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1 # On-line Solid-Phase Extraction Coupled Liquid
2 Chromatography-ESI-Ion Trap-Mass Spectrometry for Analysis of
3 Abamectin and Ivermectin Residues in Milk

4

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20 ## Abstract

21 A solid-phase extraction column was on-line coupled to the liquid chromatography
22 electrospray ionization ion-trap tandem mass spectrometer to perform the on-line
23 sample pretreatment and simultaneous analysis of Abamectin and Ivermectin residues
24 in milk samples. Multiple-reaction monitoring of secondary mass spectrometry in
25 positive mode was used to detect both Abamectin and Ivermectin. The matrix-fortified
26 external standard calibration curves of the secondary mass spectrometry in the
27 concentration range 0-15 ng mL^{-1} for Abamectin and Ivermectin shows good linearity.
28 The limit of detection/quantification (LOD/LOQ) for Abamectin and Ivermectin was
29 0.67/2.23 ng mL^{-1} and 0.63/2.11 ng mL^{-1}, respectively. Quantification of five
30 different brands of milk samples by the standard addition method with the assistance
31 of statistical *t*-test indicates that two brands of milk samples do contain trace amount
32 of Abamectin and Ivermectin. Abamectin and Ivermectin in the other three brands of
33 milk samples are all not detected. The accuracy of the two brands of milk samples for
34 Abamection and Ivermectin was 82-88%; the precision was 4.8-9.7%. The running
35 time for one sample was 10 min.

36

37 **Keywords**: Abamectin; Ivermectin; Milk; On-line solid-phase extraction; Liquid
38 chromatography-tandem mass spectrometry

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39 ## 1. Introduction

40 Abamectin (ABA) is widely used as herbicide, insecticide, and anthelmintic and
41 Ivermectin (IVE) is an antiparasitic drug. Both of them belong to Avermectins which
42 are macrocyclic lactone in structure, thus they are called macrolide antibiotics [1,2].
43 The extensive use of ABA and IVE as veterinary drug in animal husbandry endangers
44 people's health indirectly through food chain. Since the consumption of milk and
45 dairy products has been increased significantly recently in Taiwan accompanied by
46 the rapid growth of economy, the supervision, monitoring, and regulation to prevent
47 the veterinary drug abuse that may cause large amount drug residues in foods derived
48 from food-producing animals to affect human health are important and necessary.

49 The methods of investigation and analysis of macrolide antibiotics have been
50 reviewed based on thin layer chromatography, immunochemical methods, and liquid
51 chromatography (LC) with UV detection, fluorescence (FL) detection, and mass
52 spectrometry (MS) [3]. Due to high liposolubility of macrolide antibiotics, veterinary
53 drug residues in food product obtained from an animal are regulated and monitored by
54 the maximum residue limits (MRLs). ABA and IVE are zero tolerance in lactating
55 species in European Union (EU) because they are not authorized for use [4] in cattle
56 producing milk for human consumption. Since ABA is considered a pesticide, no
57 MRL is set for milk by Codex Alimetarius, while IVE is set at 10 μg kg^{-1} in milk by

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58 Codex Alimetarius [5]. In Taiwan, the MRL set by Taiwan Food and Drug
59 Administration (TFDA) in milk is 10 ng mL^{-1} for IVE and it is “not permitted” for
60 ABA. Therefore, considering the non-volatility and sensitivity, liquid chromatography
61 coupled to FL detector [6-12] after analyte derivatization and tandem mass
62 spectrometry (MS/MS) with various mass analyzers [6,13-25] have been widely used
63 for the determination of ABA and IVE in milk.
64 The sample pretreatment procedures of the current determination methods for the
65 veterinary drugs in milk include the use of acetonitrile extraction followed by
66 solid-phase extraction (SPE) cleanup [8,9,11,12,17,20,26], the QuEChERS (Quick,
67 Easy, Cheap, Effective, Rugged, and Safe) method [18,19,21,23], the liquid-liquid
68 extraction (LLE) [6,7,14,15,25] or dispersive liquid-liquid microextraction (DLLME)
69 [13], and off-line SPE [20,24]. Except for off-line SPE and DLLME, all other sample
70 pretreatment procedures usually consume solvent. Comparing off-line SPE and
71 DLLME with on-line SPE, they are somehow tedious and subject to sample loss and
72 contamination. Since on-line SPE that utilizes a column switching valve to combine
73 parallel a SPE column with an analytical LC column has all the advantages over other
74 sample preparations described above, on-line SPE coupled to LC-MS has been
75 applied for the determination of illicit or therapeutic drugs [27,28], antibiotics or
76 macrolide antibiotics [29-32], pesticides [33], and steroids [34] in environmental

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77 water, biological fluids, milk, and cell culture. However, there is no record shown in
78 literature that on-line SPE coupled to the LC-MS has been applied for the
79 determination of macrolide lactone type veterinary drug residues in milk.

80 In this paper, five local brands of whole milk samples have been investigated for
81 the determination of veterinary drug residues Abamectin and Ivermectin using on-line
82 SPE coupled LC-ESI-Ion Trap-MS/MS system by the standard addition calibration
83 method to ensure a sensitive, accurate, precise, and practical analysis.

84

85 ## 2. Experimental

86 ### 2.1 Reagents and chemicals

87 Reagent grade Abamectin ($C_{48}H_{72}O_{14}$, 98%, B_{1a}) with structure shown in Fig. 1A
88 was bought from Chem Services (West Chester, PA, USA). Reagent grade Ivermectin
89 ($C_{48}H_{74}O_{14}$, 98%, B_{1a}) with structure shown in Fig. 1B was purchased from Sigma (St.
90 Louis, MO, USA). LC grade methanol (CH_3OH), reagent grade zinc acetate
91 ($Zn(CH_3COO)_2$), potassium hexacyanoferrate(II) trihydrate ($K_4Fe(CN)_6{\bullet}H_3O$), and
92 glacial acetic acid (CH_3COOH, >99%) were all supplied by Merck (Darmstadt,
93 Germany). Both nitrogen gas produced from liquid nitrogen (N_2, 99.999%) and
94 helium gas (He, 99.999%) were provided by Tai Yuan Gas Co., Ltd. (Toufeng,
95 Taiwan). Five different local brands of whole milk sample were obtained from local

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96 supermarket or convenient store (Chunan, Taiwan). Deionized water (18.2 MΩ) was
97 purified from tap water using Millipore Synergy 185 ultrapure water system (Billerica,
98 MA, USA).

