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Real-time and non-destructive control of the freshness and viability of live mussels through portable near-infrared spectroscopy

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ABSTRACT

This study introduces the application of a novel, rapid, and non-destructive method employing portable near-infrared (NIR) spectroscopy to assess the freshness and viability of live mussels throughout their shelf-life. NIR spectra ranging from 908 to 1676 nm were collected from 150 Mediterranean mussels (*Mytilus galloprovincialis,* L.) over a 10-day storage period. Simultaneously, measurements of weight loss, intravalvular liquid pH, and nonprotein nitrogen levels were taken. A predictive model was established by correlating the multivariate information derived from NIR spectral data with days of storage under refrigerated conditions using orthogonal partial least squares regression (OPLSR) analysis. While physical and chemical parameters (except for gradual weight loss due to water leakage) showed no distinct trends throughout storage, the fourth derivative-preprocessed NIR spectra enabled the construction of an OPLSR model that, following cross-validation, exhibited a correlation coefficient of 0.86 and, following external validation with new mussel samples, an average accuracy (root mean square error of prediction) of just 1.3 days. The predictive power of the model was primarily attributed to specific NIR wavelengths associated with key chemical features, including unsaturated fatty acids, nitrogen compounds, water content, glycerol, and ATP-related compounds that, collectively, constituted a distinctive fingerprint for forecasting mussel storage duration. The performance of the tool, along with its environmentally friendly, non-invasive, real-time, and cost-effective characteristics, align with industry control procedures and inspection needs, allowing effective freshness and viability assessment of live mussels across the food supply chain.

1. Introduction

Mussels represent prized and esteemed seafood commodities, with their consumption being widespread worldwide. Two prominent species, namely Atlantic mussel *(Mytilus edulis* L.) and Mediterranean mussels (*Mytilus galloprovincialis* L.), are extensively harvested along the European coastlines, holding significant economic value and being of particular interest to stakeholders in the seafood industry (EUMOFA, 2022).

Mussels, like other bivalve mollusks, can filter large amounts of water, which makes them vulnerable to the accumulation of microbes and environmental pollutants. Furthermore, following harvest, these products are highly perishable and prone to spoilage. These aspects raise significant concerns regarding the safety and health implications potentially associated with their consumption. In Europe, specific regulations have been established to ensure that mussels meet microbiological and maximum chemical contaminant criteria, while also adhering to sensory characteristics associated with freshness and viability (European Commission, 2004). Hence, for food business operators involved in storing and transporting live mussels and other bivalve mollusks, maintaining appropriate temperatures that do not compromise their viability is of paramount importance from a commercial perspective and from a hygiene and health standpoint.

Usually, mussels demonstrate a remarkable ability to endure outside the aquatic environment for more than ten days under refrigerated conditions (Babarro et al., 2007; Isani et al., 1995). Even under chilling storage conditions, the prolonged preservation of live mussels becomes compromised due to the occurrence of hydrolytic, proteolytic, and

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lipolytic reactions, which can be attributed to both endogenous and bacterial enzymes. These biochemical processes can boost the proliferation of spoilage and pathogenic microorganisms and result in undesirable sensory alterations of the mussels (Erkan, 2005; Mueda et al., 2019). Within this context, the European regulatory framework permits the replacement of the expiration date on the label for bivalve mollusks with a statement indicating that "these animals must be alive when sold" (European Commission, 2004).

Official methods for assessing the freshness of fish and seafood typically rely on sensory (sight, smell, odor, and tactile) evaluation. If sensory evaluation raises doubts about freshness, then the quantification of specific freshness-related chemical indicators is adopted (European Commission, 2004, 2019). However, these methods are subjective, often time-consuming, reliant on specialized skills, and their accessibility across various stages of the fishery processing can be limited (Prabhakar et al., 2020). Additionally, official procedures for assessing the freshness of seafood using both sensory and chemical indicators have been defined for only a restricted selection of fish, crustacean, and cephalopod species, but not for bivalve mollusks (Council of the European Union, 1996; European Commission, 2019). Consequently, the evaluation of mussel freshness and viability heavily depends on the expertise of the official veterinarians, who typically conduct examinations for clean and intact shells, test the response of the mollusk to percussion, and observes the presence of normal intravalvular liquid. Nonetheless, this method may be influenced by inherent biases.

Within this framework, the integration of near-infrared (NIR) spectroscopy into the fishery supply chain may represent a highly promising and advantageous approach. This powerful technique enables rapid and non-destructive sample analysis, generating a fingerprint absorbance spectrum that comprehensively captures the chemical and physical properties of the sample. Consequently, by exploiting NIR spectra analysis, it becomes theoretically feasible to establish a quantitative relationship between the spectral data and the biochemical changes occurring during preservation, enabling an objective assessment of food freshness. Evidence supporting the effectiveness of NIR spectroscopy in assessing freshness and predicting the shelf life of various animal-derived foods has been well-established through several studies involving pork (Arias et al., 2022; Kucha & Ngadi, 2020; Tejerina et al., 2021) and lamb (Zhang et al., 2022). While applications to fish and seafood products have been explored, they have primarily focused on the freshness evaluation of fish (Khojastehnazhand et al., 2014; Kimiya et al., 2013; Li et al., 2022; Sivertsen et al., 2011), with only one instance focusing on the freshness assessment of shelled oyster (Madigan et al., 2013). Hence, despite the proven utility of NIR spectroscopy in the field of food freshness assessment, a notable research gap remains unaddressed. Existing literature reports have indeed highlighted the capability of NIR radiation to penetrate calcium carbonate shells, as observed in eggs, thereby offering valuable insights into the organic composition of the underlying albumen and yolk (Aboonajmi & Abbasian Najafabadi, 2014; X. Dong et al., 2018; Lima Brasil et al., 2022). However, the potential application of NIR spectroscopy on intact live bivalves, specifically to record information though the shell concerning the underlying edible soft tissue, has not been explored yet.

Considering the aspects mentioned above, this study aimed to employ a novel approach, utilizing a portable and miniaturized NIR spectroscopy instrument, for the assessment of freshness and viability live mussels during their shelf life under typical refrigeration conditions, ultimately enabling the prediction of their storage duration.

