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A mass spectrometry method for sensitive, specific and simultaneous detection of bovine blood meal, blood products and milk products in compound feed

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ABSTRACT

Feed sustainability is one of the biggest challenges for the next few years. Solutions have to be found that take feed quality and safety into account. Animal by-products are one valuable source of proteins. However, since the bovine spongiform encephalopathy (BSE) crisis, their use has been strictly regulated. The objective of this study was to propose a routine, sensitive and specific method using ultra-high performance liquid chromatography coupled to tandem mass spectrometry for the detection of blood-derived products and milk powder in feed. Contaminated aquafeeds were analysed in order to evaluate the sensitivity and specificity of the method. This new method meets both selectivity and sensitivity (0.1% (w/w)) requirements imposed by the European Commission for animal proteins detection methods. It offers an innovative and complementary solution for the simultaneously identification of authorised and unauthorised animal by-products such as processed animal proteins (PAPs).

1. Introduction

Animal by-products represent a source of high-quality proteins available for animal feed production (Pinotti & Dell'Orto, 2011). In the mid 90's, their use was banned following the bovine spongiform encephalopathy ("mad cow disease") crisis. The prohibition of the use of animal proteins in animal feed depended on three factors: by-product type (tissue origin), taxonomic origin and final destination use (pets, fur animals or other farmed animals). Regulation (EC) No. 999/2001 (European Union, 2001) describes this prohibition. Since then, prevention and control plans have raised the possibility of a gradual lifting of the feed ban. In 2013, the first step was taken with the reauthorisation of non-ruminant processed animal proteins (PAPs) in fish feed. For other species (terrestrial animals), the ban is still in force. Compared to other alternative sources, PAPs represent good resources for fish feed which do not require the production of any new ingredients, as PAPs and animal fat are by-products of the meat-processing industry. They are produced from materials that are fit for human consumption at the point of slaughter but do not enter the human food chain for commercial reasons (European Fat Processors, 2016).

In order to ensure feed safety, analytical approaches are required to monitor fraudulent inclusion of unauthorised PAPs in livestock feed. For fish feed, the main objective is to prevent the use of ruminant PAPs. Official control of aquafeed is based on two analytical methods: light microscopy (LM) and polymerase chain reaction (PCR). LM is able to distinguish between terrestrial and fish PAPs based on the detection and identification of particles (e.g. bones, scales). This provides information on the by-product type, but the taxonomic origin of these particles cannot be determined. On the other hand, PCR requires amplification of DNA targets to allow their detection (Fumière et al., 2009; Liu et al., 2011; Plouvier et al., 2012). The European Union Reference Laboratory for Animal Proteins in feedingstuffs (EURL-AP) has developed and validated two PCR assays for the detection of ruminant (Fumière, Marien, & Berben, 2016) and pig DNA (Fumière, Marien, Maljean, & Berben,

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2016). Other targets could be added in the future in the framework of the lifting of the feed ban. PCR enables the detection of animal products and the determination of their taxonomic origin. However, this information cannot be linked to a by-product type, as the result merely gives a global overview of the sample DNA content. These methods must be applied in accordance with operational schemes described in the EURL-AP Standard Operating Procedure (European Union Reference Laboratory for Animal Proteins in feedingstuffs, 2013). In most cases, a combination of these methods makes it possible to confirm the absence of unauthorised by-products, but in some cases it is not possible to distinguish between authorised by-products (e.g. milk products) and unauthorised ones (e.g. bovine blood meal). A typical example is that of, firstly, an aquafeed containing authorised porcine PAP together with authorised milk products, and secondly, an aquafeed containing authorised porcine blood meal together with unauthorised bovine PAP: their analyses will give the same results. There is therefore a crucial need for a complementary method to address this analytical gap.

Blood meal and haemoglobin powder of porcine origin is one of the animal by-products used in aquafeed. Since the lifting of the feed ban, routine analyses have revealed that some aquafeeds containing blood-derived products give positive results in PCR for ruminant targets. One of the arguments of the feed sector to explain these unexpected results is that milk protein can be used as a carrier for feed additives. The use of immunoassay methods for the detection of milk proteins was initially investigated using immunological kits developed for allergen detection in food (Dumont et al., 2010), as the absence of milk may indeed suggest that the ruminant DNA source is unauthorised. However, as this is an indirect approach, in case of a positive result the presence of unauthorised products cannot be excluded.

Mass spectrometry (MS) is the method of choice to fill in this analytical gap as it provides simultaneous information about the tissue and species of origin (Rasinger et al., 2016). In a previous study, specific bovine blood biomarkers (Lecrenier et al., 2016) were identified by high resolution MS. However, the extraction method was quite lengthy and the MS analysis was not adapted for sensitive feed monitoring. Besides that, a ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) method has been developed for the detection of milk allergens in food (Planque et al., 2016). This method has been developed by some authors of this study. The sample preparation protocol was designed to make this method simple and fast. Moreover, the instrument used, combining UHPLC and a triple quadrupole mass spectrometer, is a widely available instrument in feed testing laboratories (Grundy et al., 2007).

