Complete validation according to current international criteria of a confirmatory quantitative method for the determination of nitrofuran metabolites in seafood by liquid chromatography isotope dilution tandem mass spectrometry

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19 **Abstract**

20 Despite the ban of nitrofurans (NFs) for use in food production in many countries in the
21 1990s, NF metabolites in food are still regularly detected during import control testing. We
22 have developed a confirmatory routine method for the detection and quantification of NF
23 metabolites in seafood using LC-MS/MS and validated the method according to the strict
24 criteria in European legislation and Codex Alimentarius. Method characteristics were found to
25 fulfill the criteria. We report for the first time a new false positive for 1-amino-2,4-
26 imidazolidinedione (AHD), the metabolite of Nitrofurantoin (NFT). By using optimized
27 washing procedures, the non tissue bound false positives can be minimized. The results from
28 the validation on both lean and fatty fish and crustaceans, results from proficiency tests and
29 routine use over many years, demonstrates that the method is fit for purpose to determine NF
30 metabolites in the seafood category.

31

32 Keywords: nitrofurans; LC-MS/MS; seafood; false positive

33

34 **1. Introduction**

35 During World War II it was discovered that Schiff base derivatives of nitrofuraldehyde
36 were effective against pathogenic bacteria, leading to extensive research and synthesis of such
37 compounds (McCalla, 1983). Nitrofurans (NFs) are comprised of a Sciff base of a
38 nitrofuraldehyde and are part of the large family of hydrazide compounds characterized by the
39 $RC(=O)NRNR_2$ functional group (Moss, Smith, & Tavernier, 1995). NFs are broad-spectrum
40 synthetic antibiotics, effective against both Gram-negative and Gram-positive bacteria. Under
41 the World Health Organisation (WHO) Anatomical Therapeutic Chemical (ATC)
42 classification system they are categorization both as antiinfectives for systemic use (J01XE),
43 dermatologicals - antiseptics and disinfectants (D08AF) and antiparasitic products,
44 insecticides and repellents - antiprotozoals - agents against leishmaniasis and trypanosomiasis
45 (P01CC) (WHO). One of the advantages of using NFs is that resistance appears to develop
46 slowly towards this class of antibiotics. NFs have been used as feed additives for growth
47 promotion in domesticated animals and have been used both prophylactically and
48 therapeutically to treat gastrointestinal infections, such as bacterial enteritis caused by
49 *Escherichia coli* and *Salmonella*.

50 The main NFs used in animal production are furazolidone (FZD, 3-{(E)-[(5-nitro-2-
51 furyl)methylene]amino}-1,3-oxazolidin-2-one), furaltadone (FTD, 5-(4-morpholinylmethyl)-
52 3-{(E)-[(E)-(5-nitro-2-furyl)methylene]amino}-1,3-oxazolidin-2-one), nitrofurazone (NFZ,
53 (2E)-2-[(5-nitro-2-furyl)methylene]hydrazinecarboxamide) and nitrofurantoin (NFT, 1-{(E)-
54 [(5-nitro-2-furyl)methylene]amino}-2,4-imidazolidinedione). Upon ingestion, these NFs are
55 metabolized within minutes to their respective NF metabolites; 3-amino-1,3-oxazolidin-2-one
56 (AOZ), 3-amino-5-(4-morpholinylmethyl)-1,3-oxazolidin-2-one (AMOZ),
57 hydrazinecarboxamide (usually referred to as semicarbazide (SEM)) and 1-amino-2,4-
58 imidazolidinedione (usually referred to as 1-aminohydantoin (AHD)). These NF metabolites

59 are more persistent than the parent NFs and can prevail for weeks after treatment, covalently
60 bound to muscle protein in treated animals.

61 In 1991 the U.S. Food and Drug Administration (FDA) withdrew the approvals for
62 FZD and NFZ, and in 1995 the use of NFs in livestock production was banned in the EU due
63 to concerns regarding the carcinogenic and mutagenic properties of NFs and potential harmful
64 effects on human health (EC, 1993, 1995). Following their ban, a minimum required
65 performance level (MRPL) was established for methods to be used in official control of NF
66 metabolites at 1 μg/kg in the EU. The MRPL is implemented in the analytical method as the
67 decision limit (CCα) which is the limit at and above it can be concluded with an error
68 probability of α that a sample is non-compliant. The implications of this is that any detections
69 of NF metabolites, above the CCα of the confirmatory method used for the determination, is
70 evidence of illegal use of NFs. Codex Alimentarius Commission (CAC) has developed risk
71 management recommendations for FZD and nitrofural (another name for NFZ) to prevent
72 residues of these NFs in food (CAC, 2018).

