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Feasibility of a portable NIR-based PAT approach for discriminating fresh and thawed octopus along the supply chain

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ABSTRACT

The distinction between fresh and thawed octopus is a critical issue in seafood quality control, due to the high commercial value of fresh products and the risk of fraudulent practices. Although other techniques, such as histological analysis, have been evaluated as a routine screening technique, their destructive nature makes them unsuitable for routine on-site inspections along the supply chain. Near-infrared (NIR) spectroscopy combined with chemometrics represents a promising non-destructive alternative; however, industrial variability and process-oriented implementation must be considered.

In this study, the performance of a portable MicroNIR system was evaluated for the discrimination of fresh and thawed *Octopus vulgaris* under realistic supply chain conditions. The dataset was designed to reflect industrial variability, including different suppliers, fishing areas, fishing methods, and thawing procedures. The Partial Least Squares Discriminant Analysis (PLS-DA) models achieved classification rates of approximately 85% in calibration and cross-validation, while external prediction performance decreased to approximately 75%, particularly for thawed samples. To translate the model results into an operational quality control strategy, a Process Analytical Technology (PAT)-oriented decision logic was developed based on the mean and variability of replicate predictions. An acceptance zone was defined for fresh samples, ensuring that no thawed sample was incorrectly accepted as fresh in the external test set.

These results demonstrate the feasibility of integrating portable NIR spectroscopy into a risk-based and online PAT framework for octopus authenticity assessment, prioritizing fraud prevention and robust decision-making.

1. Introduction

Octopus is a seafood product of high commercial and nutritional value widely appreciated in Mediterranean countries where its consumption is deeply rooted in culinary tradition (Pita et al., 2021). Within the European market, fresh octopus is generally perceived as a premium product, whereas frozen octopus represents a more affordable alternative. However, the availability of fresh octopus is constrained by the seasonality of catches, resource management measures and logistical limitations, resulting in a strong reliance on imported frozen products from non-EU countries (EUMOFA, 2021, pp. 1–47; Tinacci et al., 2020).

The coexistence of fresh and frozen octopus along the supply chain is associated with a market price differential between the two product categories (ISMEA, 2019). In recent years, this gap has been further amplified by declining catches, linked to environmental factors and increasingly restrictive management policies, combined with continued high consumer demand (Zamuz et al., 2023). In this context, the higher economic value attributed to fresh octopus represents a potential risk factor for fraudulent practices.

One of the most common types of fraud in the cephalopods sector consists in the marketing of frozen-thawed octopus as fresh product (Chiesa et al., 2020). This practice compromises market transparency, causes economic damage to consumers and violates European legislation requiring accurate and non-misleading labelling of fishery products with respect to their state of preservation (Regulation No 1169/2011, 2011; Regulation No 1379/2013, 2013). Despite the regulatory framework, the verification of label compliance remains challenging, particularly in the absence of rapid, objective and non-destructive analytical tools that

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can be applied directly along the supply chain (Pennisi et al., 2021).

In the case of octopus, this issue is further complicated by the intrinsic characteristic of the product and by post-capture handling practices. From an operational perspective, octopus intended for sale as fresh is generally subjected to immediate chilling and ice storage to delay spoilage and preserve sensory quality (Bremner, 2016). However, in cephalopods, post-mortem deterioration is largely driven by autolytic processes, which may occur early and independently of microbial growth (Vaz-Pires & Barbosa, 2004). Ice storage has been shown to induce structural and physicochemical modifications in muscle tissue, including loss of compactness, changes in fibre organisation and alterations in water retention (Badiani et al., 2013; Vaz-Pires & Barbosa, 2004). Although such changes are compatible with the commercial definition of fresh product, they may partially overlap with the effects induced by a cycle of freezing and subsequent thawing.

As a consequence, the discrimination between fresh and thawed octopus based on sensory or visual assessment is inherently unreliable. Sensory grading schemes and quality indices, as well as other rapid freshness evaluation tools originally developed for fish, can provide useful information on product quality and shelf life but are not suitable for detecting undeclared freezing–thawing events in cephalopods (Hassoun et al., 2020).

More reliable discrimination can be achieved through analytical methods targeting structural tissue alterations induced by freezing. Histological analysis has proven highly effective in this respect and specific indicators for *Octopus vulgaris* have been identified and validated (Tinacci et al., 2020), leading to recent standardization efforts for fish products (UNI 11862, 2022). Nevertheless, despite their accuracy, histological methods are destructive, time-consuming and incompatible with routine, on-site control activities along the commercial chain.

In this context, near-infrared spectroscopy (NIR), combined with multivariate data analysis, has emerged as a promising non-destructive and rapid alternative (Hassoun et al., 2020). Several studies have demonstrated the ability of NIR spectroscopy to discriminate fresh and thawed cephalopods. Pennisi et al. (2021) compared three NIR devices—FT-NIR, a portable device, and a miniaturised NIR—on cuttlefish and musky octopus (Eledone spp.), reporting classification accuracies ranging from 82.3% to 97.1%, depending on the species and instrumentation. However, the limited sample size (50 samples per species) and the absence of independent validation suggest that further investigation is warranted.

Furthermore, Sannia et al. (2019) and Currò et al. (2022) evaluated portable NIR instruments for the authentication of cuttlefish (*Sepia officinalis*). Even if a representative numerosity and variability was considered, these studies were conducted under controlled conditions, using samples from individual suppliers or processing plants and standardised freezing protocols.

In any case, none of them examined *Octopus vulgaris*, which is the most commercially significant species in the Mediterranean market and among those most exposed to fraudulent practices (Chiesa et al., 2020; EUMOFA, 2021, pp. 1–47).

Finally, the cited studies focus on method development and classification accuracy, whereas limited attention has been paid to the integration of NIR-based models into an industrial quality control framework. In particular, the use of NIR spectroscopy within a Process Analytical Technology (PAT) perspective, where analytical results are interpreted as process indicators rather than as isolated measurements (Grassi & Alamprese, 2018), remains largely unexplored in the cephalopod sector.

