



# Identification of specific bovine blood biomarkers with a non-targeted approach using HPLC ESI tandem mass spectrometry



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## ABSTRACT

Animal by-products are valuable protein sources in animal nutrition. Among them are blood products and blood meal, which are used as high-quality material for their beneficial effects on growth and health. Within the framework of the feed ban relaxation, the development of complementary methods in order to refine the identification of processed animal proteins remains challenging. The aim of this study was to identify specific biomarkers that would allow the detection of bovine blood products and processed animal proteins using tandem mass spectrometry. Seventeen biomarkers were identified: nine peptides for bovine plasma powder; seven peptides for bovine haemoglobin powder, including six peptides for bovine blood meal; and one peptide for porcine blood. They were not detected in several commercial compound feed or feed materials, such as blood by-products of other animal origins, milk-derived products and fish meal. These biomarkers could be used for developing a species-specific and blood-specific detection method.

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## 1. Introduction

In the mid-1980s, the emergence of several transmissible spongiform encephalopathies (TSEs) was recorded in animals. The consumption by cattle of meat and bone meal (MBM) produced from carcasses of infected animals was incriminated (Bradley, 1991). In order to prevent the further spread of bovine spongiform encephalopathy (BSE), a ban on feeding farmed ruminants with mammalian MBM was introduced in 1994. The partial ban was extended in 2001 to an EU-wide suspension of the use of processed animal proteins (PAPs) in feed for any farmed animal, with a few exceptions, such as the use of fish meal for non-ruminants (Plouvier et al., 2012). These prevention and control efforts have raised the possibility of the ban being gradually lifted. The reintroduction of non-ruminant PAPs in feed for aquaculture was authorised in 2013 (European Commission, 2013).

The prohibition of the use of animal proteins in animal feed depends on three factors: by-product type, species of origin; and final destination (pets, fur animals or other farmed animals). Regulation (EC) No. 999/2001 (European Union, 2001) describes this prohibition.

In addition to PAPs legislation, the EU banned intra-species recycling through the so-called animal by-product legislation (European Union, 2002), repealed and replaced by Regulation (EC) No.1069/2009 (European Commission, 2009b). In this regulation, animal by-products are defined as: “entire bodies or parts of animal origin or other products obtained from animals, which are not intended for human consumption, including oocytes, embryos and semen.” In addition, edible animal products are defined as “products intended for human consumption” (e.g., meat, blood, offal) and edible animal co-products as “parts of animals not directly suitable for human consumption but which can be later processed for use in human food” (e.g., bones and skin for gelatine and collagen production, sheep intestines for sausage casings, raw fat tissue for edible fat and greaves) (Food Standards Agency, 2011). Regulation (EC) No. 1774/2002 (European Union, 2002) also introduced the classification of animal by-products into three categories based on their potential risk for animals, the public and the environment, and determined how each category could be used. These classifications are summarised in Fig. 1.

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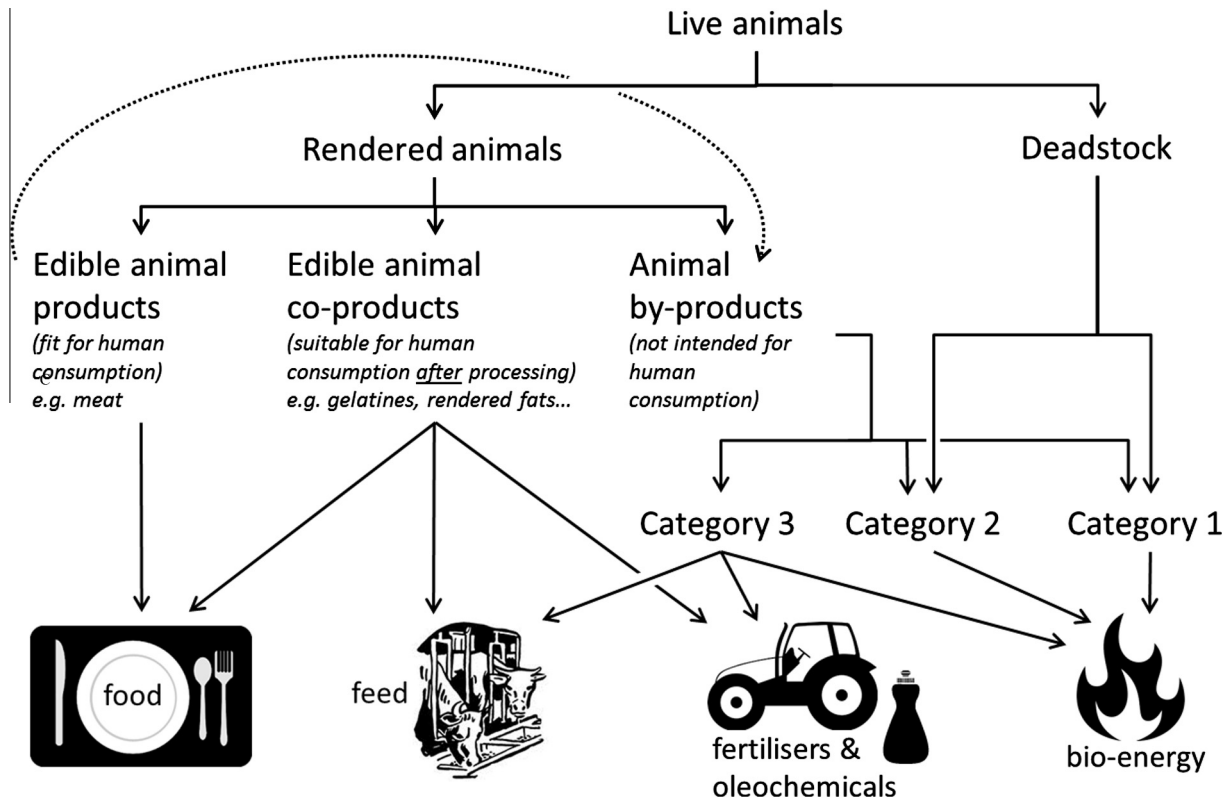


