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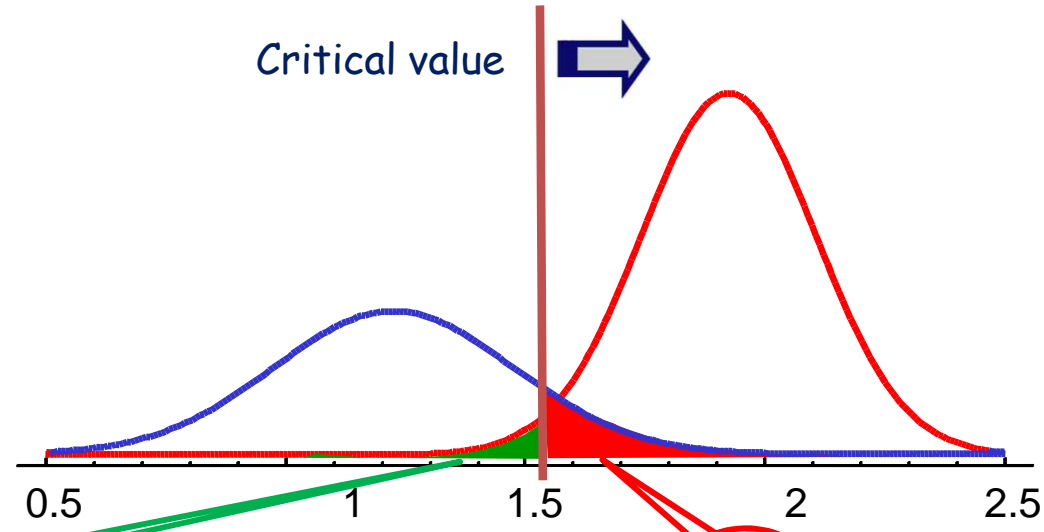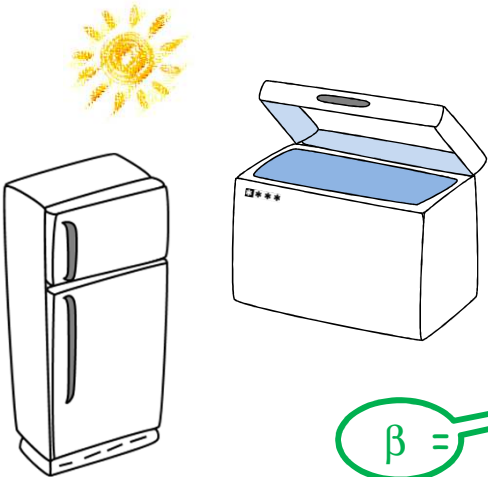
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H_0: The sample belongs to Class 1 (the cold chain has not been broken)

H_a: The sample belongs to Class 2 (the cold chain has been broken)

Critical value

β = pr {to accept H_0/H_0 is false}

α = pr {to reject H_0/H_0 is true}

DETECTION OF COLD CHAIN BREAKS USING PARTIAL LEAST SQUARES-CLASS MODELLING BASED ON BIOGENIC AMINE PROFILES IN TUNA

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DETECTION OF COLD CHAIN BREAKS USING PARTIAL LEAST SQUARES-CLASS MODELLING BASED ON BIOGENIC AMINE PROFILES IN TUNA

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Abstract

The maintenance of the cold chain is essential to ensure foodstuff conformity and safety. However, gaps in the cold chain may be expected so designing analytical methods capable to detect cold chain breaks is a worthwhile issue. In this paper, the possibility of using the amount of nine biogenic amines (BAs) determined in *Thunnus albacares* by HPLC-FLD for detecting cold chain breaks is approached. Tuna is stored at 3 different temperature conditions for 8 storage periods. The evolution of the content of BAs is analysed through parallel factor analysis (PARAFAC), in such a way that storage temperature, BAs and storage time profiles are estimated. PARAFAC has made it possible to observe two spoilage routes with different relative evolution of BAs. In addition, it has enabled to estimate the storage time, by considering the three storage temperatures, with errors of 0.5 and 1.0 days in fitting and in prediction, respectively. In addition, a class-modelling technique based on partial least squares is sequentially applied to decide, from the amount of BAs, if there has been a cold chain break. Firstly, samples stored at 25 ºC are statistically discriminated from those kept at 4 ºC and -18 ºC; next, frozen samples are distinguished from those refrigerated. In the first case, the probabilities of false non-compliance and false compliance are almost zero,

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whereas in the second one, both probabilities are 10%. Globally, the results of this work have pointed out the feasibility of using the amount of BAs together with PLS-CM to decide if the cold chain has been maintained or not.

Abbreviations: AGM, agmatine; BA, biogenic amine; CAD, cadaverine; CORCONDIA, core consistency diagnostic; CV, critical value; HIS, histamine; PARAFAC, parallel factor analysis; PCA, principal component analysis; PHE, 2-phenylethylamine; PLS, partial least squares; PLS-CM, partial least squares-class modelling; PUT, putrescine; SPD, spermidine; SPM, spermine; TYR, tyramine; TRP, tryptamine.

Keywords: Biogenic amines; Cold chain; Partial least squares - class modelling; HPLC-FLD; Parallel factor analysis; Spoilage of tuna.

1. Introduction

Data reported in a recent review [1] may serve as an indicator of the scale of the challenge of appropriate food conservation. The total cost associated with food waste is calculated in €143.000 million for the EU in 2012, whereas $218.000 million (1.3% of GDP) were spent in USA on growing, processing and transporting this food. It is estimated that food wasted annually in Canada is nearly 2% of GDP, around $25.000 million.

The cold chain, a series of storage and distribution activities, is the system designed to keep food within given temperature ranges and used for storing food in good conditions. The maintenance of the cold chain is essential to ensure foodstuff conformity and safety. The food supply chain provides better quality products by preserving nutritional and sensory qualities and extending shelf life [2]. As a consequence, there is an increasing interest in revisiting the

quality and management of the storage and distribution of food, especially with respect to the maintenance of the cold chain [3].

This deals with the regulations in force [4] and progress in monitoring the cold chain maintenance [5] such as the proposal of technological Internet-of-Things based route planning systems. These developments also integrate Taguchi experimental designs and evolutionary algorithms to formulate the distribution route monitoring and planning on the basis of a multi-temperature criterion where the priority reference is the time it takes food to spoil. The application of neural network based techniques provides more accurate predictions of perishable food temperatures along the supply chain using a limited number of temperature sensors, and therefore the estimation of the delivery routing and traveling time [6].

The modelling of the storage at distribution centres has been approached [7], together with the wholesale transport (overland, sea and air transport). Likewise, it has also been addressed the display at retail and transportation and storage by consumers [3]. New regulations may provide the necessary driver for the improvement of some of these food industry practices. In April 2016, the U.S. Food and Drug Administration (FDA) issued a rule which includes specific procedures to ensure that the food is consistently preserved at the desired temperature.

The cold chain monitoring of fish supply chain from the fish farm to the point of sale has been used to provide the shelf life predictions [8]. The consumer should be aware of these data in the form of remained shelf life for the purchased fish. This information could supplement the actual mandatory information [9]: 'Date of freezing' or 'Date of first freezing' included storage conditions (temperature) and one of the following information available as 'expiry date', 'best before date' or 'use by date'. Likewise, the phase of consumers' involvement in the cold chain from the point of sale to the final preparation of food was analyzed in reference [8], with

various options of time and temperatures scenarios to be used for remained shelf life calculations.

These technological developments in the cold chain monitoring make it possible to expand the fish delivery capability to greater distances and longer storage periods, which contribute to sustainability of food consumption. This, in turn, makes essential to have an effective control of the cold chain breaks that increases the risk of food spoilage, i.e. of obtaining a product undesirable or unacceptable for consumption. Biogenic amines (BAs) are formed during the storage of fish through microbiological activity and could cause health problems when present in high concentrations.

The determination of these compounds is a constant analytical challenge as is supported by the 100 references reported in a recent review [10], where the progress in this issue over the last ten years is shown. The toxicological effects of the BAs and their use as markers of freshness of fish have been extensively reviewed by Prester [11]. In addition, the bodies responsible for human health have regulated their presence in food; for example, the European legislation has established thresholds for histamine in fishery products and other BAs are receiving attention due to their possible toxicity and because some of them may potentiate the negative effects of others [12].

It is well known that temperature conditions and time of storage influence the evolution of the amount of BAs, particularly in the case of fish. In fact, the attempt to define a freshness index for fish on the basis of the amount of BAs has been continuous [13, 14, 15]. However, the validity of these indexes was questioned [16]. The need to consider other factors that change the evolution of the BA profile was reported in [12]. Mendes [17] showed the absence of correlation between the content of histamine or other amines and the spoilage degree of fish. Koral and Köse [18] observed that several indexes were not valid to estimate the degree of

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sensorial, chemical and microbiological spoilage of anchovy at different chilled storage conditions.

In this context, a recent work by Ortiz et al. [19] showed that both time and temperature of storage, and their interaction, significantly result in mathematically modelling changes on the profile of the amount of BAs in swordfish. It described two different ways of evolution of the content of BAs related to temperature and time of storage, which explained why the quality indexes based on sums or ratios between BAs were not able to describe the evolution of the spoilage as was already reported by other authors.

