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Advanced Solvent Signal Suppression for the Acquisition of 1D and 2D NMR Spectra of Scotch Whisky

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Keywords: NMR, ¹H, ¹³C, complex mixture, Scotch whisky, solvent suppression, alcoholic beverages

Short Title: Signal Suppression for the Acquisition of Scotch Whisky NMR Spectra

Abstract

A simple and robust solvent suppression technique that enables acquisition of high quality 1D ¹H NMR spectra of alcoholic beverages on cryoprobe instruments was developed and applied to acquire NMR spectra of Scotch Whisky. The method uses three channels to suppress signals of water and ethanol, including those of ¹³C satellites of ethanol. It is executed in automation allowing high throughput investigations of alcoholic beverages. Based on the well-established 1D NOESY solvent suppression technique, this method suppresses the solvent at the beginning of the pulse sequence, producing pure phase signals minimally affected by the relaxation. The developed solvent suppression procedure was integrated into several homo- and heterocorrelated 2D NMR experiments, including 2D COSY, 2D TOCSY, 2D band-selective TOCSY, 2D *J*-resolved spectroscopy, 2D ¹H, ¹³C HSQC, 2D ¹H, ¹³C HSQC-TOCSY and 2D ¹H, ¹³C HMBC. A 1D chemical-shift-selective TOCSY experiments was also modified. The wealth of information obtained by these experiments will assist in NMR structure elucidation of Scotch Whisky congeners and generally the composition of alcoholic beverages at the molecular level.

Introduction

Scotch Whisky is a culturally significant and high value commodity. Chemical analysis of Scotch Whisky is therefore essential to gain a better understanding of the production processes and maturation chemistry^[1,2] as well as for addressing food safety challenges,^[3-5] and authenticity concerns.^[6-11] Scotch Whisky is produced by the fermentation of a cereal source, distillation to below 94.8% (v/v) ethanol, and maturation in oak casks in Scotland for a minimum of three years.^[12] Its production follows strict guidelines outlined in the Scotch Whisky Regulations (2009)^[13], and yet every distillery produces a unique spirit.

Traditionally, chemical analysis of whisky has used standard MS techniques, in particular GC- and LC-MS, to identify and quantify its compounds, referred to as congeners.^[12,14] These methods are labour

intensive to establish, often compound specific, and require several instruments and skilled operators to prepare the samples and interpret the results.

Lately, several studies have been carried out using high-end MS techniques to characterise whisky.^[8,15] Our recent FT-ICR MS study^[15] revealed the presence of thousands of compounds in a large set of curated whisky samples. Even though we have managed to associate over 72% of MS peaks with unique molecular formulae, the structures of these compounds and their concentrations cannot be determined by this technique, which in addition fails to detect many low molecular weight congeners prevalent in whisky samples.

NMR spectroscopy, on the other hand, represents a means to detect, identify, and quantify all major components of whisky by a single technique. It is a non-selective and non-destructive method, allowing for simultaneous profiling of different compound classes, albeit with a limited dynamic range and sensitivity. Accepting the NMR detection limits, whisky is still a complex mixture that, in addition to this complication, presents additional challenges. In common with other alcoholic beverages, whisky primarily comprises of two protonated compounds – water and ethanol. Their signals dominate the standard ^1H spectra of spirits and need to be suppressed to allow observation of important, but lower-abundance, congeners.

Numerous Pulsed Field Gradient (PFG) based methods have been developed for solvent suppression, most notably H_2O in biological samples.^[16–19] Signal suppression of H_2O is relatively straightforward, as its NMR signal is a singlet. Solvent suppression is more challenging when several multiplets need to be suppressed - a case typical for LC-NMR using non-deuterated solvents.^[20,21] In these situations, n -solvent peaks are peak picked in a 1D ^1H spectrum, and appropriate shaped pulses are generated for their suppression. These techniques, when performed in automation, are limited by a few factors, mostly due to the non-optimised suppression methodology. Significantly, they will not decouple the ^{13}C satellites of a carbon-bonded solvent's proton by default. Similarly, the presence of ethanol makes the solvent suppression in whisky samples challenging. The ^1H NMR spectrum of ethanol consists of a triplet and quartet, alongside a residual $-\text{OH}$ signal, which under certain circumstances appears separately from the water signal. In addition, the non-labile ethanol protons show ^{13}C satellite signals, which are not insignificant relative to the concentration of the congeners. A solution containing 40% ethanol has ethanol concentration of 6.85 M. The ^{13}C - $^{12}\text{C}_5\text{H}_5\text{OH}$ isotopomers of ethanol with natural abundance of ^{13}C are therefore present at approximately 75 mM. For comparison, a major whisky congener, isobutanol, occurs at a concentration of approximately 4 mM in malt Scotch Whisky.^[14] It is clear that any successful attempt to suppress ethanol must also remove its carbon satellites during the solvent suppression.^[21]

In this work, we present an automated ethanol/water solvent suppression methodology allowing for the acquisition of high resolution, high sensitivity 1D ^1H NMR spectra of whisky with minimal sample preparation. The developed 1D method uses the well-established 1D NOESY pulse sequence, which in combination with signal presaturation yields very good water suppression,^[22] as documented by its widespread use in NMR studies of biofluids.^[23–26] This solvent suppression method is also used as the basis for even more challenging solvent suppression in 2D homo- and heteronuclear NMR experiments. Several examples of these experiments are presented here.

Materials and Methods

Samples were analysed in 5mm Wilmad 535-PP-7 Precision NMR tubes rated for 600 MHz. D_2O (99%), acetic acid- d_4 (99.5%), and sodium acetate- d_3 (99%) were acquired from Sigma-Aldrich Co. Samples (500 μL) of Scotch Whisky, provided by the Scotch Whisky Research Institute, were mixed

with D₂O/sodium acetate/acetic acid buffer solution (100 μ L) containing DSS-d₆ as an internal standard. The final concentrations of buffer and internal standard were 25 mM and 1 mM, respectively. Samples of very high ethanol strength (>80% v/v) were diluted using UHQ water (1:1) before mixing with the buffer solution. All spectra were acquired at 300.0 K.

NMR experiments were developed and implemented on 4-channel Avance III HD 600 and 800 MHz Bruker spectrometers equipped with a TCI cryoprobe with automated matching and tuning and a 16-slot sample changer (600 MHz only). All spectra presented here were acquired at 600 MHz unless otherwise noted. Acquisition of a 1D ¹H spectrum is preceded by the acquisition of three preliminary spectra in automation using IcoNMR and Python scripting. 1D ¹H spectra were acquired in full automation, while subsequent 2D experiments were acquired in manual mode.

The first preliminary experiment serves to locate the exact resonance frequency of water and to calibrate 90° ¹H pulses on the first and third channels. The water signal is located using a 360° pulse in a single scan experiment. The backwards linear prediction of the first 4096 points used during the processing removes the broad component of the water signal, allowing for the accurate determination of the frequency of the sharp component. The determined value is then used to irradiate the water signal on the 3rd channel. Only the first 512 points are back predicted for samples with a high ethanol concentration, due to fast decay of their water signal.

The second preliminary experiment determines the exact chemical shift of CH₂ and CH₃ signals of ¹²C isotopomers of ethanol by establishing the corresponding chemical shift in ¹³C isotopomers in a non-refocused reverse INEPT^[27,28] experiment. This approach was chosen as the radiation damping prevents accurate determination of the chemical shifts of ethanol protons in ¹²C isotopomers. The reverse INEPT spectrum is acquired using acquisition and relaxation times of 6.06 and 4 seconds, 4 dummy scans and 16 scans with ¹H and ¹³C carrier frequencies of 2.2 and 40.0 ppm, respectively. The FID is zero-filled once and Fourier transformed using exponential broadening of 0.2 Hz. The antiphase satellite ethanol signals are peak picked and the chemical shifts of CH₂ and CH₃ signals of ¹²C isotopomers are calculated by adding 1.53 and 1.11 Hz to the corresponding chemical shifts of ¹³C isotopomers, respectively, to compensate for the ¹³C isotope shift. A frequency modulated rectangular shaped pulse, *EthanolShape*, is generated starting from a square pulse (1000 points). This pulse inverts the on-resonance CH₂ protons and the off-resonance CH₃ protons. Its exact length, to be around 50 ms, is calculated to allow $2\pi n$ rotation for the off-resonance CH₃ protons. This experiment also stores the centre position of the CH₂ signal as the carrier frequency for the final experiment. The power used for this pulse corresponds to $\gamma B_1/2\pi$ equal to 28 Hz.

