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Improved approach based on MALDI-TOF MS for establishment of the fish mucus protein pattern for geographic discrimination of *Sparus aurata*

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ABSTRACT

Food fraud is still a recurrent practice throughout food supply chains. In the case of seafood, misidentification of species and products repackaging constitute the most common frauds. Therefore, the development of appropriate analytical approaches to be used against food fraud is necessary. The present study goal is to explore for the first time, the possibility to differentiate between *Sparus aurata* from two different mariculture farms located in Madeira Island (Caniçal and Ribeira Brava), using the mass fingerprint of fish mucus obtained from MALDI-TOF MS and analyzed using Mass-UP software for multivariate statistical analysis and biomarker identification. It was possible to establish, from the mucus protein fraction, a set of potential biomarkers for each location in a total of 35 peaks, being 17 peaks specific to Caniçal located farm and 18 to Ribeira Brava. The proposed analytical approach revealed a useful strategy providing accurate and fast results for fish geographical origin discrimination.

1. Introduction

Food frauds constitute a global concern not only at the economic level but can also have severe adverse health, societal, and sustainability effects. Even though considered illegal and several regulatory guidelines are in practice to enforce food protection, is still a recurrent action towards the increase of financial profits, in violation of legal rules. Throughout history, there are registers of several events, with different impacts, from loss of human life to economic damage, some examples are the "rapeseed oil" fraud (1981), milk adulterated with melamine in China (2008), and more recently the horsemeat scandal (2013) (Di Pinto et al., 2015). All of them illustrate the negative impact of food fraud, including both authenticity and adulteration (Hassoun et al., 2020). This type of incident affects the confidence in the food system with a strong impact on the internal and external market, leading the consumers to lose trust in foods and food chain, the companies to lose money as well credibility, and the authorities to lose trustworthiness. Different types of food fraud had evolved with the improvements in the production and detection processes, from formulation to procedures or data documentation. The lack of control or proper monitorization, due to a shortage in human resources or inappropriate technical support, makes such activity still prevalent and economically feasible (Medina, Pereira, et al., 2019).

In the case of fisheries and aquaculture products, the most common frauds are related with masking poor product quality, replacing of product constituents, species substitution, geographical provenance, mislabeling, repackaging of products, and tax evasion (Hassoun et al., 2020; OECD, 2013; Reilly, 2018). The complex structure of the seafood chain also contributes to fraudulent activities, being the main nodes reviewed by Fox and coworkers (Fox et al., 2018). These practices increased the pressure on food laboratories to develop fast and reliable screening methods to fight food fraud (Verrez-Bagnis et al., 2019). Anatomical and morphological analysis for fish identification is limited, not only due to similarities between some species but also due to the loss of physical traits during seafood processing (Freitas et al., 2020). Even though European Union presented several directives and regulations for the labeling and commercialization of fishery and aquaculture products, having also in mind to guarantee food safety, the control of such measures is limited by the capacity to analyze the product with proper methodologies to confirm the authenticity (Danezis et al., 2016) or traceability of the information (Badia-Melis et al., 2015).

The development of "foodomics" platforms contributed for the

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knowledge of several food properties that can be used as different layers of control to improve food quality and confirm the authenticity of the claims made throughout the supply chain. The approach is similar to metabolomics, which is based on the analysis of organism molecules (or metabolites), providing information about it at a certain time and specific conditions (Medina, Perestrelo, et al., 2019).

Fish mucus is a complex matrix with variable composition as a response to biotic (*e.g.*: growth stage, species, pathogens) and abiotic factors (e.g.: exposure to toxic elements; temperature), for example, incrementing the number of proteins related with immune response or reproduction (Reverter et al., 2018). It is a thin viscous, colloid layer, generated by the goblet cells which produce mucous granules that migrate to the cell surface and spread their content, covering the fish epithelial surface. The continuous superposition of mucous layers leads to the formation of a dynamic coating that allows the first interactions between the fish and the environment (Dash et al., 2018). Therefore, a diverse range of molecules can be found: fatty acids, lysozyme, immunoglobulin, histones, ribosomal proteins, and antimicrobial peptides (Jurado et al., 2015).

