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The Effect of Acids on Alkaloid Yield in Pressurized Water Extraction of *Narcissus Pseudonarcissus*

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Highlights:

- Pressurized water (PW) extraction is an alternative method for extracting galanthamine from the plant matrix at a high yield.
- A high-pressure condition in PW extraction of galanthamine is not necessarily required.
- A high yield of galanthamine as a major product (ca. 74%-w) was obtained.
- Other *Narcissus* alkaloids, i.e. lycoramine, *O*-methyloduline, norgalanthamine, epi-norgalanthamine, narwedine, oduline, haemanthamine, *O*-methyllycorenine, and a haemanthamine derivate, were also extracted, at lower yield.
- PW extraction of galanthamine followed by subsequent purification steps of alkaloids has good economic prospects for industrial application.

Abstract. Pressurized water (PW) extraction of galanthamine from *Narcissus pseudonarcissus* bulbs was performed. The obtained yield was compared with the yield from conventional acidified water extraction and methanolic Soxhlet extraction. Both PW and conventional acidified water extraction were followed by a subsequent purification step for the alkaloids. The PW extraction (70 °C, 150 bar, 45 min) yielded as much galanthamine as methanolic-Soxhlet extraction (ca. 3.50 mg/g). Meanwhile, acid-base extraction with 1% of HBr (v/v) at 65 °C for 3 h gave a lower yield (ca. 2.65 mg/g). A higher PW temperature did not significantly increase the galanthamine yield. Pressure increase is not necessary since more water-soluble compounds such as proteins and polysaccharides are co-extracted, resulting in high viscosity of the water extract solution, which hampers the filtration process. Hence, the acidity of the solution is highly important both in the case of PW extraction and acidified water extraction. Besides galanthamine, the total alkaloid profile following *Narcissus* alkaloids was also obtained. Lycoramine, *O*-methyloduline, norgalanthamine, epi-norgalanthamine,

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narwedine, oduline, haemanthamine, *O*-methyllycorenine, and a haemanthamine derivate were identified. Although a high yield was obtained from PW extraction, the further purification needs to be improved to obtain an economically feasible industrial extraction process.

Keywords: *acid-base extraction; alkaloids; galanthamine; narcissus; water extraction.*

1 Introduction

Narcissus ranks third in popularity of common planted ornamental flowers in the Netherlands. *Narcissus* belongs to the Amaryllidaceae family and is well known for its ornamental flower value but also for its alkaloids, an important group of compounds for drug development [1]. Several alkaloids are found in *Narcissus* such homolycorine, lycorenine [2], galanthamine, narwedine [3,4], norgalanthamine [5], haemanthamine lycoramine [6], narciclasine [7] as well as other minor alkaloids. The *Narcissus* alkaloid, galanthamine, is the most well-known bioactive compound because of its anticholinesterase activity [8]. Galanthamine is used for the treatment of nervous diseases as well as Alzheimer's disease [9]. Reminyl is the name under which galanthamine is sold as medicine by Shire and Jansen in many countries [10]. Chemical synthesis is used for the production of galanthamine. Hence, the synthesis of galanthamine has been the subject of intense research over the past few years. Despite the efforts for chemical synthesis, natural sources of galanthamine still have great interest for the production. Several plants have been investigated as a resource of galanthamine, e.g. *Leucojum* and *Galanthus* [11]. The *Narcissus* bulb has received great attention as a promising resource due to the ease of large-scale agriculture using available production systems [12]. In addition, well-established cultivation or post harvesting systems have been developed, especially in the Netherlands, which makes the *Narcissus* a more sustainable resource for alkaloids than any other plant.

For drug development, the complex step of isolating the targeted natural products (NPs) for further study, such as structure activity studies and studies on pharmacological, and toxicological effects, is the most essential step. Extraction methods that can meet all the requirements related to safety, yield, selectivity, etc. are crucial. Finding an efficient extraction process, including purification, could lead to a sustainable and economically feasible green production process of galanthamine. The pharmaceutical companies holding the patents for the medicine have also patented the synthesis process. However, as both patents have run out, generics-producing companies do have an interest again in natural resources for producing galanthamine. Hence in the past years, an increased interest has developed in the optimization of the extraction of galanthamine from bulbs.

