Factors affecting galanthamine production in Narcissus

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Chapter IV

Effect of Pre-analytical parameters on Alkaloid contents of *Narcissus* Bulbs analyzed with ^{1}H-NMR spectroscopy.

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Abstract:

Narcissus plants produce a number of different alkaloids with a diverse range of biological activities. Galanthamine, haemanthamine, lycorine and narciclasine are the most extensively studied alkaloids. The benzazepine alkaloid galanthamine has been under investigation since a long time. In recent years it was developed as a medicine for treating the symptoms of Alzheimer's disease (AD), because of its inhibition of the enzyme acetylcholine esterase. Chemical synthesis and extraction from plant material are both used for the production of galanthamine at commercial scale. Though not the plant species with the highest levels of the alkaloid, Narcissus bulbs are the major commercial natural source of galanthamine as they are already under cultivation at large scale in The Netherlands and UK. These bulbs are produced for planting in home gardens and large-scale flower production. The use of the bulbs as natural source for galanthamine isolation would add extra value to the existing crop.

Aim of the current study was to measure the effects of some pre-analytical treatment parameters on the observed metabolome using 1H-NMR spectroscopy. Among these pre-analytical parameters, special consideration was given to the effect of extraction conditions on various alkaloid yields in general and galanthamine quantification specifically. The extraction yields of the various alkaloids were found to be influenced by some of the pre-analytical variables, such as sample/solvent ratio, number of bulbs in the sample and sonication time. The 1H-NMR spectroscopy was used because it provides an overview of differences in the amounts of major metabolites extracted in a very short analysis time. It is a fast and robust method when compared with other targeted methods for screening larger numbers of samples. Moreover, it can also be used for the identification and absolute quantitation of the various alkaloids present.

Keywords: *Narcissus*, alkaloids, extraction, pre-analytical, NMR spectroscopy, metabolomics.

Introduction:

Narcissus (daffodil) is a spring flowering plant and a high value ornamental crop after roses and tulips. *Narcissus* belongs to the family Amaryllidaceae. It is mostly grown for decorative purposes (flowers) but bulbs of *Narcissus* also have great economic value due to their use as a propagation material. Recently interest in the production of *Narcissus* as a medicinal crop has been growing due to its biological activities. Many studies have shown several useful biological activities such as antimalarial [1], antiviral [2], antitumor [3], antifungal and insect antifeedant activity [2] of different *Narcissus* alkaloids. Till date about 300 alkaloids have been identified from *Narcissus* species with different biological activities at varying concentrations. These alkaloids also include galanthamine as a major compound, closely followed by haemanthamine and narciclasine (**Figure 4.1**) in an extract from the plants. Galanthamine was isolated by Proskurina and Yakovleva for the first time in 1952 and later it was used as a medicine in Eastern Europe.

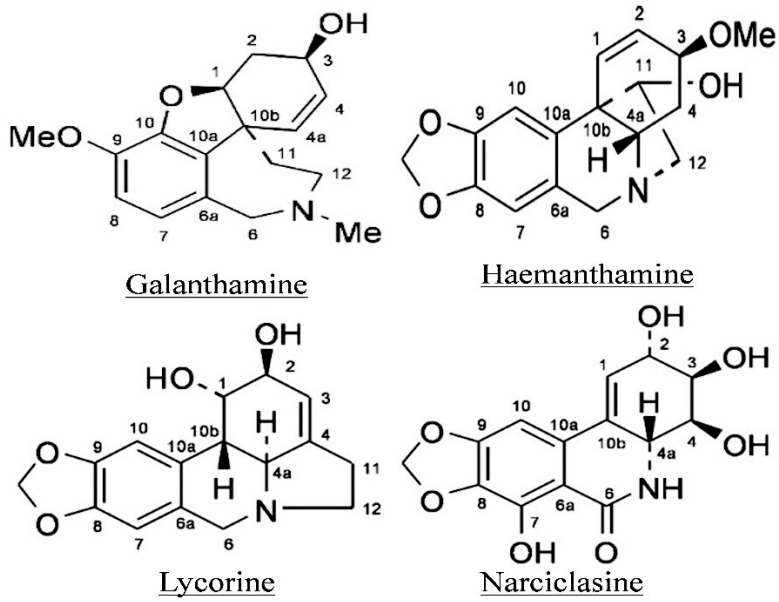

Figure 4.1. Chemical structures of the major alkaloids isolated from *Narcissus*.

The main alkaloid of *Narcissus*, galanthamine is a long-acting, selective, reversible and competitive acetylcholinesterase (AChE) inhibitor, which is now marketed as a medicine

in the form of its hydrobromide salt [4]. 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. The estimate is, that the amount of people affected by dementia will rise to 42 million by 2020 [5]. While there is no cure or preventive medicine yet for this disease, galanthamine is used for the symptomatic treatment of the disease, improving the patient's quality of life. Studies have also shown, that galanthamine can act as a mild analeptic, having an analgesic power as strong as morphine. It is also reported, that galanthamine can help in relieving jet lag and it also works against fatigue syndrome, male impotence, and alcohol dependence. It also reduces the intraocular pressure when applied in eye drops.

To produce galanthamine, *Narcissus* species and varieties are of great interest to the scientists as a natural source for commercial extraction. Besides galanthamine, there are several other alkaloids which can be found in *Narcissus*. Narciclasine is one of these alkaloids and it is a known plant growth inhibitor which can be isolated from *Narcissus* bulbs [8]. It exhibits a wide range of inhibitory effects on plant growth including seed germination, seedling growth, plastid development and growth of excised radish cotyledons as well as the development of chloroplast [9]. Although the precise mode of action of narciclasine is not known, it has been shown to inhibit protein synthesis [10], isocitrate lyase, and hydroxypyruvate reductase activities in glyoxysomes and peroxisomes [9], respectively. Moreover, it also exhibits some antimitotic [11] and antiviral [12] activities.

Haemanthamine is another Amaryllidaceae alkaloid, which is present in all plant organs except the flowers. Haemanthamine originates from intramolecular para-para oxidative phenol coupling of the common *Narcissus* alkaloid's precursor. It has hypersensitive and cytotoxic activity against a variety of in-vitro cultured cells. Haemanthamine also exhibits cell growth inhibitory activities [6, 7] and antimalarial activity [8].

Generally, small molecules that take part in wide-ranging metabolic reactions are labeled as metabolites. These metabolites are mostly required for various functions such as maintenance, growth and regular function of a cell. A complete set of these metabolites

in an organism is termed as metabolome. The identification and quantification of metabolomes in a biological system is defined as metabolomics [9]. The sizes of metabolomes are highly dependent on the specific organism under study. Some scientists have suggested a comparable number of metabolites and genes in an organism [10]. However, it should also be noted that generally there is no direct link between every gene involved in metabolism and a given metabolite. Different estimates propose up to 15,000 distinct metabolites within a given plant species [11-13], Verpoorte et al. [14] mentioned a number of about 30,000, based on the assumption that a plant probably produces a similar number of compounds as it has genes. The plant kingdom is known to produce a wide diversity of chemical molecules overall. More than 300,000 metabolites are now catalogued in the Dictionary of Natural Products most of which come from the plant kingdom [15]. It concerns very diverse structures with a broad range of physical chemical properties. Some compounds, such as sugars and lipids, have important nutritive value and can occur in very large amounts. While compounds involved in signaling, e.g. defense and resistance mechanisms, may be present in only in trace amounts(from fmol to mmol) [16]. Metabolomics aims at the qualitative and quantitative analysis of the metabolites in a certain biological sample. However, the metabolome of a plant consists of compounds that differ in many aspects. [17]. Even with the most advanced methods, a complete survey of all metabolites that are present in a crude plant extract is not possible [18]. The major limiting step is the extraction as there is no universal solvent that can extract all compounds from a biological sample. Other limitations are the detection limits of the analytical equipment and the stability of the compounds.

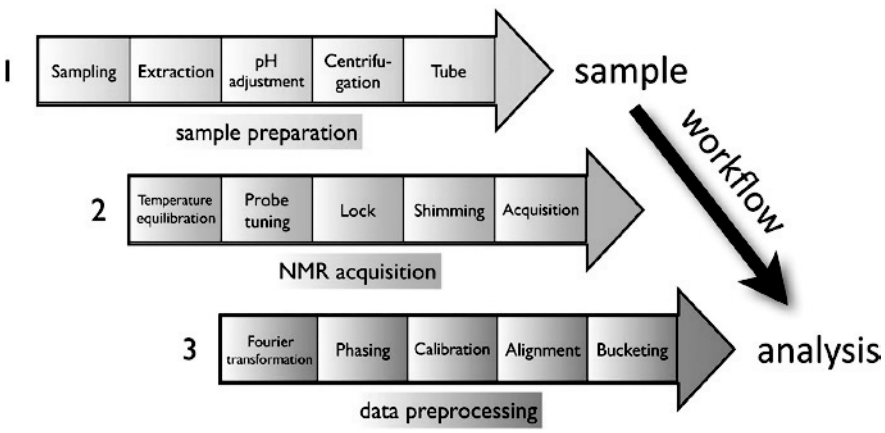

Figure 4.2: ^{1}H-NMR metabolomic workflow of plant extracts. 1, sample preparation; 2, NMR acquisition; 3, data preprocessing

Until recently, most plant metabolites have been analyzed and detected with targeted methods for very specific purposes such as diversity, quantitation and chemical variations due to different factors [19]. With the advent of powerful modern analytical methods and the development of multivariate data analysis approaches, these metabolites can now be comprehensively analyzed in complex natural extracts [20]. NMR metabolomic profiling has been widely used in the plant sciences [21-26]. Its advantages are the universal detection of organic compounds with a high dynamic range, a good reproducibility, a relatively simple implementation for the screening and quantification of a range of major metabolites, and a provision of structural information for compound identification. In NMR-based metabolomics data collection and spectral processing are also important to ensure that, for example, replicate samples provide identical NMR fingerprints. In practice small differences in line shape and chemical shift will be observed. The differences in line shape can be minimized by using exactly the same sample volume in identical NMR tubes and by optimizing the magnetic field homogeneity before data acquisition [27]. To compensate for differences in linewidth, the line broadening parameter can be varied during processing [28]. To minimize the misalignment of NMR signals, there should be a stringent control of sample preparation, especially to avoid differences in pH or ionic strength [29].

