Factors affecting galanthamine production in Narcissus

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Chapter VII

Effect of Bulb Age on Alkaloid Contents of *Narcissus pseudonarcissus* Bulbs

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Abstract

Narcissus is mostly grown for horticultural/decorative purposes in the fields and home gardens. Previously, galanthamine isolation (from the bulbs to produce medicine for the treatment of Alzheimer's disease) has been established as a novel use. Moreover, recent studies have also shown that *Narcissus* bulbs are also a source of several other interesting biologically active compounds and particularly some alkaloids. Various metabolites are produced at different time points during the growth cycle of plants. Also, for *Narcissus* bulbs it is important to find from an economical point of view the optimum time point for harvesting in its growth cycle, as the levels of galanthamine might be correlated with the age of the *Narcissus* bulbs. The aim of this study was to investigate the differences in the metabolites and specifically in the alkaloid contents based on a 1, 2- or 3-years harvesting cycle of *Narcissus* bulbs. Moreover, the effect of the cultivation site was studied for *Narcissus pseudonarcissus* cv Carlton which is well known for galanthamine production. Different methods were used for the analysis of these alkaloids. Untargeted NMR based metabolomics was used for overall metabolic changes and galanthamine quantitation. Whereas targeted GC-FID and GC-MS were used respectively for quantitative and qualitative analysis of major and minor alkaloids. An alkaloid specific acid-base extraction method was used in the GC-MS and GC–FID analysis. The results show that the contents of the metabolites vary significantly with the age of the bulbs. The contents of galanthamine were highest in two years old bulbs. It was also found that the alkaloid contents from the same variety grown at different locations differ from each other. Results presented here clearly indicate that] age of the bulb are important factors to consider for

the industrial production of certain alkaloids from the plants as yields of alkaloids may vary, certain conditions may lead to an increase of the yield with 10-15%.

Keywords: *Narcissus*; Galanthamine; Haemanthamine; Homolycorine; Lycorenine; Tazettine; Metabolite variation.

Introduction

The *Amaryllidaceae* is one of the families from the monocotyl order Asparagales comprising about 870 species from around 50 genera of herbaceous and bulbous plants. *Narcissus* is one of the few bulbous spring flowering plants from this family. *Narcissus* is mostly grown for horticultural/decorative purposes. But recently there is a growing interest in this plant due to the medicinal use of one of the major alkaloids of this genus: galanthamine. Moreover recent studies have shown that a few other alkaloids also possess some interesting biological activities such as antimalarial [1], antiviral, antitumor [2] antifungal [3, 4] and insect anti-feedant activity [5]. Galanthamine was first isolated by Proskurina and Yakovleva in 1952. It is the main alkaloid of many *Narcissus* species. Galanthamine is a long-acting, selective, reversible and competitive acetylcholinesterase (AChE) inhibitor, which is marketed in the form of its hydrobromide salt [6]. Due to its AChE inhibitory activity, galanthamine is used to treat the patients with mild to moderate Alzheimer's disease (AD). Alzheimer's disease (AD) is a disorder associated with progressive neurodegeneration and is the most common cause of dementia. It is estimated that the amount of people affected by dementia would be more than 40 million till the end of 2020 [7]. While there is no cure or preventive medicine for this disease, galanthamine is used as a symptomatic treatment of this disease which in turn helps in improving the patient's quality of life. Studies have also shown that galanthamine can act as a mild analeptic agent, having an analgesic power as strong as morphine. It is also reported, that galanthamine can help in relieving a jet lag. Alongside with these activities, it works against fatigue syndrome, male impotence, and alcohol dependence. It also reduces the intraocular pressure when applied in eye drops [8, 9]. Because of its use as medicine, great interest in *Narcissus* species and varieties has risen as an industrial source for the production of galanthamine.

Besides galanthamine, there are a number of other alkaloids found in *Narcissus* such as lycorine, lycorenine, homolycorine, haemanthamine, narciclasine and tazettine to name a few. Lycorenine can be found in plants of the Amaryllidaceae family. This alkaloid exhibits a variety of biological properties, such as antifungal- [10] anti-parasitic- [11], and anti-inflammatory activity [12]. Other than these beneficial uses, lycorenine is also suspected to cause poisoning of humans and animals [13, 14]. Among the other alkaloids

of *Narcissus,* homolycorine is structurally quite similar to lycorine but it has different biological activities. It originates from an *ortho-para* oxidative phenol coupling. It is one of the most ubiquitous alkaloids throughout the whole growing cycle of the plant. This alkaloid shows some cytotoxic effects against non-tumoral fibroblastic LMTK cells [15] and also moderately inhibits the growth of many types of tumor cells in *in-vivo* and *in-vitro* cultures [16, 17]. Additionally, it induces some delayed hypersensitivity in animals [18] and exhibits high antiretroviral activity [19].

Haemanthamine is another alkaloid from *Narcissus* which is present in all plant organs except flowers. Haemanthamine belongs to the crinine-type of alkaloids and it originates from a *para-para* oxidative phenol coupling. It was previously reported that plants enzymatically convert haemanthamine to haemanthidine in an irreversible manner. It has a hypersensitive and cytotoxic activity against a variety of *in-vitro* cultured cells. It also shows cell growth inhibitory activities [20, 21] and antimalarial activity [22].

Tazettine is a mildly active alkaloid against certain tumor cell lines with only a slight cytotoxicity when tested on fibroblastic LMTK cell lines. Tazettine also displays weak hypotensive and antimalarial activities and interacts with DNA. While it's chemically labile precursor, pretazettine is far more interesting due to its antiviral and anticancer activities [11]. All these alkaloids can be found in most of the Amaryllidaceae plants with homolycorine as the least common.

These alkaloids are present in *Narcissus* at almost all stages of their growth in almost all the parts although the amounts vary during the season. Brieterova et al., [23] evaluated 40 taxa for their alkaloid profile and to find out the best sources of galanthamine, haemanthamine and lycorine for industrial scale production. From the evaluated 40 taxa, they found out that Sundisc (galanthamine), Jenny (lycorine), and Sealing (haemanthamine) are best taxa for industrial scale cultivation. Different factors affect the amount of alkaloids present in the *Narcissus* plants. Among those factors, age in years at harvesting time and cultivation site are relatively important for the yield of bulbs. Different approaches are used for the bulb production in different countries. In The Netherlands bulbs are lifted annually while in the United Kingdom (UK) they are lifted biannually where half the bulbs are left in the field to decrease production costs of the

bulbs. However, in terms of alkaloid production there is no insight in what the best time is for harvesting in a three years growth cycle. Neither what is the best cultivation site alkaloid production. Though there is some information available regarding the effect of environment of the first flowering of Narcissus. Bock et al., [24] tested the effect of environment on the first flowering of 26 Narcissus cultivars over a 27-year time period. They found out that temperature play a major role in the first flowering and senescence of most cultivars. Though there is not any information available on the effect of environment on bulb yield and metabolite production.

Narcissus is naturally a slow propagated plant with propagation rate of 1.6 fold per annum [25] which is consistent with the Fibonacci sequence. During propagation small new bulbs (bulblets) are formed on the outer-side of the older bulbs. These new bulbs initiated from the older mother bulb are called daughter bulbs. After initiation of these daughter bulbs, there is a rapid growth period of three to four months during spring-summer and a slow growth period in the following autumn-winter. Two years after the daughter bulb initiation, the bulbs reach their peak in dry weight [22]. In the following year, there is some weight loss in the mother bulb as again new daughter bulbs are formed. Although the maximum dry weight is obtained after two years, it would be important to find out the time point of maximum yield of alkaloids.

