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Determination of sodium nifurstyrenate and nitrovin residues in edible food by liquid chromatography–tandem mass spectrometry after ultrasound-assisted extraction

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ABSTRACT

A specific and sensitive method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed for the determination of nitrovin and sodium nifurstyrenate residues in muscle and liver of swine and chicken and in muscle of fish. Sample preparation procedure includes ultrasound-assisted extraction with acetonitrile, defatting with *n*-hexane and final clean-up with solid phase extraction (SPE) on Oasis HLB cartridges. The analytes were detected in multiple reaction monitoring (MRM) under negative scan mode acquiring two diagnostic product ions for sodium nifurstyrenate and under positive mode for nitrovin. The averaged decision limits (CCα; α 1%) ranged 0.09–0.26 μg/kg while the detection capability (CCβ; β 5%) was 0.33–0.97 μg/kg in the tissues. Reasonable recoveries (71–110%) spiked in muscle and liver showed excellent relative standard deviation (RSD). The validated method was simple, rapid, sensitive, and complied with the regulations for the determination of nitrovin and sodium nifurstyrenate residues in food matrices.

1. Introduction

Sodium nifurstyrenate (NFS), 4-[2-(5-nitro-2-furyl) vinyl] benzoic acid sodium salt (Fig. 1A), is an antibacterial nitrofuran widely used for prevention and treatment of bacterial infections in fish, with its good antibiotic action for colon bacillus, staphylococcus, salmonella, part of proteus species and comma bacillus. NFS was absorbed quickly for oral administration, and could access the protective screen of placenta and blood-cerebrum [1]. In addition, NFS showed obvious mutagenicity under the dosage of 0.05–1 nmol/utensil in mutation test [2].

Nitrovin (NIT), 1,5-bis(5-nitro-2-furanyl)-1,4-pentadien-3-one (Fig. 1B), is a furan drug, which is a kind of orange yellow or red stable powder. It was widely used in veterinary medicine to control and prevent infectious diseases in livestock and aquatic products, for it could refrain or even kill all kinds of microorganism by suppressing actively the utilization of pathogenic microorganism for glucose [3,4].

NFS and NIT were banned in many countries, including Europe Unit (EU) [5] and China, because of their toxic effects on consumers by means of residues in edible tissues. But NFS and NIT were misused seriously. Therefore it is necessary to develop a qualitative and quantitative method to analyze NFS and NIT residues in animal tissues.

At present there were only three articles about NFS residues determination. Horie et al developed an HPLC method using an Inertsil ODS column with detection at 265 nm, 50 μl injection volume and the LOD of NFS was 100 μg/kg [6]. Another simple HPLC method for quantification of NFS in yellowtail serum was validated by Segawa et al. After extracted with acetonitrile, analytic sample was injected directly into a C_{18} column and detected at 420 nm with the LOD of NFS 25 μg/kg [7]. Zhan Chun-rui developed a LC–MS/MS method for NFS residues in animal tissues and the LOD was 5 μg/kg [8].

As for nitrovin there were three papers about the method for feed [9–11]. J.R. Wang reported a method to determine the amount of furazolidone, furaltadone, nitrofurazone, and nitrovin in feeds simultaneously by HPLC and LC–MS. Samples were extracted with a mixture of acetonitrile and methanol with ammonia solution, and cleaned-up by HLB and the LOD was 200 μg/kg [10]. And an improved method [11] was developed for the determination of nitrovin in feeds by HPLC. After extraction with a mixture of dimethyl formamide–acetonitrile–methanol (50:25:25, v/v/v), the sample was cleaned up with reversed-phase solid phase extraction cartridge and the LOD was 50 μg/kg.