Within a PAT approach, NIR measurements can support decision-making processes by providing real-time information on product conformity, enabling screening activities, identification of recurring criticalities and risk-based management of suppliers and logistics (Grassi & Alamprese, 2018). This is particularly relevant for biological products such as octopus, where natural variability, post-harvest handling and transport conditions may influence both product quality and model performance (Zamuz et al., 2023).

In light of these considerations, the present study aims to evaluate the feasibility of using a portable NIR spectrometer combined with Partial Least Squares Discriminant Analysis (PLS-DA) models to discriminate between fresh and thawed *Octopus vulgaris* under realistic supply chain conditions. The objective is not only to assess classification performance, but also to discuss the limitations of the approach in relation to ice storage and product variability, and to explore its potential application as a decision support tool within a PAT-oriented strategy for quality control and fraud prevention.

2. Materials and methods

2.1. Sample collection and experimental design

The experimental design was set up to evaluate the ability of a MicroNIR system to discriminate between fresh and thawed octopus under conditions representative of the commercial supply chain. The samples were divided into a training set and a test set, as shown in Fig. 1.

The training set comprised a total of 160 samples of *Octopus vulgaris*. Specifically, 60 samples of fresh octopus and 50 samples of tenderized octopus were analysed upon arrival at a large retail chain (Esselunga, Italy) in the period March 2025 – September 2025. The samples were balanced in terms of weight class, with 50% of the samples weighing less than 700 g and the remaining 50% weighing more than 800 g. After the first spectroscopic acquisition, these samples were frozen and stored at temperatures below −20 °C for a period of 2 or 4 months, then thawed and analysed again using a MicroNIR (Viavi Solutions, USA).

The remaining 50 samples in the training set were commercial frozen octopus' samples, analysed immediately after thawing. The frozen samples were thawed using two methods commonly employed in industry: air thawing and water thawing, applied in approximately equal proportions. Again, the samples were evenly distributed between the two weight classes (<7 00 g and >800 g).

Two spectroscopic replicate measurements were performed at each sampling point in the training set, resulting in540 spectracollected from 270 sample conditions generated from 160 individualoctopuses).

The test set consisted of 70 independent samples, including 35 fresh tenderized octopus' samples and 35 frozen octopus samples analysed upon arrival at a large retail chain (Esselunga, Italy) in the period October 2025 – November 2025. The test set samples were also balanced in terms of weight class. The samples collected for the test set were analysed by collecting 3 spectral measurements for each individual octopus to assess the robustness of the model on data not used in the calibration phase.

The frozen samples were thawed using two methods commonly employed in industry: air thawing and water thawing, applied in approximately equal proportions.

All samples came from different commercial suppliers (n = 14) and different fishing areas (FAO 34, 27 VII a, 27 VIII a, 27 VIII b, 27 IX a, 37.1.1, 37.2.2, and 37.3.1), using different fishing gear (hooks and lines, pots and traps, and trawl nets). These factors were included in the experimental design in order to introduce variability representative of actual supply conditions, without being the specific subject of analysis.

2.2. MicroNIR instrumentation and spectral acquisition

Spectral acquisitions were performed using a portable MicroNIR Pro 1700 instrument (Viavi Solutions, USA), equipped with an uncooled InGaAs detector. Before the measurement sessions, the instrument was calibrated by acquiring dark and white references using a Spectralon™ reflective standard. The reference measurements were repeated at regular intervals during the acquisitions, to ensure the stability of the instrumental response.

The spectra were acquired in the wavelength range between 908 and 1676 nm, with a spectral resolution of 6.2 nm. For each acquisition, 50

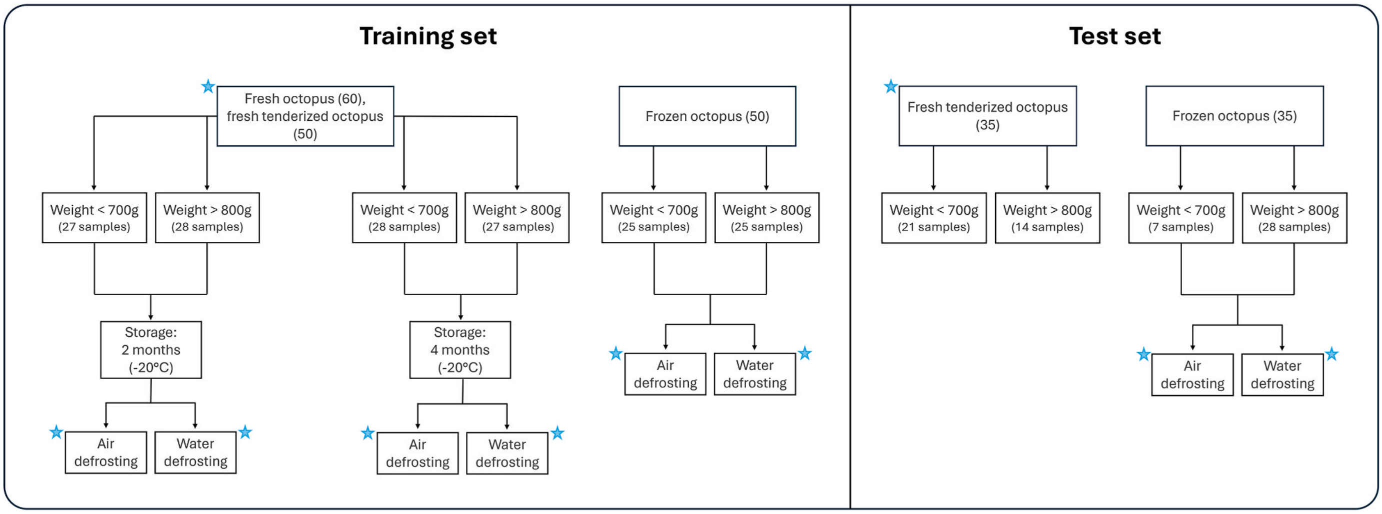

Fig. 1. Schematic representation of the experimental design. Fresh, tenderized and commercially frozen *Octopus vulgaris* samples were allocated to a training set (n = 160) and an independent test set (n = 70). MicroNIR spectral acquisitions were performed at each sampling point (marked with stars), in both fresh and thawed conditions.

consecutive scans were averaged, with an integration time of 10 ms. In accordance with the experimental design, two independent spectral acquisitions were performed for each sample belonging to the training set, whereas three acquisitions were performed for the test set samples.

