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Monitoring beer during storage by fluorescence spectroscopy

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Abstract

The present study demonstrates the use of fluorescence spectroscopy and multivariate data analysis for monitoring changes in beer during storage. Total luminescence spectra and synchronous scanning fluorescence spectra were recorded for fresh beer and beer samples stored in clear glass vials for three weeks in darkness at 4 °C and at 22 °C, and exposed to light at 22 °C, respectively. A pronounced decrease of fluorescence features ascribed to riboflavin was observed in samples exposed to light as compared to those kept in the dark. Principal component analysis of synchronous scanning fluorescence spectra revealed clear clustering of samples according to storage conditions. Successful classification of differently stored samples was accomplished using both the nearest neighbour method (*k*NN) and linear discriminant analysis (LDA).

Keywords: Beer; Fluorescence; Photo-oxidation; Chemometrics; Riboflavin

1. Introduction

Beer is a complex mixture consisting mainly of water and ethanol with about 0.5% of dissolved solids (Cortacero-Ramirez, de Castro, Segura-Carretero, Cruces-Blanco, & Fernandez-Gutierrez, 2003). Beer analysis is important for evaluation of its organoleptic characteristics, quality, nutritional aspects, and safety. In response to these needs, a wide variety of methods have been developed and optimized to characterize and quantify various constituents. In most cases, these methods require pre-treatment of samples in order to separate and concentrate the target compounds.

In recent years, increasing efforts have been undertaken to develop rapid and informative methods and to explore direct analysis of foods, thus avoiding the need for specific fractionation procedures, which may alter the nature of the sample and be accompanied by loss or dilution of certain compounds. The usefulness of spectroscopic methods in this respect has been increasingly recognized for their non-invasiveness, rapidity, and sensitivity to a wide range of compounds in a single experiment. Several research groups have discussed the potential of fluorescence for direct analysis of food products. Fluorescence spectroscopy was used for monitoring oxidation in fish and meat, and for analysis of cheeses, milk and edible oils (Baunsgaard, Andersson, Arndal, & Munck, 2000; Bro, 1999; Bro et al., 2002; Engelsen, 1997; Wold, Jorgensen, & Lundby, 2002).

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We have recently applied total fluorescence spectroscopy and synchronous fluorescence spectroscopy for characterization and differentiation of various kinds of beers (Sikorska, Gorecki, Khmelinskii, & Sikorski, 2004; Sikorska, Gorecki, Khmelinskii, Sikorski, & De Keukeleire, 2004). This paper highlights our continuous interest in exploring applications of fluorescence methods for food analysis. Total fluorescence and synchronous scanning fluorescence techniques were used for monitoring changes occurring in beer during storage under different conditions.

2. Experimental

2.1. Materials

Two lager beers A and B were acquired in a local supermarket. The expiry dates exceeded the duration of the experiments. Samples were degassed in an ultrasonic bath before measurements.

2.2. Storage of beers

After opening of the bottles, beers (15 ml) were transferred into transparent glass vials (20 ml), which were then firmly stoppered. The samples were divided into three groups. In one group, beer was stored in darkness at 4 °C, while beers in the other two groups were stored at 22 °C, one in darkness and the other one under diffuse light. All samples were stored for 21 days.

2.3. Fluorescence measurements

Fluorescence spectra were obtained on a Fluorolog 3-11 Spex-Jobin Yvon spectrofluorometer. A xenon lamp source was used for excitation. The excitation and emission slit widths were 2 nm. The acquisition interval and the integration time were maintained at 1 nm and 0.1 s, respectively. A reference photodiode detector at the excitation monochromator stage compensated for source intensity fluctuations. Individual spectra were corrected for wavelength response of the system. Right-angled geometry was used for beer samples diluted in water (3% v/v) in a 10 mm fused-quartz cuvette. Back-face geometry was used for bulk beer samples in a triangular fused-quartz cuvette.

Three-dimensional spectra were obtained by repeatedly recording the emission spectra in the range from 290 to 700 nm, at excitation wavelengths from 250 to 450 nm, spaced by 5 nm intervals in the excitation domain. Fully corrected spectra were then concatenated into an excitation-emission matrix.

