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Analytical strategy for the determination of non-steroidal anti-inflammatory drugs in plasma and improved analytical strategy for the determination of authorised and non-authorised non-steroidal anti-inflammatory drugs in milk by liquid chromatography tandem mass spectrometry

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4 1 **Analytical strategy for the determination of non-steroidal anti-**
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6 2 **inflammatory drugs in plasma and improved analytical strategy for**
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9 3 **the determination of authorised and non-authorised non-steroidal**
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11 4 **anti-inflammatory drugs in milk by LC-MS/MS**
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10
11
12 **Abstract**

13 A sensitive and selective method for the determination of 6 non-steroidal anti-
14 inflammatory drugs in bovine plasma was developed. An improved method for the
15 determination of authorised and non-authorised residues of 10 non-steroidal anti-
16 inflammatory drugs in milk was developed. Analytes were separated and acquired by
17 HPLC coupled with an electrospray ionisation tandem mass spectrometer (LC-ESI-
18 MS/MS). Target compounds were acidified in plasma, and plasma and milk samples
19 were extracted with acetonitrile and both extracts were purified on an improved SPE
20 procedure utilising Evolute™ ABN SPE cartridges. The recovery of the methods for
21 milk and plasma was between 73 and 109 %. The precision of the method for
22 authorised and non-authorised NSAIDs in milk and plasma expressed as % RSD , for
23 the within –laboratory repeatability was less than 16 % and for authorised NSAIDs of
24 meloxicam, flunixin and tolfenamic acid at their associated MRLs in milk was less
25 than 10 % however for hydroxy flunixin was less than 25%. The methods were
26 validated according to Commission Decision 2002/657/EC.

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10 **Keywords:** Non-steroidal Anti-Inflammatory Drugs; Plasma; Milk; Liquid Chromatography
11 Tandem Mass Spectrometry; Method Validation

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16 34 **Introduction**

17
18 35 Carprofen (CPF), diclofenac (DCF), ibuprofen (IBP), ketoprofen (KPF), mefenamic
19
20 36 acid (MFN), phenylbutazone (PBZ), flunixin (FLU), hydroxy-flunixin (FLU-OH),
21
22 37 tolfenamic acid (TLF) and meloxicam (MLX) are non-steroidal anti-inflammatory
23
24 38 drugs (NSAIDs) and their structures are shown in Fig.1. Over the past number of
25
26 39 years, residues of NSAIDs in food are a cause for concern. Studies have illustrated
27
28 40 that the second most prescribed class of drugs after microbials is NSAIDs [Sundlof et
29
30 41 al. 1995]. Dairy farmers and veterinarians are using NSAIDs in dairy animals more
31
32 42 frequently [US Code, 1988] and studies have shown that their increased use [Kopcha
33
34 43 et al. 1992] poses a threat to human health as permitted residue levels are being
35
36 44 violated [Smith et al. 2008]. In 2007 the EC Rapid Alert System for Food and Feed
37
38 45 reported alert notifications in relation to horse meat for these substances. The
39
40 46 European Council recommend rigorous control of NSAIDs in food producing
41
42 47 animals [SANCO 2000] because of the health effects in humans such as aplastic
43
44 48 anaemia, gastrointestinal disorders, agranulocytosis [Insel 1990] and changes in renal
45
46 49 function [Goodman et al. 1992]. Long term exposure to PBZ has caused kidney
47
48 50 tumors in mice and liver tumors in rats [Kari et al. 1995]. In recent years the COX-II
49
50 51 inhibitor class of NSAIDs has been implicated in cardiovascular harm in humans
51
52 52 [Staa et al. 2008; Debabrata et al. 2008]. According to EU law, all drugs for
53
54 53 veterinary use need to be included in Annexes 1-3 of Regulation 2377/90

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3 54 [Commission Decision 1990]. This regulation establishes lists of compounds that
4
5 55 have a fixed maximum residue limit, MRL (Annex I), that need no MRL (Annex II)
6
7
8 56 or that have a provisional MRL (III). There are no MRL's set in plasma as is not an
9
10 57 edible matrix. The recommended minimum concentration for NSAIDs in plasma is set
11
12 58 at 5 ng mL⁻¹ [SANCO 2007]. In milk FLU, FLU-OH, TLF and MLX are included in
13
14
15 59 Annex I. CPF has been included in Annex II of the regulation only for bovine milk
16
17
18 60 [European Commission 2005]. DCF was not authorised for use in animals that
19
20 61 produce milk for human consumption [European Commission 2004] until recently
21
22 62 [EMEA]. KPF is listed in Annex II of the regulation. PBZ, MFN and IBP are
23
24
25 63 considered as prohibited substances and are not included in Annexes 1-3 and have no
26
27 64 maximum residue limit (MRL) established however the minimum recommended
28
29 65 concentration for analysis of NSAIDs with no MRL set in milk is 5 ng mL⁻¹. The
30
31
32 66 widespread use of NSAIDs presents a risk to the consumer if food containing residues
33
34 67 enter the food chain. In Ireland plasma and milk are some of the target matrices
35
36 68 chosen to identify the misuse of NSAIDs in animal production. The advantages of
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38
39 69 using plasma in regulatory control are that it is an easy matrix to handle for analysis
40
41 70 and PBZ residues can be found in this matrix for a long time (personal
42
43 71 communication with the CRL). Therefore the analytical method developed in this
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45
46 72 study in plasma concentrated on the analysis of 6 NSAIDs in bovine species. Methods
47
48
49 73 have been reported for the analysis of NSAIDs in plasma by LC-UV [De Veau, 1999;
50
51 74 Kvaternick et al. 2007; Luo et al. 2004; Hardee et al. 1982; Neto et al. 1996; Grippa et
52
53 75 al. 2000; Jedziniak et al. 2007; Singh et al. 1996; Gowik et al. 1998; Quintana et al.
54
55 76 2004; Fiori et al. 2004], GC-MS [Neto et al. 1996; Singh et al. 1991; Hines et al.
56
57 77 2004; Gonzalez et al. 1996; Jaussaud et al. 1992], LC-MS [Luo et al. 2004; Miksa et
58
59 78 al. 2005; Vinci et al. 2006; Quintana et al. 2004; You et al. 2008] and capillary

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3 79 electrophoresis [Gu et al. 1997]. The majority of methods that have been cited to date
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5
6 80 have been developed in equine plasma alone or in combination with other matrices
7
8 81 with limits of detection ranging from 0.1 ng mL^{-1} to 5 ug mL^{-1} [Miksa et al. 2005;
9
10 82 Luo et al. 2004; Hardee et al. 1982; Neto et al. 1996; Grippa et al. 2000; Singh et al.
11
12 83 1991; Hines et al. 2004; Gonzalez et al. 1996; Gowik et al. 1998; Vinci et al. 2006;
13
14 84 Gu et al. 1997; You et al. 2008]. Other methods exist for the determination of
15
16 85 NSAIDs in bovine plasma are available but the limits of detection range from 20 ng to
17
18 86 3.4 ug mL^{-1} [De Veau et al. 1999; Miksa et al. 2005; Jedziniak et al. 2007; Gowik et
19
20 87 al. 1998; Vinci et al. 2006; Quintana et al. 2004; Fiori et al. 2004]. Only two methods
21
22 88 are available in equine plasma to date capable of meeting the 5 ng mL^{-1} requirement.
23
24 89 A method by Luo et al [Luo et al. 2004] for a single residue had a limit of detection of
25
26 90 0.1 ng mL^{-1} for FLU. A multi-residue method by Gonzalez et al [Gonzalez et al.
27
28 91 1996] had a limit of detection of 5 ng mL^{-1} for IBP, FLU, DCF, TLF but limits of
29
30 92 detection of only $10\text{-}25 \text{ ng mL}^{-1}$ could only be achieved for KPF, MFN and PBZ.
31
32 93 Therefore no methods are available to date for KPF, MFN and PBZ in plasma that can
33
34 94 meet the target level of 5 ng mL^{-1} . A disadvantage of the method developed by
35
36 95 Gonzalez et al [Gonzalez et al. 1996] is that the method monitors 3 ions and this is not
37
38 96 a confirmatory method according to Commission Decision 2002/657/EC [European
39
40 97 Commission Decision. 2002] and a second analytical technique is required. Overall
41
42 98 there is a paucity of methods in the literature that are available for the analysis of
43
44 99 NSAIDs in bovine plasma and of those available, the methods are not sensitive
45
46 100 enough to meet the minimum required concentration of analysis set at 5 ng mL^{-1} .
47
48 101 Milk is the second target matrix analysed in this study and is important in food safety
49
50 102 because sampling can often be restricted to sampling of meat, milk, eggs and honey as
51
52 103 in the case of retail import/exports. Milk also allows the detection of drugs in live
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3 104 animals prior to slaughter. There are few analytical methods for the determination of
4
5 105 authorised and non-authorised NSAIDs in milk and usually analyse for only a few
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7
8 106 residues. Those that have been described use LC-UV [Martin et al. 1983; Gallo et al.
9
10 107 2008; Feely et al. 2002; Rubb et al. 1995; De Veau et al. 1996], LC-MS [Gallo et al.
11
12 108 2008; Boner et al. 2003; Daeseleire et al. 2003; Malone et al. 2009; Dowling et al.
13
14 109 2009] and GC-MS [Dowling et al. 2008; Rubb et al. 1995]. A method by Gallo et al
15
16 110 [Gallo et al. 2008] is capable of analysing 16 NSAIDs in milk using two separate
17
18 111 analytical techniques and involves using a screening LC-DAD method with limits of
19
20 112 detection (LOD) of between 2- 15 ng mL⁻¹ and a runtime of 35 min with an
21
22 113 equilibration time of 15 min per injection. Confirmation is achieved using an LC ESI-
23
24 114 Iontrap -MS/MS method with an LOD of 5 ng mL⁻¹ except for flurbiprofen with a
25
26 115 runtime of 40 min per injection. The LC-MS method does not meet the requirements
27
28 116 for a confirmatory method according to Commission Decision 2002/657/EC and a
29
30 117 third analytical technique is required. A method by Stolker et al [Stolker et al. 2008] is
31
32 118 capable of analysing 20 NSAIDs in milk using a quantitative screening method
33
34 119 (UPLC-TOF-MS) with LOD's for specific NSAIDs such as NAP, PBZ and DCF at
35
36 120 12.5, 25 and 6.3 ng mL⁻¹ and a runtime of 8.5 min per injection. The method cannot
37
38 121 meet the 5 ng mL⁻¹ for NAP and PBZ or 0.1 ng mL⁻¹ recently set for DCF and
39
40 122 additionally the analysis by TOF-MS, medium to high resolution of approximately
41
42 123 10,000 FWHM is not included in Commission Decision 2002/657/EC.
43
44 124 Other methods for the determination of NSAIDs in milk have limits of detection of 20
45
46 125 ng mL⁻¹ for PBZ [Martin et al. 1983], 0.2 ng mL⁻¹ for FLU and FLU-OH [Boner et al.
47
48 126 2003], 0.5 ug kg⁻¹ for FLU, FLU-OH and 1 ug kg⁻¹ for KPF [Daeseleire et al. 2003],
49
50 127 53.05, 15.82, 61.39, 45.04 ng mL⁻¹ for TLF, MLX, 4-MAA and FLU-OH [Malone et
51
52 128 al. 2009], 0.46-2.86 ng mL⁻¹ for CPF, DCF, MFN, niflumic acid (NIFLU), naproxen
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4 129 (NAP), oxyphenylbutazone (OXYPHEN), PBZ and suxibuzone (SUXI) [Dowling et
5
6 130 al. 2009] 0.59, 2.09, 0.90 and 0.70 ng mL⁻¹ for IBP, KPF, DCF and PBZ [Dowling
7
8 131 et al. 2008], 1 ng mL⁻¹ for FLU [Feely et al. 2002], 1.7 ng mL⁻¹ for FLU [Rubb et al.
9
10 132 1995] and lowest fortification in matrix was 25 ng mL⁻¹ for PBZ [De Veau et al.
11
12 133 1996]. The objective of this study was to develop an analytical strategy for the
13
14 134 determination of NSAIDs in bovine plasma and for the determination of authorised
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16 135 and non-authorised NSAIDs simultaneously in milk that meet the EU target levels set
17
18 136 and validate according to Commission Decision 2002/657/EC.