The spectra were acquired using two different operating procedures. In the first procedure, the spectra were acquired directly on the sample as is, without any preliminary preparation, allowing for the possible presence of residual water on the product surface. This procedure was adopted to simulate measurement conditions that are more representative of real conditions. In the second procedure, before spectral acquisition, the sample was lightly blotted to remove surface water. Blotting was performed using dry, clean paper towels, avoiding pressure on the muscle tissue; the sample was then placed on a clean surface.

All acquisitions were performed at an ambient temperature of 21.7 ± 3 °C. At the time of measurement, the weighted mean temperature of the product was 4.7 °C, in accordance with the typical refrigerated storage conditions of octopus intended for sale as a fresh product or immediately after thawing.

2.3. Data analysis

All data analyses were performed in the MATLAB environment (MathWorks Inc., Natick, MA, USA) using the PLS Toolbox (Eigenvector Research Inc., Seattle, WA, USA). Exploratory and classification analyses were conducted on MicroNIR spectral data to evaluate data structure and sample variability.

Firstly, MicroNIR spectra were subjected to spectral range selection and preprocessing. The spectral region was restricted to the range between approximately 927 and 1658 nm, corresponding to the effective working range of the instrument and excluding noisy edge regions. Different spectral pretreatments were evaluated, including smoothing (Savitzky Golay, 3 window points, third polynomial order), Standard Normal Variate (SNV) correction and first derivative (Savitzky Golay, 3 window points, third polynomial order) transformation, applied either individually or in combination, in order to reduce undesired variability related to scattering effects and baseline shifts (Rinnan et al., 2009).

Then, Principal Component Analysis (PCA) was applied as an unsupervised exploratory technique to investigate the distribution of spectral acquisition in the reduced multivariate space and to identify major sources of variability within the dataset. PCA was used to visualize similarities and differences among samples through score plots, and to evaluate the contribution of spectral variables to the observed variability through loading analysis (Wold et al., 1987). PCA was also employed as a tool for outlier detection. Potential anomalous samples were identified by evaluating their distance from the centre of the multivariate space defined by the selected principal components, using the Mahalanobis distance as a statistical criterion (Todeschini et al., 2013). Samples identified as outliers were excluded from subsequent multivariate analyses, including PCA visualization and supervised modelling.

Subsequently, Partial Least Squares - Discriminant Analysis (PLS-DA) was applied as a supervised classification method (Ballabio & Consonni, 2013) to discriminate between fresh and thawed octopus' samples, encoding class membership as a categorical response variable.

Model development was performed at the spectral level, treating each replicate acquisition as an individual observation; replicate spectra from the same biological sample were never split between the training and cross-validation sets, ensuring complete independence at the sample level. Model complexity was optimized by cross-validation to avoid overfitting. Classification performance was evaluated in terms of sensitivity (TP/(TP + FN)), specificity (TN/(TN + FP)), and correct classification percentage. Model robustness was finally assessed through external validation using an independent test set not involved in the calibration phase.

For each sample of the independent test set, three replicate NIR spectra were acquired and independently projected onto the selected PLS-DA model to obtain predicted Y values. Sample-level metrics were then calculated as the mean predicted value and the standard deviation across replicate measurements, reflecting both classification tendency and measurement repeatability.

To support robust decision-making, results were visualized in a two-dimensional space defined by the mean predicted Y value and its associated standard deviation. This representation enables simultaneous assessment of class separation and intra-sample variability, which is critical in PAT applications where decisions must be resilient to measurement fluctuations.

Based on the empirical distribution of fresh and thawed samples in the external prediction set, a "conservative acceptance zone for fresh samples" was defined prioritizing the prevention of false acceptance of thawed samples over exhaustive classification.

3. Results and discussion

3.1. Spectral characteristics and interpretation of relevant NIR features

The spectral characteristics of fresh and thawed octopus were investigated to identify key absorption features in the NIR region potentially associated with differences in the state of preservation. Fig. 2 reports average spectra, of the unblotted samples, of the two classes (fresh and thawed) in the 927 – 1658 nm range after smoothing (Fig. 2A), smoothing combined with SNV correction (Fig. 2B), and smoothing combined with d1 transformation (Fig. 2C).

In the smoothed average spectra (Fig. 2A), fresh and thawed octopus have similar overall spectral profiles. However, systematic differences in absorption intensity can be observed across the entire spectral range. These differences are mainly attributable to light scattering phenomena and baseline variations related to the physical and structural properties of muscle tissue, such as surface conditions, fibre organisation and water distribution. As widely reported for octopus and other fish product matrices, these effects are not directly related to chemical composition, but rather to the physical state of the sample (Varrà et al., 2022).

After applying the SNV to the smoothed spectra (Fig. 2B), the effects of multiplicative dispersion are substantially reduced, leading to better spectral alignment and greater comparability between classes. Under these conditions, the differences between fresh and thawed samples become more apparent in specific wavelength ranges, particularly in the regions between 950 and 1000 nm, 1100 – 1200 nm, and 1400 – 1500 nm. The absorption characteristics observed around 960–980 nm are commonly associated with O–H bond harmonics and are mainly related to the moisture content and structure in muscle tissue (Fasolato et al., 2012). Variations in this region may reflect differences in water distribution and mobility, which can be influenced by ice storage and freeze-thaw processes.

The regions around 1100 – 1200 nm are related to the second harmonics of C-H vibrations (Sannia et al., 2019). Changes in these spectral regions have been associated with structural and compositional changes in muscle tissue, including lipid reorganisation and proteolytic phenomena that may also influence matrix-water interactions with a different fraction of free or bound water species (Sannia et al., 2019).