Synchronous fluorescence spectra were collected by simultaneously scanning the excitation and emission monochromator in the 250–700 nm range, with constant wavelength differences $\Delta\lambda$ between them. Spectra were recorded for $\Delta\lambda$-values of 10, 30, and 60 nm for each of the samples. Fluorescence intensities were plotted as a function of the excitation wavelength. Fluorescence measurements were done in triplicate for each sample.

2.4. Data analysis

Multivariate data analysis was performed on synchronous fluorescence spectra measured at $\Delta\lambda = 10$ and 60 nm for both beers A and B. Twelve spectra were analysed for fresh beers and for each of the storage conditions, yielding a total of 48 spectra for each beer at a given $\Delta\lambda$. Exploratory data analysis was performed using principal component analysis (PCA) (Wold, Esbensen, & Geladi, 1987). PCA is a multivariate technique acting in unsupervised manner and it is used to analyse inherent structure of the data. PCA reduces dimensionality of the data set by finding an alternative set of coordinates: principal components, PCs. The PCs are linear combinations of original variables, orthogonal to each other and designed in such a way that each one successively accounts for the maximum variability of the data set. When the principal component scores are plotted, they reveal relations existing between the samples, such as natural clustering in the data or outlier samples. This technique provides insight into how effective pattern recognition algorithms are in classifying the data.

Two methods of discriminant analysis were used for the purpose of multiple group classification: nearest neighbours method (kNN) (Wu & Massart, 1997) and linear discriminant analysis (LDA) (Kemsley, 1996; Roggo, Duponchel, Ruckebusch, & Huvenne, 2003). The k-nearest neighbours method is a well known non-parametric classification method. In principle, the test object is assigned to the cluster, which is most represented in the set of k nearest training objects. For each data point, the closest data points, called 'nearest neighbours' are searched for and then decision is made according to the values of these neighbours. kNN is one of the simplest learning techniques – the learner only needs to store the examples, while the classifier does its work by observing the examples most similar to the one to be classified. The k values were chosen in the range of $k = 1, \ldots, 10$ due to the size of our sample set, which was too small for larger values of k. This non-parametrical method was used because common parametrical methods like linear discriminant analysis and quadratic discriminant analysis are often unsuitable for datasets with a number of variables exceeding the number of objects, due to observation matrix singularity or to non-normality of the data set. The nearest neighbours method allows to analyse entire spectra, without any reduction of the datasets.

Additionally, linear discriminant analysis (LDA) was performed on simplified data sets. For this purpose, six

wavelengths were extracted from each synchronous spectrum measured at a particular $\Delta\lambda$ and analyzed. The LDA method is straightforward in calculation and interpretation.

The bootstrap method was used to estimate the classification error (Efron, 1983; Efron & Tibshirani, 1997). In this method, the dataset was randomly split into two independent sets: training and test. The training set was used to construct a rule and the test set to probe the rule. This procedure was repeated many times. Version 0.632+ of the method was applied. It has low bias and variance, and 50 bootstrap replications were found sufficient. More bootstrap replications did not improve the classification error estimates.

Data analysis was performed using The Unscrambler version 9.0 (Camo AS, Oslo, Norway) and Matlab 6.5.

3. Results and discussion

3.1. Changes in beer fluorescence characteristics during storage

Fig. 1 shows the total luminescence spectra (TLS) of beer A both in bulk and diluted with water. Back-face geometry was used for bulk samples and the right-angled geometry for diluted samples. Such arrangements, as demonstrated previously, enable to observe an intense short-wavelength fluorescence in diluted samples and a longer-wavelength emission having a considerably lower intensity in bulk samples (Sikorska et al., 2003).

The short-wavelength fluorescence, with excitation at 250 nm and emission at 350 nm, tentatively attributed to aromatic amino acids, was clearly observed in diluted samples, along with a second very weak emission band, with excitation at 350 nm and emission at 420 nm. The spectra of fresh beer and beer stored in light are rather similar. The short-wavelength emission could not be observed in the bulk beer, while the longer-wavelength emission was fairly intense. As we have recently found, this emission may originate from components of the vitamin B group (Sikorska et al., 2003). The changes occurring during storage are more pronounced than those detected in diluted samples. Comparison of fluorescence characteristics of fresh beer with that stored in light reveals main differences at 450 nm in excitation and between 500–600 nm in emission. This zone is characteristic for vitamin B_2 (riboflavin) emission and, in general, can be attributed to flavins present in beer. This emission disappears in beer exposed to light, in accordance with the well-known photoinstability of flavins (Heelis, 1982). The total luminescence characteristics of beers stored in the dark at 4 and 22 °C (not shown) were almost identical to those of fresh beers for both diluted and bulk samples. Thus, the total luminescence spectra, which represent all absorption and emission changes during storage, led us to conclude that the most pronounced effect is observed in the flavin emission zone for beers exposed to light.

