Talanta 189 (2018) 166–173

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Synchronous fluorescence spectroscopy for detecting blood meal and blood products

Marie-Caroline Lecrenier[a,b,*], Vincent Baeten[a,c], Aurélien Taira[c], Ouissam Abbas[a]

[a] Food and Feed Quality Unit of Agricultural Product Valorisation Department – Walloon Agricultural Research Centre, Gembloux, Belgium
[b] University of Liège - ULiège, Liège, Belgium
[c] Catholic University of Louvain - UCL, Louvain-la-Neuve, Belgium

ARTICLE INFO

Keywords:
Detection
Hemoglobin
Animal feed
Synchronous fluorescence spectroscopy
SFS

ABSTRACT

Fluorescence spectroscopy is a powerful method for protein analysis. Its sensitivity and selectivity allow its use for the detection of blood meal and blood products. This study proposes a novel approach for the detection of hemoglobin in animal feed by synchronous fluorescence spectroscopy (SFS). The objective was to develop a fast and easy method to detect hemoglobin powder and blood meal.

Analyses were carried out on standard reference material (hemoglobin and albumin) in order to optimize SFS method conditions for hemoglobin detection. The method was then applied to protein extracts of commercial feed material and compound feed. The results showed that SFS spectra of blood meal and blood products (hemoglobin powder and plasma powder) could be used to characterize hemoglobin. Principal component analysis (PCA) applied to area-normalized SFS spectra of artificially adulterated samples made it possible to define a limit of detection of hemoglobin powder or blood meal of 0.5–1% depending on the feed material. The projection in the PCA graphs of SFS spectra of real commercial compound feeds known to contain or to be free from blood-derived products showed that it was possible to discriminate samples according to the presence of hemoglobin.

These results confirmed that SFS is a promising screening method for the detection of hemoglobin in animal feed.

1. Introduction

Nowadays, feed safety is an important issue for consumers. In Europe, since the “mad cow disease” crisis, the use of animal by-products in animal feed has been highly regulated. Ruminant blood-derived products may not be used in feed for farm animals. In contrast, blood meal and blood products of porcine origin are both authorized in aquafeed, while only porcine blood products are allowed to be used in feed intended for other non-ruminants. The detection of these banned products uses a combination of two methods: polymerase chain reaction (PCR) and optical microscopy [1]. Until now, official controls have been based exclusively on these two methods. However, the planned lifting of the feed ban requires the development of a strategy to assess the absence of illicit materials in feedingstuffs.

Several options have been studied in order to help the official control to complement the strategy by improving the identification of animal by-products in general and blood-derived products in particular. Imaging techniques such as near-infrared microscopy (NIRM) [2–4], light microscopy [5,6] or fluorescence in situ hybridization [7] have been extensively studied. Some combinations of methods have also been evaluated [8,9]. More recently, novel developments have focused on protein-based methods such as immunological assays [10,11] and proteomics [12–16].

Another way to identify these by-products could be based on another intrinsic characteristic of these materials: their fluorescence. The literature associates the fluorescence of hemoglobin with aromatic amino acids (mainly tryptophan and to a lesser extent tyrosine and phenylalanine) as well as with the hemic group [17–19]. The structure of bovine hemoglobin contains 6 tryptophan residues and 10 tyrosine, 34 phenylalanine and 4 heme moieties. Pig hemoglobin consists of 6 residues of tryptophans, 8 tyrosines, 30 phenylalanines and 4 hemic groups [20].

Constant-wavelength synchronous fluorescence spectroscopy (SFS) consists of scanning both the excitation and emission monochromators simultaneously at the same speed and with a fixed wavelength offset between them. In comparison with conventional fluorescence methods,

Abbreviations: SFS, synchronous fluorescence spectroscopy; PCR, polymerase chain reaction; HorsF, horse feed; PigF, pig feed; AQF, aquafeed; DLA, DIGE labelling buffer; DTT, dithiotreitol; PCA, principal component analysis

* Corresponding author at: Food and Feed Quality Unit of Agricultural Product Valorisation Department – Walloon Agricultural Research Centre, Gembloux, Belgium.
E-mail address: m.lecrenier@cra.wallonie.be (M.-C. Lecrenier).

https://doi.org/10.1016/j.talanta.2018.06.076
Received 23 March 2018; Received in revised form 20 June 2018; Accepted 25 June 2018
Available online 27 June 2018

SFS generates spectra with highly improved resolution, as the spectrum of each component is simplified and the bandwidth is narrowed. Complex overlapping peaks are reduced to one or few narrow peaks, and any interfering Rayleigh and Raman scattering is avoided [21]. SFS is an excellent method for characterizing proteins. Its advantage is that it is a very sensitive and non-destructive way of tracking biological molecules such as hemoglobin. For proteins, the usual aim is to identify tyrosine and tryptophan residues. The literature showed that use of a low offset (15 nm, 17 nm or 20 nm) makes it possible to characterize tyrosine residue, while large offset values (60 nm or 80 nm) correspond to tryptophan residue in the 200–600 nm spectral range [22–25]. Moreover, Hirstch [17] reported that, for hemoglobin, an offset of 17 nm was also considered for heme-destruction species and the micro-environment of tyrosine and tryptophan [22]. The spectral resolution is often set to 1 nm, 5 nm or 10 nm [22,26,27], depending on the spectral resolution needed. On the other hand, the concentration of hemoglobin and the solvent have a considerable effect on the spectra recorded and so have to be considered. The hemoglobin concentration is generally low ($< 5\,\mu M$) in order to avoid quenching phenomena [17]. The solvent commonly used is phosphate buffer pH 7.2, Tris buffer with pH between 1.9 and 11.5 and acetate buffer at pH 5 [19,27,28]. It should be noted that a variation of pH significantly influences fluorescence spectra [29].

The aim of this study was to develop a fast and easy method, based on synchronous fluorescence spectroscopy (SFS), for detecting any use of blood meal and blood products through the identification of hemoglobin signature. Based on the literature review, the SFS acquisition parameters were selected and used in order to define a hemoglobin fluorescence signature on standards and on feed materials. The SFS method was then applied on adulterated feeds in order to evaluate the limit of detection of the method by the development a PCA model. The study was conducted on various types of feed in order to evaluate the robustness of the method. Finally, this PCA model was applied on a larger amount of commercial feeds to evaluate the applicability on real adulteration conditions.

