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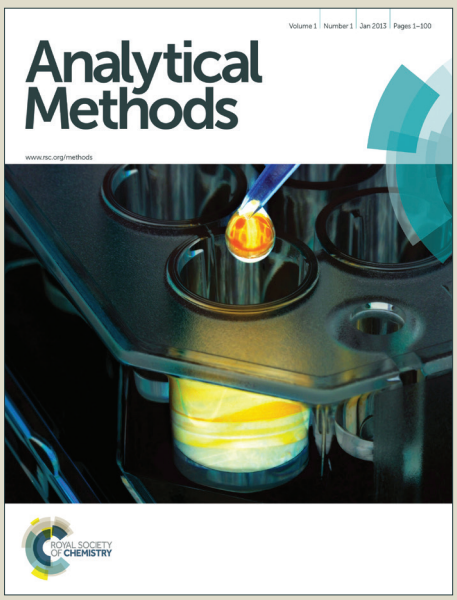

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Liquid chromatographic fingerprints and profiles of polyphenolic compounds applied to the chemometric characterization and classification of beers

Clara Pérez-Ràfols and Javier Saurina

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In this paper, liquid chromatography with UV-vis detection was used to generate compositional fingerprints of beers to be exploited for the characterization and classification purposes. Chromatographic profiles recorded at 280 nm contained features mainly associated to polyphenolic components such as phenolic acids and flavonoids. Beers from different styles and elaborated in various countries were analyzed by the proposed method and data generated was treated chemometrically to assess characterization and classification models. Three different types of data sets based on chromatograms, peak areas and concentrations were explored by principal component analysis (PCA) to evaluate their performances to discriminate among ale and lager beers. The use of raw chromatographic profiles required a comprehensive pretreatment to improve the data quality. When dealing with peak areas, single and complex integrated peaks of known and/or unknown compounds were used as the source of analytical information. In this two approaches (chromatographic fingerprints and peak areas), calibration was not necessary so the sample analysis was simplified. In the case of concentrations, selected phenolic acids and flavonoids were considered as the data to discriminate among beer types. Differences in the polyphenolic composition were relevant and some components resulted in efficient markers of beer classes. Further studies based on partial least squares discriminant analysis (PLS-DA), soft independent modelling of class analogy (SIMCA) and other methods were used to discriminate beers according to brewing styles. Classifications were highly satisfactory in terms of selectivity and sensitivity as, in general, beers of test set were correctly assigned to their actual classes.

Introduction

Instrumental fingerprints and compositional profiles of compounds naturally occurring in food products have recently been exploited for characterization, classification and authentication purposes.[1-4] Hence, apart from organoleptic, nutritional and medical implications of some small molecules such as amino acids, biogenic amines, polyphenols, volatile organic compounds (VOC) and sugars, their potentiality in exploratory and predictive tasks cannot be underestimated. The present study is mainly concerned in the generation of fingerprints and compositional profiles of beers by liquid chromatography with UV-vis detection as the source of analytical information. The concept of fingerprint is coined to define a complex instrumental signal that may contain mixed contributions from several known or unknown components while the term (compositional) profiling refers to concentrations of components of interest.

The vast majority of beers produced all over the world can be classified according to the brewing process into top (ale) and

Department of Analytical Chemistry. Faculty of Chemistry. University of Barcelona. Martí i Franquès, 1 - 11, 08028-Barcelona.

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bottom (lager) fermentation. Nowadays, lager beers represent the 90% approx. of worldwide production and are very popular in countries such as USA, France, Czech Republic and Spain. In contrast, traditional ales, with less trade impact, are highly appreciated and consumed in Britain, Germany and Belgium.[5] The composition of beer strongly depends on the raw materials used as well as the brewing practices followed. If so, it is not surprising that beer fingerprints and compositional profiles have great impact in description and discrimination issues. For instance, ale beers are produced at warmer temperature than ale ones (typically between 16°C and 24°C) which allows yeasts to generate higher amounts of VOCs such as esters, thus providing characteristic flavor notes.[6] In parallel, the extraction of components from the raw materials is also favored by temperature so, in general, richer extracts in terms of variety and quantity of soluble molecules are commonly found in ale beers.

