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NMR and Chemometrics in the Determination of Chemical Profiles for the Distinction of Brazilian Ale and Lager Beers

Lázaro S. de Jesus,[a] *Igor S. Flores,*[b] *Vinícius S. Pinto,*[c,d] *Gislane O. Ribeiro,*[d] *Karla C. R. C. Morais*[d] *and Luciano M. Lião*[*,d]

[a]*Instituto Federal de Roraima, 69365-000 Caracaraí-RR, Brazil*

[b]*Instituto Federal de Goiás, 75402-556 Inhumas-GO, Brazil*

[c]*Instituto Federal de Goiás, 73813-816 Formosa-GO, Brazil*

[d]*LabRMN, Instituto de Química, Universidade Federal de Goiás, 74690-900 Goiânia-GO, Brazil*

Determining chemical profiles of complex matrices, such as beers of different styles, can highlight regional characteristics and provide robust literature on the variability of this product, improving quality control. A practical application is to unequivocally attribute the authenticity of the product even in the face of variations in composition due to inadequate transport and storage. This work used proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and chemometrics in the semi-quantitative study of Brazilian ale and lager beers. The results demonstrated the success of applying ^{1}H NMR in characterizing chemical profiles and as a statistical database to distinguish ale and lager beers. Using principal component analysis (PCA) of the NMR data, it was possible to identify that carbohydrate content was responsible for the separation tendency between these beer styles. Ale beers had a higher residual carbohydrate content, according to the integrals of the carbohydrate hydrogens. This is expected, as these beers are obtained by fermentation at higher temperatures for shorter fermentation times. The paper also described soft independent modelling of class analogies (SIMCA) as applied to the NMR data. This class model made it possible to correctly classify 90% of the samples as ale and 100% as lager.

Keywords: Brazilian beer, beer analysis by NMR, chemometrics, PCA, SIMCA

Introduction

Beer is a popular fermented alcoholic beverage with wide acceptance worldwide. It is produced by mixing malt, water, hops, and yeasts.[1,2] Malt plays a central role in beer production since it is the primary source of fermentable sugars consumed by the yeasts during brewing.[3,4] During the fermentation process, the yeasts convert the wort sugars into ethanol and carbon dioxide, and in lower concentrations, metabolites such as organic acids and higher alcohols.[5] Thus, the crucial step in the brewing process is malt choice, as sensory characteristics such as aroma, color, texture, and flavor are directly influenced by the types and proportions of carbohydrate sources.[6]

Chemically, beer is characterized as a mixture of water (90-95%), residual extract (2-6%), ethanol (2-6%), and carbon dioxide (0.35-0.50%). Substances such as amino acids, organic acids, mineral salts, proteins, and secondary alcohols may be present in smaller amounts.[7-9] The difference in chemical composition is associated with the fermentation process, which has, for example, variables such as the type of yeast and temperature used in the process, and can be used to classify beer as "ale" or "lager".[10] The ale (high fermentation) is a type of beer brewed using a warm fermentation method (12-15 ºC), which results in beverages with more perceptible flavors (full-bodied), with the Pale, Brown, Mild, Bitter, Stout, and Porter styles being the most representative. The lager type (low fermentation), which encompasses Pilsner, Dortmunder, Vienna, Muchen, and Bock styles, refers to beverages that have been brewed and conditioned at low temperature (5-10 ºC), and is the most consumed type of beer worldwide, responsible for about 99% of sales.[11,12]

Through Normative Instruction (NI) 65/2019 of the Ministry of Agriculture, Livestock and Supply (MAPA),[13]

*e-mail: lucianoliao@ufg.br
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the Brazilian Legislation establishes identity and quality standards for brewery products and the physical-chemical, microbiological, and organoleptic parameters allowed in the Brazilian beverage market. However, although robust, the analytical tests described in this normative may not be sufficiently informative to allow the correct classification of beers of different types (e.g., ale and lager) due to the natural chemical complexity of this type of sample. Thus, it is essential to implement methods capable of determining qualitative and quantitative chemical profiles to identify different types of beers and also help identify fraud.