99 **2.2 Instruments and equipment**

100 The schematic diagram of on-line SPE coupled LC-UV/ESI-ion Trap-MS/MS
101 system is shown in Fig. 2, which includes an Agilent 1100 series HPLC system
102 (Agilent Technologies, Santa Clara, CA, USA), consisting of a binary pump (pump 1,
103 G1312A), an isocratic pump (pump 2, G1310A), a degasser (G1379A), an
104 autosampler (G1376A), a column oven (G1316A), a SPE column (Agilent SB-C18,
105 30 × 2.1 mm i.d., 3.5 μm) with a 0.2-μm filter disc attached to the front inlet, an
106 analytical column (Agilent SB-C18, 75 × 2.1 mm i.d., 3.5 μm), and a six-port
107 column-switching valve (G1158A). The SPE column, the analytical column, and the
108 column-switching valve were assembled in the column oven to maintain at certain
109 temperature. The HPLC system was coupled to an ultraviolet/visible (UV) detector
110 (G1315B) and an electrospray ionization (ESI) ion trap MS (Agilent 1100 series Ion
111 Trap VL) for signal detection (Fig. 2). Signals and data were processed by Agilent
112 Chemstation signal processing system.

113 **2.3 Sample preparation**

114 **2.3.1 Preparation of Carrez's reagent**

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115 Carrez's reagent (I) was prepared by dissolving 21.9 g zinc acetate in 3 mL
116 glacial acetic acid which was further diluted with deionized water to 100 mL. Carrez's
117 reagent (II) was prepared by dissolving 10.6 g potassium hexacyanoferrate(II)
118 trihydrate in 100 mL deionized water [35]. Then these two Carrez's reagents are used
119 for the protein precipitation.

120 **2.3.2. Preparation of milk sample**

121 Five commercial local brand whole milk samples were bought and stored in the
122 refrigerator at 4°C. When analysis, they were taken out from the refrigerator and put
123 on the bench to reach room temperature. Five milliliters of milk sample were
124 transferred to a 15 mL centrifuge tube and 0.5 mL Carrez's reagent (I) were added,
125 then they were shaken for 1 min to homogeneity. Subsequently, 0.5 mL Carrez's
126 reagent (II) were added and shaken for another 1 min. Thereafter, 4 mL methanol
127 were added and shaken for 1 min. The homogenized sample was centrifuged 5 min at
128 a speed 3500 rpm. The supernatant was syringe filtered through a 0.45 μm syringe
129 filter membrane. The clear filtrate was used for analysis.

130 **2.4 Column-switching procedure for on-line sample pretreatment**

131 For finding the suitable column-switching time, ABA and IVE was detected by a
132 UV detector at a wavelength 245 nm. A 20 μL of the prepared milk sample containing
133 1 μg mL^{-1} of ABA and IVE standard, respectively, was loaded and injected with an

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134 autosampler (AS) into the SPE column at the switching valve position shown in Fig.
135 2A. Mobile phase 1 (methanol/water, 90/10, v/v) was delivered by Pump 1 at 0.2 mL
136 min^{-1} to the SPE pretreatment column. At the time ABA and IVE is about to be eluted
137 out from the SPE column, the switching valve was turned to the analysis position (Fig.
138 2B) to back flush the milk sample with mobile phase 2 (acetonitrile/water, 10/90, v/v)
139 which was delivered by Pump 2 at 0.2 mL min^{-1} to the analytical column. The
140 switching valve was then switched back to the sample pretreatment position (Fig. 2A)
141 at the time that both ABA and IVE have just gone into the analytical column. Mobile
142 phases were filtered through a 0.45-μm membrane filter prior to use and the SPE
143 column, switching valve, and analytical column were controlled at temperature 40°C
144 by the column oven. After separation by the analytical column, ABA and IVE were
145 delivered to the ESI interface, next, to the ion trap mass spectrometer for detection.

146 **2.5 LC-MS/MS measurement conditions**

147 The MS detection for ABA and IVE was in positive mode. The instrumental
148 conditions for the mass spectrometer were as follows: nitrogen gas pressure of the
149 nebulizer was 40 psi, dry nitrogen gas flow was 8 L min^{-1} and at 350°C; voltage of the
150 capillary tube at the entrance was 5000 V; and, the helium buffer gas pressure of
151 ion-trap was set at 70 psi. Parameters of the ion trap MS for the mass scan range, the
152 number of ions collected in one batch, and the accumulated ion collection time were

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153 *m/z* 600–800, 30000 ions, and 10 ms, respectively. However, the number of ions
154 collected in one batch and the accumulated ion collection time overrode each other
155 depending on which parameter value was reached first. Other parameters were
156 adjusted automatically to the optimal value using the software smart mode.

157 The qualitative identification of compounds with the low-resolution LC-ESI-ion
158 trap-MS/MS is performed by the LC retention time and the match between precursor
159 ions and the relative abundance of two product ions in multiple-reaction monitoring
160 (MRM). Therefore, the identification of ABA was confirmed by the precursor ion (*m/z*
161 895.4) at the retention time of the extracted ion chromatography (EIC) and the
162 presence of product ion fragment (*m/z* 751.3). The identification of IVE was
163 confirmed by the precursor ion (*m/z* 897.5) and its product ion fragment (*m/z* 753.4).

164 **2.6 Matrix-fortified standard calibration curve**

165 The 1,000 μg mL^{-1} standard stock solution of ABA and IVE was prepared by
166 dissolving 10 mg each of ABA and IVE standard in 10 mL methanol. The two stock
167 solutions were stored at -20°C for later use. The stock solution was further diluted
168 with methanol to prepare respectively five standard solutions of concentration 0.3, 0.6,
169 0.9, 1.2, and 1.5 μg mL^{-1}. They were further individually diluted with the blank milk
170 sample to give five standard solutions in milk matrix of concentration 3.0, 5.9, 8.9,
171 11.9, and 14.9 ng mL^{-1}. Theses standard samples plus one blank milk sample were

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172 treated with Carrez's reagent (I) and (II) and extracted using methanol as described in
173 section 2.3.2. The matrix-fortified standard calibration curves of ABA and IVE were
174 then produced with the six standard solutions. The linear calibration equations of ABA
175 and IVE were obtained by using linear least squares regression method with MS
176 intensities of ABA or IVE versus their corresponding standard concentrations.

