Application of diffusion-edited and solvent suppression ^{1}H NMR to the direct analysis of markers in valerian-hop liquid herbal products

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Abstract

The rising trend to consume herbal products for the treatment and/or prevention of minor ailments together with their chemical and pharmacological complexity means there is an urgent need to develop new approaches to their quality and stability. This work looks at the application of one-dimensional diffusion-edited ^{1}H NMR spectroscopy (1D DOSY) and ^{1}H NMR with suppression of the ethanol and water signals to the characterization of quality and stability markers in multicomponent herbal medicines/food supplements. The experiments were performed with commercial tinctures of *Valeriana officinalis* L. (valerian), expired and non-expired, as well as its combination with *Hummulus lupulus* L. (hops), which is one of the most popular blends of relaxant herbs. These techniques did not require purification or evaporation of components for the qualitative analysis of the mixture, but only the addition of D_2O and TSP. The best diagnostic signals were found at δ 7 ppm (H-11, valerenic acid), δ 4.2 ppm (H-1, hydroxyvalerenic acid) and δ 1.5-1.8 ppm (methyl groups in prenylated moieties, α-acids/prenylated flavones). This work concludes on the potential value of 1D DOSY ^{1}H NMR to provide additional assurance of quality in complex natural mixtures.

Keywords: herbal medicinal product, quality marker, nuclear magnetic resonance, diffusion edited spectroscopy, *Valeriana officinalis, Humulus lupulus* L.

1 Introduction

The control tests on herbal drug preparations -which also include extracts- must be such as to allow the qualitative and quantitative determination of the composition of the active substances and eventually other components such as diluents and preservatives. These tests are usually performed by TLC for fingerprinting and conformation of identity/stability studies, or hyphenated techniques such as HPLC-UV for the quantitative determination of one or two quality markers. These methods are quite convenient and straight forward when applied to monocomponent herbal products. The increasing registration of multi-component herbal products, i.e. containing two or more herbal drugs as active ingredients, means that the manufacturer has to either resort to more than one analytical protocol, each targeting one of the herbal drugs, or to develop and validate a new protocol able to analyse in one run all phytomarkers. This is not only time consuming but usually the extreme differences in polarity of the phytomarkers make it challenging to achieve with the above mentioned instrumental techniques.

The implementation of a pan-European directive on the registration of traditional herbal medicinal products opens a door for the use of alternative approaches applied to the total quality control of herbal medicinal products. In fact, the Working Party of Herbal Medicinal Products of the European Medicines Agency established in its guidelines that ”New analytical technologies, and modifications of existing technologies, are continuously being developed. Such technologies should be used when they are considered to offer additional assurance of quality, or are otherwise justifiable” (European Medicines Agency, 1999).

Alternatively, Nuclear Magnetic Resonance (NMR), Near Infrared and Mass Spectrometry could give additional chemical information of the sample without any previous preparation steps (Politi et al., 2008, 2009); In particular NMR has now become an important tool for the qualitative and quantitative analysis of complex mixtures such

herbal medicines either alone (Gilard, Balayssac, Malet-Martino, & Martino, 2010) or as a complement of HPTLC (Booker, Johnston, & Heinrich, 2015).

Valerian roots (*Valeriana officinalis*) in combination with hops (*Humulus lupulus* L), are classic cases where the choice of markers is in permanent discussion for both pharmacological and economical reasons. Valerenic acids (**1**)(**2**) (Lazarowych & Pekos, 1998) and α-acids such as humulone (**3**) together with prenylated chalcones such as xanthohumol (**4**) (Hoek, Hermans-Lokkerbol, & Verpoorte, 2001) are currently regarded as the best quality markers for valerian and hops, respectively (Fig. 1).

However, reference standards of these phytochemicals are very expensive and on limited supply. For example, 15 mg of valerenic acid USP reference standard are sold at $959 in the US (Pharmacopoeial, 2015) and fetch as much as £945 in Europe (Sigma-Aldrich, 2015). As a result, many manufacturers are tempted to evaluate the quality of their valerian and hops products in terms of total content of essential oils as specified in many pharmacopoeias (American Herbal Pharmacopoeia, 1999; European Directorate for the Quality of Medicines & HealthCare, 2013) or resort to unspecific phenolic substances such as flavonoids.

Taking into account the cost of these reference materials, NMR services provided by either private companies or universities are becoming a more affordable alternative and could be used for the direct detection of such marker in both herbal drug extracts and final products at a lower cost. Therefore we here explore the application of direct 1D NMR analysis to assess the quality and stability of commercial valerian-hops tinctures.

2 Material and Methods

2.1 *Herbal drugs and herbal medicinal products.*

Different batches of commercial tinctures from the same manufacturer (Bioforce, A. Vogel, Switzerland) of were purchased in a health shop in London. All were labelled as organically grown and consisted in tinctures of *V. officinalis* L. root, one within its expiry date (alcohol strength 56% *v/v*;) and a second that had been expired for over 9 months (alcohol strength 67% *v/v*); A preparation containing 50% *V. officinalis* root and 50% fresh *H. lupulus* herb tinctures was also purchased (alcohol strength 61% *v/v*).

2.2 *Solvents and chemicals*

Chloroform-*d* and DMSO-d_6 (99.8%) were from Sigma Aldrich Chemie GmbH (Germany). Methanol-d_4 (99.8%), ethanol-d_6 (99.8%) and were from Cambridge Isotope Laboratories (USA). D_2O (99%) and sodium 3-trimethylsilyl [2,2,3,3-D4]propionate (TSP) were from Goss Scientific Instruments Ltd. (UK). HPLC-grade ethanol 99.7% was obtained from VWR International Ltd. (UK). Hydroxyvalerenic and valerenic acids were from Extrasynthese (France) whilst rutin hydrate from Sigma Chemical Co. (Germany). Purified water was obtained from a Milli Q gradient system from Millipore (UK).