2. Materials and methods

2.1. Material

2.1.1. Reference material

Lyophilized bovine hemoglobin, porcine hemoglobin and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Diegem, Belgium) and used for standards preparation. These reference materials represent the main proteins present in blood (blood plasma and blood cells included).

2.1.2. Feed material and compound feed

Feed materials and compound feeds were selected from the sample bank of the European Union Reference Laboratory for Animal Proteins in Feedingstuffs (http://eurl.craw.eu/) coordinated by the Walloon Agricultural Research Centre (CRA-W). The samples are originated from various European industries.

A total of 15 feed materials were selected: 11 blood products (9 hemoglobin powders (including Hb pwd01 and Hbpwd02) and 2 plasma powders) and 4 blood meals (including Blm01 and Blm02). These blood-derived products were of porcine, bovine or mixed origins (porcine and bovine). These species represent the main sources of blood-derived products in terms of volume. Their common fluorescence signature has been investigated.

Three types of compound feed were used in this study (Table 1): One feed intended for horse (HorsF), one feed intended for pig (PigF) and 16 feeds for aquaculture (AQF). For AQF, the feeds were classified in two categories depending on the absence (AQF01 to AQF09) or presence (AQF10 to AQF16) of blood-derived products as feed material. Before starting the sample preparation, sub-samples of the feeds were ground to 2 mm with a rotor mill (ZM200, Retsch, Haan, Germany) previously decontaminated with DNA Erase™ (MP Biomedicals Europe N.V., Belgium). Feed materials were not ground as the particle sizes were lower than 2 mm.

Table 1
List of compound feeds, their types and concentration of blood derived products.

Destination	Blood derived product type	Concentration (%)	Name
Horse feed		0%	HorsF
Pig feed		0%	PigF
Aquafeed		0%	AQF01
		0%	AQF02
		0%	AQF03
		0%	AQF04
		0%	AQF05
		0%	AQF06
		0%	AQF07
		0%	AQF08
		0%	AQF09
	Blood meal	7%	AQF10
	Hemoglobin powder	10%	AQF11
	Hemoglobin powder	10%	AQF12
	Hemoglobin powder	11%	AQF13
	Hemoglobin powder	Unknown	AQF14
	Hemoglobin powder	Unknown	AQF15
	Hemoglobin powder	Unknown	AQF16

2.2. Sample preparation

2.2.1. Preparation of the standards

Standards were prepared by dissolving reference materials in phosphate buffer pH 7.2 (Fisher Scientific, New Jersey, USA) or in DIGE labelling buffer (DLA) (urea 7 M (Merck KGaA, Darmstadt, Germany), thiourea 2 M (GE Healthcare, Chalfont, UK), Tris 30 mM (Merck KGaA, Darmstadt, Germany) and CHAPS 4% (VWR, Ohio, USA)), at a final concentration of 3 µM for hemoglobin standards and 10 µM for BSA standard to avoid any quenching effect.

2.2.2. Preparation of the adulterated feeds

Adulterated feeds (HorsF, PigF and AQF09) were prepared using the stepwise dilution procedure [30] in order to ensure homogeneity in the test portions. Seven increasing levels (0%; 0.01%; 0.05%; 0.1%; 0.5%; 1%; 5%; 10% (w/w)) were prepared. HorsF was adulterated with two different types of hemoglobin powder (Hb pwd01 and Hb pwd02) and blood meal (Blm01 and Blm02) while PigF and AQF09 were adulterated with one hemoglobin powder (Hb pwd01) and one blood meal (Blm01). This sample set is summarized in Table 2.

2.2.3. Protein extraction

All test portions from feed materials, compound feeds and adulterated feeds were prepared in duplicate and extracted separately. The protocol used was based on the protocol described by Lecrenier, Marbaix, Dieu, Veys, Saegerman, Raes and Baeten [12] with minor modifications. Extraction was performed in 2 ml test tubes containing 200 mg of test portion in which 1.8 ml of trichloroacetic acid (Acros organics, Geel, Belgium) / acetone solution (Merck KGaA, Darmstadt, Germany) (10:90 v/v) with 0.3% dithiotreitol (DTT) (Merck KGaA, Darmstadt, Germany) was added. The tubes were stored overnight at −20 °C. The test tubes were then centrifuged for 10 min at 16000 g at 4 °C. The supernatants were discarded and the 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 at −20 °C for 1 h, 30 min and 30 min respectively. Tubes were centrifuged after each washing. The supernatants were discarded and the final pellets were air-dried for a few minutes followed by the addition of 1 ml of DLA in each tube. Pellets were dispersed using ultrasonication (UIS250V, Hielscher, Ultra-sound

Table 2
Sample set of adulteration samples.

		Compound feeds		
		HorsF	**PigF**	**AQF09**
Adulteration levels	**0.01**	Blm01/Blm02/Hb pwd01/Hb pwd02	Blm01/Hb pwd01	Blm01/Hb pwd01
	0.05	Blm01/Blm02/Hb pwd01/Hb pwd02	Blm01/Hb pwd01	Blm01/Hb pwd01
	0.1	Blm01/Blm02/Hb pwd01/Hb pwd02	Blm01/Hb pwd01	Blm01/Hb pwd01
	0.5	Blm01/Blm02/Hb pwd01/Hb pwd02	Blm01/Hb pwd01	Blm01/Hb pwd01
	1	Blm01/Blm02/Hb pwd01/Hb pwd02	Blm01/Hb pwd01	Blm01/Hb pwd01
	5	Blm01/Blm02/Hb pwd01/Hb pwd02	Blm01/Hb pwd01	Blm01/Hb pwd01
	10	Blm01/Blm02/Hb pwd01/Hb pwd02	Blm01/Hb pwd01	Blm01/Hb pwd01
# adulterated samples:		28	14	14

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, after a centrifugation for 10 min at 16000*g* at 12 °C, the supernatants were stored at −20 °C until analyses. For SFS analysis, 20 µl of each protein extract was diluted in 2.98 ml of DLA.

