Rapid Determination of Bitterness in Beer Using Fluorescence Spectroscopy and Chemometrics

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ABSTRACT

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Two fluorescence spectroscopic methods with the aim to develop a fast quantitative determination of bitterness in beer were tested. The first method was based on autofluorescence of the diluted and degassed beer samples without any further processing. A total of 21 dark and light beer samples were analyzed and multivariate Partial Least Squares (PLS) regression models to bitterness in form of international bitter units (IBU) were performed. A prediction error in the form of Root Mean Square Error of Cross-Validation (RMSECV) of 2.77 IBU was obtained using six PLS components. Focusing only on the light beer samples the RMSECV was reduced to 1.81 IBU. The second method developed was based on addition of europium to induce delayed fluorescence signals in the beer samples. PLS models yielded an RMSECV of 2.65 IBU for all beers, while a model on the light beer samples gave an RMSECV of 1.75 IBU. The obtained prediction errors were compared to the errors given in the literature for the traditional extraction method of determining IBU.

Key words: Bitterness, chemometrics, europium, fluorescence spectroscopy.

INTRODUCTION

Bitterness is an essential quality parameter in modern breweries, and analysis of bitterness in beer and wort is conducted as a routine analysis throughout the brewing industry. The bitterness in beer is largely determined by alpha acids, which are resinous constituents of the hops. In the wort boil they undergo an isomerisation reaction to produce iso-alpha acids, which are dominating for the bitter taste in beer. Analysis of bitterness in beer is thus based on a quantification of these bitter acids, which has been a subject to a variety of methods, as reviewed by Verzele and De Keukeleire in 1991[26]. The traditional and international recommended analysis of bitterness in beer[1,16] in terms of international bitter units (IBU), is carried out by a spectrophotometric measurement at 275 nm of an acidic solvent extract of beer. The technique is costly, time-consuming, and involves the use of undesirable organic solvents; also a high uncertainty is introduced in the manual extraction step. The absorbance at 275 nm is the sum of all species extracted from beer into iso-octane that absorb UV light, and minor contributions from species not contributing to the bitterness, such as polyphenols can appear[21]. The IBU method can thus be considered as a rather crude technique, which also lacks the ability to discriminate between different iso-alpha acid species. Although all iso-alpha acids possess the same chromophore, their UV spectra are not exactly the same. Furthermore different stereo-isomers have been shown to possess different absorptivities and absorption maxima wavelengths[26].

Despite the limitations, the IBU method is widely used as an indicator of the bitterness in quality control. Several efforts towards an automation and reducing the time of analysis has been performed throughout the last three decades[11,23] and by applying flow injection techniques to the analysis[22]. The analysis precision reported from the modified methods analyses has been acceptable and in accordance with the IBU method[2,15]. High performance liquid chromatography (HPLC) techniques can also be used to measure the amount of iso-alpha acids in beer[9]. HPLC methods can provide more detailed chemical information on the composition of bitter acids compared with the IBU method[26]. However, the time of analysis plus the technical experience and instrumentation required to operate the HPLC, does not at first makes the technique well-suited for routine production use. Stir Bar Sorptive Extraction applied to HPLC has been suggested to improve the technique with respect to handling and costs[14].

Autofluorescence spectroscopy

Fluorescence spectroscopy is an analytical method with high sensitivity and specificity. 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 fluorophors in the form of aromatic amino acids, vitamins, cofactors and a variety of flavouring compounds makes the technique 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 in 1982 by Jensen et al.[17]. Autofluorescence of food systems can

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make up a complex chemical fingerprint of the sample, comprising both fluorescence and quenching phenomena. The use of chemometrics in the form of multivariate analysis has proven beneficial to this kind of data with respect to noise reduction, handling of interferents and outlier control[8]. Fluorescence spectroscopy applied directly on food samples evaluated with chemometrics, has among others been suggested for analysis of sugar[7,19] and the oxidative stability of various dairy products[5,10,27].