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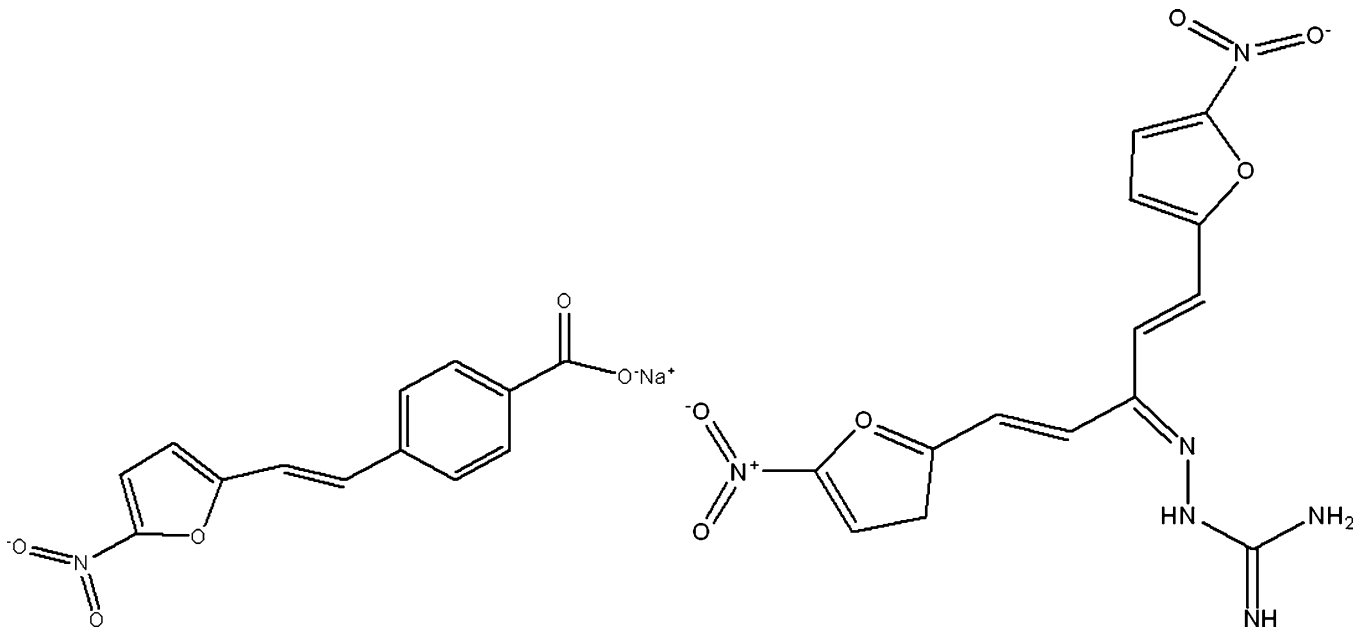

Fig. 1. The structures of sodium nifurstyrenate (A) and nitrovin (B).

However, these above methods cannot meet the requirement of the current EU legislation due to relative low sensitivity. Moreover, methods that are based on chromatographic analysis followed by mass spectrometric detection are becoming the normal way of confirming identity and determination concentration. Sensitivity- and selectivity-related problems have prompted us to develop a method with LC/MS/MS to analyze NFS and NIT in tissues.

Another critical challenge for the valid determination of trace-level NFS and NIT in complicated matrices appears in the extraction and cleanup procedure. An ideal leaching procedure should be exhaustive with respect to the target compounds, fast, simple, inexpensive, environmental friendly and amenable to automation for routine analysis. Based on ultrasound-assisted extraction (UAE), extraction methods for NFS and NIT have been developed in order to drastically decrease the volume of extract solvent and shorten the process time. Ultrasound procedure also improved the efficiency of extraction. It has been reported a lot that the UAE method was applied into the analyses of veterinary residues in animal tissues [12–14].

Therefore, the aim of this work was to develop an analytical method based on UAE extraction, followed by LC/MS/MS for the determination of NFS and NIT residues in different matrices. Optimization of UAE was conducted by varying extraction parameters such as extraction solvents, temperature and extraction time. Clean-up was based on a HLB cartridge. This novel developed method was successfully applied to real tissue sample analysis.

2. Experimental

2.1. Chemicals and reagents

Standards of NFS and NIT were purchased from Dr. Ehrenstorfer GmbH. The two standards were of over 99% purity. Methanol, acetonitrile and *n*-hexane were HPLC grade (VWR International, Zaventem, Belgium). Dimethylsulfoxide (DMF) was purchased from Acros Organics (Geel, Belgium). Water was obtained from a Milli-Q Gradient system (Milli-Q; Millipore, Bedford, MA, USA). Oasis HLB (60 mg, 3 ml) were obtained from Waters Corporation (Milford, MA, USA). C_{18} SPE (100 mg, 3 ml) were purchased from Varian (Harbour City CA, USA).