2.3 *Preparation of samples for analysis.*

All the tinctures (0.65 mL) were directly analysed by NMR after adding 0.05 mL of D_2O (0.05% TSP) as a solvent for internal lock. In addition, a volume of 0.65 mL of each tincture was measured and transferred to a microcentrifuge tube, carefully dried under oxygen-free nitrogen (BOC, UK) and the residue completely redissolved in different deuterated solvents.

Standard solutions of valerenic acid (VA) and hydroxyvalerenic acid (HOVA) in EtOD/ D_2O (60%) were similarly analysed by NMR: a volume of 0.65 mL was transferred to an NMR tube and 0.05 mL of D_2O (0.05% TSP) was added.

2.4 *NMR experiments*

NMR spectra were obtained on Bruker AVANCE 500 MHz spectrometer equipped with a CP QNP multinuclear cryoprobe head. The TOPSPIN v1.3 software was used for spectra acquisition and processing. Spectra acquisition parameters: ^{1}H NMR (Pulse program: zg30; Acquisition mode: DQD; Time domain: 65536; Number of scans: 512; Spectral width 20.66 ppm / 10330.58 Hz; Acquisition: 3.17 s; Fidres: 0.158 Hz); Multiple-solvent suppression (Same parameters as ^{1}H NMR but different pulse program: lc1pnfr and 128 scans); water presaturation (Same parameters as ^{1}H-NMR but different pulse program: zgpr; scans: 512; Spectral width: 16.02 ppm / 8012.82 Hz; Acquisition: 4.09s; Fidres: 0.122266 Hz); 1D DOSY (Pulse program: ledbpgp2s1d; Acquisition mode: DQD; Time domain: 32768; Number of scans: 512; Spectral width 20.66 ppm / 10330.58 Hz; Acquisition: 1.59 s; Fidres: 0.131 Hz) using gradient strength (gpz6) 80, little delta (p30) 1000 ms and big delta (d20) 0.2 s. 1D ^{1}H NOESY pulse sequence (lc1pnfr) with multiple offset presaturation using frequency list was used to suppress water and ethanol signals of the samples. Presaturation was carried out with a 2 s relaxation delay (d1) and 0.8 s mixing time (d18). In both cases the numbers of scans was 64 and the partial suppression of the water signal around 4.77 pm and ethanol signals at 3.65 ppm and 1.17 ppm was achieved.

3 Results and discussion

3.1 *Effects of processing on the chemistry of the samples*

Herbal extracts are composed of a number of different compounds with different polarity and solubility. The simple process of drying the commercial tinctures and redissolving the residue in different deuterated solvents leads to changes in the chemical composition as revealed by standard ^{1}H-NMR experiments (Fig. 2). A total lack of signals after redissolving in chloroform (spectra not shown) was somehow anticipated, but the subtle variability of the diagnostic aromatic regions after dissolving

in methanol or ethanol may lead to potential bias depending on the analytical protocols applied.

3.2 *Optimisation of direct one dimension diffusion-ordered (1D DOSY) ^{1}H NMR experiments*

1D DOSY ^{1}H NMR experiments required at least a 500 MHz apparatus. Similar experiments run in a 400 MHz apparatus (Bruker) resulted in poor spectra without the necessary resolution to differentiate relevant peaks of the quality markers (Data not shown).

Three different experimental DOSY conditions were tested in which bipolar gradients and pulses were used to enhance the diffusion of molecules, according to their molecular weight. The gradient used was between 80% and 60%, and little and big delta had to be increased in order to compensate for the gradient changes: DOSY1 (GPZ6=40%; d20=0.4 s; P30=2 ms); DOSY2 (GPZ6=80%; d20=0.2 s; P30=1 ms); and DOSY3 (GPZ6=60%; d20=0.4 s; P30=2 ms). In all cases the number of scans was 512.

The spectra resulting from DOSY1 and DOSY2 experiments were considerably better (Fig. 3) than those from DOSY3 (spectrum not shown). The differences in resolution between DOSY1 and DOSY2 are small, but DOSY2 provides with more intense signals than DOSY1 although DOSY1 experiments benefit from a better baseline than DOSY2. However, there is a loss of signals in the DOSY1 spectrum, compared to DOSY2, particularly in the aromatic region. These experiments reveal that optimisation of the signal detection comes from a complex balance between the gradient strength, little delta and big delta, as increasing or decreasing GPZ6 alone seems not to be directly related to the result. After all these considerations, we decided to choose the conditions set in DOSY2 as the standard protocol for our DOSY ^{1}H NMR experiments.

3.3 *Assignment of the proton signals of valerian phytochemical standards in EtOD/D_2O (60% v/v)*

The alcoholic strength of the different tinctures was in the range 58 to 67% *v*/*v*. As a compromise, we chose to assign the shift values for each proton of valerenic and

hydroxyvalerenic acids in EtOD/D_2O (60% *v*/*v*) based on ^{1}H NMR COSY experiments. All signals appear in the spectrum between δ 1-4 ppm apart from H-11, which resonates at much lower field (δ 7 ppm) and the distinctive H-1 of hydroxyvalerenic acid (δ 4.2 ppm).

