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1 Enantiomer fraction evaluation of the four stereoisomers of second-
2 generation anticoagulant rodenticides in biological matrices with
3 polysaccharide-based chiral selectors and liquid chromatography tandem
4 mass spectrometry

5

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9

10 Abstract:

11 Numerous cases of wildlife exposure to five second-generation anticoagulant
12 rodenticides have been reported worldwide, and residues of these chiral pesticides in
13 biological matrices are still quantified by achiral liquid chromatography methods.
14 However, they are a mixture of cis- and trans-diastereomers, thus a mixture of four
15 stereoisomers. Their persistence must be evaluated in a differentiated way in the
16 food chain of concerned predator species in order to reduce the environmental
17 impact.

18 This article presents an evaluation of the chiral selectivity of five polysaccharide-
19 based chiral selectors for the four stereoisomers of bromadiolone, difenacoum,
20 brodifacoum, flocoumafen and difethialone. Different chromatographic parameters,
21 influencing the chiral separation, such as organic modifier (acetonitrile, methanol),
22 percentage of formic acid and water content in the mobile phase are systematically
23 tested for all columns. It was shown that little amount of water added to the
24 acetonitrile mobile phase may influence the retention behaviors between reversed
25 phase and HILIC-like modes, and consequently the enantiomer elution order of the
26 four stereoisomers. On the contrary, reversed phase is always the observed mode for
27 the methanol water mobile phase. A suitable combination of all these parameters is
28 presented for each second-generation anticoagulant rodenticide with a description of
29 the enantioresolution, the enantiomer elution order and the retention times of the
30 respective stereoisomers. A method is validated for all stereoisomers of each

31 second-generation anticoagulant rodenticide with chicken liver and according to an
32 official bioanalytical guideline. As an example, the enantiomer fraction is evaluated in
33 the liver of a raptor species (rodent predator) exposed to bromadiolone and
34 difenacoum. The results showed that only one enantiomer of trans-bromadiolone and
35 one enantiomer of cis-difenacoum is present in hepatic residues, although all four
36 stereoisomers are present in bromadiolone and difenacoum rodenticide baits.

37

38 Keywords:

- 39 • Enantiomeric fraction
- 40 • Chiral rodenticides
- 41 • Stereoisomers hepatic residues
- 42 • Polysaccharide-based-chiral stationary phases
- 43 • LC-MS/MS

44

45 1. Introduction

46 Second-generation anticoagulant rodenticides (SGARs) are chiral pesticides used
47 worldwide for vertebrate pest control and are responsible for many unintentional
48 exposures. Environmental issues are due to their persistence in the food chain,
49 resulting in secondary exposure of non-target wildlife, mainly raptors and rodent
50 predatory mammals [1-11]. This has led European authorities to identify these
51 molecules as “candidates for substitution”. Despite the importance of
52 enantioselectivity in environmental and biological processes [12,13], stereochemistry
53 is still not of major concern in the use of racemic pesticides. Nowadays, SGARs are
54 used in urban and suburban settings, agriculture, species conservation and island
55 restoration projects [14], and, for all these pesticide or biocide uses, they are
56 considered as a single molecular entity. However, SGARs are a mixture of four
57 stereoisomers assembled into two pairs of diastereoisomers (cis-diastereoisomers or
58 trans-diastereoisomers), each pair containing two enantiomers (RR/SS or RS/SR)
59 [15,16]. SGARs are derivatives of 4-hydroxy-coumarin (bromadiolone, difenacoum,
60 brodifacoum, flocoumafen) or 4-hydroxy-thiocoumarin (difethialone). Their respective
61 chemical structure with the two stereogenic centers and the differential lateral group

62 R are presented in figure 1. Differences in persistence, assessed by achiral
63 chromatography methods, between the diastereoisomers of SGARs have been
64 suggested in previous studies [16-21]. Indeed, the presence of a single
65 diastereoisomer pair has been shown for some SGARs like bromadiolone and
66 difenacoum in the liver of rat [16,17], or even of rodent predatory wildlife species [18-
67 20]. In these different studies, trans-bromadiolone was found to be more persistent
68 than cis-bromadiolone and cis-difenacoum more persistent than trans-difenacoum in
69 the livers of Sprague-Dawley rat, wild rat (*Rattus norvegicus*), and in rodent predators
70 like the red kite (*Milvus milvus*), and fox (*Vulpes vulpes*). Residues of brodifacoum
71 were found in the liver of the Réunion harrier (*Circus maillardi*), but cis-brodifacoum
72 was more present than trans-brodifacoum (respective proportions 89.5/10.5%),
73 although both diastereoisomers are present in rodenticide baits in similar proportions
74 [20]. However, an analytical tool to assess the individual persistence of the two
75 enantiomers of trans-bromadiolone, cis-difenacoum, cis-brodifacoum and trans-
76 brodifacoum was lacking. More recently, the residue levels of the four stereoisomers
77 of difethialone were evaluated with a chiral-LC-MS/MS method in reversed-phase
78 mode in biological matrices of Sprague-Dawley rats treated with a racemic mixture of
79 difethialone stereoisomers, and their persistence was different in liver, blood, plasma
80 or faeces samples [22]. This method has also been used to compare their biological
81 properties in rats [23] and mice (*Mus musculus*) [24], and to try to establish a new
82 formulation with an innovative mixture of stereoisomers that could improve the
83 environmental quality of the rodenticide without compromising bait efficacy. This
84 suggested the same behavior could occur for the other SGARs. In this context,
85 enantioselective bioanalytical methods are mandatory to understand ecotoxicity
86 issues of all SGARs, which are all widely used in the world as biocides or pesticides.

87 In the past recent years, polysaccharide-based chiral selectors have been used for
88 the separation of enantiomers of chiral chemical standards with pure organic or
89 aqueous-organic mobile phases [25-27], including the four stereoisomers of
90 difenacoum [25]. Chiral chromatographic methods show great promise for
91 bioanalytical studies with mass spectrometry detectors, and for the development of
92 applications to assess low levels of chiral drug and pesticide residues in biological or
93 environmental samples [12,13,22,28]. The purpose of this article is to describe the
94 analytical development of enantioselective methods to evaluate enantiomer fractions

95 (EF) of the five SGARs marketed worldwide: bromadiolone, difenacoum,
96 brodifacoum, flocoumafen and difethialone. Polysaccharide-based chiral selectors
97 (figure 2) were used to achieve enantioresolution of the four stereoisomers of each
98 SGAR with good enantioresolution (R_s). The influence of the chiral selector, the
99 nature of the organic solvent (acetonitrile or methanol) or the role of the water content
100 and the acidic additive concentration (formic acid) in the mobile phase on the
101 enantioresolution and occasionally on the enantiomer elution order (EEO) was
102 evaluated. The nature of hydrogen-bonding interactions in “HILIC-like” (HILIC for
103 hydrophilic liquid chromatography) or reversed phase (RP) mode and its influence on
104 chiral separation are discussed. These chromatographic methods are compatible
105 with tandem mass spectrometry detection to achieve specificity and sensitivity. The
106 extraction method described by Fourel et al. [22] is used to process chicken livers
107 and produce liver extracts. This allowed the validation of six quantification methods
108 according to the EMEA bioanalytical guideline [29]. These protocols may then be
109 applied to the liver of rodent or non-target species to assess the enantiomer fraction
110 of the four stereoisomers of the five SGARs. An example of evaluation of the hepatic
111 enantiomer fraction of the four stereoisomers of SGARs is given to demonstrate their
112 differential persistence in the food chain of a rodent predatory species (raptor)
113 exposed via secondary exposure.

114

115 2. Experimental

116 2.1. Chemical and reagents

117 Difethialone was provided by Liphatech (Pont de Casse, France). Bromadiolone,
118 difenacoum, brodifacoum and flocoumafen were supplied by Sigma-Aldrich (St
119 Quentin Fallavier, France) with the ratios of the diastereoisomers described on the
120 batch certificates of analysis. These ratios of cis/trans diastereoisomers were useful
121 for assessing the enantiomeric fraction of each stereoisomer in the chemical
122 standards used, which are reported in table 1. For EEO evaluation, the pure
123 stereoisomers (called E1, E2, E3 or E4) of each SGAR were obtained individually by
124 respective separation and collection with the chiral chromatography methods
125 described below in this work.

126 HPLC-grade acetonitrile and methanol, acetone, dichloromethane and hexane for
127 analysis were supplied by Merck (Darmstadt, Germany), formic acid was purchased
128 from Fluka (Steinheim am Albuck, Germany).

129 The cartridges used for solid-phase extraction in the liver extract preparation process
130 were Oasis®HLB (Hydrophilic-Lipophilic-Balanced) (1 mL) purchased from Waters
131 (Milford, Massachusetts, USA). The filters used for filtration of the liver extracts prior
132 LC-MS/MS injection were 0.2 µm phenex filters from Phenomenex (Torrance, CA,
133 USA).

134

135 2.2. Instrument and analytical conditions

136 Chromatographic analysis was performed on an Agilent 1200 series HPLC (Agilent
137 Technologies, Santa Clara, CA, USA). Chiral chromatographic separation was
138 evaluated with polysaccharide-based chiral selectors as presented in figure 2 (all
139 150*2 mm in size and packed with 3 µm particles) from Phenomenex (Torrance, CA,
140 USA) at constant temperature (25°C), at isocratic mode and with various mobile
141 phases as described in the results and discussion section.

