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Short communication

Determination of neonicotinoid insecticides residues in bovine tissues by pressurized solvent extraction and liquid chromatography–tandem mass spectrometry

Zhiming Xiao, Xiaowei Li, Xiaolin Wang, Jianzhong Shen, Shuangyang Ding*

Department of Pharmacology and Toxicology, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

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ABSTRACT

A rapid, sensitive, and environmental-friendly method has been developed for the simultaneous determination of seven neonicotinoid insecticides residues in bovine muscle and liver. The sample preparation procedure was based on a high automated pressurized solvent extraction (PSE) combined with solid-phase extraction (SPE) clean-up. The target compounds were identified and quantitatively determined by liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) operated in multiple reaction monitoring mode. Average recoveries of the seven analytes from fortified samples ranged between 83.2% and 101.9%, with relative standard deviations (RSDs) lower than 10.8%. The limits of detection (LODs) and quantification (LOQs) for neonicotinoids were in the ranges of 0.8–1.5 $\mu g\,kg^{-1}$ and 2.5–5.0 $\mu g\,kg^{-1}$, respectively. This validated method was successively applied to the determination of neonicotinoid insecticides in real samples from markets.

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1. Introduction

Neonicotinoid insecticides, one of the fastest growing pesticides, are widely applied to treat crops as well as livestock animals against a broad range of commercially important sucking and chewing pests [1–3]. Commercialized neonicotinoids include nitenpyram, dinotefuran, thiamethoxam, imidacloprid, clothianidin, acetamiprid, and thiacloprid. Neonicotinoid insecticides act as agonists at the insect nicotinic acetylcholine receptors (nAChRs), which plays an important role in synaptic transmission in the central nervous system [4,5]. Due to its high selective affinity to insect nAChRs over vertebrate, neonicotinoid insecticides are always considered to be low toxicity for mammals. However, recently toxicological studies in mice have revealed that thiamethoxam may cause an increased incidence of liver tumors, and may have a relevance to humans [6,7]. Furthermore, in a study by Vesile Duzguner et al. suggest that imidacloprid may cause oxidative stress and inflammation in central nervous system and liver in non-target organisms in rats [8]. Even though the predicted studies indicate a risk, a suitable analytical method to monitor the occurrence of these insecticides in animal tissues is missing.

For screening purposes, immunoassays (ELISA) [9,10] and chromatographic technique such as high-performance liquid chromatography with diode-array detection (HPLC-DAD) [11–13] have been developed. However, the confirmation of suspect positive samples must be performed by mass spectrometry coupled to the adequate chromatographic separation [14]. Several methods utilizing gas chromatography with mass spectrometry (GC–MS) for the analysis of neonicotinoids in biological samples have been published [15,16], but chemical derivatization was needed because of low volatility of these compounds. Liquid chromatography–mass spectrometry (LC–MS) was applied by Fidente et al. for the determination of acetamiprid, imidacloprid, thiacloprid, and thiamethoxam in honey [17], fruit and vegetables [18], and drinking water [19], but only one ion was monitored for each target compound. Recently, ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) has been proved to be a very sensitive and rapid technique for determining neonicotinoids in biological samples [20]. From the literature review, we found that no method for the determination of the seven neonicotinoids in liver samples has been published so far. Since the nature of the liver matrix is considered to be different and more difficult compared with muscle for isolation of the analytes and removal of possible matrix interferences, the opportunity to develop a sample preparation strategy for liver matrix is evident.

To date an automated extraction technique pressurized solvent extraction (PSE), also called pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE), has attracted more and more attentions in sample preparation procedures. Originally, the use of PSE was mainly focused on the extraction of environmental pol-

* Corresponding author. Tel.: +86 10 6273 2802; fax: +86 10 6273 1032.
E-mail address: dingsy@cau.edu.cn (S. Ding).

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lutants present in soil matrices, sediments, and sewage sludge. However, more recently this technique is being exploited in diverse areas, including biology and food industries, and the use of PSE in these areas has recently been reviewed by Carabias-Martínez et al. [21]. PSE is attracting interests for its short extraction time, low solvent volume consumption, and its high automation level.