Other than the age of the bulb, soil is also important for bulb and alkaloid production. The Netherlands and United Kingdom (UK) are the largest producers of *Narcissus* bulbs as about 70% of global bulb production comes from these two countries. Although these bulbs are grown in different parts of these countries, particularly Lincolnshire (UK) and Lisse (The Netherland) are the places which produce most of the bulbs. The environmental conditions of these two places are quite similar, though the soil of these places is different. The soil in Lincolnshire (UK) is sandy and loamy while in The Netherlands it is mostly sandy. Moreover different types of crop rotations are used in these two countries. These factors can affect the nutrients present at each location which in turn can affect the production of bulbs in total. Thus it is important to learn more about alkaloid levels in bulbs grown on such different localities. Various methods are used for the determination of alkaloids in the *Narcissus*. Enzyme immunoassays or radioimmunoassay were used for the quantitative determination of galanthamine in

unpurified plant extracts, [26, 27]. Chromatographic methods include the use of high-performance liquid chromatography with UV detector [28-30]. Abou-Donia et al., [31] described the use of high-performance thin-layer chromatography (HPTLC) for determination of galanthamine in plant extracts. Lubbe *et al.,* [32] used ^{1}H-NMR spectroscopy for the determination of galanthamine content in *Narcissus* bulbs. Berkov with coworkers [33] developed a rapid method for the determination of galanthamine in *Leucojum aestivum* and *Narcissus ssp* with the help of GC-MS. Nevertheless, there are a few drawbacks of this method such as lack of information about the unstable compounds and low stability of some compounds due to thermal degradation but on the other hand it is very effective in identification and quantification of multiple compounds at the same time.

The aim of the present study was to investigate the effect of different growth cycles (1, 2 and 3 years) and of the site of cultivation on the alkaloid content and the metabolic profiles of bulbs of the same species and cultivar.

Experimental

Chemicals and reagents

Chemicals for extraction such as MeOH (HPLC Grade), EtOAc, Sulfuric acid, and aqueous ammonia (Analytical grade 25% v/v) were purchased from Biosolve (Valkenswaard, The Netherlands) while BSTFA was purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). Reference compounds of haemanthamine, homolycorine, lycorenine and tazettine were generously provided by Dr. Bastida from the department of Natural Products, Plant Biology and Soil Science, Faculty of Pharmacy, University of Barcelona, Spain. The standard compound for Galanthamine hydrobromide was purchased from Sigma-Aldrich (Steinheim, Germany).

For the ^{1}H-NMR analysis CH3OH-d4 (99.80%) from Cambridge Isotope Laboratories (Andover, MA, USA), and phosphate (KH2PO4) buffer (pH 6.0) in (D2O) deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France) containing 0.01% trimethylsilylproprionic acid sodium salt-d4 (TMSP, w/w) as an internal standard for quantitation and calibration of the chemical shifts.

Plant Material

Bulbs of *Narcissus pseudonarcissus* Cultivar 'Carlton' (Amaryllidaceae) were obtained from Holland Biodiversity B.V. (Lisse, The Netherlands). Bulbs were collected from growers in two locations in The Netherlands (Lisse and Noordwijk) and two locations in the UK (Lincolnshire). To measure the effect of age (1, 2 and 3 years old), all bulbs were collected from Lisse. To measure the mother and daughter bulbs, three year old bulbs were harvested. To determine the location effect, two year old bulbs were collected from the above mentioned four locations. Replicates represent different bulbs.

Metabolite Extraction

The metabolites were extracted following the previous method of Torras-Claveria *et al*. [34] with slight modifications. The *Narcissus* bulbs were frozen in liquid nitrogen and ground with a Waring laboratory blender (Waring Products Inc., Torrington, CT, USA). The ground plant materials were freeze-dried for 48 hours. Samples were macerated in methanol for 72 hours, filtered and the solvent was evaporated under vacuum. Dried methanolic extract (100 mg) was derivatized by adding 50 µL of pyridine and 50 µL of BSTFA. Samples were then kept at 40 ˚C for 90 minutes. Then the samples were analyzed by GC-MS. Replicates were from different bulbs.

For alkaloid extraction, 200 mg of the dry residue was dissolved in 2% (v/v) sulfuric acid (3 mL). The neutral compounds were removed with diethyl ether by 3 times extraction with 5 mL each. The solution was basified with 25% aqueous ammonia to pH 9-10. The alkaloids were extracted with ethyl acetate 3 times (5 mL each). Ethyl acetate was evaporated from the extract and the dried residue was dissolved in 1 mL of methanol for GC- FID analysis (5 replicates of each sample).

For NMR analysis, 60 mg of freeze-dried plant material was transferred to a 2-mL microtube and vortexed at room temperature for 1 minute with 1.5 mL of a mixture of KH2PO4 buffer (pH 6.0) in D2O containing 0.05% trimethyl-silyl-propionic acid sodium salt (w/w) (TMSP) and methanol-d4 (1:1). The samples were then sonicated for 20 min and centrifuged at 13,000 rpm for 10 minutes. An aliquot of 800 µL of the supernatant

was used for NMR analysis. ^{1}H-NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany).

GC- FID

Quantitative analysis was performed on an Agilent GC 6890 series equipped with a 7683 autosampler, a flame ionization detector (FID) (Agilent Technologies Inc., Santa Clara, CA, USA) and a DB-5 column (30 m length, 0.25 mm internal diameter, film thickness of 0.25 µm) (J&W Scientific Inc., Folsom, CA, USA). The temperature of the injector was set to 250 ºC, a split ratio of 1:20 and a carrier gas (N_2) flow rate of 1 ml/min. The temperature of the oven was programmed at 300 ºC. The initial temperature began at 100 ºC for 1 minute, increased from 100 ºC to 180 ºC at 15 ºC/min, 180 ºC to 300 ºC at 5 ºC/min and held at 300 ºC for 10 minutes. The FID detector temperature was set to 300 ºC (5 replicates representing different bulbs).

GC–MS

Qualitative analysis was performed on an Agilent 7890A series gas chromatograph equipped with a 7693 autosampler, a DB-5 column (30 m length, 0.25 mm internal diameter, film thickness 0.25 µm, Agilent Technologies Inc., Santa Clara, CA, USA) and a single quadrupole mass spectrometer 5975C. The MS source was set to 250 ˚C, the single quad temperature was 150 ˚C, and the transfer line temperature was set to 280 ˚C. The GC-column was linked to the MS via a quick swap (Agilent Technologies Inc., Santa Clara, California, USA) and restrictor (0.11 mm internal diameter, Agilent Technologies, Santa Clara, USA). The injector temperature was 230 ˚C with an injection volume of 1 µl, a split ratio of 1:20 and a carrier gas (He) flow rate of 1 ml/min. The oven temperature program was the same as the GC–FID. The mass range analyzed by the mass spectrometer was 50–500 amu. The GC–MS was controlled by Enhanced Chemstation software version E.02.00.493 (Agilent Technologies Inc., Santa Clara, California, USA). The NIST library version 2.0 (Standard Reference Data Program of the National Institute of Standards and Technology, Distributed by Agilent Technologies) was used to assist compound identification.

Data processing and analysis

^{1}H-NMR spectra were Fourier transformed (LB = 0.3 Hz). Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to δ 0.0. For quantitative analysis of galanthamine, integration of the proton signal at δ 6.17 (galanthamine H-4a) was performed. The ratio of this integral to that of the internal standard was used to calculate the amount of galanthamine per mg material. For multivariate 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 δ 4.7-5.0 and δ 3.30-3.34 were excluded from the analysis because of the presence of the residual water and methanol signal, respectively. Principal component analysis (PCA) was performed with SIMCA-P software (version 12.0 Umetrics, Umeå, Sweden) with Pareto scaling method. Analysis of variance (ANOVA) followed by Pairwise Multiple Comparison (Tukey Test) was performed to compare average galanthamine levels between treatments using SPSS (version 18, PASW Statistics, Chicago, IL, USA).

Results and Discussion

Plant extracts were analyzed by NMR to identify possible age dependent differences for the metabolites present. Visual inspection of the spectra (**Figure 7.1**) of bulbs of different ages shows marked differences in the signal intensities of various metabolites in the *Narcissus*. Important signals for distinguishing the bulbs of different ages were assigned to different metabolites (**Table 7.1**) by comparison with previous studies as well as with the in-house library of pure compounds analyzed under the same conditions.

