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Research on the Removal of Levamisole Residues in Bovine, Ovine, Caprine, Porcine and Poultry Tissues

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Abstract: In this paper, we set the waiting time for the elimination of levamisole residues at a safe level from tissues (muscles and organs) from animals treated with levamisole 10%—oral solution. We studied the depletion of levamisole residues by high performance liquid chromatography with a mass spectrometer (limit of quantification 2 µg/kg) in the tissues of bovine, ovine, caprine, porcine and poultry (chickens and pigeons) after administration of levamisole (10 mg levamisole/kg body weight for cattle, sheep, goats, pigs and 20 mg levamisole/kg body weight for birds). We found that in cattle, sheep and goats at 7 days after treatment, the residues of levamisole in the liver and at 14 days and in the other tissues were below the established limit value. In pigs at 7 days, residues from all tissues were below the set limit value. Residue depletion in chickens treated with levamisole was rapid, such that 3 days after treatment, the residues in all tissues were below the set limit. Residue depletion was slower in turkeys than in chickens.

Keywords: antihelmintic; chromatography; mass spectrometry

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1. Introduction

Levamisole hydrochloride, the hydrochloride salt of levamisole, derived from imidazothiazole, with anthelmintic activity, also has immunomodulatory activity. In immunosuppressed states, levamisole can restore immune function by: stimulating antibody formation, stimulating T-cell activation and proliferation, potentiating monocyte and macrophage phagocytosis, and increasing neutrophil mobility [1,2].

Structure of levamisole: (S)-6-Phenyl-2,3,5,6 tetrahydroimidazo [2,1b] [1,3] thiazole hydrochloride (Figure 1).

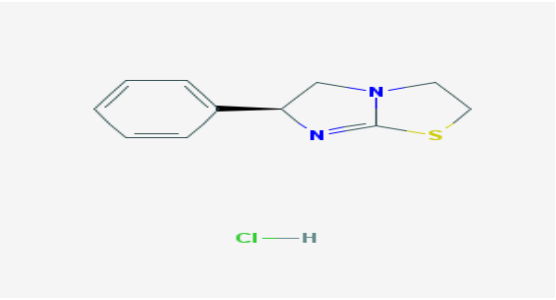

Figure 1. Chemical structure of levamisole [1].

Levamisole is used as an anthelmintic as well as in other indications, including adjuvant therapy in the treatment of cancer [2], and currently, its use has been focused on its immunomodulatory effects [3]. It can also be used to control gastrointestinal and pulmonary nematodes in cattle, sheep and pigs, acting as a nicotine-like ganglion stimulant [3].

Levamisole is a broad-spectrum antiparasitic with a long history of use in cattle, sheep, birds and pigs [4]. Levamisole remains in veterinary use for the deworming of production

animals. The probability of acute intoxication due to residues and metabolites in products of animal origin is very low [5].

The frequent use of antibiotics can favor the development of drug-resistant microorganisms, immunosuppression and residue accumulation in animals.

Levamisole has been shown to have immunostimulating properties for humans and animals and to enhance in vitro antibody production, superoxide anion and phagocytic activity [6]. It has been used in the treatment of chronic infections, cancers [7] and autoimmune diseases, including rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus, nephrotic syndrome and vitiligo, as well as in the therapy of warts and hepatitis B virus infection [8–10].

Levamisole contributes to the initiation of the local humoral response against intestinal pathogens such as enterotoxigenic Escherichia coli. Levamisole exhibits cytotoxic activity [11].

Due to the risk of serious side effects and the availability of other more effective drugs, levamisole was withdrawn as a human drug from the United States and Canadian markets in 2000 and 2003, respectively. The serious toxic effect is agranulocytosis, a severe depletion of white blood cells, leaving patients vulnerable to infection. Levamisole re-mains in veterinary use for deworming production animals. Recently, levamisole was discovered as an adulterant of cocaine after reports emerged of drug users with agranulocytosis and vasculitis. As the prevalence of cocaine use has increased over the past 15 years, the measurement of levamisole in human samples has become increasingly important [12,13].

However, in the case of some veterinary medicines, there is the possibility of chronic long-term intoxication by accumulation, with allergic, mutagenic, teratogenic or carcinogenic effects, which are difficult to evaluate [14–16]. In order to protect the population from the risk of levamisole residues in edible tissues from treated animals, strict waiting times should be observed. Adherence to the waiting period for the residues to reach concentrations below the safety limits protects the person from exposure to toxic substances added through food [17].

Orally administered levamisole is easily absorbed into the body (t_{max} about 3 h). Levamisole is rapidly and extensively metabolized to a large number of metabolites. In vitro studies in dogs, pigs, sheep, cattle and human hepatocytes suggest similar pathways of qualitative metabolism in all these species. Levamisole excretion is rapid with 40% of the dose excreted in the urine and 34% in the feces within 12 h of treatment. Urine and fecal excretion were virtually complete at 24 h (46% of dose) and 48 h (40% of dose), respectively [16,18–20].

Absorption and excretion of levamisole is rapid and is not affected by the route of administration, as it is highly soluble. It is mainly metabolized in the liver and usually excreted in the urine (83 and 84%) and feces (11 and 9%) when administered orally.

Levamisole is rapidly absorbed in the gastrointestinal tract after an oral dose of 50 mg. The maximum plasma concentration of 0.13 µg/mL is reached between 1.5 and 2 h after administration. The half-life of levamisole is 3 to 6 h. Less than 5% of the dose is excreted in unmetabolized form in the urine, and less than 0.2% in the feces. Levamisole is intensively metabolized in the liver into many metabolites, which are subsequently excreted predominantly renally (approximately 70% in 3 days) and to a lesser extent in feces (5%). A main metabolite present in urine is p-hydroxylevamisole and its glucuronide-conjugated derivatives (12% of the dose).

In sheep, after oral administration at a dose rate of 7.5 mg/kg, levamisole produced peak plasma concentrations of 0.7 microgram/mL [21]. Bioanalytical methods available for the determination of levamisole were gas chromatography coupled with specific thermionic nitrogen-selective detection and nitrogen–phosphorus detection, as well as high-performance liquid chromatography coupled with ultraviolet detection. Gas chromatography–mass spectrometry (GC-MS) and high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS, GC-MS) methods are currently described. GC-MS appears to be the method of choice, but recent developments in LC-MS/MS make research on levamisole residues in

animal tissues an attractive alternative. The merits of LC-MS/MS for the determination of levamisole are evaluated based on sample preparation, chromatographic separation conditions, and analytical performance.

The LC-MS/MS analysis presented in this paper on the depletion of levamisole residues in bovine, ovine, caprine, porcine and poultry tissues when using an oral solution with 10 mg levamisole/kg body weight for cattle, sheep, goats, pigs and 20 mg levamisole/kg body weight for birds led to the establishment of the waiting time required for the elimination of levamisole residues at a safe level from tissues (muscles and organs) from treated animals [22–24].