142 MS/MS detection was carried out by an Agilent Technologies Triple Quadrupole
143 6410B equipped with an ElectroSpray Ionization source (ESI) in negative mode. MS
144 conditions were as follows: drying gas temperature 350°C, drying gas flow 8L/min,
145 nebulizer pressure 40psi, and capillary voltage 4000V. Fragment ion spectra were
146 recorded in Multiple Reaction Monitoring (MRM) and their mass spectrometry
147 detection parameters for all SGARs were described in Fourel et al., 2017 [17]. Data
148 collection and processing were performed with the Masshunter™ Work-station from
149 Agilent Technologies.

150

151 2.3. Sample processing

152 The preparation of liver extracts was adapted from Fourel et al, 2020 [22]. Solid-
153 Liquid Extraction (SLE) was firstly used to extract SGARs from liver. 0.50(±0.01) g of
154 liver was weighed and homogenized with 10 mL of acetone using an UltraTurax. The
155 mixture was centrifuged at 3000 rpm for 5 min. The liquid part was evaporated to
156 dryness at 40 °C under a gentle stream of nitrogen. The residues were reconstituted
157 in 1 mL of acetonitrile and washed twice with 1 mL of hexane to discard the lipids.

158 The remaining acetonitrile phase was evaporated to dryness in the same manner as
159 above. The residues were reconstituted in 0.5 mL methanol and 0.5 mL ultrapure
160 water was added, the resulting mixture was then saved at room temperature for
161 further purification. Secondly solid phase extraction was performed on Oasis® HLB
162 sorbent cartridges using a vacuum extraction device. The cartridges were
163 sequentially conditioned with 1 mL dichloromethane, 1 mL methanol and 1 mL
164 ultrapure water. The reconstituted saved samples were deposited on the cartridges
165 and left to migrate by gravity. Then, the wash step was performed with 1 mL of a
166 mixture of methanol and water (90:10). The cartridges were dried under vacuum for 1
167 min precisely. Elution was performed by gravity with 1 mL of a solution of
168 dichloromethane with 10% methanol. The extracts were evaporated to dryness under
169 nitrogen. Residues were reconstituted with 200 µL of methanol, and filtered through a
170 0.2 µm phenex filter prior to chiral-LC-MS/MS analysis.

171 2.4. Validation procedure

172 For validation purposes, chicken liver samples were purchased from supermarket
173 store, and left at -20°C until processing. The validation of residue levels quantification
174 in liver samples was done according to the bioanalytical method validation guideline
175 published by the European Medicines Agency (EMA) [29] with respect to specificity,
176 carry-over, Lower Limit Of Quantification (LLOQ), calibration curve, accuracy,
177 precision, dilution integrity, matrix effect and stability and this is detailed below.

178 2.4.1. Specificity

179 The specificity of the method was evaluated by analyzing blank chicken liver extracts
180 using 6 different chicken liver samples. The evaluation of interferences response
181 should be less than 20% of the LLOQ [29].

182 2.4.2. Calibration curves and Lower Limit Of Quantification (LLOQ)

183 Blank liver of chickens were spiked to obtain six different concentrations (n=2) over
184 the expected calibration range. Calibration curves were established by plotting peak
185 areas versus the expected concentrations. A polynomial regression and a correlation
186 coefficient ($r^2 > 0.99$ required) were determined for each compound.

187

188 2.4.3. Precision and accuracy

189 The within/between-run precision and accuracy were determined by analysing five
190 replicates at four different Quality Control (QC) levels, on two different days for the
191 between-run precision/accuracy. Precision (expressed as the coefficient of variation
192 (CV)) and accuracy (expressed as the percentage of the nominal value) should not
193 exceed 15% (20% for LLOQ) and $\pm 15\%$ ($\pm 20\%$ for the LLOQ) respectively [29].

194 2.4.4. Extraction recovery and matrix effect

195 Extraction recovery and matrix effect were calculated using the same set of blank
196 samples of six different chicken livers. Extraction recovery was assessed by
197 comparing processed spiked samples and blank samples spiked after processing.
198 Matrix effect was measured by comparing the response of the processed blank
199 samples and non processed samples (reconstitution solution) spiked at the same
200 level. The CV of matrix effect for the six chicken livers should not exceed $\pm 15\%$ [29].

201 3. Results and discussion

202 3.1. Acetonitrile-water mixtures as mobile phases: "HILIC-like" or RP mode

203 3.1.1. Influence of water content, and consequence on EEO

204 Chiral discrimination on polysaccharide stationary phases is based on hydrogen-
205 bonding and Π - Π type interactions between CSPs and chiral analytes [30]. A balance
206 between hydrophilic ("HILIC-like") or hydrophobic (RP mode) interactions may define
207 the importance of hydrogen bonding. This dual behavior that may have
208 polysaccharide-based CSPs with acetonitrile-water mixtures as mobile phases has
209 been previously reported [25]. Pure acetonitrile is an aprotic solvent, but also works
210 as a proton acceptor. Consequently, it should moderately interfere with hydrogen-
211 bonding interactions. On the contrary, water added to the organic mobile phase
212 strongly modifies these interactions and induces competition between hydrogen
213 hydrophilic bonding and hydrophobic interactions, and therefore between the mobile
214 phase, the CSP and the analytes [25]. Consequently, it induces a shift in the
215 retention of chiral analytes. This phenomenon occurs in two steps. By adding a small
216 amount of water to the pure acetonitrile mobile phase, the retention decreases up to
217 a certain water content between 5 and 20%. When the water content is further
218 increased, the retention starts to recover again and may reach very large retention
219 times (figures 3 - 8). In the first step, hydrogen bonding seems to be prevalent and
220 the system may act with "HILIC-like" behavior. In the second step, hydrophobic
221 interactions and then reversed phase mode should be predominant. The chiral

222 selectivity may not be the same in both modes for enantiomers or stereoisomers,
223 which may result in a change of the enantiomer elution order [25]. This will be
224 described in more detail in this manuscript.

225 This dual behavior has been previously observed for analytes such as chiral weak
226 acids and sulfoxides [25,26]. In the present work, it was observed for the SGARs on
227 the polysaccharide-based CSPs tested, although enantioresolution was not always
228 achieved. The present work involved finding the right combination of the
229 polysaccharide-based CSP and percentage of acetonitrile in the acetonitrile-water
230 mixture, and with an appropriate concentration of formic acid. As is often the case in
231 chiral method development, all CSPs were tested by varying different analytical
232 conditions in a systematic manner [13]. In the present case, enantioresolution of the
233 four stereoisomers of SGARs were evaluated with all five CSPs, with 0.005 or 0.02%
234 formic acid, and with 40, 25, 20, 15, 10, 5, 3, 0% water content, corresponding
235 respectively to 60, 75, 80, 85, 90, 95, 97 and 100% of acetonitrile. It should be noted
236 that there was often a large difference in the chiral discrimination between pure
237 organic acetonitrile and a acetonitrile/water mobile phase at very low water content of
238 3% (figures 3 - 8). It was therefore important to test very low water contents.

239 The objective was to achieve enantioresolution and reasonable retention times for
240 the four stereoisomers of the five SGARs, and to use the resulting methods for the
241 quantitative determination of their residue levels in biological sample sets. The
242 separation mode may be either "HILIC-like" or RP mode, and is described below for
243 the individual SGARs. A modification of EEO has been observed previously for other
244 chiral compounds, and it may correspond to a change in the prevalence of the
245 "HILIC-like" or RP mode [25]. This phenomenon was observed for some SGARs on
246 the tested CSPs after injection of pure enantiomers. The success of enantioresolution
247 with acetonitrile-water mixtures as the mobile phase is presented in the table 1, and
248 in the figures 3-9 for the four stereoisomers of bromadiolone on Lux Cellulose-1, for
249 flocoumafen on Lux Cellulose-2 and Lux Cellulose-4, for difethialone on Lux
250 Cellulose-3 and Lux Cellulose-4, for difenacoum on Lux Cellulose-1, and for
251 brodifacoum on amylose-2.

252

253 3.1.2. Enantioresolution of the four stereoisomers of bromadiolone:
254 selection of Lux Cellulose-1 CSP in RP mode

255 With Lux Cellulose-1 CSP and 0.02% formic acid in the mobile phase,
256 enantioresolution of all four stereoisomers of bromadiolone is achieved with a mobile
257 phase of 97 to 60% of acetonitrile. Decreasing from 100 to 97 and then to 95%
258 acetonitrile reduces all retention times. Then decreasing from 95% to 60%
259 acetonitrile in the mobile phase increases the retention times and maintains the
260 enantioresolution for all four stereoisomers (figure 3). Enantioresolution is also
261 achieved with a high water content of 40% (and perhaps higher), but with much
262 larger retention times and associated peak broadening that are not realistic for
263 application with biological matrices and many samples to be analyzed.

