Nuclear Magnetic Resonance methodology for the analysis of regular and non-alcoholic lager beers

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ABSTRACT

The presence of seven main agents responsible for beer aroma and taste (*n*-propanol, isobutanol, 3-methylbutanol, tyrosol/tyrosine, ethyl acetate, isoamyl acetate, and acetaldehyde) is determined by different NMR techniques (^{1}H PRESAT, zTOCSY, HSQC, and HMBC) in five regular and five low- or free-alcoholic beers. The new methodology includes the identification of the ^{1}H and ^{13}C NMR chemical shifts of the analytes by a standard addition method, and the consequent identification of the compounds studied in regular and non-alcoholic beers. The chemical composition is different depending on whether the beer is regular or non-alcoholic**,** therefore affecting the organoleptic characteristics of each type of beer.

Keywords: lager beer, NMR, non-alcoholic beer, free-alcoholic beer, beer compounds

1. INTRODUCTION

Beer is an alcoholic drink obtained by fermentation of a starch-rich wort coming from cereal grain such as malted barley, wheat, maize and rice. According to the fermentation process, beers are classified as top or high, and bottom or low fermentation beers. Lagers, the most consumed type of beer, are produced by ‘‘low” fermentation, which is carried out under refrigeration (usually between 6 and 15 ºC). After fermentation, yeast cells deposit at the bottom of the fermenter and are usually removed. In contrast, ale type beers are produced by ‘‘high” fermentation, occurring between 16 and 24 ºC. (Bamforth, 2003).

Although the main steps of their processing are common, there is a wide variety of lager beers with pronounced differences among them. However, several differential aspects in the composition and organoleptic characteristics among the different lager styles may be established. These differential features depend both on the raw material used, and on the parameters employed in the subsequent steps of the production processes. Thus, some taste defects in alcohol-free beer come from the alcoholic absence (Blanco et al. 2014; Andrés-Iglesias et al. 2016).

Each country has established its required alcohol by volume (ABV) maximum thresholds, which are diverse. In the United States, alcohol-free beer (AFB) means that there is not any alcohol present, while the upper limit of the so-called non-alcoholic beer or ‘‘near-beer’’ is 0.5% ABV. However, in most of the EU countries beers with low alcohol content are divided into free-alcohol beers, which contain less than 0.5% ABV, and low-alcohol beers, with less than 1.2% ABV (Brányik et al. 2012). In Spain, beers with low alcohol content have less than 3% ABV, whereas non-alcoholic beers must contain no more than 1% ABV. Commercially, non-alcolholic beers are divided into "0,0%", which is the label for those beers containing less than 0.1% alcohol; and "free", where the alcohol content must not exceed 1% (R. D. 53/1995).

Nowadays, low-alcoholic beers are generating an increasing technological and economic interest. Some of the dealcoholization processes so far described (vacuum distillation, reverse osmosis, evaporation, fermentation control...) expose the beer to severe conditions. This may cause the loss of the original aroma, since the chemical and/or physical processes when high temperatures are used may transform the original aroma compounds. Hence, the sensorial quality of the final brew may be distinct from the original one, what is not recommended, since the success of low alcohol drinks lies on an aroma profile as close as possible to the original/alcoholic brew (Sohrabvandi et al. 2010).

Recently, the consumption of low- and alcohol-free beer is being significantly increased. This may be explained considering health reasons, safety rules at the workplaces or driving, or even strict social regulations. Moreover, alcohol consumption is completely forbidden by law in some countries (Sohrabvandi et al. 2010).

As fresh flavour is one of the most appreciated sensory characteristics of beer (Bravo et al. 2008), flavour stability is one of the main quality criterias for beer, and as well a concern for the brewing industry (Caballero et al. 2012; Moreira et al. 2013).

Beer aroma profile is made by many volatile organic compounds at very low concentration (ppm level), which are responsible for its unique flavor (Catarino et al. 2007). Levels of different chemical compounds, such as alcohols, esters, aldehydes, ketones, organic acids and phenols, can be found on beer composition, giving a specific flavor that contributes to the overall organoleptic properties of the final beer (Karlsson and Trägårdh 1997). Among them, esters and alcohols are the main groups of aroma compounds.

The sensorial evaluation of the beer organoleptic characteristics, such as color, taste, appearance, flavor, and aroma is the usual method to evaluate the beer quality control. Some analytical measurements, such as through photometry (for color and bitterness), enzymatic analyses (for organic acids), and gas chromatography (for higher alcohols) should contribute also to this evaluation (Bamforth 2003).

At present, traditional analytical reference methods tend to be replaced by others faster and more economical. Screening methods seem to be the most advantageous for this purpose, since they guarantee a very high sample throughput. Conventional methods are often focused on the analysis of few specific components, but in contrast, Nuclear Magnetic Resonance (NMR) spectroscopy enables to register most of the constituents of the foodstuff in a single experiment (Lachenmeier et al. 2005). Thus, the advantages of NMR is the rapid information which provides, when compared to other common analytical tools, such as high pressure liquid chromatography, gas chromatography or mass spectrometry (Marcone et al. 2013).

The combination of mass spectrometry analysis with multivariate statistical analysis as a suitable method to find out differential metabolites between regular and non-alcohol beers has been previously reported by us (Andrés-Iglesias, Blanco, Blanco and Montero, 2014). We have also described a simulation program which predicts the flavor compounds present in beer once dealcoholized via vacuum distillation (Andrés-Iglesias et al. 2015). Here we are extending these studies by using NMR spectroscopy in order to recognize the differences between regular and non-alcohol beers.