Applying the first derivative to the smoothed spectra (Fig. 2C) further improves the spectral characteristics by reducing broadband contributions and emphasising subtle variations in the shape and the position of the bands. In Fig. 2C, clear derivative peaks are observed around approximately 1150 nm and 1400–1450 nm. Since the absorption maxima in the original spectra correspond to zero-crossing points in the derivative spectra, these features can be associated with the main absorption bands of aliphatic C–H groups and water-related vibrations. The sharper and more pronounced derivative signals observed for thawed samples have been linked to altered molecular environments, likely due to increased free water, protein unfolding, and changes in tissue microstructure induced during freezing and thawing processes (Sannia et al., 2019).

Overall, the observed spectral differences between fresh and thawed octopus are mainly attributable to changes in water-related O-H absorptions, while regions related to C-H vibrations should be interpreted as indirect markers of structural modifications of the muscle matrix.

3.2. PCA results

To further investigate the spectral differences observed between fresh and thawed octopus, PCA was applied to both datasets to explore the main sources of variability and to assess the natural clustering of samples before supervised classification.

Potential outliers were identified based on the 95% confidence limit of the Mahalanobis distance in the PCA score plot, i.e. in the space of Hotelling's T^2 residuals, represented also in the plot of PC1 scores against PC2 (Fig. S1). Twelve outlier samples, comprising 7 thawed samples and 5 fresh samples, were identified and excluded from the subsequent supervised modelling. Their balanced distribution across the classes confirms that the exclusion did not introduce any systematic bias.

Fig. 3 shows the results obtained from d1 pre-treated NIR spectra acquired on unblotted samples after the removal of outliers.

In the score plot coloured according to the state of the sample at the time of analysis (Fig. 3A), fresh and thawed samples show a partial overlap, indicating that preservation state alone does not fully explain the variability in the dataset. This behaviour may be related to the transportation and storage of fresh samples on ice, which could have induced structural and compositional modification in octopus muscle tissue (Vaz-Pires & Barbosa, 2004).

When samples are coloured according to storage history (Fig. 3B), including fresh samples, samples frozen and thawed after 2 or 4 months of storage, and samples commercialised as frozen and analysed after thawing, an overlap between the groups is still observed. No clear separation based on freezing duration is evident, suggesting that storage time is not a dominant source of spectral variability under the investigated conditions. A slight tendency for sample commercialised ad frozen to distribute along a diagonal trend at negative PC1 values can be observed.

No clear clustering was observed in the PCA score plots when considering other sources of variability, such as fishing area, different thawing methods, or different producers, indicating that these factors contribute minimally to the overall spectral variance (data not shown).

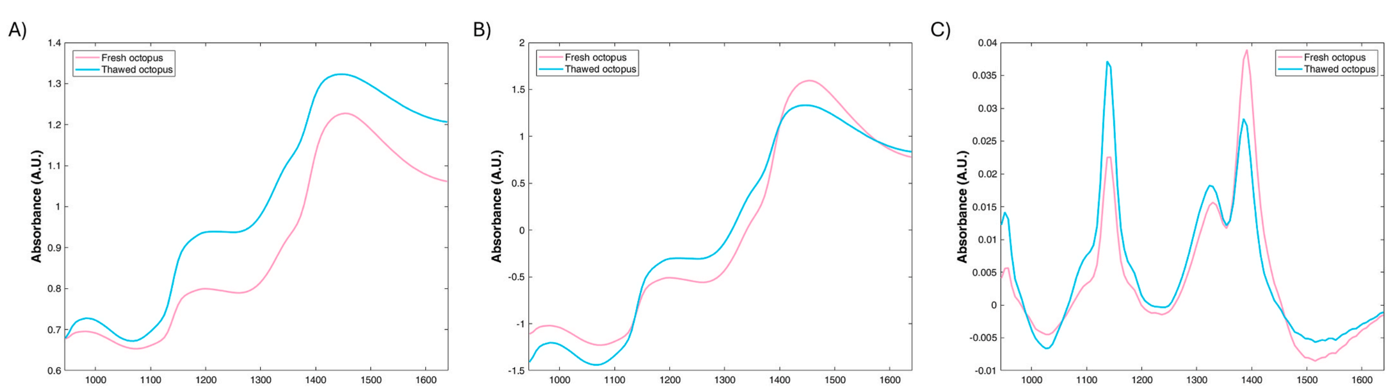

Fig. 2. Average NIR spectra (927–1658 nm) of unblotted fresh and thawed *Octopus vulgaris* samples after different preprocessing techniques: (A) smoothing, (B) smoothing combined with Standard Normal Variate (SNV) correction, and (C) smoothing combined with first derivative transformation (d1). Fresh samples are shown in pink, thawed samples in light blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

