Using experimental design and 13C satellites to optimize the measurement of the absolute concentration of ethanol in wine by 1H NMR spectroscopy

Food Research International 214 (2025) 116598

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Using experimental design and ^{13}C satellites to optimize the measurement of the absolute concentration of ethanol in wine by ^{1}H NMR spectroscopy☆

Mattia Sozzi [a], Violetta Aru [b], Anna Mascellani Bergo [c], Nicola Cavallini [a], Francesco Savorani [a], Havlik Jaroslav [c], Bekzod Khakimov [b], Søren Balling Engelsen [b,*,1]

[a] Department of Applied Science and Technology, Polytechnic of Turin, Corso Duca degli Abruzzi 24, 10129 Turin, Italy
[b] Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C, Denmark
[c] Department of Food Science, Czech University of Life Sciences Prague, Kamycka 129, 165 00 Prague 6, Suchdol, Czech Republic

ARTICLE INFO

Keywords:
Ethanol absolute quantification
qNMR
^{1}H NMR metabolomics
Wine
Beverages
^{13}C NMR satellites

ABSTRACT

Proton (^{1}H) NMR spectroscopy is routinely used for targeted food analysis. In alcoholic beverages, ethanol content is a key quality parameter. While ^{1}H NMR's inherently quantitative nature makes it ideal for absolute quantification of ethanol, challenges arise in complex matrices like wine. High ethanol concentrations, orders of magnitude larger than other constituents and co-solvents, can hinder accuracy due to phenomena such as radiation damping and intermolecular interactions. The present work investigates factors influencing absolute ethanol quantification by ^{1}H NMR, including ethanol concentration (0–40 % *v*/v), solvent for signal lock (D_2O *vs.* DMSO), pulse sequence (w/wo water suppression), NMR signal (main triplet, its ^{13}C-satellite or the combination), quantification method (multivariate curve resolution *vs.* integration by raw sum), and sample type (red *vs.* white wine). FT-IR was employed as reference method for ethanol absolute quantification. Our results indicate that the highest accuracy for the absolute quantification of ethanol in ethanol-water solutions is achieved by measuring D_2O-containing ethanol solutions with water suppression, and raw sum for quantification of the ethanol main triplet and its satellites. . As for wine samples, wine type affected the quantification the most (ASCA effect size = 60 %, $p < 0.001$). Based on our results, the best approach to obtain accurate absolute quantification of ethanol in wines samples entails the use of (1) an external standard with its satellites (*i.e.*, TSP), (2) a correction factor k_{ext}, calculated from measurements of a known ethanol reference sample to compensate for misquantifications due to matrix effects, and (3) combining the intensities of the main triplet and its satellites for absolute quantification, in order to compensate for different $^{13}C/^{12}C$ ratios in wines. Using this method a correlation coefficient (R^2) of 0.99 and RMSE of 0.23 % (*v*/v) was achieved for absolute quantification of ethanol in wine samples.

1. Introduction

Several analytical techniques are routinely employed for the quantification of chemical compounds in foods, from cumbersome classical wet chemistry methods, over chromatographic methods, to "green" spectroscopic techniques, such as infrared (IR) and near-infrared (NIR) spectroscopy (Alahmad, Kaya, Cetinkaya, Varanusupakul, & Ozkan, 2023; Czaja & Engelsen, 2025; Hassoun et al., 2023; Pallone, Caramês, & Alamar, 2018). Proton (^{1}H) Nuclear Magnetic Resonance (NMR) spectroscopy is an inherently quantitative method, which is routinely used for the quantitative analysis of molecules in diverse biological samples in a wide range of research and industrial fields, including food quality control. The NMR signal intensity is directly proportional to the number of protons giving rise to the signal, and absolute quantification by ^{1}H NMR can thus be achieved using an internal/external reference compound of known concentration or using a calibrated artificial signal (Burton, Quilliam, & Walter, 2005; Farrant et al., 2010; Giraudeau, Tea, Remaud, & Akoka, 2014; Wider & Dreier, 2006). When performing absolute quantitative ^{1}H NMR (qNMR) analysis, parameters optimization, including calibration of the 90° pulse (p1) and recycle delay (d1),

☆ This article is part of a Special issue entitled: 'Magnetic Resonance in Food Science' published in Food Research International.

* Corresponding author at: University of Copenhagen, Department of Food Science, Rolighedsvej 26, 1958 Frederiksberg C, Denmark.
E-mail address: se@food.ku.dk (S.B. Engelsen).
[1] 0000–0003–4124-4338.

https://doi.org/10.1016/j.foodres.2025.116598
Received 30 January 2025; Received in revised form 1 April 2025; Accepted 4 May 2025
Available online 8 May 2025

are essential in order to obtain reliable quantifications (Giraudeau, 2017; Torres & Price, 2016). However, despite the careful instrumental calibration, absolute quantification of high concentration solvents still represents a challenge for ^{1}H NMR spectroscopy due to physical phenomena, such as radiation damping (RD) (Krishnan & Murali, 2013).

A notable case is the absolute quantification of ethanol in alcoholic beverages. Ethanol concentration in commercial wines is measured by distillation but can also be measured (predicted) with high accuracy using the WineScan, which is based on Fourier-Transform Infra-Red (FT-IR) spectroscopy (WineScan FT 120, FOSS A/S, Hillerød, Denmark). Prediction errors are around or just below 0.1 % (*v*/v) (Bauer et al., 2008), despite broad and overlapping signals. NMR is commonly used for wine authentication and metabolite profiling but considered too advanced (expensive) for plain determination of ethanol. Curiously, this advanced technique, with nearly baseline separated ethanol signals, is inferior to FT-IR in quantitative performance. Among the studies focusing on the use of ^{1}H NMR spectroscopy for the absolute quantification of ethanol, only few have reported measurement uncertainty values, which were ranging from ±0.5–8 % (*v*/v) (Farine et al., 2024; Teipel et al., 2020; Ün & Goren, 2017; Zuriarrain, Zuriarrain, Villar, & Berregi, 2015). In 2009, Lopez et al. developed a qNMR method for ethanol quantification in wines using its main and satellite resonances (López-Rituerto et al., 2009). Their method validation was based on distillation and FT-IR (WineScan). Differently, Caleja-Ballesteros et al. focused on the development of a novel qNMR method for ethanol quantification in distilled spirits using non-deuterated solvents (Caleja-Ballesteros, Ballesteros, & Villena, 2021). In this case, method validation was performed by gas chromatography with flame ionization detection (GC-FID). Among the commercially available NMR-based solutions for wine screening is the FoodScreener™ from Bruker (Bruker Biospin, Rheinstetten, Germany). To the best of our knowledge, specific uncertainty values for ethanol quantification using the FoodScreener™ are

