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Research Article

Rapid Determination of Total Thujone in Absinthe Using ^{1}H NMR Spectroscopy

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^{1}H NMR spectroscopy is utilized to quantify total thujone (sum of α- and β-isomers) in absinthe. For sample preparation, a simple dilution with buffer is required. Thujone produces a distinct peak of the CH_2 group in the cyclopentanone moiety in the 2.13–2.11 ppm range. No overlap with other typical constituents such as anethole or fenchone occurs. The detection limit of 0.3 mg/L is adequate to control the EU maximum limit. The relative standard deviation was 6%, and linearity was observed from 1 to 100 mg/L. Applicability was proven by analysis of 69 authentic absinthes. The correlation between NMR and our previous method consisting of liquid-liquid extraction followed by GC/MS was significant ($P < 0.0001, R = 0.93$). The simple and cheap NMR method can be used for rapid screening of absinthes for total thujone content while chromatographic techniques are recommended for more specific (α- and β-thujone isomers) analysis if required.

1. Introduction

Absinthe was one of the most popular alcoholic beverages in 19th century Europe. The bitter spirit is produced using different herbs (most notably wormwood, *Artemisia absinthium L.*). Wormwood may contain the bicyclic monoterpene ketone thujone [1]. In recent years there is a discussion in the literature regarding the actual thujone content in alcoholic beverages and its impact on public health as it is often associated with the syndrome absinthism [2]. An overview of history, toxicity, and analytics of absinthe was recently provided by Lachenmeier et al. [1].

Historically applied methods for the thujone determination (e.g., iodometric titration [3] or use of color reagents [4]) were found to be unspecific and had inappropriate detection limits. Nowadays the most common technique for the quantification of thujone is gas chromatography (GC) combined with flame ionization detection [5, 6] or mass spectrometry (MS) [7]. Approaches for sample preparation include liquid/liquid extraction (LLE) [5, 7, 8], solid-phase extraction (SPE) [9], or headspace sampling combined with solid-phase microextraction (HS-SPME) [10, 11].

These methods are very sensitive and can provide reliable and accurate determination of both α- and β-thujone in bitter spirits. Nevertheless, in spite of the undoubted advantages, some difficulties exist. For instance, in some cases other compounds can coelute with thujone. Moreover, the sample preparation is often very complex and time-consuming.

Therefore, from an analytical point of view, it is important to develop a fast, reliable, and accurate method for thujone determination. Another important issue is the simplicity of sample preparation and measurement procedures. NMR spectroscopy nowadays possesses all these advantages. However, our literature search indicates that direct spectroscopic techniques (such as ^{1}H NMR) have not been studied for thujone determination so far.

This work presents for the first time an NMR-based method with minimal sample preparation for determination of total thujone in absinthes. In this paper we also compare the proposed technique with conventional GC/MS methodology.

2. Experimental Section

2.1. Reagents and Materials. Terpene standards (α-thujone, α-/β-thujone-isomer mixture, α- and β-fenchone, α-/β-linalool-isomer mixture and anethole) were purchased from Fluka (Buchs, Switzerland). 69 absinthes were analyzed using NMR including commercially available products and historic absinthes (for further details see [12]).

2.2. NMR Method and Sample Preparation. All ^{1}H NMR measurements were performed on a Bruker Avance 400 Ultrashield spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm SEI probe with Z-gradient coils, using a Bruker Automatic Sample Changer (B-ACS 120). All spectra were acquired at 300.0 K. An 8-fold suppression of all the signals of water and ethanol was conducted. The data were acquired automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany), requiring about 12 min per sample.

The NMR protocol applied was based on the Bruker standard protocol established for NMR screening of wine (Bruker BioSpin GmbH Rheinstetten, Germany). The optimization of the protocol for the analysis of spirits was described elsewhere [13]. In short, the two successive ^{1}H NMR experiments used for the acquisition of each sample are as follows.

