Delft University of Technology

# Novel Methods for the Extraction of Galanthamine from Narcissus pseudonarcissus Bulbs

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Orchidea Rachmaniah

# Novel Methods for the Extraction of Galanthamine from *Narcissus pseudonarcissus Bulbs*

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TU Delft

TU Delft

Propositions,
behorende bij het proefschrift "Novel methods for the extraction of galanthamine from *Narcissus pseudonarcissus* bulbs" door Orchidea Rachmaniah.

1. Besides producing beautiful flowers, and being the source of galanthamine, a medicine to treat Alzheimer patients, Narcissus bulbs have great potential for developing more alkaloid medicines for treating various diseases *(this thesis)*.

2. The development and exploitation of the potential of NADES is hampered by a lack of insight in their un- elucidated physico-chemical behavior *(this thesis)*.

3. Despite its robust and versatile properties, SC-CO2 has a major shortcoming in the narrow range of the extracted compounds. That leaves space for novel solvents such as ionic liquids and NADES.

4. The solubility and extractability of compounds from natural materials using solvents are not necessarily correlated.

5. Some NADES are attractive as room temperature mRNA vaccin storage and stabilisation media *(Lamya Al-Fuhaid, personal communication)*.

6. Even at high water concentrations of above 50 wt%, a NADES is formed near surfaces from a NADES constituent containing solution (*Andreia Farinha, personal communication)*.

7. When adding water on top of a NADES in a test tube, a third liquid phase develops from the interface. The absolute value of the additional heat involved in this new phase formation is in the order of one kJ per mole and is therefore measurable with nanocalorimetry. *Experiment described in Y. Dai, Natural Deep Eutectic Solvents, dissertation Leiden University, 2013 p. 158*

8. Supercritical carbon dioxide combined with NADES can provide technology to formulate medicine particles of tunable sizes.

9. Measurements of ultra-low trace element concentrations in water even far below any toxicity limit are highly useful.

10. Bikers carrying baggage on their back instead of mounted on for instance on a rack is comparable with car passengers carrying their baggage during the drive.

*Deze stellingen worden opponeerbaar en verdedigbaar geacht en zijn als zodanig goedgekeurd door de promotoren prof.dr. G.J. Witkamp, prof.dr. R. Verpoorte en dr. Y. Choi.*

**Novel Methods for the Extraction of Galanthamine from *Narcissus pseudonarcissus* Bulbs**

Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology
by the authority of the Rector Magnificus Prof.dr.ir. T.H.J.J. van der Hagen
chair of the Board for Doctorates
to be defended publicly on
Monday 8 May 2023 at 10:00 o'clock

by

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This dissertation has been approved by the promotors.

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## Summary

A large number of studies on *Narcissus*, a member of the Amaryllidaceae family, have been published. In particular on *Narcissus* species, their alkaloids content, their structures including with MS-fragmentation patterns, their preparative extractions, and the analysis of these compounds covering GC-MS, LC-MS, HPLC-DAD, as well as $^1$H-NMR have been intensively reported. However, aspects on pre-analytical steps, extraction in bulk quantities using green alternatives solvent have not been reported widely, hence leaving space for investigation.

In this thesis, the sustainable production of galanthamine from *Narcissus pseudonarcissus* cv. Carlton bulbs, a relatively cheap biological left-over matrix from the agricultural-flower industry, was investigated within joint collaboration between the former Process Equipment Laboratory, and the Biotechnology department of Delft University of Technology, and the Natural Product groups of Institute Biology of Leiden University. The aim of the project was gaining insight into the extraction of *N. pseudonarcissus* alkaloids, especially galanthamine, by means of using green solvents instead of using volatile organic solvents (VOCs) as in the conventional process. Both supercritical fluid (SCF) (c.q. supercritical carbon dioxide) and natural deep eutectic solvents (NADES) which are recently considered as green solvents were applied. Classical alkaloids extraction methods by means of acid-base purification steps of alkaloids as well as an exhaustive Soxhlet extraction as a benchmarking method were also conducted for comparison. It was investigated whether the proposed method provides high yield and selectivity of the targeted compound. The described study must be considered as the first step for further studies on the commercial production of galanthamine from the biological matrix; to address the challenges met in the bulk quantity production of galanthamine, a *N. pseudonarcissus* alkaloid.

Prior to doing the supercritical $CO_2$ ($scCO_2$) extraction, a literature study was carried out. According to the previous studies on the secondary metabolites (SMs) extractions by using $scCO_2$, many aspects were found essential for the success of the extractions process. They are divided mainly into pre-extraction, extraction, and post-extraction step, particularly when dealing with the plant's matrices. Grinding and impregnation of the grinded material are important in this step as well as the drying of the material to keep the water level around 5-10% of dry weight. The selectivity of alkaloids is largely affected by adjusting the $CO_2$ density which can be tuned by controlling the pressure and temperature of the $scCO_2$ as well as by modifier addition. In the extraction step, particle size, porosity, contact surface area, and solubility of target compounds combining with the process systems, i.e. batch or continuous, play a major role. An integrated process including $scCO_2$ extraction as well as fractionation in the post-extraction step seems to be a promising strategy to enhance the yield and selectivity of targeted compounds.

It is known that the $scCO_2$ extraction of secondary metabolites (SMs), i.e. alkaloids, from its plant matrices is challenging. Therefore, a $scCO_2$ extraction method of galanthamine extraction was developed by applying those essential aspects obtained from literature studies. Particle size, $CO_2$ density, $CO_2$ flow rate, plant material pre-treatment, as well as modifier addition were included in the study of $scCO_2$ extraction of

galanthamine from *N. pseudonarcissus* bulb. Until now research on the *Narcissus* alkaloids extraction using $scCO_2$ has not been reported.

The *N. pseudonarcissus* alkaloids extracts of $scCO_2$ were identified using gas chromatography-mass spectrometry (GC-MS) and quantified using gas chromatography-flammable ionisation detector (GC-FID). They were galanthamine, lycoramine, *O*-methyloduline, norgalanthamine, narwedine, oduline, haemanthamine, *O*-methyllycorenine, and haemanthamine derivative. Due to the different polarity between the non-polar nature of $CO_2$ and the medium-polarity of targeted alkaloids the solubility of galanthamine in $scCO_2$ is low.This situation becomes worse with the presence of the alkaloids as a protonated (salt form) in the plant and the strong interaction between the matrix and the analyte. Hence, a low yield of galanthamine was obtained when it was extracted solely by $scCO_2$ without any pre-treatment of plant material. Thus, either a modifier or a pre-treatment to alter the alkaloids form into its free form is needed.

It was found that a pre-treatment was necessary to minimise these drawbacks instead of using a modifier for having an appropriate yield of galanthamine. In fact, the presence of fatty acids (FFA) in the bulb, as a storage organ for the *Narcissus* plants, triggered the formation of fatty acid methyl esters (FAME) at the final extracts. Thus, MeOH addition for the modifier was not appropriate. The higher solubility of FAME in $scCO_2$ than the galanthamine, resulted in a competitive solubility between galanthamine and the esterlowering yield of galanthamine.

Pre-treatments of plant material using $NaHCO_3$ (10%, w/w), diethylamine (DEA), and $NH_4OH$ (25%, v/v) as well as methanol, and water addition in different proportions were applied. It was found that the yield of galanthamine and the compositions of the extracts were largely influenced by both the applied pre-treatment and modifiers. A pre-treatment using $NaHCO_3$ (10%, w/w) gave more selection towards alkaloids (galanthamine, haemanthamine, *O*-methyllycorenine, and haemanthamine derivate) though a low yield was obtained (ca. ~18 µg/g DW). On the other hand, a pre-treatment with $NH_4OH$ (25%, v/v) seemed to be the most appropriate for increasing the yield of galanthamine. Three alkaloids were quantified: 300, 75, and 100 µg/g for galanthamine, haemanthamine and *O*-methyllycorenine, respectively, at $NH_4OH$ (25%, v/v) pre-treatment, 70 $^oC$, 220 bars, and 3 h. It seems that the free bases of *N. pseudonarcissus* alkaloids are highly soluble in $scCO_2$ at a high pH as opposed to the slightly soluble salt form in which they are generally found in plants. The desorption of *N. pseudonarcissus* alkaloids from the plant material rather than the solubility of the alkaloids in the $scCO_2$ plays a major role in this $scCO_2$ extraction, which was also revealed by scanning electron microscope (SEM). These treatments and the extraction conditions proved to be optimum for the extraction of galanthamine.

Since the $scCO_2$ extraction results seem to be promising, we found it of interest to compare this method with some of the classical alkaloids extraction methods which are generally used, and to discuss its possible advantages and applications. Following, a conventional method using acidified extraction followed with subsequent acid-base purification step of alkaloids was conducted as well as hot pressurised water (HPW) extraction. By applying this HPW method, a benefit of high pressure condition and acidic condition which was formed by the formation of carbonic acid (by using $CO_2$ for

pressurising the vessel), an improvement on the matrix penetration and swelling of *N. pseudonarcissus* bulbs were expected. A higher galanthamine was yielded, 2600 µg/g DW (HBr 1% v/v, 65 $^oC$, 3 h) though a lower selectivity was observed. Broader alkaloids selectivity clearly predominated in all extractions. Covering all the alkaloids which were identified in the $scCO_2$ extract, epi-norgalanthamine was also included in these water extracts. Instead of higher yield, a stable emulsion always hampered purification steps. Although both acidified water and HPW extractions gave higher yield of galanthamine, they were so far not considered better than $scCO_2$ extraction, regarding the generated emulsion problem and the broader selectivity. This implies that further purification steps are necessary.

Lately, Natural deep eutectic solvents (NADES) have been reported as green solvents and they have been recently found to be suitable for some SMs such as phenolics, natural colourants, taxol, etc. Instead of being called DES, they are called NADES because they consist of primary metabolites such as sugars, sugar alcohols, amino acids, organic acids, and choline derivatives in different proportions. Because of the various combinations and the potential applications of these solvents (Yuntao DAI, dissertation Leiden 2013), alkaloids solubilities in NADES were studied as well. Thus, NADES is an appropriate green solvent candidate for galantamine extraction. NADES are composed of primary metabolites considered as polar to medium polar solvents, corresponding to *N. pseudonarcissus* alkaloids which are also medium-polar compounds.

Prior to the application of galanthamine extraction using NADES as a solvent, a high-performance liquid chromatography (HPLC) method for analysing galanthamine in NADES matrix was developed. The presence of organic acids, i.e. citric acid, malic acid, and lactic acid, in the NADES influences the quantitative analysis of galanthamine. At low pH, i.e. acidic condition, galantamine will be totally ionised. Interactions with anions, in this case, can be generated from trifluoroacetic acid (TFA, an ion-pairing reagent) from the mobile phase, and organic acids from the NADES components. A reproducible retention time of the targeted compound was impossible to achieve and peak broadening and/or tailing were also observed. A method with an addition of sufficient organic base, i.e. triethylamine (TEA), to achieve pH 11 was found to be the best for eliminating these problems. An optimum chromatographic separation was achieved using a high pH resistant stationary phase column with MeOH: $H_2O$ (50:50, v/v) with addition of 0.3% (v/v) of TEA. In addition, appropriate sample preparation steps were necessary when working with plant extracts of NADES, as some water-soluble polysaccharides (WSPs) and lectin were co-extracted. This was indicated by a steep increase in back pressure of the reverse-phase column due to the precipitation of these compounds in column after numbers of injection of NADES extracts.

Following these findings of a successful HPLC analysis method of galanthamine in NADES matrix, a solubility test of galanthamine-HBr was conducted. Twenty-eight of NADES was selected based on previous reported NADES by our group. They were tested for the solubility study of alkaloids in which galanthamine-HBr was used as an alkaloid model. The solubility test data was analysed by multivariate analysis data using principal component analysis method (PCA). It was clearly shown that alkaloid i.e. galanthamine-HBr solubility in NADES was positively correlated to the ionic type of NADES, acid, and

basic composition of NADES. Amino acid, water, eutectic type of NADES, and sugar gave a negative correlation. Galanthamine-HBr solubility was intensely affected by the ionic type and the acid composition of NADES, while basic composition had only a slight effect. Based on this we conclude that NADES containing acid and ionic type is the best type of NADES for solubilising galanthamine-HBr.

A part of the solubility study of galanthamine-HBr in NADES, an application of extraction of *N. pseudonarcissus* alkaloids from the dried bulbs powder was performed using NADES as solvent in high pressurised extraction (HPE) apparatus. Following a selection approach based on the previous solubility test results. Though a hypothesis that an acid and ionic type is the best combination of NADES for solubilising of galanthamine-HBr; neutral, basic, and amphoteric types of NADES were also chosen for extracting the galanthamine from it biological matrix.