Valerenic acid (**1**) COSY ^{1}H NMR (400 MHz) in EtOD/D_2O (60% *v*/*v*): H-1a *δ* 1.80 (2H, m), H-1b *δ* 1.57 (2H, m); H-2a *δ* 1.75 (1H, t), H-2b *δ* 1.38, (1H, t), H-5 *δ* 3.53 (1 H, m), H-6 *δ* 1.85 (2H, m), H-7 *δ* 1.43 (2H, m), H-8 *δ* 1.96 (1H, m), H-9 *δ* 2.90 (1H, brs), H-10 *δ* 1.60 (3H, q), H-11 *δ* 7.10 (1H, d, *J* = 9.85 Hz), H-13 *δ* 1.80 (3H, s), H-15 *δ* 0.78 (3H d, *J*=7.00 Hz)

Hydroxyvalerenic acid (**2**) COSY ^{1}H NMR (400 MHz) in EtOD/D_2O (60% *v*/*v*): H-1 , *δ* 4.2 (1H, brs); H-2a *δ* 2.5 (1H, d, *J*= 14.0 Hz), H-2b *δ* 2.16 (d, 1H, *J*= 14.0 Hz), H-5 *δ* 3.6 (1 H, m), H-6 *δ* 1.85 (2H, m), H-7 *δ* 1.40 (2H, m), H-8 *δ* 2.1 (1H, m), H-9 *δ* 2.7 (1H, brs), H-10 *δ* 1.6 (3H, q), H-11 *δ* 7.00 (1H, d, *J* = 9.85 Hz), H-13 *δ* 1.90 (3H, s), H-15 *δ* 0.98 (3H d, *J*=4.25 Hz)

3.4 *One dimension diffusion-ordered ^{1}H NMR (1D DOSY) and ^{1}H NMR with solvent suppression of valerian phytochemical standards in EtOD/D2O (60% v/v)*

Difficulties in phasing the spectrum resulted in many signals being distorted or lost under the obscuring effect of the solvent in the ethanol and water multiple-solvent suppression experiments (Data not shown). The 1D DOSY experiments benefit from a better baseline with a high ratio signal-to-noise. This allowed the unequivocal assignment of the chemical shifts observed in the COSY experiments to the corresponding protons of valerenic and hydroxyvalerenic acids in ^{1}H NMR 1D DOSY spectra. The distinctive H-1 of hydroxyvalerenic acid (δ 4.2 ppm) shows as a prominent signal that may facilitate its detection in complex tinctures (Fig. 4).

3.5 *Identification of quality and stability markers in the spectra of valerian and hops liquid products*

The commercial samples were submitted to direct NMR experiments after addition of D_2O as internal lock. Figure 5 show experiments using DOSY ^{1}H NMR and ^{1}H NMR with multiple-solvent suppression. The three major phytochemicals groups contributing to the complexity of the spectra of valerian and hops liquid extracts are phenolic

158 compounds, terpenes and sugars (Houghton, 1988; Hoek, Hermans-Lokkerbol, &
159 Verpoorte, 2001). All these components contributed to a busy spectrum, but in DOSY
160 and multiple-solvent suppression experiments the region around δ 7 ppm was free from
161 any obscuring signals, thus facilitating the detection of the H-11 of valerenic acid. In the
162 same region some signals could be tentatively assigned to H2, H5 and H8 of quercetin as
163 predicted by ACD/I-Lab (ACD Labs, Toronto, Canada) (Fig. 6). Although some
164 manufacturers may be using flavonoids as a cheap and easy markers for quality control
165 purposes, these metabolites should not be considered valid markers due to its ubiquity
166 in herbal medicines.

167 Furthermore, a signal consistent with the vinyl proton of prenylated moieties attached
168 to aromatic rings is clearly visible in the valerian-hops tincture at δ 5.2 despite the
169 residual solvent signal. The aromatic region was more populated than in the valerian
170 tincture samples, probably due to the deshielded protons present in the hydroxyphenyl-
171 2-propen-1-on moiety characteristic of chalcones such as xanthohumol. The tentative
172 assignments of the signals by comparison with predicted values from ACD/I-Lab (ACD
173 Labs, Toronto, Canada) are presented in Figure 6.

174 Additionally, Figure 6 shows some signals appearing from δ 1.8 to δ 1.4 ppm in the
175 spectrum of the valerian-hop tincture which are not seen in valerian tinctures. These
176 signals are shown in detail in Figure 7. They are consistent with the characteristic
177 protons from methyl groups in prenylated flavonoids and α-acids present in *H. lupulus*,
178 such as xanthumol and humulones, which give singlets between δ 1.2 and δ 1.6 (Hoek et
179 al., 2001; Khatib et al., 2007). They apparently belong to three different prenylated
180 moieties. To unequivocally identify the parent structures further experiments with
181 standards of iso-cohumulone, humulone, adhumulone and xanthumol dissolved in
182 60%*v*/*v* hydroethanolic solutions would be needed.

183 Finally, a proton resonating at δ 4.15 ppm consistent with H-1 of hydroxyvalerenic acid
184 appears in DOSY experiments with expired tinctures only (Fig. 8). This is consistent with
185 previous reports proposing hydroxyvalerenic acid as a degradation marker for *V.*
186 *officinalis* (Goppel & Franz, 2004).

4 Conclusions

Analysis of complex chemical mixtures, such as tinctures and infusions of plant material traditionally requires the separation of dozens of different single chemical entities. Furthermore, preparative steps prolong experimental time and increase the risk of both alteration of the chemistry and/or contamination of the sample. This work demonstrates the application of an alternative protocol to analyse multi-component liquid herbal medicines with minimum sample preparation. This approach exploits 1D NMR methods based on ^{1}H NMR DOSY and ^{1}H NMR with multiple-solvent suppression experiments. The aim was to obtain a fast and clear indication of the presence or absence of the characteristic chemical markers for metabolites of interest. Adding a second dimension for the diffusion coefficients 2D DOSY (Barjat, Morris, & Swanson, 1998; Nilsson et al., 2004; Otto & Larive, 2001; Viel, Ziarelli, & Caldarelli, 2003) would certainly provide further useful data but this technique adds complexity to the interpretation of the spectra.

In summary, we here developed a simple approach which could be easily processed and interpreted in the same manner of a 1D HPLC chromatogram or a TLC plate. The application of 1D DOSY to *valerian* and hops products successfully reveals the presence of the characteristic peaks of valerenic acid and prenylated moieties from α-acids in fresh tinctures as well as hydroxyvalerenic acid only in expired/degraded ones. Therefore direct NMR may be used as a rapid technique to provide additional information in the quality control of herbal constituents of complex herbal pharmaceutical products.