Experiment 1 (standard water suppression, Bruker sequence ZGPR): first, a standard Bruker water presaturation pulse program was used to suppress only the signal of OH-protons. This reference spectrum is required to measure the exact frequencies of ethanol and water in each specific sample. These are the input values to optimize the shaped pulse necessary for multiple suppression in the subsequent second experiment. A 25 Hz RF-field was used for presaturation. The relaxation delay (RD) and acquisition time (AQ) were set to 4 s and 3.99 s, respectively, resulting in a total recycle time of 7.99 s. After application of 4 dummy scans (DS), 8 free induction decays (FIDs) (number of scans, NS = 8) were collected into a time domain (TD) of 65536 (65 k) complex data points using a 20.5187 ppm spectral width (SW) and a receiver gain (RG) of 1. The FIDs were multiplied with an exponential function corresponding to a line broadening (LB) of 1 Hz prior to Fourier transformation.

Experiment 2 (8-fold suppression of water and ethanol, Bruker sequence NOESYGPPS1D): a one-dimensional ^{1}H NMR pulse sequence with suppression of the water and the ethanol signals, that is, $RD - t_{G1} - P(90^\circ) - 4\,\mu s - P(90^\circ) - t_m - t_{G2} - P(90^\circ)$—*acquisition* of the free induction decay (FID). The settings for the parameters RD, P(90°), AQ, and TD were kept similar to the ones from experiment 1, $DS = 4$ and $NS = 32$ were used, and the mixing time t_m was set to 10 ms. A shaped pulse was applied during RD with a frequency spectrum of 8 individual bands (frequencies 1–8) to achieve simultaneous and highly selective suppression of the water singlet (frequency 1, RF-field strength 25 Hz) and the seven individual lines of the ethanol quartet (frequency 2–5, RF-field strength ~4 Hz each) and triplet (frequency 6–8, RF-field strength ~4 Hz each) while leaving the rest of the spectrum undistorted. The RG was set to 28.5, which resulted in a considerable gain in SNR compared to experiment 1 (ZGPR). Additional defocusing gradients G1 and G2 were applied during $t_{G1}, t_{G2} = 1$ ms. FIDs were multiplied with an exponential function corresponding to LB = 0.3 Hz prior to Fourier transformation.

For analysis, 300 μL of beverage is mixed with 240 μL of ethanol (70% v/v) and 60 μL of NMR buffer (pH = 7.4, 1.5 M KH_2PO_4 in D_2O, 0.1% 3-(trimethylsilyl)-propionate acid-d4 (TSP), 3 mM NaN_3) (Bruker Biospin, Rheinstetten, Germany). With this sample preparation, clouding of solutions due to precipitation of essential oils is avoided, which is not desirable for NMR measurements. The mixture is then poured into an NMR tube and is directly measured. All NMR spectra were phased and baseline corrected.

For quantification of total thujone content, the singlet in the 2.13–2.11 ppm region was integrated. For calibration, thujone solutions were freshly prepared in ethanol (70% v/v).

2.3. ^{1}H NMR Spectra Calculations. ^{1}H NMR spectra calculations were carried out using ChemBioDraw 12.0 software (CambridgeSoft, Cambridge, UK). Chemical shifts are estimated for all hydrogen atoms for which additivity rules are available. Following a hierarchical list, the algorithm first identifies key substructures of a molecule. A substructure provides the base value for the estimated shift. For details see [14].

2.4. Validation Studies. For the validation, standard solutions as well as authentic alcoholic beverage samples were analyzed several times daily (intraday, $n = 5$) and over several days (interday, $n = 8$). The linearity of the calibration curves was evaluated between 1 and 100 mg/L. The limit of detection (LOD) and quantification (LOQ) were determined as signals for which the signal-to-noise ratios (SNR) were *3* and 10 correspondingly. For all calculations statistical significance was assumed at below the 0.05 probability level.

3. Results and Discussion

3.1. Rationale for Application of NMR to Thujone Analysis. As mentioned in the introduction, GC is currently the state-of-the-art for thujone determination in alcoholic beverages. Despite being very sensitive and accurate, these methods are time consuming and expensive due to personnel costs.

The sample preparation is the most challenging part of the established analytical methods. For example, LLE, SPE, or HS-SPME must be used for sample preparation. Limits of detection as low as 0.08 mg/L [7] were possible. However, even after these preparation techniques, the peaks of other substances could overlap with the thujone peak on the GC chromatograms [15]. It is not surprising because bitter spirits can contain up to 200 different compounds [16].