87 Nowadays, NMR is a leading technique (Mattaruchi et al. 2010), which is being applied to a wide
88 range of liquid and solid matrices. The main advantages of NMR are: easy sample preparation (sample
89 degassing and occasional pH adjustment were not necessary and were not used, even though both
90 methods have been previously reported), and rapid analysis, thus envisaging potential industrial
91 applications. This technique also enables to carry out a rapid and non-invasive characterisation of foods
92 and beverages, and therefore provides information about the compounds therein present (Belton et al.
93 1996; Monakhova et al. 2012). However, compound quantification remains less than straightforward
94 application in complex mixtures such as foodstuffs, even though NMR is a quantitative technique. This
95 may be solved by the traditional method of NMR signals integration vs. the signal area of a reference
96 compound, an approach previously described for vinegars (Caligiani et al. 2007), wines (Lopez-Rituerto
97 et al. 2009), juices (Berregi et al. 2007) and beer (Almeida et al., 2006; Petersen et al., 2013). However,
98 using internal references for quantification in complex mixtures has potential difficulties, such as signal
99 overlapping or formation of chemical interactions between the reference and the sample components.
100 Both may lead to changes of the integration with subsequent erroneous quantification.
101 Therefore, extensive compositional information in just a few minutes (Duarte et al. 2002) and
102 automation or low injection technology (Lachenmeier,et al. 2005) are the main advantages of NMR
103 technology. The analysis method has been already adapted for routine beer analysis, and high-resolution
104 NMR and hyphenated NMR (LC-NMR and LC-NMR/MS) have enabled to establish a significant
105 database of compounds found in beers, with particular emphasis on carbohydrates (Duarte et al. 2003)
106 and aromatic compounds (Gil et al. 2003). This technique has also found broad applications in the wine
107 industry (Giménez-Miralles et al. 1999; Ogrinc et al. 2001), and has now emerged as an important tool for
108 wine quality control. In the case of beer, ethanol and water D/H ratios have been measured by deuterium
109 NMR, and have been correlated to beer quality parameters, such as the beer geographical origin
110 (Rossmann 2001), environmental factors (Franconi et al. 1989), or characteristics of the raw materials and
111 of the brewing process (Franconi et al. 1989). In brewing science, NMR has mostly been applied so far to
112 solve specific problems, such as the identification and quantitation of malt and hop constituents, like
113 polyphenols (Friedrich and Galensa 2002) or isohumulones (Nord et al. 2003).
114 NMR spectroscopy may give a direct and fast overview of the chemical composition of beer (Duarte
115 et al. 2002), which can be obtained without any pre-treatment, aside from degassing. Assigning signals in
116 mono- and bidimensional NMR spectra from beer samples has facilitated the identification of *ca*. 30

compounds, including organic acids, amino acids, and alcohols, or even higher molecular weight compounds, such as lipids, and large aromatic compounds, as polyphenols. However, as indicated above, a full assignment of the beer spectra is not possible, mainly due to strong signal overlap, even though techniques like diffusion-ordered spectroscopy (DOSY) have been used for this purpose (Gil et al. 2004)

We have found only one previous report where non-alcoholic beers, along with ales and lager beers, are included. The study describes the combination of NMR and FTIR data to provide information about different factors affecting beer production (Duarte et al. 2004). However, no previous studies have been performed on the specific use of NMR to differentiate between regular and no- alcohol beers. In this work, this new approach focuses on the compounds, mainly alcohols and esters, which are responsible for the characteristic flavour of regular beer. Thus, high-resolution NMR spectroscopy is here used to identify the presence of selected compounds in different commercial alcoholic and low- or non-alcoholic beers.

2. Materials and methods

2.1. Beer samples

Ten different commercial national lager beers, five regular beers, labeled as A, two 0.0% beers (1B, 4B) and three alcohol-free (2B, 3B, and 5B) were used for this study (see Table 2). All analyses were carried out from newly opened bottles.

2.2. Sample preparation and spectra measurement.

2.2.1. Sample preparation

Sample preparation (both for the standard addition method and for test samples) was carried out as follows: a 500 µl aliquot of commercial beer was transferred to a 5 mm NMR tube, and then 50 µl of D_2O was added for internal lock. The samples thus prepared were then used without any additional treatment. One sample per beer was analyzed Degasification was not performed, as we have verified that the NMR spectra of degassed samples and of non-degasified samples were exactly the same. The absolute value lock deuterium was used as reference for the chemical shifts.

NMR spectra were recorded on an Agilent DD2 500 instrument equipped with cryoprobe, operating at 499.81 MHz for ^{1}H and at 125.69 MHz for ^{13}C.

All experiments were performed at 25ºC using the ^{1}H PRESAT pulse sequence by selective low-power irradiation in order to suppress both the water (4.71 ppm), and the ethanol (3.46 and 0.99 ppm) signal resonances. Obviously, for free-alcohol beers, only the residual water resonance was irradiated.

Homonuclear 2D experiment zTOCSY (TOtal Correlation SpectroscopY) with a zero- quantum filter was used for artifact suppression (Trippleton and Keeler, 2003). Heteronuclear 2D experiments HSQC (Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation) with gradient coherence selection CRISIS (Hu and Krishnamurthy, 2008) having BIP (Broadband Inversion Pulse) pulses in both ^{13}C and ^{1}H channels were used. These homo- and y heteronuclear 2D experiments allowed to determine unequivocally the ^{1}H and ^{13}C chemical shifts.

In order to completely identify the signals, a standard addition method was designed previously to record the sample beer tests. The acquisition parameters for these two experiments are different, since sample tests required a higher number of transients to be sensitive, whereas fewer transients are required for the standard addition method, since the target compounds are more concentrated. The acquisition parameters are summarized in the following paragraphs.

2.2.2. Standard addition method

A 50 µl aliquot of a $5 \cdot 10^{-2}$ M ethanolic solution of the compound to be determined was added to a sample of 1A. This method was used for all the compounds collected in Table 2, except for tyrosol/tyrosine and acetaldehyde, which could be assigned directly from the spectra.

Each 1D PRESAT ^{1}H spectrum was recorded with 16 transients, a spectral width of 8012 Hz, 16384 acquired points, and an acquisition time of 2.044 s. Selective irradiation at water and ethanol frequencies during the recycle delay was carried out during 2 s.

zTOCSY experiments were acquired in the phase sensitive mode with PRESAT solvent suppression. A total of 4 transients for each of the 300 t1 increments were collected, using a spectral width of 8012 Hz for both dimensions, with a mixing time of the DIPIS2 spin lock of 100 ms. The data were apodized with gaussian functions window in both dimensions.

Gradient CRISIS2 HSQC (gc2hsqc) were recorded with inverse detection and carbon decoupling during acquisition in the phase sensitive mode with PRESAT solvent suppression. A nominal value of 146 Hz was used for one-bond coupling constants J_{CH}. A total of 8 transients for each of the 256 t1 increments were collected, using spectral widths of 8012 Hz in the F2 dimension, and 25133 Hz in the F1 dimension. The data were apodized with gaussian functions windows in both dimensions.

2.2.3. Sample tests

Each 1D PRESAT ^{1}H spectrum was recorded with 128 transients, a spectral width of 8012 Hz, 16384 acquired points, and an acquisition time of 2.044 s. Selective irradiation at water and ethanol frequencies during the recycle delay was carried out during 2s.

zTOCSY experiments were acquired in the phase sensitive mode with PRESAT solvent suppression when necessary. A total of 12 transients for each of the 200 t1 increments were collected, using a spectral width of 5707 Hz for both dimensions, with a mixing time of the DIPIS2 spin lock of 100ms, and an acquisition time of 300 ms. The data were apodized with gaussian window in both dimensions.

