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Analysis of Metabolic Variation and Galanthamine Content in *Narcissus* Bulbs by ^{1}H NMR†

Andrea Lubbe, Barbora Pomahačová, Young Hae Choi* and Robert Verpoorte

ABSTRACT:

Introduction – Galanthamine is a benzazepine alkaloid used as a drug to relieve symptoms of Alzheimer's disease. For pharmaceutical use this natural product has been extracted from the plant *Leucojum aestivum* (Amaryllidaceae) or produced synthetically. Limited supply of the natural source and high cost of synthetic production has led to a search for alternative sources of galanthamine. The bulbs of *Narcissus pseudonarcissus* (Amaryllidaceae) have been identified as a potential source of raw material for galanthamine extraction. Since inconsistent chemical composition can be an issue with medicinal plant material, it is of interest to know whether large variations occur between *Narcissus* bulbs grown in different geographical locations.

Objective – To evaluate whether large differences exist in the overall metabolic profiles of *Narcissus* bulbs grown in the two most important cultivation regions.

Methodology – ^{1}H NMR and principal component analysis were used for an unbiased comparison of the bulb samples.

Results – Overall metabolite profiles were quite similar, but galanthamine levels could slightly discriminate samples by geographical region. ^{1}H NMR was used for quantitation of galanthamine, and was found to be comparable to quantitation by HPLC. Compared with conventional chromatographic methods, sample preparation for ^{1}H NMR analysis is simple and rapid, and only a small amount of plant material is required.

Conclusions – Since useful qualitative and quantitative information about the metabolic state of *Narcissus* bulbs can be obtained by ^{1}H NMR, this method is useful for agricultural applications, and for quality control of raw material used in the pharmaceutical industry. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Galanthamine; *Narcissus pseudonarcissus* bulbs; regional variation; ^{1}H NMR; metabolomics

Introduction

Galanthamine is a benzazepine alkaloid produced by some members of the Amaryllidaceae plant family (Fig. 1). This natural product possesses a number of biological activities (reviewed by Bastida *et al*., 2006) that have been under investigation since the 1950s (Heinrich and Teoh, 2004). The discovery of its long-lasting, selective, reversible and competitive inhibitory effect on the enzyme acetylcholinesterase has led to its application to Alzheimer's disease (AD) (Moghul and Wilkinson, 2001). In 2004, 1.5 million people were estimated to suffer from AD worldwide, and this number is predicted to increase three-fold in 50 years (Forman *et al*., 2004). Further studies identified other bioactivities of galanthamine relevant to AD, such as stimulation of pre- and postsynaptic nicotinic receptors and protection against β-amyloid toxicity (Arias *et al*., 2004; Matharu *et al*., 2009; Scott and Goa, 2000; Sramek *et al*., 2000). While there is currently no preventative or curative treatment available for AD, galanthamine is used therapeutically to provide relief of disease symptoms. It has been registered as a drug (Nivalin® and later Reminyl®) since 1996 and provides good pharmacological profiles with fewer side effects than other acetylcholinesterase inhibitors such as tacrine and physostigmine (Giacobini, 2000; Heinrich and Teoh, 2004).

The plant *Leucojum aestivum* was the main source of galanthamine for some time (Heinrich and Teoh, 2004). Recently the more widespread licensing of galanthamine throughout the world has caused a need for alternative sources. Synthetic methods have been developed and used to produce galanthamine for the pharmaceutical industry (Tiffen, 1997), but high costs and increasing demand make extraction from plant sources an attractive option. A potential source for large-scale extraction of the alkaloid is members of the genus *Narcissus*. The long tradition of *Narcissus* cultivation for ornamental use means that extensive knowledge of the cultivation and breeding, as well as large stocks of various cultivars is already available (Kreh, 2002). Investigations into the secondary metabolites of *Narcissus* species have led to the isolation of almost 100 alkaloids classified into different skeletal types, many of which have interesting biological activities (reviewed by Bastida *et al*., 2006). Galanthamine