The third preliminary experiment produces a single scan 1D ¹³C spectrum acquired with inverse-gated ¹H decoupling. The spectrum is acquired using an acquisition time of 2.04 s with ¹H and ¹³C carrier frequencies set to 2.20 and 40 ppm, respectively. The spectrum is peak picked and the positions of the ethanol carbon signals are used to calculate a shaped pulse, *CarbonShape*, for decoupling of the ethanol satellites in the final experiment. The ¹³C carrier is placed exactly in the middle of the CH₂ and CH₃ ¹³C chemical shifts. A 180° rectangular cosine modulated shaped pulse consisting of around 500 points and length approximately 2.5 ms is generated – the exact number of points and length is optimised per sample. Its exact length is calculated to allow $2\pi n$ rotation for both resonances. The power level was set to $\gamma B_1/2\pi$ equal to 333 Hz and the pulses were phase cycled using m4p5 scheme, where m4 and p5 are {0°, 0°, 180°, 180°} and {0°, 150°, 60°, 150°, 0°}, respectively.^[29,30]

The final experiment imports the various offsets, calculated pulse shapes and power levels to acquire a 1D ¹H spectrum with water and ethanol suppression. For water presaturation, the power

level was set to $\gamma B_1/2\pi$ equal to 20 Hz. The receiver gain is set to 45.2, and spectra are obtained using 4 dummy scans and 32 scans. The FID is acquired with digitised sampling of 128k time domain points over 16 ppm, yielding the acquisition time of 6.82 s; a 4.5 s relaxation/presaturation delay was used. The FID was zero filled once and Fourier transformed using an exponential line broadening of 0.2 Hz

These experiments were performed in automation and take approximately 15 minutes per sample, including loading and shimming. In-house Python scripts were used for batch processing of spectra including phasing and baseline corrections.

The pulse sequence of a magnitude DQF COSY experiment (Bruker pulse sequence *cosygpppqf*) was modified to include solvent signal presaturation during a 3 second relaxation delay as outlined for the acquisition of 1D ^1H NMR spectra. 2D COSY spectra were acquired using 1024 and 4096 complex points in F_1 and F_2 using spectral widths of 10 and 13 ppm in F_1 and F_2 yielding t_1 and t_2 acquisition times of 85.2 and 262 ms, respectively. Four scans were accumulated per one increment resulting in the overall acquisition time of 3 hours 52 min. A forward linear prediction to 2048 points was used in F_1 and zero filling to 8192 was applied in F_2 . A sine square window function was used for apodization prior to Fourier transformation in both dimensions.

Similar modifications to those described for the COSY experiments were implemented into a phase-sensitive TOCSY experiment using a z-axis DIPSI-2 spin-lock^[31] starting from the Bruker pulse sequence *dipsi2esgpph*. A 40 ms spin-lock was applied at $\gamma B_1/2\pi$ of 10 kHz. The PFGs surrounding the DIPSI spin-lock were applied at 1 and 3%. The spectrum was acquired using 768 and 4096 complex points in F_1 and F_2 using spectral widths of 10 and 13 ppm in F_1 and F_2 yielding t_1 and t_2 acquisition times of 63.9 and 262 ms, respectively. Sixteen scans were accumulated per one increment resulting in the overall acquisition time of 8 hours 8 min. A forward linear prediction to 2048 points was used in F_1 and zero filling to 8192 was applied in F_2 . A cosine square window function was used for apodization prior to Fourier transformation in both dimensions.

A band-selective 2D TOCSY experiment^[32] was designed starting from the Bruker pulse sequence *dipsi2gpphzs*. Two 2D band-selective TOCSY spectra were acquired focusing on methyl and methylene regions centred at 0.741 and 1.289 ppm, respectively, using the relaxation time of 2 s, mixing time of 80 ms and eight scans per increment. As these regions do not include solvent signals, no signal suppression was necessary. A zero-quantum suppression was achieved using 20 ms CHIRP pulse with a simultaneous PFG (11%) before and after mixing according to Thrippleton *et al.*^[33]

The methyl focussed experiment was acquired using the following parameters: 128 and 4096 complex points in F_1 and F_2 , respectively, spectral widths of 0.083 and 7.0 ppm in F_1 and F_2 , yielding t_1 and t_2 acquisition times of 487 ms and 1.28 s, respectively. The overall acquisition time was 1 hour and 16 min. A forward linear prediction to 256 points was used in F_1 followed by zero filling to 512 points. Zero filling to 8192 was applied in F_2 . A cosine square window function was used for apodization prior to Fourier transformation in both dimensions.

The methylene focussed band-selective TOCSY experiment was acquired using the following parameters: 576 and 4096 complex points in F_1 and F_2 , respectively, spectral widths of 0.832 and 8.4 ppm in F_1 and F_2 , yielding t_1 and t_2 acquisition times of 576 ms and 405 ms, respectively. The overall acquisition time was 4 hours and 14 min. A forward linear prediction to 1024 points was used in F_1 . A zero filling to 8192 was applied in F_2 . A cosine square window function was used for apodization prior to Fourier transformation in both dimensions.

A magnitude mode *J*-resolved experiment (Bruker pulse sequence *jresqf*) was modified to include the presaturation elements as above. Spectra were acquired using 96 and 4096 complex points in F_1

and F_2 , respectively. 16 scans were acquired per increment using spectral widths of 40 Hz and 13 ppm in F_1 and F_2 , yielding t_1 and t_2 acquisition times of 0.26 and 1.2 s, respectively. The overall experimental time was 2 hours and 20 minutes. A forward linear prediction to 256 points was used in F_1 , followed by zero filling to 512 points. A zero filling to 8192 was applied in F_2 . A sine square window function was used for apodization prior to Fourier transformation in both dimensions.

A 1D chemical-shift-selective filter (CSSF) TOCSY experiment^[34] using DIPSI-2 spin-lock^[31] and zero-quantum suppression^[33] was modified to include solvent signal presaturation during the relaxation delay. The spin-lock at $\gamma B_1/2\pi$ of 10 kHz was applied from 10 ms to 160 ms. Selective inversion of a selected proton was achieved using a 40 ms Gaussian pulse applied on channel 1 after switching to the desired frequency from the frequency of the CH₂ ethanol signal used during the presaturation. The spectra were obtained with 32 increments (2.6 ms) of the CSSF (total length 83.2ms) with 2 scans each. The 1D TOCSY spectra were acquired using 32k time domain points and a spectral width of 14 ppm, yielding an acquisition time of 1.46 s; a 2.0 s relaxation delay was used. The FID was zero filled once and Fourier transformed using exponential line broadening of 0.3 Hz.