264 Thus, it appears that enantioresolution is achieved with 97% to 60% acetonitrile
265 content, but with variability in retention times. The chiral Lux Cellulose-1 selector
266 exhibits "HILIC-like" rather than reversed-phase behavior when used with mixtures of
267 acetonitrile and low water content in the mobile phase. This is associated with a
268 decrease in the retention of all four stereoisomers as the water content increases
269 from 0% to about 3%, and then a reversal of this retention behavior as the water
270 content continues to increase. The recovery of the retention for 95% acetonitrile in
271 the mobile phase does not correspond to a change in EEO as observed for other
272 chiral compounds [25], but to a change in the balance of different interactions
273 involved. Therefore, it means that enantioresolution of the four stereoisomers of
274 bromadiolone appears to be achievable in both modes without changing the chiral
275 discrimination. For bromadiolone, 80% acetonitrile is chosen (RP-mode) for further
276 analysis in biological matrices because the enantioresolution ($R_s > 3.74$) is good and
277 the retention times are less than 12 min (table 1). Under these conditions, injection of
278 the four pure stereoisomers and the two pure diastereoisomers defined the EEO as
279 E1-trans-bromadiolone, E2-trans-bromadiolone, E3-cis-bromadiolone, and E4-cis-
280 bromadiolone (table 1). The 90% acetonitrile in the mobile phase gave good
281 enantioresolution ($R_s > 2.41$) and smaller retention times (so shorter analysis times),
282 but matrix effects were observed during the validation process with liver samples,
283 which was not the case with 80% acetonitrile in the mobile phase.

284 With the variation of water content in the mobile phase, the same trend is still
285 observed with the other Lux Cellulose-based CSPs, but the enantioresolution is not

286 achieved or not as good as with the Lux Cellulose-1 CSP. However, it is interesting to
287 highlight a reversal of EEO with Lux Cellulose-4 CSP (0.005% formic acid). Indeed,
288 the EEO corresponds to E2-trans-bromadiolone, E4-cis-bromadiolone, E3-cis-
289 bromadiolone, E1-trans-bromadiolone with 7% water content in HILIC-like mode, and
290 E2-trans-bromadiolone, E4-cis-bromadiolone, E1-trans-bromadiolone, E3-cis-
291 bromadiolone with 25% water content in RP mode. This means that a reversal of
292 elution occurs between E3-cis-bromadiolone and E1-trans-bromadiolone from one
293 mode to the other (figures 4 and S1), which illustrates the change in the nature of the
294 interactions involved when the reversal of retention times occurs. In addition, EEO is
295 different with Lux Cellulose-4 and Lux Cellulose-1, and consequently depends on the
296 Lux Cellulose CSP used.

297

298 3.1.3. Enantioresolution of the four stereoisomers of difethialone:
299 selection of Lux Cellulose-3 CSP in RP mode

300 We have previously used a chiral-LC-MS/MS method to measure the enantiomeric
301 fraction of the four stereoisomers of difethialone in rat biological matrices, and this
302 with Lux Cellulose-3 CSP and a mobile phase composed of acetonitrile with 20%
303 water and 0.1% formic acid [22-24]. Indeed, with Lux Cellulose-3, the baseline
304 enantioresolution is obtained with 85 to 75% acetonitrile in the mobile phase with a
305 respective increase of the retention times, and this regardless of the 0.1, 0.02 or
306 0.005% formic acid content (figure 5). Therefore, enantioresolution of the four
307 stereoisomers of difethialone with Lux Cellulose-3 and a water-acetonitrile mixture
308 seems to be achievable only in RP mode. A percentage of 80% acetonitrile in the
309 mobile phase corresponds to good resolution ($R_s > 1.58$) for all stereoisomers and
310 retention times were less than 16 min (table 1). Under these conditions, injection of
311 the four pure stereoisomers and the two pure diastereoisomeric pairs defined the
312 EEO as being E1-trans-difethialone, E2-cis-difethialone, E3-cis-difethialone, and E4-
313 trans-difethialone [18]. There appears to be no retention time inversion in the case of
314 difethialone with Lux Cellulose-3 and the other Lux Cellulose-based CSPs when the
315 water content in the mobile phase is varied. Enantioresolution is also obtained in RP
316 mode but with different EEO (E4-trans-difethialone, E2-cis-difethialone, E3-cis-
317 difethialone, E1-trans-difethialone) on Lux Cellulose-4 (0.005% formic acid) and a
318 water content above 10% in the mobile phase but with longer retention times. For

319 example, with 80% acetonitrile in the mobile phase, the analysis time was greater
320 than 40 min for Lux Cellulose-4 instead of 16 min for Lux Cellulose-3. Unlike Lux
321 Cellulose-3 CSP, with Lux Cellulose-4 CSP the enantioresolution depends on the
322 formic acid concentration (figure 5). The trend observed was that retention increased
323 with decreasing formic acid concentration, and baseline enantioresolution was not
324 achieved at 0.02% content. This meant that it seemed difficult to anticipate the
325 enantioresolution behavior and reinforced the choice to test the CSPs columns with
326 different variables in a systematic way. However, on both columns, EEO was not
327 influenced by the concentration of formic acid in the mobile phase although it was
328 observed for other compounds on CSPs columns [31,32] (figure 5).

329

330 3.1.4. Enantioresolution of the four stereoisomers of flocoumafen:
331 selection of Lux Cellulose-4 CSP in “HILIC-like” mode

332

333 Enantioresolution of all four stereoisomers of flocoumafen was obtained with both
334 Lux Cellulose-2 and Lux Cellulose-4 CSP at low water content in the mobile phase
335 (0.005 % formic acid) (figure 6). The enantioresolutions were overall better and with
336 lower retention times for Lux Cellulose-2 ($R_s > 2$) than for Lux Cellulose-4 ($R_s > 1.14$)
337 (table 1). It should be noted that these two CSPs have similar structures (figure 2),
338 and that they appear to behave similarly for chiral discrimination of the four
339 stereoisomers of flocoumafen because the EEO was equivalent with both columns.
340 For both columns, it appears that enantioresolution is possible in either “HILIC-like” or
341 RP mode, and retention time reversal occurs around 90% acetonitrile in the mobile
342 phase. In addition, no reversal of the EEO was observed. Enantioresolution is also
343 obtained with 60% acetonitrile in the mobile phase with much longer retention times
344 (34-70 min and 40-110 min for Lux Cellulose-2 and Lux Cellulose-4, respectively) .
345 Injection of the four pure stereoisomers, and the two pure diastereoisomers defined
346 the EEO as being E1-trans-flocoumafen, E2-cis-flocoumafen, E3-cis-flocoumafen,
347 and E4-trans-flocoumafen (table 1).

348 It appears that, in this case, the concentration of formic acid has a significant effect
349 on enantioresolution. Although enantioresolution of all four stereoisomers of
350 flocoumafen is achieved at low water content with 0.005% formic acid in the mobile

351 phase, E2 and E3 or E1, E2 and E3 co-elute with 0.02% formic acid on both columns
352 (data not shown). The mobile phase with 0.0025% (instead of 0.005%) formic acid
353 was even tested for Lux Cellulose-4 and gave better Rs. In contrast to previous work
354 [31,32], no effect of formic acid content in the mobile phase on EEO was found. A
355 large difference in the chiral discrimination of the four stereoisomers of flocoumafén
356 was observed between pure organic acetonitrile and an acetonitrile / water mobile
357 phase at very low water content of 3% (figure 6), illustrating that the introduction of
358 water induces a significant change in the kind of interactions involved.

359

360 3.1.5. Enantioresolution of the four stereoisomers of difenacoum and 361 brodifacoum

362 There are few differences in chemical structure between difenacoum, brodifacoum
363 and difethialone (figure 1). Brodifacoum and difethialone have the same R side group
364 but difethialone is derived from 4-hydroxy-thiocoumarin and brodifacoum from 4-
365 hydroxy-coumarins. They may have a similar chiral affinity with the different
366 polysaccharide-based CSP, but this is not always the case. This means that chiral
367 separation is apparently very sensitive. For example, although chiral discrimination of
368 the four stereoisomers of difethialone was possible with Lux Cellulose-3 CSP at 15-
369 25% water content in the acetonitrile/water mobile phase (figure 5), it was not
370 obtained for the four stereoisomers of brodifacoum under the same conditions.

371 Difenacoum and brodifacoum differ only in the presence of a bromine atom at the
372 terminal position of the side group R. For both, enantioresolution was obtained with
373 amylose-2 CSP and 0.005% formic acid. For difenacoum, it was obtained with 60%
374 acetonitrile in the mobile phase, and with retention times between 16 and 50 min for
375 all four stereoisomers (data not shown). For brodifacoum, enantioresolution was also
376 obtained with amylose-2 CSP, with acetonitrile content between 60 and 80%, and
377 retention times between 30 and 110 min (60% acetonitrile) and 7 and 19 min (80%
378 acetonitrile) for the four stereoisomers (figure 7, table 1). For both molecules, the
379 retention times with 60% acetonitrile in the mobile phase did not appear realistic for
380 quantification of residue levels in many biological samples. With 80% acetonitrile in
381 the mobile phase, injection of the four pure stereoisomers of brodifacoum and the two

382 pure diastereoisomers defined the EEO as E4-trans-brodifacoum, E3-cis-
383 brodifacoum, E1-trans-brodifacoum, and E2-cis-brodifacoum (table 1).

384 A similar case with 60% acetonitrile in the mobile phase was observed for
385 difenacoum and brodifacoum with Lux Cellulose-4 with very large and thus
386 inadequate retention times (data not shown).