Fig. 1. Production flow of animal products, co-products or by-products from farm to end use.

Category 3 materials are low-risk materials. This category includes by-products derived from healthy animals fit for human consumption, but no longer intended for human consumption (e.g., for commercial reasons). Only Category 3 materials can be used in the production of feed following appropriate treatments in approved processing plants. Animal blood collected at slaughterhouses can be one of these by-products. Depending on the raw material used and the processing applied to the blood, two types of materials can be produced: blood meal or blood products. Blood meal consists of processed animal proteins derived from Category 3 materials other than those materials referred to in Article 10 (n), (o) and (p) of Regulation (EC) No. 1069/2009 (European Commission, 2009b). This material has to undergo heat treatment in accordance with Section 1 of Chapter II of Annex X of Regulation (EU) No. 142/2011 (European Commission, 2011). In contrast, in order to produce blood products, only blood referred to in Articles 10(a) and 10(b)(i) of Regulation (EC) No.1069/2009 can be used and blood products must have been treated in accordance with Section 2 of Chapter II of Annex X of Regulation (EU) No. 142/2011. Blood products include dried/frozen/liquid plasma, dried whole blood, dried/frozen/liquid haemoglobin or fractions and mixtures thereof.

Blood meal and blood products are a valuable protein source in feed. From a legislative standpoint, blood meal and blood products derived from ruminants are forbidden, regardless of their destination. With regard to blood derived from non-ruminants, blood meal is authorised only in fish feed, whereas blood products can be used in feed for non-ruminants and fish. In practice, blood meal and haemoglobin powder are used in fish feed, whereas plasma powder (also known as spray-dried plasma) is used in piglet feed and poultry diets. In addition to its nutritional value, plasma powder has positive effects on growth, feed intake and feed efficiency, and has a protective effect against gut pathogens, especially during vulnerable periods such as weaning (Kats et al., 1994; Lallès, Bosi, Janczyk, Koopmans, & Torrallardona, 2009).

The method of analysis for determining constituents of animal origin for the official control of feed is described by Commission Regulation (EC) No. 152/2009 (European Commission, 2009a). Using light microscopy, particles such as muscle fibres, cartilage, bones, horn, hair, bristles, feathers, egg-shells and scales are identified on the basis of morphologically identifiable characteristics. This technique is very sensitive, with a limit of detection ranging up to 25 ppm, depending on the matrix and the type of PAPs (Veys, Berben, & Baeten, 2010). One of the restrictions of light microscopy, however, is that it is unable to determine the species or higher taxonomic ranks of origin. The second official method, polymerase chain reaction (PCR), was introduced when the feed ban was lifted in 2013. This method is based on the amplification of a particular DNA sequence specific to a species or group of species. The developed method allows ruminant DNA to be detected even in highly processed materials (Fumièrre, Dubois, Baeten, von Holst, & Berben, 2006). It is also very sensitive and reaches the same limit of detection as light microscopy. PCR identifies the species from the DNA present in the analysed matrix, however, regardless of cellular origin (e.g., leucocyte, osteocyte or myocyte). Due to the limitations of each method, even by combining the results, it is not always possible to draw conclusions about the presence of ruminant PAPs (Veys, Berben, Dardenne, & Baeten, 2012). In compound feed for fish, for example, if animal particles of terrestrial origin are microscopically detected and if a positive reaction is obtained using the official ruminant probe by PCR, it is currently impossible to determine whether the feed contains PAPs of porcine origin and milk products (both authorised in aquaculture) or PAPs of bovine origin (prohibited in aquaculture) and porcine blood meal (authorised). In such cases, additional analyses are needed to determine both the species and source of the animal products.

