| SPi | Journal Code | | | | Article ID | | | | Dispatch: 12.11.15 | CE: |
|---|---|---|---|---|---|---|---|---|---|---|
| | P | C | A | | 2 | 6 | 0 | 3 | No. of Pages: 7 | ME: |

Research article

Phytochemical Analysis

Received: 14 March 2015, Revised: 11 October 2015, Accepted: 14 October 2015 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/pca.2603

# Application of Diffusion-edited and Solvent Suppression $^1$H-NMR to the Direct Analysis of Markers in Valerian-hop Liquid Herbal Products

Q1 Jose M. Prieto,[a]* Maria Mellinas-Gomez[a] and Mire Zloh[a,b]

Q3 Q2 **ABSTRACT: Introduction – 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.**
**Objectives – 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 characterisation of quality and stability markers in multi-component herbal medicines/food supplements.**
**Material and Methods – 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.**
**Results – 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).**
**Conclusion – This work concludes on the potential value of 1D DOSY $^1$H-NMR to provide additional assurance of quality in complex natural mixtures. Copyright © 2015 John Wiley & Sons, Ltd.**

**Keywords:** Herbal medicinal product; quality marker; nuclear magnetic resonance; diffusion edited spectroscopy; *Valeriana officinalis*; *Humulus lupulus* L



## 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 mono-component 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 earlier-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 as herbal medicines either alone (Gilard *et al*., 2010) or as a complement of HPTLC (Booker *et al*., 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 and Pekos, 1998) and α-acids such as humulone (**3**) together with prenylated chalcones such as xanthohumol (**4**) (Hoek *et al*., 2001) are currently regarded as the best quality markers for valerian and hops, respectively (Fig. 1). F1

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 United States (US Pharmacopoeia, 2015) and fetch as much as £945 in Europe

* Correspondence to: Jose M. Prieto, Department of Pharmaceutical and Biological Chemistry, The School of Pharmacy, University of London, 29–39 Brunswick Square, London, WC1N 1AX, UK.
E-mail: j.prieto@ucl.ac.uk

[a] Department of Pharmaceutical and Biological Chemistry, The School of Pharmacy, University of London, 29-39 Brunswick Square, London, WC1N 1AX, UK

[b] Department of Pharmacy, University of Hertfordshire, College Lane, Hatfield, AL10 9AB, UK

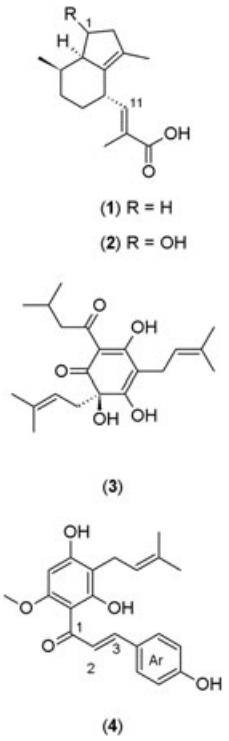



**Figure 1**. Chemical structures of the specific phytomarkers present in herbal products containing valerian and hops.

(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 and 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 one-dimensional (1D) NMR analysis to assess the quality and stability of commercial valerian-hops tinctures.

## Material and methods

### Herbal drugs and herbal medicinal products

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

### Solvents and chemicals

Chloroform-*d* and deuterated dimethyl sulfoxide (DMSO-$d_6$) (99.8%) were from Sigma Aldrich Chemie GmbH (Germany). Methanol-$d_4$ (99.8%) and ethanol-$d_6$ (99.8%) were from Cambridge Q5 Isotope Laboratories (USA). Deuterium oxide ($D_2O$) (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).

### 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 and hydroxyvalerenic acid in ethanol-$d_6$/$D_2O$ (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.

### 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: 4.09 s; 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. One-dimensional (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.