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21
22 137 In this study an improved purification procedure was developed using Evolute
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24 138 ABNTM solid phase extraction cartridges for the analysis of a wider range of NSAIDs
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26 139 including authorised and non-authorised NSAIDs in bovine milk. The developed
27
28 140 procedure was suitable for the purification of 6 NSAIDs in bovine plasma. An
29
30 141 improved liquid chromatography tandem mass spectrometry detection technique was
31
32 142 developed to analyse 10 NSAIDs simultaneously with a run-time of 15 min. The
33
34 143 methods in each matrix were comprehensively validated according to Commission
35
36 144 Decision 2002/657/EC. The methods were implemented into the National Monitoring
37
38 145 Programme in Ireland for veterinary drug residues and accredited according to the
39
40 146 ISO17025 Standard. The proposed method in milk does not cover the glucuronides.
41
42 147 This is the first time that suitably sensitive methods for the analysis of NSAIDs in
43
44 148 plasma and for the analysis of the selected range of authorised and non-authorised
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46 149 NSAIDs using Evolute ABNTM solid phase extraction cartridges simultaneously in
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48 150 bovine milk are available.
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Materials and Methods*Materials and reagents*

Water, ethanol, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid (37 %), n-hexane and iso-octane (HiPerSolv grade) were obtained from BDH (Merck, UK). CPF, DCF, IBP, MFN, FLU, KPF, MLX, TLF and PBZ were purchased from Sigma (Sigma Aldrich, Ireland). FLU-OH was obtained as a gift from The Community Reference Laboratory for NSAIDs in the EU in Germany. d₁₀-PBZ was obtained from Cambridge Isotope Labs (Cambridge Isotope Labs, USA). d₃-MLX, d₃-IBP and d₄-DCF were obtained from CDN Isotopes (CDN Isotopes, Canada). d₃-FLU was obtained from Witega (Witega, Germany). d₄-TLF was obtained as a gift from Stormont, (Stormont, UK). Primary stock standard solutions (stable for 12 months) were prepared in ethanol at a concentration of 1 mg mL⁻¹. Intermediate single standard solutions (stable for 6 months) were prepared in methanol at a concentration of 10 µg mL⁻¹. CPF, DCF, IBP, MFN, FLU, FLU-OH, KPF, TLF, MLX and PBZ standard fortification solution for plasma (stable for 6 months) was prepared in methanol at a concentration of 500 ng mL⁻¹ from the 10 µg mL⁻¹ intermediate stock solution. Internal standard fortification solution for milk or plasma containing d₃-MLX, d₄-DCF, d₃-IBP d₃-FLU d₄-TLF and d₁₀-PBZ was prepared at a concentration of 1.25 µg mL⁻¹. CPF, DCF, IBP, MFN, KPF and PBZ standard fortification solution for milk (NMRL) was prepared in methanol at a concentration of 500 ng mL⁻¹ from the 10 µg mL⁻¹ intermediate stock solution. MLX, FLU, FLU-OH and TLF standard fortification solution for milk (MRL) was prepared in methanol at a concentration of 1.5, 4, 4 and 5 µg mL⁻¹. All standards were stored at 4 °C in the dark. Isolute™ Evolute ABN 50 µm solid phase extraction cartridges (10 mL, 100 mg) were obtained

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3 176 from Biotage (Biotage, UK). Methanol:water (10:90, v/v) and 10 mM ascorbic acid
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5 177 were used as solid phase extraction wash solvents. N-hexane:diethyl
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7
8 178 ether:acetonitrile: methanol (45:45:7:3,v/v) was used as the solid phase extraction
9
10 179 elution solvent. Injection solvent was water:acetonitrile (90:10, v/v).
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15 181 *LC-MS/MS conditions*

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17 182 The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B
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19 183 Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven
20
21 184 (Agilent Ireland). The NSAIDs were chromatographed on a 1.8 µm Agilent Eclipse
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23 185 Plus C₁₈ column (3.0 × 50 mm) (Agilent, Ireland) and the column temperature was
24
25 186 maintained at 55 ° C. A gradient was applied with water containing 0.001 M acetic
26
27 187 acid and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B) (Table
28
29 188 1). The total run time was 15 minutes. The injection volume was 15 µL. The mass
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31
32 189 spectrometer used was a QTRAP 4000 with a TurboIonSpray source from Applied
33
34 190 Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by
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36 191 version 1.5 of Analyst software. The described LC-MS/MS system was shown to be
37
38 192 suitable for the analysis of NSAIDs in plasma (Figure 2-7) and milk (Figure 2-11).
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45 194 *MS/MS parameters*

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47 195 The analysis was performed using negative ion electrospray MS/MS in multiple
48
49 196 reaction monitoring (MRM) mode. The collision voltages were optimised as shown
50
51 197 (Table 2). Each transition was performed with a 13 msec dwell time and a pause time
52
53 198 of 3 msec. The MS/MS detector conditions were as follows: Ion mode electrospray
54
55 199 negative; curtain gas 45 psi; ion spray voltage 4400 V; temperature 650 °C; ion source
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3 200 gas one 70 psi; ions source gas two 70 psi; Interface heater on; entrance potential 10
4
5 201 V; Resolution Q1 unit; Resolution Q2 unit; CAD gas =high
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10 203 *Plasma/milk samples*
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12 204 Plasma/milk obtained for use as negative controls was separated into 50 mL aliquots
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14
15 205 and stored at -20°C . The plasma/milk was analysed in previous batches and
16
17 206 plasma/milk found to contain no detectable residues of NSAIDs were used as negative
18
19 207 controls.
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24 209 *Sample extraction and clean-up*
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27
28 210 Plasma Extraction
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31 211 Plasma samples (5 mL) were aliquoted into 50 mL polypropylene tubes. The plasma
32
33 212 aliquots (5 mL) were fortified with internal standard at levels corresponding to 15 ng
34
35 213 mL^{-1} by adding a 60 μL portion of a 1.25 ug mL^{-1} mix solution of d_3 -MLX, d_4 -DCF,
36
37 214 d_3 -IBP, d_3 -FLU, d_4 -TLF and d_{10} -PBZ. Samples were fortified at levels corresponding
38
39 215 to 5, 7.5 and 10 ng mL^{-1} by adding 50, 75 and 100 μL portions of a 500 ng mL^{-1}
40
41 216 solution of CPF, DCF, IBP, MFN, KPF and PBZ. After fortification, samples were
42
43 217 held for 15 min prior to extraction. Hydrochloric acid (500 μL , 1 M) was added to the
44
45 218 plasma samples and they were left to stand at room temperature (10 min). Acetonitrile
46
47 219 (5 mL) was added and the samples were vortexed (30 sec), centrifuged ($3568 \times g$, 10
48
49 220 min, 4°C) and the supernatant was transferred to a clean polypropylene tube. 10 mM
50
51 221 ascorbic acid (15 mL) was added and the samples were vortexed (30 sec) and the
52
53 222 pH of the samples were checked to ensure they were at pH 3 before proceeding to the
54
55 223 solid phase extraction (SPE) stage.
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3 224 Milk Extraction
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7 225 Milk samples (5 mL) were aliquoted into 50 mL polypropylene tubes. The milk
8
9 226 aliquots (5 mL) were fortified with internal standard at levels corresponding to 15 ng
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11 227 mL⁻¹ by adding a 60 µL portion of a 1.25 ug mL⁻¹ mix solution of d₃-MLX, d₄-DCF,
12
13 228 d₃-IBP, d₃-FLU, d₄-TLF and d₁₀-PBZ. Samples were fortified at levels corresponding
14
15 229 to 5, 7.5 and 10 ng mL⁻¹ by adding 50, 75 and 100 µL portions of a 500 ng mL⁻¹
16
17 230 solution of CPF, DCF, IBP, MFN, KPF and PBZ (NMRL solution) and at 7.5, 15
18
19 231 and 22.5 ng mL⁻¹ with MLX, at 20, 40 and 60 ng mL⁻¹ with FLU and FLU-OH and
20
21 232 at 25, 50 and 75 ng mL⁻¹ with TLF by fortifying with 25, 50 and 75 µL portions of a
22
23 233 1.5, 4, 4 and 5 ug mL⁻¹ of MLX, FLU, FLU-OH and TLF (MRL solution). After
24
25 234 fortification, samples were held for 15 min prior to extraction. Acetonitrile (5 mL)
26
27 235 was added and the samples were vortexed (30 sec), centrifuged (3568 × g, 10 min, 4
28
29 236 °C) and the supernatant was transferred to a clean polypropylene tube. The sample
30
31 237 pellet is re-extracted with 5 mL of acetonitrile and the supernatants are combined. 10
32
33 238 mM ascorbic acid (20 mL) and 1 M hydrochloric acid (0.2 mL) were added to the
34
35 239 extracts and the pH of the samples were checked to ensure they were at pH 3 before
36
37 240 proceeding to the solid phase extraction (SPE) stage.
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46 241 Solid phase extraction
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50 242 The sample extracts were purified by SPE using Evolute ABN™ SPE cartridges.
51
52 243 Sample extracts were loaded onto the cartridges (preconditioned with 3 mL of n-
53
54 244 hexane:diethyl ether (50:50, v/v) 3 mL of methanol and 5 mL of ascorbic acid. The
55
56 245 samples were loaded onto cartridges under gravity. The cartridges were washed with 3
57
58 246 mL of methanol:water (10:90, v/v). The cartridges were dried under vacuum (15 min).
59
60 247 The cartridges were eluted with 2 × 1.5 mL of n-hexane:diethyl ether: acetonitrile:

1
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3 248 methanol (45:45:7:3, v/v). The eluates were reduced to dryness under nitrogen
4
5
6 249 without heat before re-dissolving in 150 μL of water:acetonitrile (90:10, v/v) and
7
8 250 vortexed (1 min). An aliquot (15 μL) was injected on the LC column.
9