2. Materials and methods

2.1. Sample collection and storage

Live Mediterranean mussels (*Mytilus galloprovincialis* L., Bivalvia: Mytilidae) were obtained from harvesting plants situated in the northern Adriatic Sea (FAO fishing area 37.2.1). The mussels were collected within 1 h after harvesting, directly from an offshore local dispatch center responsible for reception, conditioning, washing, cleaning, grading, wrapping, and packaging of live bivalve mollusks. The production area of origin for the mussels was classified as "class A″ by the relevant authority, indicating compliance with regulations regarding the microbiological and chemical quality of the production waters. Therefore, no purifying procedures were applied to the mussels included in the experiment. Specifically, five different batches, each weighing 2 kg and containing approximately 100–120 mussels per bag, were collected and put in cool insulated polystyrene boxes with ice bricks included, avoiding the direct contact with the ice bricks such that it did not compromise their vitality. The samples were promptly transported to the laboratory and individually labeled with white enamel. Each of the five mussel batches was randomly split into two separate groups. One group (i.e., Group 1) consisted of 30 specimens and was assigned for daily non-destructive NIR analysis. The other group (i.e., Group 2), comprising the remaining 70–90 specimens, was designated for destructive chemical analyses. From Group 2, another subset of 30 mussels (6 specimens from each batch) was randomly chosen for biometric analysis which involved using a precise caliper for measurements. Average shell dimensions of the samples are summarized in Table 1.

Throughout the entire experimental storage period of 10 days all the samples were stocked at a temperature of 5.5 ± 2 °C and retained in their original nets to avoid prolonged contact with the draining intervalvular liquid, thereby replicating standard commercial storage conditions. The timepoints considered for physicochemical and NIR analysis, from the day of collection to the end of the storage period at intervals of 24 h, were as follows: T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, and T10. At each timepoint, survival rate and viability of the mussels belonging to Group 1 were assessed by verifying that the shells which had opened were able to reseal upon being tapped in a brief timeframe.

2.2. Weight loss and pH measurement during storage

Each of the five batches of mussels of Group 1 designated for NIR analysis was weighted individually using a precision balance (KERN EW1500-2M, Kern & Sohn GmbH, Balingen, Germany) at each timepoint considered, in order to measure the weight loss caused by the gradual drainage of intravalvular liquid during storage. The weight loss was calculated for each batch by subtracting the new weight from the initial weight (T = 0) and expressing it as a percentage relative to the T0.

The measurement of pH was performed on intravalvular liquid collected from a pool of 5 specimens for each mussel batch belonging to Group 2 after stabilization at room temperature and measured at T0, T2, T4, T6, T8, and T10 using a portable pH meter (edge ® HI2002-02, Hanna Instruments, Columbus, OH, USA).

Statistical differences of weight and pH over the storage period were evaluated by performing an ANOVA test followed by a Tukey's post hoc test (considering as significant a p value ≤ 0.05) by using the software package OriginPro 2023 (v. 10.0.0.154, Origin Lab Corporation, USA).

Table 1
Biometric measurements of the five batches of mussels (*Mytilus galloprovincialis* L.) considered in the present study [a].

Batch N.	A	B	C	D	E	Average
Width (cm)	3.3 ± 0.34	3.4 ± 0.17	3.4 ± 0.31	3.3 ± 0.18	3.2 ± 0.11	3.3 ± 0.36
Length (cm)	6.7 ± 0.41	6.3 ± 0.48	6.6 ± 0.43	6.7 ± 0.36	6.6 ± 0.26	6.6 ± 0.42
Thickness (cm)	2.4 ± 0.31	2.2 ± 0.12	2.4 ± 0.27	2.5 ± 0.25	2.4 ± 0.15	2.4 ± 0.25

[a] Values refer to mean ± standard deviation calculated on n = 6 specimens per batch.

2.3. Nonprotein nitrogen

Nonprotein nitrogen (NPN) content was determined following the AOAC 991.21-Kjeldhal method (AOAC (Association of Official Analytical Chemists), 2012) with slight modifications. Briefly, at six storage time points (T0, T2, T4, T6, T8, and T10), a 4 g pooled aliquot of mussel soft tissue from each batch of Group 2 was homogenized with distilled water using an Ultra-Turrax homogenizer (IKA T18, IKA-Werke GmbH and Co. KG, Staufen, Germany). After centrifugation at 10,000×*g* at 5 °C for 20 min, the samples were filtered through Whatman™ No. 1 filter paper (1 μm pore size, 180 μm thick). Then, 20 ml of the filtrate was mixed with 20 ml of 10 % trichloroacetic acid and subjected to another round of centrifugation and filtration. Next, 20 ml of the final solution obtained was digested with 17 ml of a 95 % solution of RS grade sulfuric acid and phospho-sulfuric acid (both from Carlo Erba Reagents SpA, Rodano, Italy). The digested samples were distilled using a Gerhardt® Vapodest 50 distillation apparatus (Gerhardt GmbH & Co. KG, Königswinter, Germany) with 20 ml of 0.1 N hydrochloric acid (ConvoL Normadose®, VWR Fontenay-sous-Bois, France) as the receiving solution, followed by titration using 0.1 N sodium hydroxide (ConvoL Normadose®, VWR Fontenay-sous-Bois, France). Final results were expressed as g 100 g^{-1} of NPN. Statistical differences over the storage period were evaluated by performing an ANOVA test followed by a Tukey's post hoc test (statistically significant differences at a p value $\leq$ 0.05) using the software package OriginPro 2023 (v. 10.0.0.154, Origin Lab Corporation, USA).

2.4. Portable NIR spectroscopic analysis

Live mussels of Group 1 were analyzed at T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, and T10 using the portable MicroNIR OnSite-W device (Viavi Solutions, Santa Rosa, CA, USA), coupled with the spectral acquisition software MicroNIR Pro™ (v.3.1, Viavi Solutions, Santa Rosa, CA, USA). The instrument is equipped with two vacuum tungsten lamps as illumination sources, a linear variable filter as a dispersing element, and a 128-pixel indium gallium arsenide photodiode array detector. Absorbance spectra were obtained by summing 100 scans, with an integration time of 10 ms, in the NIR range of 908–1676 nm (resulting in a spectral apparent resolution of 6.1 nm). Overall, the approach involved adapting a previously established method and implementing a workflow that optimized the NIR data acquisition process, aiming to enhance experimental productivity and ensure greater reproducibility of the recorded spectra (Varrà et al., 2022).