177 The limit of detection (LOD) and limit of quantification (LOQ) of ABA and IVE
178 for the on-line SPE-LC/MS/MS which follows the criteria of three times
179 signal-to-noise ratio (3*S/N*) and ten times signal-to-noise ratio (10*S/N*) were estimated
180 by preparing the matrix-fortified standard calibration curve with standard solution
181 concentrations of 0, 3.0, 5.9, and 8.9 ng mL^{-1} [36]. However, the LOD and LOQ of
182 ABA and IVE for the on-line SPE-LC/UV were estimated by preparing the
183 matrix-fortified standard calibration curve with standard solution concentrations of 0,
184 0.2, 0.4, and 0.6 μg mL^{-1}.

185 **2.7 Quantitative analysis of milk sample by standard addition method**

186 Five different brands of whole milk were separately pretreated by the procedure
187 described in section 2.3.2 and six 5 mL aliquots of the pretreated milk samples were
188 obtained, respectively. Then, six 50 μL of the matrix-fortified standard solutions with
189 concentration of 0, 3.0, 5.9, 8.9, 11.9, and 14.9 ng mL^{-1} were separately added to the
190 six 5 mL aliquots of the pretreated milk samples. The six standard solution added milk

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191 samples were analyzed by the on-line SPE-LC/MS/MS. For each brand of milk
192 sample, the standard addition calibration curve of ABA and IVE was separately
193 prepared [37] to find the concentration of ABA or IVE in the unknown milk sample.
194

195 # 3. Results and discussion

196 **3.1 Selection of column-switching time**

197 The on-line sample pretreatment by SPE column to eliminate most matrices
198 needs the coordination of column-switch technique to transfer the pretreated analyte
199 components to the analytical column for separation and further to the detector for
200 detection. The selection of suitable column-switching time is important because it
201 concerns about the accuracy of quantitative analysis. In this study, the UV detector
202 was convenient for use to select the column-switching time. Different
203 column-switching time intervals were tested and the average peak areas of 5 repeated
204 measurements and their standard deviations for the peak ABA and IVE in the UV
205 chromatogram were compared, respectively. For the selection of first
206 column-switching time, 0.8, 0.9, 1.0, and 1.1 min were tested for changing the
207 switching valve position and the time at 2.6, 2.7, 2.8, and 2.9 min were checked for
208 the second column-switching time The sixteen signal intensities for the sixteen
209 combinations of column-switching time intervals were compared by one-way analysis

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210 of variance (ANOVA) and the least significant difference (LSD) test to show the
211 differences among them [36]. Then the selection of an ideal optimal column-switching
212 time interval is according to a maximum peak area and a minimal standard deviation
213 of peak area. The comparison results demonstrate that the best column-switching time
214 interval for simultaneous analysis of both ABA and IVE was 0.9-2.9 min.

215 **3.2 Qualitative analysis of ABA and IVE**

216 Since the molecular weight of ABA and IVE is 873 and 875 g mol^{-1}, respectively,
217 the base peak in the primary mass spectrum shown in Fig. 3A and Fig. 3C for ABA
218 and IVE was *m/z* 895.4 ($[M + Na]^+$) and *m/z* 897.5 ($[M + Na]^+$), respectively, which is
219 formed by chelating a sodium ion (Na^+), and is also the parent ion. Fig. 3B was the
220 secondary mass spectrum obtained from the precursor ion *m/z* 895.4 in Fig. 3A and
221 the base peak *m/z* 751.3 $[M{-}144{+}Na^+]$ was the product ion. Similarly, the secondary
222 mass spectrum shown in Fig. 3D was obtained from the precursor ion *m/z* 897.5 (Fig.
223 3C) and the base peak *m/z* 753.4 $[M{-}144{+}Na^+]$ was its product ion. The loss of a 144
224 Da ion fragment from the precursor ions is due to the loss of a pyranose moiety from
225 the original macrocyclic structure of ABA and IVE, respectively [38]. Another
226 product ion with *m/z* 607.2 shown in Fig. 3B is produced from the loss of another
227 pyranose moiety from the product ion *m/z* 751.3 and the loss of two pyranose moieties
228 from the precursor ion *m/z* 895.4 shown in Fig. 3A. Similarly, the product ion with

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229 *m/z* 609.3 shown in Fig. 3D is obtained by losing one pyranose moiety from the
230 product ion *m/z* 753.4 or losing two pyranose moieties from the precursor ion *m/z*
231 897.5 shown in Fig. 3C. Since the two product ions, *m/z* 751.3 and *m/z* 753.4, are the
232 strongest signal in the secondary mass spectrum of ABA and IVE, respectively, the
233 parent ion *m/z* 895.4 and the product ion *m/z* 751.3 of ABA and the parent ion *m/z*
234 897.5 and the product ion 753.4 of IVE were used for mass spectrometric analysis.
235 Accordingly, the mass scan range was set from m/z 500 to m/z 900 to shorten the
236 scanning time and increase the number of scan for raising the detection sensitivity.

237 **3.3 Estimation of LOD and LOQ**

238 The limit of detection (LOD) and the limit of quantification (LOQ) of the
239 proposed analytical method for ABA and IVE in milk samples were estimated from
240 the secondary mass spectrometry according to the matrix-fortified calibration curve
241 prepared by matrix-fortified samples containing both ABA and IVE standards of 3.0,
242 6.0, and 9.0 ng mL^{-1} plus the blank sample extract. The linear standard calibration
243 equations for ABA and IVE were $y = 139.58x – 18.27$ ($r^2 = 0.9978$) and $y = 239.85x +$
244 0.77 ($r^2 = 0.9980$), respectively. The LOD was then calculated by dividing 3 times the
245 standard deviation ($s_{y/x}$) of the linear calibration curve with the slope of the linear
246 calibration equation and the LOQ was calculated by dividing 10 times of $s_{y/x}$ with the
247 slope of the linear calibration equation [36]. Thus, the LOD (LOQ) of analysis for

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248 ABA in milk was 0.67 ng mL^{-1} (2.23 ng mL^{-1}) and for IVE in milk was 0.63 ng mL^{-1}
249 (2.11 ng mL^{-1}) which are listed in Table 1. The LODs (LOQs) of ABA and IVE are 55
250 and 54 folds better than those of the UV detection.

