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Method development and validation for the simultaneous determination of 21 antiviral drugs by ultra-high performance liquid chromatography-tandem mass spectrometry in chicken muscle and liver

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ABSTRACT

The recent unauthorization of antiviral drugs in food-producing animals according to Commission Delegated Regulation (EU) 2022/1644 have increased the need for food control laboratories to develop analytical methods and perform official controls. In this work, a simple and fast analytical methodology was developed for the simultaneous determination of 21 antiviral drugs in chicken muscle and liver by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Chromatographic separation was achieved by an HILIC BEH amide column; followed by detection with a electrospray ionization source in positive and negative modes. Based on extraction efficiencies, critical parameters affecting sample treatment were optimized including the evaporation and clean up steps to extract the largest number of antiviral drugs and reduce interferences. The method was validated according to Commission Implementing Regulation (EU) 2021/808 in chicken muscle and liver. Most compounds showed a linearity of R^2>0.9800, while decision limits were between 0.18 and 7.05 μg kg^{-1} and 0.19 and 36 μg kg^{-1} for chicken muscle and liver, respectively. Trueness and within-lab reproducibility were determined at three levels ($n = 7$) and the results showed values ranging from 81 to 133 % and 4.2–57 % for chicken muscle, and 71–136 % and 4.6–106 % for chicken liver, respectively. The applicability of the developed method was demonstrated by the analysis real samples. 20 samples from the National Residue Control Plan in the Netherlands were analyzed and although none of targeted compounds were detected it is important to continue the analysis of larger set of samples to address any possible food safety risks.

1. Introduction

Viruses can infect humans, animals and plants through different routes, such as contact, air and food [1]. Since the approval of idoxuridine in 1963, antiviral drugs have been widely applied to prevent and treat viral infections in humans, and companion animals under the cascade principle of EU regulation No. 2019/6 [2]. In the past, human antiviral drugs have been misused in food-producing animals for the treatment of avian influenza and African swine fever, two of the most common viruses in food-producing animals worldwide [3,4]. One of the main concerns is that the misuse of antiviral drugs in food-producing animals lead to drug-resistance virus strains, indicating that the effectiveness of antiviral drugs for the treatment of human viruses decreases and may result in deaths [5,6]. In the 2000s, the widespread misuse of amantadine, an antiviral drug against influenza A, was firstly reported in Chinese poultry and has led to large-scale resistance problems [7–9]. Consequently, China has banned the use of antiviral drugs for veterinary purposes in 2005 [10] followed by the Food and Drug Administration in the United States that prohibited the extralabel use of antiviral drugs for the prevention or treatment of influenza A virus in chickens, turkeys and ducks in 2006 [11,12]. From 2014, human immunodeficiency virus (HIV) drugs were misused in Ugandan porcine and chickens [13,14]. As of 2022, antiviral drugs have been included in Commission Delegated Regulation (EU) 2022/1644 under the prohibited or unauthorized pharmacologically active substances in food-producing animals (group A) and have increased the need for food control laboratories in the

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European Union to develop analytical methods for their determination and perform official controls [15].

In the last decade, antiviral drugs have been included in various sensitive analytical methods for the analysis in animal-derived matrices [4]. Amongst others, screening assays and sensors based on specific antibody-interactions, such as enzyme-linked immunosorbent assay (ELISA) [16–18], immunochromatographic assay (ICA) [19,20] and lateral flow immunoassay (LFIA) [21,22], and spectroscopy, such as surface-enhanced Raman spectroscopy (SERS) [23,24], have been popular. While these methods are simple, rapid, sensitive and in most cases suited for on-site analysis, they only provide suspect results and are limited to individual antiviral drugs, i.e. amantadine, oseltamivir and ribavirin, or group-specific antiviral drugs, i.e. adamantines [18,21,25]. To provide quantitative confirmatory results and cover a larger number of antiviral drugs in a single method, liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) is the required technique [21, 26,27]. Although Mu *et al.* included 14 antiviral drugs in their method by using Quick Easy Cheap Effective Rugged Safe (QuEChERS) [28] and Douillet et al. 15 antiviral drugs by performing a solid-liquid extraction followed by dilution [29], most of the available LC−MS/MS methods have been developed for only one to three antiviral drugs from the same type of class, such as influenza A [30–32] or human immunodeficiency virus (HIV) drugs [13,14]. This fact is likely due to the very different chemical and physical properties in terms of pKa and LogP values of these compounds [4,29] that hinder the simultaneous determination of different antiviral classes in a single method.

Herein, the present work aimed to develop a simple and rapid LC−MS/MS method for the simultaneous determination of 21 antiviral drugs in chicken muscle and liver. The selection of the 21 target compounds was focused to include antiviral drugs used for the treatment of influenza A virus, herpes virus, human immunodeficiency virus, immunomodulator or many other viruses due to broad-spectrum activity. To the best of our knowledge, this is the first time that 21 antiviral drugs with diverse chemical and physical properties have been simultaneously determined in one single method. To assess the performance, the developed method was fully validated for chicken muscle and partially validated for chicken liver according to Commission Implementing Regulation (EU) 2021/808 and subsequently applied to real samples from the National Residue Control Plan in the Netherlands.

2. Materials and methods

2.1. Reagents and standards

Milli-Q water was produced by a water purification system at a resistance of at least 18.2 MΩ cm (Millipore, Billerica, MA, USA). Ultra LC−MS grade methanol and acetonitrile were purchased from Biosolve BV (Valkenswaard, NL), and dimethylsulfoxide from Honeywell (Charlotte, NC, USA). Ammonium acetate and formic acid were supplied by Sigma Aldrich (San Luis, MO, USA). Ammonium acetate 1 M was prepared by dissolving 7.7 g of ammonium acetate in 100 mL of Milli-Q water.

The target compounds (acyclovir, ACV; amantadine, AMT; arbidol hydrochloride, ARB; arbidol sulfone, ARBS; efavirenz, EFV; ganciclovir, GCV; imiquimod, IMQ; laninamivir, LNV; lopinavir, LPV; memantine, MMT; moroxydine hydrochloride, MOR; nevirapine, NVP; oseltamivir, OST; oseltamivir acid, OSTA; penciclovir, PCV; peramivir, PMV; ribavirin, RBV; rimantadine hydrochloride, RMT; saquinavir mesylate, SQV; viramidine hydrochloride, VRM; zanamivir, ZNV) and their isotopically labelled internal standards (acyclovir-d_4, ACV-d_4; amantadine-d_{15} hydrochloride, AMT-d_{15}; arbidol-d_6 hydrochloride, ARB-d_6; ganciclovir-d_5, GCV-d_5; lopinavir-d_8, LPV-d_8: memantine-d_6, MMT-d_6; moroxydine-d_8 hydrochloride, MOR-d_8; oseltamivir-d_3 acid, OSTA-d_3; penciclovir-d_4, PCV-d_4; ribavirin-$^{13}C_5$, RBV-$^{13}C_5$; saquinavir-d_9, SQV-d_9; zanamivir-$^{13}C_5$,$^{15}N_2$, ZNV-$^{13}C_5$,$^{15}N_2$) were purchased from LGC Standards (Teddington, Middlesex, UK). The chemical structure, acronym and chemical formula of target compounds selected for the different classes are shown in Fig. 1. Individual standard stock solutions of ACV, GCV, IMQ, PCV, VRM, ACV-d_4, GCV-d_5, RBV-$^{13}C_5$, PCV-d_4 were prepared in dimethylsulfoxide at a concentration of 1000 mg L^{-1}, except MMT and MMT-d_6 with a concentration of 970 and 100 mg L^{-1}, respectively. ZNV, ZNV-$^{13}C_5$,$^{15}N_2$, RBV and LNV were prepared in Milli-Q water at 100 mg L^{-1}. The remaining analytes were prepared in methanol at 100 mg L^{-1} for NVP, LPV, SQV, PMV, EFV, AMT-d_{15}, OSTA-d_3, LPV-d_8 and SQV-d_9, and 1000 mg L^{-1} for AMT, ARB, ARBS, MOR, OSTA, OST, RM, ARB-d_6 and MOR-d_8. Further diluted standard solutions were prepared in methanol. All the standard stock solutions were stored at −80 °C and the intermediate standard solutions at −20 °C. In some cases, ultrasonication was needed to dissolve the intermediate standard solutions before their use to prepare working standard solutions.

2.2. Samples and sample preparation

Raw chicken muscle and liver samples were obtained from the National Residue Control Plan in the Netherlands and anonymized for this study. Homogenized chicken muscle and liver were weighed to 1.00 g in 7 mL sample tubes (LA Biosystems, Waalwijk, NL) and 40 µL of internal standard solution (LPV-d_8 at 0.0125 mg L^{-1}; SQV-d_9 at 0.025 mg L^{-1}; MMT-d_6 and MOR-d_8 at 0.05 mg L^{-1}: ACV-d_4, AMT-d_{15}, and ARB-d_6 at 0.075 mg L^{-1}; OSTA-d_3 and GCV-d_5 at 0.25 mg L^{-1}; PCV-d_4 at 0.5 mg L^{-1}; and ZNV-^{13}C,$^{15}N_2$ and RBV-$^{13}C_5$ at 0.625 mg L^{-1}) was added. After 15 min, to ensure the absorption of analytes in the sample, a cup of 2.3 mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) and 4 mL of acetonitrile/Milli-Q water (80/20, *v/v*) were added to each tube. The samples were extracted using an Omni Bead Ruptor (Omni Bead Ruptor 24, Omni International, Kennesaw, GA; USA and Biotage Lysera, Uppsala, SE), with a speed of 5.65 m s^{-1} during two cycles, 0.45 min $cycle^{-1}$ and pause dwell of 0.50 min; and centrifugated at 2689 x g for 10 min (Centrifugue 5810, Eppendorf, HH, DE). Once centrifugated, 1 mL of supernatant was transferred to a test tube and evaporated to dryness under a nitrogen stream (around 20–25 kPa) at 40 °C (Zymark TurboVap L Evaporator, Marshall Scientific, Hampton, NH, USA). The dried extracts were reconstituted in 200 µL of acetonitrile/methanol/Milli-Q water (4/3/1, *v/v/v*) and the tubes were vortexed for 5 s. Subsequently, centrifugation was performed at 2000 x g for 10 min at 4 °C (Rotanta 460R, Hettich zentrifugen, Tuttlingen, DE) before the extracts were transferred to injection vials. A filtration step was required before analysis using hydrophobic polytetrafluoroethylene syringe filters (PTFE, 0.45 µm, 13 mm, Chromatography Direct Ltd., Wellingbough, UK). For analysis, 2 µL of the extract were injected into the LC−MS/MS system.