The intrinsic fluorescence or autofluorescence of undiluted as well as diluted beer was investigated in 2002 by Apperson et al.[4]. Inner-filter effects were shown to appear in the fluorescence signal from the undiluted beer samples, expressed by the fact that protein fluorescence was only obtained upon dilution with distilled water. Similar observations were found in the present study, which is why fluorescence measurements were performed on diluted beer samples, in order to obtain the most ideal fluorescence signal with respect to dependency of the concentration of the intrinsic fluorophores. The complex fluorescence characteristics of diluted beer obtained by Apperson and coworkers was suggested to arise from complex polyphenols, protein and iso-alpha-acids. The idea of using the fluorescence signal from the iso-alpha acids to determine the bitterness, was first reported in a 1995 patent[24], where multivariate analysis was used to separate the relevant bitterness fluorescence from the background signal. In the patent no dilutions were suggested; in this way, a rapid bitterness determination was possible, with no need for sample preparation. However, in Apperson et al.[4], no multivariate analysis was performed to calibrate the signals to the reference values and in the patent[24] no results are given regarding the performance of the method.

Europium-delayed fluorescence

An alternative to measure the intrinsic fluorescence of beer is delayed fluorescence induced by adding a lanthanide to the beer sample. This approach implies an extra, but simple and easily automated sample preparation step. However, the technique holds several advantages to the intrinsic fluorescence methods with respect to handling of interferents and separating the relevant fluorescent signal from the background fluorescence signal. Thus, the delay in time and the fluorescence emission characteristics of the lanthanide-induced fluorescence separates it from the autofluorescence signal.

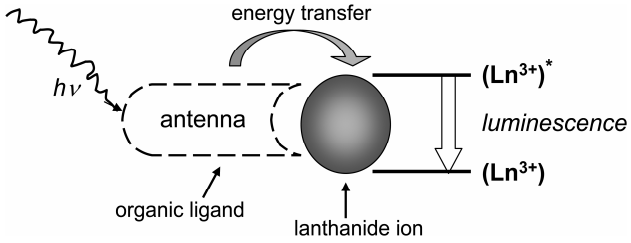

Fig. 1. The basic principles in lanthanide complex formation that exhibit efficient fluorescence. The europium (lanthanide) ion forms a complex with organic ligands, which in this case are the iso-α-acids. The ligands absorb light at a specific wavelength. The energy is then transferred to the europium ion, which emits characteristic delayed fluorescence.

The delayed fluorescence method is dependent of the selective chelation of the lanthanide, europium to the β-carbonyl structure in the iso-α-acids and the unique fluorescent properties of the resultant europium complex. As illustrated in Fig. 1, radiation is absorbed at a wavelength characteristic of the iso-α-acids, which acts as "antenna" in the complex. Energy is then transferred from the excited state of the iso-α-acids to the europium ion. The europium ion emits its characteristic delayed fluorescence with emission of Eu (III) around 613 nm. Europium will not fluoresce unless it is bound to a ligand. The complex has fluorescent decay times in the order of milliseconds compared to the nanoseconds decay time of the background fluorescence. When the time-resolved or time-gated fluorescence is measured the background fluorescence has already decayed while the europium complex is still emitting.

This technique was first introduced for bitterness determination in beer by Tomlison et al.[25]. However, a satisfactory correlation between the intensity of the delayed fluorescence (only one wavelength) and the content of iso-α-acids was not found. This study was based on a univariate approach and a calibration standard curve performed on a set of model solutions. By measuring whole emission spectra, evaluating them with multivariate data analysis and performing the calibration on real beer samples, we explore the method further and hope to improve its performance. Harms et al.[13] and Nitzsche and Harms[18] also used europium in a HPLC post column reagent in order to get a better separation of the iso-α-acids, still univariate, though.

The present study

The purpose of the present study was to explore the possibilities for a new rapid determination of bitterness in beer, which could make up an alternative to the widespread method based on extraction and a spectrophotometric determination although still using this method as a reference method. The presented analytical techniques were applied in order to investigate two different approaches:

1. Autofluorescence with multivariate evaluation, as suggested but not documented in a patent by Takhar et al.[24]
2. Europium delayed fluorescence with multivariate evaluation.

