Fluorescence Spectroscopy for Characterization and Differentiation of Beers

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ABSTRACT

J. Inst. Brew. 110(4), 267–275, 2004

Total luminescence and synchronous scanning fluorescence spectroscopic techniques were applied for characterization of the intrinsic fluorescence of eight different beers. Spectra were measured using different geometries to reveal the presence of similar fluorescent components. The total luminescence and synchronous fluorescence spectra exhibit a relatively intense short-wavelength emission ascribed to aromatic amino acids and less intense emission in the long-wavelength region, which may originate from B vitamins. Classification of beers based on their synchronous fluorescence spectra was performed using non-parametrical k nearest neighbours method and linear discriminant analysis. Very good discrimination was obtained in both methods with a low classification error. The results demonstrate the potential of fluorescence techniques to characterize and differentiate beers.

Key words: Beer, discriminant analysis, synchronous fluorescence spectroscopy, total luminescence spectroscopy.

INTRODUCTION

Beer is a very complex mixture of many constituents varying widely in nature and concentration levels[1–3]. Raw materials including water, yeast, malt, and hops contain a wide range of different chemical components that react and interact at all stages of the brewing process. The interest in studying the chemical composition of beer is prompted by its importance for the assessment of beer quality. It is important to develop fast analytical methods without sample pre-treatment. Multidimensional fluorescence techniques, such as total luminescence and synchronous scanning fluorescence are particularly useful for the

analysis of complex food matrices[4]. Total luminescence spectroscopy (TLS) involves simultaneous acquisition at multiple excitation and emission wavelengths. The resulting emission-excitation data matrix (EEM) provides a total intensity profile of the sample over the range of excitation and emission wavelengths scanned. Synchronous fluorescence spectroscopy takes advantage of the ability to vary both the excitation and the emission wavelengths during analysis, thereby maintaining a constant wavelength difference. Both techniques have been successfully used in the analysis of crude oils, pharmaceuticals, polycyclic aromatic hydrocarbons, motor oils, and humic matter in water[4–7]. Fluorescence spectral data and lifetimes in diluted beers have been explored recently[8]. We have reported on the application of total fluorescence spectroscopy to characterize beers[9]. The objective of the present study was to investigate the intrinsic fluorescence of beers for the purpose of discrimination by applying statistical methods to their synchronous fluorescence spectra.

EXPERIMENTAL

Materials

Eight different beers (seven lagers, beers A–G, and one ale, beer H) from different breweries were bought in a local supermarket. Samples were degassed in an ultrasonic bath and diluted in water. The pH values were between 3.5 and 4.5.

Phenylalanine, tyrosine, tryptophan and riboflavin were purchased from Aldrich.

Fluorescence measurements

Fluorescence spectra were obtained on a Fluorolog 3-11 Spex-Jobin Yvon spectrofluorometer. A xenon lamp was used as an excitation source. 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 the wavelength response of the apparatus. The right-angled geometry was used for diluted beer samples in a 10 mm fused-quartz cuvette. Front-face and back-face geometries were used for bulk beers and measurements were performed in a triangular fused-quartz cuvette.

Three-dimensional spectra were obtained by measuring the emission spectra in the range from 290 to 700 nm at excitation wavelengths from 250 to 500 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 monochromators in the 250–600 nm range with constant wavelength differences $\Delta\lambda$. Fluorescence intensities were plotted as a function of the excitation wavelength. Four spectra were recorded for each sample with $\Delta\lambda$ of 10, 30, 60, and 80 nm, respectively.

Statistical methods

For statistical analysis, twelve synchronous fluorescence spectra with different $\Delta\lambda$ (10, 30, 60, 80 nm) were recorded for each sample. Two methods of discriminant analysis were used for the purpose of multiple group classification: nearest neighbours method (kNN)[10] and linear discriminant analysis (LDA)[11].

The k-nearest neighbours method (kNN) is a well-known non-parametric classification method[10]. 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 used in the analysis. The k values were chosen between 1 and 10 due to the small size of the sample set. The kNN non-parametrical method was preferred, because 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-orthogonality of the data set. The kNN method allows performing analyses using entire spectra, without any prior reduction of the spectral data.

Additionally, linear discriminant analysis (LDA) was performed on reduced data sets. For this purpose, six wavelengths were extracted from the synchronous spectra recorded at a particular $\Delta\lambda$ and analysed. Discriminant coordinates were determined for the purpose of graphical presentation, with the two discriminant coordinates used in the plots.

The bootstrap method was applied to estimate the classification error. The dataset was randomly split into two independent sets (training and test). Version 0.632+ of this method was used (low bias and variance) and 50 bootstrap replications were performed. Larger numbers of bootstrap replications gave no further improvement of the classification error estimates[12].