Conflict of interest

The authors declare that there are no conflicts of interest.

5 References

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255 mixtures using chromatographic stationary phases. *Proc Natl Acad Sci USA* **100**:9696–
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Caption of Figures

Fig. 1. Chemical structures of the specific phytomarkers present in herbal products containing valerian and hops.

263 **Fig. 2. 500 MHz ^{1}H-NMR spectrum of valerian tincture 60% *v/v* EtOH dried and redissolved in different deuterated solvents.**

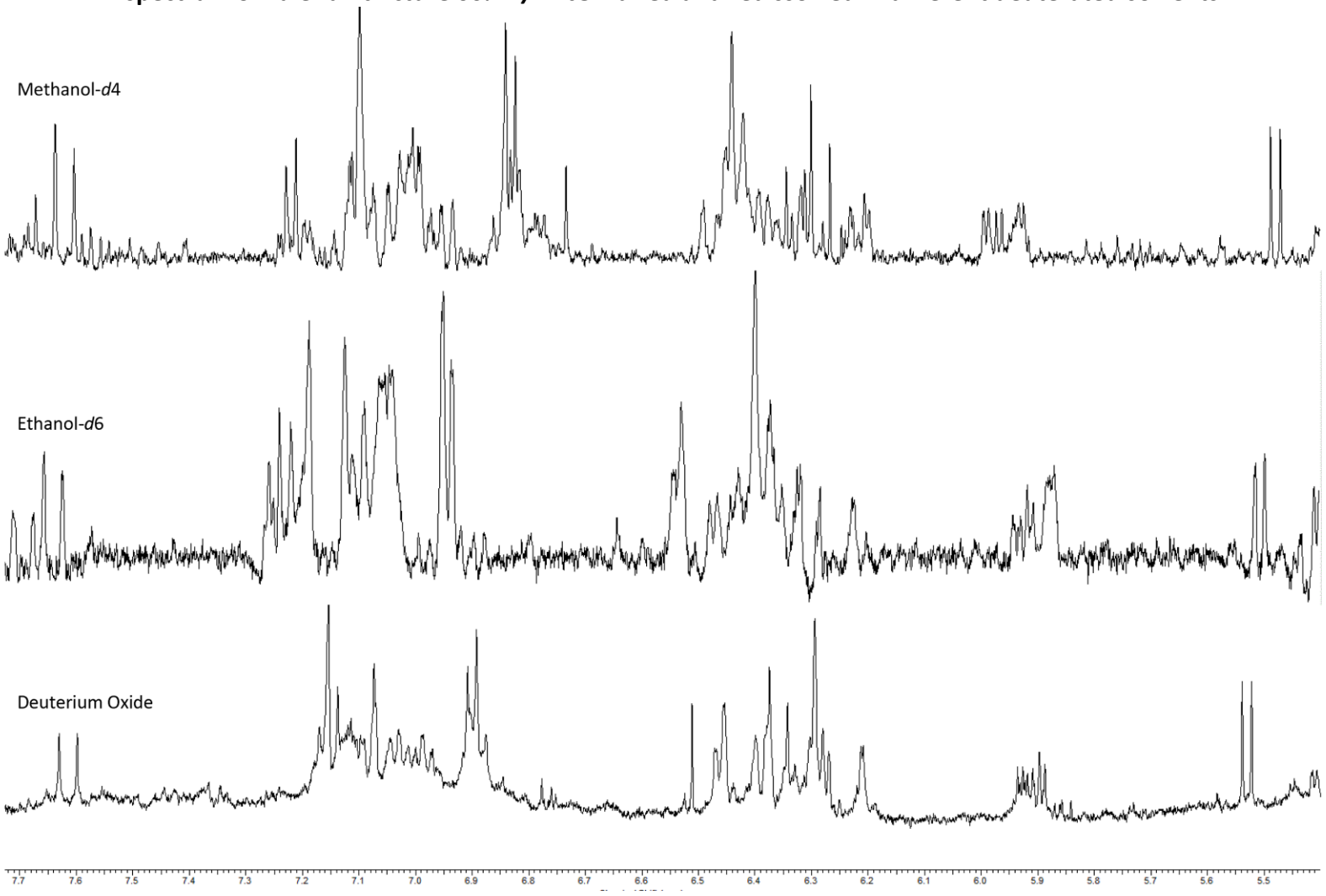

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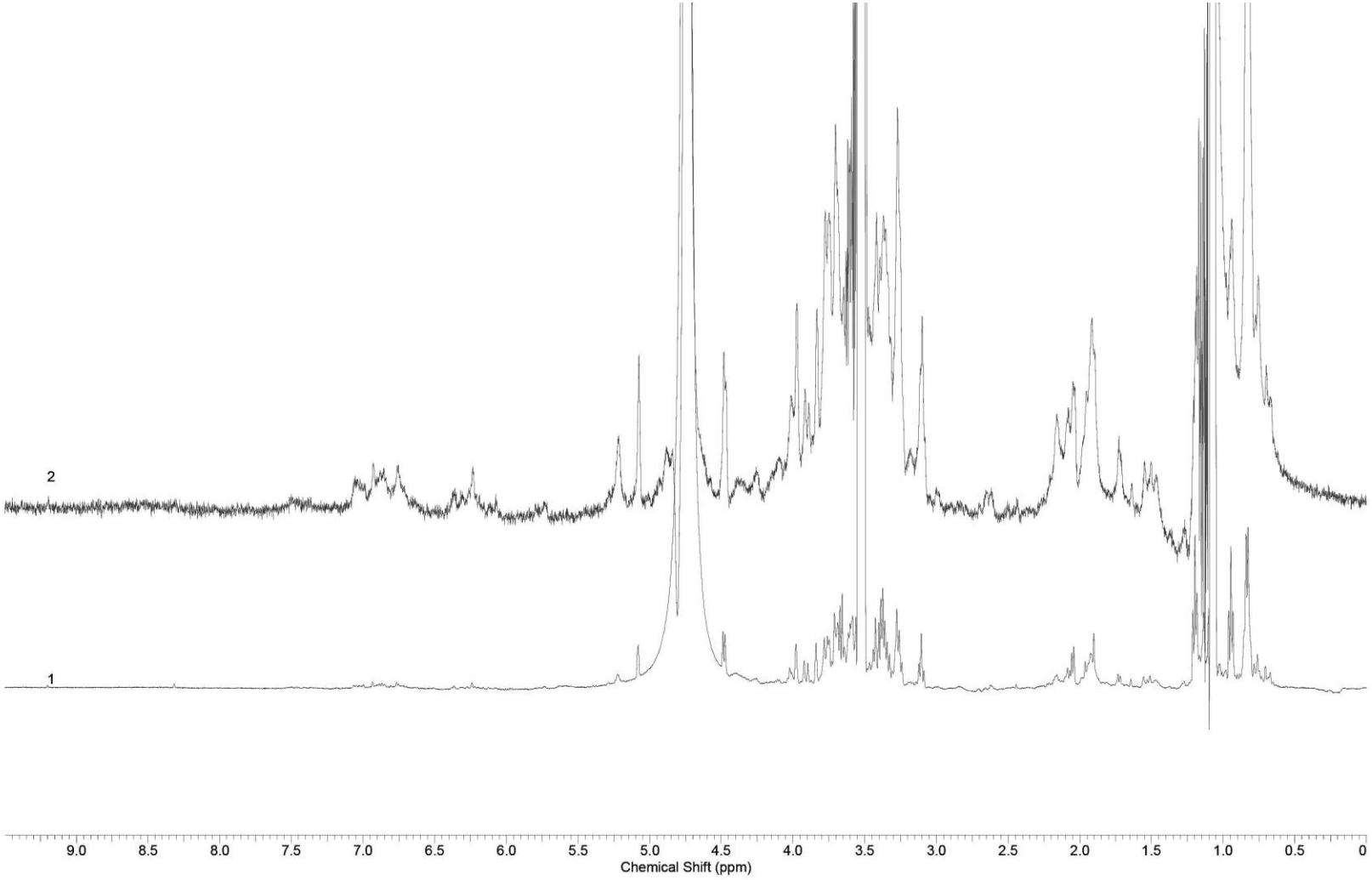
Fig. 3. 500 MHz DOSY ^{1}H NMR spectra of valerian tincture (EtOH 60% *v/v*) under different conditions. (1) (GPZ6=40%; d20=0.4 s; P30=2 ms); (2) (GPZ6=80%; d20=0.2 s; P30=1 ms.