## Results and discussion

### 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 F2 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.

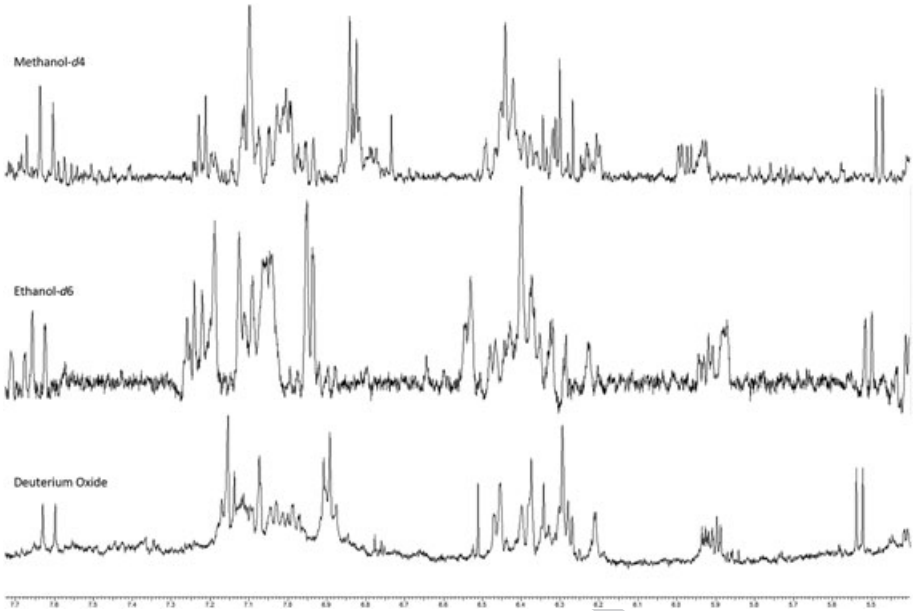



**Figure 2**. 500 MHz $^{1}$H-NMR spectrum of valerian tincture 60% *v/v* ethanol dried and redissolved in different deuterated solvents.

### Optimisation of direct one-dimensional diffusion-ordered (1D DOSY) $^{1}$H-NMR experiments

One-dimensional (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); 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 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

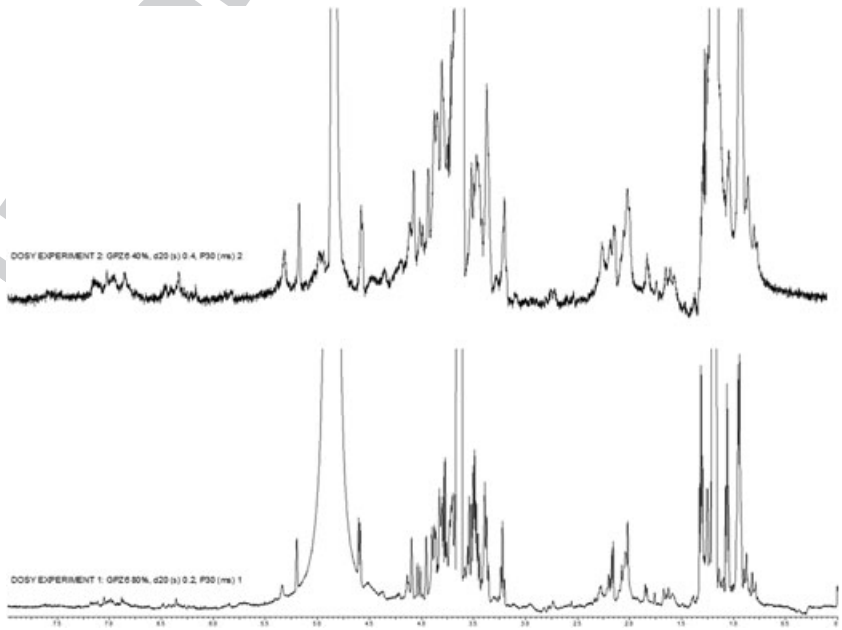


**Figure 3**. 500 MHz DOSY $^{1}$H-NMR spectra of valerian tincture (ethanol 60% *v/v*) under different conditions: (1) (GPZ6 = 40%; d20 = 0.4 s; P30 = 2 ms); (2) (GPZ6 = 80%; d20 = 0.2 s; P30 = 1 ms.

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.

### Assignment of the proton signals of valerian phytochemical standards in ethanol-$d_6$/$D_2O$ (60% v/v)

The alcoholic strength of the different tinctures was in the range 58–67% *v/v*. As a compromise, we chose to assign the shift values for each proton of valerenic and hydroxyvalerenic acids in ethanol-$d_6$/$D_2O$ (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 ethanol-$d_6$/$D_2O$ (60% *v/v*): H-1a $\delta$ 1.80 (2H, m), H-1b $\delta$ 1.57 (2H, m); H-2a $\delta$ 1.75 (1H, t), H-2b $\delta$ 1.38, (1H, t), H-5 $\delta$ 3.53 (1 H, m), H-6 $\delta$ 1.85 (2H, m), H-7 $\delta$ 1.43 (2H, m), H-8 $\delta$ 1.96 (1H, m), H-9 $\delta$ 2.90 (1H, brs), H-10 $\delta$ 1.60 (3H, q), H-11 $\delta$ 7.10 (1H, d, $J$ = 9.85 Hz), H-13 $\delta$ 1.80 (3H, s), H-15 $\delta$ 0.78 (3H d, $J$ = 7.00 Hz).