Several methods have been investigated in order to address these shortcomings, including near infrared microscopy (NIRM) (Baeten et al., 2005; Boix, Fernández Pierna, von Holst, & Baeten,

2012; Yang, Han, Fernández Pierna, Dardenne, & Baeten, 2011), light microscopy (Liu et al., 2011; van Raamsdonk et al., 2011), fluorescence *in situ* hybridization (Lecrenier et al., 2014), immunological assays (van Raamsdonk, Margry, Van Kaathoven, & Bremer, 2015), a combination of microdissection and PCR (Axmann et al., 2015) and a combination of NIRM and PCR (Fumière, Marien, Fernández Pierna, Baeten, & Berben, 2010). For the detection of blood in particular, several studies have been conducted on developing monoclonal antibody-based immunoassays targeting bovine thermostable blood proteins (Ofori & Hsieh, 2007), but, as yet, no robust method is available.

Proteomics is another interesting area to investigate. This approach has already been used for food quality and safety purposes (Cunsolo, Muccilli, Saletti, & Foti, 2014; D'Alessandro & Zolla, 2012; Piras, Roncada, Rodrigues, Bonizzi, & Soggiu, 2015; Sentandreu & Sentandreu, 2011) and within the framework of feed safety (Buckley, Collins, Thomas-Oates, & Wilson, 2009; Fernandez Ocana et al., 2004; Marbaix et al., 2016; Reece et al., 2012). Proteomics is particularly suitable for detecting constituents of animal origin because it provides information about the tissue and species of origin. Peptide sequences, as in the case of DNA, have specific polymorphisms that can be traced to their species of origin (Claydon, Grundy, Charlton, & Romero, 2015). Although the DNA content of some by-products is similar, in some cases these by-products could be distinguished based on their protein content. Although the genome remains broadly unchanged in different tissues of the same organism, cells express specific proteins in radically different ways, depending on their differentiation and function. Another important characteristic of proteins is the relatively good resistance of their primary structure to processing. Buckley et al. (2009) reported that protein sequences survive better than DNA in archaeological and heated samples.

The aim of this study was to identify and characterise specific bovine blood biomarkers with a non-targeted approach using high-pressure liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC ESI MS/MS). This work was conducted within the framework of the development of complementary methods for the detection of PAPs in feedingstuffs.

## 2. Materials and methods

### 2.1. Samples

The compound feeds and materials of animal origin analysed were industrial samples provided by several producers, sellers, laboratories and governmental agencies. These samples were part of the EURL-AP sample bank (<http://eurl.craw.eu/>) and were stored at 4 °C. Parts of samples were characterised by PCR analysis (Fumière et al., 2006).

Three sample sets were created – Set A, Set B and Set C – consisting of materials of animal origin, compound feeds and spiked feed, respectively.

A total of 36 feed materials of animal origin were selected for Set A. They were grouped into five categories: blood products (plasma and haemoglobin powder); blood meal; fresh plasma; milk products; and fish meal. The blood products and blood meal were of bovine, porcine or poultry origin. Various types of milk products were chosen: whey powder; skimmed milk powder; and milk powder. Porcine and bovine blood samples were also collected in a 4.5 ml citrate tube (BD Vacutainer 367704, BD-Plymouth, UK) using venepuncture. After centrifugation at 1600g for 15 min at room temperature, fresh plasma was stored at –20 °C. The composition of Set A is summarised in Table 1.

Sample Set B included commercial compound feeds. One horse feed, three chicken feeds and one aquafeed (AQF01) were used as

**Table 1**  
Sample Set A: materials of animal origin.

Sample	Species of origin	Number of samples
Blood products		
Plasma powder	Bovine	2
	Porcine	8
Haemoglobin powder	Bovine	2
	Porcine	6
Blood meals		
	Bovine	2
	Porcine	2
	Poultry	7
Fresh plasma		
	Bovine	1
	Porcine	1
Milk products		
Whey powder	Bovine	1
Skimmed milk powder	Bovine	1
Milk powder	Bovine	1
Fish meal	Fish	2

blank feed because they were known to be free of terrestrial animal proteins. In order to test products directly affected by the re-introduction of non-ruminant PAPs in aquafeed, four other aquafeeds (AQF02, AQF03, AQF04 and AQF05) containing animal blood protein were also analysed. According to the labelling, AQF02 contained 7% spray-dried blood meal, AQF03 and AQF04 both 10% haemoglobin powder and AQF05 11% haemoglobin powder. All feeds were ground at 0.5 mm with a rotor mill (ZM200, RetschH, Haan, Germany). Between each grinding, the grinder was disassembled and all the pieces were decontaminated with DNA Erase™.

For sample Set C, adulterated feeds were prepared by spiking. The horse feed was adulterated at two levels (1% and 10%) with bovine plasma powder, haemoglobin powder or blood meal. These levels correspond to the classic level range of non-ruminant blood products or blood meal used in feedingstuffs.