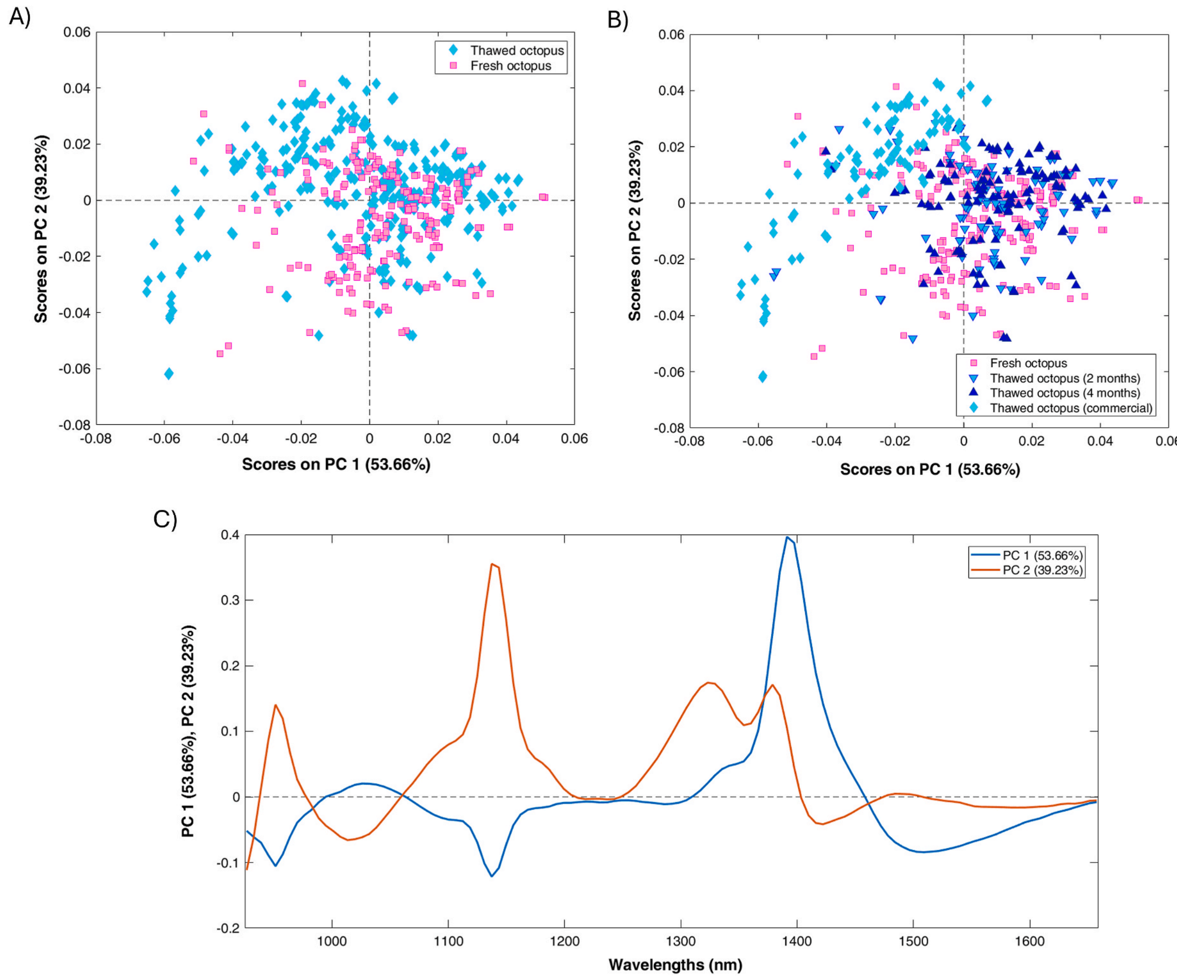

Fig. 3. PCA of first derivative (d1) NIR spectra of unblotted octopus' samples. Score plots of PC1 vs. PC2 coloured according to the sample state at the time of analysis (A) and according to sample type (fresh, fresh tenderized, thawed) (B). Loading plots of PC1 and PC2 are shown in (C).

PC1 accounted for most of the variance (53.66%) and was mainly associated with spectral characteristics around 1400 nm (Fig. 3C). As discussed in Section 3.1, this region corresponds to–CH_3 vibrations of aliphatic hydrocarbons and water-related vibrations. Variations in this region may be related to changes in lipid organisation, proteolytic activity, and water-protein interactions occurring during freezing and thawing (Sannia et al., 2019; Varrà et al., 2022).

PC2 explained 39.23% of the variance and was mainly related to peaks at 960–980 nm, associated with O–H bond harmonics and muscle moisture content (Fasolato et al., 2012), as well as the 1100–1200 nm region, corresponding to second harmonics of C–H vibrations (Sannia et al., 2019). These spectral characteristics reflect structural and compositional changes in muscle tissue.

Overall, these results suggest that preservation state and sample condition contribute to the main source of spectral variability, although their effects partially overlap. This supports the subsequent development of supervised PLS-DA models to achieve discrimination between fresh and thawed samples.

3.3. PLS-DA classification performance and model robustness

Based on the exploratory results of PCA, supervised classification models were developed using PLS-DA to distinguish fresh octopus samples from thawed ones. Two models were constructed: one using spectra acquired directly on the surface of the sample as it is, and one using spectra acquired on blotted samples. In both cases, the models were developed after removing the twelve anomalous samples previously identified during PCA, and the NIR spectra were pre-treated by smoothing and d1.

The PLS-DA models were calibrated by a training set consisting of 516 spectra (258 biological samples measured in duplicate), while an independent test set of 210 spectra (70 biological samples measured in triplicate) was used for external prediction.

The training set was specifically designed to include paired measurements relating to fresh and thawed samples derived from the same biological specimens, as well as commercially frozen samples, in order to expose the model to state-dependent spectral variations whilst controlling for inter-individual variability. The external test set consisted of completely independent biological samples collected at a different time, confirming the interpretation that the observed discrimination reflects real spectral differences between fresh and thawed samples, rather than effects related to the history of the samples.

The preprocessing strategy adopted — smoothing combined with first derivative transformation — was selected following systematic evaluation of multiple combinations, including SNV correction applied individually and in combination. The selected strategy represented the best-performing option in terms of overall classification performance and, in particular, in terms of specificity towards fresh samples in cross-validation. A detailed comparison of classification results obtained with the alternative preprocessing strategies is reported in Supplementary Table S1.

The model was optimized by cross-validation, using 10 latent variables (LVs) for the unblotted model and 9 LVs for the blotted model.

The complexity of the selected model was confirmed by comparison with reduced models (5 latent variables): a smaller number of latent variables led to a substantial decline in performance in terms of calibration and cross-validation and, above all, an unbalanced performance trend on the prediction set, with sensitivity towards thawed samples falling to 70.5% for the unblotted model and to 65.7% for the blotted model. However, the increased number of LVs did not lead to model overfitting.

The results of the PLS-DA predictions are shown in Fig. 4. The figure illustrates the classification of NIR spectra of thawed and fresh samples for both models. In both cases, there is a tendency towards correct class assignment, although there are some classification errors. Quantitative classification performances for calibration, cross-validation, and prediction are reported in Table 1. In calibration and cross-validation, both models achieved comparable and balanced classification rates for spectra collected from thawed and fresh samples, with correct classification percentages generally around 85%, indicating good model stability. In the external prediction, a decrease in performance was observed for both models, focusing on the spectra collected from thawed samples, whose correct classification rates fell to around 75%.