251

252 *Matrix-Matched Calibration*

253 Matrix matched calibration curves were prepared and used for quantification. Control
254 plasma/milk previously tested and shown to contain no residues was prepared as
255 above (2.4). One control plasma sample and one control milk was used for each
256 calibration standard level. Plasma samples (5 mL) or milk samples (5 mL) were
257 aliquoted into 50 mL polypropylene tubes. Individual plasma or milk samples were
258 fortified with internal standard at levels corresponding to 15 ng mL^{-1} by adding a 60
259 μL portion of a 1.25 ug mL^{-1} mix solution of d_3 -MLX, d_4 -DCF, d_3 -IBP, d_3 -FLU, d_4 -
260 TLF and d_{10} -PBZ. Plasma samples were fortified at levels corresponding to 0, 5, 7.5,
261 10 and 20 ng mL^{-1} by adding 0, 50, 75, 100 and 200 μL portions of a 500 ng mL^{-1}
262 standard solution of CPF, DCF, IB, KPF, MFN and PBZ. After fortification, plasma
263 samples were held for 15 min prior to the extraction procedure as described above
264 (2.5). Milk samples were fortified at levels corresponding to 0, 5, 7.5, 10 and 20 ng
265 mL^{-1} by adding 0, 50, 75, 100 and 200 μL portions of a 500 ng mL^{-1} standard solution
266 of CPF, DCF, IBP, MFN, KPF and PBZ (NMRL solution) and at levels
267 corresponding to 0, 7.5, 15, 22.5, 30, 60 ng mL^{-1} of MLX, 0, 20, 40, 60, 80 and 160
268 ng mL^{-1} FLU and FLU-OH and 0.25, 50, 75, 100 and 200 ng mL^{-1} of TLF.
269 by adding 0, 25, 50, 75, 100 and 200 μL portions of a 1.5, 4, 4, 5 ug mL^{-1} standard
270 solution of MLX, FLU, FLU-OH and TLF (MRL solution). After fortification, milk
271 samples were held for 15 min prior to the extraction procedure as described above
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4 272 (2.5). Calibration curves of plasma or milk were prepared by plotting the response
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6 273 factor as a function of analyte concentration (0 to 20 ng mL⁻¹) to quantify samples.
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11 275 *Method validation*

12
13 276 For estimation of accuracy, blank plasma samples were fortified with CPF, DCF, IBP,
14
15 277 MFN, KPF and PBZ at 5, 7.5 and 10 ng mL⁻¹. For estimation of accuracy, blank milk
16
17 278 samples were fortified with CPF, DCF, IBP, MFN, KPF and PBZ at 5, 7.5 and 10 ng
18
19 279 mL⁻¹ and at 7.5, 15 and 22.5 ng mL⁻¹ with MLX, at 20, 40 and 60 ng mL⁻¹ with FLU
20
21 280 and FLU-OH and at 25, 50 and 75 ng mL⁻¹ with TLF. Six replicate test portions, at
22
23 281 each of the three fortification levels, were analysed. Analysis of the 18 test portions
24
25 282 was carried out on three separate occasions for each matrix. For the estimation of the
26
27 283 precision of the method, repeatability and within-laboratory reproducibility was
28
29 284 calculated. For unauthorised substances the decision limit (CC α) of the method was
30
31 285 calculated according to the calibration curve procedure using the intercept (value of
32
33 286 the signal, y, where the concentration, x is equal to zero) and 2.33 times the standard
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35 287 error of the intercept for a set of data with 6 replicates at 3 levels. The detection
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37 288 capability (CC β) was calculated by adding 1.64 times the standard error to the CC α .
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39 289 For authorised substances the decision limit (CC α) of the method was calculated
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41 290 according to the ISO 11843 calibration curve procedure by plotting the corresponding
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43 291 concentration at the permitted limit plus 1.64 times the standard deviation of the
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45 292 within laboratory reproducibility for a set of data with 6 replicates at 3 levels. The
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47 293 detection capability (CC β) was calculated by adding 1.64 times the standard deviation
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49 294 of the within laboratory reproducibility to the CC α .
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296 **Results and Discussion**

297 *Development/optimisation experiments*

298 The ionisation of all NSAIDs was studied in negative and positive mode. Most
299 NSAIDs can be detected by ESI-MS both in the negative mode and the positive mode,
300 showing different ionisation efficiencies. The optimum parameters (polarity mode,
301 declustering potential, collision energy, collision cell exit potential) were determined
302 for each drug and the best diagnostic ions for MS/MS analysis are shown in Table 2.
303 Negative ion mode was chosen as the required sensitivity was obtained for all
304 compounds and less baseline noise was obtained. The MS/MS method was developed
305 to monitor one precursor ion (parent mass) and two daughters (corresponding to
306 strong and weak ion) which is a suitable confirmatory method yielding 4
307 identification points in accordance with 2002/657/EC. Only one daughter ion could be
308 obtained for KPF and IBP but two daughter ions could be obtained for all other
309 compounds investigated in the study. A previous method developed at the author's
310 laboratory utilising GC-MS/MS was capable of obtaining 2 daughter ions for these
311 substances after derivatisation [Dowling et al. 2008]. The LC method developed in
312 this study was based on a method developed at the author's laboratory for the
313 determination of 8 banned non-steroidal anti-inflammatory drugs but was not suitable
314 for incorporation of the new range of analytes in this study [Dowling et al. 2009].
315 Chromatographic tests were carried out to evaluate the suitability of the 1.8 μm
316 Agilent Eclipse Plus C_{18} column (3.0 \times 50 mm) and the LC mobile phase utilised in
317 this study when additional NSAIDs were added. The tests showed that the internal
318 standards of DCF and FLU overlapped in each internal standard transition when the
319 analytes were eluted with a flow rate of 750 $\mu\text{L min}^{-1}$ and a run time of 6.5 min per
320 injection. A study was performed using the same composition of mobile phase A and
321 B. The times in the gradient were adjusted, the flow rate was reduced and the

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3 322 chromatographic runtime was extended to separate the internal standard of DCF and
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5 323 FLU. This resulted in the NSAIDs being eluted with good peak shape when using a
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8 324 mobile phase of water containing 0.001 M acetic acid and acetonitrile (90:10, v/v) (A)
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10 325 and acetonitrile (B) with a flow rate of 0.5 $\mu\text{L}\cdot\text{min}^{-1}$ and a runtime of 15 min. The
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12 326 internal standards d_4 -DCF and d_3 -FLU were completely separated under these
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15 327 conditions. As a result a batch of 30 samples can be analysed using the developed
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18 328 LC-MS/MS method for 10 NSAID residues in 7.5 hours allowing the running of up to
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20 329 3 batches of extracted samples within a 24 hour period. The extraction of the
21
22 330 NSAIDs from plasma was based on methods developed by Gowik et al. (Gowik et al.
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24 331 1998) and by Vinci et al (Vinci et al. 2006) but modified with the addition of
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27 332 acetonitrile. The extraction of NSAIDs in milk was based on a method previously
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29 333 developed at the author's laboratory [Dowling et al. 2009]. The extraction procedures
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31 334 were found to be satisfactory in the extraction of the NSAIDs from milk and plasma
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33 335 in this study. The purification of NSAIDs from the plasma and milk extracts was
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35 336 investigated initially using a solid phase extraction procedure previously developed at
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37 337 the authors laboratory using Evolute ABNTM cartridges but the original procedure
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39 338 was not suitable for the additional range of new analytes in this study. The NSAID
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41 339 FLU-OH was poorly recovered when the method previously developed at our
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43 340 laboratory was utilised. Elution studies were performed to ascertain where losses were
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45 341 occurring. The cartridges were eluted with different compositions and volumes of
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47 342 solvents including, 3 mL diethyl ether:hexane:acetonitrile (45:45:10, v/v/v), 3 mL
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49 343 diethyl ether:hexane, acetonitrile:methanol (45:45:5:5, v/v/v/v), 1.5 mL diethyl ether:
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51 344 hexane (80:20, v/v-elution 1) and 1.5 mL acetonitrile:methanol (90:10, v/v- elution
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53 345 2), 1.5 mL acetonitrile:methanol 90:10, v/v-elution 1) and 1.5 mL diethyl ether:
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55 346 hexane 50:50, v/v-elution 2), 3 mL methanol and 3 mL diethyl

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3 347 ether:hexane:acetonitrile:methanol (37.5:37.5:20:5 v/v/v/v). The results showed that
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5 348 elution of the cartridge with a solvent composition containing n-hexane:diethyl ether:
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8 349 acetonitrile: methanol (45:45:7:3, v/v-2 × 1.5 mL) gave the best results for all the
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10 350 analytes tested in this study. This is the first time to the best of our knowledge that
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12 351 CPF, DCF, IBP, KPF, MFN and PBZ residues have been purified from bovine
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14 352 plasma using Evolute ABN™ solid phase extraction cartridges. The methodology is
15
16 353 capable of meeting the minimum concentration of 5 ng mL⁻¹ set for NSAIDs in
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18 354 plasma. Moreover the values determined for the decision capability (CC_α) in this
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20 355 study were lower than those recorded for these substances in the literature in plasma
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22 356 by LC-MS/MS to date. This is the first time to the best of our knowledge that FLU,
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24 357 TLF, FLU-OH, IBP, KPF and MLX have been purified from milk using Evolute
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26 358 ABN™ solid phase extraction cartridges. The method meets the target level of 5 ng
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28 359 mL⁻¹ for IBP and KPF in milk for the first time. There are no analytical methods that
29
30 360 monitor for authorised and non authorised NSAIDs in milk as well as FLU and FLU-
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32 361 OH simultaneously in milk that meet the stringent validation requirements according
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34 362 to Commission Decision 2002/657/EC. The primary advantage of the developed
35
36 363 analytical strategy in this study is that the quantitation and confirmation can be carried
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38 364 out using a single analytical technique according to Commission Decision
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40 365 2002/657/EC [Commission Decision 2002] except for IBP and KPF. Confirmation of
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42 366 these residues using a second analytical technique is described elsewhere [Dowling et
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44 367 al. 2008]. After validation of this method the EU changed the legislation for DCF and
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46 368 a MRL of 0.1 ng mL⁻¹ was set in milk. Preliminary spiking studies at the new MRL
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48 369 for DCF were carried out using the developed analytical strategy in this study. Results
49
50 370 showed that this analytical strategy was sensitive enough to detect DCF at this level.
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52 371 The same sample extract was also analysed on an Applied Biosystem 5500 triple
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4 372 quadrupole mass spectrometer and the response using this system gave a better signal-
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6 373 to-noise ratio compared to the 4000 QTRAP. This analytical strategy with detection
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8 374 using 4000 QTRAP or 5500 Applied Biosystems LC-MS technology shows for the
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10 375 first time during initial studies that the new MRL set for DCF in milk at 0.1 ng mL^{-1}
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12 376 can be achieved. A chromatogram of the response for milk fortified at 0.1 ng mL^{-1}
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15 377 using Applied Biosystems 5500 is shown in Figure 12.
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20 379 *Validation study*

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22 380 Validation of the method in plasma and milk was according to procedures described
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24 381 in Commission Decision 2002/657/EC [Commission Decision 2002] covering
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26 382 specificity, calibration curve linearity, recovery (accuracy), precision, decision limit
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28 383 ($CC\alpha$) and detection capability ($CC\beta$).
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34 385 Specificity

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36 386 The technique of LC-MS/MS itself offers a high degree of selectivity and specificity.
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38 387 To establish the selectivity/specificity of the method, a variety of plasma and milk
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40 388 samples were fortified with analytes and internal standards and non-fortified samples
41
42 389 were also analysed. No interfering peaks were observed at the retention time of the
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44 390 analytes. To further test specificity in plasma and milk, samples were also fortified
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46 391 with 5.0 ng mL^{-1} of naproxen (NAP), niflumic acid (NIFLU), oxyphenylbutazone
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48 392 (OXYPHEN) and suxibuzone (SUXI). No interfering peaks were observed at the
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51 393 retention window of the analytes.
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395 Linearity of the response

396 The linearity of the chromatographic response in plasma was tested with matrix
397 matched curves using 5 calibration points in the concentration range of 0 to 20 ng mL⁻¹
398 ¹ when fortified with CPF, DCF, IBP, KPF, MFN and PBZ. The linearity of the
399 chromatographic response in milk was tested with matrix matched curves using 5
400 calibration points in the concentration range of 0 to 20 ng mL⁻¹ when fortified with
401 CPF, DCF, IBP, KPF, MFN and PBZ (NMRL substances). For MRL substances the
402 linearity of the chromatographic response in milk was tested with matrix matched
403 curves using 5 calibration points in the concentration range of 0 to 30 ng mL⁻¹ for
404 MLX, 0 to 160 ng mL⁻¹ for FLU and FLU-OH and 0 to 100 ng mL⁻¹ for TLF. Overall
405 the regression coefficients (r^2) were ≥ 0.98 except for FLU-OH. The regression

406 Accuracy

407 The accuracy was determined using bovine plasma fortified at 5.0, 7.5 and 10.0 ng
408 mL⁻¹ with CPF, DCF, IBP, KPF, MFN and PBZ. Mean corrected recoveries (n = 18)
409 determined in three separate assays in plasma (Table 3) were between 99 and 109 %.
410 The accuracy was determined using bovine milk fortified at 5.0, 7.5 and 10.0 ng mL⁻¹
411 with CPF, DCF, IBP, KPF, MFN and PBZ and the mean corrected recoveries (n = 18)
412 determined in three separate assays in milk (Table 4) were between 74 and 109 %.
413 The accuracy was determined using bovine milk fortified at MRL levels of 7.5, 15
414 and 22.5 ng mL⁻¹ for MLX, at 20, 40 and 60 ng mL⁻¹ with FLU and FLU-OH and
415 fortified at 25, 50 and 75 ng mL⁻¹ with TLF and the mean corrected recovery (n = 18)
416 of the analytes, determined in three separate assays in milk (Table 4) were between 73
417 and 102 %.