269 **Fig. 4**

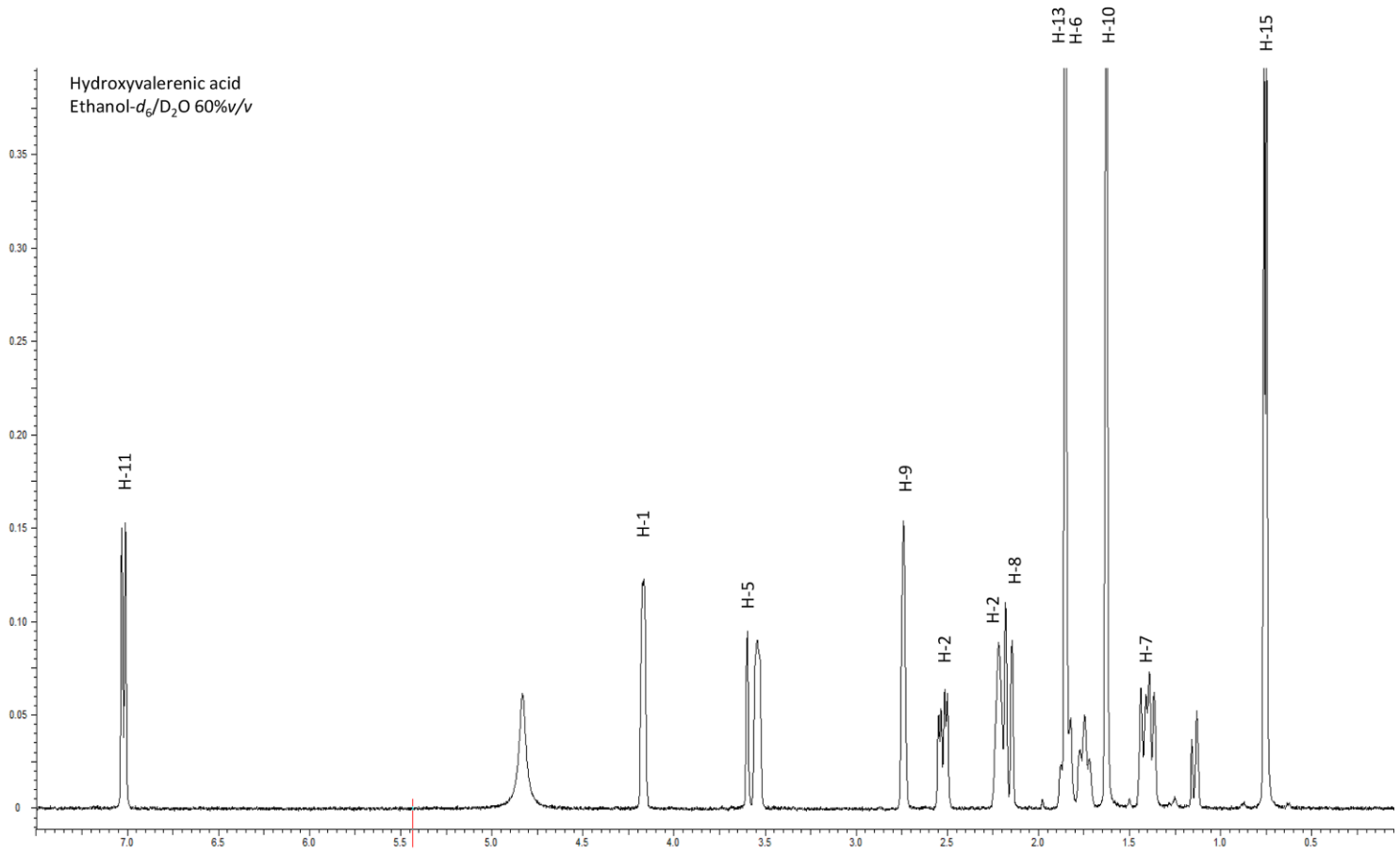

Hydroxyvalerenic acid
Ethanol-d_6/D_2O 60%*v/v*
H-11
H-1
H-5
H-9
H-2
H-2
H-8
H-13
H-6
H-10
H-7
H-15
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5

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Fig. 5. Direct NMR experiments with valerian tincture. 500 MHz ^{1}H NMR with multisolvent (ethanol and water) suppression (bottom spectrum); ^{1}H-NMR (middle spectrum); DOSY ^{1}H NMR (upper spectrum) (EtOH/H2O, 54%).

