Investigation of pharmaceuticals in processed animal by-products by liquid chromatography coupled to high-resolution mass spectrometry.

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Abstract

There is an on-going trend for developing more sustainable salmon feed in which traditionally applied marine feed ingredients are replaced with alternatives. Processed animal products (PAPs) have been re-authorized as novel high quality protein ingredients in 2013. These PAPs may harbor undesirable substances such as pharmaceuticals and metabolites which are not previously associated with salmon farming, but might cause a potential risk for feed and food safety. To control these contaminants, an analytical strategy based on a generic extraction followed by ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) using quadrupole time-of-flight mass analyzer (QTOF MS) was applied for wide scope screening. Quality control samples, consisting of PAP commodities spiked at 0.02, 0.1 and 0.2 mg/kg with 150 analytes, were injected in every sample batch to verify the overall method performance. The methodology was applied to 19 commercially available PAP samples from six different types of matrices from the EU animal rendering industry. This strategy allows assessing possible emergent risk exposition of the salmon farming industry to 1005 undesirables, including pharmaceuticals, several dyes and relevant metabolites.

KEYWORDS: aquaculture, processed animal products, salmon, liquid chromatography, screening, undesirables, pharmaceuticals, dyes, quadrupole time-of-flight.

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INTRODUCTION

Estimated global production of farmed salmon (including *Salmo salar, Oncorhynchus kisutch, O. tshawytscha*) in 2010 was approximately 1.8 million metric tonnes with expected production of around 2.9 million metric tonnes in 2020 (Tacon et al., 2008). Furthermore, consumption of all species of farmed fish is expected to exceed that of feral fish (FAO, 2014). Traditional commercial feed for farmed salmon and rainbow trout are based on marine feed ingredients extracted from pelagic fish stocks. Concern of pressure on feral fish stocks and limited fish meal and fish oil availability to supply a rapidly growing aquaculture industry has led to the development of aquafeeds in which marine resources are replaced with alternative feed ingredients to develop more sustainable marine aquafeeds (Tacon et al., 2008; Torrisen et al., 2011; Waago et al., 2013). On a global basis, processed animal products (PAP) from the rendering industry constitute one of the largest sources of high quality animal protein available for animal feed production (Toldra et al., 2012). The use of PAPs such as feather meal, poultry by-product meal, pork meat and bone meal, and poultry and pork blood meal have been shown to be relevant nutritional replacements of fish meal for many cultured fish species including salmonids (Brandsen et al., 2001; Rosenlund et al., 2001; Yanik et al., 2003; Rahnema et al., 2007; Wilson et al., 2007; Friesen et al., 2008; Poppi et al., 2011; Burr et al., 2012). However, following the peak outbreaks of transmissible spongiform encephalopathies (TSE) in the UK in the early 1990's, the use of PAPs in all animal feed was banned in the European Union (EU) in 2001 (EC, 2001; EC, 2003a). Following a bovine spongiform encephalopathies (BSE) risk assessment by the European Food Safety Authorities (EFSA) (EFSA, 2011), the EU set out a working plan for the re-authorization of the use of non-ruminant PAPs in animal feed, initially for aquafeeds in 2013 (EC, 2013a).

In EU, the use of veterinary drugs is regulated according to EU legislation. Permitted residue levels of pharmaceutical substances (EC, 2009a) and mandatory monitoring activity (EC, 1996) have been established for all food producing animals. These regulations affect the legal addition of pharmaceuticals to animal feed, including the prohibition of substances in feeds that have hormonal or thyreostatic action as well as β-agonist (EC, 2003b). In addition, the supplementation of coccidiostats or histomonostats as feed additive causing unavoidable carry-over in non-target feed is regulated in the EU (EC, 2009b). In Norway, the use of pharmaceuticals in Atlantic salmon farming is under strict control and all sales of pharmaceuticals in animal farming have to be reported to the Norwegian Food Safety Authority. Open and reliable statistics on the consumption of therapeutic agents in aquaculture is only available from some nations. As an example, the registered use of pharmaceuticals in Norwegian aquaculture in 2014 included the antimicrobials florfenicol and oxolinic acid, the anti-parasitic agents azametifos, cypermetrin, deltametrin, diflubenzuron, teflubenzuron, emamectin, praziquantel and hydrogen peroxide, as well as the anaesthetic agents benzocaine, metacaine and isoeugenol (available at http://www.fhi.no/artikler/?id=114175) (Grave et al., 2008).

The use of PAPs in salmon feed can potentially introduce new chemical undesirables that have not been previously associated with farmed Atlantic salmon. Of special interest are pharmaceuticals such as antibacterials, which are used in all sectors of farming, including poultry and swine production (Toldra et al., 2012). Other pharmaceutical agents used in terrestrial farmed animals include antiparasitic agents such as coccidiostats, which are added to poultry feed to cope with protozoa as well as enhancing animal growth (Ruff, 1999; Chapman, 2014). It should be kept in mind that the number of pharmaceuticals used in terrestrial animals is more diverse than those used for fish, and that there are substantial differences in the prescribing patterns of

97 veterinary agents between countries and regions (EMA, 2011). Earlier screening studies
98 on feather meal from the USA (10 samples) and China (2 samples) for 59
99 pharmaceuticals, showed the presence of six classes of antimicrobials including
100 fluoroquinolones, tetracyclines, folic acid antagonists, and streptogramins (Love et al.,
101 2012). Two of the main antibacterials were enrofloxacin and ciprofloxacin, and studies
102 in EU also showed the occurrence of these substances in poultry and pork PAPs
103 (Berntssen et al., 2014). In addition to potentially direct adverse health effects of
104 pharmaceutical residues in food and non-compliance with food legislation, concern has
105 been raised for the development of resistant pathogenic microorganisms when exposed
106 to low non-clinical residue levels (Reig et al., 2009; Blazquez et al., 2012; Gillings et
107 al., 2013).

108 The list of prohibited and allowed antibiotics in the EU includes many substances which
109 may co-occur in samples of animal origin. This requires the use of comprehensive
110 screening methods for detection of these substances (Bohn et al., 2013; Nácher-Mestre,
111 et al., 2013; Masiá et al., 2014; Turnipseed et al., 2014; Boix et al., 2014). Therefore,
112 generic sample treatment is advisable to cover as many compounds as possible during
113 the experimental process. High resolution mass spectrometry (HRMS) allows the
114 acquisition of full-spectrum accurate-mass data using analyzers such as quadrupole
115 time-of-flight (QTOF). The coupling of liquid chromatography (LC) with QTOF MS is
116 nowadays one of the most efficient analytical tools to face the investigation of large
117 number of medium-high polar organic contaminants and residues in food-safety and
118 related fields (Ibañez et al., 2012). The present study reports a wide-scope qualitative
119 screening approach for 1005 permitted and prohibited pharmaceutical residues (also
120 including metabolites) and dyes in commercially available EU produced PAPs with
121 potential use in aquafeed.