Although TLS provide a full picture of emission, single emission and synchronous fluorescence spectra may be more apt for detecting minor changes occurring during beer storage. On the other hand, TLS measurements take profit of the possibility to select appropriate λ_{em} (emission wavelength), λ_{ex} (excitation wavelength), and $\Delta\lambda$ (emission-to-excitation difference) values for subsequent monitoring of excitation, emission and synchronous scanning fluorescence spectra, respectively.

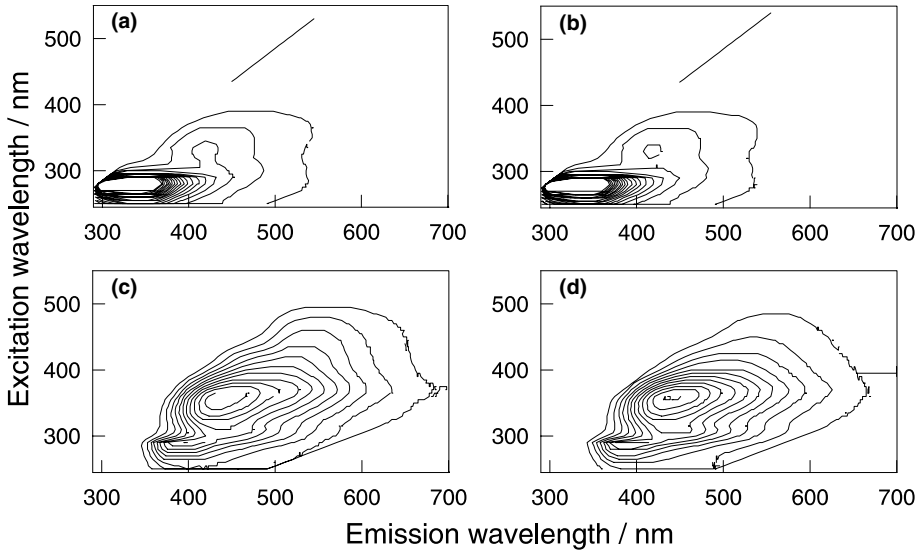

Fig. 1. Contour maps of fluorescence of beer A measured in two different arrangements: right-angled geometry, diluted sample (3% v/v, beer:water) — fresh beer (a) and beer stored for 21 days in light (b); back-face geometry, bulk beer — fresh beer (c) and beer stored for 21 days in light (d).