73 The number of detections of NF metabolites has decreased since the peak years at the
74 beginning of the millennium, possibly due to a combination of increased food import control
75 and decreased use. Residues of NFs are still regularly detected during EU import control of
76 aquaculture products such as *e.g.* fish and crustaceans as reported in the Rapid Alert System
77 for Food and Feed (RASFF) (RASFF). The main NF metabolites detected are SEM and AOZ,
78 whereas AMOZ was more prominent in the early 2000s compared to present days. For SEM
79 there is a known problem of false positives in foods due to the use of additives in both food
80 packaging and food, which will result in identical analytical response (de la Calle & Anklam,
81 2005).

82 While early methods focused on the determination of the parent NFs, the lack of
83 stability of the parent NFs has forced a change of analytical methodology focus towards

84 methods which determine NF metabolites. Because NF metabolites have high polarity and
85 low molecular mass the detection and quantification is usually carried out after derivatization
86 with 2-nitrobenzaldehyde (2-NBA). Following derivatization of the metabolites, 3-[(*E*)-(2-
87 Nitrobenzylidene)amino]-1,3-oxazolidin-2-one (NP-AOZ), 5-(4-Morpholinylmethyl)-3-[(*E*)-
88 (2-nitrobenzylidene)amino]-1,3-oxazolidin-2-one (NP-AMOZ), (2*E*)-2-(2-
89 Nitrobenzylidene)hydrazinecarboxamide (NP-SEM) and 3-[(*E*)-(2-Nitrobenzylidene)amino]-
90 2,4-imidazolidinedione (NP-AHD) are obtained. Sample workup and derivatization of NF
91 metabolites are usually carried out based on the published methods from Hoogenboom *et. al.*
92 and Leitner *et. al.* (Hoogenboom, Vankammen, Berghmans, Koeman, & Kuiper, 1991;
93 Leitner, Zollner, & Lindner, 2001).

94 Different instrument setups for the detection and quantification of NF metabolites
95 have been published using LC with either ultraviolet (UV) or mass spectrometry (MS) as
96 detectors (Conneely, Nugent, O'Keeffe, Mulder, van Rhijn, Kovacsics, et al., 2003; Hu, Xu, &
97 Yediler, 2007). Whereas UV is more cost beneficial, the use of MS is more selective and may
98 fulfill requirements for unequivocal identification of the metabolites. Both MS, MS/MS and
99 ion-trap MS have been used for the determination of NF metabolites. The use of MS/MS is
100 often preferred due to higher selectivity compared to single quadrupole MS and better
101 reproducibility compared to ion-trap-MS (Saari & Peltonen, 2004). Using two transitions for
102 the identification and quantification of NF metabolites in MS/MS, fulfills the point system for
103 unequivocal detection of an illegal substance (CAC, 2014; EC, 2002).

104 Several methods for the determination of various NF metabolites in foods such as egg,
105 chicken, pork, poultry, dairy products, feed, bakery products and baby food have been
106 developed (Vass, Hruska, & Franek, 2008). No method for the determination of all NF
107 metabolites demonstrated to be applicable to seafood as a food category has previously been
108 published, but methods for the LC-MS/MS determination of AOZ in prawns, SEM in

109 crayfish, prawns, shrimp and fish, FZD and AOZ in fish and AOZ, AMOZ, AHD and SEM in
110 shrimp has been published (Chu & Lopez, 2005; Conneely, et al., 2003; Hoenicke,
111 Gatermann, Hartig, Mandix, & Otte, 2004; Hu, Xu, & Yediler, 2007; Saari & Peltonen, 2004;
112 Valera-Tarifa, Plaza-Bolanos, Romero-Gonzalez, Martinez-Vidal, & Garrido-Frenich, 2013).
113 Important validation parameters such as *e.g.* recovery and/or CCα/CCβ are also missing in
114 several of the methods published earlier. We hypothesized that it would be possible to
115 develop a horizontal method applicable to seafood as a food category and report here our
116 findings after comprehensive validation of a method for the determination of NF metabolites
117 in seafood, carried out according to the current EU decision 657/2002/EC and Codex
118 Guideline 71-2009 (CAC, 2014; EC, 2002).

119

120 ## 2. Material and methods

121 ### 2.1 Samples

122 Samples of seafood were selected to represent both variety in matrix composition and seafood
123 where NF metabolites are often detected, such as crustaceans. Atlantic salmon (*Salmo salar*)
124 was used as a matrix representing fish with high fat content and atlantic cod (*Gadus morhua*)
125 represented fish with low fat content. Shrimp (*Pandalus borealis*) was used as a proxy for
126 crustaceans. Validation samples were obtained from regular monitoring samples collected
127 routinely by our institute and the Norwegian Food Safety Autority. Samples did not contain
128 NF metabolites. The wild cod and shrimp were collected by fishermen authorized for
129 commercial fishing by the Norwegian Directorate of Fisheries. Both wild cod, shrimp and
130 farmed salmon were treated according to Norwegian laws regulating handling and euthanasia
131 fish and seafood.