It is known that NMR spectroscopy has excellent selectivity to qualify and quantify main constituencies of complex mixtures [17]. Therefore, we hypothesized that direct NMR spectroscopy might be applicable instead of complex chromatographic separation techniques. To identify specific resonances of thujone that are not overlapped with other

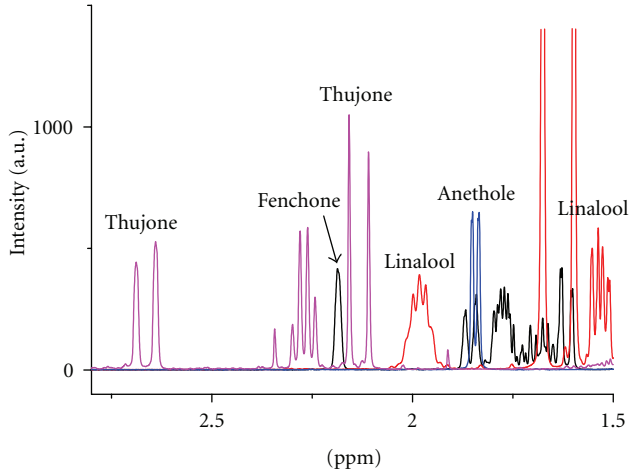


Figure 1: NMR spectra (aliphatic range) of common terpenes occurring in absinthe.

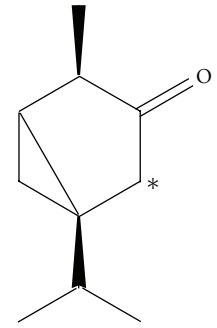
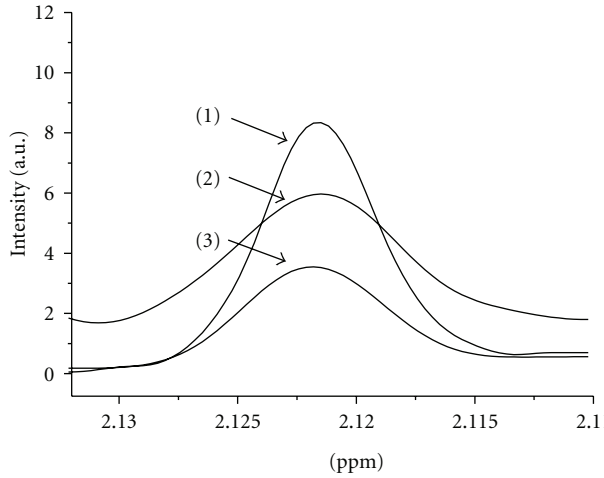


Figure 2: Chemical structure of α-thujone (the marked CH_2 group is leading to the ^{1}H NMR shift at 2.13–2.11 ppm range, which is used for quantification).



(1) 31 mg/L total thujone

(2) Sample with 22 mg/L total thujone

(3) 14 mg/L total thujone

Figure 3: ^{1}H NMR spectra of an authentic absinthe containing 22 mg/L of total thujone and two thujone standards (14 and 31 mg/L).

compounds we measured spectra of several terpenes (thujone, fenchone, linalool, and anethole) that are expected to be present in absinthes under the same conditions. In our case, all investigated terpenes have characteristic signals in the aliphatic range (Figure 1). It can be seen that thujone has resonances at 2.70–2.67, 2.65–2.62, 2.35–2.33, 2.30–2.23, 2.19–2.15, and 2.13–2.11 ppm. It can be further seen that only one resonance of thujone at 2.19–2.15 ppm can overlap with fenchone peak (especially in case of random pH variations). This fact can prevent accurate quantitative analysis of thujone at this chemical shift. Moreover, it can happen that other compounds can have signals in the same range as the thujone resonances at 2.70–2.67, 2.65–2.62, 2.35–2.33, or 2.30–2.23. After close inspection of NMR spectra of 69 absinthes we found that the signal in the 2.13–2.11 ppm range suits best for quantification purpose as it was not interfered in any case.