In this study, we are exploring the utilization of PSE coupled to LC–MS/MS for the rapid extraction and simultaneous determination of neonicotinoids in bovine tissues. Optimum conditions with regard to PSE parameters, solid-phase extraction (SPE) clean-up and enrichment using Oasis HLB cartridges were investigated. The proposed method was validated by measuring linearity, intra- and inter-day repeatability, and the limits of detection (LODs) and quantification (LOQs). To confirm the proposed method's effectiveness, the PSE procedure combined with LC–MS/MS was utilized to detect neonicotinoids in real samples from markets.

2. Experimental

2.1. Chemicals and reagents

Certified pesticide standards (99%) for nitenpyram, thiamethoxam, imidacloprid, clothianidin, acetamiprid, thiacloprid, and the deuterated internal standard [$^2H_4$]-imidacloprid (imidacloprid-d_4) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Dinotefuran (99%) was obtained from Dr. Ehrenstorfer Gmbh (Augsburg, Germany). HPLC grade methanol, acetonitrile, and formic acid were purchased from Dima Technology Inc. (Muskegon, MI, USA). Diatomaceous earth was supplied by Applied Separations Co. (Allentown, PA, USA). Ultra-pure water was obtained using a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA). Some 500 mg Oasis HLB cartridges (Waters, Milford, MA, USA) were used for solid-phase extraction (SPE) clean-up procedure. To filter the concentrated extracts, 0.2 μm nylon syringe filters (Tengda, Tianjin, China) were used. Individual neonicotinoids and deuterated internal standard stock solutions of 1.0 mg mL^{-1} were prepared in methanol and stored at −20 °C. Working standard mixtures and a mixed internal standard working solution in methanol were used for spiking samples.

2.2. Sample preparation

The extractions of bovine samples were performed using a pressurized solvent extractor (PSE, Applied Separations Co., USA), equipped with 11 mL stainless steel cells. 2.5 g of bovine samples were spiked with 125 μL of internal standard solution (1.0 μg mL^{-1}) and vortexed for 30 s. After 30 min of equilibration, the samples were grounded with 2.0 g diatomaceous earth in a 100 mL mortar. The mixture was loaded onto the 11 mL cell which had been placed a cellulose filter disk and a 10 μm frit at the bottom, and then the cell was caped and placed on the extractor. For collection of the extracts 60 mL glass vials were used. The extraction conditions were as follows: pure water as extraction solvent, static extraction time of 5 min, two static cycles, extraction temperature at 80 °C, and extraction pressure at 10 MPa.

The collected PSE extracts were kept at −20 °C for 15 min in a freezer. After centrifugation at 3800 rpm for 5 min, the supernatant was loaded onto an Oasis HLB cartridge previously conditioned with 5 mL of methanol and 5 mL of pure water. After the sample extracts passed through the columns under gravity, the cartridges were sequentially rinsed with 5 mL of water and 5 mL of methanol–water (20:80, v/v). The analytes were eluted with 3 mL of methanol and the eluate was evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 1 mL of methanol–water (30:70, v/v) and syringe filtered using a 0.2 μm nylon filter into an autosampler vial.

2.3. Instrumental conditions

The HPLC analyses were performed using a Waters Alliance 2690 LC system, equipped with a degasser and an autosampler. Chromatographic separation was achieved on a Waters Symmetry Shield^{TM} RP C18 column (150 mm × 2.1 mm, I.D., 3 μm particle size) at ambient temperature. The injection volume was 20 μL and the flow rate was 0.2 mL min^{-1}. The mobile phase was acetonitrile (A) and water, both acidified with 0.1% formic acid. The insecticides were separated with the following LC gradient program: 0–3 min, 5–32% A; 3–6 min, 32% A; 6–8 min, 32–60% A; 8–10 min, 60% A; 10–11 min, return to 5% A; 11–20 min, equilibration of the LC system.

The MS/MS system consisted of a Quattro LC triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an ESI interface. For all compounds, the MS instrument was operated in the ESI positive ion mode using a desolvation temperature of 300 °C and a source temperature of 80 °C. Nitrogen was used as desolvation gas at flow rate of 450 L/h. Collision-induced dissociation was performed using argon as the collision gas at the pressure of 2.5 × 10^{-3} mbar in the collision cell. The MS detection

Table 1
Transitions and optimal conditions used for MS/MS analysis.