Most laboratories use their own specific method of sample preparation. In metabolomic studies it is important that the reproducibility of the procedure is as best as possible [29]. An ^{1}H-NMR metabolomic workflow begins after the harvest of plant samples and consists of four steps: sample and extract preparation, spectra acquisition, spectra and data processing and metabolite identification (**Figure 4.2**). Concerning extraction, semi-polar methanolic extracts are a good compromise for accessing both major semi-polar primary and specialized metabolites [22], but specific adaptations to plant samples are often required to deal with ionic composition and its interaction with major organic acids such as malic, citric or fumaric acid [21, 30-32]. A quantity of 20 to 100 mg of powder (best grinding quality with particle size of 70 to 150 µm or 100 to 200 mesh) is recommended. Tests must be performed to optimize extract concentration by checking for the linear response of exploitable spectral information

(signal-over-noise ratio, S/N ratio) to powder quantity, but also for spectral quality. A compromise must be found, on the one hand, one should not dilute too much because extracts with low concentrations are easier to shim but provide a lower number of resonances detected. On the other hand, one should not concentrate too much extracts because concentrated extracts are more difficult to shim with broader resonances due to higher viscosity [33]. All sources of variation should be minimized. That is important in the sample selection, preparation and during measurement [34].

Metabolomics analysis consists of three distinct experimental steps. The first step is the preparation of the sample, the second is the acquisition of data using analytical chemical methods while the third and final step are data processing along with the analysis by using appropriate chemometric methods [35]. Although all these steps are strongly interrelated with each other, the first two are particularly interconnected. It is due to the reason that the analytical method used depends on the properties of the analyte and thus determines the way the sample should be prepared. This makes the sample preparation a very critical step with important consequences for the compounds isolated and the accuracy of the results. To obtain better results, all practical considerations on the process should be taken into account from the very beginning like the sample collection, drying and grinding of plant material, extraction of metabolites and the final measurement [36].

A crucial step in the pre-analytical processing is the sampling and sample preparation including the extraction of the plant material. For the sampling, the first thing to do is to establish the natural variability between different individual plants. This is needed to be able to distinguish between the natural variation and experimental variations in metabolic profiles. In the extraction process, the ratio between sample and solvent is a crucial factor. During extraction, whatever solvent is chosen, there will always be compounds that are poorly soluble in that particular solvent and will be dissolved to their saturation level. That means that a major part of these compounds may still be left in the plant material even after extraction. In the case of NMR based metabolomics where the extract is measured as such, these poorly soluble compounds will be present at the same saturation level (concentration) in all the measured samples. While at the same time well-dissolved compounds will be fully extracted and thus may show differences between different samples, i.e. some compounds will show different concentrations and other will remain

at the same level for all samples. Of course, there is also a possibility in between these extremes, there are some compounds that will be present at just below the saturation level and the full width of their changes will not be observed. Thus using different amounts of solvent to extract a certain amount of plant material, could give different results. For example, if a compound can be fully extracted with 1 mL of solvent, doing the extraction with the double amount of solvent will result in half concentration of this compound. However, the saturated compounds may become fully extracted though the concentration may remain the same. Then if a certain fixed aliquot (e.g. 0.5 mL) is taken from each of the two solutions, the absolute amount of the soluble compound in the 0.5 mL aliquot of the 2-mL extract will be half of that in the 0.5 mL aliquot of the 1 mL extract. While at the same time the poorly soluble compound would have the same amount in both samples regardless of solvent. In the case of extracting with a fixed volume of solvent but increasing amount of biomass, the amount of the soluble compound in the extract will increase, whereas the amount of extracted poorly soluble compound remains constant. Strictly following protocols is thus a must in metabolomics analysis.

Table 4.1. Possible combinations with varying amount of sample and solvent ratios.

	Soluble compound A	Non-Soluble compound B
More solvent same amount of sample	Concentration down In aliquot lower amount	Concentration the same In aliquot same amount
Amount of sample up, solvent the same	Concentration up In aliquot higher amount	Concentration the same In aliquot same amount

During extraction, it is important to consider a few factors such as the sample size to minimize the effect of inter-sample variation, particle size of powdered plant material, amount of sample used for extraction, amount of solvent used for extraction, sonication (a process used to disrupt cell walls for metabolite extraction) and how much sample is vortexed during extraction. The reason for consideration is that all these factors can affect the number as well as amount of metabolites which can be extracted from the same plant material. To extract alkaloids from *Narcissus*, several studies have been done on the

extraction method as this must be in accordance with the chromatographic method used. Analysis of galanthamine and other alkaloids from plant material has been carried out using diverse methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) in combination with mass spectroscopy (MS) [37-40]. These methods are mostly chromatography-based which require a selective alkaloid concentration step. Using an acid-base extraction method for alkaloids was shown to be successful for preparing samples for gas chromatography-mass spectroscopy (GC-MS) analysis. Although these methods are better to specifically determine the alkaloids from a plant sample, quite some time (4-72 hours) is required to fully complete the process. This is not helpful for a quick analysis. Reference compounds are indispensable for absolute chromatography based quantitative analysis, as calibration curves for each individual alkaloid are required.

If an unbiased or non-targeted metabolic profile is intended, nuclear magnetic resonance spectroscopy (NMR)-based metabolic profiling is the best approach to assess the effects of cultivation practices on plant metabolism. NMR spectra can give useful qualitative and quantitative information about a sample in a single measurement, without being compound class selective [41].

The aim of the present study was to further establish the robustness of the method described earlier [42]. For this purpose, different variables were considered, for example, does the number of bulbs mixed in a sample have any effect on the alkaloid yields, and does the sample/solvent ratio affect alkaloid yields due to saturation in case of poor solubility. We thus studied the effect of the number of bulbs extracted, i.e. the sample size, as well as different ratios of sample/solvent. Also the effect of the sonication time on the extracted metabolome and the yields of alkaloids was studied.

Materials and methods

Chemicals and solvents:

For the NMR analysis methanol-d_4 (99.80%) from Cambridge Isotope Laboratories (Andover, MA, USA), and phosphate (KH_2PO_4) buffer (pH 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France) containing 0.01% trimethylsilylproprionic

acid sodium salt-d_4 (TMSP, w/w) as an internal standard for quantitation and calibration of chemical shift was used.

Plant Material and Metabolite Extraction:

Bulbs of *Narcissus pseudonarcissus* Cultivar 'Carlton' (Amaryllidaceae) were obtained from Holland Biodiversity B.V. (Lisse, The Netherlands). Bulbs were collected from the field after the full season and stored at 4 ℃ before extraction.

Plant material was prepared for NMR analysis according to the method of Kim et al. [22]. Bulbs were rinsed after dusting to fully remove soil particles. Roots were removed along with small part of the basal plate. After that, a number of variations have been tested to optimize the extraction method and monitor changes in metabolites levels.

To monitor the effect of inter bulb variations in metabolite contents, a different number of bulbs were mixed during the grinding process and then a representative sample of the mixed powder was taken. For this purpose, 1, 2, 4, 6, 8 and10 almost identical bulbs in size and weight were selected and then chopped together before grinding into powder form and then freeze dried for 2 days. From this freeze-dried powder 50 mg plant material was taken into a 2 mL microtube and extracted with 1.5 ml of a mixture of phosphate buffer (pH 6.0) and methanol-d_4 (1:1). Samples (5 replicates for each sample) were ultrasonicated for 30 minutes, followed by centrifugation at 13,000 rpm for 10 minutes.

To monitor the changes in metabolites levels due to the extraction process, bulbs were chopped and frozen in liquid nitrogen before each bulb was ground to fine powder with a Waring laboratory blender (Waring Products Inc., Torrington, CT, USA). Powdered bulb material was dried in a freeze-drier for 2 days. Samples were taken from this dried bulb material to check the effect of sample weight, amount of solvent and sonication time. For the effect of sample weight, dried bulb material (25, 50, 75, 100, 125, 150, 175, 200 mg) was weighed into a 2 mL microtube and extracted with 1.5 mL of a mixture of phosphate buffer (pH 6.0) and methanol-d_4 (1:1) making a sample solvent ratio of 1/60, 1/30, 1/20, 1/15, 1/12, 1/10, 1/8.6 and 1/7.5 respectively where internal standard was part of the solvent. Samples were ultrasonicated for 30 minutes, followed by centrifugation at 13000 rpm for 10 minutes. For the optimization of the amount of solvent, 50 mg dried

bulb material was weighed into a screw cap tube and extracted with a different amount (1.1, 1.2, 1.3 1.4, 1.5, 1.6, 1.7, 1.8 mL) of a mixture of phosphate buffer (pH 6.0) and methanol-d_4 (1:1) thus making a sample solvent ratio of 1/22, 1/24, 1/26, 1/28, 1/30, 1/32, 1/34 and 1/36 respectively. Samples were ultrasonicated for 30 minutes, followed by centrifugation at 13,000 rpm for 10 minutes. For the sonication time optimization, dried bulb material (50 mg) was weighed into 2 mL microtubes and extracted with 1.5 mL of a mixture of phosphate buffer (pH 6.0) and methanol-d_4 (1:1). Samples were ultrasonicated (15, 30, 45, 60, 75, 90, 105 and 120 minutes) followed by centrifugation at 13,000 rpm for 10 minutes (5 replicates for each sample).

