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Experimental design for the determination of polyphenols by liquid chromatography. Application to the chemometric characterization and classification of beers

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This paper describes the development and application of a simple, rapid and inexpensive chromatographic method to determine polyphenols in beers. The separation was optimized by experimental design considering both resolution and analysis time as the objectives. Figures of merit were established under the selected experimental conditions. In general, repeatabilities of peak areas were better than 2%, detection limits were in the order of magnitude of 0.01 mg L⁻¹ and quantitative recovery percentages were about 100 ± 5. Differences in the polyphenolic composition among beer types were encountered to be relevant. This finding was exploited to perform the characterization of commercial beers using chemometric methods such as principal component analysis. Results indicated that coumaric and ferulic acids were more abundant in lager while syringic and gentistic acids were typical of some ale varieties. Concentrations of polyphenols in the samples were used to build classification models to discriminate among lager and ale classes. Models proved to be highly efficient in terms of sensitivity and specificity. It was found that all the samples were correctly assigned to their actual classes.

Introduction

Polyphenols consist of a wide family of compounds occurring as secondary metabolites of plants. Polyphenols are often classified into four main families according to the number of phenol rings that they contain as well as the structural elements that bind these rings together [1-3]. Among these families, flavonoids and phenolic acids are the most abundant in food products, representing a 70 and 30%, approximately, of total polyphenols. Two minor classes comprise stilbene and lignane derivatives. Polyphenolic compounds are found as single molecules, the so-called aglycones, or conjugated with one or more sugar residues thus resulting in the corresponding glycosides [1]. Also, polyphenols can form polymeric molecules such as condensed catechins (e.g., dimmers, trimmers, etc. of catechins) or hydrolysable tannins (e.g., esters of gallic and other phenolic acids) [3].

Polyphenols are highly relevant in food analysis because of their organoleptic, nutritional and medical implications. Recently, the role of these compounds as chemotaxonomical descriptors of food features has also been pointed out. Hence, polyphenol profiles have been exploited as a source of analytical data to characterize, classify and authenticate food products and beverages such as fruits, wines, etc. [4-8].

The determination of polyphenols in beer can be tackled by liquid chromatography (HPLC and UHPLC) [6, 8]. In general, these methods rely on the separation of analytes by reversed-phase mode. The separation by HILIC is less extended although this mode may be of interest for dealing with the most polar analytes. Regarding detection, UV-Vis spectroscopy at 280 nm is suitable for a wide range of analytes. Fluorescence and redox properties of polyphenols can also be exploited for more specific determinations [9, 10]. In the last years, liquid chromatography with mass spectrometry (LC-MS) methods have been proposed to enhance both selectivity and detectability. Besides, MS offers excellent possibilities for the unambiguous identification of phenolic compounds [5, 11]. Apart from LC, alternative methods have been proposed such as those based on gas chromatography (GC), capillary electrophoresis (CE) and electronic tongues [12-15].

To date, few papers have been published on the characterization and classification of beers through compositional profiles of polyphenols and other chemical species. Marova and coworkers have reported the quantification of 11 polyphenols by LC-UV-MS in Czech and foreign lager beers [16]. In another study, the overall polyphenolic contents and other chemical parameters (e.g., chloride, phosphate, sulfate, total amino acids, pH) has been exploited to discriminate blond beers [17]. Complex instrumental signals such as, for instance, NMR spectra have been applied to the study of beers. It has been found that aromatic signals are more appropriate to discriminate among brewing styles [18,19]. Similar instrumental approaches combined with chemometric data treatments have been considered to assess beer quality and
In this paper, we established and validated new analytical methods for the determination of polyphenols. The method offers some advantages over other methods published in the literature such as short analysis time, simplicity, low cost, and excellent reproducibility and accuracy. Beers of different types and manufactured in several countries were analyzed and polyphenolic compounds were quantified. Compositional data were evaluated to try to find markers of brewing styles. The huge amount of data generated was analyzed chemometrically. Principal component analysis (PCA) was used for exploratory studies to facilitate the extraction of relevant information. Results indicated that lager and ale samples were distinguishable according to the polyphenolic profiles. Besides, further classification studies by partial least square regression – discriminant analysis (PLS-DA) were highly satisfactory.