The interaction presence between analyte and matrix in the plant as well as the different form of alkaloids in the plant material provided different results. Eleven candidates were selected from the provided list of solubility-test in NADES. The high pressurised extraction (HPE) set-up was used to demonstrate the ability of NADES to extract the *N. pseudonarcissus* alkaloids. The set-up was able to perform the extraction. The NADES extraction gave ca. 1900-9400 µg/g DW of galanthamine with choline chloride-citric acid (CCCA) and malic acid-sucrose (MAS) as a NADES. A part of general alkaloids profile which were generated from the extracts, multivariate data analysis (principal component analysis) was applied to reveal a clear correlation between the extracted alkaloid and the NADES types. Similar alkaloids profiles were determined for both NADES and water extracts while these were noticeable difference with methanol extract. This excluded β-Alanine-malic acid (βAMA), due to the extraction of unidentified compound ($m/z$ 345 ($M^+$)). In case of selectivity, narwedine was more extracted using choline chloride-sucrose (CCS), norgalanthamine extraction was quantitatively with fructose-sucrose (FS), and homolycorine was selectively only with CCCA and MAS. Typical *Narcissus* alkaloids, galanthamine, lycoramine, oduline, and haemanthamine were quantitatively extracted with malic acid-glucose (MAG), citric acid-sucrose (CAS), and choline chloride-malic acid (CCMA). Thus, NADES offer a promising selective *Narcissus* alkaloids extraction.

The knowledge gained in this thesis can be beneficial to the application of environmentally friendly industrial processes involving $scCO_2$ and NADES, such as enhanced yield of polar compounds and their selectivity to the alkaloids as the targeted compound. The outcome of this thesis can also be extended to the application of selective alkaloids extraction in bulk quantity production.

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# CHAPTER 1

## Introduction

### 1. Natural Products

Plants are one of the most important resources for a wide range of medicines. Nearly 50% of modern medicines approved by the Food and Drugs Administration (FDA) have been derived from natural sources, dating from January 1981 through December 2010 (Newman and Cragg, 2012). - Several new drugs were developed from plants such as galanthamine (Reminyl®)(Sabbagh, 2009), huperzine A from *Huperzia serrata* (Sabbagh, 2009; Sramek *et al.*, 2000), artemisinin from *Artemisia annua* (Sabbagh, 2009; Saklani and Kutty, 2008), and Sativex® containing tetrahydrocannabinol (THC) and cannabidiol (CBD) from *Cannabis sativa* (Harvey, 2008; Saklani and Kutty, 2008), etc. This proves that natural products, in particular plants are still important as a resource of bioactive compounds.

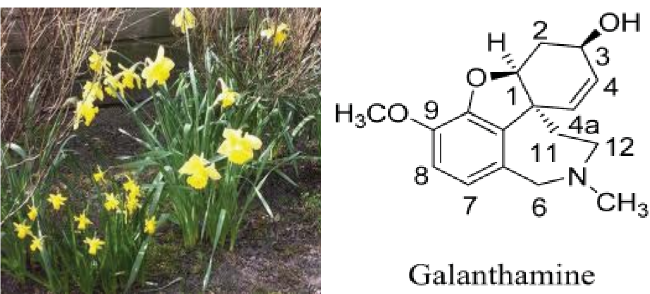


**Figure 1.** Galanthamine (right) was developed from *Narcissus* plants (left: picture of *Narcissus pseudonarcissus*).

The enormous variety of compounds (metabolites) that are produced by plants can be categorized as primary metabolites (PMs) and secondary metabolites (SMs). Primary metabolites are organic compounds essential for the metabolism involved in all vital processes e.g. amino acids, carbohydrates, lipids, fats, proteins, nucleic acids, and organic acids and bases. On the other hand secondary metabolites are the compounds involving diverse physiological roles, often differentially distributed in specific species in low amounts. The chemical diversity mainly come from secondary metabolites and most plant-originated drugs are from them. They were initially thought to be waste products of plants, without any apparent function in the plant. So though in principle, they did not

seem essential for the normal functions of a living cell, they were found to play a role in plant survival in its ecosystem. Also, many SMs have been found to play a role in the interaction of an organism with its environment, e.g. in plant defence against predators (herbivores, and pathogens), for interspecies competition and to facilitate the reproductive process (attracting pollinators).

Although the role of most of SMs are still not clear they are invaluable resources of biological active compounds for pharmaceuticals, cosmetics, and food development. Moreover, SMs play an important role in the quality traits of plants, such as aroma, taste, and colour. However, when attempting to use these metabolites as medicines, cosmetics or food ingredients it is common to encounter a number of difficulties. Of them the first hurdle is designing an efficient large scale extraction and isolation process.

## 2. Green Solvent in Natural Products Extraction/Production

In the process of drug development, the first step, i.e., the extraction and isolation of the targeted NPs is essential for further studies such as pharmacological and toxicological evaluation. It is thus crucial to develop extraction methods that can meet all requirements such as safety, yield, and selectivity for the targeted NPs as well as economic efficiency. Metabolites, particularly SMs, cover a wide range of polarity because of the diversity of chemical structures. This structural diversity combined with their presence in low amounts in the original matrices makes them difficult to isolate. Furthermore, there is a great variety of approaches in the isolation techniques of SMs as compared to macromolecules, i.e. proteins, nucleic acids, and polysaccharides.

Extraction is a step in which the targeted NPs are transferred from their original matrices into an accessible phase, often an organic or aqueous phase, obtaining a highly concentrated extract of targeted compounds. The selection of the extraction method to be applied for a certain biological matrix depends on the type of raw material, the selected NP and co-existing chemicals. There is neither a single universal method nor any standard extraction method for obtaining NPs; each method has advantages and disadvantages. At the industrial scale, the yield and the selectivity are key properties, but these are not the only factors to be considered. The effect of the process on the environment (in terms of waste generation), health and safety issues should also be taken into account, particularly for large-scale extraction. Consequently, there is a limitation to the choices of solvents that can be used. In order to solve this problem, many methods have been designed including the recent trend in developing "green solvents" which are believed to be more environmentally friendly and safe while having high selectivity to ensure the highest purity of the targeted compound. Hence, it could be possible to minimize or even completely replace the volatile organic solvents (VOCs) in the industrial processes. A few alternatives for such extraction techniques for NPs as well as their comparison to conventional methods are briefly discussed.

Hot water extraction - such as used for making teas or to isolate natural plant dyes - is the oldest technique for obtaining plant extracts (Martín *et al.*, 2011). However,

the resulting extracts can be diverse in composition and are not always reproducible. In addition, the extraction capability of water is limited to polar metabolites and is very low for medium or lipophilic chemicals. Consequently, extraction methods involving the use of VOCs were developed. Along with the development of organic solvents, liquid/liquid extraction (LLE) based on the differential partitioning of the target compound between two immiscible solvents was developed. This includes using changes in partitioning behaviour of the compounds at different pH; in the case of alkaloids for example, the successive removal of non-alkaloids or alkaloids by immiscible organic solvents from acidified or basified aqueous solution, respectively, is possible. It also allows isolation of compounds which have a large pH dependent polarity shift, like acids and bases. Thus, alkaloids can be selectively purified according to their $pK_a$ by varying the pH of the aqueous solution. However, this kind of extraction method only allows the polar, hydrophilic or basic compounds to be extracted, as hydrophobic neutral or acidic compounds cannot be extracted.

Soxhlet extraction, soaking extraction (i.e. maceration, percolation, and infusion) as well as steam distillation are well known as conventional extraction techniques (Rostagno and Prado, 2013). Soxhlet extraction is a classical extraction method involving a repetitive or continuous solid-liquid extraction (SLE). It is used as a reference extraction method for evaluating SLEs methods due to its simplicity, low cost (per sample), well-established use and robustness. The saturation of the solvent does not occur because fresh solvent reaches the sample in each cycle; it is therefore an exhaustive extraction method. The major disadvantage is that the liquid extract is for a long time heated, causing seriously problems of artefact formation and degradation of compounds. Moreover, this method is not applicable to volatile components such as essential oils. In this case, at an industrial scale, steam-distillation or hydro-distillation are mostly used for extracting volatile compounds because it is cheap and extensive know-how in this field has been achieved. The difference between them lies in the plant-matrix placement. In case of steam-distillation, the plant-matrix is supported on a perforated grid or tray which is not in direct contact with water while at hydro-distillation, it is immersed (Rostagno and Prado, 2013). Despite the advantages, these classical extraction techniques are laborious, time consuming (hours, or even days) and consume large amount of volatile often toxic and flammable, organic solvents. Furthermore, the extracted SMs require further purification processes.

Extraction methods can also be classified according to the number of solvents involved; they may use a single or multi solvents, a gradient of two or more solvents such as the comprehensive extraction method developed by Yuliana *et al*. (2011), or multi solvent that form two-phase solvent systems e.g. $CHCl_3$/MeOH/$H_2O$ (Kim *et al*., 2010).

Other features that characterize an extraction method are the temperature and/or pressure as well as the use of mechanical assistance all of which can be used to improve the efficiency of the extraction. Microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) are examples of the latter, and are applied to

increase the extraction efficiency (Choi and Verpoorte, 2014). Solid-phase extraction (SPE) is not classified as an extraction method, but rather considered to be a post-extraction treatment, used to clean-up and/or concentrate the targeted compounds (Mushtaq *et al*., 2014). Among the new type of solvents that can be used, ionic liquids (ILs), deep eutectic solvents (DES), as well as supercritical fluids (SCFs) are the best known to date.

When applying the common extraction methods for natural products, the resulting crude extracts always contain a wide variety of chemicals including impurities from the organic solvents themselves such as plasticizers; they may also contain artefacts resulting from the extraction process itself (King, 2002; Langezaal *et al*., 1990; Maltese *et al*., 2009).

In the field of green solvents, supercritical fluids (SCF), and particularly supercritical $CO_2$ ($scCO_2$) ($T_c$ = 31.1 °C, $P_c$ = 72 bar) has found many industrial applications in green extraction methods in the past decades. Green solvents are solvents which have a minimum effect on human health, are safe and environmentally friendly in their preparation, utilization, and disposal (Deetlefs and Seddon, 2010). Supercritical $CO_2$ is considered to be a green solvent as it is harmless and leaves no traces in the extracts which can therefore be considered solvent-free. Other benefits are that $CO_2$ is readily available and has a low cost.

Other emerging candidates for green solvents aside from supercritical $CO_2$ are ionic liquids (ILs) and deep eutectic solvents (DES). These solvents have been labelled as "green" because of their negligible vapour pressure and non-flammability as compared with VOCs (Deetlefs and Seddon, 2010; Domínguez de María and Maugeri, 2011; Gorke *et al*., 2010; Wood and Stephens, 2010). Ionic liquids and DES are mixtures of salts that are liquid at room temperature and possess physical-chemical properties that can be fine-tuned for selectivity by combining different cations and anions.

Ionic liquids typically consist of synthetic cations such as dialkylimidazolium and alkylpyridium derivatives, and anions such as chloroaluminate and other metal halides (Domínguez de María and Maugeri, 2011). When necessary, these water-reactive anions can be replaced with halides or anions ($BF_4$-or [PF6-]$PF_6^-$) which are more stable to water and air (Gorke *et al.*, 2010) resulting in unreactive ILs which tolerate water and air better than typical ILs.

On the other hand, DES, a more recently developed alternative to ILs, consist of a mixture of organic compounds and may also have an ionic character. These DES have similar characteristics to ILs, i.e. liquid at room temperature, and can also be tailor made, but they are cheaper to produce due to the lower cost of their raw materials (Domínguez de María and Maugeri, 2011). Moreover, they are mostly less toxic than ILs, and are often biodegradable. DES' final purities are determined by their starting materials, therefore they do not need further purification method after the synthesis as no new compound is formed. Choline chloride and urea were reported as forming DES (Abbott *et al.*, 2003). There are also synthetic DES that can be made with alkyl sulphates, alkyl phosphates, and inorganic salts ($NH_4Cl$, or $CaCl_2$) (Imperato *et al.*, 2005).

More recently, Choi *et al.* (2011) and Dai *et al.* (2013) introduced another type of green solvents, the natural deep eutectic solvents (NADES). They can include both ILs and DES that are made of common primary metabolites occurring in all living organisms. These researchers have hypothesized that NADES could in fact act as a third liquid phase in living organisms, with a broad variety of roles. These solvents can be prepared from natural bases such as choline, betaine, quaternary ammonium derivatives in combination with a uncharged hydrogen bond donor such as urea (Abbott *et al.*, 2003), carboxylic acids (e.g. citric acid (Abbott *et al.*, 2004), polyalcohol (e.g. glycerol (Abbott *et al.*, 2004; Gutiérrez *et al.*, 2009), sugars or sugar analogues, and amino acids (Choi *et al.*, 2011; Dai *et al.*, 2013).

The benefits and greenness of (at least some) synthetic ILs have been challenged because of high large-scale production costs (Gorke *et al.*, 2010), toxicity and disposal issues, and their unknown long term stability. Moreover, their often high viscosity is a disadvantage. DES needs further study to have a better insight and understanding of their properties before being ready for application in industry. Thus, $scCO_2$ and NADES are the only fully green solvents. $CO_2$ is non-toxic and widely available at low cost. While primary metabolites which are constituents of NADES are natural compounds, available at large scale at low cost. Thus NADES are biodegradable, biocompatible, and their disposal is straightforward and inexpensive as they can safely be used as feedstock.

Due to the imposed environmental regulations, the necessity of minimizing energy consumption and public health requirements, there is an urgent need for green chemical processes in e.g. drugs production. The selection of a production process to obtain target compounds with a selectivity level that ensures high purity is a critical need. Despite of using a green solvent like ethanol (EtOH) in the production process, eliminating the use of VOCs, or reducing the amount of VOCs is an important objective for the development of green technologies. Therefore, selective and cheap green solvents technologies for industrial processes like pharmaceuticals are of great interest.

