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

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Received 09 March 2026
Accepted 01 April 2026 *DOI: 10.59957/jctm.v61.i3.2026.3*

ABSTRACT

A fast, sensitive and selective method has been developed for quantitative determination of residues of six avermectins - abamectin, doramectin, moxidectin, ivermectin, eprinomectin and emamectin in milk 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 substances are extracted by liquid extraction with 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. Chromatographic separation is achieved using Agilent Eclipse XDB - C18 (150 x 4.6 mm, 5 µm) column, equipped with a guard column of the same packing in 15 min. Gradient elution is applied with mobile phase A (acetonitrile) and mobile phase B (water) and a fluorescence detector works with wavelengths at λex = 365 nm and λem = 460 nm. 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).

Keywords: avermectins, HPLC - FLD, method validation, derivatization, milk.

INTRODUCTION

Avermectins are veterinary medicinal products and are widely used for the prevention and treatment of parasitic infections. In Commission Regulation (EU) No 37/2010 are established maximum residue limits (MRLs) in foodstuffs of animal origin for these substances. Eprinomectin and moxidectin have defined MRLs in the Regulation (EU) No 37/2010 and accordance with it are permitted for use in animals which milk is produced for human consumption [1]. For the other substances (abamectin, doramectin, ivermectin and emamectin) no MRL values have been established for this matrix and therefore, for control purposes, the cascade MRLs are applied in accordance with Regulation (EU) 2018/470 [2, 3]. Avermectins are usually identified by their main component B1a, which is used as a marker residue 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 and ivermectin (Fig. 1) is one of the most well - known representatives and first introduced in 1981 for the treatment of animals [5].

The method has been developed to control residual amounts of avermectins in raw milk samples by high - performance liquid chromatography with fluorescence detection (HPLC - FLD). Avermectins are lipophilic

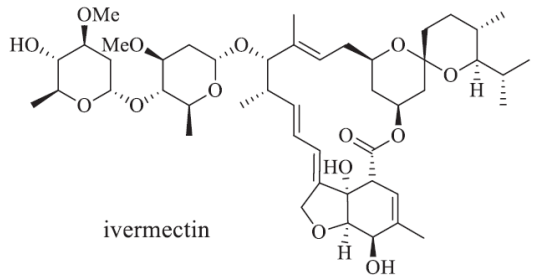

Fig. 1. Chemical structure of ivermectin is presented by Ōmura et al [5].

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 milk. Several methods have been published for the determination of avermectins in various biological matrices such as milk and liver. Sample preparation included extraction with an organic solvent (acetonitrile) and purification by solid phase extraction (SPE) [6]. Avermectins do not naturally fluoresce, and it is necessary to perform derivatization of the samples before instrumental analysis with a fluorescence detector (FLD).

The aim was to develop an analytical method for the determination and control of residual amounts of six avermectins - abamectin, doramectin, moxidectin, ivermectin, eprinomectin and emamectin in milk 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 [7]. The following criteria: linearity, calibration curves, selectivity, trueness (recovery), precision (repeatability and within laboratory reproducibility), stability, ruggedness, decision limit (CCα) and detection capability (CCβ) were covered.

EXPERIMENTAL

Materials and methods

Standards and reagents

The analytical standards of abamectin, doramectin, emamectin, eprinomectin, ivermectin and moxidectin were acquired from Dr. Ehrenstorfer (Augsburg,Germany) and the internal standard nemadectin was purchased from Toronto Research Chemicals (Toronto, Canada). The organic solvent used for the analysis was acetonitrile with high analytical purity (HPLC grade). For sample preparation and for the mobile phase Ultra - purified water was prepared by using a Millipore Mili - Q system (Milford, CT, USA). The other chemicals 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}. Acetonitrile was used as the solvent. The 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 with acetonitrile, to the following concentrations: abamectin (1 μg mL^{-1}), doramectin (4 μg mL^{-1}), moxidectin (4 μg mL^{-1}), ivermectin (3 μg mL^{-1}), eprinomectin (2 μg mL^{-1}), emamectin (10 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, 5 g raw milk were weighed and 20 μL of IS (nemadectin, 1 μg mL^{-1}) were added. The samples were extracted with 15 mL acetonitrile, homogenized with vortex 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 on a 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).

Solid phase extraction (SPE)

The next step was purification and concentration of the samples for which solid - phase extraction (SPE) was used with Strata C18 - E cartridges (200 mg/3 mL, Phenomenex, USA). The cartridges were conditioned with 1 volume acetonitrile and 1 volume of the reconstitution solvent. After that the sample was transferred to the cartridge, was 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 of the sample preparation was derivatization of the samples. For this purpose, 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 derivatized samples were transferred into vials and 25 µL were injected into the chromatographic system.

Instrumental analysis

Instrumental analysis was performed with HPLC - Agilent 1100 Series liquid chromatography system (Agilent Technologies, USA) equipped with a fluorescence detector (FLD). Chromatographic column by Agilent Eclipse XDB - C18 with parameters 150 x 4.6 mm, 5 µm was used and a guard column with the same packing material. The composition of the mobile phase included (phase A) acetonitrile and (phase B) ultra - purified water 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 chromatographic system 25 µL from the sample was injected, the flow rate was 2 mL min^{-1} and the column temperature was 40°C. Avermectins were detected with an excitation wavelength λex = 365 nm and emission wavelength λem = 460 nm.