2.3. Synchronous fluorescence spectroscopy

Synchronous fluorescence spectra were obtained with a Fluoromax®-4 spectrofluorometer using FluorEssence™ V3.5 software (Horiba Scientific via Acal BFi Nederland B.V). The analytical parameters used were based on a review of the literature in which acquisition parameters were investigated. The excitation and emission slits were fixed at 5.5 nm and 6 nm respectively. The excitation and emission wavelengths were scanned simultaneously using a wavelength resolution of 1 nm in the 200–600 nm range between them. Appropriate conditions (solvent) and constant wavelength interval (offset) were determined for hemoglobin detection during the optimization work. Standards and all extracts of feed materials were analyzed in triplicate. Duplicate measurements were made on 79% of the compound feed extracts and data were averaged in order to have one spectrum per extract. The rest has been measured only once due to technical issues. That was possible regarding the good repeatability of fluorescence spectroscopy measurements. The relative standard deviation for repeatability was equal to 1.43%. It has been calculated on the basis of values of the intensity of the most important band of hemoglobin at 485 nm. This value is due to common errors of solution preparation that induces variation in fluorescence intensity but still in the range defined for pharmaceutical compounds (relative standard deviation of 1.0% for a drug substance and of 2.0% for a drug product assay) [31].

2.4. Statistical analysis

Data were area normalized then treated by Principal component analysis (PCA). This method has the advantage of extracting the systematic variations in one data set [32]. PCA is oriented towards modeling the variance/covariance structure of the data matrix into a model which represents significant variations and which considers noise as an error [33]. It is an unsupervised exploratory method in which classification may be done on the basis of the scores, and the characteristics of each species are established by the interpretation of these specific loadings.

2.5. Software

ORIGIN software was used for synchronous fluorescence spectra collection.

Chemometric treatments (Area normalization and PCA) were performed by the UNSCRAMBLER software version X.5 from CAMO (Computer Aided Modeling, Trondheim, Norway).

3. Results and discussion

3.1. Optimization of acquisition parameters of fluorescence spectra

For this study, preliminary analyses were conducted with standards, mainly to determine the nature of the appropriate solvent and the most suitable wavelength offset. Because compounds are usually diluted in buffer solutions, phosphate buffer was tested, as well as DLA as this was the solvent employed for protein extraction. SFS spectra were then collected with a low (17 nm) and a high (80 nm) offset value.

The spectra obtained by SFS analysis of the standard solutions (BSA, porcine and bovine hemoglobin) in the phosphate buffer (pH = 7.2) and DLA (pH = 10.5) using the offset values of 17 nm and 80 nm are shown in Fig. 1. The spectra of DLA and phosphate buffer are also presented.

From this figure, we can observe that:

- Spectra of hemoglobin standards (Fig. 1a) diluted in DLA collected with an offset of 17 nm show four bands at 360 nm, 406 nm, 484 nm and 538 nm. Fluorescence bands of BSA are pointed at 360 nm and 406 nm with a small shoulder at 484 nm. DLA presents almost the same spectrum as that of BSA. The only difference is the absence of the small shoulder visible at 484 nm. The bands observed at 484 nm and 538 nm are therefore characteristic of hemoglobin.
- Spectra of hemoglobin standards (Fig. 1b) diluted in DLA recorded with an offset of 80 nm show bands at 440 nm and 518 nm with a shoulder at 548 nm. On the spectrum of BSA, only one band is observed at 427 nm with a shoulder at 518 nm. DLA presents a broad band with two maxima at 410 nm and 440 nm in addition to a shoulder pointed at 518 nm. With this offset, it seems that the characteristic band of hemoglobin appears at 518 nm with the shoulder at 548 nm. However, the problem is the overlap with the fluorescence, even if it is weak, of BSA and DLA, which is also visible at 518 nm.
- Spectra of hemoglobin standards (Fig. 1c) diluted in phosphate buffer obtained with an offset 17 nm exhibit a fluorescence band at 308 nm close to that of BSA visible at 302 nm. Hence this region cannot be employed for hemoglobin detection. The weak band composed of two maxima pointed at 445 nm and 470 nm can be used for the detection of hemoglobin, but the problem is its weakness which makes detection difficult at low concentrations.
- Spectra of standards (Fig. 1d) diluted in phosphate buffer collected with an offset of 80 nm show an overlap between bands of hemoglobin diluted in the buffer and BSA: hemoglobin has a fluorescence band at 352 nm while that of BSA is at 355 nm. This band cannot be used for the identification of hemoglobin. On the other hand, at higher wavelengths (400–500 nm), a remarkable overlap between hemoglobin and BSA still exists which makes it difficult to assign a specific band to the detection of hemoglobin. However, this region could be interesting if the aim is to discriminate porcine hemoglobin and bovine hemoglobin, which seem to have different spectral profiles in this region. Finally, the band at 518 nm could be used, but it is very weak and hence unsuitable when working with very low concentrations.

From these results, DLA and the 17 nm offset were selected as the best parameters for the purpose of this work: the detection of hemoglobin in feed material. The combination of these two factors makes