Compounds	Precursor ions (*m*/*z*)	Product ions (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
Nitenpyram	271.2	225.0[a]	15	13
		189.1	15	13
Dinotefuran	203.1	129.0[a]	15	13
		113.0	15	14
Thiamethoxam	292.2	211.1[a]	19	12
		131.9	19	13
Clothianidin	250.0	168.9[a]	16	11
		131.8	16	11
Imidacloprid	256.1	209.0[a]	20	15
		175.0	20	18
Acetamiprid	223.1	126.0[a]	25	16
		56.0	25	14
Thiacloprid	253.1	125.9[a]	21	17
		186.0	21	14
Imidacloprid-d_4	260.1	213.1[a]	20	14
		179.1	20	14

[a] Ion used for quantification.

was obtained using multiple reaction monitoring (MRM) mode. Selection and tuning of MRM transitions were performed by direct infusion of a 1.0 μg mL^{-1} standard solution of each analyte. Optimized MS/MS transitions as well as specific cone voltages and collision energies are summarized in Table 1. Two transitions were measured for the neonicotinoids identification and confirmation among which one was used for quantification.

2.4. Linearity, accuracy and precision

Isotope labeled internal standard imidacloprid-d_4 accompanied by matrix-matched solution was used to evaluate the linearity of the method. All calibration curves were constructed at the concentrations of 0, 2.5, 5, 10, 50, 100, and 500 μg kg^{-1} in blank bovine samples. The recovery experiments were carried out by spiking the samples in six replicates with working standard solutions at three concentration levels. The spiked samples were analyzed and the recoveries were calculated by comparing the measured concentration to the spiked concentrations. The precision in the conditions of intra-day repeatability (one analyst prepared six replicates of spiked samples at three different levels on a single day) and inter-day repeatability (different analysts prepared six replicates of spiked samples at three levels on three different days), expressed as relative standard deviations (RSD), was determined over four weeks.

3. Results and discussion

3.1. LC–MS/MS conditions

In order to achieve good separation of these neonicotinoids with high sensitivity, two different mobile phases acetonitrile and methanol, were compared in this study. It was found that acetonitrile could provide better ionization conditions than methanol. As described in experimental section, the gradient program could produce the best sensitivity and peak shape for all of the seven analytes.

The MRM transitions and associated acquisition parameters were optimized for the maximum abundance of fragmented ions under ESI positive mode conditions by infusing standard solutions of the target compounds into the tandem mass spectrometer via a syringe pump. Full scan mass spectra were recorded in order to select the most abundant m/z value. For each analyte the protonated molecular ion $[M+H]^+$ was determined and chosen as precursor ion. Then dissociation with argon was induced and different collision energies were tested in order to find the most abundant product ion. MRM parameters for the optimal yield of daughter ions are shown in Table 1.

3.2. Sample preparation

In order to improve the PSE extraction efficiency, extraction temperature, pressure, static extraction time, and static cycle were investigated. Bovine samples were extracted at 50, 65, 80, 100, 120, and 150 °C, respectively. Large increases of the extraction efficiency with higher temperature could observe for bovine samples (data not shown). These results were in accordance with the usual conclusion that PSE extraction efficiency would increase with the temperature's elevation [22]. For all of the seven analytes, except clothianidin and imidacloprid, the highest extraction efficiencies were obtained at 80 °C (average recovery 86%). When the temperature in excess of 120 °C, thermal degradation may have occurred because of the recoveries of the analytes decreased sharply at these conditions (data not shown). Additionally, within the range of temperature elevated, the optimum temperature appears to be somewhere between 80 and 100 °C. Based on the results we obtained, 80 °C was selected as the optimum temperature. Further instrumental parameters such as pressure, static extraction time, static cycle, and flush volume did not significantly influence the extraction efficiency, and thus they were not considered as critical experimental parameters in this work. The optimized PSE parameters are described in experimental section, under current conditions one sample can be extracted within 15 min and up to six separate samples can be extracted simultaneously.