Sensitivity and specificity values confirm these trends. In calibration and cross-validation, both models showed balanced sensitivity and specificity values, consistently above 84%. In external prediction, sensitivity towards spectra collected from thawed samples remained comparable for the two models (≈75%), while specificity towards spectra collected from fresh samples was higher for the unblotted model (79.05%) than for the blotted model (72.38%). Overall, the unblotted model showed slightly more balanced and better performance. A more detailed analysis of the misclassified spectra in the prediction set showed that some samples were affected by a single misclassification reading. For the unblotted model, three fresh samples and five thawed samples had one misclassified replicate, while for the blotted model, two fresh samples and six thawed samples were affected.

At the sample level — after averaging the replicate predicted Y values for each specimen —PLS-DA results gave comparable performance to the spectral-level model, with correct classification rates of 74.29% for fresh samples and 77.14% for thawed samples in external prediction (Supplementary Table S2).

The classification performances obtained in this work appear lower than those reported under more controlled experimental conditions by previous studies. Our fully independent external validation on octopus specimens provides a more realistic assessment of performance under real-world variability, despite the expected decrease in accuracy, and therefore leads to results that differ from those reported in the literature. Pennisi et al. (2021) reported sensitivity and specificity values of 100% and 97.1%, respectively, for the classification of fresh and thawed musky octopus using MicroNIR spectra modelled by PLS-DA with 7 latent variables. However, their study included only 25 specimens; specifically, the same samples were analysed both as fresh and after freezing and thawing. Although five sampling days were planned over a representative period, no information was provided regarding suppliers,

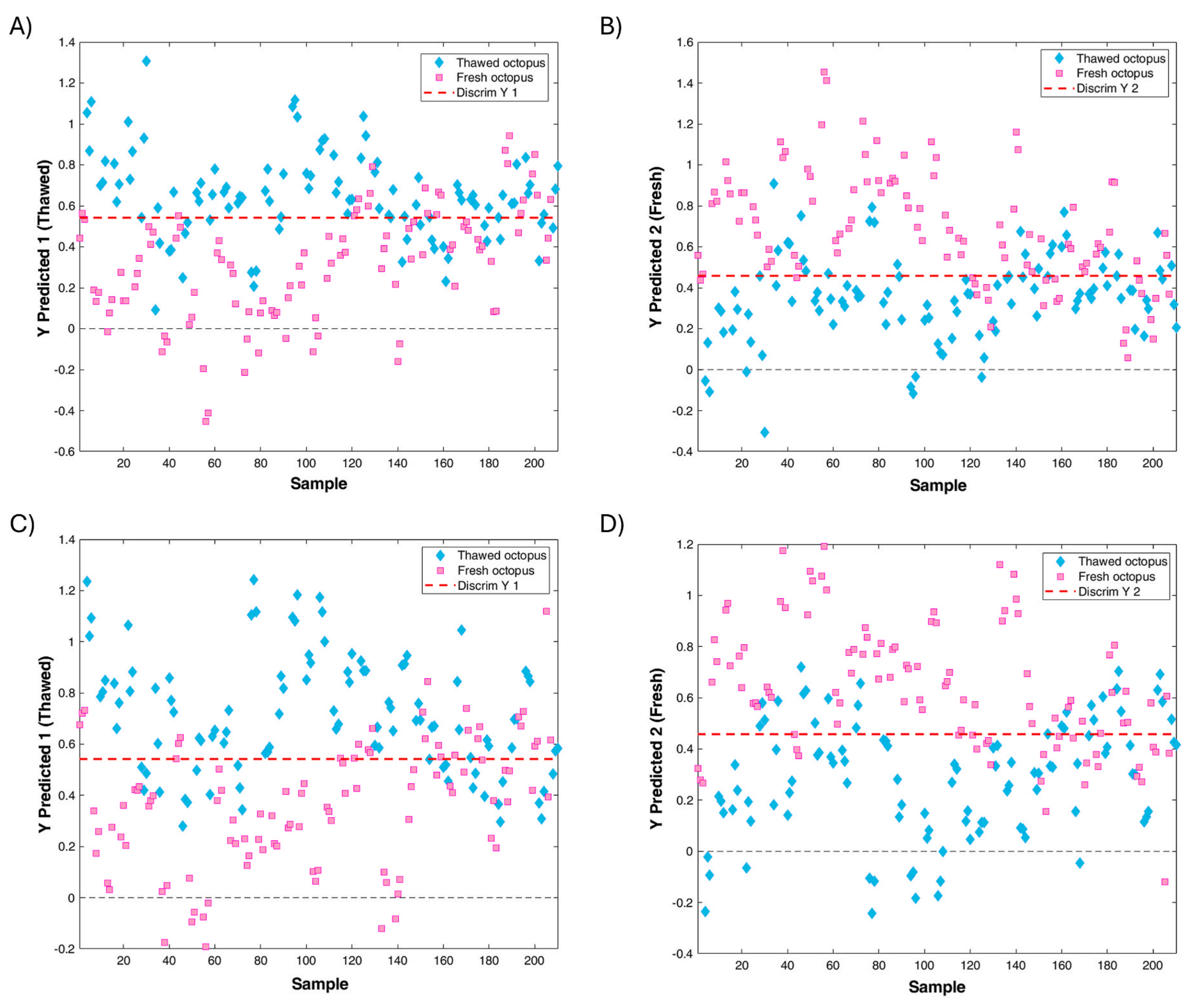

Fig. 4. PLS-DA predicted Y-values for *Octopus vulgaris* samples from the external test set (n = 70). Panels A and B show predictions for unblotted samples modelled as Y = thawed (A) and Y = fresh (B). Panels C and D show predictions for blotted samples modelled as Y = thawed (C) and Y = fresh (D). The dashed horizontal line indicates the discrimination threshold. Fresh samples are shown in pink, thawed samples in light blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
PLS-DA classification results for octopus' NIR measurement, showing the percentages of correct predictions and the number of spectral measurements (in brackets) for each class. Calibration, cross-validation and prediction results are reported for models built on unblotted and blotted samples. T: thawed; F: fresh.

