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Article

Application of ^{1}H Nuclear Magnetic Resonance Spectroscopy as Spirits Screener for Quality and Authenticity Control

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Abstract: Due to legal regulations, the rise of globalised (online) commerce and the need for public health protection, the analysis of spirits (alcoholic beverages > 15 % vol) is a task with growing importance for governmental and commercial laboratories. In this article a newly developed method using nuclear magnetic resonance (NMR) spectroscopy for the simultaneous determination of 15 substances relevant for the quality and authenticity assessment of spirits is described. The new method starts with a simple and rapid sample preparation and does not need an internal standard. For each sample a group of ^{1}H-NMR spectra is recorded, among them a 2D spectrum for analyte identification and 1D spectra with suppression of solvent signals for quantification. Using the Pulse Length Based Concentration Determination (PULCON) method, concentrations are calculated from curve fits of the characteristic signals for each analyte. The optimisation of the spectra, their evaluation and the transfer of the results are done fully automatically. Glucose, fructose, sucrose, acetic acid, citric acid, formic acid, ethyl acetate, ethyl lactate, acetaldehyde, ethanol, methanol, n-propanol, isobutanol, isopentanol, 2-phenylethanol and 5-(hydroxymethyl)furfural (HMF) can be quantified with an overall accuracy better than 8 %. This new NMR-based targeted quantification method enables the simultaneous and efficient quantification of relevant spirits ingredients in their typical concentration ranges in one process with good accuracy. It has proven to be a reliable method for all kinds of spirits in routine food control.

Keywords: NMR; alcoholic beverages; ethanol; methanol; acetaldehyde; screening; validation; food control; PULCON

1. Introduction

The analysis of spirits, which are defined in the European Union (EU) as alcoholic beverages based on some form of distillate and exceeding an alcoholic strength of 15% vol [1], is an important task for governmental food control laboratories as well as for spirits producers and for commercial contract laboratories working for all stages of trade. The control of spirits encompasses the control of compliance with food laws e.g. food declaration laws regarding alcoholic strength labelling [2] and specific spirits laws regarding volatile and non-volatile composition [1]. An overview on legal demands for spirits is provided by Lachenmeier et al. [3]. The investigation of spirits becomes more and more important for public health protection due to large-scale poisoning outbreaks due to counterfeiting and admixture, specifically with methanol [4,5]

For conventional spirits analysis, several different methods have to be applied. The EU reference procedures for determination of alcoholic strength are distillation followed by

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pycnometry, electronic densimetry or electrostatic balance. The reference procedure for volatile composition (including methanol) is based on gas chromatography (GC) with flame ionization detection, and the determination of non-volatile composition (mainly sugars and compounds due to wood cask storage) is based on liquid chromatography [6]. Some laboratories also apply enzymatic analysis for sugars and some other compounds [7,8]. The important contaminant ethyl carbamate, found in stone fruit spirits and cachaça, is typically analysed with GC combined with mass spectrometry [9]. Special analyses for some specific spirit types, such as anethol in raki or ouzo or thujone in absinthe, may require even further assays [7,8,10,11].

Typically, even for a small spirits analysis, at least three different methods are applied. Even the mentioned small selection of potential methods for spirits analysis indicates that such an analysis is time-consuming and expensive too. Several efforts have been undertaken in the past to find a suitable screening technology combining the highest possible number of analytes into a single and preferably quick assay (screening analysis). For a long time, the screening method of choice has been based on infrared spectroscopy (IR) technologies, which can determine typically up to 10 analytes in a single assay (such as ethanol, methanol, propanol and total sugar content) [12,13]. The restriction of IR is its low sensitivity and signal overlap, making a direct analysis impossible, necessitating indirect calibration approaches based on partial least squares regression models combining reference analysis data with a huge number of spectral data. The sensitivity of IR was also too low to detect analytes such as acetaldehyde or 5-(hydroxymethyl)furfural (HMF) typically occurring at lower ppm levels.

In the last two decades nuclear magnetic resonance spectroscopy (NMR) has been proven as a primary reference method for quantitative measurement [14,15] and has been successfully introduced as a screening technology for food analysis [16]. NMR-screeners for wine [17], fruit juice [18], olive oil and honey are commercially available. As there is no NMR-screener for spirits available so far, the idea of this study was to develop such a spirits screener, combining as many quantitative parameters as feasible in one assay. This screener should be able to get insight into the quantitative composition of spirits with little manual intervention, meaning that spectra processing and evaluation need to be fully automatic, similar to the screening method already developed for alcohol-free beverages [19]. The NMR sample preparation and measurement protocol is based on previous research [20,21]. This study only deals with quantitative analysis, while non-targeted analysis, e.g. for brand assignment, has been described elsewhere [21].

Table 1. Evaluated analytes in spirits.

#	Substance	Legal limit (mg/L)	Relevant working range (mg/L)
1	1-Propanol	*	200 – 5000
2	2-Phenylethanol	*	30 – 2000
3	Acetaldehyde	*	10 – 1000
4	Acetic acid	*	10 – 5000
5	Citric acid	*	100 – 20000
6	Ethyl acetate	*	20 – 10000
7	Ethyl lactate	*	500 – 2000
8	Formic acid	*	10 – 5000
9	Fructose	*	200 – 150000
10	Glucose	*	200 – 150000
11	HMF		100 – 1000
12	Isobutanol	*	200 – 5000
13	Isopentanol	*	200 – 5000
14	Methanol	40 [1]	
15	Sucrose	*	200 – 150000

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[1] (for 40% strength vodka)

*while no legal limit for the substance exists, there are group limits for some spirits requiring a minimum of volatiles, and a maximum of acidity, esters, and sugar content (see e. g. annex II of [1] or [22])

2. Materials and Methods

2.1. Reagents, standards and samples

All Reagents and standard compounds were of analytical or HPLC grade (≥ 99 %). Acetic acid, acetaldehyde, ethanol, formic acid, D-glucose, mannitol and methanol were obtained from Merck, Darmstadt, Germany; HMF was provided by Acros, Geel, Belgium; succinic acid was sourced from VWR, Darmstadt, Germany; isobutanol, isopentanol and 2-phenylethanol were acquired from Alfa Aesar (ThermoFisher, Karlsruhe, Germany); citric acid, ethyl acetate, ethyl lactate, fructose, 1-propanol, sodium benzoate, sodium propionate, sucrose, and 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt ("TSP", 98 atom% D) and the reagents for the buffer potassium dihydrogen phosphate and potassium hydroxide and sodium azide were bought from Sigma-Aldrich, Steinheim, Germany; deuterium oxide (D_2O, 99.9 atom% D) came from Deutero, Kastellaun, Germany. All aqueous solutions were made using demineralised water.

2.1.1. Buffer solution

During method development, buffer systems were tested to ensure a uniform sample pH even from spirits with different pH values without the need for further pH adjustments or titration. The optimised buffer was prepared by dissolving 20.4 g KH_2PO_4, 19.5 mg NaN_3 and 100 mg TSP in approx. 90 mL D_2O. KOH flakes were added to adjust the pH to 7.4 (at 20 °C). Finally the solution's volume is adjusted to exactly 100 mL with D_2O.

2.1.2. Quantification reference solution *("QuantRef")*

Three aqueous solutions of known concentrations were prepared from certified reference materials containing sodium benzoate (4 g/L), sodium propionate (1 g/L) and mannitol (4.5 g/L). The exact weighed portions were noted. A precise 1:1:1 mixture of these stock solutions was made, from which 900 µL were taken and mixed with 100 µL of buffer (as above) resulting in a dilution factor of 30%, compared to the initial concentrations. 600 µL of this solution were then filled into an NMR tube which was subsequently fused shut. By repeated weighing over a week the leak-tightness of the fused tube was confirmed. With this long-term stable QuantRef solution the spectrometer's response (the ERETIC factor, see below) is determined with each new sample series.