In the specific case of *Sparus aurata* most of the mucus based works have as objective the identification of proteins for welfare status determination (Fernández-Alacid et al., 2018; Guardiola et al., 2014, 2016; Sanahuja & Ibarz, 2015) or use two-dimensional electrophoreses coupled with spectrometric techniques for proteins identifications (Guglielmetti et al., 2020; Jurado et al., 2015). In order to study complex metabolomic matrices, as in the case of fish mucus, mass spectrometry has arisen as one of the most relevant high-resolution techniques. Mass spectrometry allows the identification of different compounds through the measurement mass-to-charge ratio of ions resulting in fragmentation patterns that can be used to identify different compounds (Mazzeo & Siciliano, 2016). The most emergent methodology is the Matrix-Assisted Laser Desorption Ionization- Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Through this method it is possible to determine the species authenticity (Mazzeo et al., 2008; Mazzeo & Siciliano, 2016), the origin of the product (Yoon et al., 2017), and the identification of food allergens and other hazards (Calvano et al., 2021), also fulfilling other prerequisites such as short time of analysis, flexibility, and reliability (Siciliano et al., 2015).

Since environmental factors can affect the proteins that are present in the fish mucus, this work aims to define the mass pattern from the protein fraction of mucus complex matrice to determine the fish geographical origin, from two different mariculture farms, using the MALDI-TOF MS method based on untargeted Mass Fingerprint analysis.

2. Material and methods

2.1. Sample collection and geographical areas

The samples were collected from two farms, one located at Baía d'Abra, in Caniçal (CN), Madeira (32°44′31.4″N 016°41′26.7″W); and the other located at Campanário, in Ribeira Brava (RB), Madeira (32°39′38.1″N 017°03′22.1″W) (Fig. 1). Four sampling groups were formed according to the time of arrival to the sea farm cages. Due to operational constraints (e.g.: weather, capture schedules, available time frame, company covid restrictions) and alternated production cycles between farms, the number of lots to be sampled was restricted and it was not possible to occur with the predicted frequency. Therefore, the number of new lots studied for CN was one, while for RB was three. The new arrived lots were sampled from the arrival moment, after 3 months (3 M), and up to 6 months (6 M).

The CN lot, upon arrival (August 2020) was divided into two cages, as a company standard procedure due to cage size constraints and for better management of the fish. These two cages (a and b) were equally sampled, at arrival, after 3 months (3 M), and after 6 months (6 M).

The RB new four lots (a, b, c, d), with arrival in June 2020 were not divided being directly transferred to the cages. Due to company schedules and covid-19 operational constraints, it was not possible to continuously follow the four lots from arrival to 6 M. To surpass these constraints the samples were analyzed not individually but under the group's categorization.

The group samples with approximately 12 months (12 M), were composed by ready to capture fishes (around 300–400 g or higher) from different pre-existing lots. The time needed to be ready for capture could vary between 10 and 13 months, influenced by the environmental conditions, specifically water temperature. These captures were taken in 5 different moments. At RB location the samples were collected in