Hydroxyvalerenic acid (**2**) COSY $^1$H-NMR (400 MHz) in ethanol-$d_6$/$D_2O$ (60% *v/v*): H-1 , $\delta$ 4.2 (1H, brs); H-2a $\delta$ 2.5 (1H, d, $J$ = 14.0 Hz), H-2b $\delta$ 2.16 (d, 1H, $J$ = 14.0 Hz), H-5 $\delta$ 3.6 (1 H, m), H-6 $\delta$ 1.85 (2H, m), H-7 $\delta$ 1.40 (2H, m), H-8 $\delta$ 2.1 (1H, m), H-9 $\delta$ 2.7 (1H, brs), H-10 $\delta$ 1.6 (3H, q), H-11 $\delta$ 7.00 (1H, d, $J$ = 9.85 Hz), H-13 $\delta$ 1.90 (3H, s), H-15 $\delta$ 0.98 (3H d, $J$ = 4.25 Hz).

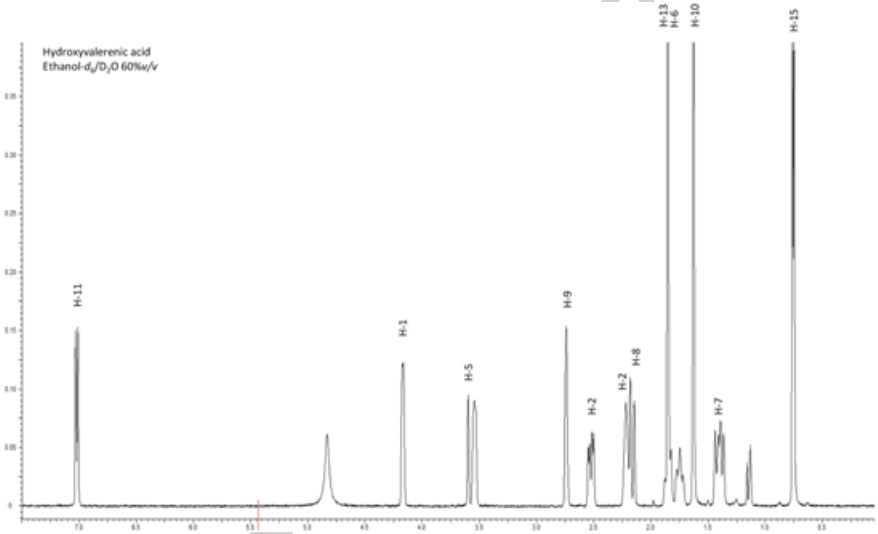



**Figure 4**. Assignment of the proton signals of hydroxyvalerenic acid in ethanol-$d_6$/$D_2O$ (60% *v/v*).

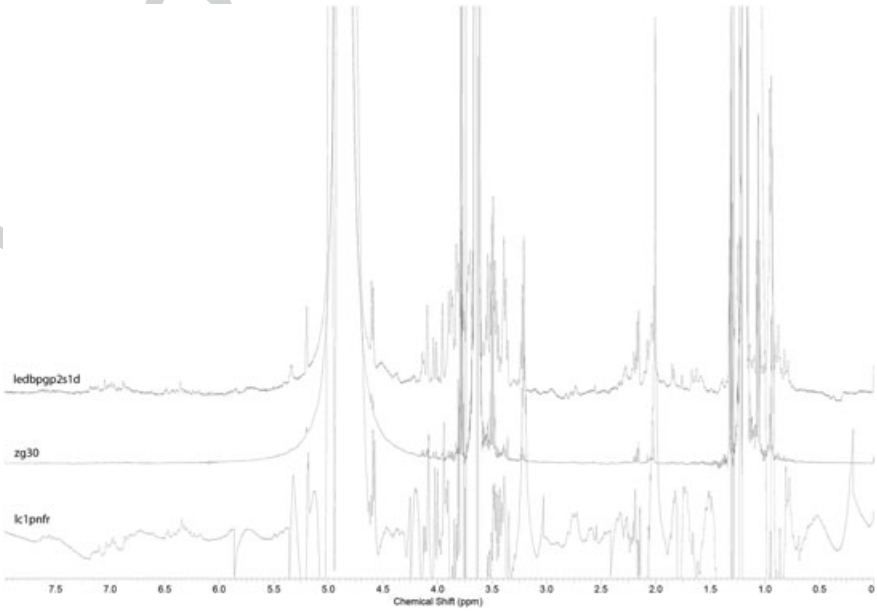



**Figure 5**. Direct NMR experiments with valerian tincture. 500 MHz $^1$H-NMR with multisolvent (ethanol and water) suppression (bottom spectrum); $^1$H-NMR (middle spectrum); DOSY $^1$H-NMR (upper spectrum) (ethanol/$H_2O$, 54%).

