Studies of iso-alpha-acids: analysis, purification, and stability.

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CHAPTER 7

Application of Two Dimensional J-Resolved Nuclear Magnetic Resonance Spectroscopy to Differentiation of Beer

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ABSTRACT

A number of ingredients in beer that directly or indirectly affect its quality require an unbiased wide-spectrum analytical method that allows for the determination of a wide array of compounds for its efficient control. ^{1}H nuclear magnetic resonance (NMR) spectroscopy is a method that clearly meets this description as the broad range of compounds in beer is detectable. However, the resulting congestion of signals added to the low resolution of ^{1}H NMR spectra makes the identification of individual components very difficult. Among two-dimensional (2D) NMR techniques that increase the resolution, *J*-resolved NMR spectra were successfully applied to the analysis of 2-butanol extracts of beer as overlapping signals in ^{1}H NMR spectra were fully resolved by the additional axis of the coupling constant. Principal component analysis based on the projected *J*-resolved NMR spectra showed a clear separation between all of the six brands of pilsner beer evaluated in this study. The compounds responsible for the differentiation were identified by 2D NMR spectra including correlated spectroscopy and heteronuclear multiple bond correlation spectra together with *J*-resolved spectra. They were identified as nucleic acid derivatives (adenine, uridine and xanthine), amino acids (tyrosine and proline), organic acid (succinic and lactic acid), alcohols (tyrosol and isopropanol), cholines and carbohydrates. Unfortunately, the presence of iso-α-acids can not be detected by applying this method.

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7.1. INTRODUCTION

Beer is a fermented beverage made from malted barley, hop, yeast and water. There are several factors which affect the character of beer, such as ingredients, production processes and storage conditions (Baxter and Hughes, 2001). Even minor differences in those factors can largely influence the taste and quality of beer. Thus, an investigation into the complete chemical composition of beer has become an important issue for evaluating beer quality.

The control of the quality of this type of product is aimed at providing a guarantee on one side of a certain flavour and fragrance which is characteristic of a particular brand and on the present, its stability during the established shelf-time. Furthermore, it must ensure that these parameters are similar if not identical from batch to batch. It is a known fact that up to the moment, no single compound has been found to be responsible for these characteristics, but rather to a very complex association of diverse compounds, the presence or absence of which, and/or their relative content will lead to particular and unique flavours. A very interesting approach to deal with this problem is to develop a method which could provide an accurate analysis of the composition of the whole matrix of the product, or if that were not possible, of at least a part of it which reflects and identifies each compound. The differences and/or similarities would therefore allow for the establishment of a certain pattern related to very particular characteristics of brews or brands.

The existing analytical methods, generally aimed at contributing to the determination of the chemical composition of beer, allow the qualitative and quantitative detection of specific compounds or groups of compounds. Early investigations reported the determination of phenolic compounds in beer by colorimetric methods (Jende-Strid, 1988). As known, a colorimetric analysis only allows the quantitation of a group of compounds but not that of an individual component of that group. Individual phenolic compounds have been analysed by thin layer chromatography (TLC) (Jende-Strid, 1988), though the utility of this technique is limited by its insufficient resolution. High performance liquid chromatography (HPLC) and gas chromatography (GC) have been applied to the analysis of beer in order to obtain higher resolution and quantitative results for several compounds. Several of these methods have been published, including the GC or HPLC detection of amino acids (Jin *et al.*, 1999), phenolic compounds (De Pascual-Teresa *et al.*, 1998;

Stevens *et al*., 1999), vitamins (Andrés-Lacueva *et al*., 1998), carboxylic acids (Miwa and Yamamoto, 1996), sulphur compounds (Hill and Smith, 2000) and bitter acids (Hermans-Lokkerbol and Verpoorte, 1994a; Raumschuh *et al*., 1999), using a DAD or MS detector for the qualitative and quantitative analysis of these components. Capillary electrophoresis has also been used recently for the determination of amino acids, bitter acids, carbohydrates, peptides, phenolic acids, proteins and vitamins in beer (Cortacero-Ramírez *et al*., 2003). Enzyme linked sorbent analysis has a limited application for protein analysis (Rasmussen, 1998).