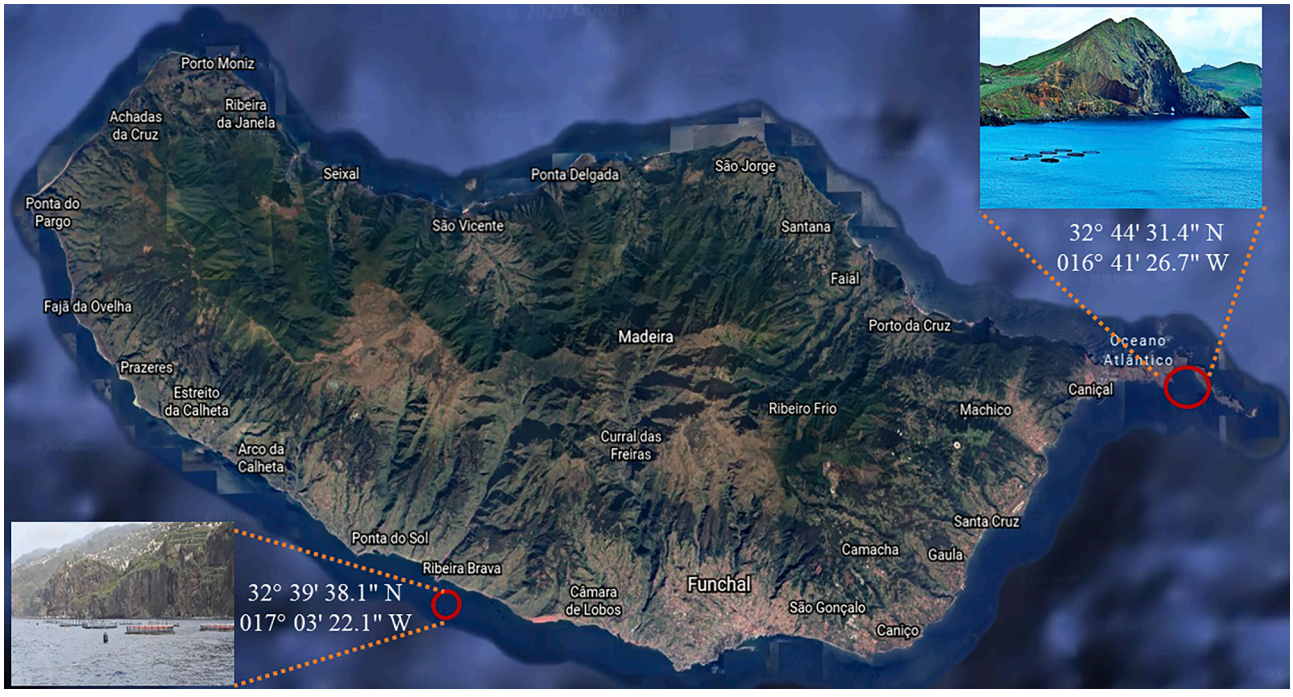

Fig. 1. Geographical indication of the sea-farm locations.

October 2019, February 2020, May 2020, August 2020, and October 2020. At the CN location, the samples were taken in October 2019 and from June 2020 (a and c are different cages) to July 2020 (b and d are different cages).

Between 5 and 10 ml of mucus was collected at each sampling moment. The mucus was gently scraped from the fish dorsal and lateral area above the pectoral fin. The samples were stored in ice until the laboratory arrival and then at −20 °C with a solution of 35% glycerol for preservation until the analysis. All mucus collected for the arrival, 3 M and 6 M groups, were from fishes sampled during control routines. Due to fish size and the number used for the control routine (approximately 60–80 fishes), an anesthetic solution was used, to reduce fish stress and possible injuries. Due to the size of the fish, a number between 40 and 55 fishes were necessary to collect 5–10 ml of mucus. The 12 M group samples were collected during the fish capture for commercialization. A mixture of ice and water was used for the capture. The fishes that were in the ice water mixture for more than 5 min were not sampled. A number between 20 and 25 fishes were used for mucus collection.

2.2. Sample preparation for MALDI-TOF MS analysis

Samples were cleaned using precipitation with chloroform/methanol to remove detergents, salts, and for protein concentration. Briefly, to 100 μL of mucus sample, 400 μL of methanol were added and vortexed. 100 μL of chloroform were added to the previous solution and vortexed, followed by the addition of 300 μL of water. After mixing in the vortex, the solution was centrifuged at 14,000 *g* for 1 min. The top aqueous layer was removed, the remaining pellet was vortexed, stored (-20 °C), and used for analysis on MALDI-TOF MS analysis.

2.3. MALDI sample preparation and spectra acquisition

For dried droplet crystallization, 1 μL of sample extract and 1 μL of HCCA matrix (10 mg/mL in MeCN:H_2O:TFA, 50:45:5 v/v) were pre-mixed. Then 0.5 μL of this mixture was deposited on a ground steel target plate and dried at room temperature (20 ± 1 °C) for 30 min. Calibration for MALDI-TOF MS analysis was carried out using insulin ($[M + H]^+$, 5734.51 *m/z*), ubiquitin I ($[M + H]^+$, 8565.76 *m/z*), cytochrome *C* ($[M + H]^+$, 12360.97 *m/z*; $[M + H]^{2+}$, 6180.99 *m/z*), and myoglobin ($[M + H]^+$, 16952.30 *m/z*; $[M + H]^{2+}$, 8476.65 *m/z*) from the protein calibration standard I kit (Bruker Daltonics, Germany).