MATERIAL AND METHODS

Reagents and chemicals. 150 reference standards were purchased from Acros Organics (Geel, Belgium), Aventis Pharma (Madrid, Spain), Bayer Hispania (Barcelona, Spain), Cerilliant (Round Rock, TX, USA), Fluka (Buchs, Switzerland), Dr. Ehrenstorfer (Augsburg, Germany), Fort Dodge Veterinaria (Gerona, Spain), National Measurement Institute (Pymble, Australia), Riedel-de Haën (Seelze, Germany), Sigma Aldrich (St Louis, MO, USA), Vetoquinol Industrial (Madrid, Spain), and Witega (Berlin, Germany). All reference materials had purities higher than 98% (w/w), except for marbofloxacin and pefloxacin, which had purities higher than 93%.

HPLC-grade water was obtained from a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). HPLC-grade methanol, HPLC-supragradient acetonitrile and acetone for residue analysis were purchased from Scharlab (Barcelona, Spain). Formic acid (HCOOH, content > 98%), ammonium acetate (NH_4Ac, reagent grade) and sodium hydroxide (NaOH, reagent grade) were supplied by Scharlab. Leucine enkephalin was purchased from Sigma Aldrich.

Samples. Commercially available PAPs were studied in this work. A total of 19 available non-ruminant PAPs from 6 different types of matrices were provided by the European Fat Processors and Renderers Association (EFPRA). The samples had been produced in different rendering factories. All PAPs were produced according to EU regulation for PAPs intended for use as feed ingredients (EC, 2001; EC, 2009c). All PAPs were “category 3 products” which are fit for human consumption at the point of slaughter as defined by EU-legislation (EC, 2009c). The PAPs obtained included six different matrices: poultry blood meal (n=4), poultry meal (n=4), feather meal (n=3), pork blood meal (n=3), pork meal (n=3) and pork greaves (n=2). Samples were stored at

-20°C until analysis (within one week). The PAPs included are all produced in central Europe and are firmly considered to be used for future aquafeeds after the lift of the ban on these products in the EU food supply chain.

Instrumentation. A Waters Acquity UHPLC system (Waters, Milford, MA, USA) was coupled to a quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), with a Z-spray-ESI interface, operating in both positive and negative ionization modes. An Acquity UHPLC BEH C18 1.7 µm particle size analytical column 2.1×100 mm (Waters) at a flow rate of 300 µL/min was employed for chromatographic separation. Mobile phase consisted of water/methanol gradient both with 0.01% HCOOH and 0.1mM NH_4Ac. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10 %; 14 min, 90 %; 16 min, 90 %; 16.01 min, 10 %; 18 min, 10 %. The column temperature was set at 40 ºC. Hybrid TOF MS resolution was approximately 20,000 at full width half maximum (FWHM), at *m/z* 556. MS data were acquired in the *m/z* range of 50-1000. A capillary voltage of 0.7 kV in positive mode and 2.5 kV in negative mode were used with a cone voltage of 20 V. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 650 ºC and the source temperature at 130 ºC.

For MS^E experiments (also known as all-ion fragmentation or broadband collision-induced dissociation by other manufacturers), two sequential acquisition functions were created: in the low energy function (LE), a collision energy of 4 eV is selected, obtaining a conventional full spectrum where intact (de)protonated molecules/adducts are commonly observed ; in the high energy function (HE), a linear collision energy ramp from 15 to 40 eV is applied in order to induce ion fragmentation. In this way, fragmentation information is obtained in advance for all compounds in a single run

without the need for re-injecting the sample in MS/MS mode. Scan time of 0.4 s was selected.

Calibrations were conducted daily from *m/z* 50 to 1000 with a 1:1 mixture of 0.05M NaOH:5% HCOOH diluted (1:25) with acetonitrile:water (80:20), at a flow rate of 20 µL/min. For automated accurate mass measurement (i.e. real-time calibration), a solution of leucine enkephalin (10 mg/L) in acetonitrile:water (50:50) at 0.1% HCOOH was used as lock mass and pumped at 30 µL/min through the lock-spray needle. The (de)protonated molecule of leucine enkephalin at *m/z* 556.2771 in positive mode and *m/z* 554.2615 in negative mode was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time. MS data were acquired in centroid mode and processed by the ChromaLynx XS application manager (within MassLynx v 4.1; Waters Corporation).

Recommended analytical procedure. A generic sample procedure has been expanded to PAPs following a screening strategy previously validated for aquaculture matrices (Nácher-Mestre et al., 2013) and feed samples (Boix et al., 2014). Briefly, PAPs were thawed at room temperature and 2.5 g of sample (dry weight) was accurately weighed (precision 0.01g) in centrifuge tubes (50 mL) and homogenized in a Vortex with 10 mL acetonitrile:water (80:20, v/v) 0.1% HCOOH. After shaking the samples for one hour, the tubes were placed in an ultrasonic bath during 15 minutes followed by centrifugation at 4500 rpm for 10 min. Then, an aliquot of 1 mL of supernatant extract was transferred to an eppendorf vial, diluted with 1 mL of HPLC-grade water and stored in a freezer (minimum 2 hours) to precipitate proteins. After that, the extract was again centrifuged at 12000 rpm for 10 min and supernatant was transferred to another vial.

196 Finally, 20 µL of the extract (final composition acetronitrile:water (40:60, v/v) 0.05%
197 HCOOH) was injected into the UHPLC-QTOF MS.

198

199 **Quality assurance.** Sample batches consisted of 20-30 vials, including 4 standards
200 (only for qualitative purposes) and quality control samples (QCs). The reliability of the
201 qualitative screening was assessed by the analysis of three QCs per matrix at different
202 concentration levels. To this aim, six different PAPs (each representing the matrices
203 studied, see samples section) were spiked with 150 pharmaceutical agents (including 4
204 dyes, see Sup. info for more details) at three different concentration levels (0.02, 0.1 and
205 0.2 mg/kg), and analyzed together with their respective "blanks". Additionally, two
206 method blanks were analyzed to assure that no laboratory contamination was introduced
207 in the procedure. This approach was carried out following the spirit of European
208 guidelines (EC, 2002; EC, 2013b).

209

210 **RESULTS AND DISCUSSION**

211 **UHPLC-QTOF MS screening.** Satisfactory chromatographic peaks, i.e. at least 10
212 data points per peak, for all 150 compounds were obtained. Formic acid (0.01 %) was
213 added to both water and methanol mobile phase solvents, favoring the formation of the
214 protonated molecule in positive ionization mode. The addition of ammonium acetate in
215 the mobile phase allowed improving peak shapes (Gracia-Lor et al., 2010). In general,
216 the best results in terms of sensitivity were obtained under positive ionization mode
217 selecting the protonated molecule as the main diagnostic ion.