To confirm the differences observed in metabolites in relation to the age of the bulb, ^{1}H-NMR spectra of all the samples were subjected to principal component analysis (PCA). The aim of the PCA analysis was primarily to identify the main areas from the bucket tables responsible for the differences in plant samples while reducing the complexity of the data. An eight-component model explained 94% of the variance in the samples.The first two components accounted for about 70% of the variance. As it is evident from the score plot of the PCA presented in **Figure 7.2A**, there is a clear separation between samples based on the age of the bulbs. A loadings scatter plot of PCA (**Figure 7.2B**) shows how different areas of the *Narcissus* bulbs spectra contribute to the variation and

grouping of the samples. Signals from these areas were identified and assigned to different compounds based on the previous reports and an in-house library.

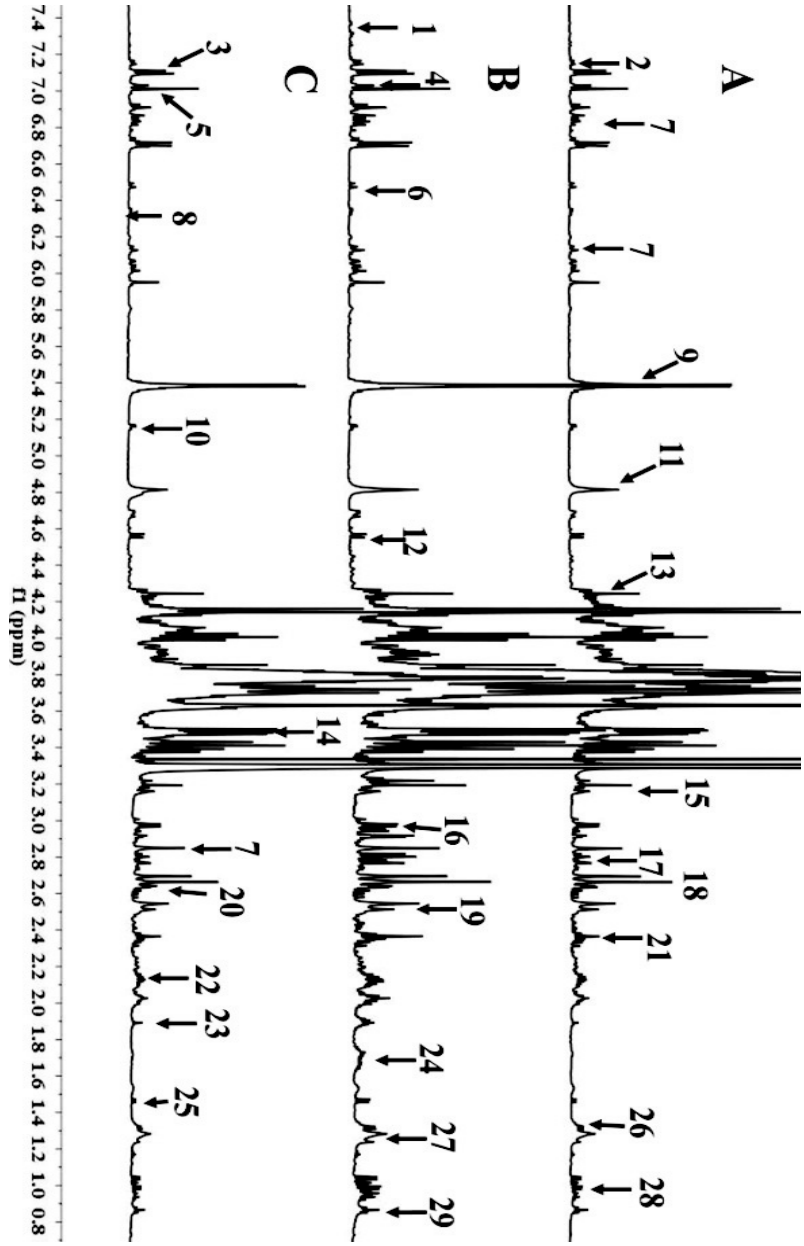


Figure 7.1. ^{1}H-NMR spectra (500 MHz) of phosphate buffer (pH 6.0) - MeOD (1:1) extracts of *Narcissus pseudonarcissus* Cultivar 'Carlton' bulbs A) one-year old bulbs, B)

two years old bulbs and C) three years old bulbs without the smaller out growing daughter bulbs.

Table 7.1. Chemical shifts (δ) and coupling constants (Hz) of identified metabolites in NMR of phosphate buffer (pH 6.0) - MeOD (1:1) extracts of *Narcissus*. Numbers correlate to signals in Figure 7.1

No.	**Metabolite**	**Abbreviations**	**Chemical shift (δ) and coupling constant (Hz)**
1	Phenylalanine	PHE	7.42-7.33 (m), 3.09 (dd) J=14.8, 8.3.
2	Tyrosine	TYR	7.18 (d) J=8.4, 6.85 (d) J=8.4.
3	4-hydroxyphenylpyruvate	4-HPP	7.13 (d) J=8.4, 6.73 (d) J=8.4, 3.02 (d) J=13.6, 2.98 (d) J=13.6.
4	Lycorenine	LYC	7.06 (s), 7.04 (s), 6.02 (s), 5.74 (brs).
5	Haemanthamine	HAE	7.06 (s), 6.71 (s), 6.51 (d) J=10.3, 6.36 (dd) J=10.3, 5.0, 5.97 (brs).
6	*cis*-aconitic acid	*cis*-AA	7.03 (s).
7	Galanthamine	GAL	6.94 (d) J=8.3, 6.88 (d) J=8.3, 6.16 (d) J=10.5, 6.1 (dd) J= 10.5, 5.0, 2.86 (s).

8	Narciclasine	NARCI	6.73 (s), 6.22 (m), 6.09 (dd) J=4.5, 5.56 (d) J=3.8, 5.27 (d) J=3.8, 4.39 (m), 4.32 (m).
9	Sucrose	SUC	5.41 (d) J=3.8, 4.17 (d) J=8.7, 4.03 (t) J=8.3, 3.78-3.83 (m), 3.75 (t) J=9.5, 3.66 (s), 3.51 (dd) J=9.9.
10	Maltose	MAL	5.14 (d) J=1.5.
11	Rhamnose	RHA	5.11 (d) J=1.5, 4.87 (d) J=0.7, 1.28 (d) J=4.9, 1.26 (d) J=6.3.
12	Glucose	GLC	5.19 (d) J=3.8, 4.58 (d) J=7.9, 3.20 (dd) J=8.9, 8.8.
13	Raffinose	RAF	5.41 (d) J=3.8, 5.27 (d) J=3.8.
14	Mannose	MAN	5.40 (d) J=3.9, 5.17 (d) J=3.8.
15	Choline	CHO	3.21 (s)
16	Aspartic acid	AspA	2.82 (dd) 17.0, 8.5, 2.63 (dd) J=17.0, 9.5.
17	Asparagine	ASP	3.94 (dd) J=8.0, 4.0, 2.95 (dd) J=17.0, 3.8,

			2.81 (dd) J=17.0, 8.23.
18	Malic Acid	MA	2.68 (dd) J=15.7, 3.4, 2.36 (dd) J=15.7, 10.4, 4.28 (dd) J=10.4, 3.2.
19	Citric acid	CA	2.71 (d) J=15.8, 2.56 (d) J=15.8.
20	Glutamic acid	GA	2.39 (td) J=7.1, 2.5, 2.10-1.28 (m).
21	Ornithine	ORT	3.71 (t) J=5.8, 3.24 (t) J=, 1.92 (m), 1.65-1.78 (m).
22	Acetic Acid	AA	1.91 (s).
23	Arginine	ARG	7.21 (s), 6.68 (s), 3.76 (dd) J=6.1, 5.9, 3.22-3.25 (m), 1.88-1.92 (m), 1.72-1.76 (m), 1.63-1.68 (m).
24	Alanine	ALA	1.49 (d) J=7.2.
25	Threonine	THR	4.22 (m), 1.34 (d) J=6.6.
26	Fatty acid	FA	1.31 (brs), 0.89 (t) J=7.1.