The anthelmintic levamisole was determined in human and animal plasma and tissues by gas chromatography using a specific nitrogen-selective thermionic detector. The detection limit was 5 ng, contained in 1 mL of plasma or in 1 g of the various tissues, and the recoveries were sufficiently high (79–86%) [25].

Depletion studies of levamisole residues in chicken tissues, eggs and plasma by HPLC with ultraviolet (UV) detection at 225 nm, when using an oral solution of 40 mg levamisole/kg body weight, led to the establishment of a withdrawal time higher than in the other tested species due in part to a higher dose of levamisole. It took 9 days for levamisole in eggs to be below the MRL and 18 days for tissues from medicated birds to be safe for human consumption [26].

Comparable results for the determination of levamisole in the liver of animals were also obtained by liquid–liquid extraction and gas chromatography–mass spectrometry, an equally simple and stable method without tedious sample preparation and suitable for the determination of levamisole in animal liver. Qualitative and quantitative analyses of levamisole were performed by a gas chromatography–mass spectrometry (GC-MS) system. The characteristic fragments m/z 148, 176 and 204 were selected and m/z 204 was used as the quantitative ion. The limit of quantification (LOQ) for levamisole was 5 µ/kg [27].

2. Materials and Methods

Levamisole residue analysis was performed by liquid chromatography with tandem mass spectrometry (LC-MS-MS). The limit of quantification (LOQ) was 2 µg/kg.

Description of the method: Extraction of the sample with ethyl acetate under alkaline conditions and purification by liquid–liquid partition by passing from the organic phase to the aqueous phase and vice versa at basic and acidic pH, respectively. Analysis by LC-MS-MS, positive electrospray ionization (ESI^+).

2.1. Reagents Used

- Levamisole hydrochloride, reference standard, Fluka, Germany.
- Acetonitrile HPLC, gradient-grade, Merck.
- Methanol HPLC, Merck.
- Ethyl acetate HPLC, Biosolve.
- Potassium hydroxide p.a.
- Hydrochloric acid conc., d = 1.19 g/L.
- Formic acid 98–100%, Riedel-de Haen.
- Hydrochloric acid 0.5 M: 41.2 mL HCl conc. dilute to 1 l with water.
- Potassium hydroxide 50%.
- Chloroform p.a.
- Anhydrous sodium sulfate p.a.
- Levamisole stock standard solution (0.1 mg/mL): Weigh accurately 11.8 mg of levamisole hydrochloride, reference standard, Fluka, dissolve in dimethylsulfoxide and make up to 100 mL with methanol. The solution is stable for up to 2 months in the refrigerator.
- Standard solutions (1 µg/mL): Prepare by diluting 1 mL of stock solution to 100 mL with methanol.

– Working standard solutions (0.01–0.10 µg/mL): Prepare at least 5 working standard solutions in the respective range by diluting the standard solution of 1 µg/mL to 1 mL with purified sample extract witness (matrix), such as in Table 1:

Table 1. Preparation of standard solutions.

No. Standard Solution	1	2	3	4	5
Levamisole concentration, µg/mL	0.01	0.025	0.050	0.075	0.10
Standard solution (1 µg/mL), µL	10	25	50	75	100
Final volume, mL	1	1	1	1	1

2.2. Apparatus and Materials Used

– Analytical balance KERN Abj.
– Waters 2695 (Waters Corporation, Milford, MA, USA) high performance liquid chromatograph, equipped with automatic injection system, XBridge RP 18 analytical column (150 mm × 2.1 mm, 3.5 µm), thermostat with controlled temperature at 40 °C, pump system with ternary gradient, solvent degasser.
– Quatro micro MS–MS detector (Micromass) equipped with ESI interface. The mass spectrometer is controlled by MassLynx software, version 4.1 (Waters Corporation, 34 Maple Street, Milford, MA, USA).
– Heidolph Reax Control vortex agitator.
– Ultraturax IKA T25.
– Centra MP 4R refrigerated centrifuge, with 40 mL and 15 mL polypropylene centrifuge tubes.
– Sonorex RK 100H ultrasonic bath.
– Ultrapure water production system SG GmbH.
– Mixer, type "Moulinette" Moulinex.
– Turbo-Vap Evaporator (Zymark, Germany).

2.3. Sample Preparation

– Freshly taken specimens are kept cold before and during shipment to the laboratory. Once they arrive at the laboratory, if they cannot be analyzed on the day of reception, they are kept in the freezer (−10 °C) until the time of analysis.
– If the samples are frozen, let them thaw, but keep them as cold as possible. The fat and related tissues are removed from the kidney or liver. Finely chop or chop the tissue in a mixer with knives. Store in the freezer (−10 °C) before analysis.

2.4. Extraction and Purification

– About 10 g of minced and homogenized tissue, at mixer, type "Moulinette" Moulinex. DD55210, 600 W, at room temperature, weighed to the nearest 0.01 g, were transferred into a 50 mL polypropylene centrifuge tube with a lid.
– For recovery, it was fortified to the desired concentration. The spiking was performed after homogenization. The spiking was performed on the 10 g of homogeneous sample transferred to the centrifuge tube. The fortification was carried out with levamisole standard solution (0.1 mg/mL) prepared as follows: 11.8 mg of levamisole hydrochloride, reference standard, Fluka, Germany. Dissolve in dime-thylsulfoxide and make up to 100 mL with methanol. It was allowed to sit for approximately 15 min to ensure that the fortification solution was absorbed.
– Five grams of anhydrous sodium sulfate was added to the sample and 1 mL of 50% KOH.
– Fifteen milliliters of ethyl acetate was added and homogenized in the mixer at maximum speed. The homogenized mixture is stirred with a horizontal shaker for 10 min and then left to stand for another 10 min.
– Centrifuged at 4000 rpm for 15 min at room temperature.
– The upper organic layer was transferred to another centrifuge tube.

- The sample was re-extracted under the same conditions (10 minutes stirring in the ultrasonic bath. Sonorex RK 100H), with another 15 mL of ethyl acetate and centrifuged for 5 min.
- Five milliliters of 0.5 M HCl was added to the combined ethyl acetate extracts.
- After centrifugation, the organic layer was discarded and the acid layer was transferred to a polypropylene tube.
- The solution was brought to alkaline pH (10.5) by the addition of 1 mL of 50% KOH.
- Two milliliters of chloroform was added and centrifuged at 2500 rpm for 5 min.
- The chloroform layer was transferred to a test tube and evaporated to dryness under a stream of nitrogen.
- The residue was dissolved in 0.6 mL mobile phase consisting of 0.1% formic acid in water-acetonitrile (50:50, v/v) by stirring. Heated to 50 °C in a water bath for 5 min. After verting in a vortex, it was made up to 1 mL with mobile phase. After cooling to room temperature, the purified sample extract was filtered through a 0.2 µm filter PTFE into an autosampler vial.

