CEN).

The method presented in this manuscript is one of the analytical methods targeted within the second Commission mandate to CEN (M 382/2005).

A multi-analyte method based on liquid chromatography–tandem mass spectrometry previously developed in this laboratory [12] would allow the determination of semduramicin within the scope of this work. Due to the wide concentration range of the targeted coccidiostats covered in the referred paper the sample preparation required an extract clean-up.

However, being the scope of the mandate limited to only one of the analytes covered within the paper (Commission mandate M 382/2005), the method appeared far too sluggish. To our knowledge this is up to know the only published analytical method suitable for the direct determination of semduramicin in feed at authorized level.

Consequently, we have decided to develop a new analytical method which would better fit the scope requested in the Commission mandate. The method presented in this manuscript is more time and cost effective than the former multi-analyte, and thus constitute a much better option for the routine control of only semduramicin in poultry feedingstuffs. Additionally the transferability assay of the method to tandem mass spectrometry instrumentation ensured its potential implementation in all the most common mass spectrometry facilities currently available in the European laboratories.

2. Experimental

2.1. Standards, reagents and test samples

2.1.1. Coccidiostats standards

Semduramicin (SEM), which is not freely commercially available, has been kindly provided by Phibro Animal Health (Fairfield, NJ, USA); monensin sodium (MON) and nigericin (NIG) (used as internal standard for the LC–MS measurement) were obtained from Calbiochem (Merck Sciences, Darmstadt, Germany), maduramicin (MAD) by Alpharma (Willow Island, USA) and salinomycin (SAL), narasin (NAR) and lasalocid (LAS) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Table 1
Cross-contamination levels established for the ionophore coccidiostats authorized as feed additives in poultry feed.

	Maximum content in mg kg^{-1} (ppm) relative to a feedingstuff with a moisture content of 12%[a]
Lasalocid sodium	1.25
Narasin	0.7
Salinomycin sodium	0.7
Monensin sodium	1.25
Maduramicin ammonium alpha	0.05

[a] Extracted from Ref. [14].

2.1.2. Reagents

Ammonium formate HPLC grade, acetonitrile (ACN) HPLC gradient grade, minimum 99.9% purity and methanol (MeOH) hypergrade LC–MS were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Pure water (18.2 MΩ cm quality) used for the preparation of all the aqueous solutions was obtained from a MilliQ Plus 185 System (Millipore, Molsheim, F).

2.1.3. Test materials

The assays performed for the development and optimization of the analytical method have been carried out with a compound feedingstuff for poultry, containing typical ingredients using a realistic recipe, that was obtained from the European FP5 project SIMBAG-FEED [13]. The absence of the target analytes has been previously confirmed by chemical analysis [12].

In order to enlarge the number of diets tested, the assays intended to perform the single-laboratory validation of the developed analytical method have been carried out using a commercial poultry feed which has been acquired in the local facilities (AVEVE laying hens). The absence of the target analytes involved in the quantification approach, i.e. semduramicin and nigericin in the commercial feed, has been initially checked using the label information provided and further confirmed by chemical analysis before their use.

Different aliquots of the blank poultry feed have been then fortified with semduramicin at three different concentration levels, ranging between half of the minimum authorized concentration, and twice the maximum authorized concentration. The test material also contained the other ionophore coccidiostats (monensin, salinomycin, narasin, maduramicin and lasalocid) at the carry-over levels (Table 1) established by the European Commission [14], as potential interferents, in order to investigate the impact of the presence of these compounds on the quantification of semduramicin.

2.2. LC–MS conditions

All chromatographic measurements were performed with a BioLC system coupled to a single quadrupole mass spectrometer (MSQ) (Dionex, Benelux). Separation was performed on a Grace Davison Discovery Sciences (Lockeren, Belgium) Alltima HP C18 (150 mm × 2.1 mm; 5 μm particle size) analytical column equipped with an Alltima HP C18 (7.5 mm × 2.1 mm; 5 μm particle size) guard column. The mobile phase used for the elution of the coccidiostats was a binary mobile phase constituted by a mixture of methanol: ammonium formate 20 mM (90 + 10 v:v). The elution of the target analytes was performed at 25 °C in isocratic mode and the flow rate was set at 0.25 ml min^{-1}.

For the detection, the electrospray positive ionisation mode (ESI+) was selected and the adducts were monitored in single ion-monitoring (SIM) mode. The following MS conditions were applied. The needle voltage was set at 2.7 kV, the probe temperature was 250 °C, and the optimum cone voltage was found to be 60 V. The acquisition has been carried out using a dwell time of 0.5 s and

a mass span of ±0.25. Finally the correspondent measurements for the transferability of the method were carried out in an HPLC Accela coupled to a Quantum Ultra triple stage quadrupole mass spectrometer (ThermoFisher Scientific Co., San Jose, USA).

2.3. Preparation of standard solutions

Stock solutions of the target analyte SEM (1.0 mg ml^{-1}), the internal standard NIG (1.0 mg ml^{-1}) and the potential interferent coccidiostats MON (1.0 mg ml^{-1}), MAD (1.0 mg ml^{-1}), LAS (1.0 mg ml^{-1}), NAR (1.00 mg ml^{-1}), SAL (1.0 mg ml^{-1}) were prepared by dissolving the compounds in methanol. These stock solutions were freshly prepared each 3 months and stored at −20 °C in amber vials until use.