Among a wide range of natural alkaloids, nitrogen containing heterocyclic compounds in a broad sense are extracted by acid-base extraction due to their own basicity. Type of solvent, temperature, extraction time, particle size, and solvent to feed ratio (S/F) as well as natural characteristics of the raw materials [13,14] are the process variables that affect the extraction efficiency apart from the extraction technique itself. Alkaloids can be extracted from plants using three approaches: firstly, pre-treatment of the plant with alkaline to liberate alkaloids in free base form so extraction can be done more easily with water immiscible organic solvents. This approach is successfully applied when galanthamine is extracted with supercritical CO_2 because its relatively low free base form has lower polarity than its salt form [14]. Secondly, alkaloids can be extracted as their salts using either water or aqueous alcohol containing a dilute acid such as 0.1% trifluoroacetic acid (v/v). Unfortunately, some soluble polar compounds will be produced as impurities. Lastly, water soluble organic solvents such as methanol (MeOH) or ethanol (EtOH) can also be applied, extracting both salts and free bases of alkaloids.

For *Narcissus* alkaloids, extraction at laboratory scale is mostly done with MeOH as solvent because with this method a broad range of alkaloids can be extracted [15-17]. Generally, a small amount of dried plant material is used, typically in the range of ca. 150 mg to 5 g. Sagdullaev [18] conducted ethanolic-extraction of galanthamine from leaves of *Ungernia victoris*, followed by purification and crystallisation steps, yielding galanthamine-hydrobromide (galanthamine-HBr) as final product. Liquid-liquid extraction (LLE) with petroleum ether of basified dried bulbs of *N. pseudonarcissus* cv. Carlton was conducted, producing galanthamine-HBr, which was recrystallized by isopropanol [19]. Moreover, Agroceuticals Products (Wales, UK) claims to produce galanthamine from daffodils. Unfortunately, they have not clearly disclosed the extraction process. Compared with lab-scale extraction, little information has been published on the production of commercial galanthamine from bulbs. One cannot avoid the problems of the consumption of large amounts of toxic solvents. However, so far there is very limited information on the efficiency, yield, toxicity of residual and consuming solvents in the commercial production of galanthamine from plants.

In the extraction, not only the solvent or solute (target compounds) but also the plant matrix greatly influences the extraction efficiency. Interactions between the solvent-matrix are an important factor, just like the solvent-solute interactions. The swelling or damaging of the matrix should be considered in extraction studies. As discussed in Rachmaniah, *et al*. [14], the matrix structure of the *N. pseudonarcissus* bulb and localization of alkaloids in the cells hinders mass transfer by solvents (the effect on $scCO_2$). Therefore, it is challenging to find an extraction method that can solve all the encountered problems when extracting galanthamine from its plant matrix in view of enhancing the yield.

Water was chosen as solvent in this study. Apart from investigating the influence of the matrix, water can also well penetrate the cell membrane due its high polarity, thus swelling or damaging the plant matrix. Hence, pressurized water (PW) extraction was selected. By maximizing the swelling and the destruction of plant material under pressurized conditions, the galanthamine yield was expected to be enhanced. For comparison purposes, classical acidified water extraction and methanolic-extraction of the galanthamine from the plant matrix under the same extraction conditions (20 °C, 3 h) were conducted as well as methanolic Soxhlet-extraction. The extraction selectivity of the *Narcissus* alkaloids was determined by performing alkaloid profiling for each extract, both by GC-FID and MS.

2 Materials and Methods

2.1 Materials and Chemicals

Dried powder of *Narcissus pseudonarcissus* cv. Carlton bulbs was kindly supplied by Leenen BV (Sassenheim, The Netherlands). Reference compound of galanthamine-HBr (GAL-HBr) was provided by Tiofarma BV (Oud-Beijerland, The Netherlands). HPLC grade of acetonitrile (ACN) and methanol (MeOH), trifluoroacetic acid (TFA, >99%), glacial acetic acid (HAc), dichloromethane (DCM) of analytical grade, ammonium water (NH_4OH 25%, v/v), carbonate-bicarbonate buffer and 2 N of HBr solution were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Extractions

2.2.1 Pressurized Water (PW) Extraction

A high-pressure vessel system equipped with a shaft-impeller, a jacket-heating system, and temperature and pressure controllers was used. The first two extraction conditions were 40 °C and 70 °C at 150 bar. For comparison, 70 °C at ambient pressure (1.35 bar) was used for the extraction. Each extraction was performed twice. One % (w/w) of water was used for the extraction of *Narcissus pseudonarcissus* bulb powder, providing 25% space volume of vessel extraction to allow for homogenous mixing of solvent and plant. The total extraction time for PW extraction was 45 min, which consisted of 15 min for pressurizing and preheating, and 30 min for the extraction process. Carbon dioxide was used to pressurize the vessel, except for the autoclave condition (70 °C, 1.35 bar). No CO_2 is needed for pressurizing the vessel. Time point t = 0 was set so that the solution reached the predetermined temperature and pressure as shown by the indicators. After the extraction time (30 min) had elapsed, the vessel was depressurized, and the resulting solution was collected and vacuum filtered before going to the further purification step.