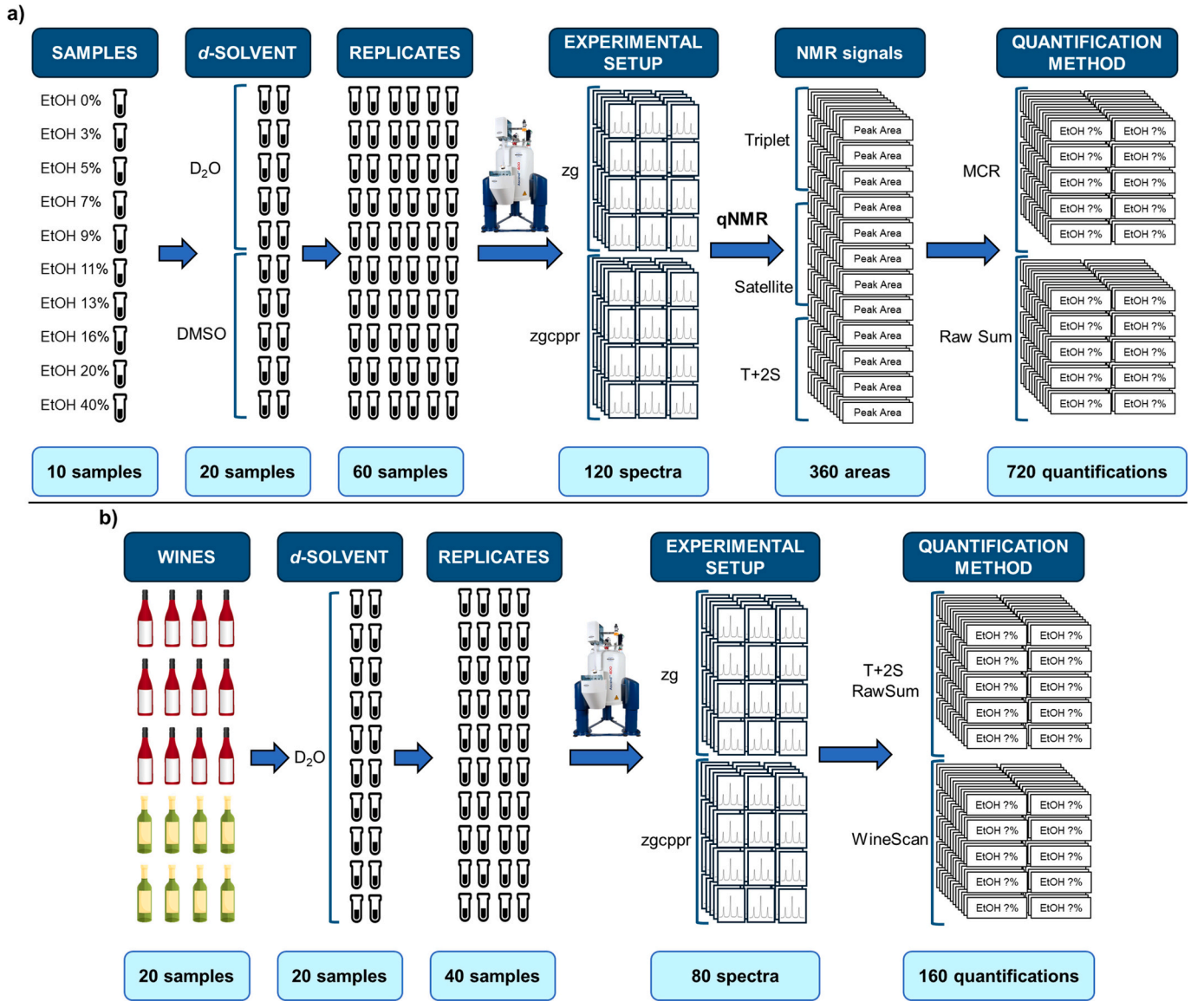

Fig. 1. Experimental design. Part 1 (a) Ten concentrations of ethanol in water were prepared using two different deuterated solvents and measured in three replicates using two different pulse sequences, with and without water suppression. In the data analysis step, two different NMR signals (the triplet -T- and its ^{13}C satellites - S) were monitored alone (T or S) and in combination (T+2S). Two approaches for signal quantification were used. In total, 720 quantifications were made. Part 2 (b) Application of the optimal parameters from (a) on a real case study on wines. 20 table wines (12 red wines and 8 white wines) were prepared in two replicates with D_2O and measured using two pulse sequences (with and without water suppression). Ethanol quantification was performed both on the ethanol triplet peak (T) and on the triplet and its satellites (T+2S) using the raw sum approach. The WineScan (FT-IR) instrument was used as reference method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not publicly available.

The applications of qNMR in beverage analysis are extensive. In addition to quantifying ethanol, qNMR can be used to identify and quantify diverse arrays of metabolites, including sugars, amino acids, phenolic compounds, pigments, organic acids, and vitamins, among others, making it an ideal tool for beverage analysis (Belton et al., 1996; Duarte et al., 2002; Mac et al., 2023). Recent applications of qNMR in beverage analysis include quality control and authentication of spirit drinks (Teipel et al., 2020), influence of terroir in wine quality (Lopez-Rituerto et al., 2012), authentication and traceability of craft beer (Palmioli, Alberici, Ciaramelli, & Airoldi, 2020), but also fermentation monitoring during winemaking (López-Rituerto et al., 2022), where ethanol measurements are of utmost importance.

The present study investigates parameters that can potentially affect the absolute quantification of ethanol in alcoholic beverages obtained using ^{1}H NMR spectroscopy including signal type, deuterated solvent type, matrix effect and quantification method. The study consists of two parts: (1) investigation of the impact of different measurement parameters such as pulse sequence/water suppression, type of deuterated-solvent used for signal lock, NMR signal used for quantification (methylene, methyl and/or their respective ^{13}C satellites) and quantification method (raw sum *vs.* multivariate curve resolution – MCR (de Juan, Jaumot, & Tauler, 2014)) on the quantification of ethanol in pure ethanol-in-water solutions at different concentrations (Fig. 1a); (2) application and evaluation of the best operating conditions on case study based on white and red wine samples (Fig. 1b). Trimethylsilyl-propionic acid (TSP) – a widely used reference compound for NMR analysis – was used as internal standard for ethanol quantification in the ethanol-in-water solutions and as external standard in wine samples, to overcome its analytical limitations of not being inert (Nagana Gowda, Hong, & Raftery, 2021).

2. Experimental

2.1. Chemicals

Deuterium oxide (D_2O, 99.9 atom % D), dimethyl sulfoxide (DMSO-d_6, 99,9 atom % D), potassium phosphate monobasic (KH_2PO_4, P ≥ 98 %), sodium 3-trimethylsilyl-propionate-2,2,3,3-d_4 (TSP-d_4, 98 atom % D), and ethanol (P ≥ 99.8 %) were purchased from Sigma-Aldrich (Darmstadt, Germany). The water used throughout the study was purified using a Millipore lab water system (Merck KGaA, Darmstadt, Germany) equipped with a 0.22 μm filter membrane.