On this basis and taking into account the amounts of BAs found over time in tuna stored at different temperatures, detection of cold chain breaks was approached in the present work. Histamine (HIS), putrescine (PUT), cadaverine (CAD), tyramine (TYR), 2-phenylethylamine (PHE), tryptamine (TRP), agmatine (AGM), spermidine (SPD) and spermine (SPM) were determined by HPLC-FLD in samples of tuna (*Thunnus albacares*) stored at three different temperature conditions (-18 ºC, 4 ºC and 25 ºC) for eight different storage periods (between 1 and 18 days). A class-modelling technique based on PLS (PLS-CM) was used. This technique searches for the BA profile which characterizes a class of samples and helps to decide whether or not a test sample belongs to that class. The PLS-CM was sequentially applied to detect if the cold chain was broken over the experiment.

2. Theory

2.1 Parallel factor analysis

In order to study the underlying complex structure, principal component analysis (PCA) is a frequently used multivariate technique for decomposition of two-way data. Parallel factor

analysis (PARAFAC) is a generalization of PCA to n-way data. In the case of three-way data, PARAFAC decomposes a data cube $\underline{\mathbf{X}}$ with dimension $I \times J \times K$ into three loading matrices $\mathbf{A}$, $\mathbf{B}$ and $\mathbf{C}$, being their columns $\boldsymbol{a_f}$, $\boldsymbol{b_f}$ and $\boldsymbol{c_f}$, $f = 1,\ldots,F$, respectively [20,21]. The trilinear PARAFAC model is given by Eq. (1), where x_{ijk} is the element in the position i, j, k of the three-way cube $\underline{\mathbf{X}}$, F is the number of factors, and e_{ijk} is the residual cube.

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \ ; \ i = 1, 2,\ldots, I; \ j = 1, 2,\ldots, J; \ k = 1, 2, \ldots, K \qquad (1)$$

Data are trilinear when the experimental data cube is compatible with the structure in Eq. (1). The core consistency diagnostic (CORCONDIA) is an index that measures the degree of trilinearity of the experimental data cube and should be close to 100% [22]. If the data are trilinear and the appropriate number of factors is chosen to fit the model, then the PARAFAC decomposition provides unique profile estimates. PARAFAC has been widely used due to this highly attractive uniqueness property [23], which could be used for the unequivocal identification of each factor that helps to understand and make the internal structure of the three-way data clearer.

2.2 PLS-CM (Partial least squares - class modelling)

Among the well-established class modelling methods, PLS-CM [24,25] consists of using the probability distribution of the response of a PLS model for estimating the probabilities of error related to the decision of assigning a sample into a certain class. The response for the samples that belong to a class is coded as '1' and the rest as '2'. In a first stage, a PLS regression model is fitted to predict this binary response. Next, a probability density function is fitted with the values predicted by the PLS model for both class 1 and class 2. And finally, the class-modelling decision of assigning a sample to one or the other class is taken through a hypothesis test; whether the sample belongs to class 1 (H_0, null hypothesis) or to class 2 (H_a,

alternative hypothesis). The probability, α, of rejecting H_0 when it is true is set and the critical value of the hypothesis test, CV, is determined through the distribution fitted for class 1. The cumulative probability of the distribution for class 2 until the CV is the probability β of accepting H_0 when it is false. When PLS-CM is applied to the data vector of a test sample, if the response value is larger than the CV, then H_0 is rejected, i.e. the sample is assigned to class 2.

3. Experimental and software

3.1 Reagents, samples and solutions

Histamine dihydrochloride (99%), putrescine dihydrochloride (98%), spermidine trihydrochloride (98%) and dansyl chloride (99%) were supplied by Sigma (Steinheim, Germany). Tyramine (99%), tryptamine hydrochloride (99%), cadaverine (95%) and 2-phenylethylamine (98%) were purchased from Aldrich (Saint Quentin Fallavier, France). Spermine dehydrate (99.5%) was supplied by Fluka (Barcelona, Spain). Agmatine sulphate (97%) was obtained from Alfa Aesar (Kandel, Germany). Acetonitrile (LiChrosolv® isocratic grade for liquid chromatography) was supplied by Merck (Darmstadt, Germany). Water was deionised using the Milli-Q gradient A10 water purification system from Millipore (Bedford, MA, USA). All other reagents used were analytical grade. Frozen tuna was obtained from a nearby supermarket.

Working solutions were prepared in 0.4 M perchloric acid by diluting stock solutions of BAs (1000 mg L^{-1}), which were previously prepared in 0.1 M hydrochloric acid. A set of standard solutions were prepared to contain concentrations of BAs in the ranges: from 0 to 24 mg L^{-1} of AGM, TRP and TYR, from 0 to 234 mg L^{-1} of HIS, and from 0 to 7 mg L^{-1} of the remaining

BAs. All solutions were stored at 4 ºC and protected from light. A 0.5% solution of dansyl chloride in acetone was prepared daily.

3.2 Experimental procedure and instrumental

Frozen tuna was homogenized after the purchase and the content of the 9 BAs was immediately determined in six portions of 5 g. These were the control samples (coded as 0). Additionally, portions of 5 g of the tuna meat were transferred into 96 centrifuge tubes: 32 tubes were frozen at -18 ºC, 32 were refrigerated at 4 ºC and the remaining 32 samples were incubated at 25 ℃ in a Heraeus Function Line model B6 laboratory incubator (Haraw, Germany). The tubes were stored at those temperatures over different periods of time from 1 to 18 days. 8 time-points were set; 1, 4, 6, 8, 11, 13, 15 and 18 days. At each time-point, 4 centrifuge tubes were removed from each of the storage container and the concentration of the BAs was determined. The stored samples were coded with two digits; the first digit indicates the storage temperature (1: -18 ºC; 2: 4 ºC; and 3: 25 ºC) and the second one the time-point (1 to 8).

The procedure previously optimized [26] was followed to extract BAs from tuna and to obtain the derivative products, as summarized here. 10 mL of 0.4 M perchloric acid were added to each tuna sample, the mixture was then vortexed for 2 min using a Velp Scientifica RX3 Vortex shaker (Milan, Italy) and centrifuged at 5000 g for 10 min at 4 ºC on a Sigma 2–16 K refrigerated centrifuge (Osterode, Germany). The supernantant was collected and the residue was extracted again. Both supernatants were filtered and combined. For the derivatization of BAs, 10 mL were neutralized with 10 M sodium hydroxide and mixed with 2 mL of carbonate buffer (pH 10.5). 1 mL of the buffered solution was derivatized by addition of 2 mL of 0.5% dansyl chloride and, after incubation for 30 min at 40 ºC in the dark, using a water bath

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equipped with a Digiterm 200 immersion thermostat from JP Selecta S.A. (Barcelona, Spain), 100 µL of 25% ammonium hydroxide were added. The solution was left for 30 min at room temperature in the dark and the volume was adjusted to 5 mL with acetonitrile. Next, the sample was centrifuged at 3000 g for 5 min and filtered.

Analyses were carried out on an Agilent 1260 Infinity HPLC chromatograph (Santa Clara, CA, USA) equipped with a quaternary pump, an autosampler (injection volume: 10 µL), a thermostatic column compartment maintained at 40 ºC and a fluorescence detector which worked at 350 nm (excitation wavelength) and 520 nm (emission wavelength). Data acquisition and peak integration were performed using the HP ChemStation software. A Kinetex EVO-C18 column (150 mm x 4.6 mm, 5 µm) was used for the separation of BAs. Deionized water (solvent A) and acetonitrile (solvent B) were used as components of the mobile phase in the gradient elution program: solvent B was increased from 40 to 70% over 12.5 min and held at 70% for 1.5 min, next it was increased to 100% over 2 min and then decreased to 40% over 4 min (20 min run time). The flow rate was maintained at 1.0 mL min⁻¹.

3.3 Data arrangement

Firstly, 3 out of 4 stored samples measured at each time-point (72 samples, 24 samples preserved at each temperature) were selected for training. One of the samples, stored at 25 ºC for 8 days, was found as outlier and removed; therefore, only 71 samples were considered. The concentrations of BAs of these 71 samples, together with that of the 6 control samples, comprised the first training set (training set A, of dimension 77×9). The remaining stored samples, i.e. 24 samples (8 samples stored at each temperature), are the external test set.

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Next, the amounts of BAs found for the 77 samples were arranged in a dataset with 30 rows and 9 columns. 6 rows corresponded to the values found for the control samples, and each one of the 24 remaining rows were the mean value (n=3) of the amounts of the BAs found at a certain time-point and storage temperature. This matrix comprised the training set B. Finally, the matrix B was folded in a cube **X** of dimension (8×9×3), where 8 was the number of time-points, 9 was the number of BAs, and 3 was the temperatures. This data cube constitutes the training set C. Control samples were not considered in this case.

3.4 Software

Correlation analyses, PARAFAC, PCA and PLS models were done with the PLS Toolbox [27] for Matlab 8.4.0 [28]. Regression models and class modelling were obtained with STATGRAPHICS Centurion XVII [29].

4. Results and Discussion

Bearing in mind the detection of a cold chain break, the evolution of the content of the 9 BAs was monitored in the tuna samples. Initially, tuna was of good quality, with a total BA content below 17.6 mg kg^{-1}; no histamine was found. The summary statistics of the content of BAs found for the control samples are shown in Table 1. The coefficients of variation ranged from 8.6% to 81.5%, the latter correspond to AGM. These values are related to both the inter-sample variability and the analytical procedure uncertainty. The method of analysis to quantify the BAs in this fish had been previously validated [26] but recovery when applied to tuna samples, in the calibration ranges considered, was determined in this work. Recovery rates of 38.9, 77.6, 86.8, 94.0, 92.6, 141.8, 89.3, 106.6 and 114.6% for AGM, TRP, PHEN, PUT, CAD, HIS, TYR, SPD and SPM were obtained, respectively. The value for AGM was low.

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4.1 Exploratory analysis

Initially, the analysis of correlations between the content of BAs and the study of the internal structure of the data was carried out.