RESULTS AND DISCUSSION

Results

Method validation

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

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 milk samples.

Avermectins were analyzed with the following calibration curves: 1.0, 2.5, 5 and 10 µg kg^{-1} for abamectin; 4, 10, 20 and 40 µg kg^{-1} for doramectin (Fig. 2); 10, 25, 50 and 100 µg kg^{-1} for emamectin; 3,

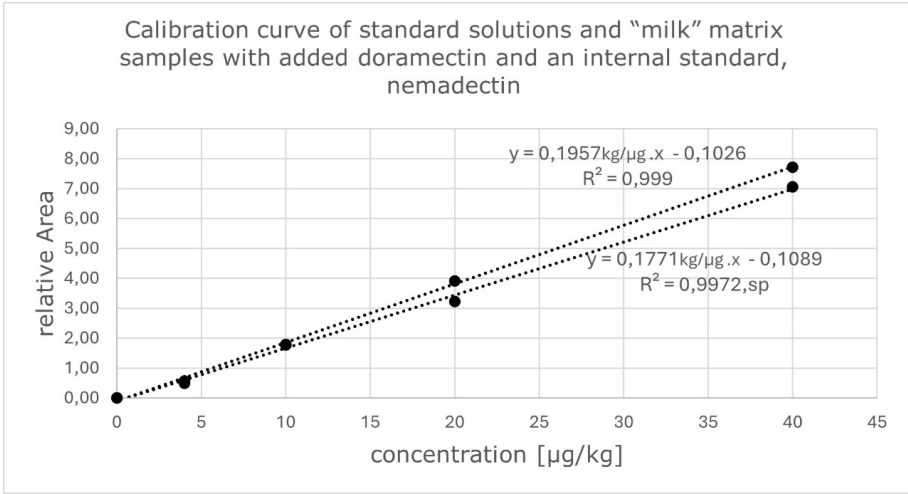

Fig. 2. Linearity, linear equation and correlation coefficient of doramectin in standard solution and fortified sample milk.

7.5, 15 and 30 μg kg^{-1} for ivermectin; 2, 10, 20 and 30 μg kg^{-1} for eprinomectin; and 4, 20, 40 and 60 μg kg^{-1} for moxidectin. The lowest calibration level (LCL) was established at 1/10 of the MRL or cascade MRL [7].

The linear correlation was determined 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. A linear correlation was established for standard solutions and fortified milk samples for all avermectins, with Fig. 2 shown as an example only for doramectin. The coefficients of determination (R^2) were ≥ 0.9779 for all substances, which indicates good linearity within the calibration curves presented in Table 1.

Trueness (recovery)

Analyses were performed using the internal standard method (nemadectin). Standard solutions of the six avermectins were added to blank milk samples to evaluate three concentration levels. For authorized substances (eprinomectin and moxidectin), 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). The mean measured concentration was calculated for each of the three levels. Trueness was determined as the ratio between the mean measured concentration and the theoretical (added) concentration for each of the three analyzed levels. 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 milk 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

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

Substance	Calibration range, μg kg^{-1}	Fortified level	Recovery, %	Repeatability (CV), %	Within-laboratory reproducibility (CV), %	R^2	CCα / CCβ μg kg^{-1}
Abamectin	0 - 10	1 LCL 2 LCL 3 LCL	100 92 98	8.3 4.5 3.3	8.4 8.9 4.8	0.9957	2.99 / 2.84
Doramectin	0 - 40	1 LCL 2 LCL 3 LCL	98 93 100	5.1 4.9 2.3	6.7 10.2 2.3	0.9972	11.52 / 11.07
Emamectin	0 - 100	1 LCL 2 LCL 3 LCL	94 98 100	8.1 5.4 1.3	8.9 8.0 1.9	0.9955	29.89 / 28.44
Eprinomectin	0 - 30	0.1 MRL MRL 1.5 MRL	102 92 93	12.1 9.0 16.1	13.2 17.3 16.7	0.9880	25.19 / 12.23
Ivermectin	0 - 30	1 LCL 2 LCL 3 LCL	98 94 101	8.4 5.0 1.7	9.9 10.6 1.7	0.9974	9.20 / 8.69
Moxidectin	0 - 60	0.1 MRL MRL 1.5 MRL	95 100 93	3.3 3.8 4.9	3.5 4.1 6.8	0.9779	42.72 / 21.10

to the lowest calibration level (LCL).

The values were determined by analysis of six replicates (n = 6) of each concentration level within one day, which was repeated in the same way two other days. A total of 18 measurements per level were performed.

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 milk 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, 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.

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 (α = 5 %) at the MRL and for non - authorized substances, CCα values were determined with (α = 1 %). The detection capability CCβ values were established with (β = 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 [8].