The purification step for further HPLC analysis followed alkaloid identification (Section 2.3.2) and quantification (Section 2.3.3). The obtained alkaloids in the aqueous soluation of PW extraction were purified applying the method described by Gotti, *et al*. [3]. The filtrate was basified to pH 9-10 in order to obtain the alkaloids as free base [3,18-21]. The subsequent alkaloid purification step was conducted with dichloromethane (DCM) to partition the alkaloids in free base form from the basified water fraction. The DCM fraction was evaporated to concentrate the alkaloids and dissolved in methanol for further analysis.

2.2.2 Conventional Alkaloid Extraction by Acidified Water or MeOH

Conventional acid extraction of alkaloids was conducted at laboratory scale using hydrobromic acid (HBr), HAc, and TFA as acidifying reagents. The extraction consisted of three-neck round bottom flask equipped with a reflux condenser and a mechanical half-moon impeller (Heidolph, Sigma-Aldrich, Zwijndrecht, The Netherlands). An overhead electric stirrer (IKA-Weke GmbH & Co.KG, Staufen, Germany), at 200 rpm, and a threaded adapter of glass bearing (Ace Trubore, Sigma-Aldrich, Zwijndrecht, The Netherlands) were also used. An oil bath was used as the heating medium. The experiments were performed using 4% (w/w) of dried *N. pseudonarcissus* bulbs to the weight of solvent at room temperature (20 °C) unless otherwise specified for 3 h. An inert nitrogen gas was streamed through the extraction system to minimize alkaloid decomposition. After the extraction, vacuum filtration was carried out over a Buchner funnel. Extraction using MeOH as solvent under the same extraction conditions was set up for comparison purposes. Each experiment was conducted in triplicate.

2.2.3 Soxhlet Extraction

Soxhlet extraction was conducted [22] using MeOH as solvent. A porous thimble 25 x 90 mm in size (Advantec TOYO 88RH, Toyo Roshi Kaisha Ltd., Tokyo, Japan) was filled with raw plant material. Extraction was conducted until colorless solvent reflux was produced.

2.3 Alkaloids Analysis

2.3.1 Galanthamine Analysis

The galanthamine yield was analyzed by high performance liquid chromatography (HPLC) equipped with a photodiode array detector detector (model 340-Varian). Prior to injection, the sample solution was filtered through a 0.45 µm membrane syringe filter. The HPLC analysis method by Mustafa *et al*. [21] was adopted. A C_{18} analytical column type 218MS54, 250 mm x 4.6 mm i.d, 5 µm, 100 Å (Vydac, Hesperia, CA, USA) equipped with a Vydac guard kit was

used. Five µL of filtered extract solution was injected into the column using an isocratic mobile phase acetonitrile: 0.1%TFA in water at a flow rate of 1.0 mL/min. UV detection was done at 210 nm and total running time was 25 min.

2.3.2 Alkaloid Profiling by GC-FID and MS

The alkaloid extract (Section 2.2.1) was also analyzed by gas chromatography-mass spectrometry (GC-MS) [2] as well as by gas chromatography-flame ionization detector (GC-FID) without derivatization step [2]. GC-MS was carried out on an Agilent 7890A GC system with a 5975C single quadruple Mass Spectrometric Detector and an Agilent 7693 Auto sampler (Agilent Technologies, Inc.). A DB-5 (30 m x 0.25 mm i.d., 0.25 µm film thickness) (JW Scientific, MA, USA) was used as GC column. It was employed with a 30-min temperature program of 200-250 °C at 2.5 °C/min, then 250-270 °C at 10 °C /min, followed by 8 min hold at 270 °C. The injector and detector temperatures were 250 and 270 °C, respectively. The alkaloid extract was dissolved in 3 mL MeOH. GC-MS and GC-FID were used to identify and quantify the alkaloids, respectively. One µL of each extract solution was injected. The flow rate of the carrier gas (Helium) was 1.5 mL/min and the split ratio was 1:20. The analysis was done in scan mode (m/z 50-350) using electron ionization at 70 eV. Identification was accomplished by comparing the measured mass spectral fragmentation data with the authentic compound (galanthamine) or with the data from the literature [2-4,6,16,23,24].

2.3.3 Alkaloids Quantification

The alkaloids were quantified by GC-FID. For this, a calibration curve was built by transferring different volumes within a 10-100 µL range of the galanthamine-HBr stock solution (78.7 µg/mL) to GC vials. After evaporation with a Speed Vac (Thermo Scientific, Waltham, MA, USA), 500 µL of internal standard solution (papaverine-HCl at 21.2 µg/mL) and 150 µL of 0.05% (v/v) HAc in MeOH were added to each vial. The sample preparation method was adopted from Gotti, *et al*. [3]. The injection volume was 1 µL of the resulting solution, following the GC method described above (Section 2.3.2). The ratio of peak area of the analyte, i.e. galanthamine free base, to the internal standard (papaverine) was plotted against the corresponding ratio of their weight to obtain the calibration graph. The linearity of the calibration curve (r^2) was 0.989, therefore the method proposed by Araujo in [25] was applied, proving that the proposed calibration curve had a positive linear correlation between ratio and peak area. By assuming a similar detector response in GC-FID, the amount of other identified alkaloids was calculated using the galanthamine calibration curve [3], expressed as µg galanthamine/g of dry weight of plant material.

