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Fluorescence spectroscopy and PARAFAC in the analysis of yogurt

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Abstract

Parallel factor (PARAFAC) analysis and fluorescence spectroscopy were applied in the evaluation of yogurt during storage. Fluorescence landscapes with excitation wavelengths from 270 to 550 nm and emission wavelengths in the range 310–590 nm were obtained from front-face fluorescence measurements directly on yogurt samples during two storage experiments over a period of 5 weeks at 4 °C. PARAFAC analysis of the fluorescence landscapes exhibited three fluorophores present in the yogurt, all strongly related to the storage conditions. The fluorescence signal was resolved into excitation and emission profiles of the pure fluorescent compounds, which are suggested to be tryptophan, riboflavin and lumichrom. Thus, it is concluded that fluorescence spectroscopy in combination with chemometrics has a potential as a fast method for monitoring the oxidative stability and quality of yogurt. Regression models between fluorescence landscapes and riboflavin content, determined by the traditional chemical analysis, were performed, yielding a root mean square error of cross-validation of 0.09 ppm riboflavin, corresponding to 7% of the mean riboflavin content in the yogurt samples. Regression models based on PARAFAC scores, Partial Least Squares (PLS) and N-PLS were compared and yielded only minor differences with respect to prediction error. Several missing values appear in the fluorescence data matrices, for all emission wavelengths below the excitation wavelength. Substituting some of the missing values with zeros was observed to have a large impact on the model solution and the computation time. It is concluded that at least 43% of the missing values in the present data set need to be substituted in order to obtain meaningful PARAFAC models.

Keywords: Fluorescence spectroscopy; PARAFAC; Yogurt

1. Introduction

Fluorescence spectroscopy is a rapid and sensitive method for characterising molecular environments and events. It can be used as a non-destructive analytical technique to provide information on the presence of fluorescent molecules and their environment in all sorts of biological samples. In food research, the presence of fluorophores in the form of aromatic amino acids, vitamins, cofactors, etc. makes the technique highly relevant and interesting. The application of autofluorescence in analysis of food has increased during the last decade, probably due to the propagated use of chemometrics, as first proposed in a food application study by Jensen et al. back in 1982 [1].

In dairy research, fluorescence spectroscopy evaluated with chemometrics has previously been investigated in a few studies to monitor structural changes in milk proteins and their physico-chemical environment during milk heating [2], milk coagulation [3], cheese manufacture [4,5] and in the evaluation of oxidative changes in processed cheese during storage [6]. Changes in vitamin A in dairy products have also been monitored in several fluorescence studies [7,8], and front-face fluorescence spectroscopy has been used for measuring light-induced oxidation in various dairy products [9], as well as proposed as a new method for rapid quantification of riboflavin [10].

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In most of the latter studies, bilinear models as principal component analysis (PCA) and Partial Least Squares (PLS) have been used for evaluation of the fluorescence measurements. With the use of PARAFAC [11], it is possible to handle fluorescence landscapes (excitation–emission matrices) keeping intact the original two-dimensional data structure of each measurements. Thus, the trilinearity of the data can ideally be utilized in a unique decomposition of the fluorescence data into scores, excitation and emission loadings according to the concentration and the physical properties of each of the fluorophores present in the analysed sample.

Several studies have explored the use of fluorescence spectroscopy evaluated by multiway data analysis, such as PARAFAC. Within food research, applications can be found in analysis of sugar, meat, fish oil and cheese. In analysis of sugar and sugar solutions [12–15], information on sugar quality at a molecular level, ascribed to the impurities in sugar, has been thoroughly investigated with fluorescence landscapes. Fluorescence spectroscopy and PARAFAC have also been suggested in a potential screening method for dioxin contamination in fish oil [16], however, based on an indirect correlation. Furthermore, the technique has been demonstrated to hold relevant information when monitoring chemical changes of Parma ham during maturation [17] and the stability of processed cheese during storage [6].