Separation and identification techniques, especially high-performance liquid chromatography (HPLC) and mass spectrometry (MS) have been successfully applied in the analytical context presented.[14-16] Detailed chemical profiles are determined quickly and unambiguously in both routines without using large amounts of solvents. However, such techniques require the use of specific standards for quantification. On the other hand, nuclear magnetic resonance (NMR) spectroscopy allows the collection of chemical profiles quickly and non-selectively, but with the advantage of not requiring specific standards for quantification due to the direct proportionality between the area of the signal and the number of nuclei responsible for the NMR signal.[14,17-21] Among the parameters recommended in NI 65/2019, through a single NMR experiment, the following can be evaluated: (*i*) alcohol content; (*ii*) presence of adjuncts such as lactose; (*iii*) counterfeiting of beverages from reference spectra; (*iv*) presence of contaminants from raw materials or process failures; (*v*) substances harmful to the consumer, produced during fermentation or by the action of unwanted microorganisms.[20]

However, the identification of counterfeit beverages using reference spectra (item *iii*) is not an easy task, as each variability factor brings unexpected changes in the chemical composition, such as changes in the recipe, parameters in the production stages, transport and storage, drastic changes in the temperature of the beverage at the commercial point, among others. These factors can generate new chemical compounds and change the signals' proportionality and consequently the sample's fingerprint.

Applying NMR to analyze complex matrices such as beers generates a range of information that may not be readily interpretable. Simple comparison methods may not recognize possible trends in the grouping or separating of samples that indicate types and non-conformities.[22-24] In the particular cases of beers rigged by the addition of compounds already present in the formulation (for example, water or ethanol) at unacceptable levels or to identify chemical profiles of different types of beer, the application of chemometrics in data processing is recommended.[3] Among the main chemometric protocols used in this analytical context were unsupervised methods for the recognition of trends, such as principal component analysis (PCA), and supervised methods used in classification and quantification, such as soft independent modelling by class analogy (SIMCA) and partial least squares regression (PLS), respectively.[3]

PCA generates information about possible sample groupings and indicates which spectral variables are decisive for the observed separation of samples by decomposing experimental information organized into data matrices. The application of PCA in the study of beer by NMR goes beyond exploratory analysis.[25] It can be used as a statistical basis for executing supervised methods, such as SIMCA. In SIMCA classification, each class is modeled using multidimensional spaces to classify new samples. The limits of each class are determined by critical values of variance typical of each model, usually represented by hyperboxes or ellipses.[26-28] Thus, the synergy between the NMR-PCA and NMR-SIMCA statistical models can be applied to separate samples from beers and classify them based on types (ale and lager), with emphasis on information on the components identified in the chemical profiles. In the analytical context discussed, this paper describes the use of ^{1}H NMR combined with chemometrics (PCA and SIMCA) applied to the study of Brazilian ale and lager beers. The experimental protocols developed were useful in determining the chemical profiles of samples of both types, as well as in identifying chemical descriptors responsible for classifying samples into ale or lager beers.

Experimental

Sample collection

Forty beer samples were analyzed in triplicate (120 analyses), divided into 20 ale and 20 lager samples, including different styles, brands, and breweries. The samples were acquired from specialized beer stores in Goiânia, Brazil. The packages (bottles and cans) were previously sanitized prior to opening. 500 µL aliquots of each sample were degassed in an ultrasonic bath as sample preparation, and the pH of each one was measured with a portable potentiometer (Toledo AG FiveGo-FG2, Columbus, USA). All pH samples ranged between 4.1 and 4.6, meeting the specifications of technical note 981.12 of the Association of Official Analytical Chemists.[29]