2.2. Experimental design

The experimental design is outlined in Fig. 1 and comprises two parts (Fig. 1a and b). The experiments of the first part were planned according to a Design of Experiment (DoE) approach based on four factors, each with two levels. To test the quantification capability of ^{1}H NMR spectroscopy with respect to increasing ethanol concentrations, ten ethanol solutions ranging from 0 to 40 % (*v*/v) were prepared in triplicates (Fig. 1a). Samples for ^{1}H NMR analysis were prepared using D_2O and DMSO as deuterated solvents for signal lock, giving a total of 60 samples (10 samples × 3 replicates × 2 d-solvents). The ethanol solutions were then analysed by ^{1}H NMR spectroscopy following two experimental setups: in the first, water suppression was employed, while in the second set up samples were measured with no water suppression. A total of 120 spectra of pure ethanol solutions were recorded. Ethanol concentrations were calculated using two different quantification methods (raw sum *vs.* MCR (de Juan et al., 2014)), leading to a total of 240 quantifications for each signal monitored in the ^{1}H NMR spectra. Three different NMR signals were tested, namely the ethanol triplet signal (methyl at 1.18 ppm), its right ^{13}C satellite peak (1.07 ppm), and a combination of main triplet and its satellites (*i.e.*, main triplet + 2 × satellite), thereby leading to a total of 720 quantifications.

For the second part of the study, a total of 20 table wines (12 red wines and 8 white wines) were selected from a larger wine collection generously donated by Vivino (Denmark). The wine sample set was selected to include table wines from different grapes, geographical origin, processing, and vintages (Fig. 1b). The selected wines spanned an ethanol range of 12–16 % (*v*/v). An overview of the wines included in the method validation experiment is given in Table S1.

2.3. Sample preparation

The ethanol solutions were prepared at different concentrations, spanning the range 0–40 % (*v*/v) (Fig. 1a). For each sample, aliquots of 1 mL were prepared by mixing the specific volumes of ethanol and ultrapure water. Deuterated solvents – D_2O or DMSO – were added to each sample (10 % v/v) and 5 mM TSP was used as internal standard. Three technical replicates of 1 mL were prepared for each sample. A total of 600 μL were transferred in 5 mm (O.D.) SampleJet NMR tubes (Bruker BioSpin, Ettlingen, Germany), which according to the manufacturer, are designed with minimal adsorptive properties.

The wine samples were prepared according to the method developed by Aru et al. (Aru, Sørensen, Khakimov, Toldam-Andersen, & Engelsen, 2018). Briefly, from each bottle 2 mL of wine were withdrawn through the cork cap using a syringe-based system replacing the wine headspace with argon (Coravin Vinitas®, Bedford, MA, US). For each sample, two replicates of a solution containing 700 μL of wine and 300 μL of 1 M KH_2PO_4 buffer in D_2O (4:1 *v*/*v*, pH = 3.50 ± 0.02) with 5 mM TSP were prepared. As before, 600 μL of the solution were transferred into 5 mm (O.D.) SampleJet NMR tubes.

2.4. Sample measurements

2.4.1. NMR measurements

^{1}H NMR spectra were recorded on a Bruker Avance III 600 operating at a proton Larmor's frequency of 600.13 MHz and equipped with a 5-mm broadband inverse (BBI) probe. Data acquisition and processing were carried out using the TopSpin software (version 4.2, Bruker, Rheinstetten, Germany). After temperature equilibration (5 min) the ^{1}H NMR spectra were measured at 298 K using two different experiments: one with a standard pulse sequence for presaturation of the water signal (*zgcppr* pulse program, Bruker nomenclature), and one experiment without the solvent suppression procedure (*zg* pulse program, Bruker nomenclature). A sweep width of 12,019 Hz (20 ppm) was employed. The 90° pulse was calibrated for each sample. Acquisition time and recycle delay (d1) were set to 3 s and 40 s, respectively. Spectral data were collected into 64 k data points, after 32 scans. The receiver gain was fixed for all the experiments to the lowest possible value. All spectra were acquired in automation using iconNMR™ (Bruker Biospin, Rheinstetten, Germany) and the SampleJet™ system (Bruker BioSpin, Ettlingen, Germany). Phase and baseline correction were performed in the TopSpin software. All the acquired spectra (both ethanol solutions and wines) were aligned using *icoshift* (Savorani, Tomasi, & Engelsen, 2010). This method aligns the NMR signals in selected intervals in order to facilitate comparison among different samples. In the present study, the selected intervals correspond to the region of the main ethanol ^{1}H NMR signals (methyl and its satellite) and the TSP signal.

2.4.2. WineScan measurements

Ethanol content in wine samples was calculated by FT-IR spectroscopy using the WineScan instrument (WineScan FT 120, FOSS A/S, Hillerød, Denmark). WineScan is a Fourier Transform interferometer, which is equipped with a 37 μm transmission measurement cell with CaF_2 windows. The scanning range is from 929 to 5011 cm^{-1} and 12 scans are averaged to produce the final spectrum. The WineScan instrument collects information about the fundamental molecular vibrations, and the spectra have been carefully calibrated to a long list of wine and grape juice parameters using advanced multivariate regression

techniques.

2.5. Data analysis

2.5.1. Ethanol quantification in DoE samples

Ethanol content in the ethanol solutions was quantified from the ^{1}H NMR spectra by processing the different ethanol signals (main triplet and its ^{13}C-satellites), using two different integration methods. First, the raw sum method was applied, where the intensity of all the data points associated with a specific signal are summed up to obtain the peak area. To minimize the systematic errors, an offset correction was applied to each signal interval to correct the baseline before calculating the area. This correction was performed on all the acquired spectra, and it consists in subtracting the lowest point of each spectrum (in terms of intensity) from all the NMR data points of that portion of spectrum. The second integration method is based on Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) (de Juan et al., 2014). Using the MCR approach, the ^{1}H NMR signals can be deconvoluted into a set of relative concentrations (C) and the resolved pure spectral profile (S, containing the individual ^{1}H NMR resonance in question). This approach is exploited similar to the Signature Mapping approach (Khakimov, Mobaraki, Trimigno, Aru, & Engelsen, 2020) where the number of components for MCR is set to 1 in order to resolve only one component (the ethanol peak) leaving noise and smaller interferences in the residuals. Non-negativity constraint was applied to both the concentrations and the resolved profile. The output of this approach is a series of relative concentration values. If MCR works, ideally only scaling is needed to provide absolute quantifications.

2.5.2. Evaluation of monitored parameters

In order to evaluate the effect of the different experimental and instrumental conditions tested, the NMR peak areas obtained for all the possible parameters combinations (2 solvent types × 2 pulse sequences × 3 signals × 2 quantification methods = 24 different combinations) were correlated to the corresponding theoretical (by design) ethanol content. The best operating conditions employable in alcoholic beverages with ethanol content ranging from 3 to 40 % *v*/v were assessed by comparing the correlation coefficients R^2 calculated for the 24 different combinations.