Gradient CRISIS2 HSQC (gc2hsqc) were recorded with inverse detection and carbon decoupling during acquisition in the phase sensitive mode with PRESAT solvent suppression when necessary. A nominal value of 146 Hz was used for one-bond coupling constants J_{CH}. A total of 32 transients for each of the 200 t1 increments were collected, using spectral widths of 5707 Hz in the F2 dimension, and of 25133 Hz in the F1 dimension. The data were apodized with gaussian functions windows in both dimensions.

Gradient CRISIS2 HMBC (gc2hmbc) were recorded with inverse detection and no carbon decoupling was applied during acquisition with PRESAT solvent suppression when necessary. A nominal value of 8 Hz was used for the multi-bond coupling constant J^{n}_{CH}. A total of 52 transients for each of the 256 t1 increments were collected, using spectral widths of 5707 Hz in the F2 dimension and of 30165 Hz in the F1 dimension. The data were apodized with sinebell window in the F2 dimension and gaussian function window in the F1 dimension.

Forward linear prediction was employed to improve digital resolution in the F1 dimensions of all 2D experiments. The resulting spectra were processed and manipulated using VnmrJ3.2 Agilent Software.

3. Results and Discussion

3.1. Experimental design

Hundreds of compounds may be detected in the ^{1}H NMR spectra of beers and other natural products. Although NMR-based urine metabolic profiling has been recently used with in order to identify and quantify a wide range of compounds (Emwas et al. 2015), the identification of individual components in beer samples is very difficult due to significant signal overlapping.

Figure 1 shows PRESAT ^{1}H NMR spectra of a regular beer (above) and an alcohol-free (below). In the regular beer spectrum the aliphatic region of the spectra (0-3 ppm) shows signals arising from alcohols (e.g. propanol, isobutanol, isopentanol), organic acids (e.g. citric, malic, pyruvic, acetic, succinic), amino acids (e.g. alanine, γ-aminobutyric, proline), and fatty acids. The contribution of fermentable sugars (e.g. glucose, maltose), and dextrins (glucose oligomers with different degrees of polymerization and branching) is observed in the midfield region (3-6 ppm). The aromatic region (6-10 ppm) shows the presence of aromatic amino acids (tyrosine, phenylalanine, tryptophan), nucleosides (cytidine, uridine, adenosine/inosine), aromatic alcohols (2-phenylethanol, tyrosol/tyrosine, tryptophol), and polyphenolic compounds. The latter give rise to underlying broad humps between 6.7 and 8.7 ppm (Lachenmeier et al. 2005).

Comparing the whole PRESAT ^{1}H NMR spectra of regular beer and alcohol-free beer, the different pattern displayed by each type of beer can be observed. Alcohol-free beers (Figure 1, below) display more signals at the 3-4 ppm region, which indicates a higher concentration of carbohydrates (sugars), and fewer signals at 1-2 ppm, where the aliphatic protons of alcohols resonate. As indicated above, the assignment of the signals of all the compounds present is not possible, except for a handful of compounds.

Some acids, acetic, succinic and pyruvic, could be readily identified from literature reports (Almeida *et al*, 2006; Duarte, Godejohann, Braumann, Spraul and Gil, 2003; Rodrigues and Gil, 2011; Rodrigues *et al*, 2011). These acids are generated from metabolic by-products or intermediates excreted by yeast cells. The assignment made could not be completed, since the two doublets observed at ca. 6.7 and 7.0 ppm may be assigned to the *para*-substituted phenyl group protons of either tyrosol or tyrosine (Almeida et al 2006; Rodrigues et al. 2011). In this case, two-dimensional (2D) NMR spectra do not help to overcome this uncertainty. Figure 2 collects the PRESAT ^{1}H NMR spectra of samples 1A and 1B divided in the three representative regions of the spectra, showing the signals of some the above mentioned compounds.

Given the complexity of the spectra, we decided to focus our work on the esters and higher alcohols, which are the main agents responsible for their aroma and taste in lager beers. The concentration of these compounds is very different depending on whether the beer is regular or non-alcoholic, thus affecting their organoleptic characteristics (Montanari et al. 2009). Table 1 collects these compounds, as well as the main features of their aromas (Kobayashi et al. 2010; Olmedo et al. 2014; Tian 2009).

Therefore, the main objective of the work is the identification of these compounds in the beers chosen for this study. Standard addition methodology has been established as a rather common practice in metabolic NMR studies for different foodstuffs (Beckonert et al. 2007). Herein, a standard addition method, supported by mono- and bidimensional spectra, has allowed the identification of these compounds in the beer samples. Once the signals of these compounds were identified, their presence in regular and non-alcoholic beers was determined. However, a realistic quantification of the compounds in the sample is precluded by significant signal overlapping, which makes impossible a reliable integration.

3.2. Standard addition method

A preliminary assignment of the main peaks may be proposed by using either existing predicting NMR data (Pretsch et al. 2001) or ChemBioDraw14.0 software. However, a more accurate determination had to be carried out, since the magnetic field and the deuterated solvent described in these sources are different from our experimental conditions. In our case, the chemical shifts should also be affected by the complexity of the matrix.

PRESAT ^{1}H NMR, zTOCSY, and HSQC experiments were recorded for samples where an aliquot of each compound had been added (see Experimental Section). Figure 3 shows the PRESAT ^{1}H NMR spectra of sample 1A before and after adding an aliquot of n-propanol. Thus the signals of the methyl and methylene groups can be identified.

This assignment was confirmed by two dimensional spectra. Figure 4 shows the zTOCSY experiment, relating the signals of the aliphatic protons, whereas the HSQC experiments are displayed in Figure 5, allowing the assignment of the ^{13}C NMR signals.

Table 1 displays the ^{1}H and ^{13}C NMR chemical shifts of the molecules subjected to this study in the complex beer matrix. They were obtained as described above for n-propanol in a sample of 1A after adding aliquots of the other molecules, except tyrosol/tyrosine and acetaldehyde, which could be assigned directly from the spectra. The latter is immediately assigned by the quartet at 9.49 ppm , but as indicated above, tyrosine could not be differentiated from tyrosol, even after two-dimensional spectra. When the chemical shifts of the targeted molecules were determined, we were ready to study their presence in regular and non-alcoholic beers.

3.3. Identification of the compounds in regular and non-alcoholic beers

The presence of *n*-propanol in the sample of regular beer 1A is detected in the HMBC spectra (Figure 6, left), whereas its absence is evident in the HMBC spectra of the non-alcoholic beer 1B (Figure 6,

right). HMBC spectra are the key experiments to unequivocally assign the signals, since they display correlations of atoms related by several bonds, and therefore the probability of different molecules displaying similar correlations is very rare. On the other hand, the possibility of spectral overlaps decreases, as the correlations occur through all the spectrum. The data supplied by HMBC spectra were also confirmed by zTOCSY experiments.

A similar method was followed to determine the presence or absence of the rest of compounds collected in Table 1, for all the Lager beers studied. The results obtained are shown in Table 2.