^{1}H NMR experiments

For each analysis, 200 µL of degassed beer were mixed

with 400 µL of D_2O (CIL, Andover, USA) containing 0.01% (m/v) 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TMSP-d_4) (CIL, Andover, USA). The resulting solution was transferred to 5 mm tubes for NMR analyses. The NMR spectra were acquired on a Bruker Avance III 500 spectrometer (Bruker, Rheinstetten, Germany) operating at 11.75 Tesla, fitted with a broadband inverse (BBI) probe at 25 °C. ^{1}H NMR spectra were acquired by the NOESYPR1d pulse sequence (Bruker) in a window of 25 ppm with 64 k points. A single 90° excitation pulse, calibrated for each sample, was used. Each sample summated 128 spectra with a relaxation delay of 16 s, an acquisition time of 3.99 s, and a pre-saturation time (D9) of 4.0 s. Inversion recovery experiments proved that this recycling time allowed for virtually complete relaxation of all the signals (more than 5 × T1). Spectra were processed in TopSpin 4.1 (Bruker). The phase and baseline were manually adjusted. The TMSP-d_4 was used as an internal reference for the chemical shift, with a calibrated signal at δ 0.0.

Chemometric analysis

Chemometric analyses (PCA and SIMCA) were performed using AMIX 3.9.15 software (Bruker). The data matrix was obtained by the spectral bucket procedure of ^{1}H NMR data, using a rectangular format with 0.01 ppm width, resulting in 900 buckets. The procedure was useful in suppressing minute variations in the chemical shifts of hydrogens that could negatively influence the statistical models. To this end, the signal range from δ 9.5 to 0.50 was considered, with the integration mode adding the intensities without the scaling process. The data matrix was mean-centered, and a 95.0% confidence interval was used.

PCA was applied for exploratory data analysis purposes. Eight principal components (PCs) were used, explaining an accumulated variance of 98.6%. The PCA underwent full cross-validation. PC1 and PC2 were used to construct the score and loading graphs. The method helped to recognize tendencies in the separation of ale and lager beers. A classification model based on the spectral data of the ale samples (AL class model) was initially created to perform the SIMCA. Seven PCs (98% explained variance) were used to model the AL class. The complete cross-validation method validated the model using all samples and a confidence interval of 95.0%. In the training stage, 75% of the sample set was used. The models' performance of classification in ale or lager beers was evaluated in the prediction step, using the samples not used in the training step (25%).

Results and Discussion

^{1}H NMR chemical profile of Brazilian ale and lager beer

Beer is a fermented product chemically characterized as a hydroalcoholic solution. This mixture contains sugars, amino acids, nucleotides, polyphenols, vitamins, and macromolecules such as polysaccharides, proteins, and nucleic acids. Figure 1 shows the ^{1}H NMR spectra of ale and lager beer samples. Due to the large amount of water in beer,