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2.4 Microscopy

Scanning electron microscopy (SEM) was used to determine the structural changes of the raw material during the extraction process. The instrument used was a JEOL JSM-840 Scanning Electron Microscope (JEOL USA, Inc., Peabody, MA), operated at 5 kV, 20 mm. Samples were prepared by mounting them on specimen stubs. Excess material was gently blown off and the samples were coated with gold in the presence of argon gas using a Hummer I sputter coater (Technics, Inc., Alexandria, VA).

3 Results and Discussion

In order to increase the extraction efficiency and reduce the toxicity and environmental hazard of the utilized solvents, pressurised water extraction was applied to the extraction of galanthamine from *N. pseudonarcissus* cv. Carlton bulbs. Galanthamine, like any other natural product originated from fine chemicals, is produced by tedious and costly steps, requiring large amounts of organic solvents, which can cause health and environmental problems. When synthesizing 1 kg of a pharmaceutical bioactive compound, approximately up to 50-100-fold of chemical waste will be generated. Therefore, water was chosen as solvent in this study. Apart from being a green solvent due to its non-toxicity, water also has good penetrability into the plant matrix, minimizing the strong interaction between the solute and the plant matrix and it is also suitable for

solubilizing alkaloids in salt form. In addition, a pressurized condition will enhance the penetration of the solvent into the matrix.

To observe the selectivity of *Narcissus* alkaloids, acidified water extraction and methanolic extraction with the same extraction condition (20 °C, 3 h) were conducted for comparison purposes apart from Soxhlet extraction with methanol (MeOH). MeOH is a class-3 solvent that is allowable to be present at a small percentage by FDA. Soxhlet extraction with MeOH was chosen as benchmark method in this study since this method possibly produces higher yields than other conventional extraction techniques [22]. A special siphon design of Soxhlet allows condensed solvent to be rinsed back into a round flask. Carrying the extracted solute into the round flask, which also functions as solvent reservoir, is called a cycle. Each cycle is an equilibrium stage and fresh solvent continuously reaches the sample during each cycle. Therefore, saturation of solvent will never occur. Thus, Soxhlet extraction is an exhaustive extraction method.

3.1 Pressurized Water (PW) Extraction

The most important factor affecting extraction efficiency is the solvent itself, e.g. polarity, viscosity, volatility, diffusivity. However, in industry the use of organic solvent is limited, only a few non-toxic solvents are preferred for use. Then, other factors related to the matrix should be considered to improve the extraction efficiency. The structure of *N. pseudonarcissus* bulb matrix and the localization of the alkaloids in the cells act as mass transfer resistance, e.g. as shown in the case of the $scCO_2$ extraction process [14]. Therefore, it is a challenge to find an environmentally friendly, non-volatile organic solvent that can minimize or even eliminate this problem. Such a solvent should improve the swelling of the bulb material so the penetration of the solvent into the biomass results in a better extraction of alkaloids.

Considering the presence of alkaloids in water soluble protonated form together with various acids in vacuole (pH 4.5-5.0) [26] water is a good candidate for extraction solvent. In addition, water penetrates the cell membrane well due to its low molecular weight and thus the mass transfer resistance is minimized. Pressure can play a key role in extraction, especially in leaching. High pressure may be favorable to leach out the trapped solute from the strong interaction of the matrix by forcing the solvent into the matrix area. Herein, the solute was subsequently solubilized into a bulk of solvent. This may not happen under atmospheric condition. Like pressure, temperature may modify the properties both of the solute and the solvent, i.e. its viscosity, diffusivity, solubility as well as the surface tension of the solvent. Generally, solubility will increase as temperature increases. However, special attention should be paid when dealing with thermosensitive solutes.