To perform the analysis, the mussels were kept outside of refrigerated conditions for a maximum of 20 min, which was the required acquisition time for analyzing 30 samples from each batch. The spectra were measured at laboratory room temperature (22 ± 2 °C) each day of preservation by positioning the acquisition window of the NIR spectrometer perpendicularly on the outer surface of one of the two valves of the live mollusk, specifically at the point of greatest convexity, while avoiding the anterior and posterior regions (Fig. 1). To account for potential inhomogeneity, four replicate NIR spectra were recorded for each intact mussel by slightly rotating the device approximately 90 °C during each measurement. Hence, a total of 600 spectra per recording day (5 mussel batches x 30 specimens x 4 spectra) were collected. Simultaneously, representative NIR spectra were recorded for a total of N = 30 shells and N = 30 soft mussels from the same specimens, with four replicates each. These spectra were exclusively employed for comparative purposes with the NIR spectra of intact mussels.

The background signal of the instrument was corrected by periodically acquiring a total absorbance (dark) and a total reflectance reference spectra (Spectralon® 99 %, LabSphere, North Sutton, NH, USA) for every series of 120 individual spectra.

Fig. 1. Acquisition of NIR spectra using the portable instrument though the shell of live mussels to predict the storage time.

2.5. Multivariate NIR data analysis: prediction model for storage time

The four NIR spectra acquired for each sample were averaged to obtain a representative mean spectrum. The complete data matrix, consisting of 1650 spectra with 125 NIR absorbance points each, was randomly divided into two separate datasets using a 75:25 partitioning criterium. The first dataset, including 1238 spectra, was designated as the calibration (training) set, while the second dataset, comprising 412 spectra, was assigned as the validation (prediction) set.

Before subjecting raw mean spectra of the training set to chemometric analysis, potential multivariate outliers were checked using the Hotelling's T^2 test statistics (95 % confidence interval) and then signal correction techniques (SNV and MSC) as well as differentiation methods (1^{st}, 2^{nd}, 3^{rd}, and 4^{th} Der) were applied to correct spectra and increase resolution. Subsequently, orthogonal partial least squares regression (OPLSR) analysis, specifically selected for its suitability in creating a calibration equation for predicting the storage time of mussels (i.e., the number of days after collection), was employed to the various sets of pre-processed spectra, each treated with distinct pre-processing techniques. The optimal combination of pre-processing methods was determined by assessing both the visual improvements in the training NIR spectra and objective statistical measures demonstrating the quality, robustness, and stability of the regression model.

The best OPLSR calibration model was thus selected, internally validated using a 7-fold cross-validation procedure, and finally externally validated by using the validation set. The statistical metrics used to evaluate the reliability of the OPLSR model included: i) r^2 (coefficient of determination) - indicating the linearity of the regression line; ii) R^2X - representing the variability of the spectral data modeled by all the

extracted latent variables; iii) R^2Y - reflecting the variability associated with class labels explained by all the extracted latent variables; iv) Q^2X - showing the variability associated with class labels predicted by all the extracted latent variables; v) RMSECV (root-mean-squared error of cross-validation) - indicating the performances of the regression model within the training data using cross-validation; vi) RMSEE (root-mean-squared error of estimation) - representing the accuracy of the model in estimating values for data points within a separate validation dataset that was not part of the model training process.; vii) RMSEP (root-mean-squared error of prediction) – indicating the predicting performances of the regression model when this is applied to completely new and independent data that was not used during model development or validation; viii) RPD (residual prediction deviation – calculated as the ratio between the standard deviation of the predicted storage days of the samples of the calibration set during CV (or of the samples of the validation set during external validation) and the RMSECV (or the RMSEP); ix) RER (range error ratio) – calculated as the ratio between the range (maximum − minimum) of predicted storage days of the samples of the calibration set during CV (or of the samples of the validation set during external validation) and the RMSECV (or the RMSEP). RPD and RER values higher than 2 and 10, respectively, can be considered adequate for prediction purposes (Lima Brasil et al., 2022; Páscoa et al., 2013; Saeys et al., 2005).

The predictive Variable Importance in the Projection (VIP) index was finally used to elucidate the key NIR wavelengths that significantly impacted on the prediction of mussel storage days, with VIP values 1 deemed as significant (Andersen & Bro, 2010).

The data pre-processing and multivariate data analysis were carried out using SIMCA® software version 17.0.2.34594 (Sartorius Stedim Data Analytics AB, Umea, Sweden).

3. Results and discussion

3.1. Survival rate and physicochemical changes of mussels during preservation

The daily observation of the samples destined to NIR acquisition revealed that all the specimens under consideration were alive up to storage day 8 (T8). The death of only two specimens was recorded on day 9 (T9), while at day 10 (T10) a total of 4 samples were found to be dead (representing 2.7 % of the inspected samples), although without any evident sensorial alteration. This high survival rate is not surprising since bivalve mollusks, when stored at an appropriate temperature, exhibit a high level of resistance, primarily attributed to their adept capacity to finely regulate their metabolic rate in order to acclimate effectively to the challenging new environment (Barrento & Powell, 2016).