218 With the previous chromatographic conditions, the screening was applied on the basis
219 of a compound database that is being continuously updated (Nácher-Mestre et al., 2013;
220 Boix et al., 2014). At this stage, new pharmaceuticals were included into our previous

lists in order to investigate their possible presence in PAP matrices of interest for salmon aquafeed. Individual standard solutions were injected to study MS fragmentation of each target analyte, achieving a final list of 150 analytes, which were also used for quality control of the qualitative screening. The selected compounds were representative from different families with quite different chemical-physical characteristics and were used to test the applicability of the screening. Briefly, 75% of the 150 compounds used in QC analysis were detected in all matrices at 0.2 mg/kg and 65% at 0.1 mg/kg, decreasing down to 30% at the lowest spiking level of 0.02 mg/kg. Many of the compounds that could not be detected in all spiked samples were however detected in some of them, sometimes even in 5 out of 6 samples. In those cases, we did not consider the screening as satisfactory at the concentration level tested, as the requirement was to have 100% of positive samples detected. This was mostly due to the high variability in matrix composition between the samples used for preparing QCs.

In addition to these 150 compounds, up to 855 compounds were searched in the samples making a total target list of 1005 compounds. The compound database used can be found in supporting information, including Retention time (Rt) (when the reference standard was available) and the elemental composition used for characterization. With so high number of compounds, it might be possible that some of them co-elute (for example, sulfaquinoxaline and sulfathiazole at 6.51 min). Under this situation, UHPLC was valuable for choosing almost co-eluting fragment ions that would correspond to the same precursor avoiding spectrum interferences that would complicate the identification process. Not only the retention time but also the chromatographic peak shape were taken into account to match each fragment ion to the correct “precursor ion”. Anyway, as a hybrid QTOF was used, additional MS/MS experiments could be performed, if necessary, in analysis of real-world samples for confirmation of positives in case of

doubt. For adequate retention time comparison, LC conditions used (LC column, mobile phase and gradient) should be the same than those used when creating the database.

Detection and identification criteria. Figure 1 illustrates the criteria used in this work. The parameters considered for a satisfactory detection and identification were Rt, mass accuracy (i.e. mass errors), q_i/Q ratios (Q: the most abundant ion –commonly the (de)protonated molecule; q_i: fragment ion –or occasionally an adduct) and the isotopic distribution pattern (especially for Cl and Br) (Hernández et al., 2015). Requirements and interpretation of results were in agreement with Commission Decision 2002/657/EC, which applies to the monitoring of certain substances and residues thereof in live animals and animal products (EC, 2002). In addition, SANCO guideline for pesticide residue analysis in food was of help in terms of mass accuracy requirements (EC, 2013b). The key parameters when testing the screening were detection and identification of the compound in the sample at the concentration levels tested. Neither recovery nor precision were calculated (contrarily to quantitative methods), which is in the line of the guidelines used nowadays in food safety or doping control analysis (EC, 2002; EC, 2013b; FDA, 2015; WADA 2010). Several situations were considered in the screening depending on the reference standard availability (Figure 1):

1) Reference standard available:

   - Detection was considered satisfactory when the most abundant ion (Q), commonly the (de)protonated molecule, was found at the expected Rt (deviation accepted ± 2.5% in comparison with the reference standard) (EC, 2002) and mass error was below 5 ppm (named as "d1") (EC, 2013b). Another likely situation for detection was to find two representative *m/z* ions (i.e. the most abundant ion (Q) and/or minor fragment/adduct ions (q)) for the target

compound at the expected Rt, but with mass errors between 5-20 ppm (named as "d2"). The latter situation seemed to occur when the signal intensity was low (favored at low analyte concentrations). In that case, an additional effort is recommended to investigate more accurate-mass ions and/or repeat sample injection, if possible.

- Identification was based on the presence of at least two representative *m/z* ions (Q, q) at the expected Rt (± 2.5%, in comparison with the reference standard) with mass errors below 5 ppm. Additionally, q/Q ratios should fit with those for reference standards within tolerance limits admitted which range from 20% to 50 % depending on the q/Q ratio (<0.1:50%, 0.1-0.2:30%, 0.2-0.5:25% and, >0.5%:20%) (EC, 2002). Identification under these conditions was highly reliable and it was considered as the ideal situation.

In both cases, the characteristic isotopic pattern (e.g. when ions as Cl or Br are present) should be observed.

2) Reference standard not available:

- Tentative identification could be made when an expected ion with mass error below 5 ppm was observed, together with its characteristic isotopic pattern. Subsequently, the fragment ions (q_i) (or characteristic isotopic ions) were evaluated. For this purpose, different possibilities are available, such as comparing experimental MS(/MS) spectra or the main fragment ions with those reported in the literature (massbank, METLIN public library), or justifying the accurate-mass fragments taking into account the structure of the molecule. So, tentative identification required the presence of one or more fragment ions (q) compatible with the chemical structure of the candidate (mass error < 5 ppm) and/or in agreement with previous data reported in literature. Retention time prediction may also help in this

process (focusing research only on "predicted" peaks) (Bade et al., 2015). Although tentative identification was strongly supported by accurate-mass data obtained, the confirmation of the identity requires the injection of the reference standard.

Screening of PAP samples. The HRMS screening developed allows research laboratories to apply a cost-effective strategy to extend their analytical scope to analytes which might potentially be present in the samples even without the need of having all reference standards (EC, 2013b). In this way, laboratories do not need to acquire all reference standards before analysis, with the subsequent problems of availability (e.g. metabolites and transformation products), costs and expiry dates. Accurate-mass full-spectrum acquisition capabilities of the TOF analyzer made feasible the investigation of many undesirables in a single run injection. In absence of standards, the only information available for data processing was the elemental composition (i.e. accurate-mass), but a tentative identification could be made if sufficient information was obtained from analyses (see Figure 1).

With the objective to support the reliability of the screening procedure, figure 2 shows a sample matrix (poultry meal) fortified with the antibiotic enrofloxacin. The standard of this fluoroquinolone was previously injected, selecting two representative *m/z* ions, the protonated molecule (Q) and one fragment ion (q) for the reliable identification (Figure 2-A). Figure 2-B shows the same *m/z* ions of a quality control sample at 0.1 mg/kg supporting the suitability of the method at this concentration level (mass deviations and q/Q were satisfactory). Finally, figure 2-C shows the identification of enrofloxacin in a PAP sample. The presence of two *m/z* ions, measured at the expected retention time, with acceptable mass deviations and appropriate q/Q ratios, allowed to identify this compound in that sample.

After applying the described screening strategy to 19 PAP samples, pharmaceutic agents like monensin, flumequine, enrofloxacin, trimethoprim, tylosin A, acetaminophen, salicylic acid, oxyphenylbutazone, and the dye leucocrystal violet (metabolite of the crystal violet dye) were found (Table 1).