^{1}H-NMR analysis:

An aliquot of 1 mL of the supernatant was collected from each sample and 800 µL transferred to a 5 mm NMR tube for ^{1}H-NMR measurement. ^{1}H-NMR measurements were carried out as described in Lubbe et al. [42]. ^{1}H-NMR spectra were recorded with a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample 64 scans were recorded using the following parameters: 0.167 Hz/point, pulse width (PW) 4.0 µs and relaxation delay (RD) = 5.0 S. FIDs were fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to 0.0 ppm for all the samples.

Data processing:

For quantitative analysis of galanthamine, haemanthamine and narciclasine, integration of the proton signals, doublet at δ 6.17 (galanthamine H-4a), doublet at δ 6.52 (haemanthamine H-1) and multiplet at δ 6.22 (narciclasine H-1) was performed. The ratio of this integral to that of the internal standard was used to calculate the amount of galanthamine per milligram material. For multivariate data analysis (MVDA), ^{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 (“buckets” or bins) 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 (v. 13.0 Umetrics, Umeå, Sweden) using the Pareto scaling method.

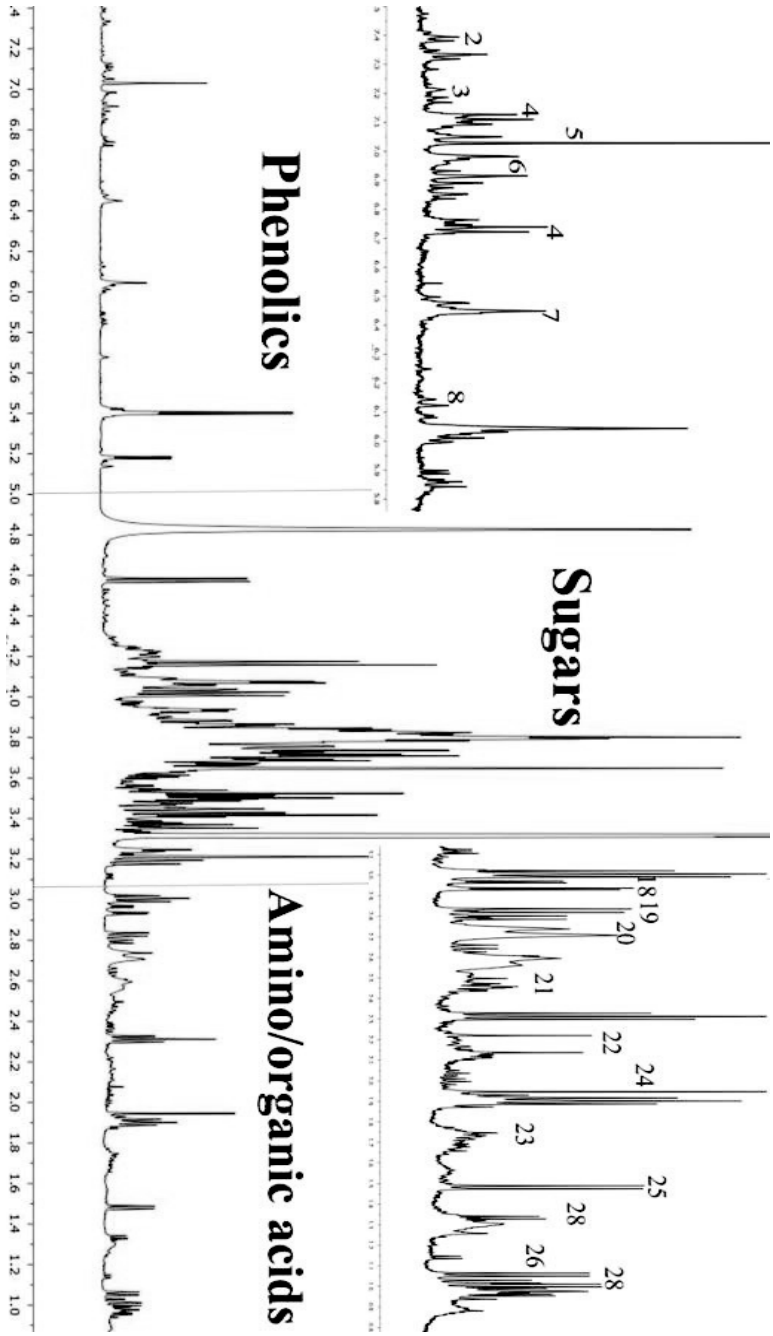

Figure 4.3. ^{1}H-NMR spectra (600 MHz) from 0.8 to 7.4 ppm of phosphate buffer (pH 6.0) and methanol-d_4 (1:1) extract of *Narcissus* bulbs divided into three regions

according to signal abundance of the compounds labeled with numbers from Table 4.2

Hierarchical cluster analysis (HCA) using Ward's minimum variance method was also done using SIMCA-P software. All statistical analyses were performed using SPSS version 20.0. An analysis of variance (ANOVA) was used for comparing the average alkaloid levels between treatments.

Results and Discussion:

Validation of the NMR-based metabolomics protocol for analysis *Narcissus* bulbs

NMR is a stable, highly reproducible tool for metabolomics, in which most variation comes from the preanalytical part of the analysis. To validate the NMR-based metabolomics as tool for measuring alkaloid contents in narcissus bulbs a number of variables in the extraction method were studied. A representative ^{1}H-NMR spectrum can be divided into three distinct regions: amino acids, carbohydrates, and phenolic region as can be seen in the ^{1}H-NMR spectra of *Narcissus* bulbs (**Figure 4.3**).

Table 4.2. ^{1}H Chemical shift (δ) and coupling constant (Hz) of *Narcissus* bulb metabolites. Measured at 600 MHz , in phosphate buffer (pH 6.0) and methanol–d_4 (1:1)

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	HAEM	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	NAR/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.

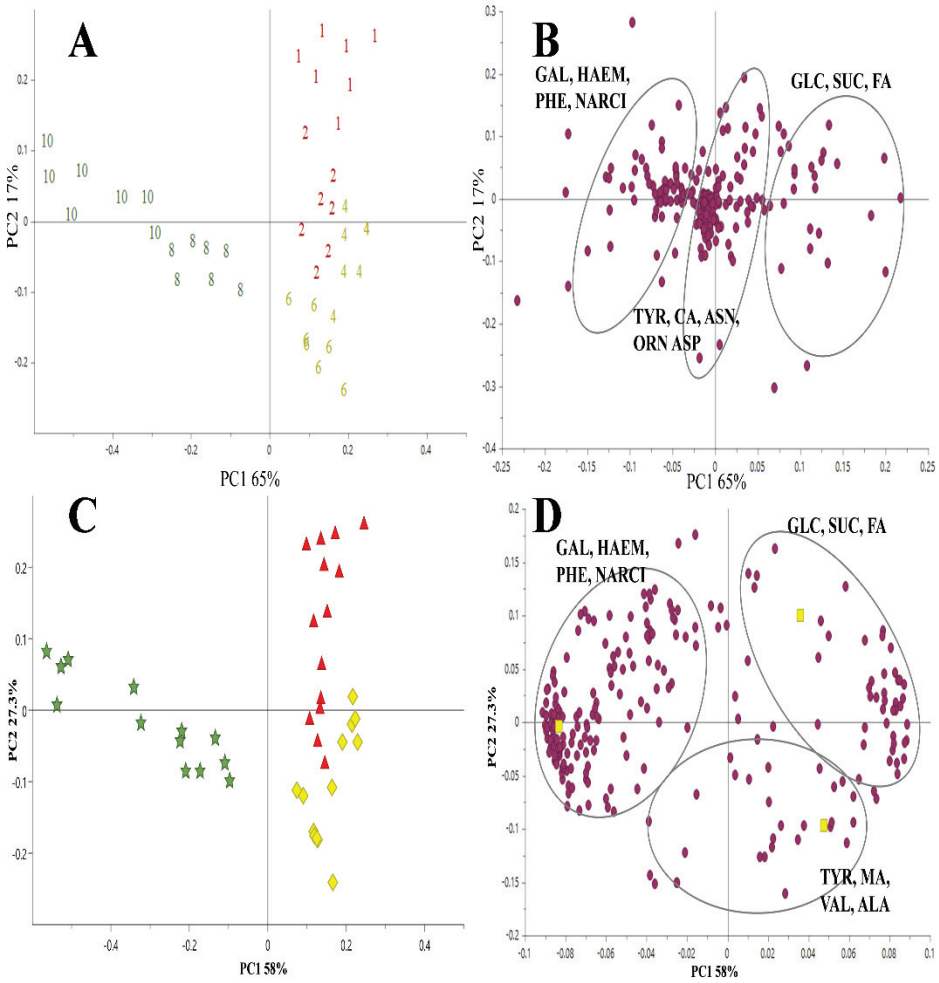

Figure 4.4. Multivariate data analysis of NMR spectra (600MHz) of *Narcissus* bulb extracts made from different number of bulbs (1, 2, 4, 6, 8, and 10).