Some interesting studies dealing with the characterization and classification of beers, published recently in the scientific literature, will be briefly commented as follows. Often, the huge amount of data generated with modern analytical instruments makes necessary the use of chemometrics to facilitate the recovery of the underlying information.[7] Among other methods, principal component analysis (PCA) is commonly applied to preliminarily sample exploration. For more specific classification and authentication studies, predictive methods such partial least squares regression – discriminant analysis (PLS-DA) can be utilized.

The potential role of some polyphenols as chemotaxonomical descriptors of food products and beverages has been pointed out by several authors.[8-11] It has been found, for instance, that phloretin and phlorizin are typical components of apple, arbutin is quite specific of pear, naringenin is commonly present in citric fruits, and ellagic acid derivatives are really abundant in pomegranate.[12-15] As a result, contents of polyphenols in foodstuffs have been envisaged as a source of information to try to discriminate among product varieties, origins, manufacturing processes, etc. This idea has been exploited by Vrhovsek *et al*.[16] to classify fruits using cluster analysis on the basis of contents of about 90 polyphenolic compounds. In a similar context, the recognition and authentication of protected designations of origin (PDO) of olive oils, such as in the case of Moroccan[17] or Italian[18] oils, has been studied extensively. A lot of work has been carried out in the field of wines. For instance, red Spanish wines from three PDOs, analyzed by HPLC-DAD-F and HPLC-ESI-QqQ-MS, have been classified using PLS-DA.[19] Conclusions on characteristic components of each PDO have been extracted. In another study, Italian Lambrusco wines belonging to three varieties have been discriminated chemometrically by using HPLC-UV data as a source of information.[20] Boselli and coworkers have studied the influence of specific polyphenols on color attributes of wines.[21] Colored components such as malvidin, petunidin and peonidin (di)glucosides, quantified by HPLC-MS/MS, have been found to be characteristic descriptors of given Italian origins. Regarding beers, in a previous publication, we worked with the contents of various phenolic acids and flavonoids to assess a preliminary classification of samples into lager and ale styles.[23] Quifer-Rada *et al.* have developed a LC-MS method for the comprehensive elucidation of polyphenolic compounds of beers.[22] Mass measurements of high accuracy and MS^2 experiments have allowed several phenolic acids and flavonoids to be identified, some of them recognised for the first time in beer. In another study, Marova *et al.* have used concentrations of 11 representative polyphenols, quantified by LC-UV-MS, to distinguish among Czech and foreign lager beers. Results have suggested that some flavonoids could have a potential use in beer authentication.[24] Mattarucchi and coworkers have reported a method for authentication of Rochefort Trappist beers from other styles.[25] In another example, additional chemical parameters including chloride, phosphate, sulfate, total amino acids, pH and overall polyphenols were exploited to characterize blond beers.[26]

Beyond compositional data, complex spectral fingerprints can be generated with instrumental techniques such as nuclear magnetic resonance (NMR),[27-29] infrared (IR),[29-32] UV-vis spectroscopies[33] and mass spectrometry (MS).[34-35] Apart from spectra, another interesting proposals for beer characterization relied on voltammetric electronic tongues.[36] Some authors proposed the combination or fusion of responses from several instruments as a way to enrich the data sets for enhancing the descriptive performance. For instance, Biancolillo *et al*. joined thermogravimetric profiles, and mid- and near- infrared and UV-vis spectra in augmented data arrangements to try to discriminate among two high quality Italian beers from other products of lower quality.[37] Focusing on chromatography, typical data consists of absorbance values recorded over time at one (or several) wavelength. Specific data pretreatments may be required to correct some drawbacks such as baseline drifts and peak shifting.[38-39] Regarding chromatography and MS hyphenation, methods of LC-MS[40-41] and GCxGG-MS[42] have been reported for beer analysis.