132

133 **2.2 Standards**

134 Unlabeled NF metabolites AOZ, AMOZ, AHD and SEM (VETRANAL, ≥ 98% purity) were
135 purchased from Sigma Aldrich (St. Louis, MO). Labelled NF metabolites AOZ-D_4, AMOZ-
136 D_5, AHD-$^{13}C_3$ and SEM-$^{15}N_2{}^{13}C$ were purchased from Witega (Berlin, DE). Stock solutions
137 used for establishing calibration curves and for spiking of control samples were prepared
138 independently. Stock solutions of unlabeled analytes used in calibration curves and control
139 samples, and labeled analytes used as internal standards, were prepared by weighing 15-25
140 mg of the compounds. The weighed amount of unlabeled AMOZ and AOZ was transferred
141 into a 20 ml volumetric flask followed by diluting to the mark with methanol (Chromasolv,
142 Sigma Aldrich). Stock solution of unlabeled AHD was prepared by transfering the weighed
143 amount into a 100 ml volumetric flask, followed by diluting to the mark with methanol. For
144 unlabeled SEM the weighed amount was transferred into a 250 ml volumetric flask, followed
145 by diluting to the mark with Milli Q water. The weighed amount of the labeled internal
146 standards were all transferred into 100 ml volumetric flasks and diluted to the mark with
147 methanol, except for SEM which was diluted with Milli Q water. All stock solutions were
148 diluted to intermediate mixture solutions by adding an appropriate amount of each stock
149 solution to a 10 ml volumetric flask and diluting to the mark with methanol. Nominal
150 concentrations (in parenthesis) for intermediate mixture solution were; calibration curves (10
151 µg/ml), internal standards (2-10 µg/ml) and control solutions (4-6 µg/ml). Working standard
152 solutions in the ng/ml range were prepared by adding an appropriate amount of the
153 intermediate solutions to 100 ml volumetric flasks and diluting to the mark with methanol.

154

155 **2.3 Reagents**

156 The extraction solution, 0.2 M hydrochloric acid (HCl), was prepared by adding 16.7 ml of
157 concentrated HCl (12 M, 37%, Merck, Darmstadt, DE) to 300 ml Milli Q water in a 1 L
158 volumetric flask followed by diluting to the mark with Milli Q water. Derivatization reagent
159 (100 mM) was prepared by weighing 76 mg 2-NBA (p.a., Sigma Aldrich) into a 5 ml
160 volumetric flask and diluting to the mark with methanol (Chromasolv, Sigma-Aldrich). The 2
161 M sodium hydroxide solution was prepared by weighing 20 g NaOH (Merck) in a 250 ml
162 volumetric flask, followed by dissolving the NaOH in Milli Q water and finally diluting to the
163 mark. The 0.3 M trisodiumfosfate buffer solution was prepared by weighing 11.4 g
164 $Na_3PO_4 \cdot 12H_2O$ (Merck) into a 100 ml volumetric flask followed by diluting to the mark with
165 milli Q water. Mobile phase A for the LC was prepared by adding 1 ml concentrated acetic
166 acid (100%, glacial, anhydrous for analysis EMSURE®, Merck) to a 1 L mixture of 900 ml
167 acetonitrile and 100 ml Milli Q water. Mobile phase B was prepared by adding 1 ml
168 concentrated acetic acid to 1 L of Milli Q water. In addition to the aforementioned Milli Q
169 water and methanol, hexane (Merck) was used for sample cleanup and ethanol (Kemetyl,
170 Kolbotn, NO) and diethyl ether (Merck) was used for the washing procedures (2.4). End
171 solution was prepared by mixing 25 ml acetonitrile with 225 ml Milli Q water and adding 250
172 µl concentrated acetic acid.

173

174 **2.4 Washing procedure for tissue bound NF metabolites**

175 Two different procedures were used for determination of tissue bound residues of NF
176 metabolites. Both washing procedures were performed after weighing of the sample, but prior
177 to the sample preparation (supplementary Figure 1) described in section 2.5.

178 Washing procedure 1 was based on the report from the 2004 Joint FAO/WHO
179 Technical Workshop on Residues of Veterinary Drugs without ADI/MRL (FAO/WHO,

180 2004). The sample was first homogenised with 5 ml ethanol/water (1/1; v/v), followed by 5
181 min 4000 rpm centrifugation. The supernatant was then discarded. The sample was further
182 washed three times with 5 ml of methanol, two times with 5 ml of ethanol and two times with
183 5 ml of diethyl ether. Each time the sample was washed by addition of the solvent, turning the
184 sample tube three times, and decanting off the solvent before the next washing step.
185 Following washing and decanting of the last washing solvent, the procedure described under
186 sample preparation (2.5) was carried out.