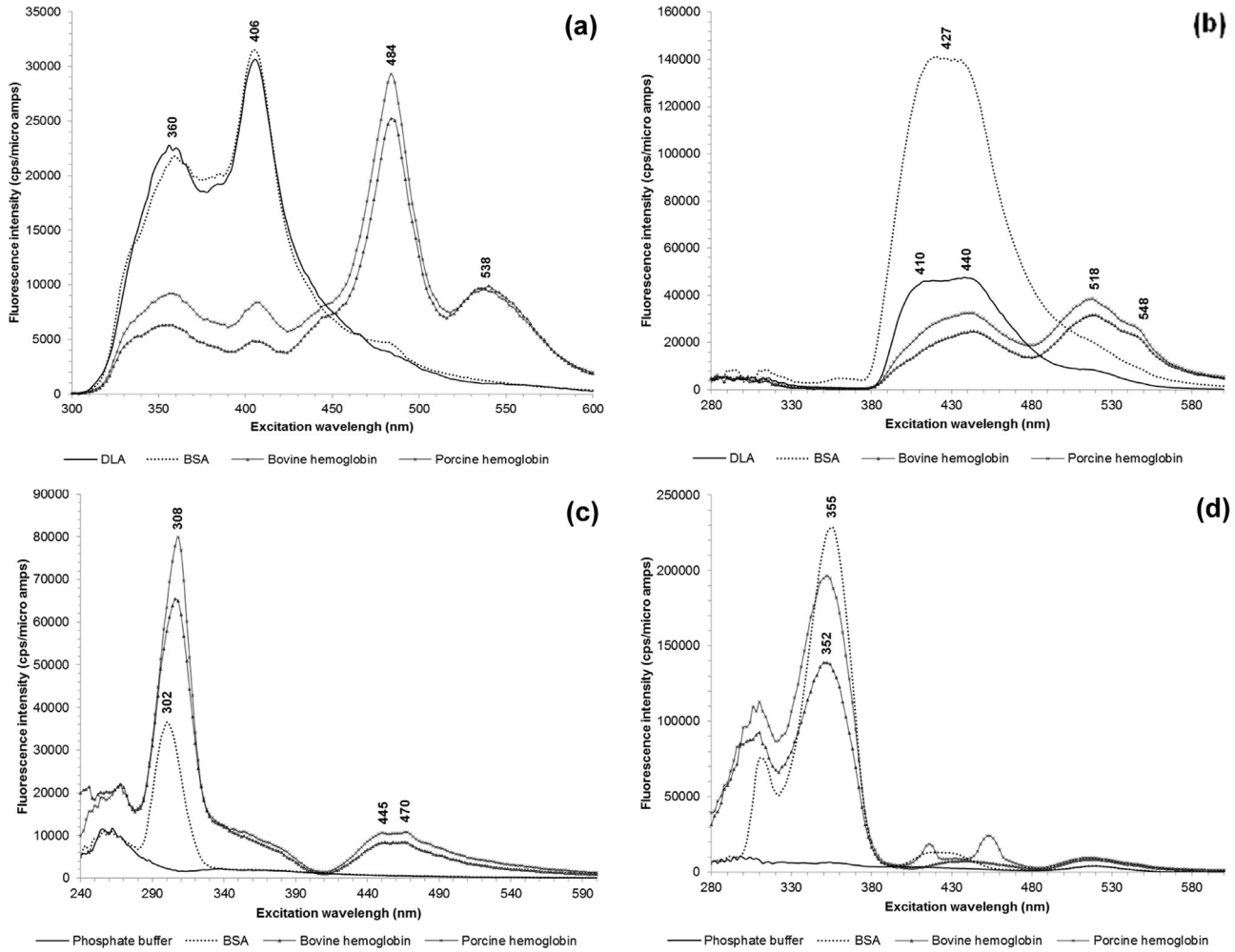

Fig. 1. Synchronous fluorescence spectra of standard solutions (BSA, porcine and bovine hemoglobin) in DLA (a & b) or phosphate buffer (c & d) using the offset values of 17 nm (a & c) or 80 nm (b & d).

it possible to highlight fluorescence bands of hemoglobin at 484 nm and 538 nm independently if it is of bovine or porcine origin.

3.2. Identification of the hemoglobin fluorescence signature

As can be observed from Fig. 1a, porcine and bovine hemoglobin standards have the same fluorescence spectral signature with an important band around 484 nm and a small band around 538 nm. Two other very small bands appear around 360 nm and 406 nm which are due to the presence of DLA as solvent. DLA has almost the same profile as BSA.

As already reported in the introduction, bands observed on hemoglobin's spectra are mainly due to fluorophores present in its structure that are aromatic amino acids (mainly tryptophan, tyrosine and phenylalanine) in addition to the hemic group [18,19]. According to the literature the maximum emission of tryptophan, tyrosine and phenylalanine is at 350 nm, 303 nm and 282 nm respectively [34,35].

In our study, using an offset of 17 nm, hemoglobin diluted in phosphate buffer showed a fluorescence band at 308 nm that can be attributed to tyrosine [22,24,36]. When hemoglobin was diluted in DLA, the spectral profile changed. Bands at 360 nm, 406 nm, 484 nm and 538 nm were pointed. DLA contains 7 M of urea (10.5 g per 25 ml of distilled water), which is a denaturing agent that can lead to a partial loss of the secondary structure of hemoglobin [37]. This denaturation may influence the hemoglobin's fluorescence [19,38], as it has been known for many years that urea is a chemical agent which induces protein denaturation by disturbing the non-covalent interactions [39–42]. Accordingly, Carvalho, Santiago and Tabak [43] reported that high concentrations of urea ($>$ 4.0 M) lead to significant structural disorder and changes in the native state, promoting oligomeric dissociation, protein denaturation and alteration of the coordination sphere of the heme iron. However, in our case, fluorescence bands of BSA and DLA are pointed at 360 nm and 406 nm, overlapping with those of hemoglobin. It is therefore difficult to observe the change in the micro-environment of tyrosine due to the presence of DLA. This means that only bands pointed at 484 nm and 538 nm are characteristic of hemoglobin. In their study of human hemoglobin structural and functional alterations and heme degradation on interaction with benzene, Hosseinzadeh and Moosavi-Movahedi [22] reported that the interval of $\Delta\lambda = 17$ nm was also used to determine the new chromophoric heme destruction species produced in the reaction samples due to the effects of benzene on hemoglobin. In our case, the same could be true; what we observe is due to conformational changes of heme after denaturation of hemoglobin by DLA.

In order to explain these bands, a parallel can be made with the study of the stability of the extracellular hemoglobin of Glossoscolex paulistus in the presence of urea. This was conducted by Carvalho, Santiago and Tabak [43], who observed absorption bands at 360 nm, 404 nm, 531 nm, 567 nm and 631 nm on the spectrum of that hemoglobin at high concentrations of urea (8 M), suggesting a mixture of several species in equilibrium: aquomet hemoglobin, penta-coordinate hemoglobin, hemichrome, and high-spin species iron complexes

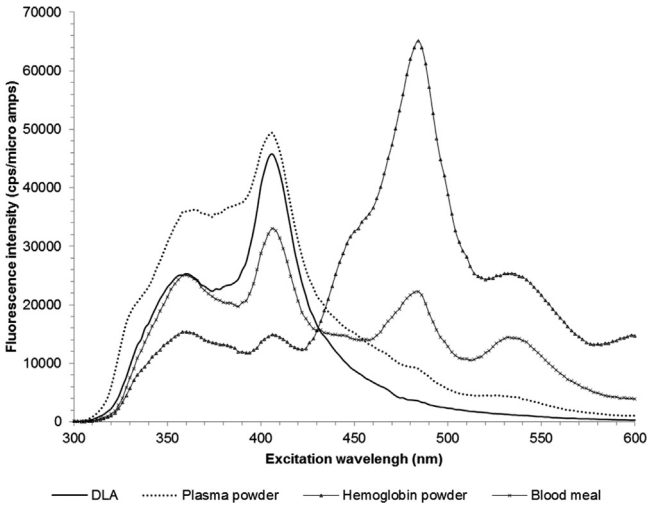

Fig. 2. Synchronous fluorescence spectra of protein extracts of commercial feed material derived from blood diluted in DLA ($\Delta\lambda = 17$ nm).