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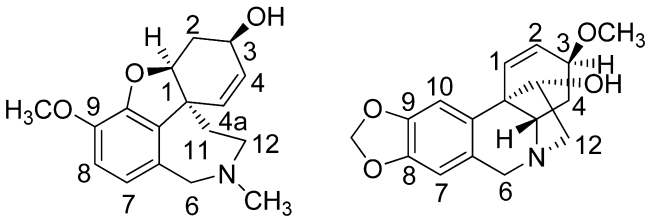

Galanthamine Haemanthamine

Figure 1. Chemical structures of galanthamine and haemanthamine.

can be found in several species, with the bulb usually accumulating the highest amount of alkaloids (Viladomat *et al*., 1986). Levels of galanthamine vary between species and cultivars, from trace amounts to as much as 2.5% of dry weight (Cherkasov and Tolkachev, 2002). In addition, levels of alkaloids in plants are known to vary during ontogenesis and also among geographical location (Elgorashi *et al*., 2002; Facchini 2001; Li *et al*., 2006).

Various methods exist for the determination of galanthamine levels in plant material. Early methods used enzyme immunoassays or radioimmunoassays for the quantitative determination of galanthamine in unpurified plant extracts (Poulev *et al*., 1993; Tanahashi *et al*., 1990). While these methods were very sensitive and precise, the need to raise antibodies and the use of radioactive substances make these methods laborious and expensive. Chromatographic methods include the use of high-performance liquid chromatography (HPLC) with UV detectors (Lopez *et al*., 2002; Mustafa *et al*., 2003; Zhang *et al*.,1999) and mass spectrometric detectors (Ptak *et al*., 2009). Methods employing gas chromatography with nitrogen/phosphorus and flame ionisation detectors (Bastos *et al*., 1996), as well as mass spectrometric detectors (Berkov *et al*., 2008; Gotti *et al*., 2006; Ptak *et al*., 2009) have also been developed. A recent publication describes the use of high-performance thin-layer chromatography (HPTLC) for determination of galanthamine in plant extracts (Abou-Donia *et al*., 2008). Gotti *et al*. (2006) used non-aqueous capillary electrophoresis to determine galanthamine levels in plant material. While these methods are validated to be precise and accurate, and mostly use a small amount of plant material (50–500 mg) for analysis, they all have some disadvantages inherent to the methods used. The chromatographic methods are not always reproducible between laboratories and over time. Extensive sample clean-up, long run times and consumption of large amounts of solvents can make these methods expensive and time-consuming. In addition, all these methods require the use of an analytical standard for quantitation.

Raw plant materials used for medicinal products are often inconsistent in chemical composition (Khan, 2006). This can have implications for the efficacy and safety of crude medicinal products, or the consistency of starting material for extraction of pharmaceutical products or precursors. Plants contain many metabolites (potentially more than 3000 in a single plant) (Lay *et al*., 2006), and various factors are involved in determining the metabolic profile of a particular plant. Apart from its genetic profile, many biotic and abiotic factors can affect the metabolism of a plant, and thereby its metabolite composition. To understand how a plant responds to all these factors, methods are needed that can provide an overview of the full metabolic profile. Metabolomics is a promising approach to deal with quality control and other issues related to the agricultural production of medicinal plants. The metabolome can be defined as the observable chemical profile of metabolites in an organism (Verpoorte *et al*., 2008), and metabolomics is the qualitative and quantitative analysis of the metabolome. The large number of metabolites as well as the huge diversity in chemical structures make it difficult to detect and quantify all metabolites with a single analytical method. Several different approaches are currently used to analyse plant metabolites. The most common are mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy-based methods in combination with multivariate statistical methods (Lindon and Nicholson, 2008; Sumner *et al*., 2003).