2.1.3. Quality assurance (control) solution

The international standard ISO 17025 demands the check and confirmation of a standard-based quantification by an independently prepared control solution. This solution was prepared by mixing 25 mL of the 4.0 g/L sodium benzoate stock solution with 20 mL of 4.5 g/L mannitol, 15 mL of 1.0 g/L sodium propionate and 30 mL of 2.0 g/L succinic acid. Of this mixture 900 µL were combined with 100 µL buffer (as above) and 600 µL of this finished preparation were filled into an NMR tube, fused shut and checked for leak-tightness by repeated weighing over a week. With this long-term stable monitoring solution the trueness of the spectrometer and the calculation algorithm are checked with each new sample series.

2.1.4. Spirits matrix samples

To check the Spirits Screener's robustness against a broad range of spirit ingredients, five types of spirit with different characteristics were used as matrix samples:

- Absinthe with its typical high essential oil and alcohol content
- Fruit spirit, having a rather high level of volatiles
- Herbal liqueur, with a high sugar content and a wide variety of plant extracts

- Whisky as a typical cask-matured spirit
- Vodka with only few minor components

2.2. *Sample preparation*

Before analysis, the spirits were filtered or centrifuged (especially required for turbid samples). For sample preparation, 500 µL spirits were mixed with 100 µL buffer solution, and 400 µL water-ethanol-mixture (190 mL + 50 mL) resulting in a dilution factor of 50 %, compared to the spirit's original concentrations. For direct measurement, 600 µL of the sample preparation were transferred into a 5 mm NMR tube (with <1 % volume variation, e.g. Wilmad Labglas Inc., Vineland, NJ, USA).

2.3. *Proton NMR experiments*

General requirements:

- To ensure a correct processing of spectra, artefacts at both ends of the spectrum, resulting from the band pass filter, shall be distanced from the spectral region of interest (ROI) by setting the spectral width (SW) wide enough, i.e. the width of empty end regions should be > 10% of the ROI's width, with a SW of >20 ppm a central region from -3 to 13 ppm can be processed and analysed correctly.
- To avoid any errors due to a non-linearity of the receiver gain (RG), all sample spectra and the spectrum of the quantitation reference (external standard) shall be acquired with the same RG setting.
- To ensure a near uniform excitation over the whole ROI, it is recommended to set the excitation frequency centrally in the ROI.
- For accurate integration or curve fitting, each signal shall be defined by a minimum of four data points in its upper half. Thus with a typical Full width at half maximum (FWHM) of 1 Hz, the resolution shall be better than 0.25 Hz

The period between pulses should be at least 5 times longer than the T1 constant of the analyte with the slowest relaxation to ensure near complete (>99 %) equilibrium, otherwise the resonance signals will be attenuated and thus concentrations will be determined too low. For a defined set of acquisition parameters this attenuation is reproducible and can be compensated empirically.

All NMR measurements were performed on a Bruker Avance III 400 Ultrashield spectrometer (BrukerBiospin, Rheinstetten, Germany) equipped with a 5-mm selective inverse probe (SEI) with Z-gradient coils, using a Bruker Automatic Sample Changer (SampleXpress).

All data were acquired under the control of Sample Track Client (BrukerBiospin, Rheinstetten, Germany), requiring about 35 min per sample.

The spectral raw data were automatically processed under TopSpin version 3.2 (Bruker Biospin, Rheinstetten, Germany) to achieve the following objectives: window multiplication and Fourier-transformation, referencing the shift scale to 0.0 ppm (using the TSP-signal) and phase correction, a baseline correction (globally and selectively between 0.001 ppm to 0.97 ppm; 1.388 to 3.4 ppm; 3.9 to 4.75 ppm and 4.95 to 9.999 ppm, the exclusion zones span the resonances of ethanol and water). Finally all processed spectral data were saved on a server.

Three different NMR experiments were measured for each sample: A 1D ^{1}H-NMR with suppression of the water signal by simple presaturation, a 1D ^{1}H-NOESY and a 2D ^{1}H-^{1}H J-resolved (JRES) (= coupling-resolved), both using shaped pulses to suppress the eight intensive signals of water and ethanol.

These experiments are based on Bruker's standard NMR experiments [17] with minor modifications, foremost among them an additional delay after acquisition allowing for full relaxation of the spins and keeping d1, the regular relaxation delay, short. During d1 the presaturation is performed, a short d1 ensures that less energy is transmitted into the sample for a maximum of sample temperature stability.

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For each sample the first experiment was used to optimize the hard 90° pulse with Bruker's PULSECAL routine and the power of the presaturation is calculated accordingly (and limited to 25 Hz), the optimized values were then stored for usage in the following experiments. Furthermore, the shaped pulse was optimized to suppress the water and ethanol peaks (8-fold suppression) in the following NOESY and JRES experiments.

Pulse program:	zgpr.mod_d7
Time Domain:	64k (= 65536 data points)
Dummy scans:	4
Number of scans:	4
Spectral width:	8.2 kHz (20.55 ppm)
AQ:	3.985 s
SFO1:	on resonance with the water signal
D1:	4 s (phase with presaturation, before excitation)
D7:	8 s (phase after acquisition, without presaturation)
Digmod:	baseopt
TE:	300.0 K (± 0.2 K)
Size (SI):	128k (= 131072 data points)
LB:	2
WDW:	EM

A 1D ^{1}H-NOESY spectrum was recorded as the second experiment, it is later used to quantify the analytes. The parameters are:

Pulse program:	noesygppr1d.comp1d7
Time Domain:	64k (= 65536 data points)
Dummy scans:	4
Number of scans:	32
Spectral width:	8.2 kHz (20.55 ppm)
AQ:	3.985 s
SFO1:	on resonance with the water signal
D1:	4 s (phase with presaturation, before excitation)
D7:	8 s (phase after acquisition, without presaturation)
Digmod:	baseopt
TE:	300.0 K (± 0.2 K)
Size (SI):	128k (= 131072 data points)
LB:	0.3
WDW:	EM

The FID of the 1D-NOESY was processed with window functions: one variant by using an exponential function to enhance the signal-to-noise ratio (SNR) for quantification and the other by using a gaussian function to improve resolution to precisely determine peak maxima. An automatic 1st order phase correction and a baseline correction (leaving out the signal regions of TSP, Ethanol and water) were applied to the spectra. Two peak lists were obtained from both processed spectra.

The third experiment performed, a 2D JRES, is used to detect the analytes' presence in the sample and the exact chemical shifts of the characteristic peaks. The parameters for the JRES are:

Pulse program:	jresgppsqf
AQ-mod:	DQD
FnMODE:	F1 QF
Time Domain:	F2 8192 F1 40
Dummy scans:	16
Number of scans:	8
Spectral width:	F2 16.70 F1 0.195
AQ:	F2 0.613 s F1 0.26 s
D1:	1 s
Digmod:	digital

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TE: 300.0 K (± 0.2K)
Size (SI): F2 16k F1 256

The JRES spectra were processed in Topspin with a squared sine bell window function, tilted and symmetrized.