27	Valine	VAL	1.06 (d) J=7.04, 1.01 (d) J=7.04.

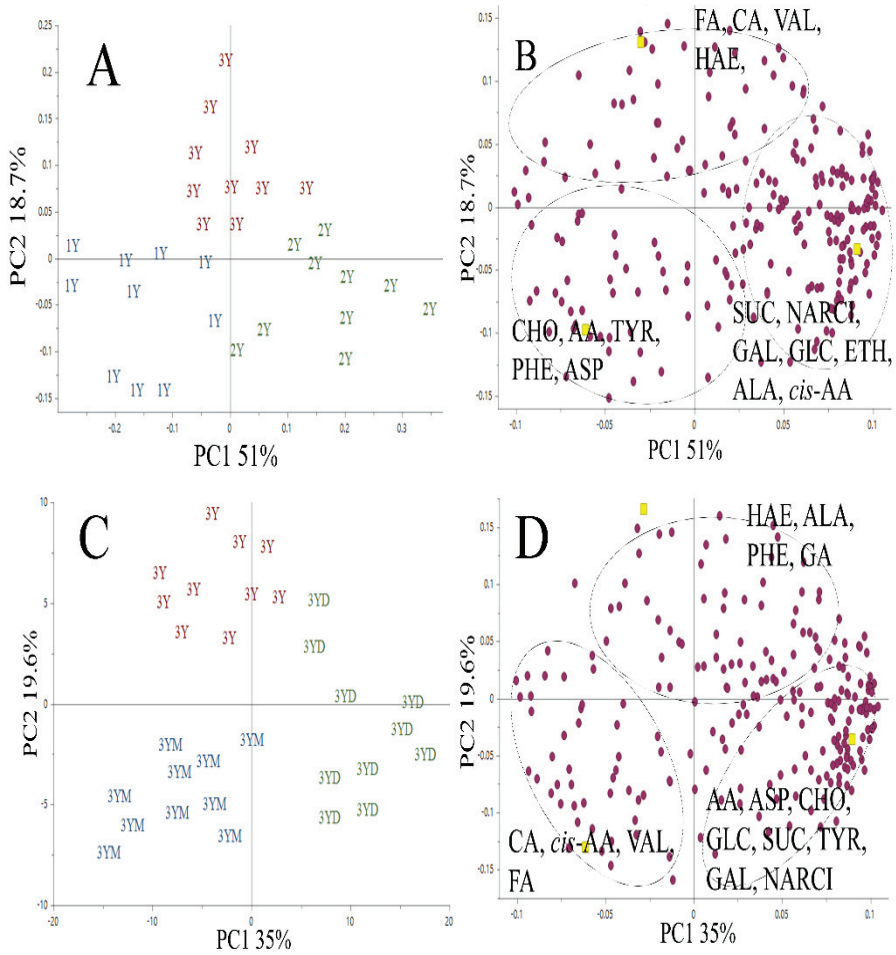


Figure 7.2. A) Score plot of PCA (PC1 vs PC2) of the ^{1}H-NMR-spectra of *Narcissus pseudonarcissus* Cultivar 'Carlton' bulbs (Figure 7.1) where numbers represent the age of the bulb.

B) Loadings scatter plot of PCA (PC1 vs PC2) of *Narcissus* bulbs. Compound abbreviations as in Table 7.1.

C) Score plot of PLS-DA for three classes: 3YD daughter bulbs separated from mother bulbs; 3YM mother bulbs, 3Y mother bulbs with daughter bulbs still attached.

D) Loadings scatter plot of PLS-DA (●) Loadings (■) average of a classes. PLS-DA models were validated by using permutation test (R^2 = 0.82, Q^2 = 0.70 with 120 permutations and 8 components)

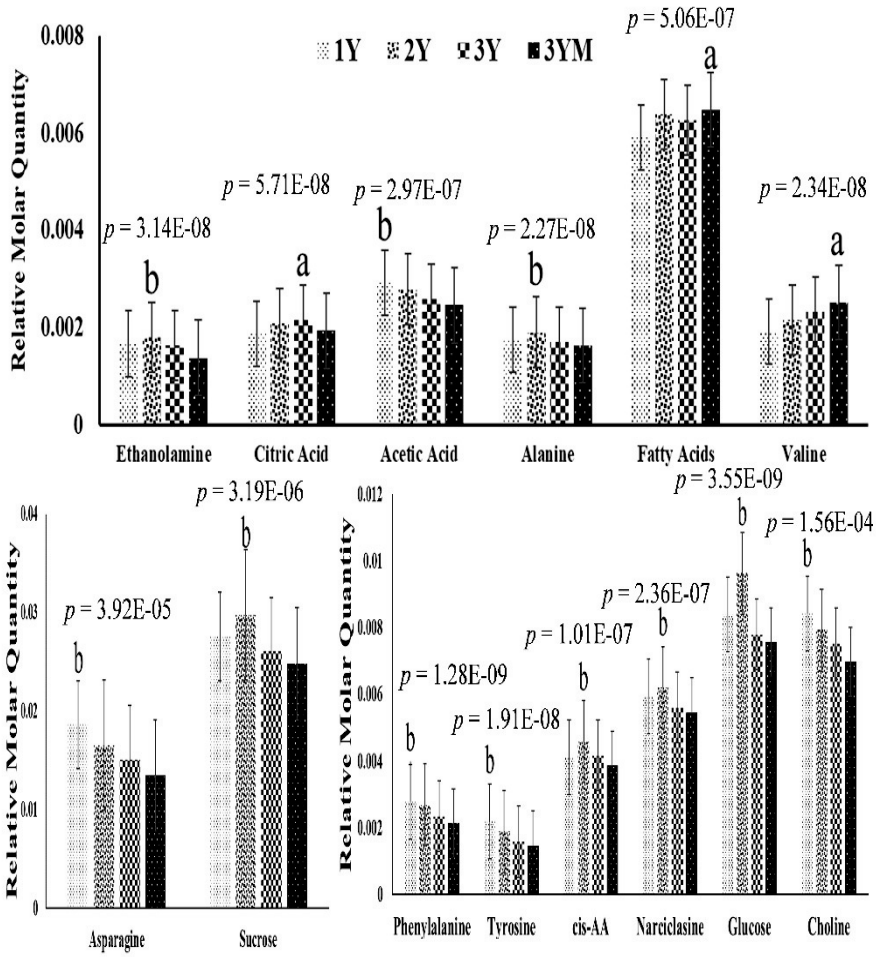


Figure 7.3. Relative quantitation of some compounds in different *Narcissus pseudonarcissus* Cultivar 'Carlton' bulbs calculated from the 500 MHz ^{1}H-NMR spectra. Chemical shifts of signals used were valine: 1.06, fatty acids: 1.31, alanine: 1.49, acetic acid: 1.91, citric acid: 2.56, ethanolamine: 3.12, choline: 3.20, asparagine: 3.91, glucose: 5.20, sucrose: 5.40, narciclasine: 6.72, cis-aconitic acid: 7.04, tyrosine: 7.20, phenylalanine: 7.36. 1Y) one-year old bulbs; 2Y) two-year old bulbs; 3Y) three-year old bulbs with daughter bulblet attached; 3YM) three-year old bulbs without daughter bulblets. One-way ANOVA along with HSD test was used to check the differences in the level of the compound between the samples (5 biological replicates, significance is shown at $P < .05$, error bars represent standard error (SEM). Different letters show

the significant comparison such as (a) denotes significant difference when compared with one-year and two-year old bulbs and (b) denotes significant difference when compared with three-year or three-year mother bulbs.

