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ANALYTICAL METHOD FOR DETERMINATION OF AVERMECTINS IN PORCINE LIVER BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A method has been developed and validated for quantitative determination of residues of six avermectins - abamectin, doramectin, moxidectin, ivermectin, eprinomectin and emamectin in porcine liver by liquid chromatography with fluorescence detection (HPLC-FLD). Nemadectin is used as an internal standard (IS). Avermectins are used as antiparasitic agents in veterinary medicine. They have maximum residue limits (MRLs) in accordance with Commission Regulation (EU) No 37/2010 in foodstuffs of animal origin. The sample preparation is held with liquid extraction by acetonitrile, followed by clean up step using solid-phase extraction (SPE) with Strata C18-E cartridges and derivatization with trifluoroacetic acid anhydride (TFAA), 1-methylimidazole and the addition of 100 % acetic acid and triethylamine (TEA), to enable fluorescence detection. For the detection a column Agilent Eclipse XDB-C18 (150 x 4.6 mm, 5 µm) has been used, equipped with a guard column of the same packing and a fluorescence detector works with wavelengths at λex = 365 nm and λem = 460 nm. Gradient elution is applied with mobile phase A (acetonitrile) and mobile phase B (water) The analytical method is validated according to the requirements of Commission Implementing Regulation (EU) 2021/808. The method has been implemented and it is used as routine method for control of residues of Avermectins in Central Laboratory of Veterinary Control and Ecology (CLVCE).

Key words: avermectins, HPLC-FLD, method validation, derivatization, liver, CLVCE.

Introduction

Avermectins are widely used in veterinary medicinal for prevention and treatment of parasitic infections. In Commission Regulation (EU) No 37/2010 there are established maximum residue limits (MRLs) in foodstuffs of animal origin for these substances. Ivermectin and doramectin have defined MRLs in the Regulation (EU) No 37/2010 and accordance with it are permitted for use in animals which porcine liver is produced for human consumption [1]. For the other substances (abamectin, moxidectin, eprinomectin and emamectin) no MRL values have been established for this matrix and therefore, for control purposes, the cascade MRLs is applied in accordance with Regulation (EU) 2018/470 [2, 3]. Marker residue is B1a their main component, which is used as a for their control. These compounds belong to the macrocyclic lactones and are characterized with a complex macrocyclic ring structure [4]. This group of substances are products of soil microorganisms of the genus Streptomyces avermectinius. Ivermectin and doramectin are well-known representatives and ivermectin was first introduced in 1981 for the treatment of animals [5].

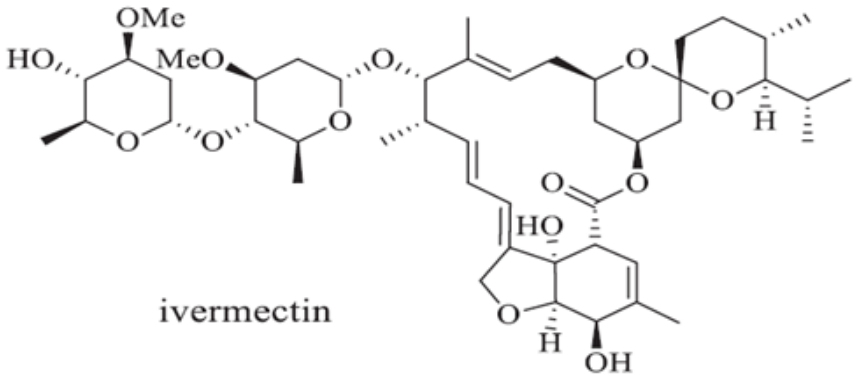

Figure 1: Chemical structures of ivermectin is presented by Ōmura et al [5].