2.4. *Automated analysis of the NMR spectra*

The TopSpin-processed NMR spectra were analysed using Matlab (version 2015b, The Math Works, Natick, MA, USA). The general process of the Matlab script runs as follows:

First the raw spectral data, the peak and peak intensity lists (generated by TopSpin) and the excitation pulse lengths of all spectra were imported into Matlab's workspace. The full data sets were then cropped leaving only the data points between -3 ppm and 14 ppm.

The presence or absence of each analyte in a spirit sample was determined by checking the 2D JRES spectrum for the analyte's specific peak pattern. Furthermore the JRES yielded the exact chemical shifts of the characteristic peaks in each sample. These exact shifts were subsequently used by the curve fitting algorithm.

The 1D ^{1}H NMR spectra were optimised by a reference deconvolution algorithm that removed minor asymmetries (due to slight field inhomogeneities) from the signals. The integral (area under the curve, AUC) of each characteristic signal was calculated with a Matlab based curve fitting algorithm focusing on the exact chemical shifts yielded by the JRES and taking into account the known coupling constants of multiplet signals.

The ERETIC factor (the spectrometer's sensitivity expressed in a.u. ^{x} ppm ^{x} L/mol) was determined from five resonances of the QuantRef sample measured in one series with the spirit samples and quantification was then performed using the ERETIC-PULCON method (see 2.4.4).

2.4.1. Shim quality and reference deconvolution

In each spectrum (QuantRef and samples) the TSP signal was fitted (using the method described in subsection 2.4.3), the fit integrated and the FWHM calculated. If the TSP-FWHM of a sample was wider than 1.3 Hz and/or the intensity of the TSP resonance fell outside of a predefined expected range, further analysis of this sample was rejected and a warning issued to the user. Potential causes for TSP resonances above the threshold value of 1.3 Hz could be e.g. an accidentally bad shim (each sample is automatically gradient-shimmed by the spectrometer), which will deteriorate all signals of the spectrum, or an aggregation of the TSP molecules e.g. as ligands around a suitable central cation. If the TSP-signal is narrower than the defined FWHM limit, it is subsequently used to achieve a reference deconvolution using the FIDDLE algorithm [23,24] on the zero-filled, Fourier-transformed and phase corrected experimental spectral data. Minor magnet field inhomogeneities will slightly deform all NMR peak shapes in a spectrum in the same way. The reference deconvolution will remove such shape errors from the whole spectrum.

By computing the inverse Fourier transformation of a) the experimental TSP peak's data and b) the optimized fit of the TSP peak (a very clean and sharp singlet) and pointwise division of a) by b) a correction function can be obtained. Pointwise division of the complete experimental spectrum's FID by the correction function and Fourier transformation then yields the deconvoluted spectrum.

Further processing of the spectra differs between the QuantRef sample and the spirits samples: Since the composition of the QuantRef is exactly known and its spectrum has only a few clearly separate peaks, signal curve fitting and subsequent integration may reliably be based on known values for the chemical shifts and coupling constants of the reference substances.

Table 2 Reference substances used in the QuantRef and the quality assurance solution

#	Substance	Signal centre (ppm)	Fit & Integration region (ppm)	Multiplicity	Coupling constants *J* (Hz)	N_H
1	Benzoate-Na [1]	7.564	7.610 to 7.525	tt	7.34 and 1.72	1
2	Mannitol (1st) [1]	3.878	3.920 to 3.840	dd	11.68 and 2.58	2

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#	Substance	Signal centre (ppm)	Fit & Integration region (ppm)	Multiplicity	Coupling constants *J* (Hz)	N_H
3	Mannitol (2nd) [1]	3.690	3.730 to 3.640	dd	11.68 and 5.96	2
4	Propionate (1st) [1]	1.060	1.120 to 1.000	t	7.66	3
5	Propionate (2nd) [1]	2.187	2.250 to 2.120	q	7.66	2
6	Succinic acid [2]	2.409	2.500 to 2.300	s	-/-	4

[1] In both solutions. [2] Ingredient of the quality assurance solution only

From each of the five integrals an ERETIC factor is calculated with formula 3 (see 2.4.4). From these five factors the arithmetic mean and standard deviations were calculated. The variation coefficient of each singular ERETIC factor must be smaller than 2 %, otherwise a warning message is issued and the evaluation aborted. If all is well, the mean ERETIC factor is stored and used for all following quantifications in this sample series.

Next step in the automated data evaluation is the calculation of the quality assurance (control) solution's recovery rate: After checking the TPS's FWHM to be below the threshold and a reference deconvolution, the reference substance's signals are curve-fitted and integrated to yield their PULCON-calculated concentrations. A successful comparison with their known concentrations (no recovery more than 10 % off) confirms that both QuantRef and spectrometer are in order.

2.4.2. Analyte identification and precise shift determination

Due to the wide variety of spirits compositions, it was expected that fixed values for the analytes' chemical shifts might sometimes differ too much from the real values in individual samples where e.g. a pH- or concentration-induced signal shift could occur.

To ensure the correct identification of an analyte in a spirit sample and the determination of its exact signal position(s) the JRES spectrum was used: Its advantage is the dispersion of multiplet peaks into the 2nd dimension resolving many overlapped signals and enabling the clear determination of coupling constants.

Common experience shows that the chemical shifts of a given analyte may vary, depending on supramolecular influences (e.g. pH, solvation and concentration), but the intramolecular scalar coupling constants have proven as reliably invariant. A JRES spectrum is basically a three dimensional dataset in a matrix where the chemical shift (F2) changes from column to column and the couplings (F1) are resolved between rows, the central row containing the spectral data with a coupling of zero (singlets). Each matrix element contains the intensity at this point in the "spectral landscape". If the multiplet type and the coupling constant *J* for a searched characteristic resonance and the acquisition parameters of the JRES are known, one can calculate from the resolution in F1 the rows which will contain the peaks of this multiplet (if the sample contains the analyte). E.g. in a JRES spectrum with 0.3 Hz resolution in F1 a triplet with $J = 7.2$ Hz will have intensity maxima in the central row and 24 "side" rows above and below the central row.

A limited subset was then temporarily extracted from the full JRES dataset, containing only one of the "side" rows where a peak of the multiplet should appear and a reduced range of chemical shifts centred on the typical value for the examined resonance. Using a publicly available Matlab function "*peakfinder*" [25] the intensity maximum was searched, thus yielding the exact column index (i.e. chemical shift). The algorithm developed by our team then checked if the intensity of the found peak was significantly higher than the other data (noise) in the evaluated row and, since multiplets must be symmetrical to $J = 0$ Hz, if the multiplet's complementing peaks could be found at the other expected positions in the same column of JRES data. After confirmation of the multiplet's existence and determination of its exact chemical shift (in this sample), precise starting values for the curve fitting were calculated.