Table 7.2. Molecular ion and fragment ions of the alkaloids of *Narcissus pseudonarcissus* Cultivar 'Carlton' with retention time from GC-MS

Alkaloids	**RT (min)**	**Molecular ion [M+] (%)**	**Ion fragmentation pattern, m/z (% abondance)**
Galanthamine	16.01	287 (85)	286 (100), 268 (12), 244 (25), 216 (30), 174 (28), 115 (15)
Haemanthamine	19.30	301 (20)	272 (100), 240 (20), 211 (18), 181 (25), 128 (18)
Homolycorine	21.21	315 (0.5)	109 (100), 108 (22), 206 (7.7), 178 (2), 94 (2), 82 (2.5), 150 (1),
Lycorenine	18.93	317 (<1)	299 (6), 191 (1), 179 (1), 109(100), 94(3),
Tazettine	19.6	331 (28)	247 (100), 70 (22), 298 (22), 115 (19), 201 (18), 181 (14), 152 (12.5), 316 (12), 230 (12), 281 (12)

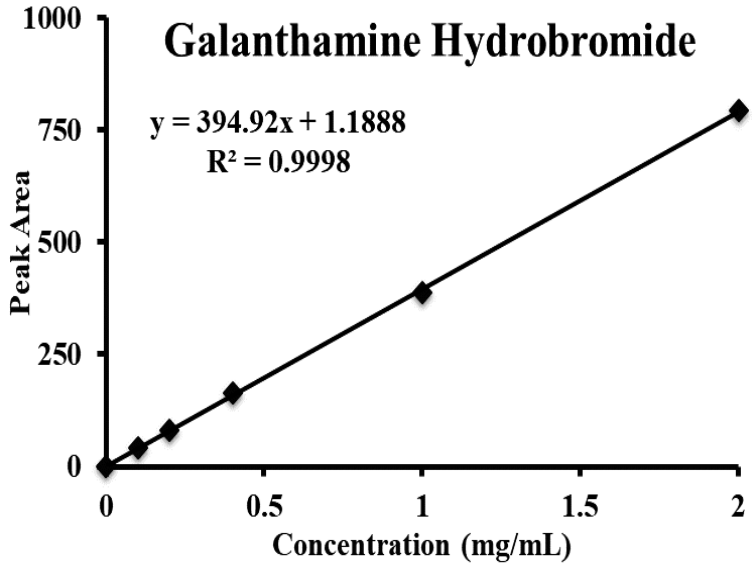


Figure 7.4. A standard curve for galanthamine hydrobromide on GC-FID obtained with a dilution series where each measuring point is based on three replicates.

From these identified signals it is clear that one year old bulbs have higher amounts of choline, acetic acid, asparagine, phenylalanine and tyrosine while two year old bulbs have higher amounts of galanthamine, sucrose, glucose, ethanolamine, *cis*-acotinic acid, alanine and narciclasine. All three year old bulbs (some with and some without daughter bulbs) group together and show a higher amount of fatty acids, citric acid, haemanthamine, and valine when compared with the other bulbs. To see the difference among the 3 years old bulbs which have daughter bulbs and which have not, samples were divided into three classes on this basis. One class was only the separated daughter bulbs, another the mother bulbs only, while the third class was the mother bulbs with daughter bulbs still attached.

The results from the PLS-DA analysis show that there are differences among these classes (**Figure 7.2C**). The resulting loadings scatter plot was analyzed in comparison with the classes to identify the corresponding metabolites (**Figure 7.2D**) responsible for these differences. It is clear from the loadings scatter plot that galanthamine and narciclasine along with sucrose and glucose are higher in the daughter bulbs than the mother bulbs as

well as the bulbs which did not have daughter bulbs. At the same time, the amount of haemanthamine along with *cis*-aconitic acid were higher in the single bulbs when compared with the mother and daughter bulbs while the amount of phenylalanine and fatty acids were higher in the mother bulbs.

To analyze the differences in metabolites, bucket areas of some compounds were quantified relatively to each other and subjected to statistical analysis. The results presented in **Figure 7.3** showed the differences in the production of different metabolites.

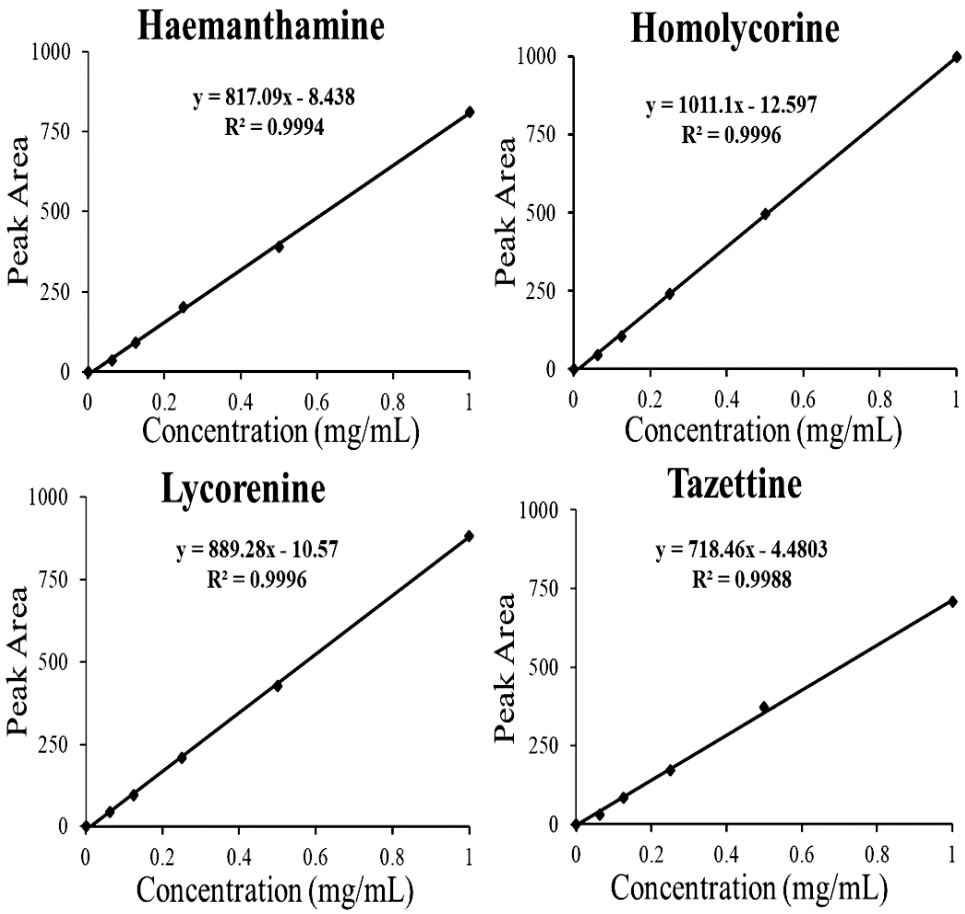


Figure 7.5. Standard curves of *Narcissus* alkaloids haemanthamine, homolycorine, lycorenine, Tazettine on GC-FID obtained with dilution series where each measuring point is based on replicates