4.1.1 Univariate correlations

Correlations between the content of BAs found for each storage temperature were estimated from dataset A. Fig. 1 shows the maps that enable the correlations for each pair of variables (content of BAs) to be assessed. When the correlation maps were studied jointly, significant differences in the correlations between the BAs depending on the storage temperature were found. All variables are plotted in all the cases although TRP, CAD and HIS were not found in the control samples (Fig. 1A), nor TRP in the frozen and refrigerated samples (Figs. 1B and 1C), and so no correlations could exist with these BAs.

Fig. 1A shows clearly that, for the control samples, AGM was positively correlated with TYR, SPD and SPM (correlation equal to 0.72, 0.68 and 0.60, respectively), whereas this correlation became negative (-0.23, -0.58 and -0.61, respectively) when the samples were frozen, that is, instead of increasing the amount of AGM with them, it decreased over time. It is also remarkable that, for the samples stored in the freezer, Fig. 1B, there was a high positive correlation between PUT-CAD, PUT-HIS and CAD-HIS (0.97, 0.85, 0.81, respectively) and 0.84 between SPM and SPD.

For the samples stored in the refrigerator (Fig. 1C), PUT and CAD were positively correlated with TYR (0.56 and 0.60, respectively) and SPD (0.75 and 0.83) and negatively correlated with HIS (-0.31 and -0.77), as opposed to the situation in the frozen samples (Fig. 1B).

Correlation of PUT and CAD with HIS shifted from positive in the frozen samples (Fig. 1B) to negative in the refrigerated samples (Fig. 1C).

No correlation between SPM and SPD (-0.04) was found solely for the samples stored at 25 ºC, while both amines were negatively correlated with the rest of the BAs, (values between -0.32 and -0.66) Fig. 1D. High positive correlations between 0.60 and 0.99 were between the remaining amines at this temperature of storage. The exception is the no significant correlation (0.07) existed between AGM and HIS.

The evolution of the samples during the storage period changed the relationships between the BAs, so choosing a single amine (or a quality index based on several BAs) to detect a cold chain break could lead to wrong conclusions. The complex paths of synthesis and transformation of these BAs have already been studied [11] and make it necessary to consider not only the correlations between the BAs but the existing internal underlying structure. Multivariate techniques that take the joint changes in the profiles of BAs into account are necessary for that [19].

4.1.2 Principal component analysis

A principal component analysis (PCA) was carried out to explore the internal relations between BAs and time and temperature of storage. PCA was performed with the training set B (data were previously autoscaled). A summary of the analysis results is shown in Table 2. The first and second principal components explained a 71.6 and 13.4% of variance, respectively. High scores on the first PC (Fig. 2A) are related to high amount of HIS, PUT, TYR, TRP, CAD and PHE, so these BAs are expected at high proportions in the samples stored at 25 ºC. Lowest scores on the first PC may be associated to high amounts of SPM and SPD, mainly related to frozen and refrigerated samples. The second PC seems to be

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related to the short-time stored samples (samples 31 to 34) versus the rest of the samples stored at 25 ⁰C (samples 35 to 38).

The percentage of variance explained by the third PC was 6.7%. This component (Fig. 2B) makes the difference between samples refrigerated over longer periods of time (between time-points 4 to 8) and the rest of samples (control, short-time refrigerated and frozen samples); higher amounts of SPD are expected in the first samples than in the second ones. The other BAs are irrelevant in the third PC. It is to be noted that the scores on the third PC for the samples stored in refrigerator increase until the time-point 6 (13 days of storage) and then decrease, this component reflecting a complex relationship between the time and the evolution of BAs. On the contrary, the scores for the first PC increase with time for the samples maintained at 25 ⁰C. This suggests the need to consider the evolution of BAs over time depending on the storage temperature.

4.1.3 Parallel factor analysis

To study jointly the effect of time and temperature of storage on tuna over the experiment, the amounts of the BAs found were arranged in the cube of training set C. The three-way structure of the data cube was analyzed by means of a PARAFAC model so that the amount of all BAs can be handled jointly with the storage time and storage temperature. Therefore, the vectors a_f, b_f and c_f of the model in Eq. (1) were the time-point, BA amount and storage temperature profiles of the f-th factor, respectively.

A PARAFAC model with two factors, which had a CORCONDIA index of 100%, was fitted. The model explained 82.85% of variance, of which the first factor explained 81.2% and 18.75% the second one. The uniqueness property of PARAFAC makes it possible the

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unequivocal identification of the evolution of BA profile with time and temperature of storage. Fig. 3 shows the loadings for the two factors in the BA, storage temperature and storage time-point profiles.

The two factors are related to two clearly different formation/transformation pathways that follow the BAs in the spoilage of tuna. The loadings of the time-point profile for the first factor (in blue) show a slightly increasing in the first days, and an overall decreasing trend over time is observed before (Fig. 3A). This pathway is mainly related to the amount of HIS and, to a lesser extent, of CAD, as loadings of the BA profile in Fig. 3C show. Contrary to the first factor, the loadings of the time-point profile for the second factor (in red) show a pronounced upward trend over time (Fig. 3A), the pathway being mainly related to CAD and, to a much lesser extent, to TRP, PHE, PUT and HIS (see loadings of the BA profile in Fig. 3C). These results explain the lack of correlation between the content of BAs and spoilage processes found in the bibliography.

There is no spoilage in the frozen samples over the experiment, as the zero loading for the two factors in the temperature profile show (Fig. 3B). The samples stored at 25 ⁰C are spoiled following the two transformation pathways, as the loadings of the temperature profile for the two factors confirm (Fig. 3B). With respect to the refrigerated samples, when the spoilage of tuna at 4 ⁰C occurs, to a much lesser extent than at 25 ⁰C, it is related only to the transformation pathway of the second factor (Fig. 3B).

Additionally, a multiple linear regression model was fitted to relate the storage time to the loadings obtained for the first two factors (l_1 and l_2) of the time-point profile of the PARAFAC model (Table 3) with the aim of assessing if it is possible to predict the days of storage of the tuna samples. The regression model of Equation (2) was obtained, the determination coefficient being 98.90 %.

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Storage days = 8.60 - 0.00225 l_1 + 0.0008 l_2 (2)

Table 3 shows the predictions for the days of storage; mean errors were of 0.5 and 1 days in

fitting and for the external test samples, respectively. In confirming the validity of the model, a

regression "calculated response" vs. "true response" was fitted both in fitting and in prediction

for the external samples (Table 4). Both regression models were significant, the correlation

coefficient being 0.99 and 0.97, respectively. The slopes and intercepts of both models were

statistically equal to 1 and 0 respectively (p-values greater than 0.05 are estimated in both

cases). As the experimental design performed in this work allows the detection of the cold

chain break, the structure highlighted by PARAFAC, related to the storage time, confirms the

different development of the profiles of BAs according to whether the cold chain is maintained

or not. As a consequence, the complete BA profile of the fish samples is needed to check the

integrity of the cold chain.

4.2 Partial least squares – class modelling

The decision about the break of the cold chain was based on using the PLS-CM technique.

The confirmation of the break was made through the sequential application of two PLS-CM

models with binary response.

Initially, a variable response was assigned for each sample of the training set A; a value of 1

for the control samples and samples stored in the freezer and refrigerator (so all these

samples constitute class 1), and a value of 2 for samples stored at 25 ºC (class 2). A test data

matrix of dimension 24×9 was obtained analogously from the external test samples.

A first PLS-CM with 4 latent variables, which explained 96.7 % of variance, was built. In Table

5, the average, the standard deviation, the coefficient of variation, the maximum and the

minimum of the predicted responses of this analysis are summarized. Fig. 4A shows the

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predictions for the samples of both training (circles) and external test (diamonds) sets. The two classes are clearly separated both in fitting and in prediction.

Once it has been established the compatibility of the predicted responses for both class 1 and class 2 with a normal distribution (the p-values of the normality tests were larger than 0.05 for both distributions), normal probability density functions were fitted with the means and standard deviations of these data. Fig. 5A shows the density functions for the PLS-CM responses estimated in fitting. The hypothesis test to model the classes was performed; H_0: The sample belongs to class 1 (the cold chain has not been broken), versus the alternative hypothesis, H_a: The sample belongs to class 2 (the cold chain has been broken). The critical value was set to 1.40, which guaranties that the probabilities of false non-compliance (α) and false compliance (β) are equal. In this case, both probabilities of error were lower than 10^{-5} for the identification of samples stored at 25 ⁰C.

Next, in a second stage, the BA contents found for the control, refrigerated and frozen samples were arranged in a new matrix of dimension 54×9. In this case, class 1 (response variable equal to 1) corresponded to the control samples and the samples stored in the freezer, and class 2 (response variable equal to 2) to those stored in the refrigerator. Once again, a test set was obtained from the external test samples, which in this case had dimension 16×9.

The second PLS-CM model fitted explained 62.0 % of variance with 5 latent variables. The summary statistics of the results of this model are shown in Table 5. In addition, the responses predicted by this model are shown in Fig. 4B. It is clear that samples of class 1 and class 2 were not as different as above, Fig. 4A.

As in the previous case, once the compatibility of the data to a normal distribution was confirmed at a significance level of 5%, normal probability density functions were fitted with

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the predicted values for both classes, and the class-modelling hypothesis test was performed.

Fig. 5B shows the density functions for the PLS-CM results. In this case, the probabilities of

false non-compliance and false compliance, when the critical value was fixed at 1.43, were

around 10%. This second decision allowed the differentiation between frozen and refrigerated

samples. Higher probabilities of false non-compliance and false compliance than above were

found because frozen samples and short-time refrigerated samples are quite similar.