387 With Lux Cellulose-1 CSP, and 0.005% formic acid in the mobile phase, a very
388 interesting and delicate enantioresolution of the four difenacoum stereoisomers was
389 observed, illustrating that chiral discrimination can change at different low water
390 contents in the mobile phase. In fact, a reversal in elution order occurred between
391 E1-cis-difenacoum and E3-trans-difenacoum when the mobile phase was changed
392 from 97% acetonitrile (HILIC-like mode) to 90% acetonitrile (presumably RP mode)
393 (figure 8). While E1-cis-difenacoum and E3-trans-difenacoum co-eluted for 95%
394 acetonitrile in the mobile phase, E3-trans-difenacoum eluted first with 97%
395 acetonitrile, and E1-cis-difenacoum eluted first with 90% acetonitrile. For this reason,
396 this enantioresolution was not retained for further analyses in biological matrices
397 although it was obtained in less than ten minutes. Also with Lux Cellulose-1 CSP,
398 enantioresolution of the four stereoisomers of difenacoum has been previously
399 reported with acetonitrile-water (50/50, v/v) and 0.1% formic acid as the mobile phase
400 [25]. However, retention times were very long, up to 270 min, which in our case is
401 hardly usable as a routine method for measuring residue levels in many biological
402 samples. A large difference in the chiral discrimination of the four difenacoum
403 stereoisomers was observed between pure organic acetonitrile and an
404 acetonitrile/water mobile phase at a very low water content of 3% (figure 8), again
405 illustrating that the introduction of water induces a significant change in the type of
406 interactions involved.

407

408

409 3.2. Methanol-water mixtures as mobile phase: RP-mode and EEO

410 Methanol is a protic solvent and interacts with the analyte and chiral selector by
411 hydrogen bonding and polar interactions. It has been shown previously that
412 polysaccharide-based CSPs used with methanol-water mixtures mostly have a

413 reversed phase based separation mode. Therefore, in contrast to acetonitrile-water
414 mixtures that have a dual behavior, the addition of water to the methanol-water
415 mobile phase would increase the retention on the CSP [25-27]. In this work, the five
416 CSPs were systematically tested under isocratic conditions, with 0.1 or 0.02% formic
417 acid and with a water content of 15, 10, 5, 3 or 0%, corresponding to 85, 90, 95, 97
418 or 100% methanol in the mobile phase. As with acetonitrile-water mobile phases, the
419 objective was to obtain enantioresolution with reasonable retention times for all four
420 stereoisomers of all SGARs. This goal was achieved for difenacoum and
421 brodifacoum (table 1, figures 7 and 8). The EEO was described when these
422 conditions were reached (table 1).

423 All four stereoisomers of difenacoum were baseline resolved under pure polar
424 organic methanol conditions ($R_s > 2.14 (\pm 0.02)$), and in less than 12 min with
425 methanol (formic acid 0.1%) on Lux Cellulose-1, as previously reported [25]. It was
426 also baseline resolved at low water content ($<10\%$) in water-methanol mixtures (0.02
427 or 0.1% formic acid) with longer retention times confirming the system was based on
428 hydrophobic-type interactions (figure 8). No reversal of retention times and no
429 change in EEO were observed by varying the composition of the mobile phase (water
430 content) because the mode of separation remained the reversed phase. Injection of
431 the four pure stereoisomers, and the two pure diastereoisomers defined the EEO as
432 E1-cis-difenacoum, E2-trans-difenacoum, E3-trans-difenacoum, and E4-cis-
433 difenacoum (figure 8 and table 1).

434 The same reversed phase behavior was observed for the four brodifacoum
435 stereoisomers on Lux Cellulose-3 at low water content ($<10\%$) in the water-methanol
436 mixture (0.02 or 0.1% formic acid), and R_s was greater than 1.09 (± 0.03) with 3%
437 water content (figure 7). Injection of the four pure stereoisomers, and the two pure
438 diastereoisomers defined the EEO as E1-trans-brodifacoum, E2-cis- brodifacoum,
439 E3-cis- brodifacoum, and E4-trans- brodifacoum (table 1).

440

441 3.3. Residue level and EF of the four stereoisomers of SGARs in liver
442 samples

443 A chiral-LC-MS/MS method has been previously validated to assess the EF and
444 residue levels of the four difethialone stereoisomers in rat and mouse biological

445 matrices using the Lux Cellulose-3 CSP in RP mode [22-24]. Such methods, with
446 appropriate CSP and mobile phase, are mandatory for all SGARs to assess residue
447 levels of the four stereoisomers in the liver of unintentionally exposed species (rodent
448 predators such as raptors for example), or to characterize their pharmacokinetics in
449 target rodents. Therefore, enantioresolution, EEO, and quantitative analysis of the
450 four stereoisomers of each SGAR were evaluated in chicken liver. Then, the EF of all
451 stereoisomers was determined for chicken livers supplemented with standard
452 solutions of SGARs, and also for the standard solutions of SGARs. The results are
453 presented and discussed hereafter.

454

455 3.3.1. Enantioresolution in biological matrices and standards solutions

456 Enantioresolution is evaluated in processed chicken livers (from a commercial store)
457 (n=4) supplemented with the standard solution of the four stereoisomers of each
458 SGAR. It is also evaluated for the same standard solutions of each SGAR. The
459 results show that enantioresolution and retention times are similar in the chicken
460 livers and standard solutions (table 1).

461

462 3.3.2. Validation of the methods, and enantiomer fraction (EF) evaluation

463 As it is not realistic to use wild animal samples for quality control, chicken liver is
464 generally used to validate analytical methods involving such samples [1,4,6,18-
465 24,33]. In this work, the methods were validated with chicken liver samples and
466 according to the bioanalytical method validation guideline published by the European
467 Medicines Agency (EMA) [29]. The four stereoisomers were named E1, E2, E3, and
468 E4 based on their respective elution order with the chiral selector and
469 chromatographic conditions chosen as the reference method for our biological
470 samples, and as described in table 1. The specificity of the method was evaluated by
471 analysing blank chicken livers, and the interference response evaluation was less
472 than 20% of the low limit of quantification (LLOQ) (figure S2). Blank chicken livers
473 were spiked to obtain six different concentrations (n=2) over the expected calibration
474 range, and calibration curves ($r^2 > 0.99$) were established by plotting the peak areas
475 versus the expected concentrations for each stereoisomer. The range was 5 to 500

476 ng/g, and the LLOQ was 5 ng/g for all stereoisomers in the liver samples. For
477 brodifacoum, two methods were used to achieve the LLOQ of 5 ng/g for all
478 stereoisomers, because for both methods the LLOQ was 15 ng/g for the two
479 stereoisomers with the largest retention times (associated to higher signal-to-noise
480 ratio) (see table 1). Therefore, for brodifacoum, both methods can be used or only
481 one depending on the study objective. Precision and accuracy were tested with
482 Quality Control (QC) samples and the respective coefficients of variation were less
483 than 15% (table S1). Extraction recovery ranged from 75 and 103% (table S1). The
484 matrix effect was measured by comparing the response of the processed blank
485 samples and non-processed samples (reconstitution solution) spiked at the same
486 level. The coefficient of variation of the matrix effect for six chicken livers did not
487 exceed $\pm 15\%$.

488 Enantiomer fractions were evaluated for both SGAR standards and chicken livers
489 supplemented with SGARs standards (table 1).

490

491 3.3.3. Examples of differential persistence of the four SGARs 492 stereoisomers in a raptor - rodent predatory species

493 Residue levels and EF of the four stereoisomers of SGARs were evaluated in the
494 liver of a raptor potentially exposed to rodenticides. Results showed that this raptor
495 had been exposed to one of the four bromadiolone stereoisomers, E1-trans-
496 bromadiolone (EF = 1, hepatic concentration 790 ng/g), and not to the other three
497 stereoisomers, namely E2-trans, E3-cis and E4-cis-bromadiolone for which EF = 0. In
498 addition, the raptor was exposed to E1-cis-difenacoum (EF = 1, hepatic concentration
499 50 ng/g), but not to the other three stereoisomers, namely E2-trans, E3-trans and E4-
500 cis-difenacoum (table 1, figure 9).

501 These results provide evidence for a different metabolism of the SGARs
502 stereoisomers in this rodent predator, as it is documented that rodenticide baits
503 contain all stereoisomers [17]. The developed chiral methods will be used in the near
504 future to study the potential enantioselective bioaccumulation of SGARs
505 stereoisomers in species of target rodent, and non-target predators. This will allow
506 the implementation of already existing data on hepatic cis-/trans-diastereoisomers
507 residues [17-21] to assess the hepatic stereoisomeric concentrations and proportions

508 (EF) of all SGARs. Achiral and multi-residual LC-MS/MS [17] can be used to search
509 for positively exposed non-target species. The new chiral-RP-MS/MS methods will
510 then be used to determine the EF of all stereoisomers.

511

512 4. Conclusion

513 The work described in this paper allows to present several chiral-LC-MS/MS methods
514 to achieve good enantioresolution (R_s) and evaluation of the EF of the stereoisomers
515 for all the SGARs. Quantification of their respective residues in liver samples is also
516 possible. Optimal enantioselectivity may be reached in HILIC-like mode or RP-mode
517 depending on the hydrogen bonding interactions with selected chromatographic
518 parameters and chiral selectors. A systematic investigation of all parameters showed
519 that enantioselectivity and EEO depend on the chiral selector (polysaccharide-
520 based), the nature of organic solvent (acetonitrile or methanol), and the water content
521 in the mobile phase (0-40%). The percentage of formic acid (0.0025 to 0.1%) can
522 influence the enantioselectivity, but does not modify EEO in the presented work.