2.3. LC−MS/MS instrumentation and working conditions

The chromatographic analysis was performed on an Acquity UPLC I-Class System equipped with a syringe pump, an autosampler, and a column oven (Waters Corporation, Milford, MA, USA). The chromatographic separation of antiviral drugs was carried out on a Acquity UPLC BEH Amide column (130 Å, 1.7 µm particle size, 150 mm x 2.1 mm) using mobile phase (A) 0.1 % formic acid and 2 mM ammonium acetate in Milli-Q water, and (B) 0.1 % formic acid and 2 mM ammonium acetate in acetonitrile/Milli-Q water (95/5, *v/v*). The gradient elution program was as follows: 0.0–2.0 min, 5 % A; 2.0–4.5 min, linear increase from 5 to 20 % A; 4.5–7.0 min, linear increase from 20 to 50 % A; 7.0–8.0 min, linear increase from 50 to 70 % A; 8.0–9.0 min, 70 % A; 9.0–9.5 min, linear decrease from 70 to 5 % A; 9.5–13.0 min, 5 % A. The flow rate of the mobile phase was 0.3 mL min^{-1}, the injection volume was 2 µL, and column oven and autosampler temperatures were held at 40 °C and 10 °C, respectively, during the chromatographic run.

The UPLC system was coupled to a Xevo TQ Absolute Triple Quadrupole Mass Spectrometer (Waters Corporation, Milford, MA, USA) equipped with a triple quadrupole mass analyzer and an electrospray

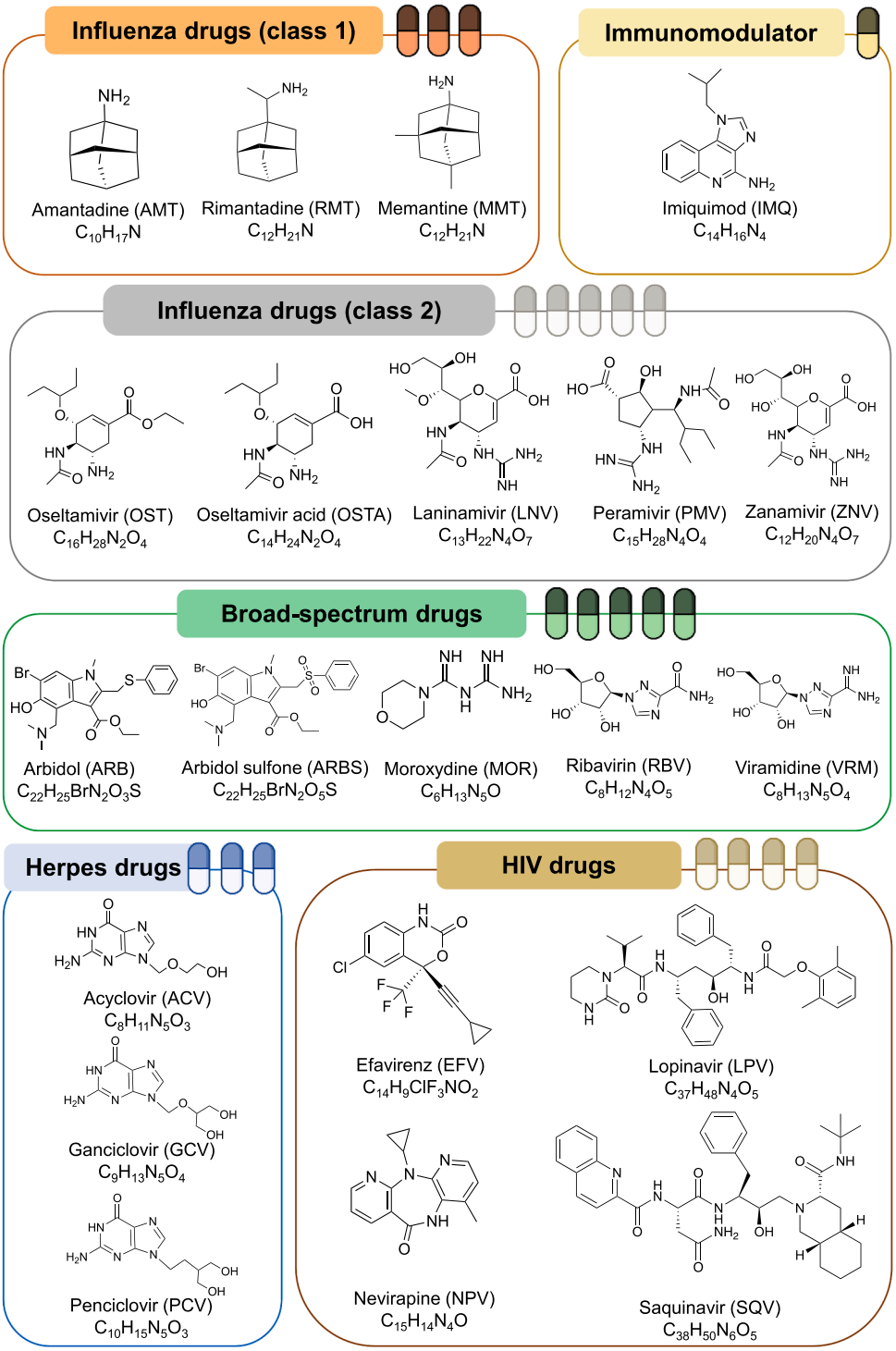

Fig. 1. Chemical structures, acronyms and chemical formula of the studied antiviral drugs from the different selected types.

ionization (ESI) source. The source and desolvation temperature were set at 150 °C and 450 °C, respectively; ESI spray voltage at 3.0/−3.0 kV and source offset at 30 V. Nitrogen was used as desolvation and cone gas at a flow rate of 600 and 150 L h^{-1}, respectively. The mass spectral data were acquired in multiple reaction monitoring mode (MRM) in positive and negative ion mode, and both quadrupoles (Q1 and Q3) operated at unit mass resolution. Argon (≥ 99.995 %) was used as a collision-induced dissociation (CID) gas at a pressure of 0.18 mL min^{-1} in the collision cell (Q2). Instrument control and LC–MS/MS data analysis were carried out using Mass Lynx v4.1 software (Waters Corporation).

2.4. Method validation

The validation of the developed analytical method has been carried out according to Commission Implementing Regulation (EU) 2021/808 with regard to the performance of the analytical methods and interpretation of results for residues of pharmacologically active substances in food-producing animals [33]. To date, there are no Maximum Residue Limit (MRL), Minimum Method Performance Requirement (MMPR) and Reference Points for Action (RPA) established by the European Union for antiviral drugs in food-producing animals because they are classified as

unauthorized substances in Commission Delegated Regulation (EU) 2022/1644 [15].

The relative matrix effect or matrix factor (MF) was evaluated at 10 μg l^{-1} and calculated for each antiviral drug by comparing the signals between the post-extraction fortified extract and standard solutions of the corresponding concentration. The absolute recovery was also evaluated and calculated as the peak area of fortified pre-extraction matrix divided by peak area of fortified post-extraction extract and multiplied by 100. In order to correct analyte loss during sample extraction and the matrix effect during ESI analysis, matrix-fortified standard curves was used for quantitation purposes and the signal of each target compound was corrected by isotope-labelled internal standards.

According to Commission Implementing Regulation (EU) 2021/808 [33], a full validation (three days) has been carried out in chicken muscle and a partial validation (one day) in chicken liver. The specificity/selectivity was assessed by analyzing the occurrence of possible interfering peaks in the retention time window expected for the analyte elution in blank samples with two multiple reaction monitoring (MRM) transitions of each analyte and one MRM transition of each internal standard. For the identification of antiviral drugs with LC−MS/MS, several identification points are required: (i) retention time by liquid chromatography; (ii) the precursor ion; (iii) the product ion corresponding to the quantification transition; (iv) the product ion corresponding to the confirmation transition. To confirm the target compound, its deviation in the relative retention time (RRT) in the sample compared to the matrix-fortified standard curve and the deviation in the relative ion ratio of the two product ions must not exceed ±1 % and ±40 %, respectively.

Linearity was calculated using the least squares method based on matrix-fortified standard curves injected at the beginning and at the end of the series at five levels corresponding to lowest calibration level (LCL) times 1, 2, 3, 4 and 6 for each target compound. The trueness and precision of the method were studied using 21 chicken muscle samples and seven chicken liver samples, and fortified at three validation levels: 1, 2, and 3 times the LCL of the target compound. Repeatability (within-day precision) was calculated for each compound at 1, 2 and 3 times the LCL by using the relative standard deviations (RSDr, %) of seven replicate extracts, each prepared from different samples, on the same day with the same instruments and operators. Reproducibility (between-day precision, RSD_{RL} %) in chicken muscle was calculated based on the mean repeatability at each LCL obtained during three days at different instruments and operators, while in chicken liver the repeatability was multiplied by 1.5. The decision limit (CCα) was calculated by fortifying 21 samples at the LCL level and according to method 3 for unauthorized or prohibited pharmacologically active substances in section 2.6 of Regulation (EU) (2021/808) [33].

