Factors affecting galanthamine production in Narcissus

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Chapter V

Effect of Enzymatic Browning on alkaloid Contents of *Narcissus pseudonarcissus* Bulbs

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Abstract:

Horticultural products have great importance for crop business in The Netherlands. Some of the commercial crops (e.g. *Narcissus)* have great interest as a resource of bioactive compounds such as galanthamine along with their production of ornamental flowers and bulbs. In the bulb production, injury can badly influence the quality of the product, because injury will give way for different micro-organisms to infect the bulb. In this connection the enzymatic browning as a symptom of injury has received much attention due to its negative effect on the quality of various products, including bulbs. Although this type of browning may be beneficial in protection the product against infections, it should be only in a very limited degree. There are no previous reports of the effect of browning on the *Narcissus* bulbs and its effect on the metabolome and alkaloid content. The current study deals with the effect of browning on *Narcissus* bulbs and its metabolites. Results indicate that browning has an effect on the metabolites profiles present of the *Narcissus* bulbs, as can be learned from the multivariate data analysis of the ^{1}H-NMR spectra. However, calculations of the absolute amounts of galanthamine only showed only very little differences.

Keywords: *Narcissus*, Browning, polyphenol oxidation, galanthamine, NMR.

Introduction

Polyphenolic compounds are secondary plant metabolites found in all plant species. These compounds are involved in many processes. Various studies showed, for example, their role in enzymatic browning caused by the polyphenol oxidases (PPO) such as tyrosinase, polyphenolase, phenolase, and cresolase [1, 2]. This oxidation is widely found in nature and, among others, in most plant tissues [3]. Because of their involvement in browning of commercial plant products, and thus affecting the quality, PPOs have received much attention from researchers in the field of plant physiology and food science. Enzymatic browning is caused by oxidation of phenolic compounds to quinones and their eventual (non-enzyme-catalyzed) polymerization to dark colored melanin pigments [4]. Even though oxidation of phenols is a normal physiological process in plants, the significance of the PPO enzyme activity in plant tissues is not fully understood. But it is at least a limiting factor in the industrial processing of crops, as peeled, sliced, bruised or diseased tissues rapidly undergo browning. The oxidation products of the phenolic compounds appear to be involved in the defense of plants against invading bacteria, fungi, and viruses. Polyphenolic compounds seem to be more toxic to potential phytopathogens than the phenolic monomers, such as chlorogenic acid, from which they are derived. The PPO-catalyzed polymerization helps to seal the injured plant tissue as the start of the healing process, analogous to the formation of fibrin blood clots in injured humans.

In the processing of some products the PPO are important, such as in the processing of black tea [5], coffee [6] and cocoa [7]. The quality of the mentioned beverages is dependent on the formation of flavors. Oxidative browning does not occur in healthy intact plant tissues, perhaps because the phenolic substrates are physically separated from the enzyme [6, 8]. Senescence or injury results in destruction of the biological barriers between PPO and polyphenols and the enzymatic oxidation of the phenolic substrates starts [9]. However, the question is whether there are further factors, besides disruption of membrane integrity, that are involved in formation of the active enzymes. The PPOs do have some selectivity as, for example, the activity of apple PPO on tyrosine is much lower than on o-diphenols [10], which is typical for PPOs. Polyphenolic compounds have

been shown to possess beneficial properties, such as antimutagenic, anticarcinogenic, anti-glycemic, and antioxidative activity [11].

Daffodils (*Narcissus*) are very popular geophytes that are sold as both bulbs and cut flowers throughout the world [12, 13]. *Narcissus* bulbs are mostly used for reproduction, so they function as underground storage organs. Underground storage organs (bulbs) can act as a buffer against adverse conditions faced by the plant [14]. Bulb size and health are critical factors in overall plant growth. But these factors are particularly important in determining the plant's ability to flower and the quality of the flowers [15]. *Narcissus* are gaining more interest due to the presence of biologically active alkaloids. There are more than 100 alkaloids identified from *Narcissus* till date. These compounds possess a wide range of pharmacological properties such as antitumor, antiviral, antimalarial and acetylcholinesterase inhibitory activity [16-18]. The most important Amaryllidaceae alkaloids are galanthamine, narciclasine and haemanthamine. Among these alkaloids, galanthamine is already on the market as an FDA and EMA approved drug for the treatment of Alzheimer's disease. Narciclasine has a potent antitumor and anti-inflammatory activity [19, 20]. An interesting anticancer agent from *Narcissus* plants is the β-crinane-type alkaloid haemanthamine, which displays significant *in vitro* cytotoxic activity [21]. The levels of these alkaloids may vary in *Narcissus* bulbs due to various factors. One of these factors could be browning, the surface discoloring process well known from handling, processing, and storage of fruits and vegetables [22]. The PPO caused oxidation can also be seen in *Narcissus* bulbs when cut and exposed to the air for some time. This wound-induced reaction increases phenylalanine ammonia lyase enzyme activity and the phenolic metabolism in plant tissues [23]. It is important to know if this process in *Narcissus* affects the alkaloid levels as these also derive from the phenylpropanoid biosynthetic pathway. So far, nothing is known about the effect on the metabolome of bulbs after wounding and browning.