Despite this wide array of analytical methods published before, they all have the characteristic of being applicable to the analysis of a certain group of compounds which are present in beer and are known or proposed to be partially responsible for certain characteristics. None of these methods, as such, can be applied for the simultaneous determination of a wide range of compounds and therefore are no suited to be used to obtain a profile of the extremely complex matrix presented by beer. An efficient method should provide a chemical fingerprint i.e., covering a broad range of metabolites. Such a method would be very useful for the quality control of beer and for the evaluation of the impact of any change in the production process. Such a method should be simple and fast – ensuring in this way the absence of any deterioration of sample components – and reproducible in order to be used at any time and any place as a tool to evaluate different samples. These requirements are true for all analytical methods, but especially for a method in which not all the detected compounds are necessarily identified, with a highly reproducible method these compounds might be identified at a later stage. Nuclear magnetic resonance (NMR) spectroscopy is such a techniques that meet these requirements. Taking into account the considerations mentioned above regarding the possible variables that affect the quality, such as the ratio of certain components, the quantitative information provided by NMR is better than that provided by all other methods mentioned as it is the only one in which the intensity of all the signals is directly correlated to the molar concentration. It means that the amount of all compounds can be directly compared, eliminating the need for calibration curves for each individual compound. NMR also has the great advantage of high reproducibility as the spectra are based on the physical properties of a molecule.

^{1}H NMR spectroscopy has recently been evaluated for beer analysis (Duarte *et al*., 2002; Duarte *et al*., 2004). In those reports, the NMR method showed the potential to cover a broad range of compounds as a rapid and informative quality control tool. However, despite the undoubted attraction of NMR, the complexity of the ^{1}H NMR spectra is a drawback for its use in the analysis of beer. The complexity of the resulting spectrum makes any attempt to identify the components very difficult. In fact, the ^{1}H NMR spectrum of beer shows a predominance of strongly overlapping signals arising from carbohydrates and amino acids (Duarte *et al.*, 2002). This problem encountered with the application of one-dimensional (1D) ^{1}H NMR can be solved in part by two-dimensional (2D) NMR methods. Diverse pulse sequencing techniques make it possible to develop numerous 2D NMR methods. Among the available two-dimensional NMR methods, the use of *J*-resolved spectroscopy considerably increases the resolution of the signals with short measuring time compared to other 2D NMR methods (Viant, 2003). In the *J*-resolved spectrum the spin – spin couplings are dispersed along the second axis. These additional variables largely improve the resolution of the NMR spectrum of a mixture.

The purpose of this study is to apply the 2D *J*-resolved NMR spectroscopic method to evaluate its potential as a method for differentiation of beer. For this, six kinds of pilsner beer were analyzed by NMR spectroscopy combined with principal component analysis.

7.2. MATERIALS AND METHODS

7.2.1. Samples

Six different brands of commercial pilsner beer were obtained from the domestic market in the Netherlands.

7.2.2. Solvents and chemicals

First grade chloroform, ethyl acetate, and 2-butanol were purchased from Merck Biosolve Ltd. (Valkenswaard, the Netherlands). $CDCl_3$ (99.9%), D_2O (99.9%), and dimethylsulfoxide-d_6 (99.9%) were purchased from Cambridge Isotope Laboratories Inc. (Miami, FL, USA). Methanol-d_4 (99.8%) and NaOD were obtained from Cortec

(Paris, France). Hexamethyldisiloxane (HMDSO) and trimethylsilane propionic acid sodium salt (TSP) were obtained from Merck (Darmstadt, Germany).

7.2.3. Preparation of samples for NMR

Sample preparation was carried out using two different methods. For direct measurement, beer samples were sonicated for 15 minutes for their degasification. One hundred microliter of D_2O containing 0.01% (w/w) TSP were added to 900 μL of the degassed sample and transferred to a NMR tube.

For liquid–liquid fractionation, 50 mL of a degassed sample was transferred to a 250 mL-flask. Fractionation was carried out by extractions with 50 mL portions of chloroform, ethyl acetate and 2-butanol, vortexing for 1 minute in each case. The organic fractions were obtained by decantation after 1 hour equilibrium, collected separately in a 250 mL-round bottom flask and taken to dryness with a rotary vacuum evaporator at room temperature.