### 2.2. Protein extraction

Protocols used for protein extraction, purification, digestion and mass spectrometry (MS) analysis were based on the protocol described by Marbaix et al. (2016) with minor changes. Extraction was performed in 2 ml micro test tubes containing test portions of 200 mg. To each micro test tube, 1.8 ml of trichloroacetic acid/acetone solution (10:90 v/v) with 0.3% dithiothreitol (DTT) was added and the tubes were then stored at –20 °C for 3 h. The micro tubes were then centrifuged for 10 min at 16,000g at 4 °C (1-15PK Refrigerated Microcentrifuge, Sigma, USA) and supernatants were discarded. The remaining pellets were resuspended in 1.8 ml of pure acetone with 0.3% DTT and stored overnight at –20 °C. After this, the micro tubes were centrifuged for 10 min at 16,000g at 4 °C and the supernatants were discarded. The remaining pellets were incubated twice in 1.8 ml of pure acetone with 0.3% DTT and once in 1.8 ml of 90% acetone with 0.3% DTT for 1 h at –20 °C each time and centrifuged after each washing. The supernatants were discarded and the pellets were air-dried for a few minutes, and then suspended in 300 µl of DIGE labelling buffer (urea 7 M, thiourea 2 M, Tris 30 mM, CHAPS 4%). For pure blood products, 300 additional µl were needed and all the test portions were dispersed using ultrasonication (UIS250V, Hielscher, Ultrasound Technology, Germany, cycle 0.5, amplitude 70%) for 3 × 10 s on ice. They were then mixed for 1 h at 1400 rpm at 12 °C in a thermomixer (Eppendorf Thermomixer comfort, Eppendorf, Germany) and stored overnight at –20 °C. The test portions were rewarmed at room temperature and centrifuged for 10 min at 16,000g at 12 °C. The supernatants were transferred into a

new tube and centrifuged at 16,000g for 10 min at 10 °C using an angle rotor. They were then transferred into 1.5 ml micro tubes and stored at –20 °C. Total protein concentration was determined by using the Pierce™ 660 nm Protein Assay (Thermo Scientific, USA) with bovine serum albumin as standard.

### 2.3. Protein purification

Protein purification was performed on 70 µg of proteins by using the 2-D Clean-Up Kit (GE Healthcare, USA). The pellets were solubilized in 23 µl of RapiGest SF Surfactant 0.2% (Waters, USA) in ammonium bicarbonate 50 mM. Protein extracts were mixed for 40 min at 15 °C in a thermomixer and then heated at 100 °C for 5 min. The micro tubes were centrifuged at 16,000g for 5 min at 10 °C and the supernatants were transferred into a new micro tube if a pellet was present. The purified protein extracts were stored at –20 °C. Total protein concentration was again determined by using the Pierce™ 660 nm Protein Assay (Thermo Scientific, USA) with bovine serum albumin as standard.

### 2.4. Protein digestion

The protein extracts were reduced by 0.2 µl of DTT 1 M and incubated at 500 rpm for 45 min at 37 °C in a thermomixer. They were then alkylated using 1.47 µl of iodoacetamide 550 mM and incubated in the dark at 500 rpm for 45 min at 37 °C. After this, 0.2 µl of CaCl<sub>2</sub> 100 mM and trypsin MS grade (Promega, USA) were added in order to obtain a final trypsin/protein ratio of 1/20 (w/w). The extracts were incubated at 300 rpm for 5 h at 37 °C. In order to degrade the RapiGest SF Surfactant and to stop trypsin digestion, trifluoroacetic acid was added (pH < 2) and the digested solutions were incubated at 300 rpm for 30 min at 37 °C. Each micro tube was then centrifuged at 12,000g at room temperature for 10 min and the supernatant was recovered and stored at –20 °C prior to MS analysis.

### 2.5. MS analysis

The peptides were analysed by using an ESI-MS/MS maXis Impact UHR-TOF (Bruker, Germany) coupled with a nano-UPLC UltiMate 3000 (Thermo, USA). The digests (corresponding to 1 µg of protein) were separated by reverse-phase liquid chromatography using a 75 µm × 250 mm C18 column (Acclaim PepMap 100 C18, Thermo, USA) in an Ultimate 3000 liquid chromatography system. The flow rate was 300 nl/min. Mobile phase A was 95% water, 5% acetonitrile and 0.1% formic acid (Myers et al., 2007). Mobile phase B was 20% water, 80% acetonitrile and 0.1% formic acid. The digest was injected and the organic content of the mobile phase was increased linearly from 4% B to 30% B in 160 min and from 30% B to 90% B in 25 min, and then washed with 90% B for 10 min. Finally, the column was reconditioned with 4% B for 20 min. The column effluent was connected to a Captive Spray source (Bruker). In a survey scan, MS spectra were acquired every 0.5 s in the mass-to-charge (*m/z*) range between 50 and 2200. The most intense peptides ions 2+ to 4+ were sequenced over a cycle time of 3 s. The collision-induced dissociation (CID) energy was automatically set according to the *m/z* ratio and charge state of the precursor ion. Thermo and maXis systems were piloted by Compass Hystar 3.2 (Bruker).