419 Precision

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4 420 The precision of the method, expressed as RSD values for the within-lab
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6 421 reproducibility of CPF, DCF, IBP, MFN and PBZ in plasma was less than 16 %
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8 422 (Table 3). No deuterated analogue was available for CPF, MFN, KPF and FLU-OH in
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10 423 our laboratory at the time of carrying out this work. d_3 -IBP was used as I.S for CPF
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12 424 and KPF, d_3 -TLF was used as I.S for MFN and d_3 -FLU was used as I.S for FLU-OH.
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15 425 The precision of the method, expressed as RSD values for the within-lab
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17 426 reproducibility of CPF, DCF, IBP, KPF, MFN and PBZ when fortified into milk was
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19 427 less than 16 % (Table 4). The precision of the method, expressed as RSD values for
20
21 428 the within-lab reproducibility of MLX, FLU and TLF when fortified into milk was
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23 429 less than 10 % except for FLU-OH which was less 25 % (Table 4). A one way
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25 430 analysis of variance was carried out at each of the fortification levels to separate out
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27 431 estimates for within run, between run and total variance of the method and the results
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29 432 are shown in Tables 3 and 4. Commission Decision 2002/ 657/EC states that the
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31 433 precision for quantitative methods for mass fractions lower than 100 ng mL^{-1} , the
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33 434 application of the Horwitz Equation gives unacceptable high values. Therefore, the
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35 435 RSD values for concentrations lower than 100 ng mL^{-1} shall be as low as possible.
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437 $CC\alpha$ and $CC\beta$

438 The decision limit ($CC\alpha$) is defined as the limit above which it can be concluded with
439 an error probability of α , that a sample contains the analyte. In general, for non-MRL
440 substances an α equal to 1 % is applied. The detection capability ($CC\beta$) is the smallest
441 content of the substance that may be detected, identified and quantified in a sample,
442 with a statistical certainty of $1-\beta$, where $\beta = 5 \%$. In the case of non MRL substances
443 $CC\alpha$ is the concentration corresponding to the intercept + 2.33 times the standard
444 error of the intercept. $CC\beta$ is the concentration corresponding to the signal at $CC\alpha$ +

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3 445 1.64 times the standard error of the intercept (i.e the intercept + 3.97 times the
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5 446 standard error of the intercept). Blank plasma was fortified at 1, 1.5 and 2 times the
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7 447 minimum required performance level of 5 ng mL⁻¹ set for CPF, DCF, IBP, KPF,
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9 448 MFN, PBZ, FLU, FLU-OH, TLF and MLX. CC α and CC β were calculated in
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11 449 plasma using the intercept (value of the signal, y, were the concentration, x is equal to
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13 450 zero) and the standard error of the intercept for a set of data with 6 replicates at 3
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15 451 levels (5.0, 7.5 and 10.0 ng mL⁻¹). CC α values of 1.80, 0.58, 0.71, 0.87, 0.70 and 1.19
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17 452 ng mL⁻¹ were determined for CPF, DCF, IBP, KPF, MFN, PBZ respectively. CC β
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19 453 values of 3.1, 0.99, 1.22 1.49, 1.20, 2.02 ng mL⁻¹ were determined for CPF, DCF,
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21 454 IBP, KPF, MFN and PBZ respectively. Non authorised substances in milk were
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23 455 fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng mL⁻¹
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25 456 set for CPF, DCF, IBP, KPF, MFN and PBZ. CC α values of 2.11, 0.83, 0.47, 1.63,
26
27 457 0.92 and 0.55 ng mL⁻¹ were determined for CPF, DCF, IBP, KPF, MFN and PBZ.
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29 458 CC β values of 3.59, 1.41, 0.80, 2.77, 1.56 and 0.94 ng mL⁻¹ were determined for CPF,
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31 459 DCF, IBP, KPF, MFN and PBZ. In the case of substances which have MRLs (MLX,
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33 460 FLU, FLU-OH and TLF) fortification was at 0.5, 1 and 1.5 times the corresponding
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35 461 MRL. The decision limit (CC α) is calculated by analysing the 18 milk samples
36
37 462 fortified at the MRL over three days, and using the concentration at the permitted limit
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39 463 plus 1.64 times the standard deviation obtained to yield CC α . The detection capability
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41 464 (CC β) of the proposed method was calculated from the CC α value plus 1.64 times the
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43 465 corresponding standard deviation. CC α values of 17.6, 42.89, 55.76 and 54.45 ng mL⁻¹
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45 466 were determined and CC β values of 20.13, 45.78, 71.50 and 58.9 ng mL⁻¹ were
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47 467 determined for MLX, FLU, FLU-OH and TLF in milk when fortified at their
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49 468 associated maximum residue limits.
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3 470 Measurement Uncertainty
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6 471 According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be
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8 472 regarded as a good estimate of the combined measurement uncertainty of individual
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10 473 methods [SANCO 2004]. For the calculation of the extended uncertainty a safety
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12 474 factor is required. The within laboratory reproducibility should be multiplied by a
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14 475 value of 2.33 and this should be used when determining the $CC\alpha$, corresponding to a
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16 476 confidence level of 99 %. As the only source of variation during the validation was
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18 477 the different days and different plasma or milk sourced from different animals it was
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20 478 decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of
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22 479 the method in plasma was estimated at 47, 12, 15, 23, 19 and 26 % for
23
24 480 CPF, DCF, IBP, KPF, MFN and PBZ. The measurement uncertainty of the method in
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26 481 milk was estimated at 50, 34, 36, 48, 20, 24, 16, 75, 24 and 30 % for CPF, DCF, IBP,
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28 482 KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX.
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34 483 This was determined by calculating the within laboratory reproducibility of the
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36 484 method, followed by multiplication of the within laboratory reproducibility by the
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38 485 safety factor of 3.0.
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44 487 *Evaluation*

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46 488 The method developed in this study has been used to evaluate the presence of CPF,
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48 489 DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX in bovine milk and CPF,
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50 490 DCF, IBP, KPF, MFN and PBZ in plasma. In monitoring for these substances in
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52 491 either matrix at our laboratory it was possible to detect the precursor ion and two
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54 492 daughter ions (at 5 ng mL^{-1}) in multiple reaction monitoring mode except for IBP and
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56 493 KPF. Furthermore the product ion ratio requirement was also met. The method has
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58 494 been carried out by different analysts under varying environmental conditions and the
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3 495 method was shown to be robust. To demonstrate the applicability of the method milk
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5 496 samples taken from animals treated with MLX and FLU-OH obtained from the
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8 497 Community Reference Laboratory in Berlin were tested. These samples had assigned
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10 498 values ranging from 5- 15 ng mL⁻¹. The samples were analysed by the method
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13 499 developed in this study and all samples were found to contain 5 ng mL⁻¹ of FLU-OH
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15 500 and 15 ng mL⁻¹ of MLX.
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502 **Conclusions**

503 A fast, simple, sensitive and selective LC-MS/MS method for the determination of
504 CPF, DCF, IBP, KPF, MFN and PBZ in bovine plasma and CPF, DCF, IBP, KPF,
505 MFN, PBZ, FLU, FLU-OH, TLF and MLX in bovine milk has been developed. The
506 LC-MS/MS method provided quantitative confirmatory data for the analysis of bovine
507 milk for CPF, DCF, MFN, PBZ, FLU, FLU-OH, TLF and MLX. The method allows
508 the analysis of a wide variety of drugs from different NSAID sub-classes such as CPF,
509 IBP and KPF (arylpropionic acid derivatives), PBZ (pyrazolidinedione derivatives)
510 DCF, MFN and TLF (anthranilic derivatives) and FLU and FLU-OH (nicotinic acid
511 derivatives) and MLX (oxicam derivative). There is no published method available to
512 the best of our knowledge for the simultaneous determination of authorised and non-
513 authorised NSAIDs such as CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF
514 and MLX in bovine milk that purifies sample extracts using EvoluteTM ABN solid
515 phase extraction cartridge procedure described in this study which is an improvement
516 on previous work carried out utilising this cartridge chemistry. This is the first time
517 that FLU, TLF, FLU-OH, IBP, KPF and MLX have been purified from milk using
518 Evolute ABNTM solid phase extraction cartridges simultaneously with other NSAIDs.
519 This is the first time that a method is available that meets the minimum requirements

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3 520 of 5 ng mL⁻¹ for IBP and KPF in milk. There is no published method available to the
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6 521 best of our knowledge for the simultaneous determination of CPF, DCF, IBP, KPF,
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8 522 MFN and PBZ in bovine plasma that purifies sample extracts using Evolute™ ABN
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10 523 solid phase extraction cartridges. This study describes the first such sensitive and
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12 524 selective methodology. This is also the first time that a rapid multi-residue
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15 525 methodology for the above authorised and non-authorised NSAIDs has been validated
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17 526 according to Commission Decision 2002/657/EC [Commission Decision 2002] and
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19 527 the measurement uncertainty of the method has been described. This methodology
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21 528 shows that suitable sensitivity was obtained and that the method performs very well in
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23 529 terms of accuracy and within-laboratory reproducibility. The developed method was
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25 530 evaluated by comparison of results when method was performed by different analysts
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27 531 under different environmental conditions, using different batches of reagents and solid
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29 532 phase extraction cartridges. The results (unpublished data) were highly acceptable
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31 533 providing evidence of the development of a rugged analytical method in this study.
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33 534 Recently it was proposed by Community Reference Laboratories (CRLs) in Europe
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35 535 that laboratories should be capable of monitoring for NSAIDs at a level of 5 ng mL⁻¹
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37 536 in EU member states where no MRL exists in plasma or milk and this study shows
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39 537 that these limits can be reached using the developed analytical strategy [SANCO
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41 538 2004]. The objective of the work to anticipate the requirements of the future where
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43 539 risks could occur due to the administration of NSAIDs by developing a method to
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45 540 monitor for authorised and non-authorised NSAIDs simultaneously has been
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47 541 achieved. The objective of the work to validate an analytical strategy for these
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49 542 residues in bovine plasma and milk that meet EU target levels according to the
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51 543 requirements in Commission Decision 2002/657/EC therefore has also been achieved
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53 544 successfully.
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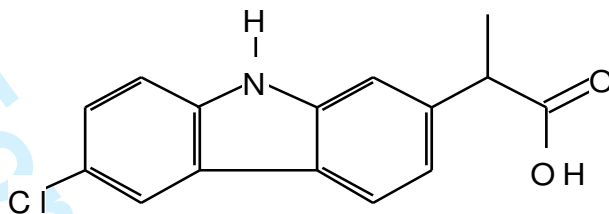
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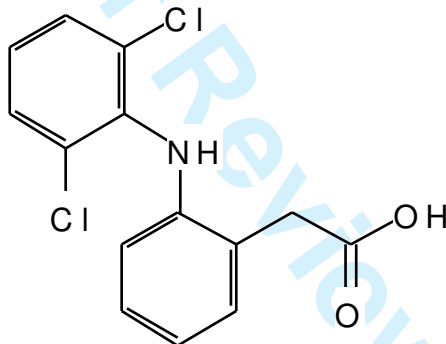
Structure a: Carprofen