A model Autofex maX device MALDI-TOF MS from Bruker Daltonics with flexControl 3.4 and flexAnalysis 3.4 software was used. This instrument is equipped with the Smartbeam-II™ laser emitting at 355 nm. MS spectra were acquired in the *m/z* range of 2–20 KDa in a positive linear mode, using 60% laser intensity and 1000 laser shots with random walk mode of the shooting position pattern at a laser frequency of 200 Hz.

2.4. Statistical analysis

The free statistical program Mass-UP (Mass-Up, Vigo, Spain), was used for pre-treatment of the MALDI-TOF spectrums, following the respective program workflow (López-Fernández et al., 2015). The spectrums were exported from flexAnalysis 3.4 software as .XML files (raw data files). The 5 replicates for each sample were filed under the appropriate sample coding.

The raw data files were uploaded to Mass-UP software, as labeled files. The following pre-process options were chosen: Intensity transformation – square root; Smoothing – Savitzky Golay; Baseline correction – Snip; Standardization - none. For peak detection, the MALDI quant package with minimal intensity established at 0.0 value was used (Gibb & Strimmer, 2012). For all samples labeled peak list was generated.

The peak matching process between peak lists was performed with the Maldiquant package with a tolerance of 0.002, for intra-sample matching and inter-sample matching. The option of generating a consensus spectrum was not selected.

The generated Labeled matched peak lists sets were then used for Principal Component Analysis (PCA) and Biomarker discovery analysis (BMD). BMD approach was used to reduce the number of peaks to be analyzed, and generate a discriminant peak list, allowing to determine which peaks were present or absent in the different groups of samples (López-Fernández et al., 2015). The obtained discriminant peak lists were used for Hierarchy Clustering Analysis (HCA). The following parameters were chosen: Cluster reference – Farthest; Distance function – Euclidean; Conversion values – Percentage of presence; Intra-sample minimum presence – 0.33.

3. Results and discussion

An example of the spectrums obtained from the pre-treatment and peak alignment procedures following the Mass-UP options is presented in Fig. 2a and Fig. 2b, respectively. This pre-process and alignment was necessary in order to clean the large data set from signal noise and identify true signals, following the software workflow (López-Fernández et al., 2015). With this pre-treatment it was reduced the *m/z* range from 20 KDa to 9 KDa, reducing the number of peaks to be analyzed from the 40,000 in the raw spectra to 100–150 of the pre-processed data.

3.1. Multivariate analysis according to time of arrival

The alteration on fish mucus was evaluated to determine if the fish mucus suffered significant alterations during the permanence in the sea cages. Taking into consideration the Madeira island specificities (e.g.: warmer waters throughout the year), the time needed to achieve a commercial size is less (10–16 months) when compared with farms located at higher latitudes that require extended periods (Caldeira et al., 2002). Therefore, sample groups were formed having in consideration both locations (CN and RB) as following: at the arrival moment (red); 3 months after arrival (3 M - green); 6 months after arrival (6 M - blue) and captures from lots with more than 12 months (12 M - yellow).

3.1.1. Principal component analysis

To gain a better perception between the similarities and differences among the groups, a multivariate approach PCA was used. To obtain better visualization between the correlations, 3D representation was performed (PCO vs PC1 vs PC2).

Figure 3 represents the PCA results for RB (Fig. 3a) and CN (Fig. 3b). For both locations, the projection of the sample forms distinct clusters. Supplementary Table 1 shows the results for each sample and principal component associated with the RB location and supplementary Table 2 for CN location.

In the case of RB, the variance associated with each component is PC0 – 33%, PC1 – 11%, PC2 – 10%, representing 54% of the data. PC0 separates the arrival samples (red) from all the others, with values between 8 and 15. For the other groups, the variations were between −1 to 1 for the 3 M, 6 M, and 12 M, (green, blue, and yellow, respectively). The PC1 presents better separation for the 3 M, 6 M and the 12 M samples, corresponding to values between −4 to −3 (green), 0.3 – 0.95 (blue) and −9.0 and −7.0 (yellow) respectively.