Monensin is an antibiotic also allowed as a coccidiostat feed additive with an established maximum residue level (MRL is 1.25 mg/kg for feed materials) for unintended cross-contamination to non-target animal feed (EC, 2009b). The antibiotics flumequine, enrofloxacin, trimethoprim and tylosin A, are authorized pharmaceutical products in the EU with set MRLs in fish products (EC, 2009a); however in Norway, these pharmaceuticals have not been registered for aquaculture use for the last decade (Grave et al., 2008). The analgesics acetaminophen (paracetamol) is allowed to use for porcine, and salicylic acid is allowed to use for all food producing species except fin fish. Oxyphenylbutazone is a non-steroidal anti-inflammatory drug (NSAID) which is not listed as an authorized drug in the EC directive (EC, 2009b), and crystal violet is a potential carcinogenic pharmaceutical triphenylmethane dye, illegal for use in food-producing animals in the EU (Serratosa et al., 2006). As opposed to the other pharmaceuticals identified in the study, crystal violet is not allowed to be used in the food production chain in the EU, and its potential source in PAPs is hence unclear. However, crystal violet can be unintendedly introduced as marker dye to identify treated farm animals. Outside the EU it is used as an antimicrobial agent to prevent the fungal growth in animal feeds (Mani and Bharagava, 2016).

Figure 3 illustrates the identification of trimethoprim, flumequine and salicylic acid in different PAP samples analyzed. Figure 3-A corresponds to trimethoprim found in feather meal. Three representative *m/z* ions with mass deviations below 5 ppm were observed together with acceptable q/Q ratios. Figure 3-B corresponds to flumequine, also in feather meal, where two representative *m/z* ions were present with acceptable

347 mass errors and q/Q deviations (<25%). However, in this sample the fragment with *m/z*
348 174.036 was not clearly observed. Finally, figure 3-C shows the identification of
349 salicylic acid in pork blood meal with again two representative *m/z* ions. The fact that
350 salicylic acid is a small molecule made that few fragments were available under the
351 experimental conditions applied. Both salicylic acid and acetylsalicylic acid are allowed
352 to be used in all food producing species except fin fish or use in animals from which
353 milk or eggs are produced for human consumption (EC, 2009a). The presence of this
354 analgesic might be related to the previous administration of acetylsalicylic acid in
355 animals, a known pain reliever, anti-inflammatory and antipyretic drug, producing
356 salicylic acid as a metabolite. Besides this, salicylic acid is also used as an agent against
357 fungal diseases in the skin of animals. However, since salicylic acid is known to be
358 found in several plants (Venema et al., 1996; Scotter et al., 2007), it could also be
359 present in feed ingredients for livestock and subsequently in by-products from livestock
360 animals. Furthermore, salicylic acid has been previously detected in man and animals
361 not medicated by salicylic acid derived therapeutics (Paterson et al., 2008).

362 Figure 4 illustrates two situations where the compounds detected in poultry blood meal
363 could not be fully identified. Figure 4-A shows two *m/z* ions for leucocrystal violet but
364 the *m/z* 239.1548 (q_1), corresponding to the fragment $C_{16}H_{18}N_2$, presented mass error
365 higher than 5 ppm and further study of other fragment ions failed. In addition, q_1/Q ratio
366 was higher than 20% (tolerance permitted for q/Q>0.5). Therefore, the identification
367 criteria were not met, and we considered this compound as detected but not identified
368 (d2 following criteria from Figure 1). A similar situation occurred with tylosin A
369 (Figure 4-B) for which three representative ions were found at the same Rt, in
370 agreement with the standard. However, it was reported only as detected (d2) because all
371 the ions presented high mass errors. This might be due to the high mass of this
372 compound, very close to the higher end of the mass axis calibration range. Although it

seemed that both compounds were present in the sample, they could not be identified in a reliable way; therefore, further analysis should be carried out for confirmation of their identity.

The data processed also revealed the suspicious presence of ciprofloxacin and dyes such as crystal violet, malachite green and leucomalachite green in PAPs, but with such a low intensity that a second target analysis with a higher sensitivity technique (e.g. LC-MS/MS with triple quadrupole) would be necessary. Thus, further work is required for those compounds identified (or tentatively identified) by this qualitative screening in order to confirm their presence and determine the concentration levels in the samples. This could be ideally made by LC-MS/MS using target quantitative methods developed for the compounds revealed by QTOF MS screening.

In conclusion, the LC-HRMS screening methodology developed in this work has revealed the presence of some pharmaceutical agents and marker dyes in re-authorized PAPs that might be introduced in novel salmon feed. The qualitative validation was performed for 150 compounds, and it was satisfactory for a notable number of analytes. Most of compounds that could not be detected in 100% of the spiked samples were however detected in several of them (in some cases up to 5 out of 6 samples), illustrating that the screening was satisfactory in nearly all matrices tested. The lack of sensitivity seemed to be the main reason for the non-detections, a drawback that can be solved with the last-generation HRMS instruments that offer much better sensitivity that the QTOF MS used in the present work.

Although the screening was tested for a limited number of compounds, it might be assumed to be applicable to the wide majority of the remaining compounds included in the database, as the contaminants used for validation covered a broad range of physicochemical properties. Obviously, the only way to ensure a completely reliable

screening would be performing a full validation for all compounds, a task that seems extremely complicated when dealing with hundreds of analytes. Yet, in the present work, one can ensure the positive findings owing to the power of accurate-mass full spectrum acquisitions in HRMS, reaching reliable tentative identifications even when the standard is not available. Nevertheless, these tentative identified compounds should be confirmed by acquiring the reference standard in a subsequent step. A major advantage of this approach is that laboratories do not need to purchase hundreds of standards for compounds that in many cases will never be found in the samples. In fact, standards only need to be purchased in a subsequent step on the basis of the tentative identifications in the samples. However, this type of screening has the drawback that false negative results may occur for the compounds that have not been subjected to validation. The proposed strategy is of interest for food safety and public health control authorities as the presence of unintended background levels of illegal pharmaceutical dyes, unreported antibiotics, or unauthorized anti-inflammatory agents in salmon feed ingredients might give cause to legal actions. The approach presented in this paper can be considered as a useful risk assessment tool for feed industry in order to widen the knowledge on novel ingredients and also traditional ingredients under use. Also of interest is the fact that accurate-mass full-spectrum acquisition allows widening the search to other compounds not included in this work. This can be done at any time in the future by retrospective evaluation of data obtained without additional analysis. In the near future, routine monitoring and quantification of the compounds detected will be carried out due to the potential implications in food safety.

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SUPPORTING INFORMATION

The compound database used in this work can be found in supporting information, including Retention time (Rt) (when the reference standard was available) and the molecular composition used for characterization.

REFERENCES

Bade, R., Bijlsma, L., Miller, T.H., Barron, L.P., Sancho, J.V., Hernández, F. 2015. Suspect screening of large numbers of emerging contaminants in environmental waters using artificial neural networks for chromatographic retention time prediction and high resolution mass spectrometry data analysis. Sci. Tot. Environ. 538, 934-941.