Variations in the weight, pH, and NPN content of the analyzed mussel specimens during the entire storage period are graphically represented in Fig. 2. Live mussels naturally respond to high stress and hypoxia during storage by opening their valves. This action aims to counteract these conditions and enable oxygen to reach the surface of the liquid but also results in the inevitable loss of the liquid (Jozić et al., 2017). In this work, a progressive decline in the overall weight of the samples due to the loss of intravalvular liquid was observed over time, as depicted in Fig. 2A, wherein the initial weight was 730 ± 17 g at T0 (average weight of the 5 batches considered) and 510 ± 14 g at T10. The weight of the samples at T2 was 619 ± 25 g, which was significantly different compared to weights recorded at T0 and T1 ($p \leq 0.05$). Indeed, at T2, the greater release of intravalvular liquid was noted, which resulted in an overall weight loss of 15 ± 5 % relative to the baseline (T0). Although delayed compared to what was expected, this outcome can plausibly be attributed to the adaptation of the mussels to the novel environmental storage conditions subsequent to their collection from the marine habitat (Jozić et al., 2017). Indeed, in the hours immediately following harvest, mussels reduce their metabolic rate and close their valves to prevent water loss. However, during prolonged hypoxia, mussels initially open their shells and switch to anaerobic metabolism to maintain a lower ATP turnover (Barrento & Powell, 2016; Powell et al., 2017). Following the T0–T2 storage interval, the weight of the mussels displayed a more gradual and linear decrease ($r^2 = 0.99$, $p \leq 0.05$), diminishing by approximately 2–3 % per day from T3 to T10 (Fig. 2A). The weight reduction observed at T3 was 19 ± 5 %, close to the range of 12–14 % that other authors have documented following a 72-h storage period at a refrigeration temperature of +5 °C (Angelidis, 2007; Jozić et al., 2017). The final weight of the samples at the end of the experimentation (T10) was found to be 30 ± 3 % lower than the baseline weight recorded at T0.

No consistent patterns of pH values of the intravalvular liquid were observed throughout the storage period, whether in terms of clear increase or decrease (Fig. 2B). As it can observed, the highest pH values were recorded at T0, being 7.03 ± 0.191. The pH measurements converged to an average of 6.86 ± 0.074 at T2, being significantly different than those measured at T0 (Fig. 2B). Consistently with our findings, an initial 7.16–7.28 pH value range immediately after mussel collection and a slight decline to 7.02–7.08 pH value range subsequent to 72 h of storage at 5 °C were previously reported in intravalvular liquid of mussel samples (Jozić et al., 2017). This reduction could be attributed to anaerobic metabolic processes and the production of carbon dioxide induced by the displacement of the mussels from the natural environment (Pastoriza et al., 2004). Nevertheless, divergent outcomes emerged from the investigations conducted by other researchers, revealing a steadily alkalinization of the intravalvular liquid from an average initial pH of 5.80 to a value of 7.00 after 72 h (Angelidis, 2007). Hence, the pH oscillations noted between T2 to T10 could potentially be attributed to the accumulation of lactic acid, carbon dioxide, ammonia, and trimethylamine as major acidic or basic metabolic byproducts (Angelidis, 2007; Aru et al., 2016), which may have induced non-uniform pH levels in the liquid throughout the duration of storage.

The trend of NPN content in the tissue of mussels over the entire storage period is plotted in Fig. 2C. The initial measurement (T0) yielded a value of 0.78 ± 0.130 g 100 g^{-1}. This content primarily comprises compounds such as TMAO, ammonia, nitrate and nitrite, free amino acids, urea, polyamines, minor peptides, creatinine, and nucleotides.

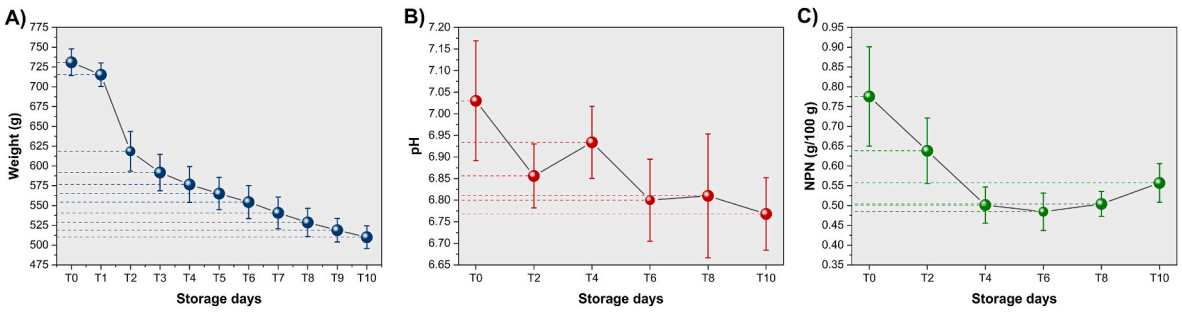

Fig. 2. Weight (A), pH (B), and NPN content (C) of live mussels measured over the 10-day storage period at + 5 °C.

These baseline NPN levels were found to be slightly higher than those reported for fresh bivalves such as striped venus clams (*Chamelea gallina*), green mussels (*Perna viridis*), and Mediterranean mussels (*Mytilus galloprovincialis*) as well (Binsi et al., 2007; Hirabayasi et al., 2022; Orban et al., 2007). Nonetheless, it is worth highlighting that the total NPN content can be affected by a multitude of factors, which encompass the physiological characteristics of the bivalve, alongside dimensions and variations due to seasonal changes (Orban et al., 2007).

Following T0, a reduction of NPN values was observed up to T4, with significant lower values at T2 (0.64 ± 0.111 g 100 g^{-1}) compared to T0 (0.78 ± 0.130 g 100 g^{-1}) and T4 (0.50 ± 0.052 g 100 g^{-1}) compared to T2 (Fig. 2C) ($p \leq 0.05$). No significant differences were found in the T4–T10 timeframe ($p > 0.05$), even though a tendency of NPN content to increase was observed. The progressive decrease in NPN content in mussels within the initial 96-h storage period is consistent with the literature, wherein other authors have associated this trend with either NPN components leaching from the soft tissue of mussels or the endogenous and/or bacterial enzymatic degradation of NPN constituents (Binsi et al., 2007). The first hypothesis finds additional support from the investigations conducted by Hirabayasi and colleagues, wherein a positive correlation was found between the reduction of NPN and the concomitant reduction in free amino acids, an important part subset of NPN constituents (Hirabayasi et al., 2022).

3.2. Estimating the storage time of mussels using portable NIR spectroscopy

3.2.1. NIR spectra features and pre-processing methods selection

While chemical tests can only be performed within a controlled laboratory setting and hinder the possibility of conducting live inspections of mussels in their natural storage environment, NIR spectroscopy offers non-destructive, fast, and remote mussel analysis, eliminating the necessity for direct mussel handling or lab transportation, providing valuable information on mussel characteristics, and addressing traditional method limitations.