[44,45].

The fluorescence bands observed in this study at 484 nm and 538 nm are very probably due to these species. These associations need to be confirmed by other analytical methods as UV–Vis absorption and/or circular dichroism techniques.

3.3. Validation on feed materials (hemoglobin powder, plasma powder and blood meal)

In order to confirm that the bands observed with standards are also present in protein extracts of commercial feed material derived from blood, the selected settings were applied to some selected feed materials.

Because blood meal contains plasma powder as well as hemoglobin, it was essential to examine their respective spectral signatures when they are extracted from feed materials. At the same time, DLA's fluorescence signal had to be considered since, as explained above, all samples studied in this work were diluted / extracted in DLA. Synchronous fluorescence spectra of these compounds were recorded and are presented in Fig. 2.

As can be observed from this figure, the spectrum of plasma powder extracts presents a band around 406 nm with a shoulder at 360 nm. A little peak can be noticed around 484 nm. DLA has the same signature as plasma powder extracts without the little peak at 484 nm. The spectrum of hemoglobin powder extracts present four signals around 360 nm, 406 nm, 484 nm and 538 nm. The most intense is the one pointed at 484 nm. Therefore, it can be concluded that the bands observed at 484 nm and 538 nm are characteristic of hemoglobin. Blood meal extracts present the four indicated bands with approximately equal intensity, with a slightly higher value for the band at 406 nm.

3.4. Application of the SFS method for the analysis of adulterated feed and commercial aquafeeds

Compound feeds (HorsF, PigF and AQF09) adulterated at various contamination levels were also analyzed in order to experimentally determine the limit of detection. The collected spectra were area-normalized and then subjected to PCA in order to differentiate samples according to both feed type and adulteration percentage.

Fig. 3 shows these samples separated according to the origin of feed independently of the adulteration material (hemoglobin or blood meal). In both cases of adulteration, samples are distributed according to the second component, corresponding to 14% and 26% of the explained variance for hemoglobin and blood meal adulterated compounds respectively. The highest values of explained variance are due to the percentage of adulteration: Figs. 4a and 5a show the distribution of samples according to the adulteration rate (from 0.01% to 10%) along the first component, corresponding to 79% and 57% of the explained variance for hemoglobin- and blood meal-adulterated compounds respectively. This may indicate that whatever the type of feed, adulteration with hemoglobin or blood meal can be detected and has a significant effect.

Fig. 4a shows the PCA applied on extracts of feed samples adulterated with hemoglobin from 0.01% to 10%. As observed, the most highly contaminated samples (5% and 10%) are clearly distinguished. However, through the traced line, it can be observed that adulteration can be detected from lower percentages of between 0.5% and 1% depending on the sample. The next step in verifying if real feed samples can be evaluated on the basis of this PCA model is presented in Fig. 4b, in which the samples have been projected in the space of the PCA hemoglobin adulteration model. Commercial aquafeeds known not to contain any blood-derived products (AQF01, AQF02, AQF03, AQF04, AQF05, AQF06, AQF07 and AQF08) are situated in the zone of samples which are not adulterated or contain a maximum of 0.5% of hemoglobin. Aquafeeds with a high percentage of hemoglobin powder (10% and 11%) (AQF11, AQF12 and AQF13) are superimposed on those adulterated at 10% and even higher. Samples with an unknown percentage of adulteration (AQF14, 15, 16) seem to have a hemoglobin value of between 5% and 10%.

In order to evaluate the performance of the projection of those real samples into the PCA hemoglobin model, spectral residuals and leverage values were studied. Results show that most of projected samples are superimposed to calibration ones. The residual variance is included between 2e-07 and 1e-06 while the leverage values are between 0.016 and 0.15. However, some commercial samples (AQF01, AQF03, AQF04 and AQF07) present a high residual variance (included in the range between 2e-06 and 1e-05) meaning they are less described by the original model. That observation is expected because commercial samples, even they are all aquafeeds, are different from the calibration

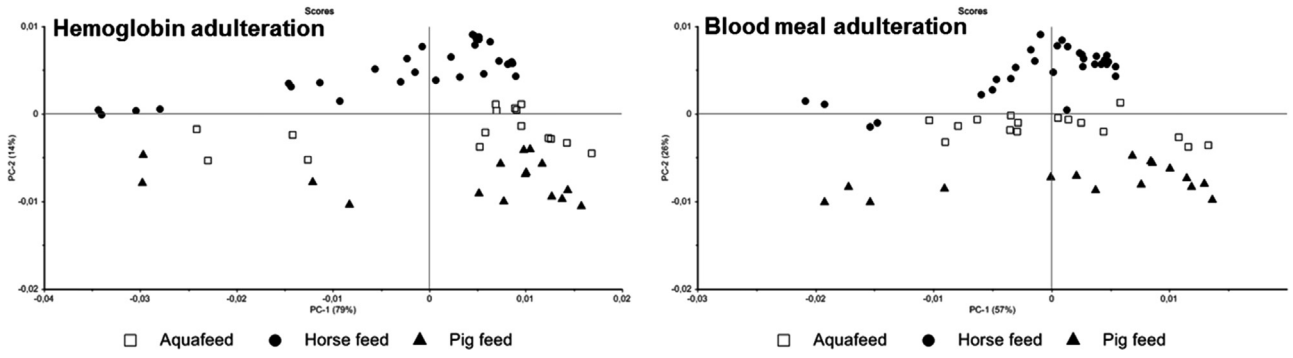

Fig. 3. PCA applied to area-normalized SFS data of extracts of feed samples adulterated with hemoglobin and blood meal.