The purpose of this study was to merge these two approaches in order to develop a multi-target UHPLC-MS/MS method for the simultaneous detection of multiple by-products (bovine blood-derived products and milk) in feed. The haemoglobin biomarkers previously identified (Lecrenier et al., 2016; Planque et al., 2016) were evaluated. A porcine haemoglobin biomarker was also added to the study in order to check the presence of porcine blood in commercial feed known to contain this type of authorised by-product. Concatenated labelled peptides were designed and used as internal standards so that the results could be compared independently of the retention time variation due to the matrices effect. This method was tested on various aquafeeds in order to assess the robustness and the applicability of the method in feed control and evaluate whether it could reach the detection limit imposed by the European Commission (EC) for analytical methods.

2. Materials and methods

2.1. Materials and reagents

Urea, ammonium bicarbonate, tris(hydroxymethyl)aminomethane (TRIS), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), iodoacetamide (IAA), and trypsin from bovine pancreas (T8802) were purchased from Sigma-Aldrich (Bornem, Belgium). Acetonitrile (UHPLC–MS/MS grade), formic acid and water (ULC/MS grade) were purchased from Biosolve (Valkenswaard, the Netherlands). Acetic acid was obtained from Acros Organics (Geel, Belgium) and hydrochloric acid was purchased from Fisher Chemical (Loughborough, UK). Clean-up was performed with sep-pak tC18 solid phase extraction (SPE) columns (WAT036790, 6cc, 500 mg) from Waters (Milford, Massachusetts, USA).

2.2. Sample preparation

The samples used were provided by several producers, distributors, laboratories and official control laboratories. They were part of the EURL-AP sample bank (http://eurl.craw.eu/) and were stored at 4 °C. All samples were characterised by light microscopy and PCR analysis as described by Regulation (EC) No. 152/2009 (Commission, 2009). Each test portion was prepared in triplicate and treated as an independent sample. This represents a total of 96 test portions. Samples consisted of feed materials of animal origin, aquafeeds and adulterated feeds.

Four industrial feed materials of animal origin were used: two blood-derived products of bovine origin (bovine haemoglobin powder (BvHb) and bovine blood meal (BvBlm)), one mixed blood meal of porcine and bovine origin and one milk powder.

Two types of commercial aquafeed were included in the study and were used to prepare the adulterated feeds in order to evaluate the specificity and sensitivity of the method. The first set was composed of two aquafeeds (AQF01 and AQF02) known to be free of terrestrial animal proteins and the second of two aquafeeds (AQF03 and AQF04) known to contain porcine haemoglobin powder as feed material. All feeds were ground at 2 mm with a rotor mill (ZM200, Retsch, Haan, Germany). Between each grinding, the grinder was dismantled and all the components were decontaminated with DNA EraseTM (MP Biomedicals Europe N.V., Belgium).

Adulterated feeds were prepared by weighing and direct spiking of the respective amount of feed material into the feed matrix. AQF01, AQF02, AQF03 and AQF04 were adulterated at three levels (0.1%, 0.5% and 1% (w/w)) with bovine haemoglobin powder or bovine blood meal or at 0.1% (w/w) with milk powder.

AQF02 were also adulterated at 1% (w/w) with the mixed bovine and porcine blood meal to be used for the Multiple Reaction Monitoring (MRM) design. AQFs adulterated with 1% (w/w) BvBlm were used as reference samples for bovine blood biomarkers and AQF01 adulterated with 0.1% (w/w) milk powder was used as reference sample for milk biomarkers.

2.3. Internal standards

Two heavy-labelled concatamers (conCAT) were synthesised and each contained two peptide internal standards. ConCAT are artificial proteins designed as a linear concatenation of peptides (usually tryptic). The peptide sequences were selected from sequences previously validated regarding their specificity (Lecrenier et al., 2016; Planque et al., 2016). The Hb-conCAT aminoacid sequence corresponds to the concatenation of one bovine haemoglobin biomarker (AAVTAFWGK) and one porcine haemoglobin biomarker (EAVLGLWGK) whereas Milk-conCAT encodes for one casein biomarker (FFVAPFPEVFGK) and one beta-lactoglobulin biomarker (LSFNPTQLEEQCHI). All peptides were isotopically labelled using heavy amino acid in order to obtain a minimum of +7 Dalton in mass to charge for each single standard as detailed in Table 1. They were synthesised by Life Technologies Europe BV (Ghent, Belgium) with at least 95% purity. Stock solutions were prepared at 1 mg/mL by dissolving 1 mg of each conCAT in 100 µL DMSO and then adding 900 µL of 0.1% (v/v) formic acid in water. ConCAT working solutions were prepared by diluting stock solutions in 0.1% (v/v) formic acid in water for a final concentration of 10 µg/mL.

Table 1
Concatenated peptide sequences.