In our study, beers of different types and manufactured in several countries were analyzed chromatographically. For each sample, data of different nature was obtained, including beer fingerprints consisting of absorbance values recorded at 280 nm over the entire chromatogram, areas of selected major and minor peaks, and concentrations of relevant polyphenolic compounds. Preliminary screening of beers by PCA displayed interesting patterns dealing with brewing styles, especially when using concentration data. Further beer classifications by PLS-DA, SIMCA and other methods were investigated for all types of data sets constructed. The most accurate predictions were obtained by PLS-DA when working with concentrations although results from corrected chromatographic fingerprints were also highly satisfactory.

Experimental

Chemicals and standards

Unless specified, analytical grade reagents were used. The mobile phase was prepared with Milli-Q water (Millipore, Milford, MA), formic acid (99% w/w, from Merck, Darmstadt, Germany) and methanol (MeOH, from Panreac, Barcelona, HPLC grade). Phenolic acids and flavonoids, including caffeic, coumaric, 2,5-dihydroxybenzoic, ferulic, gallic, 4-hydroxibenzoic, protocatechuic, salicylic and vanillic acids, (+)-catechin, (-)-epicatechin, quercetin and rutin, to be used as standards were purchased from Sigma-Aldrich (St. Louis, MO).

Samples

Beers of various styles and produced in several countries were purchased from several supermarkets in Barcelona. The set of samples considered in this study was composed of 42 lager and 21 ale beers. Beers were filtered and diluted (1:1, v:v) prior to injection into the chromatograph. Each sample was analyzed in triplicate and results of each independent replicate were used to evaluate the repeatability of the method as well as the success of the correction data pretreatment procedures. It is important to mention that the

set of beers was not analyzed in a same working session but in groups of 6 - 10 samples for a period of 2 months, approx.

Liquid chromatographic method

The chromatograph consisted of Agilent 1100 Series HPLC instrument equipped with a G1311A quaternary pump, a G1379A degasser, a G1392A autosampler, a G1315B diode-array detector and a PC with the Agilent Chemstation software (Rev. A 10.02), all of them from Agilent Technologies (Waldbronn, Germany). The separation column was a Kinetex C18 (100 mm × 4.6 mm i.d., particle size 2.6 µm) furnished with a SecurityGuard C18 cartridge (both from Phenomenex, Torrance, CA).The separation was based on the following gradient using 0.1% (v/v) formic acid aqueous solution and MeOH as the components of the mobile phase: 0 to 11.5 min, 5% → 26% MeOH; 11.5 to 19 min, 26% → 60% MeOH; 19 to 20 min, 60 → 90% MeOH. After cleaning the column at 90% MeOH for 3 min the solvent percentage retuned to the initial value. The flow rate was 1 mL min^{-1} and the injection volume 10 µL. Chromatograms were recorded at 280 nm.

Data analysis

Solo from Eigenvector Research was used for calculations with Principal Component Analysis (PCA), Partial Least Squares - Discriminant Analysis (PLS-DA), Soft Independent Modelling of Class Analogy (SIMCA) and other chemometric methods.[43] A detailed description of theoretical background of these methods is given elsewhere.[44]

For exploratory studies by PCA, three different data matrices were constructed using chromatographic profiles, peak areas and polyphenol concentrations from a set of 63 samples analyzed by triplicate. Various data pretreatment procedures were investigated in order to ascertain which conditions led to the best description and classification performance. Among other, peak synchronization, baseline correction by asymmetric least squares (AsLS), normalization through the chromatographic domain, Pareto scaling, and autoscaling were assayed.

