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Article

Nuclear Magnetic Resonance and Headspace Solid-Phase Microextraction Gas Chromatography as Complementary Methods for the Analysis of Beer Samples

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Abstract: Chemical analysis of the organic components in beers has applications to quality control, authenticity and improvements to the flavor characteristics and brewing process. This study aims to show the complementary nature of two instrumental techniques which in combination can identify and quantify the majority of organic components in a beer sample. Nuclear Magnetic Resonance (NMR) was used to provide concentrations of twenty five different organic compounds including alcohols, organic acids, carbohydrates, and amino acids. Calorie content was also estimated for the samples. NMR data for ethanol concentrations were validated by comparison to a Fourier Transform Infrared Spectrometry (FTIR) method. Headspace Solid-Phase Microextraction (SPME) Gas Chromatography Mass Spectrometry (GCMS) was used to identify a range of volatile compounds such as alcohols, esters and hop derived aroma compounds. A simple and inexpensive conversion of a Gas Chromatography Flame Ionization Detector (GC FID) instrument to allow the use of Solid-Phase Microextraction was found to be useful for the quantification of volatile esters.

Keywords: beer; nuclear magnetic resonance; solid-phase microextraction; gas chromatography

1. Introduction

Beer is the most popular alcoholic beverage in America [1]. Interest in craft beers in the United States has led to the substantial increase in small scale breweries that typically cannot afford sophisticated analytical techniques. A market potentially exists, therefore, for independent entities which have access to laboratories and expensive instrumentation to provide analytical services to breweries. Analysis of organic chemicals in beer samples has quality control and authenticity applications and can also provide information to the brewer to improve the efficiency of the process, quality of product and discover the source of problems in the system.

Beer is a complex mixture of over 800 organic molecules ranging from the ng/L to percent concentrations [2]. The major aroma and taste influencing compounds are considered to be the esters and alcohols formed during the fermentation process. Esters contribute to a beer's bouquet but can result in off-flavors when present at higher concentrations. Higher alcohols are formed in the Ehrlich pathway which proceeds when amino acids in the wort are taken up by yeast. Information about the amino acids in beer, therefore, may be used as indicators for fermentation performance. Esters are

produced as a result of yeast metabolism. Resulting ester and alcohol concentrations can provide information about wort composition, fermentation parameters, and yeast strains.

Nuclear Magnetic Resonance (NMR) is a common chemical characterization technique. The instrument is typically used to provide structural information about organic compounds. It also has the capability to generate quantitative information for components in complex mixtures. The instrument utilizes a strong magnetic field to cause the alignment of a fraction of the nuclear spins in a sample which are then capable of absorbing radio frequency radiation. The frequency absorbed depends on the chemical environment of the absorbing nucleus. The strength of absorption at a given frequency depends on the concentration of the chemical and number of nuclei in an identical chemical environment. Therefore, if the number of identical nuclei are known for a given molecule, the concentration can be determined by comparison to an internal standard of known concentration. Nord et al. [3] used proton NMR to quantify organic and amino acids in beer samples and compared their results to those obtained by High Performance Liquid Chromatography (HPLC) and capillary electrophoresis. The methods were found to be in good agreement. Duarte et al. [4] investigated multivariate analysis of NMR and Fourier Transform Infrared Spectrometry (FTIR) data as a potential tool for the quality control of beer. They applied Principal Component Analysis (PCA) to data obtained from 50 beer samples to be able to group beers to common characteristics. The same group used PCA of proton NMR data in order to be able to investigate effects of brewing site and date of production [5]. A similar approach has been reported by Lachenmeier et al. [6] for potential application to quality control of beer.

A limitation of NMR is the relatively poor detection limits. Typically sample concentrations below approximately 10 mg/L are not observable. For volatile substances at lower concentrations (such as alcohols, esters, aldehydes, ketones, and sulfur compounds) headspace capillary Gas Chromatography (GC) is commonly used. Headspace Solid-Phase Microextraction (SPME) is a simpler and less expensive alternative to static headspace. The method involves exposing a fiber to the headspace in order to concentrate the volatile analyte on the fiber surface. The analyses are subsequently thermally desorbed from the fiber in the GC inlet. SPME has been applied to a wide variety of sample types including beer. Jelen et al. [7] compared static headspace and SPME for the analysis of alcohols and esters in beers. They found the two methods to be highly correlated and suggested SPME as an inexpensive alternative to automated static headspace. Horák et al. [8] compared SPME to Stir Bar Sorptive Extraction for the analysis of selected esters in beer. They found the two methods to have similar performance with high repeatability and good linearity. Several others have demonstrated the application of SPME GCMS for the analysis of volatile compounds in beer [9-11].