ConCAT name	Concatenated sequence	Internal standard	Heavy labelling	Shift
Hb-conCAT	AAVTAFWGK*EAVLGLWGK*	AAVTAFWGK*	*Lysine $^{13}C_6$ $^{15}N_2$	+8 Da
		EAVLGLWGK*	*Lysine $^{13}C_6$ $^{15}N_2$	+8 Da
Milk-conCAT	FFVAPFPEVFGK*LSFNPTQL*EEQCHI	FFVAPFPEVFGK*	*Lysine $^{13}C_6$ $^{15}N_2$	+8 Da
		LSFNPTQL*EEQCHI	*Leucine $^{13}C_6$ ^{15}N	+7 Da

2.4. Protein extraction

Protocols used for protein extraction, digestion, peptide purification and mass spectrometry (MS) analysis were based on the protocol described by Planque et al. (2016) with the following minor changes. Extraction was performed in 50 mL test tubes containing 2 g test portions. Each conCAT was spiked (100 µL) in each tube prior to the addition of 20 mL of extraction buffer (200 mM TRIS-HCl, pH 9.2, 2 M urea). Tubes were shaken at 20 °C for 30 min (Agitelec, France) followed by sonication for 15 min at 4 °C. Tubes were then centrifuged at 4660 g for 10 min at 4 °C and 10 mL of supernatant was transferred into new tubes.

2.5. In-solution digestion

The protein extracts were diluted with 10 mL of 200 mM ammonium bicarbonate and reduced with 1 mL of 200 mM DTT at 20 °C for 45 min. They were then alkylated using 1 mL of 400 mM IAA for 45 min in the dark at 20 °C. Subsequently, trypsin digestion was performed by adding 1 mL of trypsin (1 mg/mL in 50 mM acetic acid). The extracts were incubated for 1 h at 37 °C. The choice of these digestion parameters results from a prior optimisation study (data not shown). The trypsin action was stopped by the addition of 300 µL of 20% (v/v) formic acid in water. Tubes were then centrifuged at 4660 g at 4 °C for 10 min.

2.6. Peptide purification

Digested proteins were purified by reversed-phase extraction using Sep-Pak tC18 cartridges (Waters – Milford, Massachusetts, USA). Cartridge pre-conditioning was performed with 18 mL acetonitrile followed by equilibration with 18 mL of 0.1% (v/v) formic acid in water. Digested supernatant (20 mL) was loaded on the column. Next, 18 mL of 0.1% (v/v) formic acid in water was used to flush out impurities. Elution was then performed with 6 mL of acetonitrile/0.1% (v/v) formic acid in water 80/20 (v/v). Before evaporation at 40 °C under a nitrogen flow, 30 µL of DMSO was added to each tube to prevent dryness. Finally, the pellets were resuspended in 750 µL of 0.1% (v/v) formic acid in water/acetonitrile 95/5 (v/v) and centrifuged at 4660 g for 10 min at 4 °C. The supernatants were transferred into a new tube and centrifuged at 14,480 g at 4 °C for 5 min before being transferred into polypropylene vials. Extracts were then analysed by UHPLC–MS/MS.

2.7. MS analysis

Samples were analysed using a Xevo TQS triple quadrupole system with a positive electrospray and MRM mode coupled with an Acquity system (Waters – Milford, Massachusetts, USA). The digests were separated by reverse-phase liquid chromatography using a C18 Acquity BEH130 Waters column (2.1 × 150 mm). The column compartment and thermal autosampler were set at 40 °C and 10 ± 5 °C, respectively. After the injection of 12 µL of extracts, a gradient applied for 26 min (at 0.2 mL/min) allowed the separation of the peptide biomarkers. Mobile phase A was 0.1% (v/v) formic acid in water (ULC/MS grade) and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. Elution was carried out as follows: 0–3 min: 92% A; 3–18 min: 92–58% A; 18–18.10 min: 58–15% A; 18.10–22.50 min: 15% A; 22.50–22.60 min: 15–92% A, 22.60–26 min: 92% A. A 150 l/h cone flow and a 650 l/h desolvation flow of nitrogen were then applied. The capillary voltage was set at 3.0 kV and the collision gas flow was set at 0.20 mL/min. The source and desolvation temperatures were set at 150 and 350 °C respectively. The cone voltage was fixed at 35 V. The acquisition and processing of data were carried out by MassLynx software (v. 4.1, Waters). The peptide biomarkers described in previous studies (Lecrenier et al., 2016; Planque et al., 2016) were evaluated and selected based on their peak intensities. The peptide biomarkers described by Rao, Li, Yang, Ma, and Wang (2015) were also evaluated. The selection of the MRM transitions and the optimisation of the collision energies for each peptide biomarkers were made using the open-source software Skyline (https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view).

2.8. Statistical analysis

Statistical analyses were performed using Matlab R2007b (The MathWorks Inc, Natick, USA). The Lilliefors test and Levene's test were used for assessing the normality and equality of variances respectively. The non-parametric Kruskal-Wallis test was used for multiple mean comparisons to evaluate the matrix effect, and Tukey's procedure was then used for pairwise comparisons. The level of statistical significance for all tests was set at $P < .05$ (Petrie & Watson, 2013).