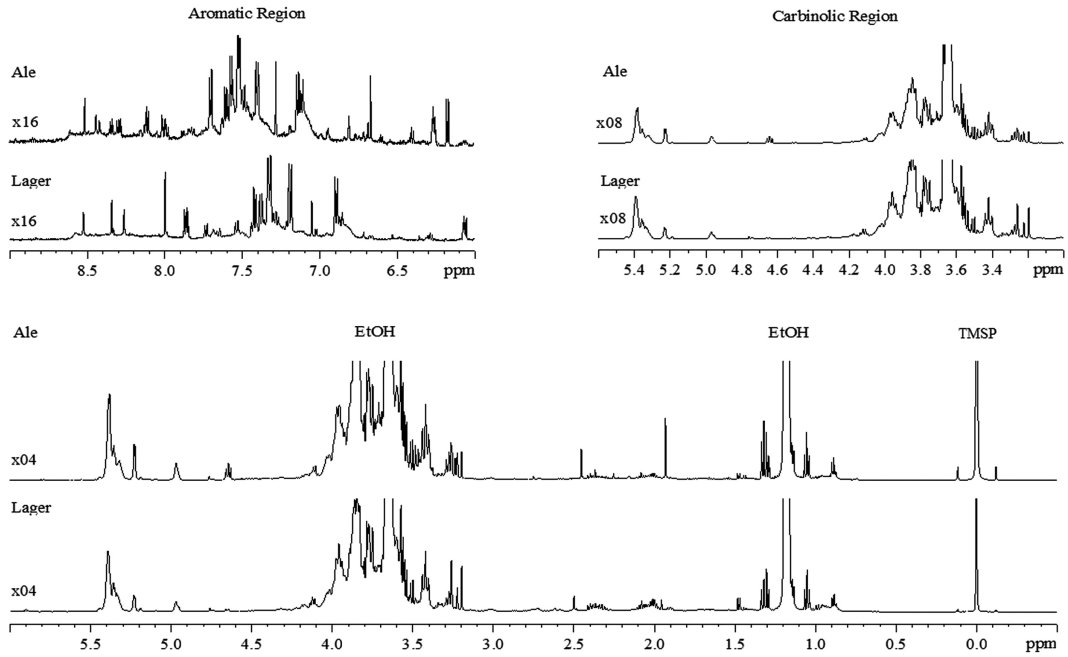

Figure 1. ^{1}H NMR spectra (500 MHz, D_2O) of Brazilian ale and lager beers. Expansions of the carbinolic and aromatic hydrogen regions are highlighted.

the region at δ 4.70 was irradiated to suppress the water hydrogen signal. Only this region was suppressed to avoid compromising signals of interest very close to the irradiated area, as happens with higher alcohols overlapping with the ethanol triplet signal, the second component in the highest proportion in the mixture. To facilitate the visualization and consequent interpretation of the spectra, expansions of the aromatic (δ 10.0-5.50) and carbinolic (δ 5.50-3.40) regions were presented. All the signals described below were identified in both types of beers. The signal assignments were corroborated by the literature.[3,14,18-20,26]

Beer spectral analysis is generally complex, with signals observed in almost all spectral ranges. In addition, the signals from ethanol and water (residuals) are intense. The peak-to-peak assignment is difficult, especially for carbohydrate hydrogens, since they have close chemical shifts, favoring overlapping signals. A summary of the spectral information was organized in Table 1 with the pertinent assignments of the aromatic (δ 10.0-5.50), carbinolic (δ 5.50-3.40), and aliphatic (δ 3.40-0.50) regions.

Table 1. ^{1}H NMR spectral data assignment for identified compounds in all beer samples (500 MHz, D_2O)

Spectral region	Compound	δ ^{1}H (multiplicity;[a] *J* / Hz) / ppm
Aliphatic	isobutanol	0.87 (d; 6.67)
	isopentanol	0.88 (d; 6.67)
	propanol	0.90 (t; 7.10)
	leucine	0.95 (t; 6.41)
	valine	1.00 (d; 7.00), 1.06 (d; 7.00)
	isoleucine	1.06 (d; 7.00)
	2,3-butanediol	1.12 (d; 7.00)
	ethanol	1.17 (t; 7.06), 3.65 (q; 7.06)
	lactic acid	1.29 (t; 7.00), 4.17 (q; 7.00)
	3-methyl-butanol	1.43 (q; 6.91)
	alanine	1.46 (d; 7.27)
	proline	1.97-2.11 (m), 2.33-2.41 (m), 3.37-3.45 (m)
	acetic acid	1.97 (s)
	pyruvic acid	2.36 (s)
	succinic acid	2.57 (s)
	choline	3.18 (s)
	betaine	3.25 (s)
Carbinolic	glycerol	3.55 (dd; 6.50; 11.70)
	β-glucose	4.64 (d; 8.00)[b]
	maltotriose and maltose	5.5-4.5 (m),[b] 3.0-4.5 (m)
	trehalose	5.18[b] (d; 4.00)
	α-glucose	5.23[b] (d; 3.55)
	α-(1→6)-branched dextrin	4.96 (m)
	α-(1→4)-linear dextrin	5.40 (m)
Aromatic	uracil	5.59 (d; 7.78)
	uridine	5.88 (d; 9.60), 7.88 (d; 9.60);
	guanosine	5.90 (d; 4.08), 8.00 (s)
	cytidine	6.08 (d; 6.55);
	tyrosine	6.88 (d; 8.50), 7.18 (d; 8.50)
	phenylalanine	7.31 (d; 8.00), 7.38 (m); 7.40 (m)
	tryptophan	7.52 (d; 8.00), 7.75 (d; 8.00)
	guanosine	8.10 (s)
	hypoxanthine	8.22 (s)
	inosine	8.26 (s)
	formic acid	8.42 (s)
	acetaldehyde	9.66 (q; 3.00)

