

Development and validation of a quantitative method for 15 antiviral drugs in poultry muscle using liquid chromatography coupled to tandem mass spectrometry

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Abstract

The objective of this work was to develop a quantitative multi-residue method for analysing antiviral drug residues and their metabolites in poultry meat samples. Antiviral drugs are not licensed for the treatment of influenza in food producing animals. However, there have been some reports indicating their illegal use in poultry. In this study, a method was developed for the analysis of 15 antiviral drug residues in poultry muscle (chicken, duck, quail and turkey) using liquid chromatography coupled to tandem mass spectrometry. This included 13 drugs against influenza and associated metabolites, but also two drugs employed for the treatment of herpes (acyclovir and ganciclovir). The method required the development of a novel chromatographic separation using a hydrophilic interaction chromatographic (HILIC) BEH amide column, which was necessary to retain the highly polar compounds. The analytes were detected using a triple quadrupole mass spectrometer operating in positive electrospray ionisation mode. A range of different sample preparation protocols suitable for polar compounds were evaluated. The most effective procedure was based on a simple acetonitrile-based protein precipitation step followed by a further dilution in a methanol/water solution. The confirmatory method was validated according to the EU 2021/808 guidelines on different species including chicken, duck, turkey and quail. The validation was performed using various calibration curves ranging from 0.1 $\mu\text{g kg}^{-1}$ to 200 $\mu\text{g kg}^{-1}$, according to the analyte. Depending on the analyte sensitivity, decision limits achieved ranged from 0.12 $\mu\text{g kg}^{-1}$ for arbidol to 34.7 $\mu\text{g kg}^{-1}$ for ribavirin. Overall, the reproducibility precision values ranged from 2.8% to 22.7% and the recoveries from 84% to 127%. The method was applied to 120 commercial poultry samples from the Irish market, which were all found to be residue-free.

Keywords

Antiviral drug residues
Influenza
HILIC
LC-MS/MS
Poultry muscle

1. Introduction

40 Influenza is a viral infectious disease that causes around 1 billion infections and between 300,000 and
41 500,000 deaths a year in humans globally [1]. The most common influenza symptoms are fever,
42 headache, dry cough and nasal congestion, but complications can arise for vulnerable individuals
43 causing more serious illness or death [2]. In humans, there are three types of influenza virus named type
44 A, B and C. Influenza is primarily described as a seasonal epidemic form, but pandemic normally occurs
45 every 10-50 years. They are due to antigenic drifts that lead to the emergence of very different influenza
46 strains to compare with previous ones [3]. These new strains were found to be of zoonotic origin, being
47 a combination of human and animal viruses such as avian influenza. Avian influenza outbreaks are a
48 concerning issue for both intensive poultry production and wild bird species. A number of different
49 strains of avian flu have been reported and can spread to humans causing high fatalities [4]. Scientists
50 concluded that the major influenza pandemics that led to more than 50 million deaths during the 20th
51 century were caused by strains resulting from a combination of animal and human origin [5]. More
52 recently, H5N1 has been spreading worldwide since 2004, and a new strain, namely H10N3, has
53 recently been found to infect humans in China [6]. With the continuous emergence of new strains, which
54 are more aggressive and more resistant to antiviral drugs [7], there are have been increasing concerns
55 about a global outbreak of avian influenza [8].

56 To counteract outbreaks, various drugs for treating influenza have been developed over the years. These
57 drugs can be grouped into different classes depending on their chemical structures or supposed mode
58 of action, neuraminidase inhibitors, fusion inhibitors, adamantanes, guanosine nucleoside analogues or
59 pyrazine-derived [9, 10].

60 Despite antiviral drugs not being licensed for the treatment of influenza infections in food producing
61 animals, amantadine has been previously detected in Chinese poultry [11-13]. As a consequence, China
62 has issued a ban on the use of amantadine in poultry farms [14]. The U.S. Food and Drug Administration
63 (FDA) banned the use of amantadine, rimantadine, zanamivir and oseltamivir in poultry in the United
64 States [15]. In the EU, antiviral drugs are not currently listed in the framework of residues that have to
65 be analysed in foods of animal origin, but it is suggested that this will likely change in newly proposed
66 legislation on National Residue Control Plans.

67 There have been a number of reports of toxicological effects following the administration of amantadine
68 or ribavirin in humans [16, 17]. However, the major concern over antiviral drugs is that their usage in
69 animal production could lead to the development of more drug resistant strains of viruses, which could
70 decrease the effectiveness of human medicines. For example, it has been proposed that the abuse of
71 amantadine was an important driver to the emergence of new H5N1 strains [18]. Furthermore, the
72 spreading of poultry litter contaminated with antiviral drug into the environment could potentially lead
73 to ingestion of these drugs by avian species like waterfowls and therefore increase the incidence of
74 antiviral resistant strains [19, 20].

75 To control the illegal use of antiviral drugs in poultry, it is necessary to develop and update sensitive
76 analytical methods quantifying antiviral drugs used against influenza in poultry tissue. An extensive
77 range of tests have been recently developed by Asian researchers for analysing antiviral drug residues
78 in food using various screening techniques. These methodologies have been mainly applied to detect a
79 selection of drugs such as amantadine or rimantadine [21], oseltamivir [22] and ribavirin [23] in poultry
80 tissue. Examples of the detection techniques applied include antibody-based surface-enhanced Raman
81 spectroscopy, immunochromatographic strip tests and molecularly imprinted polymer-based
82 chemiluminescence methods [24, 22, 21]. While these methods are fast and sensitive, they are often
83 specific to one drug or a structurally similar molecule, and provide non-quantitative results. Liquid
84 chromatography coupled to tandem mass spectrometry (LC-MS/MS) is nowadays the most widely used

85 technique for the analysis of drug residues in food of animal origin. Several LC-MS/MS methods have
86 been reported in literature for antiviral drug residue analysis but many of these methods include only a
87 limited range of analytes [25-27]. This is likely due to the highly polar nature of many antiviral drugs,
88 which hinders their inclusion in multi-residue methods. Some researchers have reported more
89 comprehensive methods, but excluded the most polar neuraminidase inhibitor drugs [28, 13], while
90 Chan *et al.* [29] developed a screening method for eight drugs, from highly polar to non-polar.
91 Berendsen *et al.* [30] reported a quantitative method for seven polar drugs, but employed a complicated
92 dual solid-phase extraction (SPE) and a combination of two liquid chromatography columns.

93 This paper reports a fast sample extraction combined with LC-MS/MS detection for the quantitative
94 and confirmatory analysis of 15 antiviral drugs against influenza and their metabolites in poultry
95 muscle. This includes a broad range of analytes from highly polar to non-polar compounds. The
96 developed method includes a fast solid-liquid sample extraction followed by a simple dilution step.
97 Separation was achieved using an amide HILIC based column that provided better retention,
98 chromatographic peak shape and separation of the analytes. Matrix effects were also more suitable when
99 using a HILIC type column. The method was fully validated following new EU guidelines [31] and
100 applied to poultry samples purchased on the Irish retail market. This is the first time that this range of
101 compounds has been quantified in food using a single method.

102 **2. Experimental**

103 *2.1. Reagents and Apparatus*

104 Laninamivir (LNV), viremagine HCl (VMD), arbidol sulfone (ABTS), arbidol sulfoxide (ABDS),
105 amantadine-d₁₅ HCl (AMT IS), arbidol-d₆ (ABD IS), oseltamivir-d₃ acid (OSTA IS), oseltamivir-d₃
106 phosphate (OST IS), ribavirin ¹³C₅ (RBV IS), ganciclovir-d₅ (GCV IS) and zanamivir-¹³C¹⁵N₂ (ZNV
107 IS) were supplied by Toronto Research Chemical (North York, ON, Canada). Amantadine HCl (AMT),
108 rimantadine HCl (RMT), oseltamivir phosphate (OST), acyclovir (ACV), ganciclovir (GCV),
109 zanamivir hydrate (ZNV), ribavirin (RBV), arbidol hydrochloride (ABDS), oseltamivir acid (OSTA),
110 favipiravir (FVP) and peramivir (PMV) were sourced from Carbosynth (St Gallen, Switzerland).