The results obtained show that alcohols such as n-propanol, isobutanol, 3-methylbutanol, or tyrosol/tyrosine, were detected in regular beers. The latter was also present in all non-alcoholic beers.

The analysis of the data obtained for non-alcoholic beer show that the three alcohol-free beers show a similar behavior; since n-propanol was not detected, although it was detected in the 0,0 beer 4B. Isobutanol is present in the three alcohol-free beers, and detected in one of the 0,0 beers (again 4B). Finally, 3-methylbutanol is present in the alcohol-free beers, but absent in 0.0 samples

As indicated above, this method is unable to differentiate tyrosol from tyrosine. Tyrosol is an intermediate product of microbial tyrosine metabolism and hence its presence in beer is chiefly attributable to the action of the yeasts on the tyrosine present in beer wort. Therefore the amount of tyrosol present in the non-alcoholic beer can be used to distinguish between beers produced by elimination of alcohol and those beers produced by restricted alcohol fermentation method.

As regards the esters studied, isoamyl acetate was not detected in any of the beers studied, whereas ethyl acetate is present in the regular beers, and absent in all the non-alcoholic beers.

Acetaldehyde is present in all the regular and alcohol-free beers, but absent in the 0.0 beers.

Finally, it should be reminded that the taste of beer depends not only on the content of its compounds, but also of their ratio. The optimum higher alcohols-to esters ratio for lagers according to Smogrovicova (2004) is from 4.1 to 4.7. The absence of esters in non-alcoholic beers analyzed modifies this proportion and therefore the flavor of these beers will be affected.

5. Conclusions

A new methodology, based on the use of NMR, allows to determine the presence of key compounds responsible for flavor or aromas in regular and non-alcoholic beers. All the alcohols studied are present in regular beers. Only one of the 0.0 beers studied (1B) contains none of the alcohols, whereas the other 0.0

beer (4B) does contain the same alcohols as any of the regular beers. Alcohol-free beers contain isobutanol and/or 3-methylbutanol. None of the beers studied contain isoamyl acetate, whereas ethyl acetate is present in regular beers and absent in all the non-alcoholic, which can be due to its removal during dealcoholization process. Acetaldehyde is present in all regular and alcohol-free beers, but absent in 0.0 beers. We believe that these results are very valuable for brewers and researchers in this field who seek to improve the quality of non-alcoholic beers.

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Compliance with Ethical Standards

Conflict of interest

Cristina Sánchez-Estébanez, Sergio Ferrero, Celedonio M. Alvarez, Fernando Villafañe, Isabel Caballero and Carlos A. Blanco declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors

Informed Consent

Informed consent was obtained from all individual participants included in the study as authors of it. Although this article does not contain any study-experiment with human participants.

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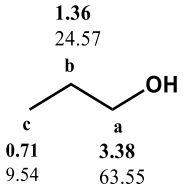
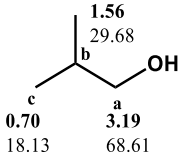
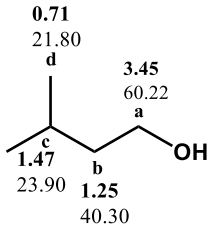
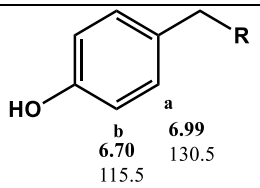
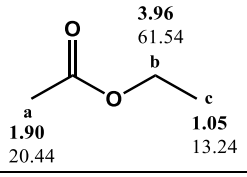
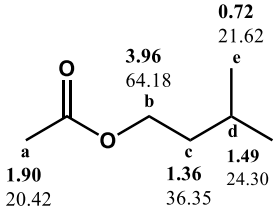
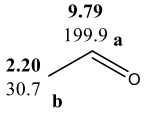
441 **Tables**

442 Table 1. Main compounds responsible for the aromas with their description, and ^{1}H and ^{13}C NMR
443 signals of each molecules (in bold above, and plain below, respectively), recorded on samples of 1A.
444

Type	Compound	Aroma	Structure and chemical shifts
Alcohols	*n*-Propanol	Alcohol, ripe fruit	
	Isobutanol	Alcohol, wine, nail polish	
	3-methylbutanol	Oil, alcohol, wine, banana	
	Tyrosol	Bitter	 R = CH_2OH (Tyrosol) $CH(NH_2)CO_2H$ (Tyrosyne)
Esters	Ethyl acetate	Sweet, fruit	
	Isoamyl acetate	Sweet, fruit, banana	
Aldehydes	Acetaldehyde	Unripe fruit, winery, mold	

445

446 Table 2. Identified compounds in the beers studied (+)

447
448

Beer	ABV	n-propanol	Iso-butanol	3-methyl-butanol	Tyrosol (or Tirosine)	Ethyl acetate	Isoamyl acetate	Acetal-dehyde
1A	5.5	+	+	+	+	+		+
2A	5.5	+	+	+	+	+		+
3A	5.0	+	+	+	+	+		+
4A	5.4	+	+	+	+	+		+
5A	4.6	+	+	+	+	+		+
1B	0.0				+			
2B	0.8		+	+	+			+
3B	0.9		+	+	+			+
4B	0.0	+	+	+	+			
5B	<1		+		+			+

449

Figures captions

Fig. 1 PRESAT ^{1}H NMR spectra of 1A (above) and 1B (below) displayed at the same intensity.

Fig. 2 PRESAT ^{1}H NMR spectra of samples 1A (above) and 1B (below) divided in the three representative regions.

Fig. 3 PRESAT ^{1}H NMR spectra of 1A and assignation of the *n*-propanol signals: (a) 0.6-1.5 ppm; and (b) 2.9-3.65 ppm. In each part, the regular sample is shown below, whereas above is the spectra after adding an aliquot of *n*-propanol. The spectra are displayed at the same intensity, and the assignment is shown.

Fig. 4 TOCSY spectra of 1A after adding an aliquot of *n*-propanol showing the signals assigned to protons labeled as **a**, **b**, and **c**.

Fig. 5 HSQC spectra of 1A after adding an aliquot of *n*-propanol showing the crosspeaking signals assigned to proton and carbon atoms labeled as **a**, **b**, and **c**.

Fig. 6 HMBC spectra of samples 1A (left) and 1B (right) showing the presence (up: ^{13}C signal of C^{a} at 63.55 ppm correlates with ^{1}H signal of H^{c} at 0.71 ppm; bottom: ^{13}C signal of C^{c} at 9.54 ppm correlates with ^{1}H signal of H^{a} and H^{b} at 3.38 and 1.36 ppm) and the absence of n-propanol, respectively.