2.4.3. Curve fitting

The signals to be evaluated were fitted with a pseudo-Voigt curve

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$$I_{\text{fit}} = \eta \cdot \frac{A}{1 + \left(\frac{x - x_{\max}}{\gamma}\right)^2} + (1 - \eta) \cdot A \cdot e^{-\ln 2 \cdot \left(\frac{x - x_{\max}}{\sigma}\right)^2} \quad (1)$$

Where:
I_{fit} is the fitted NMR signal intensity
η is the weight of the Lorentzian contribution (1 = fully Lorentz-shape, 0 = fully Gauss-shape),
A is an amplitude factor to adapt the fit to strong and weak peaks
x is a variable value on the chemical shift scale
$x_{\max}$ is the function's center, the chemical shift of the maximum of the peak to be fitted
γ is the half width at half maximum of the Lorentzian contribution
σ is the half width at half maximum of the Gaussian contribution of the peak to be fitted

These pseudo-Voigt profiles were fitted to the data points using a simple least squares cost function:

$$C = \sum_{x_{\text{start}}}^{x_{\text{end}}} [I_{\text{fit}}(x) - I_{\text{raw}}(x)]^2 \quad (2)$$

Where:
C is the sum of all residues (over the whole range of the fit)
x is a variable value between the fit region's borders on the chemical shift scale
$I_{\text{fit}}(x)$ is the fitted NMR signal intensity at x
$I_{\text{raw}}(x)$ is the measured NMR signal intensity at x

Using a publicly available Matlab function "*Fminsearchbnd*" [26] the parameters η, A, $x_{\max}$, γ and σ were iteratively adapted to optimize the fit, arriving at a minimum of C. To focus the optimization on the relevant signal even in a region congested with irrelevant other peaks, constraints were determined and applied to the fit: Each signal chosen for evaluation has characteristics such as chemical shift, FWHM, multiplet type and (where applicable) coupling constant(s) and roof effect ratios. For these characteristics, upper and lower boundaries were set: The fit may adapt $x_{\max}$ ±0.01 ppm from the chemical shift found by evaluation of the sample's JRES spectrum (or the defined values in case of the QuantRef), the widths (FWHM) can be varied between 0.5 and 2.5 times the initial value of 1 Hz. Coupling constants used to find the maxima of a multiplet may be changed less than ±5 % from the values determined from the JRES.

To fit a multiplet of n peaks, we simply fitted a sum of n pseudo-Voigt curves with different $x_{\max}$ (calculated from the multiplet's splitting) and different coefficients to A taking into account the Pascal's triangle intensities pattern and possible roof effects (dependent on the spectrometer's basic acquisition frequency and thus empirically determined). See figures 1 and 2 for examples and a visual explanation.

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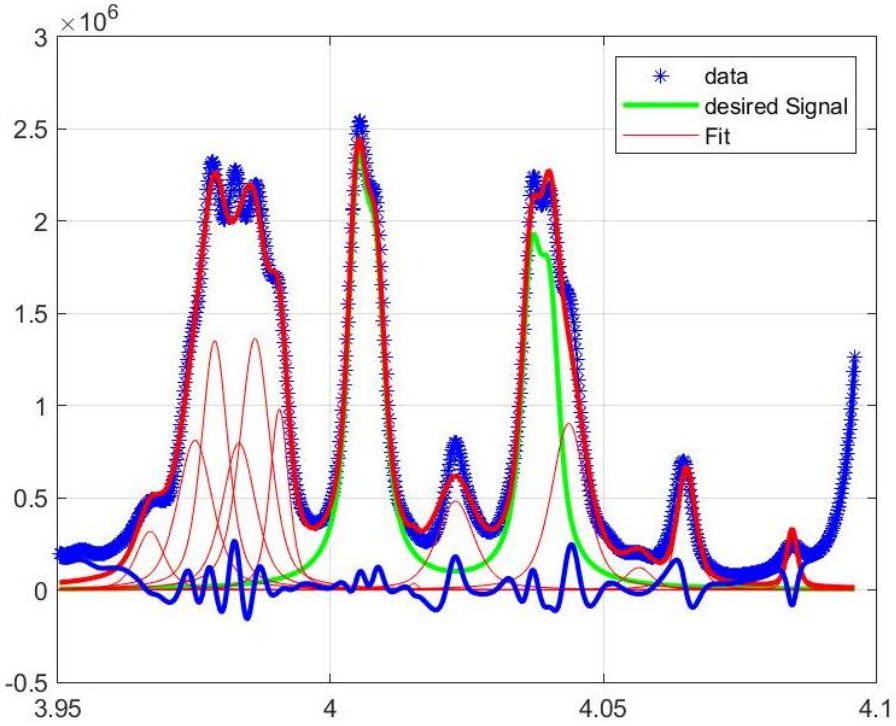

Figure 1. On demand Matlab output graphs of the curve fits, in this case the first signal of fructose. The green curve is the desired fit, the sum of four pseudo-Voigt profiles with the constraints typical for this signal. To mimic the curve of the original data (blue asterisks), Matlab adds more singlet profiles (thin red lines), all combined yield the thick red line. The residues are shown as a thick blue line.

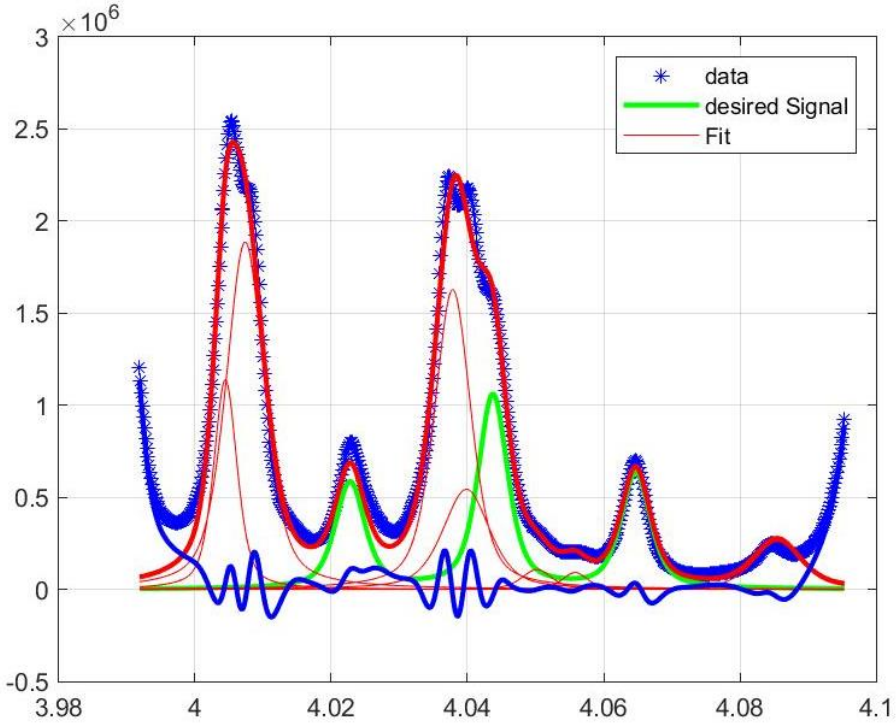

Figure 2. Curve fit of the sucrose triplet (thick green line). Although the resonance is not at all separated from the fructose double-doublet (compare fig. 1), the algorithm can again separate the desired signal from interfering signals (approximated as simple singlets, the thin red lines) arriving

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at a good representation of the original data (blue asterisks) with acceptably low residues (thick blue line)

2.4.4. Quantification

Analyte concentrations were calculated from the intensities (integrals) of their characteristic resonances using the PULCON method (*Pulse Length Based Concentration Determination*) [27,28].

Basically this well proven method uses an external standard of known concentration(s), called QuantRef (= quantification reference solution), to determine the NMR spectrometer's response to a specific number of resonating nuclei under given acquisition conditions. This normalised response/sensitivity is called ERETIC factor (*Electronic Reference To access In-vivo Concentrations*) [28].