All statistical procedures were implemented in Matlab 6.5.

## RESULTS AND DISCUSSION

Total luminescence spectra

Undiluted beer exhibits high UV-Vis absorption, thus fluorescence measured using the right-angled geometry is severely distorted due to primary and secondary inner-filter effects. Front-face geometry should avoid filter effects, although other phenomena including energy transfer and collisional quenching are not eliminated. Appropriate dilution of samples is, therefore, required. On the other hand, dilution reduces fluorescence intensities arising from such components, which are either present in low concentrations or have low fluorescence quantum yields. Bearing in mind the advantages and disadvantages of the different measurement techniques, we tested three different geometries: front-face and back-face for bulk beers and right-angled for diluted beers.

Fig. 1 shows the total luminescence spectra of a beer for different instrumental arrangements. Contour maps of beer luminescence were constructed such that one axis
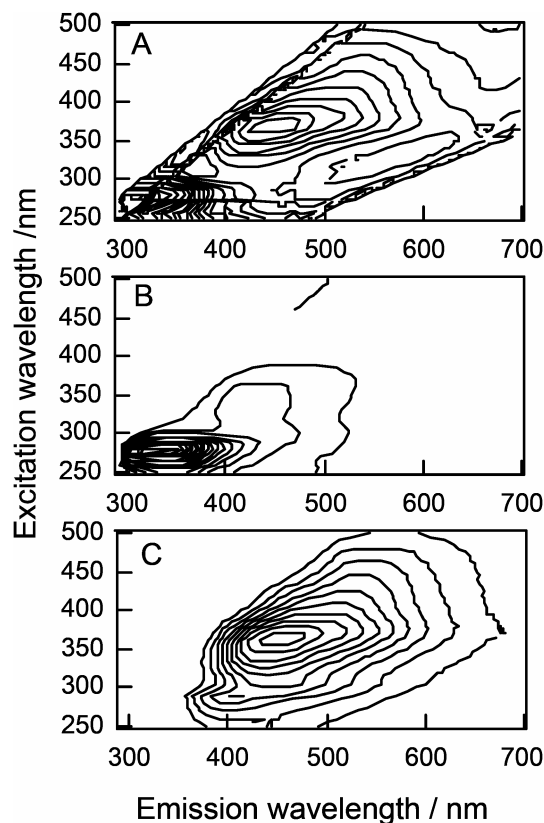
Fig. 1. Contour maps of the fluorescence of a lager beer (Beer G): (A) Front-face geometry, bulk beer; (B) Right-angled geometry, diluted beer, 3.2% in water (v/v); (C) Back-face geometry, bulk beer.
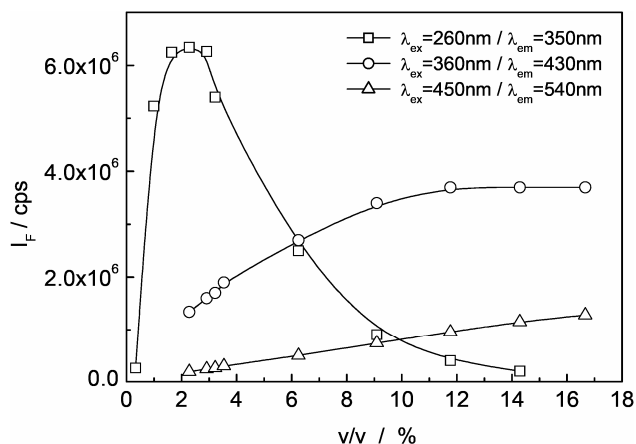
Fig. 2. Fluorescence intensity as a function of lager beer (Beer G) concentration; right-angled geometry.
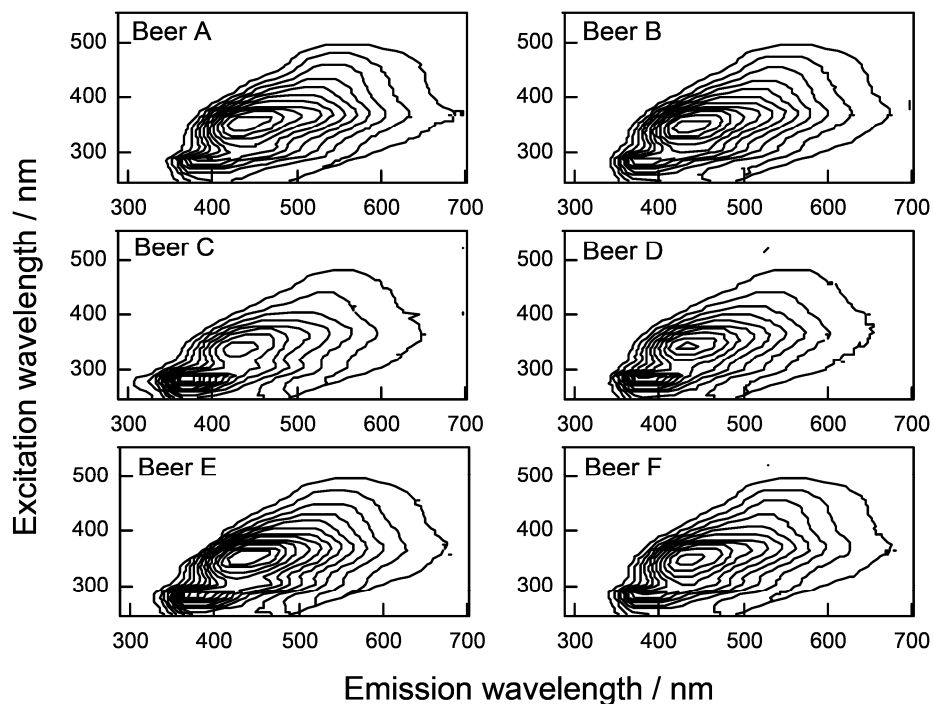
Fig. 3. Contour maps of fluorescence of various beers.