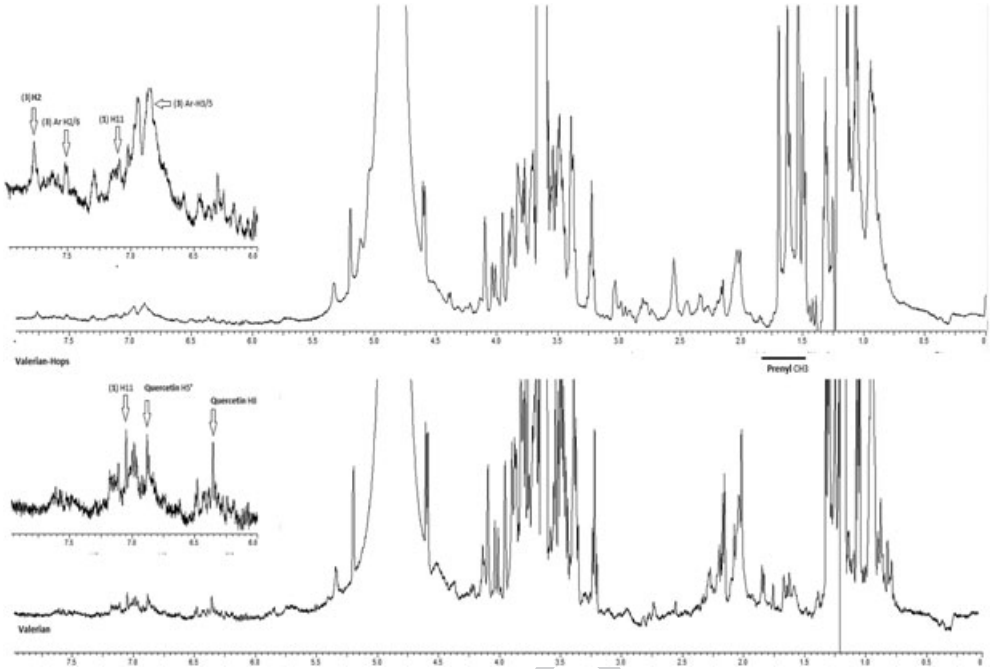



**Figure 6**. 500 MHz DOSY $^{1}$H-NMR experiments showing signals consistent with different phytomarkers in both valerian and valerian-hops tinctures.

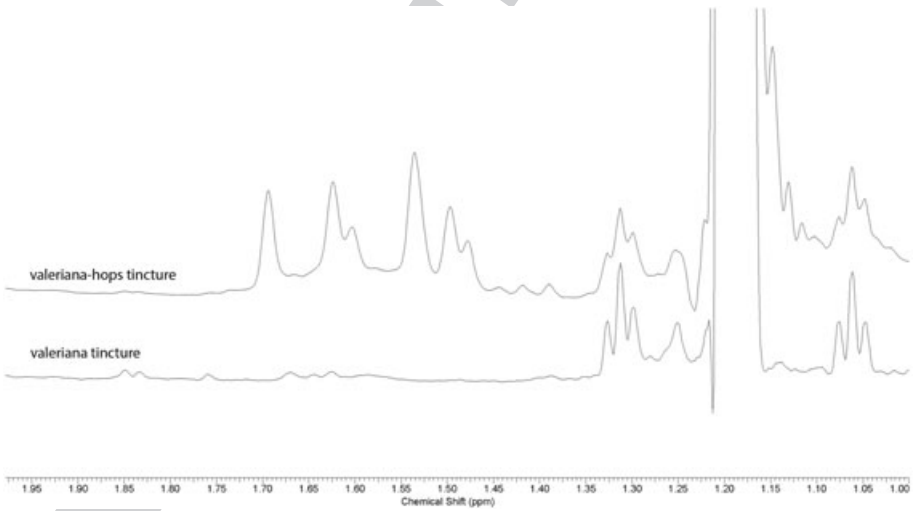



**Figure 7**. 500 MHz DOSY $^{1}$H-NMR experiments showing signals consistent with prenylated moieties.



### One-dimensional diffusion-ordered $^{1}$H-NMR (1D DOSY) and $^{1}$H-NMR with solvent suppression of valerian phytochemical standards in ethanol-$d_6$/$D_2O$ (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 signal-to-noise ratio. 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 F4 that may facilitate its detection in complex tinctures (Fig. 4).