**Experimental**

**Chemicals and standards**

Unless specified, analytical grade reagents were used. Eluent solutions were 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). Caffeic, coumaric, 2,5-dihydroxybenzoic, ferulic, gallic, 4-hydroxibenzoic, protocatechuic, salicylic syringic and vanillic acids, (+)-catechin, (-)-epicatechin, quercetin and rutin were purchased from Sigma-Aldrich (St. Louis, MO). Molecule structures are given in Fig. 1. Individual stock solutions of each polyphenol were prepared at a concentration of 5 mg mL\(^{-1}\) in dimethylsulfoxide (DMSO, from Merck). Stock solutions were stored in dark vials at 4°C. Polyphenol standard mixtures for the assessment of quality parameters and quantification, with concentrations ranging from 0.05 to 20 µg mL\(^{-1}\), were prepared in DMSO/water (1:1, v/v).

**Samples**

A total of 63 beers were purchased from several retail stores of Barcelona. Among them 42 were lager and 21 ale. Regarding the manufacturing countries, 35 were Spanish and the rest from other European countries. Samples were analyzed in triplicate in several working sessions (approx. 6 to 10 samples were analyzed in each series) throughout 2 months. The sample treatment consisted of beer dilution with DMSO (mixing 0.5 mL beer + 0.5 mL DMSO) and filtering through 0.45 µm PTFE membranes (Scharlab, Barcelona, Spain) prior injection to the chromatograph.

**Liquid chromatography**

The chromatographic system 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). A Kinetex C18 (100 mm x 4.6 mm i.d., particle size 2.6 µm) furnished with a SecurityGuard C18 cartridge (both from Phenomenex, Torrance, CA) was used as the analytical column.

The elution gradient was created from 0.1% (v/v) formic acid aqueous solution (solvent A) and methanol (solvent B). The elution gradient was as follows: time range 0 to 11.5 min, 5% → 26% B (linear increase); 11.5 to 19 min, 26% → 60% B (linear increase); 19 to 20 min, 60% → 90% B (linear increase); 20 to 23 min, 90% B (constant, cleaning step); 23 to 23.1 min, 90% → 5% B (linear decrease); 23.1 to 25 min, 5% B (constant, equilibration steps). The flow rate was 1 mL min\(^{-1}\) and the injection volume 10 µL. Chromatograms were recorded at 280, 310, 370 and 520 nm.

**Data analysis**

MATLAB (Version 6.5) was used for calculations. Principal Component Analysis (PCA) and Partial Least Square - Discriminant Analysis (PLS-DA) were from the PLS-Toolbox [32]. A detailed description of these methods is given elsewhere [33].

For exploratory studies by PCA, the data matrix to be treated consisted of concentrations of quantified polyphenols of each sample (see section 2.2). The dimension of the corresponding matrix was 189 (63 samples by triplicate x 13 analytes). Data was autoscaled to provide similar weights to all the analytes. The plot of scores, showing the distribution of the samples on the principal components (PCs), was used to reveal patterns of sample styles. The plot of loadings, depicting the distribution of variables, provided information dealing with correlations and dependences of polyphenols on beer properties.