Berntssen, M.H.G., Valdersnes, S., Lunestad, B.T., Hatlen, B., Alm, M., Waagbo, R., Buttle, L. 2014. Residue levels of enrofloxacin and ciprofloxacin in processed animal by-products used in Atlantic salmon feeds and their long-term carry-over to the edible part of the fish. Aquac. Nutr. 20, 712-721.

447 Blazquez, J., Couce, A., Rodriguez-Beltran, J., Rodriguez-Rojas, A. 2012.
448 Antimicrobials as promoters of genetic variation. Curr. Opin. Microbiol. 15, 561-
449 569.

450 Bohn, T., Pellet, T., Boscher, A., Hoffmann, L. 2013. Developing a microbiological
451 growth inhibition screening assay for the detection of 27 veterinary drugs from 13
452 different classes in animal feedingstuffs. Food Addit. Contam. Part A-Chem. 30,
453 1870-1887.

454 Boix, C., Ibáñez, M., Sancho, J.V., León, N., Yusá, V., Hernández, F. 2014. Qualitative
455 screening of 116 veterinary drugs in feed by liquid chromatography-high resolution
456 mass spectrometry: Potential application to quantitative analysis. Food Chem. 160,
457 313-320.

458 Bransden, M.P., Carter, C.G., Nowak, B.F. 2001. Effects of dietary protein source on
459 growth, immune function, blood chemistry and disease resistance of Atlantic salmon
460 (Salmo salar L.) parr. Anim. Sci. 73,105-113.

461 Burr, G.S., Wolters, W.R., Barrows, F.T., Hardy, R.W. 2012. Replacing fishmeal with
462 blends of alternative proteins on growth performance of rainbow trout
463 (Oncorhynchus mykiss), and early or late stage juvenile Atlantic salmon (Salmo
464 salar). Aquaculture 334, 110-116.

465 Chapman, H.D. 2014. Milestones in avian coccidiosis research: A review. Poult. Sci.
466 93, 501-511.

467 EC, 1996. Council Directive 96/23/EC of 29 April 1996 on measures to monitor
468 substances and residues thereof of in live animals and animal products and repealing
469 Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and
470 91/664/EEC. Official Journal of the European Communities L125, 10.

471 EC, 2001. Regulation (EC) Nº 999/2001 of the european parliament and of the council
472 of 22 May 2001 laying down rules for the prevention, control and eradication of
473 certain transmissible spongiform encephalopathies. Official Journal of the European
474 Union L 147, 1-38.

475 EC, 2002. Commission Decision (EC) Nº 2002/657 implementing Council Directive
476 96/23/EC concerning the performance of analytical methods and the interpretation of
477 results. Official Journal of the European Communities L221, 8-36.

478 EC, 2003a. Commission regulation 1234/2003 of 10 July 2003 amending Annexes I, IV
479 and XI to Regulation (EC) Nº 999/2001 of the European Parliament and of the
480 Council and Regulation (EC) Nº 1326/2001 as regards transmissible spongiform
481 encephalopathies and animal feeding. Official Journal of the European Union L 173,
482 6-13.

483 EC, 2003b. Regulation (EC) Nº1831/2003 of the European Parliament and of the
484 Council of 22 September 2003 on additives for use in animal nutrition Commission
485 directive 2009/8/EC.

486 EC, 2009a. Commission regulation (EU) No 37/2010 of 22 December 2009 on
487 pharmacologically active substances and their classification regarding maximum
488 residue limits in foodstuffs of animal origin. Official Journal of the European Union
489 L 15, 1-72.

490 EC, 2009b. Commission Directive 2009/8/EC of 10 February 2009 amending Annex I
491 to Directive 2002/32/EC of the European Parliament and of the Council as regards
492 maximum levels of unavoidable carry-over of coccidiostats or histomonostats in
493 nontarget feed. Official Journal of the European Union L 40, 19.

494 EC, 2009c. Regulation (EC) N° 1069/2009 of the European Parliament and of the
495 Council of 21 October 2009 laying down health rules as regards animal by-products

496 and derived products not intended for human consumption and repealing Regulation
497 (EC) Nº 1774/2002 (Animal by-products Regulation).

498 EC, 2013a. Commission regulation (EU) Nº 56/2013 of 16 January 2013 amending
499 Annexes I and IV to Regulation (EC) Nº 999/2001 of the European Parliament and of
500 the Council laying down rules for the prevention, control and eradication of certain
501 transmissible spongiform encephalopathies. Official Journal of the European Union
502 21, 3-16.

503 EC, 2013b. SANCO/12571/2013. Guidance document on analytical quality control and
504 validation procedures for pesticide residues analysis in food and feed.

505 EFSA, 2011. Scientific Opinion on the revision of the quantitative risk assessment
506 (QRA) of the BSE risk posed by processed animal proteins (PAPs). EFSA Panel on
507 Biological Hazards (BIOHAZ). EFSA Journal 9, 1947.

508 EMA, 2011. Trends in the sales of veterinary antimicrobial agents in nine European
509 countries Reporting period: 2005-2009. (EMA/238630/2011),
510 http://www.ema.europa.eu/docs/en_GB/document_library/Report/2011/2009/WC500
511 112309.pdf.

512 FAO, 2014. The State of World Fisheries and Aquaculture 2014. FAO Fisheries and
513 Aquaculture Department Food and Agriculture Organization of the United Nations,
514 Rome (2014). http://www.fao.org/3/a-i3720e.pdf.

515 FDA 2015, FVM Executive Council 2015. Foods and Veterinary Medicine Science and
516 Research Steering Committee. Guidelines for the Validation of Chemical Methods
517 for the FDA FVM Program. 2nd Edition. US Food & Drug Administration, Office of
518 Food and Veterinary Medicine.

519 Friesen, E.N., Ikonomou, M.G., Higgs, D.A., Ang, K.P., Dubetz, C., 2008. Use of
520 terrestrial based lipids in aquaculture feeds and the effects on flesh organohalogen

521 and fatty acid concentrations in farmed Atlantic salmon. Environ. Sci. Technol. 42,
522 3519-3523.

523 Gillings, M.R., 2013. Evolutionary consequences of antibiotic use for the resistome,
524 mobilome, and microbial pangenome. Front. Microbiol. 4, 1-19.

525 Gracia-Lor, E., Sancho, J.V., Hernández, F. 2010. Simultaneous determination of
526 acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure
527 liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1217, 622-632.

528 Grave, K., Hansen, M.K., Kruse, H., Bangen, M., Kristoffersen, A.B., 2008.
529 Prescription of antimicrobial drugs in Norwegian aquaculture with an emphasis on
530 "new" fish species. Prev. Vet. Med. 83, 156-169.

531 Hernández, F., Ibáñez, M., Botero-Coy, A.M., Bade, R., Bustos-López, M.C., Rincón,
532 J., Moncayo, A., Bijlsma, L., 2015. LC-QTOF MS screening of more than 1,000 licit
533 and illicit drugs and their metabolites in wastewater and surface waters from the area
534 of Bogotá, Colombia. Anal. Bioanal. Chem. 407, 6405-6416.