111 Methanol (MeOH) and acetonitrile (MeCN) were supplied by Romil (Cambridge, UK). Ultra-pure
112 water (18.2 MΩ cm⁻¹) was prepared in-house using a Merck Millipore (Cork, Ireland) water purification
113 system. Dimethyl sulfoxide (DMSO) was obtained from Sigma Aldrich (Dublin, Ireland). Ammonium
114 acetate (LC-MS grade) was purchased from VWR International (Leuven, Belgium), while formic acid
115 was sourced from Honeywell Fluka (Seelze, Germany).

116 Polypropylene tubes (15 and 50 mL) with screw caps were sourced from Sarstedt Ltd (Wexford,
117 Ireland). PTFE membrane syringeless filters 0.2 μm (Mini-UniPrep™) were purchased from GE
118 Healthcare (Buckinghamshire, UK). A Merris Minimix Vibrational Shaker was obtained from Merris
119 Engineering (Milltown, Co. Galway, Ireland). The Rotanta 460R refrigerated centrifuge was supplied
120 by Hettich (Kirchlengern, Germany).

121 *2.2. Standard Preparation*

122 Individual stock standard solutions of 1 mg mL⁻¹ were prepared by dissolving each compound in the
123 appropriate solvent. Rimantadine, oseltamivir acid, oseltamivir-d₃ acid, oseltamivir, oseltamivir-d₃,
124 arbidol, arbidol-d₆, amantadine, amantadine-d₁₅, arbidol sulfoxide and arbidol sulfone were dissolved
125 in MeOH. Ultrapure water was used for ribavirin, ribavirin-¹³C₅, zanamivir, zanamivir-¹³C¹⁵N₂,
126 peramivir, favipiravir, laninamivir and viremagine, while ganciclovir, ganciclovir-d₅ and acyclovir
127 solutions were prepared in DMSO.

128 One intermediate mixed solution was prepared at concentrations between 0.20 and 50 μg mL⁻¹ in

129 MeCN:H₂O (50:50, v/v), depending on the analyte. Eight working calibration and two recovery standard
130 solutions were prepared from the intermediate solution by diluting in MeCN:H₂O (50:50, v/v). The
131 concentration of these solutions for each analyte is listed in **Table S1**. A mixed internal standard
132 solution was also prepared at concentrations ranging from 20 µg L⁻¹ to 1,000 µg L⁻¹ in MeCN:H₂O
133 (50:50, v/v). All solutions were stored at -30°C and found to be stable for at least three months.

134 2.3. *Quality Control and Calibration Curve*

135 Avian muscle tissue samples found to contain no detectable residues were selected as negative controls.
136 An eight-point procedural matrix matched calibration curve was prepared by fortifying negative control
137 muscle samples with 100 µL of each working calibration standard.

138 Quality control samples (i.e. recovery controls) were prepared with each batch of analysis by spiking
139 extracts post-extraction with 100 µL of low recovery solution (n = 2) and 100 µL of high recovery
140 solution (n = 2) to monitor the extraction efficiency. The levels spiked provided a final concentration
141 equivalent to calibrant 3 and calibrant 7.

142 2.4. *Sample Preparation*

143 Muscle tissue (5.0 ± 0.05 g) was weighed into a 50 mL polypropylene tube. A 50 µL volume of the
144 internal standard solution was added to all samples, while 100 µL of the working standards were added
145 to the calibrants. Samples were subsequently let to stand for 15 minutes. A 20 mL volume of
146 MeCN:H₂O (80:20, v/v) and ceramic homogenizers were then added to each tube. The tubes were
147 shaken for 1 min on a Minimix vibrational shaker. The samples were then centrifuged at 2725 x g for
148 10 min at -4°C. One mL portions of the supernatants were subsequently transferred into 15 mL
149 polypropylene tubes, 0.6 mL volumes of a MeOH:H₂O (2:1, v/v) solution were added, and the tubes
150 vortexed for 30 s. The reconstituted samples were filtered using PTFE syringeless filter vials, and
151 injected into the LC-MS/MS system.

152 2.5. *LC-MS/MS Analysis*

153 Chromatographic analysis was performed on an Acquity I Class UPLC system from Waters (Milford
154 MA, USA). The chromatographic separation was carried out on a BEH Amide HILIC column (100 mm
155 × 2.1 mm, 2.7 µm) maintained at a temperature of 50°C. Analytes were separated using a binary gradient
156 system comprising of mobile phases (A) 0.1% formic acid and 2 mM ammonium acetate in H₂O and
157 (B) 0.1% formic acid and 2 mM ammonium acetate in MeCN:H₂O (95:5, v/v), at a flow rate of 0.3 mL
158 min⁻¹. The gradient profile was as follows: (1) 0-2 min 95% B; (2) 2-4.5 min 80% B; (3) 4.5-7 min 50%
159 B; (4) 7-8 min 30% B; (5) 8-9 min hold at 30% B; (6) 9-9.5 min 95% B and (7) 9.5-13 min hold at 95%
160 B. The injection volume was 2 µL and the autosampler temperature was set at 5°C.

161 The UPLC-MS/MS instrument was controlled by MassLynx software (V.4.2), and the results processed
162 by TargetLynx XS software. Antiviral drug residues were detected using a Xevo TQ-XS triple
163 quadrupole mass spectrometer fitted with a Z-spray electrospray ionisation source in positive mode.
164 The source and desolvation temperatures were set at 150°C and 600°C, respectively. The cone and
165 desolvation gas flows were 150 and 1000 L h⁻¹, respectively. Tuning was operated by infusion of
166 individual standard solutions with mobile phase A:B (50:50, v/v) at a flow rate of 0.3 mL min⁻¹. The
167 infusion was operated using the built-in IntelliStart fluidics system. The optimal capillary voltage was
168 set at 2.5 kV, and a cone voltage of 20 V was used for all analytes. The most sensitive/selective product
169 ions were selected from the infusion studies and included in the final method, avoiding non-specific
170 ions such as neutral loss (**Table 1**). A time sector selected reaction monitoring (SRM) method was
171 developed using a minimum of two transitions for each analyte to ensure five identification points, as

172 required by 2021/808 guidelines [31]. The dwell time of each MRM window was optimised to ensure
173 the appropriate number of data points across each chromatographic peak.

174 *2.6. Validation Procedure*

175 Validation was performed according to the 2021/808 guidelines [31]. The following criteria were
176 assessed: selectivity, trueness, repeatability (WLR), within-laboratory reproducibility (WLR) and matrix
177 effects. Decision limits (CC α) and recoveries were also evaluated. The selectivity of the method was
178 investigated by injecting each compound and each internal standard (IS) individually at a concentration
179 of 50 ng mL⁻¹ to verify the absence of cross-talk and isobaric interferences. The selectivity of the method
180 was evaluated through the analysis of samples, which were supplied by Irish veterinary inspectors or
181 purchased at shops between August 2020 and June 2021. A total of 21 different blank poultry (chicken
182 (n=15), turkey (n=3) and duck (n=3)) sample extracts were also injected to verify the absence of matrix
183 interfering peaks at the retention time of the analytes. A total of 22 different blank poultry samples
184 (chicken (n=17), turkey (n=3) and duck (n=2)) were used for the matrix effect investigation, and spiked
185 post-extraction at a concentration equivalent to the Lowest Calibration Levels (LCLs). These signals
186 were compared to the signal obtained from a solvent standard at the same concentration. As no
187 Reference Points for Action (RPA) have been established for antiviral drugs, the trueness of the method
188 was determined at 1 x LCL, 2 x LCL and 3 x LCL. The WLR study was performed on three separate
189 days by the same analyst. Seven aliquots of the same poultry muscle were fortified at each validation
190 level. The WLR study was carried out over a period of three days by three different analysts, and by
191 fortifying 63 different blank poultry samples (n=7 for each validation level). The 63 different samples
192 included 52 different chicken, six turkey, four duck samples and one quail sample, which were supplied
193 by Irish veterinary inspectors or purchased at shops between August 2020 and June 2021. Samples
194 analysed using the method and found not to contain detectable peaks at or around the retention time of
195 the analytes were selected as negative controls. CC α values were calculated in accordance with
196 2021/808 EC Method 3 (LCL + k(one-sided, 99%) x (combined) standard measurement uncertainty at
197 LCL) for unauthorised or prohibited pharmacologically active substances [31]. Absolute recoveries
198 were also assessed by comparing the signal obtained from fortified samples to that of blank samples
199 spiked post-extraction.