For the PW extraction, carbon dioxide was used as pressuring gas. Water in contact with CO_2 becomes acidic due to the formation of carbonic acid [27]. Therefore, CO_2 was used to pressurize the vessel (Section 2.2.1). It has been reported that the pH of the CO_2/H_2O system will be in the range of 2.84-2.95 (at 25-70 °C and 71-203 bar) [27]. Consequently, by applying such conditions, an aqueous acidic solution (approx. pH 3) is expected. The acidic condition is assumed to be advantageous for the extraction of alkaloids because of the presence of alkaloids in their water-soluble protonated form together with various acids in vacuole (pH 4.5-5.0) [26]. Combining an acidic aqueous solvent with high capacity of swelling, the cell membrane is expected consequentially to enhance the solubility and extractability of protonated galanthamine from its plant matrix. Two different extraction conditions were applied for PW extraction: 40 °C and 70 °C, both at 150 bar, while the third, ambient extraction, was conducted at 70 °C at 1.35 bar. The third is also called autoclave extraction, since it is pressurized at the saturation pressure of water. Therefore, no CO_2 is needed to pressurize the extraction vessel. The third condition was conducted to further know the effect of acidic condition, both on the selectivity of alkaloids and the yield of galanthamine. For that reason, autoclave extraction at 40 °C 1.35 bar was not conducted.

Unfortunately, the expected pH solution of 2.80 or 2.86 (for 40 °C and 70 °C both at 150 bar [27]), respectively, could not be achieved. A slightly acidic condition, ca. pH 5.2-5.4, was obtained for PW extraction. This was apparently because the water was not in equilibrium with the CO_2, hence limiting the dissolution of the CO_2 in the water. However, application of high pressure is required to keep water in liquid state as well as to maximize its penetration into the plant's matrix. Providing a high penetration capacity of water to the cells results in a higher yield of galanthamine. The obtained aqueous filtrate was neutralized and basified to pH 9-10, followed by extraction of the alkaloids with a non-water miscible organic solvent [3,18-21]. Kreh [28] reported quantitative extraction of galanthamine from aqueous phase at pH values >9.0. Considering the pK_a of galanthamine, it is particularly important for the purification process by means liquid-liquid extraction (LLE) to be successful in order to maintain the basified solution in the range of pH 9-0 [19], i.e. a pH slightly higher than the pK_a of galanthamine ($pH \geq pK_a+1$). A carbonate-bicarbonate buffer solution (around pH 9.6) was applied to basify the aqueous filtrates [3]. In fact, this buffer may minimize the emulsion problem, especially when an organic solvent is used for further LLE.

The free bases, including galanthamine, were in the organic layer. Meanwhile, polar non-alkaloid substances remained in the aqueous layer. Previously, a washing step of the dichloromethane (DCM) was included using aqueous carbonate-bicarbonate buffer solution (pH 9.6) [3] to remove hydrophilic

impurities. Therefore, DCM was added to extract the free bases from the basified aqueous layer. Dichloromethane is thought to be less toxic than chloroform [29]. This aqueous extraction process only requires one step to remove non-alkaloids (although more polar primary metabolites such as sugars or amino acids are extracted by water), whereas in the alcoholic extraction method an additional LLE is required to obtain the alkaloid fraction. Aqueous acidic extracts contain only small amounts of lipophilic compounds [30]. In case of extraction using an alcoholic solvent, i.e. MeOH, the next step is a liquid-liquid aqueous extraction to remove the lipophilic non-alkaloids. This can also be done after evaporation of the organic solvent. After the residue is re-dissolved in acidic water, however, the thick viscous residues make the solubilization in water more difficult.

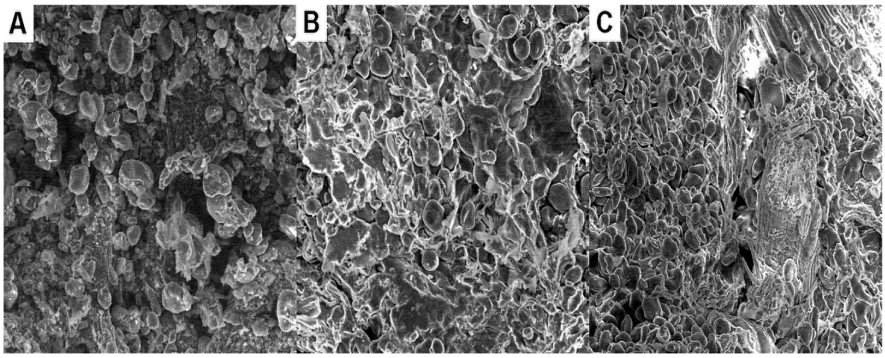

Figure 1 SEM images of Narcissus pseudonarcissus residues of (A) HBr 1% (v/v) at 65 °C, (B) MeOH, and (C) pressurized water (PW) extractions (70 °C, 150 bar) of bulb powder of Narcissus pseudonarcissus. Images are registered at 700x magnification.