The raw NIR spectra of the mussel samples analyzed in the present study are reported in Fig. 3, where Fig. 3A shows representative spectra of only the shells, Fig. 3B shows spectra of only the soft tissue of mussels, and Fig. 3C shows spectra of intact mussels (i.e., spectra recorded through the shell). As observed, no significantly prominent absorption bands were recorded when analyzing the shells, except for the mild absorption bands around the 1450 nm region typically associated to water (Workman & Weyer, 2012) and which is likely to be the consequence of the presence of humidity of the shells (Fig. 3A). Contrary to the flat spectra of the shells, the spectra of soft tissues and intact mussels exhibited similar patterns, characterized by absorption bands at the same wavelengths and with similar shapes (Fig. 3B and C). It is worth noting that the presence of the shell during the recording of the NIR spectra of the intact mussels led to an overall attenuation of absorption band intensity, likely due to light dispersion during analysis. Nevertheless, this observed attenuation should not be considered a negative outcome. Valuable spectra information related to the soft tissues underlying the shell was indeed retained, supporting the hypothesis that NIR light can effectively penetrate the outer shell of the mussel.

Prior to data manipulation, the raw NIR spectra of intact mussels underwent an assessment through the evaluation of the Hotelling's T^2 range plot (Fig. S1, Supplementary Material), which revealed that several samples at all the timepoints exceeded the 95 % confidence interval, but none of them fell above the 99 % confidence interval. Consequently, the decision was made to retain all the samples without excluding any, as none of them was deemed a significant outlier.

Due to the presence of broad and low informative bands, the raw NIR spectra of intact mussels were subsequently filtered using different pre-processing techniques. The statistical outputs resulting from 12 different cross-validated OPLSR models, built using different pre-processing techniques, are presented in Table 2, while the impact of applying these pre-processing strategies on the characteristics of the NIR spectra is illustrated in Fig. S2 (Supplementary Material). As observed from Table 2, the cumulative NIR spectral variation ($R^2X_{(cum)}$) was modeled less effectively when combining derivative methods with either SNV or MSC, as opposed to employing derivative methods alone. Similarly, the cumulative predictive ability ($Q^2_{(cum)}$) exhibited a decrease when combining 1st Der or 2nd Der with SNV or MSC, as opposed to utilizing derivative methods in isolation. Conversely, a significant enhancement was observed when the 3rd Der or the 4th Der were applied in tandem with SNV or MSC. Overall, the highest $R^2X_{(cum)}$ was achieved when exclusively employing the 2nd Der ($R^2X_{(cum)} = 0.982$), while the highest $R^2Y_{(cum)}$ and $Q^2_{(cum)}$ values (0.856 and 0.845, respectively), were attained when utilizing the 4th Der spectra (Table 2). The 4th Der spectra also yielded the lowest RMSEE and RMSECV values, measuring 1.21 and 1.25 days, respectively. The similarity between the performance metrics $R^2X_{(cum)}$, $R^2Y_{(cum)}$, and $Q^2_{(cum)}$, as well as the convergence of RMSEE and RMSECV in the OPLSR model constructed using the 4th Der spectra, can be regarded as strong indicators of the absence of overfitting (Ghidini et al., 2021; Hair et al., 2019). This assertion found further support through the visual inspection of the spectra, where the use of the 4th Der effectively mitigated light-scattering phenomena and facilitated the deconvolution of overlapping bands, all without introducing spectral artifacts or noise (Fig. S2, Supplementary Material). Fig. 4 depicts average 4th Der NIR spectra measured from the outside shell of live mussels at the beginning (T0), middle (T5), and the end of the storage time (T10). The NIR spectra of mussels displayed the highest absorption peaks within two distinct wavelength ranges: 1180–1220 nm (peak centered at 1200 nm) and 1430–1470 nm (peak centered at 1453 nm). Notably, samples nearing the end of their storage period exhibited the highest absorbance values within the first spectral range, whereas a gradual increase in absorbance was observed from T0 to T10 within the second range. Given that these absorption bands correspond to first overtone fundamental stretching vibrations of O–H functional groups typical of water molecules (Workman & Weyer, 2012), this result suggests that changes in the water content (such as simple water leakage) occurred and that these were visible in the NIR spectra (Currò et al., 2022; Sannia et al., 2019). Samples at the end of the storage time exhibited also lower absorbance values in the 1000–1040 nm and in the 1360–1400 nm spectral regions, where two other prominent peaks (at 1015 and 1390 nm respectively) were located. These peaks are associated to asymmetric stretching vibrations of N–H functional groups of primary aromatic amine and C–H functional groups of hydrocarbon chains of fatty acids (Varrà et al., 2021; Workman & Weyer, 2012). As previously reported, changes in NIR peaks in the 900–1030 nm spectral range are likely ascribed to chemical and physical transformations affecting proteins and lipids during the storage period (Pennisi et al., 2021; Sannia et al., 2019).

3.2.2. Calibration and validation of the prediction model

The OPLSR model correlating quantitative information enclosed within the 4th Der spectral data (calibration set, n = 1236) to the freshness and viability status of live mussels, was selected as the optimal model for predicting the storage time. This decision was based on the superior performance of the model, as evidenced by the highest results obtained during cross-validation (outlined in Table 2). In Fig. 5, the OPLSR score scatter plot is presented. Given the natural variability in specimen composition, there were no distinct, fully separated clusters of samples based on storage time; indeed, a considerable overlap among them was evident. Nevertheless, a remarkable trend emerged: samples consistently shifted from left to right as storage duration increased along the first predictive (horizontal) component. It is noteworthy that this component, although accounting for only 9.1 % of the total spectral variance, effectively captured this trend. Overall, this phenomenon underscored the gradual changes of chemical and physical characteristics of mussels during storage, which could be reliably tracked and monitored using NIR spectroscopy. The OPLSR calibration equation for