200 *2.7. Data Analysis and Calculations*

201 Calibration curves were plotted using the internal standard approach for seven analytes with
202 corresponding isotopically labelled standards (arbidol, oseltamivir, amantadine, ribavirin, zanamivir,
203 oseltamivir acid and ganciclovir). For all other analytes, calibration curves were plotted using peak
204 areas, as the available internal standards were not found suitable in their quantitation. Calibration curves
205 were prepared using linear regression with a 1/x weighting factor. Matrix effects were calculated by
206 dividing the signal of matrix spiked post-extraction by the signal of solvent standard at the same
207 concentration. A result below 100% indicated ion suppression while a result above 100% expressed ion
208 enhancement. Measurement Uncertainty was determined by combining the standard error of the
209 absolute recovery obtained over ten analytical runs with the coefficient of variation (CV) of the WLR
210 study at 0.5 $\mu\text{g kg}^{-1}$ using a k factor of 2. Instrument detection limits (IDL) which are specific to the
211 instrument used for validation of the method, were determined for each analytes. They were calculated
212 using the relative standard deviation of the signal in solvent standard [32].

213 *2.8. Application to Real Samples*

214 A total of 120 poultry meat samples were purchased from five different shops in the Republic of Ireland
215 during the month of August 2020. The samples were stored at -28°C until analysis. The retail study was
216 designed to cover a wide range of poultry samples from different geographical regions. Ninety-one
217 samples originated from EU/UK, while 29 samples were from outside EU/UK. Forty-seven samples
218 consisted of processed or cooked meat, while 73 were raw. Several species were encompassed in the
219 study including chicken, duck, turkey and quail.

220

221 **3. Results and Discussion**

222

223 *3.1. Selection of Analytes*

224 One of the first steps in this work was to systematically identify relevant target residues prior to
225 commencement of method development work. Amantadine and rimantadine are active against influenza
226 A and have been reported in many published methods for the analysis of antiviral drug residues in meat
227 [26, 12, 10]. There have been a number of reports of amantadine residues being found in poultry meat
228 [15, 28]. Ribavirin is a multi-purpose drug, which is active against influenza A and B, but also used for
229 Hepatitis C and the Respiratory Syncytial Virus [10]. The prodrug of ribavirin, namely viramidine, has
230 a similar activity [33]. Arbidol is another drug that is used to treat influenza A, B and C [34, 9]. Arbidol
231 sulfoxide and arbidol sulfone are two of its main metabolites found in human plasma [35]. Other drugs
232 that were identified included the neuraminidase inhibitors, namely oseltamivir, zanamivir, laninamivir
233 and peramivir, and can be used against influenza type A and B. Oseltamivir acid is the major metabolite
234 of oseltamivir [36]. Favipiravir is an RNA polymerase inhibitor used against type A, B and C influenza
235 [10]. Two non-influenza antiviral drugs were included in the method. Ganciclovir was selected because
236 it has been previously detected in chicken muscle (Mu *et al.* [28]), and also acyclovir because it is an
237 antiviral drug that has been banned from use in food producing animals in China [13].

238 *3.2. Mass Spectrometry*

239 Electrospray and UniSpray ionization sources were initially evaluated. Similar to electrospray
240 ionization, UniSpray nebulises a liquid flow using a high velocity nitrogen gas flow. However, the spray
241 is directed to a cylindrical metallic target (pin), and a high voltage (around 1kV) is applied to the pin to
242 ensure optimal ionization while redirecting the flow towards the cone. As highlighted by previous
243 studies, ionization performances are highly dependent on chromatographic parameters [37]. In this
244 work, ionization efficiency was seven-fold better using UniSpray for all studied compounds when using
245 a reversed-phased column, where the analytes elute on a higher proportion of H₂O compared to organic
246 solvent. On the contrary, when eluted in HILIC mode using higher levels of organic solvent, UniSpray
247 presented smaller improvement. Similar conclusions were drawn by Lubin *et al.* [38], who observed
248 better performance on higher aqueous proportion. Further experiments in matrix found that electrospray
249 was optimal when using HILIC at lower mobile phase flow, while UniSpray gave best sensitivity when
250 using reversed-phase at higher flow.

251 Source parameters were optimised to obtain the best sensitivity for each analyte. Harsh desolvation
252 conditions, such as high temperature and gas flow, were found to drive more efficient ionization.
253 Positive mode provided a better signal, and protonated molecules [M+H]⁺ were observed for all
254 compounds. These selected transitions were in accordance with previous published works [29, 30, 39].

255 *3.3 Liquid Chromatography*

256 One of the main challenges of this work was the development of a liquid chromatography method, due
257 to the diverse chemical properties of the analytes. The analytes include highly polar compounds like
258 zanamivir (log P= -5.8), and non-polar drugs such as arbidol (log P= 3.8). This selection of molecules
259 is also heterogeneous because they include acidic, basic and neutral functionalities, which can exist in
260 different ionic forms depending on pH. The list of the selected drugs and their physico-chemical
261 properties is provided in **Table S2**, while their structures are shown in **Fig. 1**.

262 Therefore, the primary objective of this research was to obtain satisfactory retention, peak shape and
263 adequate chromatographic separation of the analytes. The gain of adequate retention was a major
264 challenge in this work. The 2021/808 EC guidelines require the minimum acceptable retention time to
265 be twice that of the void volume of the column. Initially, a wide range of different reversed-phase
266 chromatographic columns were evaluated using a binary gradient separation comprising of 0.1% formic
267 acid in H₂O (mobile phase A) and 0.1% formic acid in MeCN (mobile phase B). While reversed-phase
268 is not the natural choice for polar compounds, a wide range of reversed-phase stationary phases have
269 been developed with embedded polar groups to improve their performance with aqueous mobile phase
270 conditions, and to provide better retention of polar molecules. However, reversed-phase separations
271 always represent a good option in the laboratory because they are easy to work with and require short
272 equilibration times between injections. C₁₈ columns such as HSS T3, CSH C₁₈, YMC Triart C₁₈ or EC-
273 C₁₈ Poroshell were among the columns tested. As expected, the most polar analytes were poorly retained
274 by C₁₈ columns. This is the case for zanamivir and ribavirin, with a retention factor of $k < 0.5$ on all
275 columns.

276 Alternative reversed-phase columns were also investigated, such as phenyl-hexyl, diphenyl, PFPs
277 (pentafluorophenyl) and PFPP(pentafluorophenyl propyl). These phases are made of less hydrophobic
278 material than C₁₈, but can provide retention of analytes using alternative mechanisms. PFPP provided
279 better retention for zanamivir and ribavirin as well as good peak shape for the other analytes (**Fig. 2**
280 **and Figure S1**). The stronger retention of the PFPP is due to the π - π interactions with phenyl functions,
281 but also to the δ^+ - δ^- or hydrogen interactions with the fluorine function that phenyl-hexyl and diphenyl
282 columns are lacking. This phase provided better retention of the antiviral drug residues because of the
283 interaction with cyclic and amine functional groups. Surprisingly, other PFP columns did not provide a
284 retention advantage over the C₁₈ columns. The inclusion of MeCN as an organic modifier is proposed
285 to reduce π - π interactions. However, the use of MeOH did not result in a change of the retention of most
286 polar analytes because they eluted in 100% aqueous. In addition, this solvent led to broader peaks for
287 the less polar analytes, such as arbidol. Similarly, the inclusion of different additives, such as
288 ammonium acetate or ammonium formate for C₁₈ columns or modified reversed-phase, did not affect
289 the retention of the early eluters.

290 During preliminary studies, ion-pairing chromatography was also evaluated to improve the retention of
291 polar compounds, which also had the advantage of improving peak shape [40]. By creating an ion pair
292 with the analyte, a hydrophobic reagent favours the interactions with the reversed-phase column. After
293 optimisation on the PFPP column, 5mM of heptafluorobutyric acid (HFBA) in H₂O was used, which
294 was found to improve the retention factor for zanamivir from 0.5 to approximately 2 (**Figure S2**).
295 However, the use of an ion-pairing agent was not chosen for this method, as it caused ion suppression
296 through source contaminations and prevented multiple applications on a single instrument. Ion pairing
297 reagent were also introduced directly in-vial as previously reported by Lehotay *et al.* [41] in order to
298 reduce the amount of HFBA in the system, but this approach did not provide sufficient and consistent
299 retention improvements.