In this study, pure water was successfully applied as extraction solvent in order to get an environmental-friendly analytical method. Our next endeavor was to clean-up the crude sample extracts since no organic solvent (acetonitrile or methanol) was used for precipitation of proteins. The extracts were stored in the refrigerator at −20 °C to eliminate lipids. This freezing step has proved to be worthwhile, since the following sample preparation becomes more reproducible and less matrix interference occur. However, the freezing step can only remove part of the interference. SPE cartridge was used to purify the samples and minimize matrix effects. Oasis HLB cartridge was chosen because of its high capacity, good and reproducible recovery for both polar and non polar compounds, and showing no loss of recovery due to dryness the cartridge. The recommended HLB method suggested using 5% methanol as the washing solvent followed by 100% methanol for elution, but this non-selective recommendation cannot sufficiently remove interferences from complex animal tissue such as liver. Different SPE washing conditions were thus compared in order to select the optimal washing solvent: spiked samples were washed successively with 5 mL of 10, 20, 30, 40, 50, 60, 70, 80, and 90% methanol in water. It was found that 20% methanol in water gave the best responses of nearly all the analytes. The first analyte eluted from the cartridge, dinotefuran, was observed with the 30%

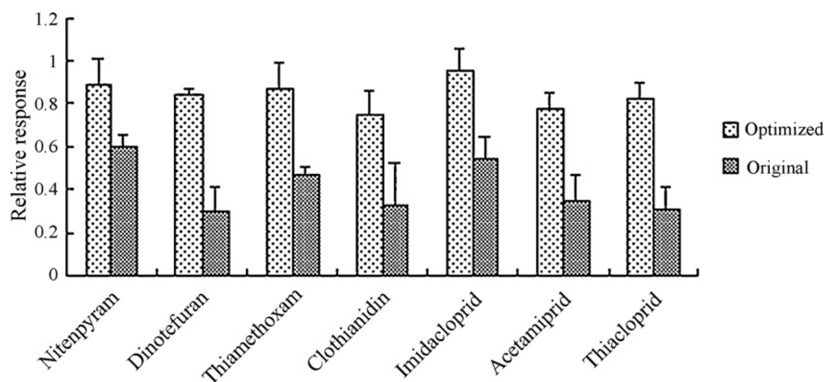

Fig. 1. Comparison of relative response (response matrix/response solvent) of the analytes in bovine liver samples between original and optimized SPE procedures. Error bars represent the standard deviation (n = 3).

Table 2
Linear regression results of matrix-matched calibration curves.

Compounds	Matrix	Intercept	Si[a]	Slope	Ss[b]	R^2
Nitenpyram	Liver	131.80	1.9657	263.09	1.5344	0.9993
	Muscle	133.19	2.2143	262.01	0.4318	0.9968
Dinotefuran	Liver	193.95	10.3167	117.95	0.02828	0.9997
	Muscle	186.65	2.6568	117.97	0.6571	0.9996
Thiamethoxam	Liver	274.58	4.3063	500.65	1.1295	0.9973
	Muscle	271.53	1.1133	497.73	1.1707	0.9982
Clothianidin	Liver	227.65	6.6452	224.66	1.4254	0.9957
	Muscle	239.42	1.6553	222.94	0.8193	0.9993
Imidacloprid	Liver	564.45	2.5173	420.14	0.1132	0.9989
	Muscle	566.23	1.2251	420.22	0.6672	0.9965
Acetamiprid	Liver	280.56	5.1830	420.14	0.07071	0.9951
	Muscle	284.22	2.7190	426.51	0.6221	0.9996
Thiacloprid	Liver	765.10	1.4142	875.69	0.2758	0.9975
	Muscle	764.16	4.8218	876.89	0.03260	0.9991

[a] Standard deviation of intercept.
[b] Standard deviation of slope.

methanol wash. Therefore, 20% methanol in water was applied for clean-up. The optimized clean-up procedure was found to be effective for the elimination of interferences. Additionally, as depicted in Fig. 1, the signal increasing was observed using the optimized method which should be attributed to the reduction of ion suppression effect.