In this work we demonstrate the complementary nature of NMR and headspace SPME GC for identification and quantification of important flavor and aroma compounds in beer samples. The techniques were applied to 9 beer samples of a variety of styles obtained from a small craft brewery. NMR ethanol data has also been compared to Fourier Transform Infrared Spectrometry (FTIR) results to provide validation of the NMR method.

2. Experimental

Nine beer samples were obtained from a craft brewery and stored in HDPE bottles at 4˚C prior to analysis. The characteristics of each sample are described in Table 1.

2.1 NMR Analysis

A volume of degassed beer samples (175 µL) was brought to a final volume of 750 µL with deuterated water. Exactly 10.0 mg of the internal quantitation standard in the form of maleic acid (Sigma Aldrich, St. Louis, MO, USA, 99.0%) was added to the samples. The samples were run under quantitative conditions on a Varian Mercury 300MVX NMR spectrometer equipped with a 5 mm ATB probe. Spectra were analyzed using Mnova (Mestrelab, Escondido, CA) software.

Components were quantified using the equation:

$$\text{Component mg/L} = 10 * ((I_{comp} / N_{comp}) / 50) * (MW_{comp} / 116.1) * (1{,}000{,}000 / 175)$$

Where 10 is the mass of maleic acid in milligrams, I_{comp} is the integration of component resonance, N_{comp} is the number of protons integrated, 50 is the integration of maleic acid/number of protons , MW_{comp} is the molecular weight of component molecule in atomic mass units, 116.1 is the molecular weight of maleic acid in atomic mass units, and the 1000000/175 factor rectifies the volumetric component of the calculation to allow mg/L to be calculated.

Calorie values were estimated based on total alcohol = 7 Cal/g, total carbohydrates = 4 Cal/g, total amino acids = 4 Cal/g and total organic acids = 4 Cal/g.

2.2 SPME GC Analysis

Identification and quantitation of aroma compounds in samples were accomplished by Solid-Phase Microextraction (SPME) of the headspace followed by Gas Chromatography (GC). For identification, the headspace was exposed to a Custodion Solid Phase Micro Extraction fiber (DVB/PDMS, 65µm) for 30 seconds prior to injection into a Torion T-9 GCMS (MXT-5 column, 50-296˚C at 2˚C/s with an initial hold time of 10 s and final hold time of 47 s).

For quantification of selected esters, a standard additions calibration method was employed followed by Gas Chromatography with an Agilent 5890 GC FID fitted with a DB 1 column (30 m, 0.53 mm id, 1.5 µm film thickness) and modified to accept the Custodian SPME fiber using a SPME septum (Merlin Microseal) and 19 ga nut. The fiber was exposed in the GC inlet for 10 seconds prior to starting the temperature program (injector 270°C, detector 280˚C, oven temperature: 50°C for 3 min, 10˚C/min, 280°C for 1 min).

Standard additions were prepared in 4 mL glass vials fitted with septa. Solutions were prepared by combining 1.5 mL of degassed beer sample with varying amounts of a mixed stock solution and adding deionized water to achieve a total volume of 2 mL. Solutions were capped and heated at 80˚C for 30 minutes while exposing the SPME fiber to the headspace. The mixed stock solution was prepared by combining 40 mL of 200 proof ethanol (Sigma Aldrich, St. Louis, MO, USA) with 40.0 mg of isoamyl acetate (TCI, Tokyo, Japan, >98%), 11.9 mg of ethyl caprylate (Acros, New Jersey, USA, 99%+), and 12.4 mg of ethyl caprate (Acros, New Jersey, USA, 99%+) in a 100 mL volumetric flask and making to volume with deionized water.