The ruggedness of the method was studied to evaluate the effect of minor changes that can occur during analysis. Ruggedness was calculated by spiking the same chicken muscle and liver sample as used for the matrix-fortified standard curve and applying three different modifications of the method ($n = 3$). The evaluated modifications were (i) evaporation temperature at 50 °C instead of 40 °C; (ii) modification of the bead ruptor parameters to a speed of 4.20 m s^{-1} during one cycle, 0.30 min $cycle^{-1}$ and pause dwell of 0.10 min; and (iii) extension of 10 min evaporation time after dryness. The method is robust to a modification if the performance, i.e. trueness, precision and number of confirmations, remains similar to the performance of the original method. The analyte stability in extract was determined by storing the matrix-fortified standard curve and the seven samples from chicken muscle at 1 times LCL at −20 °C after the second validation day and re-analyze it on the third validation day with an interval of one week. The analytes are stable in extract under the studied conditions if the performance, i.e. trueness, precision and number of confirmations, remains similar to the performance of the full validation at 1 times LCL.

3. Results and discussion

3.1. LC−MS method development

3.1.1. Liquid chromatography

The antiviral drugs selected in this work present a wide range of chemical properties, with diverse structures and functional groups that affect their behavior depending on the pH. Their pH-dependent ionization that results in different ionic forms as well as their wide polarity indicated by their logP values (Table 1) add complexity and highlight the challenges to their simultaneous chromatographic separation. For example, antiviral drugs used for influenza A virus such as AMT, RMT, MMT have logP values between 1.47 and 2.22, suggesting they are hydrophobic and non-polar. In contrast, antiviral drugs used for herpes virus like ACV, PCV, and GCV have logP values between −2.18 and −1.55, reflecting they are hydrophilic and polar. Table 1 lists the studied antiviral drugs in this work along with their pKa values, and logP values.

With the aim to achieve a simultaneous chromatographic separation of as many as possible families of antiviral drugs and considering their diverse characteristics as shown in Table 1, a hydrophilic interaction chromatography (HILIC) is suggested in this work. The mechanism of HILIC is based on the partition and adsorption of analytes between a predominantly polar organic mobile phase and a water-enriched layer of the mobile phase immobilized on the stationary phase, driven by hydrophilic and electrostatic interactions [35]. Therefore, to start with, the use of a BEH Amide (2.1 mm x 100 mm, 2.5 μm) analytical column as suggested by Douillet et al. [29] for 15 antiviral drugs was evaluated for the chromatographic separation of the target compounds, using acetonitrile/water (0.1 % formic acid and 2 mM ammonium acetate *v/v*) gradient elution program [29]. Under these conditions, a baseline chromatographic separation was observed for most target compounds in less than 13 min except for OST/AMT, PCV/GCV and RMT/MMT which partially coeluted. Douillet et al. [29] also reported the coelution between OST/AMT but this could be solved as the authors suggested by using different product ion transitions for quantification and confirmation purposes [29]. This strategy could also be applied in our method in the case of PCV and GCV coelution by individually monitoring the selected non-interfering transitions (precursor-to-product ion). Nevertheless, this is not possible in the case of RMT and MMT owing to their similarities in the structure they have same precursor ion and also yield

Table 1
Acronyms, pKa and logP values for the studied antiviral drugs.

Compound	Acronym	Strongest acidic pKa (25 °C)[a]	Strongest basic pKa (25 °C)[a]	LogP (25 °C)[a]
Acyclovir	ACV	10.16	1.49	−1.55
Amantadine	AMT	–	10.46	1,47
Arbidol	ARB	6.01	10.02	3.75
Arbidol sulfone	ARBS	6.01	9.81	2.38
Efavirenz	EFZ	12.52	−1.49	4.46
Ganciclovir	GCV	10.16	1.49	−2.18
Imiquimod	IMQ	–	2.89	2.65
Laninamivir	LNV	3.31	12.14	−5.18
Lopinavir	LPV	13.39	1.06	4.69
Memantine	MMT	–	10.45	2.07
Moroxydine	MOR	15.13	11.48	−1.58
Nevirapine	NVP	10.73	3.28	2.49
Oseltamivir	OST	14.03	9.26	1.16
Oseltamivir acid	OSTA	4.38	9.29	−1.84
Penciclovir	PCV	10.17	1.47	−2.07
Peramivir	PMV	4.18	11.70	−2.18
Ribavirin	RBV	11.88	−0.25	−2.77
Rimantadine	RMT	–	10.14	2.22
Saquinavir	SQV	13.64	7.27	3.16
Viramidine	VRM	12.42	5.34	−2.54
Zanamivir	ZNV	3.25	11.93	−5.83

[a] Predicted data calculated by playgroundcalculators [34].

common product ions under tandem mass spectrometry conditions. For this reason, different gradient elution programs together with a reduction of the analytical column temperature from 50 to 40 °C were tested without any success on the improvement of the separation resolution for RMT and MMT (Fig. S1A). Additionally, the percentage of organic content in the injection solvent was also evaluated by injecting target compounds at 0.01 μg L^{-1} under 100 % organic (ACN), 90 % organic (ACN/MeOH/water, 5/4/1 *v/v/v*) and 75 % organic (ACN/MeOH/-water, 2/1/1 *v/v/v*) conditions, Fig. S1A, B and C, respectively. In these cases, the chromatographic peak of target compounds showed slight differences in terms of baseline separation and shape. However, the resolution separation of the chromatographic peaks is not adequate since no baseline separation of target compounds was achieved in any case. Therefore, in order to improve the chromatographic separation of RMT and MMT, a longer BEH Amide (2.1 mm x 150 mm, 1.7 μm) analytical column was evaluated and same tests with the injection solvent were carried out. The retention time of both MMT and RMT was increased under these conditions from 1.8 to 3.8 min and from 1.9 to 3.9 min, respectively. Additionally, comparing to the results obtained with the 100 mm analytical column, a better resolution of target compounds was obtained improving the peak shape to two separate peaks when employing 100 % (Fig. S1D), 90 % (Fig. S1E) and 75 % (Fig. S1F) of organic content. Regarding the sensitivity of the analytes, the use of 90 % organic solvent (Fig. S1E) resulted into a slightly higher sensitivity for RMT, as indicated by the higher response of the RMT compound. Generally, a higher percentage of organic solvent also showed improved chromatographic peak shapes for all target compounds, especially for the earlier eluting compounds. In overall, these results showed that the longer analytical column as well as the 90 % of organic content in the injection solvent significantly improve the chromatographic peak shape of all target compounds, including MMT and RMT, separating MMT and RMT with Rs value of 0.8.

Furthermore, due to the mentioned coelution of a few target compounds, the suppression or enhancement of the signal response in their ionization by ESI was also studied by injecting individual standard solutions and a mixture of the target compounds at the same concentration (10 μg L^{-1}). Comparison was carried out in terms of peak areas and results showed that the differences between areas were less than 10 % and lower than the relative standard deviation (RSD, %), showing that coelution does not significantly affect the individual response of these analytes. Fig. 2 shows the chromatographic separation corresponding to a standard mixture solution at 25 μg L^{-1} of all 21 selected antiviral drugs under optimal liquid chromatography conditions (*Section 2.3*) in less than 13 min.

3.1.2. Mass spectrometry

The liquid chromatography system was coupled to a triple quadrupole mass spectrometer with H-ESI operated in both positive and negative modes. Target compounds were individually injected and all of them showed better ionization efficiencies in positive ion mode, generating the protonated molecular ion $[M+H]^+$ except for EFV which showed a better ion signal intensity in negative ion mode, with a deprotonated molecular ion $[M–H]^-$ as base peak of the mass spectra. Once the ionization mode of each target compound was selected, the cone voltage was also evaluated due to its high influence in the ionization efficiency of individual chemicals. In many cases (13 out of 33, including internal standards), a cone voltage of 30 V was optimal. For instance, some compounds, like IMQ or ARBS, would suffer from ionization efficiency, as their signal would be reduced by 20 % if the cone voltage was 15 V instead of the optimal value at 30 V. While, the antiviral drug EFV, which requires a cone voltage of 40 V, would experience a signal reduction of 15 % if the cone voltage was lowered to 20 V. Thus, these results showed the need to optimize the cone voltage for each compound to ensure the best analytical performance of each target compound.