Beer classification was attempted by PLS-DA, SIMCA and other modelling approaches. Samples available were distributed among training and test sets. In particular, two thirds of samples, approx., were devoted to the training stage while the remaining ones were used for test assays (23 lager and 13 ale for calibration, and 19 lager and 7 ale for predictions). X-data matrices of chromatographic profiles, peak areas and concentrations variables were used. As in the case of PCA, the influence of several data preprocessing approaches on the classification rates was evaluated. The assignation of samples to lager and ale classes was defined in the Y-matrices as follows: 0 was used for lager and 1 for ale.

Results and discussion

An HPLC-UV method established and validated elsewhere was here applied to the characterization and classification of beers.[22] Concentrations of some relevant polyphenols in beers were quantified to obtain the corresponding data set. The method offered an excellent repeatability in terms of peak areas and the retention time of compounds of interest, with RSD% values ranging from 0.2 to 0.7%. Regarding reproducibility, however, it was detected that the variability in the chromatograms obtained in different working sessions, evaluated from the injection of a given beer sample used as a control, was more remarkable. In particular, as shown in Fig. 1, slight variations in retention time, of ± 5 to 15 s, were observed. Peak shifting seemed to increase with time as it was more evident in the last part of the chromatogram. Additionally, the peak variability in partially overlapping peaks provoked changes in the resolution as well as in the shape of overall profiles. As commented below, this phenomenon may hinder the interpretation of further results so it should be minimized in order to obtain more reliable descriptions. Fortunately, peak areas and concentrations were less sensitive in front of the influence of the working session, especially for well-resolved components that presented RSD values below 2%. For poorly resolved systems comprising two or several overlapping compounds, peaks could hardly be integrated separately and the precision of individual peak areas was poor. Anyway, the information provided by such multi-peak systems should not be underestimated as underlying data may be relevant for descriptive tasks. Then, in order to take advantage of variance from such overlapping peak systems, they were integrated as a whole and the resulting overall areas were used as a source of highly reproducible data to be incorporated to enrich the data sets.

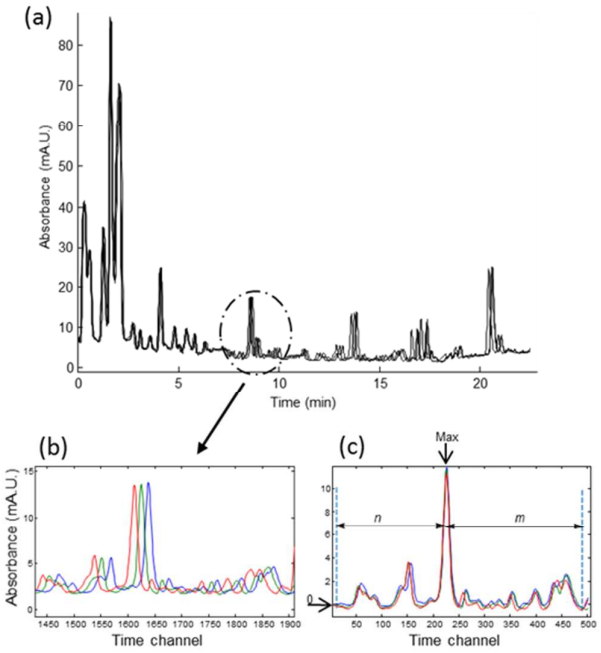

Fig. 1. Scheme of correction of chromatographic data. (a) Chromatograms of three sample replicates of a given beer; (b) Focus on the peak shifting; (c) Corrected data on the selected window taking the peak maximum as a reference.

Exploratory studies by PCA

PCA provided plots of scores and loadings, showing the distribution of the samples and variables on the principal components (PCs), respectively. The study of scores revealed patterns of sample characteristics, such as brewing style or origin, clusters of similar beers, etc. The plot of loadings displayed the distribution of variables to gain information dealing with their correlations as well as relationships of polyphenols with beer properties.