7.2.4. NMR measurements

^1H NMR and *J*-resolved spectra were recorded at 25 °C on a 400 MHz Bruker AV-400 spectrometer operating at a proton NMR frequency of 400.13 MHz. Each ^1H NMR spectrum consisted of 128 scans requiring 10 minutes acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 30 (4.0 μs), and relaxation delay (RD) = 5.0 s. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zero-filled to 32 K points. The resulting spectra were manually phased and baseline corrected, all using XWIN NMR (Version 3.5, Bruker).

Two-dimensional *J*-resolved ^1H NMR spectra were acquired using 8 scans per 32 increments that were collected into 16 K data points, using spectral widths of 5208 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin–spin coupling constant axis). A 1.0 s relaxation delay was employed, giving a total acquisition time of 14.52 minutes. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions prior to double complex FT. *J*-resolved spectra tilted by 45°, symmetrized about F1, and then calibrated, all using XWIN NMR (Version 3.5, Bruker). Data were exported as the 1D projection (F2 axis) of the 2D *J*-resolved spectra. ^{1}H–^{1}H-correlated spectroscopy (COSY) spectra were acquired with 1.0 s relaxation delay, 4194 Hz spectral width in both dimensions. The heteronuclear multiple bond correlation (HMBC) spectra were obtained with 1.0 s relaxation delay, 4401 Hz spectral width in F2 and 14500 Hz in F1.

The spectra were referenced to the solvent signal of $CDCl_3$ at δ 7.26, MeOD at δ 3.30, DMSO-d_6 at δ 2.49 for the organic solvent extracts and trimethylsilane propionic acid sodium salt (TSP) at δ 0.00 for aqueous extracts.

7.2.5. Data analysis

The ^{1}H NMR and the *J*-resolved projection spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.40–10.00. The region of δ 4.70–5.10 was excluded from the analysis because of the residual signal of water. Principal component analysis (PCA) were performed with the SIMCA-P software (v. 10.0, Umetrics, Umeå, Sweden) using mean centered scaling method. Dimension of data matrices was 241 × 18.

7.3. RESULTS AND DISCUSSION

7.3.1. Evaluation of different sample preparation methods.

Direct ^{1}H NMR measurement of beer seems to be a rapid and informative analytical tool compared to other conventional methods as it provides a clear and characteristic profile. However, most of the compounds detected in the ^{1}H NMR spectra were aliphatics and sugars, while aromatic signals which significantly influence beer quality showed relatively low intensity (Fig. 7.1A). Moreover, broad and overlapping signals resulted in difficulties in the identification of compounds in the spectra.

Liquid–liquid partition was attempted to partly purify and concentrate the aromatic compounds and thus to increase the signal intensity. Chloroform, ethyl acetate and 2-butanol were evaluated as partitioning solvents according to the efficiency for the extraction of aromatic compounds. It proved for 2-butanol to be the most adequate: its extracts exhibited the highest intensity and best resolution of aromatic signals in the NMR (Fig. 7.1B). However, the signals still showed considerable overlapping. The congestion of NMR signals was finally solved by applying 2D *J*-resolved NMR spectroscopy.