2.5.3. Ethanol quantification in wines

For the wine samples the ethanol content was quantified from the NMR spectra using the raw sum approach. The signal areas obtained were converted into molar concentrations using Eq. (1):

$$C_{EtOH} = (I_{EtOH}/I_{TSP})^{*}(N_{TSP}/N_{EtOH})^{*}C_{TSP}^{*}D \quad (1)$$

Where C_{EtOH} is the ethanol concentration in mM, I_{EtOH} is the integral of the ethanol signal, I_{TSP} is the integral of the reference compound TSP, N_{TSP} is the number of protons giving rise to the TSP signal, N_{EtOH} is the number of protons giving rise to the ethanol resonance, C_{TSP} is the known TSP concentration (1.5 mM), and D is a correction factor associated with the sample dilution. In this work its value is 1.0/0.9 because the amount of deuterated solvent was 10 % (*v*/v) per each sample.

The corresponding ethanol (EtOH) % (v/v) values was obtained by converting the molar concentrations using Eq. (2):

$$\%EtOH = ((C_{EtOH}^{*}MW_{EtOH})/d/1000)^{*}100 \quad (2)$$

Where % *EtOH* is the ethanol concentration in %(v/v), C_{EtOH} is the previously calculated ethanol concentration in mM, MW_{EtOH} is the molecular weight of ethanol (46.07 g/mol), *d* is the ethanol density (set according to the ethanol percentage in the different solutions), and 1000 was used to assess the correct unit of measurement.

All analyses of spectral data were carried out using homemade scripts in MATLAB (R2021b, Mathworks, Natick, MA, USA).

2.5.4. Effect of DoE parameters by ASCA

To evaluate the effect of the parameters on the ethanol quantification in wine samples, ANOVA Simultaneous Component Analysis (ASCA) (Smilde et al., 2005) was applied using the PLS_Toolbox (version 8.9.2, Eigenvector Research Inc., Manson, WA, USA).

Two different ASCA models were explored. The first was developed using as a response the ethanol triplet area obtained from the spectra of the wines. The second was developed instead using the corresponding TSP area as a response. For both models a DoE coded matrix was built considering three factors: the pulse sequence used, the wine type (*i.e.*, red or white) and the number of sample replicates, to evaluate also the experimental reproducibility. A fourth factor representing the ethanol content in the sample was used for the model with the TSP area set as the response. For all models, to assess the significance of the ASCA outcomes, the corresponding *p*-value was calculated setting the number of permutations to 1000.

2.6. External standard approach

An external standard approach was utilized for the absolute quantification of ethanol in wines, where TSP was used as reference compound. Briefly, two spectra were selected from the samples of the experimental design part 1: the first one ("*external standard*") is the one containing only TSP, water and D_2O; the second one ("*ethanol reference*") is obtained from the ethanol solution with EtOH 13.0 % v/v, corresponding to an average alcoholic content in wines. The pulse sequence used for these two samples was the "*zg*" (no water suppression) to prevent adding variations due to the water suppression pulse sequence. All the other NMR parameters were kept identical on all the other sample experiments. Starting from these two samples, a constant (k_{ext}) was derived using Eq. (3):

$$k_{ext} = A_{TSP}/A_{EtOH}^{*}C_{EtOH}/C_{TSP}^{*}N_{EtOH}/N_{TSP} \quad (3)$$

Where A_{TSP} is the area of the TSP peak in the *external standard* sample, A_{EtOH} is the area of the ethanol triplet peaks in the *ethanol reference* sample, C_{EtOH} is the ethanol molar concentration in *ethanol reference* sample corresponding to 13 % v/v, C_{TSP} is the TSP molar concentration in the *external standard* sample, N_{EtOH} is the number of protons described by the ethanol triplet peak (3) and N_{TSP} is the number of protons described by the TSP peak (9). The k_{ext} constant was calculated in triplicate considering the three replicates of the *external standard* sample and of the *ethanol reference* sample. The final obtained mean value (k_{ext} = 1.8681) was used to obtain the molar concentration of ethanol in all the wine samples analysed during the case study.

3. Results and discussion

3.1. Ethanol quantification from ^{1}H NMR spectra: Signal selection

In order to find the best approach for the absolute quantification of ethanol, the study first focused on assessing the different ^{1}H NMR resonances stemming from the ethanol molecule. Fig. 2 shows a representative ^{1}H NMR spectrum of a red wine in which the two ethanol signals are clearly visible: the triplet (Fig. 2a) at 1.18 ppm generated by the coupling of the methyl (–CH_3) protons with the two protons of the methylene (–CH_2–) moiety; and the quartet (Fig. 2b) at 3.69 ppm generated by the coupling of the methylene (–CH_2–) protons with the three methyl (–CH_3) protons. In addition, close to those signals, the ^{13}C-satellites peaks can be observed, which are clearly visible especially for the triplet (Fig. 2c). The generation of these small peaks is related to the coupling of the protons with the ^{13}C isotopes of the adjacent carbon atoms. Their signal shape is the same as the main proton signal and their lower intensity is due to the low natural abundance of the ^{13}C isotope, which is about 1 % of the total carbon presence.

Since compound quantification by ^{1}H NMR is related to the area or to the intensity of the peaks generated by the molecule (*i.e.*, ethanol), the

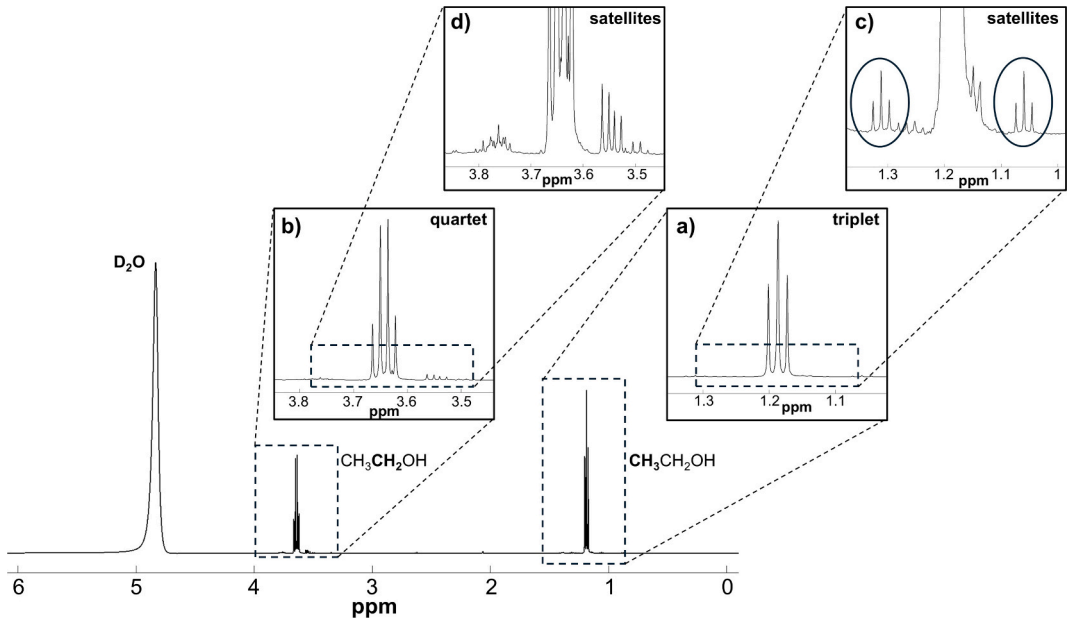

Fig. 2. Representative ^{1}H NMR spectrum of a red wine sample. The ethanol resonances are highlighted in the figure insets. The triplet (a) is related to the -CH_3 protons (1.18 ppm); the quartet (b) is related to the -CH_2- protons (3.65 ppm); the triplet and quartet satellites (c and d) are related to the couplings of the protons with the adjacent ^{13}C carbons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