The method has been developed to control residual amounts of avermectins in porcine liver samples by high-performance liquid chromatography with fluorescence detection (HPLC-FLD). Avermectins are lipophilic substances, practically insoluble in water, but readily soluble in organic solvents such as acetonitrile and methanol. These properties cause them to accumulate in fat tissue and consequently in liver. Some methods have been published for the determination of avermectins in various biological matrices such as liver. For extraction of the analytes acetonitrile is used and after that solid-phase extraction (SPE) is preformed [6, 7]. Avermectins do not nave natural fluoresce and so in order to be detected by fluorescence detector (FLD), avermectins have to be derivatizated before instrumental analysis.

The aim was to develop an analytical method for determination and control of residual amounts of six avermectins - abamectin, doramectin, moxidectin, ivermectin, eprinomectin and emamectin of porcine liver samples. High-performance liquid chromatography with fluorescence detection (HPLC-FLD) was used and the method was validated in accordance with Commission Implementing Regulation (EU) 2021/808 [8]. The following criteria: linearity, calibration curves, selectivity, trueness (recovery), precision (repeatability and within-laboratory reproducibility), stability, ruggedness, matrix effect, decision limit (CCα) and detection capability (CCβ) were covered.

Materials and Methods

Standards and reagents

The analytical standards of abamectin, doramectin, emamectin, eprinomectin, ivermectin and moxidectin were delivered by Dr. Ehrenstorfer (Augsburg, Germany). Nemadectin internal standard (IS) was purchased from Toronto Research Chemicals (Toronto, Canada). Acetonitrile with high analytical purity (HPLC grade) was used. Ultra-purified water was prepared by Millipore Mili-Q system (Milford, CT, USA), the other substances were glacial acetic acid from Fisher Chemical (USA), 1-methylimidazole from Acros Organics (Belgium), trifluoroacetic anhydride (TFAA) and triethylamine from Thermo Scientific (USA).

Standard solutions

Stock standard solutions of all avermectins and the internal standard nemadectin were prepared individually with concentration of 1000 µg mL^{-1}, dissolved with acetonitrile. Solutions were

stored in a freezer at -18°C and they are stable for 24 months. Working standard solutions of avermectins were prepared by dilution from the stock standard solutions and using acetonitrile to the following concentrations: abamectin (0.5 µg mL^{-1}), doramectin (2.5 µg mL^{-1}), moxidectin (1 µg mL^{-1}), ivermectin (2.5 µg mL^{-1}), eprinomectin (1.25 µg mL^{-1}), emamectin (2.5 µg mL^{-1}) and internal standard (IS) - nemadectin (1 µg mL^{-1}). The working solutions were stored for 12 months at temperature of 2-8°C.

Sample preparation

In 50 mL polypropylene centrifuge tube, 2.5 g raw porcine liver were weighed and 20 µl of IS (nemadectin, 1 µg mL^{-1}) were added. The samples were extracted with 15 mL acetonitrile, homogenized with shaker 1 min and centrifuged (8500 rpm, 4°C) for 3 min. The organic extract was transferred to a round-bottom flask. This step was repeated twice. The combined organic extracts were evaporated to dryness by rotary vacuum evaporator in a water bath at 40°C. The dry residue was reconstituted with 5 mL of a reconstitution solvent (mixture of water : acetonitrile (90:10, v/v) and 0.1 % TEA).

Strata C18-E cartridges (200 mg/3 mL, Phenomenex, USA) was used for solid-phase extraction (SPE). With this step purification and concentration of the samples is obtained. The cartridges were conditioned with 1 volume acetonitrile and 1 volume of the reconstitution solvent. After that the sample was transferred to the cartridge, washed with 0.1 % aqueous TEA and dried under vacuum. The substances (avermectins) were eluted with 9 mL acetonitrile and the eluate was evaporated to dryness in a stream of nitrogen in a water bath at 40°C.

Derivatization

The final step before the instrumental analysis was derivatization of the samples. To the dry residue were added 100 µL of a mixture acetonitrile: TFAA (1:1, v/v) and 100 µL of a mixture acetonitrile:1-methylimidazole (1:1, v/v). After homogenization (10 s), 50 µL acetic acid and 50 µL TEA were pipetted and after that homogenized again. The derivatizated samples were transferred into vials and 25 µL of each sample was injected into the chromatographic system.