2.5. LC-MS–MS Analysis

2.5.1. Working Parameters LC

- Analytical column: Xbridge RP 18 (150 mm × 2.1 mm, 3.5 µm); flow rate: 0.25 mL/min; mobile phase: A—Water with formic acid 0.2% v/v, B—Acetonitrile, A/B = 25:75; injection volume: 20 µL; temperature of the column thermostat: 35 °C.
- At the exit of the analytical column, the compounds are ionized by the ESI + method and analyzed in multiple-reaction monitoring (MRM) mode with MS Micromass Quatro micro Electrospray Ionization/Atmospheric Pressure Ionization (ESI/API).

2.5.2. Working Parameters MS

- MS applied in positive ESI mode; desolvation gas flow (nitrogen): 350 L/h; MS applied in positive ESI mode. Desolvation gas flow (nitrogen): 350 L/h; dissolving temperature: 350 °C; ionization source temperature: 120 °C; tuning of the device was performed by direct infusion (flow 10 µL/min) of standard solutions (concentration 1 µg/mL levamisole) using a 250 µL syringe pump (Hamilton).
- For fragmentation, the collision gas flow (argon) (2.5×10^{-3} torr) and the collision energy were adjusted to obtain an optimal fragmentation of the molecular ion.
- The retention time and optimal parameters for levamisole are given below (Table 2):

Table 2. Retention time and MS/MS parameters for levamisole.

Component	Retention Time (min)	Precursor Ion (m/z)	Fragmentary Ions (m/z)	Cone Voltage (V)	Collision Energy (eV)
Levamisole	2.85	205.2	90.5	35	38
			122.6	35	28
			177.8 *	35	20

* Quantification ion (most abundant) (dwell time 0.3 s).

- Capillary voltage: 3.5 kV; voltage at the extractor: 1 V; the MS control was performed with the MassLynx data system, version 4.1; the fragmentation ion with the highest intensity was chosen for quantification, 205.0 > 177.8.
- The evaluation of the areas, the regression analysis of the standard curve and the calculation of the concentrations were performed with the QuanLynx V4.1 program.

3. Results

3.1. Calculation of Recovery from Samples, Residue Content and Quality Control

3.1.1. Calculation of the Recovery Percentage from Samples

- The calibration curve was drawn based on the results obtained with the calibration standard solutions. The linearity of the response was checked. The mathematical relationship between the detector response and the analyte concentration in the matrix was established. For this, a series of standard solutions of levamisole prepared in matrices with concentrations between 0.01 and 1.0 µg/mL were analyzed, which under the conditions described in the working method (10 g sample/1 mL final purified extract) corresponds to 1 to 100 µg/kg. (Appendix B, Figure A9).
- The percentage of recovery from the samples was calculated by comparing the result obtained for the sample with that obtained for the standard solution of corresponding concentration.

$$\%R = \frac{x_2 - x_1}{x_{ad}} \times 100, \quad (1)$$

where:

$\%R$—percentage of recovery from the sample;
x_1—the measured value for the control;
x_2—the measured value for the treated sample;
x_{ad}—the amount added.

3.1.2. Calculation of Residue Content

The residue concentration was calculated using the calibration curve and taking into account the recovery percentages using the following formula:

$$Levamisol = c_{pr} \times \frac{1000}{10} \times \frac{100}{R} \mu g/kg, \quad (2)$$

where:

c_{pr}—concentration of levamisole in the final sample extract, read from the calibration curve, in µg/mL
R—recovery percentage.

3.1.3. Quality Control

To each series of samples was added a negative control sample that did not contain traces of levamisole and a positive control sample obtained by fortifying the control sample to maximum residue limits (MRL) [14,19,22].

The highest concentrations of levamisole residues were found in the liver in all species tested [28–31]. In cattle, sheep and goats, at 7 days after treatment, the yeast residues in the liver samples were below 100/g/kg. At 14 days, they were below 100 µg/kg in all analyzed tissues. At 21 days, residues of muscle, kidney, and fat were below the detection limit of the method (2 µg/kg). In pigs, levamisole residues in the liver 5 days after treatment were below 100 µg/kg. At 7 days, three out of four samples of muscle, kidney and fat contained residues below 10 µg/kg. In chickens treated at the recommended dose, the residue disappeared rapidly from the tissues within 24 h. Three days after treatment, two out of four muscle samples and all kidney and fat samples contained levamisole residues below 10 µg/kg. At 7 days after treatment, residues of muscle, kidney and fat were below the minimum detection limit of 2 µg/kg. In turkeys 3 days after treatment, muscle levamisole residues were 10.7–13.8 µg/kg, kidney 12.7–15.6 µg/kg and fat 9.8–13.0 µg/kg [31].

The levamisole content of the samples taken from the untreated control animals was below the detection limit of the method of analysis (2 µg/kg) (Appendix A, Figures A1–A8) [32].

From the analysis of the data obtained in the depletion studies and by applying the statistical calculation model recommended by the European Medicines Agency Veterinary

Medicines (EMEA)—Guideline EMEA/CVMP/036/95, maximum waiting times for meat and organs from levamisole-treated animals of 11 days for cattle were found, 11 days for sheep, 11 days for goats, 10 days for pigs and 4 days for birds. The recommended waiting times are in accordance with the data published in the literature for similar products [20]

4. Discussion

Validation of the method and calculation of measurement uncertainty for the determination of levamisole residues in tissues by LC-MS/MS was performed according to the requirements of the annex to Directive 2002/657/EC and is based on the method for determining levamisole residues in animal tissues by LC-MS/MS described in the specific internal procedure PS-IP-CD-16 [20].

A known amount of sample (10 g) was transferred to a centrifuge tube and extracted with ethyl acetate (2 × 15 mL) in the presence of anhydrous $NaSO_4$. The organic extract underwent purification by liquid–liquid partition in alternating acidic and basic medium. The final purified extract was concentrated under a stream of nitrogen, and the residue was taken up again in the mobile phase. An aliquot of the extract (20 µL) was injected into the LC-MS/MS system. The separation was performed on an XBridge C18 analytical column (150 mm × 2.1 mm, 3.5 µm) using as mobile phase 0.1% formic acid in water and acetonitrile with a concentration gradient with a flow rate of 0.25 mL/min. ESI+ was used with two-transition MRM data acquisition.

To validate the method, the following performance characteristics were established: confirmation of identity and specificity, limits of detection and quantification, linearity and domain of linearity, interference, accuracy and fidelity, repeatability, reproducibility in the laboratory, recovery efficiency, decision limit (CCα) and detection capacity (CCβ), storage stability, estimation of measurement uncertainty.