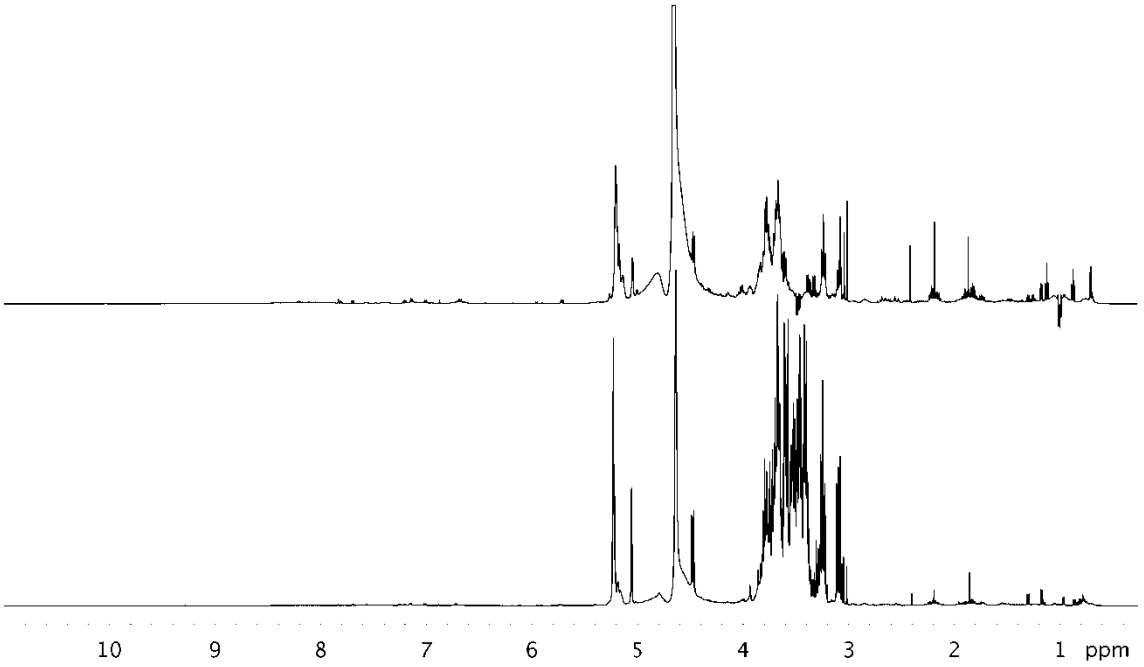
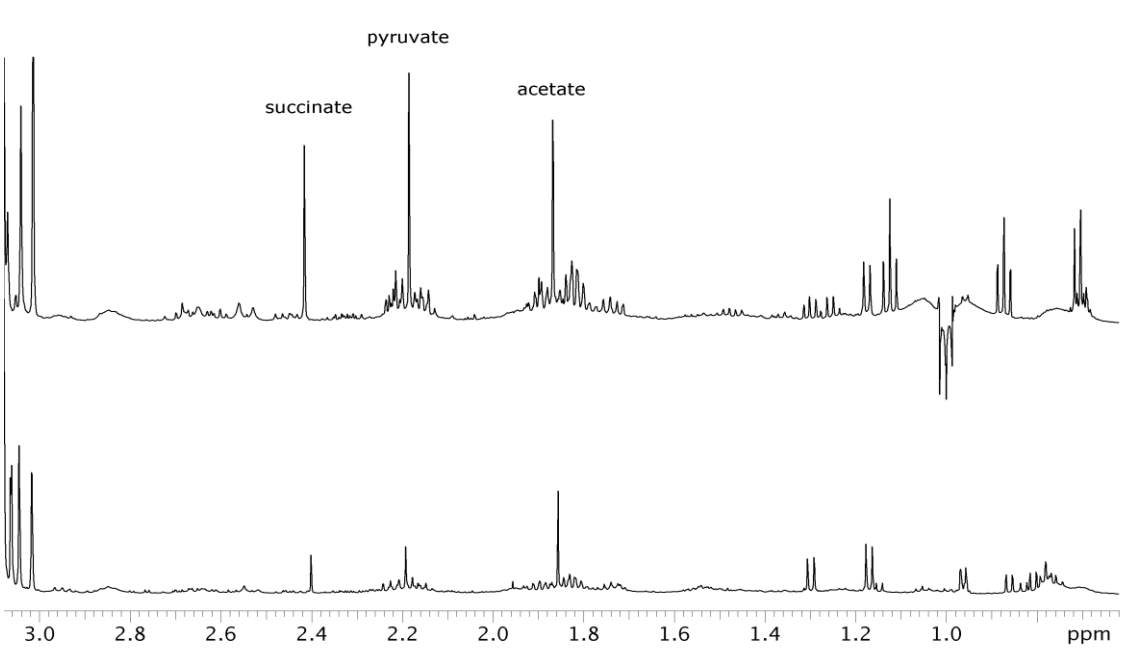


Figure 1.



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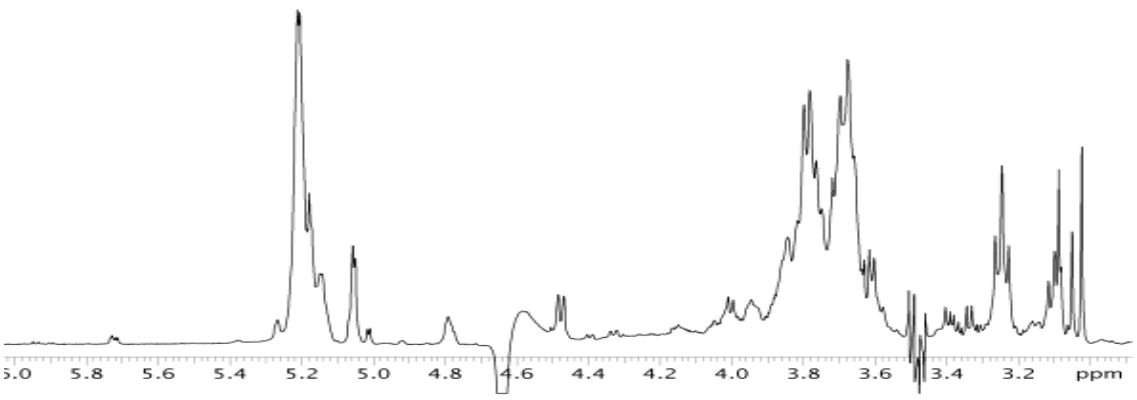