187 Washing procedure 2 was based on the method used by the European Reference
188 Laboratory (EURL) for residues of veterinary medicines and contaminants in food of animal
189 origin (Eric Verdon, Couedor, & Sanders, 2007). In this procedure the sample was washed
190 four times, first with 6 ml of methanol/water solution (1/1; v/v), then with 6 ml of
191 methanol/water solution (3/1; v/v), followed by 6 ml of methanol and finally 2 ml of Milli Q
192 water. Each time the addition of washing solvent was followed by a 15 min rotary
193 homogenization at 100 rpm, before centrifugation for 10 min at 4000 rpm and disposal of the
194 supernatant by decantation. Following the washing and decanting of the milli Q water, the
195 procedure described in sample preparation (2.5) was carried out.

196

197 **2.5 Sample preparation**

198 Sample weighing for validation and later routine use was identical. Homogenized samples
199 were weighed (1.00±0.02 g) into polypropylene (PP)-tubes. It is important to make sure that
200 the analytical test portion is sufficiently large and homogeneous to produce representative
201 results of the sample. During validation matrix blank for positive controls were spiked with
202 appropriate volumes of a 10 ng/ml working standard mixture of NF metabolites. During later
203 routine use, when the CCα of the method had been established, the matrix blank for positive

204 control was spiked with 100 µl of a 4-6 ng/ml working standard mixture of NF metabolites.
205 Calibration curve solutions were prepared by adding 10-1000 µl of a 10 ng/ml working
206 standard mixture to PP-tubes. Calibration curves with 5-6 concentrations in the range 0.1-10
207 ng of each NF metabolite were prepared fresh from the stock solutions every day. All samples
208 and calibration curve solutions were then spiked with 100 µl of a 20-100 ng/ml internal
209 standard mixture solution. The derivatization agent, 2-NBA, (50 µl of the 100 mM solution)
210 was added to all samples, matrix blanks and calibration curve solutions . Then 5 ml of 0.2 M
211 HCl were added to samples and matrix blanks and 1 ml 0.2 M HCl was added to calibration
212 curve solutions. A procedural blank was prepared by adding 50 µl of 2-NBA and 1 ml of 0.2
213 M HCl to an empty tube. All tubes were vortex mixed for 1 minute and placed in a heating
214 cabinet at 37±3 ºC overnight. The next morning samples were removed from the heating
215 cabinet and allowed to cool down to room temperature. Then 0.3 M Na_3PO_4 solution was
216 added to each sample and matrix blank (500 µl) and calibration curve solution (100 µl)
217 followed by swirl mixing. The pH was adjusted to 7 ± 0.5 by adding 2 M NaOH to the
218 samples and matrix blanks (340-370 µl), the procedural blank (340-370 µl) and calibration
219 curve solutions (80 µl), followed by swirl mixing and waiting 5 minutes before the pH was
220 checked using pH-paper. The amount of 2 M NaOH required to adjust the pH to 7 was found
221 to be matrix dependent. Typical volumes of 2 M NaOH required to reach pH 7 was 340-360
222 µl for salmon, 350 µl for shrimp, 350-360 µl for cod and 370 µl for the procedural blank.
223 After the pH adjustment, 4 ml ethyl acetate was added to the samples and matrix blanks and
224 the content of the tubes were mixed for 20 minutes using a rotator. Samples and matrix blanks
225 were then centrifuged at 4000 rpm for 5 minutes, and the ethyl acetate was transferred to a
226 new PP-tube. The extraction was repeated with 4 ml ethyl acetate and the combined extracts
227 were filtered through a 0.2 µm nylon filter. The solvent was removed by evaporation at 30 ºC
228 using a flow of nitrogen. The samples and matrix blanks were reconstituted in 250 µl of end

229 solution. Further clean up of the samples was done by adding 2 ml of hexane to the

230 reconstituted sample, vortex mixing and centrifugation at 4000 rpm for 5 minutes. The bottom

231 layer was transferred to vials for instrument determination.