The specificity of the method was demonstrated by the clear measurement of the analyte of interest in the presence of other components such as analytical reagents, metabolites and matrix constituents. To confirm the identity of levamisole by LC-MS/MS, the precursor ion and two transitions, corresponding to two fragment ions, were selected. This corresponds to four points of identification (for substances in group B (authorized), a minimum of three points is required (Directive EC 96/23)). The specific ions chosen were: the precursor ion 205.2 and two fragment ions 177.8 and 90.5. The relative intensities of the fragment ions, expressed as a percentage of the intensity of the ion with the highest intensity, were compared for the samples with those obtained for the standard solution at comparable concentrations, measured under the same conditions. The results were confirmed by maintaining the ratio within the allowed tolerance limits (Table 3).

Table 3. Confirmation of identity and specificity.

Analit	Precursor Ion $[M+H]^+$	Fragment Ions, *m/z*	The Relative Intensity of the Ions, %	Max. Tolerance Allowed, %
Levamisole	205.2	177.8; 90.5	51.3	20

Linearity and domain of linearity: the linear regression equation, y = 46329.2x + 2649 and the regression coefficient of the calibration curve (r^2) is 0.9974 (Appendix B, Figure A9). The correlation coefficient of the linear regression for the calibration curve (r^2) is greater than 0.9950, which denotes an appropriate linearity for the chosen linearity range. Limit of detection (LOD) and quantification (LOQ).

They were calculated based on the calibration curve built at low concentrations with standard solutions prepared by fortifying the control sample extract in the range 0.01–0.1 µg/mL.

The residual standard deviation of the areas, as standard deviation (σ), was calculated. The limit of detection was calculated with the formula LOD = 3.3 σ/p, where p is the slope of the curve and the limit of quantification LOQ = 10 σ/p.

LOD = 0.018 µg/mL was obtained, which in the conditions described in the work mode (10 g sample/1 mL final purified extract), corresponds to 1.8 µg/kg and LOQ = 0.054 µg/mL, which in the conditions described in the work mode (10 g sample/1 mL final purified extract), corresponds to 5.4 µg/kg (Appendix A).

Interference: blank samples of the reagents and blank samples for each matrix were analyzed, and no interference was observed in the retention times of levamisole.

Accuracy and fidelity: the accuracy of the method was established in terms of repeatability on samples treated with levamisole at the MRL level and analyzed in six replicates; the relative standard deviation of repeatability was 8.4% for muscle, 9.10% for liver, 7.5% for kidney and 19.5% for fat.

The fidelity of the method was estimated by the recovery efficiency as a percentage of the deviation of the concentration found from the theoretical concentration on control samples treated at the three levels: 0.5×MRL, 1×MRL and 1.5×MRL, and analyzed in six replicates. Mean recoveries for muscle, liver, kidney and fat were 77.9%, 80.4%, 78.6% and 74.8%, respectively. The recovery coefficients were within the limits allowed by the legislation of 70% to 125% [22].

Decision limit (CC_α) and detection capacity (CC_β)

Both parameters were determined by the calibration curve method, in which blank meat, liver and kidney samples were fortified around the MRL in equidistant increments representing 0.5×MRL, 1×MRL and 1.5×MRL. The samples were analyzed, and the signal graph was represented depending on the added amount. CCα was calculated as equal to the corresponding concentration at the MRL plus 1.64 × standard deviation of reproducibility (α = 5%). The CC_β was calculated as equal to the concentration corresponding to the decision limit value plus 1.64 times the standard deviation of the laboratory reproducibility (β = 5%).

Calculation of the decision limit (CC_α) and detection capacity (CC_β) for the determination of levamisole residues (Table 4):

Table 4. The determination of levamisole residues.

Tissue	Quantity Added (x) µg/kg	Quantity Found (y) µg/kg	y = ax +b	CC_α µg/kg	CC_β µg/kg
Muscle	10	8.89	y = 1.006x − 1.17	10.3	11.7
Liver	100	93.14	y = 1.038x − 10.66	104.4	115.6
Kidney	10	9.06	y = 1.041x − 1.35	10.4	11.8
Fat	10	8.32	y = 0.859x − 0.27	10.2	12.1

Stability was demonstrated on samples fortified at the level of 100 µg/kg and subjected to three freeze–thaw cycles. If the samples are not analyzed immediately, they can be stored for up to 3 months at −20 °C.

To estimate the measurement uncertainty, the sources of uncertainty acting on the result, having a significant influence on it, were taken into account. All these uncertainties were combined into a combined uncertainty, which by multiplying by a coverage factor, k, resulted in obtaining the extended uncertainty of the method. Quantification of measurement uncertainty was based on: fidelity studies, justice studies (bias), the identification and assessment of other contributions to uncertainty inadequately covered by fidelity and fairness studies. The result is reported as the value obtained ± the extended uncertainty. The values obtained for the extended uncertainty were between 26–36%. The expanded uncertainty was calculated using a spread factor of 2, which gives a confidence level of approximately 95% (thus, the true value is within the range of the obtained value ± the expanded uncertainty in 95% of cases). The expanded uncertainty of the method is: muscle: 2 × 0.154 = 0.308 or in percentage 30.8%, liver: 2 × 0.134 = 0.268 or in percentage 26.8%, kidneys: 2 × 0.146 = 0.292 or in percentage 29.2%, fat: 2 × 0.18 = 0.36 or in percentage 36.0% [31].

5. Conclusions

The method of analysis of levamisole residues by the LC-MS/MS technique ensures specificity and selectivity, and it is fast and accurate.

Quantification limits based on accuracy and fidelity are 5 μg/kg.

The accuracy and fidelity of the method meet the requirements for residue analysis. Thus, the recoveries on fortified samples at the level of 0.5×LMR, MRL and 1.5×LMR were higher than 75.0%.

The method is validated according to the requirements for the following parameters: confirmation of identity and specificity, limits of detection and quantification, linearity and linearity range, interferences, accuracy and fidelity, storage stability, measurement uncertainty.

In order to protect the population from exposure to levamisole residues in the edible tissues of treated animals, strict adherence to the waiting time resulting from this study is required.

Adherence to the waiting period for the residues to reach concentrations below the safety limits protects the person from exposure to toxic substances added through food. It is the responsibility of veterinarians and animal breeders to monitor the waiting period for the medicinal product before slaughter to ensure that no illegal concentrations of residues occur in products of animal origin intended for human consumption from treated animals. The study has a practical importance because it informs the consumer about the risk of consuming meat that can harm human health and brings attention to a less visible, well-known aspect that deserves to be popularized.