523 Enantioseparation with longer retention times can be used for preparative
524 chromatography. Enantioseparation with shorter retention times and good
525 enantioresolution are chosen as chiral-LC-MS/MS applications for the evaluation of
526 the enantiomer fraction of the four stereoisomers of all SGARs in liver samples. For
527 example, a raptor exposed to bromadiolone and difenacoum via consumption of
528 exposed rodents had liver residues of only E1-trans-bromadiolone and E1-cis-
529 difenacoum. Analysis of wildlife or rodent samples sets may allow detailed
530 assessment of the enantiomer fraction of any SGAR stereoisomer for ecotoxicology
531 or pharmacokinetic purposes.

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697

1 Figure Captions:

2

3 Figure 1: Structure of the five SGARs with the two stereogenic centers and the lateral
4 group R.

5

6 Figure 2: Representation of the structure of the chiral selectors (phenyl-carbamate or
7 phenyl-ester) in the polysaccharide-based (Lux Cellulose or Lux-Amylose) chiral
8 columns used in this study.

9

10 Figure 3: Dependence of retention ($\log k$) of bromadiolone stereoisomers on the
11 content of water in acetonitrile on Lux Cellulose-1 column. All mobile phases contains
12 0.02% formic acid. See section 2.2 for other analytical conditions.

13

14 Figure 4: Dependence of retention ($\log k$) of bromadiolone stereoisomers on the
15 content of water in acetonitrile on Lux Cellulose-4 column. All mobile phases contains
16 0.005% formic acid. See section 2.2 for other analytical conditions. Reversal of the
17 EEO for E1-trans-bromadiolone and E3-cis-bromadiolone between 75 and 93%
18 acetonitrile in the mobile phase.

19

20 Figure 5: Dependence of retention ($\log k$) of difethialone stereoisomers on the content
21 of water in acetonitrile on Lux Cellulose-3 and Lux Cellulose-4 columns. All mobile
22 phases contains 0.005% or 0.02% formic acid as indicated. See section 2.2 for other
23 analytical conditions.

24 Figure 6: Dependence of retention ($\log k$) of flocoumafen stereoisomers on the content
25 of water in acetonitrile on Lux Cellulose-2 and Lux Cellulose-4 columns. All mobile
26 phases contains 0.005% formic acid. See section 2.2 for other analytical conditions.

27

28 Figure 7: Dependence of retention ($\log k$) of brodifacoum stereoisomers on the content
29 of water in acetonitrile on Lux Amylose-2 and on the content of water in methanol on

30 Lux Cellulose-3 columns. All mobile phases contains 0.005% or 0.1% formic acid as
31 indicated. See section 2.2 for other analytical conditions.

32

33 Figure 8: Dependence of retention ($\log k$) of difenacoum stereoisomers on the content
34 of water in acetonitrile, and on the content of water in methanol on Lux Cellulose-1
35 column. All mobile phases contains 0.005% or 0.1% formic acid as indicated. See
36 section 2.2 for other analytical conditions. Reversal of the EEO for E1-trans-
37 difenacoum and E3-cis-difenacoum between 90 and 97% acetonitrile in the mobile
38 phase.

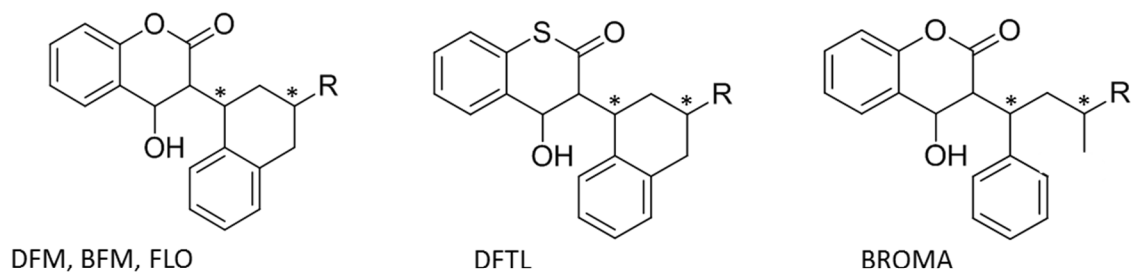
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40 Figure 9: **A**- Supplemented chicken liver sample with bromadiolone standard with EF
41 as described in Table 1, and hepatic residues of E1-trans-bromadiolone (EF=1) from
42 a raptor, without E2-trans-bromadiolone (EF=0), E3-cis-bromadiolone (EF=0), E4-cis-
43 bromadiolone (EF=0), with 80% acetonitrile in the mobile phase as described in Table
44 1. See section 2.2 for other analytical conditions. **B**- Supplemented chicken liver
45 sample with difenacoum standard with EF as described in Table 1, and hepatic
46 residues of E1-cis-difenacoum (EF=1) from a raptor, without E2-trans-difenacoum
47 (EF=0), E3-trans-difenacoum (EF=0), E4-cis-difenacoum (EF=0), with 100%
48 acetonitrile in the mobile phase as described in Table 1. See section 2.2 for other
49 analytical conditions.