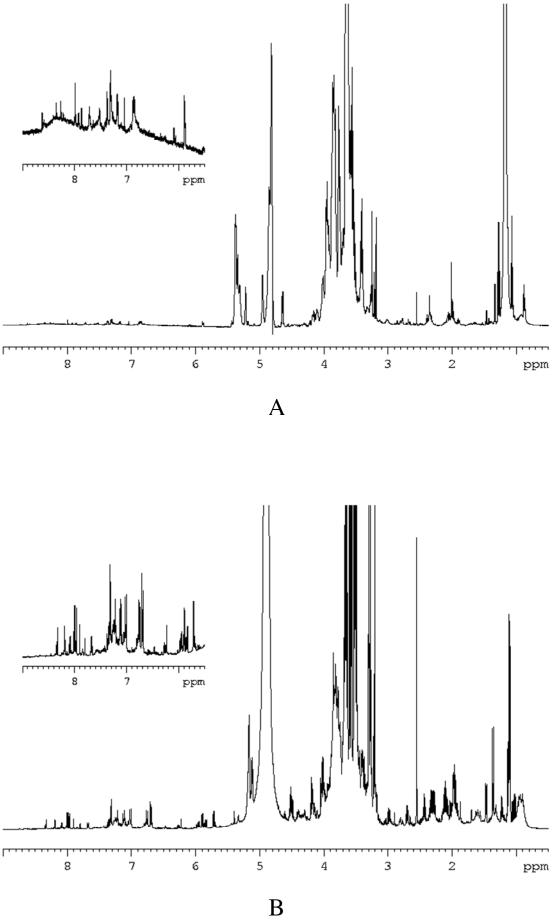
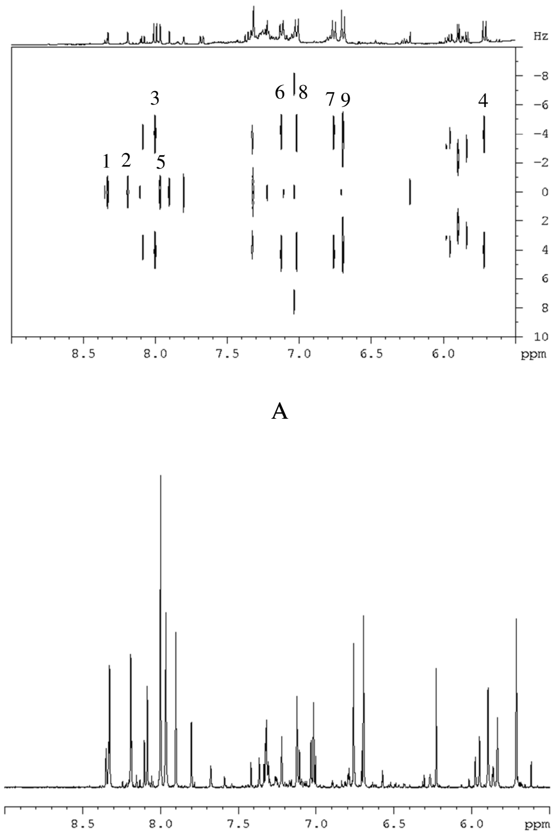


Fig. 7.1. 400 MHz ^{1}H-NMR spectrum of beer obtained by direct measurement (A) and 2-butanol extract (B) in the range of δ 0.5 – δ 9.0. Expanded spectra cover the δ 5.5 – δ 9.0 range.

Projected *J*-resolved spectra were recorded in order to reduce the complexity of ^{1}H NMR spectra (Fig. 7.2), as all protons ideally appear as a singlet in the *J*-resolved spectra using the projected spectrum on the axis of chemical shift (just like ^{13}C NMR spectra showing a fully H decoupled spectrum). The resulting enhanced resolution made it possible to directly compare and match the spectra obtained from different samples. The projected *J*-resolved spectra were applied to discriminate the beer samples through principal component analysis.



A

B

Fig. 7.2. 400 MHz 2D J-resolved (A) and its projected (B) spectra of 2-butanol extract of beer in the range of δ 5.5 – δ 9.0. 1; H-2 of adenine, 2; H-5 of adenine, 3; H-3 of uridine, 4; H-2 of uridine, 5; H-2 of xanthine, 6; H-2 and H-6 of tyrosine, 7; H-3 and H-5 of tyrosine, 8; H-2 and H-6 of tyrosol, 9; H-3 and H-5 of tyrosol.

7.3.2. Identification of minor compounds in beer using NMR

The identification of the compounds in the beer samples was performed using ^{1}H NMR, *J*-resolved, COSY and HMBC spectra. Chemical shifts and coupling constants of the compounds are summarized in Table 7.1. and the chemical structures are shown in Fig. 7.3.

Table 7.1. ^{1}H–NMR chemical shifts and coupling constants of the compounds identified in pilsner beer.