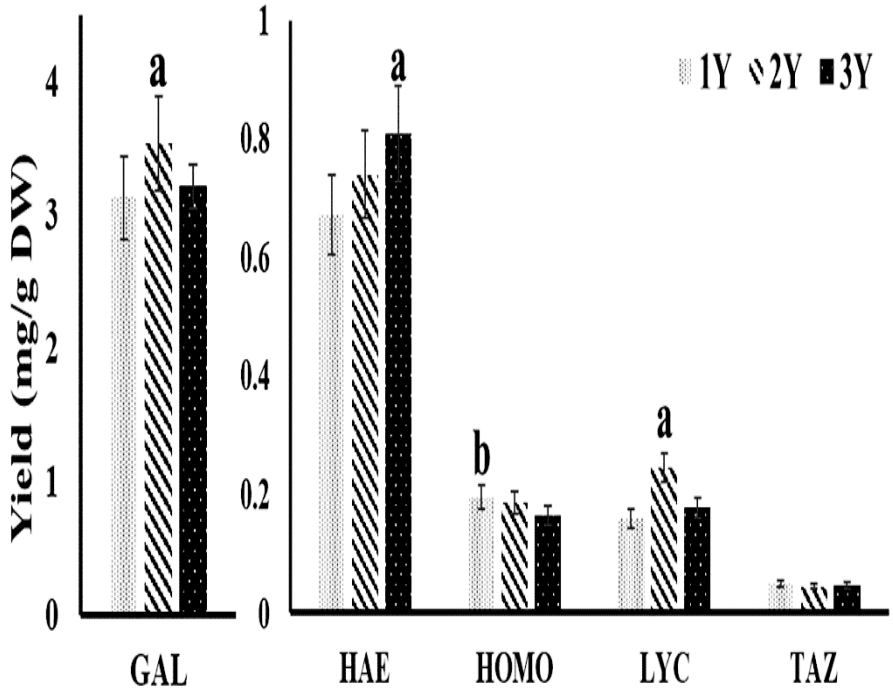
Quantified amounts of these alkaloids were subjected to one-way ANOVA followed by Tukey's HSD test for post-hoc analysis. Different parts yielded different *F* ratios and p-values (given in the **Figure 7.3**). These results confirmed that fatty acids and valine are indeed higher in the three-year old bulbs without the daughter bulbs while acetic acid along with asparagine, choline, phenylalanine, and tyrosine are higher in the one-year old bulbs as well as in the daughter bulbs. In comparison, two-year old bulbs have higher amounts of ethanolamine, alanine, sucrose, *cis*-aconitic acid and narciclasine (**Figure 7.3**).

To identify the alkaloids and quantify their levels, samples were analyzed by using gas chromatography (GC) with two different types of detectors (FID) and mass spectroscopy. The extraction method used in this experiment and the instrumental parameters of GC have been reported previously by Torras-Claveria *et al*. (2010) [34] for the metabolite profiling in *Pancratium canariense*. In this study, the same method was applied to the extraction of alkaloids and other compounds in *Narcissus*. In this work, samples from bulbs of *Narcissus pseudonarcissus* cv. Carlton were used.

Table 7.3. Inter-day and intra-day variations of the *Narcissus* alkaloids.

Sr. No.	**Name of Alkaloid**	**Interday (n=5) RSD%**	**Intraday (n=15) RSD%**
1	Galanthamine	1.4	1.9
2	Haemanthamine	2.2	1.5
3	Homolycorine	2.0	1.8
4	Lycorenine	1.7	1.6
5	Tazettine	1.8	1.6

Compounds were identified by comparing their mass spectra and retention times with those of reference compounds. The NIST library on mass spectra was also used to assist in compound identification. The derivatized extract did show peaks including mono-, di- and tri-saccharides followed by fatty acids, alkaloids, and various phosphates. Some of the peaks could not be identified, among others, because they are only found in trace amounts. GC-Mass spectrometric data (**Table 7.2**) were used for confirmation of the identification of the alkaloids in the alkaloid specific extract.



wzZ

Figure 7.6. Quantitative GC-FID analysis alkaloid contents of *Narcissus pseudonarcissus* Cultivar 'Carlton' bulbs of different age. GAL, galanthamine (P = .01582); HAE, haemanthamine (P = .02687); HOMO, homolycorine (P = .03454); LYC, lycorenine (P = .01747); TAZ, tazettine (P = .10574) (5 biological replicates, significance at $P < .05$, error bars represent standard error (SEM). Different letters show the significant comparison such as (a) denotes significant difference when compared with one-year old bulbs and (b) denotes significant difference when compared with two-year and three-year old bulbs.

The samples of different age varied in the content of alkaloids. In this experiment, we have quantified different alkaloids by using galanthamine as a single standard for

calibration. For this purpose, first calibration curves for all the pure alkaloids were run in five different concentrations (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.063 mg/mL) similar to the range present in plant extracts. All the standards of the five alkaloids galanthamine, haemanthamine, homolycorine, lycorenine and tazettine were run on the same concentrations mentioned and compared (**Figure 7.4, 7.5)**. The response of the detector was also checked by calculating the inter-day and intra-day variations.

For this, a single alkaloid extract of the sample was used. First, the alkaloid extract of the sample was run for five times a day (n=5) from the same vial to check the interday variations. To check the intraday variations, the same sample was run for the next two days, which makes in total three days of analyzing (n=15). Results of these calculations are shown in **Table 7.3**.

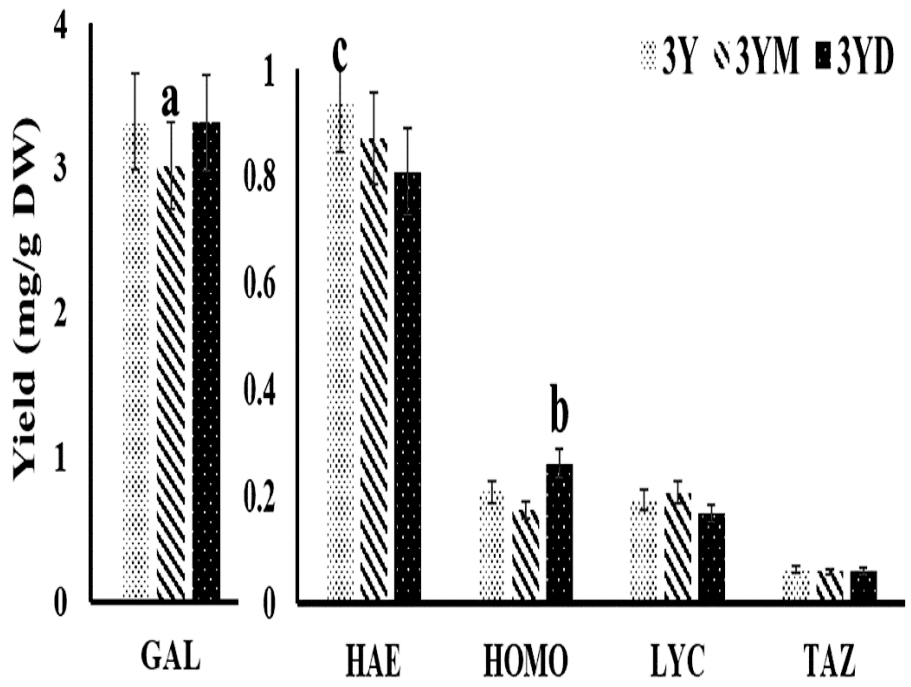


Figure 7.7. Quantitative GC-FID analysis alkaloid contents of *Narcissus pseudonarcissus* Cultivar 'Carlton' bulbs of 3 years old bulbs. 3Y, whole bulb; 3M, mother bulb; 3D, daughter bulbs. GAL, galanthamine (*P* = .01569); HAE, haemanthamine (*P* = .03542);

HOMO, homolycorine (P = .03252); LYC, lycorenine (P = .01903); TAZ, tazettine (P = .14395) (5 biological replicates, significance at P < .05, error bars represent Standard Error). Different letters show the significant comparison such as (a) denotes significant difference when compared with three-year old bulbs, (b) denotes significant difference when compared with three-year old mother bulbs and (c) denotes significant difference when compared with three-year old daughter bulbs.

Quantified amounts of these alkaloids were subjected to one-way ANOVA followed by Tukey's HSD test for post-hoc analysis. Different parts yielded different *F* ratios and p-values (given in the **Figure 7.6**). From the results of the GC-FID analyses (**Figure 7.6**) it is clear that highest amount of galanthamine and lycorenine was obtained from the bulbs which were two year old while haemanthamine was highest in the 3 year old bulbs. In the case of other alkaloids, homolycorine was high in the one-year old bulbs while tazettine was found in small amounts and did not show a significant difference for the ages.