3.3. Method validation

Eight blank bovine liver samples (collected from local farms) and 12 blank muscle samples (purchased from local markets) were analyzed to verify the selectivity of the proposed analytical method. Specificity was found to be satisfactory, with no chromatographic interference being observed around the retention time of the target compounds. Typical MRM chromatograms of fortified samples are shown in Fig. 2. The calibration was performed by use of matrix-matched calibration standards prepared as described in experimental section. The correlations of coefficient values (R^2) were all above 0.9951 and linear regression results are shown in Table 2. The accuracy and precision of the method were assessed using bovine samples fortified with three different levels (5, 50, and 100 μg kg^{-1}). Good corrected recoveries were obtained for each of the seven neonicotinoids at all fortification levels as shown in Table 3. The average recoveries ranged between 83.2% and 101.9%

Table 3
Intra- and inter-day repeatability and corrected recovery of the method.

Compounds	Fortified levels (μg kg^{-1})	Samples							
		Bovine muscle				Bovine liver			
		Intra-day		Inter-day		Intra-day		Inter-day	
		Mean (%)	RSD[a] (%)	Mean (%)	RSD[b] (%)	Mean (%)	RSD[a] (%)	Mean (%)	RSD[b] (%)
Nitenpyram	5	94.9	2.1	93.0	4.5	91.5	4.3	95.5	5.8
	50	89.4	4.4	91.8	7.6	93.0	2.3	97.9	5.1
	100	102.3	4.1	96.5	5.7	89.3	1.9	94.6	8.4
Dinotefuran	5	84.8	2.1	88.0	6.8	96.6	1.2	101.5	11.0
	50	87.9	4.5	86.3	6.0	86.3	3.2	85.7	7.4
	100	84.7	5.5	85.6	10.4	89.3	1.3	84.6	6.9
Thiamethoxam	5	96.9	4.7	97.4	7.5	87.5	3.8	98.1	7.0
	50	102.4	1.4	83.5	4.0	98.0	5.4	95.7	7.0
	100	93.2	5.6	89.9	10.6	86.5	4.4	84.0	9.6
Clothianidin	5	94.6	4.1	84.6	7.9	89.3	1.1	86.9	4.7
	50	83.5	1.3	102.4	6.7	92.6	0.4	97.7	5.7
	100	91.2	6.0	97.8	9.6	101.9	1.4	98.0	9.1
Imidacloprid	5	84.6	1.3	95.6	7.5	83.8	6.3	101.0	7.9
	50	99.1	5.3	97.8	6.6	93.2	4.5	99.9	10.8
	100	86.5	3.9	95.3	8.4	94.6	1.5	91.1	5.8
Acetamiprid	5	93.8	3.2	90.1	7.2	95.3	5.6	88.2	8.6
	50	95.3	1.7	91.9	7.8	90.8	5.7	96.6	8.2
	100	83.2	3.6	85.1	6.2	89.8	1.6	84.6	6.5
Thiacloprid	5	100.3	1.2	91.2	6.9	88.4	2.3	90.4	9.0
	50	95.1	6.9	91.9	9.8	92.0	1.6	87.7	6.8
	100	91.4	2.0	92.6	10.1	88.8	4.6	92.8	9.5

[a] $n = 6$.
[b] $n = 18$.