represents the emission and the other axis the excitation wavelengths, while the contours are plotted by linking points of equal fluorescence intensity.

In the spectra of undiluted beer, Fig. 1A, measured using the front-face geometry, a relatively intense band with excitation at about 250 nm and emission at 350 nm is observed. Additionally, a distinct emission band with excitation at 350 nm and emission at 420 nm is present, and a less intense emission band is observed with excitation at 450 nm and emission at 520 nm. Fig. 1B shows the contour map for a diluted beer sample (3.2% v/v in water). The short-wavelength fluorescence with excitation at 250 nm and emission at 350 nm is clearly observed, while only a very weak emission band exists with excitation at 350 nm and emission at 420 nm. This spectrum exhibits almost no fluorescence above 400 nm in excitation and 500 nm in emission. To observe this long-wavelength emission, we recorded the total luminescence of bulk beers using the back-face geometry. In this scheme, the optical path length of the exciting radiation is considerably reduced, resulting in a reduction of the primary inner-filter effect. The emitted light is still absorbed, as the optical path length for the emission is ca. 0.5 cm. However, the absorption at longer emission wavelengths is considerably lower than that at shorter excitation wavelengths. Fig. 1C shows that the short-wavelength emission is completely removed, while the longer-wavelength emission retains a reasonable intensity.

Fig. 2 shows the fluorescence intensity at three excitation/emission wavelength pairs: 260/350 nm, 360/430 nm, and 450/540 nm, as a function of the beer concentration, obtained in the right-angled arrangement. The short-wavelength emission has a maximum intensity at 1.6–3.2% v/v concentrations, rapidly decreasing in more concentrated solutions. In contrast, the emission intensity of

the two longer wavelength excitation/emission pairs increases with concentration (almost linearly for the longest wavelength pair). Thus, due to the large differences in fluorescence intensities of various fluorescent species, the most complete fluorescent characteristics of the beers were obtained by using the front-face geometry. However, our system utilizes a configuration, in which front-face illumination is performed using a triangular cuvette with its front-face at 45° to the incident beam. The geometry causes a large amount of light to be reflected directly into the emission monochromator, thereby distorting the emission spectra. Further measurements were conducted in diluted solutions to observe the intense short-wavelength fluorescence. Back-face geometry was used for observation of the long-wavelength fluorescence.

The emission contour maps for some beers studied are shown in Fig. 3. Although all beers exhibit very similar fluorescence characteristics, differences in band positions, shapes, and relative intensities are easily noticeable.

The intrinsic fluorescence characteristics of beers are expected to be quite complex due to overlapping of emissions from numerous species. Consequently, complete assignment of the spectral features is difficult and beyond the scope of this work. However, some tentative qualitative assignments could be made, based on comparison of the observed characteristic features with the well-known fluorescent properties of particular beer constituents.

The relatively intense band, observed in each beer studied in diluted solutions, with the excitation at ca. 250–300 nm and the emission at ca. 300–400 nm has been ascribed to amino acids. Only the aromatic amino acids are fluorescent: tryptophan (excitation/emission at 280/350 nm), tyrosine (275/300 nm), and phenylalanine (260/280 nm). Both tyrosine and tryptophan are fluorescent at 280–295 nm; however energy transfer from tryptophan to tyrosine

is quite common. Using excitation wavelengths above 295 nm, only tryptophan emits fluorescence. On the other hand, the amino acids can be excited below 280 nm[13]. In water, the quantum yield of phenylalanine fluorescence ($\Phi_{Fl} = 0.024$) is relatively low compared with that of tryptophan ($\Phi_{Fl} = 0.13$) and tyrosine ($\Phi_{Fl} = 0.14$)[14]. The fluorescence of amino acids is greatly affected by protonation and pH. A typical content of fluorescent amino acids in beer: tryptophan 3.1 mg/100 g, tyrosine 14.9 mg/100 g, phenylalanine 5.9 mg/100 g[3], although amounts may vary widely depending on the choice of raw materials, the brewing conditions, and the beer type.