As required by Commission Implementing Regulation (EU) 2021/808, confirmatory methods should provide information on the structural chemical composition of the analytes, which can be obtained through mass spectrometric detection. Therefore, tandem mass spectrometry was selected to analyze the 21 target antiviral drugs in order to enhance sensitivity and achieve accurate characterization and identification of the target compounds. The product ion scan of each target compound was evaluated at collision energies (CE, eV) ranging from 0 to 50 eV. The corresponding product ions of each target compound were characterized and the two most selective and abundant ones were selected for quantitative and confirmatory purposes when working in multiple reaction monitoring mode. Table 2 summarized the selected precursor and product ions for each antiviral drug and the respective internal

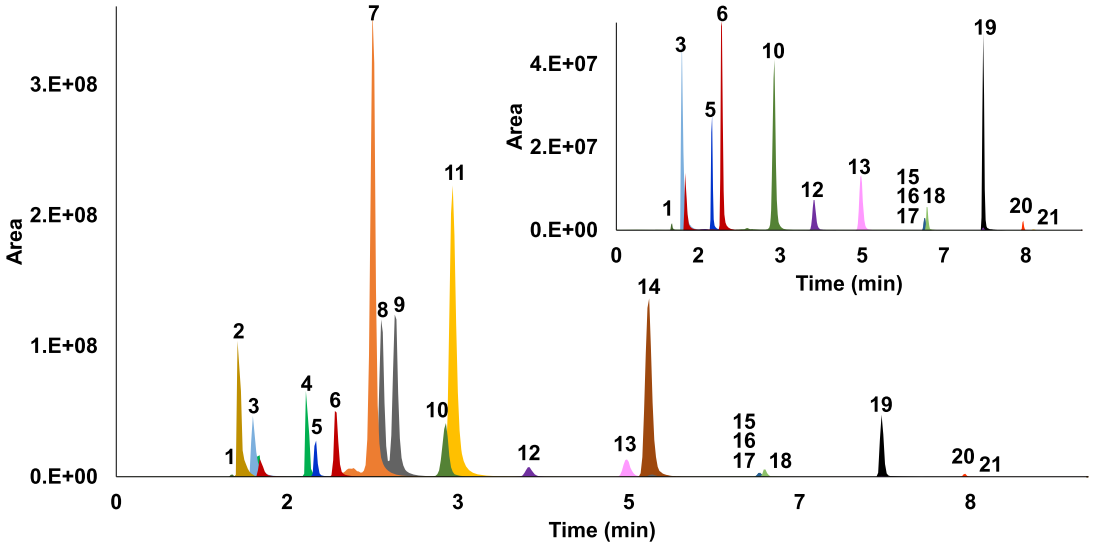

Fig. 2. UHPLC–MS/MS chromatogram corresponding to a standard mixture solution (25 μg L^{-1}) of targeted compounds obtained under optimal gradient elution conditions. 1. Efavirenz, 2. Lopinavir, 3. Nevirapine, 4. Arbidol, 5. Arbidol sulfone, 6. Saquinavir, 7. Imiquimod, 8. Memantine, 9. Rimantadine, 10. Oseltamivir, 11. Amantadine, 12. Ribavirin, 13. Acyclovir, 14. Moroxydine, 15. Penciclovir, 16. Oseltamivir acid, 17. Ganciclovir, 18. Peramivir, 19. Viramidine, 20. Laninamivir, 21. Zanamivir.

Table 2
MRM transitions used for quantification and confirmation purposes with their cone voltages and collision energies (CEs, eV) in the employed LC-MS/MS method.

Compound	Acronym	Precursor ion (m/z)	Product ion (m/z)	Cone (V)	Collision energy (V)
Acyclovir	ACV	226.1	152.1	15	**10**
			135.1	15	25
Acyclovir-d_4	ACV-d_4	230.1	152.1	15	16
Amantadine	AMT	152.2	135.1	15	18
			93.1	15	25
Amantadine-d_{15}	AMT-d_{15}	167.3	150.2	20	17
Arbidol	ARB	479.2	434.0	20	16
			281.0	20	30
Arbidol-d_6	ARB-d_6	485.2	433.9	30	15
Arbidol sulfone	ARBS	511.1	295.9	30	20
			465.9	30	30
Efavirenz	EFV	314.0	243.9	40	17
			241.9	40	17
Ganciclovir	GCV	256.2	152.1	15	10
			135.1	15	30
Ganciclovir-d_5	GCV-d_5	261.2	152.0	30	15
Imiquimod	IMQ	241.2	185.1	30	20
			168.1	30	30
Laninamivir	LNV	347.2	60.1	30	18
			288.1	30	12
Lopinavir	LPV	629.4	447.2	15	15
			183.2	15	22
Lopinavir-d_8	LPV-d_8	637.4	447.1	25	13
Memantine	MMT	180.2	163.1	15	13
			107.1	15	18
Memantine-d_6	MMT-d_6	186.2	169.2	20	15
Moroxydine	MOR	172.1	60.1	30	15
			113.1	30	15
Moroxydine-d_8	MOR-d_8	180.2	60.1	30	15
Nevirapine	NPV	267.2	226.0	30	25
			107.1	30	27
Oseltamivir	OST	313.2	166.1	20	15
			225.1	20	10
Oseltamivir acid	OSTA	285.2	94.1	30	20
			197.1	30	8
Oseltamivir acid-d_3	OSTA-d_3	288.2	200.1	30	8
Penciclovir	PCV	254.1	152.1	30	12
			135.1	30	29
Penciclovir-d_4	PCV-d_4	258.1	152.1	30	15
Peramivir	PMV	329.2	100.1	20	30
			270.1	20	20
Ribavirin	RBV	245.1	113.1	15	7
			96.0	15	20
Ribavirin-$^{13}C_5$	RBV-$^{13}C_5$	250.2	113.1	15	10
Rimantadine	RMT	180.2	163.1	15	10
			81.1	15	20
Saquinavir	SQV	671.5	570.3	15	30
			416.0	15	40
Saquinavir-d_9	SQV-d_9	680.5	570.2	25	33
Viramidine	VRM	244.2	112.1	30	10
			95.1	30	30
Zanamivir	ZNV	333.2	60.1	20	35
			121.0	20	20
Zanamivir-$^{13}C,^{15}N_2$	ZNV-$^{13}C,^{15}N_2$	336.2	63.2	20	15

standards at the optimum cone voltage and collision energies.

3.2. Sample treatment

For the analysis of antiviral drugs in food-producing animals at low concentration levels, a sample treatment is necessary prior to their determination by the developed LC−MS/MS method. Initially, the extraction procedure of Douillet et al. [29] which consisted on a solid-liquid extraction, was considered although some modification were needed since a broader range of antiviral drugs were included in the present work.

The reduction of the sample amount and the extraction solvent was necessary due to the unavailability of a minimix vibrational shaker, requiring the use of a bead ruptor as the most suitable alternative. Consequently, the sample size (5.0 g) and extraction solvent volume (20 mL) recorded by Douillet et al. [29] were reduced by a factor of five (to 1 g and 4 mL, respectively) to keep the same proportions and ensure compatibility with the bead ruptor, which has a maximum capacity of 4 mL. Furthermore, the final dilution of 1 mL of supernatant is performed with 0.6 mL of methanol instead of methanol/water (2/1, *v/v*), in order to increase the organic content (final extract solvent proportions: acetonitrile/methanol/water, 4/3/1, *v/v/v*), as the gradient elution program starts with 95 % of organic phase (B).

Considering these modifications, all experimental tests involved 15 out of the 21 studied antiviral drugs that were available (ACV, AMT, ARB, ARBS, GCV, IMQ, MMT, MOR, OST, OSTA, PCV, RBV, RMT, VRM, ZNV) and their respective internal standards (AMT-d_{15}, MMT-d_6, ACV-d_4, GCV-d_5, ARB-d_6, MOR-d_8, RBV-$^{13}C_5$, PCV-d_4, OSTA-d_3, and ZNV-$^{13}C_5$,$^{15}N_2$); which were spiked to blank chicken muscles. The target compounds were successfully extracted using the sample treatment described above and detected by the developed LC−MS/MS method in this work. However, most of the target compounds were difficult to

identify, as their sensitivity was very low, resulting in high signal-to-noise that interfered with the compounds' signals. To address this, an evaporation step was introduced to the sample treatment procedure and as an initial test, to evaluate its performance, only a quarter part of the extract (1 mL out of 4 mL of extract) was used. Therefore, 1 mL of supernatant was evaporated to dryness with N_2 at 40 °C, reconstituted with 200 µL of acetonitrile/methanol/water (4/3/1, *v/v/v*), vortexed for 30 s, and centrifuged at 2000 x g for 10 min at 4 °C. Results indicated that the evaporation step was favourable for most of the compounds, except for RMT and RBV (Fig. 3). RMT and RBV reduces 30 % and 10 % of the response factor with the addition of evaporation step, respectively. Nevertheless, since the evaporation step enable a preconcentration of target compounds, yielding an increase of the response factor from 5 to 30 % depending on the specific antiviral drug as shown in Fig. 3, the evaporation step was included for further studies in the sample treatment development. Besides, the evaporation step enhanced sensitivity, leading to cleaner chromatograms, enabling measurement at lower concentrations and significantly improving peak shapes of the target compounds.

As previously described, in the preliminary studies, only a quarter part of the extract was evaporated until dryness, meaning the final signal represented only a quarter part of the total. Therefore, to further optimise this step, the evaporation volume of the supernatant was evaluated by comparing the evaporation of the total supernatant (4 mL), 2.5 mL supernatant, 1 mL supernatant, and no evaporation. In cases where the supernatant was evaporated to dryness, it was reconstituted with the same volume (200 µL) of acetonitrile/methanol/water (4/3/1, *v/v/v*), while 0.6 mL of methanol were added to 1 mL of the supernatant that has not been evaporated in order to achieve the same proportions of acetonitrile/methanol/water (4/3/1, *v/v/v*). As can be seen in Fig. 3, in most of the target compounds, there were no significant differences between the different evaporation volumes of the supernatant (lower than 10 %) as more amount of sample also indicate more amount of matrix and its respective interferences. For those cases where the evaporation volume did influence, it was observed that for OST the highest response factor was obtained with 1 mL, for MOR with 2.5 mL, and for RMT and ACV with 4 mL. Therefore, to simplify and speed up the sample treatment procedure, 1 mL of supernantant was selected for evaporation as the compromise solution.

Despite the reduction of interferences evidenced by the cleaner chromatograms obtained after the evaporation step, a few interferences in the selected MRM transitions still hinder the correct integration and identification of target compounds such as AMT, MMT, OSTA, RBV and ZNV. Therefore, several clean-up steps were tested before the evaporation step to further reduce interferences, minimize matrix effects, and improve sensitivity and chromatographic results. Among the tested procedures, the Primary and Secondary Amine (PSA) sorbent, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) kit, and the incorporation of solid-phase extraction (SPE) cartridges were evaluated [26,28,29,36,37].