50

51

Figure 1



SGARs	R :
DFM	
BFM, DFTL BROMA	
FLO	

Figure 2

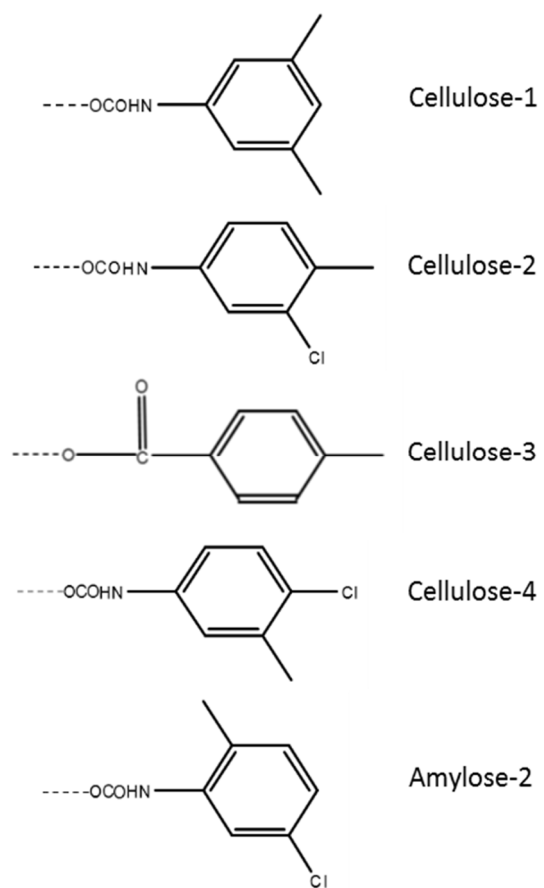


Figure 3

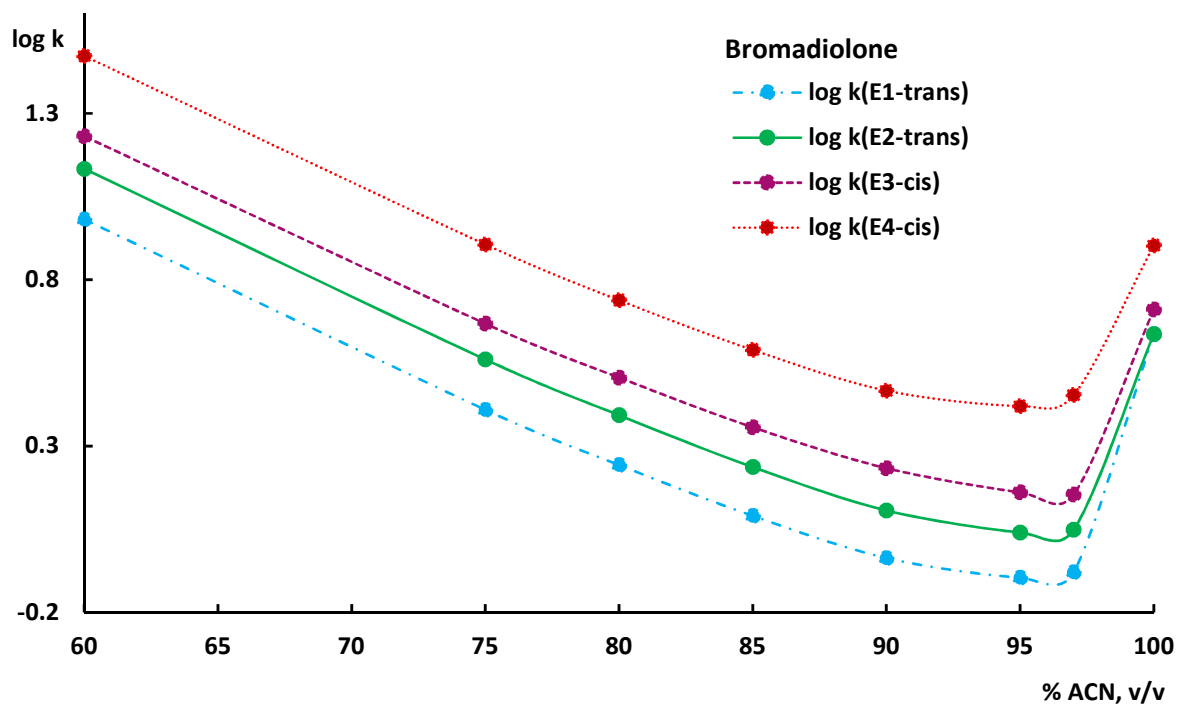


Figure 4

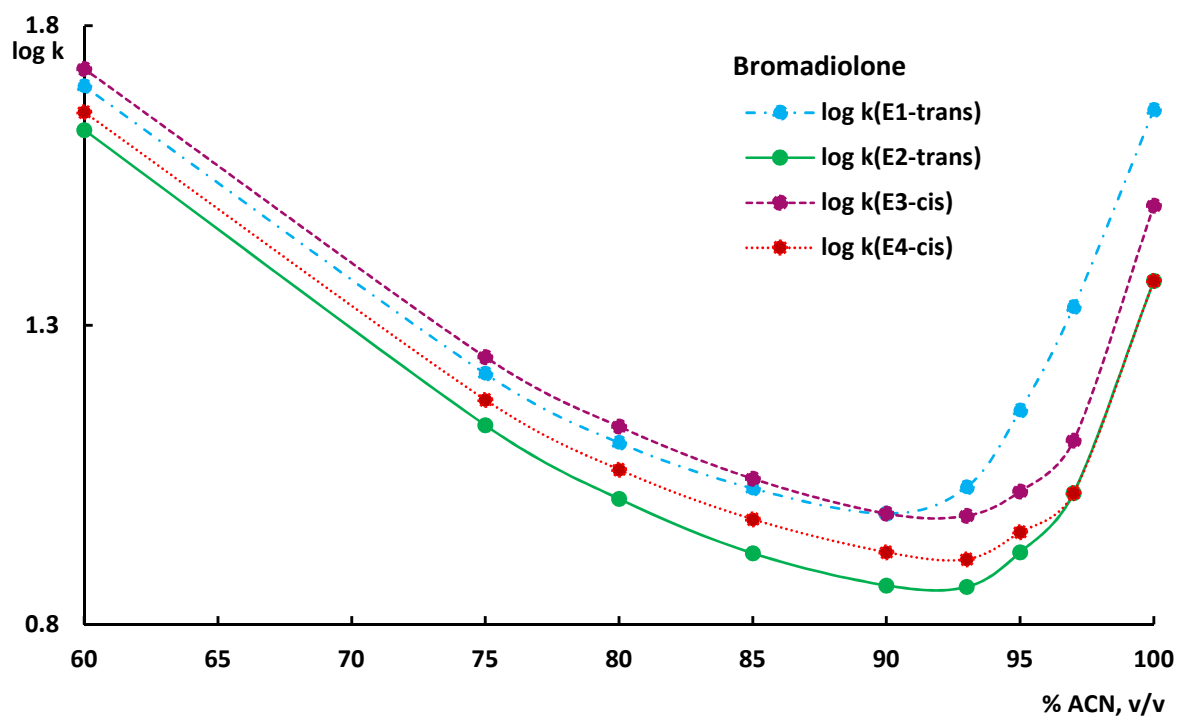


Figure 5

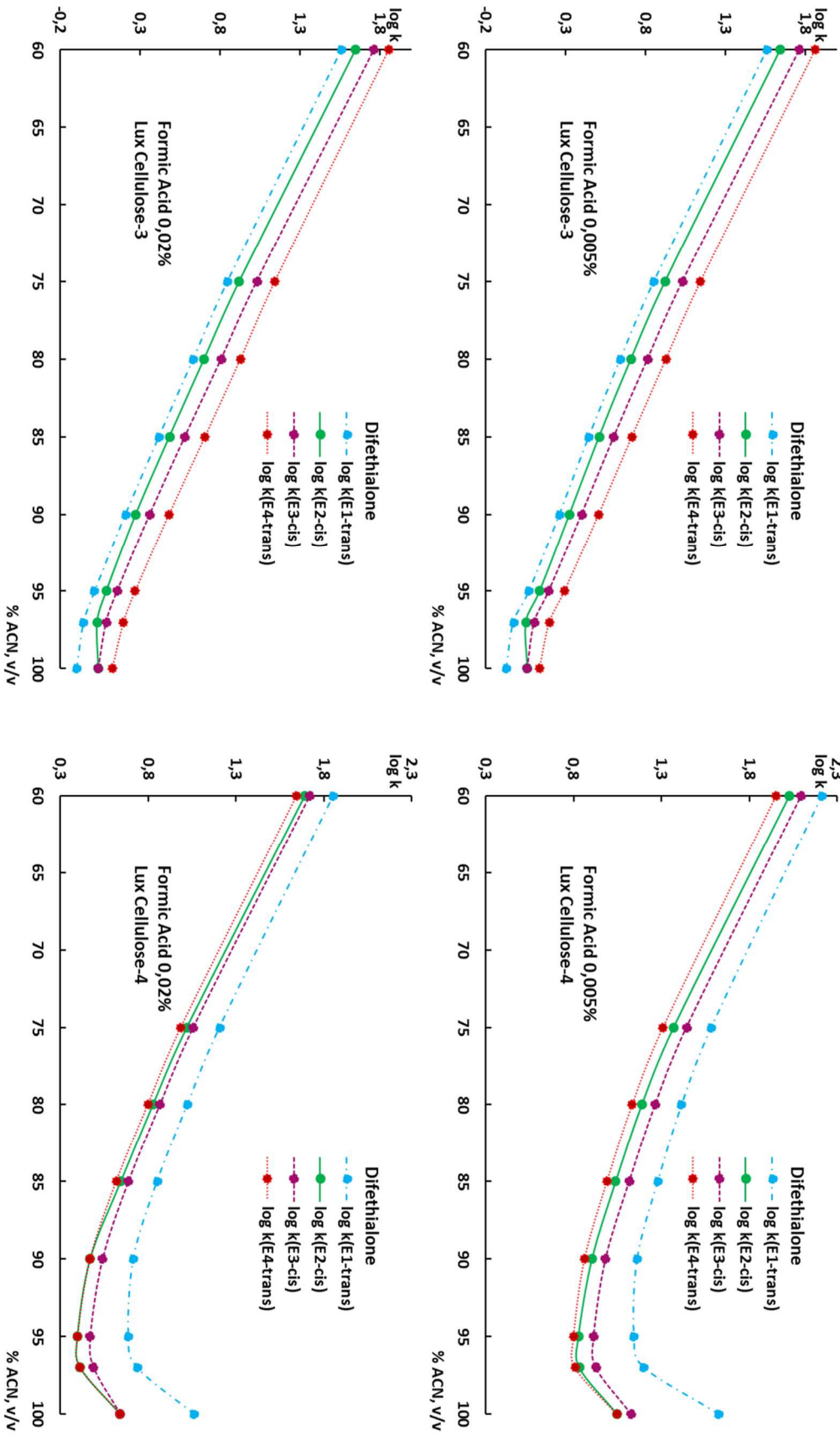


Figure 6

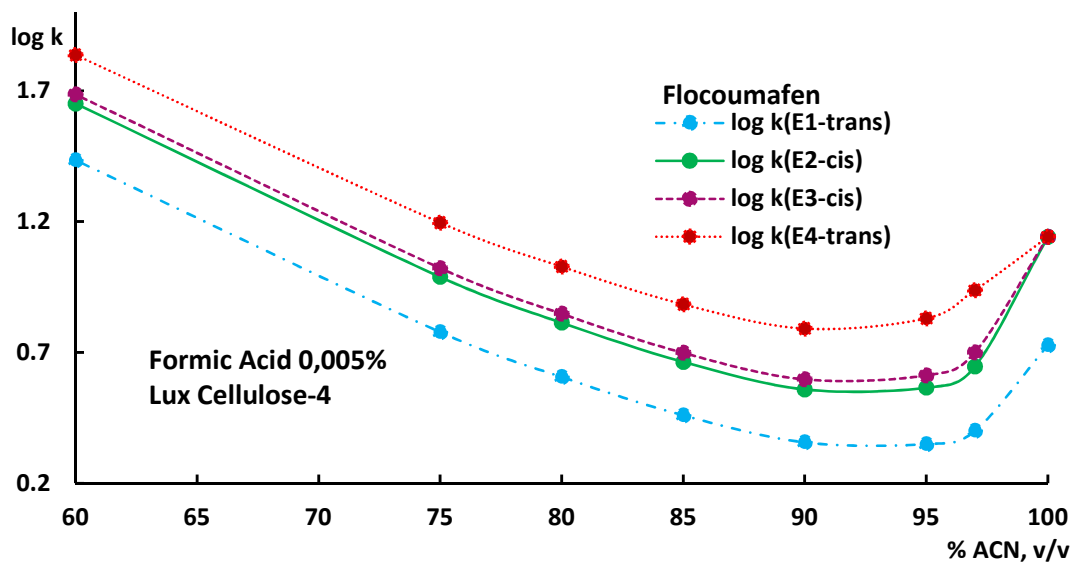
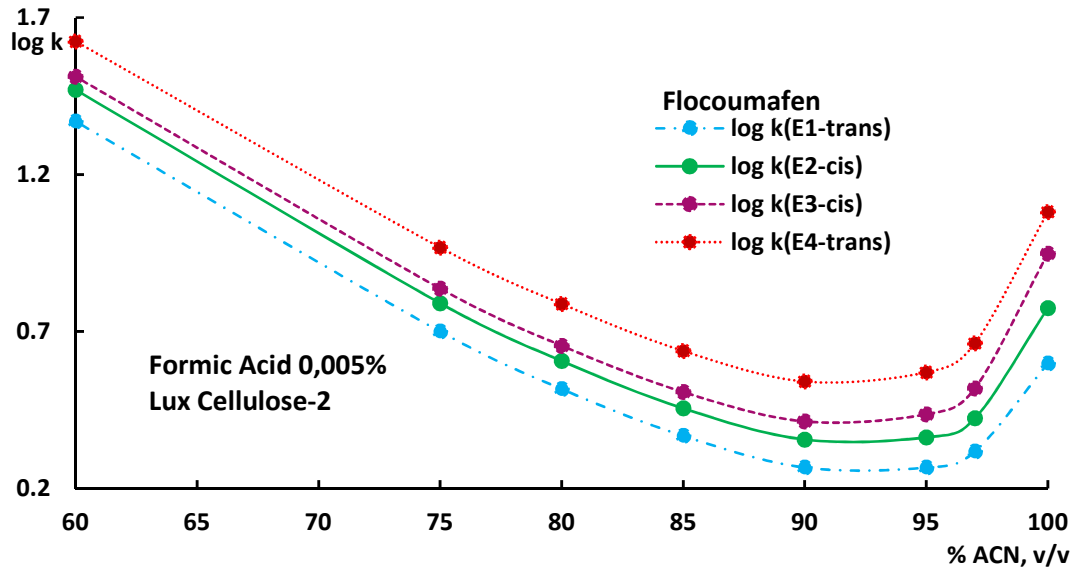