## 3. Galanthamine

Plants of Amaryllidaceae family are well known for their ornamental value but also for their bioactive metabolites (Heinrich and Lee Teoh, 2004). Due to their interesting bioactivities Amaryllidaceae alkaloids have attracted the attention of researchers for many decades resulting in the isolation, identification, and structural elucidation of a large number of approximately 150 alkaloids, including galanthamine (Jimenez *et al.*, 1976). Bastida and Viladomat (2002) have reviewed the occurrence of alkaloids in *Narcissus* species.

Galanthamine was first isolated from *Galanthus nivalis* L. in the late 1940s by a Bulgarian pharmacologist (Cronnin, 2001). It is now the most interesting Amaryllidaceae alkaloid as it is used for treating Alzheimer Disease (AD) patients. Galanthamine is an acetylcholinesterase (AChE) inhibitor with a long-lasting, selective, reversible, and competitive effect on AChEs. Furthermore, even though a number of natural compounds with an AChE inhibitory effect have been found, galanthamine and sanguinine, both

Amaryllidaceae alkaloids, are the most active (McNulty *et al.*, 2010). Sanguinine is in fact more potent than galanthamine (López *et al.*, 2002; McNulty *et al.*, 2010), but it is very low availability in nature precludes it from further development.

Galanthamine can cross the blood-brain barrier and act within the central nervous system but it also increases the release of neurotransmitters by stimulating the nicotinic receptors. This dual mode of action makes galanthamine an excellent therapeutical agent for the treatment of symptoms of neuro-degenerative diseases such as AD (Bastida *et al*., 2011). It is the first *Amaryllidaceae* alkaloid, which was approved as a prescription drug for the treatment of a human disease.

Galanthamine was introduced into the market as a hydrobromide under the proprietary name of Nivalin® in Bulgaria and was produced from *Galanthus nivalis L*. (Cronnin, 2001). Later on, it was obtained from *Leucojum aestivum* and from leaves of *Ungernia victoris* L. in (ex) USSR (Sagdullaev, 2005). However, the most economically advantageous natural source are *Narcissus* "Carlton" variety bulbs (Bastida and Viladomat, 2002; Heinrich and Lee Teoh, 2004). Despite this, limitations in the availability of this source for industrial production made the chemical synthesis the first choice. Several synthetic routes of galanthamine-HBr have been reported (Bolugoddu *et al.*, 2006; Koilpillai *et al.*, 2012; Lahiri *et al.*, 2006), and reviewed by Bulavka and Tolkachev (2002) including some of the purification processes of synthetic galanthamine (Gabetta and Mercalli, 2011). Nowadays, all commercial galanthamine is synthetic. Reminyl® is a brand name of galanthamine hydrobromide launched by Shire and Janssen in many countries (Heinrich and Lee Teoh, 2004); no information has been published for the commercial production of galanthamine from plants. The pharmaceutical companies that held the patents for the drug also patented the synthesis process. However as both patents have run out, generics-producing companies now have interest in exploring the production of galanthamine from natural sources. Hence in the past years, there is a renewed interest in the optimization of the extraction of galanthamine from bulbs.

## 4. Scope of This Thesis

The scope of this PhD project was to develop a sustainable method for the production of galanthamine from *Narcissus pseudonarcissus* cv. Carlton bulbs using green technologies. To achieve this goal, we first developed the method to quantify the galanthamine by high performance liquid chromatography (HPLC). Subsequently the following subprojects were defined:

1. Determine the maximum extractable galanthamine present in the bulbs using an exhaustive Soxhlet extraction method with methanol, considered as a benchmark method;
2. Study the efficiency of the classical acid-extraction of galanthamine followed by an acid-base liquid/liquid extraction (LLE) for the purification of alkaloids, and explore the effect of an acidifier on the selectivity of the extraction of the *Narcissus* alkaloids;

3. Study the use of pressurized water (PW) extraction of galanthamine from the *Narcissus* bulb matrix, exploring the effect of temperature on the selectivity of the *Narcissus* alkaloids, and observing the degree of cell destruction (if any) of the plant material;
4. Develop an extraction method of galanthamine using a supercritical $CO_2$ ($scCO_2$), evaluating the effect of the following variables on its yield: particle size, $CO_2$ density (temperature and pressure), $CO_2$ flow rate, pre-treatment of plant material, as well as the addition of modifiers and observing the presence of cell destruction (if any) of plant material. Determine the selectivity for the *Narcissus* alkaloids.
5. Develop and validate an HPLC method for the analysis of galanthamine in the NADES plant extracts; explore the interference (if any) of NADES constituents on the analysis;
6. Study the solubility of galanthamine-HBr in different types of NADES and explain the existing correlation (if any) between constituents of NADES and galanthamine-HBr solubility;
7. Develop a method for the extraction galanthamine using the novel natural deep eutectic solvents (NADES), and explore the effect of types of NADES on the yield of galanthamine and the selectivity for the different *Narcissus* alkaloids.

## 5. Thesis Outline

**Chapter 1**, the introduction, provides general background knowledge about the extraction methods of secondary metabolites (SM) from plant materials. This includes a brief overview about the utilization of plants as sources of drug entities for therapeutical drug development and the history of galanthamine production.

In **Chapter 2**, supercritical $CO_2$ ($scCO_2$) extraction technology is reviewed especially for SMs extraction, e.g. terpenoids, essential oils, carotenoids, vitamins, natural pigments, phenolics, and alkaloids. This chapter identifies the parameters that must be considered and optimized for a successful, efficient $scCO_2$ extraction of the compound of interest. In this review, key points for an effective $scCO_2$ extraction of alkaloids from plant matrices are identified.

In **Chapter 3**, the development of a $scCO_2$ method for galanthamine extraction is described. The effect of parameters such as particle size, $CO_2$ density, $CO_2$ flow rate, pre-treatment of plant material, as well as modifier addition on the yield of galanthamine were evaluated. In addition, scanning electron microscopy (SEM) was used to observe the presence of cell destruction (if any) caused by the application of high pressure during the $scCO_2$ extraction.

In **Chapter 4** describes the results of alkaloid extraction using acidified water followed by acid-base liquid/liquid extraction (LLE) for purification of alkaloids as a classical extraction method of alkaloid. This technology is widely applied on laboratory scale for separating and purifying alkaloids. In addition, an exhaustive methanol extraction using Soxhlet apparatus was also performed to determine the total extractable galanthamine amount present in the material. Furthermore, pressurized water extraction

(PW) was conducted to learn more about possible strong interactions between galanthamine and the plant matrix, which could be broken under the extreme conditions used in PW, increasing the yield. This was thought to occur since the high pressure and acidic conditions which were produced by the formation of carbonic acid (formed as a result of the $CO_2$ used to pressurize the vessel), could improve the penetration of the extraction solvent into the matrix and swell the *N. pseudonarcissus* bulbs.

In **Chapter 5**, several NADES were tested in a possible alternative green process for galanthamine extraction. An HPLC analysis method was developed in order to be able to analyse the galanthamine in the NADES extracts.

In **Chapter 6**, the solubility of galanthamine-HBr in different NADES was determined, to achieve a selective extraction of galanthamine with NADES; then use these results to identify NADES solvents that provide the highest yield of galanthamine when applies to the extraction of bulbs material using a pressurised extraction set-up.

In **Chapter 7,** the production cost of galanthamine (sc$CO_2$ extraction process) was estimated and compared with that of the classical extraction. This should reveal the major bottleneck in the different extraction methods that can then be the subject for further optimization.

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# CHAPTER 2

## Supercritical $CO_2$ Extraction of Secondary Metabolites: A Review

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**Abstract**

Supercritical carbon dioxide is a promising green solvent increasingly attracting attention for the extraction and processing of natural materials. A question is to what extent it is suitable for the process of extraction or purification of natural products, and how much it improves the performance and greenness compared with classical solvent based processes. In many cases, supercritical fluid extraction is shown to be an attractive technology for secondary metabolites extraction, especially for volatile or non-polar compounds with a low molecular weight. Therefore, the objective of this chapter is to compare reported applications of supercritical fluid extraction for plant chemicals, particularly for bioactive secondary metabolites, with conventional techniques, and to evaluate strengths and weaknesses of supercritical fluid extraction. Literature on supercritical fluid extraction of different groups of secondary metabolites (e.g. phenolics, terpenes and steroids, and alkaloids) including some commercial application of supercritical fluid extraction were searched and evaluated. Additionally, the critical steps for doing supercritical fluid extraction of secondary metabolites from their biological matrix, the various factors affecting extraction features of supercritical $CO_2$ extraction, as well as its modelling are briefly discussed (size of the matrix particles; pre-treatment of matrix, e.g. acid/base treatment, soaking; modifier addition to supercritical fluid; type of process: static, dynamic, or combination; ratio of plant material to solvent; density of supercritical fluid (pressure and temperature of the supercritical fluid); duration of extraction; flow rate of supercritical fluid; and fractionation conditions of extract; covering pre-extraction treatment step and extraction process of SFE). Modifier is mandatory for the successfully SFE of alkaloids to bring the alkaloids in the non-protonated form.

**Keywords**: plant materials; secondary metabolites; supercritical carbon dioxide

## 1. Introduction

In the period 1981 to 2010, 1130 new approved drugs have entered the market, of which almost 50% is a natural product or derivative or analogue (Newman and Cragg, 2012), among them particular antiviral and anticancer drugs (42% of 206 new anticancer drugs) (Newman *et al.*, 2003; Newman and Cragg, 2012), including Taxol® (Paclitaxel) from *Taxus brevifolia*. Other examples from plants are acetylcholine esterase inhibitors, Reminyl® with galanthamine (*Galanthus nivalis*), and Huperzine A (*Huperzia serrata*) (Sabbagh, 2009; Sramek *et al.*, 2000), which are used for the symptomatic treatment of Alzheimer's disease (**Fig. 1**). Tetrahydrocannabinol and/or cannabidiol (*Cannabis sativa*) containing drugs as Sativex® are registered for relieve of pain, symptoms of HIV or chemotherapy. Hemoxin, a mixture of four Nigerian plant materials (*Piper guineense, Pterocarpus osun, Eugenia caryophyllum*, and *Sorghum bicolor*), for sickle cell anaemia treatment, was approved as medicine in 2006 (Newman and Cragg, 2007).

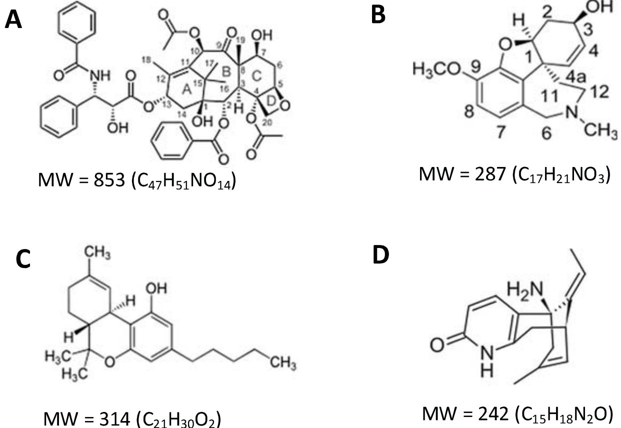



**Figure 1**. Some examples of natural products: (**A**) Paclitaxel (Taxol®) from bark of *Taxus brevifolia* (Pacific yew tree), (**B**) galanthamine from *Galanthus nivalis*, (**C**) tetrahydrocannabinol from *Cannabis sativa*, and (**D**) huperzine from *Huperzia serrata*.

Natural product related processes start from making an extract of biomass, followed by further purification of the active compounds, initially at a scale of kilograms of raw material but reaching multi-ton scale in full production. Therefore, costs and environmental issues are important. The natural products of interest here are small organic molecules, molecular weight approx. <1500 amu, which are also called secondary metabolites (SMs); a subject of interest in this review. Isolation of a secondary metabolite (SM), which are main bioactive chemicals, is different from a primary metabolite or biological macromolecule. Secondary metabolites are usually present in low amounts in the original matrix and have a wide range of chemical structures with diverse chemical and physical properties compared with primary metabolites such as proteins, nucleic acids, and carbohydrates. Hot water extraction such as tea decoction or plant dye is the

oldest extraction technique and still most popular for plant extraction (Peter *et al.*, 2006). Water, however, is a poor extracting medium for non-polar SMs, and heating may cause degradation. Therefore, alternative extraction and enrichment methods including those with organic solvents were developed such as Soxhlet extraction, maceration, percolation, steam distillation, infusion, decoction and liquid-liquid extractions (LLE), often utilizing changes in partitioning behaviour at different pH (alkaloids and organic acids). However, these organic extraction methods are laborious and/or consume large amounts of volatile and often toxic and/or inflammable organic solvents. Emulsions are a common problem in LLE of crude extracts (Dean, 2009). The co-eluted compounds often cause a problem to obtain high purity of SMs. Crude extracts always contain natural pigments, chlorophyll, fatty acids, primary metabolites as well as impurities from the organic solvents such as plasticizers and artefacts from the extraction process itself, and thus require further purification (Maltese *et al.*, 2009; Silva *et al.*, 1998).

Alternative methods have been developed to diminish the above-mentioned shortcomings of conventional methods. For example, many other mechanical methods have been developed including pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and solid phase extraction (SPE). Though MAE and UAE are efficiently applied to destruct the plant matrix, MAE may trigger chemical reactions or cause isomerization of the analytes (Destandau *et al.*, 2013) while degradation of unstable compounds, e.g. carotenoids, was observed in UAE (Joana Gil-Chávez *et al.*, 2013), thus reducing the product yield. In addition, a balance between yield and purity of the targeted compound must be found with low consumption of energy when it will be applied in industrial scale. Whilst SPE is suited for analytical scale separation, due to the high costs of stationary phase and organic solvents it is not suited for large scale process.