For the CN samples, cluster formation was also achieved, when the 3 components variance is analyzed, corresponding to PC0 – 25%, PC1 – 20%, and PC2 – 15% with a cumulative value of 60%. In this case, the PC0 separates between all the different clusters, each one with a distinct quadrant. The projection of arrival samples formed a cluster in 10 to 5 quadrant (red), 3 M samples in the quadrant 5 to 0 (green), 6 M samples between −3 to −2, and 12 M samples between 0 and −2. PC1 improved the resolution between the 6 M, 3 M, and 12 M clusters.

These results demonstrate the evolution of the mass fingerprint of fish mucus, influenced by the new environmental conditions in which they are inserted (Reverter et al., 2018). The separations between the arrival samples, mainly in the RB group samples, could reflect the

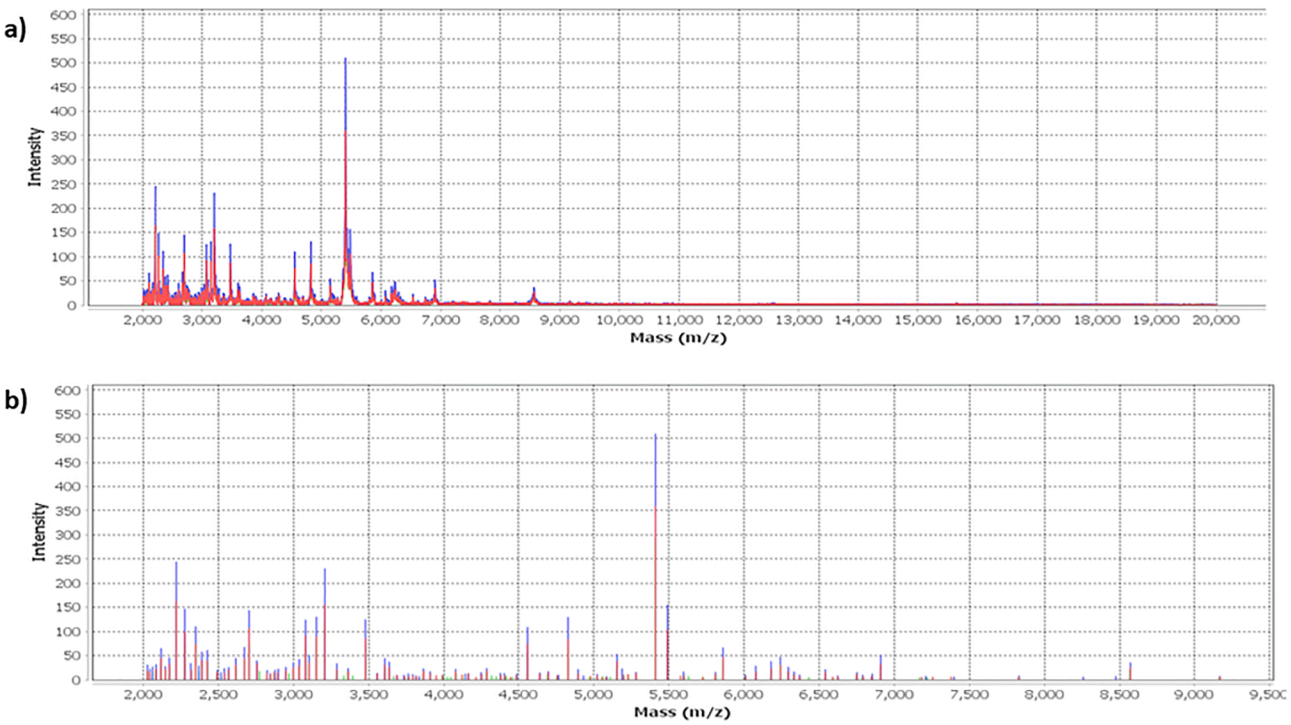

Fig. 2. Example of MALDI-TOF MS spectra on the Mass-UP software. a) After the pré-process treatment; b) after peak detection and peak alignment.