The broad emission observed in bulk beer samples, with decreased intensity upon dilution, originates presumably from several fluorescent components. In particular, compounds of the vitamin B group may contribute to this emission. Beer is a rich source of water-soluble B-vitamins and it typically contains: B_1 (0.003–0.006 mg/100 g), B_2 (0.02–0.04 mg/100 g), B_3 (0.65–1.1 mg/100 g), and B_6 (0.03–0.08 mg/100 g)[2,3].

Vitamin B_3 (niacin) includes nicotinic acid, nicotinamide and their coenzyme forms: nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Reduced forms NAD(P)H fluoresce at around 470 nm with excitation maxima at 260 and 340 nm[15].

Vitamin B_6 consists of three closely related pyridine derivatives: pyridoxine, pyridoxal and pyridoxamine and their corresponding phosphates. Vitamins B_6 have the following excitation/emission maxima: pyridoxine – (332,340)/400 nm, pyridoxal – 330/385 nm, and pyridoxamine – 335/400 nm, pyridoxic acid – 315/425 nm, pyridoxal 5′-phosphate – 330/400 nm. Vitamin B_{12} (cobalamin) emits at 275/305 nm[13].

The emission band at ca. 450/500–600 nm could be ascribed to the vitamin B_2 emission[15]. The principal forms of vitamin B_2 found in nature are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Vitamin B_2 contents in beer were reported as: riboflavin – 169–508 μg/l; FAD – 19–65 μg/l; FMN – 8.1 μg/l[16].

The spectral characteristics of the various beers studied are generally similar to those reported previously by Apperson et al.[8], who measured single-emission spectra. By comparing the fluorescence spectra of beer with those of tea and hops, they inferred that proteins, complex polyphenols, and iso-alpha-acids can contribute to the intrinsic fluorescence of beer, although contribution of polyphenols is minimal since their removal by polyvinylpolypyrrolidone (PVPP) had not greatly affected the observed fluorescence. The emission at 295/315 nm has been attributed to tyrosine and not β-catechin or epicatechin – the proanthocyanidin monomers. The fluorescence maxima for both catechin and epicatechin occur at 315 nm, when excited at 265 and 280 nm, respectively, hence, they may overlap tyrosine and tryptophan fluorescence[8].
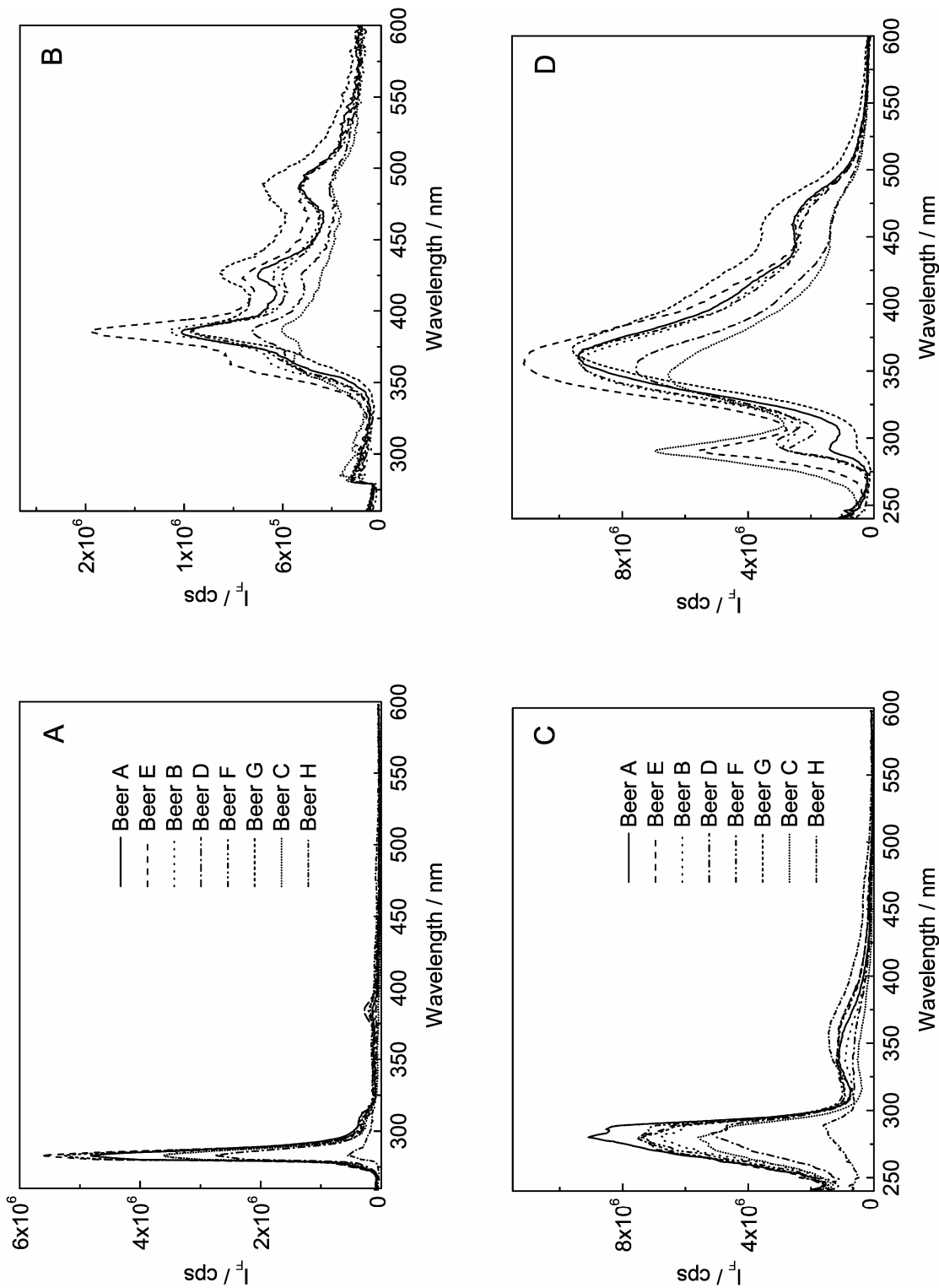
Synchronous fluorescence spectra