From these stock solutions an intermediate mixture solution containing the interferent coccidiostats at the appropriate concentration ratio were prepared in acetonitrile (ACN) in order to allow the fortification of the blanks with the interferent coccidiostats in a single addition. The mixture of interferent coccidiostats was prepared weekly and stored at +4 °C in amber vials until use.

Working individual solutions of the target analyte (SEM) and the internal standard (NIG) at a concentration of 2 μg ml^{-1} were prepared weekly from the respective stock solutions by dilution in acetonitrile (ACN). All working solutions were kept in amber vials at +4 °C until use.

2.4. Sample preparation

A 5.0 ± 0.1 feedingstuff sample was placed in an appropriate container, the powder was then thoroughly mixed with 40 ml ACN in order to wet all the feed and submitted to head over heels agitation for 60 min. After that, the mixture was centrifuged at 2900 g for 10 min at 20 °C. Subsequently about 5 ml of the supernatant was filtered through a syringe Nylon 0.2 μm filter. A 200 μl aliquot of the filtered supernatant was collected and diluted up to 10 ml in a volumetric flask with ACN. The diluted extract was then used to perform the quantification of the unknown sample.

2.5. Standard addition solutions

In order to quantify the unknown samples using the standard addition approach the following standard addition solutions were prepared for each unknown sample.

Six 900 μl aliquots of the diluted extract prepared in Section 2.4 were placed into 1.5 ml glass vials and labelled as S0, S1, S2, S3, S4 and S5, respectively.

25 μl of the nigericin sodium working solution (2 μg ml^{-1}) was added to each of the vials labelled as S0, S1, S2, S3, S4 and S5, respectively.

Subsequently 0, 15, 30, 45, 60 and 75 μl of the semduramicin spiking solution (2 μg ml^{-1}) were added to the vials labelled as S0, S1, S2, S3, S4 and S5, respectively.

Finally, in order to have a final volume of 1 ml in all the vials, 75, 60, 45, 30, 15 and 0 μl of acetonitrile were added to the vials labelled as S0, S1, S2, S3, S4 and S5, respectively.

All the vials were then capped and shaken vigorously before injection into the chromatographic system.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

Adverse matrix effects often occur when applying mass spectrometry (MS) to quantify a target analyte in complex matrices, especially due to the impact of matrix components coeluting with the target analyte on the processes in the ion source of the MS. One option to overcome this limitation is to quantify using matrix matched standards. However, this is not possible for official control laboratories due to the common lack of feed material that would be strictly identical to the test samples to analyse, but not containing the target analytes (blank feed samples). Alternatively, the "standard addition" technique is used, which increases significantly the required work load. This is due to the fact that this technique requires the analysis of a subsample of the test material as such and additional subsamples from the same test material, but after fortification with known amounts of the target analyte at different concentration levels. Here we applied an alternative approach, where the standard addition is applied on aliquots of the extract from a single sample and not on different subsamples of the same test material, reducing thus the workload. This is possible, as we expect the ionisation process in the MS to be the major factor for matrix effects rather than the sample preparation step.

In order to overcome a well-known phenomenon of potential run to run and/or day by day changes of the detector response due to the variations within the ionisation source, the quantification of semduramicin was carried out using an internal standard, namely nigericin.

The quantification of semduramicin in the test sample was therefore carried out by plotting the area ratio, response (y-axis) versus the added concentration of semduramcin (x-axis) for each of the standard addition solutions prepared as detailed in Section 2.5 (Eq. (1)). The regression line was then calculated as usual and extrapolated to the point on the x-axis at which $y = 0$. This negative intercept on the x-axis corresponds to the semduramicin concentration in the test sample (S0).

$$\text{Response} = \frac{ApSEM}{ApNIG\ (\text{I.S.})} \quad (1)$$

where, *ApSEM* is the absolute signal (area) of the semduramicin quantification adduct; *ApNIG* (I.S.) is the absolute signal (area) of the nigericin quantification adduct.

3.1.2. Optimization of the chromatographic conditions

In this work, we have optimized the chromatographic conditions in order to adapt them to the scope of the final application namely to establish trade-off conditions for achieving a reliable method suitable for the determination of semduramicin at authorized level and avoiding any adverse effect from the presence of the potential interferents.

The optimization of the chromatographic separation was focussed in getting a complete resolution between the target analyte (SEM), the internal standard (NIG) and the group of the other cocciodistats. As no quantification is required for the other coccidiostats, their co-elution was not considered as a relevant issue if no spectral interferences occurred.

The best compromise was found when performing the elution in isocratic mode using a binary mobile phase NH_4COOH 20 mM:MeOH (10:90) at 30 °C and a flow rate of 0.25 ml min^{-1}. Fig. 1 depicts the obtained chromatograms of a mixture of all the tested coccidiostats under those conditions at equivalent concentration level.

3.1.3. Selection of the quantification adduct

In order to establish the role of the different adducts within the scope of this work the following assay was performed. Individual solutions of the seven coccidiostats at the same concentration level were prepared in ACN from the respective stock solutions. This mixture was analyzed using two different mobile phases, which are HCOOH:MeOH:H_2O 0.1:90:10 (phase A) and NH_4COOH 20 mM:MeOH 90:10 (phase B) in order to favour the formation of the molecular sodium or the molecular ammonium adduct, respec-