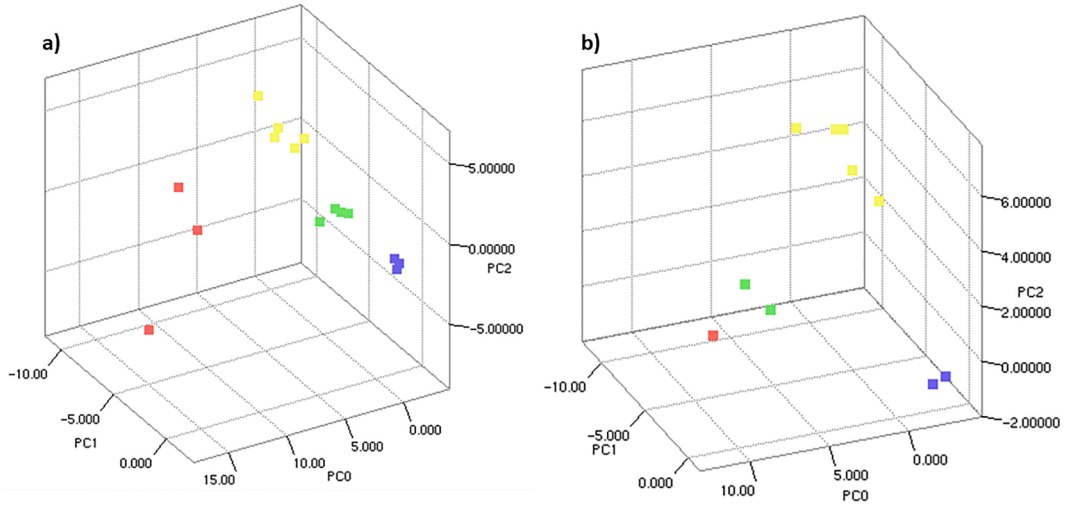

Fig. 3. 3D representation of the PCA results. a) Ribeira Brava (RB); b) Caniçal (CN). Red – arrival samples; green – 3 M samples; blue – 6 M samples; yellow – 12 M samples.

different origins of the fish lots that are frequently imported from Spanish or French suppliers.

3.1.2. *Hierarchy clustering analysis*

HCA was used to obtain other different representations from the data projection, establishing relations between the cluster samples. The aligned peaks list used for PCA analysis was subject to HCA analysis. Fig. 4a and Fig. 4b show the cluster analysis for each group of samples according to the location, CN and RB respectively. It is possible to see that, for both locations, the arrival cluster forms a distinguished cluster from the other ones. For the 3 M, 6 M, and 12 M samples, for both locations, they form clusters with a closer association between them, having the 3 M and 6 M samples a closer relation than with the 12 M.

These results could be an indication that each moment of capture reflects a specific environmental condition, with an impact on the mass-fingerprint (Dash et al., 2018; López-Fernández et al., 2015). A closer analysis of the 12 M samples from RB (Fig. 4b), reveals that having into consideration the month of the capture, the samples associated with captures from August and October have proximate relations than the ones from May or February, even if they were done in different years. However, confirmation of such relations requires bigger data set for pattern verification.

The 12 M cluster formation associated with the CN location (Fig. 4a) is influenced by fish lot distribution in two different cages, due to fish density constraints. This procedure could explain the closer relations between the samples, nevertheless, as in the previous case, more fish lots need to be followed to increase the data set for verification of this pattern.