Compound	Proton number	Chemical shift (ppm)	Multiplicity [1]	Coupling constant (Hz)
Adenine	2	8.32	s	-
	5	8.19	s	-
Uridine	3	8.00	d	8.0
	2	5.72	d	8.0
	1’	5.90	d	
Xanthine	2	7.90	s	-
Tyrosine	2,6	7.12	d	8.5
	3,5	6.76	d	8.5
Tyrosol	2,6	7.02	d	8.5
	3,5	6.70	d	8.5
	7	3.05	t	5.1
Carbohydrates		3.00-4.00		
Cholines	*N*-Methyl	3.20, 3.28	s	-
Succinic acid	2,3	2.56	s	-
Proline	2	4.03	m	-
	3	2.43, 2.12	m	-
	4	2.00	m	-
Lactic acid	2	4.18	q	7.0
	3	1.36	d	7.0
Isopropanol	1,3	1.12	d	8.0

[1] s; singlet, d; doublet, t; triplet, q; quartet, m; multiplet.

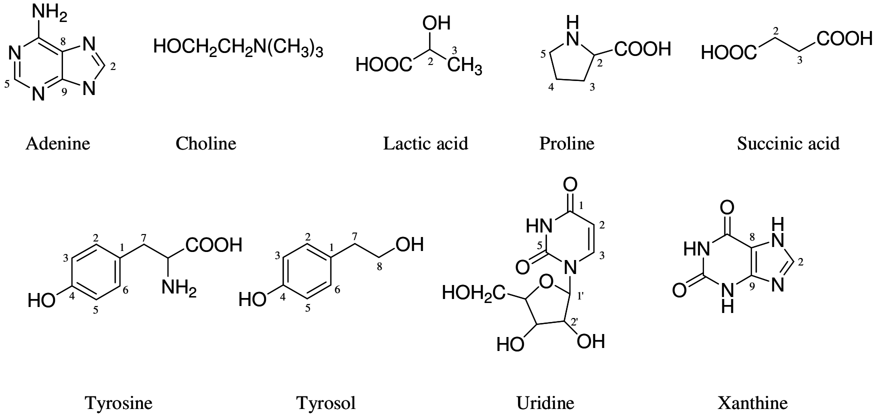


Fig. 7.3. Chemical structures of compounds identified in beer

The signals in the aromatic region were assigned to adenine, uridine, xanthine, tyrosine and tyrosol. The characteristic signals of adenine and xanthine are detected around δ 8.0. Adenine shows two singlets (H-2 and H-5) at δ 8.32 and 8.19 which can be confirmed by the *J*-resolved spectrum (Fig. 7.2A). H-2 correlates with C-8 (δ 120.6) and C-9 (δ 149.9) in the HMBC spectrum (Fig. 7.4). The correlation between H-5 and C-10 at δ 157.2 is also clearly detected in the HMBC spectrum. Xanthine shows a singlet at δ 7.90 correlating with C-8 at δ 108.5 in the HMBC spectrum. Two doublets (J = 8.0 Hz) at δ 8.00 and 5.72 are assigned to H-3 and H-2 of uridine, respectively, which is confirmed by the *J*-resolved spectrum. In the HMBC spectrum, the correlations of H-3 with the carbonyl group at δ 166.3 and C-5 at δ 152.5 of the uracil moiety and with C1′ of the D-ribofuranose moiety at δ 90.4 are detected as well (Fig. 7.4). Two doublets (J = 8.5 Hz) at δ 7.02 and 6.70 are typical resonances of 1,4-disubsituted benzene ring and were assigned to tyrosol by COSY, *J*-resolved and HMBC spectra. Other characteristic doublets at δ 7.12 and 6.76 were assigned as those of tyrosine. The HMBC spectra show the correlation between those doublets of tyrosine with C-1 (δ 131.6) and C-4 (δ 157.8) of tyrosine, and between the doublets of tyrosol with C-1 (δ 130.8) and C-4 (δ 156.6) of tyrosol. In addition, the correlations of H-7 at δ 3.05 of the ethanol moiety of tyrosol with C-1 and C-8 at δ 75.9 are also clearly detected.