Chromatographic data

First beer characterization was attempted using raw chromatographic profiles (i.e., absorbance over time) as the analytical data. Chromatograms were processed in different ways to

find out the best pretreatment conditions, including AsLS de-trending, normalization, Pareto scaling and autoscaling. The aforementioned variations on the overall shape of chromatographic profiles depending on the working session affected the performance of the PCA model. Results in Fig. 2a, corresponding to autoscaled data, indicated that the distribution of beers was poorly structured as a function of brewing style. More precisely, some replicates of beers appeared dispersed on the map of samples and similar beers belonging to a same class were widely spread on the plot of scores. It was then deduced that samples were mainly distributed according to the working session so all beers analyzed in a same day tended to form a cluster regardless the brewing style. Similar inefficient models were obtained with the other preprocessing procedures. The study concluded that raw chromatographic data was quite inefficient for beer characterization.

Further work was focused on stablishing corrective mechanisms to reduce the chromatographic variability while enhancing the overall quality of data. First, time ranges 0 - 1.5 min and 20 - 25 min were removed from the data set as they corresponded to the death volume front and cleaning step. Subsequently, chromatograms were synchronized using various representative peaks distributed throughout the chromatogram were chosen as references. It should be mention that the use of an only peak for repositioning the whole chromatogram was not sufficient for an accurate correction since, although peaks were well aligned in the close vicinity of the reference point, some shifting still remained in the furthest regions. Asymmetric time windows were defined by taking an adjustable number of time channels (n and m) to the left and right of the peak maximum selected (see scheme in Fig. 1). If baseline drifts occurred, AsLS could be applied for signal de-trending. After preprocessing, sections were assembled to obtain the reconstructed chromatogram. The resulting data set was referred to as "Corrected chromatogram". In comparison with raw data, the overall descriptive performance of this data matrix was improved and the plot of scores from PCA denoted clear patterns depending on the brewing style. As depicted in Fig. 2b (model with autoscaled data), it was deduced that PC1 was related with the overall beer body (possibly related with the dry extract percentage as well as the alcoholic content). Non-alcoholic and light beers were mainly located to the left while stronger ones were to the right part. PC2 allowed a reasonable discrimination among brewing styles as samples belonging to each class were mostly distributed around specific areas (e.g., in general, lager beers had higher scores than ales). Of course the separation between the two classes was not strict and some samples were confounded. On the other hand, lager beers formed a more compact group of samples while ales exhibited higher diversity in agreement with the wider variety of ale subclasses.

Peak area data

Areas of 27 peaks were integrated to generate the so-called "Peak area" data set. Data was pretreated in different ways (see Data analysis section) to equalize the influence on the model of minor and major peaks. The map of scores using autoscaling as the pretreatment (Fig. 2c) indicated that replicates appeared in close positions, thus suggesting that data variability was acceptable. As in the previous case, PC1 described the behavior regarding the beer body and PC2 showed that lager and ale beers appeared predominantly in top and bottom areas, respectively. To conclude, data from peak areas was found to be of potential interest as a source of information for tackling characterization issues.

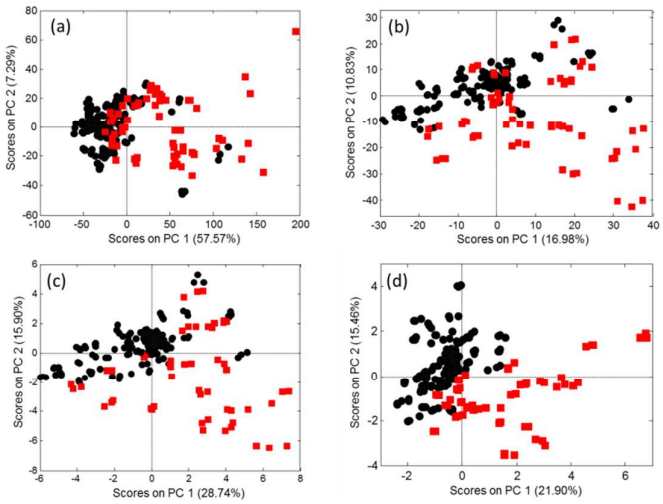

Fig. 2. Scatter plot of scores of PC1 versus PC2 corresponding to raw chromatograms (a), corrected chromatograms (b), peak areas (c) and concentrations (d). Symbols: Circle = lager; square = ale.