The aim of this study was to measure possible effects of browning of the bulbs on the total metabolome and on the levels of the individual alkaloids. The ^{1}H-NMR based metabolomics protocol (see previous Chapter 4) was used to measure the effect of browning after different types of cutting the *Narcissus* bulbs on the metabolome and the alkaloid levels.

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. [24] and extract was prepared according to the optimized procedure in the last chapter. Bulbs were rinsed after dusting to fully remove soil particles. Roots were removed along with small part of the basal plate. After that, some different methods have been used for wounding, after which the changes in metabolites levels were measured.

Bulbs were cut in three distinct ways. a bulb was cut vertically in half, or in 4 parts quarter. Some bulbs were fully chopped to a size of roughly 2×3 mm (more than 25 pieces) using a knife. Each type of cut bulbs were left for (2, 15, 30, 60 and 120 minutes) to observe the discoloration of the surface and its effect on the metabolites. For each treatment and each time point the bulbs were ground and dried in a freeze-drier for 2 days (5 replicates for each treatment and time point). Dried bulb material (50 mg) was weighed into a 2 mL microtube and extracted with a mixture of phosphate buffer (pH 6.0) and methanol-d_4 (1:1). Samples were ultrasonicated for 30 minutes, followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was used for further analysis.

^{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. [25]. ^{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:

Quantitative analysis of galanthamine, haemanthamine and narciclasine was performed by integration of the following 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). The ratio of the 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. 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:

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 studying alkaloid contents in narcissus bulbs several 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 5.1**).

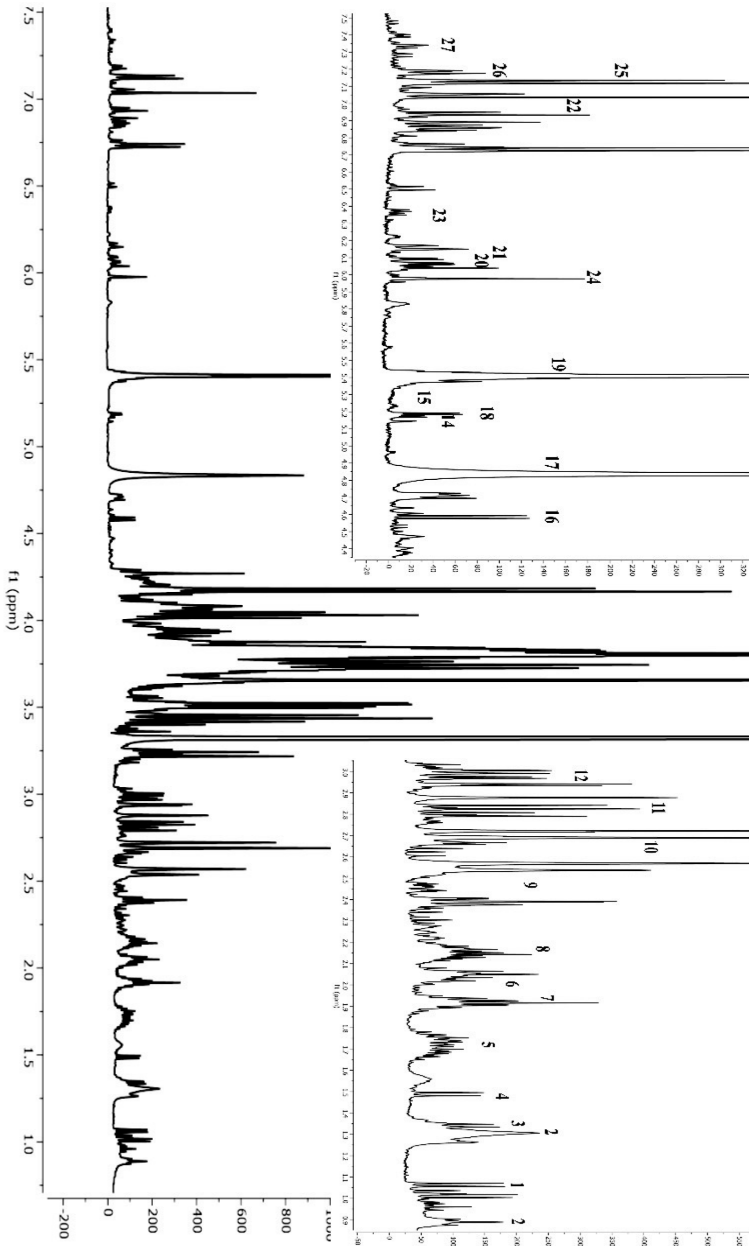

Figure 5.1. ^{1}H NMR spectra (600 MHz) from 0.8 to 7.5 ppm of phosphate buffer (pH 6.0) and methanol-d_4 (1:1) extract of *Narcissus* bulbs divided into three regions

according to signal abundance of the compounds labeled with numbers from Table 5.1.