In the present study, yogurt samples stored under different light and packaging conditions were measured by front-face fluorescence spectroscopy applied directly on the yogurt samples, with the purpose to monitor the oxidative stability and chemical changes during storage in order to compare the packaging materials. The underlying fluorescence structure of the fluorescence landscapes was investigated using PARAFAC. PARAFAC ideally decomposes the fluorescence landscapes presented in a three-way array into trilinear components according to the number of fluorophores present in the yogurt samples. The retrieved scores and loadings can then be directly related to the relative concentration (scores) and the fluorescence characteristics of the present fluorophores, which means that the excitation and emission loadings can be used in interpretation and identification of the found fluorescence phenomena. The obtained fluorescence PARAFAC results were compared with the riboflavin content as a chemical validation of the model results. Riboflavin—a strong fluorophore—plays a key role in the photo-oxidation of dairy products, and can be considered an important marker of early oxidation in milk and dairy products.

A few studies have recently focussed on some practical aspects of PARAFAC modeling of fluorescence data [18,19]. Especially light scattering effects and the large amount of missing values in fluorescence landscapes due to the fact that emission below the excitation wavelength does not give any physical meaning can be a challenge in the PARAFAC modeling. In this study, the strategy of inserting zeros instead of missing values, as suggested by Ref. [19] in order to stabilize the decomposition, will be explored.

Thus, the present study aims at three investigations:

- The potential of using PARAFAC models of fluorescence data from measurements applied directly to an untreated complex food system like yogurt.
- Handling missing values by inserting zeros in fluorescence data in the PARAFAC analysis
- The potential of front-face autofluorescence spectroscopy as a new rapid method for riboflavin quantification.

2. Materials and methods

2.1. Yogurt storage and packaging materials

Plain yogurt (3.5% fat) was obtained from Arla Foods amba (Viby, Denmark). The yogurt was filled into cups of two rigid packaging materials, polylactate (PLA) and polystyrene (PS), with PS having the higher oxygen permeability and light transmission. The packages contained 180 and 155 g yogurt for PLA and PS, respectively, resulting in a headspace volume of 35 ml for each package. The cups were sealed by a transparent and colorless laminate. The packed samples were stored at 4 °C for 5 weeks under a radiant flux of approximately 3500 lx of fluorescent light (Philips fluotone, TLD 18W/830) or in darkness. The samples stored in light were illuminated both from above through the lid and from the sides. Throughout the storage period, the samples were randomly interchanged to minimize unequal temperature fluctuations and light conditions. For further details on packaging materials, see Becker et al. [10].

2.2. Experimental design

Two batches of yogurt were examined in two identical storage experiments. Analyses were performed after 0, 7, 14, 21, 28 and 35 days of storage, giving a total of 21 different samples (5 days×2 light conditions×2 packaging materials+1 starting sample) for each batch. Thus, 42 independent samples were studied in all. Three cups from identical conditions for each of the two batches were selected and considered as triplicates. All analyses were carried out in duplicate. Prior to the measurements, the yogurt was mixed in the cup.

2.3. Fluorescence spectroscopy

Fluorescence landscapes were measured directly on the yogurt by filling 15 g of yogurt in a 30-ml plastic cup and recording fluorescence by dipping the measuring probe 1 mm into the yogurt sample. All samples were measured on a BioView spectrofluorometer (Delta Light & Optics, Denmark) using a pulsed xenon lamp for excitation and

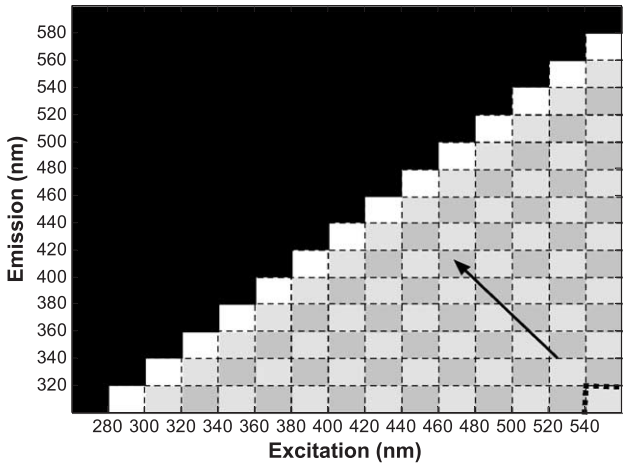

Fig. 1. Diagram of the recorded fluorescence data matrices. The black area indicates the range in which the fluorescence signal is recorded. The rest of the data points remain as missing values during the measurement. The colours indicate the strategy of inserting zeros in the modelling phase from the lower right corner and up to the diagonal line, stepping one diagonal data point at a time. This approach gives a total of 15 different amounts of inserted zeros.

equipped with an optical light conductor and a measuring probe, giving an open-end 180° measuring geometry. Fluorescence landscapes were obtained with excitations from 270 to 550 nm and emission wavelengths from 310 to 590 nm, with intervals and bandwidths of 20 nm, giving a total of 15 excitation and 15 emission wavelengths. Only emission wavelengths 40 nm above the excitation wavelength were recorded, resulting in 120 data points obtained for each measurement, as indicated in black in Fig. 1.