2.3 FTIR Analysis

Samples were prepared as for SPME GC analysis i.e. 1.5 mL of beer was measured into 4 mL glass vials and 0.5 mL of analyte solution at various concentrations added. An FTIR (Thermo Nicolet iS5) fitted with

an iD7 ATR accessory was used to measure the area of the C-O stretching band between 1064 cm^{-1} and 1030 cm^{-1}.· Ethanol concentrations were determined by a standard additions calibration method.

3. Results

3.1 NMR Results

A typical NMR spectrum of a beer sample is shown in Figure 1. The concentrations of several alcohols, organics, carbohydrates, and amino acids can be determined from a single spectrum using maleic acid as an internal standard. Concentrations were calculated by comparison of integrals, knowing the number of chemically identical protons in each compound, the number of moles of maleic acid and the volume of sample. Concentrations of major organic compounds are listed in Table 2.

3.2 SPME GC Results

Figure 2 shows a typical chromatogram of a beer sample demonstrating clear resolution of common alcohol and ester compounds allowing several aroma compounds to be identified. Table 1 shows quantitative information for three common ester compounds. Several compounds were found to be below the detection limit for the method. Further optimization of the method may be possible to produce lower detection, however, the values determined are in a typical range for beer samples [12, 13]. Highest isoamyl acetate values were detected in samples 1 and 9 which were both characterized as Belgium style ales. Lowest ester concentrations were seen for the stout, Hefeweizen and English brown styles (samples 4, 5 and 6).

3.3 Ethanol Content

In order to validate the NMR method for ethanol content, the %ABV (Alcohol By Volume) quoted by the brewery was compared to the NMR values and FTIR values determined by a standard additions method. An ANOVA test at 95% confidence suggests a significant difference between the methods. Paired t-tests at 95% confidence show significant differences between the stated values and NMR method and the stated values and FTIR results, however, no statistical difference was found between the FTIR and NMR methods. Generally the stated values were lower than NMR and FTIR values.

4. Discussion

The combination of NMR and Headspace SPME GC provides good coverage of organic compounds in beer samples. The NMR technique has the ability to measure and easily quantify multiple compounds in the mg/L to percent range in the liquid sample providing a good deal of information allowing the brewer to gain an understanding of the brewing process. In this study, 25 different compounds were measured in a single run including organic acids, carbohydrates, alcohols, and amino acids. Figure 3 provides an example of one utilization of the data. In this case, malt and lactose concentrations are shown clearly, revealing that lactose has been added as a sweetener to the stout. It also demonstrates the higher malt content of styles such as the Scotch ale, Belgium ale, and stout, compared to Patersbier, Hefeweizen, and English brown ale styles. The NMR technique also provides %ABV values and allows calorie content to be determined via a simple calculation. To validate the NMR method for %ABV, the values obtained

were compared to the values stated by the brewer and obtained by standard additions attenuated total reflectance infrared spectroscopy. The FTIR and NMR values were in good agreement which provides validation of the NMR method. Both NMR and FTIR methods provided values generally the same or higher than the stated values. This may indicate that some of these unfiltered samples continued to ferment in the barrel. The number of identifiable compounds by NMR could be increased and the detection limit lowered by freeze drying the sample.

The headspace SPME GC method is a nice complement to NMR as it provides information about volatile compounds which are present at concentrations that are too low for NMR to measure. In this work we focused on ester compounds but any volatile compound in the sub mg/L to percent range could potentially be measured. We used a GCMS instrument after headspace SPME sample preparation to identify volatile compounds. Some of the compounds identified are listed in Table 3. A relatively simple and inexpensive conversion of a GC FID instrument provides a cheaper method for identifying and quantifying compounds. In this case, however, known standards need to be available and identification is only possible by comparison of retention times. Quantification is also much more complex and time consuming than the NMR method. Initial attempts to quantify by external standards and internal standards calibration proved unsuccessful. Therefore, standard addition calibration was employed. Values obtained for three ester compounds were within expected ranges [12, 13]. Isoamyl acetate is known to be produced in higher amounts by Hefeweizen and Belgian yeast [14]. In this study, the two Belgium style beers (samples 1 and 9) did show higher isoamyl acetate values. The Hefewiezen (sample 4) however, did not show a measureable isoamyl acetate concentration. We do not have an explanation for this discrepancy. More compounds could be identified and detection limits lowered by a thorough optimization of the headspace SPME sample preparation. For example, Jeleń et al. [7] found that the addition of 28% sodium chloride had a significant effect on extraction efficiency via the salting out effect. Similarly, Horák et al. [8] found that adding 5 g of sodium chloride to their 10 mL sample improved recoveries of esters from beer. Extraction time, temperature, and sample volume could also be optimized to improve performance.