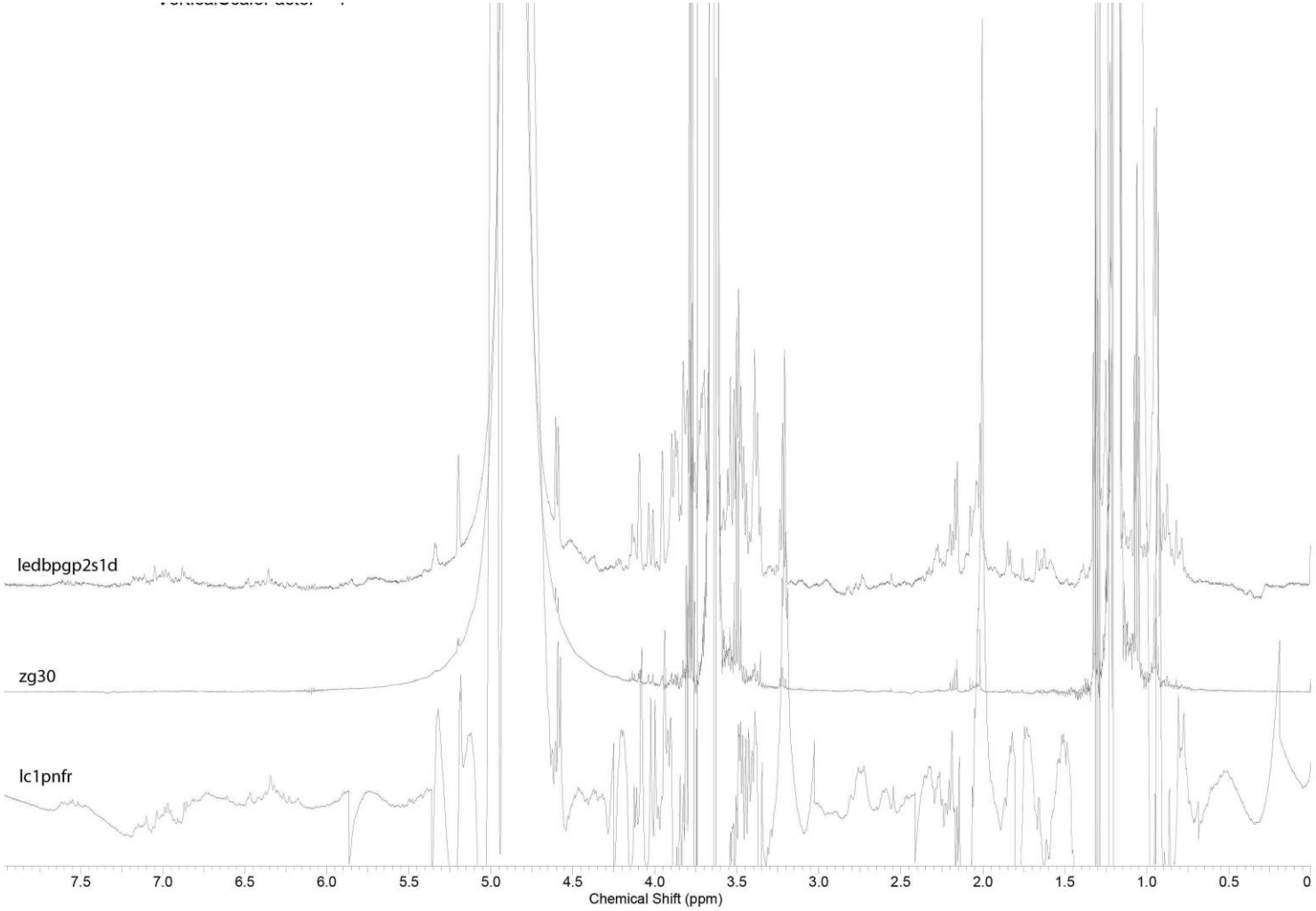
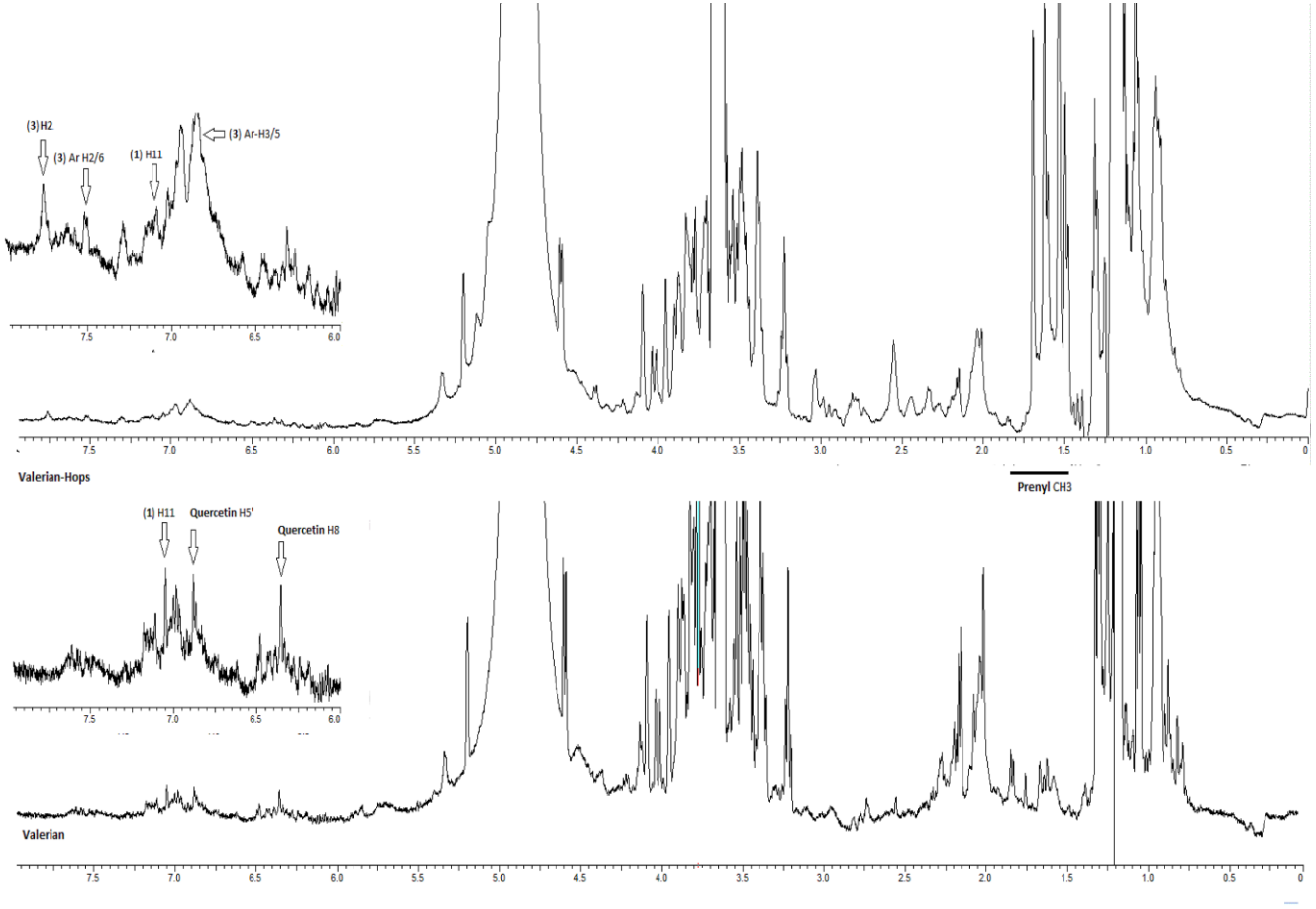