The following equation was used to calculate the ERETIC factor:

$$f_{\mathrm{ERECTIC}} = \frac{I_{\mathrm{Ref}} \cdot SW_{\mathrm{Ref}} \cdot M_{\mathrm{Ref}}}{SI_{\mathrm{Ref}} \cdot \rho_{\mathrm{Ref}} \cdot N_{\mathrm{Ref}}^{\mathrm{H}} \cdot f_{\mathrm{QR}}^{\mathrm{dil}}} \quad (3)$$

Where:

I_{Ref} is the absolute integral (Ref = reference) of a selected resonance of the reference substance,
SW_{Ref} is the spectral width (20.5504 ppm),
M_{Ref} is the reference substance's molecular weight (144.11 g/mol for sodium benzoate, 182.17 g/mol for mannitol, and 96.06 g/mol for sodium propionate),
$N_{\mathrm{Ref}}^{\mathrm{H}}$ is the number of protons (per molecule) generating the selected resonance,
ρ_{Ref} is the reference substance's exact mass concentration,
SI_{Ref} is the size of the real spectrum, which shows the number of data points after Fourier transformation (131072),
$f_{\mathrm{QR}}^{\mathrm{dil}}$ is the QuantRef's dilution factor (0.3) resulting from its preparation.

Evaluating the sodium benzoate peak between δ 7.61 ppm and 7.525 ppm, $n^{\mathrm{H}} = 1$, the mannitol doublets between δ 3.92 and 3.84 ppm, $n^{\mathrm{H}} = 2$ and between δ 3.73 and 3.64 ppm, $n^{\mathrm{H}} = 2$, the sodium propionate quartet at δ 2.25 to 2.12 ppm, $n^{\mathrm{H}} = 2$ and its triplet at δ 1.12 to 1.00 ppm, $n^{\mathrm{H}} = 3$ an average ERETIC factor was calculated from these signals and used for quantification. The relative deviation between the five individual ERETIC factors shall be lower than 2 %. Otherwise the QuantRef solution needs to be checked and possibly be freshly prepared.

The analyte concentration ρ_X in unknown samples was calculated using the following PULCON equation:

$$\rho_X = \frac{I_{\mathrm{X}} \cdot SW_{\mathrm{X}} \cdot M_{\mathrm{X}} \cdot NS_{\mathrm{QR}} \cdot P1_{\mathrm{X}}}{SI_{\mathrm{X}} \cdot f_{\mathrm{ERETIC}} \cdot N_{\mathrm{X}}^{\mathrm{H}} \cdot NS_{\mathrm{X}} \cdot f_{\mathrm{X}}^{\mathrm{dil}} \cdot P1_{\mathrm{QR}}} \quad (4)$$

Where:

I_{X} is the absolute integral of the evaluated peak,
$f_{\mathrm{X}}^{\mathrm{dil}}$ is the sample's dilution factor (0.50),
NS_{X} and NS_{QR} are the number of scans for spectrum of sample and number of scans for the reference respectively,
SI_{X} is the size of real spectrum (131072),
$P1_{\mathrm{X}}$ and $P1_{\mathrm{QR}}$ are the respective pulses,
f_{ERETIC} is the ERETIC factor (see eq. 1).

If more than one signal was be used for the determination of an analyte, the Matlab script automatically checked that the standard deviation stayed under a threshold (20 % for fructose, 8 % for sucrose and glucose, 10 % for isobutanol and 5 % for all other analytes). If the values passed this test, the mean of the calculated concentrations was saved as the results, otherwise the analyte was noted as "not quantifiable".

As output the Matlab script reports the results as excel and text (txt) files for direct import in the laboratory's LIMS system (Limsophy, AAC Infotray AG, Winterthur, Switzerland).

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2.5. *Quality assurance*

The QuantRef's calculated concentrations are documented in a control chart and shall not vary more than ±5 % from their original values. Another internal quality control of each sample's measurement is the FWHM of the TSP (internal standard) peak. It shall not exceed 1.3 Hz, otherwise the measurement or even the sample preparation has to be repeated. A FWHM higher than 1.3 Hz could result e.g. from a substandard shimming or a turbid sample.

For quality assurance the last sample in each measurement series is the control solution (see above). From each new measurement the concentrations of the control solution's substances are calculated and may not vary more than ±5 % from the original values at preparation (recovery rate between 95 % and 105 %)

2.6. *Validation experiments*

Based on previous experience in validating quantitative multicomponent NMR assays [29], validation was performed "in matrix" and not just in blank aqueous/ethanolic solutions spiked with reference substance.

2.6.1. Working range and measurement uncertainty

For each analyte, a stock solution was prepared in an ethanol/water mixture (190 mL + 50 mL, ca. 21 % vol). To determine linearity, the limit of detection (LOD) and the limit of quantification (LOQ), samples at 9 different concentration levels were prepared by spiking a 35 % vol ethanol-water mix with the stock solutions and measured by NMR. Appropriate concentrations were chosen regarding the typical concentration ranges for the selected analytes in spirits or taking into account the maximum content as defined by legal food regulations (see Table 1). At each concentration level four sample preparations were done and measured to evaluate the preparation variance.

The Measurement uncertainty was then calculated with an in-house QM-approved Excel script. Variances of the four repetitions at each concentration level were calculated and a weighting function was determined using a best-fit function. The weighted measurement results were then used to determine the upper and lower confidence intervals at each concentration point..

To determine the relative measurement uncertainty over the working range, the uncertainties were extrapolated against the concentration 0 mg/L and the blank value $\beta(0)$ found was used as the offset for the measurement uncertainty. The function $\Delta\beta = \beta_{found} \times 8\% + \beta(0)$ was then used as the concentration-dependent measurement uncertainty. This function is stored in the Matlab script. Thus, Matlab will output the calculated concentration value and the associated uncertainty.

2.6.2. Recovery and matrix effects

To check for matrix effects, the five different spirit matrices (see above) were spiked with the analyte stock solutions to prepare four different concentration levels in the range typical for each analyte in each spirit matrix. Each of the five spirit matrices was also measured without spiking to yield blank values. If a matrix contained an analyte beforehand, a series of five dilutions was prepared by adding a 35 % (vol) ethanol-water mix to the spirit matrix.

2.6.3. Specificity and selectivity

From NMR-spectra of spirit matrix samples spiked with analytes the signals specific for each analyte were identified and integration regions were determined which were not overlapping with other signals.

2.6.4. Sample stability

For laboratories with a high sample throughput the stability of a sample over time is important, because measurement does not always immediately follow sample preparation. To determine the

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sample stability over time, three samples of each matrix were prepared and each sample was measured four times at intervals of about one day. The stability was evaluated by the slope of the compensation line of all four measuring series.

3. Results

3.1. Relevant analytes and their characteristic NMR signals

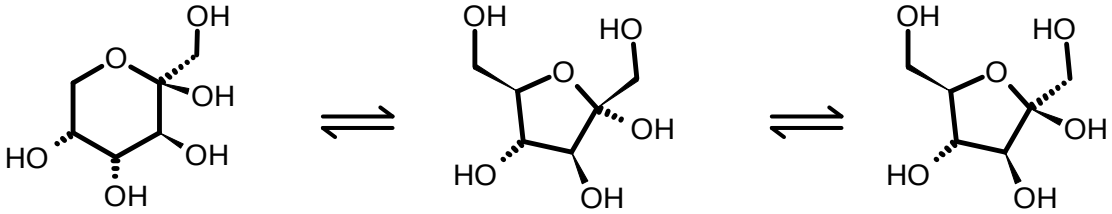
A vast variety of spirits is available on the market, and their compositions (both qualitative and quantitative) differ considerably. Of the 20 different spirits ingredients initially identified as potential analytical targets, 15 were found to be accessible by NMR analysis; these are summarized in tables 1 and 3.