2.2. Preparation of standard solutions

Accurately weighed solid portions of NFS and NIT were dissolved in dimethylsulfoxide to prepare stock standard solutions at 1.0 mg/ml. Mixed working standard solution (0.1 mg/ml) was prepared by dilution of the stock standards in dimethylsulfoxide. Then, mixed working standard solution was serially diluted with acetonitrile–water (30:70, v/v) to prepare six calibrators with the concentrations of 0.5, 1, 2, 5, 10 and 50 μg/l. Stock standard solutions were stable for 1 year stored in glass tube at −20 °C in the absence of light, and mixed working standard solution was freshly prepared every day.

2.3. Sample preparation

Animal products including muscles (swine, chicken and fish) and livers (swine and chicken) were collected from local markets in Wuhan (Hubei, China). Typically, 500 g tissue samples were first minced by a kitchen homogenizer and stored at −18 °C until further preparation.

2.4. Extraction by ultrasonic bath and by shaking

Approximately 5 g of ground tissue were weighed accurately into 50 ml polypropylene centrifuge tube. After addition of 15 ml acetonitrile, the mixtures were positioned for 15 min in ultrasound-assisted extraction (Kunshan Instruments Company, Kunshan, PRC; power: 100 W, frequency: 40 kHz). The temperature was maintained at 50 °C. After extraction, the suspension was centrifuged at 8000 rpm for 10 min, and the supernatant was transferred to a polypropylene tube.

Shaking extraction, approximately 5 g of ground tissue were weighed accurately into 50 ml polypropylene centrifuge tube. After addition of 15 ml acetonitrile, the residues were extracted by mechanical shaking in an Erlenmeyer flask at 25 °C for 10 min. After extraction, the suspension was centrifuged at 8000 rpm for 10 min, and the supernatant was transferred to a polypropylene tube. Then another 15 ml acetonitrile was added to extract the residues again. The twice extracts were combined to be evaporated.

Table 1
The optimal parameters of multiple reaction monitoring.

Compound	M_W	Retention time (min)	Precursor ion (m/z)	Scan mode	Qualitative ions (m/z)	Quantitative ions (m/z)	Collision energy (V)
NIT	361	3.7	361.1	ESI+	361.1 > 301.8, 361.1 > 222.1	361.1 > 222.1	28; 19
NFS	281	6.7	257.8	ESI−	257.8 > 213.9, 257.8 > 183.5	257.8 > 213.9	20; 15

2.5. Clean-up

The supernatant was evaporated to dryness under a gentle nitrogen stream at 45 °C. The flask was rinsed twice with 3 ml of 50% acetonitrile/water (v/v). The supernatants were combined, and partitioned with 5 ml *n*-hexane. The extracted solution was cleaned-up by offline SPE on an Oasis HLB cartridge. The SPE column (60 mg, 3 ml, Milford, MA, USA) was pre-conditioned with methanol (3 ml) and water (3 ml). All flow rates for conditioning and washing were set at 1 ml/min. The column was washed with 20% methanol (6 ml), and then dried by purging air at the rate of 10 ml/min. The analytes were eluted with 1% DMF–methanol (2 ml) at a flow rate of 1 ml/min into a 5 ml glass tube and evaporated to dryness under a stream of nitrogen at 45 °C. Five hundred microliters of mobile phase was added to dissolve the residues and the tube was mixed for 30 s. The solution was then transferred to tapered micro-vial and centrifuged at 12,000 rpm for 15 min, 10 μl of supernatant was injected into LC–MS/MS.

2.6. LC–MS–MS analysis

Analysis was carried out using a Thermoelectron TSQ Quantum Access triple quadrupole mass spectrometer coupled to a Surveyor LC pump and autosampler. The separation was achieved by on a Thermo Hypersil Gold C_{18}, 100 mm × 2.1 mm, 5 μm column (100 mm × 2.1 mm, 5 μm) at 40 °C. The mobile phase A was acetonitrile, while the mobile phase B was water. The gradient was as following: 0.0 min: A/B (30/70, Flow rate: 0.20 ml/min); 6.0 min: A/B (35/65, Flow rate: 0.20 ml/min); 10 min: A/B (30/70, Flow rate: 0.20 ml/min).