Compared to these methods, SFE in particular when using supercritical carbon dioxide (SC-$CO_2$) has many prominent advantages. $CO_2$ is cheap, non-toxic, non-flammable, environmentally friendly and almost inert, and allows supercritical operations at relatively low pressures (>73 bar) and near–room temperature (>32 °C) (Kim *et al.*, 2001; Selva *et al.*, 2007). In addition, it can easily be removed from the product after the extraction by simple expansion to the ambient conditions, leaving no traces. Therefore, the extracts are free of toxic solvents residues which is attractive to comply with the restrictions for residual organic solvents in pharmaceutical, food, and nutraceutical products extraction. SC-$CO_2$ can be used at low temperature, i.e. 30—40 °C, for extracting thermally labile or easily oxidize-able compounds. Thus, degradation of thermal labile compounds can be minimized. Traditionally $CO_2$ has been thought to be suitable for extracting non-polar, and/or low molecular weight compounds such as hydrocarbons as well as medium polar compounds, like alcohols, esters, aldehydes, and ketones (Reverchon and De Marco, 2006). The solubilizing properties of SC-$CO_2$ are to some extent comparable to *n*-heptane. By adding minor amounts of water (its solubility is typically in the order of a gram of water per kg of $CO_2$), or modifiers such as ethanol (EtOH) or methanol (MeOH), the solubility of protic, and more polar substances can be improved, as will be discussed later. In case of SC-$CO_2$ extraction of caffeine (**subsection 3.1.1**), water

was successfully used as modifier (Cardozo *et al.*, 2007; Cassel *et al.*, 2010; Saldaña *et al.*, 2000; Saldaña *et al.*, 2002a; 2002b; Tello *et al.*, 2011) though its molar solubility (y) in SC-$CO_2$ is low, i.e. 6900x$10^{-6}$ (at 50 ºC and 202 bar) (Gupta and Shim, 2007), due to high solubility of caffeine in water. Hence, depending on the properties of targeted compound. The goal of this chapter is to compare reported applications of supercritical fluid extraction (SFE) for plant secondary metabolites (SM) with more established techniques, and to evaluate strengths and weaknesses of SFE. Literature on SFE of different groups of secondary metabolites was searched, evaluated and analysed: phenolics, terpenes and steroids, and alkaloids.

## 2. Supercritical Fluid Extraction

2.1 General Many parameters need to be optimized for a successful application of SFE for the extraction, which can be divided into physical-chemical and technical properties including $CO_2$ density (pressure and temperature), duration of batch type of extraction, flow rate in continuous extraction, the type of sample, method of sample preparation, water content, modifiers, and type of process. Concerning the sample, the particle size, and void volume of the matrix are important factor. The compounds one may extract from the matrix are typically non- to medium polar and low molecular weight compounds (MW: ca. 100-1000). Many high molecular weight compounds show insignificant solubility in SC-$CO_2$ especially at low $CO_2$ density with more gas-like behaviour.

The plant material loaded in the SC-$CO_2$ extraction vessel can be considered as a fixed fluidised bed. In case of the development of SC-$CO_2$ extraction from the plant material one needs to consider three steps: pre-extraction treatment of the matrix, extraction, and post-extraction purification.

### *2.1. Pre-extraction treatment of the matrix before supercritical $CO_2$ extraction*.

The particle size of the material and impregnation of the ground plant material with chemicals and/or solvents is important in the pre-extraction treatment as well as the water content of the plant material. An average particle size of 0.4-0.8 mm was suggested as optimal by Laurent *et al.* (2001) while Reverchon and De Marco (2006) suggested a wider range, ca. 0.25-2.00 mm. Attention should be taken when milling plant materials rich in essentials oils. Avoiding loss of volatile compounds, i.e. essential oils, during milling of the plant material is necessary. A cryogenic process has been reported in which the material is processed in liquid nitrogen or liquid $CO_2$ (Meghwal and Goswani, 2010). In addition, a mixed bed of particles differing in both size and density will tend to segregate during the extracting process resulting in the formation of two different beds of different densities which provides a better contact between the plant matrix and SC-$CO_2$ (Lang and Wai, 2001; Reverchon and De Marco, 2006; Richardson *et al.*, 2005). Rachmaniah *et al.* (2014) found a decrease of galanthamine yield when a wider distribution of particle size, ca. 53-1000 µm was applied. A lower yield may result from hydrodynamic effects for instance through a less favourable fluidization. Hawthorne *et al.* (1995) highlighted the

hydrodynamic effects resulting from interaction of particle size and SC-$CO_2$ flow rate on the extraction yield.

The SM-matrix interaction which influences the dissociation of the SM from the matrix should be also taken into consideration. Choi *et al.*, (1999a; 1999b) and Rachmaniah *et al.*, (2014) revealed that in some cases the alkaloid desorption rate from the matrix plays a more important role than the solubility of the SM in SC-$CO_2$. Desorption can be improved by adding modifiers to the SC-$CO_2$ and often requires extra treatments (commonly for alkaloids and glycosides) prior to the extraction process to alter the chemical form of the SM (e.g. transformation into a free base). The modifiers can be part of the solvent in case of a semi continuous or continuous process, or can be used in the pre-treatment of the material prior to a batch extraction process or a combination of these two. Addition of modifier is often more efficient for changing the solubility in sc$CO_2$ than increasing the pressure (Del Valle *et al.*, 2005). A high amount of modifier will change the critical parameters of the solvent and in fact can be considered as a mixture of solvents. Disadvantages of modifiers concern issues like safety, health, toxicity, and environment. Improving the solubility of polar and ionic compounds in SC-$CO_2$ can also be achieved by dissolving the SM through ion-pairing, esterification, organometallic compound formation, complex formation, or reverse-micelle formation as discussed by Jiménez-Carmona and Luque de Castro (1998) and Luque de Castro and Tena (1996).

The appropriate water content in plant materials was determined to be ca. 3-10% (w/w) (Martínez and Vance, 2007). The water is needed to expand and swell the cell structure, facilitating mass transfer of solvent and solute through the solid matrix. Silica beads or diatomaceous earth are commonly mixed with fresh plant material to eliminate the excessive water content; moreover enhancing the surface area. A very low water content causes the plant material to shrink, consequently hindering the release of the targeted compound from the plant matrix (Laurent *et al.*, 2001; Reverchon and De Marco, 2006). Too high water content in plant material functions as a mass transfer resistance for SFE as well as causes technical problems such as clogging. Due to the fact that water has low solubility in SC-$CO_2$, it may cause a phase separation, even at 60 $^oC$ and 340 bar (Kim and Yoo, 2000; Choi *et al.*, 1999b). In case of working with plant materials, a coalescence phenomenon among the particles may occur when they contain too much water hampering the extraction.

### *2.2. Extraction process with supercritical $CO_2$.*

Among many SC candidates, $CO_2$ is the most preferred as a green solvent of supercritical fluids. Regarding its low polarity it is particularly suited for non-polar compounds. Thus, essential oils, oils and fatty acids are relative easily extracted. The extraction of alkaloids and glycosides, is much more challenging. The density of $CO_2$ is a variable that can be changed and influences the selectivity of the extraction. Dependent upon the density it can be liquid-like, gas-like, or in between .By manipulating temperature and/or pressure a gradient extraction can be achieved (i.e. fractionations). The commonly used batch process will result in a number of compounds to be extracted only partly, because of the low solubility of these compounds. This can be avoided by applying a continuous process.

The extraction rate is mainly determined by particle size distribution, porosity and contact surface. The mass transfer between phases is an important parameter, as well as the solubility of the target compound. Moreover, plant material preparation in the extraction vessel such as packing and the use of, trays, or basket is also affecting the effectiveness of mass transfer.

### *2.3. Post-extraction purification.*

This step is often required to further purify the SM. To obtain a high purity compound. SFE can be followed by fractionation of extracts or other purification processes in an integrated process. In fact, the full potential of a supercritical fluid (SCF) process can only be achieved by using an integrated process approach. An integrated approach should improve the process selectivity and the recovery of targeted compounds, and offers a strategy for yielding multi-products from the same plant material. Integrated processes concerning SFE of SMs were developed for the winning and separating of hydrocarbon monoterpenes and oxygenated hydrocarbon compounds (Akgün 2011; Fang *et al*., 2004; Martín *et al.*, 2011), terpenoid-cannabinoids (Brunel, 2011), carotenoids, tocopherol, (Ibáñez *et al.*, 2000), phenolics (Bernardo-Gil *et al.*, 2001; Ruen-ngam *et al.*, 2012; Serra *et al*., 2010; Yu *et al.*, 2007) and alkaloids (Then *et al.*, 2000; Palma *et al.*, 2000). Higher yields of high quality target compounds can be obtained by integrating SFE with separation processes (e.g. column chromatography, fractionation, and distillation) as well as reaction processes such as in case of hesperidin (Ruen-ngam *et al*., 2012) and squalene (Akgün, 2011). Coupling hydrolysation process under SC-$H_2O$ and SC-$CO_2$, hesperidin was hydrolysed into more valuable products, hesperetin-β-glucoside and hesperetin (Ruen-ngam *et al*., 2012). While squalene was obtained by esterifying olive oil deodorizer distillate (OODD) in supercritical MeOH followed with SC-$CO_2$ extraction (Akgün, 2011).

### *2.4. Modelling of SFE*

Natural compounds often have a so-called crossover point or region, meaning that for some solute-solvent combinations there is a region where at equal pressure the solubility increases with temperature, while in another zone this trend is reversed. Examples are limonene (Pourmortazavi and Hajimirsadeghi, 2007), cannabinoids (Brunel *et al.*, 2010) and rose-hip seeds oil (Machmudah *et al.*, 2008). Because of the complexity of the system, a fully predictive model taking into account all heterogeneities, matrix binding effects and transport limitations does not exist yet, only some simplified process steps have been described or modelled in several studies (Bravi *et al.,* 2002; Machmudah *et al.,* 2006; Meyer *et al.,* 2012; Reverchon *et al.,* 2000; Reverchon and Marrone, 2001; Sovová, 2005; Zizovic *et al.*, 2007). Machmudah *et al.* (2008) assumed two steps: desorption of targeted compounds from the plant matrix dominated by diffusion followed by compound elution in the same manner as in frontal elution chromatography.

For dealing with the process where the compounds are liberated from the plant matrix, two concepts have been used for modelling SFE: the shrinking core (SC) model and the broken and intact cells (BIC) model. In the shrinking core model, it is assumed that an irreversible desorption process of analytes from their matrices takes place followed by

diffusion through the pores in the solid, analogous to a dissolving particle. SC models have been developed for describing solid-fluid interaction phenomenon in desorption of the solute (Goto *et al.*, 1996; Machmudah *et al.*, 2006) and extraction column as a packed bed system (Ajchariyapagorn *et al.*, 2009). This model has successfully been applied for developing SFE of nutmeg oil (Machmudah *et al.*, 2006). The development this process for an extractor (modelled as fixed bed extractor) has been described by Ajchariyapagorn *et al.* (2009) based on previous work by Goto *et al.* (1996).

The Broken-Intact Cell (BIC) model introduced first by Sovová *et al.* (1994) assumes that analytes are easier extracted from crushed materials. However, it seems that this model only functions for lower concentrations of compounds (Machmudah *et al.*, 2006). Broken cells are mostly located at the surface of the material, where they have been damaged by mechanical actions e.g. grinding, cutting, chopping etc., whereas in the core of the particles intact cells are still present (Sovová, 2005). Therefore, initial fast extraction from broken cells is followed by slower extraction from intact cells. Fiori (2009) combined the two models of 'broken and intact cells' and the 'shrinking-core' model.

According to Huang *et al.* (2012), modelling of extraction is widely applied. However, it only works well for non-polar and volatile compounds using broken cell (BC) or BIC models such as essential oils which has been subject of extensive studies on SFE, including modelling work. Further improved models should include: the structure of the plant matrix and solute sorption and desorption on- and in the plant matrix, the location of the targeted compounds, the breakage of the cell structure, and the size and shape of particles (Reverchon and De Marco, 2006; Sovová, 2012a). Analyzing a static batch type of process, the following processes can be seen to play a role: desorption/sorption of compounds from the matrix, this includes parameters as partitioning coefficients; mass transfer factor and concentrations; diffusion rate in solvent; and contact between surface matrix and solvent. Obviously in a dynamic continuous extraction, the flow rate is a further parameter that affects the concentration both in matrix and solvent which in turn affects the diffusion rate. The two models, SC and BIC, are also applied in different ways in a batch or continuous process.

# 3. Application of Supercritical Fluid Extraction to Secondary Metabolites Purification

The compounds present in plants cover the whole range from non-polar, to polar. The non-polar compounds are mainly lipids and terpenoids; the medium polar compounds comprise phenolics, alkaloids, polyketides and highly oxygenated terpenoids; the polar compounds are the primary metabolites and glycosides of the medium polar compounds. The polarity range of the extracted compounds is shown by using comprehensive extraction (Yuliana *et al.*, 2011).