Figure 7

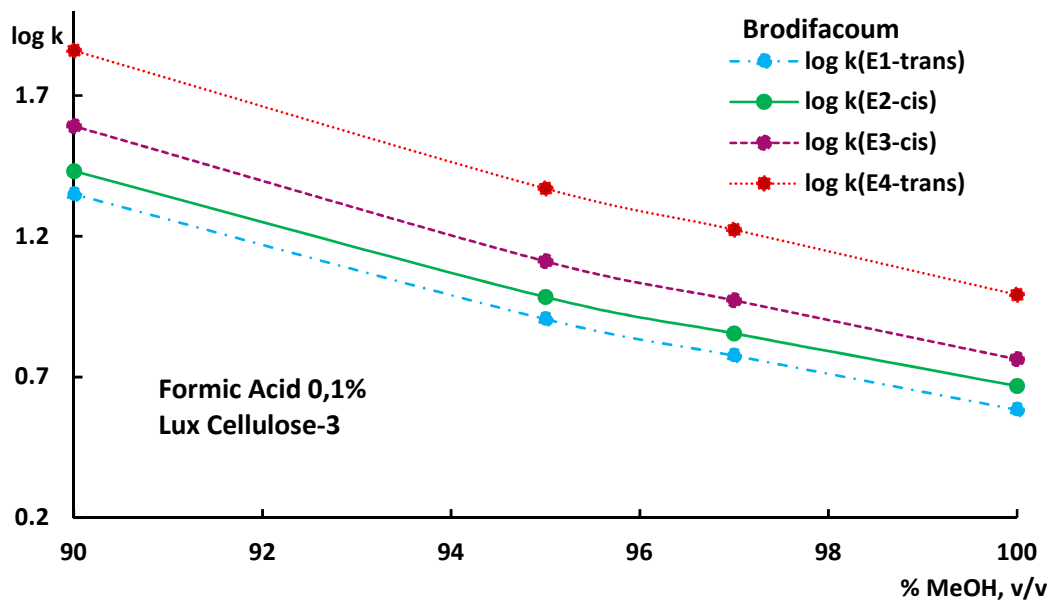
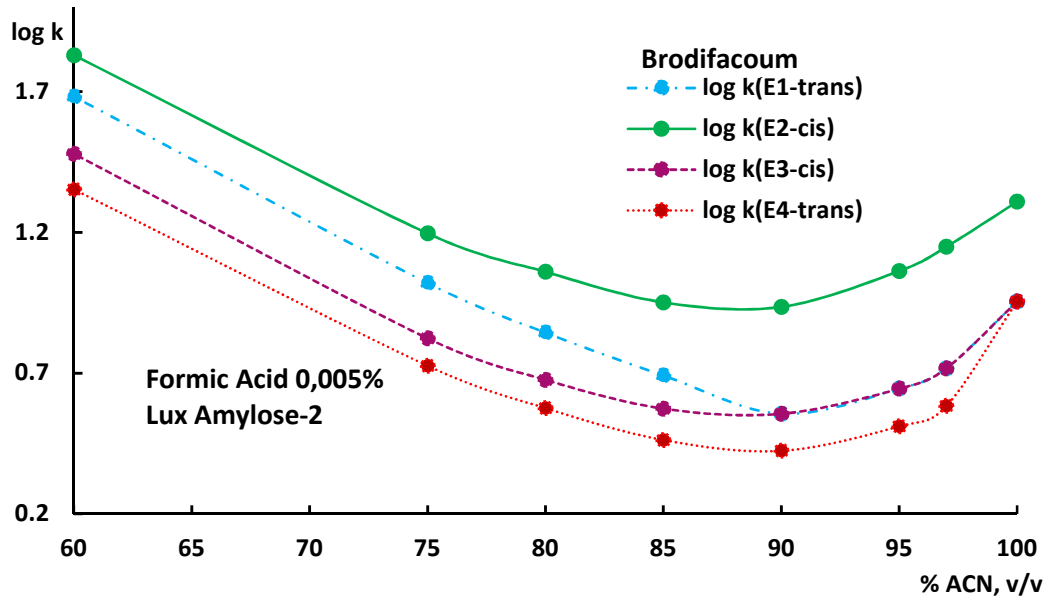


Figure 8

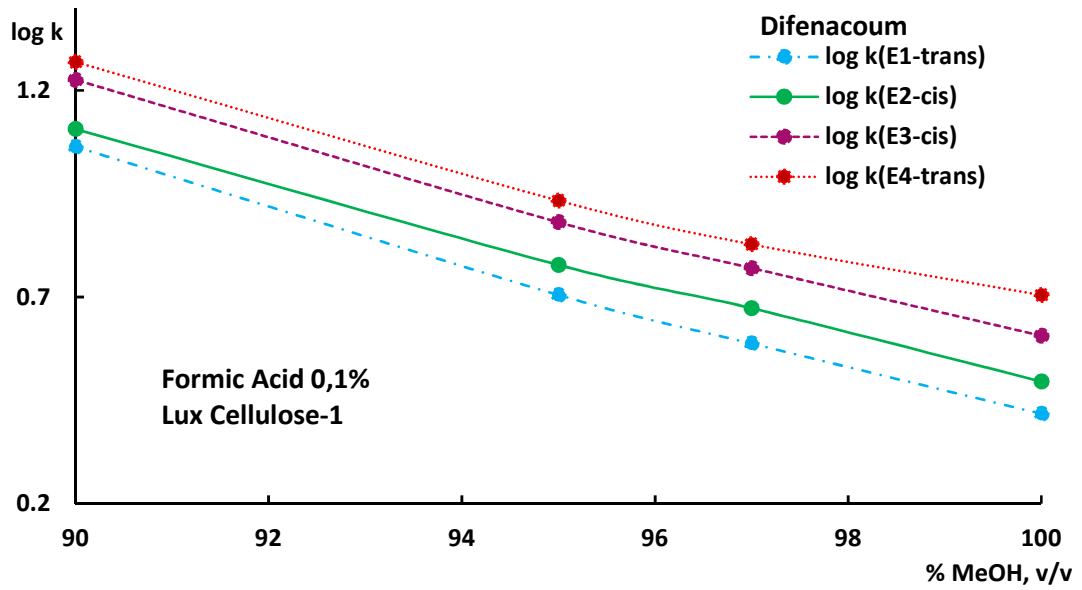
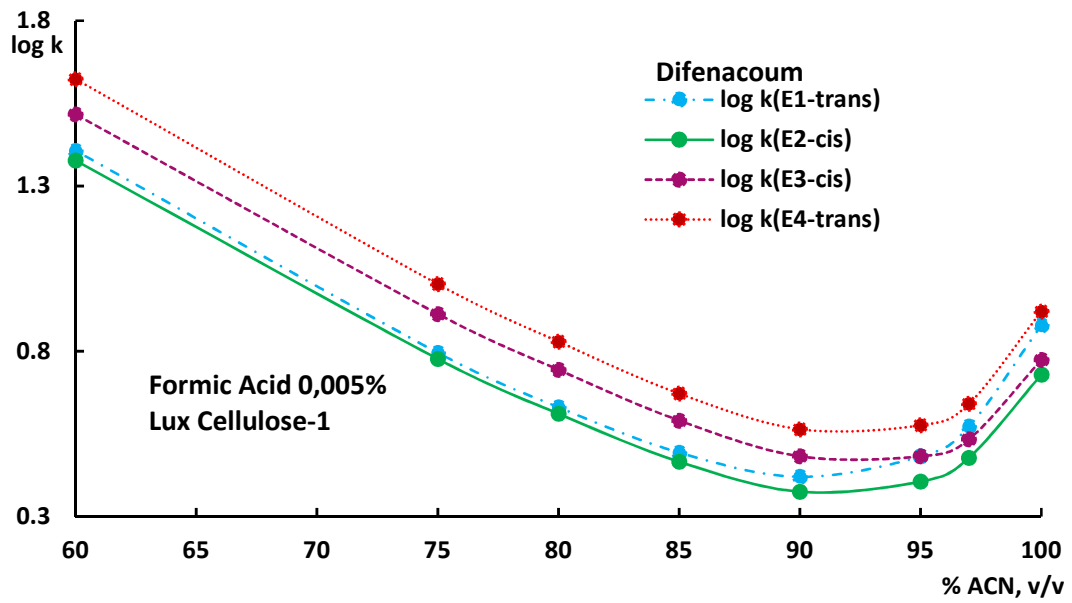
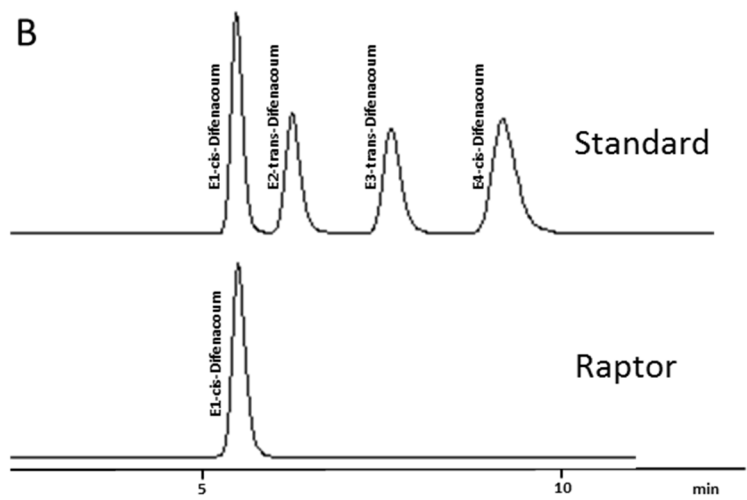
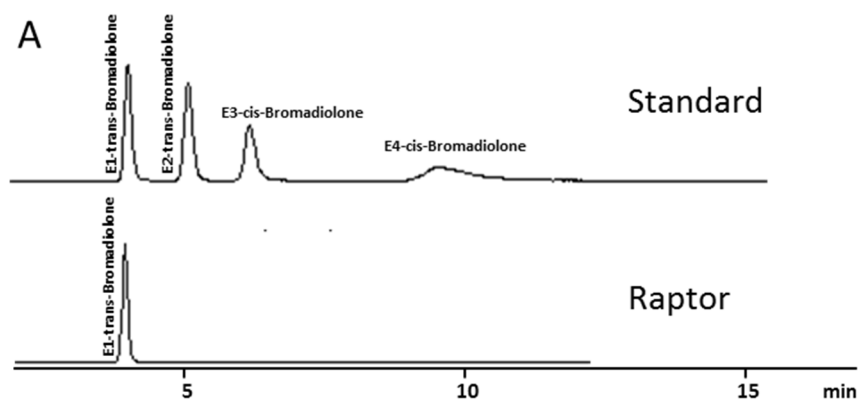