The source parameters were optimized by monitoring the MS and MS/MS spectra of the NFS and NIT. Multiple reaction monitoring (MRM) was performed on each of the analytes using the parameters. Segment 1 (during the first 5.0 min) for the analysis of NIT was detected in positive (ESI+) electrospray ionization mode. Ionspray voltage was 4.5 kV. Capillary temperature was set at 350 °C. The Sheath Gas and Aux Gas Pressure were carried out using nitrogen at 20psi and 25arb, respectively. And Segment 2 (during the last 5.0 min) for the analysis of NFS was operated detected in negative (ESI−) electrospray ionization mode. Ionspray voltage was set at −3.5 kV. The other parameters were the same as NIT. The parameters on the m/z and collision energy of precursor ions and quantitative product ions from NFS and NIT are shown in Table 1.

2.7. Validation of method

The method was validated with reference to the implemented validation procedure for residues in food animal products as described in EU Commission Decision 2002/657/EC [15]. The validation of specificity, linearity, CCα, CCβ, recovery and precision for the method were determined by spiking 5 different types of matrixes (muscles and livers of swines, chicken; and muscles of fish) with a mixture of NFS and NIT.

2.7.1. Specificity

The specificity of the method was demonstrated by testing all matrices available. Muscle and liver blank samples from thirty different subjects were studied in the present test with identical concentration of NFS and NIT spiked at 1 μg/kg. The results were evaluated by the presence of interfering substances around the NFS and NIT retention time.

2.7.2. Matrix effects and calibration curves

Matrix effects on the ionization of analytes were evaluated by comparing the peak area of standard solution with that of the matrix extract solution. Matrix effect was calculated by the formula: matrix effect = $[1 - (a_{matrix}/a_{standard})] \times 100$, where a_{matrix} and $a_{standard}$ are the slopes of calibration straight lines for standard solution and matrix matched calibration graphs. To achieve a better quantitative result, the matrix effects were compensated by using matrix-matched standard curves.

Blank samples of swine muscle, chicken liver, and fish muscle were used as matrices for calibration curve study. The matrix-match calibration curves were made by fortified tissues with six levels of 0, 0.10, 0.25, 0.5, 1.5, 2 and 5 μg/kg. The tissues spiked with NFS and NIT were operated with complete extraction and purification procedure. The calibration curves were constructed using a peak area from six concentrations versus the concentration of analytes.

2.7.3. CCα and CCβ

The decision limit (CCα) is the lowest concentration at which a method can discriminate with a statistical certainty of $1 - \alpha$ that the analyte is present. In the case of NFS and NIT, CCα was established by the following steps: 20 blank samples of swine muscle and liver, chicken muscle and liver, fish muscle were analyzed and the signal–noise ratio (S/N) is calculated at the time window in which the analyte is expected. CCα values were defined as three times of S/N. The detection capability (CCβ) is the concentration at which the method is able to detect truly contaminated samples with a statistical certainty of $1 - \beta$. CCβ was calculated by analyzing 20 blank samples of swine muscle and liver, chicken muscle and liver, fish muscle spiked with the NFS and NIT at CCα and then the CCα value plus 1.64 times the corresponding standard deviation is equal to CCβ (β = 5%).

2.7.4. Trueness and precision

Swine muscle, chicken liver and fish muscle, which were non-contaminated, served as blank matrices. Trueness and precisions (intra-day, inter-day, and within-laboratory) were calculated from the determination of five aliquots each tissue fortified at three levels (0.5, 1.0, 2.0 μg/kg). The analyses were finished by the same operator in triplicate in a 2-week period. Within-laboratory was carried out in the same laboratory, but performed by two different operators. The recovery was calculated by the following formula: (the measured level/the fortified level) × 100%. The precision was expressed as the RSD.

3. Results and discussion

3.1. Optimization of LC–MS/MS conditions

NFS and NIT are both shown good retention on reversed-phase column in the previous research [6–11]. This method was conducted to obtain the best separation of the analytes in the shortest time by optimizing the chromatographic separation. After the optimization experiment, the analytical separation of NFS and NIT was

Table 2
Sensitivity (μg/kg) and matrix effect (%) of the method for different animal tissues.

Compound	Sensitivity and matrix effect (%)	Swine muscle	Chicken muscle	Fish muscle	Swine liver	Chicken liver
Sodium nifurstyrenate	CCα (μg/kg)	0.09	0.11	0.10	0.25	0.23
	CCβ (μg/kg)	0.33	0.40	0.29	0.59	0.51
	Matrix effect (%)	10.6	6.9	8.8	25.5	26.4
Nitrovin	CCα (μg/kg)	0.20	0.18	0.14	0.26	0.19
	CCβ (μg/kg)	0.60	0.58	0.36	0.89	0.97
	Matrix effect (%)	10.0	9.3	12.0	28.1	29.8

performed with a reverse phase C_{18} column. And acetonitrile and water were chosen as mobile phase under gradient elution.