simultaneous presence of signals generated by other molecules (interferences) in the same spectral region can affect the quantification accuracy. In this work, the ethanol quartet was discarded due to the heavy overlap with the signals from glycerol at 3.45 ppm and other minor resonances in the same spectral region. These overlaps can be potentially disruptive for the quantification, especially when using the raw sum method without applying complex curve fitting methods. For the same reason, the quartet ^{13}C-satellite signal was not included in the analysis. Thus, ethanol quantification was performed on the ethanol main triplet (T) signal, its right ^{13}C satellite (S_H), and main triplet and its rigth satellite together (T+2S_H) assuming satellite symmetry (Fig. 2c).

3.1.1. *Correction factor for ^{13}C-satellite signals*

A previous study showed the possibility of using the ^{13}C-satellite peaks for quantification purposes (López-Rituerto et al., 2009). The quantitative nature of ^{1}H NMR is due to the fact that the area under each ^{1}H NMR peak (*i.e.*, ^{1}H main triplet and ^{13}C satellite ethanol signal) is proportional to the number of equivalent protons contributing to that signal. However, since different materials can have different ^{13}C/^{12}C ratios, the ^{13}C-satellite signals must be scaled to their main ^{1}H NMR signals to obtain quantitative areas for specific samples (Guyon et al., 2015; Hoffman & Rasmussen, 2022).

When attempting to quantify ethanol from only one satellite signal it is necessary to calculate a correction factor *k*. In this work this factor was calculated from the ^{1}H NMR spectra of ethanol solutions with ethanol content ranging from 7 % *v*/v to 16 % v/v – similar to the % v/v ethanol content in wines.

In order to evaluate the influence of the DoE on the ratios between the raw sum area of the triplet's ^{13}C satellite signals and the main ethanol triplet the ratio has been calculated for the four combinations of deuterated solvent and experimental setup which is shown in Fig. 3 (see also Fig. S1 for the isotopic ratios in the wine samples). The average satellite-to-triplet ratios (2S/T) vary from $k = 0.0111$ to $k = 0.0113$, meaning that the single satellite signal has an area of approximately 0.5 % of the main ethanol triplet, which can be considered as a good attenuation for the ethanol signals to enter a dynamic range aligned with the other wine metabolites.

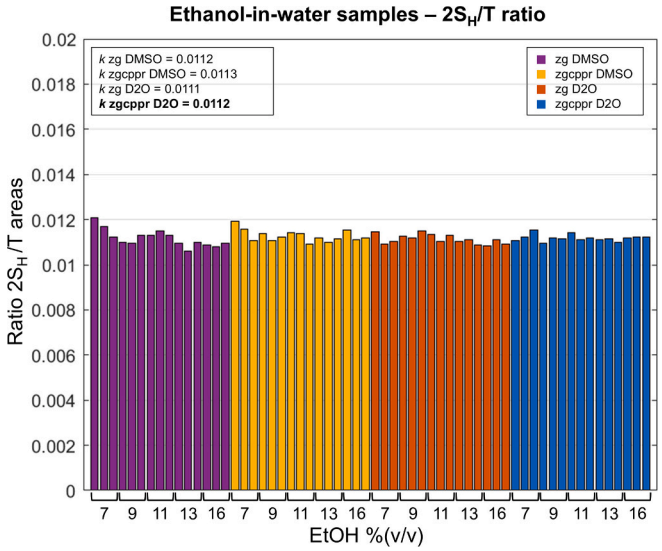

Fig. 3. Bar plot showing the satellites-to-triplet (2S_H/T) raw sum areas ratios in ethanol solutions. Bars are coloured according to the experimental setup. Samples are ordered based on the increasing ethanol concentrations. For each concentration, replicates are highlighted at the bottom of the graph using square brackets. For each experimental condition, the correction factor (*k*) is shown in the inset in the top-left corner of the figure.

3.2. *Optimization of the measurement conditions*

The ethanol areas for all the combination of parameters tested - 10 samples × 2 deuterated solvents for signal lock × 3 replicates × 2 pulse sequences × 3 ^{1}H NMR signal intervals × 2 quantification methods = 720 calculated areas - were plotted against the real ethanol content according to the study design to better evaluate the optimal experimental conditions. For each combination, the corresponding correlation coefficient was calculated and, to better visualize the results, all the correlation coefficients are plotted together in Fig. 4 – individual correlation plots are shown in the Supporting Information (Figs. S2–25). As