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Carprofen

Structure b: Diclofenac

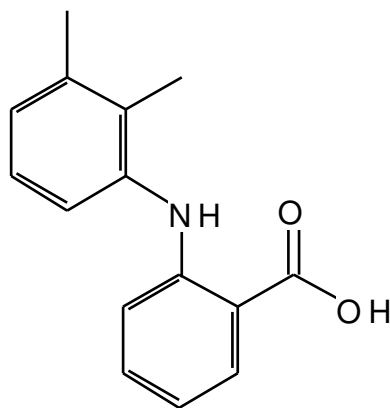


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Diclofenac

Structure c: Mefenamic Acid

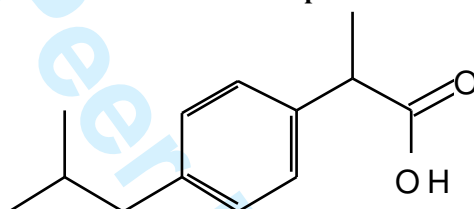
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Mefenamic acid

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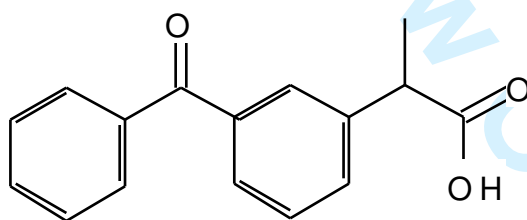
Structure d: Ibuprofen



Ibuprofen

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Structure e: Ketoprofen

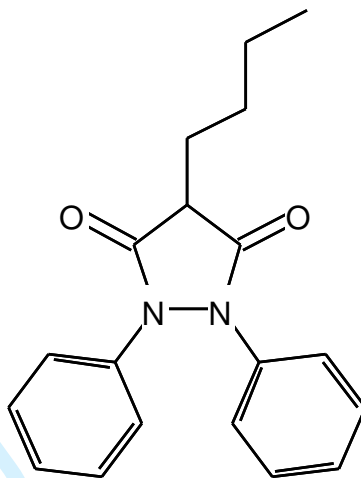


Ketoprofen

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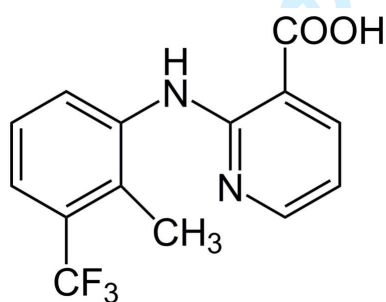
Structure f: Phenylbutazone



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Phenylbutazone

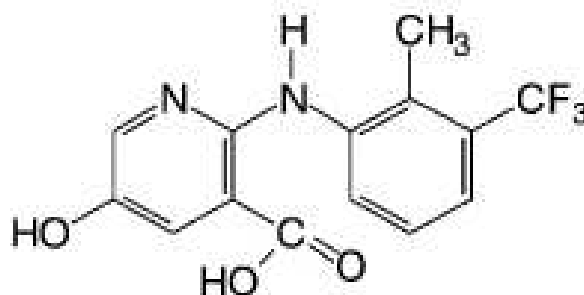
Structure g: Flunixin



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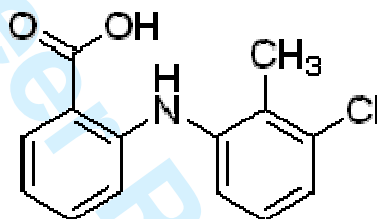
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Structure h: Hydroxy-Flunixin



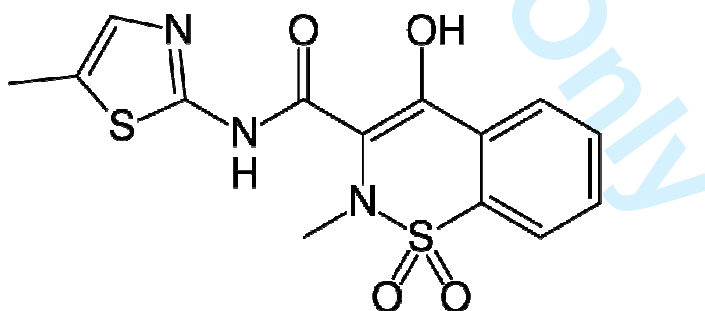
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Structure i: Tolfenamic Acid



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Structure j: Meloxicam



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Fig. 1 Structures of the NSAIDs

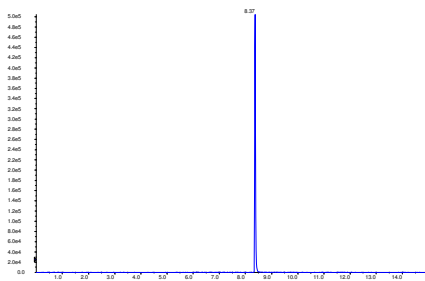
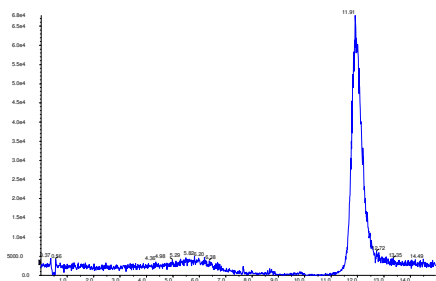
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For Peer Review Only

2 A

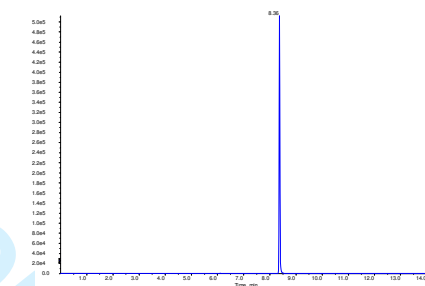
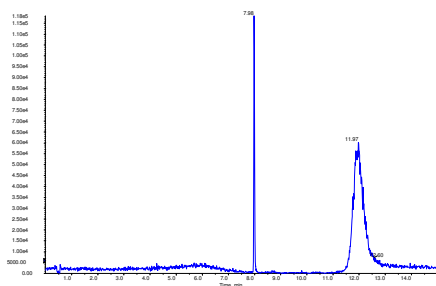
Intensity, cps



d3-Ibuprofen

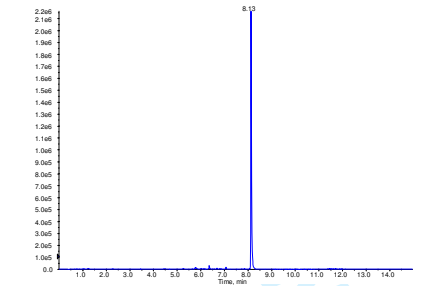
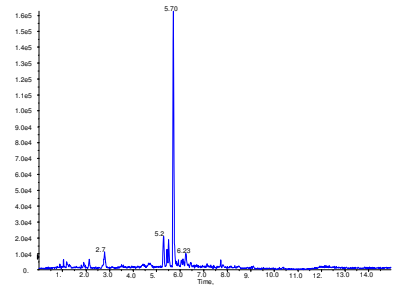
2 B

Intensity, cps



2 C

Intensity, cps



2 D

Intensity, cps

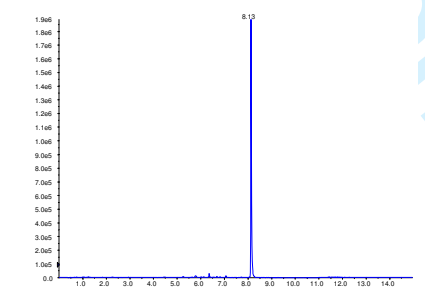
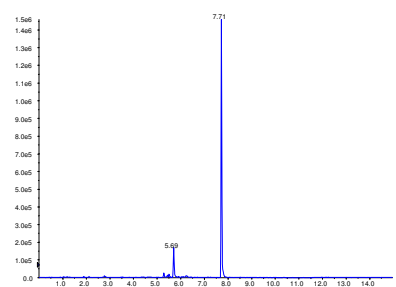


Fig. 2A. Chromatogram of negative control milk (2A) and plasma (2C) fortified at 15 ng mL⁻¹ with internal standard d₃-IBP and fortified with 5 ng mL⁻¹ of CPF in milk (2B) and plasma (2D)