NMR spectra and signal assignments of thujone were published by Sirisoma et al. [18]. It can be concluded from their findings and our observations that the peak at 2.12 assigned to thujone is part of an AB spin system (proton 2 alpha) splitted by a geminal spin-spin coupling constant. For additional confirmation, we calculated the NMR spectrum of thujone and found out that the CH_2 group at the cyclopentanone moiety is responsible for this chemical shift (Figure 2). Our results therefore confirm the previous data [18].

An example of thujone NMR peaks from an authentic commercial absinthe in comparison with two reference spectra is presented in Figure 3. Unfortunately, NMR spectroscopy is not selective for thujone isomers, therefore we cannot differentiate between α- and β-thujone isomers by NMR. The same finding was established for fenchone isomers. For control purposes, this is not a large problem as the EU legislation specifies limits for the sum of isomers.

3.2. Method Validation and Measurement of Authentic Samples. Table 1 summarizes the method validation results for thujone. As we apply external calibration along with internal standardization (TSP), there is obviously a direct linear relation between our chosen NMR signal and the thujone concentration, which makes it suitable for quantitative analysis. The ^{1}H NMR assay was linear in the concentration range of 1–100 mg/L ($r = 0.99$). The limit of detection (LOD) and limit of quantification (LOQ) are 0.3 and 0.9 mg/L correspondingly, which is expectedly higher than what can be reached with chromatographic methods but is definitely sufficient for the control of the legal limit of 35 mg/kg. Validation of the method was further conducted by repeated sample preparation of standard samples and authentic absinthes. For the standard samples, the relative standard deviations (RSD) were always below 5%. For the authentic absinthes the RSDs were slightly higher but still acceptably below 10%.

Table 1: Results of method validation for thujone.

Parameter		Result
Linear range (mg/L)		1–100
LOD (mg/L)		0.3[a]
LOQ (mg/L)		0.9[a]
Precision (%)[b]	Intraday	4.0[c]; 5.8[d]
	Interday	4.9[c]; 6.7[d]

[a]Limit of detection (LOD) and quantification (LOQ) were determined as signals for which the signal-to-noise ratio (SNR) were 3 and 10 correspondingly.
[b]Precisions are expressed as relative standard deviation (RSD; %): intraday ($n = 5$), interday ($n = 8$).
[c]Standard solution.
[d]Authentic sample.

Table 2: Results of the quantitative determination of total thujone by NMR ($n = 69$).

Thujone content (mg/L)	Number of samples found in the category[a]
<2	16 (23%)
2–10	5 (7%)
10–35	32 (46%)
>35	16 (23%)

[a]Includes several preban and illegally marketed samples. The distribution is not representative for the current market in the EU. We chose the samples to get an equal distribution over the whole range of possible thujone concentrations.

Furthermore, NMR results for 69 absinthes were compared with values previously obtained by our conventional LLE-GC/MS method [7, 12, 19]. Statistical analysis between results from GC/MS and NMR revealed that the linear correlation is significant ($P < 0.0001, R = 0.93$). No systematic or proportional differences between both methodologies were found, as the standard deviations of both the y-axis and the slope were encompassing 0 or 1.

The results of our investigations of the 69 authentic absinthes are presented in Table 2. From the analyzed 69 samples 53 (77%) contained thujone above the detection limit and 16 (23%) had levels above 35 mg/L. The total thujone content of 53 samples (which corresponds to 77% of total number of investigated samples) falls in the range between not detectable and 35 mg/L. The average and median concentrations of total thujone in all investigated samples were 27.5 and 22.3 mg/L, correspondingly. These values are in full agreement with our previous findings [7, 12, 19].

4. Conclusions

In this article ^{1}H NMR spectroscopy was applied for the analysis of total thujone content (sum of α- and β-thujone) in absinthes.

We can conclude that the routine application of NMR is a useful addition to standard analytical methods for thujone quantification. ^{1}H NMR spectroscopy has proven to be a robust analytical tool yielding highly reliable quantitative results regarding thujone in a very short time. The approach is advantageous as it minimizes sample preparation and allows to analyze a large number of samples without human intervention (120 samples in a batch). In case of positive results NMR results should be supplemented by the more selective GC/MS analysis as it allows differentiation of isomers that are not separated by NMR spectroscopy.

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