### 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 compounds, terpenes and sugars (Houghton, 1988; Hoek *et al*., 2001). All these components contributed to a busy spectrum, but in DOSY and multiple-solvent suppression experiments the region around δ 7 ppm was free from any obscuring signals, thus facilitating the detection of the H-11 of valerenic acid. In the same region some signals could be tentatively assigned to F5

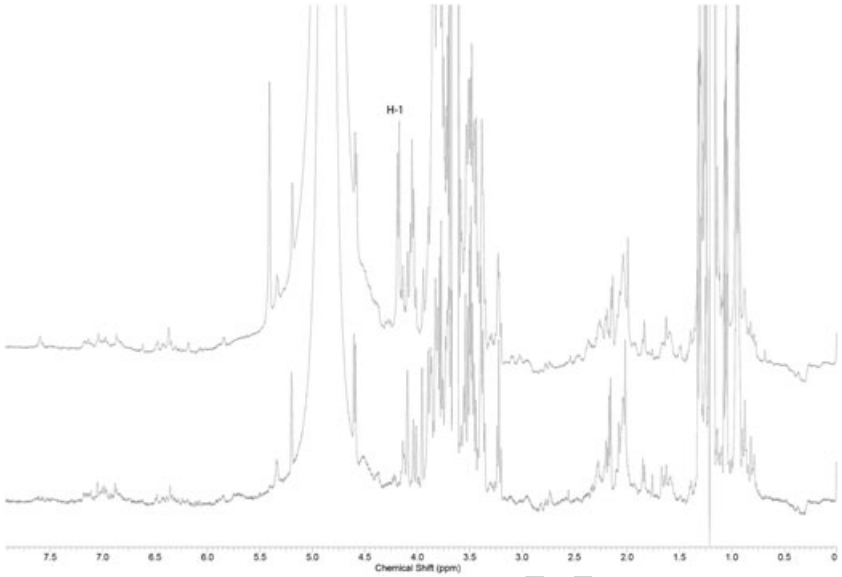



**Figure 8**. 500 MHz DOSY $^{1}$H-NMR spectra of expired valerian tincture (upper spectrum) and non-expired valerian tincture (lower spectrum) in ethanol/$H_2O$ (60%) showing the appearance of H-1 of hydroxyvalerenic acid.

H2, H5 and H8 of quercetin as predicted by ACD/I-Lab (ACD Labs, F6 Toronto, Canada) (Fig. 6). Although some manufacturers may be using flavonoids as cheap and easy markers for quality control purposes, these metabolites should not be considered valid markers due to its ubiquity in herbal medicines.

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

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

F7

Finally, a proton resonating at δ 4.15 ppm consistent with H-1 of hydroxyvalerenic acid appears in DOSY experiments with expired F8 tinctures only (Fig. 8). This is consistent with previous reports proposing hydroxyvalerenic acid as a degradation marker for *Valeriana officinalis* (Goppel and Franz, 2004).

## 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 *et al*., 1998; Otto and Larive, 2001; Viel *et al*., 2003; Nilsson *et al*., 2004) 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.

## References

American Herbal Pharmacopoeia. 1999. Valerian root (Valeriana officinalis): analytical, quality control, and therapeutic monograph. In *American Herbal Pharmacopoeia*, Upton R (ed.). Q6

Barjat H, Morris G, Swanson A. 1998. A three-dimensional DOSY-HMQC experiment for the high-resolution analysis of complex mixtures. *J Magn Reson* **131**: 131–138.

Booker A, Johnston D, Heinrich M. 2015. Value chains of herbal medicines—ethnopharmacological and analytical challenges in a globalizing world. In *Evidence-Based Validation of Herbal Medicine*. Mukherjee PK (Ed.). Elsevier: Amsterdam; 29–44.