In the case of PSA, the addition of different amounts of sorbent (50, 100 and 200 mg) to the initial extract (4 mL) as well as simultaneously the addition of formic acid in the extraction solvent acetonitrile/water (80/20, *v/v*) were evaluated. Briefly, results showed that higher PSA quantities resulted in higher signal intensity of target compounds (up to 90 % higher with the addition of 200 mg of PSA comparing to 50 mg) except for VRM and OSTA (Fig. S2A) whereas the addition of formic acid in the extraction solvent reduced the signal response for most of the target compounds by 50 % except for VRM (*data not shown*). Additionally, comparing to the procedure without clean-up steps (Fig. 4), ARB, ARBS, IMQ, MMT, RMT, OST, AMT, ACV, and VRM showed significantly lower signal responses using 200 mg of PSA as clean-up step (70–90 % lower). On the other hand, when using QuEChERS, the initial extract (4 mL) is mixed with a sorbent composed of PSA (400.1 mg), C18 (400.1 mg) and $MgSO_4$ (1199.8 mg). Besides, the effectiveness of magnesium sulfate ($MgSO_4$) as a desiccant was also assessed by adding it to the 4 mL supernatant before the first centrifugation, which generally resulted in worse signals (signal reduction between 25 and 90 %) except for ARB, ARBS, RMT and OST with which increased up to 40 % (Fig. S2B). Additionally, QuEChERS led to the loss of ZNV and a significant decrease in signal response for compounds such as MMT, RMT, AMT, ACV, GCV, PCV and VRM compared to the procedure without this clean-up step (Fig. 4). Additionally, the SPE evaluated in this study was carried out by diluting the supernatant (4 mL) with Milli-Q water with formic acid to achieve a pH of 0–2, and to protonate all the analytes and retain them on the cation exchange SPE column (MCX Oasis cartridge). The MCX Oasis SPE cartridges were washed several times using water (pH 0–2) and target compounds were eluted using 3 mL of acetonitrile or acetonitrile/water 80/20 *v/v* containing 5 % ammonium hydroxide (NH_4OH). In this study, different volumes of water, 4 mL and 12 mL, were tested to dilute the 4 mL supernatant, resulting in final water/acetonitrile ratios of 60/40 *v/v* and 80/20 *v/v*, respectively. Additionally, several acetonitrile proportions in the elution solvents (100 % acetonitrile and acetonitrile/water 80/20 *v/v*) were evaluated. However, neither approach resulted in significant signal differences (*data not shown*). Comparing the two different cation exchange SPE cartridges (MCX Oasis and Strata X-C) tested in this work, the Strata XC cartridges showed better response factor for most target compounds, except for ARB (Fig. S3). Despite that,

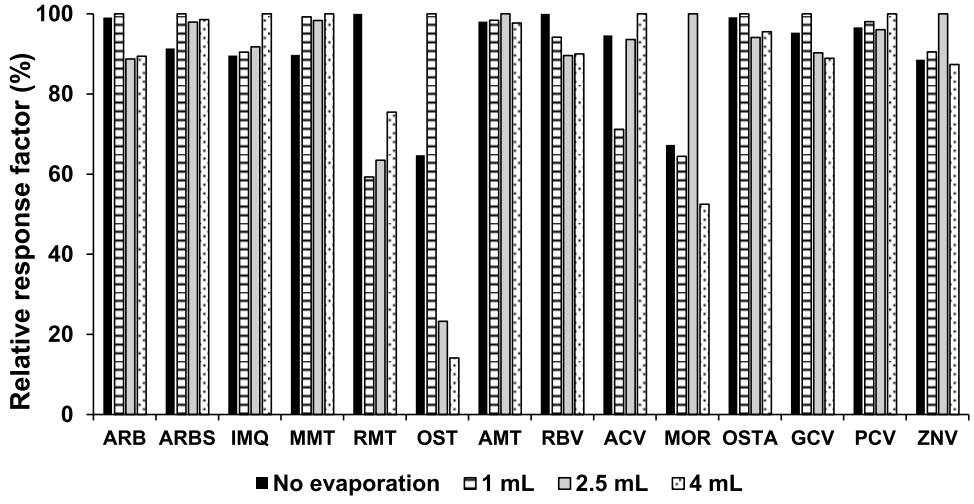

Fig. 3. Relative response factor of 14 target compound taking into account no-evaporation or the use of 1 mL, 2.5 mL or 4 mL of sample for evaporation in the sample treatment.

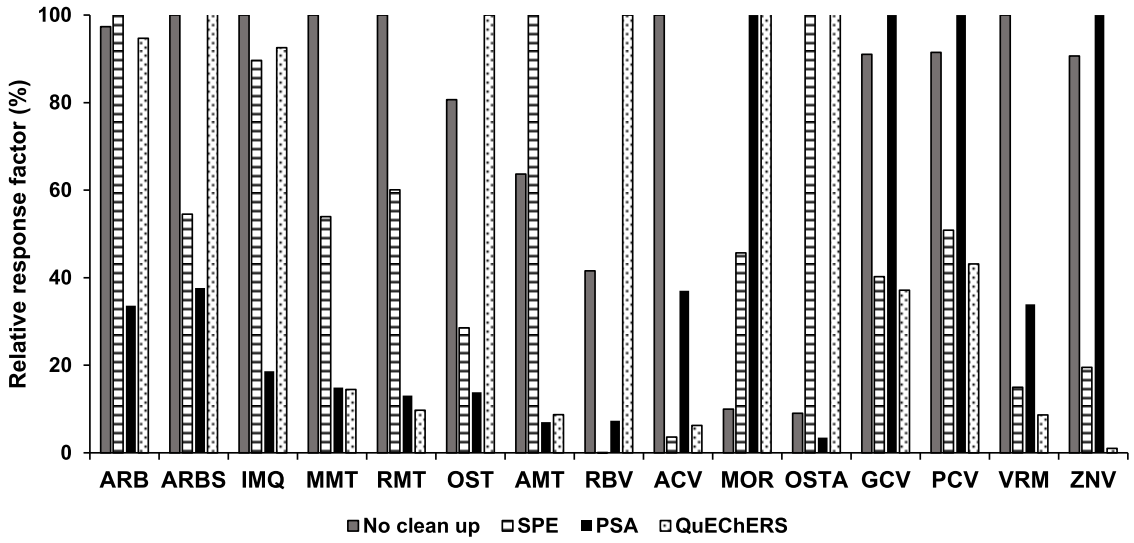

Fig. 4. Relative response factor of 15 target compound taking into account no-evaporation or the use of SPE (MCX Oasis cartridge, supernatant dilution with 4 mL water, elution with 100 % acetonitrile), PSA (200 mg, no formic acid in extraction solvent) and QuEChERS as clean-up step in the sample treatment.

RBV could not be detected with the SPE clean-up step whereas the rest of target compounds show substantially lower responses (compared to the method without clean-up steps (Fig. 4).

As last attempt, to reduce the possible lipid interferences in the used biologically derived samples, two additional SPE columns were tested: the Captiva EMR-Lipid SPE (effective enhanced matrix removal lipid, Agilent, USA) and the Finetech NH_2 sorbent SPE cartridge (40–75 μm, 70 Å, Finetech, TW). Regarding the Captiva EMR-Lipid cartridge, designed for lipid removal through size exclusion and hydrophobic interactions [38], it was proved to be ineffective, as it caused the lose of most of the studied compounds. In contrast, the NH_2 SPE sorbent cartridge, which removes negatively ionized interferences through interaction with ammonium ions (NH_3^+) [39], successfully extracted most compounds, although MOR, VRM, RBV, and ZNV were exceptions (*data not shown*). Therefore, it was not possible to include a clean-up step in the sample treatment procedure as it did not enable the detection of all target compounds in this study (Fig 4).

To evaluate the performance of the optimized sample treatment (Section 2.2.) in the developed LC–MS/MS method, the absolute recovery and matrix effect of all the 21 target compounds was studied at a concentration of 10 μg L^{-1}. As described in Section 2.4, the signals between the pre-extraction and post-extraction fortified extract and standard solutions of the corresponding concentration were compared, respectively in chicken muscle and liver. Some of the compounds such as EFV, LNV, RBV and ZNV could not be studied due to their lack of sensitivity at this concentration. Regarding the absolute recovery (Fig. S4A), all target compounds showed values above 60 % in both chicken muscle and liver except PMV and VRM which showed absolute recovery values of 42 and 51 %, respectively in chicken liver. Additionally, when evaluating the matrix (Fig. S4B), either ion enchancement or ion suppression could be observed in all the remaining antiviral drugs with the developed method. For this reason, to cope the matrix effect observed in the developed and proposed analytical method, the signal of each target compound is corrected by the corresponding internal standard and the quantification of the analytes were carried out by means of matrix fortified calibration curves.

3.3. Method validation

Antiviral drugs have been recently classified as unauthorized pharmacologically active substances in food-producing animals according to Commission Delegated Regulation (EU) 2022/1644 [15]. To date, no Maximum Residue Limits (MRL), Minimum Method Performance Requirement (MMPR) or Reference Point of Action (RPA) have been established for antiviral drugs in chicken muscle and liver. For this reason, the Lowest Calibration Level (LCL) of the target compound was applied for the validation and selected based on the feasibility of target compound with the developed analytical method.

3.3.1. Chicken muscle

As stated in Commission Implementing Regulation (EU) 2021/808 [33], the performance characteristics selectivity/specificity, identification, linearity, trueness, repeatability, within-lab reproducibility, CCα, ruggedness and stability were evaluated for the full validation of antiviral drugs in chicken muscle (*Section 2.4*.). From a food safety perspective, chicken muscle is relevant to monitor as it is an edible matrix commonly consumed by the human population and also, infections of chickens with influenza A virus have been very common in the past years, indicating that farmers could notice the use of antiviral drugs as a solution.