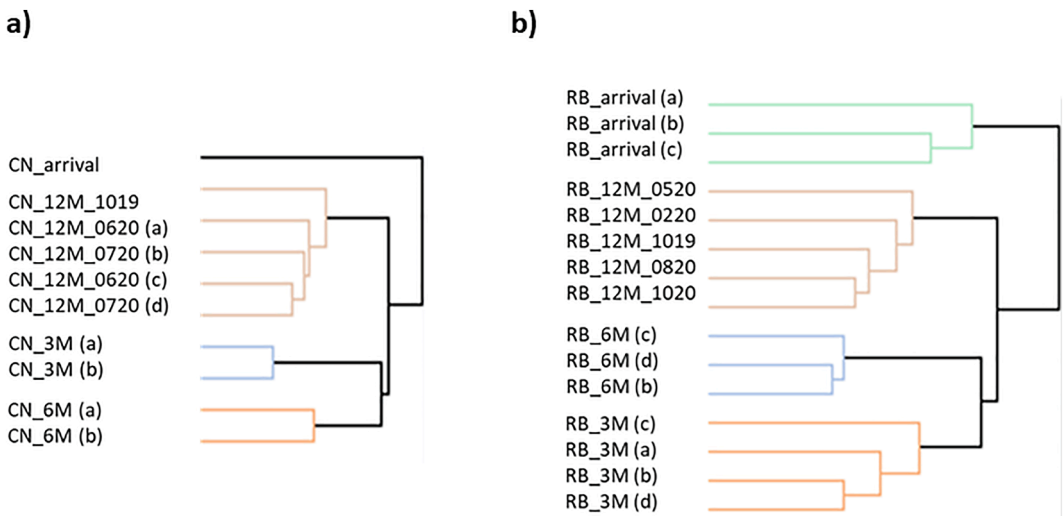

Fig. 4. Hierarchy cluster analysis results for each group according to the location. a) Caniçal (CN); b) Ribeira Brava (RB).

3.2. *Multivariate analysis according to farm location*

Even considering the analysis of the samples based on the time of arrival revealed promising results, the main objective is to access the feasibility of the approach in determining a specific group of mass peaks, to be used as biomarkers for each location (RB and CN). For this part, only the samples taken at captures moment of lots with commercialization size were taken into consideration (300–400 g or higher).

3.2.1. – *Principal component analysis and biomarker discovery*

Figure 5 presents the PCA results for the capture samples from CN and RB. The variance results to the three principal components are PC0 – 51%, PC1 – 8%, and PC2 – 7%, representing a cumulative value of 66%. Without the influence of the other samples (arrival, 3 M and 12 M), the component PC0 clear projects the samples in distinct clusters, the CN cluster (blue) being placed between the quadrants −9 to −6 and the RB (red) cluster between 10 and 9. Supplementary table 3 summarizes the values for all samples and principal components results.

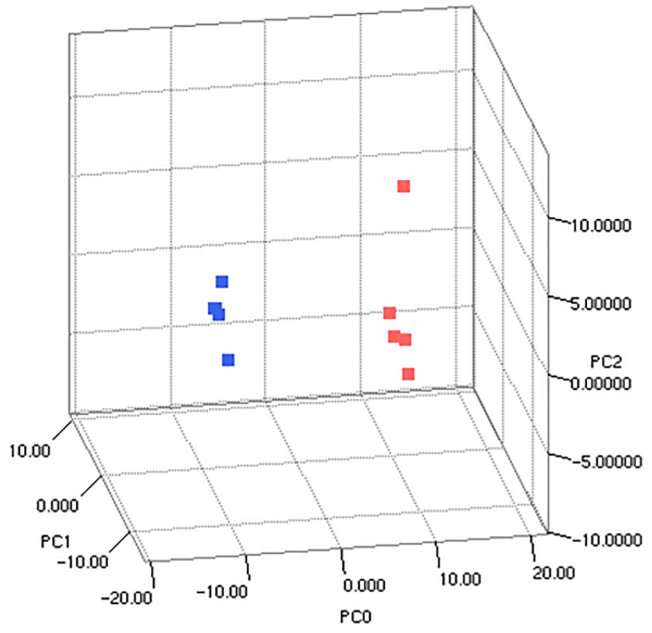

Fig. 5. PCA results of the capture samples (12M group) from Caniçal (blue) and Ribeira Brava (red).

For the determination of peaks responsible for clusters discrimination, the same dataset was used for biomarkers discovery analysis in the Mass-Up software (BMD). First, the data set was processed to obtain a list of peaks intersection between the samples (option BMD-intersample). For exclusion of insignificant peaks, the presence thresholds parameters were set to 35% as maximum absence and 50% as minimum presence in the samples. The used percentages are associated with the peak occurrence in the samples. Also, the algorithm calculates, for each peak, the p-value based on Fisher's exact test of independence and the q-value using the Benjamini Hochberg FDR (López-Fernández et al., 2015). A list with 772 peaks was generated and the p-values variated between 0.008 and 1.0, while for the q-values the variance was between 0.12 and 1.0. Supplementary table 4 presents the peaks with p-value < 0.05 and q-value < 0.4, corresponding to 161 peaks.

Reduction of peak number for analyses was processed under BMD-intrasample analysis. In this case, the RB group samples, and CN group were uploaded simultaneously. This allowed refining the peaks search analysis, through comparison between groups, and determine the ones that are present or absent simultaneously. The threshold conditions were set as previously (35% maximum absence and 50% minimum presence). The resultant analysis allowed the creation of a smaller discriminant peak list of 43 peaks, 22 relatively to RB and 21 to CN, with the following p-values < 0.008 and q-value < 0.10. The selected peaks correspond to the first 43 peaks in supplementary table 4.