For the initial evaluation whether a quantitative NMR spectroscopy approach is suitable for the potential analytical targets, proton spectra of commercially available pure compounds were acquired and compared to spectra of real spirits samples. For the majority of the tested substances, direct quantification by simple integration of their resonance signals was not possible due to insufficient separation from other signals or due to minor signal shifts depending on the analyte's concentration or the solutions pH-value. Especially the mid-field region (around 3.3 to 4.1 ppm) of ^{1}H-NMR spectra of multi component mixtures is rather crowded, the strong and often (partially) overlapping signals of carbohydrates (mostly sugars) and other substances appear there. Strong neighbouring signals of major components can hide comparably small peaks of other, lower concentrated minor ingredients. This is a well-known problem and has been described earlier, for example for the analysis of honey by using NMR spectroscopy [30].

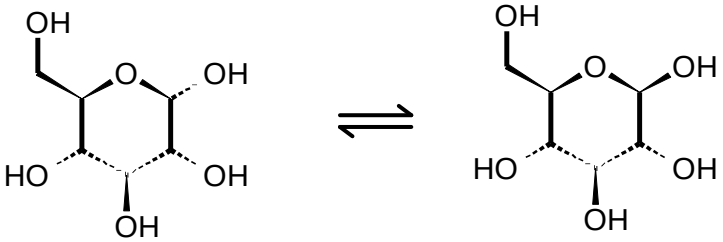
Attempts to evaluate JRES data for quantification were not satisfying in spite of the advantages of JRES: its increased spectral dispersion in a second dimension and the clear information about the *J*-coupling. On the other hand, the JRES' lower sensitivity and the overlap of the dispersive tails of nearby signals are obstacles to the quantification (especially at lower concentrations) [31].

Thus we found the 1D ^{1}H NMR spectrum more suitable for quantification. Nevertheless, in the current context the JRES experiment has proven valuable and reliable in confirming the presence of analytes in the needed concentration range and determining the exact chemical shifts of the analytes in each sample. The information obtained from the JRES was then subsequently used for the curve fitting to analyse the 1D ^{1}H NMR spectrum.

For five compounds (anethol, furfural, malic acid, 1-pentanol and isopropanol) recoveries were found to be unreliable or too low (< 80%), LOD and LOQ too high, or the separation of their signals from other resonances was unreliable. Finally, for the fifteen compounds listed in Tables 1 and 3 the test method was validated with results fit for purpose.



(**a**)



(**b**)

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Figure 3 The predominant forms of fructose and glucose in aqueous solution: (**a**) fructose equilibrium 76 % β-D-Pyr, 4 % α-D-Fur, 20 % β-D-Fur; (**b**) glucose equilibrium of 36% α-D-Pyr, 64 % β-D-Pyr

In aqueous solutions the carbohydrates glucose and fructose exist in equilibrium of different anomeric and tautomeric forms (Figure 3). For glucose, the α-and β-anomers of glucopyranose predominate with 36 % and 64 % respectively. Fructose exists in at least five tautomeric forms in aqueous solution [32]. Furthermore, the presaturation used to suppress the water resonance slightly attenuates the surrounding spectral area as well, especially for the anomeric glucose peaks located around 4.6 and 5.2 ppm [33]. Taking into account both the signal attenuation by the water suppression and the anomeric / tautomeric equilibria, the calculation correction factors for glucose and fructose were determined by analysing spirits samples spiked with varying concentrations of glucose and fructose.

Figures 4 and 5 show spectra with enlargements of the characteristic resonances used for identification and integration of the fifteen analytes. Table 3 lists the chemical shift ranges of the characteristic signals, their multiplicity, coupling constants and the number of protons (per molecule) giving rise to each signal.

Table 3 Characteristic NMR signals of the analytes in spirits.

#	Substance	search range (ppm)	Multiplicity	Coupling constants *J* (Hz)	N_H
1	1-Propanol	1.58 – 1.52	Hx	7.2	2
		0.92 – 0.89	T	7.38	3
2	2-Phenylethanol	2.90 – 2.80	T	6.92	2
3	Acetaldehyde	9.72 – 9.68	Q	2.94	1
		2.27 – 2.24	D	2.94	3
4	Acetic acid	1.925 – 1.9	S	–	3
5	Citric acid	2.72 – 2.65	D	15.05	2
		2.62 – 2.49	D	15.05	2
6	Ethyl acetate	4.18 – 4.12	Q	7.2	2
		2.10 – 2.07	S	–	3
7	Ethyl lactate	4.40 – 4.32	Q	6.95	1
		4.26 – 4.18	Q	7.13	2
8	Formic acid	8.48 – 8.44	S	–	1
9	Fructose[1]	4.08 – 3.99	Dd	12.6 and 1.35	1
		4.01 – 3.95	Pe	1.69	1
10	Glucose[2]	5.25 – 5.20	D	3.76	1
		4.66 – 4.58	D	7.96	1
		3.26 – 3.21	Dd	8.60 and 0.68	1
11	HMF	9.50 – 9.45	S	–	1
		7.60 – 7.50	D	3.85	1
		6.75 – 6.65	D	3.85	1
12	Isobutanol	3.375 – 3.35	D	6.62	2
		1.77 – 1.71	No	6.7	1
		0.89 – 0.75	D	6.74	6
13	Isopentanol	1.70 – 1.62	No	6.74	1
		1.455 – 1.41	Q	6.8	2
		0.915 – 0.895	D	6.68	6

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#	Substance	search range (ppm)	Multiplicity	Coupling constants *J* (Hz)	N_H
14	Methanol	3.375 – 3.35	S	–	3
15	Sucrose	5.46 – 5.38	D	3.85	1
		4.25 – 4.18	D	8.72	1
		4.10 – 3.99	T	8.47	1

[1] In aqueous solution fructose exists in five tautomeric forms [32]. Their different signals are partially discernible in the NMR spectrum and the intensities reflect the ratio between tautomers. Thus correction factors were empirically determined by evaluating fructose spectra at different concentrations: 1.49 for the double doublet and 1.51 for the pentet.

[2] In aqueous solution glucose exists in different anomeric forms. To calculate the glucose concentration, signals 1 and 2 (originating from the α and β anomeric protons respectively) were summed. Signal 3 originates from the C-2 proton of the β anomer only, a correction factor of 1.47 was determined (as for fructose).