251 **3.4 Determination of ABA and IVE in milk by standard addition method**

252 Five domestic brands of milk samples named anonymously as A, B, C, D, and E
253 were selected for the determination of Abamectin and Ivermectin residues by the
254 standard addition method described in section 2.7. The TIC of purified water spiked
255 with 15 ng mL^{-1} each of ABA and IVE is shown in Fig. 4A. The EICs for the product
256 ion *m/z* 751.3 of ABA and for the product ion *m/z* 753.3 of IVE are shown in Fig. 4B
257 which illustrates the detection time for ABA and IVE is around 4.1 min and 5.8 min,
258 respectively. We also need to mention that trace ABA and IVE were not found in the
259 secondary mass spectra of purified water.

260 Ten standard addition calibration curves were prepared and used for the
261 determination of ABA and IVE in the five different brands (A, B, C, D, and E) of milk
262 samples, respectively. The ten linear equations and their corresponding linear
263 correlation coefficients (r^2) are listed in Table 2. Since milk samples of brand A and
264 brand E contained both ABA and IVE residues while brand B, C, and D did not, the
265 concentrations and their corresponding standard deviations [37] of ABA and IVE
266 residues in milk samples of brand A and E are calculated and listed in Table 1 and

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267 Table 2.

268 Because the amount of both ABA and IVE residues in milk samples of brand A
269 and E was all very small, a *t*-test was used to compare their concentrations with their
270 corresponding LODs to see whether there is difference between them or not and was
271 used to judge if ABA and IVE exist in milk. The comparison results are listed in Table
272 1. Since the result of *t*-test shows no difference of the determined concentrations for
273 both ABA and IVE residues in milk samples of brand A and E with their
274 corresponding LODs, we conclude with a 95% confidence that the milk samples of
275 brand A and E do contain trace amount of ABA and IVE residues, even though the
276 two determined concentrations of ABA in brand A and E seem smaller than the
277 corresponding LOD. These odd results were simply due to the intrinsic large
278 measurement error when the determined quantities are close to the LOD.

279 The corresponding secondary mass spectra of ABA and IVE for milk samples of
280 brand A and E are shown in Fig. 5. The mass signal *m*/*z* 751.3 and *m*/*z* 753.4 shown in
281 Fig. 5A and 5B, respectively, indicates the existence of ABA and IVE in brand A milk
282 sample. Also, the mass signal *m*/*z* 753.1 and *m*/*z* 607.2 shown in Fig. 5C and the mass
283 signal *m*/*z* 753.4 shown in Fig. 5D prove the existence of ABA and IVE in brand E
284 milk sample. The complication of the mass spectra in Fig. 5A-5D was due to the
285 relative high background signals of deionized distilled water by the corresponding

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286 very low amount of ABA and IVE in the samples.

287 **3.5 Determination precision and accuracy**

288 The measurement precisions of ABA and IVE residues in milk samples of brand
289 A and E by the developed on-line SPE-LC/MS/MS method are listed in Table 3. The
290 precisions at concentration levels near their LODs for ABA and IVE residues were all
291 less than 10%. Therefore, the determination of ABA and IVE residues in milk samples
292 using the developed method has very good reproducibility.

293 Standard ABA and IVE with concentration approximately the same as the
294 determined concentration were spiked into the milk samples of brand A and E to
295 estimate the analytical accuracies. An overall average concentration from a total of 25
296 measurements was obtained and also listed in Table 3. The spiked recoveries were
297 calculated and transferred to the analytical accuracy according to the equation,
298 accuracy (%) = 100% – |recovery (%) – 100%|. The analytical accuracies of ABA and
299 IVE residues in milk sample were from 82% to 88% which demonstrate satisfactory
300 analytical accuracy for the developed method.

301 **3.6 Comparison of methods and performance**

302 Table 4 shows the performance of the developed method compared to other
303 methods of recent advances to obtain an overall understanding of these methods for
304 the analysis of ABA and IVE residues in milk sample. The most sensitive method in

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305 our references was the use of LC-APCI(–)-IT-MS/MS coupled with an off-line
306 DLLME [13] which has a LOD (LOQ) of 0.12 (0.40) ng mL^{-1} for ABA and 0.03 (0.10)
307 ng mL^{-1} for IVE; this method also shows a very good analysis accuracy (90.2-97.1%).
308 The LOD (LOQ) of our method with an on-line SPE sample pretreatment is about the
309 same as most other methods at the tenth ng mL^{-1} (ppb) level which can fulfill the
310 detection requirement of MRL set by the regulation authorities. Most of methods
311 including ours possessed an analysis precision less than 10% except the method using
312 LC-ESI(+)-QqQ-MS/MS with an off-line liquid-liquid extraction followed by a low
313 temperature purification (LLE-LTP) [6] and the method using
314 UPLC-ESI(+)-QqQ-MS/MS with an off-line LLE [15]. Most of methods including
315 ours demonstrated ideal analysis accuracy (> 80%) and the most accurate method was
316 HPLC-FL (99.2% and 98.8%) coupled with an off-line LLE-LTP [6] at a quite high
317 spiked concentration of 5 ng mL^{-1}. The analysis accuracy for both the two methods,
318 LC-ESI(+)-QqQ-MS/MS with an off-line LLE-LTP sample pretreatment [6] and
319 UPLC-ESI(+)-QqQ-MS/MS with an off-line SPE sample pretreatment [17], was less
320 than 80% at a spiked concentration larger than 5 ng mL^{-1} (5-50 ng mL^{-1}). The running
321 time of one sample for all the LC-MS methods is quite rapid within 12 min with the
322 fastest analysis time of 4 min [17]. The analysis time for HPLC-FL method was
323 slowest at 35 min [6]. Therefore, the comparison of performance of our on-line

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324 SPE-LC-ESI(+)-IT-MS/MS method with other recent developed methods indicates
325 that overall our method is sensitive, precise, accurate, and practical and can meet the
326 requirement of MRL set by regulation authorities such as EU, US, and Taiwan.
327

328 4. Conclusion

329 The present methods for analysis of veterinary drugs such as Abamectin and
330 Ivermectin residues in milk are all coupled with off-line sample pretreatment. The
331 development of an on-line solid-phase extraction coupled liquid
332 chromatography-tandem mass spectrometry for analysis of Abamectin and Ivermectin
333 residues in milk samples sold in retail stores is the first study shown in literature
334 which demonstrates a convenient, rapid, sensitive, precise, and accurate method. The
335 developed on-line SPE-ESI(+)-IT-MS/MS method for analysis of Abamectin and
336 Ivermectin residues in milk can be used as a standard inspection method to meet the
337 requirement of MRL set by regulation authorities and can be extended to other
338 macrolide antibiotics.
339

340 Acknowledgements

341 The authors gratefully acknowledge the financial support of this study from
342 National Science under grant number NSC 101-2113-M-033-001 and from Chung

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343 Yuan Christian University. The providing of necessary LC-MS facility for this
344 research from Animal Technology Institute Taiwan is also acknowledged.