A) Score plot of PCA (PC1 vs PC2) of extracts made from samples prepared from different number of bulbs. The labelled number (1, 2, 4, 6, 8 and 10) represent the number of bulbs in the sample that were used for extraction. For all the samples 50 mg material was extracted with 1.5 mL solvent. 7 biological replications were made for each sample mixture. B) Loadings scatter plot of PCA (PC1 vs PC2) of the bulb's mixtures showing the metabolites that correspond with the different regions of the score plot A.

C) PLS-DA scatter plot where the bulb samples were grouped in 3 classes (▲) the class of samples containing first two samples i.e. single bulb samples and 2 bulbs mixture, (◆) class of samples containing 4 and 6 bulbs in the mix of each sample and (★) class of samples containing 8 and 10 bulbs in the mix of each sample. D) Loadings scatter plot showing the metabolites that correspond with the different regions of the PLS-DA score plot C. where (■) represent average of the classes.

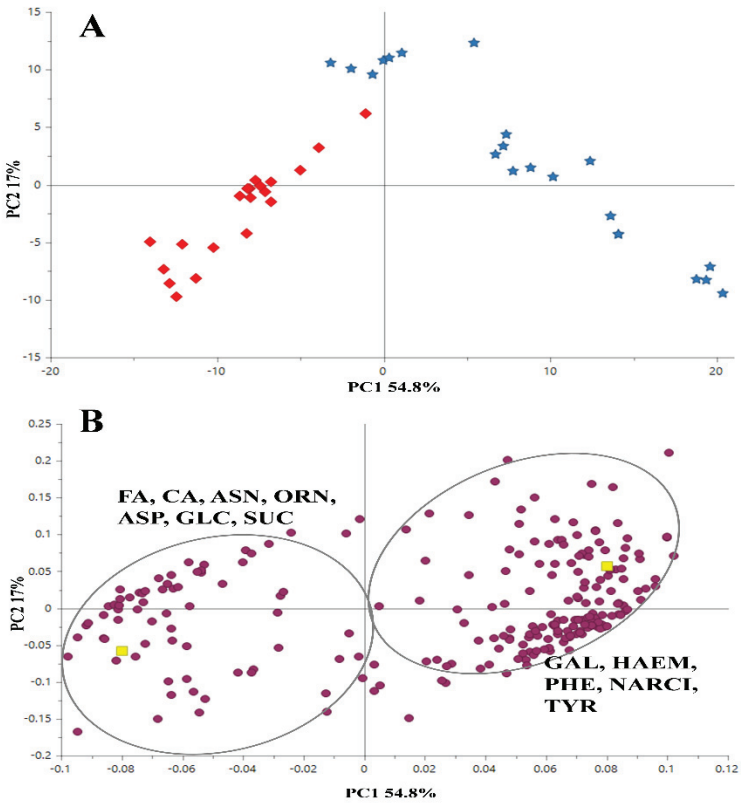

Figure 4.5. Multivariate data analysis of NMR spectra (600MHz) of extracts of *Narcissus* bulb samples made with different number of bulbs.

A) PLS-DA scatter score plot of mixed bulbs samples which are divided into two distinct classes where (◆) represent class of samples of less than 5 bulbs and (★) represent class of samples that have more than 5 bulbs (with 5 biological replicates each) B) Loadings scatter plot showing the metabolites that correspond with the different regions of the PLS-DA score plot A. PLS-DA models were validated by using permutation test (R^2 = 0.86, Q^2 = 0.73 with 100 permutations and 5 components)

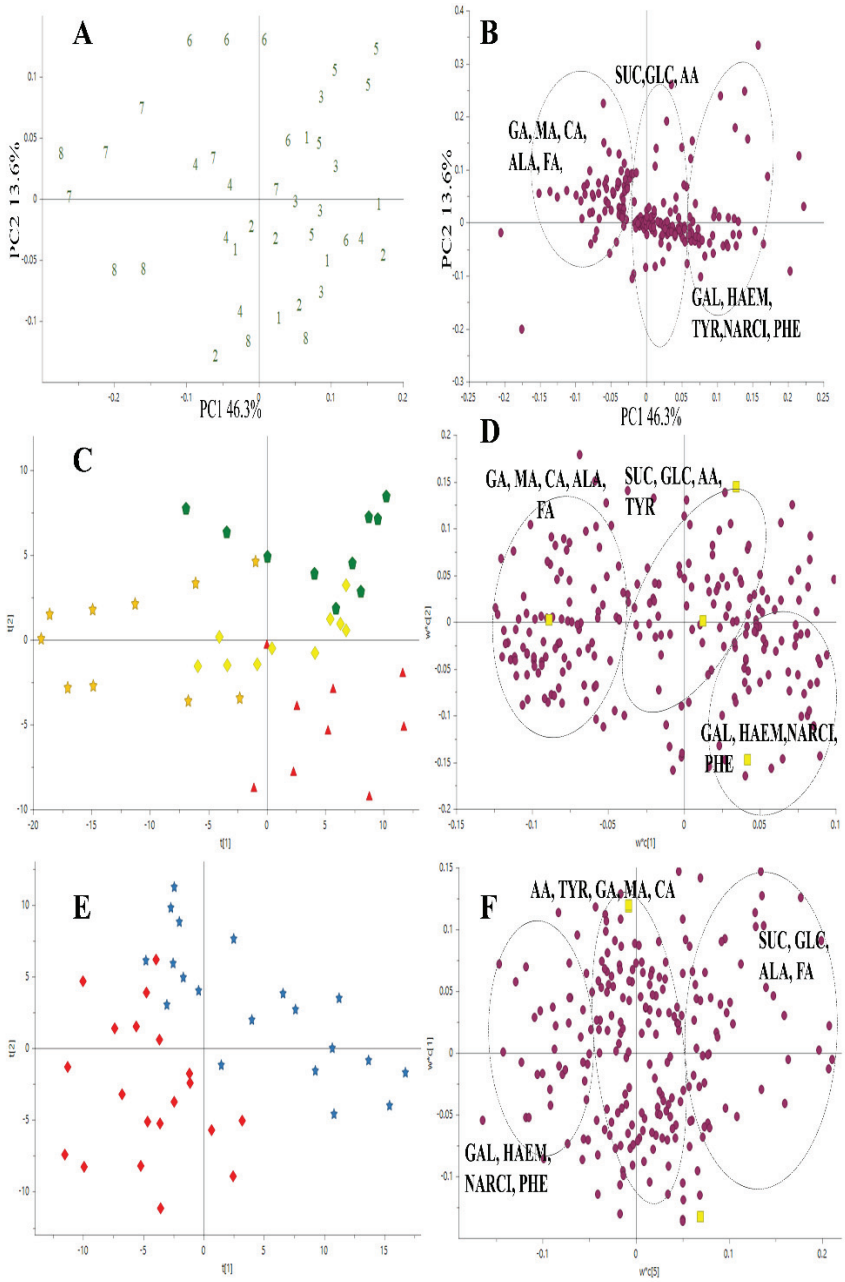

Figure 4.6. Multivariate data analysis of NMR spectra (600 MHZ) of *Narcissus* bulb extracts from different amounts of the same bulb sample extracted with the same amount of solvent.

A) Score plot of PCA (PC1 vs PC2) of bulb samples based on the weight of sample, numbers from 1-8 represent sample weight of 25, 50, 75, 100, 125, 150, 175 and 200 mg respectively. Each sample was extracted with 1.5 mL of solvent. Five biological replicates were extracted for each sample. B) Loadings scatter plot showing the metabolites that correspond with the different regions of the PCA score plot A.

C) PLS-DA scatter plot with (▲) class which contains 25 mg and 50 mg samples, (♦) class which contains 75 mg and 100 mg samples, (★) class which contains 125 mg and 150 mg samples and (●) class which contains 175 mg and 200 mg sample weight. D) PLS-DA loadings plot of *Narcissus* bulb samples (●) metabolites that correspond with the different regions of the PLS-DA score plot C (■) average of classes in the loadings plot.

E) PLS-DA scatter plot with (♦) class of samples containing the weight of 100 mg or less and (★) class of samples weighing more than 100 mg. F) PLS-DA loadings showing the metabolites that correspond with the different regions of the PLS-DA score plot of E. (■) represent the average of the classes in the loadings plot. PLS-DA models were validated by using permutation test (R^2 = 0.75, Q^2 = 0.69 with 120 permutations and 8 components)

Visual inspection of the spectra of bulb samples showed that there was a marginal increase in the signals of some compounds. The results show that these extracts were dominated by high concentrations of primary metabolites such as amino acids, sugars, and organic acids. Although NMR is quite extensively used for metabolite analysis, sometimes overlapping signals and low signal intensity create problems in identification of compounds.

This problem is overcome using different 2D techniques like J-resolved, ^{1}H–^{1}H COSY, HMBC, and HSQC, which provide extra information regarding the molecular structures. Thus, the application of 1D ^{1}H-NMR spectroscopy in combination with different 2D techniques resulted in the identification of quite a few *Narcissus* metabolites, covering a wide range of structural and functional diversity. The identified compounds belong to different classes including amino acids, organic acids, carbohydrates, and alkaloids. All NMR signals discussed (**Table 4.3**) were assigned by comparison of spectra with previous reports as well as 1D and 2D NMR spectra of common metabolites in our in-house library.