The lowest galanthamine yield was obtained by applying the autoclave method (70 °C, 1.35 bar). In this case, water soluble compounds were more effectively extracted from the plant material, e.g. proteins and water-soluble polysaccharides [30]. A substantial amount of colloidal substance was indeed observed in the filtrate. The lower yield was probably due to the absence of an acidic condition in the solution; like in the autoclave method no CO_2 was present in the vessel. Consequently, the pH of the aqueous solution was around neutral, ca. pH ~6.1.The extraction yield can be increased not only by changing the acidity but also through the matrix effect. The cell structure of the *N. pseudonarcissus* residue from PW extraction (Figure 1C) was slightly altered compared to the residue from MeOH extraction (Figure 1B). The globular granules seem more prominent. SEM of the autoclave extraction residue was not possible because of the formed muddy paste residue, which is not suitable for SEM preparation.

Table 1 Alkaloid content of pressurized water (PW) extracts after subsequent purification step.

Alkaloids name	Formula (MW) [1]	Alkaloids content [a] ± SD [b]		
		Autoclave (70 °C, 1.35 bar)	PW1 (40°C, 150 bar)	PW2 (70°C, 150 bar)
Galanthamine	$C_{17}H_{21}NO_3$ (287)	1836.3±33.3	3223.6±101.6	3467.4±71.6
other alkaloids		914.4	1416.8	1217.5
Lycoramine	$C_{17}H_{23}NO_3$ (289)	1.9±0.6	7.9±0.6	6.5±1.3
O-Methyloduline	$C_{18}H_{21}NO_4$ (315)	90.2±2.5	214.3±6.2	196.6±37.5
Norgalanthamine	$C_{16}H_{19}NO_3$ (273)	38.0±8.9	22.4±1.1	15.7±3.0
epi-norgalanthamine	$C_{16}H_{19}NO_3$ (273)	32.0±5.4	27.9±2.4	49.9±11.8
Narwedine	$C_{17}H_{19}NO_3$ (285)	6.5±2.1	27.3±0.6	25.6±4.9
Oduline	$C_{17}H_{19}NO_4$ (301)	29.9±15.2	66.2±33.3	13.1±3.2
Haemanthamine	$C_{17}H_{19}NO_4$ (301)	513.7±1.3	717.7±5.7	588.7±6.8
O-Methyllycorenine	$C_{19}H_{25}NO_4$ (331)	169.6±0.8	242.7±3.8	264.6±54.8
Haemanthamine derivate [2]	N. D [c]	32.6±0.3	63.4±3.0	56.8±11.3

[a] Yield of alkaloids other than galanthamine are expressed as galanthamine (μg/g of dry weight); [b] Standard deviation (SD) calculated from three replicates; [c]N.D = not determined.

3.2 Conventional Extraction Method of Alkaloids

Acidified water extraction as common analytical-scale alkaloid extraction method was also performed, as well as methanolic Soxhlet extraction. Instead of H_2SO_4 or H_3PO_4 other acidifiers were used, such as hydro bromic acid (HBr), trifluoroacetic acid (TFA), and acetic acid (HAc). Their effects on the extraction process parameters, i.e. the yield of galanthamine and the selectivity of the alkaloids were studied. However, MeOH was the sole solvent used for Soxhlet extraction in this study, due to its broad use for laboratory scale extraction of alkaloids [3,18]. Hydrobromic acid was chosen because galanthamine is usually produced as HBr-salt, while TFA was chosen because Mustafa, *et al*. in [21] effectively extracted galanthamine from its plant matrix with 0.1% (v/v) of TFA. In addition, HAc is believed to better soak and swell the bulb matrix. Moreover, methanol (MeOH) extraction of dried powder of *N. pseudonarcissus* with the same condition as acidified water extraction was conducted as control because the methanolic Soxhlet extraction of *N. pseudonarcissus* gave the highest yield of galanthamine (Table 2). Four percent of dry weight of *N. pseudonarcissus* bulb powder in acidified water was used in the extraction. This ratio is needed to provide a properly mixed material in the extraction process. An inappropriate solid-liquid ratio may prevent effective mixing since the plant material swells during the extraction.

A high yield of galanthamine was obtained both by applying PW and acidified water extraction (Tables 1 and 2), even higher than the extraction control condition with MeOH. Apparently, its yield is like that of methanolic-Soxhlet extraction (Table 2). One percent (v/v) HBr, TFA, and HAc as acidifying

substance all gave a high yield of galanthamine (Table 2) though the yield was not as high as with exhaustive Soxhlet extraction. Undoubtedly, acidic water is an excellent solvent for the extraction of alkaloids from its biological matrix. Particularly other alkaloids had much higher yields with TFA than any of the other extraction methods. This is probably due to the more appropriate selectivity of the TFA to the alkaloids compared to the other acidifying substance.