[a]Multiplicities: d: doublet, dd: doublet of doublet, m: multiplet, q: quartet, s: singlet, t: triplet; [b]anomeric hydrogens.

Statistical analysis of ale and lager beer samples

Analyzing intensities, multiplicities, and the number of signals in each spectral subdivision presented in Figure 1, it was impossible to recognize patterns that would allow the distinction between the ale and lager samples since all the assigned signals (Table 1) are present in both beer styles. The set of samples, represented by the ^{1}H NMR spectra of the ale and lager samples, was subjected to exploratory analysis by PCA (Figure 2). The accumulated variance explained by the first two components used in the construction of Figure 2 was 85.6%. The PCA score plot was useful to identify trends in the separation of beers by type, as described in Figure 2a.

In the PC1 *versus* PC2 score plot (Figure 2a), a clear trend of separation between ale and lager beer samples was identified, with the group of beer samples arranged along the negative PC1 scores. In contrast, the lager group was disposed on positive PC1 scores. Along PC2, whose tendency for samples to separate was less evident, it was observed that a large part of the lager samples was arranged in negative PC2 scores, while the ale samples were arranged in positive and negative PC2 scores. The analysis by the loading plot (Figure 2b) indicated the main chemical descriptors (NMR signals) responsible for separating the samples. The statistical region where the ale samples were located was mainly influenced by signals close to δ 3.83, 3.84, 3.85, and 3.86. In this chemical shift range, signals of hydrogens from glucose units of sugars (e.g., maltotriose and maltose) were characterized. The negative PC1 scores were influenced by signals close to the region of δ 4.82. This range of chemical shifts is commonly associated with the anomeric hydrogens of sugars. The sample spread

observed along PC1 and PC2 with the strong influence of fermentable sugar signals was interpreted in terms of the extension of the fermentation process that each type of beer (ale and lager) was subjected to.

Fermentable sugar contents vary according to the type of beer. Lagers, in general, are more fermented than ale and, consequently, have fewer residual carbohydrates.[30] During fermentation, the yeast first absorbs and ferments all the glucose and then the maltose. Some yeasts used in the production of the lager beers also use maltotriose.[31] Therefore, it is plausible to infer that the concentrations of residual fermentable sugars were responsible for the sample separation observed in the PCA. This conclusion was corroborated by comparing the areas of the NMR signals indicated in the PCA loadings, as shown in Figure 3. Assuming that the area of a ^{1}H NMR signal is proportional to the number of atoms responsible for this signal and, therefore, the concentration of the substance associated with the nucleus in question,[32] it was possible to expand the compositional comparison between ale and lager. In Figure 3, the signal areas of the main descriptor identified as responsible for the distinction of ale and lager beers along PC1 were presented and referenced by the TMSP-d_4 signal area.