3. Results and discussion

3.1. Biomarker selection and MRM design

For bovine blood detection, the haemoglobin biomarkers identified by Lecrenier et al. (2016) were evaluated and the four peptide biomarkers given the most intense transitions were selected. As the signal obtained with the porcine blood biomarker described in the same study was poor, another peptide from the same haemoglobin subunit described by Rao et al. (2015) was used. The three most intense transitions for each peptide were retained and collision energy was optimised for each one. To improve the specificity of the MRMs, only y-ion or b-ion fragments higher than 4 amino acids in length were selected so that the majority of the selected product ions had *m/z* values above the *m/z* value of the precursor ion. For milk detection, all casein and beta-lactoglobulin biomarkers previously described by Planque et al. (2016) were selected and the published MRM design was used. Table 2 summarises the selected peptide biomarkers, transitions and their optimised MRM parameters.

3.2. Validation of the method on aquafeed

3.2.1. Specificity

Although the specificity of the biomarkers was validated in previous studies against the entire NCBInr or Uniprot database and in spite of the high specificity of triple quadrupole analyses, non-specific MRM signals can be observed due to the complexity of matrices such as animal feed (Lange, Picotti, Domon, & Aebersold, 2008). In order to avoid false positive identification, strict acceptance criteria had to be applied to consider a signal as positive. As no legal evaluation criteria already

Table 2
Peptide biomarkers, specific transitions and Multiple Reaction Monitoring (MRM) parameters for the identification of haemoglobin, casein and beta-lactoglobulin. Product ions were classified by decreasing peak intensity and the most intense ions are in bold.

Target	Protein	Uniprot AC number	Peptide	Precursor ion: *m/z* (z)	Product ion: *m/z* (fragment^{z})	CE (V)	Estimated RT (min)	Estimated area ratio
Bovine blood	Haemoglobin α chain	P01966	VGGHAAEYGAEALER	510.6 (3+)	**617.3 (y5^{+})**	16	7.46	0.55
					745.4 (y7^{+})	14		
					622.3 (b7^{+})	14		
	Haemoglobin β chain	P02070	AAVTAFWGK	475.8 (2+)	**709.4 (y6^{+})**	12	10.74	0.44
					608.3 (y5^{+})	13		
					537.3 (y4^{+})	11		
			EFTPVLQADFQK	711.9 (2+)	**523.3 (y9^{2+})**	19	11.62	0.63
					1045.6 (y9^{+})	19		
					849.4 (y7^{+})	22		
			VVAGVANALAHR	393.2 (3+)	**490.3 (y10^{2+})**	9	8.26	0.18
					454.8 (y9^{2+})	9		
					681.4 (y6^{+})	13		
Porcine blood	Haemoglobin β chain	P02067	EAVLGLWGK	486.8 (2+)	**560.3 (y5^{+})**	16	12.31	0.71
					673.4 (y6^{+})	14		
					503.3 (y4^{+})	11		
Milk	Casein αS1	P02662	HQGLPQEVLNENLLR	587.3 (3+)	**436.2 (b4^{+})**	17	11.58	0.67
					758.4 (y6^{+})	16		
					790.4 (b7^{+})	17		
			FFVAPFPEVFGK	692.9 (2+)	**920.5 (y8^{+})**	18	15.86	0.37
					991.5 (y9^{+})	18		
					676.4 (y6^{+})	28		
			YLGYLEQLLR	634.4 (2+)	**771.5 (y6^{+})**	20	14.76	0.78
					658.4 (y5^{+})	21		
					934.5 (y7^{+})	21		
	Casein αS2	P02663	NAVPITPTLNR	598.3 (2+)	**285.1 (b3^{+})**	12	9.66	0.68
					911.5 (y8^{+})	17		
					456.3 (y8^{2+})	14		
	Beta-lactoglobulin	P02754	VYVEELKPTPEGDLEILLQK	771.7 (3+)	**627.8 (y11^{2+})**	20	13.50	0.59
					790.9 (y14^{2+})	19		
					912.0 (y16^{2+})	19		
			VLVLDTDYK	533.3 (2+)	**853.4 (y7^{+})**	15	10.37	0.56
					754.4 (y6^{+})	14		
					641.3 (y5^{+})	16		
			LSFNPTQLEEQCHI	858.4 (2+)	**1254.6 (y10^{+})**	26	12.18	0.52
					928.4 (y7^{+})	27		
					627.8 (y10^{2+})	27		

AC number = accession number; *m/z* = mass/charge; z = ion charge; CE = collision energy; V = volt; RT = retention time.

existed for this type of application (i.e. detection of animal proteins in feedingstuffs), it was decided to draw inspiration from the criteria described by Picotti and Aebersold (2012), using the tolerances permitted for the monitoring of certain substances and their residues in live animals and animal products (European Commission, 2002).

In order to report the detection of one peptide biomarker, the observation of at least three transitions was required at the estimated retention time (RT) at a tolerance of ± 2.5%. A perfect "co-elution" of all transitions derived from the same biomarker had to be observed. When the corresponding internal standard was available (by example AAVTAFWGK* used as internal standard for AAVTAFWGK), the RT of the standard was used. When no standard was available, the RT was estimated as the mean RT obtained with the reference samples.