## *3.1. Commercial Application of SFE to SM Extraction*

The isolation and purification by SFE of the alkaloids caffeine and theobromine (Lack and Simándi, 2001; McNally, 2000), hop bitter acids (Langezaal *et al.*, 1990; Zhu *et al.*, 2006),

fatty acids, fats, and sterols (King, 2002; Morgan, 2000) takes place on a commercial scale. In terms of annual capacities and investment costs, decaffeination of coffee and tea is the largest application of SC-$CO_2$ extraction of SM followed with the extraction of hops, including smaller capacity in aromas, colorants, and dietary lipids (Laurent *et al.*, 2001).

#### ***3.1.1. The decaffeination process.***

The successful decaffeination process of both coffee and tea served as a model for other caffeine extractions e.g. from leaves of *Ilex paraguariensis* known as herba mate (Cardozo *et al.*, 2007; Cassel *et al.*, 2010; Saldaña *et al.*, 2000), guarana seeds *Paullinia cupana* (Saldaña *et al.*, 2002a;b) and coffee husks (*Coffea canephora*) (Tello *et al.*, 2011), see **Fig. 2**. The process key is the fact that caffeine has a high water solubility (21.6 g/L at 25 °C), therefore either SC-$CO_2$ saturated with water or moistened coffee beans are used in the extraction. In addition, hydration of coffee materials helps in hydrolytic rupture of hydrogen bonds which link the caffeine to the natural matrix, swells the cell membrane and sequentially enhances the caffeine diffusion out of the plant matrix (Brachet *et al.*, 2000). The extracted caffeine will be dissolved into water in the separator, and crystallization of caffeine-water mixture can be performed for caffeine purification. Without water the caffeine is incorporated too strongly in the matrix and thus difficult to extract.

Caffeine
MW = 194 ($C_8H_{10}N_4O_2$)

**Figure 2**. Chemical structure of caffeine which is containing in some plants: (A) coffea plant: coffee beans and coffee husks, (B) tea plant, and (C) *Ilex paraguariensis* (herba mate).

#### ***3.1.2. The extraction of hop bitter acids.***

In case of SC-$CO_2$ extraction of hop (**Fig. 3**), the SC-$CO_2$ extract has several advantages for beer: better foam value because tannins are not extracted by $CO_2$ (Van Cleemput *et al.*, 2009), resulting in reduced precipitation of proteins; more stable extract product i.e. non-isomerized compounds which can be directly added to the brewing kettle; and better long term stability of beer (Palma *et al.*, 2000). Langezaal *et al.* (1990) obtained a high yield of bitter acids and volatile compounds, ca. 107.5 mg/g, from cones and leaves of hop (*Humulus lupulus*) with SC-$CO_2$ extraction (40 °C, 200 bar, 3 h). The extract contained 65%, 30%, and 5% w/w, respectively, of α- and β-acids, β-myrcene, β-caryophyllene, and α-humulene, and δ-cadinene, and β-caryophyllene epoxide. Nearly all hop extraction plants in US, Europe and Australia operate with $CO_2$. The industrial plants are working at 40-65 °C and 300-350 bar, under these conditions all the hop bitter acids are extracted

quantitatively (Lack and Simándi, 2001). This shows that the conditions applied are not quite different from what is reported by Langezaal *et al.* (1990).

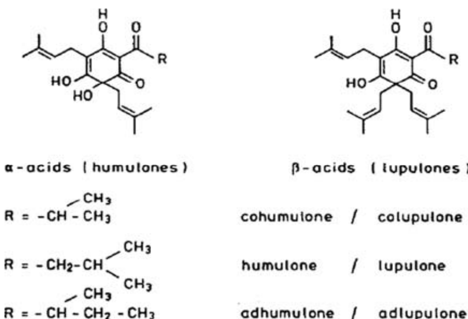



**Figure 3**. Chemical structures of the main hops bitter acids extracted from hop (*Humulus lupulus*) (Hermans-Lokkerbol and Verpoorte, 1994).

The distribution of publications (source: Scopus database) on SFE of different types of SMs is shown in **Fig. 4**. The search was conducted with the key words 'supercritical AND fluid AND extraction' combined with either terpenoids, essential oils, vitamins, pigments, glycosides, phenolics, or alkaloids. The focus is on SFE of terpenoids including essential oils, vitamins and natural pigments, phenolics, and alkaloids to give a perspective for new industrial applications. Extractions of highly polar secondary metabolites like glycosides are also briefly discussed while lipids/fatty acids and fats, non-polar compounds, will not be discussed further. This because Sahena *et al*. (2009); Temelli (2009); Temelli *et al.* (2007) already published a comprehensive review about SFE of fats and oils in food processing including the perspectives of the development.

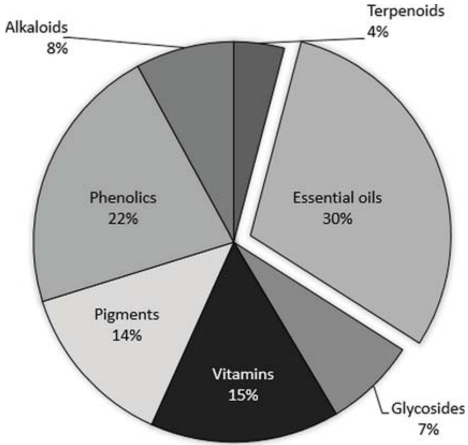



**Figure 4.** Distribution of publications on SFE applications for plant secondary metabolites within 1982-2013 time span (the pie diagram does not represent the number of SFE

applications but the number of publications, and serves to illustrate that there are still ample opportunities and challenges for exploring and studying SM by SFE. Total number of publications is 14,977).

**Table 1**. Details of selected SFE conditions for Terpenoids and Phenolics

| Extracted compounds | Matrix | Yield[a] | Extraction conditions | | | | Modifier | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | | Temp (°C) | Press (bar) | $CO_2$ flow[b] | Time[c] | | | |
| ***Glycosides*** | | | | | | | | | |
| β-amyrin and β-sitosterol | Leaves of Dandelion (*Taraxacum officinale*) | 4.46 and 1.23 mg/g of β-amyrin and β-sitosterol respectively | 65 | 450 | 35-53 kg $CO_2$/kg leaves | - | - | -moisture content of the dried material was 12.47%-w<br>-extraction was stopped when increased yield <0.1% | (Simándi *et al.*, 2002) |
| Amygdalin and β-sitosterol | Loquat seed (*Eribotrya japonica*) | ◦ *Roasted seed*<br>0.5635 and 0.315 mg/g of Amygdalin and β-sitosterol respectively.<br>◦ *Unroasted seed*<br>2.457 and 0.177 mg/g of Amygdalin and β-sitosterol respectively. | 80 | 200 | 3 | - | - | -$d_p$ 0.3 mm (roasted); $d_p$ 1.7 mm (unroasted) | (Kawahito *et al.*, 2008) |
| Digoxin | Leaves of *Digitalis lanata* | 100% recovery of digoxin was achieved[d] | 100 | 380 | 1 | 45 | MeOH-$CO_2$ (20:80, v/v) | -leaves were soaked 24 h into $H_2O$-EtOH = 80:20 v/v, and lyophilized prior the SFE. | (Moore and Taylor, 1996) |
| Glycosylglucose (GG) | *Vitis vinera* | 51.37 mMole/g of sample of GG | 40 | - | 1.5 | 20 | MeOH-$CO_2$ (20:80, v/v) (in dynamic state) | -extraction was conducted at 0.95 g/mL of $CO_2$ density<br>-static time was 15 min with 2 mL addition of MeOH<br>-sand was used as an inert matrix<br>-water 8 mL was used as liquid trap at 30 °C, and 50 °C restrictor temperature | (Palma *et al.*, 2000) |

**Table 1**. Details of selected SFE conditions for Terpenoids and Phenolics (*continued*)

| Extracted compounds | Matrix | Yield[a] | Extraction conditions | | | | Modifier/ treatment | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | | Temp (°C) | Press (bar) | $CO_2$ flow[b] | Time[c] | | | |
| ***Terpenoids*** | | | | | | | | | |
| Essential oil | *Carum coticum* | 8.53, 26.16, and 23.43 mg/g of γ-terpinene, p-cymene, and thymol respectively. | 35 | 304 | 0.3-0.4 | 30 | MeOH (10%, v/v) | -20 min static time<br>-glass beads mixed with plant material for SFE | (Khajeh *et al.*, 2004) |
| | *Mentha spicata* | 1340 mg/g of essential oils | 40 | 200 | 5 g $CO_2$/ min | 180 | EtOH (20%, v/v) | -dp 0.362 mm<br>-moisture content 10.7% w/w<br>-density of $CO_2$ 0.841 g/cm$^3$ | (Almeida *et al.*, 2012) |
| | Leaves and flowers of wormwood (*Artemisia absinthium*) | 47120, 23370, 12140, and 18050 mg/g of Z-epoxycimene, chrysanthenol, chrysanthenyl acetate, and nonacosane | 40 | 180 | 1.08 kg/h | 11 h | - | -dp 0.56 mm | (Martín *et al.*, 2011) |
| Squalene | Olive oil deodorizer distillate (OODD) | 237.76 mg/g | 50 | 105 | 7 | 180 | - | -Two steps of SFs were used. FFA was first esterified by SC-MeOH. | (Akgün, 2011) |
| Cannabinoid | *Cannabis sativa* | 197.4, 21.9, and 13.7 mg/g respectively for $\Delta^9$-THC, CBN, and CBG[e] | 40 | 230 | 6 kg/h | 180 | - | Raw material was decarboxylated first prior the SFE | (Brunel, 2011) |
| 10-Deacetylbaccatin III (10-DAB) | Needles of *Taxus baccata*. | 0.718 mg/g | 45 | 400 | - | 30 | MeOH (25%, v/v) | -dp 0.3 mm | (Kayan and Gizir, 2009) |

**Table 1**. Details of selected SFE conditions for Terpenoids and Phenolics (*continued*)

| Extracted compounds | Matrix | Yield[a] | Extraction conditions: Temp (°C) | Press (bar) | $CO_2$ flow[b] | Time[c] | Modifier/ treatment | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***Vitamin and Natural Pigments*** | | | | | | | | | |
| Chlorophyll a, chlorophyll b, β-carotene, and lutein | Leaves of stinging nettle (*Urtica dioica*) | 0.73, mg/g of chlorophyll a<br>0.10 mg/g of chlorophyll b<br>0.39 mg/g of lutein | 40 | 280 | 0.52 g/min | 2-12 h | EtOH 5.7%-w<br>EtOH 4.3%-w<br>EtOH 7.1%-w | -$d_p$ 0.2-0.4 mm<br>-void fraction of the particle bed was 0.63 | (Sovová *et al.*, 2004) |
| | | 0.24 mg/g of β-carotene | 60 | 280 | 0.52 g/min | 2-12 h | EtOH 4.4%-w | | |
| α-tocopherol and β-carotene | Pepper (*Capsicum annum*) by product[f] | 97 and 68% yield respectively for α-tocopherol and β-carotene[g] | 60 | 240 | 2000 mL/h | 120 | - | -freeze-dried material with <1% of humidity<br>$d_p$ 0.2-0.5 mm | (Romo-Hualde *et al.*, 2012) |
| Tocopherol (Vit.E) | Olive pomace[h] | 0.001747, >6.0, and >40.0 mg/g respectively for α-tocopherol, β-tocopherol, and γ-tocopherol[i] | 50 | 350 | 2000 mL/h | 180 | EtOH at 100 mL/h | -combination of SFE and two fractionation columns. | (Ibáñez *et al.*, 2000) |
| Lycopene and β-carotene | Fresh tomatoes | 0.1492 and 0.0194 mg/g of Lycopene and β-carotene | 40 | 268 | 500 | 30 | 1 mL of chloroform | -the material was mixed with silica gel to absorb the moisture | (Cadoni *et al.*, 2000) |
| | | 0.0235 and 0.0031 mg/g of Lycopene and β-carotene | | | | | 1 mL of hexane | | |
| | Tomato skins and seeds | 0.6441 and 0.3488 mg/g of Lycopene and β-carotene | 80 | 268 | 500 | 30 | - | -air dried skins and seeds (dried 24 h at 35°C), grounded and stored at -5°C. | (Cadoni *et al.*, 2000) |

**Table 1**. Details of selected SFE conditions for Terpenoids and Phenolics (*continued*)

| Extracted compounds | Matrix | Yield[a] | Extraction conditions: Temp (°C) | Press (bar) | $CO_2$ flow[b] | Time[c] | Modifier/ treatment | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***Vitamin and Natural Pigments*** | | | | | | | | | |
| Lycopene | Tomato paste waste | 0.17 mg/g of lycopene | 55 | 300 | 4 kg/h | 120 | EtOH 5%-v | -tomato waste was dipped in 5% sodium metabisulfite sol., dried for 5 days, and sieved with 3 mm sieve | (Baysal *et al.*, 2000) |
| Vitamin $D_3$ | Powder and granulated samples | 42.0 and 417 IU/g of vit.$D_3$ respectively for powder and granulated sample | 40 | 281 | 2 | 60 | $Et_2O$[j] 0.25 mL | -samples were mixed with diatomaceous earth<br>-modifier was added 10 min before extraction | (Gámiz-Gracia *et al.*, 2000) |
| ***Phenolics*** | | | | | | | | | |
| Naringin | Grapefruits (*Citrus paradise*) seeds | 0.18 mg/g | 50 | 483 | 5 | 30 | EtOH 30%-v | -two stages of extraction<br>-shells of dried seeds were used for the extraction, while the kernels were discarded | (Yu *et al.*, 2007) |
| | | 0.2 mg/g | 60 | 414 | 5 | 30 | EtOH 20%-v | | |
| Flavonoids | Ginkgo leaves (*Ginkgo biloba*) | 1.342 mg/g flavonoids | 60 | 312 | 5 | - | EtOH 24%-v | -total used volume of $CO_2$ was 300 L | (Chiu *et al.*, 2002) |