### 2.6. Data base searching and protein identification

Peak lists were created using DataAnalysis 4.2 (Bruker) and saved as MGF files for use with ProteinScape 3.1 (Bruker), using Mascot 2.4 as the search engine (Matrix Science). The following parameters were used: taxonomy focused on mammals; trypsin

digestion; maximum of two missed cleavages; monoisotopic peptide; mass tolerance of 10.0 ppm; and MS/MS tolerance of 0.05 Da. Carbamidomethylation of cysteine, oxidation of methionine and conversion of glutamine in pyroglutamate were allowed as variable modifications. The peak lists were searched against a homemade database (616,520 entries) that contained four taxonomic groups (class Aves, order Rodentia and suborders Ruminantia and Suina) with an automatic decoy database search and a False Discovery Rate (FDR) of 1%. Scaffold 4.3 (Proteome Software) was used to validate the MS/MS-based protein and peptide identifications. The peptide identifications were accepted if they could be established at greater than 95% probability by the Scaffold local FDR algorithm. Protein identifications were accepted if they could be established at greater than 5% probability in order to achieve an FDR less than 1% and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller, Kolker, & Aebersold, 2003).

### 2.7. Sequence alignment

Candidate biomarkers were searched against the NCBI nr database (<http://www.ncbi.nlm.nih.gov>) using the standard protein BLAST alignment research tool and blastp algorithm for species specificity.

## 3. Results and discussion

The methodology used in this study for identifying blood biomarkers involved three steps: a selection of the proteins of interest; an initial selection of candidate biomarkers; and a final selection of the biomarkers based on their specificity. The biomarkers were then challenged by the analysis of commercial feed samples known to contain blood ingredients.

### 3.1. Selection of the target proteins

For this selection, MS data obtained from blood materials of bovine origin (bovine plasma powder, haemoglobin powder, blood meal and fresh plasma) was used. The proteins were not selected according to their physiological abundance because the aim of the study was to identify biomarkers for processed blood. The resistance of the proteins to thermal treatment was therefore an important parameter and the selection was done with samples processed under industrial conditions (Fumière et al., 2009). Thus, proteins with the highest sequence coverage were selected.

The selected proteins are presented in Table 2. Five proteins were selected: alpha-2 macroglobulin; apolipoprotein A1; fibrinogen; haemoglobin; and serotransferrin. While serum albumin, immunoglobulins and complement component 3 represented highly abundant plasma proteins, these proteins were not retained because they are also present in milk-derived products (Boehmer, Bannerman, Shefcheck, & Ward, 2008; Swaisgood, 1995).

### 3.2. Candidate biomarker selection

For the initial selection of biomarkers, two protein groups were created (Table 2). The first group, which included alpha-2 macroglobulin, apolipoprotein A1, fibrinogen and serotransferrin, was chosen for the selection of plasma powder biomarkers. For the second group, haemoglobin was chosen for haemoglobin powder and blood meal biomarker selection.

In order to focus the selection on the more abundant peptides, the study was limited to those peptides detected in the spiked samples (Set C). These peptides were studied in terms of their presence or absence in pure bovine plasma powder, pure bovine

**Table 2**

Selected proteins in bovine plasma powder, haemoglobin powder blood meal and fresh plasma.

Protein or protein subunit	Uniprot accession number	Average percentage of sequence coverage (%)			
		BvPl	BvHb	Bvblm	BvFPI
Alpha-2 macroglobulin <sup>a</sup>	Q7SIH1	44	20	/	50
Apolipoprotein A1 <sup>a</sup>	P15497	72	33	42	66
Fibrinogen alpha chain <sup>a</sup>	P02672	39	18	5	36
Fibrinogen beta chain <sup>a</sup>	P02676	45	19	/	49
Fibrinogen gamma chain <sup>a</sup>	P12799	58	21	/	57
Haemoglobin alpha <sup>b</sup>	P01966	56	90	97	60
Haemoglobin beta <sup>b</sup>	P02070	49	84	87	59
Serotransferrin <sup>a</sup>	Q29443	60	19	2.7	66

BvPl, Bovine plasma powder; BvHb, Bovine haemoglobin powder; Bvblm, Bovine blood meal; BvFPI, Bovine fresh plasma; /, not detected.

<sup>a</sup> Protein selected for plasma powder detection.<sup>b</sup> Protein selected for haemoglobin powder and blood meal detection.

haemoglobin powder, pure bovine blood meal and spiked horse feed. The presence of plasma powder biomarkers was also checked in the sample of fresh bovine plasma. In order to be considered as relevant, peptides had to be sequenced in all related samples.

Another selection parameter was the presence of amino acid modifications or missed cleavages. Peptides with missed cleavages were discarded. With regard to the modifications, only methionine oxidation was allowed. A total of 11 peptides met the criteria for plasma powder: three alpha-2 macroglobulin peptides (LSFVTVDNSLR, SLFTDVVAEK and SNSFVYLEPLPR); four apolipoprotein A1 peptides (LLDNWDTLASTLSK, LSPLAQELR, VAPLGEFR and VSILAAIDEASK); and four serotransferrin peptides (DNPQTHYYA VAVVK, HSTVFDNLPNPEDR, TSDANINWNNLK and TYDSYLGDD YVR). For haemoglobin powder, 13 peptides met the criteria: six