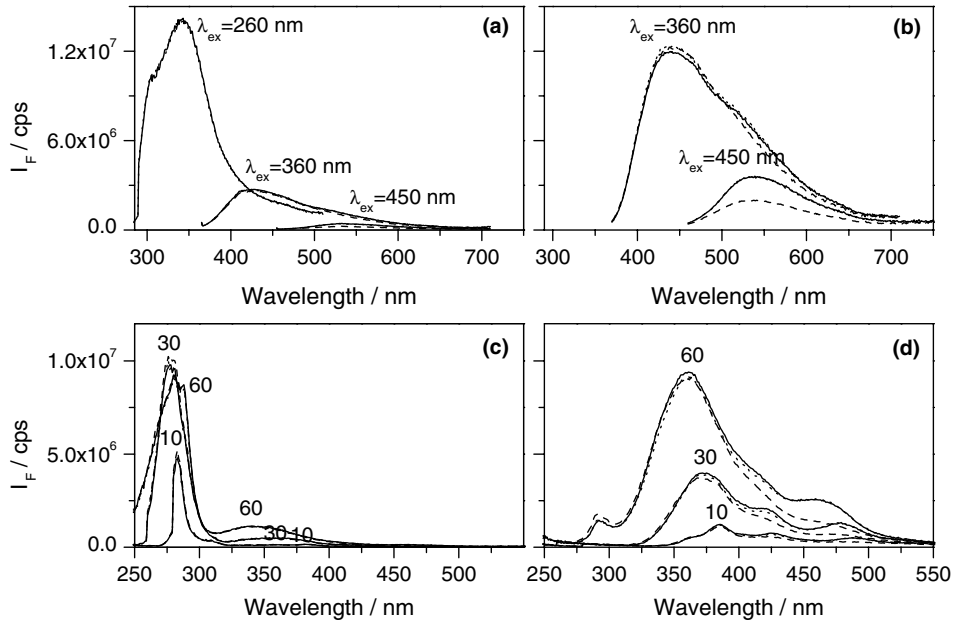

Fig. 2. Changes in emission spectra of beer A during storage: (a) diluted beer (3% v/v, beer:water), right-angled geometry; (b) bulk beer, back-face geometry. Changes in synchronous fluorescence spectra of beer during storage: (c) diluted beer(3% v/v, beer:water), right-angled geometry; (d) bulk beer, back-face geometry; $\Delta\lambda$ = 10, 30, and 60 nm. Storage conditions: darkness at 4 °C (solid); darkness at 22 °C (dot); light at 22 °C (dash).

Figs. 2(a) and (b) show emission spectra of diluted and bulk beers, recorded at selected excitation wavelengths and extracted from TLS. As was already concluded from TLS measurements, no differences were noted between fresh and stored beers in spectra, recorded with excitation at 260 and 360 nm. Noticeable changes appear in the emission spectra, recorded with excitation at 450 nm, more specifically, a decrease of fluorescence intensity in beer exposed to light. Alterations were more evident in the emission spectra of bulk beers, showing a pronounced decrease in intensity on excitation at 450 nm and some small changes on excitation at 360 nm.

Synchronous scanning fluorescence (SSF) spectroscopy is a valuable technique for analysis of mixtures of fluorescent compounds. Both excitation and emission characteristics are accessible by simultaneously scanning excitation and emission wavelengths at a constant difference between both. Such scanning improves the selectivity for individual components and allows collecting superior information on mixtures of fluorescent compounds. Since TLS of beers leads to major overlap of the emission spectra of particular fluorescent beer constituents, SSF may well be the method of choice.

Figs. 2(c) and (d) present the SSF spectra of beer A recorded at $\Delta\lambda$ = 10, 30, and 60 nm. SSF spectra of the diluted beer for $\Delta\lambda$ = 10 nm show a sharp, intense band with a maximum at 283 nm and a weak band with a maximum at 384 nm. The short-wavelength emission band was broadened for $\Delta\lambda$ = 30 nm with the maximum shifted to 275 nm. Additionally, the intensity of both bands increased. A further increment of $\Delta\lambda$ to 60 nm reduced the intensity at 275 nm, thereby simultaneously increasing the intensity of the broad band having its maximum shifted to the blue. No differences were observed between beers stored under varying conditions.

Fig. 2(d) shows the SSF spectra of bulk beer A, recorded in the back-face geometry. As observed in TLS, the short-wavelength emission was absent as a result of intense absorption in this spectral region. Three overlapping bands with maxima at 386, 428, and 489 nm are prominent for λ = 10 nm. For $\Delta\lambda$ = 30 nm, the fluorescence intensity of all bands increased, changes in their relative intensities were noted, and maxima were blue-shifted to 380, 422, and 480 nm, respectively. A further increase of intensity and band broadening was apparent for $\Delta\lambda$ = 60 nm, resulting in only two bands with maxima at 367 and 460 nm. In addition, a short-wavelength band appeared with a maximum at about 295 nm. The fluorescence intensity of the long-wavelength bands has decreased significantly in the beer exposed to light, while spectra of samples stored in the dark remained mainly unaltered.

Both in the present work and in our previous studies, it seems feasible that the long-wavelength bands originate from flavin fluorescence (Sikorska et al., 2003; Sikorska et al., 2004). To confirm this feature, spectra of riboflavin in water have been recorded. Fig. 3 presents the SSF spectra of riboflavin along with its excitation and emission spectra.