An echo/anti-echo DEPT-edited ¹H, ¹³C HSQC experiment, Bruker pulse sequence *hsqcetgpcisp2.3* was modified to include explicit adiabatic bilevel decoupling^[35-41] and the solvent suppression as described for the acquisition of 1D ¹H NMR spectra. Standard Bruker broad-band CHIRP ¹³C pulses were used including 500 and 2000 μ s long inversion and refocusing pulses, respectively. Analogous 2D ¹H, ¹³C HSQC-TOCSY with DIPSI-2 spin lock but without the multiplicity selection was also modified (standard Bruker pulse program *hsqcdietgpcisp.2*) to include signal suppression. The heterocorrelated spectra were acquired using 1024 and 3072 complex points in F_1 and F_2 , respectively. Eight scans (HSQC) or 16 scans (HSQC-TOCSY) were acquired per increment using spectral widths of 15 and 165 ppm in ¹H and ¹³C dimensions yielding t_1 and t_2 acquisition times of 21 and 176 ms, respectively. The ¹³C carrier frequency was set to 80 ppm. The spectrum was zero filled to 8192 and 2048 points and the cosine square window function was used before Fourier transformation; a 40 ms spin-lock was applied at $\gamma B_1/2\pi$ of 10 kHz in the HSQC-TOCSY experiment.

A constant-time phase sensitive gradient-selected ¹H, ¹³C HMBC experiment with two-fold low-pass *J*-filter to suppress one-bond correlations and no decoupling during acquisition,^[42,43] Bruker standard pulse program *hmbcctetgpl2nd*, was modified to include signal presaturation during the relaxation delay, as described above. Additional ethanol CH₂ and water signal saturation ($\gamma B_1/2\pi = 28$ Hz) were applied during the long-range evolution delay optimised for ⁿJ_{CH} of 6 Hz. A two-step low pass *J*-filter was applied optimised for the ¹J_{CH} couplings of ethanol (125.7 and 142.6 Hz). The 90° ¹³C pulses of the filter were implemented as composite 90°_x-90°_y pulses. 2D ¹H, ¹³C HMBC spectra were acquired using 910 and 4096 complex points in F_1 and F_2 , respectively. Sixteen scans were acquired using spectral widths of 14 and 200 ppm in ¹H and ¹³C dimensions yielding t_1 and t_2 acquisition times of 15 and 243 ms, respectively. The ¹³C carrier frequency was set to 100 ppm. The spectrum was zero filled to 8192 and 4096 points and the cosine square window function was used before Fourier transformation. Full example spectra for all experiments are included in the Supplementary Information.

Results and Discussion

Sample preparation

A sample preparation protocol was designed to minimise time and costs to allow high-throughput acquisition of spectra. Scotch Whisky is an acidic solution (pH ca. 4), with acidity increasing during maturation.^[14] To minimise the chemical shift changes due to pH variations between samples, a buffer solution was added to each sample. Deuterated sodium acetate/acetic acid buffer (25 mM)

1
2
3 was used as acetic acid is naturally occurring in whisky at a typical concentration of 1.5mM, and its
4 pK_a is close to whisky's natural pH. The residual signal of the undeuterated acetic acid (0.5%, 125
5 μM) at 2.08 ppm thus contributes to the naturally occurring signal. The final buffer concentration of
6 25 mM was decided upon as a compromise for achieving sufficient buffering capacity, whilst
7 minimising the reduction in sensitivity of cryoprobes caused by salt.^[44] Using these conditions, the
8 pH variations were limited to few hundredths of pH units. In addition, it was observed that changes
9 in ethanol strength also contributed to the movement of signals, but no attempt was made to
10 equalise it, as this would substantially increase the complexity of sample preparation.
11

12 Ethanol and water suppression in whisky samples

13
14 The acquisition of proton NMR spectra of high-alcohol strength samples is severely hindered by the
15 high concentrations of both water and ethanol. To overcome this issue, it is possible to dry the
16 sample and re-dissolve it in a deuterated solvent,^[45] however this has obvious drawbacks in
17 increased labour and loss of volatile compounds and thus working with original liquids is preferred.
18 Excitation sculpting^[17] is a very efficient water suppression technique that has been adopted for the
19 suppression of multiple signals.^[46] However, there are issues with this approach, as the intensity of a
20 wider range of resonances is affected by applied selective inversion pulses. Employment of longer
21 pulses, which partially addresses this issue, leads to unwanted J -modulation of multiplets. The latter
22 problem can be addressed by the perfect-echo pulse sequence^[19] that nevertheless can induce
23 intensity variations due to relaxation over the extended periods spins spend in the transverse plane.
24 Methods that deal with the solvent suppression prior to spin excitation are therefore preferable.
25 Water suppression enhanced through T_1 effects or WET^[47,48] is one such method. It has been
26 recently successfully applied to acquire 1D and 2D spectra of strong distilled spirits produced from
27 grape pomace.^[49] This study was performed using a room temperature probe, where WET performs
28 well. Nevertheless, the efficiency of WET is known to deteriorate on cryoprobe instruments and a
29 modification has been put forward that improves its performance.^[50] Another water suppression
30 based method that suppresses solvent signals at the beginning of the pulse sequence is based on a
31 1D NOESY experiment.^[51] This technique widely used in metabonomic studies and has also been
32 adopted for multiple solvent suppression. Working with high alcohol strength spirits, Monokhova *et al.*
33 presented an automated eightfold (the OH singlet, the ethanol triplet and quartet) one-channel
34 signal suppression technique using a single combined shaped pulse.^[52] A similar method was
35 implemented by Ragone *et al.* to examine wine samples.^[53] Described as line-selective methods they
36 nevertheless do not achieve solvent suppression by irradiating selected lines of ethanol multiplets
37 only. As demonstrated by Kupce *et al.*, when shaped pulses targeting very close frequencies are
38 combined in a single selective pulse, they do not work in a line-selective manner; instead, they will
39 saturate a broader region.^[54] We have therefore decided to abandon the "line-selective" approach
40 and focused on the multiplet-selective solvent suppression instead within the framework of the 1D
41 NOESY pulse sequence. In addition, we have also introduced suppression of ^{13}C satellites that is
42 compatible with cryoprobes. The proposed pulse sequence is shown in Fig. 1.
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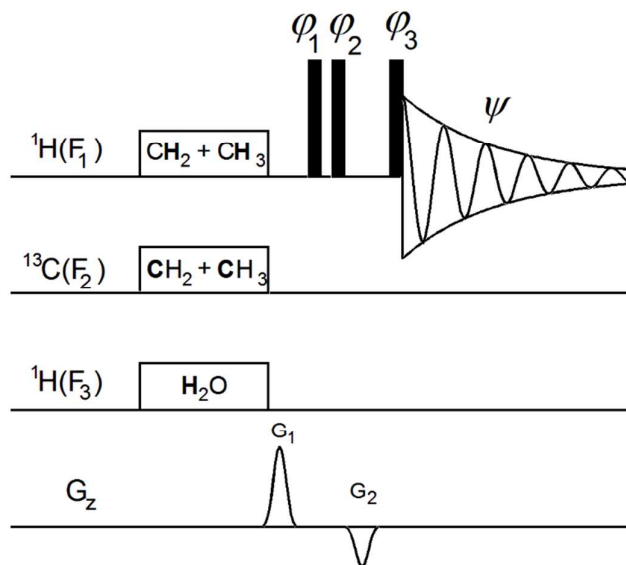


Fig. 1 – Pulse sequence for simultaneous suppression of water, ethanol and ^{13}C ethanol satellites in whisky samples. Narrow filled rectangles represent 90° pulses. 1 ms CHIRP-shaped PFGs were applied at $G_1 = 50\%$ and $G_2 = -11\%$ followed by a 200 μs gradient recovery delay. The following phase cycle was used $\varphi_1 = x, -x$; $\varphi_2 = 8(x), 8(-x)$; $\varphi_3 = 2x, 2(-x), 2y, 2(-y)$ and $\psi = x, 2(-x), x, y, 2(-y), y, (-x), 2x, (-x), (-y), 2y, (-y)$. For more details see Materials and Methods, Bruker pulse program included in Supplementary Information.