Although visual inspection and metabolite identification were helpful in checking the differences in different samples, still a more statistical approach was required to confirm the significance of changes. The changes considered in metabolite profiles were due to

inter-bulb variations, sample weight, amount of solvent, sonication time and browning effect. For this purpose, multivariate data analysis (MVDA) was used to determine the differences between samples. Principal component analysis (PCA) is a commonly used MVDA method in which clustering is used to reduce the dimensionality of a multivariate dataset. It is an unbiased and unsupervised method, in which the principal components can be represented graphically and any grouping or outliers among the samples can be easily observed in a score plot of PCA. The corresponding loadings plot shows the variables (NMR chemical shifts) responsible for the separation or grouping on the score plot. From these variables, with the assistance of 1D and 2D NMR, the involved metabolites can be identified. To highlight the metabolic differences based on bulb mix, sample weight, amount of solvent, sonication time and effect of browning, all samples were compared with each other as well as with the standard sample preparation protocol. Principal component analysis of mixed bulbs to find out the inter-bulb variations and its effect on alkaloid levels showed that there were certain differences between different mixes of bulbs. In the PCA of bulb mix samples, the first two components accounted for 72% of the variation in the data set. The score scatter plot of the unbiased PCA analysis is shown in the **Figure 4.4A**. A loadings plot is used to see which spectral areas contribute to the grouping of samples **Figure 4.4B**.

Principle Component Analysis (PCA) is an unsupervised method and gives a general overview of the maximal variation in data. The partial least square (PLS) is a supervised version of PCA and next step in the analysis of multivariate data. The aim of PLS modeling is to model two or more sets of data and predict results by comparison with the response. **Figure 4.4B** shows the results of PLS modelling in the case of bulbs mix when they are divided into three different classes, based on the number of bulbs mixed to obtain the sample. There is a clear separation between the classes based on the numbers of mixed bulbs. The results show that when there was a larger number of bulbs in the mixture, there was a pronounced effect on the metabolites and specifically on alkaloids. When samples were divided into two groups i.e. five or less than five bulbs in the mix or more than five bulbs in the mix there was a clear separation between these two groups (**Figure 4.5A**). **Figure 4.5B** shows the responsible chemical shifts for the separation of the compounds involved.

In terms of specific metabolites, the results (**Figure. 4.5)** show that there is a higher level of carbohydrates along with other primary metabolites in the lower number of bulbs mixtures, whereas alkaloids galanthamine, haemanthamine and narciclasine as well as other phenolics (tyrosine and phenylalanine) are higher in the high number of bulbs in the mixture. Thus, the primary metabolites such as fatty acids, ornithine, citric acid and asparagine were at lower levels when the number of bulbs is larger in the sample. The PLS model was validated by using the response of a permutation test which assesses the statistical significance of the predictive power calculated by cross validation. The results of the current PLS model are valid when tested through 100 permutations.

The PCA analysis of the extraction process of one batch of plant material shows that there are differences when varying the amount of sample weight, volume of extraction solvent and duration of sonication. In the case of different sample weight, the score scatter plot of PCA analysis is shown in **Figure 4.6A**. From the results, it is evident that although samples vary from each other, there is not enough separation between different weight groups if compared to the variation of the replicates for each weight sample, to draw any conclusion. Loadings scatter plot of PC1 vs PC2 (**Figure 4.6B**) shows the metabolites connected with the different regions of the score plot. The trend is that low weight samples extracts contain relatively more alkaloid, though in the high weight samples the variability was high for these compounds. Similarly, the primary metabolites seem to be higher in medium and higher weight samples. The higher weight samples seem to be more variable than the lower weight ones (**Figure 4.6A**).

As the PCA was unable to show any meaningful difference between different sample weights, it was decided to apply a supervised method (PLS-DA) to identify the effect of sample weight during the extraction of metabolites when the amount of solvent was the same for each sample. The samples were divided into four classes based on weight. The results (**Figure 4.6C**) showed that sample weight did have some effect on the metabolites extracted.

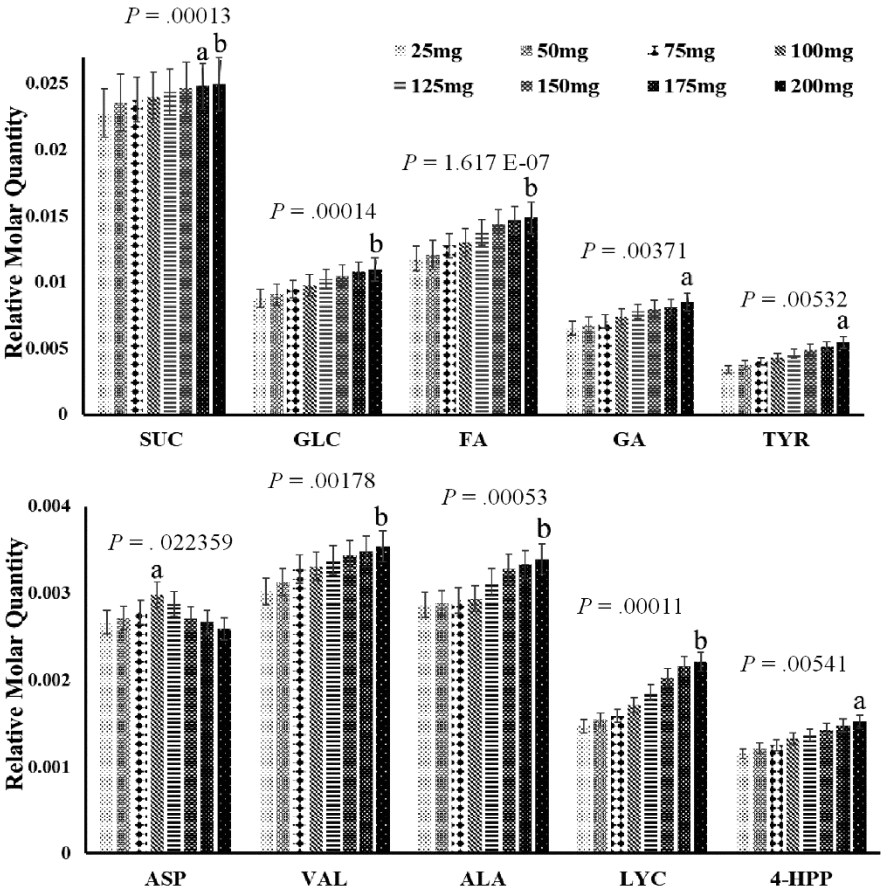

Figure 4.7. Relative molar quantities of some metabolites calculated from the ^{1}H-NMR spectra (600 MHz) of the extracts of different amounts of sample after normalizing all to the same amount of sample (50 mg). The molar amount is calculated based on the internal standard in the NMR-spectra.

Samples weighing 25, 50, 75, 100, 125, 150, 175 and 200 mg respectively (i.e. with sample/solvent ratio of 1/60, 1/30, 1/20, 1/15, 1/12, 1/10, 1/8.6 and 1/7.5) were labelled accordingly. One-way ANOVA was used for statistical analysis and Tukey's HSD (honestly significant difference) test (post-hoc test) for comparisons with 50mg sample (control sample) with 5 biological replicates and significance at (**a**) $p < .05$ and (**b**) $p < .01$ where error bars represent standard error (SEM).

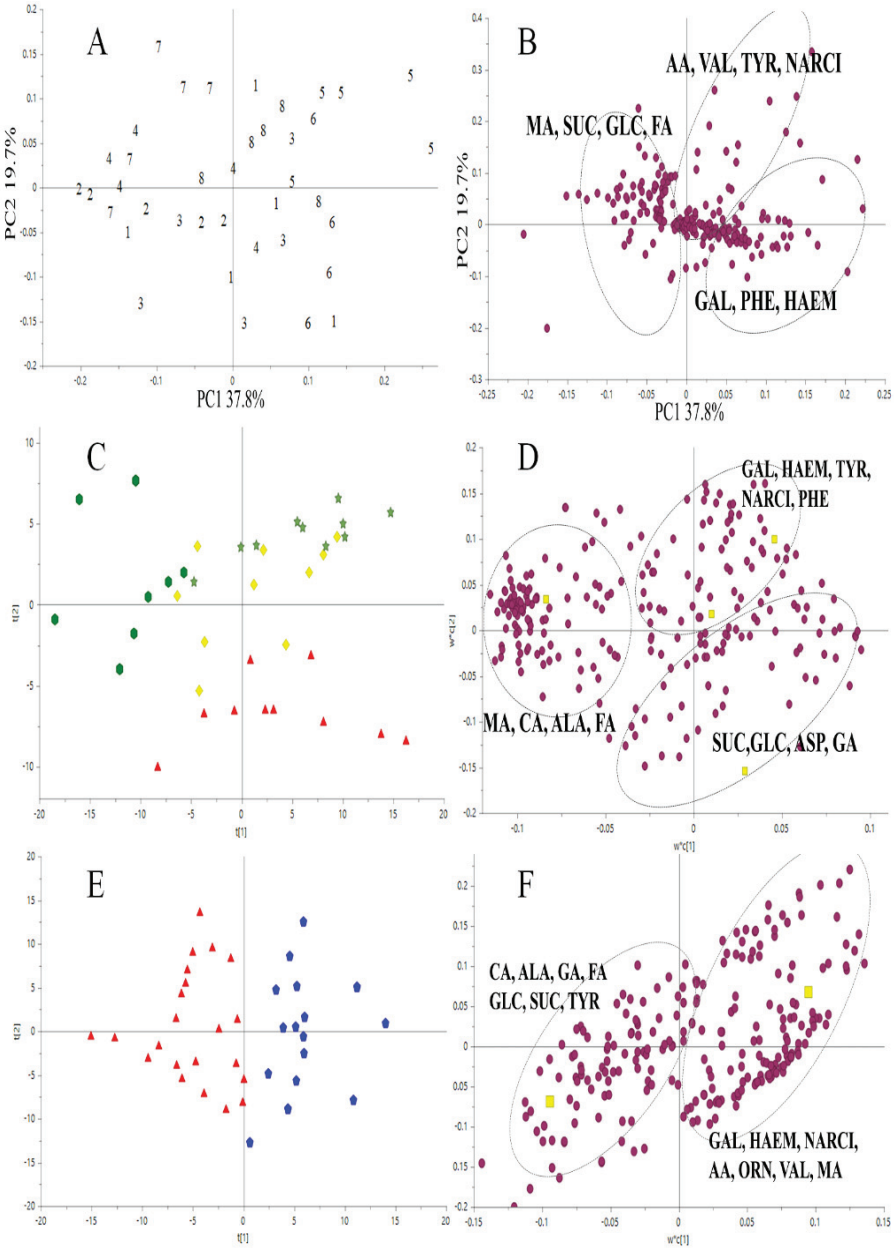

Figure 4.8. Multivariate data analysis of ^{1}H-NMR spectra (600 MHZ) of *Narcissus* bulb extracts made with different amounts of solvent with the same amount of sample.