From the analysis of the data obtained in this research, it appears that in cattle, sheep and goats, 7 days after treatment, levamisole residues in the liver were below the value established for the MRL, and at 14 days, they were below the MRL in the other tissues as well. In pigs at 7 days, the residues in all tissues were below the MRL. Residue depletion in chickens treated with levamisole was rapid, such that 3 days after treatment, the residues in all tissues were below the MRL. In turkeys, the depletion of residues was slower than in chickens. By applying the statistical calculation model recommended by the EMEA (Guideline EMEA/CVMP/036/95), maximum waiting times for meat and organs from animals treated with the levamisole 10% product were found to be 10.95 days for cattle, 10.75 days for sheep, 10.95 days for goats, 9.96 days for pigs and 3.98 days for poultry. The results obtained fall within the limits provided for and reconfirmed by Commission Regulation (EU) no. 37/2010.

Author Contributions: Conceptualization: V.C.C.; methodology: V.C.C., C.O.R. and V.V.S.; investigation: V.C.C., C.O.R. and V.V.S.; validation: V.C.C. All authors have read and agreed to the published version of the manuscript.

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Appendix A. Chromatograms

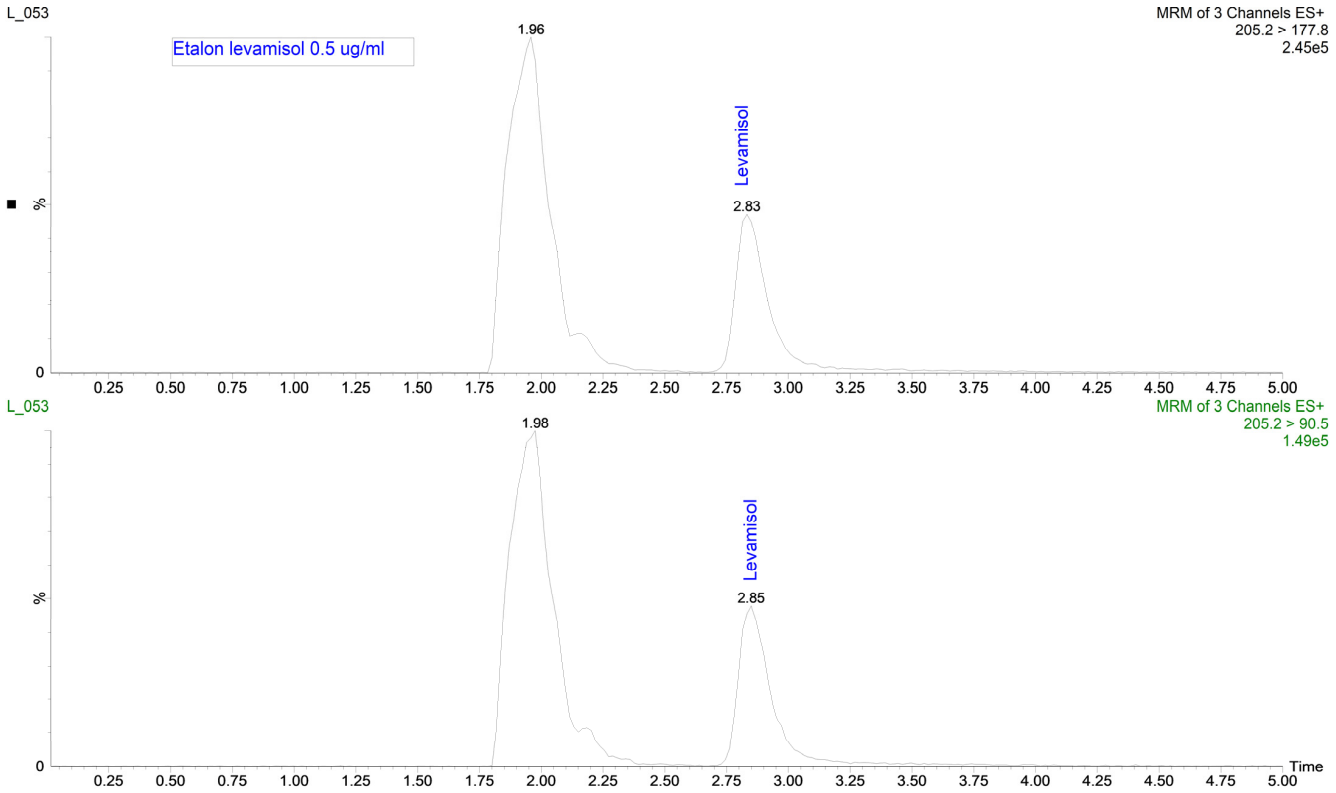

Figure A1. Chromatogram of standard levamisole solution 0.5 μg/mL.

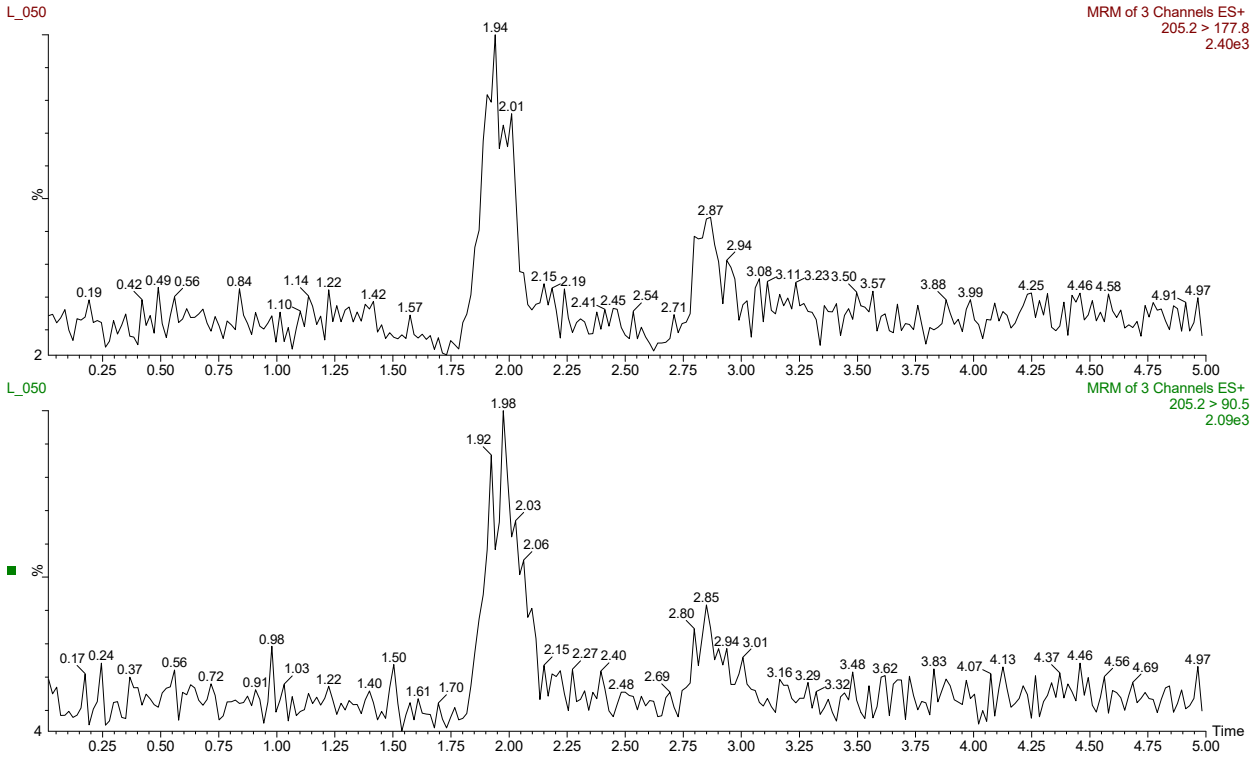

Figure A2. Chromatogram of the liver control sample.