The strategy followed in the optimization of the MS–MS method was based on multiple reaction monitoring and the scan mode was tested. ESI was chosen since NFS and NIT are medium polarity. For NIT, there are some alkaline radical in its structure, so the mode of scan with ESI+ was appropriate. Under the ESI+ mode, the precursor ion is 361.1 and two product ions are 301.9 and 222.1 *m*/*z*. While for NFS, the mode of scan with ESI− was selected due to its negative ion formation in solution. Under the ESI− mode, the precursor ion is 257.8 and two product ions are 213.9 and 183.5 *m*/*z*, respectively, which is consistent to the document [8]. So the scan mode method was performed in two segments, one of which was ESI+ scan mode during the previous 5 min for NIT and the other ESI− scan mode during the later 5 min for NFS. The optimal values for each parameter and triterpenic compound are shown in Table 1. This parameter fulfills the European Commission requirements (at least 3 identification point) [15].

3.2. Optimization of extraction and clean-up

As for NIT, previous studies [3,9–11] have shown that the acetonitrile, or acetonitrile containing 1% ammonia solution, or acetonitrile containing DMF exhibit good solubility of NIT. While as for NFS, the solubility of it is higher in organic solvents such as acetone, methanol and acetonitrile under acidic condition. It has been reported that methanol with 2% metaphosphoric acid or acetonitrile with phosphoric buffer (pH 7.0) was used to extract NFS [6,7]. The latest report has shown that acetonitrile exhibit good solubility towards trace analysis of NFS residues in tissues [8]. Therefore, acetonitrile was chosen as the main extraction solvent for trace analysis of NIT and NFS. Ultrasound-assisted extraction (UAE) can be used as an extraction method applied to veterinary residues in animal tissues. The most important purpose of optimization experiments for UAE was to improve the extraction efficiency, achieved by changing the extraction time (min), temperature (°C) and the amount of extraction solution (ml), with minimum solvent consumption and minimum duration of the extraction time. The UAE results are shown in Fig. 2.

To compare the extraction efficiency of shaking extraction and ultrasonic-assisted extraction, we have performed these two procedures respectively according to Section 2.4 (liver was selected as the representative tissue). The compared results of recoveries between shaking extraction and ultrasonic-assisted extraction are listed in Table 3. The result indicated that the recovery of NFS and NIT obtained by ultrasonic extraction was better than shaking extraction. What's more, the employment of ultrasound is easy to handle and time-saving.

Biological sample matrices, especially edible animal tissues, are complex and often contain elements that can interfere with the compounds of interest. Therefore direct analysis may not always be possible. Moreover, concentrations of analytes are generally low in these samples, and it is usually necessary to purify and concentrate the analytes prior to analysis. The utilization of solid phase cartridges greatly simplifies the pre-workup process. Previously, this technique has been used to the clean-up of NFS extracts from