Crucial to the quality of solvent suppression is the determination of the exact frequencies of solvent signals. However, due to the high Q factor of cryoprobes, even a very short r.f. pulse produces radiation damping^[55], which severely distorts ethanol multiplets and makes this task impossible when focusing on ^{12}C isotopomers of ethanol. This is not an issue for their much weaker ^{13}C satellite signals, which can be acquired as the first trace of a non-refocused gradient-selected HSQC^[56] or non-refocused reverse INEPT^[57] experiment. The former method produced distorted multiplets affected by the evolution of proton-proton couplings, while the latter yielded pure antiphase multiplets with respect to $^1J_{\text{CH}}$. Despite the lower sensitivity of the latter method, signal-to-noise > 4000 was routinely obtained, which is more than adequate for accurate frequency determination. The level of the suppression of the main signals (< 25% of the height of the satellite signals) was also sufficient. The chemical shifts of ^{12}C isotopomers of ethanol were therefore obtained based on the frequency of ^{13}C isotopomers and considering the proton $^{13}\text{C}/^{12}\text{C}$ isotope shift as detailed in the Materials and Methods.

A double-selective pulse of approximately 50 ms, saturating both CH_2 and CH_3 signals, was used to suppress ethanol signals. Its exact length was calculated for each sample taking into account the exact chemical shift difference between the CH_2 and CH_3 signals and allowing the off-resonance signal (CH_3 in this implementation) to make exactly a multiple of 2π rotation. We find that this is essential for excellent suppression of the off-resonance signal as illustrated in Fig. 2. Here, for a difference of 1470 Hz between the CH_2 and CH_3 signals, a 50094 μs pulse produces $74 \times 2\pi$ rotation of the off-resonance CH_3 signal (the first, left spectrum in Fig. 2). When the pulse length was recalculated to allow additional precession in 20° increments the suppression of the CH_3 signal became progressively worse. Lengthening the pulse by as little as 38 μs , or 0.076% of the original pulse length, which corresponds to the time needed to generate such as 20° phase shift, has a noticeable effect.

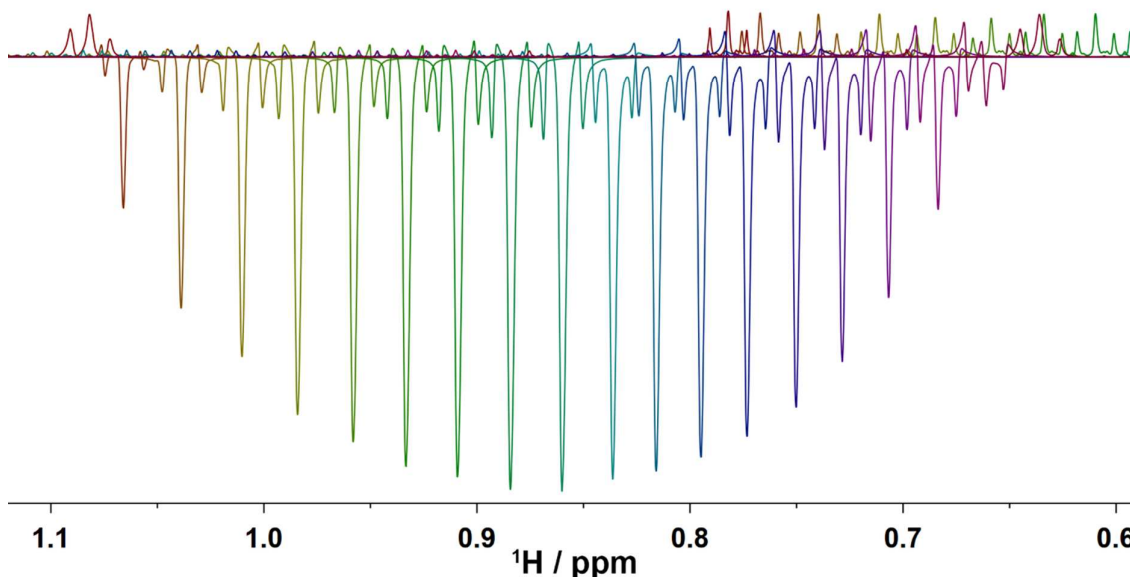


Fig. 2 – Residual signal of CH_3 protons of ethanol obtained using the pulse sequence of Fig. 1, as a function of the pulse length of the presaturation pulse. On the left, the pulse length, $50094 \mu\text{s}$, was calculated to allow $74 \cdot 2\pi$ rotations, while the subsequent experiments increased this value by $38 \mu\text{s}$. Each recalculated shape allowed for an additional phase shift increment of 20° . The range of 360° is shown in 17 increments. Note that the central line of the CH_3 triplet is disproportionately taller.

The exact rotation of only one off-resonance signal can be achieved in this way; therefore, another channel was used to suppress the water signal as described in the Materials and Methods.

Acquisition of ^{13}C satellites can serve another purpose - quantification of the ethanol content of the sample. Indeed, calibrating the ethanol strength using water/ethanol mixtures based on integration of CH_2 and CH_3 signals for standard alcohol strengths in the region of 20 to 70% ABV yielded a standard deviation of 4.5% ABV. This error was mostly due to baseline and phase distortions from the attenuation of the ^{12}C isotopomer signal.

The next spectrum to be acquired was a 1D ^{13}C NMR spectrum using inverse-gated decoupling. This spectrum also serves a dual purpose. High ethanol concentrations yield single-scan spectra with a signal-to-noise ratio $> 740:1$ at 43% ABV, that are suitable for the quantification of the ethanol concentration. Indeed, this spectrum provides better quantification than the reverse INEPT, yielding a standard deviation for the same calibration of only 1.5% ABV. As the ^{13}C signals displays ethanol concentration dependent chemical shift changes and line broadening, these are peak picked and integrated using individually adjusted integral regions. The ^{13}C chemical shift of the CH_2 resonance varies up to 0.32 ppm (or 48 Hz at 150 MHz) between 20-70% ABV, shifting down field at higher alcohol strengths. The ^{13}C CH_3 resonance shifts by up to 0.20 ppm (or 30 Hz at 150 MHz) between 20-70% ABV, shifting up field at higher alcohol strengths. These chemical shift and line widths changes may be explained, in part, by the changing nature of water-ethanol solutions at different ethanol concentrations, including hydrogen bond effects and ethanol aggregates.^[58-60]

The major reason for acquiring 1D ^{13}C NMR spectra is to determine the exact ^{13}C chemical shifts of ethanol resonances. This information is required for setting up the parameters for the decoupling of ^{13}C satellites of ethanol in the ^1H spectrum of whisky. As it is not possible to apply broadband ^{13}C -decoupling during a six second acquisition of ^1H spectra on a cryoprobe instrument, decoupling of ethanol signals was performed in a selective manner. For this purpose, a cosine modulated rectangular pulse of approximately 2.5 ms was generated at the frequency midway through the two

ethanol signals. As with the ^1H presaturation pulse, its exact length was calculated to allow a $2\pi n$ rotation of both signals, as detailed in the Materials and Methods. This treatment resulted in a residual signal $< 1\%$ of the original satellite signal height (Fig. 3).