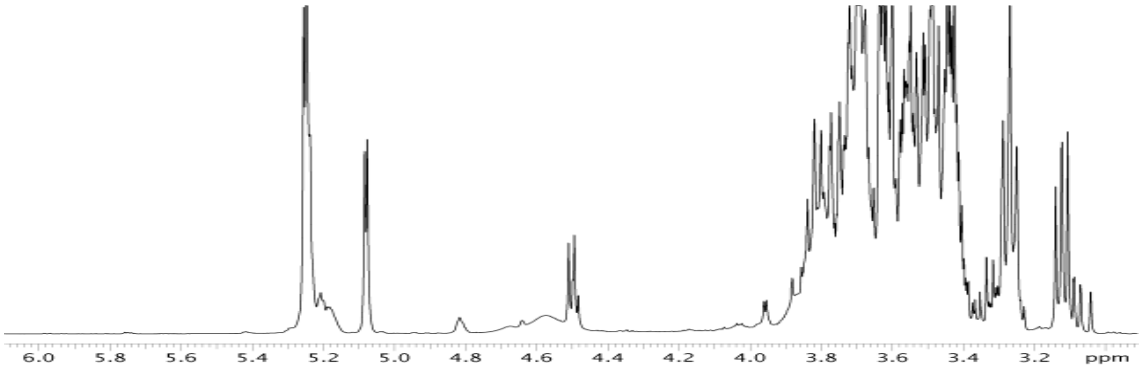

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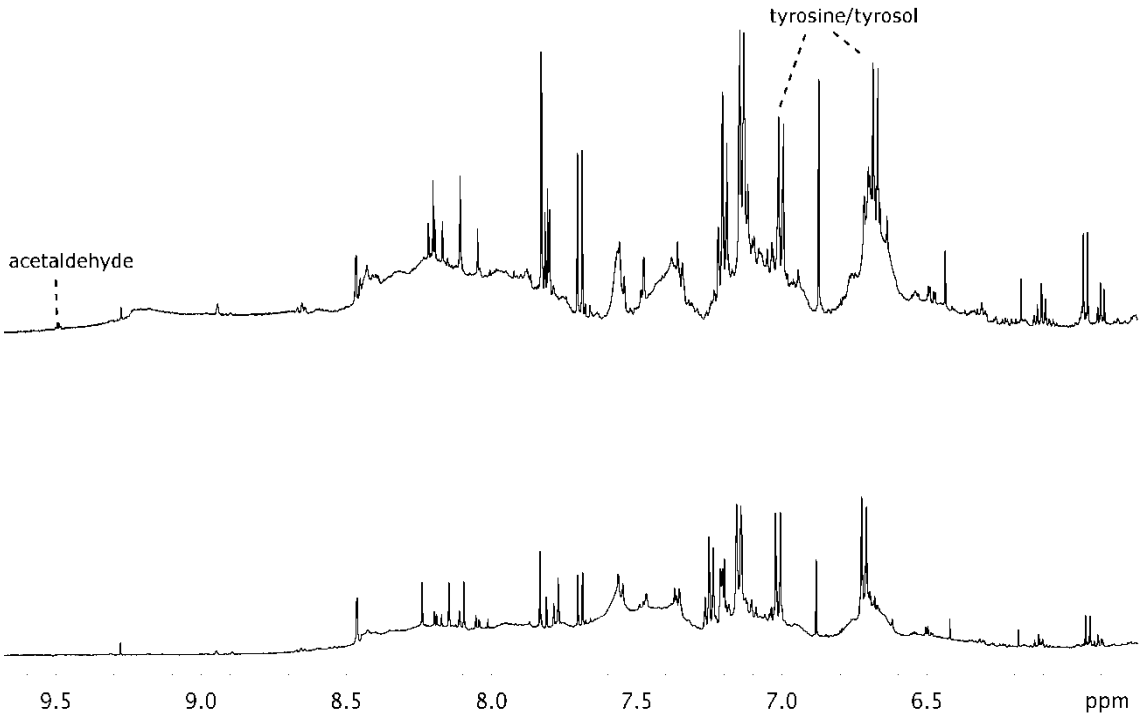

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476 Figure 2

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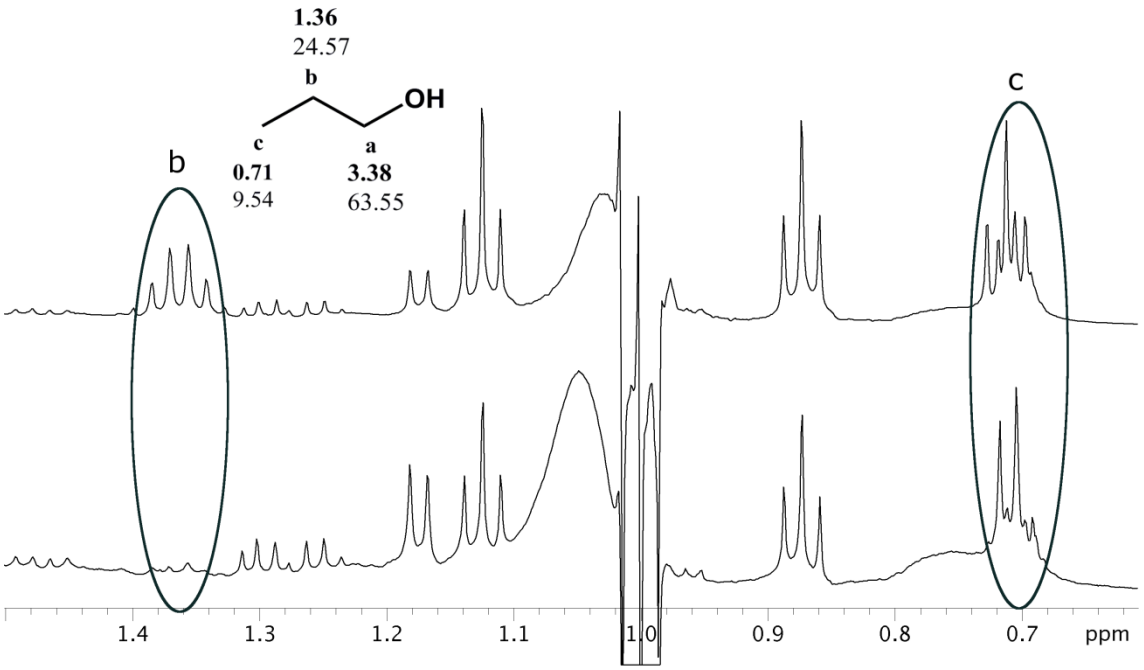
a)


1.36
24.57
b
OH
c
a
0.71
9.54
3.38
63.55
b
c
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
ppm

b)