Synchronous scanning fluorescence spectroscopy is a very useful technique for the analysis of mixtures of fluorescent compounds. Both excitation and emission characteristics are included in the spectrum by simultaneously scanning excitation and emission wavelengths thereby
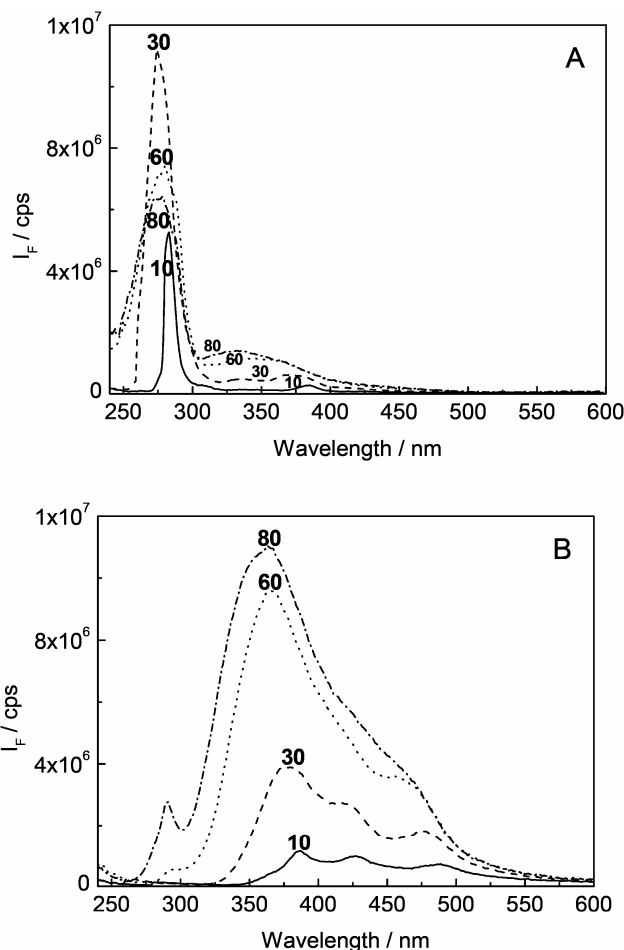
Fig. 4. Synchronous fluorescence spectra of a lager beer (Beer G) recorded at $\Delta\lambda$ = 10 nm, 30 nm, 60 nm, and 80 nm: (A) Diluted beer, 3.2% in water (v/v); (B) Bulk beer.

holding a constant difference between them. As a result, the selectivity for individual components is considerably improved and much additional information on mixtures of fluorescent compounds is gained. The synchronous scanning fluorescence method is a very simple and effective means of obtaining data for several compounds present in mixture in a single scan. Although it provides less information than the TLS, in many cases, it may present a viable alternative due to its inherent simplicity and rapidity.