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 also no relevant changes when the samples were analysed immediately after preparation or after storage for 2 - 3 days.

Selectivity

To determine the selectivity of the method, twenty blank milk samples from different locations and animal species were analysed. The analytical method was considered to have good selectivity as no interfering peaks were detected at the retention times of the six avermectins and the internal standard nemadectin.

Discussion

The sample preparation procedure was optimized to achieve high selectivity and effective extraction of the analytes from the complex milk matrix (Fig. 3, chromatogram 3). The preparation and method development were selected due to the properties of avermectins such as lipophilicity, structure and the lack of natural fluorescence. The avermectins were extracted with organic solvent acetonitrile and cleaned up and concentrated by solid - phase extraction (SPE) with Strata C18 - E cartridges to improve chromatographic results by eliminating the matrix effects. The derivatization of the samples was performed to provide fluorescent properties to the substance and was done with TFAA and 1 - methylimidazole, to which 100 % acetic acid and TEA were added to stabilize the reaction. This was a significant part of the method, as it resulted in better peaks of the substances and made their identification and quantification easier. The derivatization is stable and allows many samples to be analyzed in a single day, which is important for routine analyses.

An Agilent Eclipse XDB - C18 column with the following parameters 150 x 4.6 mm, 5µm was used for chromatographic separation. 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, chromatogram 1, 3). 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

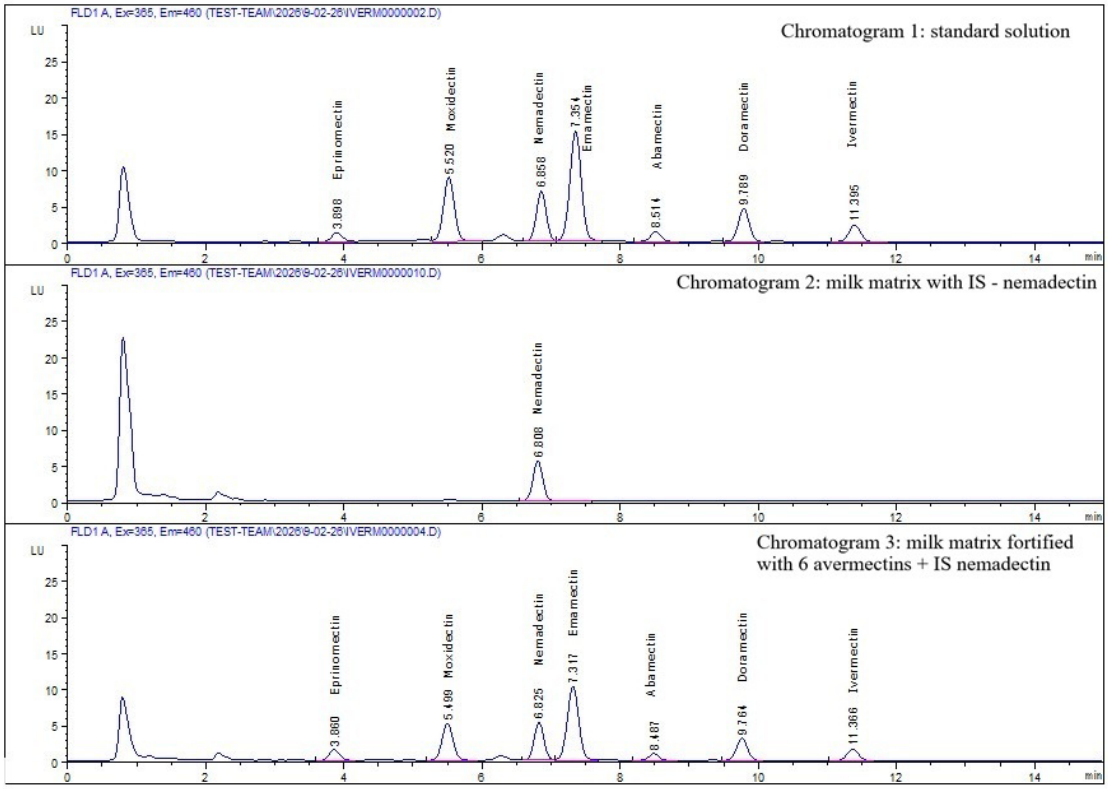

Fig. 3. HPLC - FLD chromatograms of standard solution, blank sample milk and fortified milk with 6 avermectins - (Eprinomectin - 10 μg kg^{-1}; Moxidectin - 20 μg kg^{-1}; Emamectin - 25 μg kg^{-1}; Abamectin - 2.5 μg kg^{-1}; Doramectin - 10 μg kg^{-1}; Ivermectin - 7.5 μg kg^{-1} and IS - Nemadectin 4.0 μg kg^{-1}.

relative time in the instrumental signal.

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, decision limit for confirmation (CCα) and detection capability (CCβ).

- Sample preparation of the method provides effective extraction, purification of samples and chromatograms without interfering peaks.
- Instrumental analysis shows good resolution between peaks and optimal analysis time of 15 min for 6 substances.
- The method is suitable for food safety control and the determination of avermectin residues in milk.

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 milk.

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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