The signal areas expressed the correlation between the yeast contact time and the concentration of residual fermentable sugars indicated as a descriptor of the PCA. Lagers are a group of beers obtained by fermentation at low temperatures, which require a longer fermentation time. This means that the yeast used in the production of the lager beers has more time to convert fermentable sugars into compounds such as ethanol and carbon dioxide, resulting in a lower concentration and, therefore, a lower integral value (8.45) for the carbinolic hydrogen signals (Figure 3). On the other hand, the ale-type beer production process, which involves higher fermentation temperature, is conducted with a shorter contact time between the yeast and the wort. This causes the conversion of fermentable sugars to occur to a lesser extent, resulting in a higher residual concentration and, in addition, a higher integral value (12.94) for the carbonic hydrogen signals. The comparison between the areas of the anomeric hydrogens of the residual sugars at δ 4.82 was not considered due to the imprecision in the integration caused by the proximity of the water signal suppression region.

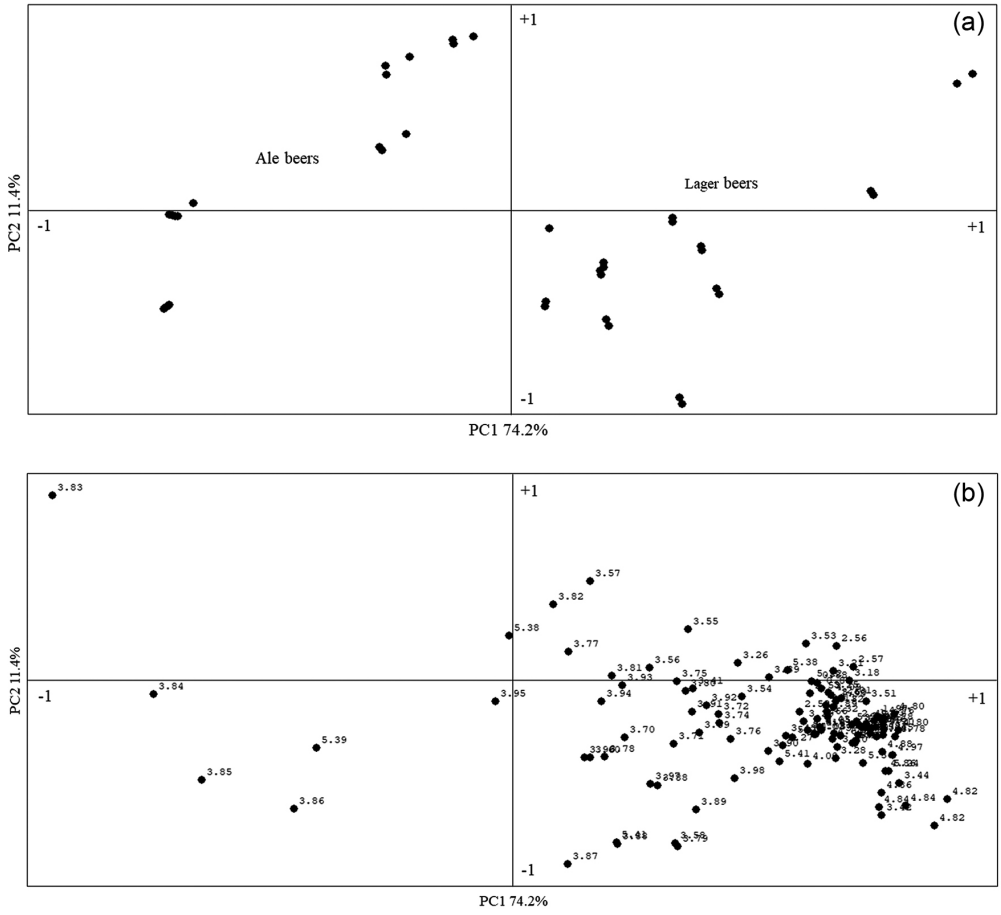

Figure 2. Score plot (a) and loadings (b) of PC1 *versus* PC2 obtained from the ^{1}H NMR data of ale and lager beer samples. In (b) the numbers above each dot represent the chemical shifts (in ppm) of the ^{1}H NMR signals involved in the separation between ale and lager beer.