The synchronous fluorescence spectra of a lager beer (undiluted and diluted in water, 3.2% v/v) are shown in Fig. 4. As is evident from Fig. 4, the shape and intensity of synchronous spectra depend on the difference between the excitation and emission wavelengths $\Delta\lambda$, which defines overlapping of absorption and emission bands. An effective bandwidth reduction is observed at the smallest $\Delta\lambda$ = 10 nm, when compared to the emission band. Synchronous fluorescence spectra of a diluted beer in the right-angled geometry obtained at a small $\Delta\lambda$ ($\Delta\lambda$ = 10 nm) show a sharp, intense band with a maximum at 283 nm and a very weak band with a maximum at 384 nm. The short-wavelength emission band is broadened at $\Delta\lambda$ = 30 nm and its maximum is shifted to 275 nm, accompanied by an increased intensity of both this band and of the

Fig. 5. Comparison of synchronous fluorescence spectra of beers measured, at (A) $\Delta\lambda = 10$ nm, diluted samples, 3.2% in water (v/v); (B) $\Delta\lambda = 10$ nm, diluted samples, 3.2% in water (v/v); (C) $\Delta\lambda = 60$ nm, diluted samples, 3.2% in water (v/v); (D) $\Delta\lambda = 60$ nm, bulk samples; (D) $\Delta\lambda = 60$ nm, bulk samples.

broad band between 300 nm and 450 nm. Further increasing the Δλ values to 60 nm and 80 nm led to a decrease of fluorescence intensity at 275 nm; simultaneously, the long-wavelength broad band grows in intensity and its maximum shifts to the blue.

Fig. 4B shows the synchronous spectra of undiluted beer samples recorded using the back-face geometry. As was shown for the total luminescence spectra, the short-wavelength emission is not observed when using this geometry, due to the high absorption of both the excitation and the emission light. Three overlapping bands with maxima at 386, 428, and 489 nm are observed at Δλ = 10 nm. An increased Δλ of 30 nm results in increased fluorescence intensity of all the bands and in changes of their relative intensities, while their maxima were slightly shifted to the blue, to 380, 422, and 480 nm, respectively. A further increase of intensity and broadening of bands are observed at Δλ = 60 nm and 80 nm, accompanied by disappearance of the 422 nm band and appearance of a 295 nm band.

Similar spectral profiles were obtained for the other beers, see Fig. 5. In the same way as in the previous discussion of the total luminescence spectra, the intense short-wavelength emission band for diluted beer samples was attributed to amino acid fluorescence. The long-wavelength emission in the synchronous spectra of bulk beers should originate in the vitamin B group. The existence of more than one band suggests that several substances emit in this region. To test this assignment, synchronous fluorescence spectra of standard compounds were measured in aqueous solutions, at pH = 4.

Fig. 6. shows spectra of three aromatic amino acids and vitamin B_2 recorded at Δλ = 10 nm. Phenylalanine, tyrosine and tryptophan exhibit single, narrow bands in the short-wavelength region with respective maxima at 263, 283 and 296 nm. Riboflavin emission occurs in the long-wavelength region with a maximum at 489 nm. The very good matching of fluorescence of tyrosine and riboflavin with the respective emission bands of beers provides support for the identification of these two fluorophores. Further studies are needed to confirm assignments of the other fluorescent bands as well as to establish a quantitative relationship between the chemical composition of a beer and its fluorescence characteristics.
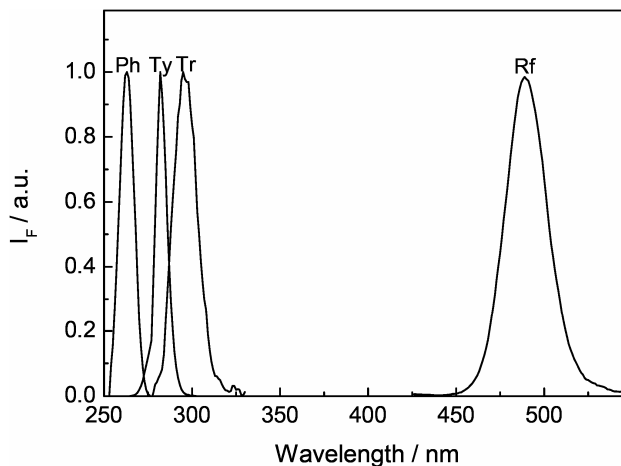
Fig. 6. Synchronous fluorescence spectra, recorded at Δλ = 10 nm, of phenylalanine (Ph) tyrosine (Ty) tryptophan (Tr) and riboflavin (Rf) in water, at pH = 4; fluorescence intensity normalized to 1.