5. Conclusions

NMR and SPME GC have been demonstrated to be complementary methods in order to identify and quantify a range of organic compounds in beer samples. The information obtained can provide a host of useful information to the brewer. NMR has the capability of measuring a large number of organic compounds in the liquid phase as long as the concentrations are high enough. Lower concentration volatile compounds, such as those responsible for aroma, can be identified by headspace SPME GCMS. Quantification can be accomplished by standard additions calibration using headspace SPME with a modified GC FID instrument.

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Figure Captions

Figure 1: Proton NMR spectrum of an IPA style beer (sample 2).

Figure 2: Headspace SPME chromatogram of an IPA style beer (sample 2).

Figure 3: Comparison of malt and lactose concentrations for nine beer samples.

Table Captions

Table 1. Beer sample styles.

Table 2: Concentrations of organic compounds in beer determined by NMR.

Table 3: Concentrations of common ester compounds and other compounds identified.

Table 4: Comparison of methods for the determination of ethanol concentrations.

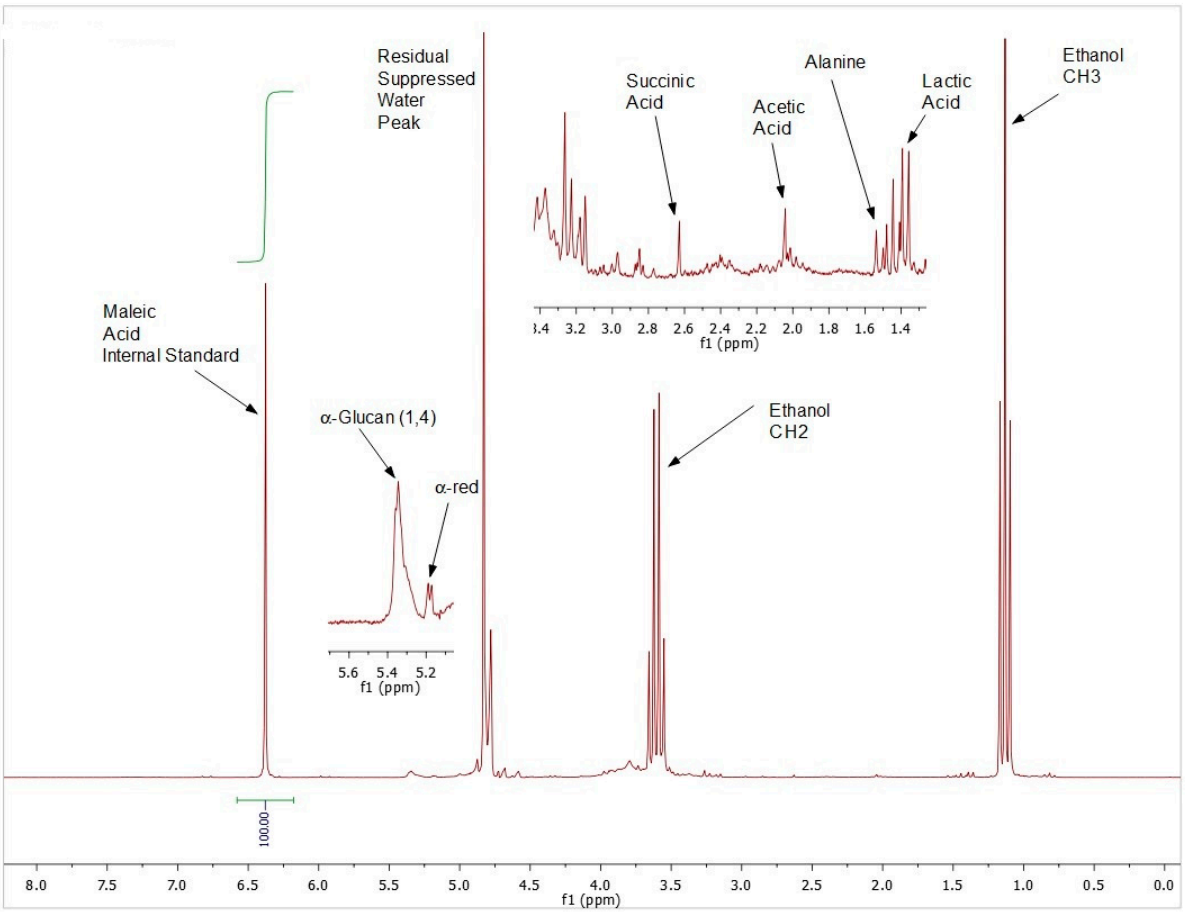

Figure 1: Proton NMR spectrum of an IPA style beer (sample 2).

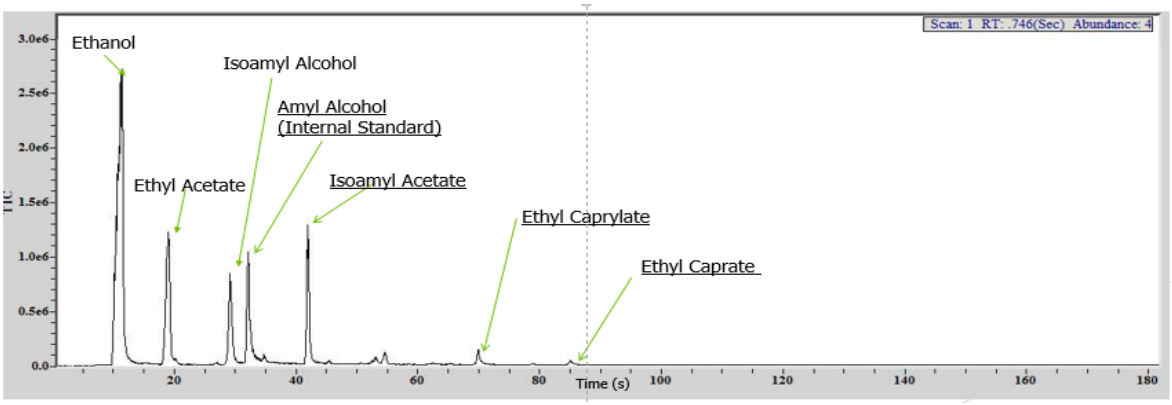

Figure 2: Headspace SPME chromatogram of an IPA style beer (sample 2).

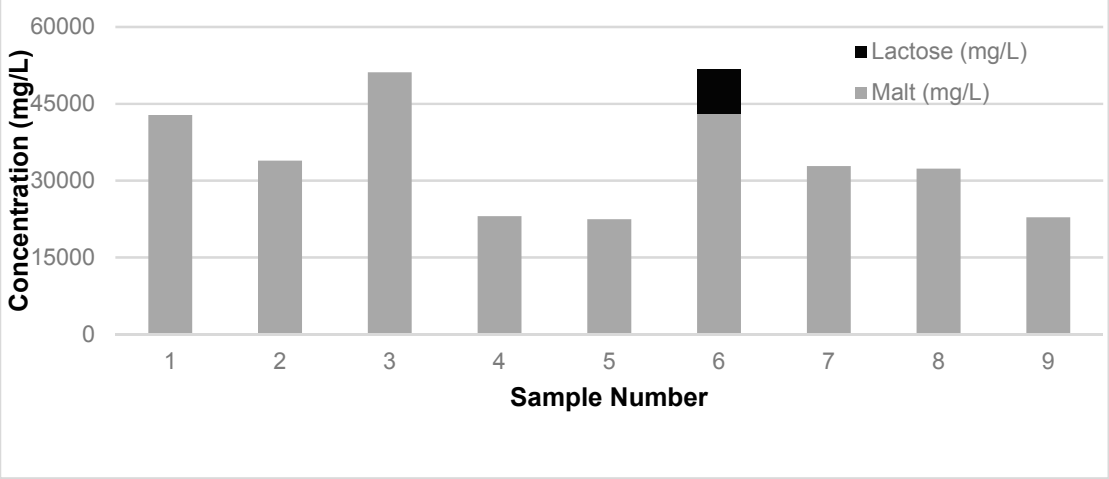

Figure 3: Comparison of malt and lactose concentrations for nine beer samples.