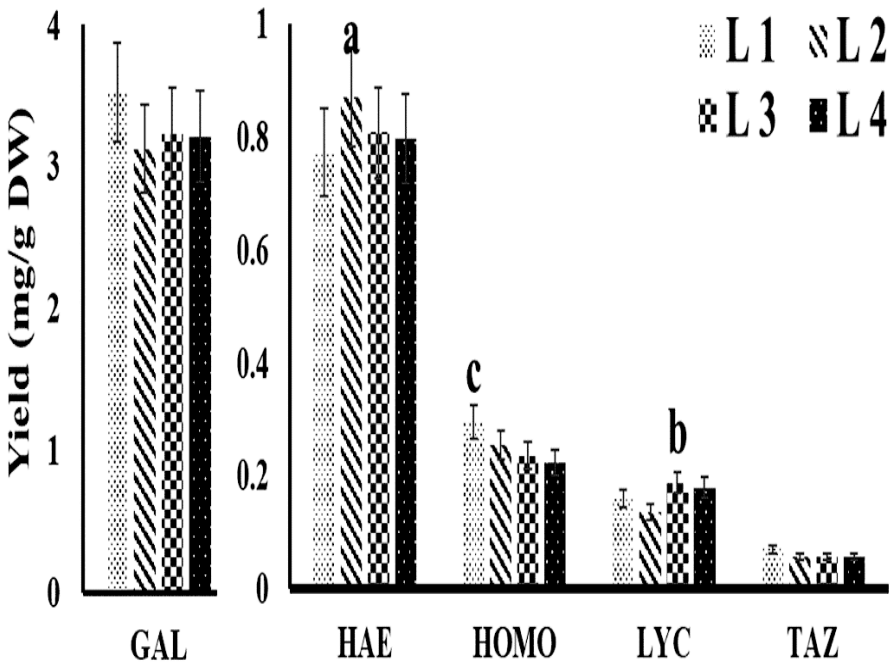


Figure 7.8. Quantitative GC-FID analysis alkaloid contents of *Narcissus pseudonarcissus* Cultivar 'Carlton' bulbs grown at different locations. L1 Lisse; L2 Noordwijk; L3 Lincolnshire 1; L4 (Lincolnshire 2. GAL, galanthamine (P = .05594), HAE,

haemanthamine (P = .04281), HOMO, homolycorine (P = .01468), LYC, lycorenine (P = .02079), TAZ, tazettine (P = .0584), (5 biological replicates, significance t-test at P < .05, error bars represent standard error (SEM). Different letters show the significant comparison such as (**a**) denotes significant difference when compared with location 1 bulbs, (**b**) denotes significant difference when compared with location 2 bulbs and (**c**) denotes significant difference when compared with location 3 or 4 bulbs.

The separation of the three-year old bulbs into mother bulbs and daughter bulbs (**Figure 7.7**), the whole bulbs as well as daughter bulbs have a higher amount of galanthamine, in fact almost similar to each other. The differences in galanthamine contents in these bulbs were statistically significant. At the same time, haemanthamine was highest in the whole bulbs as compared to the mother and daughter bulbs. Homolycorine was found to be high in the daughter bulbs while lycorenine was high in the mother bulbs in comparison with other bulbs.

In terms of the effect of the cultivation site on the levels of galanthamine and other metabolites, Lubbe et al., [32] developed a ^{1}H-NMR spectroscopy method to quantify galanthamine in bulbs of *Narcissus*. To confirm these results in another production year and to also see the difference in levels of other alkaloids, samples from four different locations were quantified by using GC-FID. These four locations consists of two locations from The Netherlands and two locations from the UK. The Dutch locations include Lisse and Noordwijk while UK locations were both in Lincolnshire. The results show that the samples from Lisse (The Netherlands) contained the maximum amount of galanthamine among the four locations. Although the difference when compared with other locations was not statistically significant. In ranking, the samples from Lincolnshire (UK) were second highest, while the samples from Noordwijk (The Netherlands) ranked third in terms of galanthamine quantity. These results are in accordance with the previous study by Lubbe et al. They also found galanthamine contents to vary for the locations though also not statistically significant.

The same was the case with the level of homolycorine, highest quantity was obtained from the bulbs obtained from Lisse while other three locations have minor differences in levels of this alkaloid. Haemanthamine and lycorenine show a different pattern from galanthamine and homolycorine. In the case of haemanthamine, highest amount among

the locations was found in the samples obtained from Noordwijk, while in case of lycorenine highest amount was in the Lincolnshire samples. Tazettine shows minor differences between all the locations with the highest amount in the Lisse samples.

These results indicate that there are differences between bulbs of different ages as well as from different cultivation sites. The differences in alkaloids on the basis of age of the bulbs can be attributed to the development of the bulb over time. As it was mentioned before, *Narcissus* are perennials and, in their growth, cycle the first two seasons bulb weight is increasing, while in the third year daughter bulbs are formed. The higher production of galanthamine per hectare is mainly due to the growth of bulbs as production and storage site of galanthamine.

The difference between cultivation sites can be due to the type of soil as well as different amounts of macronutrients in the field. In terms of soil type, soils in The Netherlands for bulb production are mostly sandy while in the UK, they are clay. So it can be that sandy soils help in providing better aeration as well as better soil nutrients. The theory about macronutrients was tested by Lubbe et al. [32]. They applied different amounts of Nitrogen, Phosphate and Potassium to the field and found that the amount which is normally given in the field produces the best results. Other than the amount of macro nutrients, micro-nutrients can also be an important factor for the difference in the amount of alkaloids. As higher amounts of Zinc are present in The Netherlands soil while the soils of UK are rich in Calcium, Magnesium, and Iron. So inorganic ions could be a further factor involved in the different amounts of alkaloids found in the bulbs.

Conclusion

Narcissus is economically an important plant, which contains several alkaloids like galanthamine. These compounds could add economical value to this crop plant. Hence, it is important to know which cultivation practices are better to get maximum overall yield from the plants and of the alkaloids, and to see if the site of cultivation is an important factor. To measure this, we developed a simple GC-FID method of analysis for the alkaloids, in which all alkaloids can be quantified with galanthamine as the standard. Using this method, it was shown that the alkaloid contents in two year old bulbs are

highest. In the third year no higher levels are found, instead daughter bulbs are formed. Some alkaloids are found to be in higher level in younger bulbs than in the older bulbs. The alkaloid contents of bulbs from the same variety grown at different locations differ but mostly no significant differences were found. Both age of the bulb and location of the cultivation are thus factors affecting galanthamine levels in the bulbs. Yields of alkaloids may vary, under certain conditions a 10-15% higher yield may be obtained. For optimizing the total alkaloid production per hectare further studies are needed in the field in which also costs factors like fertilizers, crop protectants and labor are considered. An important consideration is what the main target of the *Narcissus* production will be, the ornamental or the medicinal products. Or that an integrated production can be aimed at. It will also depend on the volumes of bulbs needed for either application.

References

1. Jin, Z., Amaryllidaceae and Sceletium Alkaloids. Natural Product Reports, 2003. **20**(6): p. 606-614.

2. He, M., C. Qu, O. Gao, X. Hu, and X. Hong, Biological and Pharmacological Activities of Amaryllidaceae Alkaloids. Rsc Advances, 2015. **5**(21): p. 16562-16574.

3. Hotchandani, T. and I. Desgagne-Penix, Heterocyclic Amaryllidaceae Alkaloids: Biosynthesis and Pharmacological Applications. Current Topics in Medicinal Chemistry, 2017. **17**(4): p. 418-427.

4. Hu, Y.F., X.F. Na, J.L. Li, L.J. Yang, J. You, X.L. Liang, J.F. Wang, L. Peng, and Y.R. Bi, Narciclasine, a Potential Allelochemical, Affects Subcellular Trafficking of Auxin Transporter Proteins and Actin Cytoskeleton Dynamics in Arabidopsis Roots. Planta, 2015. **242**(6): p. 1349-1360.

5. Evidente, A., O. Arrigoni, R. Liso, G. Calabrese, and G. Randazzo, Further Experiments on Structure-Activity Relationships among the Lycorine Alkaloids. Phytochemistry, 1986. **25**(12): p. 2739-2743.

6. Maelicke, A., Pharmacokinetic Rationale for Switching from Donepezil to Galantamine. Clinical therapeutics, 2001. **23**: p. A8-A12.