300 HILIC chromatography has been developed to provide improved retention of highly polar molecules
301 on a polar stationary phase and where elution occurs between 5% and 50% organic solvent [42]. In this
302 work, HILIC separation of the analytes was carried out on PFPP, HILIC-Z and Amide columns. It was
303 found that the PFPP column retained most analytes based on its cation exchange retention mechanism
304 but not ribavirin, which is a neutral molecule. In contrast, HILIC-Z and BEH Amide retained the
305 analytes based on a partitioning retention mechanism, and showed better results than PFPP. As a
306 consequence, the Amide column was selected because it was found to show better overall retention,
307 especially for early eluting compounds such as favipiravir, arbidol and its metabolites (**Fig. 3**).
308 However, further work was required to reduce broad peaks and tailing. Therefore, combinations of
309 formic/acetic acid and ammonium formate/acetate were evaluated. It was shown that oseltamivir,
310 rimantadine and amantadine peaks were relatively wide and/or tailing with acetic acid or formic acid
311 only in the mobile phase. HILIC separation often requires high additive concentrations (i.e. ammonium
312 salts) to give better retention and peak shape, although this can reduce analytical sensitivity. A
313 compromise was reached by using 0.1% formic acid and 2mM ammonium acetate, which provided
314 satisfactory peak shapes for all compounds and also enhanced sensitivity. The same concentration of
315 additives was included in both mobile phases to reduce the required equilibration time.

316 Ion suppression values were calculated when using a reversed-phase (PFPP) column, and compared to
317 results obtained from a HILIC column (BEH Amide) under the same conditions, as shown in **Figure**
318 **S3**. It could be observed that ZNV, PMV, LNV, GCV and VMD were advantaged in HILIC mode as
319 they have a negative Log P. On the contrary, the signal for less polar analytes such as AMT, RMT and
320 ABD was more suppressed in HILIC. HILIC chromatography was previously reported by Periat *et al.*
321 [43] as causing higher matrix effects. However, in this work, this was found to be more suitable for
322 highly polar molecules, as it enhanced chromatographic retention and reduced signal suppression.

323 3.3. Sample Preparation

324 The broad range of polarities of antiviral drugs has forced other researchers to use very selective sample
325 preparation procedures that use more than one SPE clean-up to purify extracts [29, 30]. Therefore, the
326 aim of this work was to develop a simple sample preparation for the efficient isolation of antiviral drug
327 residues from tissue samples. A number of generic sample preparation procedures have been developed
328 to isolate residues from biological samples, including QuEChERS (Quick, Easy, Cheap, Effective,
329 Rugged and Safe), QuPPE (Quick Polar Pesticide method) and simple solvent extraction. However, not
330 all of these methods are compatible with highly polar molecules, and their suitability needs to be carefully
331 evaluated. For example, Mu *et al.* [28] used QuEChERS to extract 14 antiviral drugs from chicken
332 muscle. In the current work, it was found that QuEChERS provided low recovery for highly polar
333 compounds (**Fig. 4**), indicating that the analytes were trapped in the aqueous phase. A modified QuPPE
334 method, which was originally designed to extract polar pesticide residues, was also experimented [44].
335 Results showed that the QuPPE method provided less matrix effects, especially for the polar compounds.
336 However, the extraction efficiency was much lower for all residues (**Fig. 4**). This was probably due to
337 the fact that the freezing step, which was necessary to crash out the matrix interferences resulting from
338 the MeOH extraction, also affected analyte recoveries. The use of pure MeCN or isopropanol also
339 showed lower extraction efficiency for zanamivir and laninamivir (**Fig. 4**). As a result, alternative
340 protocols developed for the extraction of highly polar molecules from food samples were investigated,
341 such as MeOH or MeCN:H₂O extraction solvents. These solvents have been previously employed for
342 the extraction of antiviral drugs in a number of published methods [29, 30, 45]. Improved extractability
343 was found with MeOH or MeCN:H₂O (80:20, v/v) but the latter was finally chosen because it led to
344 more matrix enhancement or less matrix suppression for most of the compounds.

345 Subsequently, different extraction techniques that can be used to assist the extraction of antiviral drug
346 residues from tissue were investigated, including probe blending, vibrational shaking (with or without
347 a ceramic homogenizer), horizontal shaking, vortexing and ultrasound-assisted extraction. Vibrational
348 shaking with a ceramic homogenizer was selected as the most effective technique, as samples could be
349 adequately homogenized in one minute. The vibrational shaking apparatus used for this is of relatively
350 low cost, provides unattended processing of 36 samples at a time, and eliminates sample cross-
351 contamination because the homogenization is carried out separately in sealed tubes [46]. The other
352 techniques that were evaluated showed lower extraction efficiency, or were more laborious and time
353 consuming.

354 During sample clean-up, it is desirable to selectively isolate the analytes of interest from matrix
355 interferences. The main goal of this work was to develop a simple method for both polar and non-polar
356 compounds while also avoiding the use of SPE clean-up cartridges. Ultra-filtration using cellulose-
357 based devices with different molecular weight cut-offs (cut-offs 3kDa and 30kDa) were investigated as
358 an additional protein removal strategy. However, although satisfactory recoveries were achieved, the
359 procedure did not decisively negate matrix effects. Similarly, low temperature treatment at -30°C, which
360 was previously used to reduce lipophilic matrix effects when analysing pesticides in fat, was evaluated
361 [47]. However, the extraction efficiency for polar zanamivir and laninamivir was close to zero because
362 the H₂O present in the sample extracts froze during the freezing step, and trapped the polar compounds.
363 Various dispersive solid-phase extraction (dSPE) sorbents (50 mg) were evaluated to purify extracts,
364 including C₁₈, Z-sep+, PSA and C₈. The majority of these sorbents are generally used to remove
365 lipophilic matrix interferences from sample extracts. Results showed that with dSPE sorbents, matrix
366 effects and extraction rates were equivalent or worse when compared to the results obtained from
367 extracts injected without any dSPE treatment (**Fig. 5**). The only exception was when using C₁₈, Zsep or
368 C₈ sorbent on arbidol group analytes, however these improvements were offset by the partial adsorption
369 of the compounds by the sorbents. Therefore, a decision was made not to use any dSPE, as it was found
370 to be unnecessary. The injection solvent composition was also evaluated, showing that optimal peak
371 shape could be achieved when using MeCN:MeOH:H₂O (50:25:25, v/v/v). This solvent composition
372 also improved signal-to-noise (S/N) for some compounds such as laninamivir and zanamivir, possibly
373 due to better analyte solubility in the extract. These results were in agreement with what previously
374 reported by Chan *et al.* [29], who also found a MeCN:MeOH:H₂O mixture to be the optimal for the
375 reconstitution of the final extracts.

376 3.4. Validation Results

377 As stated by the 2021/808 guidelines, five identification points are required for unauthorised or
378 prohibited substances. One point was obtained from separation (LC), one point from the precursor ion
379 and 1.5 points from each of the two product ions. All the ion ratios were found to be within the limit of
380 $\pm 40\%$ ion ratio tolerance. All retention times were also within the 1% limit for the compounds with an
381 IS, and within the 0.1 minute limit for the compound for which an IS was not used. Calibration curves
382 were constructed using nine points including eight calibrants and the zero level with a 1/x fit.
383 Satisfactory linearity was achieved during all analytical runs, with $R^2 \geq 0.988$ for all analytes, and
384 residuals within the $\pm 20\%$ range from the calibration curve.

385 When injecting standards separately (not as a mixture), isobaric interference was observed between
386 viramidine and ribavirin. This is likely due to the second major isotope of viramidine, which has a mass
387 similar to ribavirin. However, this interference did not impact on the capability of the method to
388 accurately identify and quantify the drug residues, as the chromatographic peaks were well resolved,
389 with a 3 min interval between the elution of the two compounds. The selectivity study also showed

390 some matrix interferences in the viramidine qualifier retention region, but it had no significant impact
391 on the ion ratio. A chromatogram of calibrant 2 is displayed on **Fig. 6**, and compared to a chromatogram
392 of a blank matrix sample. The matrix effects study showed that the ionization of analytes were generally
393 suppressed in the presence of matrix, with the exception of laninamivir and zanamivir, which were
394 enhanced by an average of 337%, and 913%, respectively (**Table 2**). Ribavirin was the most suppressed
395 compound, with an average matrix effect of 15%. Ion suppression is a more serious problem because it
396 reduces sensitivity, while enhancement can be advantageous because it improves sensitivity. However,
397 matrix effects were compensated by the use of a procedural matrix calibration curve. The coefficients
398 of variation calculated were below guidelines authorised limits of 20% for compounds with IS, and
399 below the in-house specified limits of 30% for compound without an IS. The use of an internal standard
400 was particularly important for zanamivir, which helped to reduce the matrix effect coefficient of
401 variation from 37.4% to 4.9%.