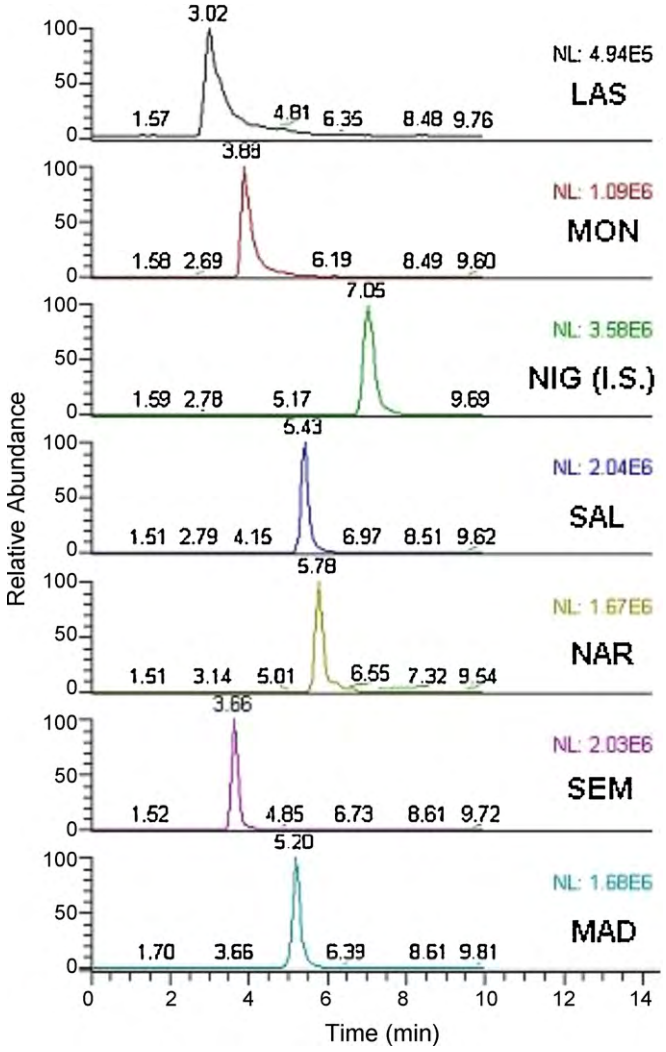

Fig. 1. Chromatograms of semduramicin along with the other five ionopohore coccidiostats in poultry feed plus the internal standard used for quantification (nigericin). Standard mixture of the seven compounds at equivalent concentration in acetonitrile. Selected ion monitoring (SIM) of the respective molecular ammonium adducts. Mobile phase NH_4COOH 20 mM:MeOH (10:90); flow rate 0.25 ml min^{-1}; probe temperature 375 °C; cone voltage 80 V; needle voltage 2.7 kV.

tively. In both cases the respective sodium and ammonium adducts for each target coccidiostats were monitored.

The performed tests lead to the following conclusions:

- In both mobile phases it is possible to detect the sodium and ammonium adducts of each coccidiostat.
- As expected, most of the coccidiostats showed the sodium adduct as dominant adduct when using phase A and the ammonium adduct when using phase B. However monensin lead to the sodium adduct as dominant adduct in both mobile phases.
- The ratios between the dominant and the secondary adducts widely differed among the coccidiostats (Table 2)
- The absolute signals from the respective dominant ions for each coccidiostat in each mobile phase are different from one coccidiostat to the other (Fig. 2).

In summary, the performed tests revealed that the most intense signals for SEM, NIG and MAD are obtained with the ammonium adducts when using phase B while for LAS, MON, SAL and NAR higher signals are obtained with the sodium adducts when using phase A.

The full scans of the individual solutions revealed a spectral interference between NAR and NIG for the *m*/*z* corresponding to the sodium and ammonium adducts of NIG when using phase A and with the sodium adduct of NIG when using phase B (Fig. 3). However in the case of phase B both critical analytes elute at different retention times, whereas a complete coelution of the critical compounds occurs when phase A is used (Table 2). As a result a mixture of the seven coccidiostats at equivalent concentration shows a chromatographic profile with two signals at the *m*/*z* corresponding to the ammonium adduct of NIG, i.e. one from each critical coccidiostat when the elution is carried out with phase B while the same mixture shows a single signal, i.e. from the combined contribution of both coccidiostats, when the elution is carried out with phase A.

Based on the obtained results it was decided to perform the determination using phase B and selecting the ammonium adduct for quantification purposes while the sodium adduct would also be monitored for diagnosis purposes, i.e. qualitative assessment.

The mass spectrometric determination of semduramicin was carried out by recording the respective molecular adducts in selected ion monitoring mode (SIM). Under these conditions we can enhance the signal of our target analyte (SEM) as well as diminishing the effect of some of the potential interferents (MON, LAS, SAL and NAR) which have the highest potential carry-over levels.

Furthermore this approach leads to an analytical method with at least three identification points for each target analyte namely retention time, *m*/*z* of the molecular ammonium adduct and the respective *m*/*z* of the molecular sodium adduct which is an evident added value for identification purposes.

3.1.4. Mass spectrometry detection

Once the chromatographic separation has been optimized, the relevant instrumental parameters related to the single mass spectrometer namely probe temperature, cone voltage and needle voltage have been also optimized.

Fig. 4 depicts the influence of the two most relevant variables, cone voltage and probe temperature, in the signal of each target coccidiostat and in the signal ratio. Additional criteria such as between injection repeatability, i.e. relative standard deviation among injections and sensitivity at the target concentration level,

Table 2
Ratio between dominant and secondary molecular adducts for each respective coccidiostat in the two different tested mobile phases.