535 Ibáñez, M., Portolés, T., Rúbies, T., Muñoz, E., Muñoz, G., Pineda, L., Serrahima, E.,
536 Sancho, J.V., Centrich, F., Hernández, F., 2012. The power of hyphenated
537 chromatography/time-of-flight mass spectrometry in public health laboratories. J.
538 Agric. Food Chem. 60, 5311-5323.

539 Love, D.C., Halden, R.U., Davis, M.F., Nachman, K.E., 2012. Feather meal: A
540 previously unrecognized route for reentry into the food supply of multiple
541 pharmaceuticals and personal care products (PPCPs). Environ. Sci. Technol. 46,
542 3795-3802.

543 Mani, S., Bharagava, R.N., 2016. Exposure to Crystal Violet, Its Toxic, Genotoxic and
544 Carcinogenic Effects on Environment and Its Degradation and Detoxification for
545 Environmental Safety. Rev. Environ. Contam. T. 237, 71-104.

546 Masiá, A., Campo, J., Blasco, C., Picó, Y., 2014. Ultra-high performance liquid
547 chromatography-quadrupole time-of-flight mass spectrometry to identify
548 contaminants in water: An insight on environmental forensics. J. Chromatogr. A
549 1345, 86-97.

550 Nácher-Mestre, J., Ibáñez, M., Serrano, R., Pérez-Sánchez, J., Hernández, F., 2013.
551 Qualitative screening of undesirable compounds from feeds to fish by liquid
552 chromatography coupled to mass spectrometry. J. Agr. Food Chem. 61, 2077-2087.

553 Paterson, J.R., Baxter, G., Dreyer, J.S., Halket, J.M., Flynn, R., Lawrence, J.R., 2008.
554 Salicylic acid sans aspirin in animals and man: Persistence in fasting and
555 biosynthesis from benzoic acid. J. Agric. Food. Chem. 56, 11648-11652.

556 Poppi, D.A., Quinton, V.M., Hua, K., Bureau, D.P., 2011. Development of a test diet
557 for assessing the bioavailability of arginine in feather meal fed to rainbow trout
558 (Oncorhynchus mykiss). Aquaculture 314, 100-109.

559 Rahnema, S., Borton, R., 2007. Determination of the effects of fish vs plant and feather
560 meal-based diets on the growth and health of rainbow trout. J. Appl. Anim. Res. 32,
561 113-117.

562 Reig, M., Toldra. F. Veterinary Drugs and Growth Promoters Residues in Meat and
563 Processed Meats. Springer, 2009, 233 Spring Street, New York, Ny 10013, United
564 States.

565 Rosenlund, G., Obach, A., Sandberg, M.G., Standal, H., Tveit, K., 2001. Effect of
566 alternative lipid sources on long-term growth performance and quality of Atlantic
567 salmon (Salmo salar L.). Aquac. Res. 32, 323-328.

568 Ruff, M.D., 1999. Important parasites in poultry production systems. Vet. Parasitol. 84,
569 337-347.

570 Scotter, M.J., Roberts, D.P.T., Wilson, L.A., Howard, F.A.C., Davis, J., Mansell, N.,
571 2007. Free salicylic acid and acetyl salicylic acid content of foods using gas
572 chromatography–mass spectrometry. Food Chem. 105, 273-279.

573 Serratosa, J., Blass, A., Rigau, B., Mongrell, B., Rigau, T., Tortades, M., Tolosa, E.,
574 Aguilar, C., Ribo, O., Balague, J., 2006. Residues from veterinary medicinal
575 products, growth promoters and performance enhancers in food-producing animals:
576 A European Union perspective. J. Rev. Sci. Tech. Off Int. Epizoot. 25, 637-653.

577 Tacon, A.G J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in
578 industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285,
579 146-158.

580 Toldra, F., Aristoy, M.C., Mora, L., Reig, M., 2012. Nutritional pork meat compounds
581 as affected by ham dry-curing. Meat Sci. 92, 290-296.

582 Torrissen, O., Olsen, R.E., Toresen, R., Hemre, G.I., Tacon, A.G.J., Asche, F., Hardy,
583 R.W., Lall, S., 2011. Atlantic Salmon (Salmo salar): The “Super-Chicken” of the
584 Sea?. Rev. Fish Sci. 19, 257-278.

585 Turnipseed, S.B., Lohne, J.J., Storey, J.M., Andersen, W.C., Young, S.L., Carr, J.R.,
586 Madson, M.R., 2014. Challenges in implementing a screening method for veterinary
587 drugs in milk using liquid chromatography quadrupole time-of-flight mass
588 spectrometry. J. Agr. Food Chem. 62, 3660-3674.

589 Venema, D.P., Hollman, P.C.H., Janssen , K.P.L.T.M., Katan, M.B., 1996.
590 Determination of acetylsalicylic acid and salicylic acid in foods, using HPLC with
591 fluorescence detection. J. Agric. Food Chem. 44, 1762-1767.

592 Waagbo, R., Berntssen, M.H.G., Danielsen, T., Helberg, H., Kleppa, A.L., Lea, T.B.,
593 Rosenlund, G., Tvenning, L., Susort, S., Vikesa, V., Breck, O., 2013. Feeding
594 Atlantic salmon diets with plant ingredients during the seawater phase - a full-scale

net production of marine protein with focus on biological performance, welfare, product quality and safety. Aquac. Nutr. 19, 598-618.

WADA - World Anti-Doping Agency. International Standard for Laboratories. Identification criteria for qualitative assays TD2010IDCR.

Wilson, C.M., Friesen, E.N., Higgs, D.A., Farrell, A.P., 2007. The effect of dietary lipid and protein source on the swimming performance, recovery ability and oxygen consumption of Atlantic salmon (*Salmo salar*). Aquaculture 273, 687-699.

Yanik, T., Dabrowski, K., Bai, S.C., 2003. Replacing fish meal in rainbow trout (Oncorhynchus mykiss) diets. Isr. J. Aquac-Bamidgeh 55, 179-186.

Figure captions.

Figure 1. Scheme of detection and identification criteria for HRMS screening used in this work.

Figure 2. nw-XICs for enrofloxacin in: (A) standard in solvent at 250 ng/mL; (B) quality control of poultry meal A at 0.1 mg/kg; (C) poultry meal C sample. In each case, chromatograms for the LE function (bottom) and HE (top) are shown to illustrate the presence of the protonated molecule (LE) and fragment ions (HE). ✓ Accurate mass deviations within tolerance limits (below 5 ppm). ✓ q/Q ratio tolerance accepted by Com. Decision 2002/657/EC.

Figure 3. nw-XICs illustrating the identification of: (A) trimethoprim in feather meal; (B) flumequine in feather meal; (C) salicylic acid in pork blood meal. ✓ Accurate mass deviations within tolerance limits (below 5 ppm). ✓ q_i/Q ratio tolerance accepted by Com. Decision 2002/657/EC.