Table 5.1. ^{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	Valine	VAL	1.06 (d) J=7.04, 1.01 (d) J=7.04.
2	Fatty acid	FA	1.31 (brs), 0.89 (t) J=7.1.
3	Threonine	THR	4.22 (m), 1.34 (d) J=6.6.
4	Alanine	ALA	1.49 (d) J=7.2.
5	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).
6	Acetic Acid	AA	1.91 (s).
7	Ornithine	ORT	3.71 (t) J=5.8, 3.24 (t) J=, 1.92 (m), 1.65-1.78 (m).
8	Glutamic acid	GA	2.39 (td) J=7.1, 2.5, 2.10-1.28 (m).

9	Citric acid	CA	2.71 (d) J=15.8, 2.56 (d) J=15.8.
10	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.
11	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.
12	Aspartic acid	AspA	2.82 (dd) 17.0, 8.5, 2.63 (dd) J=17.0, 9.5.
13	Choline	CHO	3.21 (s)
14	Mannose	MAN	5.40 (d) J=3.9, 5.17 (d) J=3.8.
15	Raffinose	RAF	5.41 (d) J=3.8, 5.27 (d) J=3.8.
16	Glucose	GLC	5.19 (d) J=3.8, 4.58 (d) J=7.9, 3.20 (dd) J=8.9, 8.8.
17	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.
18	Maltose	MAL	5.14 (d) J=1.5.
19	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.
20	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).
21	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).
22	cis-aconitic acid	cis-AA	7.03 (s).
23	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).
24	Lycorenine	LYC	7.06 (s), 7.04 (s), 6.02 (s), 5.74 (brs).
25	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.
26	Tyrosine	TYR	7.18 (d) J=8.4, 6.85 (d) J=8.4.
27	Phenylalanine	PHE	7.42-7.33 (m), 3.09 (dd) J=14.8, 8.3.

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 may create problems in the identification of compounds.

This problem can be overcome using different 2D techniques like J-resolved, ^{1}H–^{1}H COSY, HMBC, and HSQC, which provides 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 5.1**) 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 the 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 the type of bulb cutting, sample weight, amount of solvent, sonication time and effect of browning, all samples were compared

with each other as well as with the control non-treated standard sample. Principal component analysis was used to determine the inter-bulb variations and the effects of the treatments. Specific quantitative analysis of the major alkaloids showed that there were differences between differently cut bulbs.

The spectra of the different bulb samples obtained from the NMR-based metabolomics method were subjected to PCA analysis to get an overview of the changes in the metabolite profile after the different types of cutting and after different time points after cutting. The first two principle components for this PCA analysis model accounted for 62% of the variation in the data set. The score scatter plot of PCA analysis is shown in **Figure 5.1**. There is quite some variation between the replicates for the different treatments and time points, but for the different types of cutting one can observe some separation, the half and quarter are separated mainly in PC2, whereas the full chopping separates from these mainly in PC1. For the exposure time the separation is not clear.

Because of all overlapping samples in the unsupervsied PCA, a supervised MVDA was applied. By defining 2 or 3 different classes the effect of the experimental conditions can be separated from the noice of the biological variation. The supervised PLS-DA method was applied to identify first the effect of exposure time after cutting the bulbs when the type of the cut was ignored. The samples were divided into five classes depending on the exposure time after the cut.

The results (**Figure 5.3 A**) show that exposure time after the cut does influence the extracted metabolite profiles. Different classes based on the exposure time can be seen in groups but there is still some overlap. To identify the compounds involved in these variations, a loadings scatter plot (**Figure 5.3 B**) was used which showed that some carbohydrates along with galanthamine and other phenolic compounds were higher in the samples after about 30-minute exposure after cutting the bulbs. While some amino acids, organic acids, and high fatty acids were responsible for the grouping of samples when exposure time after cut was higher than 30 minutes. To clarify the differences based on the exposure time from the **Figure 5.3 A**, two different analyses were performed where samples were divided into three classes each. In the first PLS-DA analysis (**Figure 5.3**

C) samples were divided into three different classes i.e. 2 minutes, 15 minutes, and 30 minutes while other samples were ignored.