MATERIALS AND METHODS

Beer samples and bitterness determination

A sample set of 21 lager beer samples provided from Carlsberg Breweries was investigated. The sample set included 16 light beers and five dark beers. Colour and bitterness in terms of international Bitter Units (IBU) were determined in duplicates according to Analytica EBC[3]. All analyses were performed the same day; spectral measurements were performed the following day.

Autofluorescence

Three hundred µl degassed beer and 2.7 mL water were mixed in a 5 mL quartz vial. Fluorescence landscapes (excitation-emission matrices) were measured on a Varian

Cary Eclipse Fluorescence Spectrofluorometer with excitation from 230–400 nm, and a step size of 10 nm. Emission spectra were recorded for every nm from 240 to 600 nm. Excitation and emission slits were set to 5 nm.

Europium-induced delayed fluorescence

A 0.050 M aqueous solution of europium (III) chloride hexahydrate (Aldrich Chem. Co.) was prepared. Three hundred µl europium solution and 2.7 mL degassed beer were mixed in a 5 mL quartz vial. The europium concentration and mixture ratio used were in agreement with the optimal findings of Tomlinson et al.[25]. Front face fluorescence emission spectra were measured on a Varian Cary Eclipse Fluorescence Spectrofluorometer using the accessory solid sample holder equipped with a cuvette holder and an incident light angle of 60 degrees. The measurement was started exactly 30 s after mixing of the sample and the europium solution. Delayed fluorescence measurements were performed with excitation 275 nm. The delay time was 0.10 ms with a gate time of 2.0 ms and total decay time of 0.1 s. Emission spectra were recorded for every nm from 575 to 715 nm. Excitation and emission slits were set to 5 nm.

Data analysis

Partial Least Squares (PLS) regression models[29] were applied for the comparison of autofluorescence spectra and europium-induced delayed fluorescence spectra with traditional determination of IBU, respectively. PLS is a predictive two-block regression method based on estimated latent variables and it is applied to the simultaneous analysis of the two data sets **X** (spectra, independent variables) and **y** (reference analysis, dependent variable).

Interval PLS (iPLS)[20] was applied in order to compare different spectral sub-regions in the autofluorescence measurements. In this study the spectral sub-regions according to each excitation wavelength were chosen for the iPLS calculations. The prediction performance of each of the local models and the global (full spectrum) model was compared.

Full cross-validation was applied for all regression models. Cross-validation[28] is a strategy for validating calibration models based on systematically leaving out groups of samples in the modeling and testing the left out samples in a model based on the remaining samples. In this case each of the samples was left out one by one (full cross-validation), meaning that 21 sub-models were calculated based on 20 samples plus one global model based on all 21 samples. For each of the models, the bitterness of the sample left out was predicted, and the prediction was compared with the reference value and used as a term for the validated performance of the regression model. The regression model was evaluated using the correlation coefficient (r), and the validation parameter, Root Mean Square Error of Cross-Validation (RMSECV) as a term to indicate the error of the model. The RMSECV is defined as in equation (1):

$$\mathrm{RMSECV} = \sqrt{\frac{\sum_{i=1}^{N} (y_i^{\mathrm{pred}} - y_i^{\mathrm{ref}})^2}{N}} \qquad (1)$$

where y_i^{pred} is the predicted value for sample i in the cross-validation, y_i^{ref} is the corresponding reference value and N is the number of samples.

Data analysis were performed in MATLAB 6.5 (MathWorks, Inc.) with the iToolboox 1.0 (www.models.kvl.dk), and Unscrambler 9.1 (CAMO, Norway).

RESULTS AND DISCUSSION

Traditional analyses

In Table I the results of the reference measurements on the 21 beer samples are given. The IBU values are in the range 3.1–31.5 representing large variation for lager beers, but within normal occurring values. The beer colours can be divided into two major groups; 16 light beers with values around 5 colour units and 5 darker beers with colour values above 20. The darker beers can be used to investigate the effect of different colours on the fluorescence signal and its correlation to bitterness in beer.