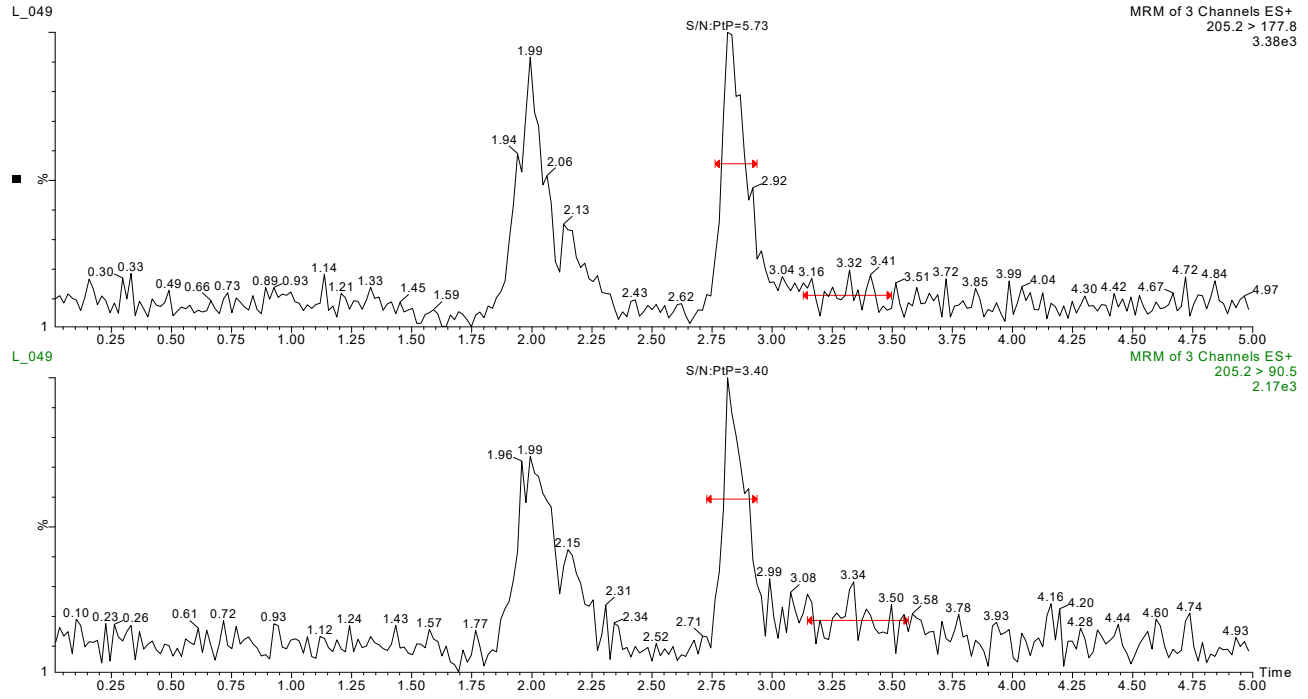

Figure A3. Chromatogram of the liver sample treated at detection limit (LOD) level.

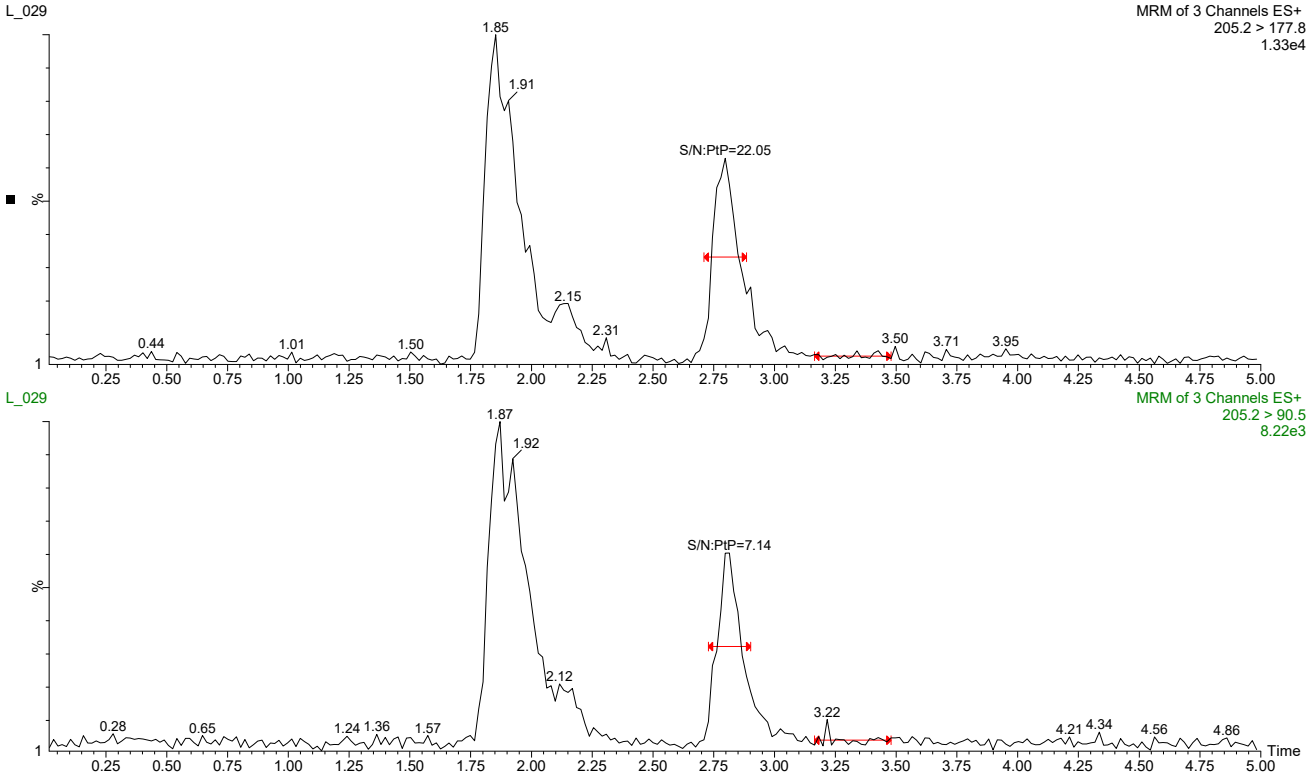

Figure A4. Chromatogram of the liver sample treated at the limit of quantification (LOQ) level.