When comparing the results for the first and second analyses by PLS-CM, in Table 5, it may

be concluded that centralisation, dispersion and range values obtained in prediction with the

external set are similar to those obtained in fitting.

Finally, the application of the decision rule to a new tuna sample would involve the

measurement of the concentration of the 9 BAs and the prediction of the response using the

first PLS-CM model; if the predicted value is larger than the critical value 1.40, then it will be

concluded that the sample has been spoiled because of cold chain break. On the contrary, if

the predicted value for the response is not larger than this critical value, then the response is

predicted through the second PLS-CM model; if the value is larger than the critical value 1.43,

then it will be concluded that the freezing chain has been broken.

5. Conclusions

The principal component analysis has shown the complex evolution of the 9 BAs studied in

the samples of tuna stored over time at different temperatures. Two spoilage routes, with

different relative evolution of BAs, have been observed through the PARAFAC

decomposition. Their description by means of two factors of a PARAFAC model has made it

possible to estimate the storage time by considering the three storage temperatures, with

errors of 0.5 and 1 days in fitting and in prediction, respectively.

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The determination of the content of 9 BAs together with two PLS-CM models applied sequentially have enabled to assess the cold chain break in samples of tuna. The procedure developed has provided probabilities of false non-compliance (α) and false compliance (β) lower than 10^{-5} to distinguish the samples stored at 25 ºC from the rest of samples (control, frozen and refrigerated). Whereas β increased to 0.10 when control and frozen samples were distinguished from refrigerated samples. These risks were confirmed when the procedure was applied to a set of external samples.

Globally, the results of this work are a proof of concept for the feasibility of using the BA profile and its changes, modelled by PLS-CM, for controlling cold chain breaks. This could be used to verify the proper time-temperature operation management that should be given by the sensors and predictive algorithms developed for the proper management of storage and distribution of foods.

Acknowledgements

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Figure Captions

Figure 1. Correlation maps of the content of the 9 BAs found for the control samples (A) and the samples stored in the freezer (B), in the refrigerator (C) and at 25 ⁰C(D).

Figure 2. Biplot of the principal component analysis showing the projection of loadings (blue triangles) and scores (diamonds). Scores are coded by two digits: the first digit is for the storage temperature (0: control samples, in cyan; 1: freezer, in green; 2: refrigerator, in red; 3: storage at 25 ⁰C, in magenta) and the second one for the storage time-point (1 to 8), in the planes: A) PC1-PC2 and B) PC1-PC3.

Figure 3. Loadings for the two-factor PARAFAC model fitted. A) Time-point profile, B) storage temperature profile and C) BA profile. Loadings are in blue diamonds for the first factor and in red circles for the second one.

Figure 4. Responses predicted from the first PLS-CM model in fitting (training samples, grey circles) and in prediction (external test samples, red diamonds) versus sample number. A) class 1 (control, frozen and refrigerated samples) and class 2 (samples stored at 25 ºC); and B) class 1 (control and frozen samples) and class 2 (refrigerated samples). Control samples are coded as 0; the remaining samples are coded by two digits: the first one indicates the storage temperature (1: freezer, 2: refrigerator, 3: storage at 25 ºC), and the second one the time-point (1 to 8).

Figure 5. Normal density functions fitted with the PLS-CM response predicted for the two classes. A) class 1 (control, frozen and refrigerated samples) in blue and class 2 (samples stored at 25 ºC) in red; and B) class 1 (control and frozen samples) in blue, and class 2 (refrigerated samples) in red.

Table 1. Summary statistics of the content of BAs (expressed as mg kg^{-1}) found for the control samples.

	AGM	TRP	PHE	PUT	CAD	HIS	TYR	SPD	SPM
Average	6.83	-	0.260	0.094	-	-	1.42	2.36	8.64
Median	6.03	-	0.243	0.097	-	-	1.40	2.41	9.03
Std. dev.	5.59	-	0.074	0.011	-	-	0.12	0.22	1.25
Min.	0.00	-	0.172	0.077	-	-	1.29	1.91	6.23
Max.	17.10	-	0.358	0.104	-	-	1.62	2.50	9.50
C.V. (%)*	81.50	-	28.60	12.10	-	-	8.57	9.49	14.40

*C.V.: Coefficient of variation

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Table 2. Summary of the principal component analysis for BAs.

Principal component	Eigenvalue	Explained variance (%)	Cumulative variance (%)
1	6.45	71.62	71.62
2	1.20	13.35	84.97
3	0.60	6.69	91.66

Table 3. Loadings for the two factors in the time-point profile of the PARAFAC model and estimated days of storage and average errors achieved, both in fitting and in prediction (external test set).

Days of storage	1st factor loading	2nd factor loading	Estimated days of storage	
			In fitting	In prediction
1	1750	-469	0.81	1.36
4	2237	-42	3.22	3.25
6	2033	316	6.63	5.49
8	1688	508	8.99	9.30
11	1679	740	10.92	8.63
13	1302	928	13.32	14.94
15	1179	1025	14.39	13.38
18	1585	1541	17.72	16.87

Average error (in days)	0.485	1.01

Table 4. Parameters of the validation lines fitted and p-values obtained for the model significance test, both in fitting and in prediction (external test set).

Prediction	Slope	Intercept	r	Standard error of estimation	P-value*
In fitting	0.989	0.105	0.994	0.652	$<10^{-4}$
In prediction	0.938	0.235	0.970	1.53	$<10^{-4}$

* Null hypothesis: the linear model is not significant.

Table 5. Summary statistics of PLS-CM results both in fitting and in prediction (external test set). Classes for the first PLS-CM: class 1 (control samples and samples stored in freezer and refrigerator) and class 2 (samples stored at 25 ºC). Classes for the second PLS-CM: class 1 (control samples and samples stored in freezer) and class 2 (samples stored in refrigerator).

PLS-CM	Statistics	In fitting		In prediction	
		Class 1	Class 2	Class 1	Class 2
First	Count	54	23	16	8
	Average	1.013	1.967	1.017	2.017
	Median	1.013	2.003	1.023	2.118
	Std. dev.	0.0445	0.166	0.0343	0.244
	C.V. (%)	4.39	8.44	3.38	12.10
	Minimum	0.928	1.644	0.959	1.587
	Maximum	1.136	2.246	1.069	2.223

	Count	30	24	8	8
	Average	1.172	1.784	1.303	1.692
	Median	1.223	1.765	1.328	1.728
Second	Std. dev.	0.193	0.301	0.194	0.250
	C.V. (%)	16.46	16.87	14.90	14.80
	Minimum	0.776	1.28	1.03	1.26
	Maximum	1.591	2.441	1.578	2.160