During the assessment of the selectivity/specificity, the blank chicken muscle extracts did not show any interfering peaks in the retention time window of the targeted antiviral drugs with the two selected MRM transitions for each compound, except for ACV and EFV. In these cases, an interfering peak was presented at one transition in a few blank samples, meaning that ACV and EFV could not be confirmed as the deviation of ion ratio between the two MRM transitions exceeded 40 % in at least two samples at all three validation levels. Also, ARB, ARBS, LNV, NVP, RBV, SQV, VRM and ZNV could not be confirmed for similar reasons as ACV and EFV. As consequence, these 10 target compounds are defined as screening compounds in chicken muscle. For ZNV, the applied 1*LCL (25 μg kg^{-1}) turned out to be too low to screen it as no clear signal was visible in many of the analyzed chicken muscle samples, and therefore 2*LCL (50 μg kg^{-1}) will be selected as the 1*LCL for future analyses. For screening compounds, in case of a suspect result, multilevel standard addition needs to be applied on the suspect sample to confirm the target compound in the sample.

The linearity of the analytical response within the matrix-fortified standard curve resulted for most target compounds in correlation coefficients (R^2) higher than 0.998, except for ARB, NVP, and VRM with R^2 of 0.974, 0.953 and 0.970 respectively. The accuracy, repeatability and within-lab reproducibility was determined at three validation levels (1, 2 and 3 times the LCL) based on 21 chicken muscle samples divided over three days (*Section 2.4*). Table 3 summarizes the performance characteristics of all target compounds in chicken muscle at the corresponding validation level. Comparing the LCL of each compound in this method

Table 3
Performance characteristics of the developed LC−MS/MS method per analyte in chicken muscle ($n = 21$).

	Concentration (μg kg^{-1})	Accuracy (%)	Repeatability (RSD, %)	Within lab reproducibility (RSD, %)	N° confirmation	CCα (μg kg^{-1})
ACV	0.5	103	7.5	7.5	16 / 21	–
	1	103	7.3	7.8	19 / 21	
	1.5	101	4.2	4.2	19 / 21	
AMT	0.15	104	7.0	7.4	20 / 21	0.18
	0.3	100	7.4	7.4	21 / 21	
	0.45	100	4.1	4.2	21 / 21	
ARB	1	102	24	24	19 / 21	–
	2	104	15	16	21 / 21	
	3	94	27	30	20 / 21	
ARBS	1	87	15	45	20 / 21	–
	2	118	29	29	20 / 21	
	3	99	22	22	17 / 21	
EFV	10	125	38	46	19 / 21	–
	20	115	34	45	19 / 21	
	30	111	37	57	18 / 21	
GCV	5	94	12	13	21 / 21	6.4
	10	99	14	14	21 / 21	
	15	96	10	12	21 / 21	
IMQ	0.15	102	12	12	21 / 21	0.19
	0.3	108	13	14	21 / 21	
	0.45	106	11	11	21 / 21	
LNV	20	99	21	25	18 / 21	–
	40	94	15	18	20 / 21	
	60	90	14	15	20 / 21	
LPV	0.15	104	14	15	21 / 21	0.20
	0.3	104	8.2	8.3	21 / 21	
	0.45	101	9.3	9.3	21 / 21	
MMT	0.15	98	7.8	9.1	21 / 21	0.18
	0.3	100	9.6	11	21 / 21	
	0.45	95	8.1	9.5	21 / 21	
MOR	0.5	98	8.8	9.0	21 / 21	0.60
	1	99	7.4	7.4	21 / 21	
	1.5	98	6.5	8.6	21 / 21	
NVP	1	81	23	33	19 / 21	–
	2	105	26	34	20 / 21	
	3	121	22	47	21 / 21	
OST	0.5	102	12	13	21 / 21	0.65
	1	101	14	15	21 / 21	
	1.5	99	14	16	21 / 21	
OSTA	2	103	14	17	21 / 21	2.8
	4	105	15	16	21 / 21	
	6	103	7.7	7.9	21 / 21	
PCV	2	100	14	14	20 / 21	2.6
	4	109	11	12	20 / 21	
	6	107	14	17	21 / 21	
PMV	5	92	18	19	21 / 21	7.1
	10	95	16	19	21 / 21	
	15	95	14	18	21 / 21	
RBV	25	81	24	28	16 / 21	–
	50	87	16	22	17 / 21	
	75	91	14	16	17 / 21	
RMT	1	103	9.5	11	21 / 21	1.3
	2	97	8.4	15	21 / 21	
	3	99	8.4	12	21 / 21	
SQV	0.15	99	15	25	19 / 21	–
	0.3	97	15	18	19 / 21	
	0.45	97	9.7	9.9	21 / 21	
VRM	5	123	22	27	21 / 21	–
	10	129	20	23	19 / 21	
	15	133	17	17	20 / 21	
ZNV	25	*	*	*	*	
	50	101	42	47	19 / 21	–
	75	98	48	51	19 / 21	

* Not possible to screen ZNV due to absence of signal in many chicken muscle samples, and therefore performance characteristics could not be determined. The 1*LCL is therefore changed to 50 μg kg^{-1} for future analyses.

with Douillet et al.'s study [29], the LCL for ACV, OST, OSTA, and RBV are the same.

The accuracy, expressed in percentage (%), were between 81 and 118 for most target compounds. Only EFV, NVP, and VRM showed accuracies higher than the maximum acceptable criteria of 120 % according Commission Implementing Regulation (EU) 2021/808. The repeatability showed acceptable relative standard deviation (RSD, %) values ranging from 4.1 to 18 % ($n = 21$), while acceptable within-lab reproducibility results were between 4.2 and 29 % ($n = 21$), except for the compounds ARB, ARBS, EFV, LNV, NVP, RBV, VRM, and ZNV whose repeatability values and within-lab reproducibility exceeded at some validation levels the maximum acceptable criteria of 17–20 and 25–30 % depending on the concentration of the target compound in chicken muscle. As a result, the 8 target compounds that exceeded the

criteria for trueness and/or precision are defined as qualitative compounds in chicken muscle. In case of a suspect result, multi-level standard addition needs to be applied on the suspect sample to correctly quantify the target compound in the sample. The CCα can be only calculated based on the validation results for target compounds whose method is quantitative confirmation. For 11 out of 21 antiviral drugs, the CCα were determined between 0.18 and 7.05 μg kg^{-1}. In the case of CCα reported by Douillet et al. [29], the values for OST and OSTA are practically the same, while some CCα values are half as high (RMT) or one-third as high (PMV, GCV) compared to those determined in the present study.

In summary, the validation results showed that the developed method in chicken muscle can be used as a quantitative confirmatory method for 11 out of the 21 studied antiviral drugs (AMT, GCV, IMQ, LPV, MMT, MOR, OST, OSTA, PCV, PMV, RMT), as quantitative screening for 2 analytes (ACV, SQV) and qualitative screening for 8 analytes (ARB, ARBS, EFV, LNV, NVP, RBV, VRM, ZNV).

The ruggedness of the method was examined by applying minor modifications to the sample treatment procedure as described in Section 2.4. The analytes ACV, ARB, ARBS, LNV, and NVP showed to be affected by one to three of the applied clean-up modifications as the trueness (%) and RSDr (%) values deviated significantly compared to the sample treatment without modification. In conclusion, the bead ruptor program, evaporation time and temperature are critical steps in the sample treatment, and must be carried out properly.

Additionally, the stability of the target compounds in extract was evaluated as described in Section 2.4. The results from the reanalysis showed similar results in comparison to the full validation, indicating that the target compounds are stable in extract for a week when stored in the freezer (−20 °C).

3.3.2. Chicken liver

For chicken liver, a partial validation of the analytical method was conducted, evaluating parameters such as selectivity/specificity, identification, linearity, trueness, repeatability, within-lab reproducibility, and CCα in accordance with Commission Implementing Regulation (EU) 2021/808 [33]. Additionally, to simplify the workflow and increase the laboratory's throughput, as Arrizabalaga-Larrañaga et al. suggested for the determination of sedatives in animal tissues [40], the possibility of applying the chicken muscle as matrix-fortified calibration curves for quantifying the target compounds in chicken liver samples was assessed. For these purpose, performance characteristics in chicken liver were calculated based on matrix-fortified standard curves in (i) chicken liver and (ii) chicken muscle (Table S1). These studies showed that the matrix-fortified standard curve of chicken liver yields better results for chicken liver analysis in accuracy (%), repeatabilty (RSD, %), and within-lab reproducibility (RSD, %). Hence, in this case, it is not possible to use a single matrix-fortified standard curve for the determination of antivirals drugs in other animal tissues and it requires its respective tissue. During the assessment of the selectivity/specificity, the blank chicken liver extracts did not show any interfering peaks in the retention time window of the targeted antiviral drugs with the two selected MRM transitions for each compound. Therefore, most antiviral drugs could be confirmed with the method as the deviation in ion ratio and retention time was <40 % and <1 %, respectively, except for ACV, AMD, LNV and PMV.