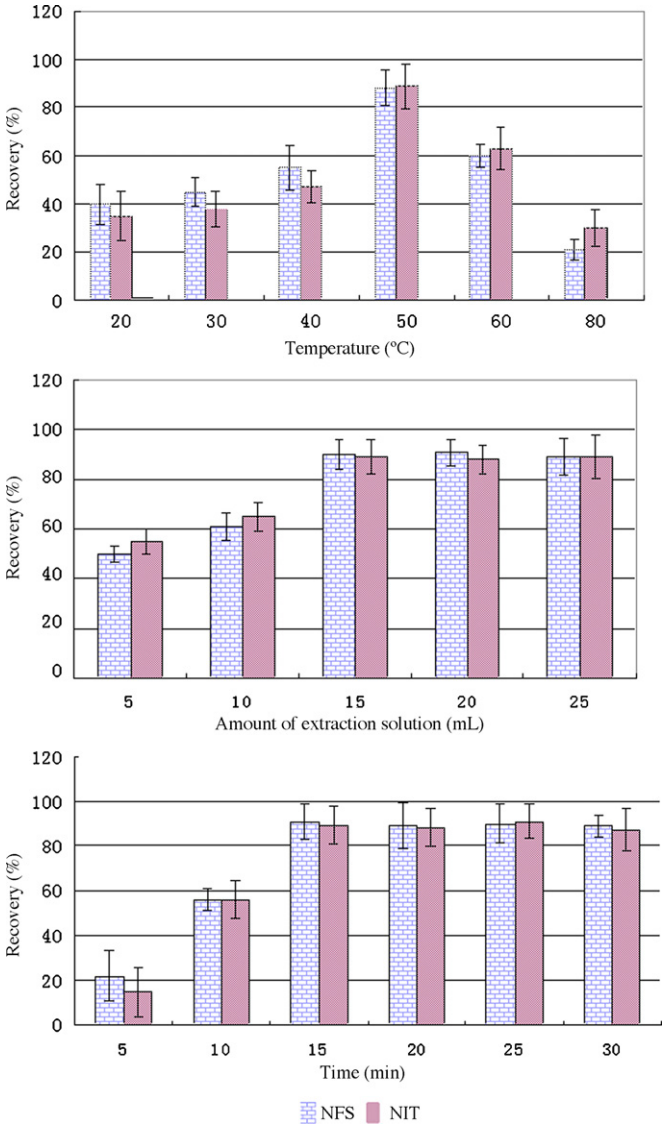

Fig. 2. Effect of time (A), temperature (B) of sonication and (C) amount of extraction solution on the recovery of NFS and NIT ($n = 5$).

Table 3
Effect of different extraction method and solid phase extraction columns on the recovery of sodium nifurstyrenate and nitrovin (n = 5).

Sample preparation	Spiked level (μg/kg)	Recovery % ± SD%	
		Sodium nifurstyrenate	Nitrovin
Shaking	2	76.3 ± 7.4	78.9 ± 10.6
Ultrasonic-assisted extraction	2	89.9 ± 6.4	88.3 ± 9.5

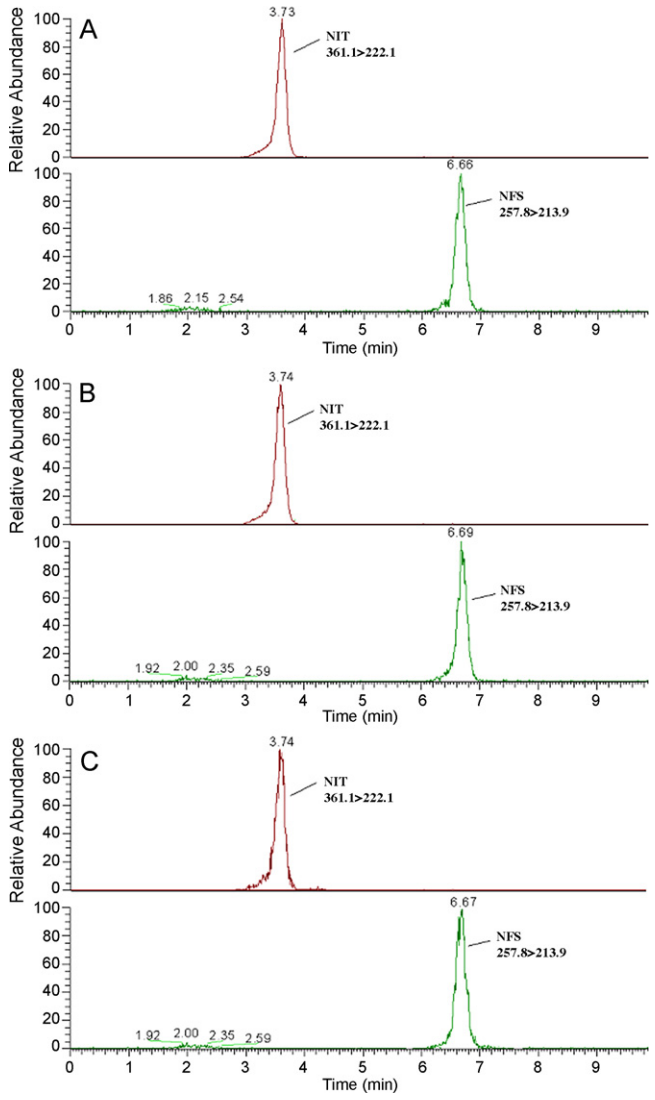

Fig. 3. (A) LC–MS/MS MRM chromatogram of standard solution (1 μg/l for NFS and NIT). (B) SRM LC–MS/MS chromatograms of: extract of blank swine liver spiked with 1 μg/kg for NFS, NIT equivalent to the CCβ. (C) SRM LC–MS/MS chromatograms of: extract of blank fish muscle spiked with 0.5 μg/kg for NFS, NIT equivalent to the CCβ.