402 Due to the absence of certified reference material, trueness and precision were evaluated from fortified
403 samples. To demonstrate the robustness of the methods, 63 different samples were used for the
404 reproducibility assay. The high number of different samples as well as the use of various species allowed
405 the procedure to test the reproducibility of the method through the investigation of potential matrix
406 interferences. . In relation to the repeatability studies, all trueness values were within the acceptable
407 limits, as they ranged from 85% to 109% (**Table 3**). Similarly, the WLR CVs were $\leq 10.8\%$, while the
408 maximum acceptable values were 16.7% and 20% for levels $>10 \mu\text{g kg}^{-1}$ and $<10 \mu\text{g kg}^{-1}$, respectively.
409 As expected, trueness values (84 - 127%) for WLR were spread on a wider range than repeatability
410 trueness values. These values were all acceptable except for acyclovir, for which the values on two
411 levels were slightly above 120% (125% and 127%). This is mainly due to the coefficient of variation
412 of the matrix effect (29.2%) observed for this analyte. This issue could be potentially addressed in future
413 works by including a suitable internal standard for this compound. The precision values associated to
414 the reproducibility study were all satisfactory. WLR precision values ranged from 2.8% to 22.7%, while
415 the maximum acceptable values were of 25% and 30% for levels of $>10 \mu\text{g kg}^{-1}$ and $<10 \mu\text{g kg}^{-1}$,
416 respectively.

417 The robustness of the method was verified by varying different parameters during the validation studies.
418 These parameters were: (1) the time of homogenisation on the vibrational shaker (1 min vs 5 min); (2)
419 the use of different pipettes for spiking or transferring (air vs positive displacement pipettes); (3) and
420 the final filtration achieved by different means (syringeless vial vs syringe filtration).

421 As outlined in the experimental section, Method 3 (2021/808) for CC α calculation was based on the
422 LCL and the combined standard measurement uncertainty at this concentration. The calculated CC α
423 values ranged from 1.09 to 1.4 times their respective LCL.

424 3.5. Occurrence in Real Samples

425 Among the 120 samples selected from the Irish market, none were found positive for any of the targeted
426 antiviral drugs. These results suggest the relatively low occurrence of antiviral drug residues in poultry
427 meat and on the European market.

428 4. Conclusion

429 In this research, a LC-MS/MS method for the confirmatory and quantitative analysis of 15 antiviral
430 drug residues in poultry muscle was developed and validated. This included highly polar, non-polar,
431 acidic, basic and neutral analytes. The sample preparation strategy was optimised by evaluating various
432 solvents and methods of extraction as well as different clean-up options. The final procedure consisted

433 of an MeCN-based protein precipitation step, followed by a dilution in a MeOH:H₂O mixture.. Different
434 chromatographic modes were assessed including reversed-phase, ion pairing and HILIC
435 chromatography on the basis of retention, separation and matrix effects. Satisfactory results were
436 achieved using a BEH Amide column in HILIC mode. This method is advantageous over current
437 published procedures for its simplicity and cost-efficiency, while also including a broader range of
438 analytes. The high-throughput sample preparation protocol allows a single analyst to process 50
439 samples, along with calibration and control samples, in one day. The method was validated according
440 to the new EU guidelines 2021/808 at levels as low as 0.1 µg kg⁻¹. Trueness and precision calculated
441 from the WLR studies were found to be satisfactory, and ranged from 84 to 127% and from 2.8 to
442 22.7%, respectively. No residues of antiviral drugs were found in the 120 samples analysed using the
443 proposed method.

444 **Competing financial interests**

445 The authors declare no competing financial interests.

446

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452

453 **Table and figure captions**

454 **Fig. 1.** Chemical structures of antiviral drugs.

455 **Fig. 2.** Retention factor of zanamivir, ganciclovir and ribavirin using different reversed-phase columns.

456 **Fig. 3.** LC-MS/MS HILIC mode chromatograms of avian muscle tissue fortified with antiviral drugs,
457 injected using column (A) Poroshell 120 HILIC-Z (2.1 x 100 mm, 2.7 µm); (B) BEH Amide (2.1 x
458 100 mm, 2.5 µm) and (C) UCT selectra PFPP (2.1 x 100 mm, 3.0 µm). The analytes were labelled as
459 (1) favipiravir; (2) arbidol; (3) arbidol sulfone; (4) arbidol sulfoxide; (5) rimantadine; (6) ribavirin; (7)
460 amantadine; (8) acyclovir; (9) oseltamivir; (10) viramidine; (11) ganciclovir; (12) peramivir; (13)
461 oseltamivir acid; (14) laninamivir and (15) zanamivir. The flow rate was set at 0.3 mL min⁻¹.

462 **Fig. 4.** Average absolute recoveries (and standard deviations shown as error bars) obtained using
463 QuEChERS, Quppe, MeCN, MeOH, isopropanol and MeCN:H₂O (80:20, v/v) as extraction solvents
464 (n=3).

465 **Fig. 5.** Average matrix effects (and standard deviations shown as error bars) obtained using the dSPE
466 sorbents C₁₈, PSA, Zsep and C₈ compared to extraction without any dSPE clean-up (n =3).

467 **Fig. 6.** Chromatograms of avian muscle tissue sample fortified at calibrant 2 level (A, B and C) and
468 chromatograms of blank muscle tissue sample (D, E and F).

469 **Table 1** LC-MS/MS conditions for antiviral drugs.

470 **Table 2** Matrix effect, LOD, and LOQ values for antiviral drugs in poultry muscle tissue and IDL
471 specific to the instrument used.

472 **Table 3** Validation results for antiviral drugs in poultry muscle tissue.

