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Microscopy in combination with image analysis for characterization of fishmeal material in aquafeed

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ABSTRACT

The aim of this study was to investigate the use of microscopy in combination with image analysis (IA) measurements for the characterization of fish bone lacunae in aquafeed-extracted material. For this purpose two experiments have been conducted.

In experiment (exp.) 1, six samples of fish meal based aquafeed were analyzed by the microscopic method, according to Annex VI of Regulation 152/2009. Sediment fractions of each sample were observed with a compound microscope at ×40. Two hundred and fifty eight bone fragment lacunae images were recorded and processed through IA software. Accordingly, on each lacuna 30 geometric variables have been obtained and measured. The geometric variables have been grouped in two main families, namely size descriptors and derived shape descriptors.

In exp. 2 measurements obtained from 1081 bone lacunae (644 for mammals and 437 for poultry) acquired from 14 mammalian and 7 poultry samples have been merged with the aquafeed dataset (258 bone lacunae). Results obtained in exp. 1 indicated that nearly two thirds of the descriptors presented differences among the analyzed samples. Differences in observed values were not systematically distributed among the six samples. Nevertheless, in all analyzed samples features of lacunae have shown an overlap. By contrast the comparison of fish bone lacunae with avian and mammalian bone lacunae (exp. 2), has revealed a large gap between terrestrial and aquatic animals in the case of several descriptors (16 primary and 8 secondary descriptors). Therefore, it can be concluded that combining light microscopy and image analysis: (i) fish material in aquafeed appears quite homogenous in term of bone features; (ii) fish material can be distinguished from avian and mammalian materials by selecting specific descriptors.

1. Introduction

The recent revision of the feed ban rules (European Commission, 2013b), which re-authorized from 1 June 2013 processed animal proteins (PAPs) from non-ruminants for use as feed or feed ingredient in aquaculture, is a new scenario in both animal nutrition and feed analysis. In fact, reintroducing non-ruminant PAPs in feed for aquaculture represents a big challenge for

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the feed sector, since old and new analytical and characterization requirements need to be addressed. In the EU only two methods are allowed within the frame of official controls for the detection of animal proteins in feed, namely light microscopy and polymerase chain reaction (PCR), which delivers information on the species origin of the detected PAPs (Regulation (EC) No 152/2009; Regulation (EU) No 51/2013). Both methods have been validated for proper implementation of the feed ban. Light microscopy in combination with computer image analysis (IA), which is based on the identification of bone particles or tissues in feedingstuffs, has been also proposed (Ottoboni et al., 2014; Pinotti et al., 2013; van Raamsdonk et al., 2007). Findings in these studies have indicated that the use of the microscopic method in association with computer image analysis to identify the origin of PAPs appears promising, especially as a complementary method to the DNA-based ones. Other methods are also applied by the feed sector, such as immunoassays and near-infrared microscopy (NIRM) (Tena et al., 2014). In this respect, Tena et al. (2014) have recently investigated a near-infrared microscopy method using partial least squares discriminant analysis (PLS-DA) to differentiate between meat and bone meal and fishmeal. In this study the evaluation of the spectra confirmed the conclusions from former studies that demonstrated that the higher content of polyunsaturated fatty acids in fishmeal compared to meat and bone meal is an important factor for the differentiation of these groups.

However, most of these methods developed and implemented for PAPs identification have been focused on the feed ban for terrestrial animal (Veys et al., 2014), and therefore fish meal characterization, especially in microscopy, was limited and mainly descriptive. In the literature (Makowski et al., 2011) it is reported that lacunae in fish bone fragments can be elliptical or elongated, whereas they can be oval to elliptical in terrestrial animals. With respect to fish material, herring and sardine have elliptical or elongated lacunae with clearly visible canaliculae, with irregularly shaped bone fragments (Makowski et al., 2011; van Raamsdonk et al., 2012). Fish bones of cod and its relatives are normally parallel sided, and show nearly linear lacunae without visible canaliculae, orientated parallel to the sides of the bone fragment. However, several species of fish (e.g., tuna and salmon) have bone lacunae resembling those of land animals (van Raamsdonk et al., 2012) making difficult the discrimination from other animal classes.

In light of this, it is important to implement methods of fishmeal characterization not only in the case of pure material, but also in practical conditions such as in aquafeed. This goal can be achieved in different ways, including the use of methods already developed and applied to terrestrial PAPs. Accordingly, the aim of this study was to investigate the use of microscopy in combination with image analysis measurements for the characterization of fish bone lacunae in aquafeed-extracted material.

2. Material and methods

2.1. Experiment 1

For this experiment, 6 samples of commercial compound fish feeds containing fish meal have been used.

2.1.1. Chemical analysis

Samples have been analyzed for dry matter (DM), crude oils and fats (CF), neutral detergent fiber (NDF), crude protein (CP) and ash. Specifically, the DM of feeds was determined by an oven-drying method, at 130 °C for 2 h, as proposed by the European Commission (Commission Regulation No. 152/2009) and CF was determined by the Soxhlet method, with prior hydrolysis, as proposed by the European Commission (Commission Regulation No. 152/2009). Neutral detergent fiber analysis was performed according to procedures of the AOAC (2005): method 2002.04, using an Ankom 220 fiber analyzer (Ankom^{TM} technology, Fairport, NY, USA); Neutral detergent fiber was measured using heat stable amylase and expressed exclusive of residual ash (aNDFom). Crude protein (CP) content has been measured according to the Kjeldahl method (proc. 2001.11; AOAC, 2005), while ash has been measured by using a muffle furnace at 550 °C (proc. 942.05; AOAC, 2005).

2.1.2. Microscopy and image analysis

For microscopy and image analysis, samples have been previously ground with mortar and pestle. Five grams of ground material were transferred into a separation funnel and treated with 50 ml of tetrachloroethylene in order to obtain the sediment. The total sediment of each sample was dried. If the sediment consisted of many large particles it was sieved in two fractions according to the official procedure (European Commission, 2013a). Subsequently several microscopic slides for each sample were prepared with the sediment fraction using Norland Optical adhesive 65 as embedding agent as reported in the official protocol for the detection of animal particles in compound feed (European Commission, 2013a). After drying each sample was observed using a compound microscope (Olympus BX41; Tokyo, Japan), at several magnifications. In order to guarantee a randomized image acquisition, at least 40 bone fragment lacunae images were randomly acquired at ×40 in each sample without any pre-selection. Specifically, sediment material originating from extracted feeds have been used for preparing at several slides for each sample. Using a digital camera (Retiga 2000R, Fast 1394, QImaging) and image analysis software (Image-Pro Plus 7.0; Media Cybernetics Inc., Rockville, MD, USA), a total of 258 bone fragment lacunae images at ×40, have been collected from the 6 samples. Images were acquired according to Pinotti et al. (2013). After acquisition, each image was processed in order to obtain a monochrome mask for each lacuna, on which thirty geometric variables have been measured (Pinotti et al., 2013). All lacunae measurements were collected in Excel files and used for dataset assembly. According to Ottoboni et al. (2014) geometric variables have been grouped in two groups, namely: size descriptors and derived shape descriptors. The size descriptors, also termed as dimension (primary) descriptors, represent