peptides of the haemoglobin alpha chain (AVEHLDDLPGALSELSD LHAHK, FLANVSTVLTSK, LLSHLLVTLASHLPSDFTPAVHASLDK, MFLSFPTTK, TYFPFDLSHGSAQVK and VGGHAAEYGAEALER); and seven peptides of the haemoglobin beta chain (AAVTAFWVGK, EFTPVLQADFQK, FFESFGDLSTADAVMNNPK, LHVDPENFK, LLGNLVVVVLAR, LLVVYPWTQR and VVAGVANALAHR). For blood meal, eight peptides met the criteria: four peptides of the haemoglobin alpha chain (AVEHLDDLPGALSELSD LHAHK, FLANVSTVLTSK, MFLSFPTTK and VGGHAAEYGAEALER); and four peptides of the haemoglobin beta chain (AAVTAFWVGK, EFTPVLQADFQK, FFESFGDLSTADAVMNNPK and VVAGVANALAHR). These peptides were part of those selected for haemoglobin powder. Even when the protein coverage in pure blood meal samples was high and similar to the haemoglobin powder samples, fewer peptides were identified in feed spiked with bovine blood meal. This difference with regard to haemoglobin powder could be explained by the stronger processing of blood meal. The selected candidate biomarkers are summarised in the second column of Table 3 (this Table is discussed in more detail in Section 3.3.).

Although the focus of this study was bovine blood detection, one porcine blood biomarker was identified. This porcine blood biomarker (VLQSFSDGLK) is a peptide from the porcine haemoglobin beta chain (Uniprot accession number: P02067). It was observed in all porcine haemoglobin powders (6/6) and blood meal (2/2), as well as in 7 out of 8 porcine plasma powders. Its presence was also positively checked in the fresh porcine plasma sample.

### 3.3. Specificity evaluation

Species specificity of the candidate biomarkers was evaluated against feed materials of animal origin and blank matrices used in animal feed (Table 3). The presence or not of the candidate biomarkers was therefore assessed in porcine meal samples (eight

**Table 3**

Evaluation of the candidate bovine blood biomarkers with regard to their specificity. Presence/absence in porcine blood materials, milk products, poultry blood meal, fish meal and blank feed.

Protein name or protein subunit	Peptide sequence	PcPl (8)	PcHb (6)	PcBlm (2)	PcFPI (1)	Milk products (3)	PoultryBlm (7)	Fish meal (1)	Blank feed (5)
Alpha-2-macroglobulin	<b>LSFVTVDNSLR</b>	–	–	–	–	–	–	–	–
	<b>SLFTDVVAEK</b>	–	–	–	–	–	–	–	–
	<b>SNSFVYLEPLPR</b>	–	–	–	–	–	–	–	–
Apolipoprotein A1	<b>LLDNWDTLASTLSK</b>	–	–	–	–	–	–	–	–
	<b>LSPLAQELR</b>	–	–	–	–	–	–	–	–
	<b>VAPLGEFR</b>	–	–	–	–	–	–	–	–
	<b>VSILAAIDEASK<sup>a</sup></b>	+(8/8)	+(4/6)	+(2/2)	+(1/1)	–	–	–	–
Serotransferrin	<b>DNPQTHYYAVAVVK<sup>a</sup></b>	+(7/8)	+(3/6)	–	+(1/1)	–	–	–	–
	<b>HSTVFDNLPNPEDR</b>	–	–	–	–	–	–	–	–
	<b>TSDANINWNNLK</b>	–	–	–	–	–	–	–	–
	<b>TYDSYLGDDYVR</b>	–	–	–	–	–	–	–	–
Haemoglobin alpha chain	<b>AVEHLDDLPGALSELSD LHAHK</b>	–	–	–	–	–	–	–	–
	<b>FLANVSTVLTSK<sup>a</sup></b>	+(8/8)	+(6/6)	+(2/2)	+(1/1)	–	–	–	–
	<b>LLSHLLVTLASHLPSDFTPAVHASLDK</b>	–	–	–	–	–	–	–	–
	<b>MFLSFPTTK<sup>b</sup></b>	–	–	–	–	–	–	–	–
	<b>TYFPFDLSHGSAQVK<sup>b</sup></b>	–	–	–	–	–	–	–	–
	<b>VGGHAAEYGAEALER</b>	–	–	–	–	–	–	–	–
Haemoglobin beta chain	<b>AAVTAFWVGK</b>	–	–	–	–	–	–	–	–
	<b>EFTPVLQADFQK</b>	–	–	–	–	–	–	–	–
	<b>FFESFGDLSTADAVMNNPK</b>	–	–	–	–	–	–	–	–
	<b>LHVDPENFK<sup>b</sup></b>	–	–	–	–	–	–	–	–
	<b>LLGNLVVVVLAR<sup>a</sup></b>	+(8/8)	+(6/6)	+(2/2)	–	–	–	–	–
	<b>LLVVYPWTQR<sup>a</sup></b>	+(4/8)	+(6/6)	+(2/2)	–	–	–	–	–
	<b>VVAGVANALAHR</b>	–	–	–	–	–	–	–	–

PcPl, Porcine plasma powder; PcHb, Porcine Haemoglobin powder; PcBlm, Porcine blood meal; PcFPI, Porcine fresh plasma; PoultryBlm, Poultry blood meal.

(#), number of samples; +, presence (ratio of positive samples); –, absence.