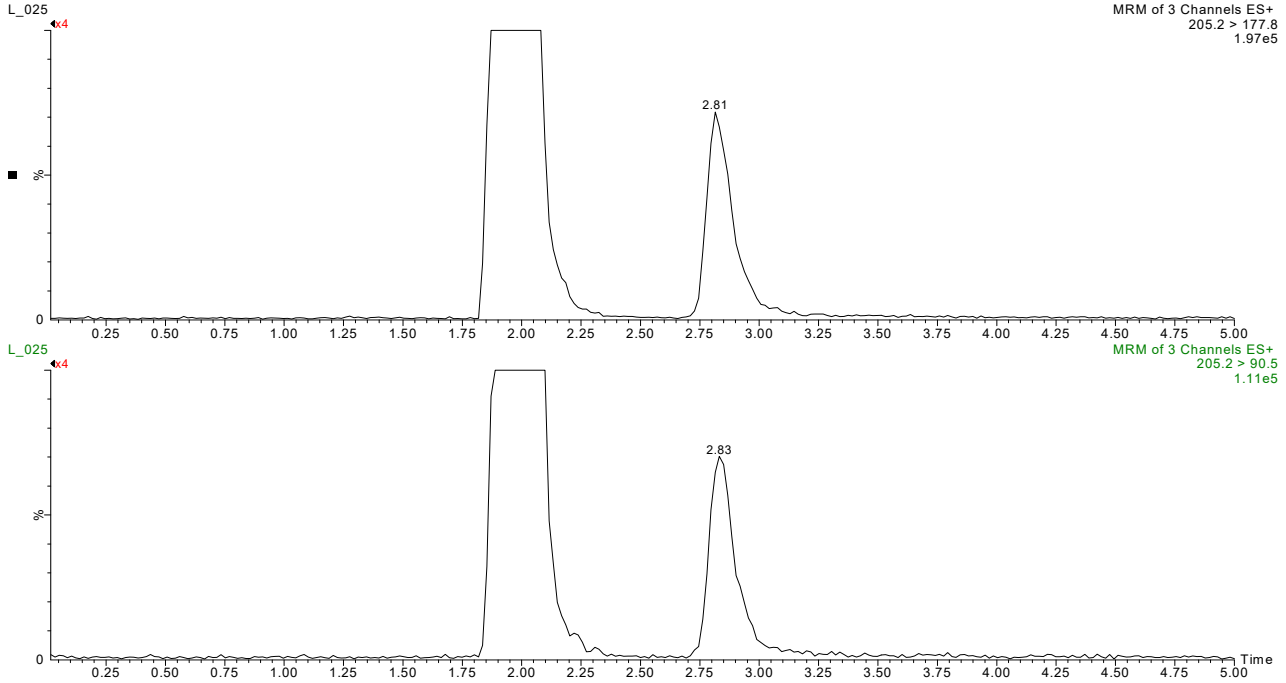

Figure A5. Chromatogram of the liver sample treated at 10 ppb.

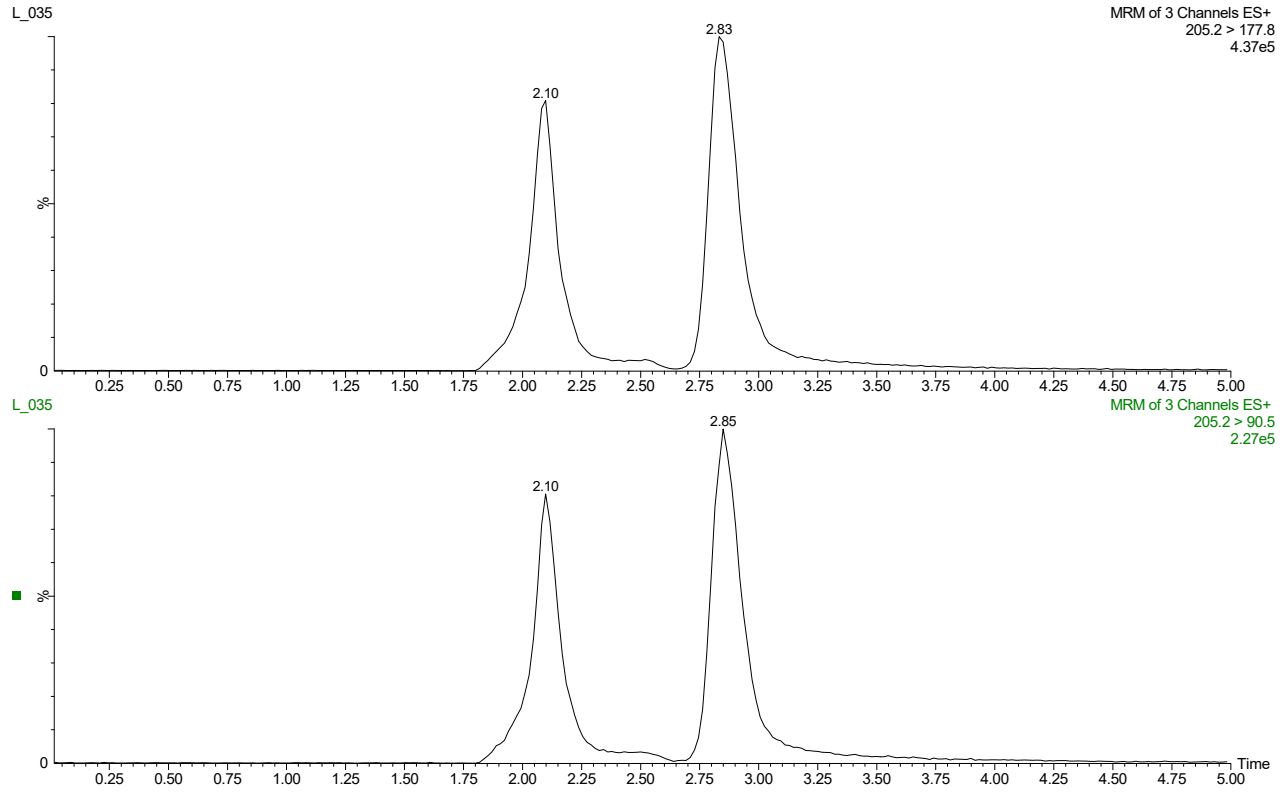

Figure A6. Chromatogram of the treated chicken liver sample 24 h after treatment.