7. Ferri, C.P., M. Prince, C. Brayne, H. Brodaty, L. Fratiglioni, M. Ganguli, K. Hall, K. Hasegawa, H. Hendrie, Y. Huang, A. Jorm, C. Mathers, P.R. Menezes, E. Rimmer, and M. Scazufca, Global Prevalence of Dementia: A Delphi Consensus Study. The Lancet, 2005. **366**(9503): p. 2112-2117.

8. Blacker, C.V.R., D.T. Greenwood, K.A. Wesnes, R. Wilson, C. Woodward, I. Howe, and T. Ali, Effect of Galantamine Hydrobromide in Chronic Fatigue Syndrome: A Randomized Controlled Trial. JAMA: The Journal of the American Medical Association, 2004. **292**(10): p. 1195-1204.

9. Ghosal, S., K. S. Saini, and S. Razdan, *Crinum* Alkaloids: Their Chemistry and Biology. Phytochemistry, 1985. **24**(10): p. 2141-2156.

10. Del Giudice, L., D.R. Massardo, P. Pontieri, and K. Wolf, Interaction between Yeast Mitochondrial and Nuclear Genomes: Null Alleles of Rtg Genes Affect Resistance to the Alkaloid Lycorine in Rho0 Petites of Saccharomyces Cerevisiae. Gene, 2005. **354**: p. 9-14.

11. Mackey, Z.B., A.M. Baca, J.P. Mallari, B. Apsel, A. Shelat, E.J. Hansell, P.K. Chiang, B. Wolff, K.R. Guy, J. Williams, and J.H. McKerrow, Discovery of

Trypanocidal Compounds by Whole Cell Hts of Trypanosoma Brucei. Chemical Biology & Drug Design, 2006. **67**(5): p. 355-363.

12. Çitoğlu, G., M. Tanker, and B. Gümüşel, Antiinflammatory Effects of Lycorine and Haemanthidine. Phytotherapy Research, 1998. **12**(3): p. 205-206.

13. Cooper, M.R., A.W. Johnson, and W.A. Dauncey, Poisonous Palnts and Fungi in Britain Second ed. 2003, London: TSO (The stationary office).

14. Dauncey, E.A., W.G. Berendsohn, T.G.J. Raynert, R. Hand, S. Ritter-Franke, V.S.G. Murray, M. Brockstedt, R. Bunjes, and M.H. Jackson, Die Giftpflanzen Deutschlands. 2000, Berlin: Springer.

15. Weniger, B., L. Italiano, J.P. Beck, J. Bastida, S. Bergoñon, C. Codina, A. Lobstein, and R. Anton, Cytotoxic Activity of Amaryllidaceae Alkaloids. Planta Med, 1995. **61**(01): p. 77-79.

16. Antoun, M.D., N.T. Mendoza, Y.R. Ríos, G.R. Proctor, D.B.M. Wickramaratne, J.M. Pezzuto, and A.D. Kinghorn, Cytotoxicity of *Hymenocallis Expansa* Alkaloids. Journal of Natural Products, 1993. **56**(8): p. 1423-1425.

17. Fennell, C.W. and V.J. Staden, *Crinum* Species in Traditional and Modern Medicine. Journal of Ethnopharmacology, 2001. **78**(1): p. 15-26.

18. Gude, M., B.M. Hausen, H. Heitsch, and W.A. König, An Investigation of the Irritant and Allergenic Properties of Daffodils (*Narcissus Pseudonarcissus* L., Amaryllidaceae). Contact Dermatitis, 1988. **19**(1): p. 1-10.

19. Szlávik, L., Á. Gyuris, J. Minárovits, P. Forgo, J. Molnár, and J. Hohmann, Alkaloids from Leucojum Vernum and Antiretroviral Activity of Amaryllidaceae Alkaloids. Planta Med, 2004. **70**(09): p. 871-873.

20. Hohmann, J., P. Forgo, J. Molnár, K. Wolfard, A. Molnár, T. Thalhammer, I. Máthé, and D. Sharples, Antiproliferative Amaryllidaceae Alkaloids Isolated from the Bulbs of *Sprekelia Formosissima and Hymenocallis × Festalis*. Planta Med, 2002. **68**(05): p. 454-457.

21. Meerow, A.W. and D.A. Snijman, Amaryllidaceae, in *The Families and Genera of Vascular Plants*, K. Kubitzki, Editor. 1998, Springer: Berlin. p. 83.

22. Şener, B., I. Orhan, and J. Satayavivad, Antimalarial Activity Screening of Some Alkaloids and the Plant Extracts from Amaryllidaceae. Phytotherapy Research, 2003. **17**(10): p. 1220-1223.

23. Breiterova, K., M. Locarek, E. Kohelova, M. Talackova, D. Hulcova, L. Opletal, and L. Cahlikova, Daffodils as Potential Crops of Biologically-Active Compounds: Assessment of 40 Ornamental Taxa for Their Alkaloid Profile and

Cholinesterases Inhibition Activity. Natural Product Communications, 2018. **13**(4): p. 419-422.

24. Bock, A., T.H. Sparks, N. Estrella, N. Jee, A. Casebow, M. Leuchner, and A. Menzel, Climate Sensitivity and Variation in First Flowering of 26 *Narcissus* Cultivars. International Journal of Biometeorology, 2015. **59**(4): p. 477-480.

25. Rees, A.R., The Initiation and Growth of *Narcissus* Bulbs. Annals of Botany, 1969. **33**: p. 277–288.

26. Poulev, A., B. Deus-Neumann, and M.H. Zenk, Enzyme Immunoassay for the Quantitative Determination of Galanthamine. Planta Med, 1993. **59**(05): p. 442-446.

27. Tanahashi, T., A. Poulev, and M.H. Zenk, Radioimmunoassay for the Quantitative Determination of Galanthamine. Planta Med, 1990. **56**(01): p. 77-81.

28. Ingkaninan, K., C.M. de Best, R. van der Heijden, A.J.P. Hofte, B. Karabatak, H. Irth, U.R. Tjaden, J. van der Greef, and R. Verpoorte, High-Performance Liquid Chromatography with on-Line Coupled UV, Mass Spectrometric and Biochemical Detection for Identification of Acetylcholinesterase Inhibitors from Natural Products. Journal of Chromatography A, 2000. **872**(1–2): p. 61-73.

29. Mustafa, N.R., I.K. Rhee, and R. Verpoorte, Rapid Method for Determination of Galanthamine in Amaryllidaceae Plants Using HPLC. Journal of Liquid Chromatography & Related Technologies, 2003. **26**(19): p. 3217-3233.

30. Zhang, J., M. Wang, Y. Shen, G. Ma, and S. Hong, Studies on Alkaloids of Amaryllidaceae Xii. Identification of Amaryllidaceae Alkaloids by Tlc and Determination of Galanthamine by Hplc. Yaowu Fenxi Zazhi 1999. **19**: p. 399–403.

31. Abou-Donia, A.H., S.M. Toaima, H.M. Hammoda, and E. Shawky, New Rapid Validated HPTLC Method for the Determination of Galanthamine in Amaryllidaceae Plant Extracts. Phytochemical Analysis, 2008. **19**(4): p. 353-358.

32. Lubbe, A., B. Pomahačová, Y.H. Choi, and R. Verpoorte, Analysis of Metabolic Variation and Galanthamine Content in *Narcissus* Bulbs by 1h Nmr. Phytochemical Analysis, 2010. **21**(1): p. 66-72.

33. Berkov, S., J. Bastida, F. Viladomat, and C. Codina, Development and Validation of a GC–MS Method for Rapid Determination of Galanthamine in *Leucojum Aestivum and Narcissus* Ssp.: A Metabolomic Approach. Talanta, 2011. **83**(5): p. 1455-1465.

34. Torras-Claveria, L., S. Berkov, O. Jáuregui, J. Caujapé, F. Viladomat, C. Codina, and J. Bastida, Metabolic Profiling of Bioactive *Pancratium Canariense* Extracts by GC-MS. Phytochemical Analysis, 2010. **21**(1): p. 80-88.