Concentration data

The use of concentrations of selected polyphenols for the characterization and classification of beers was also explored. It should be noted that obtaining such a type of data is time-consuming as prior quantification step is required. In our case, polyphenols were determined by external calibration using the HPLC-UV method. Results obtained indicated that gallic acid (~ 30 mg L^{-1}) was the most abundant and other compounds such as catechin, epicatechin and ferulic acid occurred at concentrations around 2 mg L^{-1}. The rest of polyphenols were present, in general, at levels below 1 mg L^{-1}. PCA model working with autoscaled data showed that PC1 described the overall content of polyphenols, with concentrations increasing from left to right (see Fig. 2d). PC2 provided a first rough separation of among beer types. Lager beers were mainly located to the top left part of the plot of scores while ale beers took up to the bottom right area. In accordance with the previous results, ale samples were not located compactly but were spread in broad area thus confirming the higher variability in composition and attributes. It was concluded that polyphenolic contents depended on the brewing method. In general, ale beers were 15% richer in overall polyphenols and the diversity of sub-types was also in accordance with up- or down expressed contents of given polyphenols. In particular, a subgroup of ale beer was characterized by higher amounts of epicatechin and gentistic acid (samples to the bottom), and another subgroup was represented by

rutin and syringic acid as descriptors (samples to the right). Regarding lager beers, although they typically contained lower polyphenol concentrations, ferullic and coumaric acids were 2-fold more abundant in this class.

Table 1. Results of the classification of beers by PLS-DA for the different types of data sets. Data corresponds to the number of replicates wrongly assigned in both training a test steps.

			Training		Test	
Data set	Pretreatment	Latent Variables	False +	False -	False +	False -
Raw chromatograms	Normalization	7	1	4	10	5
	AsLS detrending	2	0	0	7	0
	Pareto Scaling	8	1	3	9	6
	Autoscaling	5	0	0	5	4
Corrected chromatograms	Normalization	5	1	0	9	9
	AsLS detrending	2	0	0	6	1
	Pareto Scaling	2	0	0	7	9
	Autoscaling	2	0	0	3	3
Peak areas	Normalization	6	0	3	7	6
	Pareto Scaling	3	0	0	6	4
	Autoscaling	2	0	0	3	3
Concentrations	Normalization	3	2	3	3	12
	Pareto Scaling	2	0	0	0	0
	Autoscaling	2	0	0	0	0

Classification of beers

The preliminary inspection of the different types of data by PCA suggested they could be exploited to carry out the classification of beers according to the brewing style. For such a purpose, chemometric methods were used to assign a set of commercial beers into lager and ale classes. In all the cases, the training set consisted of 13 ale and 23 lager beers and the test set of unknown samples was composed of 7 ale and 19 lager beers. Table 1 summarizes the results obtained including the number of latent variables (LV) to be used and the number of wrong assignations to each class in both training and prediction steps. The number of LV, pre-established by cross-validation, was in agreement with that deduced from the application of a classification rate criterion relying on the number of misclassified samples.

As shown in the table, these results corresponded to the 4 types of data sets (i.e., raw and corrected chromatograms, peak areas and concentrations) under the application of several pre-processing conditions. In general, it can be seen that the use of some scaling procedures as well as baseline correction by AsLS slightly improved the predictive performance of PLS-DA models. In contrast, the application of normalization on the chromatographic domain negatively affected the quality of results. With the exception of normalization, all samples of the training set were correctly assigned to their actual classes.

When dealing with the test set, however, various wrong assignations occurred. In general autoscaling was found to be the most appropriate pretreatment. Regarding the data type, the best option corresponded to the use of concentrations from which classification models were exempt of mistakes when applying Pareto scaling or autoscaling.