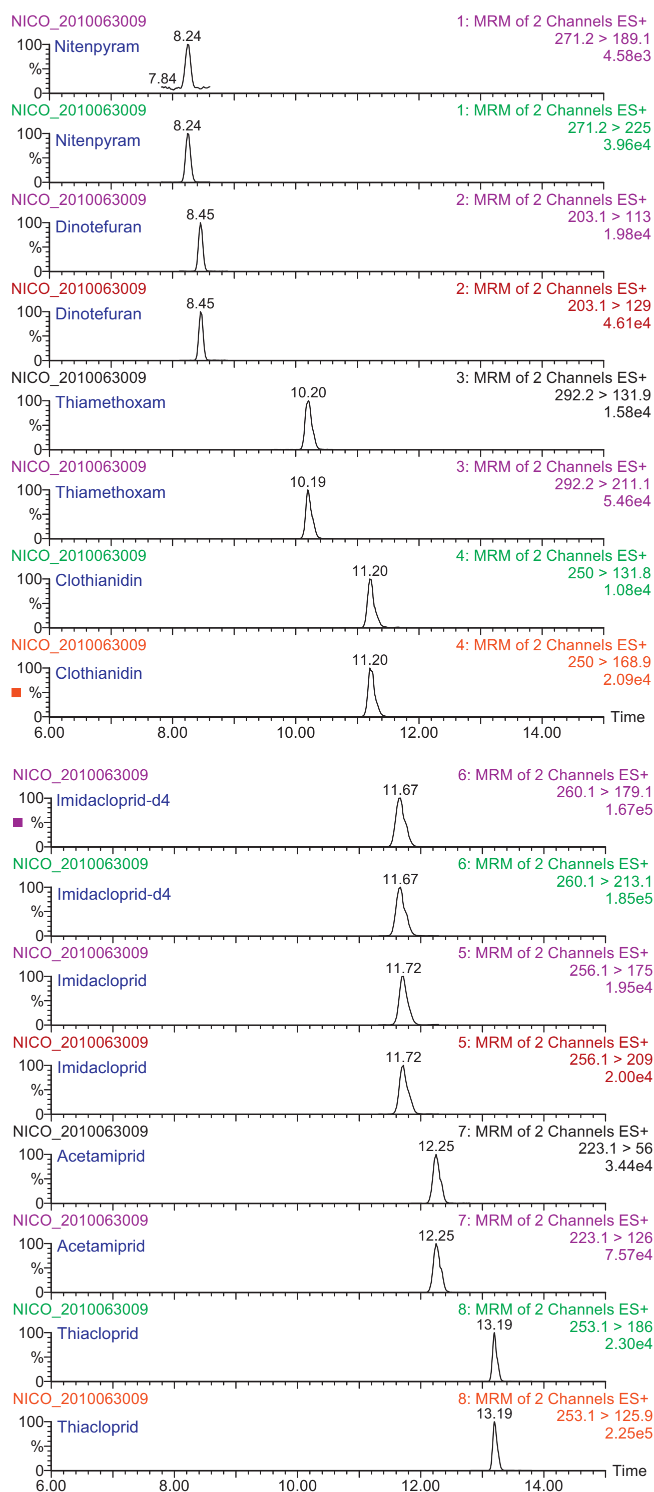

Fig. 2. Typical MRM chromatograms obtained from a bovine liver sample spiked with neonicotinoids at 5 μg kg^{-1} and internal standard imidacloprid-d_4 at 50 μg kg^{-1}.

with intra-day RSD values ≤6.9%. Inter-day repeatability was found satisfactory for the seven compounds under survey (RSD ≤ 10.8%). The most important condition to be satisfied for identification of the presence of a target compound is that at least two ion transitions give signals distinguishable from the background ion current when MS/MS detection is performed. To unequivocally identify the drugs in samples, the limits of detection (LODs) and quantification (LOQs) were defined as the concentration with a signal-to-noise ratio (S/N) of 3 and 10 using the less intensive ion transition for each analyte. This parameter was determined by analysis of a series of decreasing concentrations of the spiked sample in multiple replicates. The LODs and LOQs values obtained were 1.5 and 5.0 μg kg^{-1} for nitenpyram, thiamethoxam, and imidacloprid, 1.2 and 4.0 μg kg^{-1} for dinotefuran and clothianidin, 0.8 and 2.5 μg kg^{-1} for acetamiprid and thiacloprid, respectively. The LOQs presented here are 10–120-folder lower than the maximum residue limit (MRL) for nionicotinoids (valid for bovine and aquaculture animals) established by the European Commission [23].

3.4. Application to real samples

The effectiveness of this method in measuring trace levels of neonicotinoids was checked by analyzing 22 bovine liver samples (collected from local farms) and 28 muscle samples (purchased from local markets). Two muscle positive samples were detected, which contained 2.1 and 5.6 μg kg^{-1} of dinotefuran, respectively. However, none of the liver samples analyzed showed residues of the target compounds at detectable levels.

4. Conclusion

A rapid, sensitive, and environmental-friendly multi-residue method based on a PSE extraction procedure and LC–MS/MS analysis, has been developed and validated for bovine muscle and liver samples. The use of PSE which significantly reduced the consumption of volatile organic solvent and the simple preparation procedure represent major advantages, making this method suitable for high-throughput determination of the seven neonicotinoids in animal samples.

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