As is evident from Fig. 3, the shape and intensity of the SSF spectra depend on the difference between

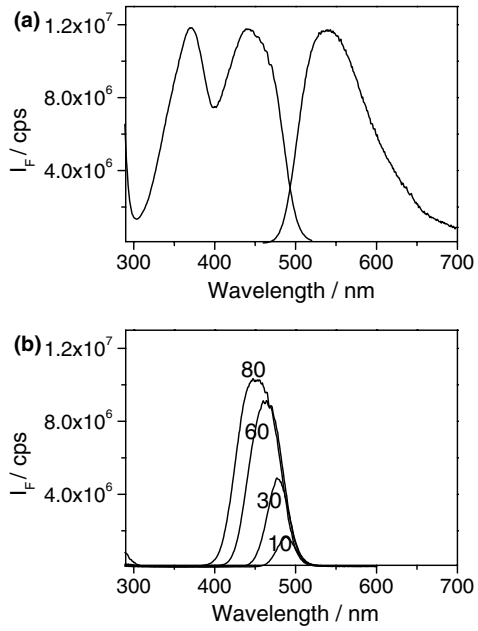

Fig. 3. (a) Excitation (λ_{em} = 535 nm) and emission (λ_{ex} = 450 nm) spectra of riboflavin in water. (b) Synchronous fluorescence spectra of riboflavin in water measured for $\Delta\lambda$ = 10, 30, 60, and 80 nm. Concentration of riboflavin in water: 1.5 μg ml^{-1}.

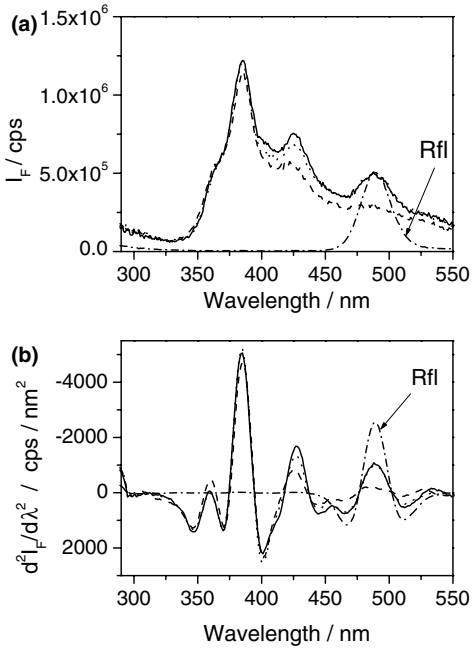

Fig. 4. Synchronous scanning fluorescence spectra (a) and second-order derivative of the SSF spectra (b) for bulk beer A stored in different conditions (back-face geometry) and for riboflavin in water (right-angled geometry) measured for $\Delta\lambda$ = 10 nm (for storage conditions, see Fig. 2).

excitation and emission wavelengths $\Delta\lambda$, which defines the extent of overlap of the absorption and emission bands. An effective bandwidth reduction was observed for $\Delta\lambda$ = 10 nm in comparison with the emission band and the SSF spectrum has a single band with a maximum at 490 nm. At increased $\Delta\lambda$, the maximum was shifted to the blue by values around half the $\Delta\lambda$ variations, dependent on the emission and absorption band shapes (to 479 and 465 nm for $\Delta\lambda$ = 30 and 60 nm, respectively). Although broadening and intensity increase were noted, a single SSF band was always apparent. Comparison shows that the maxima observed for riboflavin at different $\Delta\lambda$-values match very well the respective long-wavelength maxima in beers, thus confirming a correct assignment of the bands to riboflavin.

When overlapping emission bands appear in spectra originating from various species, improved band separation may be achieved by analysing derivative spectra instead of zero-order spectra. The most useful feature of the derivative curves is the bandwidth reduction, which provides some resolution enhancement. The even-order derivative spectra, besides of greater resolution, have another advantage over the zero-order spectra, namely reduction of background interferences such as light scattering from turbid samples. On the other hand, a typical disadvantage of the derivative spectra is their increased noise levels.