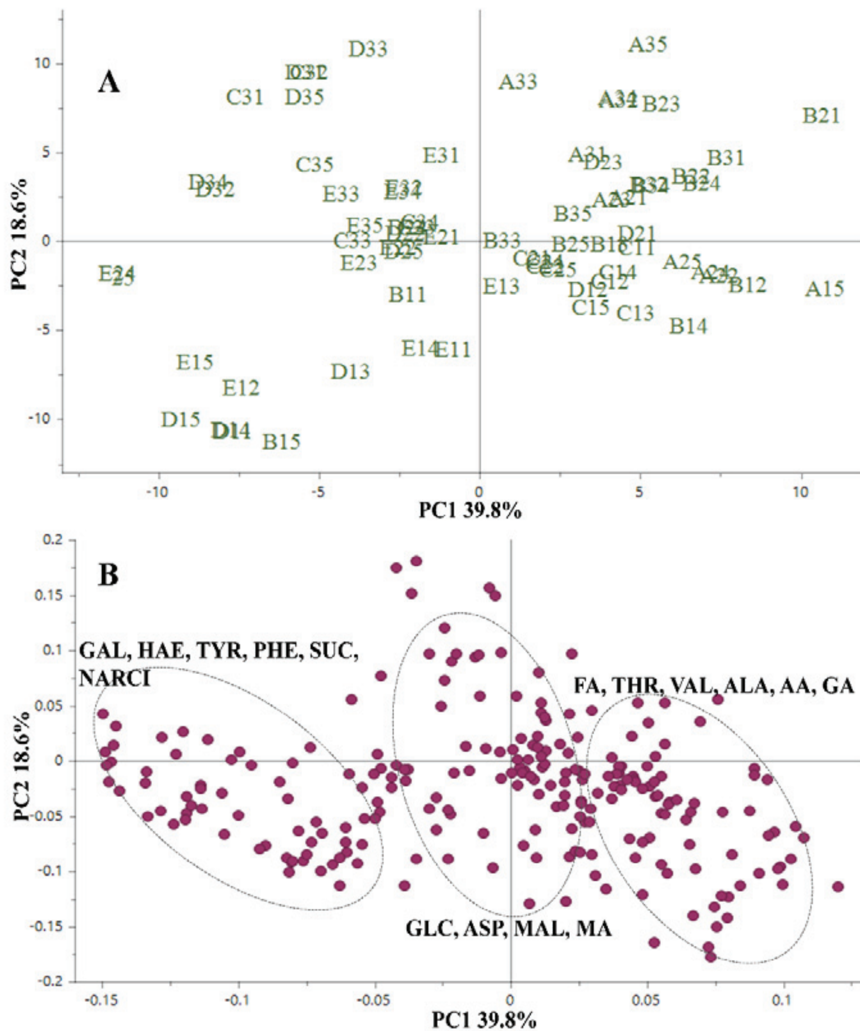

Figure 5.2. Multivariate data analysis ^{1}H-NMR data (600 MHz) of extracts of *Narcissus* bulb samples after different types of cutting.

A) PCA score plot (PC1 vs PC2) of NMR spectra of extracts of bulb samples. Labels A-E represents times (2, 15, 30, 60 and 120 minutes) after cutting, the first number (1, 2, 3) represents the three possible ways i.e. half, quarter and chopped and the second number (1-5) represents the number of biological replicates for each type of sample.