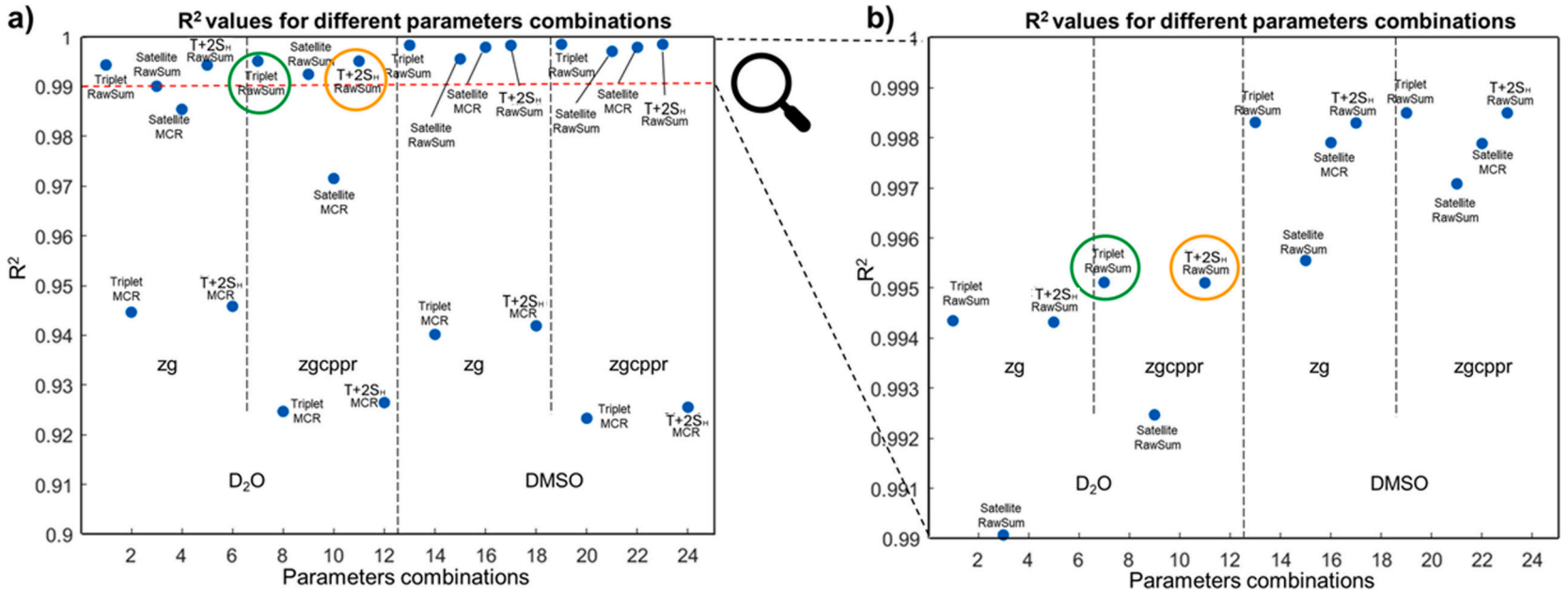

Fig. 4. Overview of the correlation coefficients (R^2) calculated between actual and estimated EtOH concentrations from the DoE depicted in Fig. 1**a.** EtOH obtained for the different parameters combinations (a) and focus only on the combinations with an R^2 higher than 0.99 (b). Highlighted are in circles are the methods that proved to be the most accurate for ethanol quantification in water-ethanol solutions. **Keys.** $2S_H$: 2 × area of the rigth ^{13}C satellite; T: area of the main ethanol ^{1}H triplet.

Table 1
Results obtained from ASCA performed using the ethanol triplet peak area as a response. The monitored factors are the pulse sequence (F1), the wine type (F2) and the replicates (F3).

Factor	Effect (%)	p-value
F1: Pulse sequence	1.67	0.043
F2: Wine type	64.25	0.001
F3: Replicates	0.07	0.710
F1 × F2	0.01	0.893
F1 × F3	0.01	0.889
F2 × F3	0.00	0.931
Residuals	33.99	/

it can be observed in Fig. 4a, MCR is inferior to the raw sum approach, especially when applied to the main triplet peak - all other combinations resulted in an R^2 higher than 0.97. This could be ascribed to the fact that MCR requires that the peak-shape is conserved across all samples. MCR is a multivariate approach, which deconvolutes complex spectra into pure analyte spectra and concentrations. In theory, MCR should be better than raw sum for quantification when overlapping signals are present. However, if the ^{1}H NMR signal to be quantified has slightly varying shapes in different samples (or is misaligned), the MCR algorithm will increase the unexplained part in the fitting, leading to larger errors. In our study, the highest ethanol concentrations led to the broadening of the ethanol signals, thereby compromising the efficacy of the MCR approach. Furthermore, physical phenomena such as radiation damping can cause signal distortion, which can further affect MCR quantification. MCR is only competitive in the case of the ethanol triplet satellite where ethanol quantification using DMSO as solvent is more consistent and unaffected by the tested factors ($R^2 = 0.998$, Fig. 4b)..

Focusing on ethanol quantification using D_2O as solvent, water suppression (*zgcppr*) gave an $R^2 > 0.99$ - higher than no water suppression (*zg*) in the same experimental conditions (Fig. 4b). More specifically, both raw sum on the main triplet (green circle in Fig. 4b) and satellites + main triplet (yellow circle in Fig. 4b) had comparable and higher accuracy (R^2 of 0.995) when compared to raw sum on the triplet satellite alone (Fig. 4b). For the sake of correctness and to compensate for possible variable $^{13}C/^{12}C$ ratios in "real" wine samples, both main triplet and satellites ($T+2S_H$) were chosen as target signals for quantification.

Since the ^{1}H NMR spectrum of residual DMSO is characterized by a multiplet resonating between 2 and 3 ppm, which overlaps with important metabolite signals in the ^{1}H NMR spectrum of wines, D_2O was chosen as the best solvent option for signal lock.

3.3. A case study on wine samples

3.3.1. Ethanol quantification using the optimized conditions

For the case study, a total of 20 table wines (12 red and 8 white, Table S1) were measured using the experimental conditions optimized in the designed experiment (Part 1, Fig. 1a), with and without water suppression. The ethanol content was quantified from the main triplet peak in the ^{1}H NMR spectra using the raw sum approach. The absolute ethanol content was calculated both in molar concentrations and in EtOH % (*v*/v) using Eqs. 1 and 2. The contents of EtOH % (v/v) were plotted against the WineScan measurements (used as a reference) for all the wine samples (Fig. S26). In contrast to the pure ethanol solutions, ethanol concentrations measured in wines from their ^{1}H NMR spectra were not as accurate as the WineScan measurements. Instead, a general underestimation of the real ethanol content was observed resulting in a lower correlation coefficient ($R^2 = 0.91$) to be compared with the correlation coefficient obtained on the ethanol in water solutions ($R^2 = 0.99$).

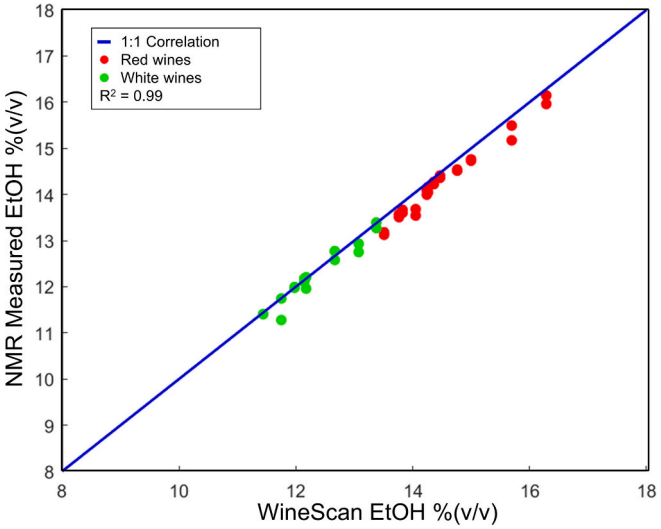

Fig. 5. ^{1}H NMR *vs.* FT-IR correlation plot. EtOH concentrations obtained from the NMR spectra using the external standard approach are plotted against the EtOH quantifications obtained from the WineScan (FT-IR) instrument, which we used asreference method. The estimated uncertainty of the ethanol measurements is RMSE = 0. 23 % (*v*/v).

Table 2
Results obtained from ASCA performed using the TSP peak area as a response. The monitored factors are the pulse sequence (F1), the wine type (F2), the replicates (F3) and the ethanol content (F4).