Despite their general similarity, the profiles of synchronous fluorescence spectra of individual beers vary significantly, producing unique spectral patterns. Apart from qualitative distinctions, the samples also differ in fluorescence intensities of particular components. These differences may reflect variation in the contents of fluorescent compounds in a particular beer; however, due to the complexity of the system, quantitative predictions require further investigation.

The ale beer measured in diluted aqueous solutions exhibited considerably lower fluorescence intensities than lager beers, which is evident from the higher absorbance of the ale beer. No fluorescence signal could be observed in the bulk ale beer.

A synchronous spectrum contains more information than conventional single-excitation or single-emission spectra, as it depends on both excitation and emission profiles of the respective fluorescent components. The synchronous fluorescence spectrum is, in fact, a spectral signature of the particular sample and may be used, for example, in qualitative analysis, for beer identification,

Table I. Classification of beers using entire synchronous fluorescence spectra in the k nearest neighbours method (diluted beers).

| | Δλ | | | |
| | 10 nm | | 60 nm | |
k	Error	Standard deviation	Error	Standard deviation
1	0	0	0.0101	0.0147
2	0	0	0.0106	0.0152
3	0.0004	0.0049	0.0077	0.0122
4	0.0012	0.0103	0.0135	0.0334
5	0.0012	0.0103	0.0103	0.0144
6	0.0124	0.0617	0.0154	0.0459
7	0.0115	0.0557	0.0142	0.0464
8	0.0107	0.0524	0.0279	0.0741
9	0.0089	0.047	0.0157	0.0352
10	0.0235	0.0696	0.0352	0.0844

Table II. Classification of beers using entire synchronous fluorescence spectra in the k nearest neighbours method (bulk beers).

| | Δλ | | | |
| | 10 nm | | 60 nm | |
k	Error	Standard deviation	Error	Standard deviation
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0.0037	0.0411	0.0037	0.0416
6	0	0	0.0033	0.0364
7	0.007	0.0542	0.0036	0.0404
8	0.0098	0.00629	0.0142	0.0749
9	0.01	0.0628	0.018	0.0831
10	0.021	0.080	0.0267	0.0989
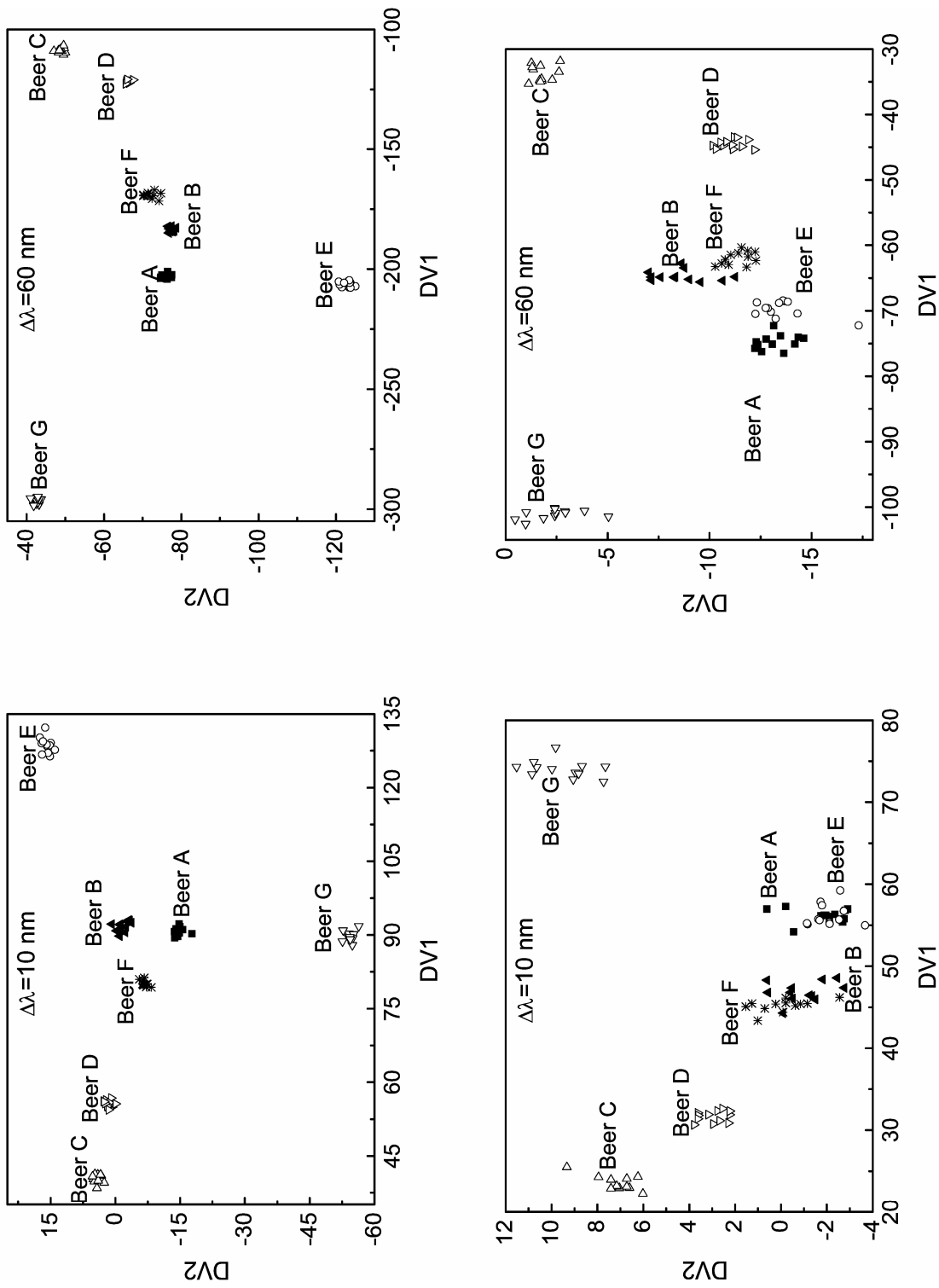
Fig. 7. Classification of beers by linear discriminant analysis. Top panels: bulk beers at $\Delta\lambda = 10$ nm and at $\Delta\lambda = 60$ nm; bottom panels: diluted beers at $\Delta\lambda = 10$ nm and at $\Delta\lambda = 60$ nm.