Due to the high acidity of the aqueous solutions, ca. pH 1.9-3.4, respectively, for HBr (1%, v/v) and TFA (0.1%, v/v), galanthamine was ionized and thus effectively extracted. A solution with a pH at least one unit below the pK_a of galanthamine (the pK_a of galanthamine is 8.32 [31]) is needed to ensure that the galanthamine is ionized. Even though TFA is less acidic compared to HBr, ion-pair formation between protonated galanthamine and ionized TFA provides effective extraction. TFA (0.1%, v/v) yielded 2.25 mg/g of galanthamine compared to only 0.75 mg/g of galanthamine with HBr (0.1%, v/v). By increasing the amount of HBr as acidifier up to 1% (v/v) and the extraction temperature to 65 °C, the galanthamine yield was increased almost five-fold. The higher acidity thus may give better penetration of the plant matrix, resulting in a higher alkaloid yield.

A similar extraction condition using MeOH, the extraction control condition, gave a lower galanthamine yield (Table 2). A possible explanation for this is that MeOH does not cause as severe swelling of the plant matrix as water. This hypothesis was proven by observation of the residual plant material using SEM (Figure 1A and 1B). Although MeOH does not swell the *Narcissus* bulbs powder as much, it is more selective towards galanthamine as well as norgalanthamine, haemanthamine, and haemanthamine than other *Narcissus* alkaloids. This MeOH selectivity is shown by the alkaloid profile of the extract from methanolic Soxhlet extraction (Table 2). Soxhlet extraction was proven to be an exhaustive extraction method for *Narcissus* alkaloids. Fresh solvent continuously reaches the sample during each cycle; no solvent saturation will occur [22]. Particularly *O*-methyllycorenine was much better extracted with HBr (1%, v/v, 65 °C) than with MeOH. Despite its capacity to swell and penetrate the cell membrane, acidified water extraction also has disadvantages. Like many other polar plant components [32-34], proteins, and phosphatides, water-soluble polysaccharides are also co-extracted [30]. As storage organs, *Narcissus* bulbs accumulate polysaccharides as their major source of reserve energy [35]. Moreover, Lubbe, *et al*. [36] also identified the presence of sugars like raffinose, sucrose; organic acids such as citric acid, acetic acid; fatty acids; and amino acids such as asparagine, aspartic acid, glutamic acid in *N. pseudonarcissus* cv. Carlton bulbs. Several primary metabolites will thus be co-extracted and usually in larger quantities than secondary metabolites.

3.3 Alkaloids Profile

We found that both pressurized water (PW) and conventional acidified water extraction have similar selectivity for *N. pseudonarcissus* cv. Carlton alkaloids. However, a higher yield of galanthamine was obtained with PW compared to acidified water extraction (Table 1). Nine alkaloids were identified in the extracts: lycoramine, *O*-methyloduline, norgalanthamine, epi-norgalanthamine, narwedine, oduline, haemanthamine, *O*-methyllycorenine, and haemanthamine derivate (Tables 1 and 2) besides galanthamine, the targeted compound.

Haemanthamine is always found as the second major alkaloid in the extracts of *N. pseudonarcissus* after galanthamine. Haemanthamine is one of the most frequently found alkaloids in the genus *Narcissus* together with galanthamine and *O*-methyllycorenine [37]. As a tertiary alkaloid (pK_a value of 6.95 [38]) haemanthamine is extracted quantitatively from the aqueous phase with dichloromethane (DCM). Haemanthamine (MW 301 [28]) will partly decompose under GC conditions even when injected without thermal stress by on-column injection at 70 °C [2] decomposition occurred. This means that decomposition occurs during chromatography on the GC-column at an the elution temperature of 260 °C. However, further isolation and spectroscopic studies are necessary for its identification and structure determination.Although epi-norgalanthamine was not extracted with MeOH under the same extraction conditions as acidified water extraction (Table 2), these results coincided with the alkaloid profile of exhaustive methanolic Soxhlet extraction, which extracted galanthamine, lycoramine, norgalanthamine, haemanthamine, and *O*-methyllycorenine. Apparently, the type of acid has a strong effect on the extraction efficiency of some alkaloids. Trifluoroacetic acid results in the highest yields of all alkaloids.

Epi-galanthamine may be formed by epimerization of galanthamine in ca. 10% (v/v) sulphuric acid at 70 °C [28]. However, epi-norgalanthamine was reported as a native alkaloid of *Narcissus leonensis* [39]. Both norgalanthamine and epi-norgalanthamine exhibit a similar fragmentation pattern, i.e. *m/z* 273, 272, 230, 202, and 174. However, their base peaks are different: m/z 272 and *m/z* 273 for norgalanthamine and epi-norgalanthamine, respectively. There are many isomers among the Amaryllidaceae alkaloids [2]. Both PW and water extraction resulted in high yields of galanthamine, ca. 3.50 and 2.65 mg/g respectively for PW (70 °C, 150 bar, 45 min) and HBr (1%, v/v, 65 °C, 3 h). Other researchers have reported much lower galathamine yields. Sun, *et al*. [40] only yielded 0.0903 mg/g of galanthamine using ultrasound assisted extraction (UAE) for extracting galanthamine from *Lycoris radiata*. Tian *et al*. [41] obtained the lowest yield, at 0.0294 mg/g of galanthamine, when galanthamine from *Lycoris aurea* was extracted with enzyme-assisted extraction.