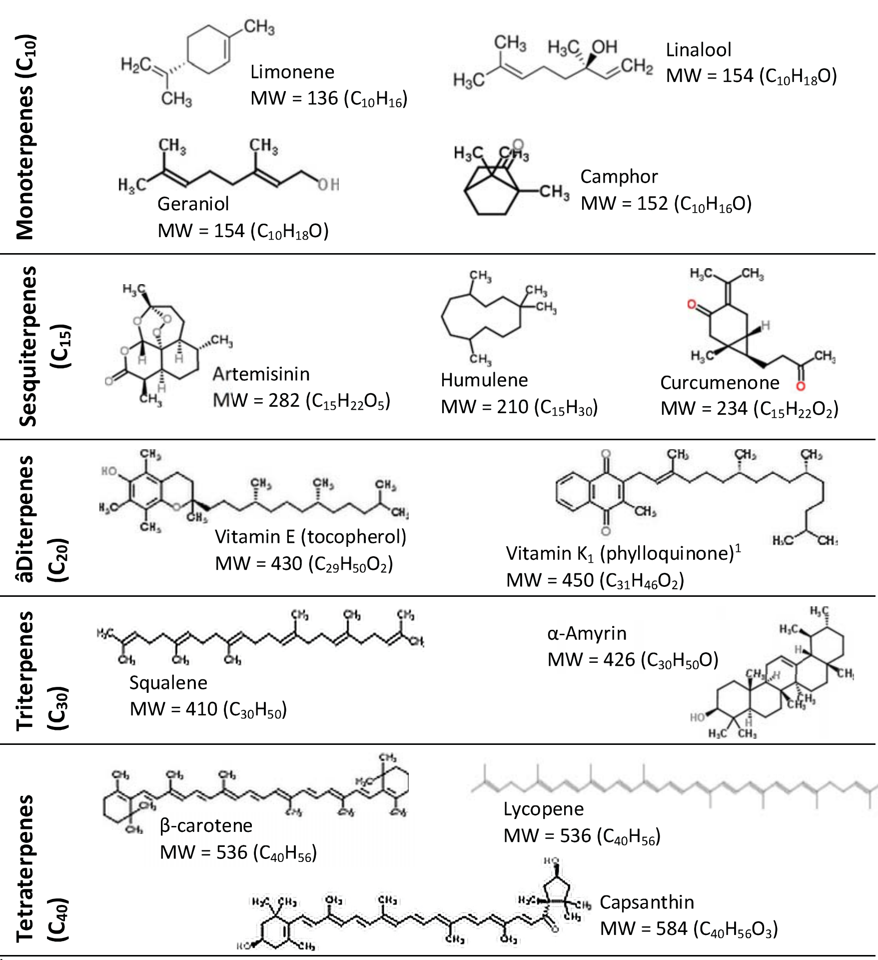
[a]Yield based on dry weight of materials.
[b]Flow rate of $CO_2$ is in mL.min$^{-1}$ unless in different units.
[c]dynamic extraction time is in minutes unless specified otherwise.
[d]The Recovery based on 0.25% recovered digoxin with liquid-liquid chloroform extraction.
[e]tetrahydrocannabinol ($\Delta^9$-THC), cannabinol (CBN), and cannabigerol (CBG)
[f]The pepper by product consisted of seeds, skin left overs and stem at 15.9, 34.7, and 49.4% in weight respectively.
[g]Yield presented on percentages based on the initial content of α-tocopherol and β-carotene in raw material.
[h]Olive pomace is semi solid residue collected from the outlet decanter after two-phase centrifugation of the olive paste for the production of olive oil.
[i]The yield was obtained after the second fractionation.
[j]Diethyl ether ($Et_2O$).

### 3.2. *Experimental and Analytical Applications of SFE to SM Extraction*

#### 3.2.1. *Terpenoids (including essential oils).*

Terpenoids or isoprenoids are derived from coupling of two or more isoprene units ($C_5$ units) and are classified according to the number of isoprene units (2, 3, 4, 6 or 8). Terpenoids form the largest class of SMs, widely diverse in size, polarity, volatility, and activity, explaining why there is not a single recipe for SFE of terpenoids. The best known terpenes are probably the essential oils (flavours and fragrances) which mainly comprise monoterpenoids ($C_{10}$) > 90% and a few sesquiterpenoids ($C_{15}$) which are all volatile and non-polar. Another major group consists of the carotenoids ($C_{40}$) which includes colorants and vitamin A. All are low polarity compounds which make them potentially suited for SC-$CO_2$ extraction. Other major groups as sesquiterpenes, diterpenes, triterpenes (**Table 2**). Selected SFE conditions for terpenoids are listed at **Table 1**.

**Table 2**. Terpenoids classification.



| Class | Compounds |
| --- | --- |
| Monoterpenes ($C_{10}$) | Limonene MW = 136 ($C_{10}H_{16}$); Linalool MW = 154 ($C_{10}H_{18}O$); Geraniol MW = 154 ($C_{10}H_{18}O$); Camphor MW = 152 ($C_{10}H_{16}O$) |
| Sesquiterpenes ($C_{15}$) | Artemisinin MW = 282 ($C_{15}H_{22}O_5$); Humulene MW = 210 ($C_{15}H_{30}$); Curcumenone MW = 234 ($C_{15}H_{22}O_2$) |
| âDiterpenes ($C_{20}$) | Vitamin E (tocopherol) MW = 430 ($C_{29}H_{50}O_2$); Vitamin $K_1$ (phylloquinone)[1] MW = 450 ($C_{31}H_{46}O_2$) |
| Triterpenes ($C_{30}$) | Squalene MW = 410 ($C_{30}H_{50}$); α-Amyrin MW = 426 ($C_{30}H_{50}O$) |
| Tetraterpenes ($C_{40}$) | β-carotene MW = 536 ($C_{40}H_{56}$); Lycopene MW = 536 ($C_{40}H_{56}$); Capsanthin MW = 584 ($C_{40}H_{56}O_3$) |

[1]Vitamin K1 contains a diterpenoidal part.

Paclitaxel (Taxol®) an important anti-cancer drug as well as its precursor, 10-Deactylbaccatin III (10-DAB), are diterpenes which can be extracted by SFE (**Fig. 5**). Taxol® was originally obtained from the trunk of Pacific Yew (*Taxus brevifolia*) in small quantities. The limited supply prompted intensive research to find alternative sources as well as its semi-synthesis using 10-DAB as a starting material which is abundantly available from leaves of different *Taxus* species. Though, 10-DAB is a rather non-polar compound, its large molecular weight (544.59 g/mol) causes its low volatility. Therefore, a modifier addition is required for extraction. Several modifiers in different polarities, i.e. MeOH, EtOH, THF, and DMSO have been tested in SFE of taxol from *Taxus baccata* needles (Kayan and Gizir, 2009). Methanol as modifier gave the highest yield (0.718 mg/g) which is even higher than the ethanolic-Soxhlet extraction (0.555 mg/g). Chun *et al*. (1996) also obtained higher yield of 10-DAB with SFE when using methanol as modifier of needles leaves of *Taxus cuspidata* compared to when it treated with n-hexane, which was produced only 0.02 and 0.071 mg/g, respectively, of paclitaxel and 10-DAB

MW = 853 ($C_{47}H_{51}NO_{14}$)

MW = 544 ($C_{29}H_{36}O_{10}$)

**Figure 5.** Chemical structures of paclitaxel (Taxol®), *left*, and its precursor 10-Deactylbaccatin III (10-DAB), *right*.

Supercritical fluid extraction of cannabinoids, has been conducted by Brunel (2011). Applying a centrifugal partition chromatography (CPC) coupled with the SFE extraction of *Cannabis sativa* cv. Bedrocan tetrahydrocannabinol ($\Delta^9$-THC), cannabidiol (CBD), cannabinol (CBN), and cannabigerol (CBG) were obtained as pure compounds (92-100%). SC-$CO_2$ extraction gave a cleaner extract compared to classical extraction since the majority of colouring matter and chlorophylls did not dissolve in SC-$CO_2$. However, some high molecular weight waxy ballast and terpenes were co-extracted. Thus, a post-extraction method either with winterization or CPC was needed to remove the waxes from the cannabinoids. SFE and hexane extraction, both after winterization, gave almost the same yields for cannabinoids: around 85, 9, and 6% for $\Delta^9$-THC, CBN, and CBG respectively (Brunel, 2011).

The classic process for the isolation of essential oils is hydro-distillation which yields a volatile oil mainly consisting of mono and a few sesquiterpenes plus oxygenated hydrocarbons derivatives (alcohols, aldehydes, ketones, acids, phenols, lactones, acetals, ethers, and esters) (Pourmortazavi and Hajimirsadeghi, 2007), some triterpenes, long chain hydrocarbons, fatty acids, and sterols. The hydrocarbon monoterpenoids have a lower boiling point (150-185 $^oC$) than the other mentioned compounds. Essential oils can

also be extracted with hydrocarbon like pentane and hexane or with SFE, yielding volatile (corresponding to the above mentioned hydro-distillate) but also non-volatile compounds like carotenoids, waxes, phenolics, and flavonoids. To obtain an essential oil by SFE that is at least similar or better than one obtained by hydro-distillation, one needs to determine the physical-chemical characteristics of the various compounds in the essential oil in order to develop an essential oil specific SFE process.

Due to their lipophilic nature essential oils can readily be extracted by means of SC-$CO_2$ at moderate temperature and pressure i.e. with a gas-like density of 400-750 kg/$m^3$ (Fornari *et al.*, 2012) due to their lipophilic nature. So modifier addition is not needed and the extraction time can be short (**Table 1**). Though in some cases, higher pressure and modifier addition were applied either to destruct the plant matrix (Fornari *et al.*, 2012) or to better solubilize and release the essential oils in high water containing materials (Almeida *et al.*, 2012). In case of essential oils extraction, duration time of an extraction (ca. >60 min) does not correlate with the scale of an extraction but is controlled by the rate of solubilisation (Aghel *et al*., 2004). More by-products, i.e. free fatty acids (FFA), alcohols, and cuticular waxes, will be co-extracted with a prolonged extraction time. These compounds are easily dissolved because of their lipophilic character and their localization on the leaf surface. Thus, they are easily solubilized in the non-polar $CO_2$ and generally obtained as co-extractants SFE of essential oils. In addition esterification of FFA and methanolysis or ethanolysis of lipids may occur with prolonged extraction time when alcohols are used as modifier (Kawakura and Hirata, 1998; Pinnarat and Savage, 2008). For example, major peaks of FFA and FAME were observed in GC-FID analysis of flower bulbs extracts when MeOH was used as a modifier (Rachmaniah *et al.*, 2014). Decomposition of some of the terpenoids present in the hydro-distilates was observed when exposed to heat, light or oxygen. Lack and Simándi (2001) and Morgan (2000) reported alteration of some essential oils obtained by hydro-destillation when comparing SFE and hydro-distillation extracts of essential oils and flavours and fragrances. A hydrolysis product of esters of linalyl acetate has been found in hydro-distillation extract of clary sage oils. SFE has been found to be gentler, and faster with less decomposition of terpenoids and gave higher yields. However, additional purification steps might be required to obtain an oil similar or better than an oil produced by hydro-distillation (Lack and Simándi, 2001). For example SFE followed by multistage fractionation.

An integrated two stage SFE process has been applied to obtain wormwood (*Artemisia absinthium*) essential oil (Ibáñez *et al.* 1999; Martín *et al.*, 2011). The fractionation separated mainly terpene hydrocarbons (lower boiling point) in the first stage and oxygenated derivatives in the second stage. Fang *et al.* (2004) used vacuum distillation coupled with SC-$CO_2$ fractionation of Bergamot oil (*Citrus aurantium Bergamia* Risso)in order to separate terpenes and oxygenated compounds from their non-volatile impurities, i.e. pigments, waxes, and coumarins (citropten). This deterpenation was successfully performed by vacuum distillation at low temperature (35 $^oC$), while oxygenated compounds and the impurities were satisfactorily separated by SC-$CO_2$ fractionation at 40-75 $^oC$. Integrated SC-$CO_2$ extraction process yielded high quality

oxygenated compounds as end product than applying a solely SC-$CO_2$ extraction method such terpene-less, pigment-less, and wax-less.

The general conclusion is that less polar compounds of volatile oils can be extracted at low pressure (vacuum condition) while more polar compounds require higher pressure (SFE). In fact the most volatile compounds are those with low molecular weight and polarity. Fornari *et al.* (2012) and Pourmortazavi and Hajimirsadeghi (2007) reviewed the isolation techniques of volatiles and essential oils including SFE.

***3.2.2. Glycosides.***

Kawahito *et al.* (2008), Moore and Taylor (1996), and Zhu *et al.* (2006) reported SFE of the glycosides-amygdalin and –digoxin (**Fig. 6**). They are moderately polar due to the attached sugar molecule, thus have a low solubility in SC-$CO_2$. Glycosides are composed of two portion: sugar (glycone) and non-sugar portion (aglycone); yielded one or more sugar upon hydrolysis.