The derivatization procedures was carried out in accordance with the official method of the German Federal Office of Consumer Protection and Food Safety (BVL) and Danaher et al [9].

Instrumental analysis

Agilent 1100 Series liquid chromatography with HPLC - system (Agilent Technologies, USA) with a fluorescence detector (FLD) was used. Chromatographic column by Agilent Eclipse XDB-C18 with parameters 150 x 4.6 mm, 5 µm and a guard column with the same packing material were used. The mobile phases included (phase A) acetonitrile and (phase B) ultra-purified water. The substances were eluted with a gradient program. The initial conditions were 90:10 (Acetonitrile: ultra-purified water). The percentage of acetonitrile was maintained at 90 % for the first 4 min, then increased to 95 % at 7 min and to 97 % at 10 min. From 10 to 15 min, the system was re-equilibrated to the initial mobile phase conditions 90:10.

In the liquid chromatographic system 25 µL from the sample was injected. The flow rate was 2 mL/min and the column temperature was 40°C. Avermectins were detected with an excitation wavelength λex = 365 nm and emission wavelength λem = 460 nm.

Results

Method validation

The analytical method was developed and validated in accordance with the requirements of Commission Implementing Regulation (EU) 2021/808 and performs the following criteria: linearity, selectivity, trueness (recovery), calibration curves, repeatability, within-laboratory reproducibility, stability, ruggedness, matrix effect, decision limit for confirmation (CCα) and detection capability (CCβ).

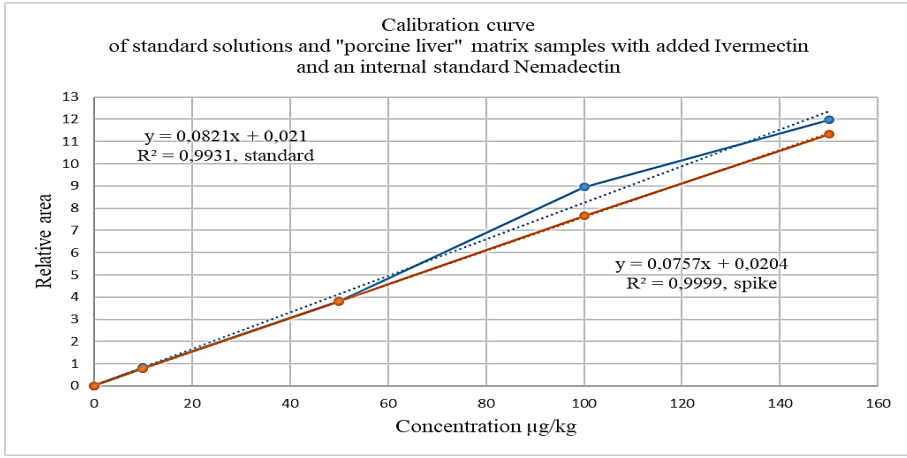

Figure 2: Linearity, linear equation and correlation coefficient of ivermectin in standard solution and fortified porcine liver samples.

Linearity and Calibration curve

The calibration curves of the method were established and prepared for the six avermectins at five concentration levels (including zero). Nemadectin is used as an internal standard (IS). Analysis was performed using standard solutions and fortified blank porcine liver samples.

Avermectins were analyzed with the following calibration curves: 2.0, 5.0, 10 and 20 µg kg^{-1} for abamectin; 10, 50, 100 and 150 µg kg^{-1} for doramectin; 10, 25, 50 and 100 µg kg^{-1} for emamectin; 10, 50, 100 and 150 µg kg^{-1} for ivermectin (Fig. 2); 5, 7.5, 10 and 15 µg kg^{-1} for eprinomectin; and 4, 10, 20 and 40 µg kg^{-1} for moxidectin. The lowest calibration level (LCL) was established at 1/10 of the MRL or cascade MRL [3,8].

For substances which do not have determined MRL (Emamectin, Moxidectin, Abamectin, Eprinomectin), the European commission is determined cascade MRL. Examination of them is needed due to possible unregulated use.