	HCOOH:MeOH:H_2O 1:900:100 predominant adduct: Na			NH_4COOH:MeOH:H_2O 100:900 predominant adduct: NH_4[a]		
	m/*z* Na adduct	R_t (min)	Ratio Na/NH_4 adduct	*m*/*z* NH_4 adduct	R_t (min)	Ratio NH_4/Na adduct
MAD	939.5	4.8	3.7	934.5	5.2	2.9
SEM	**895.5**	**3.4**	**3.1**	**890.5**	**3.6**	**3.7**
NAR	787.5	6.2	6.2	782.5	5.7	1.7
SAL	773.5	5.0	6.9	768.5	5.4	1.4
NIG (I.S.)	**747.5**	**6.2**	**1.6**	**742.5**	**7.0**	**12.2**
MON	693.5	4.2	22.3	688.5	3.9	0.7
LAS	613.5	4.3	5.6	608.5	2.9	2.0

[a] MON shows also the sodium adduct as predominant one in this mobile phase.

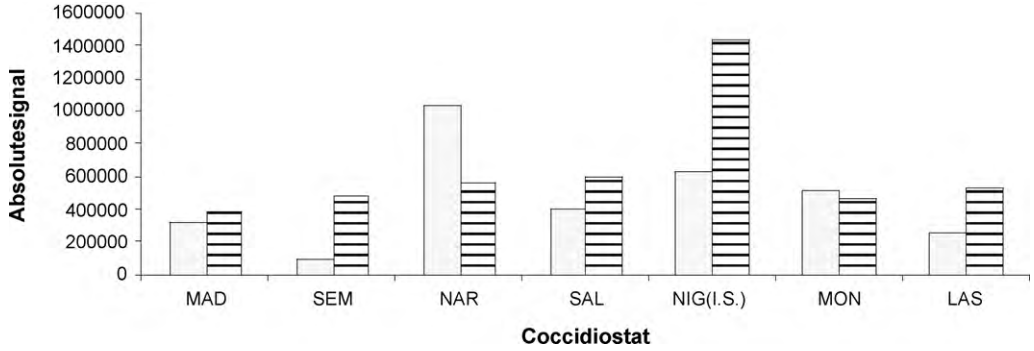

Fig. 2. Absolute signals obtained for the dominant molecular ion of each coccidiostat in both mobile phases. Dotted filling corresponds to the signal of the molecular sodium adduct when eluted with HCOOH:MeOH:H_2O (1:90:10) and stripped filling corresponds to the signal of the molecular ammonium adduct when eluted with NH_4COOH 20 mM:MeOH (10:90).

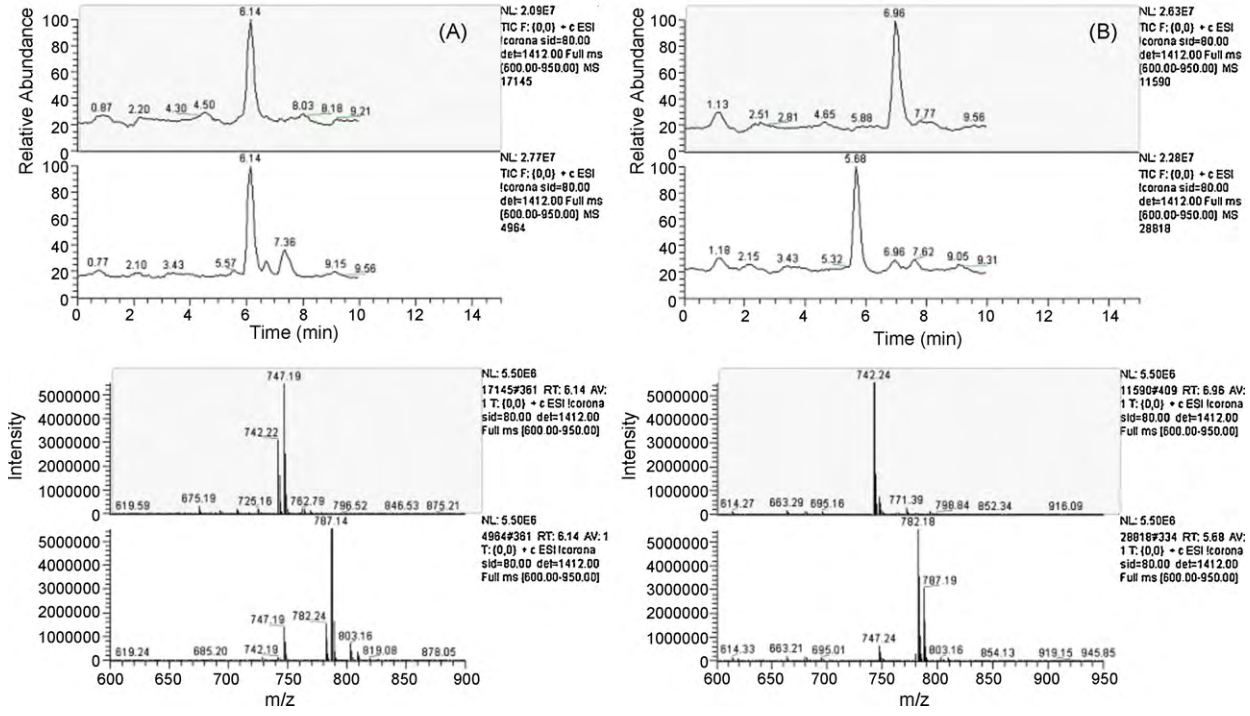

Fig. 3. Chromatograms and full scan mass spectra of individual solutions of nigericin (gray background) and narasin (white background) at equivalent concentration level. (A) Eluted with HCOOH:MeOH:H_2O (1:90:10) mobile phase and (B) eluted with NH_4COOH 20 mM:MeOH (10:90) mobile phase.