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

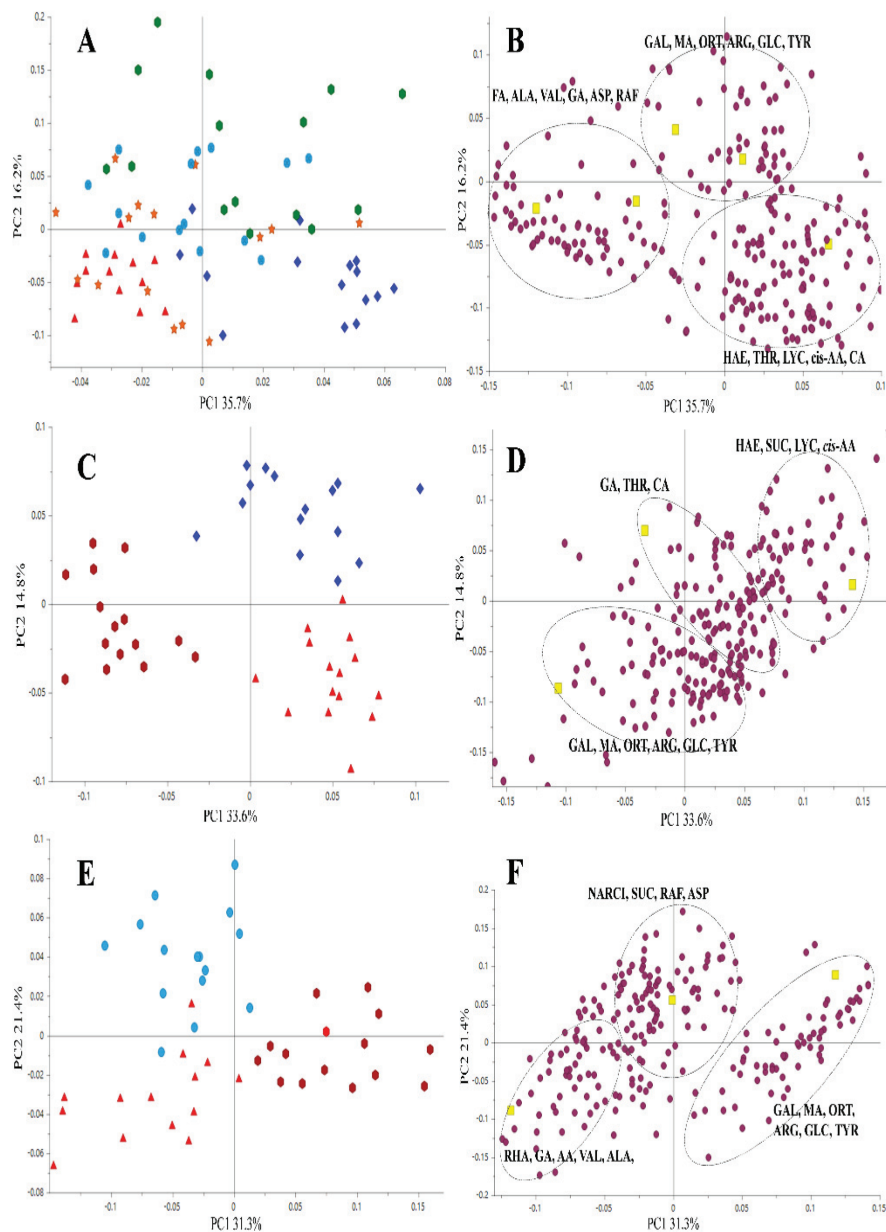

Figure 5.3. Multivariate data analysis ^{1}H-NMR data (600 MHz) of extracts of *Narcissus* bulb samples at different times after cutting. (see Figure 5.2 for PCA of all samples)

A) PLS-DA scatter score plot for 5 classes based on only time after the cut while the type of cut was ignored. (▲) class of samples left for 2 minutes after the cut, (♦) class of samples with exposure time of 15 minutes after the cut, (⬣) class of samples with exposure time of 30 minutes after the cut for each sample, (★) class of samples with exposure time of 60 minutes after the cut and (⊗) class of samples with exposure time of 120 minutes after the cut.

B) PLS-DA loadings scatter plot showing the metabolites that correspond with the different regions of the PLS-DA score plot A. (●) NMR signals (■) average of classes shown in Figure 5.3 A.

C) PLS-DA scatter score plot when samples were divided into three classes' i.e. (▲) class of samples left for 2 minutes' time after cut, (♦) class of samples of 15 minutes time after cut and (⬣) class of samples left for 30 minutes after the cut for each sample.

D) PLS-DA loadings plot of the metabolites that correspond with the different regions of the PLS-DA score plot C.

E) PLS-DA scatter score plot when samples were divided into three classes i.e. (▲) class of samples left for 30 minutes after the cut for each sample, (⬣) class of samples left for 60 minutes after the cut and (●) class of samples left for 120 minutes after the cut F) PLS-DA loadings scatter plot for longer time of exposure showing the metabolites that correspond with the different regions of the PLS-DA score plot E.

From the **Figure 5.3 C,** a clear grouping between the samples can be seen based on the exposure time after cut and loadings plot (**Figure 5.3 D**) confirms that for a shorter period a decrease in sugar and an increase of galanthamine are among the discrimination factors among the classes of samples. In the second PLS-DA analysis (**Figure 5.3 E**) samples were again divided into three classes i.e. 30 minutes, 60 minutes and 120 minutes. Again, clear grouping can be seen based on the time after cutting between all these classes thus making it clear from the loadings plot (**Figure 5.3 F**) that there were consistent variations in metabolite levels when exposure time after the cut is considered with galanthamine showing a slightly lower level after long exposure times.

To identify the variation in metabolites based on different types of cutting, samples were divided into three classes depending on the cut i.e. cut in half, cut in quarters and chopped. There is a clear separation between these groups on the cutting type basis. From the loadings scatter plot **Figure 5.4 B**, compounds responsible for the grouping can be identified. Apparently, alkaloids increase in level in the extract of bulbs subjected to multiple cutting and chopping, whereas sugars are low in this material. This might be due to the use of sugars after wounding for providing energy needed for the defence metabolism of the plant cells.