Autofluorescence

The autofluorescence spectra of three selected samples are given as contour plots in Fig. 2. The three samples span the bitterness level with sample A having a value of 8.8 IBU and sample B having a value of 28.5 IBU as well as the color with sample A and B being light beers while sample C is a dark beer. In all three samples two distinct peaks are seen with excitation/emission maxima around 290/350 nm and 340/430 nm, respectively. The first peak probably arises from protein fluorescence, mainly attributed to tryptophan, while the second peak can be related to fluorescence from complex polyphenols or iso-α-acids, as suggested by Apperson et al.[4] Sample B has a significant higher IBU value than sample A, and thus was expected to contain a higher amount of iso-α-acids. However the intensity of the peak around 340/430 nm is not very different for the two samples, indicating that the bitter acids are not the main contributors to the fluorescence signal obtained in this area. Thus, polyphenols might be a

Table I. IBU and color values for 21 beer samples.

Beer ID	IBU	Colour, EBC
1	16.3	5.7
2	20.9	5.8
3	25.6	5.8
4	31.5	5.7
5	21.4	5.5
6	17.8	5.0
7	22.1	4.9
8	18.1	4.5
9	23.2	4.6
10	28.5	4.4
11	3.1	4.8
12	6.0	5.5
13	8.8	5.1
14	11.9	5.1
15	8.0	4.9
16	9.2	5.3
17	3.2	23.1
18	6.7	23.5
19	9.8	23.9
20	13.3	23.9
21	16.1	20.3

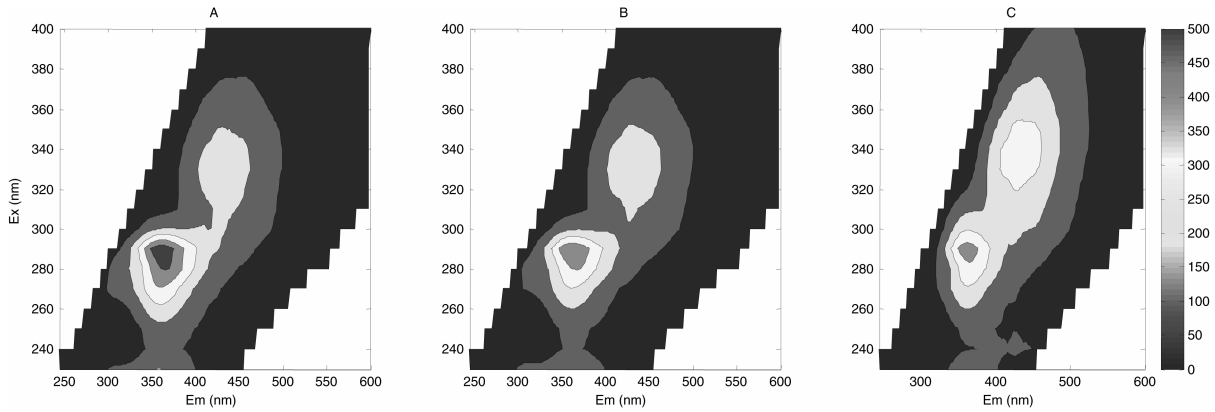

Fig. 2. Contour plots of the intrinsic fluorescence of the three diluted beer samples, A, B and C. The bitterness level of the three beers was found to be 8.8, 28.5 and 16.1, respectively. Samples A and B represent light beer samples, and sample C is a darker beer sample.