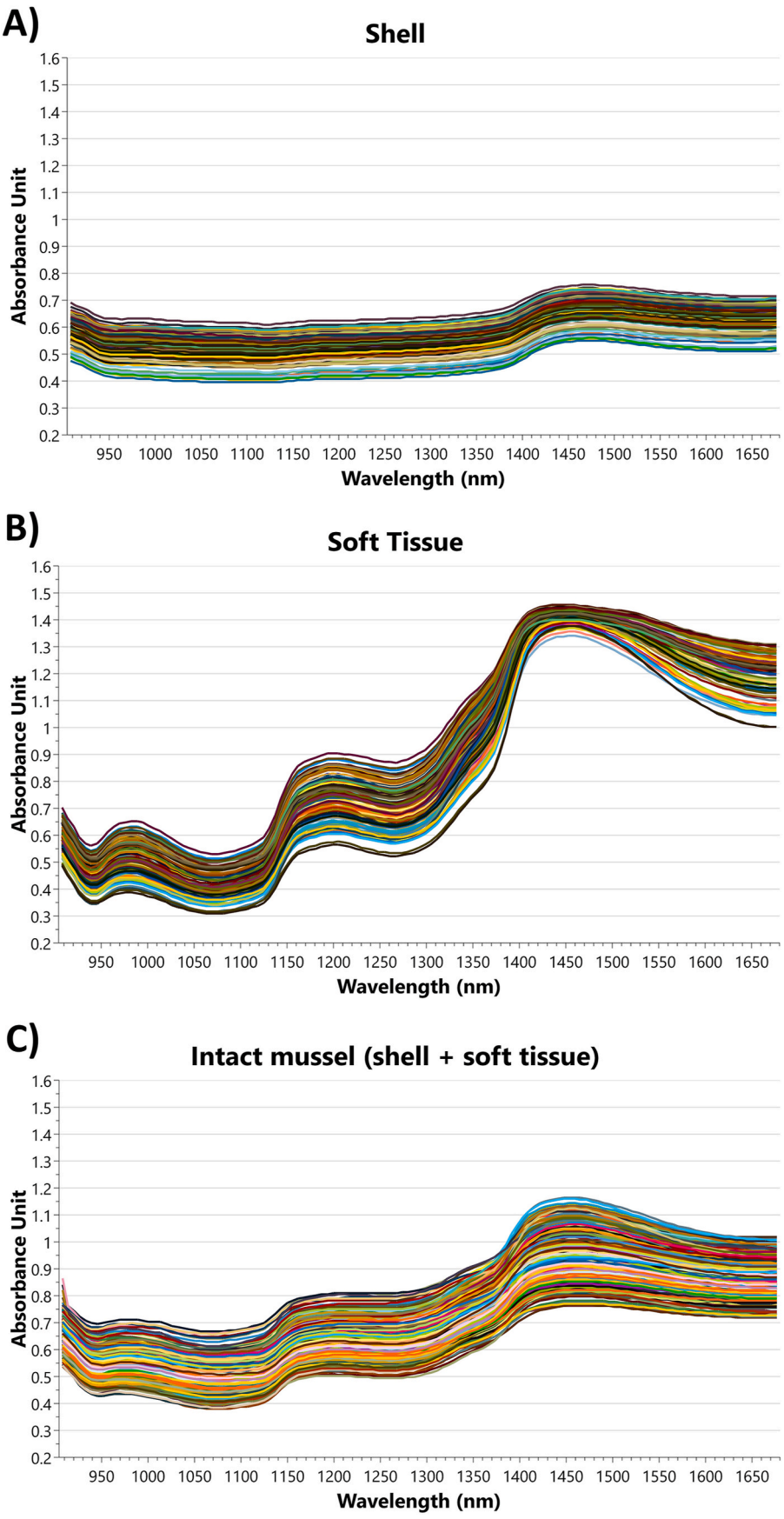

Fig. 3. Comparison among raw absorbance (log1/R) NIR spectra (908–1676 nm) of representative samples of mussel shells (A), mussel soft tissues (B) and intact mussels (shells + soft tissues) (C).

Table 2
Cross-validation results of OPLSR analysis applied to NIR spectra of live mussels (training set, n = 1238) aimed to predict the days of storage (up to 10 days).

Pre-processing	Comp	$R^2X_{(cum)}$	$R^2Y_{(cum)}$	$Q^2_{(cum)}$	RMSEE	RMSECV
1st Der	1 + 5	0.936	0.576	0.570	2.07	2.07
1st Der + SNV	1 + 3	0.797	0.556	0.551	2.11	2.12
1st Der + MSC	1 + 3	0.799	0.560	0.554	2.10	2.11
2nd Der	1 + 10	0.982	0.681	0.667	1.80	1.82
2nd Der + SNV	1 + 4	0.892	0.592	0.583	2.03	2.04
2nd Der + MSC	1 + 4	0.901	0.590	0.582	2.03	2.04
3rd Der	1 + 6	0.915	0.662	0.653	1.85	1.86
3rd Der + SNV	1 + 11	0.954	0.748	0.733	1.60	1.63
3rd Der + MSC	1 + 9	0.943	0.715	0.704	1.70	1.72
4th Der	1 + 12	0.934	0.856	0.845	1.21	1.25
4th Der + SNV	1 + 9	0.896	0.815	0.803	1.37	1.40
4th Der + MSC	1 + 9	0.903	0.810	0.796	1.39	1.43

Comp = total number predictive + orthogonal OPLS components; $R^2X_{(cum)}$ = cumulative modeled spectral variation; $R^2Y_{(cum)}$ = cumulative modeled variation associated to groups; $Q^2_{(cum)}$ = cumulative predictive variation; RMSECV = root mean square error from cross-validation (days of storage); RMSEE = root mean square error of estimation (days of storage); 1st Der = 1st order derivative; 2nd Der = 2nd order derivative, 3rd Der = 3rd order derivative; 4th Der = 4th order derivative; SNV = standard normal variate; MSC = multiplicative scatter correction.