The second acceptance criterion used was the peak area ratio. This criterion is used to distinguish the detection of low-abundant peptides from non-specific signals that elute at the exact same RT (Lange et al., 2008). This also varied depending on the internal standard availability. The peak area ratio (also named signal intensity ratio or relative ion intensity) between the most intense and the second most intense transition shall correspond to that from the standard or that from the reference samples measured under the same conditions if no standard is available. The maximum permitted tolerances were calculated as described in the Commission decision (European Commission, 2002). Table 2 summarises the estimated retention times and peak area ratios of the peptide biomarkers. Supplementary Table 1 summarises the tolerance levels depending on the peak area ratio. This second criterion is not applicable in the case of saturated signals, as the area measurement is not possible.

All results were in line with the expected results for the detection of blood biomarkers: all blank AQFs gave negative results (Fig. 1) for all (4/4) bovine blood biomarkers, whereas porcine biomarker was only detected in AQF03 and AQF04. Blank AQFs also gave negative results for milk biomarkers except for the most abundant casein biomarker (FFVAPFPEVFGK), which gave a clear positive result in AQF03 (Fig. 2) for the three replicates. However, the signal-to-noise ratio (S/N) of blank AQF03 was much lower than the S/N observed in the AQFs adulterated with 0.1% (w/w) milk powder, and the comparison of the peak areas of the best MRM transitions from AQF03 with 0.1% (w/w) milk powder and blank AQF03 gave a mean ratio of 1000. This unexpected signal could be explained by the presence of milk in AQF03. It can be assumed that milk proteins are present in this feed at a very low concentration and below the limit of detection (LOD) of other biomarkers.

In order to take account of these differences in LOD and to ensure the specificity and the reliability of the method, a minimum threshold of two peptides identified per by-product was fixed to declare a sample as positive for the related by-product. As the evaluation of the presence of porcine blood was only informative in this study, the AQFs were only checked for one porcine biomarker. To validate the method for the detection of porcine blood, at least one additional pig biomarker would be required.