		Calibration		Cross-validation		Prediction	
		T	F	T	F	T	F
Pred (unblotted)	**T**	87.25% (267)	13.80% (29)	87.60% (268)	15.20% (32)	75.20% (79)	20.95% (22)
	F	12.75% (39)	86.20% (181)	12.40% (38)	84.80% (178)	24.80% (26)	79.05% (83)
Pred (blotted)	**T**	86.27% (264)	15.24% (32)	85.62% (262)	15.24% (32)	75.24% (79)	27.62% (29)
	F	13.73% (42)	84.76% (178)	14.38% (44)	84.76% (178)	24.76% (26)	72.38% (76)

catching practices, or storage conditions (e.g., ice storage). Similarly, Sannia et al. (2019) achieved sensitivity and specificity values of approximately 91% in external validation for cuttlefish using spectra collected with a portable device (poliSPECNIR, ITPhotonics, Breganze, Italy). While the authors considered variability related to catching areas (Italy, FAO zone 37.2.1, and France, FAO zone 27), frozen–thawed samples were obtained by freezing specimens originally purchased as fresh under standardised conditions. In addition, only 25 samples were analysed, which limits the statistical robustness of the model and the generalisability of the results. The study by Currò et al. (2022) better reflects market variability, as it considered three catching seasons, 44 commercial batches, and three catching areas, for a total of 359 fresh–thawed and 221 fresh individual cuttlefish. The Support Vector Machine models developed to discriminate between fresh and frozen–-thawed samples achieved a sensitivity of 89% and a specificity of 94% in prediction (Currò et al., 2022). However, it is unclear whether the prediction step accounted for batch-level variability.

In Currò et al., 2022 work, as in this study, fresh samples were transported and stored on ice prior to analysis, reflecting standard commercial handling practices. However, in contrast to our findings, ice storage did not appear to affect classification performance. This disagrees with Vaz-Pires and Barbosa (2004), who reported that ice storage can induce structural and biochemical modifications in cephalopod muscle, potentially affecting water distribution and protein–water interactions.

In the present study, these changes may have partially affected the NIR signal, leading to overlap with the spectral effects induced by freezing and thawing. This likely resulted in increased similarity between some fresh and thawed samples and, consequently, greater classification ambiguity.

3.4. Translation of NIR – PLS-DA outputs into a PAT-oriented decision logic

To translate the results of chemometric classification based on spectral measurements into an operational decision-making strategy suitable for PAT applications, the result of the best PLS-DA model (unblotted samples) was further analysed. For each sample, the replicate NIR spectra were independently projected onto the PLS-DA model to obtain individual predicted Y values.

Sample-level decision variables were then calculated as the mean predicted Y value (Mean($\hat{Y}$)) and its standard deviation (SD) across replicate NIR measurements, reflecting both classification tendency and prediction consistency at the sample level. This approach was applied to both the training set (2 replicates per sample) and the external test set (3 replicates per samples), and results were visualized in a two-dimensional scatter plot of Mean($\hat{Y}$) versus SD (Fig. 5). This representation allows simultaneous evaluation of the classification trend and the intra-sample measurements consistency, which is relevant in at-line PAT applications where decisions must be robust with respect to measurement variability rather than based on individual spectral acquisitions (Nishal et al., 2024).

As shown in Fig. 5, fresh samples tend to cluster in a defined region characterised by low Mean($\hat{Y}$) values and limited SD, while thawed samples are predominantly distributed outside this area. Based on the training set distribution, a conservative acceptance zone for fresh samples was defined by imposing thresholds on the mean predicted value (Mean ($\hat{Y}$) $<$ 0.55) and the standard deviation of replicate predictions

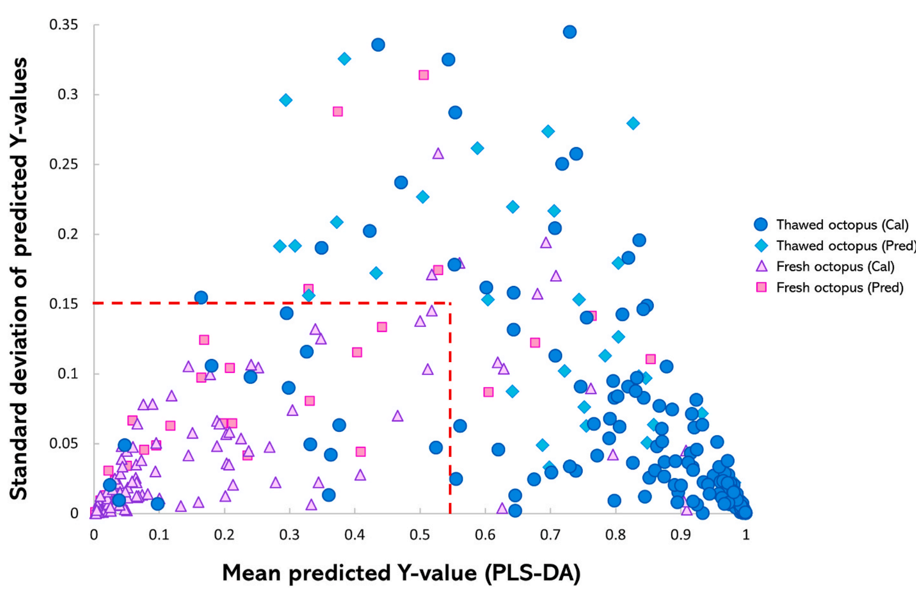

Fig. 5. Sample-level distribution of PLS-DA predictions represented as mean predicted Y-value versus standard deviation across replicate NIR measurements, for training set samples (2 replicates; n = 258) and external test set samples (3 replicates; n = 70). Each point represents one sample. The red dashed area defines the conservative acceptance zone for fresh samples (Mean($\hat{Y}$) $<$ 0.55; SD $<$ 0.15). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(SD < 0.15), selected to maximize the inclusion of fresh samples while minimising the risk of incorrectly accepting thawed samples.

When applied to the training set, these criteria resulted in only 9.15% of thawed samples being incorrectly accepted as fresh, compared to 12.75% when using standard PLS-DA classification alone, confirming the improvement achieved by the PAT-oriented approach.