^{1}H NMR metabolomics has been successfully used in the field of medicinal plant authentication and quality control (reviewed by Holmes *et al*., 2006). Crude plant extracts give rise to complex ^{1}H NMR signals. These spectra can either be analysed in full resolution or can be divided into compartments of specific spectral intervals in a process called 'binning' or 'bucketing' (Verpoorte *et al*., 2007). Many spectra can then be used for multivariate statistical analysis. Unsupervised methods such as cluster analysis and principal component analysis can group samples according to metabolic similarity, without any prior knowledge of samples. Differences between patterns of metabolites in samples can be recognised in an unbiased way. These methods can for example be applied to the authentication of plant species or cultivars (Choi *et al*., 2004; Frédérich *et al*., 2004) and the classification of plant material from different geographical locations (Tarachiwin *et al*., 2008; Wang *et al*., 2004).

One of the most important advantages of NMR as a metabolomics tool is the ease of quantitation, including the ease of quantitation of individual compounds in a crude mixture. Quantitative nuclear magnetic resonance (qNMR) has been used for the quantitation of natural products in various kinds of biological matrixes. A review by Pauli *et al*. (2005) covers more than two decades of literature on the topic, and some recent publications also use this method for plant secondary metabolites (Castilho *et al*., 2008; Li *et al*., 2009; Nazari *et al*., 2007; Rivero-Cruz *et al*., 2006; Tatsis *et al*., 2008). An advantage of qNMR over other analytical methods is elimination of the need to use analytical standards for quantitation. Also, it is a relatively inexpensive to run, fast and non-destructive method requiring only a small amount of plant material for analysis. Plant metabolites can be directly analysed in a mixture without the need for fractionation and isolation. Analysis of a target compound in a mixture also provides further advantages as other metabolites (primary metabolites, non-active marker compounds) can be analysed simultaneously (Rivero-Cruz *et al*., 2006) for applications such as plant identification and quality control.

In this study ^{1}H NMR-based metabolomics methods were used to investigate the chemical profiles of *Narcissus* bulbs. The aim was to investigate whether there are differences in the metabolic profile patterns of bulbs of the same species and cultivar grown in different locations, and, if so, to identify the metabolites responsible for the variation. Also, the ^{1}H NMR was applied to the quantitation of galanthamine, one of the most bioactive metabolites of *Narcissus* bulbs.

Experimental

Solvents and chemicals

Methanol-d_4 (99.80%) from Cambridge Isotope Laboratories (Andover, MA, USA) and the phosphate (KH_2PO_4) buffer (pH = 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France)

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containing 0.01% trimethylsilylproprionic acid sodium salt-d_9 (TMSP, w/w), as an internal quantitation reference standard and calibration of chemical shift, were used for ^{1}H NMR analysis. Acetonitrile (HPLC-S grade), trifluoroacetic acid (TFA, reagent grade >98%) and Chromasolv® water for LC-MS were obtained from Sigma Aldrich (St. Louis, MO, USA). Galanthamine hydrobromide was also obtained from Sigma Aldrich.

Plant material

Bulbs of *Narcissus pseudonarcissus* Cultivar 'Carlton' (Amaryllidaceae) were obtained from Holland Biodiversity B.V. (Lisse, the Netherlands). Bulbs were sourced from growers in two locations in the Netherlands (Lisse and Noordwijk) and one location in the UK (Lincolnshire). Plant materials were frozen in liquid nitrogen and ground with a Waring laboratory blender (Waring Products Inc., Torrington, CT, USA). Ground materials were freeze-dried for 48 h and kept at –20°C until analysis.