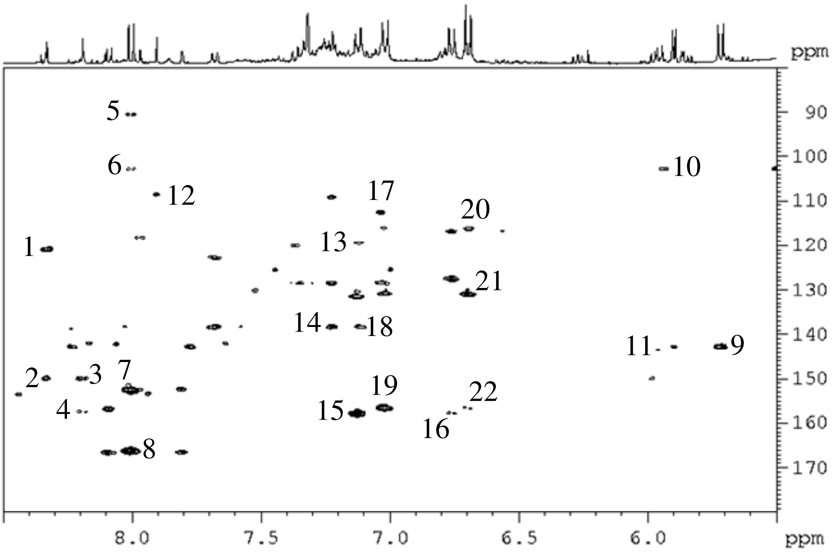


Fig. 7.4. HMBC spectrum of 2-butanol extract of beer in the aromatic region. 1; H-2/C-8 of adenine, 2; H-2/C-9 of adenine, 3; H-5/C-9 of adenine, 4; H-5/C-10 of adenine, 5; H-3/C-1' of uridine, 6; H-3/C-2 of uridine, 7; H-3/C-5 of uridine, 8; H-3/C-1 of uridine, 9; H-2/C-3 of uridine, 10; H-1'/C-2' of uridine, 11; H-1'/C-3 of uridine, 12; H-2/C-8 of xanthine, 13; H-2 and H-6/C-3 and C-5 of tyrosine, 14; H-2 and H-6/C-2 and C-6 of tyrosine, 15; H-2 and H-6/C-4 of tyrosine, 16; H-3 and H-5/C-4 of tyrosine; 17; H-2 and H-6/C-3 and C-5 of tyrosol, 18; H-2 and H-6/C-2 and C-6 of tyrosol, 19; H-2 and H-6/C-4 of tyrosol, 20; H-3 and H-5/C-3 and C-5 of tyrosol, 21; H-3 and H-5/C-2 and C-6 of tyrosol, 22; H-3 and H-5/C-4 of tyrosol.

In the aliphatic region, cholines, succinic acid, proline, lactic acid and isopropanol are identified in the NMR spectra of 2-butanol extracts of beer. Choline analogues show a singlets at δ 3.20 and 3.28 correlating with C-1 (at δ 67.0 and 68.8) and C-2 (δ 53.9 and 54.7) in HMBC spectrum. Succinic acid has a singlet at δ 2.56 which correlates with the carbonyl group at δ 176.5 in the HMBC spectrum. Multiplets at δ 2.43 and 2.12 were assigned to H-2 and H-3 of proline. Other signals of the pyrrolidine moiety of proline are also observed in the ^{1}H NMR except H-5 which overlapped with other signals in the sugar region. Proline was confirmed by the correlation between H-3 and H-2 of proline with the carbonyl group at δ 174.4 in the HMBC spectrum. Lactic acid shows the quartet of H-2 at δ 4.18 and the doublet of H-3 at δ 1.36 in ^{1}H and *J*-resolved NMR spectra. Both signals correlate with a carbonyl group at δ 179.0 in the HMBC spectrum. The doublet at δ 1.12 which correlate to δ 73.0 in the HMBC spectrum was identified as isopropanol. All of these assignments were confirmed by the NMR spectra of reference compounds.

7.3.3. PCA of beer sample

Among the multivariate techniques, principal component analysis (PCA) is one of the most widely used methods. The concept of PCA is to describe the variance in a set of multivariate data in terms of a set of underlying orthogonal variables (principal components). The original variables (metabolite concentrations) can be expressed as a particular linear combination of the principal components (Eriksson *et al.*, 2001). The principal components can be displayed graphically as a score plot. This plot is useful for observing any groupings in the data set. PCA models are constructed using all the samples in the study. Coefficients by which the original variables must be multiplied to obtain the PC are called loadings. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Eriksson *et al.*, 2001; Massart *et al.*, 2001). Thus, for NMR data, loading plots can be used to detect the compounds responsible for the separation in the data. Generally, this separation takes place in the first two or three principal components (PC1, PC2 and PC3).