European Directorate for the Quality of Medicines and HealthCare. 2013. *European Pharmacopoeia*, 7.0, 7.8 edn. Council of Europe. Q4

European Medicines Agency. 1999. *Report from the ad-hoc working group on herbal medicinal products 1997-1998.*

Gilard V, Balayssac S, Malet-Martino M, Martino R. 2010. Quality control of herbal medicines assessed by NMR. *Curr Pharm Anal* **6**: 234–245.

Goppel M, Franz G. 2004. Stability control of valerian ground material and extracts: a new HPLC-method for the routine quantification of valerenic acids and lignans. *Pharmazie* **59**: 446–452.
Hoek AC, Hermans-Lokkerbol AC, Verpoorte R. 2001. An improved NMR method for the quantification of alpha-acids in hops and hop products. *Phytochem Anal* **12**: 53–57.
Houghton PJ. 1988. The biological activity of valerian and related plants. *J Ethnopharmacol* **22**: 121–142.
Khatib A, Wilson EG, Kim HK, Supardi M, Choi YH, Verpoorte R. 2007. NMR assignment of iso-alpha-acids from isomerised extracts of *Humulus lupulus* L. cones. *Phytochem Anal* **18**: 371–377.
Lazarowych NJ, Pekos P. 1998. Use of fingerprinting and marker compounds for identification and standardization of botanical drugs: strategies for applying pharmaceutical hplc analysis to herbal products. *Ther Innov Regul Sci* **32**: 497–512.
Nilsson M, Duarte IF, Almeida C, Delgadillo I, Goodfellow BJ, Gil AM, Morris GA. 2004. High-resolution NMR and diffusion-ordered spectroscopy of port wine. *J Agric Food Chem* **52**: 3736–3743.
Otto WH, Larive CK. 2001. Improved spin-echo-edited NMR diffusion measurements. *J Magn Reson* **153**: 273–276.
US Pharmacopoeia. 2015. Reference Standards. URL [http://store.usp.org] Accessed 4 Aug 2015.
Politi M, Peschel W, Wilson N, Zloh M, Prieto JM, Heinrich M. 2008. Direct NMR analysis of cannabis water extracts and tinctures and semi-quantitative data on delta9-THC and delta9-THC-acid. *Phytochemistry* **69**: 562–570.
Politi M, Zloh M, Pintado ME, Castro PML, Heinrich M, Prieto JM. 2009. Direct metabolic fingerprinting of commercial herbal tinctures by nuclear magnetic resonance spectroscopy and mass spectrometry. *Phytochem Anal* **20**: 328–334.
Sigma-Aldrich. Valerenic acid United States Pharmacopeia (USP) Reference Standard. In [http://www.sigmaaldrich.com] Accessed 4 Aug 2015.
Viel S, Ziarelli F, Caldarelli S. 2003. Enhanced diffusion-edited NMR spectroscopy of mixtures using chromatographic stationary phases. *Proc Natl Acad Sci U S A* **100**: 9696–9698.

# Author Query Form

## Journal: Phytochemical Analysis

## Article: pca_2603

Dear Author,

During the copyediting of your paper, the following queries arose. Please respond to these by annotating your proofs with the necessary changes/additions.

- If you intend to annotate your proof electronically, please refer to the E-annotation guidelines.
- If you intend to annotate your proof by means of hard-copy mark-up, please use the standard proofing marks. If manually writing corrections on your proof and returning it by fax, do not write too close to the edge of the paper. Please remember that illegible mark-ups may delay publication.

Whether you opt for hard-copy or electronic annotation of your proofs, we recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

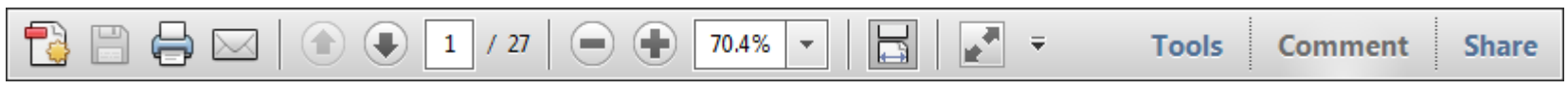
| Query No. | Query | Remark |
|---|---|---|
| Q1 | AUTHOR: Please confirm that given names (red) and surnames/family names ( green) have been identified correctly. | |
| Q2 | AUTHOR: Abstract has been split in to sub headings as journal style, please confirm or correct. | |
| Q3 | AUTHOR: Please provide a short abstract (maximum of 80 words or three sentences) summarising the key findings presented in the paper for Table of Content (TOC) entry. | |
| Q4 | AUTHOR: Please provide the city location of publisher for Reference European Directorate for the Quality of Medicines and HealthCare. 2013. | |
| Q5 | AUTHOR: Please confirm ‘Deuterium oxide (D2O)’ as in Fig. 2. | |
| Q6 | AUTHOR: Please provide the name and city location of the publisher for Reference American Herbal Pharmacopoeia. 1999. | |