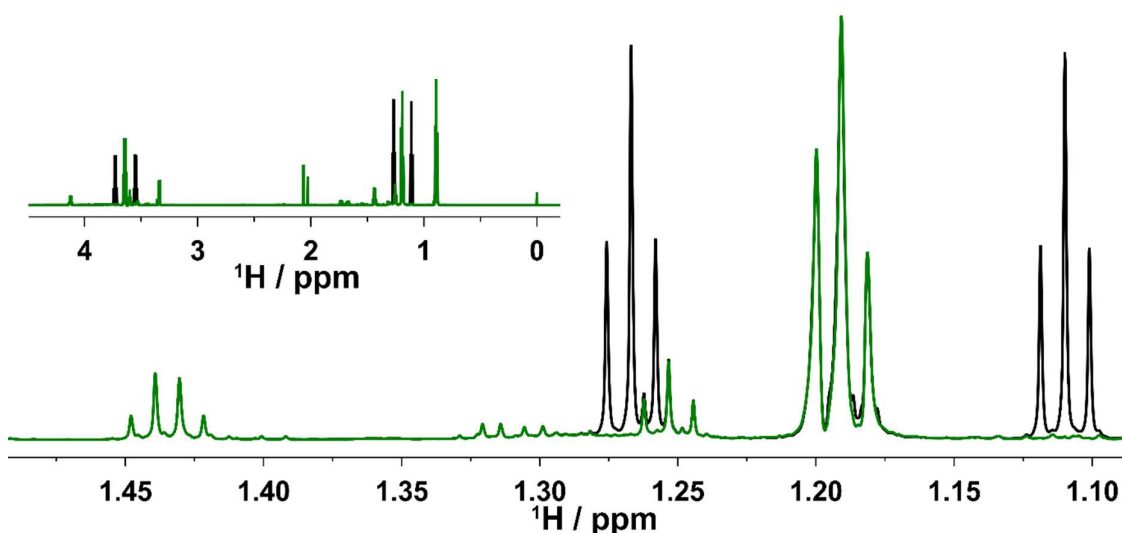


Fig. 3 – A partial ^1H NMR spectrum of Scotch Whisky sample S15-3896 acquired using the pulse sequence of Fig. 1 with (green) and without (black) ethanol signal suppressed. This spectrum was acquired at 800 MHz. The ethanol CH_3 region is shown in the main Fig., the inset shows a broader range of the spectrum including DSS at 0 ppm (1 mM) and CH_2 signals of ethanol at 3.64 ppm. Note that the signals of 3-methylbutan-1-ol (1.43 ppm) overlay perfectly, indicating lack of any heating caused by the decoupling, and the triplet at 1.255 ppm becomes clearly visible when the decoupling is activated.

The final ^1H NMR spectrum of Scotch Whisky shows remarkable suppression of water/ethanol signals with residual “solvent” signals smaller than the overlapping CH_3 signals of higher alcohols at 0.88 ppm (Fig. 4). The residual CH_2 signals of ethanol are smaller than the signal of nine CH_3 protons of 1 mM DSS used as internal standard. The level of the ethanol signals suppression can be quantified by recording a spectrum without the carbon decoupling (Fig. 3). Here the residual ^{12}C isotopomer ethanol signals have approximately the same intensity as their satellites, representing suppression of 99.5% of the signal. The CH_2 signals is typically better suppressed as its chemical shift coincides with the carrier frequency, whereas the CH_3 is off-resonance, contains three protons and displays a simpler multiplet. The water signal is typically suppressed to the level of the DSS signal.

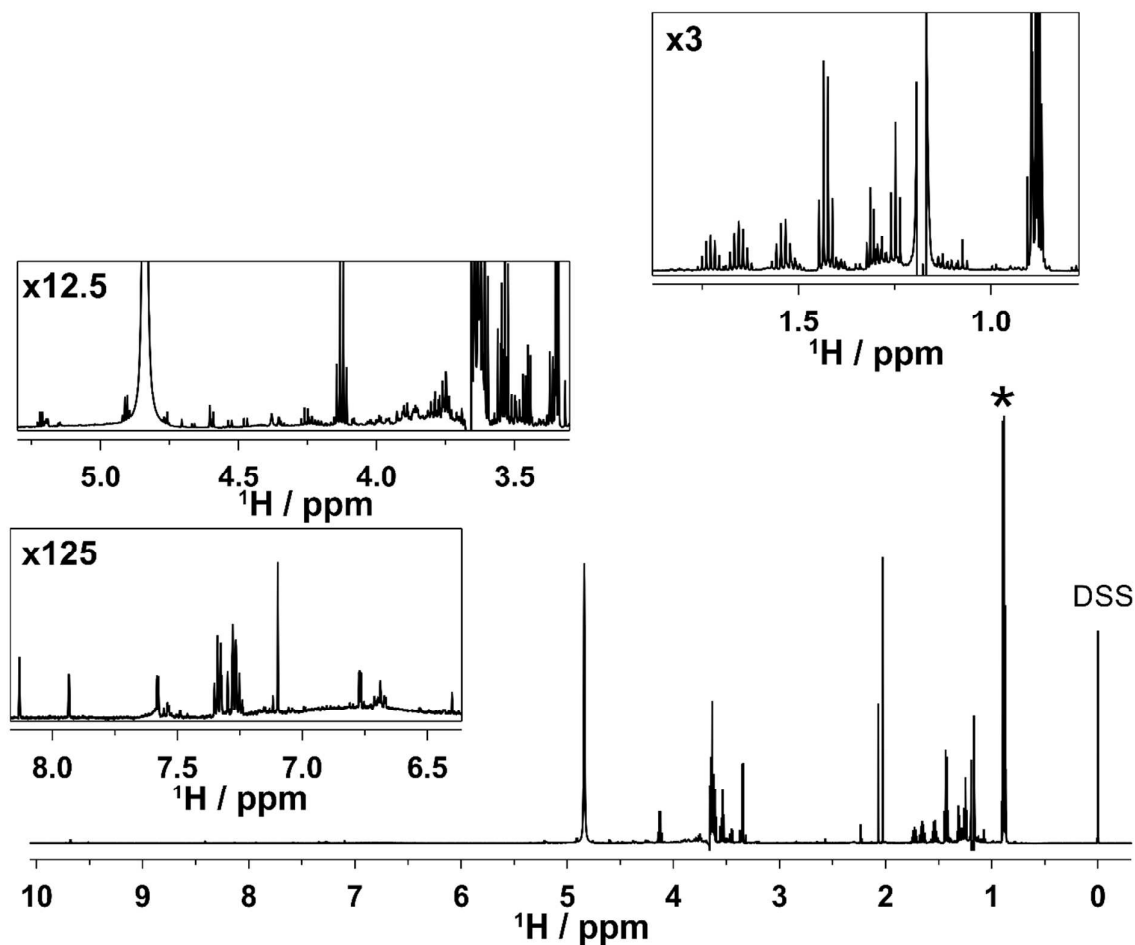


Fig. 4 - Typical ^1H NMR spectrum of Scotch Whisky sample S14-1941 with ethanol and water suppression, including the suppression of ^{13}C satellite signals of ethanol acquired using the pulse sequence of Fig. 1. Insets show three regions representing higher alcohols (0.8-1.8 ppm), carbohydrates (3-5.5 ppm) and cask extractives and aromatic compounds (6.5-8.5 ppm) indicating the vertical scale up relative to the main spectrum. The overlapped methyl signals from congeners at 0.88 ppm are labelled with an asterisk. DSS signals at 0 ppm act as a 1mM internal standard.

The final spectrum shows many congeners clearly identifiable and quantifiable, displaying pure phase multiplets as shown in Fig. 4. The signal-to-noise ratios (SNR) for signals of several compounds are summarised in Table 1. Their identity was confirmed through the analysis of 2D experiments and spike-in 1D spectra. We estimate that the limit of detection is therefore approximately 50 μM . This could be improved by increasing the number of scans or using a higher field instrument.

Table 1 - Signal to Noise Ratios for Resonances of Several Identified Compounds in Sample S14-1941

Compound	δ ppm	Concentration (mM)		Multiplicity	# H	SNR
DSS- d_6	0.00	-	1	s	9	14623
3-Methyl-1-butanol	1.43	6.8 ^a	7.1	q	2	6442
n-Propanol	1.53	2.8 ^a	3.5	m	2	1589
Acetaldehyde	4.13	2.50 ^a	2.50	q	2	465
Glucose	5.19/4.59	0.59 ^a	0.40	s	2	117/270
Furfural	6.77	0.16 ^a	0.13	dd	1	63
2-Phenylethanol	7.34/7.27	0.21 ^a	0.23	m	5	112/127
Syringaldehyde	7.3	0.059 ^a	0.051	s	2	66

^a The concentrations quoted were provided by the Scotch Whisky Research Institute.