Pre-soaking leaves of *Digitalis lanata* with 20% (v/v) of EtOH effectuated enzymatic hydrolysis of lanatoside-C into acetyldigoxin and subsequently to digoxin which could be recovered with SFE with a 77% yield as compared to a $CHCl_3$ extraction (Moore and Taylor, 1996). In fact modifier addition combined with a pre-soaking step improved the solvation power of SC-$CO_2$. Other strategies for extracting digoxin with SC-$CO_2$ were reported by Moore and Taylor (1995; 1997) including other alternative supercritical solvents, pre-extraction steps, and selective rinsing solvents for further purification of the extracts. Glycosyl-glucose (GG) and grape-glycosides have been successfully extracted from *Vitis vinera*. (Palma *et al.*, 2000) by using a similar strategy of modifier addition and solvent trap as in case of extraction of digoxin.

MW = 457 ($C_{20}H_{27}NO_{11}$)

MW = 780 ($C_{41}H_{64}O_{14}$)

**Figure 6.** Chemical structure of some of glycosides: (*left*) amygdalin from seeds of loquat fruits (*Eribotrya japonica*) (Kawahito *et al.*, 2008), and (*right*) digoxin from leaves of *Digitalis lanata* (Moore and Taylor, 1996).

Higher amounts of modifier, ca. >20% (v/v), has been successfully applied for extracting limonene from limonene-glycoside of grapefruit seeds (*Citrus paradise* Macf.) (Yu *et al.*, 2007) and flavonoids from flavonoid-glycoside of *Ginkgo biloba* leaves (Chiu *et al.*, 2002). High purity and more concentrated phenolic extracts were obtained with this application. Hydrolysis of the attached sugar molecule of glycosides compounds by

coupling SC-$CO_2$ with hydrothermal extraction (supercritical water) was the selected strategy of Ruen-ngam *et al.* (2012) for obtaining hesperetin. The hydrolysis of hesperetin-β-glucoside was possible due to the acidic conditions, formed by the dissociation of carbonic acid from the reaction of $H_2O$ and $CO_2$ at elevated temperature and pressure.

Hence, successful SC-$CO_2$ extraction of glycosides can be achieved either by removing the hydrophilic sugar molecule from the glycoside compound via hydrolysis or by enzymatic reaction, or by using a higher amount of modifier at a liquid-like density range of ca. 800-900 kg/m$^3$. Details of some SFE conditions of glycosides are summarized in **Table 1**.

### *3.2.3. Vitamins and Natural Pigments.*

SFE can only be applied to fat-soluble vitamins A, D, E, and K and to carotenoid pigments like β–carotene from carrots, tomatoes, or red pepper, lycopene from tomatoes and capsanthin from pepper. Although they are more stable than other pigments such as chlorophyll and anthocyanins, they also tend to degrade in the presence of oxygen. Carotenoids like lycopene are highly soluble in organic solvents, such as benzene, chloroform, methylene chloride, hexane, diethyl ether, and ethanol. Although they should be easily soluble in SC-$CO_2$, considering their non-polar nature their large molecular weight, ca. 500-700 g/mol, constrains their solubility in SC-$CO_2$. Therefore, an organic solvent can be added to improve the solubility (Baysal *et al.*, 2000; Cadoni *et al.*, 2000). Also the addition of vegetable oils (Ciurlia *et al.*, 2009; Vasapollo *et al.*, 2004) increases the SC-$CO_2$ extraction yield of carotenoids.

The optimal SFE conditions (729.37 kg/m$^3$ (80 °C, 268 bar), no modifier) for lycopene and β-carotene extraction from all parts of fresh tomatoes e.g. pulp, skin, and seeds yielded 0.644 and 0.349 mg/g of lycopene and β-carotene respectively, which is similar to 6 h of acetone-hexane Soxhlet extraction (Cadoni *et al.*, 2000). SFE of dried raw material gave even higher yields showing that a high water content in the fresh plant material will act as mass transfer resistance. However, fresh tomatoes having a high water content can be extracted by SC-$CO_2$ after adding silica beads to the material (absorbing the excessive water content) and solvents like chloroform or hexane, yielding 0.149 and 0.024 mg/g of lycopene, respectively (Cadoni *et al.*, 2000). There was clearly no yield benefit from modifier use. Moreover, traces of chloroform were present in the lycopene extract. Decomposition of lycopene may occur during extraction. Having 11 conjugated double bonds made lycopene susceptible to isomerization by light, heat, or chemicals (Zuknik *et al.*, 2012). Therefore sodium bisulfite was added to the waste of raw tomato paste preventing the isomerization and oxidation of lycopene. Using EtOH as modifier (850.30 kg/m$^3$ (55 °C, 300 bar)), yielded 0.167 mg/g of lycopene (Baysal *et* al., 2000), no yield increased. Solving this, Ciurlia *et al.* (2009) and Vasapollo *et al.* (2004) used hazelnut oil as modifier, either for increasing the yield and preventing the decomposition. This yielded 7.38 mg/g of lycopene (850.30 kg/m$^3$, 4 h) from dried tomatoes.

β–carotene, a pro-vitamin A, has been extracted from pumpkin (*Curcurbita moschata*) using MeOH as modifier with a yield of 79% compared to total carotene present (Seo *et al.*, 2005). Inert diatomeous earth was mixed with plant material (50:1) to increase the contact surface area between SC-$CO_2$ and the plant material. The strategy

developed by Cadoni *et al.* (2000) was also used by Gámiz-Gracia *et al.* (2000) for extracting vitamin $D_2$ and $D_3$ by using non-polar solvents as modifier, i.e. diethyl ether. Vegetable oil as modifier was also applied for vitamin E extraction which was referred as tocols, a cumulative value of tocopherol and tocotrienol (Temelli *et al*., 2013). Instead of using hazelnut oil as vegetable oil-modifier, Temelli *et al*. (2013) used barley as raw material without any modifier. Tt contained 4-8% of lipids (35% pearling flour), thus also serving as natural modifier, yielding 4.39 mg/g of tocol (913.40 kg/m$^3$ (60 °C, 450 bar)). A further fractionation process to obtain high purity of tocols was suggested. An integrated process of SC-$CO_2$ extraction of tocopherol (50 °C, 350 bar) using EtOH as modifier and with subsequent fractionation was reported (Ibáñez *et al.,* 2000). Tocopherol was collected in the second separator (25 °C, 10 bar), while the first separator (60 °C, 100 bar) produced low-polarity, high molecular weight compounds like triglycerides, waxes, and sterols. Rapidly reducing the pressure, increasing the temperature, or both, will selectively precipitate different compounds as their solubility is a function of SC-$CO_2$ density (i.e. temperature and pressure). Applying these principles on SC-$CO_2$ coupled with two separators which allows fractionation of a tocopherol extract into two fractions. Thus, an integrated purification strategy was adapted for this purification from essential oils purification process.

Clearly vegetables oils are more appropriate for use as modifier than organic solvents for SFE of fat-soluble vitamins. An explanation for this effect is that fat-soluble vitamins as well as lycopene are well extracted and more stable in the presence of fatty acids (Vasapollo *et al.*, 2004). The high solubility of fatty acids in SC-$CO_2$ also enhanced the extraction yield. Selected methods for SFE of vitamins including natural pigments from plant matrices are summarized in **Table 1**.

***3.2.4. Phenolics.***

A common group of plant compounds are the phenolics, characterized by at least one oxygen substituted benzene ring. It includes a broad variety of secondary metabolites which have otherwise not much in common, for example flavonoids (e.g. *epi*-catechin, quercetin), anthocyanins (e.g. cyaniding), lignans (e.g. pinoresinol), coumarins (e.g. umbelliferone), and tannins (e.g. gallic acid, ellagic acid) while each of these groups also contains many compounds without a phenolic function. A biosynthetic classification might in fact be more suitable, as most of the mentioned compounds are derived from the phenylpropanoid pathway. These type of compounds generally are of intermediate polarity, have a weak acidic character and are easily oxidized, making them good antioxidants. Their solubility in SC-$CO_2$ is less than the essential oils. For example, essential oils of rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*), and hyssop (*Hyssop officinalis*) were extracted at 40 °C, 115 bar (674 kg/m$^3$) while their phenolic diterpenes, carnosol and carnosic acid, were extracted at higher temperature and pressure (100 °C, 350 bar, 716 kg/m$^3$) (Babovic *et al*., 2010). Thus, different strategies of SC-$CO_2$ extraction should be applied for phenolics such as addition of modifiers, operating at conditions of liquid-like density, ca. >800 kg/m$^3$, and prolonged extraction time.

**Table 3**. Comparison of yield of Phenolics with three different extraction methods

| | Extraction methods | | | Ref. |
|---|---|---|---|---|
| | **SFE** | **Soxhlet** | **Ultrasound** | |
| **Extraction condition** | -$CO_2$ density was 0.95 g/mL; 1 mL/min<br>-static time was 20 min and 60 min of dynamic time<br>-MeOH as modifier | -MeOH-$H_2O$ = 4:1 (v/v)<br>-ratio of solid-liquid material was 24:1 mg/mL<br>-extraction time was 16 h | -MeOH as a solvent<br>-1 h extraction at 30 °C | (Palma and Taylor, 1999) |
| **Yield** | 77.6 mg/g of catechin | 63.0 mg/g of catechin | 65.6 mg/g of catechin | |
| **Extraction condition** | -60 °C, 250 bar, and 13 g/min of $CO_2$<br>-extraction time was 4 h<br>-17.5% of EtOH as modifier | -EtOH as a solvent<br>-Extraction time was 8 h | -EtOH as a solvent<br>-Extraction time was 30 min | (Oliveira *et al.*, 2013) |
| **Yield** | 2.008, 1.624, 0.263, 6.587, and 0.1521 mg/g[a] respectively for EP, GA, TA, p-BA, and VA[b] | 98.6 mg/g[a] for protocatecuic acid[c] | 1.219, 0.510, 0.642, and 5.0661 mg/g[a] respectively for EP, GA, TA, and p-BA[b] | |

[a]Yield expressed as mg of gallic acid equivalents (GAE) /g extract.
[b]EP = epicatechin, GA = gallic acid, TA = tannic acid, p-BA = p-OH-benzoic acid, and VA = vanillic acid.
[c]EP, GA, TA, p-BA, and VA were undetermined.

Residues of wine production, e.g. grape skins, seeds, and stems are known as a great source for phenolics. Caffeic acid (71%), *p*-coumaric acid (90%), *trans*-resveratrol (77%), and salicylic acid (59%) have been recovered from dry grape residues mixed with diatomaceous earth (899 kg/m$^3$ (50 °C, 350 bar), MeOH as modifier) (Tena *et al.*, 1998). In contrast, 50% lower recoveries were obtained from wet materials, showing that a high water content acts as a mass transfer resistance. The obtained yields of catechin and other phenolic compounds from white grape seeds (Palma and Taylor, 1999) and grape pomace (*Vitis vinefera* var. Merlot) (Oliveira *et al.*, 2013) by different extraction methods, i.e. SFE, Soxhlet, and ultrasound extraction, are compared in **Table 3**. Hence, SFE modified with either EtOH or MeOH gave higher yields of phenolic compounds in shorter extraction times compared with classical Soxhlet extraction. The chemical properties of alcohols such as hydrogen bonding, dipole-dipole interaction, and other interaction with phenolic compound make alcohols excellent modifiers for extraction of phenolics.

In an integrated process on SFE of phenolics both Yu *et al.* (2007) and Serra *et al.* (2010) have applied two-stage SFE extraction. Nonpolar $CO_2$-soluble compounds were exclusively eliminated in the first stage whereas higher polarity compounds including phenolics ended up in the second stage with the aid of alcohols as a modifier. A two-stage SFE strategy (without modifier) removed fatty oils of cherries (*Prunus avium*) in the first

stage (ca. 40.84% and 32.64% of linoleic acid and oleic acid respectively) and in the second stage 0.6 GAE mg/g of phenolics were isolated (expressed as Gallic acid equivalent (GAE)) (Bernardo-Gil *et al.*, 2001). Cavero *et al.* (2006) combined SFE (4%, v/v of EtOH modifier) and fractionation for successfully extracting antioxidants from Oregano (*Origanum vulgare*). A higher solvent density (879.60 kg/m$^3$ (40 °C, 250 bar)) was applied as compared to the essential oils fractionation column conditions (628.70 kg/m$^3$ (40 °C, 100 bar)).

Concluding, phenolics can be extracted by SC-$CO_2$ using a modifier like MeOH or EtOH. The phenolics can be quantitatively extracted and separated from terpenoids by applying a multistage SC-$CO_2$ extraction process yielding high-added value phenolic compounds. There is no material discharge between the stages because both extractions were carried out in the same extractor eliminating a unit operation of extraction and thereby reducing investment costs.

#### ***3.2.5. Alkaloids.***

Alkaloids are SMs containing a nitrogen atom as a part of a ring system and are biosynthetically derived from amino acids. They are basic in nature and form water soluble salts with (mineral) acids (De Keukeleire *et al.*, 2003). In plants, alkaloids occur as salts of organic acids such as acetic, oxalic, citric, malic, lactic, tannic, and tartaric acid (De, 2000) and some are neutral e.g. colchicine, xanthines and piperine. Only a few of them occur as glycosides or esters of organic acids. Alkaloids are usually stored inside the vacuoles of plant cells in the water soluble protonated form (De, 2000). This location in the cells and the protonation hamper SFE alkaloid extraction of which only a few publications could be found (**Fig. 4**). **Fig. 7** shows some examples of alkaloids extracted from different kinds of plants. In addition, **Table 4** shows a summary of studies made on SFE of alkaloids.