Table 1. Beer sample styles.

Sample Number	Beer Style
1	Belgium Ale
2	IPA
3	Scotch Ale
4	Hefeweizen
5	English Brown Ale
6	Stout
7	Double IPA
8	Amber Ale
9	Patersbier

Table 2: Concentrations of organic compounds in beer determined by NMR.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
Organic Acids									
Lactic Acid (mg/L)	553	781	470	724	623	571	839	496	316
Succinic Acid (mg/L)	299	262	161	201	98	247	201	144	448
Acetic Acid (mg/L)	135	133	205	139	119	178	111	107	88
Pyruvic Acid (mg/L)	57	97	103	94	103	83	89	74	57
Pyruvic Acid hydrate (mg/L)	0	109	103	74	126	37	123	77	54
Citric Acid (mg/L)	780	494	520	520	260	520	390	390	338
Formic Acid (mg/L)	31	0	22	0	9	40	0	0	0
Alcohols									
Ethanol (% v/v)	6.6	8.0	8.6	6.1	6.2	8.0	8.9	4.9	5.5
iso-Butanol (mg/L)	34	29	12	24	17	17	22	19	12
iso-Pentanol (mg/L)	123	126	72	77	60	77	86	49	60
1-Propanol (mg/L)	47	156	78	62	62	70	78	47	55
2,3-Butandiol (mg/L)	65	74	79	57	51	90	75	59	38
Carbohydrates									
Malt (mg/L)	42814	33910	51173	23093	22478	43165	32822	32347	22829
Lactose (mg/L)	0	0	0	0	0	8606	0	0	0

Glycerol (mg/L)	1699	2276	1667	2372	1506	2660	2724	1346	1699
Amino Acids									
Histidine (mg/L)	60	60	91	106	30	106	0	0	0
Uridine (mg/L)	24	24	95	119	24	167	214	24	143
Tryptophan (mg/L)	60	40	119	179	60	219	239	0	219
Phenylalanine (mg/L)	277	306	377	406	203	425	509	167	261
Tyrosine (mg/L)	194	247	300	380	159	362	415	203	177
Gallic Acid (mg/L)	66	50	50	0	25	83	91	0	33
GABA (mg/L)	487	643	713	668	266	558	477	362	352
Proline (mg/L)	1122	1149	1463	1274	754	1274	1301	628	870
Alanine (mg/L)	336	315	333	315	136	246	420	98	200
Valine (mg/L)	0	384	221	145	129	303	411	120	204
Cal/L	561	607	709	462	452	682	654	417	418

Table 3: Concentrations of common ester compounds and other compounds identified.

Sample	Isoamyl Acetate (mg/L)	Ethyl Caprylate (mg/L)	Ethyl Caprate (mg/L)	Other compounds identified
1	2.1	1.6	0.8	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate
2	0.2	0.2	BDL	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate, glyceraldehyde, butyl butyrate, β-pinene
3	ND	BDL	0.9	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate
4	BDL	BDL	BDL	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate, linalool
5	ND	BDL	BDL	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate
6	BDL	BDL	BDL	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate
7	1.0	BDL	0.8	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate, butyl butyrate, isoamyl propionate, β-pinene, amyl butyrate
8	0.6	0.4	0.2	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate, amyl butyrate
9	3.4	BDL	0.6	Ethanol, ethyl acetate, isoamyl alcohol, ethyl butyrate, ethyl valerate

BDL=Below Detection Limit, ND=Not Determined/Unacceptable Line Fit.

Table 4: Comparison of methods for the determination of ethanol concentrations.

Sample	Stated %ABV	FTIR %ABV	NMR %ABV
1	6.5	6.5	6.6
2	7.1	8.6	8.0
3	8.2	8.8	8.6
4	5.2	6.3	6.1
5	5.4	6.1	6.2
6	7.5	7.1	8.0
7	9.0	9.2	8.9
8	5.0	5.4	4.9
9	5.4	5.9	5.5