*C.V.: Coefficient of variation
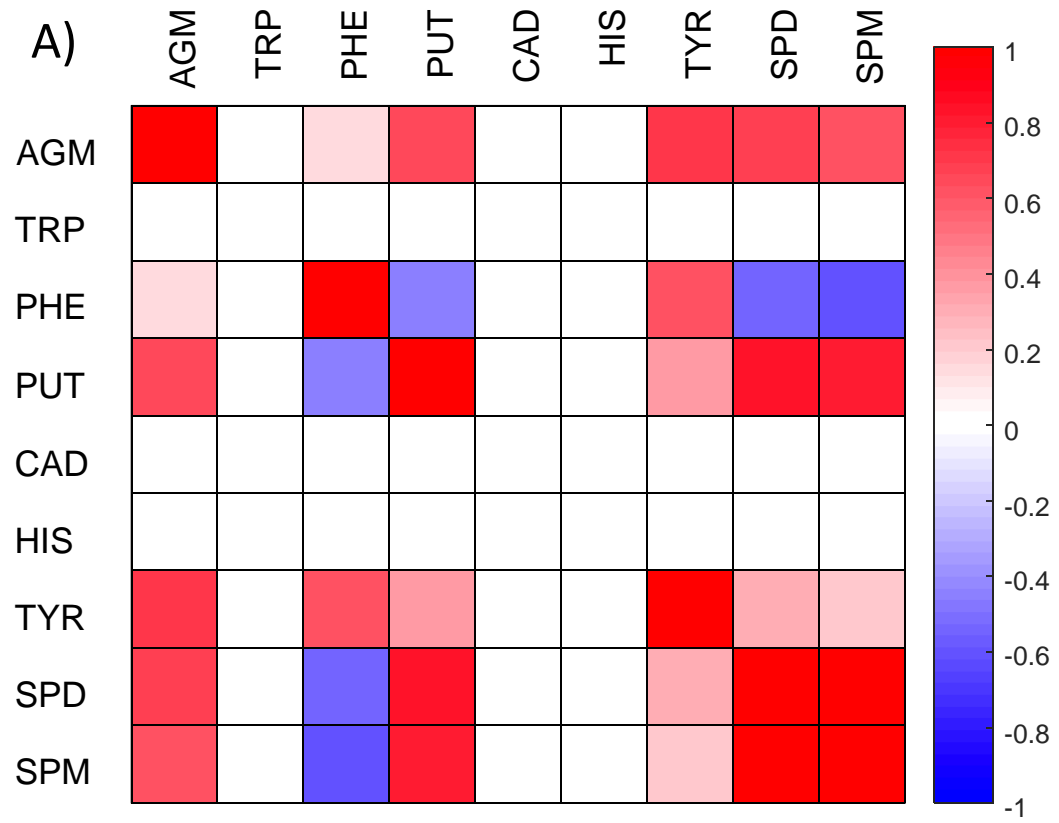
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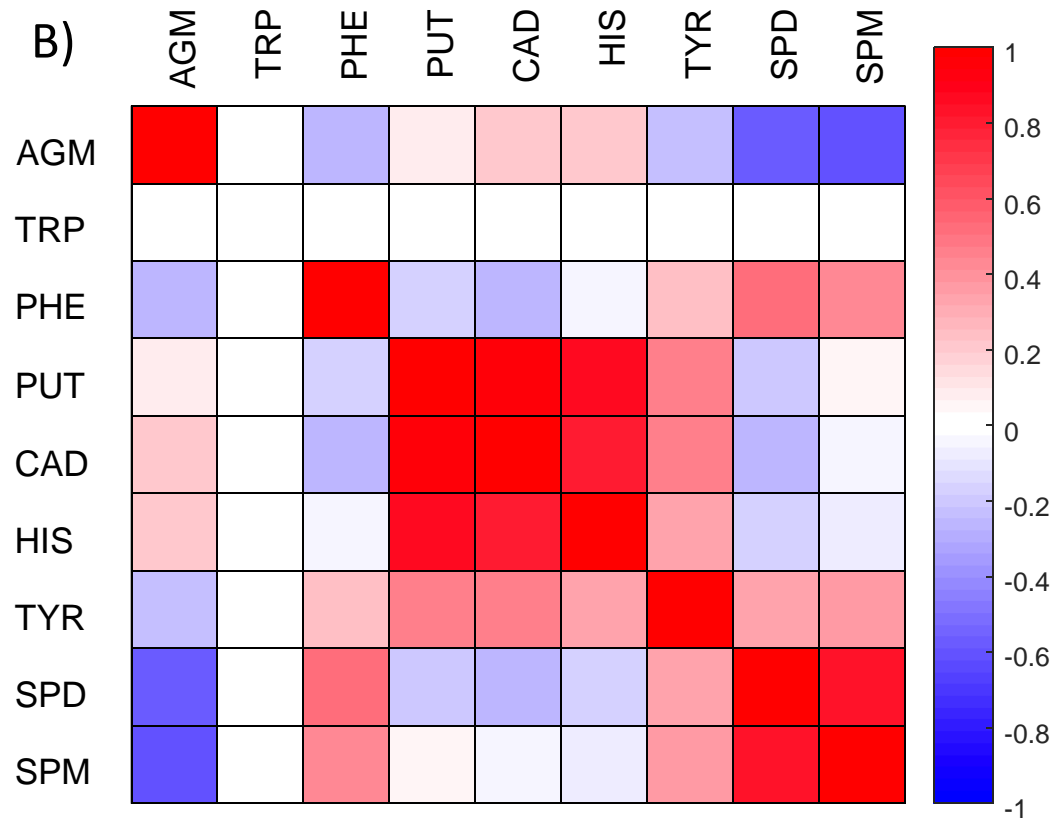
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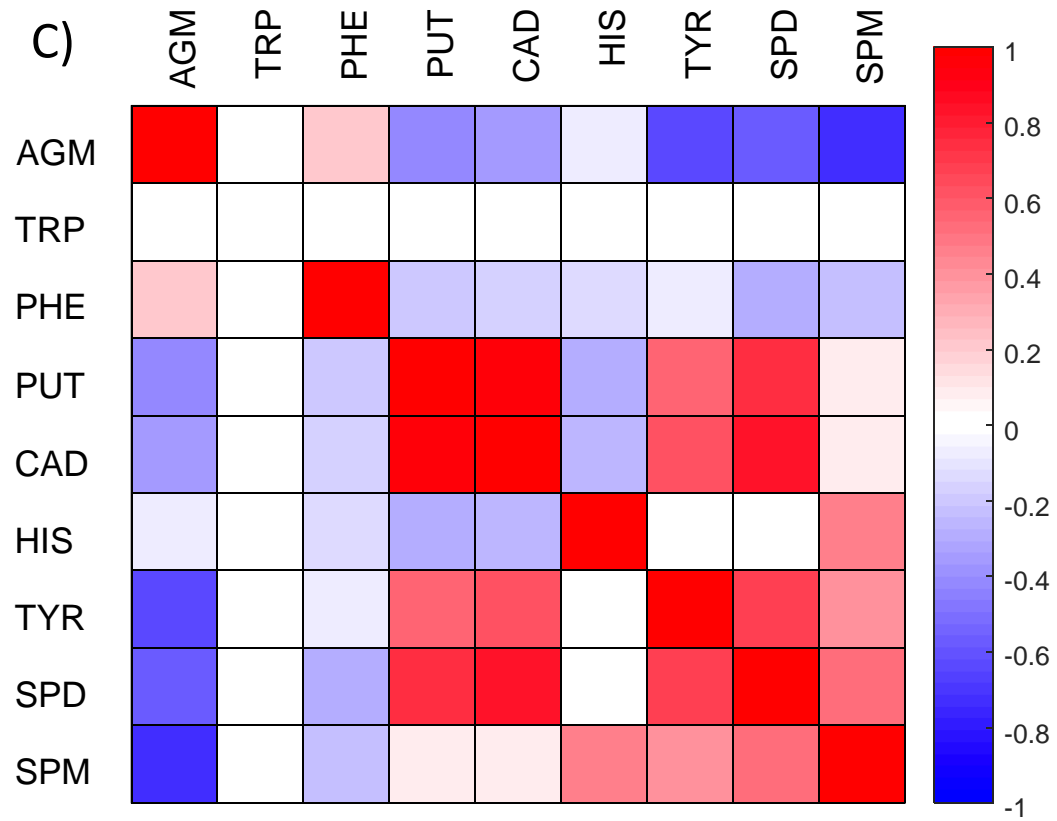
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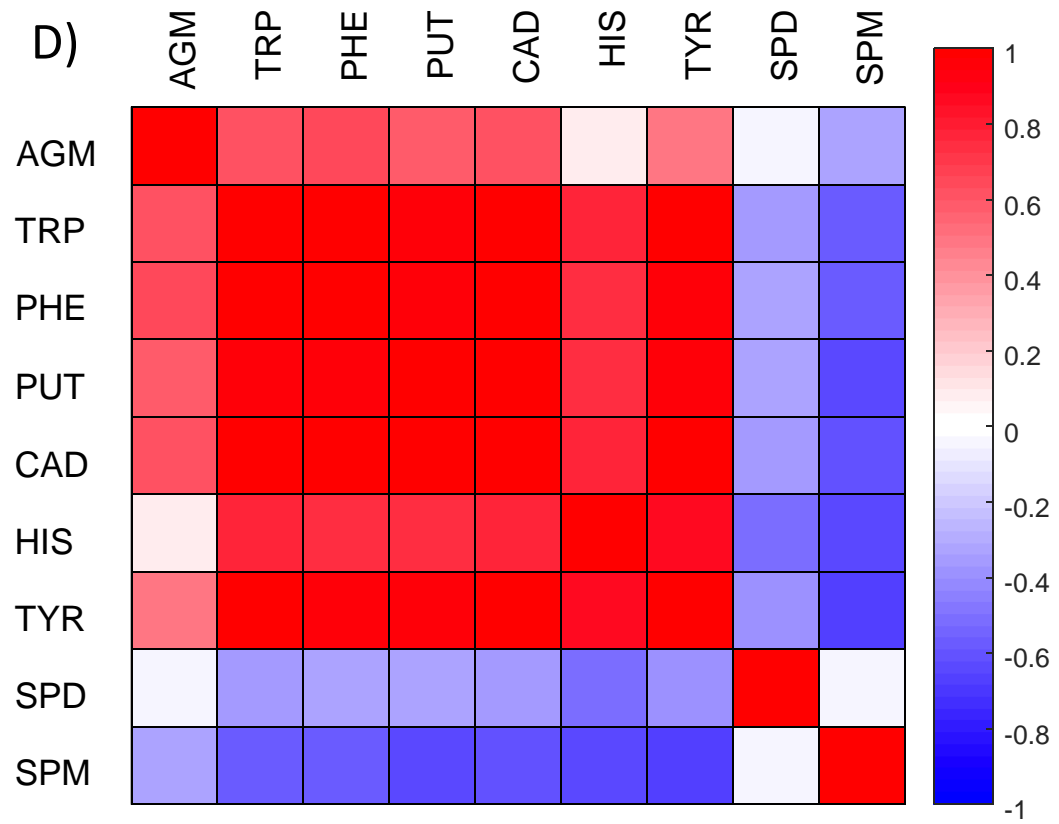
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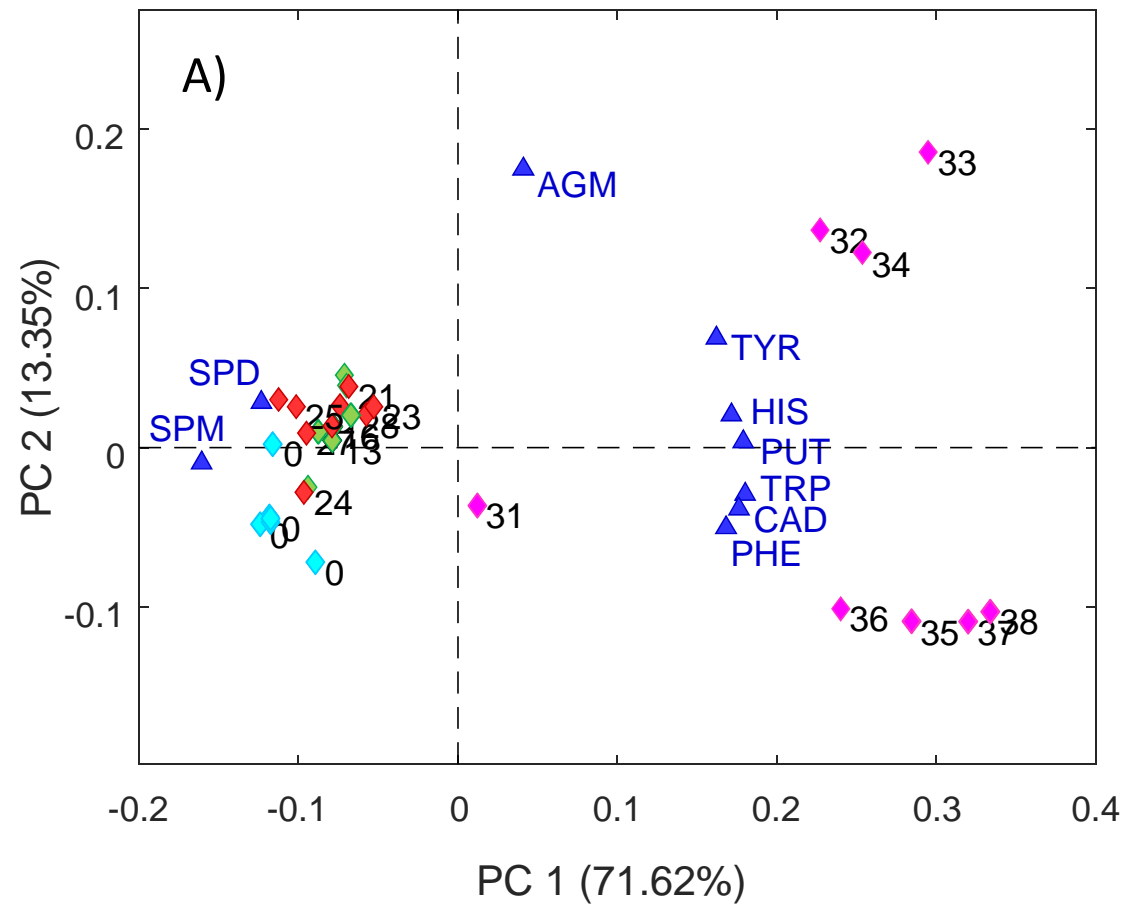
Figure 2A