345

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419 **Figure Captions**

420 Figure 1 (A) Structure of Abamectin and (B) structure of Ivermectin.

421 Figure 2 Schematic diagram of solid-phase extraction coupled liquid
422 chromatography-electrospray-ion trap-tandem mass spectrometry: (A)
423 column switching valve position for sample pretreatment with
424 solid-phase extraction column, (B) column switching valve position for
425 sample separation and analysis with analytical column.

426 Figure 3 Mass spectra of 0.1 μg mL^{-1} Abamectin standard solution: (A) the
427 primary mass spectrum, (B) the secondary mass spectrum from the
428 precursor ion *m/z* 895.4. Mass spectra of 0.1 μg mL^{-1} Ivermectin
429 standard solution: (C) the primary mass spectrum, (D) the secondary
430 mass spectrum from the precursor ion *m/z* 897.5.

431 Figure 4 Analysis of pure water by solid-phase extraction coupled liquid
432 chromatography-tandem mass spectrometry: (A) the total ion
433 chromatogram (TIC) of pure water spiked with 15 ng mL^{-1} each of
434 Abamectin and Ivermectin, (B) the extracted ion chromatograms (EICs)
435 of secondary mass spectrometry for water sample spiked with 15 ng
436 mL^{-1} each of Abamectin (black line) and Ivermectin (red line).

437 Figure 5 Secondary mass spectra of Abamectin and Ivermectin in milk samples of

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438 brand A and E: (A) Abamectin (*m/z* 751.3) of brand A, (B) Ivermectin

439 (*m/z* 753.4) of brand A, (C) Abamectin (*m/z* 751.3) of brand E, (D)

440 Ivermectin (*m/z* 753.4) of brand E.

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454 Table 1 LODs/LOQs of Abamectin and Ivermectin in milk samples and comparison results with their determined quantities in brand A
455 and E

Analyte	Brand	Measured concentration (ng mL^{-1})	n	LOD/LOQ (ng mL^{-1})	t_{calc}[a]	t_{table}[a]	Difference between measured concentration and LOD
Abamectin	A	$0.6_5 \pm 0.3_6$	5	$0.6_7/2.2_3$	0.124	2.78	no
	E	$0.5_7 \pm 0.4_7$	5		0.476		no
Ivermectin	A	$0.7_9 \pm 0.4_1$	5	$0.6_3/2.1_1$	0.873	2.78	no
	E	$1.0_4 \pm 0.4_5$	5		2.037		no

456 [a] 95% confidence level

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463 Table 2 Ten linear equations and their corresponding linear correlation coefficients (r^2)
464 of standard addition calibration curve for Abamectin and Ivermectin in the
465 five brands of milk sample

Analyte	Brand	Linear equation	r^2	Measured concentration[a] (ng mL^{-1})
Abamectin	A	$y = 180.23x + 117.98$	0.9959	$0.6_5 \pm 0.3_6$
	B	$y = 185.97x - 33.59$	0.9986	0
	C	$y = 164.45x - 51.43$	0.9948	0
	D	$y = 179.86x - 84.23$	0.9942	0
	E	$y = 195.88x + 111.28$	0.9906	$0.5_7 \pm 0.4_7$
Ivermectin	A	$y = 68.26x + 53.61$	0.9931	$0.7_9 \pm 0.4_1$
	B	$y = 75.03x - 24.95$	0.9957	0
	C	$y = 69.67x -20.09$	0.9959	0
	D	$y = 69.15x - 5.61$	0.9976	0
	E	$y = 75.70x + 78.78$	0.9941	$1.0_4 \pm 0.4_5$

466 [a] standard deviation = $\frac{s_y}{|m|}\sqrt{\frac{1}{n}+\frac{\bar{y}^2}{m^2\sum(x_i-\bar{x})^2}}$

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477 Table 3 Analytical precision and accuracy of Abamectin and Ivermectin by SPE-LC-ESI(+)-MS/MS[a]

Analyte	Brand	Original concentration (ng mL^{-1})	Spiked concentration (ng mL^{-1})	Theoretical total concentration (ng mL^{-1})	Measured total concentration (ng mL^{-1})	RSD (%)	Recovery (%)	Accuracy (%)
Abamectin	A	0.65	0.66	1.31	$1.13 \pm 0.09_0$	8.0	86	86
	E	0.57	0.53	1.10	$0.95 \pm 0.06_9$	7.3	86	86
Ivermectin	A	0.79	0.73	1.51	$1.24 \pm 0.05_9$	4.8	82	82
	E	1.04	1.01	2.04	$1.80 \pm 0.17_5$	9.7	88	88

478 [a] Each milk sample was measured five times ($n = 5$).

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486 Table 4 Comparison of methods and performance

Method	Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Precision (%)	Accuracy (%)	Analysis time (min)	Reference
HPLC-DAD[a]	Abamectin	0.5	1.7	9.9	-	12	13
	Ivermectin	0.3	1.0	8.6	-		
HPLC-FL[b]	Abamectin	0.4	1.2	3.9	99.2	35	6
	Ivermectin	1.2	4.0	3.9	98.8		
LC-MS/MS[c]	Abamectin	0.12	0.40	9.8	90.2-96.5	12	13
	Ivermectin	0.03	0.10	7.7	91.5-97.1		
LC-MS/MS[d]	Abamectin	0.6	1.9	11.2	74.3	11	6
	Ivermectin	2.9	9.7	15.1	66.6		
UPLC-MS/MS[e]	Abamectin	0.6	2.0	8-16	81-95	10	15
	Ivermectin	0.6	2.0	5-15	81-95		
UPLC-MS/MS[f]	Abamectin	0.25	0.83	6.4-8.6	62.4-74.3	4	17
	Ivermectin	0.68	2.27	6.6-7.3	64.8-79.1		
SPE-LC-MS/MS	Abamectin	0.67	2.23	8.0, 7.3	86, 86	10	This work
	Ivermectin	0.63	2.11	4.8, 9.7	82, 88		

[a] Sample pretreatment: off-line DLLME.