3.2.2. Sensitivity

In order to evaluate the sensitivity of the method and to know if the

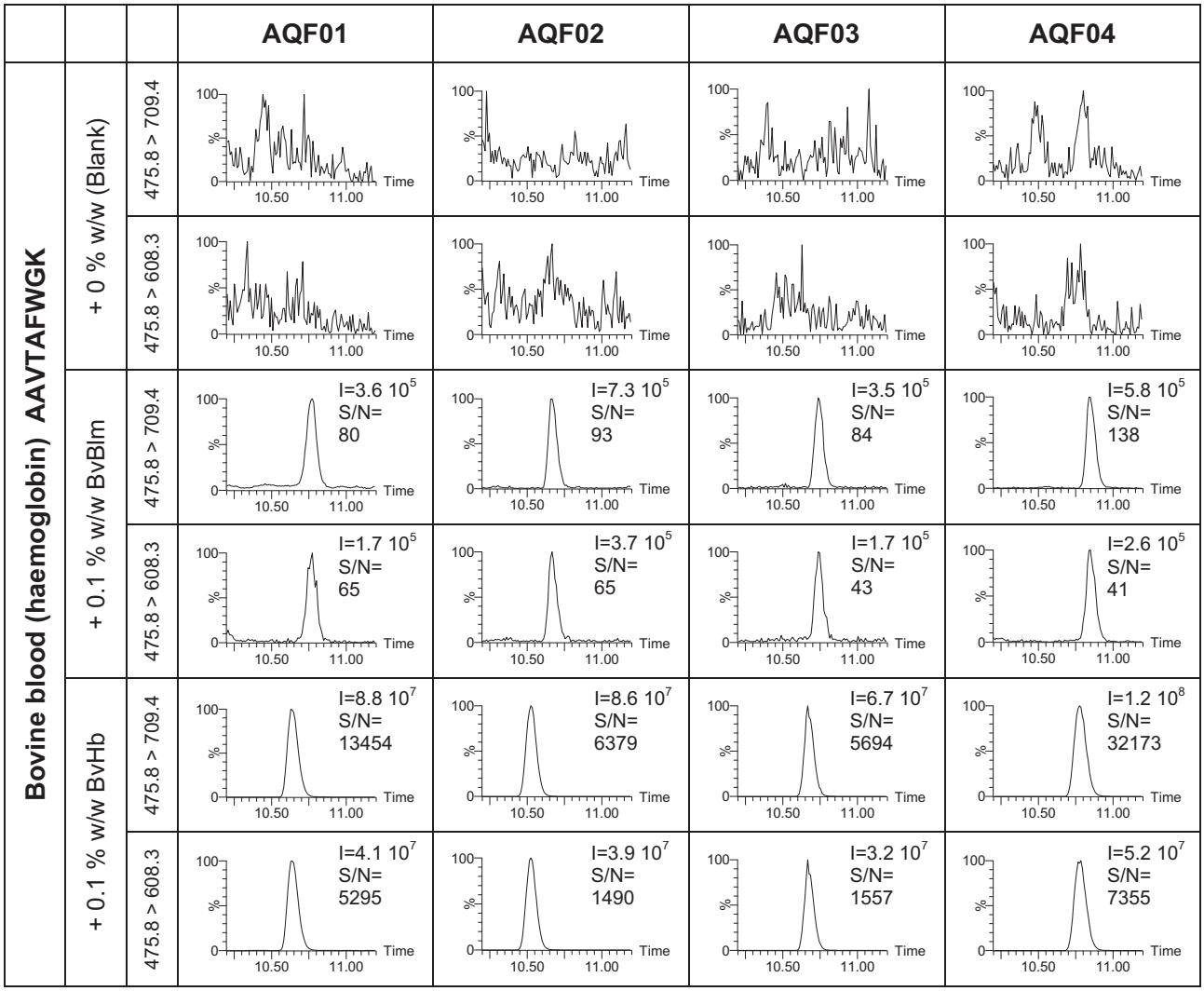

Fig. 1. Chromatograms of the two higher MRM transitions of haemoglobin peptide AAVTAFWGK in AQF01, AQF02, AQF03, AQF04 adulterated with 0% or 0.1% (w/w) of bovine blood meal (BvBlm) or bovine haemoglobin powder (BvHb). Legend: S/N = signal-to-noise ratio; I = peak intensity; Time unit = minutes.

method could reach the 0.1% (w/w) LOD level required by the EC for animal protein detection methods, decreasing amounts (1%, 0.5%, 0.1% (w/w)) of bovine blood-derived products in AQFs were analysed in triplicate as described above. For milk powder, the method was only tested at the 0.1% (w/w) level. S/N was calculated peak-to-peak in a range equal to six times the peak width at half height for three independent replicates of the targeted matrix. The threshold was fixed at a S/N of 10 for the most intense MRM transition.

Fig. 1 shows the chromatograms of the two higher MRM transitions of AAVTAFWGK in AQF01, AQF02, AQF03, AQF04 adulterated with 0% or 0.1% (w/w) of bovine blood meal (BvBlm) or bovine haemoglobin powder (BvHb). S/N ratios were far higher than the 10 ratio threshold for both adulterants. Haemoglobin powder gave higher signals than blood meal. This can be explained by the lower concentration of haemoglobin in blood meal which is produced from whole blood whereas haemoglobin powder is prepared only with the haemoglobin fraction. The processing temperature imposed by Regulation (EU) No. 142/2011 (European Commission, 2011) for the production of blood meal is also higher than for haemoglobin powder, and this may also have an influence on the preservation of the peptides. Regarding these results, it is likely that the level of detection could be lower than 0.1% (w/w) for both adulterants.

For milk detection, all test portions of AQFs adulterated at 0.1% (w/w) gave a positive result for all casein (4/4) and beta-lactoglobulin (3/3) biomarkers. Fig. 2 shows the chromatograms of the two higher MRM transitions of FFVAPFPEVFGK and LSFNPTQLEEQCHI in AQF01, AQF02, AQF03, AQF04 adulterated with 0% or 0.1% (w/w) of milk powder. Here also, the S/N ratios which were obtained for all milk biomarkers showed that the LOD could be far lower than 0.1% (w/w).

Using a threshold of a minimum of two peptides identified per by-product, the 0.1% (w/w) level was easily reached, as all test portions adulterated with BvHb or BvBlm at this level were identified as positive. In 91.7% (22 out of 24 test portions) of cases at the 0.1% (w/w) adulteration level, all bovine blood biomarkers (4/4) were identified and met all the criteria. In one replicate of AQF01 adulterated with 0.1% (w/w) BvBlm, three out of four bovine blood biomarkers (AAVTAFWGK, VGGHAAEYGAEALER and EFTPVLQADFQK) were identified, and in one replicate of AQF02 adulterated with 0.1% (w/w) BvHb, two out of four bovine blood biomarkers (AAVTAFWGK and EFTPVLQADFQK) were identified. At the 0.5% (w/w) adulteration level, all AQFs were declared as positive for all (4/4) bovine blood biomarkers. At the 1% (w/w) level, all (4/4) bovine blood biomarkers were found in 95.8% of cases (23 out of 24 test portions), and 3/4 bovine blood biomarkers (AAVTAFWGK, VVAGVANALAHR and EFTPVLQADFQK) were identified in one replicate of AQF02 adulterated with 1% (w/w) BvHb. In all cases, the misidentification was due to a shift of the retention time. The retention time dispersion of two blood biomarkers (VGGHAAEYGAEALER and VVAGVANALAHR) in AQFs