Figure 2B

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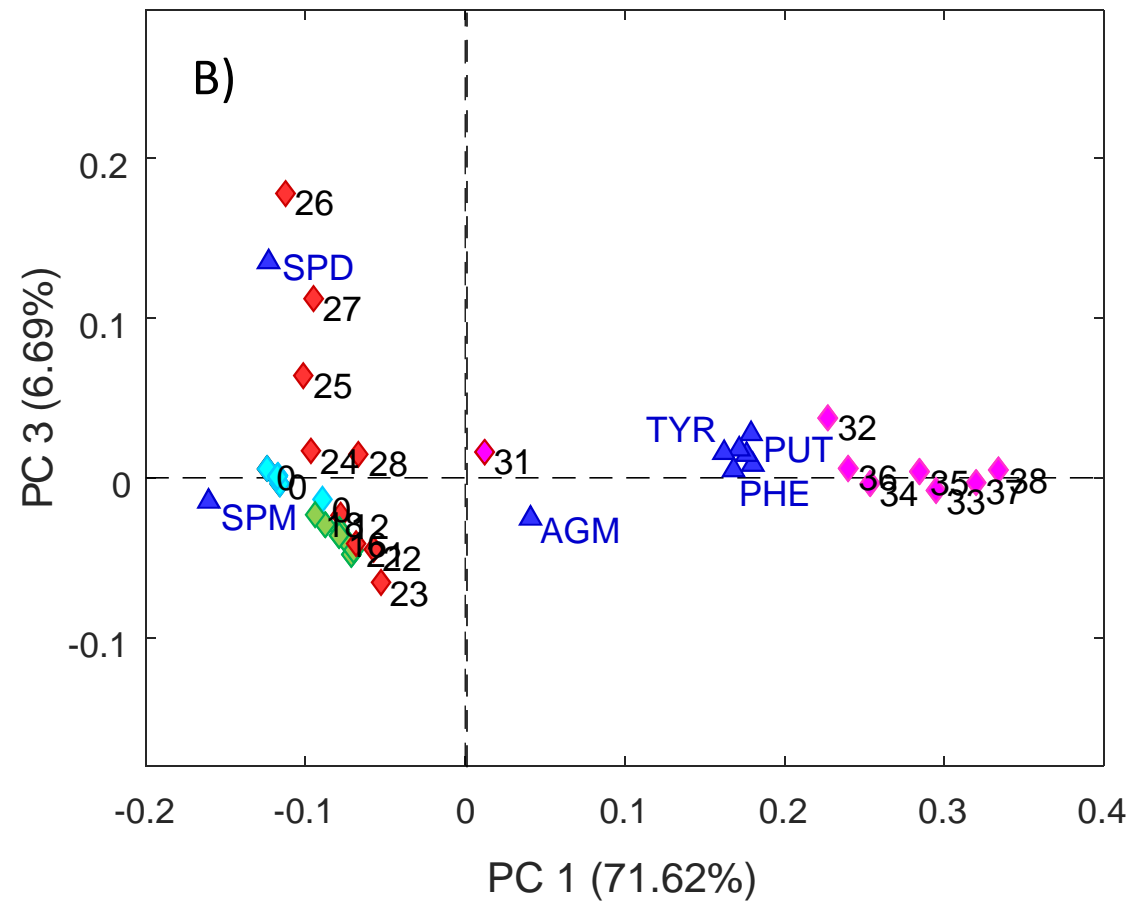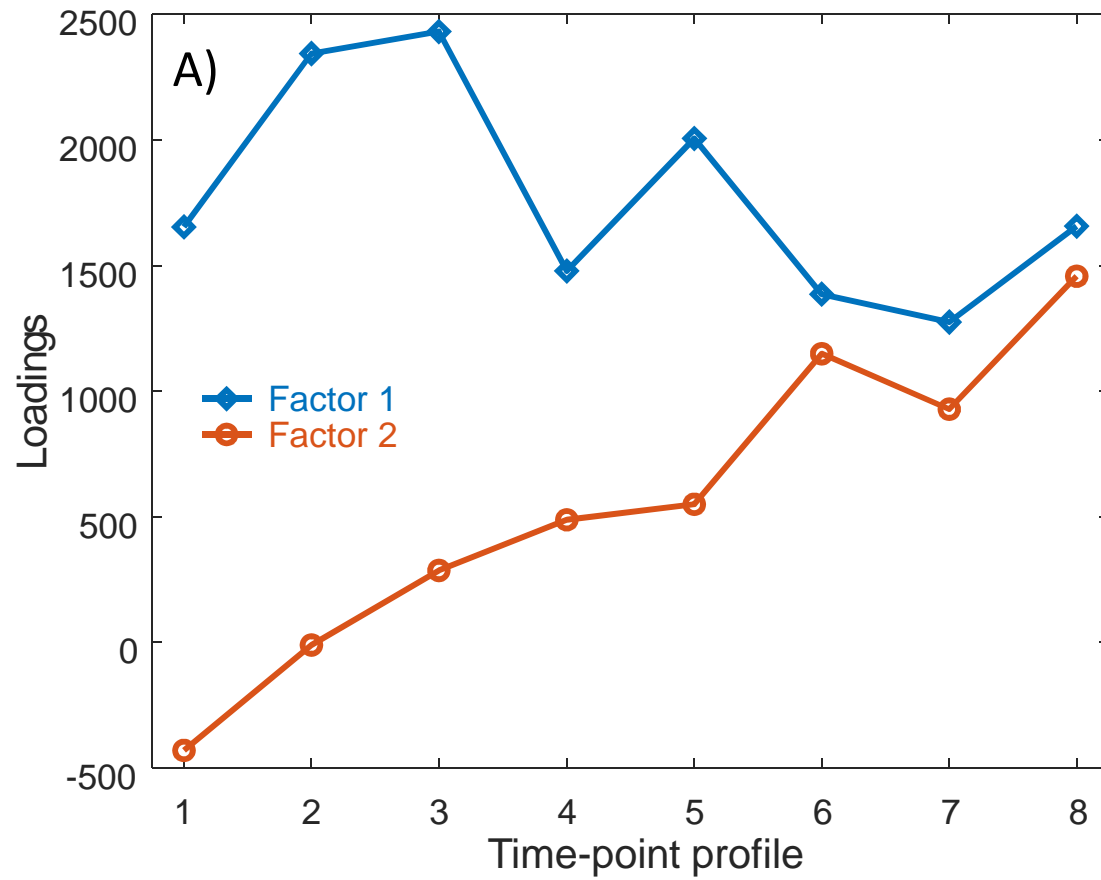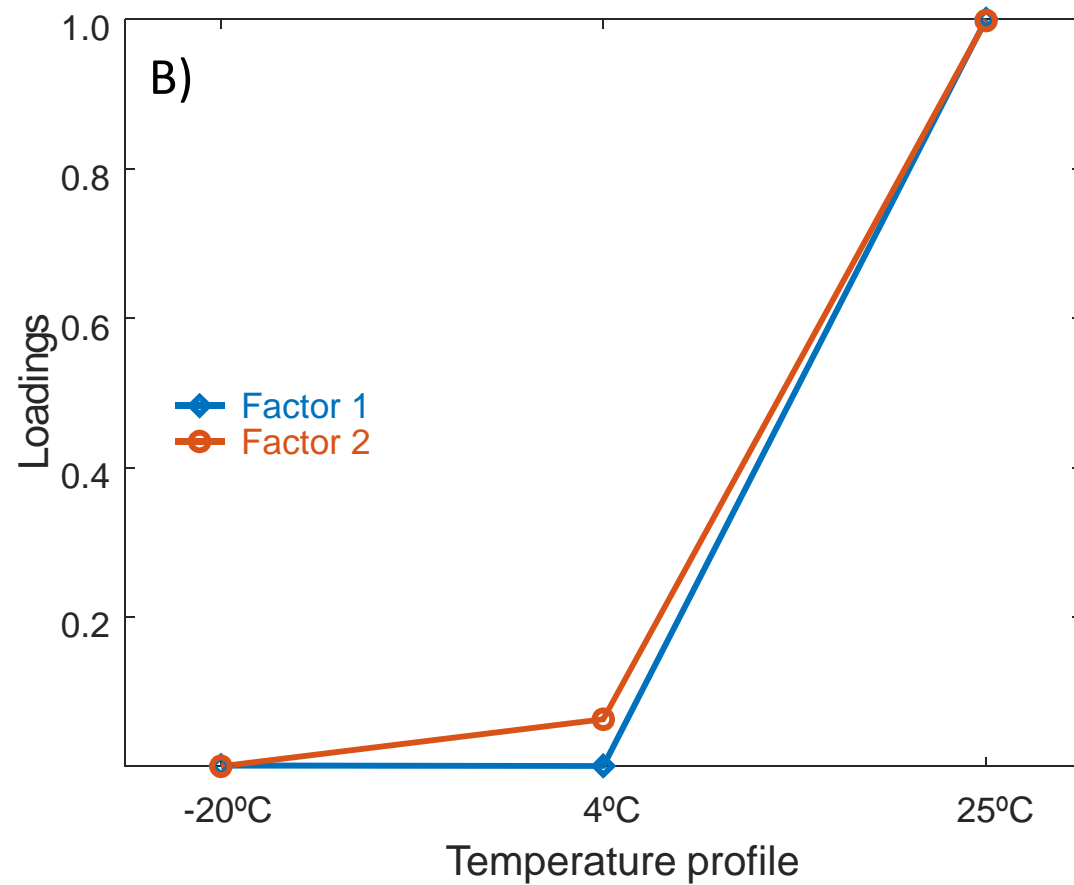
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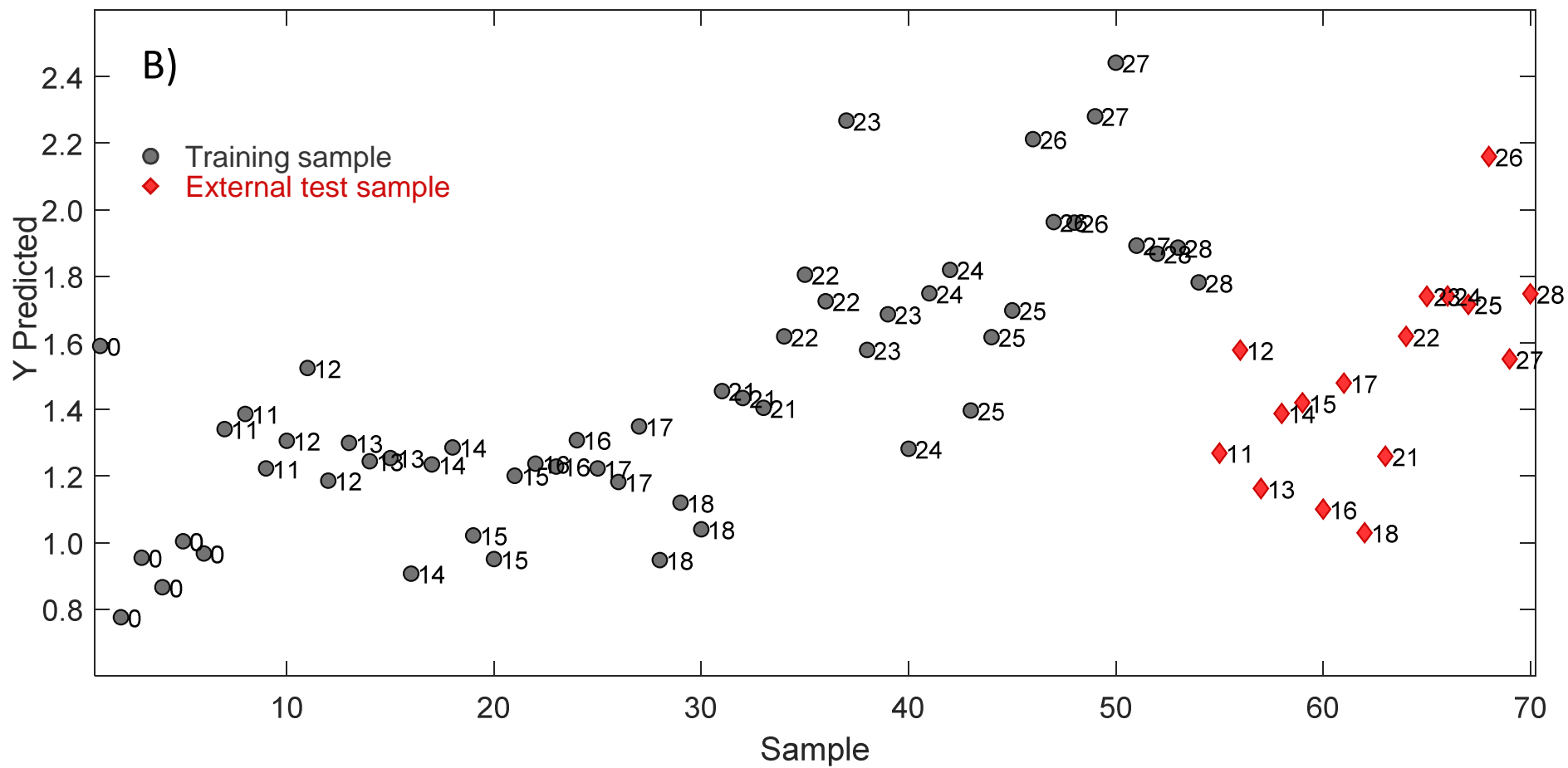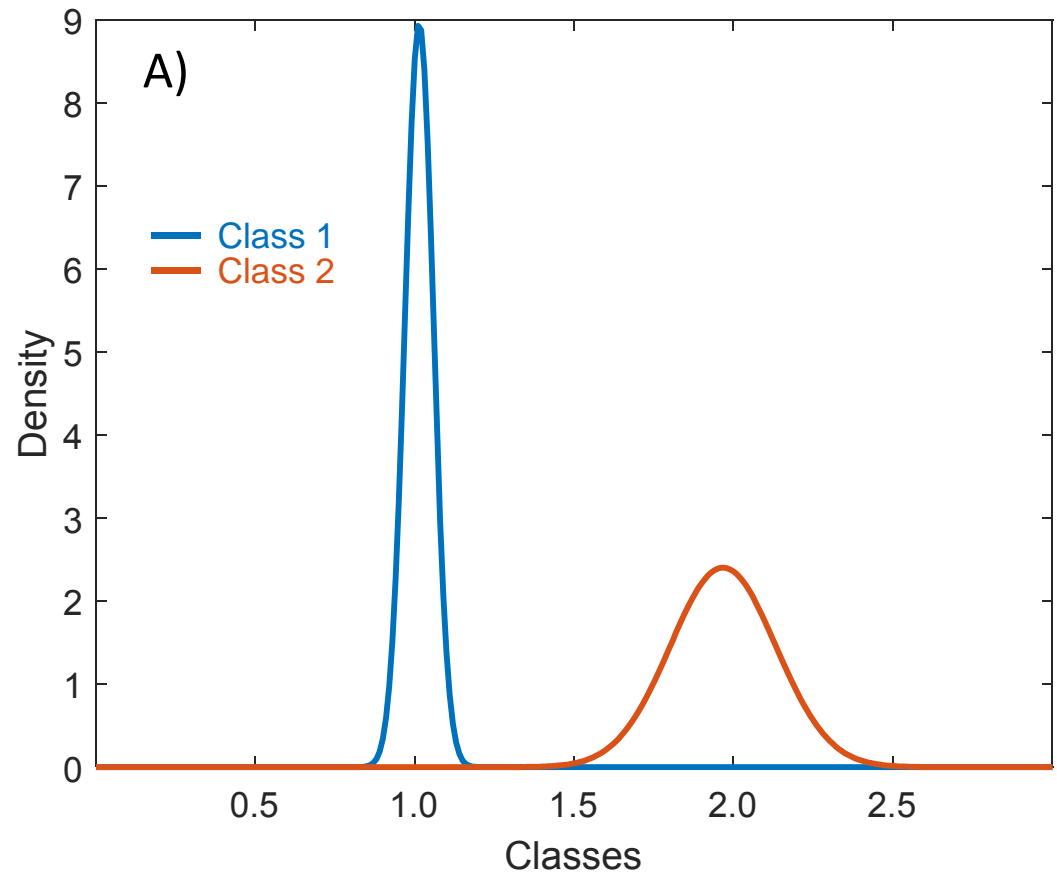Figure 3B