NMR apparatus and measurements

^{1}H NMR spectra were recorded using a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). Freeze-dried bulb material (50 mg) was transferred to a 2 mL microtube and vortexed at room temperature for 30 s with 1.5 mL of a mixture of the phosphate buffer and methanol-d_4 (1:1). Each sample was ultrasonicated for 30 min, and centrifuged at 13000 rpm for 10 min. An aliquot of 1 mL of the supernatant was collected for ^{1}H NMR analysis. For each sample, 64 scans were recorded with the following parameters: 0.167 Hz/point, pulse width (PW) = 4.0 µs and relaxation delay (RD) either short (1.5 s) or longer (5 s). FIDs were Fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied prior to integration of target regions for quantitative analysis.

Data analysis

^{1}H NMR spectra were automatically binned by AMIX software (v. 3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.32–10.0 was reduced to integrated regions of 0.04 ppm each. The regions of δ 4.7–5.0 and δ 3.30–3.34 were excluded from the analysis because of the residual water and methanol signal, respectively. Principal component analysis (PCA) was performed with SIMCA-P software (version 11.0, Umetrics, Umeå, Sweden) with Pareto scaling method. The original data of binned ^{1}H NMR are provided as Supporting Information (TableS. 1, sm001.xls).

HPLC measurements

HPLC analysis was done using a Waters 600E gradient controller pump and on-line Waters 991 PDA detector (Waters, Milford, MA, USA). The Vydac C18 low-TFA reversed-phase analytical column (5 µm particle size, 250 × 4.6 mm i.d.) was equipped with the guard column recommended by the manufacturer (Vydac, Hesperia, CA, USA). The previous method of Mustafa *et al.* (2003) was followed for HPLC analysis of galanthamine. Freeze-dried bulb material (200 mg) and 10 mL of 0.1% TFA (v/v) were added to a screw-top glass tube. After vortex mixing for 30 s, samples were ultrasonicated for 30 min and left overnight at 4°C. The following day samples were again placed in the ultrasonic bath for 30 min. A volume of 1.5 mL of sedimented supernatant was transferred to a 2 mL microtube, and centrifuged for 10 min at 13000 rpm. One millilitre of clear supernatant was collected for analysis, and 20 µL was injected for analysis. The HPLC mobile phase was 10% (v/v) acetonitrile in water containing 0.1% TFA, at a flow rate of 1.0 mL/min. Galanthamine was identified by comparison of its retention time and UV-spectra with galanthamine hydrobromide standard. Quantitative measurements were performed at a wavelength of 210 nm.

Extraction recovery

A known amount of galanthamine was added to 50 mg of ground and freeze-dried bulb material. This was done by adding precise volume of a 1 mg/mL galanthamine-HBr solution in methanol-d_4 to the plant material. After addition, the samples were dried under a stream of nitrogen to dry the solvent. Once dry, the samples were extracted for NMR analysis as described above.

Accuracy of method

Solutions of galanthamine–HBr were prepared in methanol-d_4–phosphate buffer (1:1), as described above, to give a range of galanthamine concentrations from 0.039 to 0.390 mg/mL (five different concentrations). ^{1}H NMR spectra of these solutions were recorded using two different relaxation times. Quantitative analysis was performed by integration of the area under the target signals. Calculated concentrations of galanthamine were compared with the actual concentration in solutions as weighed.

Results and Discussion

Alkaloid content is known to vary between different species of Narcissus (Bastida et al., 2006), but it is not known whether the same is true for the same species and cultivar grown in different geographical locations. In order to investigate the regional metabolic variation, bulbs of Narcissus pseudonarcissus cv. Carlton obtained from two locations in the Netherlands, and one location in UK were employed. These represent the most important regions for large-scale cultivation of Narcissus plants.

To investigate the metabolic profiles of the bulbs, a solvent was required for ^{1}H NMR analysis that would be efficient at extracting a wide range of primary and secondary metabolites. A 1:1 mixture of methanol-d_4 and KH_4PO_4 buffer in D_2O (pH 6) was chosen as this has been shown to be suitable for a wide range of metabolite extraction (Verpoorte *et al.*, 2007). Since no reports exist of galanthamine in this particular solvent, ^{1}H NMR measurements of an analytical standard were performed and resonances were assigned as indicated in Table 1. The original ^{1}H NMR, *J*-resolved, COSY, and HMBC spectra are shown in the Supporting Information, including Fig. S.1–4 (sm002.ppt, sm003.ppt, sm004.ppt, sm005.ppt, respectively).