Fig. 3 shows complementary plots illustrating the classification performance for autoscaled data based on the analysis of the areas under the ROC (receiver operating characteristic) curves. In all the cases, ROC areas were close to 1 which indicated that the classification was highly satisfactory (ROC areas were 0.90 for raw chromatograms, 0.92 for corrected chromatograms, 0.88 for areas and 1.00 for concentrations, see Fig. 3). Scores plots of the corresponding classification results are depicted in Fig. 4 with the sample distribution as a function of LV1 and LV2, with circles and squares corresponding to lager and ale classes, respectively. In accordance with the ROC results, most of the samples were located in their correct positions so that the classification was satisfactory in terms of selectivity and sensitivity. In particular, the two classes were clearly separated across the line plotted in the case of concentration data, which was the most efficient model.

As an alternative to PLS-DA classifications, SIMCA was applied to the study of the beer data sets. The probability of each sample of belonging to lager and ale classes was accounted on the basis of Q and T2 statistics. For class assignation, the significance level was set to 0.05. The best data pre-processing options previously established were here applied as recommended pretreatment prior SIMCA analyses. Results summarized in Table 2 indicated that, in general, ale beers were more efficiently classified than lager. As above, the most limited strategy corresponded to raw chromatographic data, with only 4.5 and 14.3% of correct assignations of lager and ale in the test set. In the case of corrected chromatograms the performance of the SIMCA models was more satisfactory, thus corroborating that the chromatographic treatment certainly

improved the quality of data. In this case, 50.0 and 71.4% of lager and ale samples of the test set were classified correctly. Again, the best predictions were obtained when concentrations were used as the analytical data, with 68.2 and 71.4% of success in the classification of lager and ale beers, respectively.

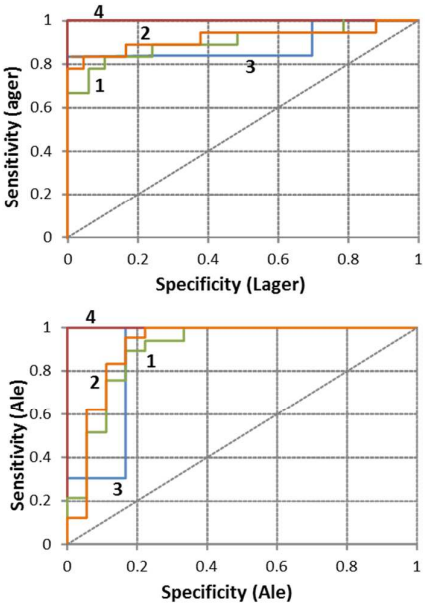

Fig. 3. Plots of receiving operating characteristic (ROC) curves of lager and ale classes. Assignments: 1, raw chromatograms; 2, corrected chromatograms; 3, peak areas; 4, concentrations. In all the cases, autoscaling was used as the data pretreatment.

From the comparison of results obtained with PLS-DA and SIMCA, it was found that predictions with PLS-DA were more accurate. PLS-DA allowed better percentages of correct assignations while the number of misclassifications was more limited.