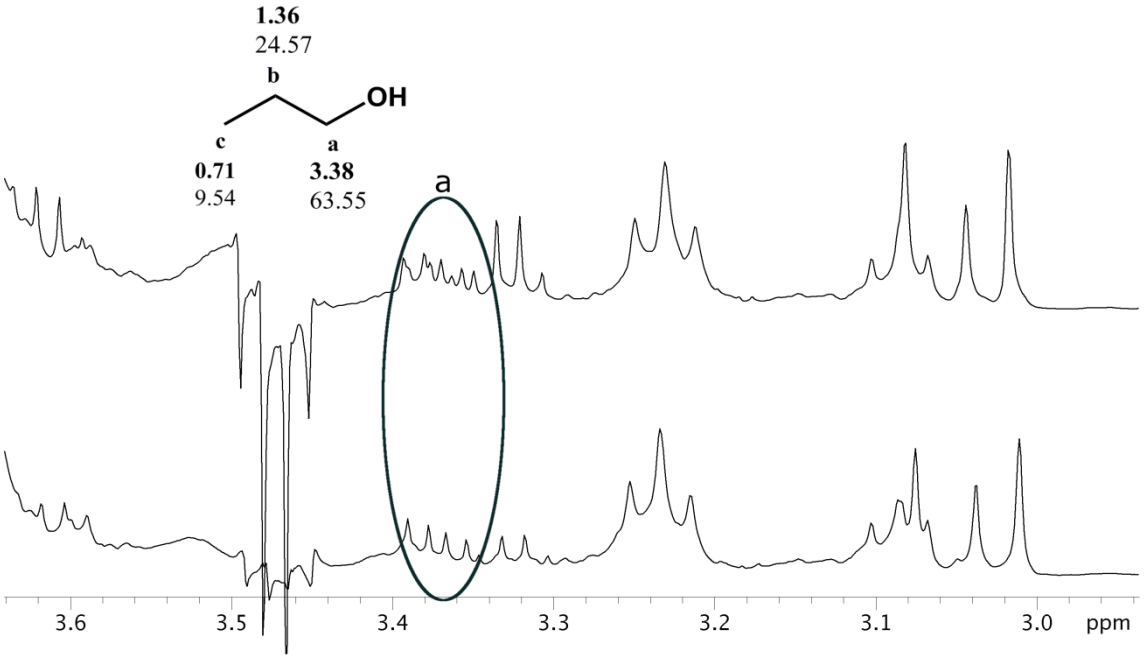

1.36
24.57
b
OH
c
a
0.71
9.54
3.38
63.55
a
3.6
3.5
3.4
3.3
3.2
3.1
3.0
ppm

Figure 3.

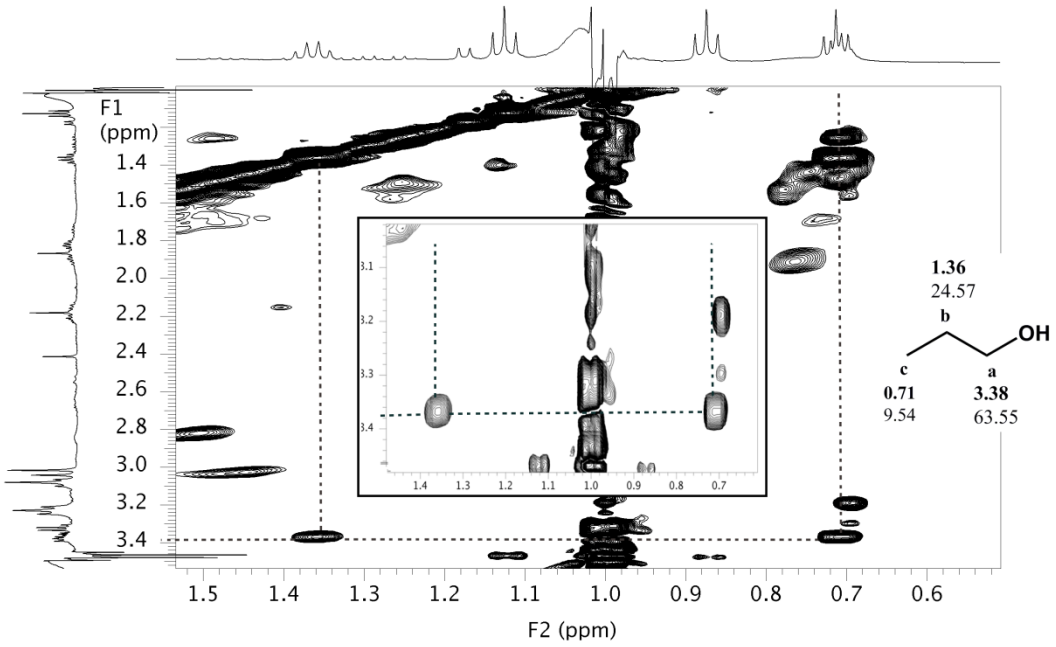

F1
(ppm)
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
F2 (ppm)
1.36
24.57
b
OH
c
a
0.71
9.54
3.38
63.55