232

233 **2.6 Instrument determination**

234 Samples were analysed using an Agilent 1200 LC, fitted with the large volume injecton kit

235 allowing for volumes up to 100 µl to be injected. The LC was connected to an Agilent 6410B

236 MS/MS fitted with a Zorbax Eclipse Plus C18 column (600 Bar), with particle size of 1.8 µm,

237 internal diameter 2.1 mm and length 150 mm. The instrument sequence was set up according

238 to Commission decision 2002/657/EC with minor adjustments. The validation sequence

239 started with calibration curve solutions followed by procedural blanks, negative matrix

240 blanks, (spiked) samples and calibration curve solutions. The sequence used for later routine

241 followed the same setup except that the negative matrix blank and spiked sample at CCα were

242 also analysed after the samples in the sequence. Injection volumes were 50 µl for all solutions

243 expect calibration curve solutions at or above 1 ng, where 20-5 µl injection volumes were

244 used. Flow rate was set at 0.25 ml/min and the column temperature was held at 45 ºC. The

245 gradient started at 17 % A, which was held for 0.1 minutes before changing rapidly to 40% A

246 in 0.01 min. This composition was held until 3.5 minutes. Then the composition was rapidly

247 returned to initial conditions during 0.01 min, which was held until 10 minutes. The MS was

248 operated in positive ESI MRM mode. Gas temperature was held at 350 ºC, gas flow was held

249 at 6 L/min, nebulizer pressure was held at 15 psi and capillary was held at 3500 (V). Detailed

250 MS-parameters are given in Table 1.

251

252 **2.7 Validation setup and calculation of validation results**

253 Validation was carried out according to Codex guideline 71-2009 and EU Commission
254 decision 2002/657/EC which lays down the parameters to be checked in the validation and the
255 criteria for the evaluation of results and fitness for purpose of the method. The outline of the
256 validation is shown in Table 2.

257 Selectivity/specificity was evaluated by investigating chromatographic separation of
258 the analytes, comparing peak shape in standards and samples. Matrix effects which could give
259 interferences were evaluated by analyzing >20 blank samples of each matrix.

260 Linear range of the method was evaluated by analysis of calibration curves and by
261 determining the decision limit (CCα). The upper limit of quantification (ULOQ) was set equal
262 to the concentration of the highest calibration curve standard.

263 CCα was determined using the three methods described in Commission decision
264 2002/657/EC. When using the first method CCα was determined by analyzing > 20 blank
265 samples of each matrix (sample blank method) and using three times signal to noise (S/N)
266 given by the formula

267

268 $$CC\alpha = 3 * S/N$$

269

270 CCα was also determined using linear regression of the matrix blanks spiked at 1.0 x MRPL,
271 1.5 x MRPL and 2.0 x MRPL as stated in Commission decision 2002/657/EC (calibration
272 curve method). The CCα was determined by calculating > 20 calibration curves and
273 determining the actual mean zero value of analyte and adding 2.33 standard deviations (SD)
274 of the intercept as given in the formula

275

276 $$CC\alpha = zero\ value_{Standard\ curve} + 2.33SD_{Intecept\ standard\ curve}$$

277

278 The CCα was also determined using the ISO 11843 procedure referred to in Commission
279 decision 2002/657/EC (ISO 11843 method) using the formula from Verdon et. al (E. Verdon,
280 Hurtaud-Pessel, & Sanders, 2006).

281

282
$$CC\alpha = t_{(\alpha,IJ-2)} \frac{\hat{\sigma}}{\hat{b}} \sqrt{\frac{1}{K} + \frac{1}{IJ} + \frac{\bar{x}^2}{\sum(x_{ij} - \bar{x})^2}}$$

283

284 where t_α is the Student's t-value at risk α and IJ-2 degrees of freedom, $\hat{\sigma}$ is the estimated
285 residual standard deviation of the regression function, $\hat{b}$ is the estimated slope of the
286 calibration curve, $\bar{x}$ is the mean of the x_{ij} values, K is the number of replicates of the real
287 state, I is the number of calibration levels and J is the number of replicates per level.

288 Detection capability (CCβ) was determined by analyzing > 20 matrix blanks spiked at
289 CCα and adding 1.64 SDs as specified in the formula

290

291
$$CC\beta = CC\alpha + 1.64\ SD_{[CC\alpha]}$$

292

293 Ion ratios of quantifier and qualifier in standards and samples were determined every day.

294 Recovery was evaluated by spiking matrix blanks at 1.0 x MRPL, 1.5 x MRPL and 2.0
295 x MRPL and calculating the recovery using the formula

296

297
$$Recovery\ (\%) = \frac{Measured\ content}{Fortification\ level} * 100\%$$

298

299 Since there were no certified reference materials available at the time of validation, the
300 trueness was evaluated by determining a proficiency test of shrimp containing SEM.
301 Following validation, the method has participated regularly in proficiency tests.

302 Precision, as repeatability, was determined by analyzing six replicates of salmon
303 muscle spiked at 1.0 x MRPL, 1.5 x MRPL and 2.0 x MRPL on three different days.
304 Intermediate precision as within-laboratory reproducibility was determined by analyzing two
305 replicates of each matrix spiked at 1.0 x MRPL, 1.5 x MRPL and 2.0 x MRPL on three
306 different days. Results from both the repeatability experiments and the intermediate precision
307 experiments were combined to calculate the within-laboratory reproducibility.