In conclusion, the presented protocol allows automated acquisition of high quality ^1H NMR spectra of Scotch Whisky in 32 scans over 15 minutes on a 600 MHz cryoprobe NMR spectrometer. The obtained spectra are suitable for chemometric analysis and quantitative analysis of congeners of Scotch Whisky or other spirits.

1D and 2D experiments with solvent suppression

To aid in interpretation of whisky spectra, several established NMR experiments were modified to include the developed solvent suppression scheme. Amongst these are homonuclear 1D and 2D experiments such as 2D COSY, 2D TOCSY, 2D band-selective TOCSY, 1D chemical-shift-selective TOCSY, and 2D J -resolved as well as heterocorrelated experiments including 2D ^1H , ^{13}C HSQC, 2D ^1H , ^{13}C HSQC-TOCSY and 2D ^1H , ^{13}C HMBC.

In the following these experiments are briefly discussed in reference to the original experiments and illustrated using partial spectra of a 12-year-old Highland single malt Scotch Whisky (S14-1941) and a 12-year-old Lowland single malt Scotch Whisky (S14-1963). The spectra presented below were generated using appropriate apodization followed by Fourier transformation and baseline correction in both dimensions. Additional measures like removal of the signal by a filter and a t_1 noise reduction produced cleaner spectra that are presented in the Supplementary material together with the full spectra.

Homocorrelated experiments

Establishing proton-proton chemical shift correlations is essential for the structure elucidation of compounds and is typically achieved using J coupling constants to transfer the magnetisation in a COSY or TOCSY type of experiments. The usefulness of through space correlations for small molecular weight congeners is limited and NOESY experiments were therefore not included in this set. Acquisition of COSY spectra in aqueous solutions is challenging^[61] and therefore a basic gradient-selected 2D COSY method (Bruker pulse sequence *cosygpppqf*) was used here. For acquisition of 2D TOCSY spectra, a phase-sensitive method with DIPSI-2^[31] and ZQ-suppression scheme^[33] applied before and after the mixing time were chosen. Both methods yielded excellent results as seen in Fig. 5. The level of the solvent suppression can be judged by inspecting vertical projections positioned at the sides of the 2D spectra. The methyl signals of higher alcohols resonating at 0.88 ppm are more intense than the residual signal of the methyl protons of ethanol. Several correlations between aliphatic protons resonating in a 1 ppm region (0.8 - 1.8 ppm) to the methyl protons around 0.88 ppm and other protons up to 5.2 ppm can be seen. These protons belong to higher alcohols, such as 2- and 3-methylbutan-1-ol, n-propanol, and iso-butanol.

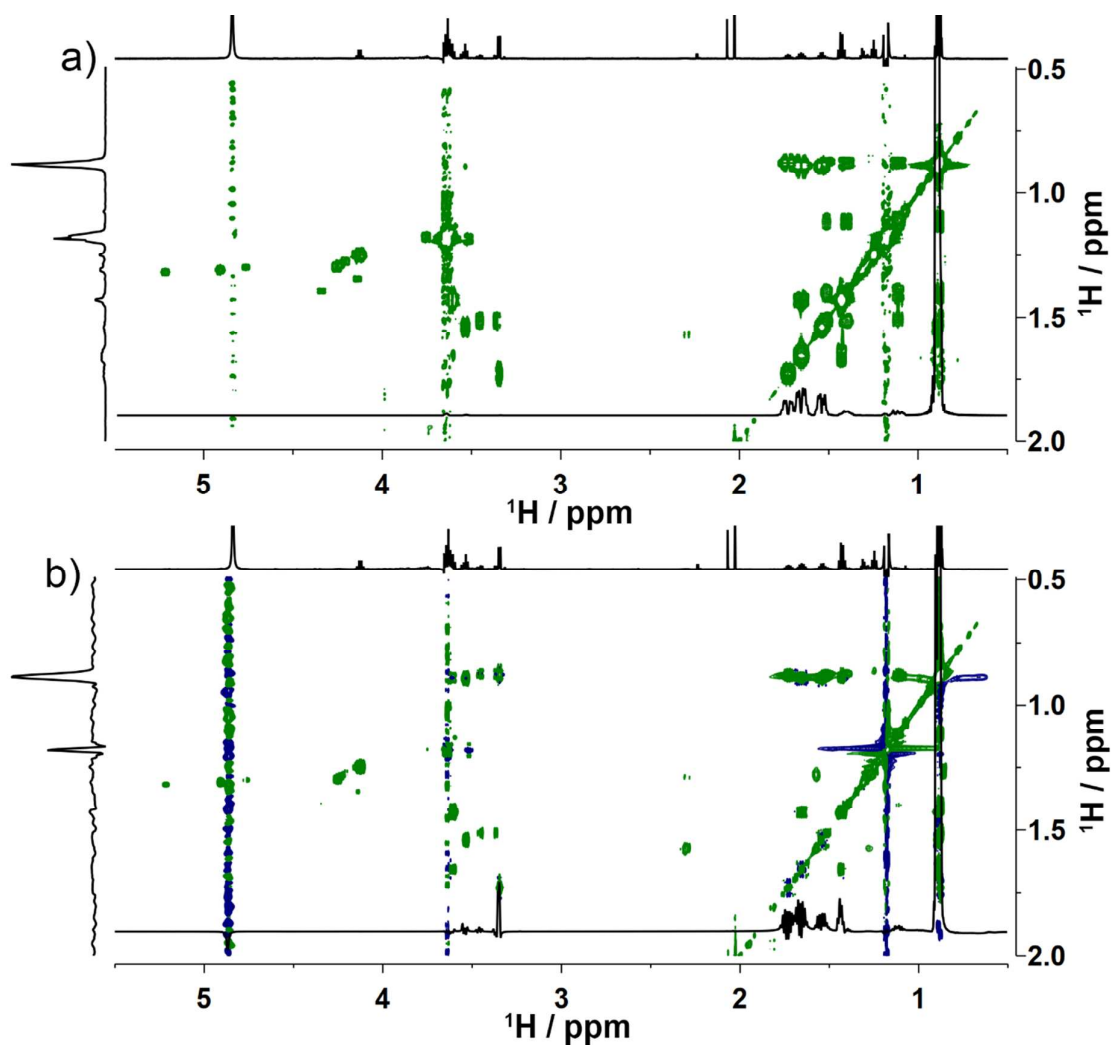


Fig. 5. Partial (a) 2D COSY and (b) 40 ms mixing time 2D TOCSY spectra of Scotch Whisky sample S14-1941 acquired using the developed solvent suppression scheme of Fig. 1. A 1D spectrum is shown at the top, scaled to show appropriate features, while vertical projections are shown on the sides. Horizontal traces taken at 0.88 ppm are scaled appropriately and overlaid to highlight spectral features.

As with every complex mixture, compounds containing very similar fragments are present at different concentrations. This leads to strong signals concealing the weak ones and preventing their identification, especially when wide proton multiplets are present. Their decoupling in one dimension is therefore highly desirable, while preserving their multiplicity in the other, thus retaining important information provided by J -couplings. Towards this end, we have implemented a solvent suppression scheme into a band-selective 2D TOCSY experiment^[32] achieving excellent resolution as illustrated in Fig. 6.