The linearity of the analytical response within the matrix-fortified standard curve resulted for most target compounds in correlation coefficients (R^2) higher than 0.998, except for VRM and LNV, with R^2 of 0.963 and 0.944, respectively. The trueness, repeatability and within-lab reproducibility was determined at three validation levels (1, 2 and 3 times the LCL) based on seven chicken liver samples on a single day (described in Section 2.4). The same LCL were set for chicken muscle and liver to minimize the complexicity in routine analysis, as the same mixed-standard solutions can be applied for both tissues. The accuracies, expressed in percentage (%), were between 71 and 120 for most target compounds. Only LNV and VRM showed accuracies beyond the maximum acceptable criteria at the corresponding validation level according to Commission Implementing Regulation (EU) 2021/808 [33]. The repeatability showed acceptable relative standard deviation (RSD, %) values ranging from 3.1 to 20 % ($n = 7$), while acceptable within-lab reproducibility results were between 4.6 and 30 % ($n = 7$), except for the compounds ARB, ARBS, EFV, LNV, PCV, PMV, VRM, and ZNV. The repeatability and within-lab reproducibility values of those compounds exceeded at some validation levels the maximum acceptable criteria of 17–20 and 25–30 % depending on the concentration of the target compound in chicken liver. The CCα can be only calculated based on the validation results for target compounds whose method is quantitative confirmation. For 11 out of 21 antiviral drugs, the CCα were determined between 0.19 and 36 μg kg^{-1}.

As a result, the validation results showed that the developed method in chicken liver can be used as a quantitative confimatory method for 11 out of the 21 studied antiviral drugs, (GCV, IMQ, LPV, MMT, MOR, NVP, OST, OSTA, RBV, RMT, and SQV), as qualitative confirmation for 6 analytes (ARB, ARBS, EFV, PCV, VRM, and ZNV), as quantitative screening for 2 analytes (ACV and AMT) and qualitative screening for 2 analytes (LNV and PMV).

3.4. Sample analysis

The developed method was applied to monitor antiviral drugs in chicken muscle and liver at the European Union Reference Laboratory (EURL) in Wageningen. A total of 10 chicken liver samples and 10 chicken muscle samples were analyzed, and resulted negative samples as none of the studied compounds were detected in any of the samples. Fig. 5 shows an UHPLC−ESI−MS/MS extracted chromatogram obtained by spiking a blank chicken liver sample at LCL level for each target compound. As observed in Fig. 5, the target compounds can be easily detected at this concentration level. On the base of these findings, the feasibility of the UHPLC−APCI−MS/MS method has been demonstrated, and it can be proposed for the reliable determination of 21 antivirals drugs in chicken muscle and liver for official controls as required by EU legislation. As it is highlighted by Sasse et al., there is a lack of data with regard to the occurrence of antiviral drugs in food-producing animals in different countries [4]. Therefore, data collection by means of sample analysis is essential for food control laboratories to determine if antiviral drugs pose a food safety risk in the world.

4. Conclusions

Recently, in 2022, antiviral drugs have been included in Commission Delegated Regulation (EU) 2022/1644 under the prohibited or unauthorized pharmacologically active substances in food-producing animals (group A). As a consequence, the need for food control laboratories in the European Union to develop analytical methods for their determination and perform official controls has increased significantly. Therefore, as the European Union Reference Laboratory, we have worked on the development of an easy, fast and sensitive method for antiviral drugs in chicken muscle and liver to assist Member States to implement it in their laboratories and evaluate whether antiviral drugs are used in food-producing animals and pose a food safety risk. The developed UHPLC–MS/MS method has proved to be a reliable and accurate method for the simultaneous determination of 21 antivirals drugs in chicken muscle and liver samples. The use of a UHPLC HILIC analytical column yielded in an efficient chromatographic separation and resolution of most target compounds in a short analysis time (< 8 min). In the mass spectrometry, all antiviral drugs showed the protonated molecule $[M + H]^+$ as the base peak, except efavirenz yielding the $[M\text{-}H]^-$ as base peak. The most relevant product ions for quantification and confirmation purposes in the determination of antiviral drugs by UHPLC−MS/MS in MRM mode were identified. A generic sample preparation procedure was required to cover the multiple classes of antiviral drugs, in which

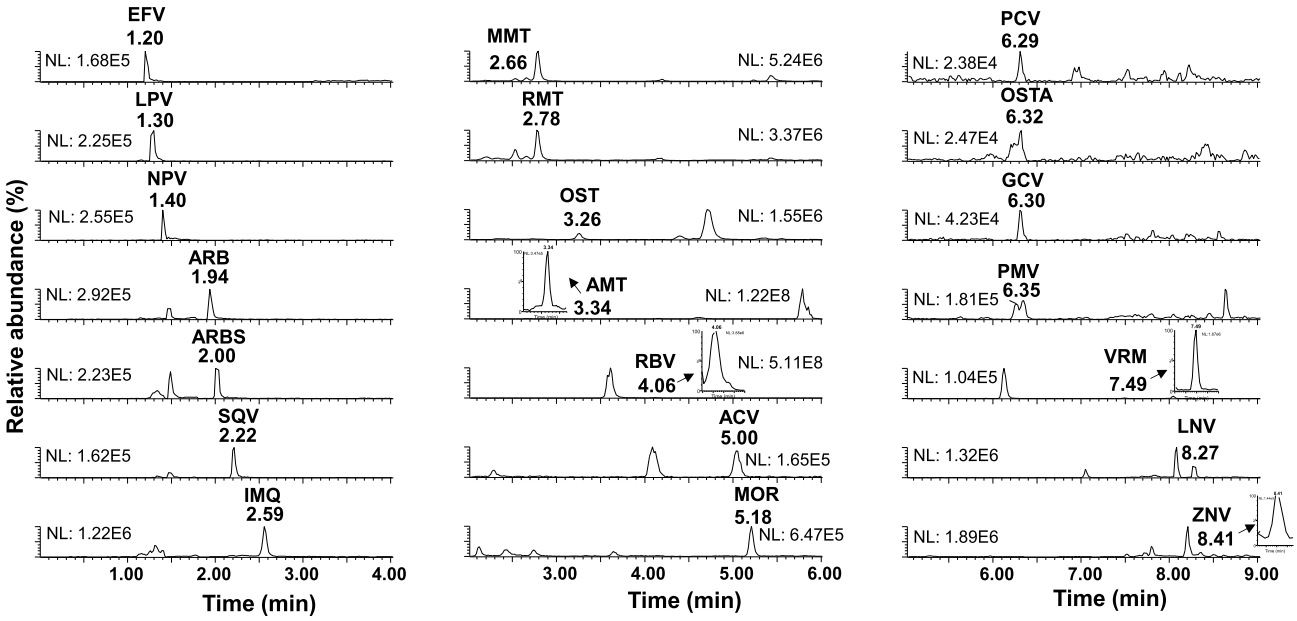

Fig. 5. UHPLC–ESI–MS/MS extracted chromatogram obtained by spiking a blank chicken liver sample at LCL level for each target compound.

the analytes were extracted from the homogenized chicken tissues using an acetonitrile: water (80:20) mixture followed by evaporation and reconstitution to obtain the highest extraction efficiencies.

The developed UHPLC-MS/MS method for antiviral drugs in chicken muscle and liver has been successfully validated according to Commission Implementing Regulation (EU) 2021/808. The obtained performance characteristics demonstrated that the method is accurate, reproducible, selective and sensitive, with lowest calibration levels at 0.15 μg kg^{-1} for amantadine, imiquimod, memantine, saquinavir and lopinavir, up to 50 μg kg^{-1} for zanamivir. The performance of the developed UHPLC–MS/MS method and the relevant results obtained in the analysis of chicken muscle and liver samples have demonstrated its applicability to determine 21 antiviral drugs under the residue control requirement established in EURL Guidance Paper. To the best of our knowledge, this is the first time that an analytical method was developed and validated with such a broad scope of antiviral drugs and has been implemented for the determination of food residues in National Control Plan, showing important steps for the future risk assessment in the European Union.

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CRediT authorship contribution statement

Ana Castiñeira-Landeira: Writing – original draft, Validation, Methodology, Investigation. **Samantha Sasse:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Melissa Broeren:** Validation, Methodology, Investigation, Conceptualization. **Saskia S. Sterk:** Funding acquisition. **Ane Arrizabalaga-Larrañaga:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.465711.

Data availability

Data will be made available on request.

References

[1] J. Louten, Chapter 5 - virus transmission and epidemiology, editor, in: J Louten (Ed.), Essential Human Virology, Academic Press, Boston, 2016, pp. 71–92.
[2] Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC. 2019/6.
[3] European Food Safety Authority. Disease profiles. Retrieved September 2023 from https://animal-diseases.efsa.europa.eu/. (2023). Accessed.
[4] S. Sasse, A. Arrizabalaga-Larrañaga, S.S. Sterk, Antiviral drugs in animal-derived matrices: a review, Heliyon 10 (18) (2024) e37460, https://doi.org/10.1016/j.heliyon.2024.e37460.
[5] Advice On the Designation of Antimicrobials Or Groups of Antimicrobials Reserved For Treatment of Certain Infections in Humans - in relation to Implementing Measures Under Article 37(5) of Regulation (EU) 2019/6 On Veterinary Medicinal Products, European Medicines Agency, 2022.
[6] J.F. Acar, G. Moulin, S.W. Page, P.P. Pastoret, Antimicrobial resistance in animal and public health: introduction and classification of antimicrobial agents, Revue Scientifique et Technique 31 (1) (2012) 8, https://doi.org/10.1016/j.aca.2024.343335.
[7] D. Cyranoski, China's chicken farmers under fire for antiviral abuse, Nature 435 (7045) (2005) 1009, https://doi.org/10.1038/4351009a.
[8] G. He, J. Qiao, C. Dong, C. He, L. Zhao, Y. Tian, Amantadine-resistance among H5N1 avian influenza viruses isolated in Northern China, Antiviral Res. 77 (1) (2008) 72–76, https://doi.org/10.1016/j.antiviral.2007.08.007.
[9] J. Parry, Use of antiviral drug in poultry is blamed for drug resistant strains of avian flu, BMJ 331 (7507) (2005) 10, https://doi.org/10.1136/bmj.331.7507.10.
[10] Ministry of Agriculture of the People's Republic of China. (2005). Announcement No. 560 of the Ministry of Agriculture of the People's Republic of China. Retrieved