The linear correlation was determined for standard solutions and fortified samples of porcine liver, using the relative value between peak areas of analytes divided to the peak area of internal standard and the concentration ranges showed good linearity presented graphically for all avermectins. Fig. 2 is an example only for linear correlation of ivermectin. The coefficients of determination (R^2) in fortified samples were ≥ 0.986 for all substances, which indicates good linearity within the calibration curves presented in Table 1.

Table 1: Summary of calibration range, recovery, precision (repeatability, within-laboratory reproducibility), coefficient of determination R^2, matrix effect, decision limit for confirmation (CCα) and detection capability (CCβ) for the quantitative determination of the avermectins in porcine liver (summarized data from 18 samples per level for the 3 days of validation).

Substance	Calibration range, µg kg^{-1}	Fortified level	Recovery %	Repeatability µg kg^{-1}	Within-laboratory reproducibility (CV), %	Matrix effect %	R^2	CCα/CCβ µg kg^{-1}
Abamectin	0 -20	1 LCL	102	0.058	3.5	5.37	0.978	2.12/2.18
		2 LCL	104	0.097	3.1			
		3 LCL	102	0.429	4.5			
Doramectin	0 -150	0.1 MRL	102	0.596	5.9	4.94	0.9999	104.22/10.98
		MRL	101	1.611	2.5			
		1.5 MRL	100	2.096	1.4			
Emamectin	0 -100	1 LCL	104	0.208	4.3	7.93	0.9991	26.10/10.73
		2 LCL	100	0.381	2.7			
		3 LCL	101	1.070	2.3			
Eprinomectin	0 -15	1 LCL	99	0.138	3.1	9.17	0.986	7.87/5.25
		2 LCL	101	0.213	3.0			
		3 LCL	111	0.527	8.4			
Ivermectin	0-150	0.1 MRL	103	0.230	3.7	3.88	0.9999	103.60/10.93
		MRL	100	0.901	3.5			
		1.5 MRL	100	1.608	1.2			
Moxidectin	0 - 40	1 LCL	101	0.110	2.7	14.13	0.9987	10.87/4.18
		2 LCL	106	0.170	1.6			
		3 LCL	105	0.559	2.7			

Trueness (Recovery)

To porcine liver samples free of avermectins Standard solutions of the six avermectins were added to evaluate three concentration levels. For authorized substances (ivermectin and doramectin), the levels were based on their Maximum Residue Limits (MRLs). For the other substances, the levels were selected according to the lowest calibration level (LCL). For each of the three levels the mean measured concentration was calculated. Trueness was determined as the mean measured concentration divided to the theoretical (added) concentration for each of the three analyzed levels. Analyses were performed using the internal standard method (nemadectin). The values are in line within the criteria of Regulation (EU) 2021/808 and are summarized in Table 1.

Precision (Repeatability and Within-laboratory Reproducibility)

Precision was determined in accordance with Regulation (EU) 2021/808 by analysis of blank porcine liver samples fortified at three concentration levels. For authorized pharmacologically active substances, concentration levels were selected according to their MRLs and for non-authorized substances, according to the lowest calibration level (LCL).

The values were determined by analysis of 18 measurements for each level divided in three days (6 replicates per day).

Repeatability

Repeatability was determined based on the results under constant conditions in a single series, performed over a short period of time, same operator, a single batch of porcine liver and the same technical equipment and are summarized and presented in Table 1.

Within-laboratory reproducibility

Within-laboratory reproducibility was determined by calculations of all data from the three series on different days with different matrices, operators and changing environmental conditions. For each concentration level mean concentration, standard deviation (SD) and coefficient of variation (CV %) were calculated. All coefficients of variation values (CV %) are within the acceptable limits from the Regulation (EU) 2021/808 and are summarized and presented in Table 1.

Matrix effect

It is calculated with 20 different samples free of avermectins, fortified after the sample preparation (at MRL or LCL levels). The results are calculated and after that compared with standard solution. The values are in line within the criteria of Regulation (EU) 2021/808 and are summarized in Table 1.