tissues [7] and NIT extracts from feed [10,11]. In the present work, in order to eliminate interferences from the matrix, C_{18} and Oasis HLB cartridges were tested for the clean-up of tissue samples. The poor recoveries, repeatability and reproducibility were obtained by the clean-up of generic C_{18} (recovery less than 70%). The Oasis HLB, made of lipophilic and hydrophilic copolymer with a balanced ratio of two monomers, was chosen for tissue samples of the clean-up. Its recoveries obtained for NFS and NIT were above 80%. So HLB cartridges were selected because of its higher recovery for the clean-up of NFS and NIT. Different washing and eluting agents were used to improve the cleanup efficiency, namely, 6 ml of 20% methanol for washing and 2 ml of methanol elution, which resulted in good clean-up and separation.

3.3. Method validation

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank tissues and blank tissues spiked with NFS and NIT. Fig. 3B–C represents chromatograms of NFS and NIT from tissues after extraction and clean-up. No interference of endogenous peaks with NFS and NIT at their respective retention time (6.7 min for NFS, 3.7 min for NIT) in blank tissues was detected.

Table 4
Accuracy and precision of the method for sodium nifurstyrenate and nitrovin in various tissues (n = 5 at each concentration, on 5 separation days).

Muscle of swine	Spiked level (μg/kg)	Recovery (%)	Within-day RSD (%)	Between-day RSD (%)
Sodium nifurstyrenate	0.5	75.2	7.2	12.2
	1	80.4	7.9	13.6
	2	85.8	5.8	10.5
Nitrovin	0.5	77.1	9.1	11.6
	1	89.1	8.7	17.2
	2	83.0	6.5	16.3
Liver of swine				
Sodium nifurstyrenate	0.5	80.1	8.1	14.9
	1	83.3	7.7	15.6
	2	87.8	6.9	9.7
Nitrovin	0.5	79.6	9.6	10.8
	1	86.7	8.8	11.9
	2	87.8	8.1	8.9
Muscle of chicken				
Sodium nifurstyrenate	0.5	74.3	5.9	8.9
	1	81.9	7.2	15.9
	2	90.4	6.2	12.6
Nitrovin	0.5	72.5	5.6	10.3
	1	81.3	8.1	11.8
	2	92.8	9.5	16.5
Liver of chicken				
Sodium nifurstyrenate	0.5	72.2	8.1	11.3
	1	85.6	9.8	10.8
	2	89.7	6.8	9.7
Nitrovin	0.5	71.4	7.2	8.6
	1	80.3	7.5	10.1
	2	84.7	8.8	11.8
Muscle of fish				
Sodium nifurstyrenate	0.5	74.9	6.7	15.6
	1	88.7	8.5	12.8
	2	91.6	10.2	14.3
Nitrovin	0.5	67.3	9.5	13.5
	1	75.3	8.3	10.8
	2	82.4	9.6	11.9

The result of matrix effect from different kinds of tissues was shown in Table 2. In muscle tissues the matrix effect was very low (6.9–12.0) while liver matrix effect was relative higher (25.5–29.8). Matrix-matched calibration curves were performed to compensate for the matrix effect and loss in sample preparation. The matrix spike curves showed good linearity (r > 0.9990) within the tested range. The results suggested that the linearity of the method would be quantitative by an external standard method. In the method, CCα and CCβ were calculated from the analysis of 20 tissue samples. From Table 2, CCα values of NIT and NFS in different tissues were 0.09–0.26 μg/kg and CCβ 0.29–0.97 μg/kg, respectively.

Fortified tissue samples at three different concentrations for each target were analyzed to evaluate the accuracy and precision of the methods. Representative chromatograms of fortified muscle and liver are represented in Fig. 3. The accuracy and precision of the method were described by means of recovery and repeatability (inter-day and intra-day). Table 4 give the overview of the results of accuracy and precision of the method on each animal tissue determined at different spiked levels. Most of the recoveries were higher than 70% at the spiking levels in muscle samples. From all of the recovery results, it is clear that the matrix composition has a great influence on the extraction and clean-up yield. Repeatability of the method tested at different spiking levels for NFS and NIT were relatively high but in most cases less than 20%.