In bold, the final selected bovine biomarkers.

<sup>a</sup> Non-specific peptides based on mass spectrometry results.<sup>b</sup> Non-specific peptides based on search against the NCBI database.

plasma powders, six haemoglobin powders, two blood meal samples and one fresh plasma sample), three milk product samples, seven poultry blood meal samples, one fish meal and five blank feed samples (horse feed, three chicken feed samples and one aquafeed sample [AQF01]) in order to check their specificity. Five candidate biomarkers were sequenced in the porcine samples. VSI-LAAIDEASK and FLANVSTVLTSK were systematically present in all tested porcine samples. DNPQTHYYAVAVVK was present in the plasma powder, haemoglobin powder and fresh plasma samples. LLGNLVVVVLAR and LLVVYPWTQR were found in the plasma powder, haemoglobin powder and blood meal samples. These five peptides were therefore kept apart, pending a confirmation of non-specificity by sequence alignment. The other 19 candidate biomarkers were absent from the porcine samples. None of the candidate biomarkers were present in milk products, poultry blood meal, fish meal or blank feed.

In order to validate the results, the sequences were searched against the entire NCBI nr database to establish the specificity of the identified candidate biomarkers. Even when the main materials of animal origin that are likely to give false positive results were tested, it was necessary to search the candidate biomarkers against all farmed species. As the legislation on animal protein use in feed

makes a distinction between PAPs of ruminant and non-ruminant origin, peptides found to be similar in non-ruminant farmed animals (i.e., pigs, horses, rabbits, chickens, turkeys) were excluded. Three other peptides were rejected due to similarities with rabbits (LHVDPENFK), with horses (TYFPFDLSHGSAQVK) or with too many species (MFLSFPTTK, more than 200 matching records). This search also confirmed the previous results for the porcine samples, except for the peptide LLGNLVVVVLAR, which was not assigned to any porcine protein. Another peptide, LLGNVIVVVLAR, was found to match the porcine haemoglobin beta chain. As leucine has the same monoisotopic mass as isoleucine, it is difficult using conventional MS to distinguish these two amino acids. This issue should be kept in mind when selecting biomarkers in order to avoid subsequent false positive results.

The specificity of the unique porcine blood biomarker was tested against bovine samples (plasma powders, haemoglobin powders, blood meal and fresh plasma), poultry samples, milk products, fish meal and blank feed. The porcine biomarker was not found in any of these samples, apart from one of the two bovine blood meal samples. This meal, however, was labelled as a blood meal with 80% ruminant origin. The origin of the remaining 20% was not given, but it probably had a porcine or poultry source

**Table 4**  
Final list of specific biomarkers for bovine and porcine blood detection and their specificities with regard to the blood of cow (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*) and pig (*Sus scrofa*) origin.

Marker	Protein name	Species (protein accession number)	Peptide sequence	Mass [M+H] <sup>+</sup>	AA position				
						<i>Bos taurus</i>	<i>Ovis aries</i>	<i>Capra hircus</i>	<i>Sus scrofa</i>
A2M01	Alpha-2-macroglobulin	Cattle (Q7SIH1)	LSFVTVDNSLR	1,250.67	350-360	■	■	■	■
A2M02	Alpha-2-macroglobulin	Cattle (Q7SIH1)	SLFTDVVAEK	1,108.59	72-81	■	■	■	■
A2M03	Alpha-2-macroglobulin	Cattle (Q7SIH1)	SNSFVYLEPLPR	1,421.74	455-466	■	■	■	■
APO01	Apolipoprotein A1	Cattle (P15497)	LLDNWDTLASTLSK	1,576.82	69-82	■	■	■	■
APO02	Apolipoprotein A1	Cattle (P15497)	LSPLAQELR	1,026.59	164-172	■	■	■	■
APO03	Apolipoprotein A1	Cattle (P15497)	VAPLGEEFR	1,017.54	142-150	■	■	■	■
SER01	Serotransferrin	Cattle (Q29443)	HSTVFDNLPNPEDR	1,640.77	230-243	■	■	■	■
SER02	Serotransferrin	Cattle (Q29443)	TSDANINWNNLK	1,389.68	457-468	■	■	■	■
SER03	Serotransferrin	Cattle (Q29443)	TYDSYLGDDYVR	1,466.64	671-682	■	■	■	■
HBA01	Haemoglobin alpha chain	Cattle (P01966)	AVEHLDDLPGALSELSDLHAHK	2,367.19	70-91	■	■	■	■
HBA02	Haemoglobin alpha chain	Cattle (P01966)	LLSHSLVTLASHLPSDFTPAVHASLDK	2,969.61	101-128	■	■	■	■
HBA03	Haemoglobin alpha chain	Cattle (P01966)	VGGHAAEYGAEALER	1,529.73	18-32	■	■	■	■
HBB01	Haemoglobin beta chain	Cattle (P02070)	AAVTAFWGK	950.51	8-16	■	■	■	■
HBB02	Haemoglobin beta chain	Cattle (P02070)	EFTPVLQADFQK	1,422.73	120-131	■	■	■	■
HBB03	Haemoglobin beta chain	Cattle (P02070)	FFESFGDLSTADAVMNNPK	2,089.95	40-58	■	■	■	■
HBB04	Haemoglobin beta chain	Cattle (P02070)	VVAGVANALHR	1,177.68	132-143	■	■	■	■
HBB01p	Haemoglobin beta chain	Pig (P02067)	VLQSFSDGLK	1,093.59	68-77	■	■	■	■

AA, Amino acid.