Fig. 7.5A and B show the comparison between the score plots of PCA based on entire range of ^{1}H NMR spectra and projected *J*-resolved spectra using PC1 versus PC3. Eight components explained 99.9% of the variance, and the first three components explained 93.1% (^{1}H NMR spectra) and 89.3% (projected *J*-resolved spectra). In the score plot, the similarity of the samples with respect to the intensity of chemical shift correlates with their grouping pattern. The separation of beer samples dramatically increase in the score plot using projected *J*-resolved spectra. All of the samples are clearly separated.

The score plot using PCA based on ^{1}H NMR spectra can only separate beer sample 6 from samples 1–5. Loading plot indicates that the signals responsible for the differentiation are at δ 3.50–3.70 (carbohydrates), δ 3.20 and 3.28 (choline analogues), δ 2.56 (succinic acid), δ 2.00 (proline), δ 1.36 (lactic acid) and δ 1.12 (isopropanol) (Fig. 7.5C).

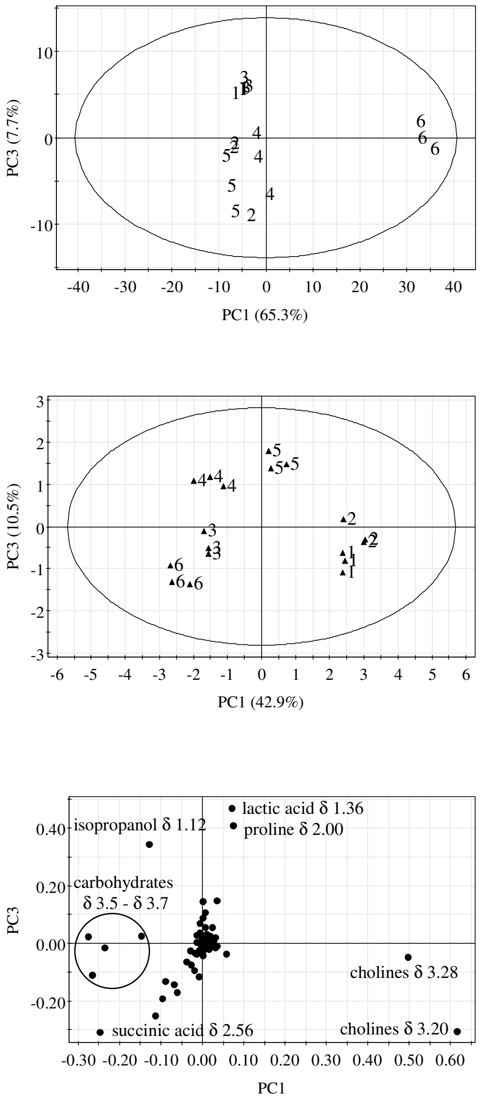


Fig. 7.5. Score plot of principal component analysis of 2-butanol extract of beer using PC1 vs PC3 based on ^{1}H NMR spectra (A), projected J-resolved NMR spectra (B), and loading plot based on projected J-resolved NMR spectra (C) using entire range of the spectra (δ 0.4 – δ 10.0).

Samples 1–2 have more cholines and less isopropanol, carbohydrates and succinic acid than samples 3–6. Whereas samples 4–5 have more lactic acid, proline and isopropanol but less succinic acid and cholines than samples 1–3 and sample 6. The non-volatile organic acids, such as succinic acid and lactic acid are both malt-derived by-products of yeast during fermentation. Succinic acid has little effect on the beer flavour except for acidity. On the other hand, lactic acid emerges as the most important non-volatile organic acid in beer since it is the only one present in beer in the above-threshold amounts (Charalambous, 1981; Hardwick, 1994). Proline is always at a high level in beer and influences the colour of final beer through the maillard reaction during wort boiling. The browning reaction is believed to be caused by maltol which is produced by the reaction between proline and maltose (Charalambous, 1981).