3. PARAFAC

PARAFAC decomposes the fluorescence landscapes into a number of trilinear components or factors, f. The principle behind the PARAFAC decomposition is to minimize the sum of squares of the residual e_{ijk}, according to Eq. (1).

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

The element x_{ijk} represents the fluorescence intensity for sample, i, excitation wavelength, j, and emission wavelength, k. The fluorescence landscapes are thus decomposed into sample scores, a_{if}, excitation loadings, b_{jf}, and emission loadings, c_{kf}, for each factor, f, also called PARAFAC components. The residual, e_{ijk}, contains the variation not captured by the PARAFAC model.

Split-half analysis of PARAFAC models [11] was performed for validation. The idea of this strategy is to divide the data set into two halves and make a PARAFAC model on both halves. Due to the uniqueness of the PARAFAC model, one will obtain the same result—same loadings in the non-split mode, e.g. excitation and emission mode—on both data sets, if the correct number of components is chosen. The split-half analysis was performed by calculating PARAFAC models for each batch at a time, subsequently comparing the results and ensuring that more or less identical excitation and emission loadings were obtained.

PARAFAC modelling was performed on all 42 samples in triplicates. One single replicate sample was removed as spectral outlier, giving a total of 125 yogurt samples in the modelling. Thus, a three-way data array of 125×15×15 was analysed.

3.1. Handling missing values—inserting zeros

The recorded fluorescence data matrices contain 47% missing values, as shown in Fig. 1, where the measuring area is indicated in black; the rest represent data points with missing values. Insertion of zeros to replace the missing values was tested. PARAFAC models were calculated from fluorescence data without any zeros inserted to replace the missing values as well as from fluorescence data with zeros completely inserted until the emission wavelength equals the excitation wavelength. All intermediate data matrices with zeros inserted up to a diagonal line were also used for calculations as indicated with the diagonal dotted lines in Fig. 1, giving a total of 15 different solutions. Thus, a gradient from 0 to 100% of zeros incorporated instead of missing values was compared. All PARAFAC models were calculated both with no constraints, and with non-negativity applied in all modes.

3.2. Chemical determination of riboflavin

The riboflavin content in the yogurt was measured by the fluorometric method established by AOAC (1990) using an Aminco Bowman series 2 luminescence spectrofotometer (SLM-Aminco, Urbana, IL, USA). This method implies a chemical extraction and cleanup of riboflavin prior to the fluorometric measurement, making the method time-consuming and the use of organic solvent necessary. The selected wavelengths were 446/525 nm for excitation/emission.

3.3. Riboflavin calibration

Regression models were performed between traditionally determined riboflavin content and fluorescence landscapes of 42 averaged samples. Leave-one-out cross-validation was performed. Regression models were evaluated using the validation parameter, Root Mean Square Error of Cross-Validation (RMSECV), as a term to indicate the prediction error of the model. Score values derived from the PARAFAC decomposition were used for regression models using Partial Least Squares (PLS) and multiple linear regression (MLR). Also, multiway calibration in the form

of N-PLS regression was performed on the fluorescence landscapes kept as three-way array, and PLS regression was performed on unfolded fluorescence emission spectra.

3.4. Software

Data analyses were performed in MatLab 6.5 (MathWorks) with the N-way Toolbox [20] (www.models.kvl.dk) and the PLS Toolbox 2.0 (www.Eigenvector.com). In the N-way Toolbox, missing values in the PARAFAC modelling are handled by expectation maximization.

Spectral data and reference riboflavin values can be downloaded in MatLab format from: http://www.models.kvl.dk.