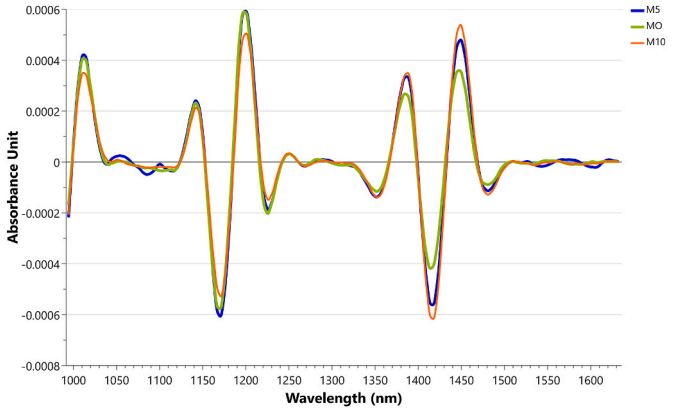

Fig. 4. Mean 908–1676 nm absorbance (log1/R) NIR spectra of mussel samples recorded at the beginning (T0), middle (T5), and the end (T10) of the storage period and transformed by 4th order derivatization.

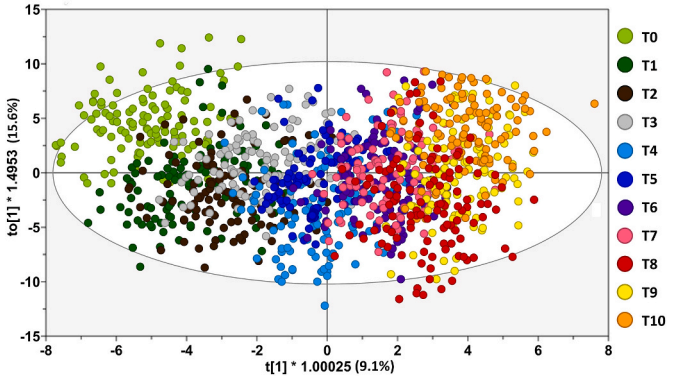

Fig. 5. Score scatter plot (first horizontal predictive component vs. first vertical orthogonal component) of the OPLSR model for the prediction of the storage time (up to 10 days) of live mussels (ellipse indicates 95°% confidence interval).

the prediction of the storage duration of mussels, along with the distribution of the training data along the regression line (i.e., the actual storage days vs. the storage days predicted using NIR spectroscopy) are visually depicted in Fig. 6. Within the observed range of 0–10 days of storage, a good degree of linearity was apparent, as evidenced by the high r^2 value of 0.86. This robust correlation suggests that the regression model effectively captured and explained the relationship between the spectral variables and the freshness status of the samples within this specific time frame. During cross-validation, the OPLSR model also exhibited a RPD value 2.74 and a RER value of 13.3, providing additional evidence of its suitability for the reliable prediction of the storage days of mussels. To enhance the understanding of the model's reliability, an assessment of residuals was conducted, as shown in Fig. S3 of the Supplementary Material. The residuals, which represent the differences between the actual and predicted values, exhibited a normal distribution pattern, closely aligning to a straight line and suggesting that the model's predictions were generally unbiased. Furthermore, the absence of any discernible outliers in the residual normal probability plot (i.e., no data points deviating significantly either below and to the right or above and to the left of the normal line by more than 3 standard deviations) provided additional assurance regarding the robustness of the model (Hair et al., 2019).

Although the promising findings achieved in cross-validation, the OPLSR model was tested for its generalization ability and further validated by predicting the storage time of the n = 412 mussel samples of the independent validation set. Remarkably, the attainment of an r^2 value of 0.83, an RMSEP value of just 1.3 days, a RPD value of 2.68, and a RER value of 11.83, closely mirroring the cross-validation metrics, affirms that the model performed well also when applied to unknown samples.

Table 3 provides further insights into the precision of the model in estimating the true storage duration of mussels within the validation set. The alignment between the predicted and actual storage days was noteworthy at all the considered time points. For instance, at T0, the model predicted an average storage day of 0.17, which remarkably mirrored the true freshness state of mussels upon harvest. As time progresses, the model's predictions consistently maintained their close correspondence to the actual days of storage of the mussels. On day 10, the model exhibited a slight underestimation of the actual storage time compared to its performance at other predicted time points. Nevertheless, predictions remained within an acceptable range, demonstrating the overall reliability of the model (Table 3).

In the context of fishery products, the application of multivariate data analysis to NIR spectra has demonstrated notable success, consistent with findings reported in the current study. For instance, regression analysis applied to NIR spectra yielded r^2 values exceeding 0.70, resulting in an overall accuracy of 1.04 days for predicting the freshness of cod (*Gadus morhua*) stored on ice for a duration of 17 days (Nilsen et al., 2002). Similarly, the integration of NIR spectroscopy with regression analysis was proven to be effective in predicting the storage days of salmon (*Salmo salar*) samples. In these investigations, r^2 values as

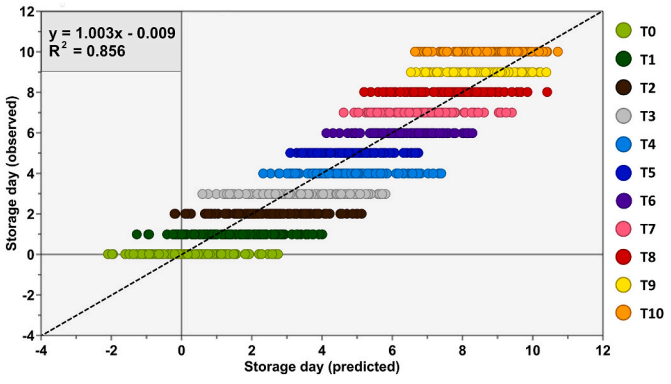

Fig. 6. Calibration line resulting from the application of the OPLSR analysis to the NIR spectra for the prediction of the storage time.

Table 3
Predicted days of storage (mean ± standard deviation, SD) of mussel samples of the independent validation set (n=412) using the calibrated OPLSR model.