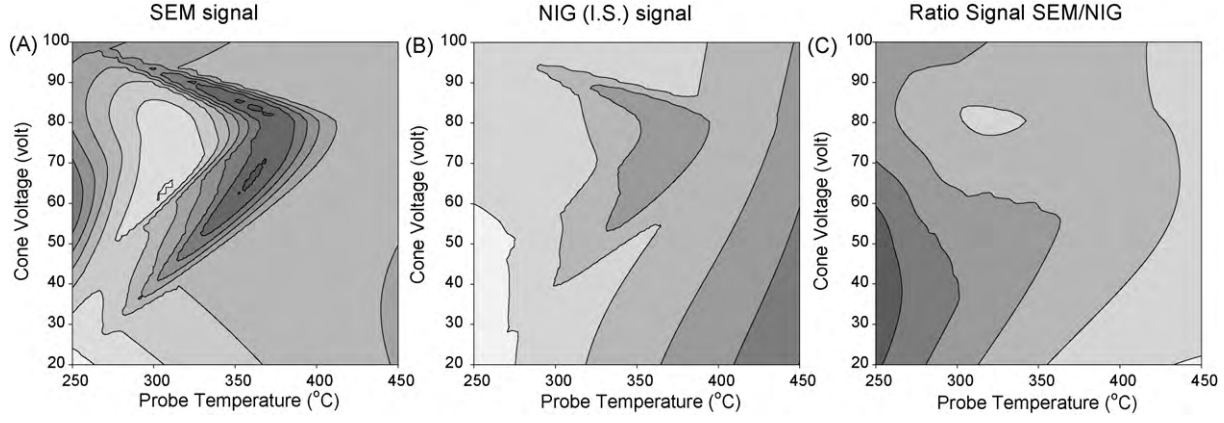

Fig. 4. Influence of the probe temperature and of the cone voltage in the absolute signals of semduramicin (A), nigericin (B) and in the signal ratio (C). Contour gray intensity plots where the darker colour corresponds to the higher signal value.

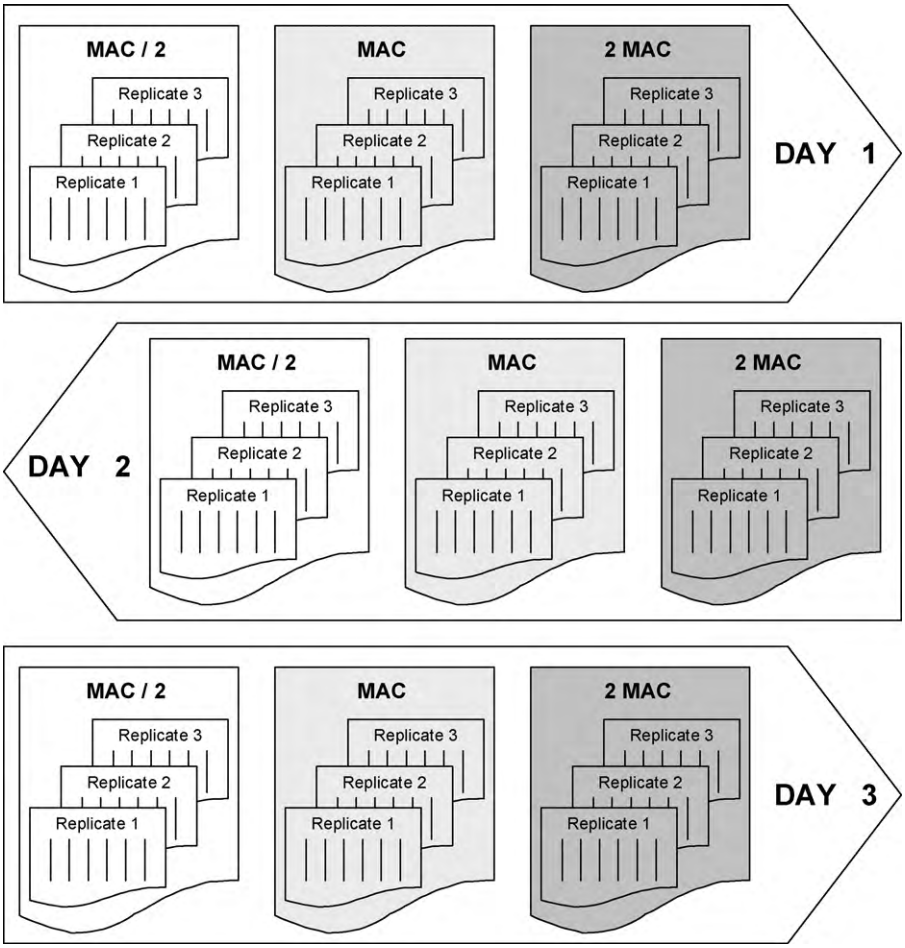

Fig. 5. Design of the single-laboratory validation assay performed to establish the analytical performance characteristics of the method.

i.e. slope of the correspondent standard addition calibration curve, have also been taken into consideration for selecting the final values for the parameters of the MS. The best trade-off conditions that better fulfilled the mentioned criteria have been found for a probe temperature of 250 °C, a cone voltage of 60 V and a needle voltage of 2.7 kV.

3.2. Analytical performance characteristics: single-laboratory validation

Within the last years a big effort within the different European organisations have been done in order to achieve common accepted criteria to be applied for establishing the analytical performance characteristics of analytical methods intended to be used for regulatory purposes. These performance criteria had been compiled in Commission Decision 657/2002 [15] which provides a well-established regulatory frame. However this document is restricted to food matrices and up to now there is no equivalent legislative frame applicable to feed.