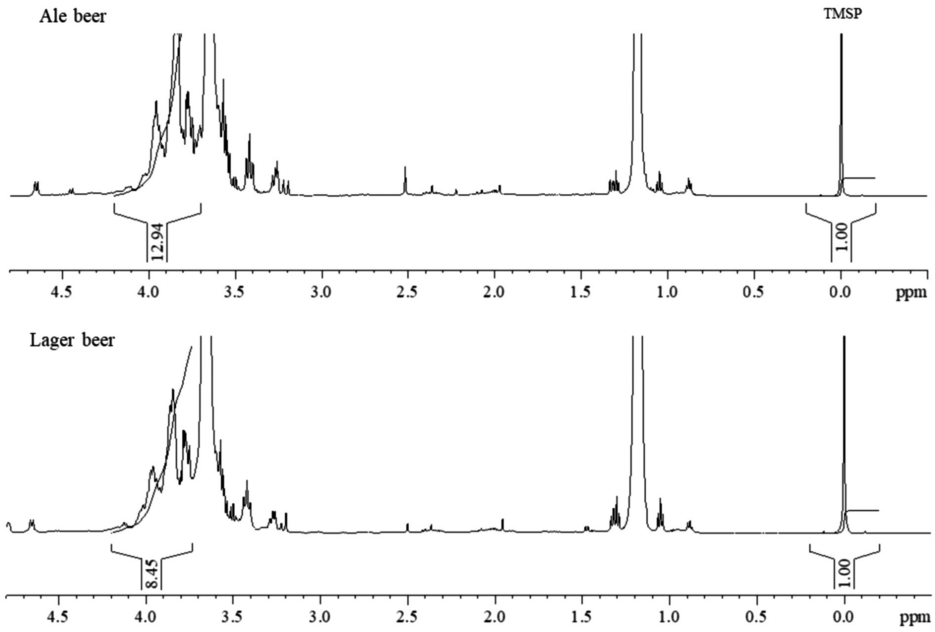

Figure 3. ^{1}H NMR spectra (500 MHz, D_2O) of ale and lager beer samples, highlighting the carbohydrates region. The signal areas were referenced to the TMSP-d_4 (δ 0.0) area.

The concentration of residual sugars is, in fact, an important variable, even in beers of the same type. In an investigation similar to the present one, da Silva *et al.*[3] were successful in discriminating samples of Brazilian lager-type beers via chemometric treatment of NMR data. In that work, the authors presented results indicating that residual concentrations of maltooligosaccharides and maltose, whose signals were identified at δ 5.22, 4.63, 3.41, and 3.27, were higher in Premium American Lager beers than in Standard American Lager.

The separation trends observed in the PCA were decisive for predictive model creation through the statistical modeling SIMCA. The possibility of classifying samples by type was evaluated using a modeling approach using the SIMCA algorithm, where 7 PCs were selected to build the ale model and the corresponding results are reported in Figure 4. Using the SIMCA model it was possible to correctly classify all beer samples in their respective set with 100% accuracy in both the calibration and prediction stages.

The graph was constructed from the square root of the residual variance (Si) *versus* the distance of each sample in relation to the center of the class model (Hi), represented by the hyperbox in the lower left corner and designated as "region 0". The class model was obtained from the spectral information of ale beers. The class model limits were determined based on Hotelling's T-square distribution (T^2), understood as a statistical generalization