308 Measurement uncertainty (MU) was evaluated since this is an important requirement
309 of ISO 17025 and accreditation. MU was calculated using the relative standard deviation
310 (RSD) from the within-laboratory reproducibility using a coverage factor of two followed by
311 adding five percent points due to the limited traceability of the trueness, using the formula

312 $$MU\ (\%) = 2\ x\ RSD\ (\%) + 5\ (\%)$$

313

314 and finally rounding up to the nearest 5%.

315 Stability of the analytes in neat solutions and in matrix was determined by storing 10
316 standards at -20 ºC in the dark, 10 standards at +4 ºC in the dark, 10 standards at room
317 temperature in the dark and 10 standards at room temperature in daylight. In addition 5 blanks
318 of each matrix was spiked at 5 ng and stored at -20 ºC in the dark. One standard and one
319 spiked matrix blank from each storage condition was analyzed after one day, one week, two
320 weeks etc. until the validation study was finished after eight weeks.

321 Ruggedness/robustness was evaluated using different batches of reagents, solutions
322 and personnel during the eight weeks of the the validation study. Experience from later
323 routine use of the method, and participation in proficiency tests, allows for a more long term
324 evaluation of the robustness of the method.

325

326 **3. Results**

327 **3.1 Selectivity/specificity**

328 Full chromatographic separation of the four analytes was achieved with equivalent peak
329 shapes in sample and standards. A typical chromatogram of standard is shown in Figure 1.
330 Isotope labelled internal standards were used in the determination to ensure robust
331 determinations since they are known to equalize matrix effects and they make it possible to
332 unambiguously identify an analyte's retention time . Ion ratios were found to be quite similar
333 from day to day and all the ratios were within the maximum permitted tolerances fulfilling the
334 criteria for unequivocal identification of all the NF metabolites (Supplementary Table 1).

335 During validation no interfering signals were detected in the blank samples. Later routine
336 analysis revealed that *e.g.* scampi could contain possible false positive signals for NP-AHD
337 with the same quantifier and qualifier ion within the expected ratio-interval as the analyte
338 (Figure 2). However, the use of the mass labeled internal standard for AHD proved that the
339 retention time for these false positive signals were not due to AHD. The signals could
340 possibly be due to other compounds with similar structures. This demonstrates the importance
341 of using isotope labeled internal standards since retention times may vary from injection to
342 injection depending on the system stability and matrix load on the column. The use of isotope
343 labelled internal standards is particularly important when determining illegal substances such
344 as NF metabolites, or other compounds where false positives may lead to serious economic

345 damage for the producer. Preferably ^{13}C or ^{15}N labeled internal standards should be used since
346 they have more similar retention time to the unlabeled analyte compared with ^{2}H labeled
347 internal standards (see *e.g.* the chromatogram of NP-AMOZ in Figure 1). It is well known that
348 SEM can contain false positive signals due to the legitimate use of food packaging or food
349 additives, but to our knowledge, this is the first time a false positive for AHD is reported.

350

351 **3.2 Calibration curves and working range**

352 Linear working ranges for the NF metabolites spanned from CCα to the highest level in the
353 calibration curve (10 ng/g). Higher concentration levels were not anticipated to be necessary
354 since samples with a concentration of any NF metabolite above CCα would be in violation of
355 the legal limit. Calibration curves were calculated using linear regression analysis with 5-7
356 concentration levels by forcing the curve through origo and weighting each observation with
357 1/x. RSDs for the calibration curves were better than or equal to 12% for all analytes, and
358 correlation coefficients were better than 0.99 on all days (Supplementary Table 2).

359

360 **3.3 Decision limit (CCα)**

361 Results for CCα determined using the three methods outlined in section 2.7; sample blank
362 method, calibration curve method and ISO 11843 method are shown in Table 3. Using the
363 sample blank method, the CCα was found to be between 0.1 ng/g wet weight (w.w.) and up to
364 0.3 ng/g w.w. for AOZ in salmon. The calibration curve method gave CCα values in the range
365 between 0.2 ng/g w.w. for AHD in shrimp up to 0.5 ng/g w.w. for SEM in both salmon and
366 shrimp and AHD in salmon. The ISO 11843 method gave CCα values between 0.2 ng/g w.w.
367 and up to 0.8 ng/g w.w. for AOZ in shrimp. CCα was always found to be under the MRPL (1
368 ng/g w.w.) for all analyte-matrix combinations using all calculation methods. The three