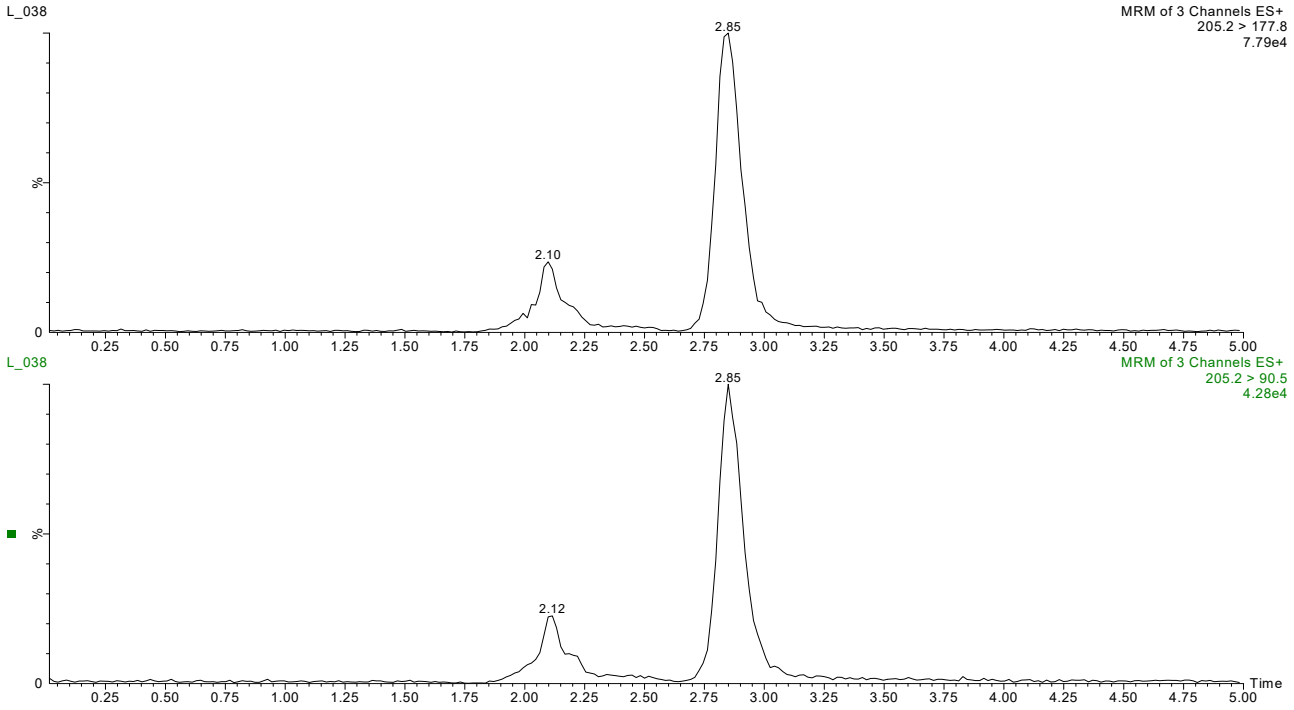

Figure A7. Chromatogram of the treated chicken muscle sample 24 h after treatment.

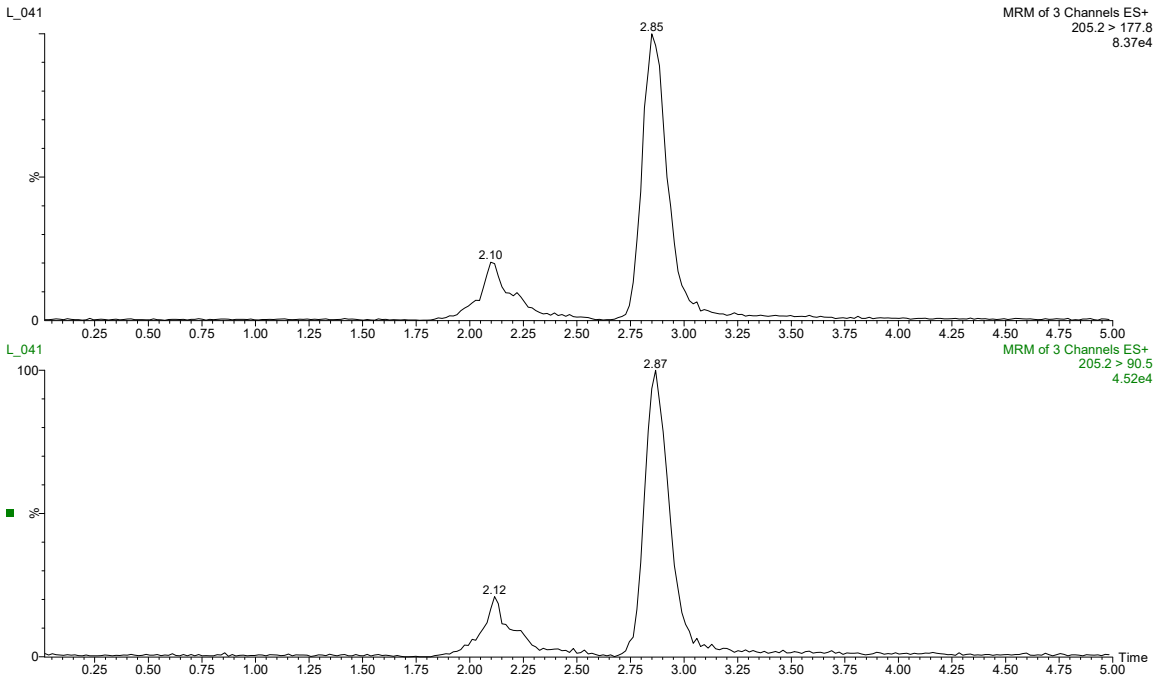

Figure A8. Chromatogram of the treated chicken kidney sample 24 h after treatment.

Appendix B

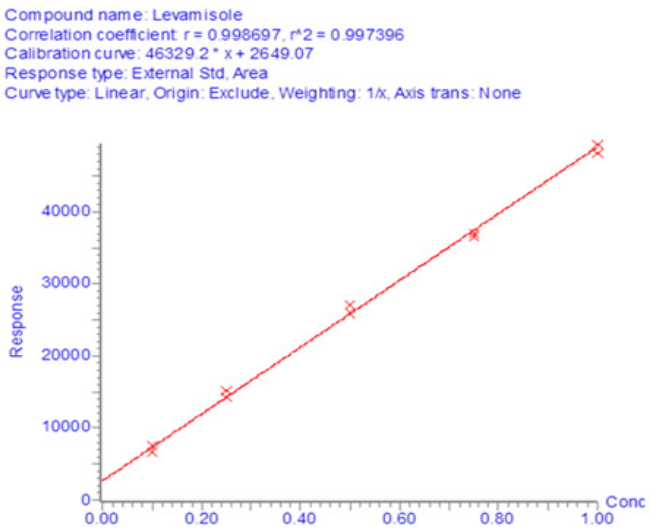

Figure A9. Calibration curve for levamisole (0–100 µg/kg).

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