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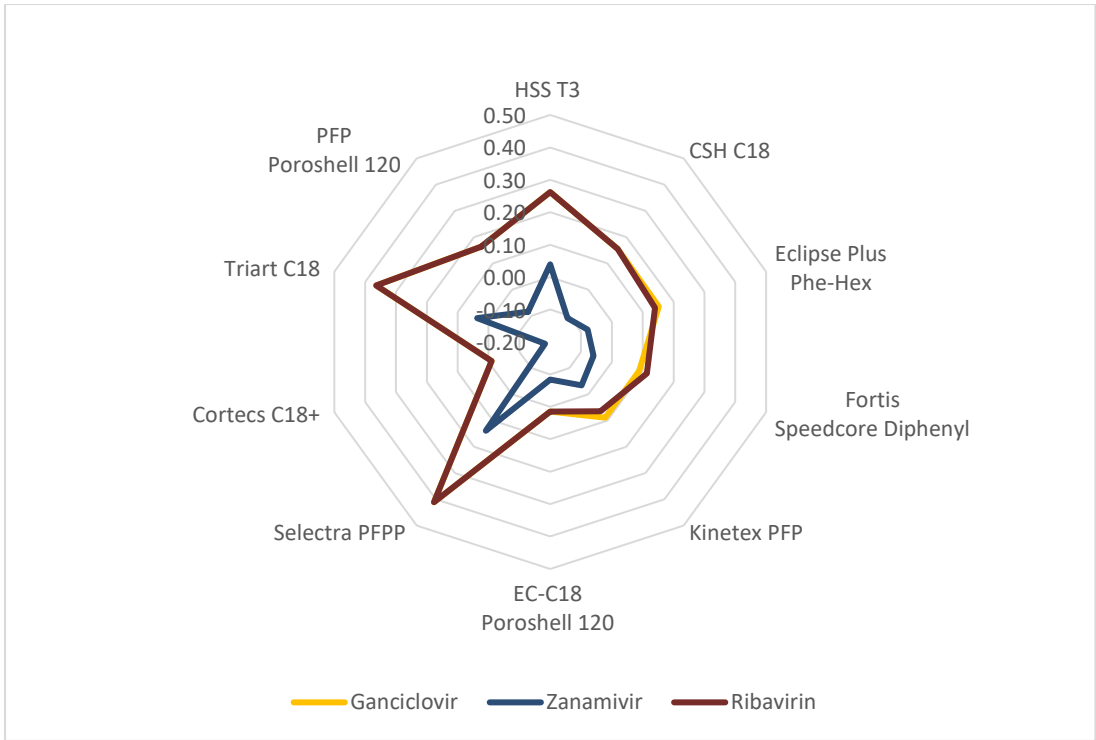
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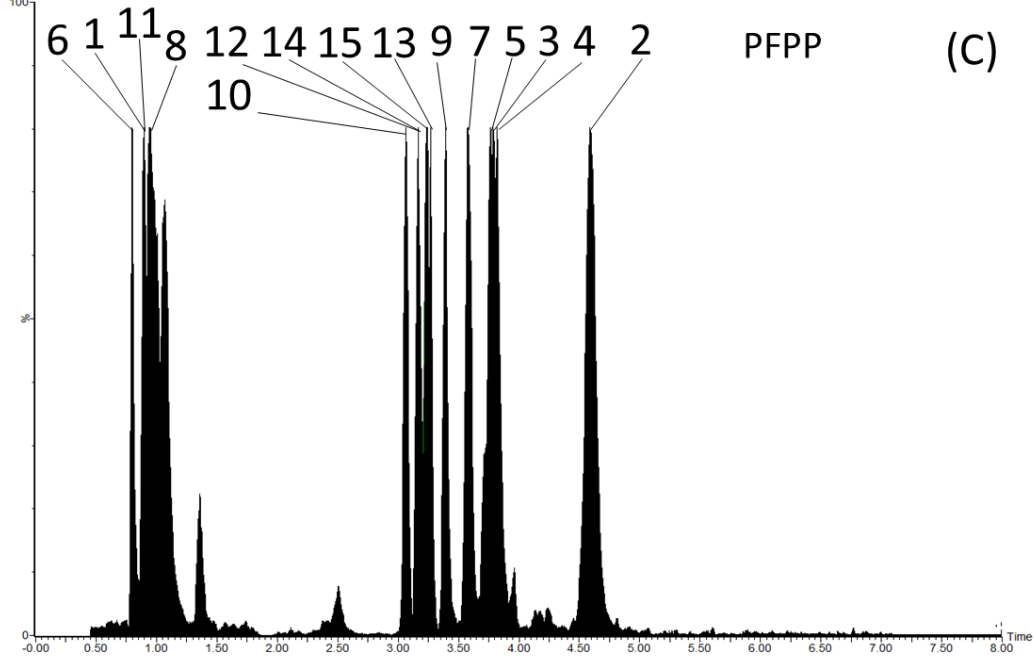
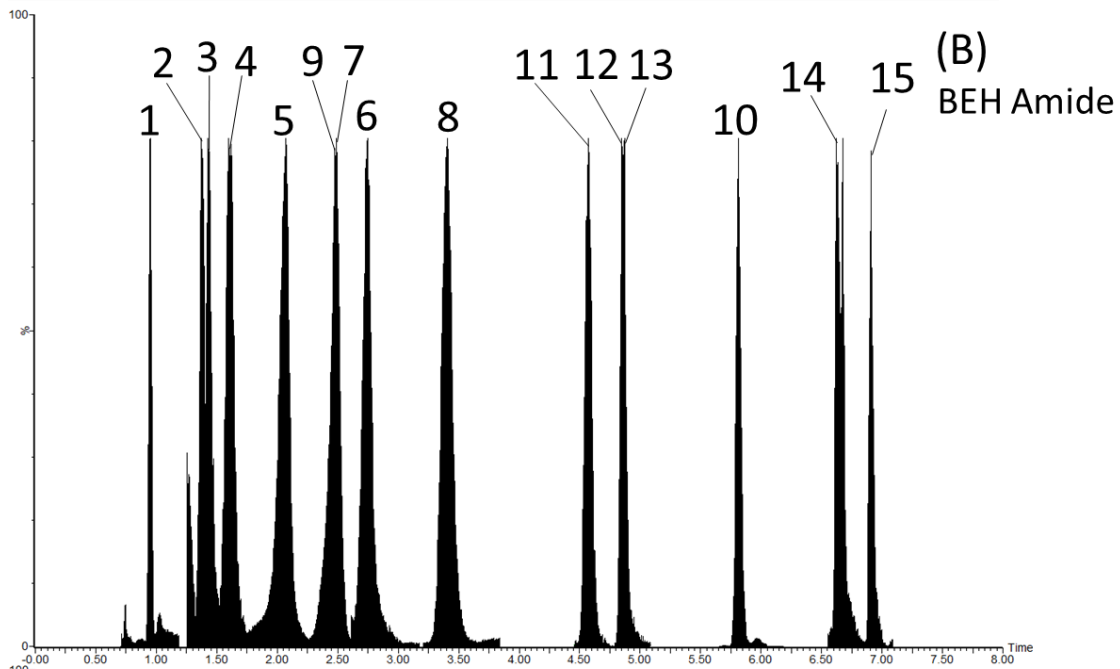
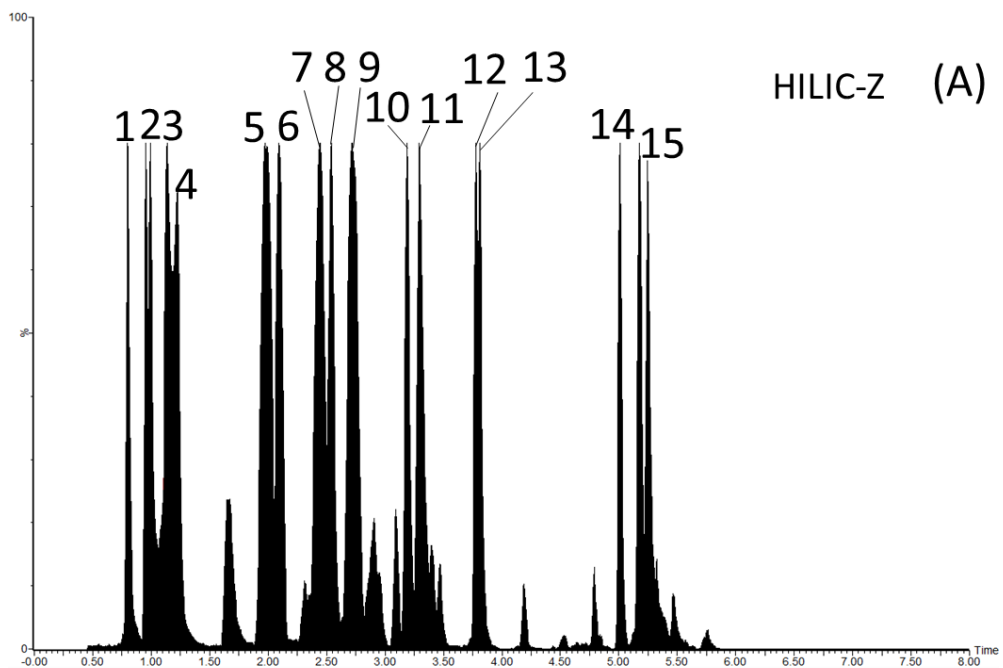
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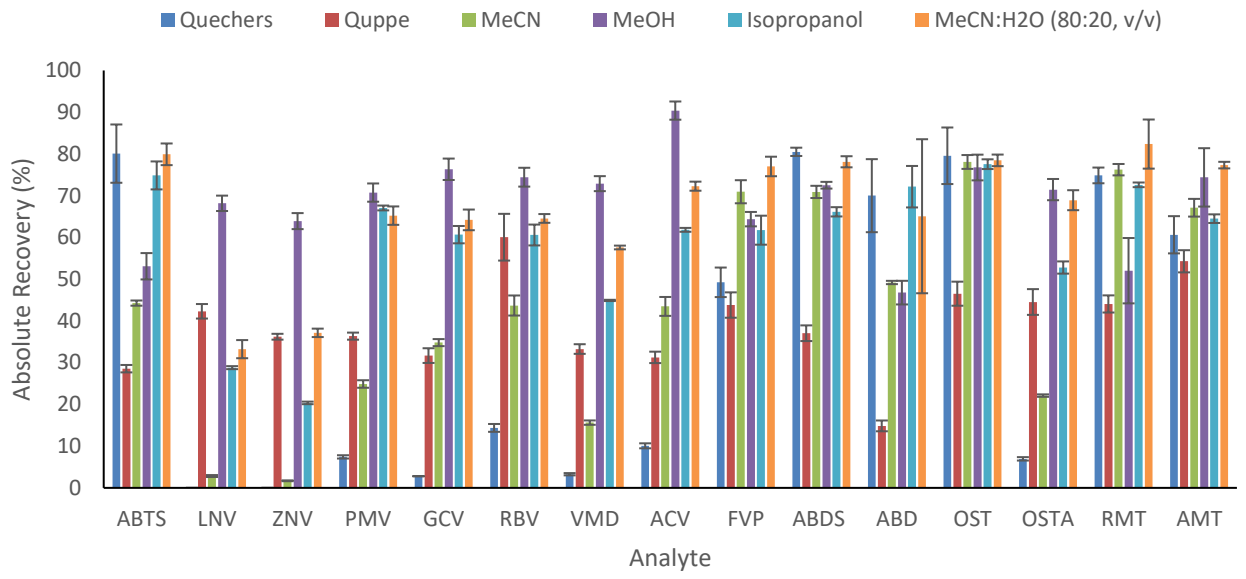