Figure 9



SGAR	CSP	Mode	Organic modifier (%)	Formic acid (%)	Figure	Matrix	Rs E1/E2 (±SD)	Rs E2/E3 (±SD)	Rs E3/E4 (±SD)	Analysis length (min)	Selected for bioanalysis	Stereoisomers EEO	Retention time (min)	LOQ (ng/g)	EF in standard ^f / chicken liver (±SD) ^g	EF / residue levels (ng / g) in raptor
Bromadiolone	Lux Cellulose-1	RP	Acetonitrile (80)	0.02	3	Standard	3.74 (±0.13)	4.46 (±0.17)	3.97 (±0.08)	12	Yes	E1-trans	4.2	5	0.300 / 0.303 (±0.008)	1 / 790
						Liver	3.85 (±0.10)	4.67 (±0.13)	3.90 (±0.12)	12		E2-trans	5.1	5	0.300 / 0.298 (±0.008)	0 / <LOQ
Difethialone	Lux Cellulose-3	RP	Acetonitrile (80)	0.02	5	Standard	1.58 (±0.05)	2.52 (±0.10)	2.48 (±0.10)	16	Yes	E3-cis	6.4	5	0.200 / 0.200 (±0.007)	0 / <LOQ
						Liver	1.58 (±0.05)	2.66 (±0.12)	2.55 (±0.09)	16		E4-cis	9.2	5	0.200 / 0.199 (±0.007)	0 / <LOQ
Difethialone	Lux Cellulose-4	RP	Acetonitrile (85)	0.005	6	Standard	1.49 (±0.05)a	2.15 (±0.06)	5.20 (±0.14)b	32	No	E4-trans	16.0	NA	0.275 / NA	NA
						Liver	1.49 (±0.07)a	2.11 (±0.02)	5.11 (±0.17)b	32		E2-cis	17.0		0.225 / NA	
Flocoumafen	Lux Cellulose-2	HILIC-like	Acetonitrile (95)	0.005	7	Standard	2.56 (±0.09)	1.96 (±0.07)	3.00 (±0.07)	10	Yes	E3-cis	21.0	NA	0.225 / NA	NA
						Liver	2.65 (±0.04)	2.00 (±0.06)	3.17 (±0.14)	10		E1-trans	30.0		0.275 / NA	
Flocoumafen	Lux Cellulose-4	HILIC-like	Acetonitrile (95)	0.0025	7	Standard	5.93 (±0.21)	1.14 (±0.03)	5.53 (±0.10)	18	No	E1-trans	4.5	NA	0.190 / 0.187 (±0.004)	NA / <LOQ
						Liver	5.81 (±0.05)	1.24 (±0.12)	5.51 (±0.09)	18		E2-cis	5.2		0.310 / 0.306 (±0.007)	NA / <LOQ
Difenacoum	Lux Cellulose-1	RP	Methanol (100)	0.1	10	Standard	2.14 (±0.02)	3.18 (±0.05)	2.78 (±0.05)	11	Yes	E3-cis	6.0	NA	0.310 / 0.313 (±0.005)	NA / <LOQ
						Liver	2.09 (±0.03)	3.23 (±0.09)	2.78 (±0.06)	11		E4-trans	7.8		0.190 / 0.195 (±0.004)	NA / <LOQ
Difenacoum	Lux Cellulose-1	RP	Acetonitrile (90)	0.005	9	Standard	1.59 (±0.03)c	1.97 (±0.03)d	3.22 (±0.01)	8	No	E1-trans	5.9	NA	0.190 / NA	NA
						Liver	1.37 (±0.08)c	2.02 (±0.02)d	2.87 (±0.03)	8		E2-cis	9.1		0.310 / NA	
Brodifacoum	Lux Cellulose-3	RP	Methanol (97)	0.1	11	Standard	1.09 (±0.03)	1.51 (±0.06)	3.14 (±0.10)	32	Yes	E3-cis	10.1	NA	0.310 / NA	NA
						Liver	1.11 (±0.05)	1.50 (±0.04)	3.31 (±0.13)	32		E4-trans	15.9		0.190 / NA	
Brodifacoum	Lux Amylose-2	RP	Acetonitrile (80)	0.005	8	Standard	1.61 (±0.06)e	1.82 (±0.07)b	1.68 (±0.13)c	28	Yes	E1-cis	5.4	NA	0.270 / 0.275 (±0.006)	1 / 50
						Liver	1.69 (±0.07)e	1.74 (±0.14)b	1.57 (±0.12)c	28		E2-trans	6.1		0.230 / 0.222 (±0.007)	0 / <LOQ
Brodifacoum	Lux Cellulose-1	RP	Acetonitrile (90)	0.005	9	Standard	1.59 (±0.03)c	1.97 (±0.03)d	3.22 (±0.01)	8	No	E3-trans	7.3	NA	0.230 / 0.234 (±0.010)	0 / <LOQ
						Liver	1.37 (±0.08)c	2.02 (±0.02)d	2.87 (±0.03)	8		E4-cis	8.7		0.270 / 0.270 (±0.002)	0 / <LOQ
Brodifacoum	Lux Cellulose-3	RP	Methanol (97)	0.1	11	Standard	1.09 (±0.03)	1.51 (±0.06)	3.14 (±0.10)	32	Yes	E1-trans	10.1	NA	0.205 / 0.213 (±0.004)	NA / <LOQ
						Liver	1.11 (±0.05)	1.50 (±0.04)	3.31 (±0.13)	32		E2-cis	11.8		0.295 / 0.292 (±0.013)	NA / <LOQ
Brodifacoum	Lux Amylose-2	RP	Acetonitrile (80)	0.005	8	Standard	1.61 (±0.06)e	1.82 (±0.07)b	1.68 (±0.13)c	28	Yes	E3-cis	15.0	NA	0.295 / 0.288 (±0.009)	NA / <LOQ
						Liver	1.69 (±0.07)e	1.74 (±0.14)b	1.57 (±0.12)c	28		E4-trans	26.3		0.205 / 0.208 (±0.020)	NA / <LOQ
Brodifacoum	Lux Amylose-2	RP	Acetonitrile (80)	0.005	8	Standard	1.61 (±0.06)e	1.82 (±0.07)b	1.68 (±0.13)c	28	Yes	E1-trans	7.2	NA	0.205 / 0.202 (±0.003)	NA / <LOQ
						Liver	1.69 (±0.07)e	1.74 (±0.14)b	1.57 (±0.12)c	28		E2-cis	18.6		0.295 / 0.297 (±0.006)	NA / <LOQ