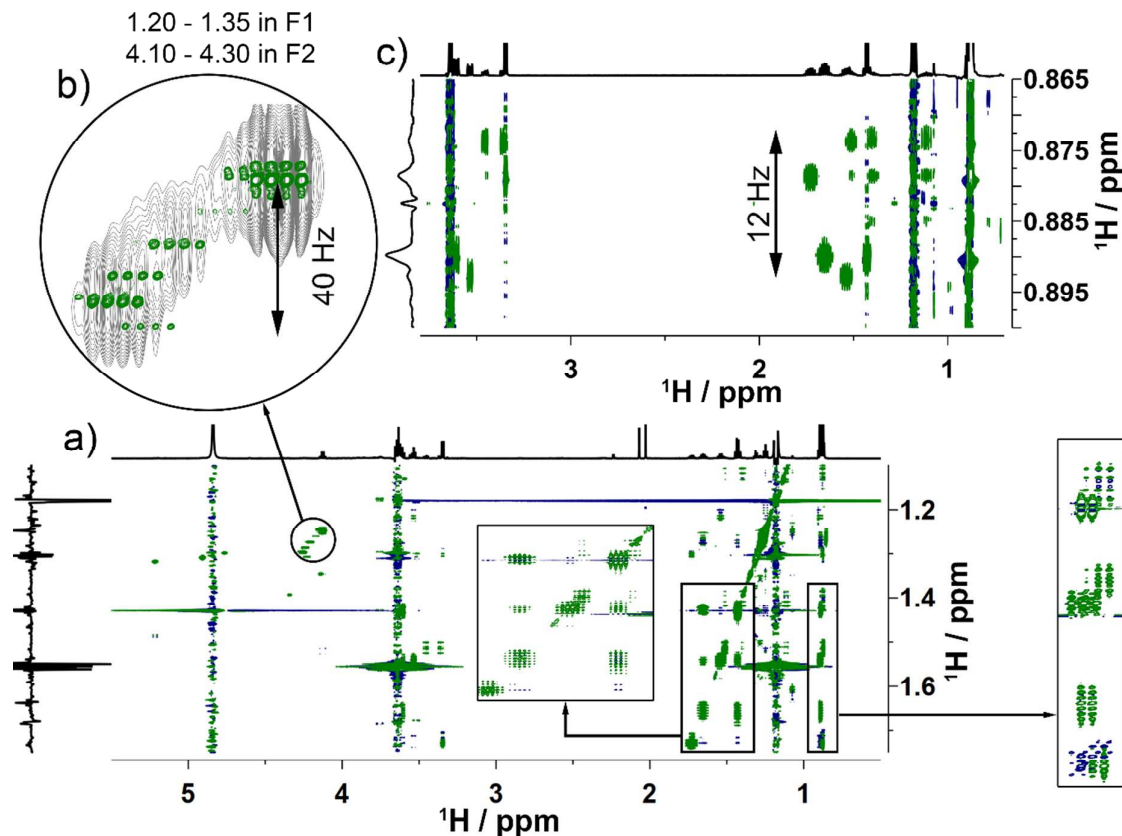


Fig. 6. Partial band-selective 2D TOCSY spectra of Scotch Whisky sample S14-1941 acquired using the solvent suppression scheme of Fig. 1 and 80 ms mixing time. Separately acquired 1D spectra are shown as external horizontal projections, while internal vertical projections are shown. (a) A region of 500 Hz centred on 1.43 ppm was inverted by a 10 ms IBurp 2 pulse. Two expansions are shown as indicated by arrows; (b) expansion of a circled region from (a) in green overlaid with the same region of 2D TOCSY spectrum shown in Fig. 5b. (c) In a separate experiment, a region of 50 Hz centred on 0.88 ppm was inverted by a 20 ms IBurp2 pulse.

In the example presented in Fig. 6a, a 500 Hz region centred at 1.43 ppm was selectively inverted, which contains some mutually coupled protons, preventing refocusing of J -couplings. Still, even this region benefits from excellent resolution afforded by a long t_1 acquisition time (576 ms) as shown in the inset of Fig. 6a. Protons from within this region resonating in a small window of 40 Hz centred on 1.13 ppm, do not have coupled partners in the inverted region, but are J -coupled to protons resonating at around 4.05 ppm. Consequently, they appear as singlets in F1 as shown in Fig. 6b. At least 10 different resonances were identified in this narrow region due to removal of the couplings in F_1 . In a separate experiment, methyl signals at 0.88 ppm resonating within 15 Hz could also be resolved in F_1 by applying a 20 ms inversion pulse that does not invert any additional resonances. Several cross peaks in two regions around 1.6 and 3.4 ppm were clearly resolved connecting the protons resonating here with the methyl groups (Fig. 6c).

Limited signal overlap in certain regions of ^1H NMR spectra of Scotch whisky allows the separation of signals in one-dimensional experiments using highly selective chemical-shift correlated experiment, chemical shift selective filter (CSSF) 1D TOCSY^[34]. The original CSSF-TOCSY pulse sequence was modified to include the solvent suppression element as described above. In an example given in Fig. 7, the anomeric proton of β -glucose at 4.52 ppm was selected and subjected to isotropic mixing of increasing length.

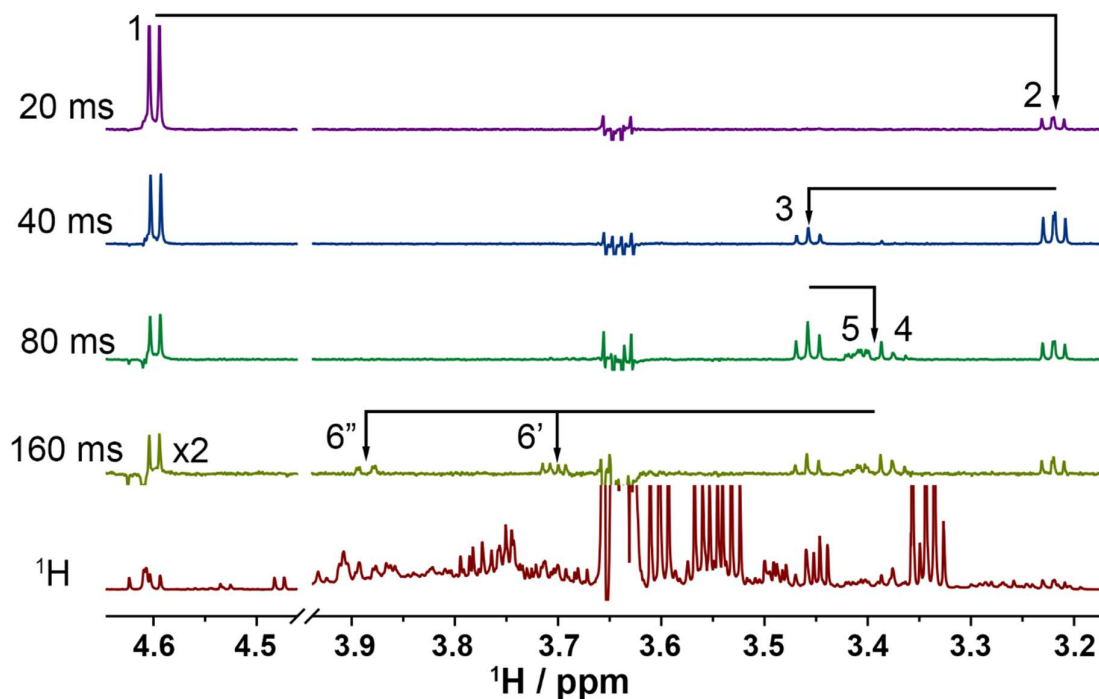


Fig. 7 - Selective 1D CSSF TOCSY of β -D-glucose in Scotch Whisky sample W16-1009 with mixing times from 20 ms to 160 ms showing TOCSY-transfer throughout the ring all the way to H-6 protons. The 160 ms mixing time spectrum had the y-scale multiplied by a factor of 2. The residual signal at 3.64 ppm belongs to CH_2 protons of ethanol. Spectra acquired at 800 MHz.

Note that a partially overlapping signal to the left of the H-1 of β -D-glucose was not excited and the protons of the glucose ring were assigned in a few minutes. Also noticeable is a remarkable suppression of the CH_2 signals of ethanol.