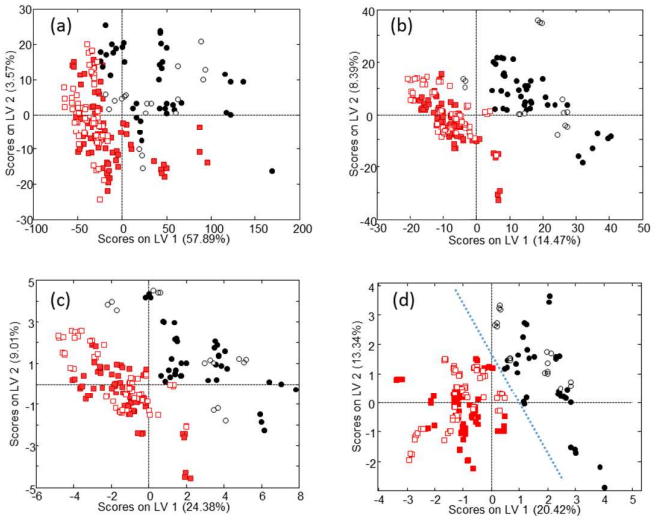

Fig. 4. Results of distribution of beers into lager and ale classes by PLS-DA. (a) Raw chromatograms; (b) Corrected chromatograms; (c) Peak areas; (d) Concentrations. Symbols: Circle = lager; square = ale; solid symbol = calibration sample; empty symbol = test sample.

Table 2. Results of the classification of beers by SIMCA for the different types of data sets. Data corresponds to the percentage of replicates wrongly assigned in both training a test steps.

			Training				Test			
Data set	Beer class	Latent Variables	Assigned	Mis-classified	Non-classified	Multiple assigned	Assigned	Mis-classified	Non-classified	Multiple assigned
Raw chromatograms	Lager	5	8.6	2.5	4.9	84.0	4.5	7.6	10.6	77.3
	Ale	2	38.5	0	2.5	59.0	14.3	0	85.7	4.8
Corrected chromatograms	Lager	5	81.5	0	3.7	14.8	50.0	0	22.7	27.3
	Ale	5	100	0	0	0	71.4	0	4.8	23.8
Peak areas	Lager	5	53.1	0	4.9	42.0	25.8	1.5	18.2	54.5
	Ale	5	100	0	0	0	71.4	0	28.6	0
Concentrations	Lager	2	76.6	0	3.7	19.7	68.2	0	4.5	27.3
	Ale	3	89.7	0	0	0	71.4	0	0	28.6

Conclusions

This paper aims at exploring the possibilities of various types of HPLC-UV data for the characterization and classification of beers. In contrast to those more expensive approaches based on LC-MS, the proposed HPLC-UV may have a great practical impact offering a simpler, faster and more robust method for quality control and routine analysis. In particular, the performance of chromatographic fingerprints, peak areas and compositional profiles was compared. Raw chromatographic profiles required pretreatment to enhance the data quality. Further exploratory sample evaluation by PCA allowed a reasonable discrimination of beers depending on their main classes (lager and ale). Regarding potential markers of each class, it was encountered that some compounds occurred at concentrations significantly higher in lager (e.g., ferulic and coumaric acids) while other were much more abundant in ale (e.g., gentisic and syringic acids). The classification of commercial beers using chemometric methods, especially PLS-DA, was highly promising. Results working with concentration data were excellent, with 100% of correct assignations to the respective lager or ale classes. We expect that this approach could be extended to other purposes such as classifications on geographical factors or authentication studies.

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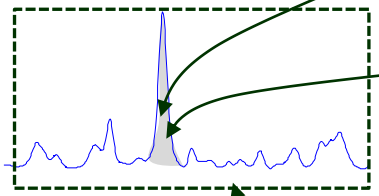
HPLC Analysis



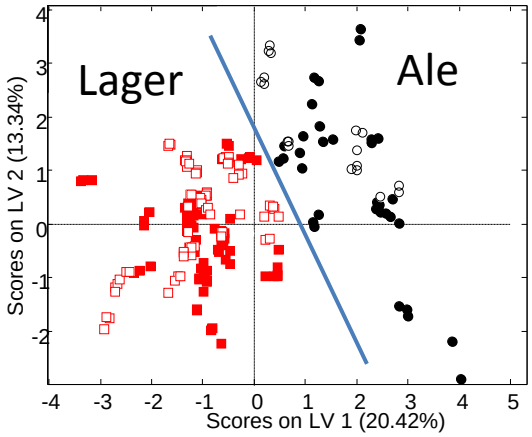
Concentrations

Peak areas

Chromatographic fingerprints

Data sets

Chemometric Analysis



PCA

PLS-DA