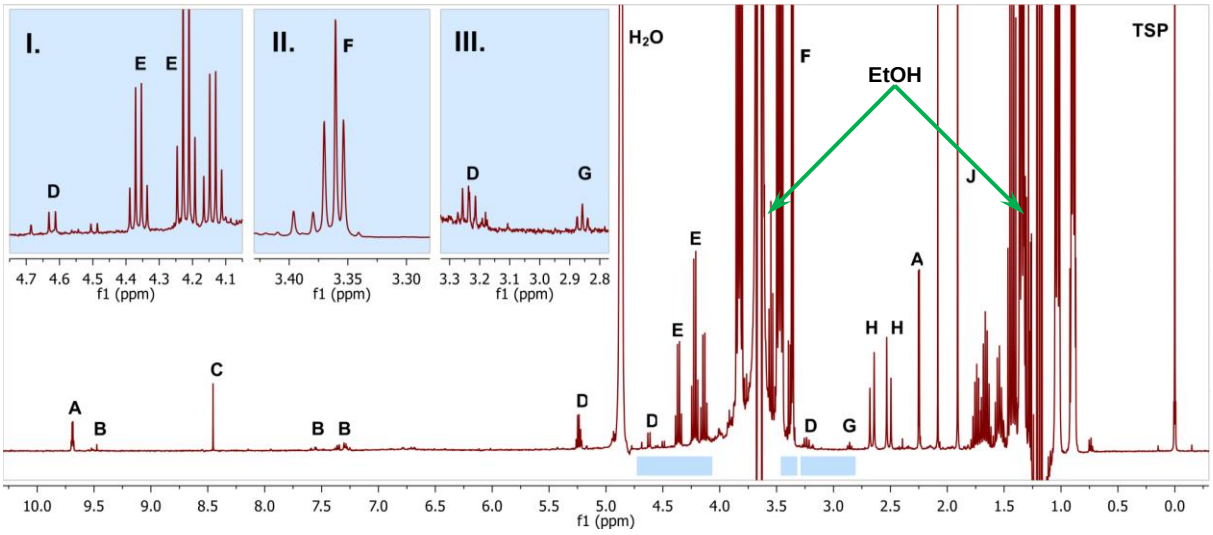

Figure 4. 1D ^{1}H NMR Spectrum of whisky spiked with several of the analytes. I, II, III: enlargements, see the blue bars under the main spectrum for the regions. The labelled signals are: A: acetaldehyde, B: HMF (positive, < LOQ), C: formic acid, D: glucose, E: ethyl lactate, F: methanol, G: 2-phenylethanol (positive, < LOQ), H: citric acid, J: acetic acid, H_2O: water (suppressed), TSP: trimethyl silylpropionate-d4; EtOH: ethanol (suppressed)

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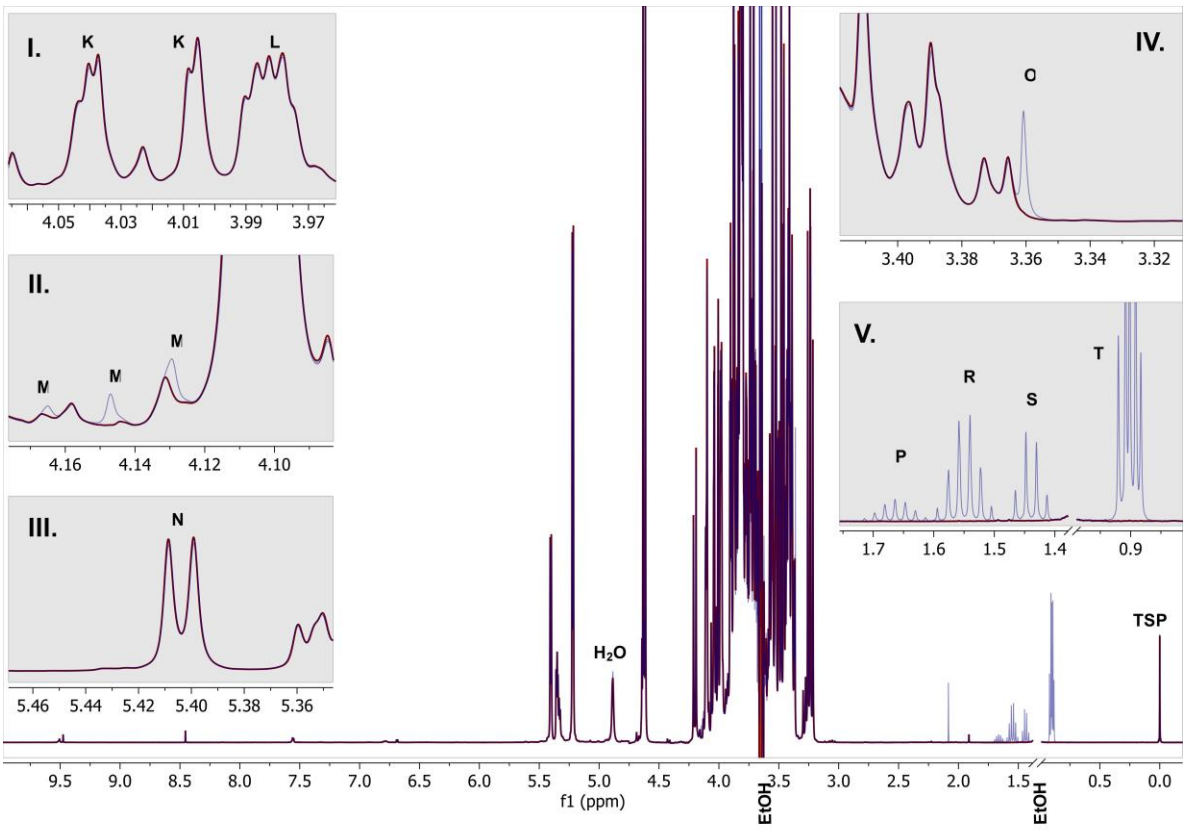

Figure 5. 1D ^{1}H NMR spectrum of herbal liqueur overlayed with spiked sample (blue). I. to V.: enlargements. The labelled signals are: K: fructose (dd), L: fructose ("pe"), M: ethyl acetate (q), N: sucrose (d), O: methanol, P: isopentanol (no), Q: 1-propanol (hx), R: isopentanol (q), S: overlayed triplet of 1-propanol and doublet of isopentanol, H_2O: water (suppressed), TSP: trimethylsilyl propionate-d4; EtOH: ethanol (suppressed, cut out)

3.2. *Analytical limits*

The calculated analytical limits for the validated analytes are listed in Table 4. They were calculated according to the German Standard DIN 32645:2008 using a validated and QM-approved Microsoft Excel worksheet.

Table 4 Analytical limits for the validated analytes (in mg/L)

#	Substance	LOD (mg/L)	LOQ (mg/L)	Range evaluated (mg/L)
1	1-Propanol	11	26	10 – 509
2	2-Phenylethanol	8	19	24 – 243
3	Acetaldehyde	4	10	5 – 96
4	Acetic acid	3	6	1 – 229
5	Citric acid	3	8	10 – 100
6	Ethyl acetate	4	9	10 – 249
7	Ethyl lactate	11	27	11 – 546
8	Formic acid	3	7	10 – 100
9	Fructose	56	132	75 – 749
10	Glucose	35	84	50 – 1001
11	HMF	23	57	23 – 2333
12	Isobutanol	25	61	92 – 920
13	Isopentanol	74	173	51 – 505

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#	Substance	LOD (mg/L)	LOQ (mg/L)	Range evaluated (mg/L)
14	Methanol	2	5	2 – 110
15	Sucrose	41	98	50 – 1002

The LODs varied in the range of 2 mg/L to 74 mg/L. The lowest value was attributed to methanol (2 mg/L) followed by formic, citric and acetic acids (3 mg/L) while the highest value was found for isopentanol. The LOQ Values were in the range from 5 – 173 mg/L for methanol and isopentanol, respectively.

Of the three sugars, fructose showed the highest LOD and LOQ values (56 and 132 mg/L). Still the LODs and LOQs of the analytes are lower or at the low end of the relevant concentration range for the analysed spirits ingredients. Thus, NMR spectroscopy can be considered as a suitable tool to monitor spirits by official food control authorities.