(■) denotes the presence of a peptide and (□) the absence of a peptide.

because these are the main species used for this type of product. PCR analysis revealed that this blood meal gave a positive result with the ruminant probe and the porcine probe, but a negative result for the poultry probe (data not shown). The porcine blood biomarker was also compared with the NCBI database and its specificity was confirmed.

Table 4 summarizes the final list of the 16 biomarkers selected for the detection of: bovine plasma powder (A2M01, A2M02, A2M03, APO01, APO02, APO03, SER01, SER02 and SER03); bovine haemoglobin powder (HBA01, HBA02, HBA03, HBB01, HBB02, HBB03 and HBB04); and bovine blood meal (HBA01, HBA03, HBB01, HBB02, HBB03 and HBB04). The porcine blood biomarker (HBB01p) is also described. The final list was intentionally kept quite large in order to increase the probability of a successful identification of unauthorised ingredients. With regard to specificity, the search against the NCBI database revealed that some of the bovine biomarkers were present in sheep and goats. These biomarkers are particularly interesting because they allow other farmed ruminant blood matching regulation (EC) No. 999/2001 (European Union, 2001) to be detected. A2M02, A2M03, APO01, APO02, APO03, SER03, HBB01 and HBB02 are shared by the three species and are therefore consistent with the legal restrictions on the use of animal by-products. A2M01, HBA02 and HBA03 are common to both cows and sheep, the major sources of ruminant blood. On the other hand, if the specific detection of bovine blood is needed, biomarkers SER01, SER02, HBA01, HBB03 and HBB04 can be used.

#### 3.4. Prospective study on real feed sample

In order to determine whether the selected biomarkers were fit for a purpose to analyse real commercial compound feeds known to contain blood products or blood meal, four other aquafeed samples (AQF02, AQF03, AQF04 and AQF05) were analysed. These samples were reported to contain 7–11% blood meal or haemoglobin powder. The species of origin of these ingredients was not mentioned, but given the legislation in force for aquafeed, it was probably of porcine or poultry origin because only blood products or blood meal of non-ruminant origin are authorised for aquafeed. By microscopic evaluation, no particles derived from terrestrial animals were detected, but fish particles were observed. These microscopic results are in accordance with the feed declarations about fish meal and blood material, which have been shown to not always be detectable by microscopy (Veys, Berben, & Baeten, 2015). PCR analyses revealed that these feed samples gave a positive result with the porcine probe and a negative result for the poultry probe. For AQF02, AQF03 and AQF04, however, PCR analyses also gave a positive result with the ruminant probe (data not shown). The origin of this ruminant DNA is not known. The hypothesis of a milk origin was investigated using enzyme-linked immunosorbent assays (ELISAs). These assays, initially developed by the CER Groupe (<http://www.cergroupe.be/fr/>) for allergen detection in food, are very sensitive in the detection of casein and beta-lactoglobulin, with a limit of quantification of 0.5 ppm and 0.25 ppm, respectively (Dumont et al., 2010). No milk proteins were detected in AQF02, AQF03 and AQF04. With regard to the MS results, all aquafeed samples gave a positive result for the presence of the porcine biomarker, but no bovine biomarkers were detected. These results are consistent with the expected results for these types of feed products, even though they cannot be linked to the PCR results with the ruminant probe. Unfortunately, it is not yet possible to explain the PCR results. Possible hypotheses are a difference in the limit of detection of both methods or a non-blood origin of the DNA targets found in the feeds. This parameter has yet to be evaluated for this protocol. Forthcoming studies will focus on this issue.

#### 4. Conclusion

In this work, candidate bovine specific biomarkers for detecting the presence of blood proteins in feed were identified. Nine biomarkers were selected for the detection of bovine plasma powder and seven biomarkers for the detection of bovine haemoglobin powder, including six biomarkers for the bovine blood meal. One porcine blood biomarker was also identified. The combination of these biomarkers will give an accurate answer about the ingredients used and the species origin of the proteins.

Efforts are now being focused on developing fast and targeted diagnostic biomarker assays that can be implemented in feed control. Such protocols will have to be adapted to triple-quadrupole MS systems. These instruments are more widely spread in routine laboratories and allow excellent analytic sensitivity for selected biomarkers. Particular attention will focus on evaluating the limit of detection and improving the extraction protocol in order to increase the sensitivity of the method with regard to the proteins of interest. An immunoaffinity column could be used for the selective purification of an extract with regard to the proteins selected in this study. In this way, their concentration in the protein extract would be increased, thus improving the limit of detection and analytic sensitivity.

#### Authors' contributions

Authors M.C. Lecrenier and H. Marbaix equally contributed to this work.

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