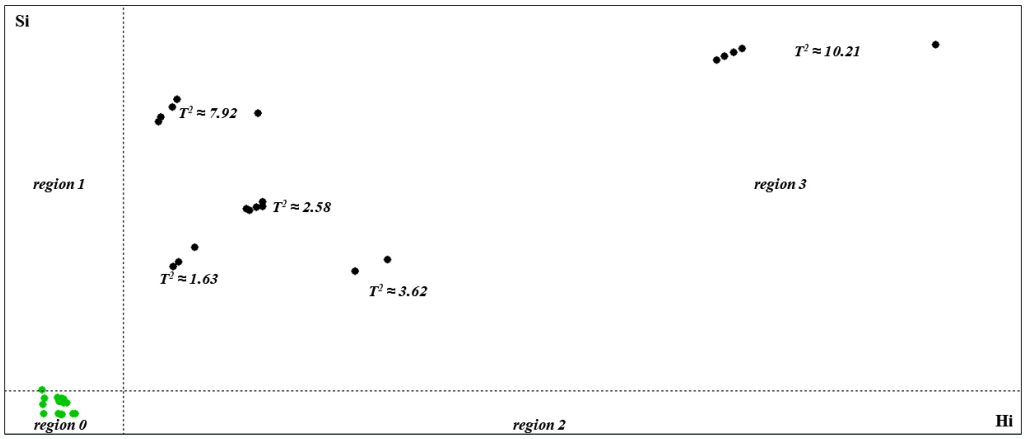

Figure 4. Si *versus* Hi plot. The hyperbox "region 0" represents the "ale type" class model. Regions 1, 2, and 3 correspond to the statistical areas for "components not classified by the model", "components significantly outside the model", and "not corresponding to the model", respectively. Ale samples were represented by green circles and lager by black ones.

of the Student's *t*-test. The more significant the difference between the sample information and the classifier (hyperbox of the class model), the greater the value of T^2.[25] Thus, only the samples arranged in the statistical area called region 0 presented T^2 values compatible with the spectral information of ale beer. In addition, the correct classification of 90% of the ale samples was observed, represented by green circles. The remaining ale samples were arranged in the statistical region called region 1. They presented T^2 values that prevented classification in their own class, characterizing two type I errors (sample not included in its own class). On the other hand, all lager beer samples, represented by black circles, showed high T^2 values (1.63, 2.58, 3.62, 7.92, 10.21), resulting in the correct exclusion of the model. No type II errors (sample included in the wrong class) were identified for lager-type beers.

This study demonstrated the applicability of ^{1}H NMR in the unequivocal identification of different ales and lagers. The results indicated that chemometric analysis using exploratory analysis (PCA) and SIMCA modeling can be employed as part of a classification procedure based on information declared by the manufacturer about the type of beer the consumer purchases. Considering that all chemometric pattern recognition methods were successful in the training and prediction stage of beer classes, the approach can also be used as a screening method for authenticating beer types. Such results may interest regulatory and inspection agencies linked to the Ministry of Agriculture, Livestock and Supply (MAPA), a governmental body responsible for verifying the authenticity and quality of foods of animal origin and beverages.

Conclusions

The results indicated that ale and lager beers have very similar chemical profiles, as all chemical components described were identified in both types. However, with the in-depth analysis of NMR data through PCA, it was possible to identify chemical components useful in distinguishing between ale and lager beers. The differentiation between ale and lager beers was due to the residual carbohydrate content, suggesting a higher consumption of sugars during the longer fermentation process for producing lager beer *versus* the higher content of residual carbohydrates in ale. Such differences were evidenced by applying the SIMCA classification model to the NMR data. SIMCA correctly classified 90% of the samples as ale and 100% as lager. The results indicate that information commonly obtained by different physical, chemical, and biological tests, widely applied in the evaluation of the quality of beers, can be obtained by the adequate chemometric treatment of ^{1}H NMR data, and these can concomitantly provide qualitative (chemical profiles) and quantitative (carbohydrate content) results.

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Author Contributions

Lázaro S.de Jesus performed the experiment and wrote the main manuscript text; Igor S. Flores contributed to the conception of the study, performed the experiment, and assisted in the interpretation of the results; Vinícius S. Pinto wrote the main manuscript text, assisted in the interpretation of the results, and prepared Figures 1-4; Karla Cristina R. C. Morais assisted in the interpretation of the results; Gislane O. Ribeiro assisted in the interpretation of the results; Luciano M. Lião contributed to the conception of the study, wrote the main manuscript, assisted in the interpretation of the results, and the funding acquisition. All authors reviewed the manuscript.

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