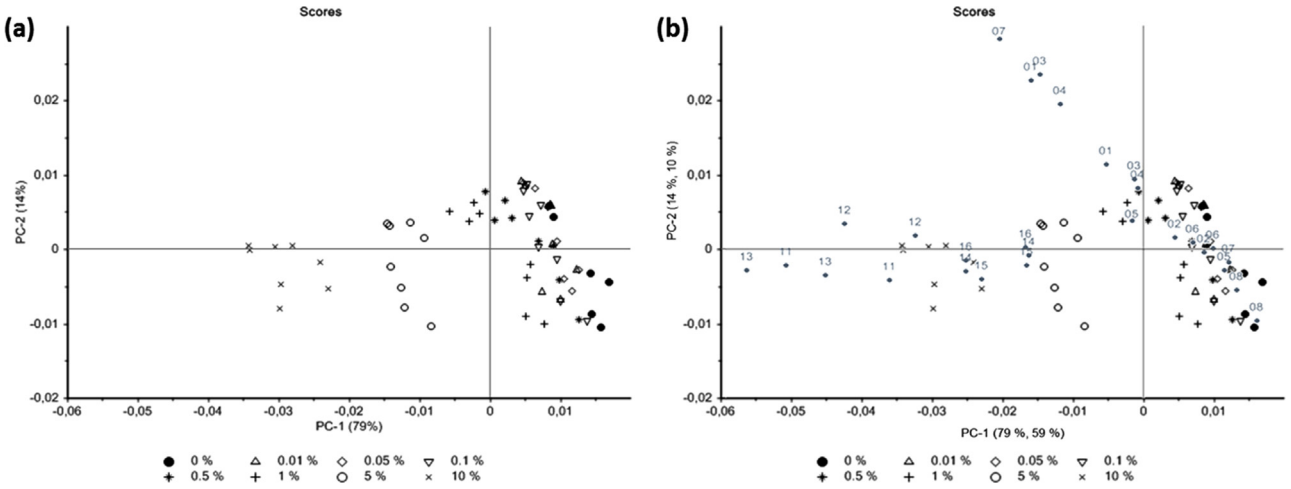

Fig. 4. PCA applied to area-normalized SFS data of extracts of feed samples adulterated with hemoglobin (a) and the projection of real samples in PCA space (b). Numbers correspond to the aquafeed names (AQF##).

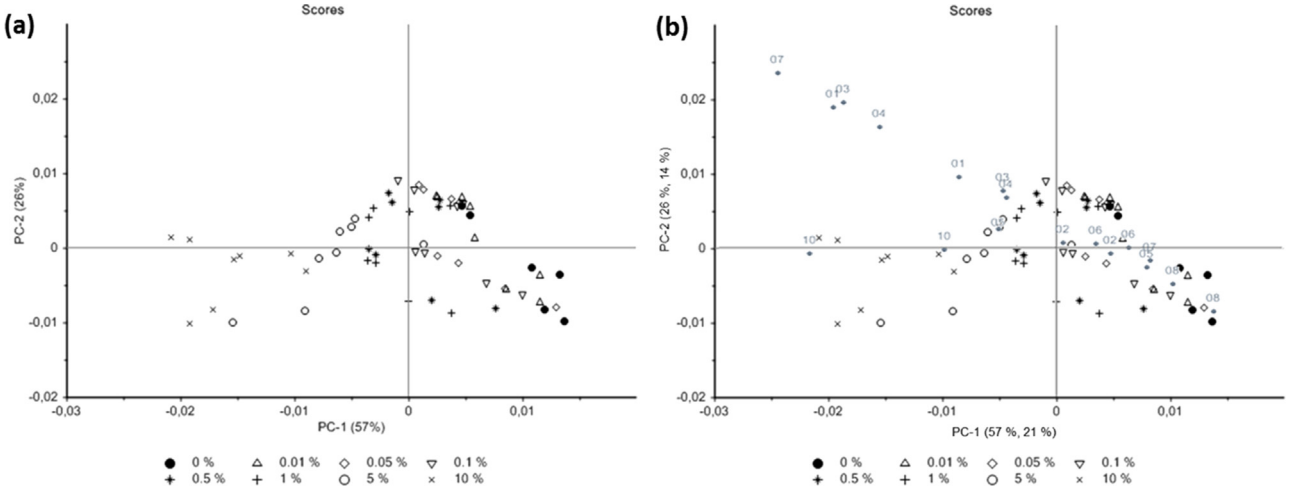

Fig. 5. PCA applied to area-normalized SFS data of extracts of feed samples adulterated with blood meal (a) and the projection of real sample in PCA space (b). Numbers correspond to the aquafeed names (AQF##).

ones; the only similarity is the absence of adulteration. That was also confirmed by their spectral profile which shows an additional shoulder around 454 nm (data not shown). Three other real samples (AQF11, AQF12 and AQF13) with high percentage of hemoglobin powder (10% and 11%) present a high leverage (included between 0.18 and 0.3). They are projected far from the center of the original model.

The same procedure was carried out for feeds adulterated with blood meal. Fig. 5 presents both the PCA plot of the distribution of extracts of feed samples according to the blood meal adulteration rate (5a) varying from 0.01% to 10% and the projection of real sample spectra in the space defined by the two principal components (5b); the same behavior is observable as with the hemoglobin adulteration model. In fact the limit of detection seems to be less easy to define: the samples are more mixed, although a slight separation from a percentage of 0.5% can be observed. When aquafeeds known to be free of blood-derived products are projected on PCA graph, here too they are located in the same area as commercial samples which are not adulterated or are adulterated at percentages lower that 0.5%. The percentage of blood meal in AQF10, which is known to be around 7%, is overestimated in this case. The sample overlaps with those adulterated at 10%.

In the same way as for adulteration with hemoglobin, performance of the projection of real samples into the PCA blood meal model, values of leverage and residual variance for the first component have been investigated. Data show that all samples are well described by the PCA model. Values of residuals belong to the range between 2e-07 and 2e-06. The leverage range varies between 0.016 and 0.15. However, four samples (AQF01, AQF03, AQF04 and AQF07) exhibit a higher residual variance values included between 4e-06 and 1e-05. These samples are the ones mentioned above with high residual values in the case of hemoglobin adulteration.

To understand how these PCA models can describe this adulteration with even hemoglobin or blood meal, the loadings corresponding to the first components of PCA graphs of hemoglobin and blood meal adulteration were examined. They present the same profile, i.e. the synchronous fluorescence spectrum of hemoglobin with its characteristic band around 485 nm and 536 nm (Fig. 6). These bands located in the negative part of the loading correspond to samples with high percentages of adulteration. A small shift can be noted if we compare with the bands of hemoglobin standard described at the beginning of the article (484 nm and 538 nm). This can be explained by the difference of environment. In the first case, hemoglobin was analyzed alone as a standard, whereas now it is included in a feed matrix.

The positive part of the loading clearly shows bands of DLA present in all studied samples. The spectral profiles of hemoglobin are well defined and guide the discrimination of samples according to their adulteration rate.