4. Results and discussion

4.1. Fluorescence spectroscopy

The fluorescence landscapes of two yogurt samples are shown in Fig. 2 in the form of contour plots. The two samples represent the extremes in the experimental plan, i.e. a fresh yogurt sample (a) and a yogurt sample stored under the most severe conditions (b). The highest fluorescence peaks for the fresh sample are seen with excitation below 300 nm and emission around 370 nm as well as for excitations between 370 and 490 nm, and emission wavelengths around 500 to 550 nm. The excitation and emission characteristics indicate that the fluorescence peaks arise from tryptophan and riboflavin, which are both expected to be present in yogurt. Tryptophan was reported to have excitation/emission wavelength maximum at 285/365 nm in pure solutions, and riboflavin to have emission maximum at 520 nm for excitations at 270, 370 and 445 nm [21]. Apparently, the excitation around 270 nm of riboflavin is not observed in this case, probably due to absorption by other molecules. The suggested tryptophan fluorescence still seems present in the yogurt sample stored in light for 35 days (Fig. 2b), whereas the riboflavin signal seems considerably decreased. Furthermore, a peak with excitation/emission maxima around 370/430 nm seems to have increased, and the fluorescence profile could correspond to the photo-chemical degradation product from riboflavin, lumichrome, with excitation/emission maxima reported somewhere around 360/450 nm in a model system [22]. These observations were further investigated by the use of PARAFAC.

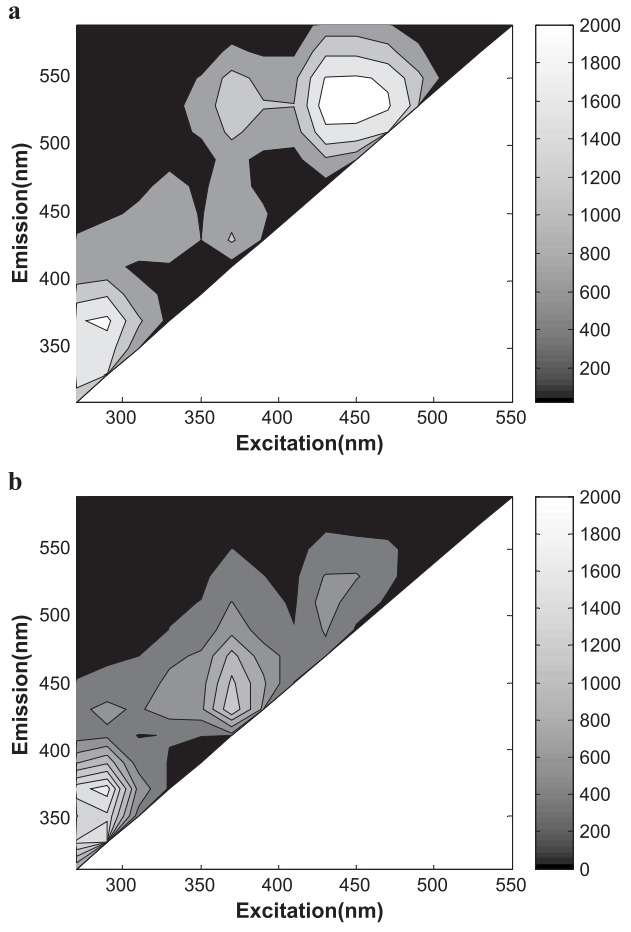

Fig. 2. Contour plot of a fluorescence landscape of a yogurt samples. (a) Fresh yogurt sample and (b) yogurt sample stored in light for 35 days and packed in polystyrene.

4.2. PARAFAC results

PARAFAC models of fluorescence landscapes were estimated with one to four factors. Based on split-half experiments and investigation of any systematics in the residuals, the PARAFAC model with three components was considered optimal, i.e. three different fluorescence phenomena were found present in the yogurts in this investigation.

The results from a PARAFAC model are shown in Figs. 3 and 4, in the form of PARAFAC loadings and scores, respectively. This result is based on fluorescence data with zeros inserted instead of the missing values for all emission wavelengths below the given excitation wavelength, corresponding to 74% of the missing data replaced with zeros (the missing data subject will be more thoroughly discussed in the next passage). In Fig. 3, the fluorescence profiles of the three resolved fluorophores are shown as fluorescence landscapes, formed by products of the derived excitation (b) and emission (c) loading vectors. The profiles of the three PARAFAC components are in agreement with the first inspection of the fluorescence data, and further indicate the fact that the obtained fluorescence signal from the yogurt samples arises from riboflavin (Fig. 3a), tryptophan (Fig. 3b) and lumichrom (Fig. 3c). The concentration level of these three PARAFAC components can be followed in the score plots (Fig. 4a and b), displaying the distribution of the samples. Thus, the development in riboflavin (score 1) and tryptophan (score 2) fluorescence are seen in Fig. 4a, showing a decrease in riboflavin throughout time for the