[b] Sample pretreatment: off-line LLE–LTP; spiked concentration: 5 ng mL^{-1}.

[c] Method: LC-APCI(–)-IT-MS/MS; Sample pretreatment: off-line DLLME; spiked concentration: 2.0, 4.0, 10 ng mL^{-1} for ABA and 0.5, 1.0, 2.5 ng mL^{-1} for IVE.

[d] Method: LC-ESI(+)-QqQ-MS/MS.

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[e] Method: UPLC-ESI(+)-QqQ-MS/MS; sample pretreatment: off-line LLE.

[f] Method: UPLC-ESI(+)-QqQ-MS/MS; sample pretreatment: off-line SPE; spiked concentration: 10, 25, 50 ng mL^{-1} for both ABA and IVE.

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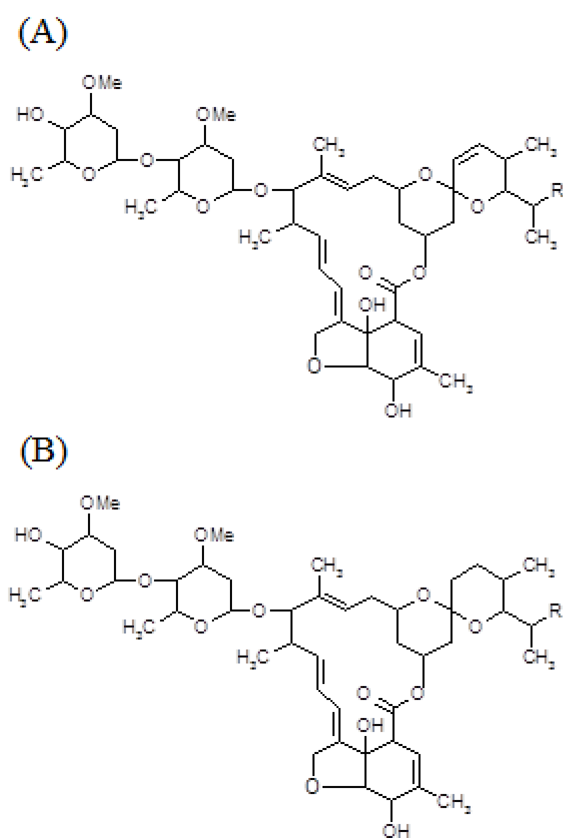

Figure 1

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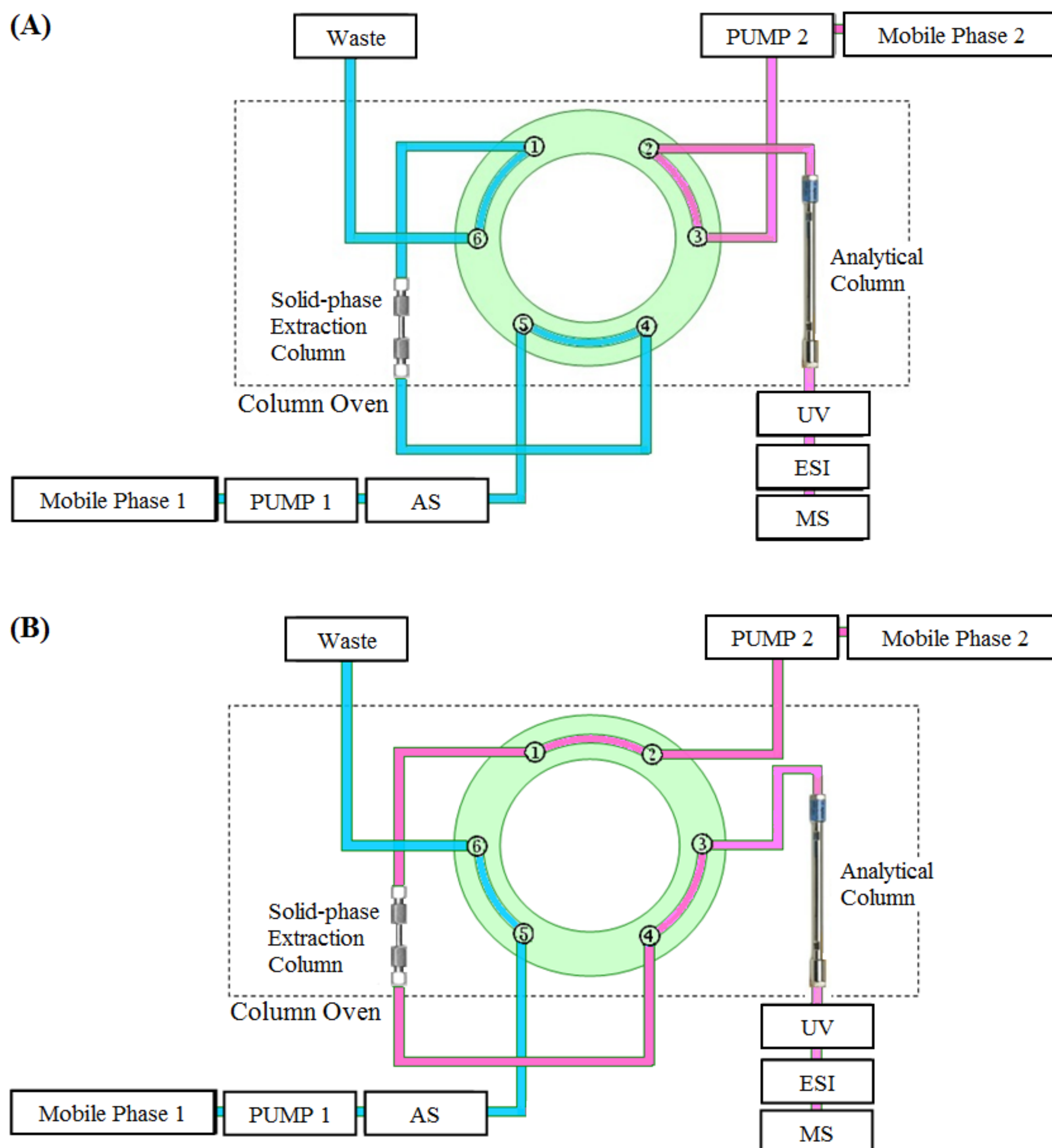