	Predicted days of storage										
Days	0	1	2	3	4	5	6	7	8	9	10
Mean	0.17	1.6	2.2	3.4	4.6	4.7	6.5	6.8	7.7	8.4	8.5
SD	1.020	1.18	1.21	1.36	0.97	1.18	0.93	1.05	0.97	1.06	1.05

high as 0.98 were accompanied by average prediction accuracies ranging from 1.2 to 2.4 days (Kimiya et al., 2013; Nilsen et al., 2002). Additionally, the application of NIR spectroscopy and PLSR analysis to bivalve mollusks, specifically Pacific oysters (*Crassostrea gigas*), was explored by Madigan and colleagues in the context of assessing the freshness over a 5-day shelf life (Madigan et al., 2013). Their findings displayed considerable promise, as they reported an r^2 value of 0.80 and a RMSECV of 0.93 days. These results notably exceeded the performance metrics observed in the current study. Nevertheless, it is essential to emphasize that the methodology introduced in the present study is set apart by the spectroscopic analysis of live mussels directly through the shell, eliminating the need for any preparatory sample treatment prior to NIR data acquisition. In contrast, the approach adopted for oysters in the aforementioned study necessitated a preliminary homogenization step before analysis (Madigan et al., 2013). The variation in sample preparation techniques may have contributed to the differences observed in the predictive performance between the two methodologies.

The remarkable accuracy of NIR in predicting storage durations may be attributed to its capacity to offer a spectral fingerprint that encapsulates information pertaining to the underlying chemical or physical alterations in the food components occurring during the shelf life of the product, including changes in water content, lipolysis, fat oxidation, protein degradation or denaturation, or carbohydrate changes associated to microbial spoilage (Wu et al., 2019). Fig. 7 showcases a comprehensive ranking of individual NIR wavelengths in terms of their influence on predicting the storage time of live mussels. This figure specifically highlights the wavelengths with the highest predictive VIP values (VIP ≥1), providing a clear visual representation of their significance in the predictive model. As observed, among the 125 original variables, a subset of 6 exhibited VIP values ≥ 2.0. Notably, the most influential variable was identified as the NIR absorption peak located at 1174 nm, which is associated with the absorption of alkene C–H double carbon bonds (HC═CH), a characteristic feature of unsaturated fatty acids (Workman & Weyer, 2012). The significant impact of the fat fraction was further evident in the overall VIP pattern, where additional peaks at 1143, 1180, 1112, 1193, 1155, 1224, 1162, 1069, 1217, and 1230 nm, indicating the absorption of carbonyl C═O, methyl C–H, and C–H alkene groups (Workman & Weyer, 2012), showed high VIP values (Fig. 7).

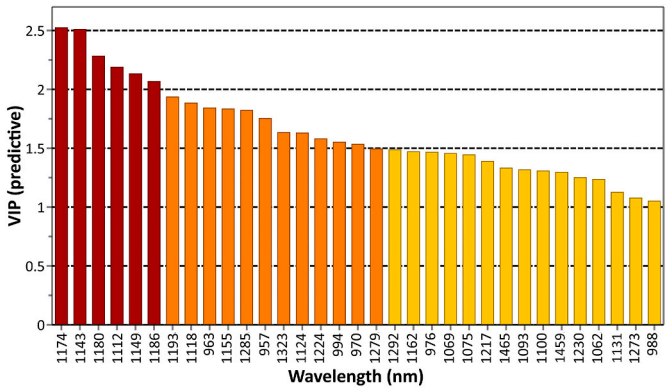

Fig. 7. Variable importance in projection (VIP) plot showing the NIR wavelength having the highest predictive power (VIP >1.0) for the identification of the days of storage of live mussels.

The NIR peaks at 1465 nm (VIP = 1.30) and 1459 (VIP = 1.33) nm underscored the impact of low molecular weight nitrogen compounds, commonly associated with the advancement of shelf life in fishery products. These peaks are related to the absorption of NIR light by N–H groups found in primary and secondary amines, as well as urea (Varrà et al., 2021; Workman & Weyer, 2012), which may serve as valuable indicators of the physiological condition of live mussels. While exerting a comparatively lesser influence, the peaks at 976 nm (VIP = 1.47), corresponding to the absorption of O–H groups in water molecules, also played a significant role in the predictive model. This contribution was consistent with the visual observation of the spectra, as discussed above (see Section 3.2.1). Lastly, the NIR peaks related to the absorption of alcoholic O–H groups (963 nm, VIP = 1.85, and 1062 nm, VIP = 1.24) also emerged as significant predictors of storage time of the mussel samples. This observation may imply that molecules such glycerol and adenosine triphosphate (ATP)-related compounds, possibly formed during the catabolism of triglycerides and nucleotides, could be significant factors contributing to the predictivity of model (Dong et al., 2023; Ricardo et al., 2017).

In conclusion, this research highlights the efficacy of NIR spectroscopy in accurately monitoring freshness and viability changes and providing precise estimates of storage time of live mussels. These findings hold the potential to improve food safety protocols and enhance supply chain management, ultimately bolstering the safety of mussel products. However, the non-specific nature of NIR spectroscopy outputs underscores the importance of future research focused on acquiring more detailed molecular insights related to changes in the freshness and viability of live mussels.

4. Conclusions

This study introduces an innovative and promising method for rapidly and non-destructively assessing the freshness and viability of live mussels using portable NIR spectroscopy. By creating a predictive regression model based on NIR spectral data collected directly from the mussel shells and employing multivariate data analysis, the storage time of live mussels over a 10-day period was successfully predicted, achieving an average error as low as 1.3 days. This groundbreaking approach prioritizes key attributes such as environmental sustainability, non-destructiveness, and objectivity, making it an ideal candidate for widespread adoption throughout the entire seafood supply chain. Furthermore, it enhances on-site inspection and enables real-time control of mussels, thereby reinforcing the assurance of safety in food of animal origin.

However, the adoption of this tool presents its own set of challenges. Further research, ongoing development, and collaborative initiatives are essential to assess its adaptability to different mussel species and shellfish from various origins. Simultaneously, the establishment of standardized protocols and guidelines is crucial to ensure consistent and reliable results across diverse settings and within various fish industries.

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CRediT authorship contribution statement

Sergio Ghidini: Conceptualization, Supervision, Writing – review &

editing. **Maria Olga Varrà:** Conceptualization, Writing – original draft, Methodology. **Davide Bersellini:** Data curation, Writing – original draft. **Mauro Conter:** Resources, Visualization. **Maria Pia Fabrile:** Formal analysis, Investigation. **Adriana Ianieri:** Project administration, Supervision. **Emanuela Zanardi:** Conceptualization, Supervision, Writing – review & editing.

Declaration of generative AI and AI-assisted technologies in the writing process

Not applicable.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110353.

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