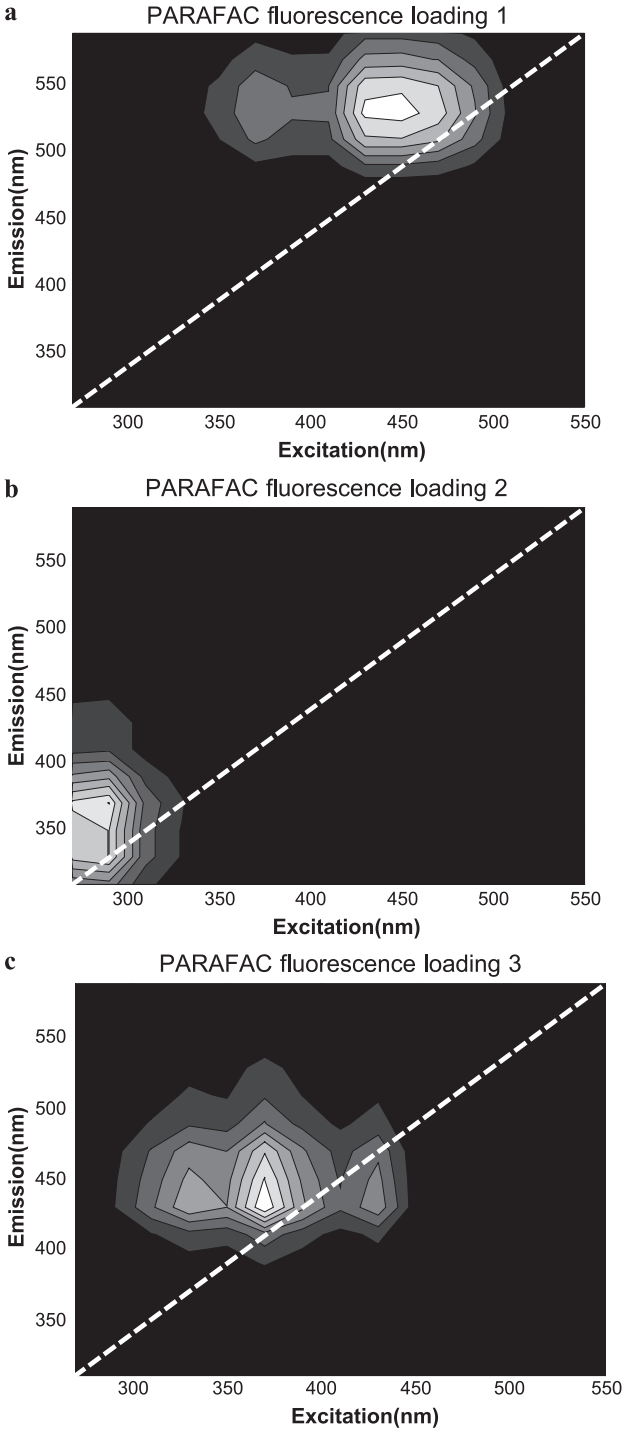

Fig. 3. PARAFAC fluorescence loadings (excitation times emission loading vectors) for the first three components (a+b+c) of a PARAFAC model based on fluoresence measurements of 125 yogurt samples and developed with zeros inserted instead of the missing values for all emission wavelengths below the given excitation wavelength, meaning that 74% of the missing values were replaced with zeros, corresponding with two steps in the diagonal line from the black measuring area in Fig. 1.

yogurt samples stored in light, and a general decrease in tryptophan throughout the storage, more or less independent of the storage conditions. The observed decrease in riboflavin fluorescence is the most pronounced for yogurts stored in polystyrene cups, which also has the highest oxygen permeability and light transmission, and therefore expected to induce a higher level of oxidation. All in all, the experimental plan is very evident in this score plot, as indicated with the dotted lines, separating the different packaging materials and light exposure, and indicating the direction of samples according to storage time.