In order to perform the classification of beers by PLS-DA, the set of 63 samples was divided into two subsets to be used for calibration and validation purposes. The training set was composed of 26 lager and 13 ale beers and the test set contained 16 lager and 8 ale samples. Concentrations of 13 compounds were used as X-data sets for training and prediction, with dimensions 37 × 3 (samples) × 13 (concentrations) and 24 × 3.
Results and discussion

Optimization of the separation conditions

Chemometric approaches based on experimental design and multicriteria decision making were here applied to help optimize the separation [34-35]. As indicated in the experimental section, an aqueous solution of 0.1% formic acid (solvent A) and MeOH (solvent B) were used to create the elution profiles. The solvent gradient consisted of two ramps increasing the MeOH percentage to tackle the separation of phenolic acids and flavonoids, respectively. The gradient profile was preliminarily established using a mixture of 13 polyphenols, each at 5 µg mL⁻¹. An experimental design of 2 factors (namely, gradient ramp time \( t_{ramp} \) and final MeOH percentage, MeOH\(_{final} \)) at three levels was planned, thus involving 9 assays to run. In all the cases, the starting MeOH percentage was 5%. Experiments for optimization of the first gradient step (ramp1) were as follows: \( t_{ramp1} = 8, 11 \) and 14 min; MeOH\(_{ramp1} = 20, 25 \) and 30%. The second step, focused on the separation of flavonoids, consisted of a linear increase of MeOH percentage (ramp2) connected to the end of ramp1. Times and solvent percentages were: \( t_{ramp2} = 6, 8 \) and 10 min; MeOH\(_{final} = 50, 60 \) and 70%. After separating a cleaning step using 95% MeOH was applied. Elution gradients were evaluated in terms of both separation quality (resolution of close peak) and analysis time (accounted from the retention time of the last analyte, here, quercetin). In particular, the resolution of syringic acid and epicatechin (Rs\(_{s/\alpha} \)), and ferulic and salicylic acids (Rs\(_{f/s} \)) were here considered. Hence, the mathematical expression of overall desirability was 

\[
D = (d_{Rss/e} \times d_{Rs/\alpha} \times d_{Rs/f})^{1/3}
\]

being \( d_{Rss/e}, d_{Rs/\alpha}, d_{Rs/f} \) desirabilities of the two resolution values and retention time, respectively. Resolution data was transformed into desirabilities considering that \( Rs > 1.3 \) corresponded to an excellent separation \((d = 1)\) and \( Rs < 1 \) were unacceptable \((d = 0)\). For retention time, limits of optimal (fast) and unacceptable (too time-consuming) were set to 10 and 25 min, respectively. Under these criteria, the response surface describing the overall desirability, i.e. the best separation, was maximal for the following gradient profile:

\[
t_{ramp1} = 11 \text{ min}, \text{ MeOH}_{ramp1} = 25\%, \ t_{ramp2} = 6 \text{ min}, \text{ MeOH}_{ramp2} = 60\%\text{ as a compromise among chromatographic separation and speed (see Fig. 2a).}
\]

The separation performance was also assessed on a lager Spanish beer (Estrella Damm) taken as an example to confirm if separation conditions established with standards were also valid for the samples. In this case, because of the higher complexity of chromatograms, the optimal separation was that providing the highest number of peaks in a reasonable analysis time. Depending on the elution conditions, peaks resolved varied from 31 to 44 and analysis times from 14.5 to 24.7 min. For reaching both objectives simultaneously, a desirability function combining our two interests was used: 

\[
D = (d_{peak} \times d_{Rss/e})^{1/2}
\]

being \( d_{peak} \) the desirability of number of peaks. Optimum and unacceptable values for \( d_{peak} \) were 50 and 25 peaks, respectively. The criterion of limits of retention time was as above. Results shown in Fig. 2b indicated that the best response was attained at the same conditions as with the standards. The gradient profile was slightly modified to improve the resolution of two intense close peaks of syringic acid and epicatechin so the elution gradient finally selected was: \( t_{ramp1} = 11.5 \text{ min}, \text{ MeOH}_{ramp1} = 26\%, \ t_{ramp2} = 6 \text{ min}, \text{ MeOH}_{ramp2} = 60\%\). 