Inspection of ^{1}H NMR spectra of bulb material extracted with the deuteriated solvent showed that some of the galanthamine resonances are detected in a non-crowded region without interference from other signals in the mixture (Fig. 2). To test the recovery of galanthamine with this extraction method, ^{1}H NMR measurements were taken of three bulb samples (in triplicate) before and after adding a known amount of galanthamine to the plant material. Galanthamine was quantified by integrating the area under the singlet methoxy peak (δ 3.89) and using the area relative to the known internal standard signal area to calculate the concentration in the sample. The average recovery was

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Table 1. ^{1}H NMR chemical shifts (δ)[a] and coupling constants (J) of galanthamine in the mixture of methanol-d_4 and KH_2PO_4 buffer in D_2O (1 : 1) (600 MHz)

Position	Chemical shift and coupling constant
H-1	4.72 (m)
H-2α	2.21 (ddd, J = 16.3, 5.3, 3.1)
H-2β	2.53 (dt, J = 16.3, 2.5)
H-3	4.28 (m)
H-4	6.07 (dd, J = 10.5, 5.0)
H-4α	6.17 (d, J = 10.5)
H-6	4.26 (dd, J = 15.2, 2.0)
H-6′	4.70 (d, J = 15.2)
H-7	6.89 (d, J = 8.5)
H-8	6.95 (d, J = 8.5)
H-11α	2.24 (dd, J = 15.5, 3.0)
H-11β	2.04 (dd, J = 15.5, 3.0)
H-12α	3.55 (dd, 13.5, 3.0)
H-12β	3.82 (td, J = 13.5, 1.0)
OMe	3.89 (s)
NMe	2.87 (s)

[a] Chemical shifts (ppm) were determined with reference to TMSP at δ 0.00.

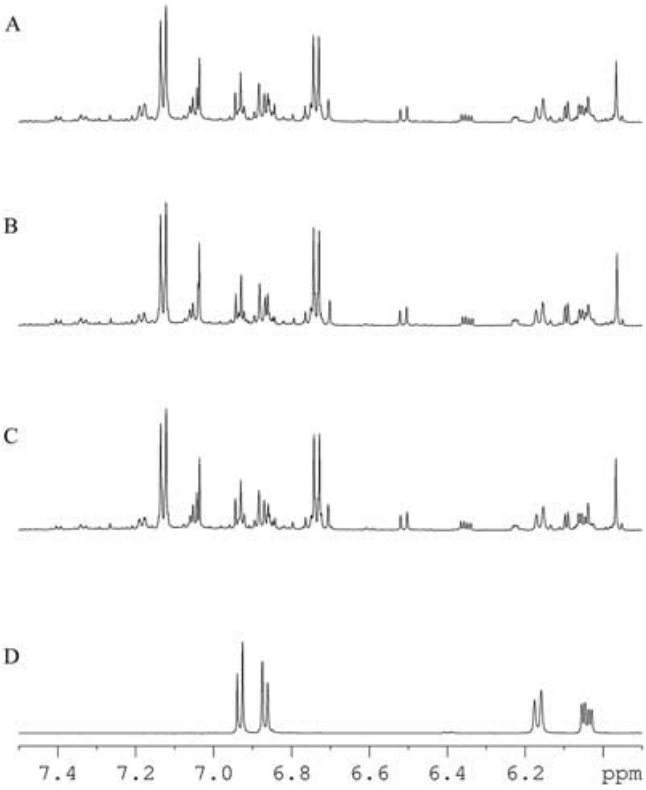

Figure 2. ^{1}H NMR spectra (600 MHz, methanol-d_4 and KH_2PO_4 buffer in D_2O, 1 : 1) of *Narcissus pseudonarcissus* bulb extracts obtained from Lisse in the Netherlands (A), Noordwijk in the Netherlands (B) and Lincolnshire in the UK (C) together with reference compound of galanthamine HBr (D) in the range of δ 5.9–7.5.

calculated as 85.4% with a relative standard deviation (RSD) of 9.1% (n = 9).