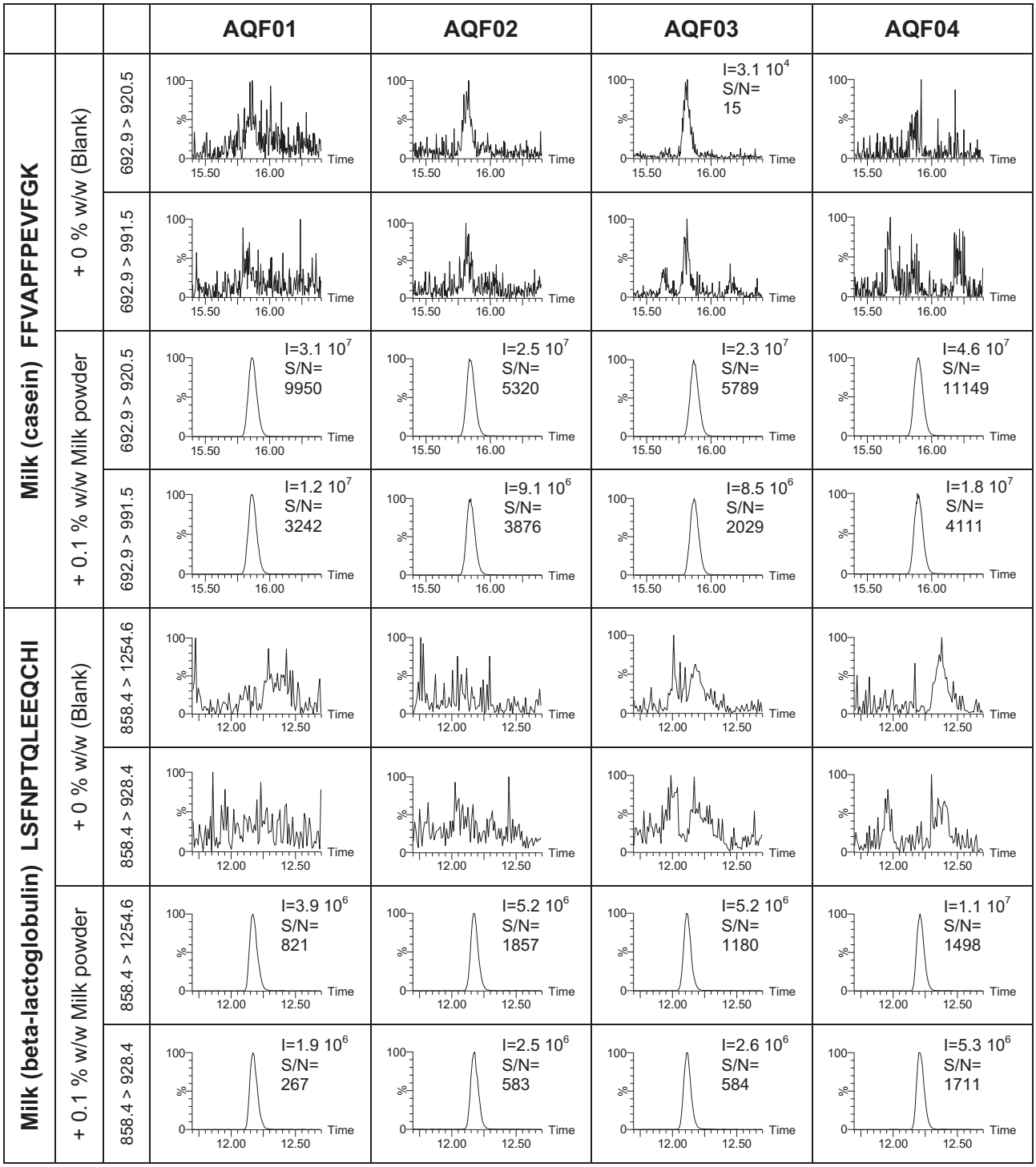

Fig. 2. Chromatograms of the two higher MRM transitions of casein peptide FFVAPFPEVFGK and beta-lactoglobulin peptide LSFNPTQLEEQCHI in AQF01, AQF02, AQF03, AQF04 adulterated with 0% or 0.1% (w/w) of milk powder. Legend: S/N = signal-to-noise ratio; I = peak intensity; Time unit = minutes.

adulterated at the three levels (0.1%, 0.5% and 1% (w/w)) with BvHb or BvBlm is represented by box plots in Fig. 3.

Although the retention time means (black dots) are stable among the different AQFs, the box plots show that the dispersion varies between the AQFs and that the variations are not the same for the two different biomarkers, in particular for AQF01. Three test portions had a retention time out of range and have already been mentioned above: one replicate of AQF01 adulterated with 0.1% (w/w) BvBlm (for VVAGVANALAHR), one replicate of AQF02 adulterated with 0.1% (w/w) BvHb (for both biomarkers) and one replicate of AQF02 adulterated with 1% (w/w) BvHb (for VGGHAAEYGAEALER). This type of non-compliance was not observed when a standard was available. This underlines the importance of working with standards to be discharged of the matrix effect if the retention time is used as an evaluation parameter.

3.3. Matrix effects

Matrix effects were assessed by comparing the peak areas of the best MRM transition for the internal standard AAVTAFWGK*. All data