WILEY

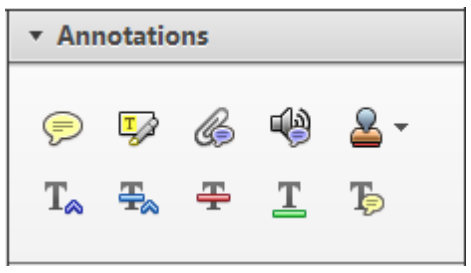
# USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

**Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)**
**The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/**

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:




This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

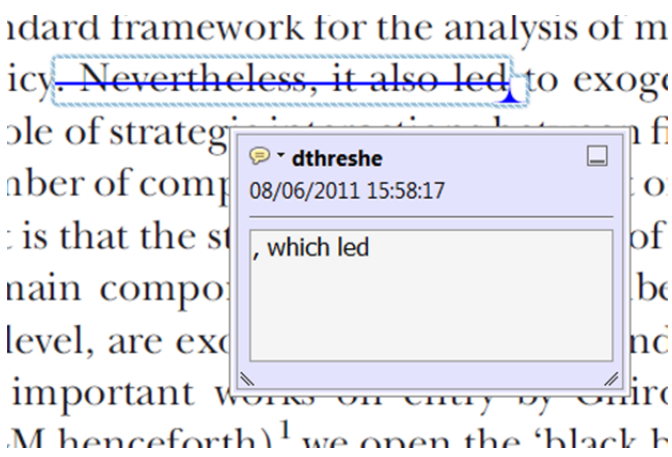



## 1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

**How to use it**

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

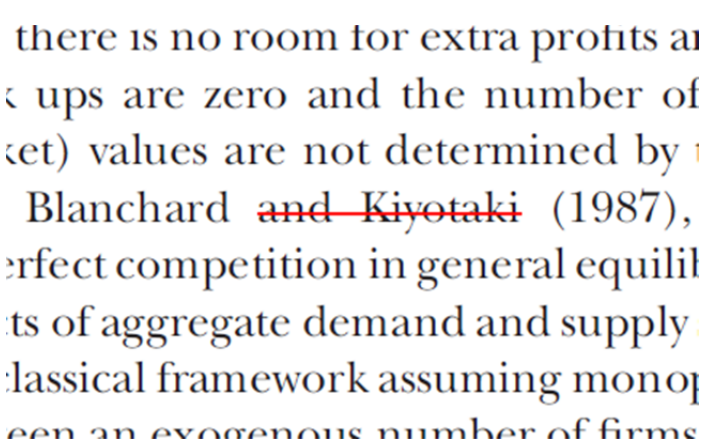



## 2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

**How to use it**

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

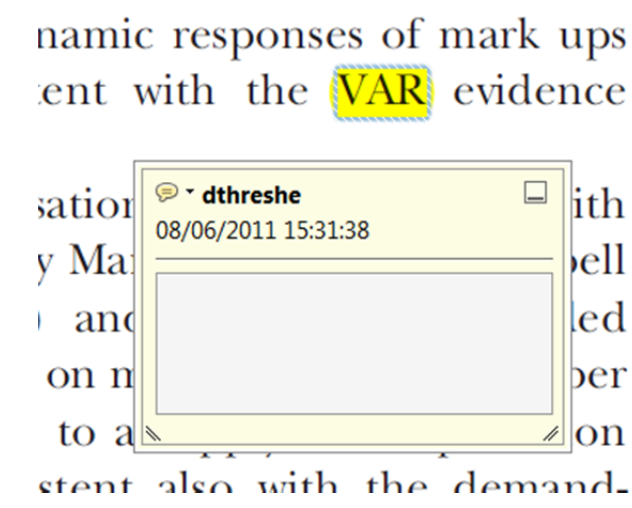


## 3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

**How to use it**

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