The lumichrom and riboflavin fluorescence can be followed in the score plot in Fig. 4b, which shows a decreasing trend in lumichrom throughout storage. Comparing each of the storage times, a negative correlation to riboflavin fluorescence is seen, though, as indicated with the dotted trend lines in the score plot. Thus, for a given storage

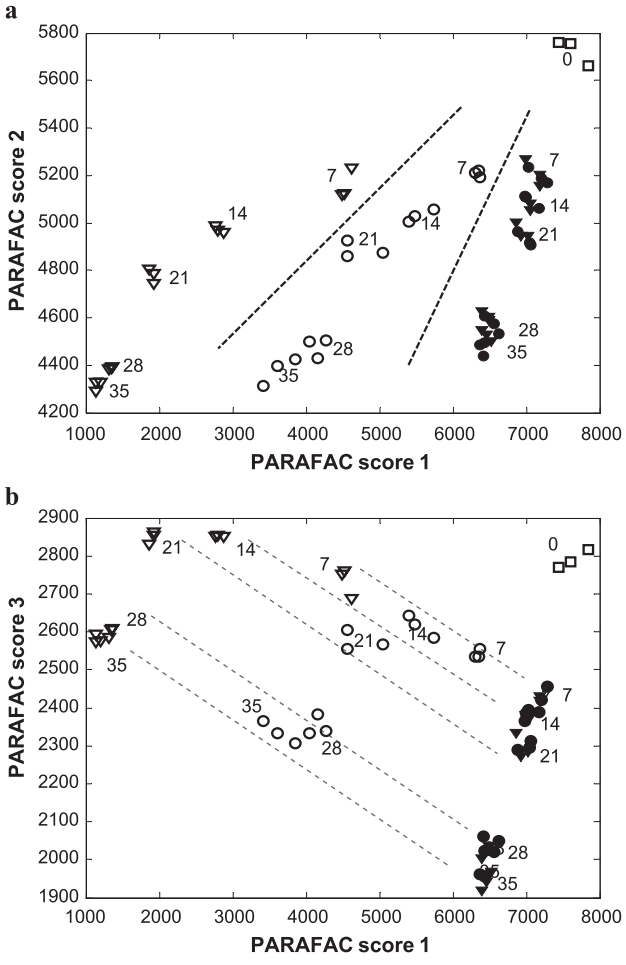

Fig. 4. Score plot of (a) scores 1 and 2, (b) scores 1 and 3 from a PARAFAC model based on fluoresence landscapes of 125 yogurt samples packaged in PLA (○) or PS (▽) at 4 °C under continuous light exposure at 3500 lx (open symbol) or in darkness (dark symbol). Samples are numbered according to days of storage, Day 0=(□). The dotted lines indicate the differences between the storage conditions (a) and the trend line for each time of storage (b). The PARAFAC model was developed with zeros inserted instead of the missing values for all emission wavelengths below the given excitation wavelength. Only samples from the first storage experiment are showed. Similar score plots were obtained for the second experiment (result not shown), with a little difference in offset, but with similar trends, comparing the two identical storage experiments.

time, the level of lumichrom fluorescence increases with decreasing riboflavin content and with storage conditions expected to induce the higher level of oxidation.

4.3. Handling missing data—inserting zeros

The measured excitation–emission matrix (landscape) contains 47% missing values, due to the physical limitations of the fluorescence measurements. The large amount of missing values can be a potential problem in the PARAFAC decomposition [19]. In order to facilitate the decomposition, insertion of zeros instead of missing values was tested.

PARAFAC component 1 is used as an example to illustrate the impact on the decomposition, when comparing PARAFAC models with different amounts of zeros replacing the missing values. In Fig. 5, contour plots of the fluorescence loadings for the first PARAFAC component are shown, based on the different PARAFAC models developed with increasing amount of zeros inserted, from the upper left corner to the lower right. It is evident that meaningful fluorescence profiles, corresponding to the fluorescence characteristics of riboflavin, only appear for the last three models, indicating that a certain amount of zeros is needed to perform a proper decomposition of these data. As indicated with the grey background, the three apparently satisfactory fluorescence loading profiles represent models with zeros inserted, leaving a band of four, two and no missing values, respectively, in the diagonal line, according to Fig. 1 (52%, 74% and 100% of zeros inserted instead of missing values). The rest of the obtained PARAFAC models yield loadings which are difficult to interpret and they all contain major artefacts in the sense that fluorescence profiles appear for emission wavelengths shorter than the excitation wavelength.