Nevertheless relevant aspects of this document together with other internationally recognised guidelines dealing with single-laboratory validation have been taken into account for setting the performance characteristics of the method developed in this manuscript [16,17]. The precision under repeatability and intermediate conditions and the trueness of the described method have been established. The validation study has been carried out at 10 mg kg^{-1}, which is half of the minimum authorized level (MAC/2), 25 mg kg^{-1}, which is the maximum authorized level (MAC) and 50 mg kg^{-1}, which is twice the maximum authorized level (2 MAC).

Additionally during the validation of the method, an estimation of the limit of detection (LOD) and the limit of quantification (LOQ) has been done, even when not critical for the target concentration range of this method, i.e. authorized level (MAC).

3.2.1. Precision assessment

The precision of the method has been estimated according to the ISO standard 5725-3 [17]. As required in the aforementioned guideline a set of experiments has been planned and carried out under *repeatability* conditions, i.e. within the same day, on the same instrument and by the same operator as well as under *intermediate* conditions, i.e. on the same instrument and by the same operator but distributed along several days.

The assay has been performed with a commercial poultry feed acquired in the local facilities (AVEVE laying hens) appropriately fortified as described in Section 2.1.

Regarding precision, fitness for purpose criteria has been taken from the Commission Decision 2002/657 [15]. As described in a previous work [12] the intermediate precision was considered acceptable whenever the obtained value was below the precision value calculated through the Hortwitz equation [18]. This requirement is expressed by the HORRAT value described as the ratio of the experimentally obtained values of the standard deviation and the target standard deviation calculated by the Horwitz equation [18]. As a fitness for purpose criterion for a single-laboratory validation the target value should be 1 but in any case below 1.3.

In order to be able to establish the performance characteristics of the method a design of the experiments has been drawn and is depicted in Fig. 5. In essence the set of experiments implies the anal-

Table 3
Method performance characteristics. Single-laboratory validation. Results from the standard addition experiments conducted on samples fortified with semduramicin (standard spike) for the individual concentrations (A) and for the pooled concentrations (B). (C) Assessment of the linearity range.

(A)

	Target value (mg kg^{-1})	Mean value (mg kg^{-1})	RR (%)	S_r	RSD$_r$ (%)	$S_{Int.}$	RSD$_{Int.}$ (%)	Horrat value
Semduramicin	10.1	10.5	104.6	0.3	3.2	0.8	7.3	0.29
	25.0	26.8	107.2	0.7	2.8	1.0	3.7	0.29
	50.0	53.4	106.8	1.5	2.9	2.7	5.1	0.33

(B)

	RR%	RSD$_{Int.}$ (%)	Working range (mg kg^{-1})
Semduramicin	106.2	5.6	10–50

(C)

	Target value (mg kg^{-1})	Linear range (mg kg^{-1})	r	p Lack-of-fit
Semduramicin	10.1	10.1–76.8	0.9990	0.52
	25.0	25.0–91.7	0.997	0.54
	50.0	50.0–116.7	0.993	0.63

S_r, standard deviation for repeatability; RSD$_r$ (%), relative standard deviation for repeatability; $S_{Int.}$, standard deviation for intermediate precision; RSD$_{Int.}$ (%), relative standard deviation for intermediate precision. The means are calculated from the 54 results obtained for each analyte/concentration combination; RR (%), mean percentage recovery rate; r, correlation coefficient; p, significance values.

ysis of three replicates at three concentration levels namely MAC/2, MAC and 2 MAC. The whole schedule of analysis was repeated in three different days. Each aliquot was injected 6 times, obtaining in total 54 results for each analyte/concentration combination. This analysis schedule is a 3 factor-nested design as specified in the ISO standard [17] and minimizes the number of samples with respect to the design proposed in the Commission Decision 2002/657 without increasing the uncertainty of the performance characteristics [19].

The model that underlies the analysis of variance of the data collected by the nested design is that each of the measurement, Y_{ijk} is defined as the sum of three variance components plus the true value (TV) of the standard material measured (fixed quantity) and was estimated as follows:

$$Y_{ijk} = \mathrm{TV} + D_i + S_{ij} + W_{ijk}$$

where D_i stands for the *between day* variability, S_{ij} for the *within day* variability and W_{ijk} for the *between injection* variability.

For the calculation of the repeatability standard deviation, reflecting the within day variability including the injection error, and of the intermediate precision, the analytical results were subjected to analysis of variance (ANOVA) using the software package MINITAB^{TM} Statistical Software for Windows (version 15).

In summary, relative standard deviation for repeatability (RSD$_r$) varying from 2.8 to 3.2% have been obtained for each of the tested concentrations whereas the relative standard deviation for intermediate precision (RSD$_{Int.}$) ranged from 3.7 to 7.3% depending on the tested concentration. As the HORRAT value was below the predefined criterion of 1.3 in all cases, the obtained precision profile of the method is considered acceptable (Table 3A).

Following the recommendation of the relevant ISO standard [17] an additional evaluation of the data has been carried out in order to evaluate whether a concentration independent trueness and precision of the method can be estimated.

The regression analysis of the absolute standard deviation of the intermediate precision against the semduramicin concentration revealed a strong correlation. However, this correlation is not observed when the regression analysis is carried out using the corresponding relative standard deviation. Therefore all the measured concentrations were first divided by the respective mean values given in Table 3A in order to get normalized data.