quality monitoring or for authentication purposes. Visual comparison of spectra could be insufficient for such applications; hence, multivariate statistical methods should be applied.

Classification of beers using synchronous fluorescence spectra

The possibility of discriminating different beers on the basis of their synchronous fluorescence spectra was investigated by using two statistical methods: the k nearest neighbours method and the linear discriminant analysis. The k nearest neighbours method was applied using the entire spectra as input. In the linear discriminant analysis, selected excitation/emission wavelength pairs were used for classification purposes.

Tables I and II present the results of the k nearest neighbours method applied to diluted and bulk samples, respectively. Discrimination between different beers using this method was very good, with zero or very low classification error and low standard deviation values. The best discrimination was achieved using synchronous fluorescence spectra of bulk beers measured at $\Delta\lambda = 10$ and 60 nm and diluted beers measured at $\Delta\lambda = 10$ nm. Discrimination using spectra of diluted beers at $\Delta\lambda = 60$ nm was still very good.

Further, linear discriminant analysis was applied to a set of selected wavelength pairs from the synchronous spectra. Such analysis, although simplified and limited to only six excitation/emission wavelength pairs, produced a satisfactory discrimination between different beers (low error and standard deviation values). These results show that it is not even necessary to record the entire synchronous spectra in order to discriminate between beers. Instead, the fluorescence intensity could be measured at selected excitation/emission wavelengths and then subjected to linear discriminant analysis.

The results of the LDA analysis are visualized on the maps plotted by using the two discriminant coordinates, DV1 and DV2 (Fig. 7).

The bulk beers could be correctly classified by using only two principal discriminant coordinates, both at $\Delta\lambda = 10$ and 60 nm values, with very good separation between all beers. For diluted beers at $\Delta\lambda = 10$ and 60 nm, some of the beers were located too close to each other.

The results indicate that, although both techniques could be applied for beer discrimination, the spectra of bulk samples appear to be more reliable than those of diluted beers. This may result from the more pronounced variations in vitamin-B contents (long-wavelength bands) than in the amino acid contents. Despite these effects, we confirmed that both the short-wavelength UV-emission and the long-wavelength visible emission can be applied to discriminate various beers.

CONCLUSIONS

Total luminescence and synchronous fluorescence spectroscopic techniques were used for direct beer analyses. Fluorescence spectroscopy provides information on the overall beer characteristics thereby enabling identification of some beer constituents. Although unambiguous attribu-

tion of fluorescence bands to beer components requires further studies, the present results show that distinct spectral ranges, such as those corresponding to amino acids and to compounds of the vitamin-B group, may be identified in the spectra and used as markers for differentiation of beers. Synchronous scanning fluorescence spectroscopy was successfully used to characterize and discriminate various beer samples. We demonstrated that it is possible to classify various beers using single synchronous fluorescence scans or even selected excitation/emission wavelength pairs.

It would be interesting to correlate the spectral characteristics of beers to known beer classifications and specific beer properties. Such investigation is currently in progress.

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