So far the analyses are based on relative values, therefore the next step was to calculate from the NMR spectra the absolute amounts of the three major alkaloids (**Figure 5.5**). First of these alkaloids was galanthamine. To check the statistical significance, bulb samples were subjected to two-way ANOVA with two factors i.e. type of cut and time after cut (exposure time). For type of cut three treatments (half, quarter and chopped) and for time after cut/exposure time, five treatments (02 minutes, 15 minutes, 30 minutes, 60 minutes and 120 minutes) were evaluated for their effect on galanthamine contents. From the statistical analysis it was clear that both main effects (type of cut and exposure time) as well as their interaction had a significant effect on the quantity of galanthamine at the .05 significance level.

The main effect of type of cut yielded an F ratio of $F(2, 60) = 7.25681$, $p = .00124$ which showed a significant effect. While the main effect of time after cut yielded an F ratio of $F(4, 60) = 4.67394$, $p = .00021$ which showed a significant effect. At the same time, their interaction (type of cut × time after cut) yielded an F ratio of $F(8, 60) = 9.41282$, $p = .00284$, indicated that difference in galanthamine contents were present when bulbs were cut in different ways and left for phenolic browning. Post-hoc (Tukey's HSD (honestly significant difference) test) revealed that chopped samples left for 30 minutes after chopping ($M = 3.686 \pm SEM = 0.18$) had the most significant variation when compared with chopped sample ($M = 3.327 \pm SEM = 0.14$) which was left for only 02 minutes (normally used method/control sample).

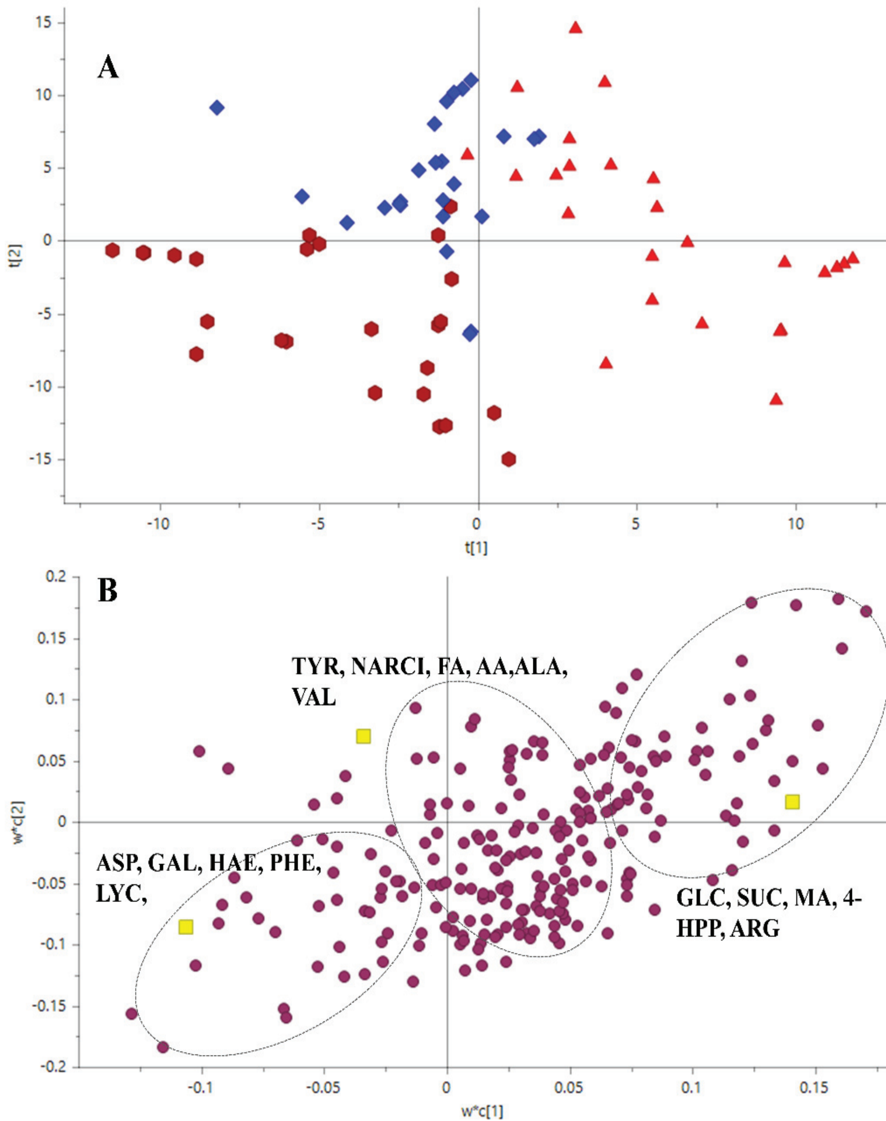

Figure 5.4. Multivariate data analysis ^{1}H-NMR data (600 MHz) of extracts of *Narcissus* bulb samples after different types of cutting.