The bulb samples from Lisse and Noordwijk in the Netherlands and Lincolnshire in UK were analysed by ^{1}H NMR and the recorded spectra were analysed by PCA. Using this unsupervised multivariate data analysis technique, major principal components (PCs) did not clearly distinguish between different geographical regions, suggesting that the separation of the regional variation is smaller than biological variation. Slight separation could be observed between samples from different geographical locations only by minor principal components such as PC2 and PC4 (Fig. 3A). PC2 separated the samples from Lisse from the other two sample groups, and PC4 separated the Lincolnshire samples from the Netherlands groups.

Additionally, in order to examine the effect of NMR parameters on the PCA results, ^{1}H NMR spectra were measured at two different relaxation times, which is one of the most important parameters for quantitative features of ^{1}H NMR experiments (Pauli *et al.*, 2005). It was evaluated whether this parameter would influence the grouping of samples in PCA analysis. In the PCA results, the

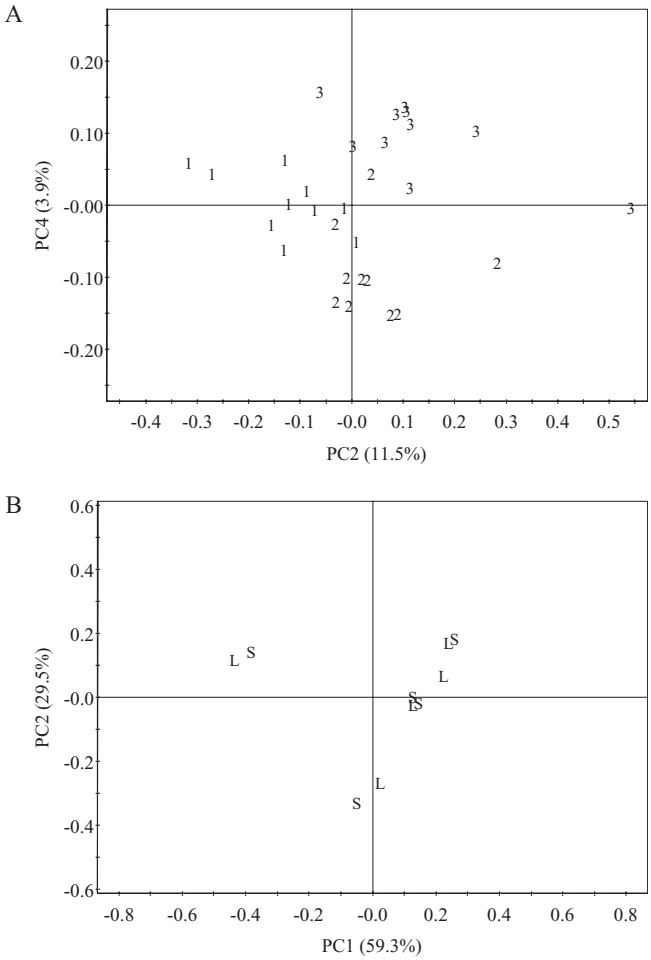

Figure 3. Score plot of principal component analysis using ^{1}H NMR spectra (PC2 vs PC4) for all the samples employed (A) and the samples obtained from Lisse, the Netherlands using two different relaxation times (B). **1**, *Narcissus pseudonarcissus* bulbs obtained from Lisse in the Netherlands; **2**, *Narcissus pseudonarcissus* bulb obtained from Noordwijk in the Netherlands; **3**, *Narcissus pseudonarcissus* bulb obtained from Lincolnshire in UK. S, short relaxation time (1.5 s); L, long relaxation time (5.0 s).