d4-Diclofenac

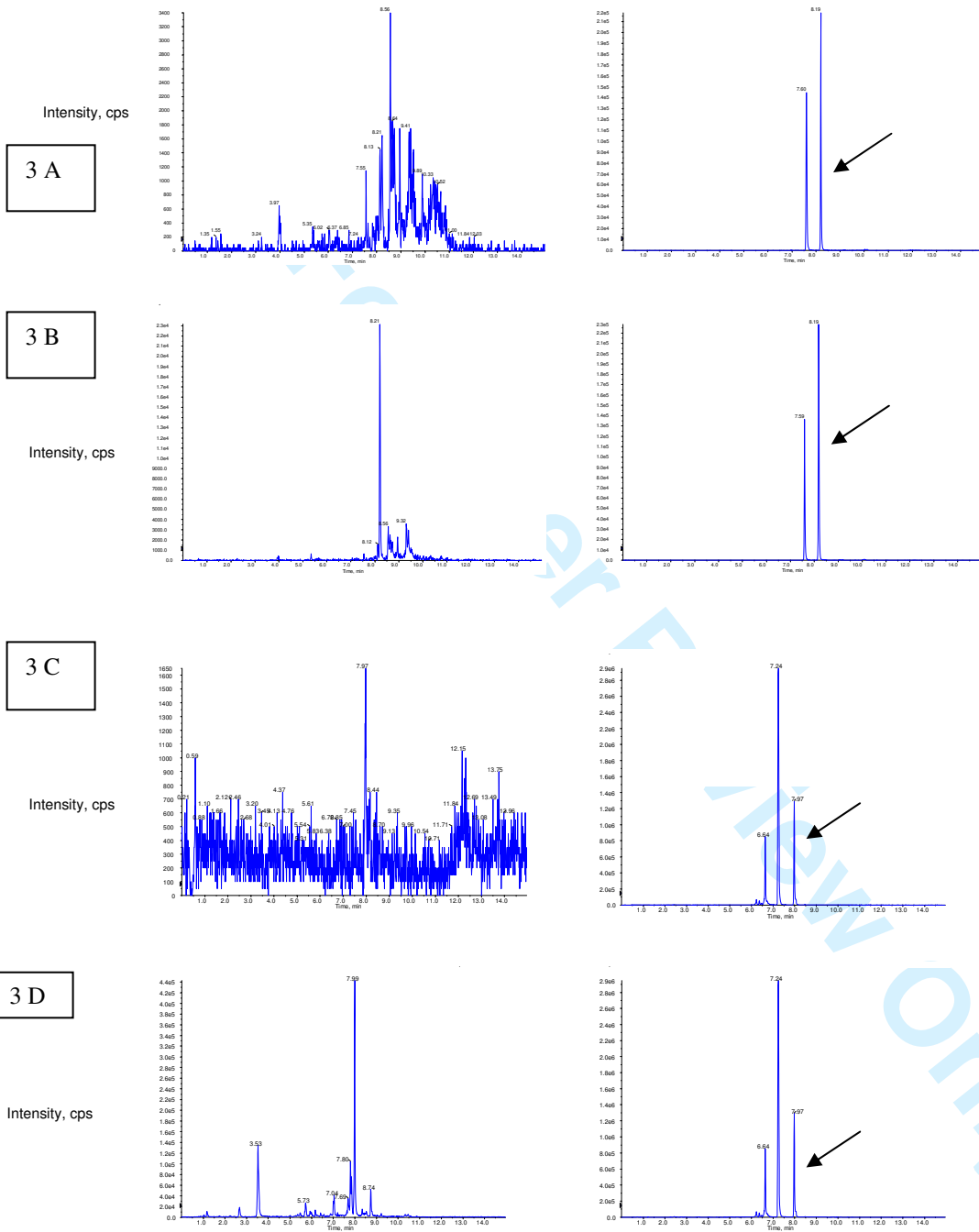
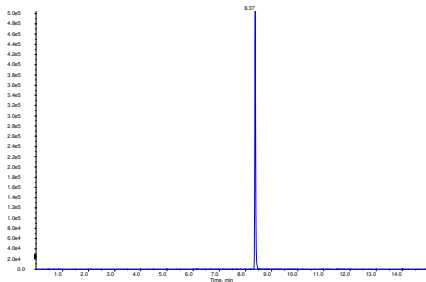
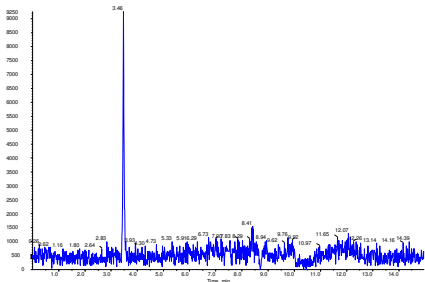


Fig. 3A. Chromatogram of negative control milk (3A) and plasma (3C) fortified at 15 ng mL⁻¹ with internal standard d₄-DCF and fortified at 5 ng mL⁻¹ with DCF in milk (3B) and plasma (3D)

d3-Ibuprofen

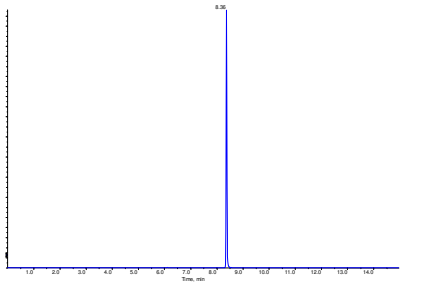
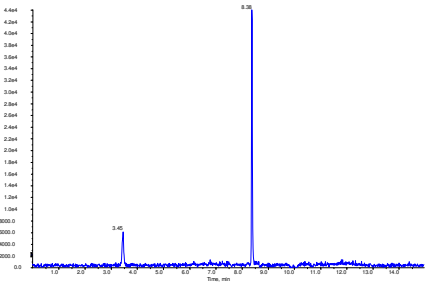
4 A

Intensity, cps



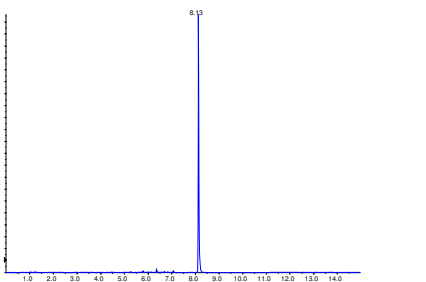
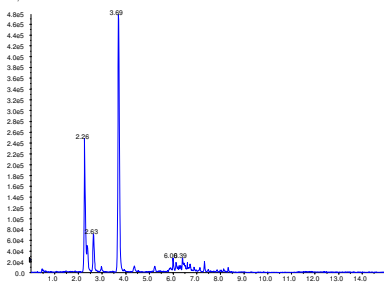
4 B

Intensity, cps



4 C

Intensity, cps



4 D

Intensity, cps

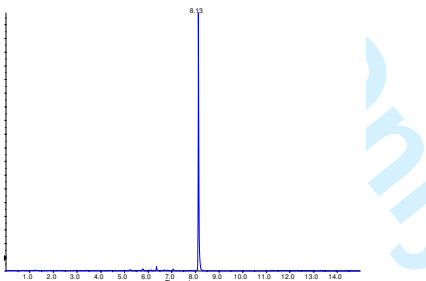
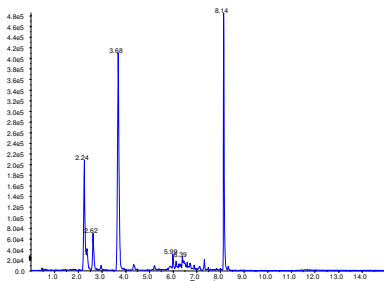
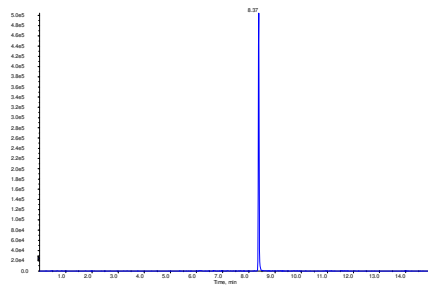
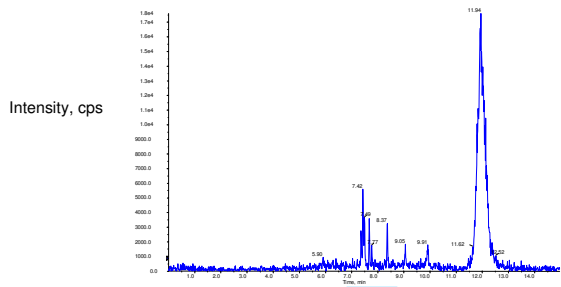


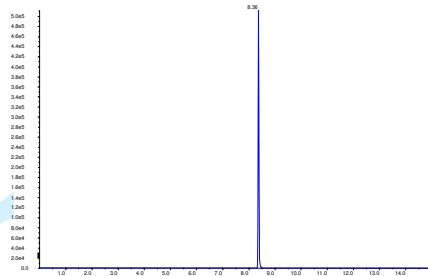
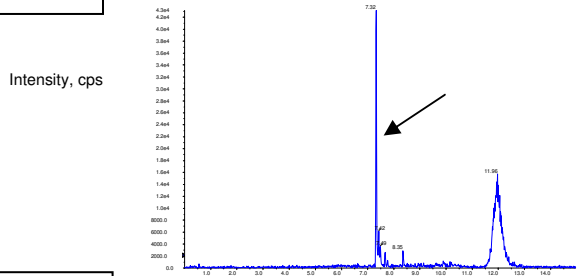
Fig. 4A. Chromatogram of negative control milk (4A) and plasma (4C) fortified at 15 ng mL⁻¹ with internal standard d₃-IBU and fortified at 5 ng mL⁻¹ with IBP in milk (4B) and plasma (4D).

5 A

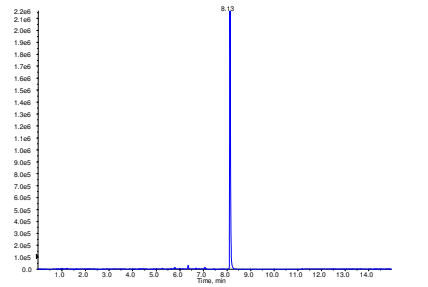
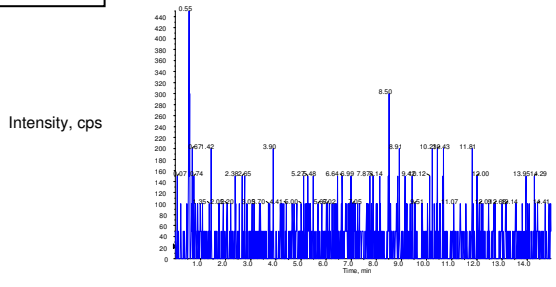


d3-Ibuprofen

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5 C



5 D

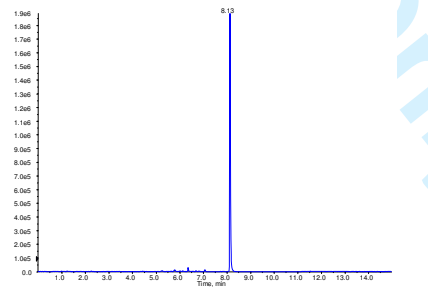
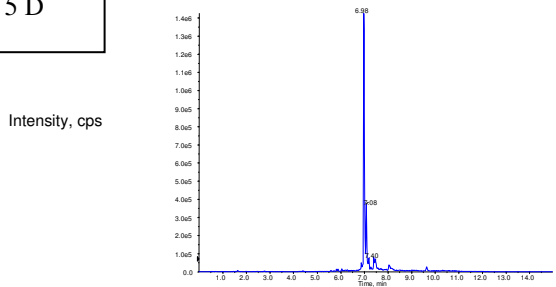
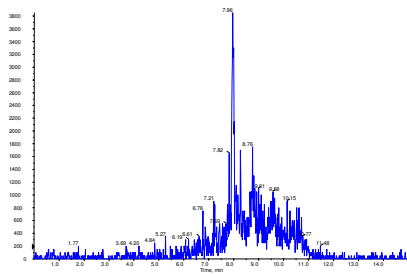
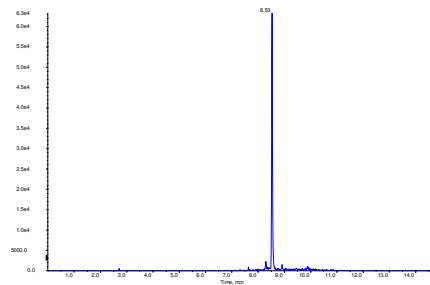


Fig. 5A. Chromatogram of negative control milk (5A) and plasma (5C) fortified at 15 ng mL⁻¹ with internal standard d₃-IBU and fortified at 5 ng mL⁻¹ with KPF in milk (5B) and plasma (5D).