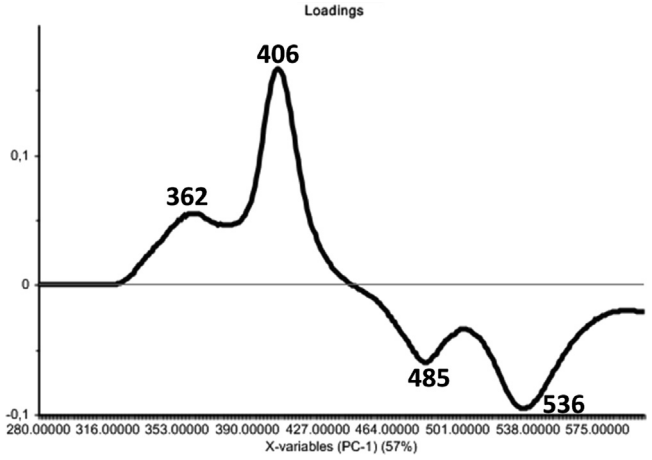

Fig. 6. Loading corresponding to the first component of PCA models established on area-normalized SFS data of samples adulterated with hemoglobin or blood meal.

4. Conclusion

In this study, an SFS method was developed for the detection of hemoglobin in various animal feeds. SFS combined with some simple chemometric tools such as PCA is appropriate for detecting the presence of hemoglobin in feed material with a limit of detection of hemoglobin or blood meal from 0.5% to 1% depending on the feed material. Projection of real commercial compound feeds on the PCA graph shows the potential of the method to estimate hemoglobin or blood meal content.

Future work will need to focus on optimizing the extraction method in order to increase the sensitivity. Another improvement would be the reduction of the extraction time in the context of a rapid method. The optimized method will then have to be applied on high number of feeds adulterated at various levels in order to build and valid a robust reference PCA model.

Acknowledgements

Acknowledgements to Quentin Arnould, Marie Collard, Benoit Scaut and Valentin Dubois for their help in carrying out this study, and to Vincent Servais for the pig picture.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] European Commission, Commission Regulation (EU) No 51/2013 of 16 January 2013 amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed Text with EEA relevance, Off. J. Eur. Union L 20, 2013, pp. 33–43.
[2] V. Baeten, C. von Holst, A. Garrido, J. Vancutsem, A. Michotte Renier, P. Dardenne, Detection of banned meat and bone meal in feedstuffs by near-infrared microscopic analysis of the dense sediment fraction, Anal. Bioanal. Chem. 382 (1) (2005) 149–157.
[3] A. Boix, J.A. Fernández Pierna, C. von Holst, V. Baeten, Validation of a near infrared microscopy method for the detection of animal products in feedingstuffs: results of a collaborative study, Food Addit. Contam. Part A 29 (12) (2012) 1872–1880.
[4] Z. Yang, L. Han, J.A. Fernández Pierna, P. Dardenne, V. Baeten, Review: the potential of near infrared microscopy to detect, identify and quantify processed animal by-products, J. Infrared Spectrosc. 19 (4) (2011) 211–231.
[5] X. Liu, L. Han, P. Veys, V. Baeten, X. Jiang, P. Dardenne, An overview of the legislation and light microscopy for detection of processed animal proteins in feeds, Microsc. Res. Tech. 74 (8) (2011) 735–743.
[6] L.W.D. van Raamsdonk, L. Pinotti, P. Veys, M. Bremer, W. Hekman, A. Kemmers, A. Campagnoli, C. Paltanin, C.B. Crespo, J. Vliege, V. Pinckaers, J.S. Jørgensen, New developments in classical microscopy; what can be expected for the official control? Biotechnol. Agron. Soc. Environ. 15 (2011) 15–24.
[7] M.C. Lecrenier, Q. Ledoux, G. Berben, O. Fumière, C. Saegerman, V. Baeten, P. Veys, Determination of the ruminant origin of bone particles using fluorescence in situ hybridization (FISH), Sci. Rep. 4 (2014).
[8] S. Axmann, A. Adler, A.J. Brandstettner, G. Spadinger, R. Weiss, I. Strnad, Species identification of processed animal proteins (PAPs) in animal feed containing feed materials from animal origin, Food Addit. Contam. Part A (2015) 1–10.
[9] O. Fumière, A. Marien, J.A. Fernández Pierna, V. Baeten, G. Berben, Development of a real-time PCR protocol for the species origin confirmation of isolated animal particles detected by NIRM, Food Addit. Contam. Part A 27 (8) (2010) 1118–1127.
[10] L.W.D. van Raamsdonk, R.J.C.F. Margry, R.G.C. Van Kaathoven, M.G.E.G. Bremer, Inter-laboratory validation study of two immunochemical methods for detection of processed ruminant proteins, Food Chem. 185 (2015) 333–339.
[11] A.C. Huet, C. Charlier, E. Deckers, H. Marbaix, M. Raes, S. Mauro, P. Delahaut, N. Gillard, Peptidomic approach to developing ELISAs for the determination of bovine and porcine processed animal proteins in feed for farmed animals, J. Agric. Food Chem. 64 (47) (2016) 9099–9106.
[12] M.C. Lecrenier, H. Marbaix, M. Dieu, P. Veys, C. Saegerman, M. Raes, V. Baeten, Identification of specific bovine blood biomarkers with a non-targeted approach using HPLC ESI tandem mass spectrometry, Food Chem. 213 (2016) 417–424.
[13] H. Marbaix, D. Budinger, M. Dieu, O. Fumière, N. Gillard, P. Delahaut, S. Mauro, M. Raes, Identification of proteins and peptide biomarkers for detecting banned processed animal proteins (PAPs) in meat and bone meal by mass spectrometry, J. Agric. Food Chem. 64 (11) (2016) 2405–2414.
[14] J.D. Rasinger, H. Marbaix, M. Dieu, O. Fumière, S. Mauro, M. Palmblad, M. Raes, M.H.G. Berntssen, Species and tissues specific differentiation of processed animal proteins in aquafeeds using proteomics tools, J. Proteom. 147 (2016) 125–131.
[15] D. Marchis, A. Altomare, M. Gili, F. Ostorero, A. Khadjavi, C. Corona, G. Ru, B. Cappelletti, S. Gianelli, F. Amadeo, C. Rumio, M. Carini, G. Aldini, C. Casalone, LC-MS/MS identification of species-specific muscle peptides in processed animal proteins, J. Agric. Food Chem. 65 (48) (2017) 10638–10650.
[16] M.C. Lecrenier, M. Planque, M. Dieu, P. Veys, C. Saegerman, N. Gillard, V. Baeten, A mass spectrometry method for sensitive, specific and simultaneous detection of bovine blood meal, blood products and milk products in compound feed, Food Chem. 245 (Suppl. C) (2018) 981–988.
[17] R.E. Hirstch, Hemoglobin fluorescence, in: R.L. Nagel (Ed.), Hemoglobin Disorders: Molecular Methods and Protocols, Humana Press, Totowa, NJ, 2003, pp. 133–154.
[18] A.H. Hegde, B. Sandhya, J. Seetharamappa, Investigations to reveal the nature of interactions of human hemoglobin with curcumin using optical techniques, Int. J. Biol. Macromol. 52 (2013) 133–138.
[19] S. De, A. Girigoswami, A fluorimetric and circular dichroism study of hemoglobin–effect of pH and anionic amphiphiles, J. Colloid Interface Sci. 296 (1) (2006) 324–331.
[20] E.Y. Adje, Hydrolyse ménagée de l'hémoglobine bovine par la pepsine porcine en mélanges hydro-alcoolique et obtention d'une nouvelle famille de peptides anti microbiens, University of Lille1 - Sciences and Technologies, 2010, p. 231.
[21] O. Abbas, C. Rébufa, N. Dupuy, A. Permanyer, J. Kister, D.A. Azevedo, Application of chemometric methods to synchronous UV fluorescence spectra of petroleum oils, Fuel 85 (17) (2006) 2653–2661.
[22] R. Hosseinzadeh, A.A. Moosavi-Movahedi, Human hemoglobin structural and functional alterations and heme degradation upon interaction with benzene: a spectroscopic study, Spectrochim. Acta A Mol. Biomol. Spectrosc. 157 (2016) 41–49.
[23] M. Makarska-Bialokoz, Interactions of hemin with bovine serum albumin and human hemoglobin: a fluorescence quenching study, Spectrochim. Acta A Mol. Biomol. Spectrosc. 193 (2018) 23–32.
[24] A. Chakrabarti, D. Bhattacharya, S. Deb, M. Chakraborty, Differential thermal stability and oxidative vulnerability of the hemoglobin variants, HbA2 and HbE, PLoS One 8 (11) (2013) e81820.
[25] J. Chou, X.G. Qu, T.H. Lu, S.J. Dong, Y. Wu, The effect of solution pH on synchronous fluorescence spectra of cytochrome c solutions, Microchem. J. 52 (2) (1995) 159–165.
[26] S. Pang, S. Liu, X. Su, A novel fluorescence assay for the detection of hemoglobin based on the G-quadruplex/hemin complex, Talanta 118 (Suppl. C) (2014) 118–122.
[27] K.R. Grigoryan, L.S. Sargsyan, Denaturation of hemoglobin in the presence of tannic acid, Proc. YSU Chem. Biol. 1 (2014) 23–27.
[28] A. Barati, M. Shamsipur, H. Abdollahi, Hemoglobin detection using carbon dots as a fluorescence probe, Biosens. Bioelectron. 71 (Suppl. C) (2015) 470–475.
[29] O. Abbas, Vieillissement simulé ou naturel de la matière organique. Apport du traitement chimiométrique des données spectroscopiques. Conséquences environnementales et aide à l'exploration pétrolière, University of Aix-Marseille III - Paul Cezanne, 2007, p. 329.
[30] G. Gizzi, C. von Holst, V. Baeten, G. Berben, L. van Raamsdonk, Determination of processed animal proteins, including meat and bone meal, in animal feed, J. AOAC Int. 87 (6) (2004) 1334–1341.
[31] United States Pharmacopeia, Fluorescence Spectroscopy, USP-NF2018, pp. 1–7.
[32] H. Martens, T. Næs, Multivariate Calibration, Wiley, New York, 1989.
[33] S. Millar, P. Robert, M.F. Devaux, R.C.E. Guy, P. Maris, Near-infrared spectroscopic measurements of structural changes in starch-containing extruded products, Appl. Spectrosc. 50 (9) (1996) 1134–1139.
[34] N. Ramanujam, Fluorescence spectroscopy of neoplastic and non-neoplastic tissues, Neoplasia 2 (1–2) (2000) 89–117.
[35] A. Rodat, Mise au point d'un système CE-LIF intégré et application à l'analyse de biomolécules, University of Toulouse III – Paul Sabatier, 2009, p. 188.