A) PLS-DA scatter score plot where samples were divided into three classes based on type of cut when exposure time was ignored, (▲) class of samples where bulbs were cut in half, (♦) class of bulb samples cut in quarters and (⬢) class of samples where bulbs were chopped into small pieces for browning to take effect.

B) Loadings plot showing the metabolites that correspond with the different regions of the PLS-DA score plot A (●) different signals and (■) average of classes from the

PLS-DA. PLS-DA models were validated by using permutation test (R^2 = 0.80, Q^2 = 0.65 with 100 permutations and 6 components)

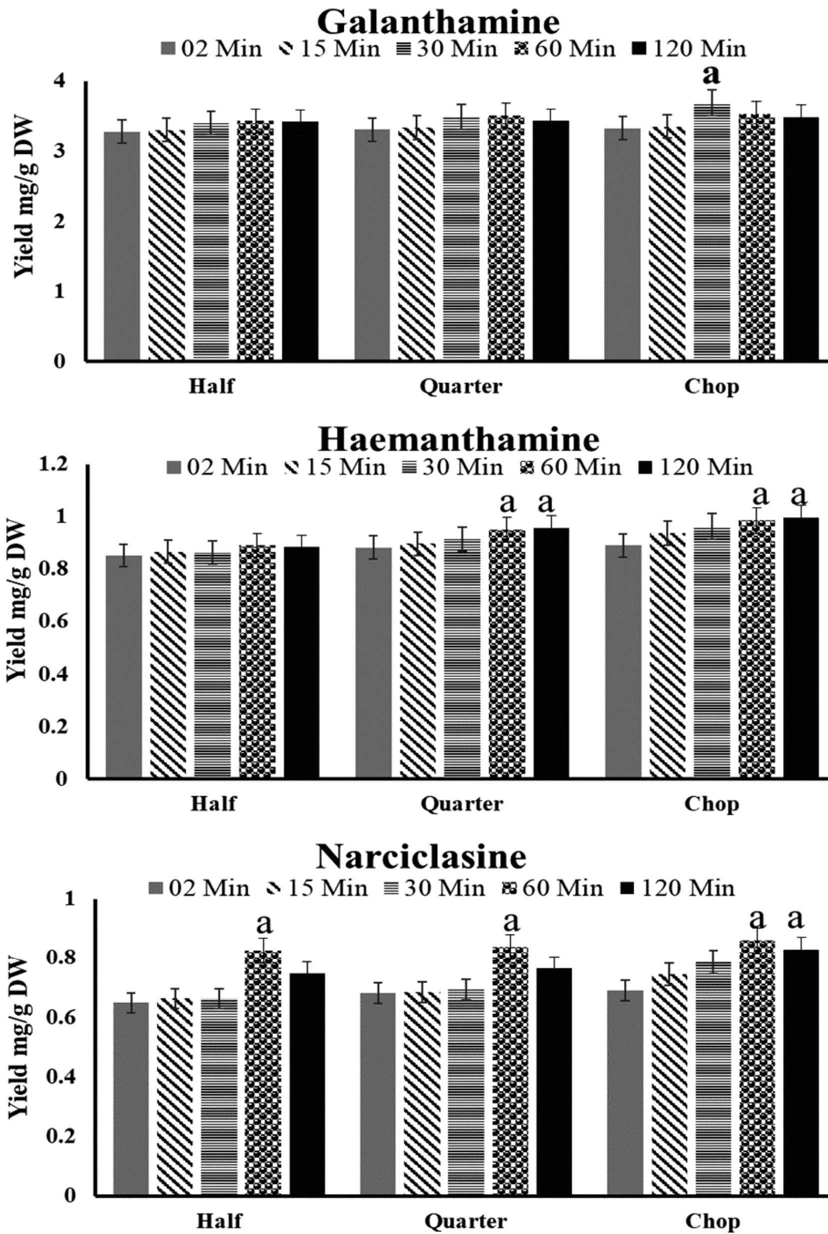

Figure 5.5. Absolute amounts of alkaloid calculated from the ^{1}H-NMR spectra (600MHz) of plant extracts (mg/g on dry weight) obtained after the different types of cuts and different exposure time for browning.

A) amount of galanthamine mg/g dry weight basis with different types of cuts at different time intervals,

B) amount of haemanthamine mg/g dry weight basis with different types of cuts at different time intervals and

C) amount of narciclasine mg/g dry weight basis with different types of cuts at different time intervals.

Statistical analysis (Two Way ANOVA) was performed along with Tukey's HSD (honestly significant difference) test (post-hoc test) for comparison purposes (samples compared with 2 minute sample in all cutting types) when 5 biological replicates were used with significance at (a) $p < .05$ and error bars represent standard error (SEM).