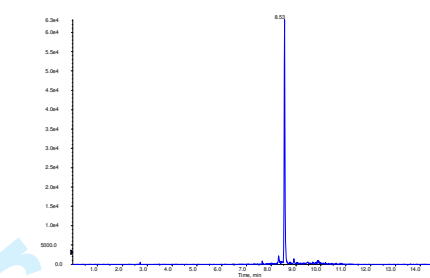
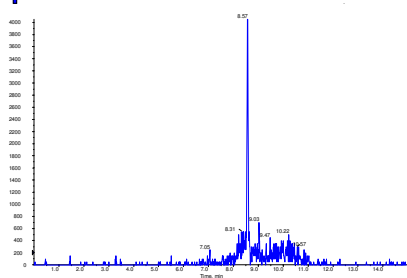
7 A

Intensity, cps

d₁₀-Phenylbutazone

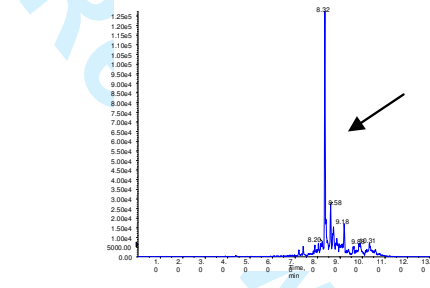
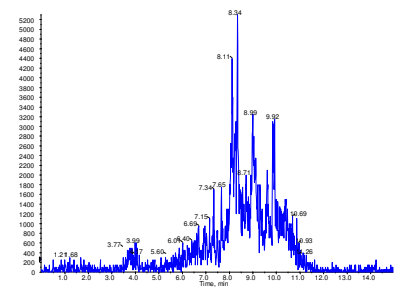
7 B

Intensity, cps



7 C

Intensity, cps



7 D

Intensity, cps

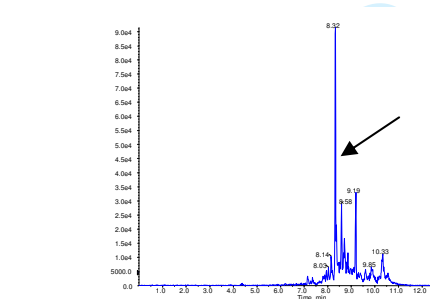
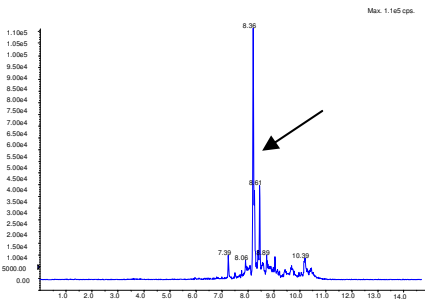
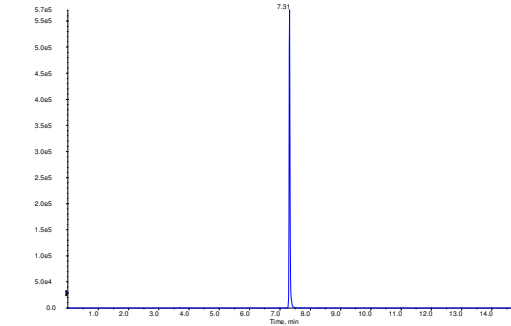
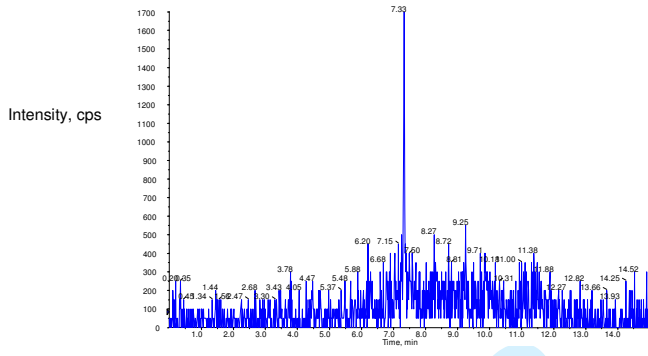


Fig. 7A. Chromatogram of negative control milk (7A) and plasma (7C) fortified at 15 ng mL⁻¹ with internal standard d₁₀-PBZ and fortified at 5 ng mL⁻¹ with PBZ in milk (7B) and plasma (7D)

8 A

d₃-Meloxicam



8 B

Intensity, cps

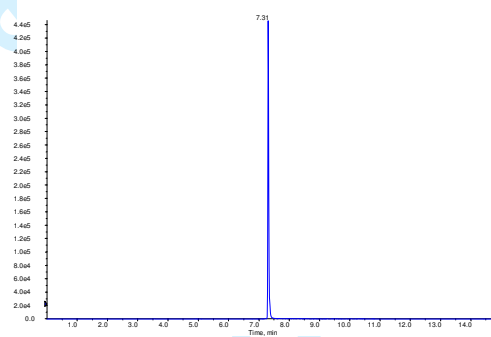
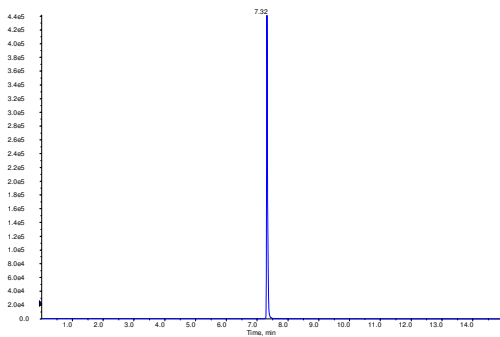
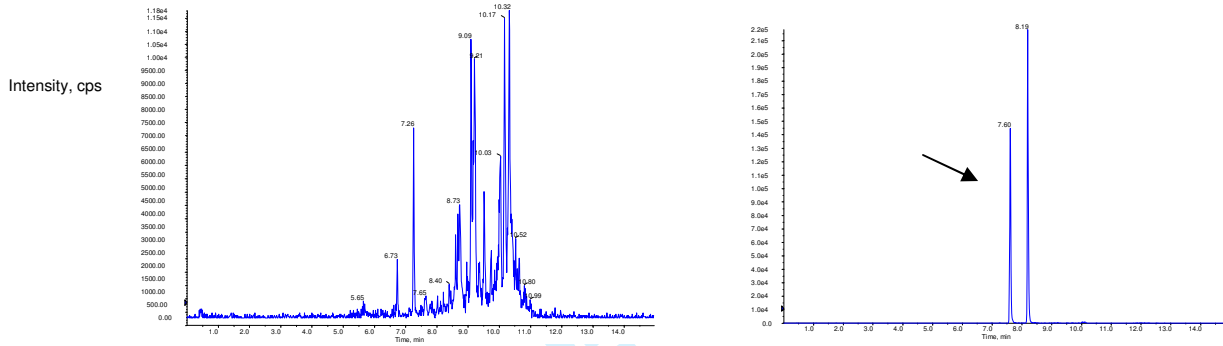


Fig. 8A. Chromatogram of Negative Control milk (8A) fortified at 15 ng mL⁻¹ with internal standard d₃-MLX and fortified at 7.5 ng mL⁻¹ with MLX (8B)

9A

d3-Flunixin



9 B

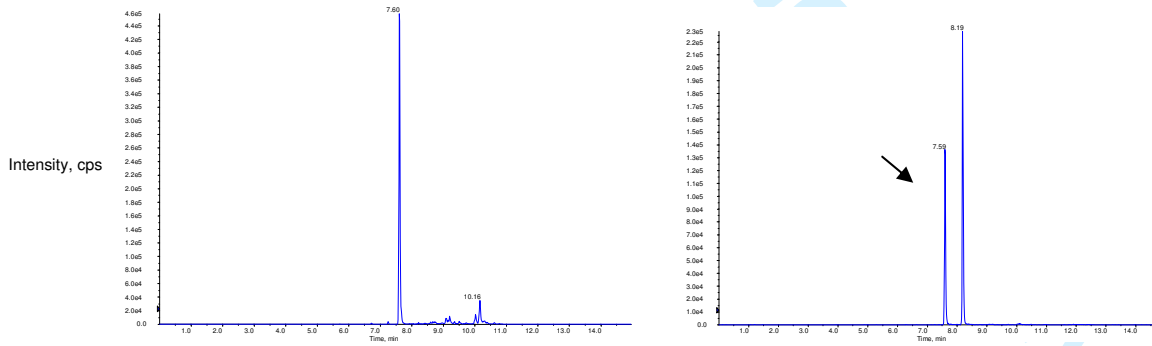
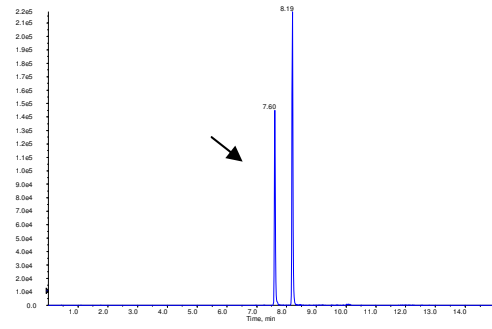
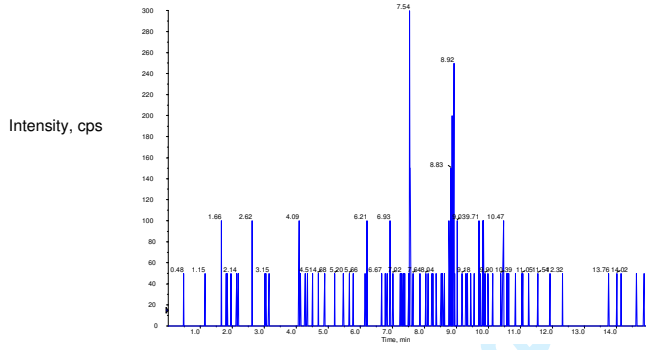


Fig. 9A. Chromatogram of negative control milk (9A) fortified at 15 ng mL^{-1} with internal standard $\text{d}_3\text{-FLU}$ and fortified at 20 ng mL^{-1} with FLU (9B)

10 A

d3-Flunixin



10 B

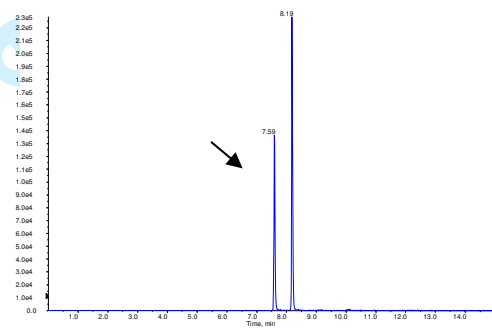
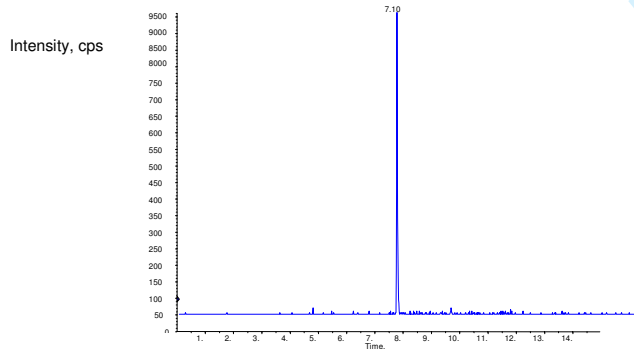


Fig. 10A. Chromatogram of negative control milk (10 A) fortified at 15 ng mL⁻¹ with internal standard d₃-FLU and fortified at 20 ng mL⁻¹ with FLU-OH (10B)