As an example, Fig. 3 depicts the chromatograms of a standard solution and a representative lager and ale beer sample. It can be seen that an excellent resolution of all polyphenol was reached in Fig. 3a. The compositional profile of the ale beer was more complex than that of the lager one as it contained a higher number of compounds (see Fig. 3b). Besides, polyphenol concentrations were, in general, higher in the ale beer. These considerations could be generalized to the rest of ale and lager samples studied.

Figures of merit

Once the method was fully optimized, figures of merit were assessed using synthetic standards prepared in DMSO/water (1/1, v/v) (see Table 1). DMSO was used as a co-solvent to enhance the solubility of less polar flavonoids. Besides, the stability of stock solutions was improved as polyphenol degradation was minimized in this hydro-organic medium. Regarding the chromatographic performance, as shown in Fig. 3a, the effect of DMSO on the distortion chromatographic peaks was negligible. The linearity was evaluated at the selected wavelengths for each polyphenol as specified in the experimental section. The method was linear within the range of concentrations assayed here, with regression coefficients better than 0.999 for most of the analytes. The sensitivity of the method, i.e., the slope of the calibration curve expressed as AU

\[
\text{AU} = \frac{\text{Area of peak}}{\text{Concentration of analyte}}
\]

This is a standard expression used to quantify the sensitivity of chromatographic methods.
Matrix effects were assessed from the comparison of calibration curves in DMSO/water (1/1, v/v) and DMSO/beer (1/1, v/v). For this purpose, two independent studies on beers (Estrella Damm and Steinburg) were carried out. Standards of each analyte were prepared in the working range 0.5 to 20 µg mL\(^{-1}\) to estimate the sensitivity in the two matrices. In all the cases, sensitivities were similar thus estimating that matrix effects were negligible (see Table 1). Hence, it was concluded that calibration with DMSO/water standards was appropriate for the quantification of polyphenols in beer.

The evolution of polyphenolic concentrations over time was evaluated once containers were opened in order to check the stability of beers regarding the possible degradation of the polyphenolic fraction. This study was devoted to ascertain if the time from the opening to the analysis had (or not) a critical impact on the composition of this family of molecules. A commercial beer (Steinburg clásica), was chosen as a model to address the study. Beers diluted 1:1 with DMSO and were stored in polypropylene vials at room temperature and 4°C for a period of 14 days. Polyphenol concentrations were quantified on different days throughout this period in order to follow their evolution over time. Within this time range, the concentration of analytes remained constant at the two temperatures of the study. The same behavior was encountered for the vast majority of analytes with the only exception of catechin. For such a compound, its concentrations remained approximately constant at 4°C although, at room temperature, a noticeable increase with time was observed. This finding was attributed to the progressive degradation of condensed tannins to release catechin monomers in the medium.

The proposed method was further applied to the determination of analytes in a set of beers as described in the experimental section. Results are summarized in Fig. 4. It was found that the most abundant compound was gallic acid (~30 mg L\(^{-1}\)). Other components 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\(^{-1}\). From these results, differences in the percentages of each polyphenol among lager and ale classes were difficult to establish although it was clearly evidenced that ale samples were, approximately, 15% richer in overall polyphenols. In general, concentrations of individual components were higher in ale than in lager (for instance, 3-fold higher for protocatechuic and gentistic acids). Conversely, only a few compounds such as ferulic and, especially, coumaric acid were more abundant in lager.

**Application of chemometric methods to beer classification**

Chromometric methods were used to try to extract reliable conclusions on the relationships between polyphenolic contents and beer features. Data was pretreated by autoscaling to provide similar weights to all analytes. PCA was preliminarily applied to explore analogies and differences among beers depending on the analyte composition.
As shown in Fig. 5a, PC1 was related to the overall content of polyphenols (concentrations increased from left to right). PC2 was mainly focused on sample description as a function of composition of some species. It was found that lager samples were mainly located to the top left part of the plot of scores while ale beers predominated to the bottom right area. Ale samples were not distributed as a compact cluster but they showed dispersion thus suggesting a higher variability in composition and attributes (in agreement with wider variety of ale styles). From this study, it was encountered that polyphenolic profiles depended on the brewing process. Ale beers were quite irrelevant in terms of beer description (Fig. 5b). A simultaneous evaluation of scores and loading indicated that a subgroup of ale beer was characterized by higher contents of epicatechin and gentisic acid (samples to the bottom), and another subgroup was richer in rutin and syringic acid (samples to the right).