Fig. 4 shows the second-order derivative of the SSF spectra recorded for Δ = 10 nm. The short-wavelength emission is resolved into two bands with maxima at 360 and 384 nm. The long-wavelength bands are much better separated than those in the zero-order spectrum. Excellent matching of the riboflavin derivative spectrum with the long-wavelength band confirms the origin of this emission. The intensity of the flavin emission clearly decreased in beer exposed to light. The impact of light is perhaps most offensive and numerous studies have addressed light-induced decomposition of beer thereby focussing on formation of sulphur-containing degradation products, in particular 3-methylbut-2-ene (MBT). The intervention of riboflavin to sensitize decomposition of beer bitter compounds (isohumulones) has been amply demonstrated and, in fact, riboflavin serves as a sacrificial compound in the process (Burns, Heyerick, De Keukeleire, & Forbes, 2001; Huvaere et al., 2004). We even proved that a number of riboflavin fragments are incorporated into radicaloid intermediates of isohumulones and derivatives (data not shown).

Additional information is gained from the present study with respect to established knowledge, i.e., photodecomposition of riboflavin and formation of MBT as a marker for the impact of light. Indeed, fluorescence alterations refer directly to subtle and minor structural changes occurring during aging. A comprehensive picture of gross features from mixtures of fluorescent compounds is obtained, although specific degrading molecules escape identification for the time being. However, results presented here provide a platform to probe into detailed reactivities. A further advantage is the fact that sample pre-treatment is not necessary, which contrasts

the tedious pre-concentration needed to assess MBT using sophisticated equipment. It is also feasible that stored and validated fluorescence spectra can serve as references for beer quality control purposes.

3.2. Multivariate analysis of synchronous fluorescence spectra

Our objective was to test whether synchronous spectra allow discrimination between differently stored samples. Synchronous spectra of bulk beers were chosen for the multivariate analysis, because significant changes in fluorescence characteristics of beer during storage only occurred in the long-wavelength emission of these spectra. PCA methods were used for exploratory spectral analysis.

The PCA results for beer A are presented in Fig. 5 and Table 1. Fig. 5 shows the score plot of PC1 versus PC2 of PCA models prepared for synchronous fluorescence spectra of beers that were either fresh or stored in different conditions. The first model includes all the storage conditions, while the second model excludes the samples exposed to light. The distribution of samples in the score plots for both models clearly displays clustering of beer samples according to freshness and storage conditions.

From the score plot of Fig. 5(a), it is concluded that the samples exposed to light are clearly distinct from fresh beer and samples stored in the dark by the negative values of the first principal component PC1. This component explains 83% of the total variance. The loading for this component shows the importance of the bands attributed to flavins, with a maximum at 489 nm, and also of the short-wavelength bands at 375–450 nm. PC2 explains 15% of the total variance and is related to the changes in the 350–440 nm bands. This component differentiates the samples stored in the dark at 22 °C from all other samples.

The second PCA model was constructed for fresh beers and beers stored in darkness, excluding the samples exposed to light. In this model, PC1 and PC2 describe 90% and 7%, respectively, of total variance. PC1 corresponds to variations in the bands with maxima at 428, 400 and 380 nm, while PC2 describes changes in the bands with maxima at 367 and 380 nm.

Comparison of the two PCA models shows that the main difference between beers stored in darkness and under light conditions corresponds to changes in the