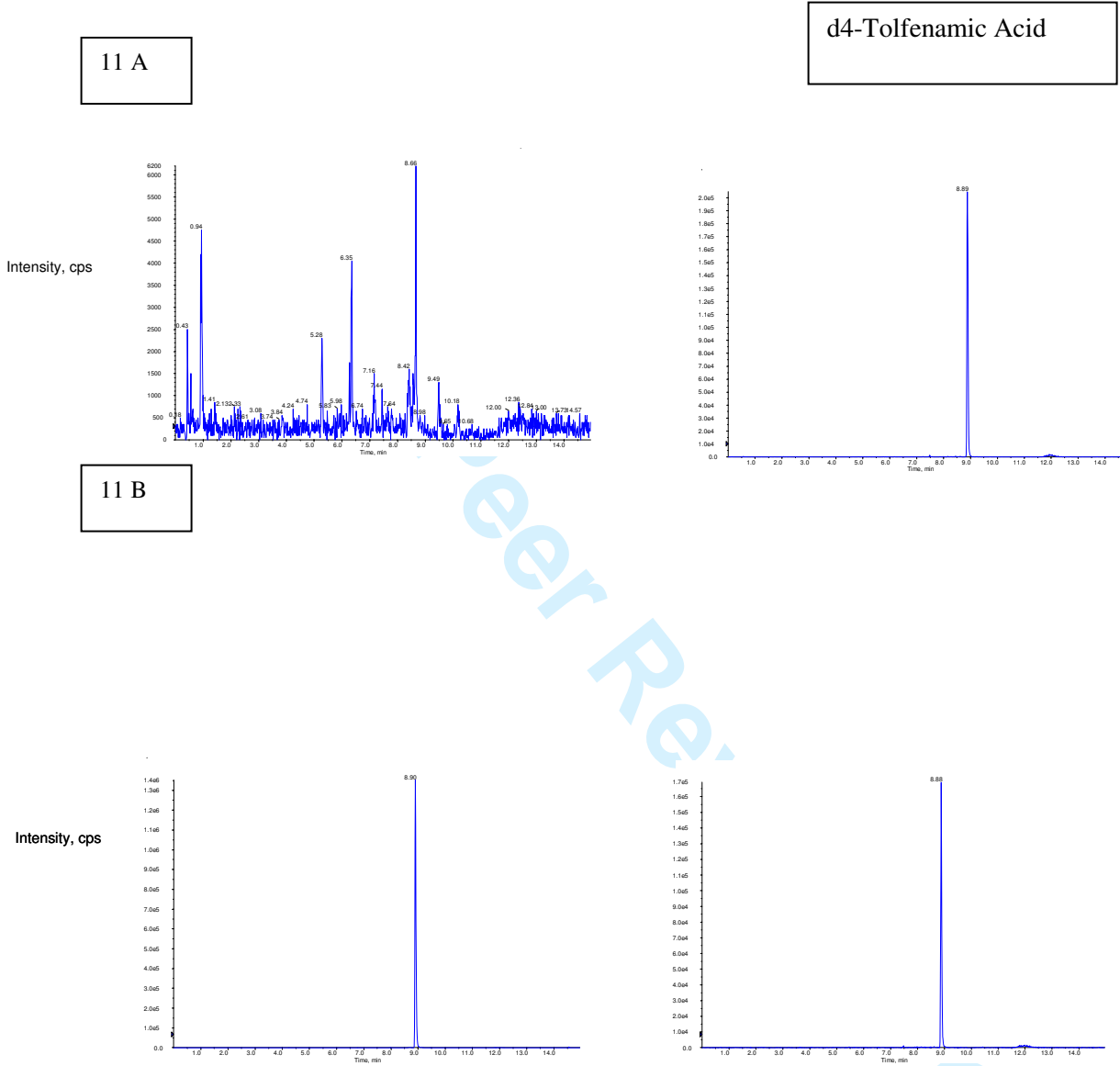


Fig. 11A. Chromatogram of negative control milk (11A) fortified at 15 ng mL^{-1} with internal standard $\text{d}_4\text{-TLF}$ and fortified at 25 ng mL^{-1} with TLF (11B)

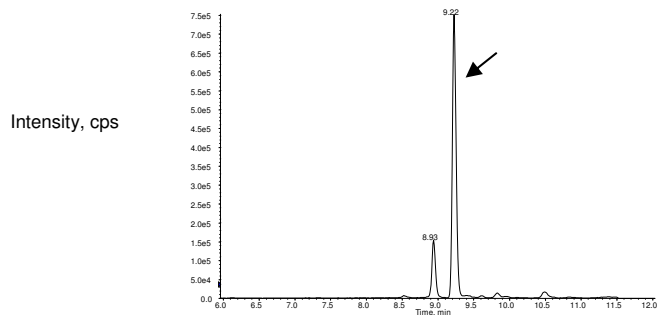


Fig. 12A. Chromatogram of negative control milk fortified at 0.1 ng mL^{-1} with DCF and analysed by Applied Biosystems 5500 LC-MS

Table 1: LC gradient profile for determination of CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX

Time (min)	Component A (%)	Component B (%)
0.0	90	10
1.0	90	10
3.5	85	15
7.5	35	65
9.5	35	65
11.0	90	10
15.0	90	10

Component A: Component A: water containing 0.001 M acetic acid + acetonitrile (90 + 10, v/v)

Component B: Acetonitrile

Table 2: MS/MS parameters for determination of CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX

Compound	Transition	Declustering Potential [V]	Collision Energy [eV]	Collision Cell Exit Potential [V]
CPF	271.8>227.9(strong)	-60	-16	-13
	271.8>225.8(weak)	-60	-38	-13
DCF	294.0>250.0(strong)	-70	-19	-15
	294.0>214.0(weak)	-70	-28	-15
IBP	205.0>161.0(strong)	-45	-14	-9
PBZ	306.9>279.0(strong)	-70	-30	-16
	306.9>130.9 (weak)	-70	23	-12
FLU	294.9>250.8(strong)	-80	-18	-15
	294.9>191.0(strong)	-80	-25	-17
FLU-OH	310.9>266.9(strong)	-65	-48	-9
	310.9>226.9(weak)	-65	-50	-9
MFN	239.8>196 (strong)	-60	-34	-11
	239.8>179.9 (weak)	-60	-18	-17
MLX	349.8>285.9(strong)	-50.	-19	-18
	349.8>145.9(strong)	-50.	-35	-12
KPF	252.8>209.0(strong)	-40.	-10	-9
TLF	259.8>215.9(strong)	-60.	-16	-5
	259.8>214.0(strong)	-60	-30	-5
d4-DCF	298.0>218.0(strong)	-70	-28	-15
d3-IBP	208.0>164.0(strong)	-45	-14	-9
d10-PBZ	316.9>289.0(strong)	-70	-25	-16
d3-FLU	298.0>254.0(strong)	-70	-14	-15
d3-MLX	353.0>289.0(strong)	-50	-15	-18
d4-TLF	264.0>220(strong)	-60	-16	-5

Note: Matrix matched curves were used for quantification of all compounds
d₃-IBP was used as internal standard (I.S) for CPF, IBP and KPF, d₄-DCF was used as I.S for DCF, d₁₀-PBZ was used as I.S for PBZ, d₃- FLU was used as I.S for FLU and FLU-OH. d₄- TLF was used as I.S for MFN and TLF. d₃- MLX was used as I.S for MLX.

Table 3: Intra- and inter-assay variation for accuracy of CPF, DCF, IBP, KPF, MFN, PBZ, in plasma

Analyte	Fortification level (ng mL ⁻¹)	Accuracy (%)	Within Run CV (%)	Between Run CV (%)	Total CV (%)
CPF	5	104	18.8	9.2	20.9
	7.5	99	7.1	9.6	12.0
	10	101	11.0	6.6	12.8
Combined Variance	5,7.5,10				15.2
DCF	5	106	4.0	2.1	4.5
	7.5	102	3.0	2.3	11.9
	10	101	3.9	1.2	12.8
Combined Variance	5,7.5,10				4.1
IBP	5	104	4.4	0.0	4.4
	7.5	103	4.3	4.2	6.0
	10	100	3.8	0.9	3.9
Combined Variance	5,7.5,10				4.8
KPF	5	108	8.4	3.8	9.2
	7.5	104	5.4	7.2	9.0
	10	103	3.2	0.0	3.2
Combined Variance	5,7.5,10				7.1
MFN	5	103	7.0	4.3	8.2
	7.5	103	4.2	4.0	5.8
	10	99	2.3	3.0	3.8
Combined Variance	5,7.5,10				5.9

Analyte	Fortification level (ng mL ⁻¹)	Accuracy (%)	Within Run CV (%)	Between Run CV (%)	Total CV (%)
PBZ	5	109	6.8	5.2	8.6
	7.5	102	10.3	4.9	11.4
	10	101	4.5	0.8	4.6
Combined Variance	5,7.5,10				8.2

Table 4: Intra- and inter-assay variation for accuracy of CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX in milk

Analyte	Fortification level (ng mL ⁻¹)	Accuracy (%)	Within Run CV (%)	Between Run CV (%)	Total CV (%)
CPF	5	103	5.2	7.1	8.8
	7.5	108	11.3	15.4	19.1
	10	101	12.6	15.0	19.6
Combined Variance	5,7.5,10				15.8
DCF	5	92	4.5	13.9	14.6
	7.5	106	5.8	9.5	11.1
	10	103	5.3	4.1	6.7
Combined Variance	5,7.5,10				10.8
IBP	5	81	2.7	15.0	15.2
	7.5	99	3.0	6.4	7.1
	10	104	3.4	12.0	12.5
Combined Variance	5,7.5,10				11.6
KPF	5	107	3.6	20.2	20.6
	7.5	93	8.1	6.3	10.3
	10	106	13.9	7.3	15.7
Combined Variance	5,7.5,10				15.5
MFN	5	74	5.3	3.3	6.2
	7.5	103	5.9	6.0	5.9
	10	109	3.1	7.8	8.1
Combined Variance	5,7.5,10				6.7

Analyte	Fortification level (ng mL ⁻¹)	Accuracy (%)	Within Run CV (%)	Between Run CV (%)	Total CV (%)
PBZ	5	79	4.5	7.5	8.7
	7.5	99	4.9	4.7	6.8
	10	104	5.8	5.4	7.9
Combined Variance	5,7.5,10				7.8
FLU	20	93	3.0	1.9	3.6
	40	102	3.9	2.1	4.4
	60	98	4.4	5.8	7.3
Combined Variance	20,40,60				5.1
FLU-OH	20	73	9.1	28.3	29.7
	40	83	12.2	20.7	24.0
	60	87	9.6	18.2	20.5
Combined Variance	20,40,60				24.7
TLF	25	97	4.7	7.2	8.6
	50	101	3.3	4.3	5.4
	75	92	2.4	8.9	9.2
Combined Variance	25,50,75				7.7
MLX	7.5	88	2.8	6.6	7.2
	15	92	4.9	9.2	10.4
	22.5	87	1.9	11.6	11.7
Combined Variance	7.5,15, 22.5				9.8

Table 5: Calculated $CC\alpha$ and $CC\beta$ values for plasma

	$CC\alpha$ (ng mL ⁻¹)	$CC\beta$ (ng mL ⁻¹)
CPF	1.80	3.07
DCF	0.58	0.99
IBP	0.71	1.22
KPF	0.87	1.49
MFN	0.70	1.20
PBZ	1.19	2.02

Table 6: Calculated $CC\alpha$ and $CC\beta$ values for milk

	$CC\alpha$ (ng mL ⁻¹)	$CC\beta$ (ng mL ⁻¹)
CPF	2.11	3.59
DCF	0.83	1.41
IBP	0.47	0.80
KPF	1.63	2.77
MFN	0.92	1.56
PBZ	0.55	0.94
FLU	42.89	45.78
FLU-OH	55.76	71.50
TLF	54.45	58.90
MLX	17.57	20.13