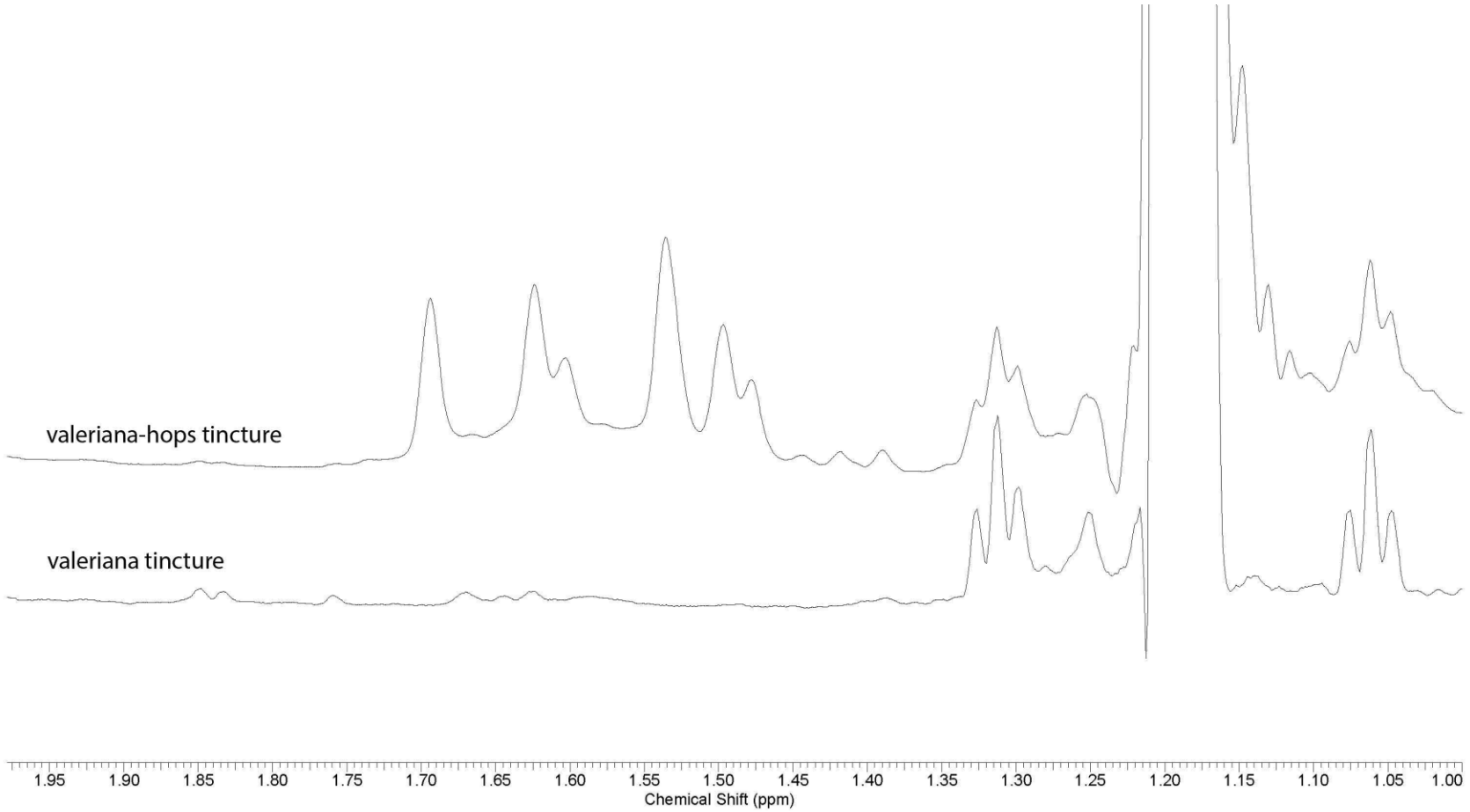