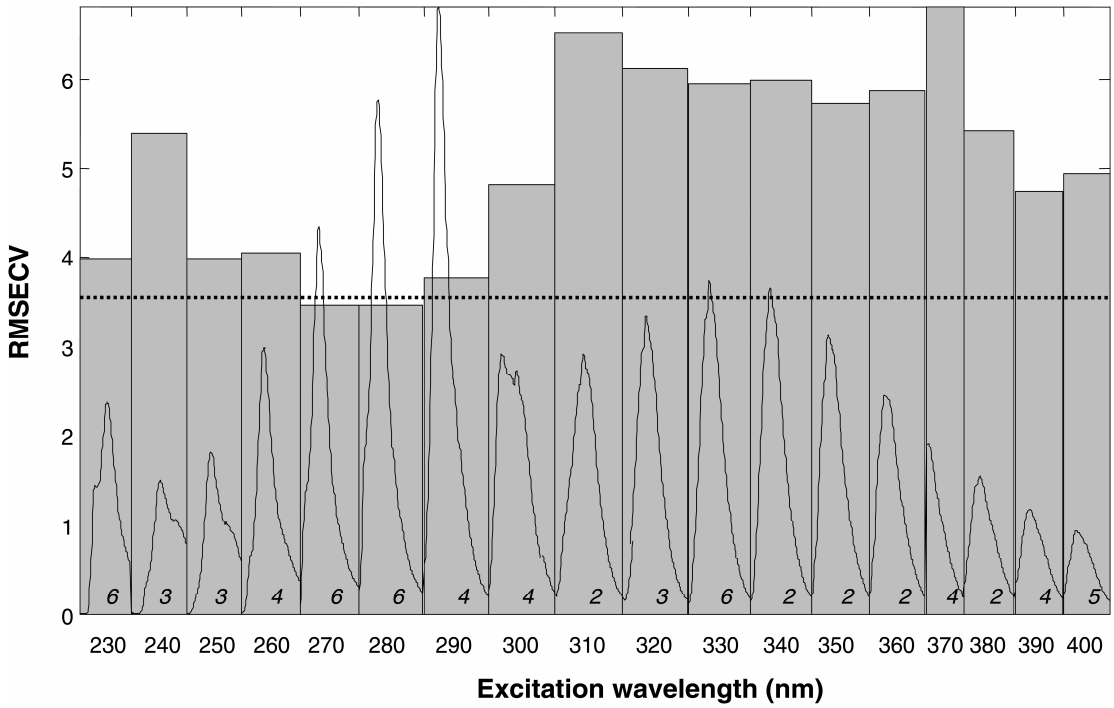

Fig. 3. Output result plot from interval-PLS analysis. Cross-validated prediction performance (RMSCEV) of PLS regression models on IBU values is plotted from a full-spectrum ("global") model with 5 PLS components (dotted line) and from 20 interval models (bars) with the optimal number of components for the given interval. The mean spectrum is shown.

better suggestion. The dark sample, C yields a little lower but broader shaped fluorescence signal. The lower intensity could suggest that inner-filter effects took place, but the differences observed are to be investigated through regression models to the assessed bitterness levels in the beer samples.

A full spectrum PLS regression model between the concatenated autofluorescence spectra and the IBU reference values yielded an RMSECV of 3.56 IBU (Table II). In this model the independent variables had the dimension 21 × 2111 variables since the data were unfolded. The optimal number of PLS components to apply as determined by full cross-validation was five and the correlation coefficient between the predicted values and the reference values is 0.90.

Table II. Results from PLS regression models between autofluorescence measurements and bitterness determined according to the IBU method. Fluorescence emission spectra from all excitation wavelengths (230–400 nm) or selected excitation wavelengths were used, as noted in brackets.

	RMSECV (IBU)	# PLSC	r
All beer samples (n = 21):			
Unfold PLS	3.56	5	0.90
Unfold PLS [Ex. 270 nm]	3.46	5	0.90
Unfold PLS [Ex. 260, 270, 290 nm]	2.77	6	0.94
Only light colored beers (n = 16):			
Unfold PLS	3.42	3	0.91
Unfold PLS [Ex. 230 nm]	2.71	5	0.94
Unfold PLS [Ex. 230 nm] without samples 1 & 4	1.81	5	0.97
Unfold PLS [Ex. 260, 270, 290 nm]	2.65	5	0.95