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difference between the spectra using different relaxation time was found to be less than the variation of the samples, that is, the variation between different groups was bigger than the variation within groups of samples measured with different relaxation times. To confirm this, PCA analysis was also conducted separately with each individual geographical group and no separation of samples were seen on the basis of relaxation time in any of the groups. In Fig. 3B two kinds of ^{1}H NMR spectra obtained from different relaxation times (1.5 and 5 s) are not separated at all.

Even though there are only minor metabolic differences between the samples employed in this study, the metabolites associated with regional variation were elucidated by loading plot. The ^{1}H NMR signals with the most influence were all found to be in the range of δ 3.2–3.9, which is the region of the spectra mostly crowded with primary metabolites such as amino acids and sugars. Interestingly, some aromatic resonances, including δ 6.84, 7.12, 6.52 and 6.32, together with galanthamine resonances were clearly related to the regional variation of the *Narcissus* samples. The original ^{1}H NMR spectra were inspected to elucidate the resonances. The data bucket including δ 6.84 contains galanthamine signals, as shown earlier with the pure compound. The doublet signals at δ 7.13 (J = 8.4 Hz) and δ 6.74 (J = 8.4 Hz) were assigned to the phenolic ethylamine tyramine. For the signals at δ 6.51 (J = 10.2 Hz) and δ 6.32 (J = 10.2, 5.4 Hz), the splitting pattern and two-dimensional correlation were similar to the signals of H-4 and H-4a of galanthamine (at δ 6.17 and 6.07), but are both more deshielded. Haemanthamine (Figure 1) is the second most abundant alkaloid in the *Narcissus* cultivar Carlton (Gotti *et al*., 2006). ^{1}H NMR spectra of haemanthamine have been reported (Bastida *et al*., 1987; Pabuccuoglu *et al*., 1989). Different NMR solvents were used in those studies so the chemical shifts and coupling constants are slightly different than in this study. However, the signal patterns are the same, and together with the fact that this is the second most abundant alkaloid in this cultivar of *Narcissus*, the above-mentioned signals most likely belong to H-1 and H-2 of this alkaloid. The compounds responsible for the slight separation seen between the Lisse samples and the others were galanthamine and tyramine. These compound signals were negatively correlated so that samples with more galanthamine contained less tyramine. Since tyramine is one of the biosynthetic precursors of galanthamine (Bastida *et al*., 2006) this is not an unexpected finding. Galanthamine content was also observed to be negatively correlated to haemanthamine content.

Little variation was seen in the overall pattern of metabolites between samples from different geographical locations. However, it seemed that galanthamine levels were able to discriminate between samples together with other metabolites. The same ^{1}H NMR spectra used for the PCA analyses were used for the quantitative determination of galanthamine in the bulb extracts. One of the galanthamine resonances at δ 6.17 (doublet, J = 10.15 Hz) was chosen as the target peak for quantitative analysis. Usually singlets are preferred as target peaks for quantitative NMR experiments, but since the methoxy singlet signal of galanthamine used previously occurred in a crowded region of the spectra, it was not suitable for this purpose. Figure 2 shows the region of the ^{1}H NMR spectra of three bulb samples containing the target galanthamine peak.

In the previous report Pauli *et al*. (2005) describe the importance of optimising the relaxation delay for qNMR experiments, as insufficient relaxation delay can result in inaccurate quantitation. To see how relaxation time affects quantitation of galanthamine, an analytical standard was dissolved in methanol-d_4 and KH_2PO_4 buffer (1 : 1) at a range of concentrations for qNMR analysis. Final concentrations of galanthamine in the samples were from 0.039 to 0.390 mg/mL. Two measurements were taken at different values of d1 (relaxation delay). The ratio of the area under the target signal (6.145–6.185 ppm) relative to the area under the internal standard peak (TMSP, −0.11 to 0.11 ppm) was used to calculate the concentration of galanthamine. Table 2 shows the difference between the two measurements, and the longer delay time gave results closer to the actual weighed amount of galanthamine. Using the short relaxation delay, the results were found to be overestimated. It might be due to the fact that the internal standard, TMSP, is not fully relaxed with short relaxation delay. The effect of relaxation delay was not detected in the PCA results because PC scores are a relative value, but the absolute quantitation results were largely affected by this parameter.