A) Score plot of PCA (PC1 vs PC2) of bulb sample (50 mg) extracted with different amounts of solvent. Numbers (1-8) represent e increasing amounts of extraction solvent (1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 and 1.8 mL with five replicates each).

B) Loadings scatter plot of PCA (PC1 vs PC2) of extracts of the bulb samples showing the metabolites that correspond with the different regions of the PCA score plot A.

C) PLS-DA scatter plot (▲) class of samples extracted with 1.1 mL and 1.2 mL of solvent, (♦) class of samples extracted with 1.3 mL and 1.4 mL of solvent for each sample, (⬢) class of samples extracted with 1.5 mL and 1.6 mL of solvent for each sample and (★) class of samples extracted with 1.7 mL and 1.8 mL of solvent.

D) PLS-DA loadings plot of bulb samples (●) ^{1}H-NMR signal buckets important for the discrimination (■) average of the classes mentioned in Figure C.

E) PLS-DA scatter plot (⬟) class of samples extracted with less than 1.5 mL of solvent while (▲) class of samples extracted with 1.5 mL or more amount of the solvent.

F) Loadings plot showing the metabolites that correspond with the different regions of the PLS-DA score plot. PLS-DA models were validated by using permutation test (R^2 = 0.75, Q^2 = 0.61 with 100 permutations and 7 components)

Although the classes can be seen in groups based on weight, there is still some overlap between the classes. The loadings scatter plot (**Figure 4.6D**) shows that some of the amino acids, fatty acids and carbohydrates are relatively higher in the extracts of the high weight samples. Alkaloids and phenylalanine levels in the extracts are relatively higher in the low weight samples.

To further clarify the separation of groups on the weight basis samples were divided into two classes, samples with 100 mg sample weight or less were put into one class, while samples with more than 100 mg sample weight were put in another class. The results (**Figure 4.6E**) show that there is a clear separation between these two classes. It also shows (**Figure 4.6F**) that as the sample weight increases, the level of some amino acids, fatty acids and carbohydrates increase in the extracts, while the level of alkaloids along with phenylalanine are highest in the lower weight samples.

The multivariate data analysis shows relative changes but does not show the real absolute quantitative changes in the amount of a compound extracted when the weight of the sample increases. Therefore, the compounds responsible for the separation were quantified by relating them to internal standard. The results (**Figure 4.6**) also show that

the absolute amounts of carbohydrates, fatty acids, and most amino acids increases with the increase of the amount of sample extracted.

In the next part of the experiment, different amounts of solvent were added to a constant amount of sample (50mg) for all samples. When the results were subjected to PCA, it was apparent that although there is a separation between samples, there was no clear grouping based on amounts of solvent used. The PCA for the amount of solvent used for extraction of samples showed that the first two components accounted for 58% of the variation.

The score scatter plot of the PCA analysis is shown in **Figure 4.8A**. From the results, it can be concluded that samples are separated along both PC1 and PC2 but there is not enough grouping between different amounts of solvent used for extraction. Corresponding loadings plot was used to identify the contribution of spectral areas to the grouping of samples based on the amounts of solvent used for extraction. A loadings scatter plot of PC1 vs PC2 (**Figure 4.8B**) showed that carbohydrates and fatty acids are responsible for the grouping when the amount of solvent was lower while amino acids and alkaloids along with organic acids were causing the separation when the amount of solvent was higher (**Figure 4.8A)**. As the PCA was unable to show any meaningful separation among different amounts of the solvent, a supervised method (PLS-DA) was used to identify the effect of the amount of solvent during the extraction of metabolites when sample weight was same for each sample.

The samples were divided into four classes based on the amount of solvent used. The results (**Figure 4.8C**) show that amount of solvent does have some effect on the metabolites extracted. The classes can be seen in clear groups after each amount of solvent was put in a specific class, but the overlap between groups was still there. The loadings scatter plot (**Figure 4.8D**) showed that some of the amino acids, fatty acids and carbohydrates are high with the lower amount of solvents while alkaloids and some phenolic compounds and alkaloids are high with the higher amount of solvent. These trends are quite similar as found in the previous part of the experiment where the amount of plant material was increased for extraction with the same amount of solvent to change the sample/solvent ratio.

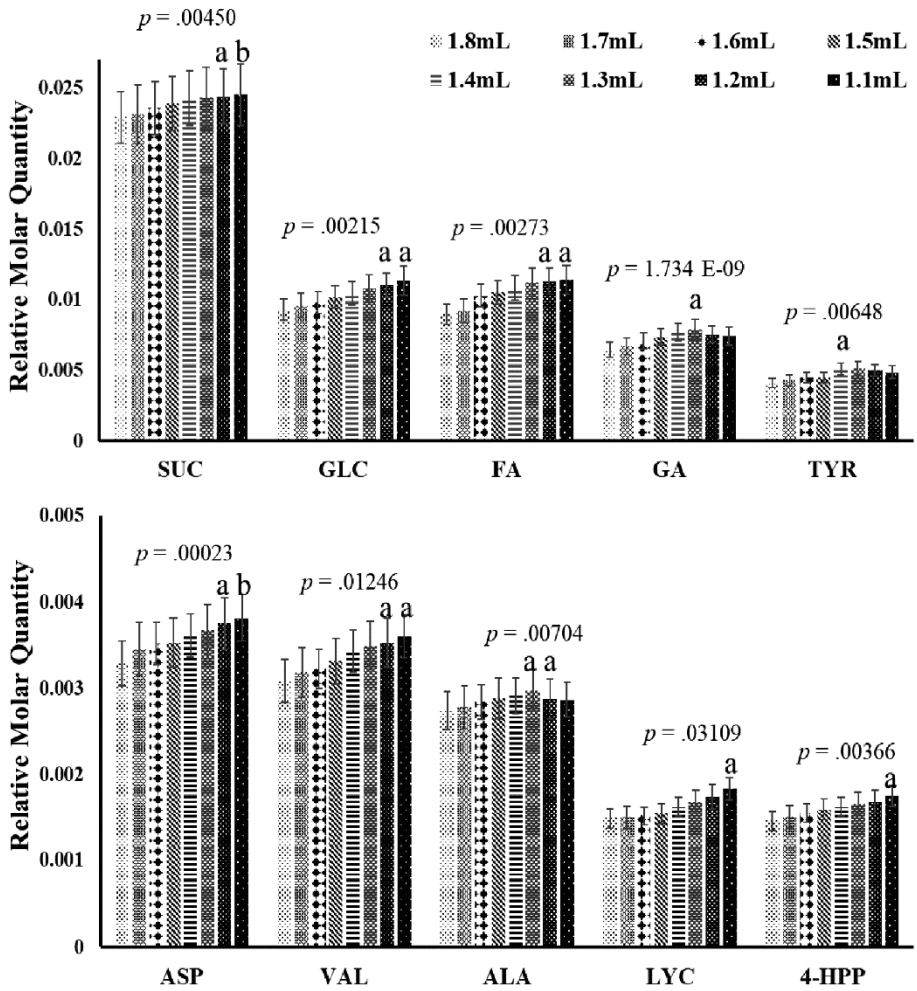

Figure 4.9. Relative molar quantities of some metabolites calculated from the ^{1}H-NMR spectra (600 MHz) obtained after extracting a fixed weight of sample with different amounts of solvent. The molar amount is calculated on the basis of the internal standard in the NMR-spectra. Varying amount of solvent was used for the extraction.

Samples were extracted with 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 and 1.8 mL solvent for 50 mg (with sample/solvent ratio of 1/22, 1/24, 1/26, 1/28, 1/30, 1/32, 1/34 and 1/36) solute respectively. Sample sequence is inversed to enable comparison with the results shown in Figure 4.6. One-way ANOVA was used for statistical analysis and Tukey's HSD (honestly significant difference) test (post-hoc test) for comparisons with 1.5mL sample (control sample) while using 5 biological replicates along with significance at (**a**) $p < .05$ and (**b**) $p < .01$ where error bars represent standard error (SEM).