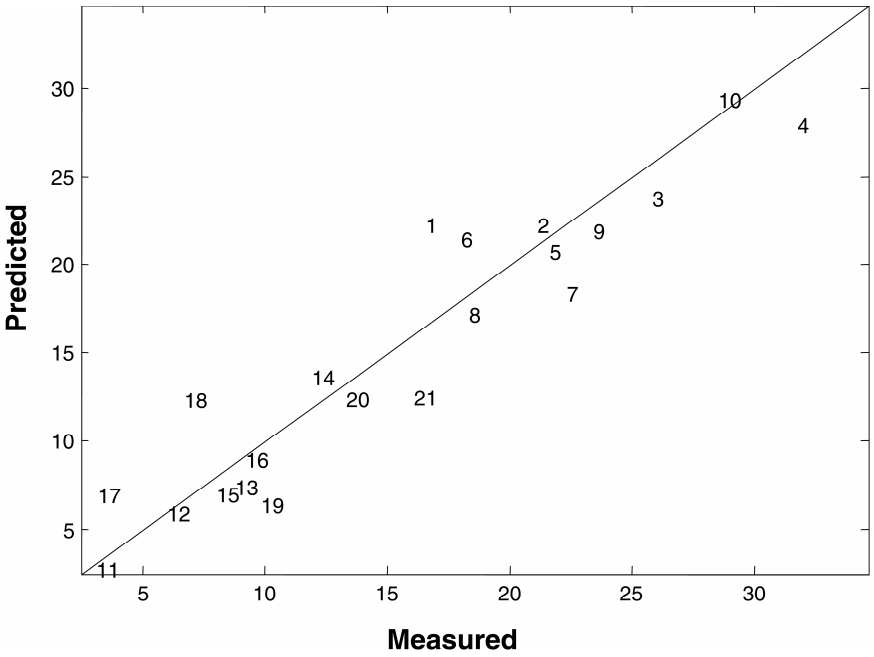

Fig. 4. Predicted versus measured IBU values of all 21 beer samples, based on a PLS regression model with 6 PLS components of autofluorescence measurement. Intervals 4, 5 and 7 of the fluorescence measurements from the iPLS analysis were included in the regression model. A multivariate regression coefficient, r of 0.94 and RMSECV of 2.77 IBU was obtained for the presented model.

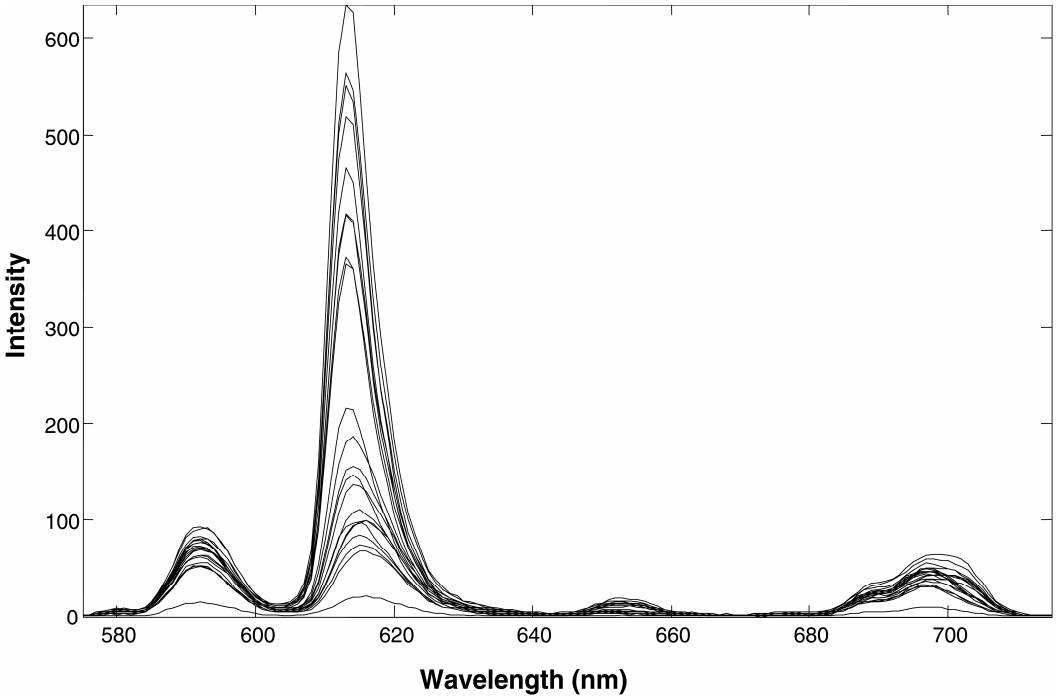

Fig. 5. Europium-induced delayed fluorescence emission spectra of all 21 beer samples. Excitation wavelength 275 nm.