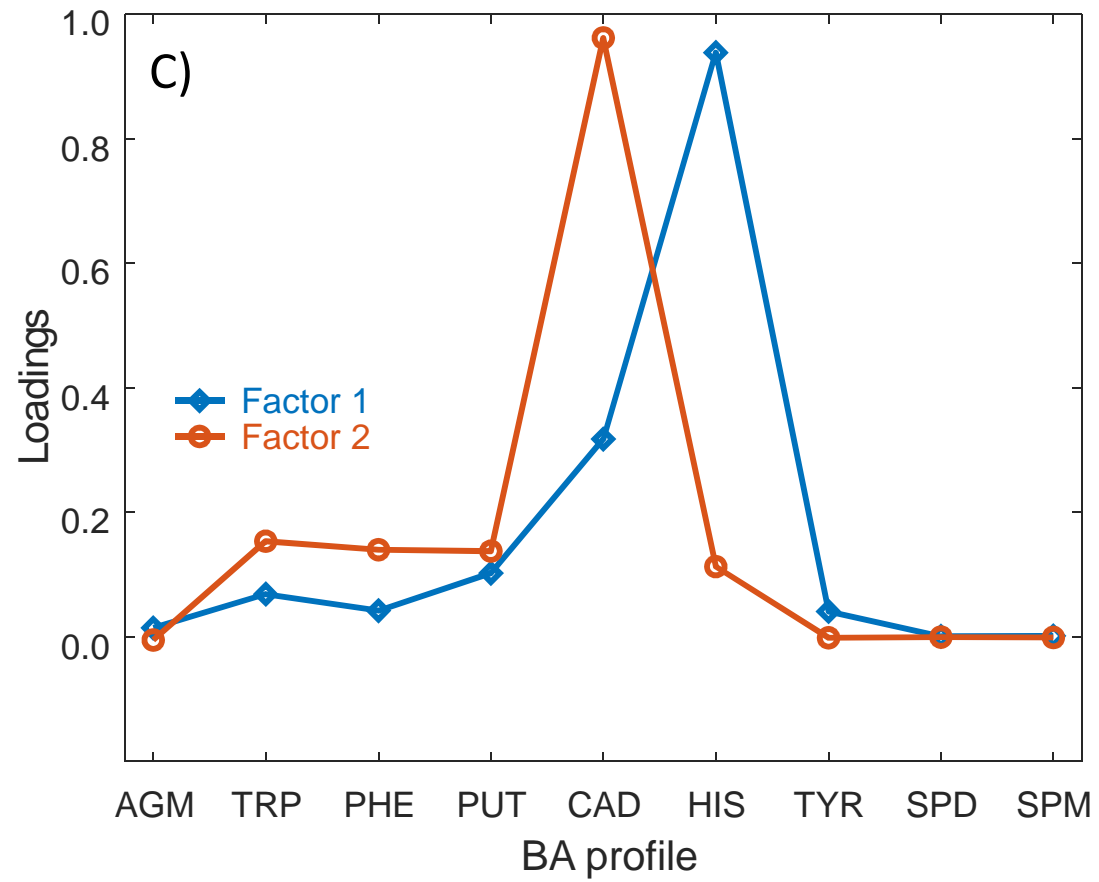
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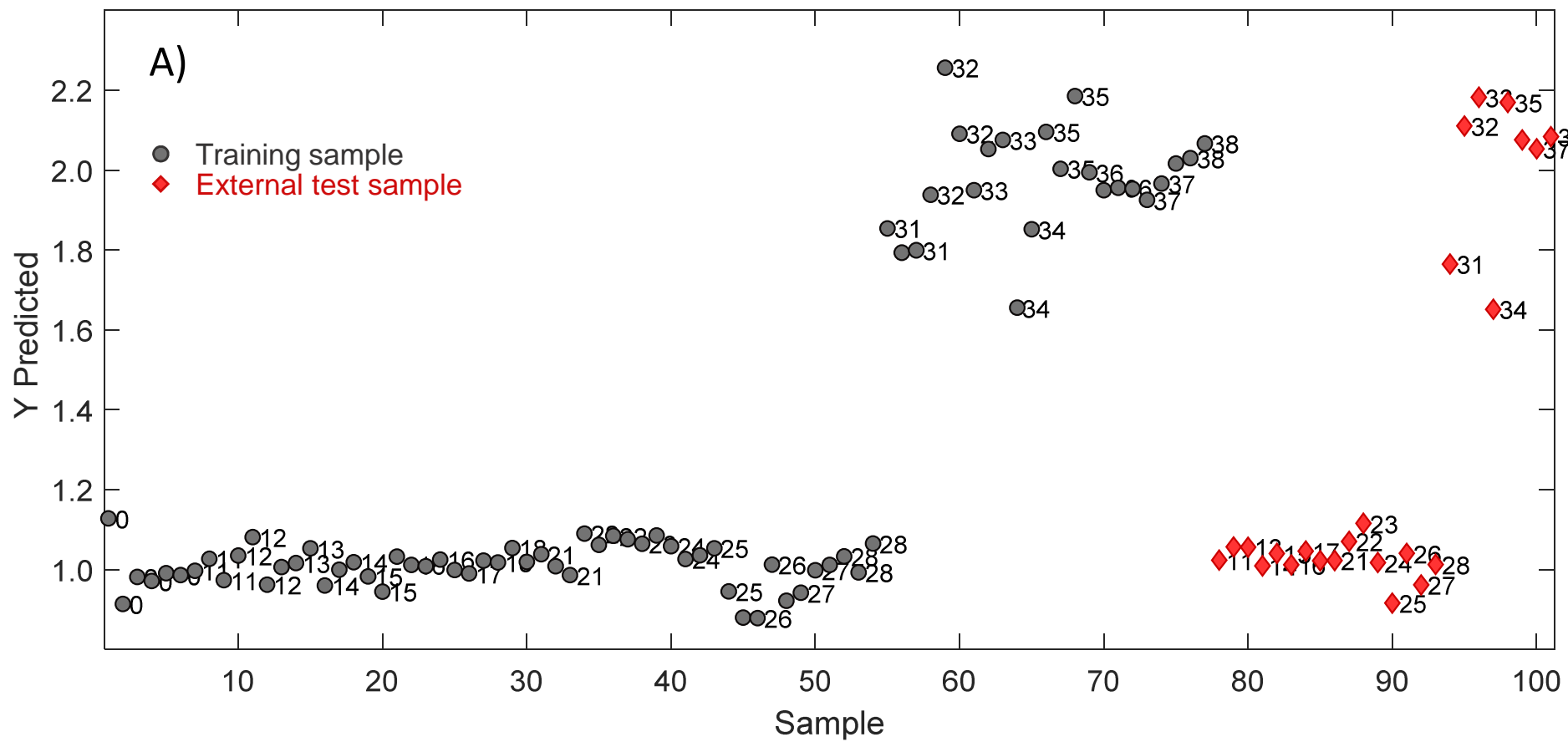
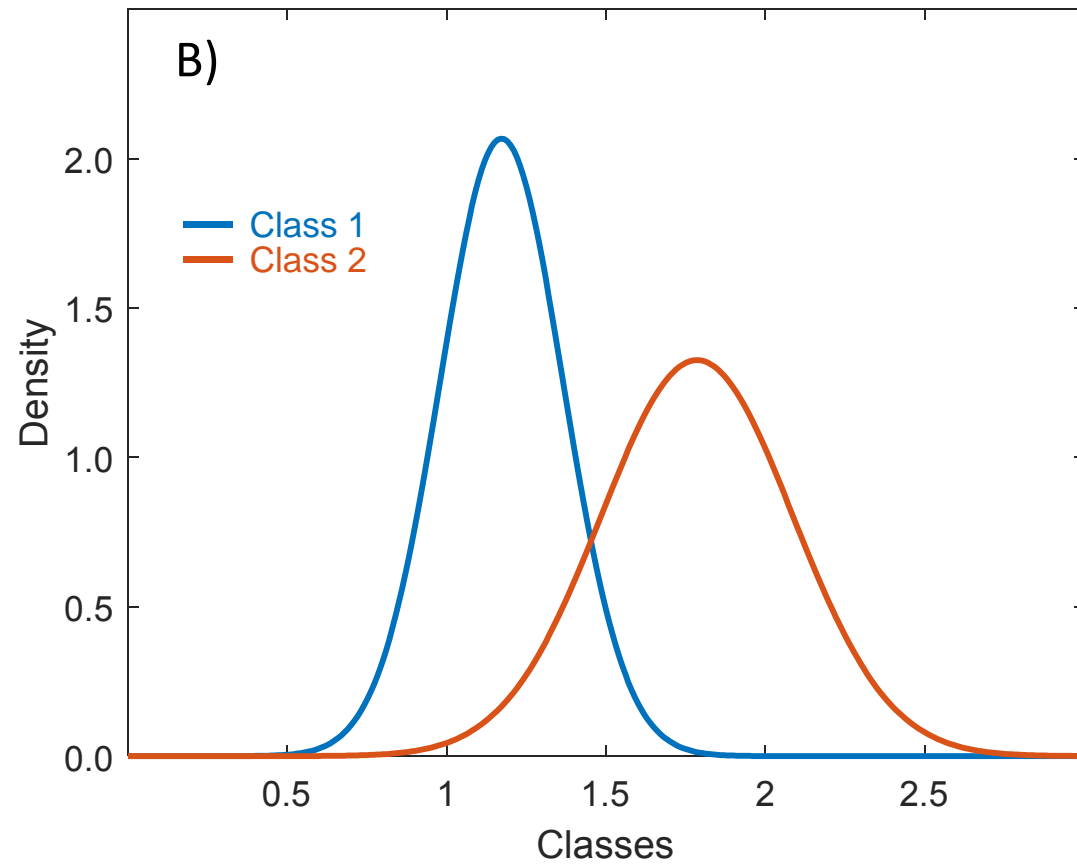Figure 4A

Figure 4B

Figure 5A

Figure 5B

Highlights:

PCA and PARAFAC show a complex BA evolution over time at diverse storage temperatures

Two different spoilage routes with different relative evolution of BAs are observed

Estimating the storage time with errors of 0.5 (in fitting) and 1 day (in prediction)

Two PLS-CM sequentially applied allow for decision making about cold chain breaks

Tuna stored at 25ºC, 4ºC and -18ºC, for different storage periods, is differentiated