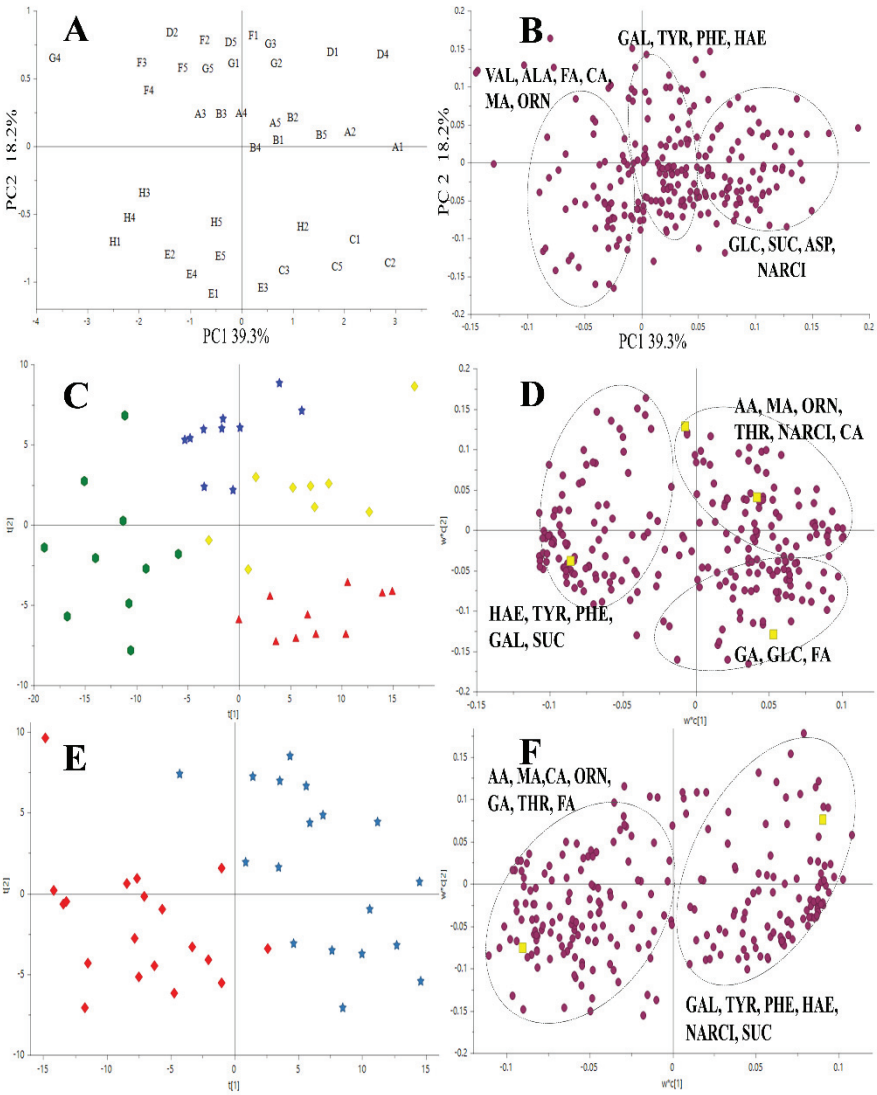

Figure 4.10. Multivariate data analysis of ^{1}H-NMR data (600 MHz) of extracts of *Narcissus* bulb samples obtained with different sonication time (15-120 min).

A) Score plot of PCA (PC1 vs PC2) NMR data bulb extracts. Labels A-H represent increasing sonication times and numbers 1-5 represent the biological replications of each time point.

B) Loadings scatter plot of PCA (PC1 vs PC2) of bulb samples showing the metabolites that correspond with the different regions of the PCA score plot A (5 replicates).

C) PLS-DA scatter plot (▲) class of samples extracted with 15 minutes and 30 minutes of sonication time, (♦) class of samples extracted with 45 minutes and 60 minutes of sonication time, (⬢) class of samples extracted with 75 minutes and 90 minutes of sonication time and (★) class of samples extracted with 105 minutes and 120 minutes of sonication time.

D) PLS-DA loadings plot showing the metabolites that correspond with the different regions of the PLS-DA score plot C (●) represents ^{1}H-NMR signal buckets from those important for the discrimination are labelled as mentioned before and (■) represents the average of classes.

E) PLS-DA scatter plot where samples are divided into two classes, (♦) class of samples extracted with 60 minutes or less sonication time and (★) class of samples extracted with more than 60 minutes' sonication time while

F) Loadings plot of the metabolites that correspond with the different regions of the PLS-DA score plot E. PLS-DA models were validated by using permutation test (R^2 = 0.89, Q^2 = 0.79 with 100 permutations and 9 components)

To compare the results to the normal/standard extraction method where 1.5 mL of solvent is used, samples were divided into two distinct classes, a first class where solvent amount used is less than 1.5 mL while second class has a solvent amount which is 1.5 mL or more than 1.5 mL.

The results (**Figure 4.8E**) show that there is a clear separation between these two classes with a very small overlap. **Figure 4.8F** shows that a higher amount of solvent for extraction, gives higher levels of alkaloids, some amino acids, organic acids and phenolics while levels of carbohydrates, fatty acids, other amino acids and organic acids increase with less amount of solvent or higher amount of sample weight i.e. by increasing sample solvent ratio. To identify the effect of the amount of solvent or sample on the absolute amounts of metabolites extracted, the relevant metabolites were quantified by comparison with the internal standard (**Figure 4.9**). Results clearly indicate that these quantities are consistent with the results from the sample weight when compared after sample/solvent ratio calculation.

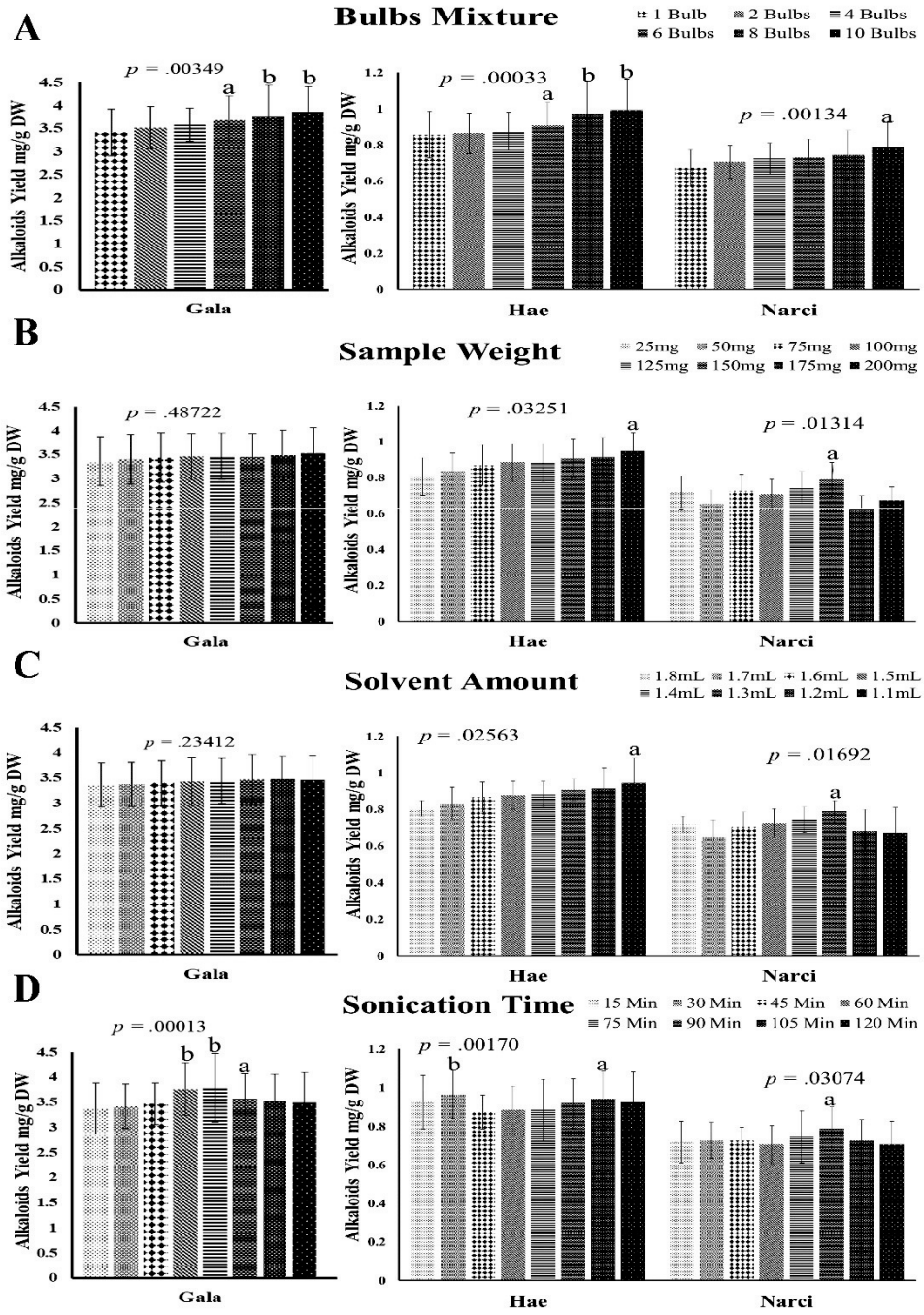

Figure 4.11. Levels of alkaloids found after different preanalytical treatments as calculated from the ^{1}H-NMR spectra of the plant material extracts after normalizing all

to the same amount of sample (50 mg). The amount is calculated in mg/g on dry weight basis on the basis of the internal standard TMSP in the NMR-spectra (600 MHz) of the extracts, (Gal = galanthamine, Hae = haemanthamine and Nar = narciclasine)

A) Mixing different number of bulbs for extraction to check the inter-bulb variation; 7 replicates for each number of bulbs

B) varying amounts of sample weight extracted by the same amount of solvent; 5 replicates for each weight

C) varying amount of solvent to extract samples of the same; 5 replications for each solvent amount.