The last homonuclear experiment discussed is a 2D J -resolved experiment. J -resolved spectra have been used in the analysis of complex mixtures^[62,63] due to their ability to resolve overlapping multiplets and expose the existence of very small coupling constants. Due to its simplicity, a magnitude mode 2D- J -resolved experiment^[64] was selected and the developed signal suppression technique was implemented. High quality spectra were obtained revealing multiplets of numerous minor compounds. The solvent suppression was very efficient, as illustrated in Fig. 8a, where a region around the CH_2 resonances of ethanol is displayed.

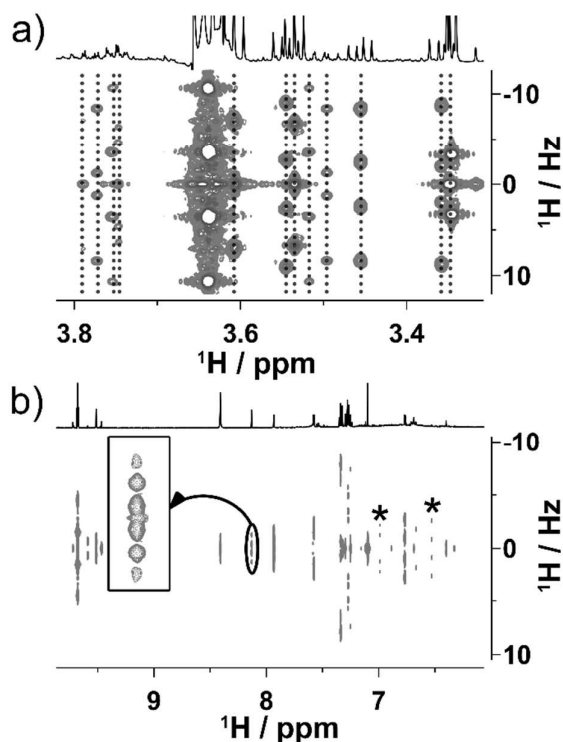


Fig. 8 - Magnitude mode 2D J-resolved spectrum of Scotch Whisky sample S14-1941 (a) region around the CH₂ signal of ethanol (b) aromatic region. The spectrum was tilted and symmetrised in F1. Vertical dotted lines have been added to a) to highlight the resolution in F2 obtained. The multiplets labelled with an asterisk in b) belong to signals that are at the noise level in the 1D spectrum. The inset in b) shows the multiplet of a signal at 8.13 ppm that is unresolved in the 1D spectrum.

Here, many minor signals are clearly resolved with only a 40 Hz wide strip lost to the t_1 noise of ethanol signal. Note the partially overlapping triplet to the right of the CH₂ signal of ethanol that is well resolved in the J-resolved spectrum. The aromatic region of the spectrum (Fig. 8b) revealed valuable details of signals that were at the noise level in the 1D NMR spectrum. In addition, the magnetic field inhomogeneity compensating capacity of J-resolved spectra uncovered splittings that are very valuable in establishing the identity of signals (see the inset in Fig. 8b).

Heterocorrelated experiments

Reflecting the 1% natural abundance of ¹³C, heterocorrelated spectra of Scotch Whisky will only show the more abundant compounds present. This simplification, together with access to ¹³C chemical shifts and heteronuclear connectivity, makes these experiments very powerful, assisting significantly with unambiguous compound identification. The challenge associated with these experiments again is the solvent suppression, this time of both ¹²C and ¹³C isotopomers of ethanol. Insufficient suppression of the former can lead to excessive t_1 ridges; while the failure to suppress the latter would produce very strong ethanol cross peaks obscuring the signals of interest.

When implemented into standard Bruker pulse programs (see Materials and Methods), the proposed solvent suppression produced excellent results. No additional measures were required for the HSQC and HSQC-TOCSY experiments, while additional solvent suppression was implemented into the HMBC pulse sequence. Partial spectra for all three methods displaying the aliphatic region are shown in Fig. 9.

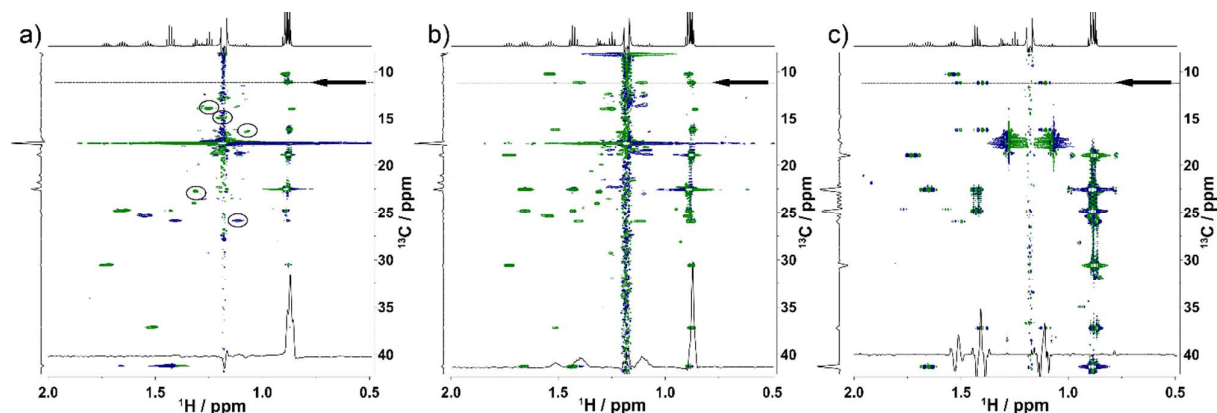


Fig. 9 - Partial 2D ^1H , ^{13}C correlated spectra of Scotch Whisky S14-1941 (a) HSQC, (b) HSQC-TOCSY and (c) HMBC showing the low ppm aliphatic region. 1D spectra are shown at the top, while projections are presented on the left-hand side of the spectra. Signals close to CH_3 protons of ethanol in both the ^{12}C and ^{13}C isotopomers are circled in the HSQC spectrum. While the residual CH_2 cross peak of ethanol is a singlet in (a) and (b), it appears as a $^1\text{J}_{\text{CH}}$ doublet in (c) as ^{13}C decoupling is not applied here. Rows taken at $\delta(^{13}\text{C}) = 11.22$ ppm highlighted by arrows are presented at the bottom of the spectra.

In all cases, the t_1 -noise is below the intensity of most of the cross peaks signals. The rudimentary decoupling of satellite signals reduced the ethanol cross peaks dramatically; nevertheless, these still are the most intense signals in the HSQC and HSQC-TOCSY spectra. At the same time, this method allowed for the observation of signals that are very close, or even overlap, with the signals of ^{12}C and ^{13}C isotopomers of the protons of ethanol. 2D CT-HMBC required minor modification to improve the solvent suppression due to the relaxation effects during the long-range proton-carbon coupling evolution interval. During this interval, which could be up to 100 ms, a CW presaturation of water and CH_2 signals at low power was applied improving their suppression.

Conclusions

We have presented a simple and robust solvent suppression technique that enables acquisition of high quality NMR spectra of alcoholic beverages on cryoprobe instruments and applied it to acquire 1D and 2D NMR spectra of Scotch Whisky. The method uses three channels to suppress signals of water and ethanol, including those of ^{13}C satellites of ethanol. It is based around the well-established 1D NOESY solvent suppression technique and suppresses the solvent at the beginning of the pulse sequence, producing pure phase signals minimally affected by the relaxation. The acquisition of 1D ^1H spectra is executed in automation allowing high throughput characterisation of Scotch Whisky. The developed solvent suppression was used to modify several homo- and heterocorrelated 2D NMR experiments that provide a wealth of information and will assist in the structure elucidation of Scotch Whisky compounds, and generally the characterisation of alcoholic beverages in their native form.

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