Using the PCA analysis based on the entire range of projected *J*-resolved NMR spectra no significant contribution is observed by the aromatic signals due to their low intensity compared to that of sugars or aliphatic compounds. In order to evaluate their effect on the differentiation, PCA was applied only to the aromatic part of the spectra. Fig. 7.6A shows the score plot thereby obtained using PC2 versus PC3. An eight component model explained 99.9% of the variance while the first three components explained 94.9%. All the beer samples are also well separated. The loading plot (Fig. 7.6B) shows signals of adenine at δ 8.32 and 8.19; uridine at δ 8.00; xanthine at δ 7.90; tyrosine at δ 7.12 and tyrosol at δ 7.02 and 6.70 were found to be the aromatic compounds responsible for the differentiation. Samples 3 and 6 were found to contain more tyrosol but less uridine and tyrosine than the other samples. While samples 1–2 have more uridine, xanthine and tyrosol but less adenine than other samples.

Those aromatic compounds responsible for the differentiation are believed to contribute the quality of beer. Nucleic acid derivatives, purines (adenosine, guanosine, adenine, hypoxanthine and xanthine) and pyrimidines (cytosine, cytidine, uridine, thymine and uracil) area all known to contribute to beer flavour (Charalambous, 1981; Hardwick, 1994). The content of amino acids affects the flavour of the beer through their participation in a variety of biochemical pathways during fermentation. Tyrosol is also an important component of the bitter flavor of beer with a flavor threshold of 200 mg/L. Above this level, tyrosol causes a harsh, lingering bitter aftertaste considered to be undesirable in beers (Hardwick, 1994). Unfortunately the presence of iso-α-acids can not be detected. A further work required optimize the detection of these acids. From this example we can see the power of *J*-resolved spectroscopy which can differentiate the samples and detect the compounds responsible for the quality of beer. This method will be useful for the product development and quality control.

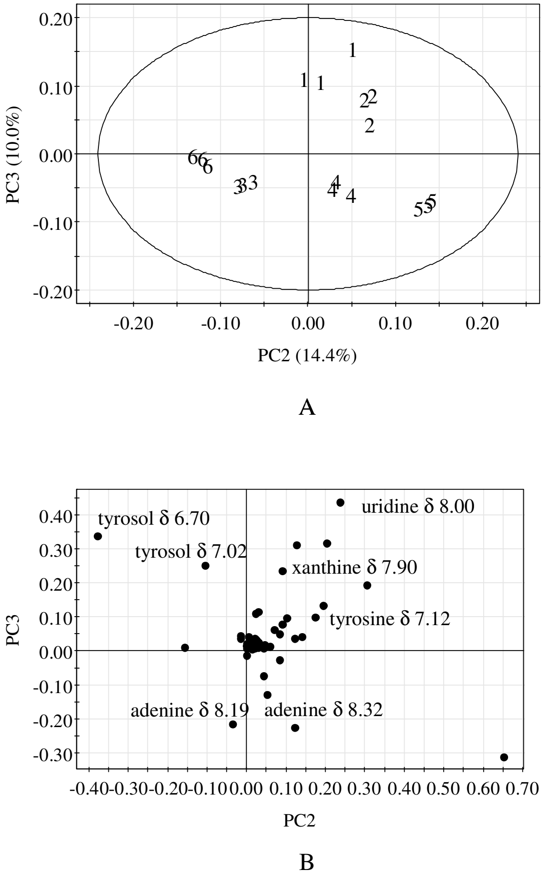


Fig. 7.6. Score plot of principal component analysis of 2-butanol extract of beer using PC2 vs PC3 (A) and loading plot (B) based on aromatic region of projected J-resolved NMR spectra (δ 6.0 – δ 10.0).

7.4. CONCLUSION

The fractionation of beer samples by liquid–liquid partition using 2-butanol provided a significant increase in the intensity of aromatic compounds in the NMR spectra. PCA analysis using projected *J*-resolved spectra for the beer extracts shows increased separation of beer samples compare to conventional ^{1}H NMR spectra. The increased resolution obtained from *J*-resolved spectra also provided further information on the structures of the compounds in beer.

Based on the chemical profiling obtained from the 1D and 2D NMR spectra it was found that adenine, uridine, xanthine, tyrosine, proline, succinic acid, lactic acid, tyrosol, isopropanol, cholines together with the most abundant compounds, the carbohydrates are responsible for the differentiation of the pilsner beers evaluated in this study. Unfortunately the presence of iso-α-acids can not be detected. Further experiment to optimize the detection of iso-α-acids is required.