3.3. *Overall measurement uncertainty*

The determined uncertainty measurement of analytes in spirit drinks are summarized in Table 5. The recovery rates were set at 100%. Any deviations in recovery are already included in the determination and verification of the measurement uncertainties. Recovery errors are considered purely statistical errors and not systematic errors. A recovery rate correction is therefore not performed. $\Delta\beta = \beta_{found} \times 8\% + \beta(0)$

Table 5 Measurement uncertainties of analytes in spirit beverages

#	Substance	Offset $\beta(0)$ (mg/L)	Measurement uncertainty $\pm\Delta\beta$ (mg/L)	Concentration range for calculation (mg/L)
1	1-Propanol	17	$\beta_{found} \times 8\% + 17$	10 – 509
2	2-Phenylethanol	11	$\beta_{found} \times 8\% + 11$	48 – 486
3	Acetaldehyde	10	$\beta_{found} \times 8\% + 10$	5 – 964
4	Acetic acid	4	$\beta_{found} \times 8\% + 4$	0.5 – 229
5	Citric acid	10	$\beta_{found} \times 8\% + 10$	10 – 498
6	Ethyl acetate	15	$\beta_{found} \times 8\% + 15$	10 – 497
7	Ethyl lactate	30	$\beta_{found} \times 8\% + 30$	11 – 1090
8	Formic acid	6	$\beta_{found} \times 8\% + 6$	10 – 1000
9	Fructose	200	$\beta_{found} \times 8\% + 200$	75 – 7491
10	Glucose	62	$\beta_{found} \times 8\% + 62$	50 – 1001
11	HMF	10	$\beta_{found} \times 8\% + 10$	23 – 4667
12	Isobutanol	30	$\beta_{found} \times 8\% + 30$	92 – 920
13	Isopentanol	90	$\beta_{found} \times 8\% + 90$	51 – 1010
14	Methanol	4	$\beta_{found} \times 8\% + 4$	2 – 110
15	Sucrose	53	$\beta_{found} \times 8\% + 53$	50 – 1002

3.4. *Stability*

The general stability of the samples is judged as sufficient for two days after preparation, see table 6 for details. The observed deviations due to ageing of the samples stay under the 8 % threshold determined as the overall uncertainty. Acetaldehyde and formic acid show a loss above threshold even after one day. If there is an objection concerning these analytes, the measurement shall be confirmed using a complementary analytical method or a NMR measurement immediately after sample preparation.

Table 6 Stability of individual ingredients in spirit drinks

#	Substance	Deviation after 1st day (%)	after 2nd day (%)	after 3rd day (%)

#	Substance	Deviation after 1st day (%)		after 2nd day (%)		after 3rd day (%)	
1	1-Propanol	3.56 mg/L	(0.6%)	4.47 mg/L	(3.8%)		
2	2-Phenylethanol	---		---		---	
3	Acetaldehyde	2.47 mg/L	(10.2%)	1.17 mg/L	(1.4%)	0.06 mg/L	(1%)
4	Acetic acid	0.57 mg/L	(5.1%)	0.33 mg/L	(2.4%)	1.87 mg/L	(6.7%)
5	Citric acid	-0.35 mg/L	(0.4%)				
6	Ethyl acetate	0.63 mg/L	(4.8%)	- 15.2 mg/L	(1.2%)	-0.8 mg/L	(0.8%)
7	Ethyl lactate	-5.97 mg/L	(2.0 %)				
8	Formic acid	5.87 mg/L	(15.7%)	2.9 mg/L	(6.7%)	0.57 mg/L	(3.5%)
9	Fructose	-94.0 mg/L	(0.4%)	-63.5 mg/L	(0.1%)	-0.5 mg/L	(1.5%)
10	Glucose	17.1 mg/L	(0.1%)	-177 mg/L	(0.3%)	-1.0 mg/L	(1.4%)
11	HMF	-1 mg/L	(0.8%)	-1 mg/L	(0.8%)		
12	Isobutanol	-2.5 mg/L	(2.5%)	1.2 mg/L	(0.6%)		
13	Isopentanol	-6.7 mg/L	(2.1%)	-8.37 mg/L	(1.5%)		
14	Methanol	0.23 mg/L	(1.4%)	1 mg/L	(0.1%)	-0.17 mg/L	(3.2%)
15	Sucrose	-22.0 mg/L	(0.1%)	-46.2 mg/L	(0.2%)	1.6 mg/L	(0.4%)

4. Discussion

Accuracy fit for purpose (the routine screening of spirits) was proven for the fifteen analytes in all spirit matrices except for methanol in herbal liqueur. Due to the typically low concentrations of methanol in herbal liqueur and the rather high glucose and sucrose contents, which disturb the baseline in the range of the methanol signal, too high concentrations of methanol were found. In case of a complaint about methanol in herbal liqueur, the baseline around the methanol resonance must be checked especially if there is a positive bias.

For ethyl lactate, a minor positive bias was found in fruit spirit and absinthe. Since the deviations are only small, further validation is not deemed necessary.

For citric acid in herbal liqueur and absinthe sometimes wrong contents were found. This is due to an extreme signal overlap by accompanying substances. For citric acid, correct values are obtained only for whisky, vodka and fruit spirit. In case of conspicuously low or high citric acid values, it is necessary to check the line shape of the citric acid signal and confirm the result with another measuring technique.

4.1. Comparison with other methods

In 2018 our lab took part in an interlaboratory proficiency test. Table 7 shows the z-scores achieved with the NMR spirit screener:

Table 7 z-scores of the 2018 proficiency test "sprits drink analysis"

#	Substance	z-score	#	Substance	z-score
1	1-Propanol	0.7	9	Fructose	–
2	2-Phenylethanol	–	10	Glucose	–
3	Acetaldehyde	-0.5	11	HMF	–
4	Acetic acid	–	12	Isobutanol	3.6
5	Citric acid	–	13	Isopentanol	1.3
6	Ethyl acetate	-1.4	14	Methanol	0.4
7	Ethyl lactate	0.7	15	Sucrose	–
8	Formic acid	–			

4.2. Application in routine analysis

With rising global trade and new products on offer every year, official food and beverage control needs to stay innovative to cope with coming analytical challenges. The increasing number of samples per year (due to centralization of official laboratories in Germany e.g.) furthermore demands for accurate and efficient high-throughput measurement and data processing workflows.

Some NMR analyser packages were already commercialised (e.g., "Juice Screener" and "Wine Screener" by Bruker [18,34]) and several current research projects are striving to deliver solutions for more analytical questions. Typically the commercial analysis packages are fully automated and installed on a NMR spectrometer in the form of a "black box" system. Users cannot enhance or adapt these test methods on their own to cope with new challenges or to apply these systems to other matrices.

The automated procedures for the analysis of spirits developed in our laboratory successfully fill a demand for an efficient simultaneous multi-analyte test method and are open for further development.

To start the automated data evaluation, the operator needs to input only the data path to the selected experimental series containing the spectra of samples, the QuantRef and the QA sample. The report for each experimental series contains concentrations of the 15 analytes in each sample as excel and txt files for direct import into LIMS. The algorithm offers a high throughput as it allows analysing a large number of samples without human intervention within reasonable time (for example, evaluation requires about 3 minutes for 25 samples).

5. Conclusions

NMR spectroscopy offers a fast, reliable and efficient methodology to quantify relevant ingredients in spirits fulfilling the requirements of DIN ISO 17025. The accuracy and detection limits allow for controlling legal maximum limits (such as methanol) as well as manufacturer declarations. Furthermore, performance parameters of the developed NMR spectroscopic method are similar to other methods used for spirits analysis. The developed automatic routine provides an integrated workflow that performs all necessary steps (spectra import, extraction of data points, curve fitting the signals of interest, integration of the optimized fit, quantification according to the PULCON principle and reporting of results) for quantitative analysis. According to the obtained results, the developed protocol provides an optimal data processing workflow for NMR analysis of spirits, which can be simply transferred to other matrices as well.

Future work will focus on streamlining and flexibilisation of the algorithm code and on improvement of the quantification of compounds not yet adequately separated from interfering signals.

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Conflicts of Interest: The authors declare no conflict of interest.

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