D) Different sonication times; for 5 replicates for each condition.

Statistical analysis (one way ANOVA) of alkaloids was performed for each part of the experiment along with post hoc (Tukey's HSD (honestly significant difference) test and compared with A: 1 bulb sample, B: 50 mg sample, C: 1.5 ml solvent Sample, D: 30 minutes sonication time sample with significance at (**a**) $p < .05$ and (**b**) $p < .01$ where error bars represent standard error (SEM).

From the molar quantitation, it is clear that some of the metabolite levels started to increase with the increase in the sample/solvent ratio. From the **Figures 4.7 and 4.9** it becomes clear that the differences observed in the PCA analyses in absolute sense are rather small. The differences are in the order of 1-15%, and only a few are statistically different from the standard extraction procedure which have been used already for many years.

The results of the extraction of samples from the same ratio of sample and solvent when using different sonication times were subjected to PCA (**Figure 4.10A**). This shows some differences among samples, although grouping for sonication time was not possible. The first two components of the PCA for sonication time accounted for 58% of the variation between samples. Corresponding loadings scatter plot of PC1 vs PC2 (**Figure 4.10B**) showed that levels of various primary metabolites are involved in the separation between samples.

However, the PCA was not helpful to show any clear or meaningful difference among different sonication times, a supervised method (PLS-DA) was applied to identify the effect of sonication time. The samples were divided into four classes based on time used

for sonication. The results (**Figure 4.9C**) showed that there is clear grouping with slight overlap among the classes.

Loadings scatter plot (**Figure 4.10D**) showed that fatty acids along with glucose and glutamic acid were responsible for separating the first class where sonication time was close to the normal extraction times. While alkaloids and other phenolic compounds were higher in the classes where longer sonication time was used. To further clarify the separation of the groups, samples were divided into two classes i.e. samples with 60 minutes or less sonication time and samples which have more than 60 minutes' sonication time. The results (**Figure 4.10E**) showed a clear separation between these two classes with very small overlap. The resulting loadings scatter plot (**Figure 4.10F**) showed that with shorter sonication time for sample extraction, the levels of amino acids, fatty acids along with organic acids are more abundant. While at the same time, levels of sucrose along with alkaloids and other phenolics were at a higher level when sonication time was longer than the usual. From these results, it seems there is a clear difference in the extraction rate of the various metabolites. Sucrose, alkaloids and phenolics appear to need more time to be solubilized in the extraction solvent.

With the purpose to specifically monitor the changes in levels of galanthamine and other alkaloids, quantitative analysis of the major alkaloids were performed for all the experiments. **Figure 4.11** shows the results of the NMR-based quantitative analysis of alkaloids from the bulbs of *Narcissus* plants. It is already well known that galanthamine is the major alkaloid in the *Narcissus pseudonarcissus* var. Carlton bulb samples, followed by haemanthamine and narciclasine. 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 4.11**). For example, the experiment in which bulbs were mixed for a single sample yielded an F ratio of $F(5, 36) = 5.15452$, $p = .00349$ for galanthamine at .05 significance level. This F ratio and p-value indicated that there were clear significant variations when different number of bulbs were mixed for a single sample. To pinpoint the highest variation, samples were subjected to Tukey's HSD test. In the case of a number of bulbs in each sample, it became clear that the concentration of galanthamine was significantly higher in samples with multiple number of bulbs in the mixture used for extraction, when compared with the

extracts from single bulb samples (**Figure 4.11A**). Which is in accordance with the results of the multivariate data analysis shown in **Figure 4.5.** The figure clearly shows a significantly higher amount in the samples where more bulbs were mixed to obtain a sample in comparison to the single bulb samples. Same was the case with the other two alkaloids where higher number of bulbs gave higher amount of alkaloids. While the amount of galanthamine was affected by the number of bulbs, there was not any significant difference in the amounts of galanthamine when sample weight was changed. It shows that galanthamine have no significant change when sample weight was increased. Though for the other two alkaloids, there were significant change when the sample weight was varied. For haemanthamine, highest amount was obtained when 200 mg sample was extracted with 1.5 mL of solvent. While highest amount of narciclasine was obtained when 150 mg of sample was extracted with the 1.5 mL of solvent (**Figure 4.11B**).

Extraction of alkaloids with an increasing amount of solvent (**Figure 4.11 C**) showed a clear increase of alkaloids yields. In the case of galanthamine yield, the difference was statistically non-significant in samples. The amount of solvent had a significant effect on the other two alkaloids. Highest amount of haemanthamine was obtained when 50 mg was extracted with 1.1 mL of the solvent. While highest amount of narciclasine was obtained when 50 mg sample was extracted with 1.3 mL of solvent. At the same time, sonication did have a significant effect on the amount of alkaloids extracted from the samples with the same amount of solvent and sample weight. **Figure 4.11 D** indicates that in case of longer sonication time (60-90 minutes), a significant increase in all the alkaloids and specifically in galanthamine yield can be obtained in comparison with the normal extraction procedure which has 30 minutes sonication time.

General aim of the metabolomics field is to identify and determine the quantity of various metabolite in complex biological samples. These biological samples consist of two main parts, animal samples and plant samples. Plants samples are generally comprised of a diverse metabolome which have metabolites that differ in many aspects [17]. This diversification of metabolites is important under various circumstances. But its side effect can be that even the most advanced methods are incapable of a complete survey of all metabolites from a crude plant extract in a single run [18]. Though NMR spectroscopy

offers a means of non-invasive structural analysis of metabolites in any given sample. In the present study, the use of ^{1}H-NMR helped in qualification and quantification of different metabolites. The metabolites identified cover a vast structural diversity and include amino acids, organic acids, sugars, phenolics and alkaloids. This clearly suggests the immense analytical capacity of NMR spectroscopy to analyze a vast range of chemically diverse metabolites as compared to other platforms, a feature that is particularly useful for metabolomic studies [43]. NMR in combination with multivariate data analyses has been widely used as a fingerprinting tool for plants. The system has proved very effective for the metabolic characterization of not only cultivars but species as well [22, 23, 44-46]. These reports clearly suggest the enormous potential of this approach in metabolic characterization of plants that in turn can be very useful to explain different physiological behaviors and distinctive characteristics of plants species [21, 23, 47]. In the current study, NMR was used for the analysis of preanalytical variations on the alkaloid yield. Though both the PCA and the quantitative NMR show clear differences between the alkaloid levels found in the different experiments. It is also clear that the original protocol is quite robust in case of galanthamine as sample weight and solvent had no significant effect. Thought the other two factors (Bulb Mixture and Sonication time) did have a significant effect but it was not economically viable. The original protocol in which 50 mg of dry plant material is extracted with 1.5 ml of the NMR solvent with 30 min of sonication seems a robust procedure. This method was thus applied in a further study on preanalytical factors that may affect alkaloid yields.

Conclusions

Optimization of an extraction process as well as sample handling and sampling method for metabolite extraction is a crucial step in the quantitative and qualitative analysis of metabolites in biological materials. Different types of metabolites require different types of extraction methods to obtain reliable qualitative and quantitative results. In the current study, different sampling methods, as well as varying extraction processes, were applied to optimize the process for alkaloid extraction in general and galanthamine production specifically. The sampling method itself is considered as important in metabolomic studies as, for example, harvesting, type of sampling and grinding affect the availability of the metabolites [35].

In the case of *Narcissus,* normally a single bulb is used for each sample. But the problem with this kind of approach is the biological variability, e.g. bulbs vary in size as well as in their interaction with the environment during their growth. This may result in different levels of secondary metabolites. From the various experiments, it is clear that different factors may affect the yield of alkaloids, though this effect is in the range of at most about 8-15%. The overall conclusion is that the alkaloids are slowly released from the plant material and conventional extraction and sonication time are not optimal for total alkaloid extraction. Short extraction time shows that extracts have a relatively higher quantity of primary metabolites. Furthermore, to first chop the bulbs and wait around 30 minutes with the extraction can increase the galanthamine yield significantly. To nullify the effect of inter-bulb variation, we have tried different mixes of the bulbs in the sampling process. In the case of galanthamine and other alkaloid's yields, there was a significant increase, though small, in the quantity of galanthamine when there was more than one bulb in the sample mix. The ratio of the amount of solvent and weight of sample was another aspect which is considered important for metabolite extraction. Though some differences were found between the different ratios of plant material and solvent, the levels detected in the plant material were similar, so apparently for none of the identified compounds saturation occurs in the range of ratios applied.

Concerning extraction efficiency, solubility and dissolution rate are two different aspects that need to be considered. A slow dissolution process can be dealt with in two different ways. By extending the period of extraction or adding some form of energy to the system like temperature increase or ultrasonic treatment [35]. Ultrasonic treatment can be useful in terms of breaking the cell walls and to release the metabolites in the solvent. From the results, it is evident that a longer sonication time does increase the extracted amount of galanthamine and other alkaloids significantly. Although an extended period of ultrasonic treatment and increase in temperature may help in obtaining a higher yield of metabolites, they also increase the risk of artifact formation [48]. Here we used the ultra-sonication to provide energy to the system for a longer time-period to get higher yields of metabolites.

The previously reported NMR-based metabolomics method [49] for the analysis of the metabolome and galanthamine specifically in *Narcissus* plant materials seems to be a robust method.

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