The thresholds were then applied to the independent external test set, where none of the thawed samples fell within the acceptance zone, even considering that the fresh samples had been subjected to transportation and ice storage prior to analysis — conditions known to induce partial spectral modifications in cephalopod muscle (Vaz-Pires & Barbosa, 2004). The effectiveness of the acceptance criteria under these real-world commercial operating conditions further confirms the soundness of the proposed decision-making framework.

The definition of an acceptance zone for fresh samples, rather than forced classification of all samples, reflects a risk-based PAT philosophy (Nishal et al., 2024). In this context, the critical quality attribute is the reliable identification of freshness, and the most critical risk is the incorrect acceptance of thawed samples as fresh. Samples that do not meet the acceptance criteria are therefore conservatively treated as likely thawed. While this approach may result in the rejection of some fresh samples, such behaviour is acceptable in a PAT context, as it prioritizes consumer protection, fraud prevention, and robust decision-making.

Based on the acceptance criteria identified in Fig. 5, a PAT-oriented decision flow was defined (Fig. 6). The flowchart integrates NIR spectral acquisition, chemometric prediction, replicate consistency assessment, and final acceptance or rejection of the sample as fresh. This workflow translates the multivariate model output into an easily implementable operational rule, suitable for at-line industrial applications and scalable quality control systems.

Following the principles of PAT design selection, this strategy aligns with the concept that PAT tools require robust, science-based decision rules derived from multivariate data and tailored to the specific risk profile of the process (Nishal et al., 2024). The proposed approach is consistent with at-line PAT implementations, where rapid measurements and simple decision criteria support timely and informed quality control decisions.

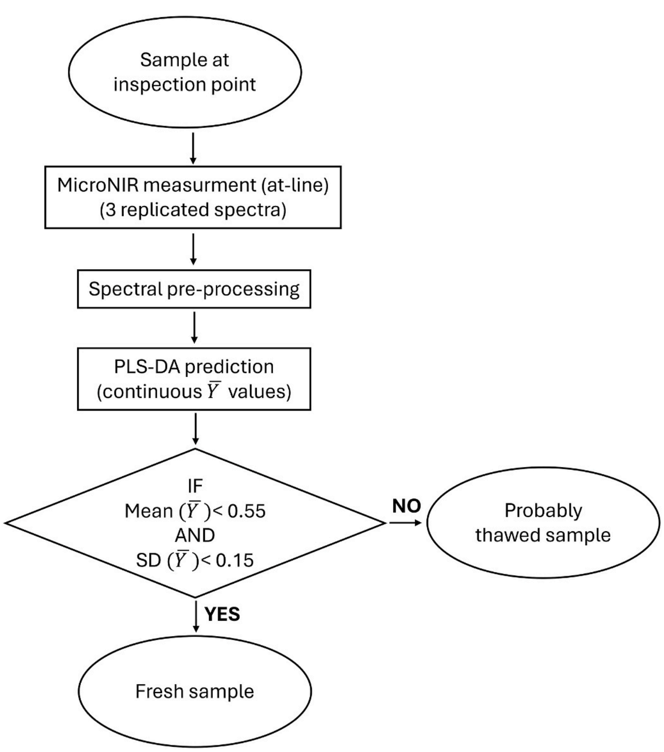

Fig. 6. PAT-oriented decision flow for freshness assessment of octopus samples. The workflow integrates miniature NIR spectral acquisition, PLS-DA chemometric prediction across three replicate measurements, and evaluation of mean predicted Y-value and standard deviation against empirically defined acceptance criteria. Samples meeting both thresholds (Mean < 0.55 and SD < 0.15) are accepted as fresh; all others are conservatively treated as likely thawed.

4. Conclusion

This study evaluated the applicability of a portable NIR spectrometer combined with chemometric modelling for discriminating between fresh and thawed *Octopus vulgaris* under conditions representative of the real-world food supply chain.

Using PLS-DA models developed on MicroNIR spectra, classification performance of approximately 85% was achieved in calibration and cross-validation for both fresh and thawed samples, indicating good model stability. In external prediction, a decrease in performance was observed, with correct classification rates for thawed samples around 75%, while specificity for unblotted fresh samples reached 79.05%. Although these values are lower than those reported in previous studies conducted under controlled conditions and with reduced sampling variability, they provide a realistic estimate of the model's behaviour in industrial settings, where ice storage, handling practices, and biological variability contribute to spectral overlap between classes.

Beyond classification accuracy, this work focuses on translating chemometric results into PAT-oriented decision logic. By analysing the mean predicted Y values and the associated standard deviation across three replicate measurements, a conservative acceptance zone for fresh samples was empirically defined. It is important to note that none of the thawed samples in the external test set fell within this acceptance zone, effectively eliminating the most critical classification error — the incorrect acceptance of thawed octopus as fresh.

This risk-based strategy reflects the core principles of PAT, in which analytical tools are designed to support robust process control. Samples that fail to meet acceptance criteria are treated as likely thawed, accepting the potential rejection of some fresh products as a trade-off to maximize consumer protection, fraud prevention, and decision reliability.

Overall, the integration of portable NIR spectroscopy with a simple and interpretable decision flow demonstrates the feasibility of implementing in-line PAT tools for seafood authenticity testing. This approach enables rapid screening, supports supplier and process monitoring, and reduces reliance on destructive confirmatory analyses. Future developments should focus on expanding the dataset and refining decision thresholds, with a focus on further enhancing the model's robustness. Notably, the proposed workflow has already been implemented in an industrial quality control setting, demonstrating its practical feasibility. Long-term monitoring will enable the assessment of aspects such as instrument drift, operator variability, environmental conditions and the consistency of the measurement location, which are recognised as critical factors for maintaining the performance of online PAT instruments.

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CRediT authorship contribution statement

Irene Locatelli: Data curation, Validation, Writing – original draft. **Fabio Benecchia:** Conceptualization, Formal analysis, Methodology. **Paolo Giuseppe Ubaldi:** Conceptualization, Methodology, Writing – review & editing. **Alberto Pianezzola:** Conceptualization, Methodology, Writing – review & editing. **Elena Santi:** Conceptualization, Methodology, Writing – review & editing. **Silvia Grassi:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2026.112477.

Data availability

The data that has been used is confidential.

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