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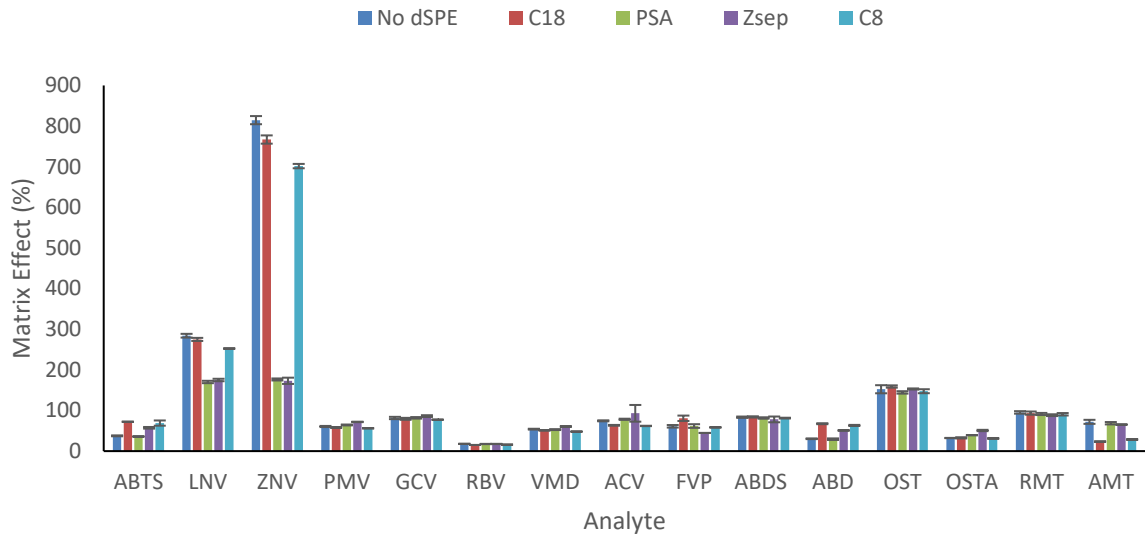
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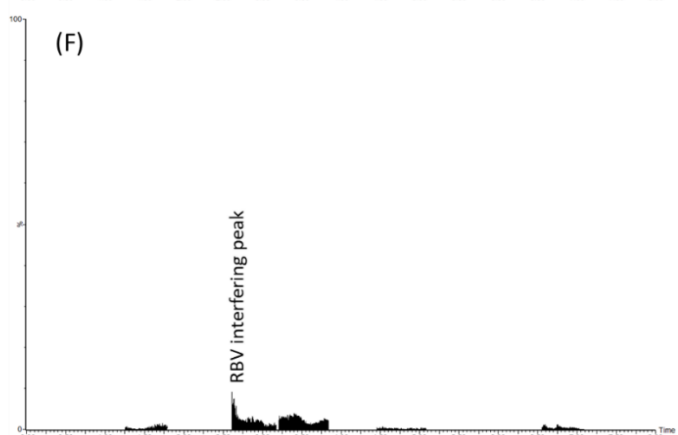
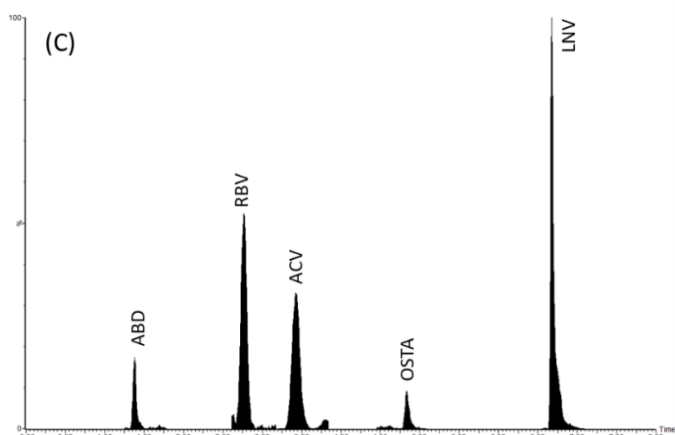
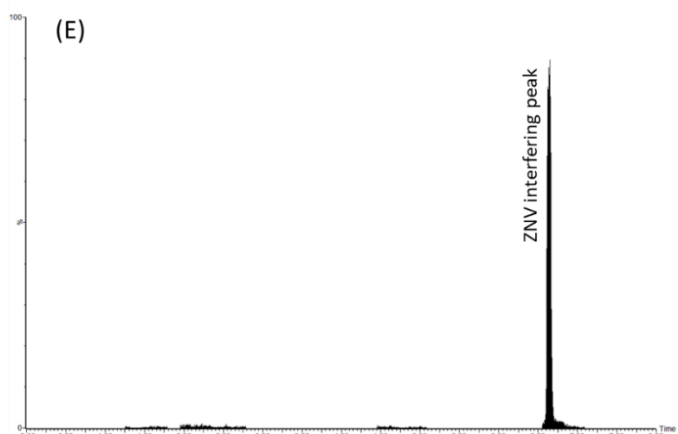
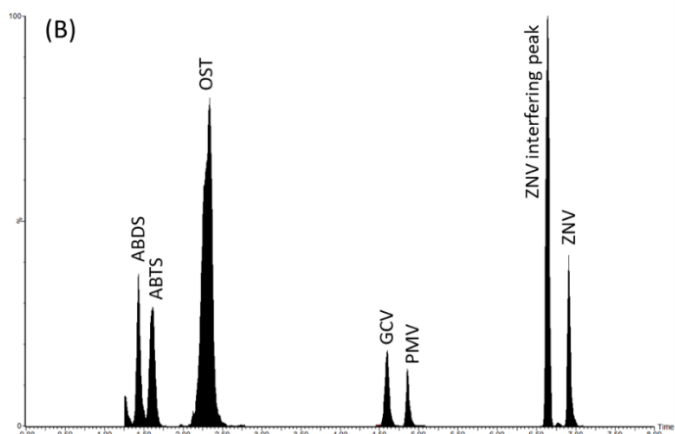
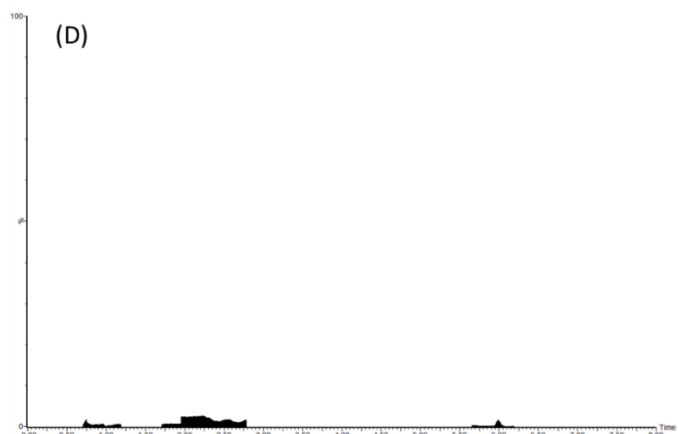
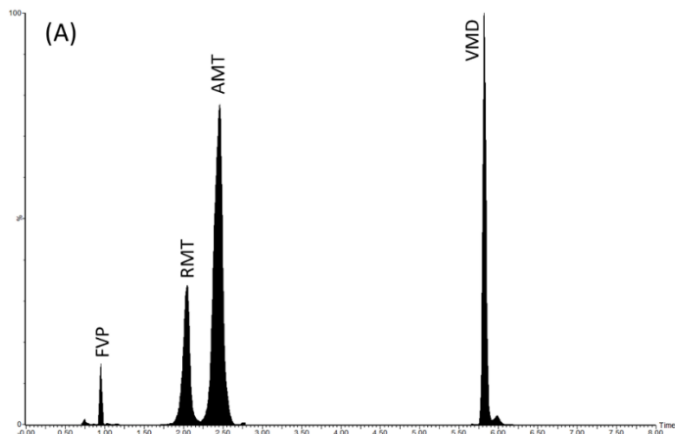