Figure 4. nw-XICs illustrating the detection of: (A) leucocrystal violet in poultry blood meal A; (B) tylosin A in poultry blood meal. ✓/× Accurate mass deviations within/without tolerance limits. ✓/× q_i/Q ratio tolerance accepted/not permitted by Com. Decision 2002/657/EC.

637 **Table 1. Compounds detected and identified from the LC-HRMS screening.**

Pharmaceuticals & Dyes	Poultry blood meal A	Poultry blood meal B	Poultry blood meal C	Poultry blood meal D	Poultry meal A	Poultry meal B	Poultry meal C	Poultry meal D	Feather meal A	Feather meal B	Feather meal C	Pork blood meal A	Pork blood meal B	Pork blood meal C	Pork meal A	Pork meal B	Pork meal C	Pork greaves A	Pork greaves B
Acetaminophen	-	-	-	-	-	d1	-	d1	-	-	-	-	-	-	-	-	-	d1	-
Enrofloxacin	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	-	-	-	-
Flumequine	-	-	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	-	-
Leucocrystal violet	d2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Monensin	-	-	-	-	-	-	d1	-	-	-	-	-	-	-	-	-	-	-	-
Oxyphenylbutazone	-	d1	-	-	-	-	-	-	-	-	-	-	-	d1	-	-	-	-	-
Salicylic acid	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	d1	d1	d1	d1	d1
Trimethoprim	-	-	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	-	-
Tylosin A	-	-	-	d2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

638 d: detected (d1 and d2 are defined in Figure 1); ✓: identified.

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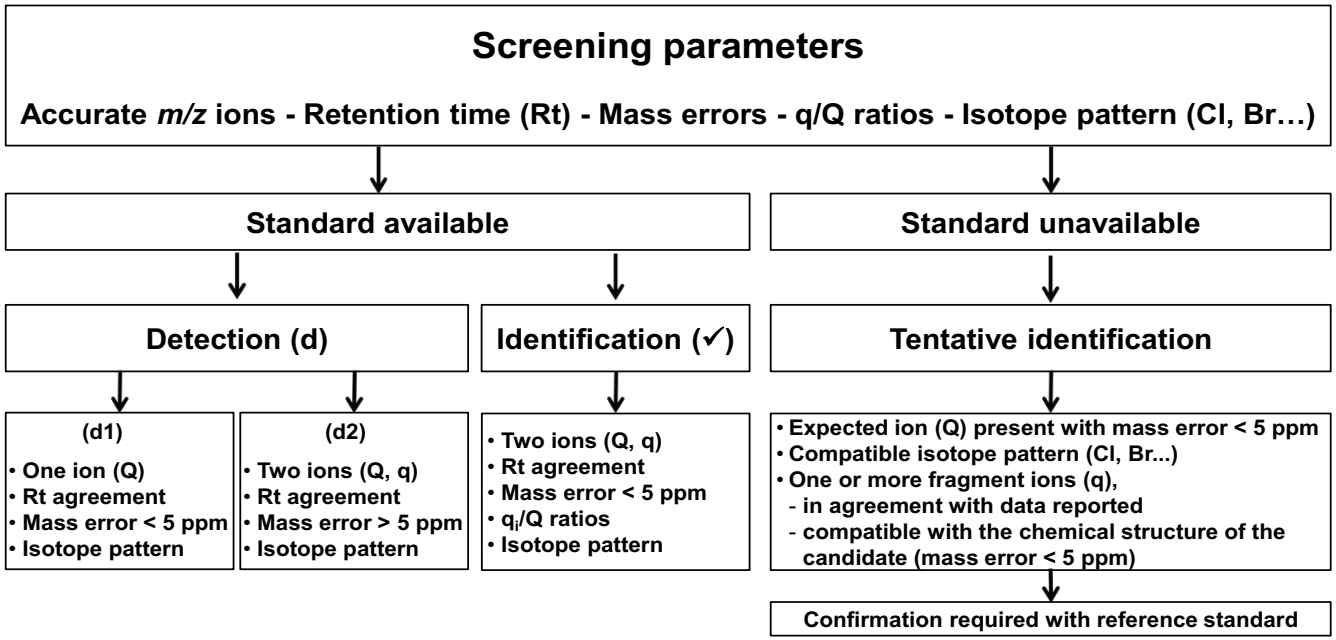

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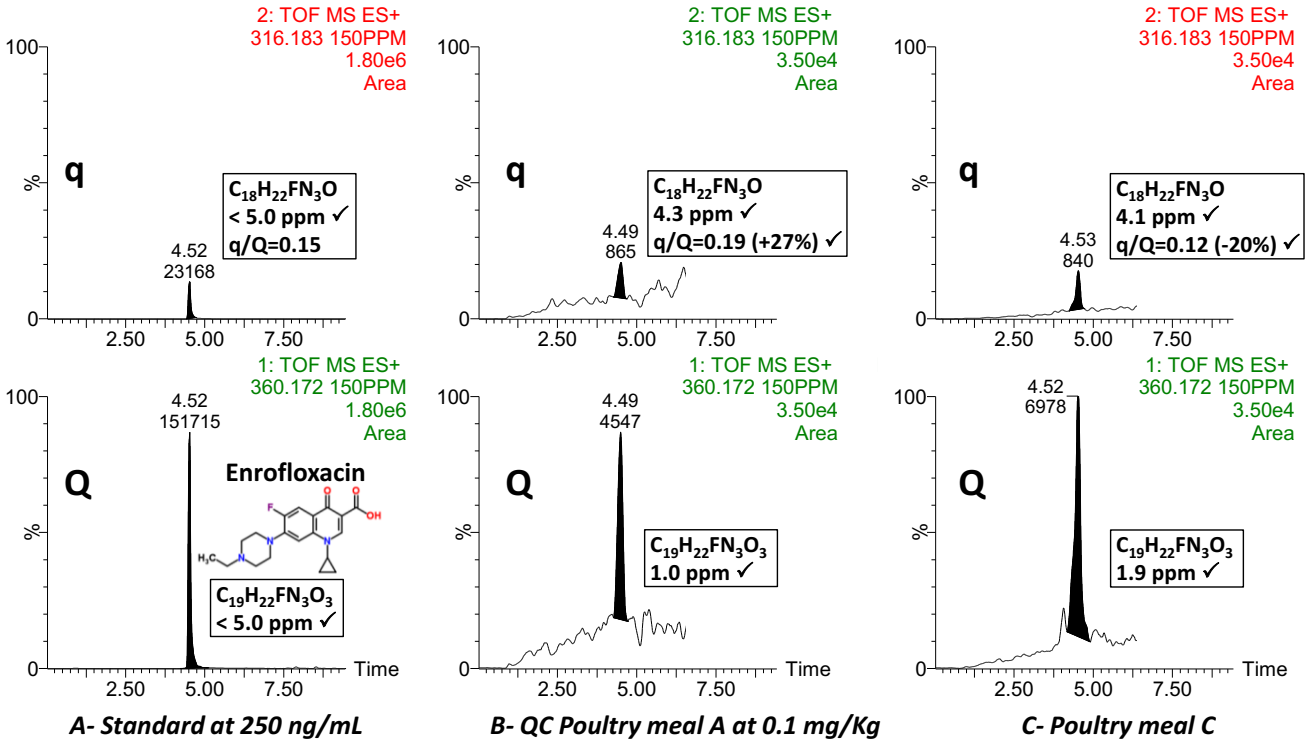
646 **Figure 1**.