Figure 2

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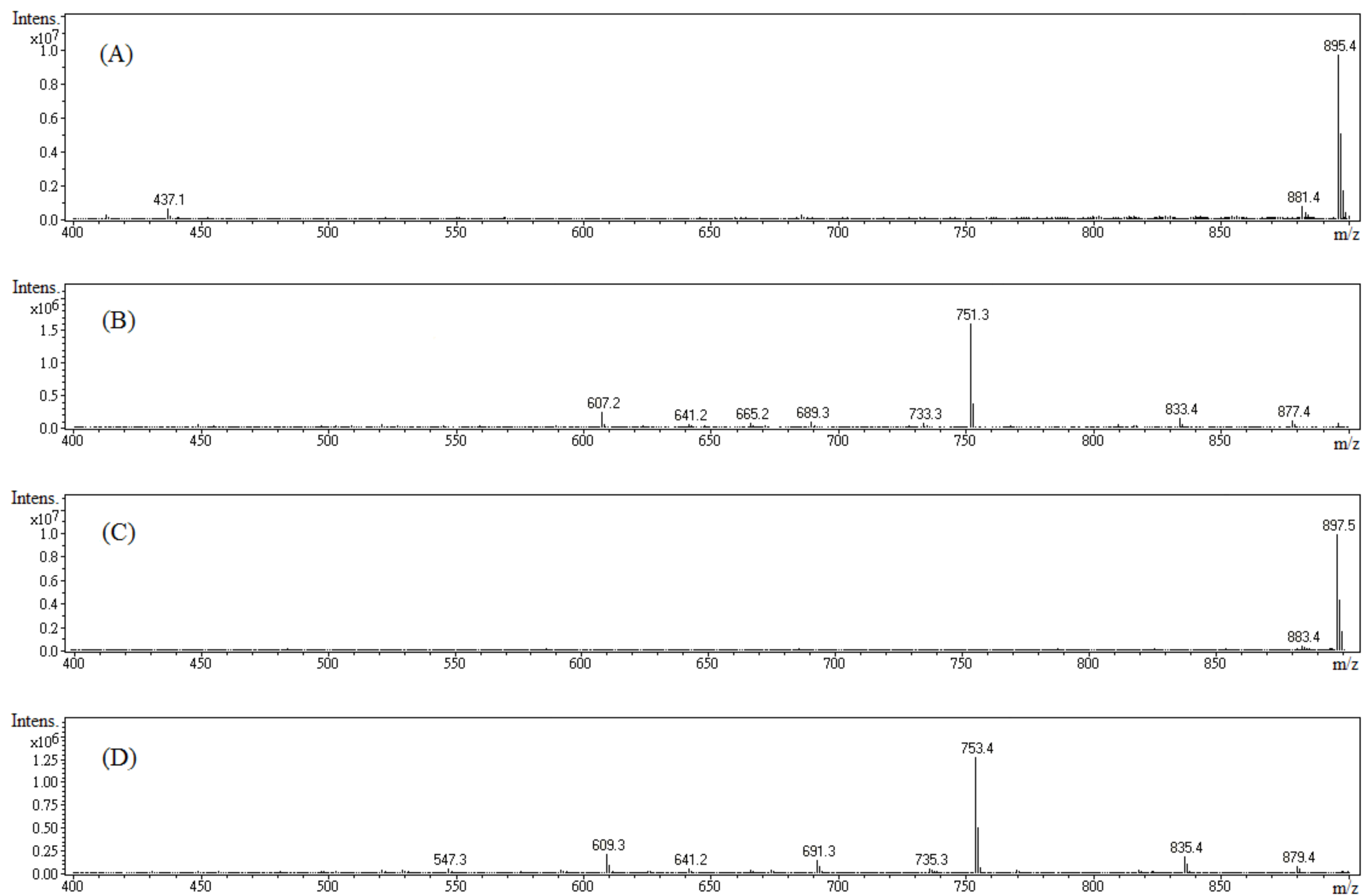

514 **Figure 3**

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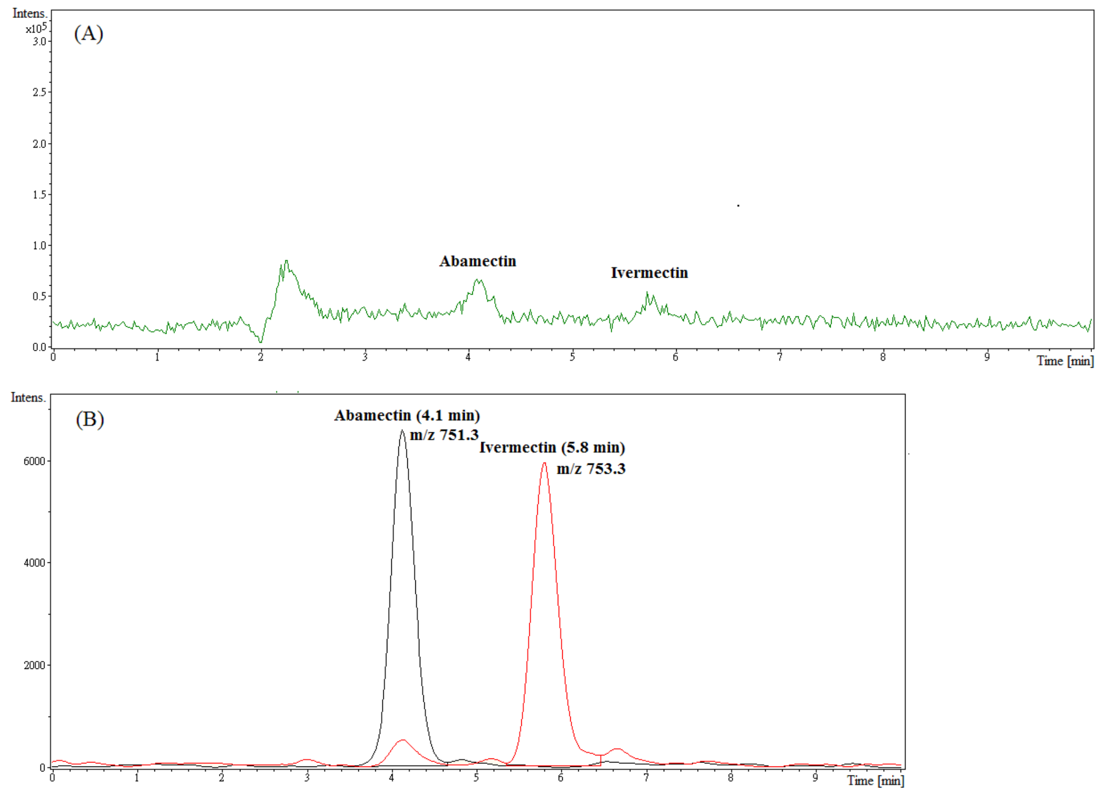