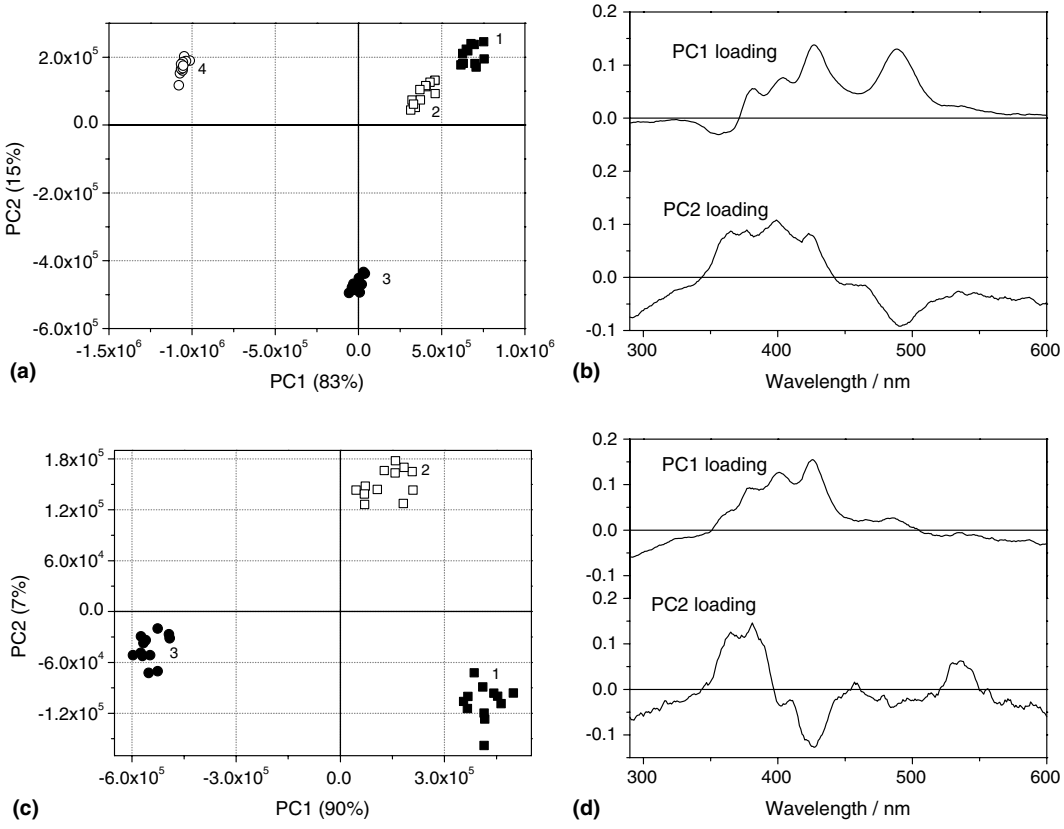

Fig. 5. Score (a), (c) and loading (b), (d) plots for two PCA models of synchronous scan fluorescence spectra of bulk beer A stored in different conditions, recorded for $\Delta\lambda = 10$ nm; 1-fresh beer and samples stored for 21 days: 2-darkness, 4 °C; 3-darkness, 22 °C; 4-exposed to diffuse light, 22 °C. Series 4 was only included in the model represented in (a) and (b).

Table 1
Results of the principal component analysis of synchronous fluorescence spectra of beers A and B

PCA model	PC1 [%]	PC2 [%]	PC1 loading [nm]	PC2 loading [nm]
Beer A				
$\Delta\lambda$ = 10 nm	83	15	383, 404, 428, 489	377, 400, 425
$\Delta\lambda$ = 60 nm	66	31	465	355, 464
Beer B				
$\Delta\lambda$ = 10 nm	94	4	404, 428, 489	382, 401, 422
$\Delta\lambda$ = 60 nm	85	12	465	410, 350

zone around 489 nm, which is attributed to the riboflavin emission. These observations agree with the well-known riboflavin photodecomposition and its stability in the absence of light (Andres-Lacueva, Mattivi, & Tonon, 1998; Duyvis, Hilhorst, Laane, Evans, & Schmedding, 2002; Heelis, 1982).

It is important to note that convincing discrimination between samples stored in darkness at different temperatures was achieved. As noted earlier, the fluorescence changes for samples exposed to light could be readily recognized from the spectra, as opposed to the more subtle differences due to variation of the temperature. Although differentiation between samples stored in darkness at different temperatures was not possible by visual inspection of the spectra, it was readily accomplished by PCA analysis.

Similar PCA models were constructed for synchronous fluorescence spectra recorded for $\Delta\lambda = 60$ nm with all four series of samples (see Table 1). The results also revealed clustering of samples according to storage conditions and confirmed the importance of emission originated from flavins in differentiating samples exposed to light from those stored in darkness.

Similar results were obtained for beer B. However, separation between fresh samples and beers stored in darkness at 4 °C was not as evident as that obtained for beer A when analyzing synchronous spectra for $\Delta\lambda = 10$ nm. Nonetheless, good separation was obtained for these samples using synchronous spectra taken for $\Delta\lambda = 60$ nm.