December 2023 from http://www.moa.gov.cn/so/s?qt=560&tab=gk; http://www.moa.gov.cn/govpublic/SYJ/201006/t20100606_1535306.htm; https://animal-diseases.efsa.europa.eu/(2005). Accessed.
[11] Food and Drug Administration. (2006). New animal drugs; adamantane and neuraminidase inhibitor anti-influenza drugs; extralabel animal drug use; order of prohibition. (Accessed November 2024) https://www.federalregister.gov/documents/2006/03/22/06-2689/new-animal-drugs-adamantane-and-neuraminidase-inhibitor-anti-influenza-drugs-extralabel-animal-drug. Accessed.
[12] Food and Drug Administration. (2023). Title 21/part 530 - extralabel drug use in animals. (Accessed November 2024) https://www.ecfr.gov/current/title-21/chapter-I/subchapter-E/part-530. Accessed.
[13] R. Nakato, J.K. Tumwine, S. Nanzigu, S. Naluyima, A. Buzibye, L. Alinayitwe, et al., Antiretroviral drugs found in pork on Ugandan market: implications for HIV/AIDS treatment, One Health 9 (2020) 100125, https://doi.org/10.1016/j.onehlt.2020.100125.
[14] D. Ndoboli, N. Frederick, B. Lukuyu, B. Wieland, D. Grace, Braun Av, et al., The misuse of antiretrovirals to boost pig and poultry productivity in Uganda and potential implications for public health, Int J One Health 7 (2021) 8, https://doi.org/10.14202/IJOH.2021.88-95.
[15] Commission delegated Regulation (EU) 2022/1644 of 7 July 2022 supplementing regulation (EU) 2017/625 of the European Parliament and of the Council with specific requirements for the performance of official controls on the use of pharmacologically active substances authorised as veterinary medicinal products or as feed additives and of prohibited or unauthorised pharmacologically active substances and residues thereof 2022/1644.
[16] D. Peng, W. Wei, Y. Pan, Y. Wang, D. Chen, Z. Liu, et al., Preparation of a monoclonal antibody against amantadine and rimantadine and development of an indirect competitive enzyme-linked immunosorbent assay for detecting the same in chicken muscle and liver, J. Pharm. Biomed. Anal. 133 (2017) 56–63, https://doi.org/10.1016/j.jpba.2016.11.009.
[17] L. Xu, S. Peng, L. Liu, S. Song, H. Kuang, C. Xu, Development of sensitive and fast immunoassays for amantadine detection, Food Agric. Immunol. 27 (5) (2016) 678–688, https://doi.org/10.1080/09540105.2016.1148667.
[18] J. Zhu, Q. Li, X. Yu, X. Zhang, H. Li, K. Wen, et al., Synthesis of hapten, production of monoclonal antibody, and development of immunoassay for ribavirin detection in chicken, J. Food Sci. 86 (7) (2021) 2851–2860, https://doi.org/10.1111/1750-3841.15789.
[19] X. Huo, S. Wang, K. Lai, J Peng, Sensitive CG-ICA based on heterologous coating antigen and mAb prepared with carbons-linker immunogen, Food Agric. Immunol. 32 (1) (2021) 727–739, https://doi.org/10.1080/09540105.2021.1987393.
[20] S. Wu, F. Zhu, L. Hu, J. Xia, G. Xu, D. Liu, et al., Development of a competitive immunochromatographic assay for the sensitive detection of amantadine in chicken muscle, Food Chem. 232 (2017) 770–776, https://doi.org/10.1016/j.foodchem.2017.04.058.
[21] Y. Gao, X. Wu, Z. Wang, P. Luo, L. Xu, Q. Zheng, et al., A sensitive lateral flow immunoassay for the multiple residues of five adamantanes, Food Agric. Immunol. 30 (1) (2019) 647–661, https://doi.org/10.1080/09540105.2019.1612331.
[22] X. Ning, J. Qiu, A semi-quantitative multi-range lateral flow immunoassay for amantadine residues in livestock and poultry products, Food Agric. Immunol. 33 (1) (2022) 817–831, https://doi.org/10.1080/09540105.2022.2137472.
[23] M. Ma, J. Sun, Y. Chen, K. Wen, Z. Wang, J. Shen, et al., Highly sensitive SERS immunosensor for the detection of amantadine in chicken based on flower-like gold nanoparticles and magnetic bead separation, Food Chem. Toxicol. 118 (2018) 589–594, https://doi.org/10.1016/j.fct.2018.06.013.
[24] J. Yang, M. Pan, K. Liu, X. Xie, S. Wang, L. Hong, et al., Core-shell AuNRs@Ag-enhanced and magnetic separation-assisted SERS immunosensing platform for amantadine detection in animal-derived foods, Sens. Actuators B 349 (2021) 130783, https://doi.org/10.1016/j.snb.2021.130783.
[25] X. Yang, J. Yang, Y. Wang, L. Li, Z. Sun, Z. Yue, et al., A lateral flow immunochromato-graphic strip test for rapid detection of oseltamivir phosphate in egg and Chicken meat, Sci. Rep. 8 (1) (2018) 16680, https://doi.org/10.1038/s41598-018-35080-5.
[26] B.J.A. Berendsen, R.S. Wegh, M.L. Essers, A.A.M. Stolker, S. Weigel, Quantitative trace analysis of a broad range of antiviral drugs in poultry muscle using column-switch liquid chromatography coupled to tandem mass spectrometry, Anal. Bioanal. Chem. 402 (4) (2012) 1611–1623, https://doi.org/10.1007/s00216-011-5581-3.
[27] Z. Liu, F. Yang, M. Yao, Y. Lin, Z Su, Simultaneous determination of antiviral drugs in chicken tissues by ultra high performance liquid chromatography with tandem mass spectrometry, J. Sep. Sci. 38 (10) (2015) 1784–1793, https://doi.org/10.1002/jssc.201401461.
[28] P. Mu, N. Xu, T. Chai, Q. Jia, Z. Yin, S. Yang, et al., Simultaneous determination of 14 antiviral drugs and relevant metabolites in chicken muscle by UPLC–MS/MS after QuEChERS preparation, J. Chromatogr. B 1023-1024 (2016) 17–23, https://doi.org/10.1016/j.jchromb.2016.04.036.
[29] C. Douillet, M. Moloney, M. Di Rocco, C. Elliott, M. Danaher, Development and validation of a quantitative method for 15 antiviral drugs in poultry muscle using liquid chromatography coupled to tandem mass spectrometry, J. Chromatogr. A 1665 (2022) 462793, https://doi.org/10.1016/j.chroma.2021.462793.
[30] R. Shen, X. Xu, Q. Shuai, L. Huang, Fast and efficient extract oseltamivir from aquatic products using magnetic covalent organic frameworks/graphene oxide composite prior to liquid chromatography-tandem mass spectrometry analysis, Food Chem. 396 (2022) 133646, https://doi.org/10.1016/j.foodchem.2022.133646.
[31] Y.L. Wu, R.X. Chen, Y. Xue, T. Yang, J. Zhao, Y. Zhu, Simultaneous determination of amantadine, rimantadine and memantine in chicken muscle using multi-walled carbon nanotubes as a reversed-dispersive solid phase extraction sorbent, J. Chromatogr. B 965 (2014) 197–205, https://doi.org/10.1016/j.jchromb.2014.06.038.
[32] H. Yan, X. Liu, F. Cui, H. Yun, J. Li, S. Ding, et al., Determination of amantadine and rimantadine in chicken muscle by QuEChERS pretreatment method and UHPLC coupled with LTQ Orbitrap mass spectrometry, J. Chromatogr. B 938 (2013) 8–13, https://doi.org/10.1016/j.jchromb.2013.08.020.
[33] Commission Implementing Regulation (EU) 2021/808 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing decisions 2002/657/EC and 98/179/EC. 2021/808.
[34] Chemaxon. (2024). Playground v1.6.2. (Accessed November 2024) https://playground.calculators.cxn.io/. Accessed.
[35] H.P. Nguyen, K.A. Schug, The advantages of ESI-MS detection in conjunction with HILIC mode separations: fundamentals and applications, J. Sep. Sci. 31 (9) (2008) 1465–1480, https://doi.org/10.1002/jssc.200700630.
[36] Y. Tsuruoka, T. Nakajima, M. Kanda, H. Hayashi, Y. Matsushima, S. Yoshikawa, et al., Simultaneous determination of amantadine, rimantadine, and memantine in processed products, chicken tissues, and eggs by liquid chromatography with tandem mass spectrometry, J. Chromatogr. B 1044-1045 (2017) 142–148, https://doi.org/10.1016/j.jchromb.2017.01.014.
[37] Y.L. Wu, R.X. Chen, L. Zhu, Y. Lv, Y. Zhu, J. Zhao, Determination of ribavirin in chicken muscle by quick, easy, cheap, effective, rugged and safe method and liquid chromatography-tandem mass spectrometry, J. Chromatogr. B 1012-1013 (2016) 55–60, https://doi.org/10.1016/j.jchromb.2016.01.016.
[38] Agilent, Sample Filtration Captiva EMR-lipid and Captiva EMR-lipid HF. https://www.agilent.com/en/product/sample-preparation/filtration/captiva-emr-lipid#howitworks, 2024 (accessed 19 August 2024). Accessed.
[39] Finetech Research and Innovation Corporation, NH2 SPE Cartridge. https://www.finetech-filters.com/nh2-spe, 2024 (accessed 19 August 2024). Accessed.
[40] A. Arrizabalaga-Larrañaga, D. van Doorn, S.S. Sterk, Towards high throughput analysis using 96-well plate solid phase extraction to determine sedatives and β-blocker residues in food control monitoring, Anal. Chim. Acta 1331 (2024) 343335, https://doi.org/10.1016/j.aca.2024.343335.