Table 1 LC-MS/MS conditions for antiviral drugs.

Analyte	RT (min)	Precursor Ion (m/z)	Product Ion (m/z)	Dwell time (s)	Collision Energy (eV)	SRM window
Favipiravir	0.91	157.9	84.9/112.9	0.163	21/17	1
Arbidol	1.35	479.4	281.2/434.2	0.034	33/19	2
Arbidol-d ₆	1.35	485.2	434.1	0.034	20	2
Arbidol Sulfone	1.43	511.3	296.1/466.2	0.034	33/20	2
Arbidol Sufloxide	1.57	495.3	325.2/370.3	0.034	25/14	2
Rimantadine	2	180.1	80.9/163.0	0.034	21/16	3
Oseltamivir	2.32	313.4	166.1/225.1	0.034	18/9	4
Oseltamivir-d ₃	2.32	316.2	167.2	0.034	19	4
Amantadine	2.4	152.2	79.0/135.1	0.034	24/14	4
Amantadine-d ₁₅	2.4	167.3	102.2	0.034	23	4
Ribavirin	2.71	245.2	96.0/112.9	0.034	25/10	5
Ribavirin- ¹³ C ₅	2.71	250.3	113.1	0.034	13	5
Acyclovir	3.41	226.2	135.0/152.0	0.163	27/12	6
Ganciclovir	4.57	256.2	135.0/152.0	0.044	31/14	7
Ganciclovir-d ₅	4.59	261.2	152.1	0.044	15	7
Oseltamivir acid	4.79	285.3	180.1/197.1	0.044	09/12	7
Oseltamivir acid-d ₃	4.79	288.2	183.3	0.044	13	7
Peramivir	4.8	329.2	99.9/141.9	0.044	30/24	7
Viramidine	5.75	244.2	94.9/112	0.163	35/14	8
Laninamivir	6.56	347.3	59.9/288.3	0.063	16/15	9
Zanamivir	6.87	333.3	59.9/121.1	0.063	17/27	9
Zanamivir- ¹³ C ¹⁵ N ₂	6.87	336.2	63.2	0.063	16	9

SRM 1 (0.70–1.20 min); SRM 2 (1.25–1.80 min); SRM 3 (1.70–2.50 min); SRM 4 (1.95–2.80 min); SRM 5 (2.61–3.18 min); SRM 6 (3.20–3.85 min); SRM 7 (4.45–5.10 min); SRM 8 (5.65–6.20); SRM 9 (6.55–7.1 min).

SRM: selected reaction monitoring, RT: retention time.

Table 2 Matrix effect, LOD, and LOQ values for antiviral drugs in poultry muscle tissue and IDL specific to the instrument used.

Analyte	IDL (ng kg ⁻¹)	Curve range (µg kg ⁻¹)	LOD (µg kg ⁻¹)	LOQ (µg kg ⁻¹)	Matrix effects (%)	C.V. matrix effects (%)	C.V. matrix effects without IS (%)
Amantadine	96.8	1 - 8	0.06	0.19	49%	4.4%	17.5%
Rimantadine	29.4	0.5 - 4	0.03	0.11	61%	6.1%	6.1%
Oseltamivir	22.7	0.5 - 4	0.05	0.18	139%	3.9%	10.2%
Oseltamivir acid	89.4	2 - 16	0.28	0.92	43%	6.9%	16.5%
Zanamivir	208	2 - 16	0.16	0.55	913%	4.9%	37.4%
Peramivir	119	2 - 16	0.19	0.62	61%	8.1%	8.1%
Laninamivir	227	10 - 80	0.44	1.5	337%	14.6%	14.6%
Ribavirin	693	25 - 200	2.5	8.3	15%	6.7%	25.6%
Viramidine	38.8	2 - 16	0.08	0.26	57%	18.5%	18.5%
Favipiravir	613	25 - 200	3.1	10.5	38%	19.1%	19.1%
Arbidol	7.4	0.1 - 0.8	0.01	0.04	32%	8.8%	11.3%
Arbidol sulfone	9.9	0.5 - 4	0.11	0.35	29%	11.2%	11.2%
Arbidol sulfoxide	3.5	0.1 - 0.8	0.01	0.03	59%	10.6%	10.6%
Acyclovir	16.7	0.5 - 4	0.07	0.22	136%	29.2%	29.2%
Ganciclovir	85.4	2 - 16	0.19	0.62	80%	5.5%	14.1%

IDL: instrument detection limit, LOD: limit of detection, LOQ: limit of quantitation, C.V: coefficient of variation. Matrix effects below 100% means ion suppression while matrix effects above 100% means ion enhancement.

Table 3 Validation results for antiviral drugs in poultry muscle tissue.

Analyte	LCL ($\mu\text{g kg}^{-1}$)	Trueness% (WLR%)			Trueness% (WLR%)			CC α ($\mu\text{g kg}^{-1}$)
		1 x LCL	2 x LCL	3 x LCL	1 x LCL	2 x LCL	3 x LCL	
Amantadine	1	104 (3.1)	102 (4.1)	104 (3.1)	97 (3.8)	97 (4.5)	97 (2.8)	1.1
Rimantadine	0.5	99 (4.1)	97 (4.0)	96 (5.7)	94 (4.1)	92 (5.6)	93 (6.6)	0.55
Oseltamivir	0.5	105 (4.6)	102 (5.0)	104 (4.5)	99 (5.5)	97 (6.6)	97 (4.9)	0.57
Oseltamivir acid	2	108 (6.5)	105 (7.1)	106 (5.6)	101 (10.3)	101 (9.6)	103 (11.7)	2.5
Zanamivir	2	102 (4.1)	101 (4.5)	104 (4.0)	93 (7.2)	98 (4.4)	100 (3.7)	2.34
Peramivir	2	98 (6.1)	95 (4.6)	99 (3.6)	105 (6.2)	104 (6.3)	106 (8.6)	2.3
Laninamivir	10	99 (2.8)	97 (2.8)	100 (2.5)	84 (13.2)	86 (16.2)	88 (11.7)	13.1
Ribavirin	25	101 (4.4)	99 (4.7)	102 (4.3)	96 (5.9)	96 (6.0)	98 (5.4)	28.5
Viramidine	2	99 (2.9)	97 (2.1)	101 (3.1)	85 (17.2)	90 (21.7)	93 (21.6)	2.8
Favipiravir	25	104 (5.3)	104 (5.1)	104 (5.5)	95 (16.6)	95 (20.7)	90 (21.2)	34.7
Arbidol	0.1	109 (6.3)	104 (6.2)	91 (4.1)	104 (7.5)	104 (7.7)	88 (9.2)	0.12
Arbidol sulfone	0.5	102 (5.4)	99 (4.9)	85 (10.8)	101 (12.4)	100 (10.6)	93 (16.3)	0.65
Arbidol sulfoxide	0.1	101 (4.3)	99 (4.6)	88 (7.9)	99 (7.2)	96 (6.6)	89 (6.5)	0.12
Acyclovir	0.5	105 (5.9)	104 (8.8)	105 (7.1)	127 (15.6)	125 (22.7)	117 (22.1)	0.68
Ganciclovir	2	105 (4.1)	102 (5.3)	103 (4.5)	97 (5.1)	100 (10.6)	100 (4.3)	2.3

LCL: lowest calibration level, WLR: repeatability, WLR: within-lab reproducibility, CC α : decision limit.