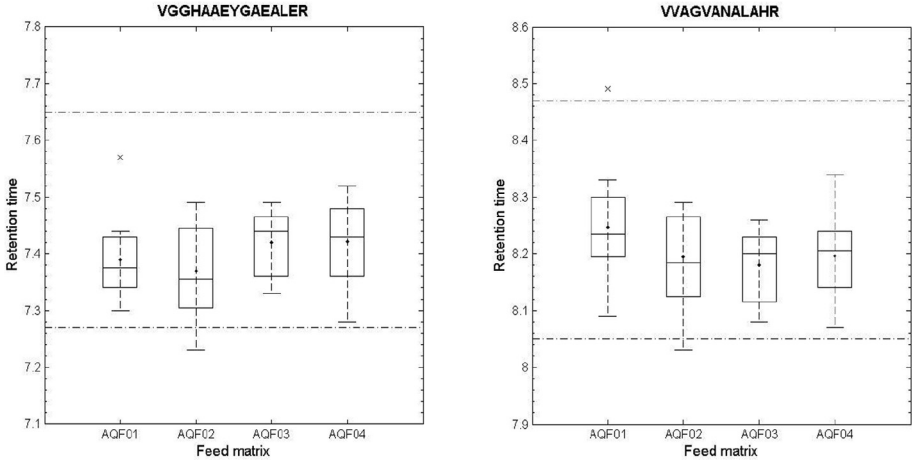

Fig. 3. Box plots of retention time of two bovine blood biomarkers (VGGHAAEYGAEALER and VVAGVANALAHR) in AQF01, AQF02, AQF03 and AQF04 adulterated at levels of 0.1–1% (w/w) with blood meal or haemoglobin powder. Horizontal dot-dashed lines represent the upper and lower acceptance limit for the retention time. Legend: Retention time unit = minutes.

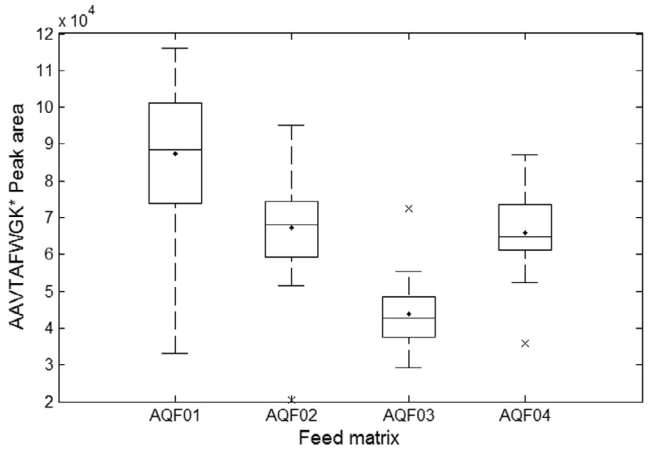

Fig. 4. Box plots of the peak area values of the bovine haemoglobin standard AAVTAFWGK [13C6, 15 N2] in AQF01, AQF02, AQF03 and AQF04.

obtained for the four different AQFs (blank or adulterated) were analysed together. Observation of the box plots (Fig. 4) revealed a high variation of the peak mean area values and of the degree of dispersion between the AQFs. Analysis of the distribution of the areas showed a normal distribution of the data. However, due to a lack of equality of variances (p-value = 0.018), non-parametric tests had to be used for variance analysis. Data were processed using the Kruskal-Wallis test in order to assess the significance of the variation of mean between feed matrices. The p-value ($5.06{\cdot}10^{-9}$) suggested that at least one AQF was significantly different from the others. Because Kruskal-Wallis did not specify which AQF was different, all feeds were compared to each other by multiple comparison of means using Tukey's procedure in order to identify which feeds were significantly different. The multiple comparison of means showed that there was no significant difference between AQF01, AQF02 and AQF04. Although the AQF01 peak area values seemed higher than the values observed for the other AQFs, the high variability of its values makes this difference non-significant. On the other hand, a significant difference was demonstrated between AQF03 and the other AQFs. These results show that the matrix can have a significant effect on the signal.

4. Conclusion

In this study, an ultra-high performance liquid chromatography (UHPLC) tandem mass spectrometry method was successfully applied on compound aquafeed for the detection of animal by-products. The sample preparation procedure used was fast (one-day protocol) and easy to apply, making this method very suitable for routine use. Concatenated labelled peptides were designed and synthesised. The use of those internal standards allowed to be discharged of the matrix effect with regard to the retention time, making the interpretation of results easier and more robust. This point is crucial for potential implementation in EU laboratories.

The method was optimised for the identification of multiple peptides in one analysis. Four blood biomarkers and seven milk biomarkers were implemented with a sensitivity reaching the 0.1% (w/w) legal requirement imposed by the EC for analytical methods. This study was intentionally limited to this legal level. However, using a minimum threshold of two peptides identified per by-product with a S/N threshold of 10 and based on the most difficult cases (S/N obtained on AQFs adulterated with 0.1% (w/w) BvBlm), a theoretical LOD for the detection of bovine blood-derived products could estimate at 0.015% (w/w). For milk products, a 0.001% (w/w) adulteration level could be reached. This assumption should obviously be verified experimentally.

Due to the promising results obtained in aquafeed, efforts are now being focused on the evaluation of the method on other types of feeds (e.g., poultry feed, porcine feed) in order to have a routine method running in all feed matrices. Moreover, in the future, others targets could easily be added to the MRM method in order to complete or extend the method for other purposes, as other by-products (e.g. gelatines, plasma powder) can also disrupt the results obtained by official methods. Similarly, this novel approach represents a way of responding rapidly to the recent regulatory change authorising the use of insect proteins in fish feed.

This study has produced an innovative way of ensuring feed safety by providing a rapid, sensitive and specific method fully adapted to routine analysis in the perspective of a future implementation of the technique in the EU national reference laboratories. Forthcoming studies will focus on the validation of the method by intra- and inter-laboratory studies in order to determine the transferability of this method to official control laboratories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.11.074.

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