As these normalized data are independent on the respective concentrations they can be pooled into one single data set. The statistical evaluation of the normalized concentrations leads to an acceptable value for the intermediate precision independent of the tested concentrations (Table 3B).

The trueness of the method, expressed as percentage recovery rate (RR), has been calculated taking into account the mean values of the results from all the experiments depicted in Fig. 5. The individual values obtained for each concentration level are between 104.6 and 106.8% (Table 3A) whereas the pooled trueness leads to a value of 106.2% (Table 3B).

The validation of mathematical model selected for quantification (linear regression) has been performed through the ANOVA lack-of-fit test. Three poultry blanks were spiked at the three tested concentrations i.e. MAC/2, MAC and 2 MAC in triplicate. The three replicates of each concentration level were then submitted to the sample preparation procedure described in Section 2.4 and quantified as described in Section 2.5. The ANOVA lack-of-fit test has been applied to each set of measurements independently in order to verify that the chosen model (regression line) adequately describes the relationship between the response and the concentration. Since the obtained P-values for lack-of-fit in the ANOVA are greater or equal to 0.10, the model appears to be adequate for each of the observed set data corresponding to the three tested concentrations (Table 3C).

3.2.2. Determination of experimental LOD and LOQ

The scope of the method detailed in this work is the determination of semduramicin in poultry feed at authorized concentration level. Consequently, its target concentration level is far above the theoretical concentration fitting the common established criteria for LOD and LOQ, i.e. 3 and 10 times the signal to noise ratio. This fact makes thus the determination of the LOD and LOQ not a key issue for this method.

However, these parameters being two of the most typical performance characteristics of an analytical method a two-steps assay has been performed in order to estimate them.

The first step involved the calculation, through the different calibration curves, of the theoretical concentration of SEM that should give a minimum signal to noise ratio of 3 (LOD) and 10 (LOQ). Theoretical concentration values of 0.22 and 0.73 mg kg^{-1} have been calculated for the LOD and LOQ, respectively.

The second step implied the experimental confirmation of the obtained theoretical values. Therefore, a poultry feed blank has been spiked in duplicate at those concentration levels of semduramicin and submitted to the analysis procedure described in this manuscript.

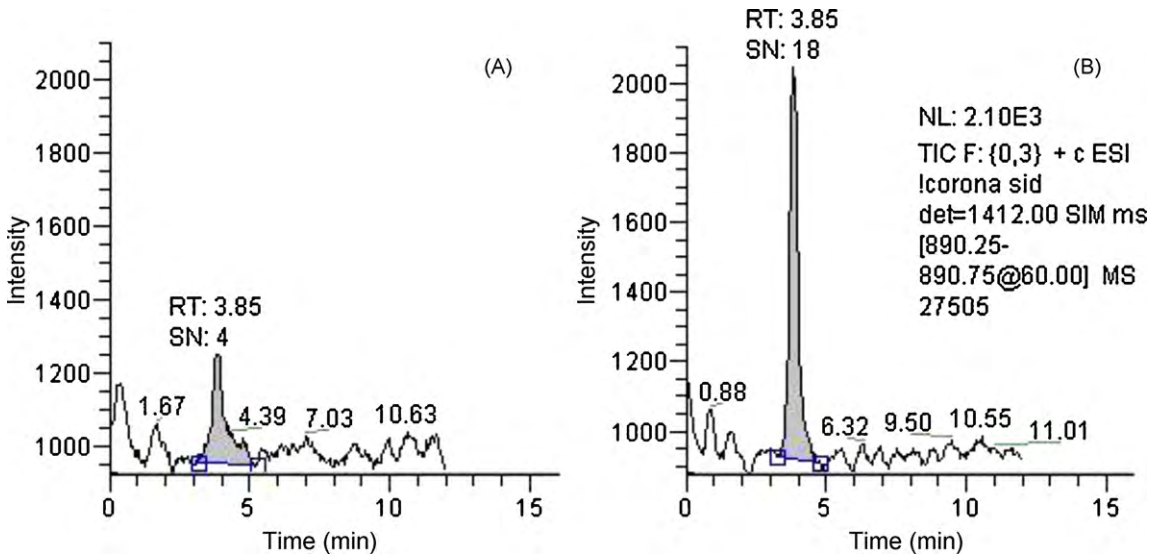

Fig. 6. Single ion monitoring chromatograms of the molecular ammonium adduct of semduramicin. Poultry feedingstuffs spiked at the concentration corresponding to the theoretical calculated (A) limit of detection (LOD) and (B) limit of quantification (LOQ). Instrumental conditions as described in the text.

The analyzed samples lead to average (between replicates) experimental signal to noise values of 3 and 13, therefore the theoretical concentration values can be established as the experimental LOD and LOQ for the analytical method. Fig. 6 shows the obtained chromatograms of the poultry blank spiked at those concentration levels. Those concentrations are around 90 (for LOD) and 27 (for LOQ) times below the minimum targeted authorized level, i.e. 20 mg kg^{-1} which confirms the aforementioned assumptions.

3.2.3. Transferability study

In order to ensure the suitability of the validated method for the mass spectrometry instrumentation commonly available in the European laboratories it has been decided to carry out a transferability study of the method from a single mass spectrometry instrumentation, where the method had been developed and single-laboratory validated to a triple quadrupole tandem mass spectrometer.