Further classification studies were conducted with PLS-DA to assign the commercial samples to lager and ale classes. The set of samples was divided into two subgroups for modelling and validation purposes, respectively. In particular, the modeling subset was composed of 8 ale and 26 lager beers and the test subset was composed of 8 ale and 16 lager beers. The prediction performance of the corresponding model was checked internally (i.e., on the set of modelling samples) and externally (i.e., on the set of test samples). The number of latent variables (LV) to build the training model was preliminarily estimated by leave-one-out cross-validation and 3 LV were found as optimal. Other validation criteria were also investigated for a better choice of latent variables as well as a better estimation of error of predictions. Results of cross validation based on random blocks using 10 data splits and 5 iterations are given in Fig. 6a, in which average errors in classification were below 2% when using 2 or 3 LV. Here, 3 factors were selected to perform further predictions and the corresponding classification in terms of specificity and sensitivity are depicted in Fig. 6b. From the receiving operating characteristic (ROC) curves, it can be seen that 100% of ale samples were correctly assigned and no false lager was detected as ale. Regarding lager beers, 100% of tested samples were correctly predicted and, again, no ale beer was seen as a lager one. As a result, classifications were entirely satisfactory in terms of sensitivity and specificity.

Conclusions

This paper aims at reclaiming the value of HPLC-UV for the determination of polyphenols in beers. In contrast to much more expensive approaches based on LC-MS that are often exploited to food analysis, the proposed HPLC-UV may have a great practical impact offering a simpler, faster and more robust method for quality control and routine analysis. A relevant point providing both the high reliability and sample throughput relies on the optimization of the chromatographic conditions using experimental design. For such a purpose, the definition of an optimization criterion taking into account separation performance and analysis time allowed the proposed objectives to be achieved. It should be noted that although selectivity of UV detection is limited, efforts devoted to improve the
separation contributed to overcome interferences from overlapping peaks. Validation results suggested that precision and accuracy of the method were highly satisfactory. Matrix effects were negligible so the quantification of polyphenols in beers can be tackled by external calibration using pure standards prepared in DMSO/water (1:1 v:v). The characterization of beers was attempted by polyphenolic profiling using analyte concentrations as the analytical data. Exploratory sample evaluation by PCA provided a reasonable discrimination of beers depending on their main classes (lager and ale). Besides, although specific chemical markers (i.e., present in one class and absent in the other) were not found, it was encountered that amounts of some polyphenols were significantly higher in one of the classes than in the other. On this basis, the classification of commercial beers using PLS-DA gave highly promising results. The calibration model assessed from a set of selected beers was further applied to an independent test set. Results were excellent, with 100% of correct assignations to the respective lager or ale classes. Therefore, this method can be clearly extended to other purposes such as beer classification based on geographical factors, row materials, brewing practices, etc. Finally, studies of authentication and detection of adulterations could be tackled in a similar way.

Fig. 5. Exploratory study of beers by PCA. (a) Plot of scores; (b) Plot of loadings. Symbols: Circle = lager; square = ale

Fig. 6. Results of classification of beers into lager and ale classes by PLS-DA. (a) Average prediction error as a function of number of latent variables; (b) Plots of receiving operating characteristic (ROC) curves of lager and ale classes.

Acknowledgements

This project has been partly founded by Generalitat de Catalunya (2014 SGR 377 project).

Notes and references

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