We have also evaluated PCA analysis of second derivatives of synchronous fluorescence spectra measured at $\Delta\lambda = 10$ nm. This transformation, however, did not improve separation of the sample groups, which could very likely be attributed to noise necessarily added by the numerical differentiation procedure.

With respect to quality monitoring, it is desirable to separate products into classes, for example, fresh and aged samples. In order to test the feasibility of such classification based on the fluorescence spectra, two statistical methods were employed: the *k*NN method, which uses entire spectra, and the LDA method, for which only six selected excitation/emission wavelength pairs were extracted from the spectra. The results of the *k*NN and LDA analyses are shown in Tables 2 and 3.

Table 2
Classification of beers using entire synchronous scan fluorescence spectra for the *k* nearest neighbour method (*k*NN)

Sample		Beer A		Beer B	
k		$\Delta\lambda$ 10 nm	$\Delta\lambda$ 60 nm	$\Delta\lambda$ 10 nm	$\Delta\lambda$ 60 nm
1	Error	0	0	3.4	0
	SD	0	0	3.3	0
2	Error	0	0	3.9	0
	SD	0	0	3.5	0
3	Error	0	0	4.4	0
	SD	0	0	3.6	0
4	Error	0	0	5.2	0.6
	SD	0	0	6.4	6.7
5	Error	0	0	4.5	0
	SD	0	0	3.5	0
6	Error	0	0.5	4.1	0.6
	SD	0	4.2	5.8	6.3
7	Error	0	1.6	4.3	0
	SD	0	10.3	6.7	0
8	Error	0.3	1.8	4.4	1.7
	SD	3.3	9.5	6.2	10.8
9	Error	0	2.2	4.7	1.1
	SD	0	9.8	7.4	8.7
10	Error	1.4	4.8	4.5	0.6
	SD	9.1	15.0	7.1	6.7

Table 3
Classification of beers using six selected excitation/emission wavelengths pairs for linear discriminant analysis (LDA)

	Sample A		Sample B	
	$\Delta\lambda$ 10 nm	$\Delta\lambda$ 60 nm	$\Delta\lambda$ 10 nm	$\Delta\lambda$ 60 nm
Error	0	0	12.0	0
SD	0	0	75.2	0

All differently stored samples of beer A were correctly classified by *k* NN for $k = 1,\ldots,7,9$ using synchronous fluorescence spectra recorded for $\Delta\lambda = 10$ nm, and for $k = 1,\ldots,5$ using spectra measured at $\lambda = 60$ nm. The classification errors increase at higher *k* values and the misclassification rates are generally higher for the spectra measured for $\Delta\lambda = 60$ nm. LDA performed on selected excitation/emission pairs provided 100% classification for both sets of synchronous spectra. The *k* NN analysis of synchronous spectra of beer B for $\lambda = 10$ nm led to 3.4–5.2% classification error depending on the *k*-value, as a consequence of poor separation between fresh beers and beers stored in darkness at 4 °C. Much better classification was achieved in the analysis of $\Delta\lambda = 60$ nm spectra; 100% rates were obtained for $k = 1, 2, 3, 5, 6, 7$. With LDA, the fraction of correct classification was about 88% for $\Delta\lambda = 10$ nm spectra and 100% for $\Delta\lambda = 60$ nm spectra.

4. Conclusions

Fluorescence spectroscopy allows monitoring changes in the chemical composition of beers during storage. Total luminescence spectrosocopy (TLS) gives a comprehensive insight into alterations, since more detailed information is gained with respect to single excitation and emission spectra. Synchronous fluorescence spectroscopy presents a suitable alternative for TLS measurements in routine analysis. The method allows obtaining information on several fluorescent constituents in a single scan. Appropriate $\Delta\lambda$-values may be chosen from TLS analysis to improve spectral resolution for strongly overlapping components and to enhance the fluorescence intensity. This feature is unique for fluorescence in comparison to other spectroscopic techniques. Thus, fluorescence characteristics serve well to monitor either selected constituents or the overall mixture and the utility, e.g., in food analysis is enhanced by using multivariate statistical data analysis methods for spectral data treatment.

Although current methods are limited to fluorescent compounds, it would be interesting to identify correlations between fluorescent constituents and other components or properties of interest, for example, association of changes in flavin concentrations with development of lightstruck flavor in beers upon exposure to light.

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