Table 2 Alkaloids content of acidified water extracts after subsequent purification step including methanol as a control.

Alkaloids name	Alkaloids content [a] ± SD [b]							
	A[c]	B	C	D	E	F	Control (MeOH)	Methanolic Soxhlet Ex.
Galanthamine	745.6±3.9	2322.9±12.7	2649.6±15.5	2111.2±19.4	1693.7±2.8	2248.1±3.3	1402.1±5.7	3451.0±0.1
Other alkaloids	593.8	1127.6	1242.6	1222.1	950	3079.3	3291.4	1622.4
Lycoramine	23.0±5.0	3.9±0.1	5.4±1.2	8.5±0.3	9.5±0.8	53.6±0.3	154.0±1.0	184.1±4.2
O-Methyloduline	21.3±12.5	119.1±5.0	165.8±16.0	141.5±32.1	79.3±2.9	229.4±0.9	130.0±0.4	N.N [d]
Norgalanthamine	2.4±0.1	21.0±1.3	11.2±1.0	55.8±0.9	59.2±5.0	414.7±0.9	567.8±1.8	165.4±0.1
epi-norgalanthamine	18.8±1.2	48.9±7.8	44.1±7.9	27.0±5.4	36.5±0.6	73.3±0.4	N.N [d]	N.N [d]
Narwedine	10.4±0.1	17.1±1.5	20.9±3.4	26.3±0.3	14.6±0.2	42.2±0.3	33.3±0.1	N.N [d]
Oduline	24.2±0.9	69.9±2.9	83.8±8.0	18.6±0.4	7.3±0.6	132.9±0.7	31.3±0.1	N.N [d]
Haemanthamine	372.2±2.8	627.6±51.3	668.7±34.0	691.6±32.0	592.9±6.7	1753.7±1.5	1728.9±9.1	1060.6±2.3
O-Methyllycorenine	78.6±0.5	190.0±14.9	219.9±21.4	224.2±6.3	111.5±1.9	198.7±0.3	30.2±0.3	212.3±0.8
Haemanthamine derivate	42.9±6.8	30.1±8.4	22.8±0.3	28.6±6.3	39.2±2.9	180.8±1.0	615.9±5.0	N.N [d]

[a] Yield of alkaloids other than galanthamine are expressed as galanthamine (µg/g of dry weight), assuming a similar detector response for all alkaloids in GC-FID.
[b] Standard deviation (SD) calculated from three replicates.
[c] Various acidifying reagent at different concentrations as well as methanol as a control. All the experiments were performed at ambient temperature (20 °C) and atmospheric condition lasted 3 h unless further explained. **Legends**: (**A**) HBr (0.1%, v/v); (**B**) HBr (1%, v/v); (**C**) HBr (1%, v/v) at 65 °C; (**D**) HBr-HAc (1%, v/v); (**E**) HAc (1%, v/v); and (**F**) TFA (0.1%, v/v).
[d] N.N = no

Thus, in term of yield, the resulted yield of galanthamine from both PW and water extraction was higher compared to $scCO_2$ extraction with NH_4OH (25%, v/v) treatment with an identical extraction time of 3 h [14] only 0.30 mg/g of galanthamine was produced [14]. Pure $scCO_2$ extraction at comparable temperature and pressure conditions as PW extraction (40 °C, 150 bar, and 70 °C, 150 bar) yielded much less galanthamine, respectively only 24 and 14 µg/g, although 3 h of extraction time was applied [14]. In spite of its low yield, $scCO_2$ extraction has the advantage of high selectivity for *Narcissus* alkaloids.

4 Conclusions

Pressurized water (PW) extraction was successfully applied for extracting selectively galanthamine from its biological matrix. Although a high amount of galanthamine yield was produced, ca. 3.50 mg/g of DW, this method (70 °C, 150 bar, 45 min) was not the method of choice, as handling of filtration was difficult due to high viscosity of the water extract solution compared to the classical acidified-water extract solution. In comparison, acidified water extraction (HBr (1%, v/v), 65 °C, 3 h) yielded ca. 2.65 mg/g of DW galanthamine. However, this extraction method needs further purification steps for enhancing the required purity of galanthamine. The acidified water extracts gave high yields of minor alkaloids. Overall, higher yields of minor alkaloids were obtained with TFA-acidified water.

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