Only every second of the calculated PARAFAC models are included in Fig. 5. The overall results are that models on data with zeros inserted and leaving a band of up to five data points of missing values result in satisfactory decomposition and meaningful loadings, which means that at least 43% of the missing values need to be substituted. Similar results were obtained for PARAFAC loadings 2 and 3 (results not shown), underlining that the missing values need to be handled in order to obtain a valid PARAFAC decomposition of these kind of fluorescence data.

Since fluoresence emission at wavelengths below the excitation wavelengths is physically impossible, it makes sense to insert zeros in this area of the excitation–emission matrix in order to facilitate the decomposition by reducing the amount of missing values to be handled in the PARAFAC algorithm. However, it is previously shown that zeros inserted for emission wavelengths slightly below the excitation wavelengths can hinder an adequate mathematical decomposition, due to the required trilinearity in the fluorescence data [18]. Apparently, this does not make up a problem in the present investigation; even zeros inserted for all the missing values (also including one emission wavelength above the given excitation wavelength) seem to facilitate a meaningful decomposition, as seen in the lower right corner of Fig. 5.

As an alternative to the insertion of zeros, one could try to cut off some of the extreme wavelengths (high excitation or low emission wavelengths) which contain many missing values, as seen by Ref. [17]. In this way, the part of missing values can be significantly reduced, but also the measuring

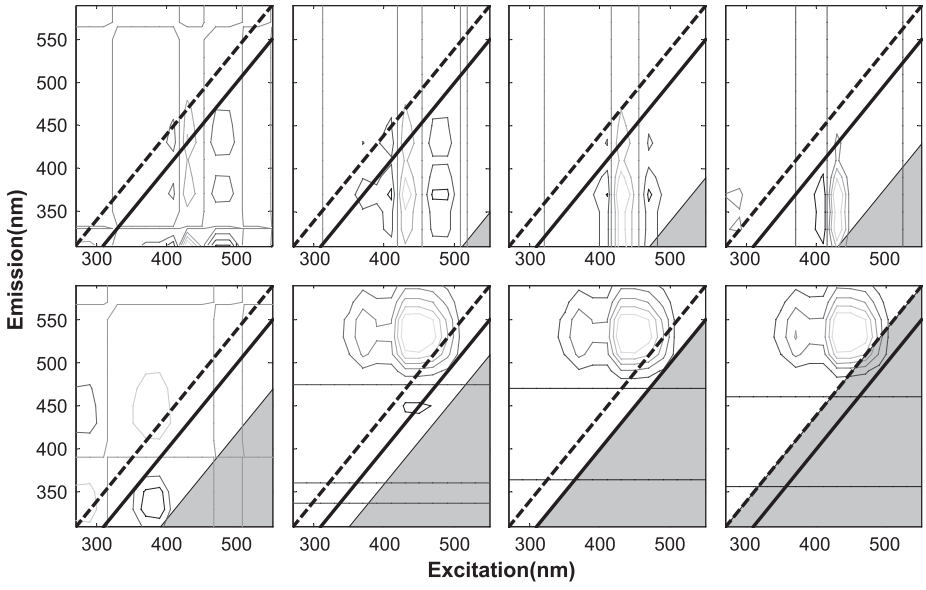

Fig. 5. Contour plots of fluorescence loadings (excitation times emission loadings) of the first component from PARAFAC models based on data with different amounts of inserted zeros, going from modelling with no insertion of zeros (upper left) to all missing values completely substituted with zeros (lower right corner). The grey-filled area represents where zeros are inserted. Loadings from every second intermediate model result according to Fig. 1 are shown. The solid diagonal lines indicate when emission equals the excitation wavelength. Fluorescence is only measured in the area above the dotted line.

spectral range will be restricted. Applying constraints to the PARAFAC model parameters has also proven to be helpful in the curve resolution and model stability [18]. However, when applying non-negativity in the PARAFAC modelling in the present study, similar results were obtained, with respect to the amount of zeros needed for a meaningful decomposition.