Factor	PCs	Effect (%)	p-value
F1: Pulse sequence	1	1.96	0.001
F2: Wine type	1	59.69	0.001
F3: Replicates	1	0.04	0.462
F4: EtOH content	1	14.48	0.001
F1 × F2	1	0.05	0.525
F1 × F3	1	0.04	0.583
F1 × F4	1	0.20	0.984
F2 × F3	1	0.07	0.465
F2 × F4	1	15.54	0.001
F3 × F4	1	0.90	0.464
Residuals	/	7.01	/

3.3.2. Factors affecting ethanol quantification in wine samples

ANOVA Simultaneous Component Analysis (ASCA) was used to identify the experimental parameters/factors which affected the areas of the ethanol triplet peak and/or of the internal standard TSP peak used for the quantification the most. The first ASCA model was developed to explore (1) the effects of the pulse sequence (with *vs.* without water suppression), (2) of the wine type (red *vs.* white) and (3) of the replicates on the ethanol triplet peak area. The results are reported in Table 1 and reveal that wine type is the factor that affects the variance in the data the most (effect size = 64 %, $p < 0.001$). A minor effect associated with the pulse sequence was also found (effect size = 1.7 %, $p < 0.05$). No significant effect was observed for the replicates.

The second ASCA model was developed to explore the same effects monitored in the first model plus a fourth parameter related to the ethanol content in the wine samples. For this model the response was the peak area of the internal standard TSP. The results are reported in Table 2. As before, a small effect was observed for the pulse sequence (effect size = 2 %, $p < 0.001$), and no significant effects related to the replicates were observed. However, large effects of 59.69 % ($p < 0.001$) and 14.48 % ($p < 0.001$) were observed for the wine type and for the ethanol content, respectively. These results suggest a significant matrix effect on the TSP peak area, which varied across the different wine samples, despite all being prepared using the same SOP. TSP is a well-known internal standard for NMR. However, the use of TSP as quantitative standard in metabolomics studies is limited owing to their peak attenuation or complete suppression due to interactions with proteins

(Nagana Gowda et al., 2021). Alternative compounds to TSP have been proposed as internal standard for human metabolomics investigations, including fumaric acid, succinic acid or maleic acid (Gowda, Gowda, & Raftery, 2015). However, these compounds can be naturally present in wines and have been avoided in this study. In simple matrices such as ethanol-water calibration solutions (see paragraph 2.2 - Experimental design, Part 1), absolute quantification using TSP as internal standard is straightforward and can be obtained by simple mathematical computations. For more complex mixtures, like wines, the use of external standards or artificial signal is recommended.

3.3.3. Ethanol quantification using an external standard

To overcome the previously observed matrix effect on TSP area, ethanol quantification was subsequently performed using an external standard (see section 2.6). Ethanol quantifications were performed using the main ethanol triplet together with its satellites ($T+2S_H$, area calculated with the raw sum approach). The areas were converted into molar concentrations using the constant k_{ext} in Eq. 3 and then converted into EtOH % v/v using Eq. 2. The obtained NMR-based quantifications were plotted against the corresponding WineScan measurements of ethanol, which were used as a reference (Fig. 5). As it can be observed, the inclusion of the ^{13}C satellites compensates for the natural abundance of ^{13}C, which is about 1.1 % (compare Fig. 5 and Fig. S26). Due to the small effect derived from the pulse sequence factor on the signal areas, only the spectra acquired using the water suppression pulse sequence (*zgcppr*) were considered.

By inspection of Fig. 5 it can be observed that the external standard approach is able to compensate for the variation in the TSP area. In particular, the strong effect of the wine type has disappeared (see Fig. S27), meaning that it was mainly generated by a matrix effect affecting the TSP peak area. The small underestimation of the ethanol concentration, still present in some samples, can perhaps be explained by the fact that the ethanol content is ranging from 11 % v/v to 16 % v/v in the wine samples, while k_{ext} constant was calculated from a sample with EtOH 13 % v/v.

4. Conclusions

In this work, a detailed experimental design was developed to explore the possibility and the limitations of using ^1H NMR spectroscopy and a common NMR standard such as TSP for the pragmatic absolute quantification of ethanol in alcoholic beverages. Even though absolute quantification of ethanol in simple ethanol-water solutions is straightforward using TSP as an internal standard, in more complex sample matrices (such as wines), where metabolite-standard interactions can negatively affect the standard's quantitative properties, the use of external standards or artificial signals is recommended to prevent potential discrepancies in accuracy and precision. Furthermore, our study highlights the importance of taking into account the isotopic $^{13}C/^{12}C$ ratio. As ^{13}C constitutes approximately 1.1 % of carbon atoms, failure to account for this isotopic distribution can lead to inaccuracies in the calculated ethanol concentration.

Based on our results, the best approach to obtain absolute quantification of ethanol in wines samples entails the use of (1) an external standard with its satellites (*i.e.*, TSP), (2) a correction factor k_{ext}, calculated from measurements of a known ethanol reference sample to compensate for misquantifications due to matrix effects, and (3) combining the intensities of the main triplet and its satellites for absolute quantification, in order to compensate for different $^{13}C/^{12}C$ ratios in wines. Using our method, the uncertainty of the ethanol measurement in wines by ^1H NMR spectroscopy is estimated to be 0.23 % (*v*/v) with a correlation of $R^2 = 0.99$.

CRediT authorship contribution statement

Mattia Sozzi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Violetta Aru:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Mascellani Bergo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Nicola Cavallini:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Francesco Savorani:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis. **Havlik Jaroslav:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Bekzod Khakimov:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Søren Balling Engelsen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Data was generated though accessing research infrastructure at the University of Copenhagen, including FOODHAY (Food and Health Open Innovation Laboratory, Danish Roadmap for Research Infrastructure). Violetta Aru and Søren Balling Engelsen would like to acknowledge Vivino and FOSS A/S for the support with wine samples and for making the WineScan available for measuring reference ethanol data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.116598.

Data availability

Data will be made available on request.

References

Alahmad, W., Kaya, S. I., Cetinkaya, A., Varanusupakul, P., & Ozkan, S. A. (2023). Green chemistry methods for food analysis: Overview of sample preparation and determination. *Advances in Sample Preparation, 5*, Article 100053.

Aru, V., Sørensen, K. M., Khakimov, B., Toldam-Andersen, T. B., & Engelsen, S. B. (2018). Cool-climate red wines—Chemical composition and comparison of two protocols for 1H–NMR analysis. *Molecules, 23*(1), 160.

Bauer, R., Nieuwoudt, H., Bauer, F. F., Kossmann, J., Koch, K. R., & Esbensen, K. H. (2008). FTIR spectroscopy for grape and wine analysis. *Analytical Chemistry, 80*(5), 1371–1379.

Belton, P. S., Delgadillo, I., Holmes, E., Nicholls, A., Nicholson, J. K., & Spraul, M. (1996). Use of high-field H-1 NMR spectroscopy for the analysis of liquid foods. *Journal of Agricultural and Food Chemistry, 44*(6), 1483–1487.

Burton, I. W., Quilliam, M. A., & Walter, J. A. (2005). Quantitative 1H NMR with external standards: Use in preparation of calibration solutions for algal toxins and other natural products. *Analytical Chemistry, 77*(10), 3123–3131.