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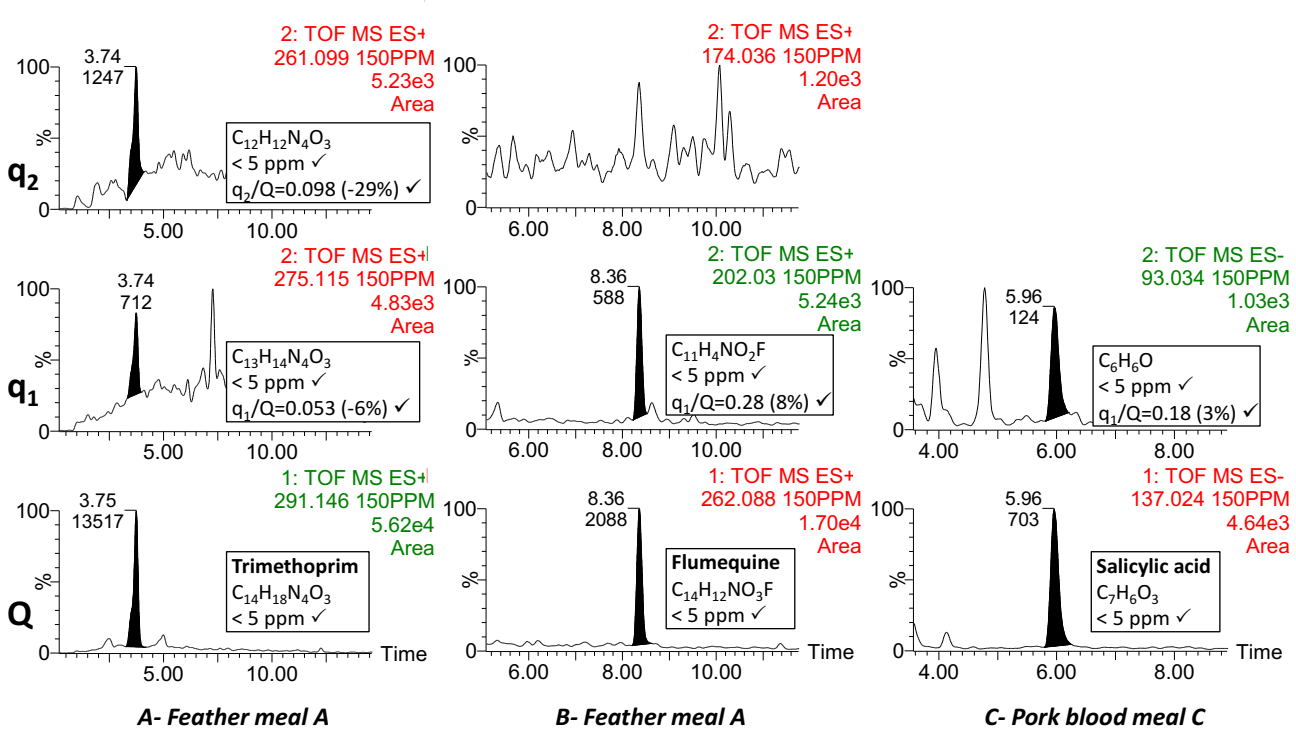

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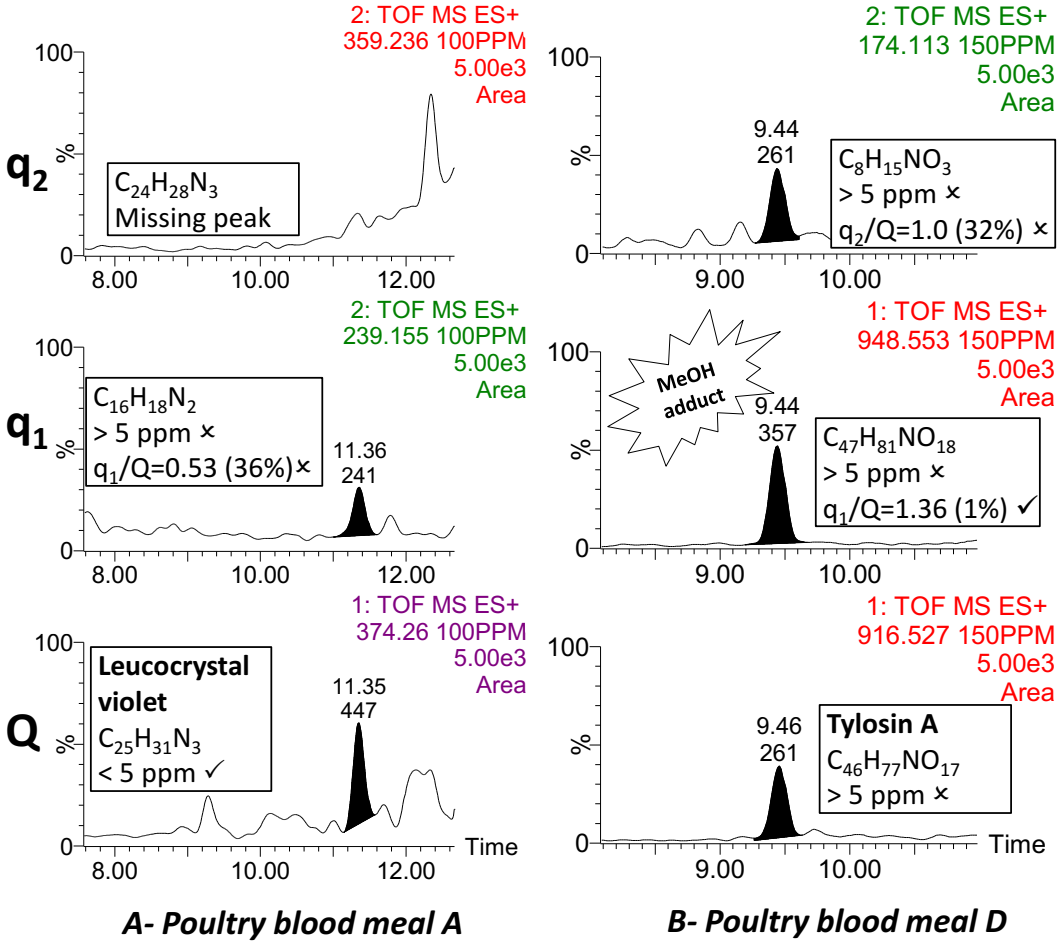
650 **Figure 3**.



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652 **Figure 4**.