3.2.2. *Hierarchy clustering analysis*

For HCA analysis the data set that was uploaded and the reduced discriminant peak list was used to restrain the analysis only to those peaks. The resultant dendrogram, the peaks used, and the correspondent heat map is presented in supplementary Fig. 1. HCA analysis results in the formation of two separated clusters, one correspondent to RB and the other to CN. However, a closer analysis of the selected peaks shows that there were 4 similar peaks between the groups, that vary <2.0 m/z units, the 2346.77–2346.59 m/z, 3629.30–3631.57 m/z, 3604.54–3605.88 m/z, and 5405–5407 m/z. Due to their similarities, the peaks were removed from the list and the HCA was repeated, given the results present in Fig. 6. The alteration on the peak list did not result in significant alterations on the HCA results, confirming that they were not significant peaks for the HCA discrimination.

Therefore, the final set of potential biomarkers consists of 17 biomarkers for CN location and 18 for RB location.

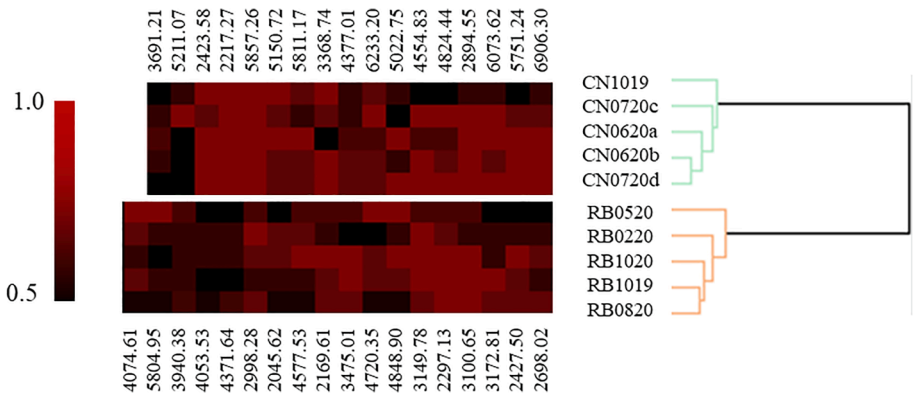

Fig. 6. HCA dendrogram and the corresponding heat map for the 35 potential biomarkers. Ribeira Brava (RB -red); Caniçal (CN - blue). Black color corresponds to 0.5% of frequency between samples and red color to 1%.

4. Conclusions

Even though fish mucus is an interesting target of study, its variable composition due to environmental and fish physiological influence creates several challenges on the analysis of its constituents. Also, the scarce number of studies on fish mucus metabolomics and dedicated databases contribute to challenges in mucus studies. However, the incorporation of omics technologies provides new prospects to uncover new knowledge that otherwise would remain unnoticed.

In this work, fish mucus was analyzed with the MALDI-TOF MS method, employed as a mass-fingerprint technique for the origin discrimination of aquaculture fish from two different mariculture farms, Caniçal - Madeira and Ribeira Brava - Madeira. The Mass-Up software was used for multivariate analysis and potential biomarkers discovery.

As result it was possible to discriminate between samples with different origins, resulting in a list of 35 potential biomarkers, being 17 biomarkers specific for Caniçal and 18 for Ribeira Brava. Also, the mass-fingerprint approach allowed to differentiate between samples with different residence times in the sea cages, confirming the alterations that fish mucus undergoes from the moment of arrival to the moment of capture. The results also confirm the potential of MALDI-TOF MS to be applied for origin discrimination studies of aquaculture fish from different farms and as a fast, reliable approach.

CRediT authorship contribution statement

Jorge Freitas: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Pedro Silva:** Conceptualization, Methodology, Validation, Writing – review & editing, Visualization. **Rosa Perestrelo:** Methodology, Validation, Investigation, Resources, Data curation, Writing – review & editing. **Paulo Vaz-Pires:** Conceptualization, Writing – review & editing, Supervision, Project administration. **José Sousa Câmara:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131237.

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