Decision limit for confirmation (CCα) and detection capability (CCβ)

The decision limit for confirmation (CCα) and detection capability (CCβ) were determined in accordance with the criteria of Regulation (EU) 2021/808. For authorized substances, the decision limits CCα were calculated from the within-laboratory reproducibility ($\alpha = 5$ %) at the MRL and for non-authorized substances, CCα values were determined with ($\alpha = 1$ %). The detection capability CCβ values were established with ($\beta = 5$ %). The calculated results are presented in Table 1.

Stability

In accordance with Regulation (EU) 2021/808, there are recommended requirements for determining stability in solution and matrix. Stability in standard solutions and matrices were determined with information from European Union Reference Laboratories (EURL) guidelines on stability studies [10].

Ruggedness

The ruggedness was determined by introducing minor changes to the experimental conditions. The method proved to be rugged, as no significant deviations were observed when temperature differences were varied during sample preparation. There were no relevant changes when the samples were analyzed immediately after preparation or after storage for 2–3 day.

Selectivity

To determine the selectivity of the method, twenty blank porcine liver samples from different locations and animal species were analyzed. The analytical method showed good selectivity as no interfering peaks were detected at the retention times of the six avermectins and the internal standard nemadectin.

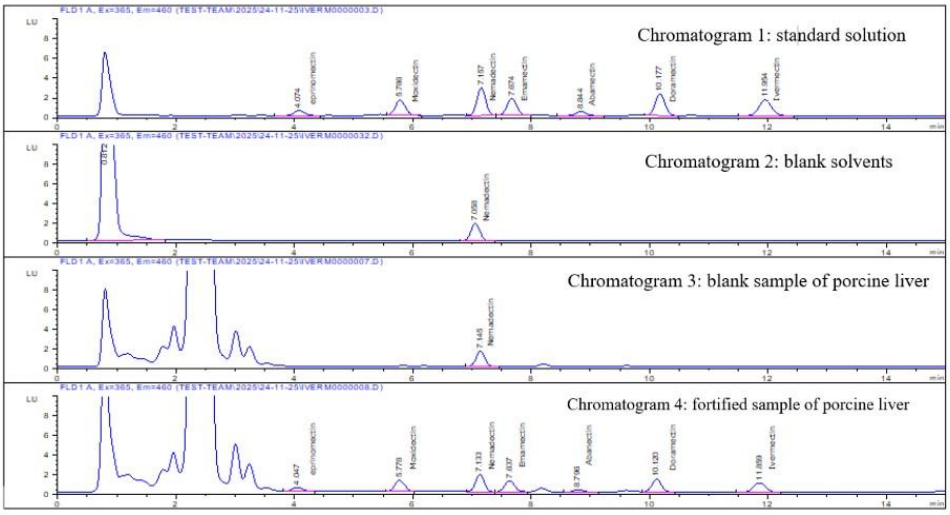

Figure 3: HPLC-FLD chromatograms of standard solution, blank solvents, blank sample of porcine liver and fortified sample of porcine liver with 6 avermectins - (Eprinomectin - 5 µg kg^{-1}; Moxidectin - 4 µg kg^{-1}; Emamectin - 10 µg kg^{-1}; Abamectin - 2.0 µg kg^{-1}; Doramectin - 10 µg kg^{-1}; Ivermectin - 10 µg kg^{-1} and IS - Nemadectin 4.0 µg kg^{-1}.