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Figure 4

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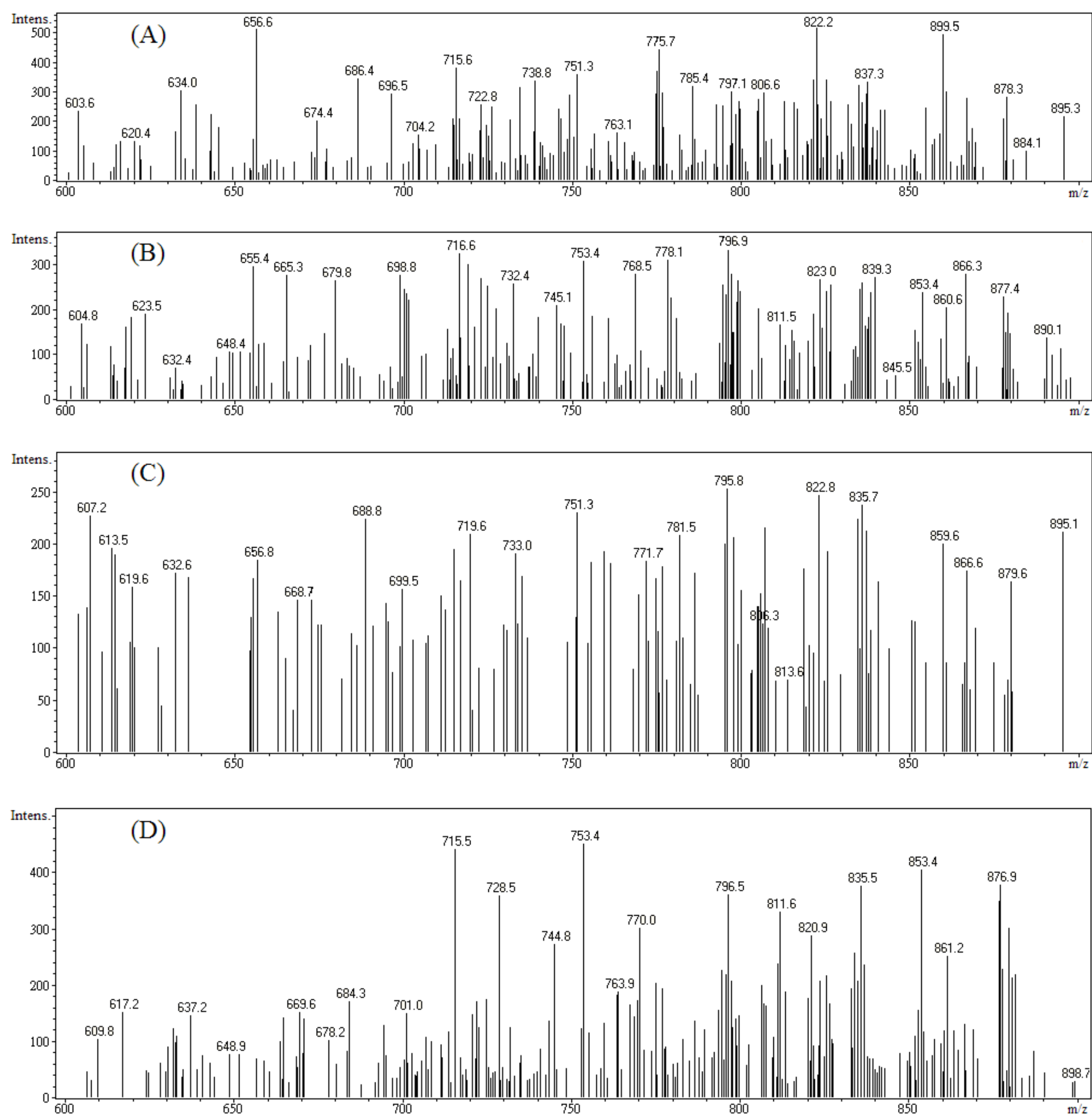

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Figure 5

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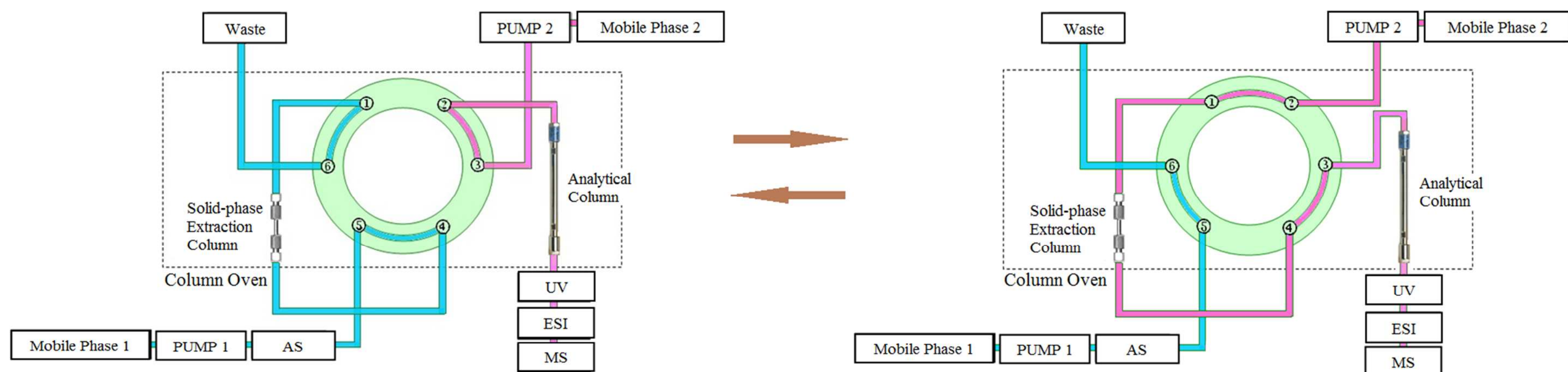

The on-line SPE-LC-ESI-ion trap/MS/MS for the simultaneously analysis of veterinary drugs Abamectin and Ivermectine residues in milk using column-switching technique.

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