4.4. Riboflavin calibration

To verify the dependency between riboflavin and fluorescence signal, various regression models were developed. At first, the score values of PARAFAC component 1, which is expected to be due to riboflavin fluorescence, are compared with the traditional determined riboflavin content. A linear regression between the PARAFAC score and riboflavin content yields a correlation coefficient, R^2 of 0.94, confirming the assumption. However, a regression model for predicting the riboflavin content from the fluorescence landscapes can be further optimized, including the other PARAFAC scores in the regression model, as listed in Table 1 and shown in Fig. 6. This is probably due to the fact that the resolved fluorescence signal of riboflavin still is somewhat influenced by the fluorescence from other fluorophores or by differences in the sample matrix.

In Table 1, the results of different regression models are shown in the form of correlation coefficient and prediction errors, RMSECV. Comparing MLR on PARAFAC scores with PLS regression of PARAFAC scores as well as unfolded fluorescence spectra and multiway PLS regression of the fluorescence landscapes, only minor differences are found in this study. Only the univariate regression model of PARAFAC score 1 proved to perform significantly poorer ($p \leq 0.015$), compared with the best performing regression model, when evaluated with an F-test of the RMSECV values [23].

Even though three fluorescent components were found in the PARAFAC model, only two components were apparently needed to obtain the optimal regression. This is both evident for the PLS and MLR models in Table 1. Thus, for MLR between PARAFAC scores of the expected riboflavin and lumichrom components and riboflavin content, a regression model was obtained with a correlation of 0.97 (R^2), and a prediction error of 0.094 ppm, corresponding to 7% of the mean riboflavin content in the yogurt samples.

Table 1
Results from different regression models between fluorescence landscapes and riboflavin content of 42 yogurt samples

Data	Method	# Comp.	R^2	RMSECV (ppm)
PARAFAC score 1	MLR	1	0.94	0.13
PARAFAC scores 1–3	MLR	3	0.97	0.096
PARAFAC scores 1–2	MLR	2	0.97	0.096
PARAFAC scores 1+3	MLR	2	0.97	0.094
PARAFAC scores 1–3	PLS	2	0.97	0.094
Unfolded emission spectra	PLS	2	0.97	0.092
Fluorescence landscapes	N-PLS	2	0.97	0.094

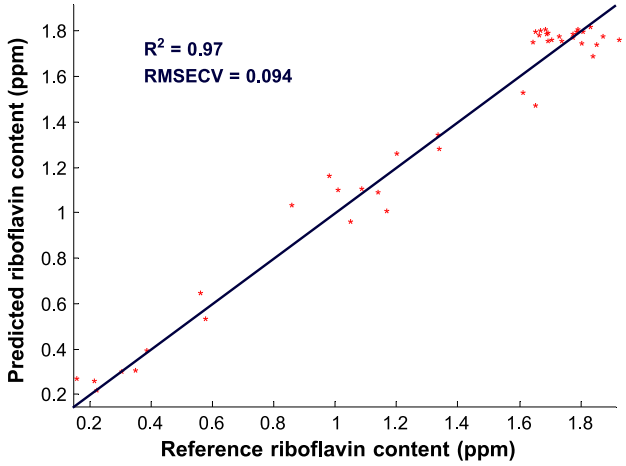

Fig. 6. Predicted vs. reference riboflavin content of 42 yogurt samples, based on a PLS regression model with two components of the scores values (1–3) from a PARAFAC model of fluorescence landscapes.

The results further indicate that the investigated non-destructive fluorescence measurements have the potential to substitute the traditional AOAC method for determination of riboflavin. The AOAC fluorometric method involves a chemical extraction while the investigated method makes use of a "mathematical extraction" of the riboflavin contribution to the fluorescence signal from the yogurt.

5. Conclusions

The present study represents an example of the application of fluorescence spectroscopy and PARAFAC to follow the oxidative quality of a food product. Thus, yogurt was monitored with rapid and non-destructive fluorescence spectroscopy, yielding information about the yogurt throughout storage at a molecular level, in the form of the development in fluorescence signal assigned to riboflavin, tryptophan, and the oxidative product of riboflavin, lumichrom.

The fluoresence landscapes in the present study contained a large amount of missing values, which make up a problem in the PARAFAC modelling, leading to model solution, which did not make sense, physically and chemically. Substituting a certain amount of the missing values with zeros facilitated a satisfactory decomposition. Thus, it was shown that at least 43% of the missing values had to be substituted in order to obtain meaningful PARAFAC models with the present data.

Regression models between the fluorescence landscapes and the riboflavin content of the yogurts confirmed the chemical causality, and underlined the potential of the method to be used for rapid determination of riboflavin.

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