369 methods used to determine CCα had small variations for most analyte-matrix combinations,
370 but the difference was larger for *e.g.* AOZ in shrimp where the difference was 0.6 ng/g w.w.
371 between the sample blank method (0.2 ng/g w.w.) and the ISO 11843 method (0.8 ng/g w.w.).
372 CCαs found using the calibration curve approach and the ISO 11843 method were always
373 higher than the CCαs found using the sample blank method. This could in part be due to the
374 very low background noise in modern MS/MS-instruments, resulting in very low S/N in
375 sample blanks. Since the instrument sensitivity will vary from day to day and from matrix to
376 matrix, the determination of CCα in every instrument sequence has been suggested (E.
377 Verdon, Hurtaud-Pessel, & Sanders, 2006). However, as our results show, CCα is also
378 dependent on the method used to calculate it. Nevertheless, all results for CCα for this method
379 will also fulfill the future criteria for reference points for action (RPA) proposed at 0.5 ng/g,
380 except when using the ISO 11843 method to calculate the CCα for AOZ in shrimp (0.8 ng/g
381 w.w.) (EC, 2019).

382

383 **3.4 Detection limit (CCβ)**

384 CCβ was determined following the determination of CCα by spiking 24 matrix blanks
385 of each matrix at CCα. Since the values of CCα was found to be both matrix, analyte and
386 calculation dependent, samples were spiked at a selected level in the ranges of the found
387 CCαs. CCβ results were then calculated for each matrix and analyte combination and the
388 results are shown in Table 3. CCβ was found to be between 0.3 ng/g w.w. for AHD in shrimp
389 and 0.9 ng/g w.w for AHD in cod.

390

391 **3.5 Recovery**

392 Recovery was evaluated by spiking experiments. Samples of salmon, shrimp and cod were
393 spiked at three levels and the results are shown in Supplementary Table 3. Recoveries for all
394 analytes were found to be in the ranges 89-101% for salmon, 93-115% for shrimp and 84-
395 112% for cod. The results were within the criteria of 50-120% for concentrations at or below
396 1 ng/g and mostly within 70-110% for concentrations above 1 ng/g and up to 10 ng/g. The
397 exceptions were 2 ng/g of AHD in shrimp (115%) and 2 ng/g AHD in cod (112%).
398 Nevertheless, such concentrations are well above the CCα and any detection at this
399 concentration would therefore be illegal.

400

401 **3.6 Trueness**

402 To evaluate the trueness of the method a proficiency test of shrimp (SEM consensus value
403 2.77 ng/g w.w.) was also analyzed during validation. We found 2.7 ng/g w.w. of SEM in this
404 sample which gave a z-score of 0.0. The method has participated in many proficiency tests
405 since it was first validated and the results have shown that other analyte-matrix combinations
406 also result in z-scores less than |2| (Figure 3). In 2016 we had an exceptional high value for
407 tissue bound AOZ in shrimp with a z-score +3.3. When this result was reported we had used
408 washing procedure 1 to report the amount of tissue bound NF metabolites. Due to our high z-
409 score in this proficiency test we decided to try to modify our washing procedure by adding a
410 stone for better mixing during shaking. At the same time we also tested a different washing
411 procedure 2 for comparison, published by the EURL for veterinary drugs (see 2.4 for details),
412 and also modified this washing procedure by adding the stone for better mixing. Following
413 the modification with the stone both washing procedures 1 and 2 produced satisfactory results
414 with z-scores of -1.4 for washing procedure 1 and -1.7 for washing procedure 2. However, we
415 noticed that our results were now on the lower side of the z-score scale, and we suspected this
416 to be due to the increased loss of sample/analyte due to adsorption to the stone. We therefore

417 compared the old washing procedure 1 and the new washing procedure 2 without adding the
418 stone (Supplementary Table 4 and 5). We found that the new washing procedure 2 was more
419 efficient in removing particularly SEM and AOZ, which could explain why we got the high z-
420 score for AOZ when using the old washing method 1. We therefore changed to the new
421 washing procedure 2 for future analyses when determining tissue bound NF metabolites.

422

423 **3.7 Precision as repeatability**

424 Repeatability (Supplementary Table 6) determined by analyzing salmon spiked at 1, 1.5 and 2
425 ng/g w.w using six replicates on three different days gave RSDs between 0.7% for AMOZ at
426 1 ng/g w.w. and 10% for AHD at 1 ng/g w.w.. Hence, repeatability of the method was within
427 the 12% criteria for half the value predicted for reproducibility by the Horwitz equation, and
428 well within the upper criteria of two thirds (15%) of the value predicted by the Horwitz
429 equation.