Galanthamine was quantified in bulb samples from Lisse and Noordwijk in the Netherlands and from Lincolnshire in the UK based on four or five replicates. A single ^{1}H NMR measurement was taken of each sample using the longer relaxation delay. The same bulbs samples were also extracted in triplicate and analysed using the HPLC method of Mustafa *et al*. (2003). The results are shown in Table 3, where the galanthamine levels of the samples are quite similar to those determined by HPLC. To assess whether there was any statistically significant difference between these results, a paired Student's *t*-test was performed comparing the ^{1}H NMR results with the HPLC results. A *p*-value of 0.066 meant there was no statistically significant difference between the results ($p > 0.05$). Both in ^{1}H NMR and HPLC results, the samples obtained from Noordwijk, the Netherlands showed the lowest level of galanthamine when compared with other samples. This is in accordance with the results obtained with PCA.

^{1}H NMR measurements together with multivariate data analysis were used to obtain a global view of metabolite profiles of *N. pseudonarcissus* cv. Carlton bulbs grown in different

Table 2. Galanthamine content of standard solutions quantified by ^{1}H NMR, using two different relaxation delays (d1), and percentage difference (±) between weighed and calculated galanthamine amount

Galanthamine (mg)	Calculated galanthamine (mg) by d1 = 1.5 s	Percentage difference	Calculated galanthamine (mg) by d1 = 5 s	Percentage difference
0.390	0.495	26.922	0.382	1.92
0.234	0.292	24.999	0.232	0.64
0.156	0.195	24.999	0.150	3.85
0.078	0.090	15.383	0.075	3.85
0.039	0.045	15.383	0.037	3.85

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Table 3. Galanthamine per dry weight of bulb samples determined by ^{1}H NMR and HPLC

Sample	^{1}H NMR galanthamine (mg/g ± SD), $n = 5$	HPLC galanthamine (mg/g ± SD), $n = 9$
Noordwijk	2.36 ± 0.13	2.23 ± 0.05
Lisse	3.32 ± 0.22	3.13 ± 0.28
Lincolnshire	3.28 ± 0.26	2.98 ± 0.27

geographical locations. The results showed that there were no big differences between the overall metabolic profiles. Samples grown in different locations could be slightly distinguished on the basis of galanthamine content. ^{1}H NMR was used to quantify galanthamine in *Narcissus* bulb material, and was shown to be an accurate and precise method. Compared with other methods, sample preparation is simple and rapid, and only a small amount of plant material is required. A further advantage is the possibility of detecting and quantifying other metabolites besides the target alkaloid, such as primary metabolite precursors or other alkaloids. Quantifying haemanthamine in plant material usually requires analytical standards (Bastos *et al*., 1996; Lopez *et al*., 2002) and decomposition of the alkaloid may occur in some methods (Gotti *et al*., 2006; Kreh *et al*., 1995). This ^{1}H NMR method can potentially quantify the second most abundant alkaloid, haemanthamine, using small amounts of plant material and requiring no analytical standards. This method can provide useful qualitative and quantitative information about the metabolic state of *Narcissus* bulbs. The same ^{1}H NMR spectra can be used for quantitation as well as for use in multivariate data analysis such as PCA. The method may be useful for agricultural applications, and for quality control of raw material used in the pharmaceutical industry.

Supporting information

Supporting information can be found in the online version of this article.

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