Therefore a set of samples has been analyzed in both instruments. Three replicates of a poultry blank (one per day) have been spiked at the three target concentration levels (MAC/2, MAC and 2 MAC). All the samples were submitted to the sample preparation procedure described in the manuscript and quantified using the standard addition design previously described. After the appropriate instrument tuning one aliquot of each solution was analyzed using the tandem mass spectrometer operated as a single mass spectrometer. In parallel the other aliquot was analyzed using a true single mass spectrometer. The instruments were operated by two different people. In both cases each solution has been injected 4 times.

The representation of the results obtained for the analyses of 12 different samples with both mass spectrometers lead to a regression line ($y = a + bx$) with a determination coefficient $R^2 = 0.997$; with an intercept $a = -1.052 \pm 1.304$ and a slope $b = 1.036 \pm 0.037$. The obtained regression coefficients do not differ significantly from zero to one, respectively (at 95% significance level) evidencing therefore that both sets of results are statistically equivalent [20].

4. Conclusions

A new simple and cost-effective analytical method perfectly tailored with the scope described in the Commission's mandate to CEN has been presented in this manuscript. The analytical performance characteristics of the developed method, calculated through a single-laboratory validation carried out according to the recognized international guidelines, demonstrates its fitness for purpose for the quantitative determination of semduramicin in complete feedingstuffs at authorized levels for official control purposes. In addition, the validation experiments confirmed the suitability of the method to be fully validated through the inter-laboratory study. The proposed method represents the best direct method available to carry out the analysis of semduramicin at authorized levels in poultry feed.

Acknowledgements

The authors are grateful to Zigmas Ezerskis for his kind assistance in the operation of the LC–MS/MS system within the transferability study.

References

[1] Commission Directive 97/6/EC of 30 January 1997 amending Council Directive 70/524/EEC concerning additives in feedingstuffs, Official J. Eur. Commun. L 35 (1997) 11–13.
[2] Council Regulation No. 2821/98 amending, as regards withdrawal of the authorisation of certain antibiotics, Directive 70/524/EEC concerning additives in feedingstuffs, Official J. Eur. Commun. L 351 (1998) 4–8.
[3] Council Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, Official J. Eur. Commun. L 268 (2003) 29–43.
[4] C.T. Elliott, D.G. Kennedy, W.J. McCaughey, Methods for the detection of polyether ionophore resides in poultry, Analyst 123 (1998) 45R–56R.
[5] D.K. Matabudul, N.T. Crosby, S. Sumar, A new and rapid method for the determination of nicarbazin residues in poultry feed, eggs and muscle tissue using supercritical fluid extraction and high performance liquid chromatography, Analyst 124 (1999) 499–502.
[6] Council Regulation (EC) No. 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene, Official J. Eur. Union L 35 (2005) 1–22.
[7] A.P. Ricketts, E.A. Glazer, T.T. Migaki, J.A. Olson, Anticoccidial efficacy of semduramicin in battery studies with laboratory isolates of coccidian, Poult. Sci. 71 (1992) 98–103.
[8] M.E. McKenzie, D.P. Conway, N.B. Logan, C.P. Wilkins, L.R. Chappel, Anticoccidial efficacy of semduramicin. 1. Evaluation against field isolates by dose titration in battery tests, Poult. Sci. 72 (1993) 2052–2057.
[9] Commission Regulation (EC) No. 1443/2006 of 29 September 2006 concerning the permanent authorisations of certain additives in feedingstuffs and an authorisation for 10 years for a coccidiostat, Official J. Eur. Union L 271 (2006) 12–18.

[10] E.A. Glazer, D.A. Koss, J.A. Olson, A.P. Ricketts, T.K. Schaaf, R.J. Wiscount Jr,.Synthetic modification of a novel microbial ionophore: exploration of antimicrobial structure–activity relationships, J. Med. Chem. 35 (1992) 1835–1839.
[11] Regulation (EC) No. 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, Official J. Eur. Union L 165 (2004) 1–141.
[12] U. Vincent, M. Chedin, S. Yasar, C. von Holst, Determination of ionophore coccidiostats in feedingstuffs by liquid chromatography-tandem mass spectrometry. Part I. Application to targeted feed, J. Pharm. Biomed. Anal. 47 (2008) 750–757.
[13] SIMBAG-FEED project funded by the European Commission, DG RTD, Competitive and Sustainable Growth (GROWTH) programme, Contract No. G6RD-CT-2000-00413.
[14] Commission Directive 2009/8/EC of 10 February 2009 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels of unavoidable carry-over of coccidiostats or histomonostats in nontarget feed, Official J. Eur. Union L 40 (2009) 19–25.
[15] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Official J. Eur. Commun. L 221 (2002) 8–36.
[16] M. Thomson, S.L.R. Ellison, R. Wood, Harmonized guidelines for single laboratory validation of methods of analysis, Pure Appl. Chem. 74 (2002) 835–855.
[17] ISO 5725-3:1994(E), Accuracy (trueness and precision) of measurement methods and results – Part 3. Intermediate measures of the precision of a standard measurement method, Geneva, ISO Standards, ICS 03.120. 30 (1994) 1–25.
[18] W. Horwitz, R. Albert, Performance characteristics of methods of analysis used for regulatory purposes. Part II. Pesticide formulations, J. AOAC Int. 74 (1991) 718–744.
[19] J. Van Loco, H. Beernaert, An alternative method validation strategy for the European Decision 2002/657/EC, in: Proceedings of Euro Food Chem. XII. Strategies for the Safe Food, vol. 1, 2003, pp. 91–94.
[20] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, 3rd ed., Ellis Horwood Limited, Chichester, UK, 1993.