Fig. 6. 500 MHz DOSY ^{1}H NMR experiments showing signals consistent with different phytomarkers in both valerian and valerian-hops tinctures.



278 **Fig. 7. Fig. 6. 500 MHz DOSY ^{1}H NMR experiments showing signals consistent with prenylated moieties.**



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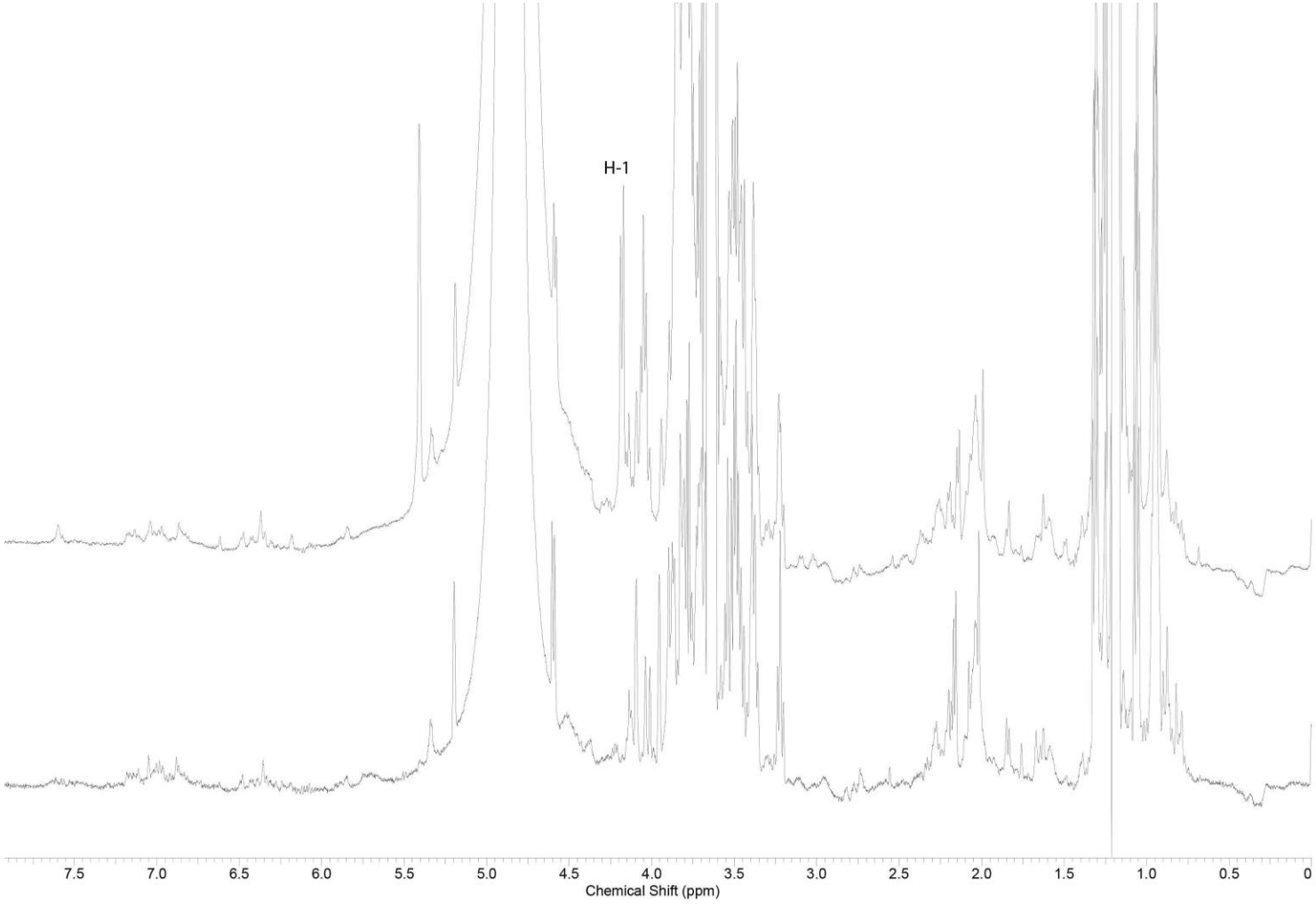
280 **Fig. 8. 500 MHz DOSY ^{1}H NMR spectra of expired valerian tincture (upper spectrum) and non expired valerian tincture (lower**
281 **spectrum) in EtOH/H_2O (60%) showing the appearance of H-1 of hydroxyvalerenic acid.**



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