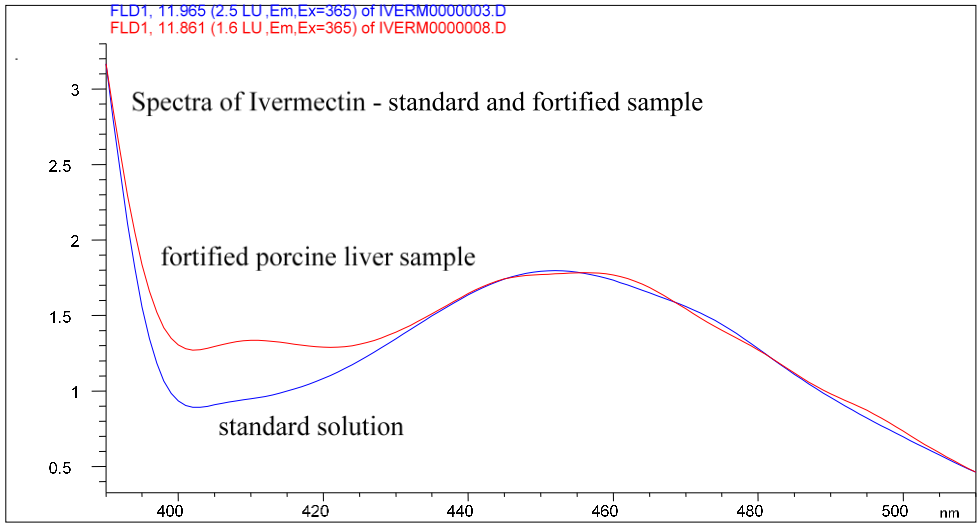

Figure 4: Spectra of Ivermectin - standard solution and fortified sample of porcine liver

Discussion

The sample preparation procedure was optimized to achieve high selectivity and effective extraction of the analytes from the complex porcine liver matrix (Fig. 3, chromatogram 4). The preparation and method development were selected due to the properties of avermectins such as lipophilicity, structure and the lack of natural fluorescence. To improve chromatographic results solid-phase extraction (SPE) was used. The derivatization of the samples was performed to provide fluorescent properties to the substance. This was a significant part of the method, as it resulted in

better peaks of the substances and made their identification and quantification easier (Fig. 3). The derivatization is stable and allows many samples to be analyzed in a single day, which is important for routine analyses.

The detection of the six avermectins and the internal standard (IS) nemadectin was optimized with a gradient program that allowed complete separation of the peaks within 15 min (Fig. 3, chromatograms 1, 4). This gradient program provided good resolution of the peaks (Fig. 3). The use of the internal standard (IS) nemadectin ensure the accuracy of the quantitative determination by compensating the losses during sample preparation and correcting variations in relative time in the instrumental signal, so the relative retention time is in the limit of 1 % according to Commission Implementing Regulation (EU) 2021/808 (Fig. 3). Also according to Commission Implementing Regulation (EU) 2021/808 there is difference between excitation wavelength λex and emission wavelength λem = 460 nm of at least 50 nm.

Until now only for components were examined (Ivermectin, Abamectin, Moxidectin and Doramectin). With the new method two more analytes were added (Eprinomectin and Emamectin), according to the requirement of the EURL- Berlin. Emamectin was included as a substance which can have unregulated use.

The analytical method was validated according to Commission Implementing Regulation (EU) 2021/808. All validated parameters are presented in Table 1. The linearity obtained from the matrix curves is $R^2 \geq 0.9779$ and above. No interfering peaks were detected in the analyses of blank milk samples, which indicates good selectivity (Fig. 3, chromatogram 2). The trueness and precision values for all substances and at all levels correspond to the permissible deviations specified by the Regulation (EU) 2021/808.

Conclusions

The proposed analytical procedure has been validated in accordance with the requirements of Regulation (EU) 2021/808, covering the criteria for linearity, calibration curves, trueness, selectivity, precision, stability, ruggedness, matrix effect, decision limit for confirmation (CCα) and detection capability (CCβ). Thera are no interfering peaks, has good sensibility and optimal analysis time. The method is suitable for determination of residues of avermectins in porcine liver and food safety control. The method has been checked by proficiency test organized by European reference laboratories (EURL) and passed it successfully.

The method has been implemented and is used in the routine practice at the Central Laboratory of Veterinary Control and Ecology (CLVCE) for the control of residual amounts of avermectins in porcine liver.

Authors' contributions

All authors contributed to every part of the work, for the research, the experimental part and the writing of this paper.

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