To investigate the performance of each excitation wavelength, i.e. from 230 nm to 400 nm with 10 nm steps, an iPLS was calculated. The result is given in Fig. 3 where the bars indicate the RMSECV obtained by a full cross-validation PLS model for each excitation wavelength, i.e. in total 18 local models have been developed. Due to the limited number of samples it was decided not to use more than six PLS components in all models even though the cross-validated results indicated a higher number of components for some models. The iPLS showed that intervals corresponding to excitation 270 nm and 280 nm performed slightly better than the full spectrum model using

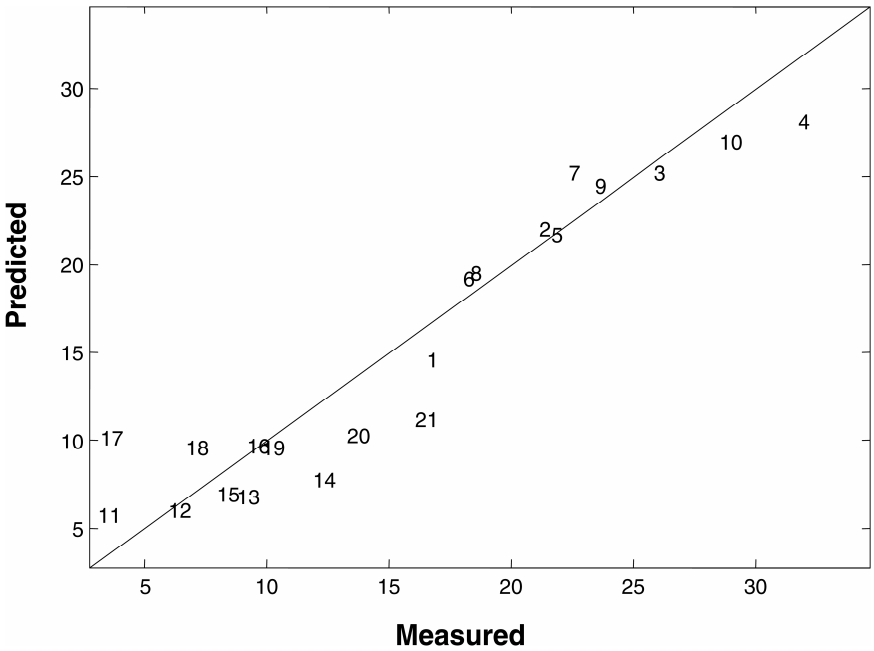

Fig. 6. Predicted versus measured IBU values of all 21 beer samples, based on a PLS regression model with 2 PLS components of europium-induced delayed fluorescence measurements. A multivariate regression coefficient, r of 0.94 and RMSECV of 2.69 IBU was obtained for the presented model.

one more PLS component. A model on the emission spectra from excitation at 270 nm gave an RMSECV of 3.46 IBU and a correlations coefficient of 0.90. As judged by an F-test[12] this is a non-significant difference from the RMSECV of the full spectrum model ($p = 0.45$). All possible combinations of two and three intervals were also calculated and the best performing combinations of intervals were excitations 260 nm, 270 nm and 280 nm with an RMSECV of 2.77 IBU, lower than the full spectrum RMSECV ($p = 0.13$) and all individual intervals. The Predicted versus Measured plot for this PLS model based on six PLS components is given in Fig. 4. Inspecting this model revealed that the autofluorescence spectra of the dark samples deviated from the corresponding light beer spectra; this was also observed in the raw data as given in Fig. 2. Models without dark samples gave the results compiled in Table II. The full spectrum model is in the level regarding the RMSECV while the interval model now shows that excitation 230 nm (interval 1) is the one with optimum performance. Furthermore, attention was drawn to samples 1 and 4 and excluding these gave an RMSECV of 1.81 IBU ($p = 0.01$ compared to RMSECV = 3.56). Samples 1 and 4 were only excluded based on their poor fit to the regression model for the remaining model; no obvious causality was found and therefore the result should be addressed rather cautiously. It was not possible to further improve the model by combinations of intervals; the optimal combination gave an RMSECV of 2.65 IBU as seen in Table II.