Figure 4

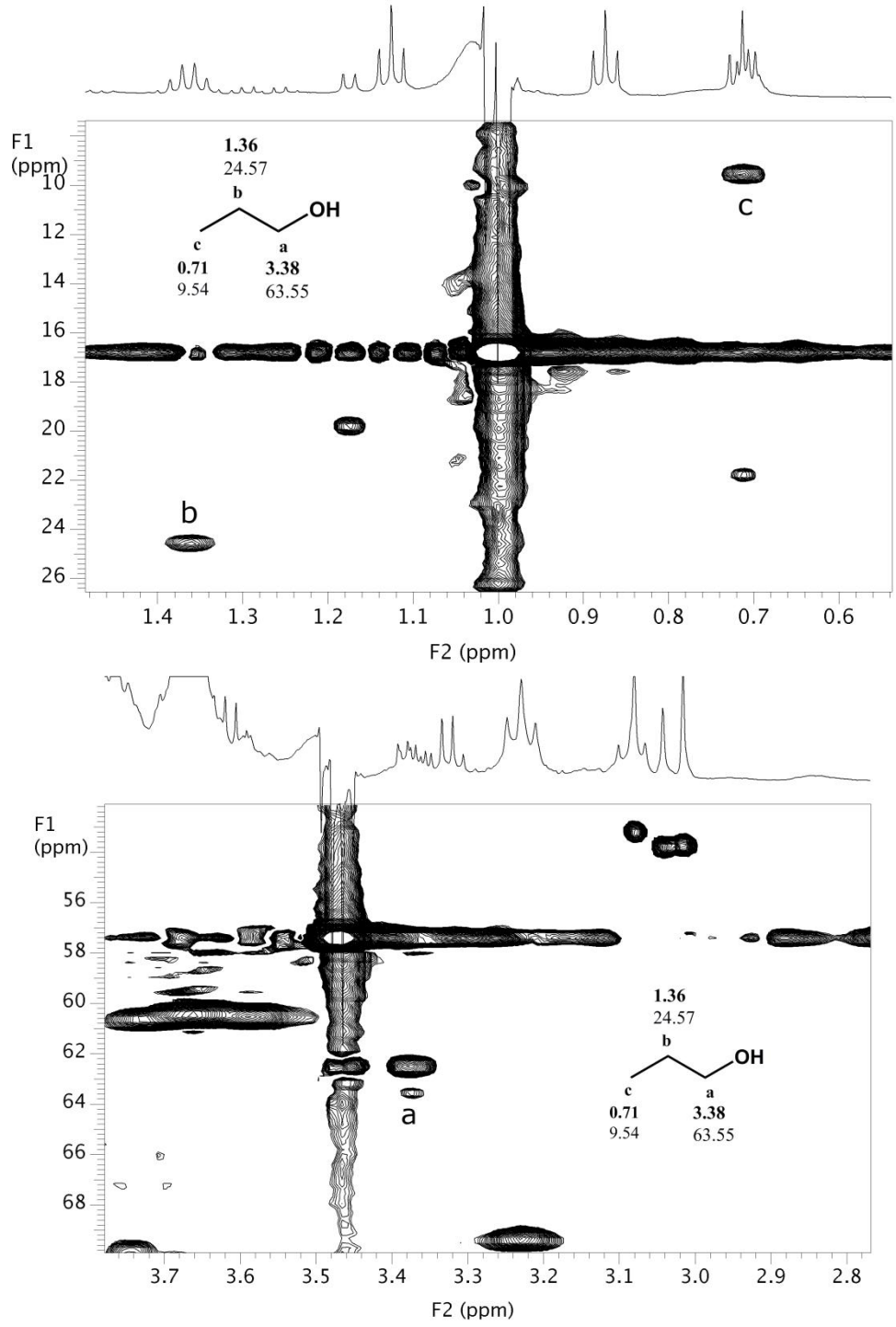
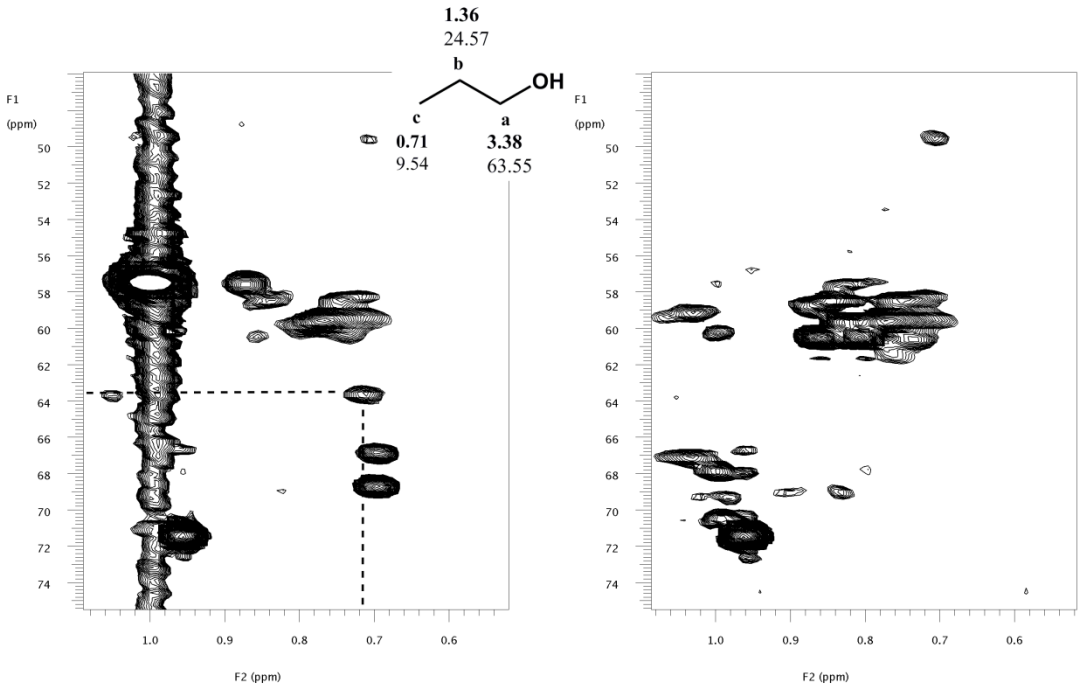
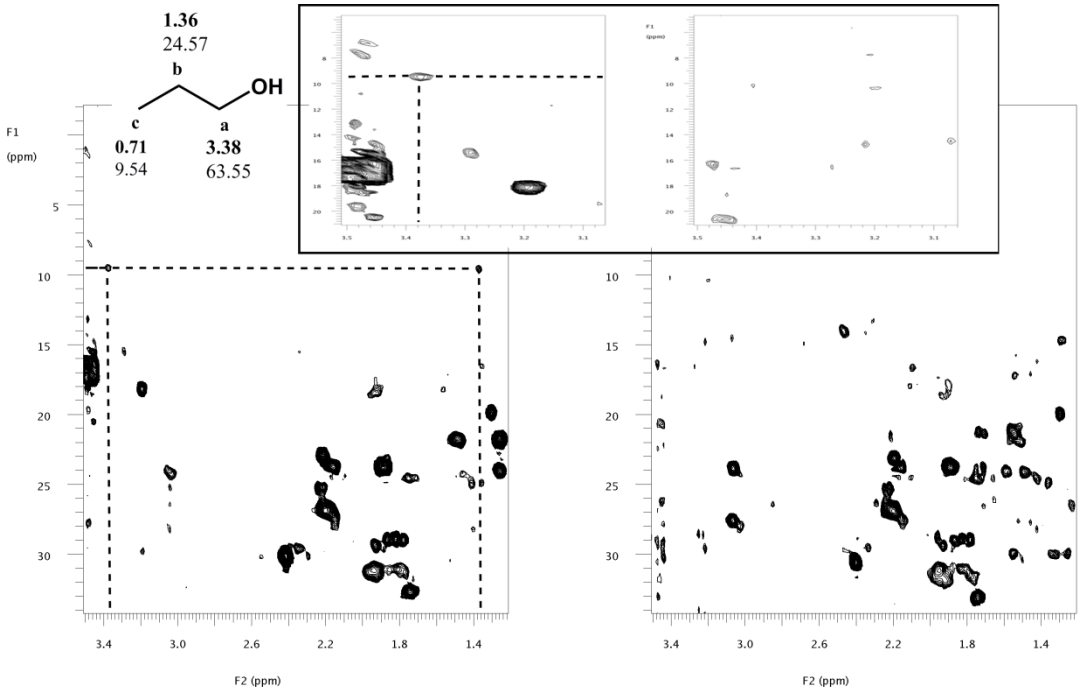


Figure 5



499



500

501 *Figure 6*

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