[36] J.N. Miller, Recent developments in fluorescence and chemiluminescence analysis. Plenary lecture, Analyst 109 (3) (1984) 191–198.
[37] J. Gonzalez-Jimenez, M. Cortijo, Urea-induced denaturation of human serum albumin labeled with acrylodan, J. Protein Chem. 21 (2) (2002) 75–79.
[38] A. Sułkowska, B. Bojko, J. Równicka, D. Pentak, W. Sułkowski, Effect of urea on serum albumin complex with antithyroid drugs: fluorescence study, J. Mol. Struct. 651–653 (Suppl. C) (2003) 237–243.
[39] C.N. Pace, The stability of globular proteins, CRC Crit. Rev. Biochem. 3 (1) (1975) 1–43.
[40] C.N. Pace, Determination and analysis of urea and guanidine hydrochloride denaturation curves, Methods Enzymol. 131 (1986) 266–280.
[41] C. Tanford, Protein denaturation, Adv. Protein Chem. 23 (1968) 121–282.
[42] F. Vanzi, B. Madan, K. Sharp, Effect of the protein denaturants urea and guanidinium on water structure: a structural and thermodynamic study, J. Am. Chem. Soc. 120 (41) (1998) 10748–10753.
[43] F.A. Carvalho, P.S. Santiago, M. Tabak, On the stability of the extracellular hemoglobin of Glossoscolex paulistus, in two iron oxidation states, in the presence of urea, Arch. Biochem. Biophys. 519 (1) (2012) 46–58.
[44] Y. Sugawara, A. Matsuoka, A. Kaino, K. Shikama, Role of globin moiety in the autoxidation reaction of oxymyoglobin: effect of 8 M urea, Biophys. J. 69 (2) (1995) 583–592.
[45] A.L. Poli, L.M. Moreira, Á.A. Hidalgo, H. Imasato, Autoxidation studies of extracellular hemoglobin of Glossoscolex paulistus at pH 9: cyanide and hydroxyl effect, Biophys. Chem. 114 (2) (2005) 253–260.