Second alkaloid was haemanthamine whose yield was subjected to two-way ANOVA. From the statistical analysis it was clear that both main effects (type of cut and exposure time) as well as their interaction had a significant effect on the quantity of haemanthamine at the .05 significance level. The main effect of type of cut yielded an F ratio of $F(2, 60) = 6.18291$, $p = .00362$ which showed a significant effect. While the main effect of time after cut yielded an F ratio of $F(4, 60) = 8.72183$, $p = .00128$ which showed a significant effect. At the same time, their interaction (type of cut × time after cut) yielded an F ratio of $F(8, 60) = 6.39857$, $p = .00584$, indicated that difference in haemanthamine contents were present when bulbs were cut in different ways and left for phenolic browning. Post-hoc (Tukey's HSD (honestly significant difference) test) revealed that chopped samples left for 60 minutes after chopping ($M = 0.985 \pm SEM = 0.06$) and 120 minutes after chopping ($M = 0.996 \pm SEM = 0.08$) had the most significant variation when compared with chopped sample ($M = 0.894 \pm SEM = 0.07$) which was left for only 02 minutes (normally used method/control sample). HSD test also revealed that bulb samples which were cut into quarters and left for 60 minutes after chopping ($M = 0.957 \pm SEM = 0.09$) as well as 120 minutes after cutting ($M = 0.948 \pm SEM = 0.07$) had the most significant variation when compared with quarter bulb sample ($M = 0.881 \pm SEM = 0.05$) which was left for only 02 minutes. It also revealed that there were less significant differences when the samples were halved and left for different time periods.

Narciclasine was the last alkaloid whose yield was subjected to two-way ANOVA. From the statistical analysis it was clear that time after cut/exposure time as well as its interaction with type of cut had a significant effect on the quantity of narciclasine at the

.05 significance level. The main effect of type of cut yielded an *F* ratio of $F(2, 60) = 1.76964$, $p = .17917$ which showed a non-significant effect. While the main effect of time after cut yielded an *F* ratio of $F(4, 60) = 3.24266$, $p = .01786$ which showed a significant effect. At the same time, their interaction (type of cut × time after cut) yielded an *F* ratio of $F(8, 60) = 5.03764$, $p = .00829$, indicated that difference in narciclasine contents were present when bulbs were cut and left for phenolic browning. Post-hoc (Tukey's HSD (honestly significant difference) test) revealed that samples left for 120 minutes after cutting ($M = 0.818 \pm SEM = 0.07$) had the most significant variation when compared with sample ($M = 0.664 \pm SEM = 0.08$) which was left for only 02 minutes after cut (normally used method/control sample).

Browning is a discoloring process which occurs mostly in fruits and vegetables due to damage during the product chain from field to customer. The main enzyme involved in the browning reaction is polyphenol oxidase causing the oxidation of phenolics, like caffeic acid derivatives in fruits and vegetables [23]. Although this reaction is studied quite extensively in fruits and vegetables, there are no reports on its effect on *Narcissus* bulbs and its metabolites during processing and storage. To study this effect, different types of cutting were applied to expose varying amounts of damaged area to this process. Moreover, different periods of exposure were also applied after cutting to find out the effect of this process on metabolite profiles in general and more specifically on the levels of galanthamine and other alkaloids. Metabolomics as a tool contributes to a better understanding of the metabolic changes caused by different factors during extraction. For metabolic characterization, NMR was used which offers a means of non-invasive structural analysis of metabolites in each sample. In the current study, the use of ^{1}H-NMR in combination with other 2D NMR techniques allowed the identification of several key metabolites of the *Narcissus* metabolome. The identified metabolites cover a vast structural diversity and include amino acids, organic acids, sugars, and alkaloids. This clearly shows the strength of analytical ^{1}H-NMR spectroscopy to quantitatively analyze a vast range of chemically diverse metabolites as compared to other platforms, a feature that is particularly useful for this type of metabolomics studies [26]. 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. The metabolic profiling of treated plants was performed using NMR spectroscopy in combination with multivariate data analysis. This approach enabled us to highlight the genuine differences among different processes as several classes of metabolites were identified by comparison of their spectra with a library of NMR spectra of standards run under identical conditions.

The differences between the alkaloid levels over time and after different types of cut were observed which showed some significant differences. A trend was observed of increasing yield of alkaloids with the increasing of exposure time before extraction. For example, a significant increase in galanthamine contents can be obtained from bulbs 30 minutes after chopping. Narciclasine and haemanthamine gave significantly higher levels in different types of cutting which were left for 1 and 2 hour after cutting. This is in accordance with the results of the multivariate data analysis. The time after chopping is apparently important for optimizing the production of a certain type of alkaloid, longer exposure times after chopping lead to an increase of the haemanthamine and narciclasine, whereas the galanthamine is slightly decreased under these conditions.

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