430

431 **3.8 Intermediate precision/within-laboratory reproducibility**

432 Intermediate precision for salmon was determined by combining the results from
433 repeatability testing (3.7) with two replicates on three different days, giving a larger dataset
434 spanning six days in total (Supplementary Table 7). For shrimp and cod two replicates were
435 analyzed on six different days. Intermediate precision RSDs ranged from 3.1% for 2 ng/g
436 w.w. SEM in shrimp up to 17% for 1 ng/g w.w. of both AHD in cod and AOZ in shrimp.
437 Hence, the within-laboratory reproducibility of the method was within the maximum
438 permitted tolerance of 23% predicted by the Horwitz equation.

439

440 **3.9 Measurement uncertainty (MU)**

441 A “worst case” approach was chosen for the estimation of measurement uncertainty by
442 selecting the highest within-laboratory reproducibility per matrix and using this RSD in the
443 calculation of MU as specified in section 2.7. Calculated values for MU are shown in
444 Supplementary Table 8. The within-laboratory reproducibility per matrix-analyte combination
445 was found to vary from 7.2% for SEM in cod to 17% for AHD in cod and AOZ in shrimp.
446 The associated calculated MU was estimated to vary between 20% for SEM in cod to 40% for
447 AHD in cod and AOZ in shrimp.

448

449 **3.10 Stability**

450 Stability of the standards were investigated as described in section 2.7. One of the samples
451 from each storage condition was worked up together with the other samples on every day of
452 the validation. Linear regression analysis of the results was performed following complete
453 validation after eight weeks and the results are shown in Supplementary Table 9. The analysis
454 revealed no significant negative trend (p-values > 0.05) during the validation period for the
455 analytes with the exception of AOZ stored in the dark at room temperature (p-value 0.05).
456 However, this significant result could be due to chance, since AOZ stored at both -20 °C and
457 +4 °C were not found to decrease significantly. The lack of significant trends should be
458 interpreted with caution since all analytes, except AHD (+0.01 ng/day) stored in the dark and
459 AMOZ in cod (0.00 ng/day), displayed negative correlation coefficients has high as -0.08
460 ng/day. The regression coefficients for SEM (average -0,05 ng/day) were significantly (p <
461 0.005) more negative than for the other NF metabolites (average -0.01 ng/day). This is
462 consistent with previous investigations where stock solutions of NF metabolites were found to
463 be stable for at least 10 months if stored at -4 °C, whereas more dilute solutions were found to

464 be stable for three months with the exception of SEM which was found to decompose at a rate
465 of 5% during 3.9 months in dilute solutions (Cooper & Kennedy, 2007; Radovnikovic,
466 Moloney, Byrne, & Danaher, 2011). Our decomposition rate for SEM was faster with an
467 overall average of 1% per day. We also found that regression coefficients for SEM in matrix
468 (average -0,08 ng/day) were significantly more negative ($p< 0.0005$) than regression
469 coefficients in solution (average -0.04 ng/day), so care should be taken when storing samples
470 with SEM.

471

472 **3.11 Ruggedness/robustness**

473 The method was found to be robust during the eight weeks of validation and has also proved
474 to be robust during routine use performed by different personnel, using different batches of
475 regents, solutions and standards over several years. The main factors, which may influence
476 the robustness, was found to be the preparation and storage of standards and the pH
477 adjustment.

478

479 **4. Conclusion**

480 The method was successfully validated and implemented for the determination of both tissue
481 bound NF metabolites and total (free + tissue bound) NF metabolite residues and has been
482 used in routine analysis for many years. Washing procedure 2 turned out to be the most
483 efficient in removing free metabolites when determining tissue bound NF metabolites,
484 particularly for SEM and AOZ. The two washing procedures we investigated differs to some
485 extent in type and amount of solvent, but the main difference appears to be the more thorough
486 homogenization, followed by centrifugation, for washing procedure 2 compared to the more
487 gentle mixing and decanting of washing procedure 1.

488 A new false positive for AHD is reported for the first time and an isotope labeled internal
489 standard is required for identifying this new false positive. This is because the false positive
490 has identical response in the mass spectrometer compared to AHD, but the slight shift in
491 retention time, compared to the isotope labeled internal standard, makes it possible to identify
492 this false positive.

493 The CCα of the method was satisfactory with respect to the MRPL using all the three methods
494 for its calculation. Small differences in CCα were found when using the different calculation
495 methods. Calculations using the sample blank method generally gave the lowest CCα of the
496 investigated calculation methods.

497 No statistically significant negative trends were seen from the stability investigations,
498 although most NF metabolites had negative correlation coefficients. SEM had the highest
499 negative correlation coefficient and decomposed at a rate of around 1% per day.

500 The analytical method was found fit for purpose to determine both bound and total NF
501 metabolites. The selection of validated matrixes, results from proficiency tests and routine use
502 over many years ensures that the method is horizontally applicable to seafood as a food
503 category.

504

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508

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