Table 5
Confirmatory and quantitative LC–MS/MS analysis for incurred samples of swine, chicken and fish tissues.

Sample	Analyte detected	Incurred samples			Matrix-matched standard		Level (μg/kg)
		Product ions	Ion ratio (%)	Retention time (min)	Ion ratio (%)	Retention time (min)	
Swine liver 5	Nitrovin	361.1 > 222.1	81 ± 4.5	3.72	78 ± 6.3	3.74	5.9 ± 0.8
Swine liver 8	Nitrovin	361.1 > 222.1	87 ± 3.5	3.71	83 ± 5.3	3.72	3.6 ± 0.5
Swine liver 11	Nitrovin	361.1 > 222.1	82 ± 6.2	3.70	79 ± 5.7	3.71	4.1 ± 0.7
Chicken muscle 7	Nitrovin	361.1 > 222.1	83 ± 4.6	3.75	81 ± 3.9	3.73	3.2 ± 0.5
Chicken muscle 15	Nitrovin	361.1 > 222.1	82 ± 7.3	3.67	82 ± 6.5	3.71	2.3 ± 0.7
Fish muscle 5	Sodium nifurstyrenate	257.8 > 213.9	72 ± 6.3	6.71	70 ± 5.8	6.72	1.5 ± 0.8
Fish muscle 9	Sodium nifurstyrenate	257.8 > 213.9	78 ± 5.1	6.69	76 ± 3.5	6.71	2.4 ± 0.5
Fish muscle 11	Sodium nifurstyrenate	257.8 > 213.9	78 ± 4.7	6.73	71 ± 3.5	6.75	3.4 ± 0.5
Fish muscle 17	Sodium nifurstyrenate	257.8 > 213.9	75 ± 3.9	6.72	72 ± 4.9	6.73	6.6 ± 0.8

Three kinds of solution including stock solutions, working standard solution (100 mg/l) and resulting extracts (Blank sample was spiked at levels of 2 μg/kg and then extracted), were analyzed. Using the same calibration set, after comparing with the background noise in various matrices, the results demonstrated that, there were no interfering peaks that could be detected on the expected retention time for these target analytes (within 2.5%). Consequently stock solutions could be stable for at least 1 year in glass tube at −20 °C, Working standard solution for 2 week and resulting extracts for 1 week at 4 °C.

3.4. Applicability of the proposed method

Using the method described above, 100 swine liver samples, 100 chicken muscle samples, 100 fish muscle samples were bought from different supermarkets in the city of Wuhan (China). All the samples were transferred to our laboratory and analyzed by LC–MS/MS with UAE and calculated. The results are given in Table 5. In accordance with the Commission Decision 2002/657/EC a sample can be confirmed as positive when the following criteria are met: (1) the relative retention time (RRT) of the analyte should correspond to that of the standard analyte, from a spiked sample, with a tolerance of ±2.5%, and (2) the relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion, must correspond to those of the reference analyte, either from calibration standards or from incurred samples, at comparative concentrations and measured under the same condition, within the needed tolerances. The ion ratios of the two product ions (relative intensities >50%) of each analyte, signal 2/signal 1 (most abundant), must not exceed the tolerance of ±20%. All criteria were fulfilled for the analysis of the real samples and spiked samples with ion ratios ranging from 67.1% to 90.5% and not exceeding the tolerance of 20%.

The results demonstrated that the residue of nitrovin was at the levels of 2.3–5.9 μg/kg in the swine liver and chicken muscle samples. And the residue of sodium nifurstyrenate was found at the levels of 1.5–6.6 μg/kg in fish muscles.

4. Conclusions

In this study, an LC–MS/MS method for simultaneous determination and confirmation of NFS and NIT residues in the muscle and liver of swine and chicken; in the muscle of fish using ESI− and ESI+ with MRM has been developed. The two analytes were extracted from tissues by ultrasonic-assisted extraction and SPE cleanup prior to the LC–MS/MS analysis. The method could fulfill the requirement of the confirmatory criteria according to European Commission Decision 2002/657/EC by four identification points obtained for each analyte with high sensitivity and selectivity. At different spiking levels for each analyte, good accuracy and precision were obtained, which indicated that the present method is highly quantitative, linear, specific and suitable for the routine analysis of the two analytes in animal tissues.

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