Europium-induced delayed fluorescence

The europium-induced delayed fluorescence spectra of all samples are given in Fig. 5. The spectra holds the typical spectral characteristic of europium with four peaks corresponding to different electron transitions and with the main peak around 615 nm, where large variations in intensity between the samples can be observed.

A full spectrum PLS model on all 21 samples gave an RMSECV of 2.69 using two PLS components. The predicted versus measured plot for this model is shown in Fig. 6. The distribution of the samples does not appear linear; it seems that the samples are almost divided into two levels with either low or high IBU value, split between 15 and 20 IBU. The lower number of components in the PLS model based on delayed fluorescence can be explained by the fact that the signal is induced by the addition of europium and the model only needed one component to compensate for interferences as opposed to the autofluorescence case where up to six PLS components were necessary to perform this compensation.

A PLS model on only light beer samples further reduced the RMSECV to 2.05 IBU while excluding samples 1 and 4 gave an RMSECV of 1.75 IBU for the remaining fourteen samples, as listed in Table III.

In the preliminary experiments replicates were measured and it turned out that it was difficult to obtain reproducible measurements for the replicates for the induced

Table III. Results from PLS regression models between europium-induced delayed fluorescence measurements and bitterness determined according to the IBU method.

	RMSECV (IBU)	# PLSC	r
All beer samples (n = 21):			
PLS	2.69	2	0.94
Only light colored beers (n = 16):			
PLS	2.05	2	0.97
PLS without samples 1 & 4	1.75	3	0.97

experiment. After addition of the europium, a precipitate was formed, leading to a rather unstable chemical system. It was decided to measure exactly after 30 s in order to standardize the procedure and minimize the replicate deviations. However, the problem with precipitation needs to be addressed, before further implementation.

Discussion on uncertainties of bitterness determinations

The reproducibility of the traditional method for bitterness determination has been evaluated several times, estimating the terms for repeatability (r95) and reproducibility (R95) as defined in ISO Standard 5725, stating that the probability that two analyses deviate up to the value of r95/R95 is approximately 95%, within one laboratory (r95) or within all tested labs (R95), respectively. The mean precision values of the IBU method in a major UK brewing company showed a repeatability of 1.0 IBU and a reproducibility of 4.1 IBU for 33 samples in the range 18–32 IBU, on the basis of 16 laboratories[25]. In 2000, the EBC Analytical Committee found the precision of the bitterness analysis somewhat lower in a ring test of six beers in the range 13–36 IBU, analysed in 13 laboratories, that resulted in mean values of 0.8 IBU for repeatability, and 3.5 for reproducibility, with both r95 and R95 proportional to the measured IBU values[6]. The optimal models for predicting the bitterness from fluorescence in the present study yielded prediction errors below 2 IBU. These findings may not seem precise enough for implementation in breweries, but compared with the reproducibility of the reference method, they appear to be in the same order of magnitude. However, the reported repeatability of the IBU method, i.e. the precision within one laboratory seems to be superior to the findings in the present study.

CONCLUSIONS

A fast reliable method for bitterness determination is needed, and it is documented that both autofluorescence spectra and europium induced delayed fluorescence spectra by the aid of multivariate modeling holds the potential to be used to predict the bitterness in beers with an error comparable to that of the reference method. Both these methods are faster than the traditional method because the extraction step is avoided. Future work should delve into the uncertainty of the reference method as well as testing the developed methods on a larger number of samples in order to reveal if local models (for example on light beers) can be developed. Also methods for handling the precipitation observed in the europium induced delayed fluorescence method should be investigated.

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