Caleja-Ballesteros, H. J. R., Ballesteros, J. I., & Villena, M. C. (2021). No-D quantitative 1H nuclear magnetic resonance spectroscopy method for the determination of ethanol in distilled spirits. *Microchemical Journal, 164*, Article 105999.

Czaja, T. P., & Engelsen, S. B. (2025). Why nothing beats NIRS technology: The green analytical choice for the future sustainable food production. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 325*, Article 125028.

Duarte, I., Barros, A., Belton, P. S., Righelato, R., Spraul, M., Humpfer, E., & Gil, A. M. (2002). High-resolution nuclear magnetic resonance spectroscopy and multivariate analysis for the characterization of beer. *Journal of Agricultural and Food Chemistry, 50*(9), 2475–2481.

Farine, G., Scilinguo, M., Calvagna, C., Romano, S., Polito, M., & Sgrò, S. (2024). Determination of ethanol content in alcoholic products by LF-NMR. *Analytica, 5*(3), 458–470.

Farrant, R. D., Hollerton, J. C., Lynn, S. M., Provera, S., Sidebottom, P. J., & Upton, R. J. (2010). NMR quantification using an artificial signal. *Magnetic Resonance in Chemistry, 48*(10), 753–762.

Giraudeau, P. (2017). Challenges and perspectives in quantitative NMR. *Magnetic Resonance in Chemistry, 55*(1), 61–69.
Giraudeau, P., Tea, I., Remaud, G. S., & Akoka, S. (2014). Reference and normalization methods: Essential tools for the intercomparison of NMR spectra. *Journal of Pharmaceutical and Biomedical Analysis, 93*, 3–16.
Gowda, G. A. N., Gowda, Y. N., & Raftery, D. (2015). Expanding the limits of human blood metabolite quantitation using NMR spectroscopy. *Analytical Chemistry, 87*(1), 706–715.
Guyon, F., van Leeuwen, C., Gaillard, L., Grand, M., Akoka, S., Remaud, G. S., … Salagoïty, M.-H. (2015). Comparative study of 13C composition in ethanol and bulk dry wine using isotope ratio monitoring by mass spectrometry and by nuclear magnetic resonance as an indicator of vine water status. *Analytical and Bioanalytical Chemistry, 407*(30), 9053–9060.
Hassoun, A., Jagtap, S., Garcia-Garcia, G., Trollman, H., Pateiro, M., Lorenzo, J. M., … Cámara, J. S. (2023). Food quality 4.0: From traditional approaches to digitalized automated analysis. *Journal of Food Engineering, 337*, Article 111216.
Hoffman, D. W., & Rasmussen, C. (2022). Absolute carbon stable isotope ratio in the Vienna Peedee belemnite isotope reference determined by 1H NMR spectroscopy. *Analytical Chemistry, 94*(13), 5240–5247.
de Juan, A., Jaumot, J., & Tauler, R. (2014). Multivariate curve resolution (MCR). Solving the mixture analysis problem. *Analytical Methods, 6*(14), 4964–4976.
Khakimov, B., Mobaraki, N., Trimigno, A., Aru, V., & Engelsen, S. B. (2020). Signature mapping (SigMa): An efficient approach for processing complex human urine ^{1}H NMR metabolomics data. *Analytica Chimica Acta, 1108*, 142–151.
Krishnan, V. V., & Murali, N. (2013). Radiation damping in modern NMR experiments: Progress and challenges. *Progress in Nuclear Magnetic Resonance Spectroscopy, 68*, 41–57.
López-Rituerto, E., Cabredo, S., López, M., Avenoza, A., Busto, J. H., & Peregrina, J. M. (2009). A thorough study on the use of quantitative 1H NMR in Rioja red wine fermentation processes. *Journal of Agricultural and Food Chemistry, 57*(6), 2112–2118.
Lopez-Rituerto, E., Savorani, F., Avenoza, A., Busto, J. H., Peregrina, J. M., & Engelsen, S. B. (2012). Investigations of La Rioja terroir for wine production using H-1 NMR metabolomics. *Journal of Agricultural and Food Chemistry, 60*(13), 3452–3461.
López-Rituerto, E., Sørensen, K. M., Savorani, F., Engelsen, S. B., Avenoza, A., Peregrina, J. M., & Busto, J. H. (2022). Monitoring of the Rioja red wine production process by H-NMR spectroscopy. *Journal of the Science of Food and Agriculture, 102*(9), 3808–3816.
Mac, H. X., Pham, T. T., Ha, N. T. T., Nguyen, L. L. P., Baranyai, L., & Friedrich, L. (2023). Current techniques for fruit juice and wine adulterant detection and authentication. *Beverages, 9*(4), 84.
Nagana Gowda, G. A., Hong, N. N., & Raftery, D. (2021). Evaluation of Fumaric acid and maleic acid as internal standards for NMR analysis of protein precipitated plasma, serum, and whole blood. *Analytical Chemistry, 93*(6), 3233–3240.
Pallone, J. A. L., Caramês, E. T. D. S., & Alamar, P. D. (2018). Green analytical chemistry applied in food analysis: Alternative techniques. *Current Opinion in Food Science, 22*, 115–121.
Palmioli, A., Alberici, D., Ciaramelli, C., & Airoldi, C. (2020). Metabolomic profiling of beers: Combining 1H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products. *Food Chemistry, 327*, Article 127025.
Savorani, F., Tomasi, G., & Engelsen, S. B. (2010). Icoshift: A versatile tool for the rapid alignment of 1D NMR spectra. *Journal of Magnetic Resonance, 202*(2), 190–202.
Smilde, A. K., Jansen, J. J., Hoefsloot, H. C. J., Lamers, R., van der Greef, J., & Timmerman, M. E. (2005). ANOVA-simultaneous component analysis (ASCA): A new tool for analyzing designed metabolomics data. *Bioinformatics, 21*(13), 3043–3048.
Teipel, J. C., Hausler, T., Sommerfeld, K., Scharinger, A., Walch, S. G., Lachenmeier, D. W., & Kuballa, T. (2020). Application of 1H nuclear magnetic resonance spectroscopy as Spirit drinks screener for quality and authenticity control. *Foods, 9*(10), 1355.
Torres, A. M., & Price, W. S. (2016). Common problems and artifacts encountered in solution-state NMR experiments. *Concepts in Magnetic Resonance Part A, 45A*(2), Article e21387.
Ün, I., & Goren, A. C. (2017). Accurate determination of ethanol in water by qNMR: Validation and uncertainty assessment. *Journal of Chemical Metrology, 11*(1), 9–15.
Wider, G., & Dreier, L. (2006). Measuring protein concentrations by NMR spectroscopy. *Journal of the American Chemical Society, 128*(8), 2571–2576.
Zuriarrain, A., Zuriarrain, J., Villar, M., & Berregi, I. (2015). Quantitative determination of ethanol in cider by 1H NMR spectrometry. *Food Control, 50*, 758–762.