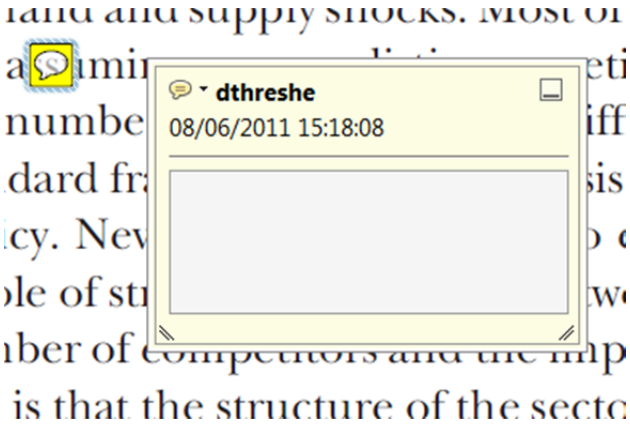



## 4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

**How to use it**

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



WILEY

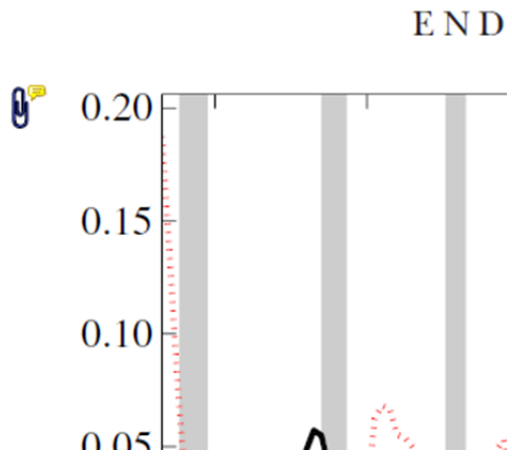
# USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

## 5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

**How to use it**

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

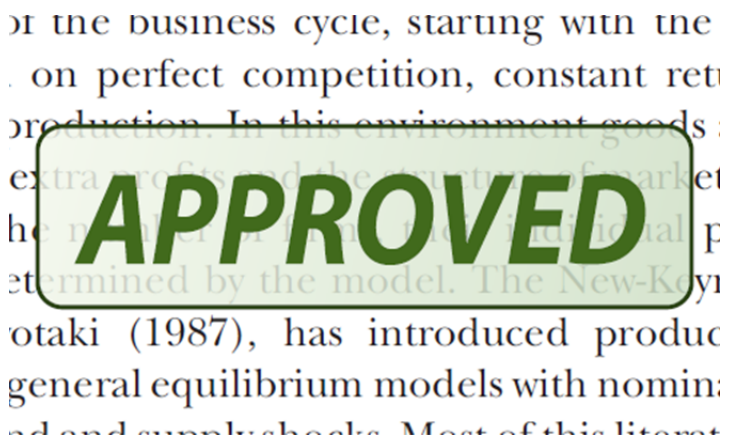


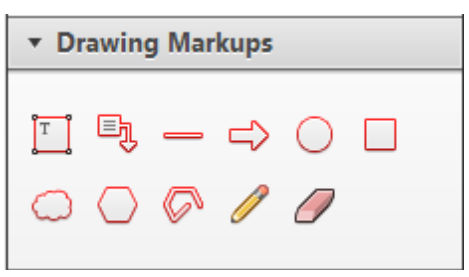

## 6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

**How to use it**

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



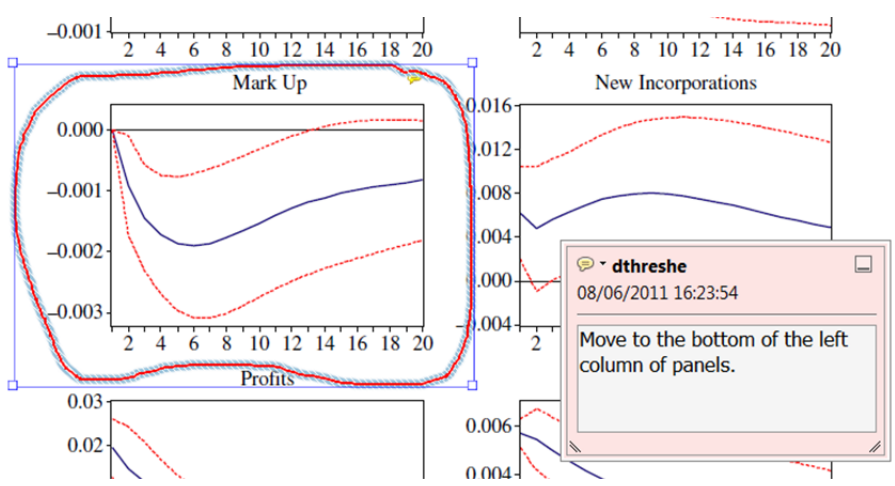




## 7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

**How to use it**

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.




**For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:**