In order to extract the alkaloids, generally two strategies were applied. Primarily, the utilization of polar solvents such as EtOH and MeOH as modifiers to enhance the $CO_2$ polarity for example in the extraction of vinblastine (VLB), pyrrolizidine alkaloids (PAs), colchicine, boldine, and cocaine. Vinblastine, a well-known anti-cancer drug, as well as a taxol, can be extracted with SFE. Due to the low content of VLB in plant material (ca. 0.002 mg/g), an optimum simultaneously extraction and concentration method is needed. Indeed, the high molecular weight of VLB (824.96 g/mol) requires the use of a modifier. The SC-$CO_2$ extraction modified with a combination of MeOH and triethylamine (TEA) of the aerial part of *Catharanthus roseus* yielded 0.047 mg/g of VLB (Choi *et al.*, 2002), while MeOH modified SFE of leaves of *Catharanthus roseus* only gave catharanthine (CTR), vindoline (VDL), and 3,4-anhydrovinblastine (AVLB) (Verma *et al.*, 2008). Instead of VLB, 0.0778 mg/g of AVLB was obtained. Compared with a solid-liquid classical extraction of alkaloids, SFE gave a quicker extraction, a simplified clean-up procedure, and cleaner extracts. Different modifiers gave different contents of extract.

Another SC-$CO_2$ extraction was applied to extract colchicine, an FDA-approved gout medicine (Colcrys®), from *Colchicum autumnale* seeds (Ellington *et al.*, 2003). The yield was about the same as for conventional extraction using maceration/sonication (1198 mg/g colchicine, 126 mg/g 3-demethylcolchicine, and 546 mg/g colchicoside) (**Table**

**4**). A combination of $CO_2$ with MeOH as modifier with mechanical destruction like sonication resulted in a high yield and cleaner extract in a shorter time.

Boldine, an alkaloid from boldo (*Peumus boldus*) leaves was extracted at a high density of $CO_2$ (944.1 kg/m$^3$ (50 $^oC$, 450 bar)) with a modifier addition of EtOH (del Valle *et al.*, 2005), yielding 0.0074 mg/g. Modifier addition is mandatory in this case. At higher pressure (600 bar, 993.7 kg/m$^3$), boldine was hardly extracted with pure SC-$CO_2$ was used. No boldine was extracted at low pressure (90 bar, 285 kg/m$^3$) but essential oil was obtained, with ca. 50% yield. Changing the polarity works more efficiently by adding small amounts of modifier rather than by increasing the pressure to a high level. Longer extraction time (more than 7h) is recommended for an exhaustive SC-$CO_2$ extraction of boldine. This indicates that the SC-$CO_2$ extraction of boldine is solubility controlled. A hot pressurized water (HPW) extraction at 110 $^oC$ was also conducted as comparison, yielding 0.0052 mg/g of boldine. The yield was lower but the antioxidant potency of the HPW extract was higher as compared with the SC-$CO_2$. Therefore, it was suggested to utilize an integrated process of multistage SC-$CO_2$ coupled with HPW extraction. Low pressure SFE was conducted first with pure SC-$CO_2$ for selective removal of fatty acids and aroma compounds, followed by SC-$CO_2$ modified with EtOH for boldine extraction, and finally the HPW extraction of the antioxidants.

Traditionally, alkaloids have been extracted from plant materials with the addition of ammonium- or sodium-hydroxide at basic conditions. Such analogue treatments were applied for the successful SFE of alkaloids such as hyoscyamine, and scopolamine (Choi *et al.*, 1999a), *Ephedra sinica* alkaloids (Choi *et al.*, 1999b; Kim and Yoo, 2000), and galanthamine (Rachmaniah *et al.*, 2014), **Table 4**. The use of alkaline modifiers also helps to minimize or even eliminate the matrix-analyte desorption problem in case of extraction of SC-$CO_2$ alkaloids.

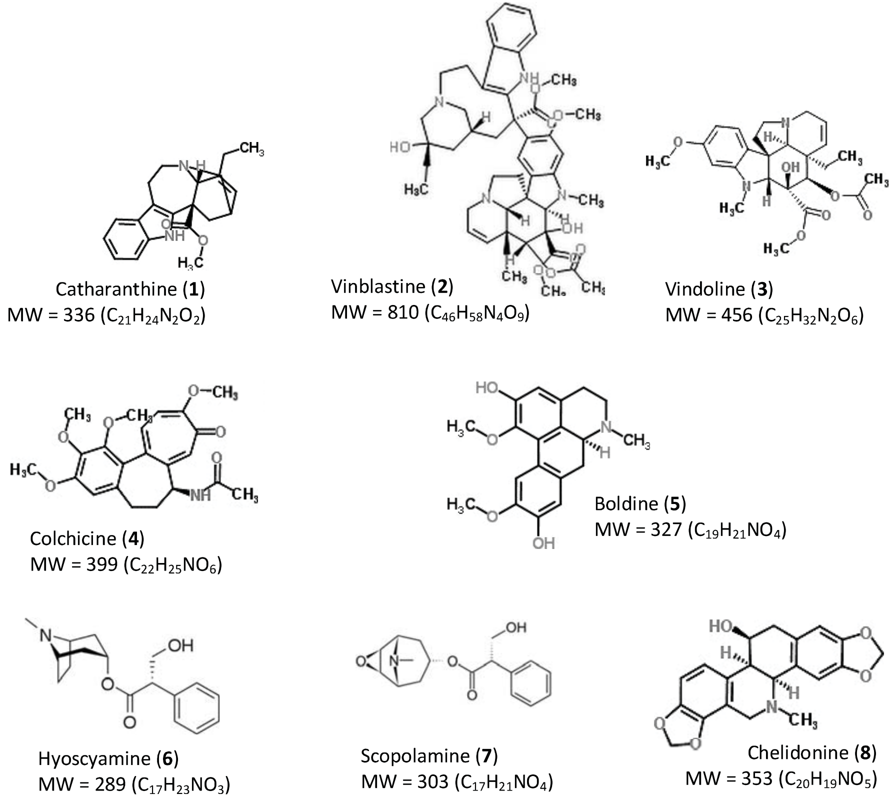



**Figure 7.** Chemical structure of some of alkaloids: catharanthine (**1**), vinblastine (**2**), and vindoline (**3**) from *Catharanthus roseus*; colchicine (**4**) from seeds of *Colchicum autumnale*; boldine (**5**) from leaves of *Peumus boldus*; hyoscyamine (**6**) and scopolamine (**7**) from *Ephedra sinica*; and chelidonine (**8**) from *Chelidonium majus*.

**Table 4.** Details of selected SFE conditions for Alkaloids

| Extracted compounds | Matrix | Yield[a] | Temp (°C) | Extraction conditions Press (bar) | $CO_2$ flow[b] | Time[c] | Modifier/ treatment | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***Alcohols (MeOH and EtOH) as modifier*** | | | | | | | | | |
| Evodiamine (EVD) and rutaecarpine (RTC) | Unripe fruits of *Evodia rutaecarpa* | 1.205 and 0.949 mg/g of EVD and RTC, respectively | 80 | 250 | 2 | 78 | 0.4 $mL.min^{-1}$ of MeOH | -5 min static time | (Liu *et al.*, 2010) |
| Pyrrolizidine alkaloids (senecionine (SNC) and seneciphylline (SNP)) | *Senecio inaequidens*<br>*Senecio cordatus* Senecio alpinus | 0.83 and 0.26 mg/g respectively for SNC and SNP<br>0.48 and 3.49 mg/g respectively for SNC and SNP | 55 | 150 | 250-350 µL/min | 60 | MeOH | -two steps extraction | (Bicchi *et al.*, 1991) |
| Colchicine (CLC), 3-demethylcolchicine (DCLC), and colchicoside (CLD) | Seeds of *Colchicum autumnale* | Total yield were 12.5, 1.72 and 4.58 mg/g of CLC, DCLC and CLD respectively | 35 | 247 | 1.5 | 30 | MeOH (3%, v/v) | -25 min static time<br>-two extraction step with $CO_2$ density kept constant at 0.90 g/mL | (Ellington *et al.*, 2003) |
| Quinolizidine alkaloids | Roots of *Sophora flavescens* | [check these high numbrs]85800, 221600, and 670230 mg/g of MT, OSC and OMT[d] respectively | 50 | 250 | 2 | 120 | 0.04 $mL.min^{-1}$ of $EtOH-H_2O$ (75:25, v/v) | -60 min static time<br>Plant material was treated with $NH_4OH$-EtOH (1:4, v/v) 24 h prior the SFE | (Ling *et al.*, 2007) |
| Boldine (aporphine alkaloid) | Leaves of boldo (*Peumus boldus*) | 0.0074 mg/g | 50 | 450 | 1.51 g $CO_2$/g substrate /min | 90 | EtOH (5%, w/w) | -dp 0.69 mm | (del Valle et al., 2005) |

**Table 4.** Details of selected SFE conditions for Alkaloids (*continued*)

| Extracted compounds | Matrix | Yield[a] | Extraction conditions | | | | Modifier/ treatment | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | | Temp (°C) | Press (bar) | $CO_2$ flow[b] | Time[c] | | | |
| ***Alcohols (MeOH and EtOH) as modifier*** | | | | | | | | | |
| Sinomenine | Vine stem of *Sinomenium acutum* | 7.34 mg/g | 60 | 300 | 0.5 | 60 | MeOH (0.4 $mL.min^{-1}$) | -5 min static time<br>-plant material wetted by 10% ammonia water and dried at 60 °C prior the SFE | (Liu et al., 2005) |
| Cocaine | Leaves of *Erythroxylum coca* | 600 mg/g | 40 | 200 | 2 | 15 | $CO_2$-MeOH-$H_2O$ (90:9:1, v/v/v) | dp 150-170 µm | (Brachet *et al.*, 2000) |
| Catharanthine (CTR), vindoline (VDL), and anhydroxvinblastine (AVLB) | Dried leaves of *Catharanthus roseus* | 0.1988, 0.2082, and 0.0778 mg/g respectively for CTR, VDL, and AVLB | 62 | 280 | 1.5 | 40 | MeOH (6.6%, v/v) | - | (Verma *et al.*, 2008) |
| ***Basified modifier*** | | | | | | | | | |
| Vinblastine | Aerial parts and roots of *Catharanthus roseus* | 0.036 and 0.005 mg/g respectively for aerial parts and roots | 80 | 340 | 1 | 50 | $CO_2$-MeOH-TEA (80:18:2, v/v/v) | - | (Choi *et al.*, 2002) |
| Nuciferine | Leaves of *Nelumbo nucifera* | 0.32554 mg/g | 70 | 300 | 1.2 | 120 | 10% v/v of modifier (1% v/v $H_2O$-DEA) | -plant material wetted by 10% $NH_4OH$ and dried at 60 °C prior the SFE | (Xiao et al., 2010) |
| Galanthamine | Bulbs of *Narcissus pseudonarcissus* cv Carlton | 0.303 mg/g | 70 | 220 | 1 | 180 | - | -plant material moistened by $NH_4OH$ (25%, v/v) | (Rachmaniah *et al.*, 2014) |

**Table 4.** Details of selected SFE conditions for Alkaloids (*continued*)

| Extracted compounds | Matrix | Yield[a] | Extraction conditions | | | | Modifier/ treatment | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | | Temp (°C) | Press (bar) | $CO_2$ flow[b] | Time[c] | | | |
| ***Basified modifier*** | | | | | | | | | |
| Hyoscyamine (HS) and scopolamine (SC) | Roots of *Scopolia japonica*.<br>Aerial parts of *Scopolia japonica*. | 6.24 and 0.24 mg/g respectively for HS and SC<br>1.17 and 0.69 mg/g respectively for HS and SC | 60 | 340 | 1 | 150 | DEA-MeOH 10% v/v | -15 min static time | (Choi *et al.*, 1999a) |
| Ephedrine derivate | ephedrine free base<br>ephedrine hydrochloride | The pseudo-solubility is 2.47 mg/mL<br>The pseudo-solubility are 0.213-0.564 mg/mL | 80 | 340 | 1 | 10 | $CO_2$-MeOH-DEA (90:9:1, v/v/v) | -15 min static time | (Choi *et al.*, 1999b) |
| ME, NE, E, and PE[e] | Aerial parts *of Ephedra sinica* | 0.37, 0.046, 3.44, and 0.40 mg/g of ME, NE, E, and PE respectively | 80 | 340 | 1 | 50 | $CO_2$-MeOH-DEA (80:18:2 v/v/v) | -15 min static time | (Choi *et al.*, 1999b; Kim and Yoo, 2000) |
| ***No addition of modifier*** | | | | | | | | | |
| Caffeine and theobromine | Leaves of *Ilex paraguariensis* | 41.5 and 0.327 mg/g for caffeine and theobromine respectively | 20 | 150 | 1 | 60 | - | -dp ≤0.35 mm | (Cardozo *et al.*, 2007) |
| | | 336 and 2.34 mg/g for caffeine and theobromine respectively | 50 | 150 | 60 | 60 | - | -dp 1.0 mm | (Cassel *et al.*, 2010) |
| Caffeine | Coffee husks (*Coffea canephora*) | 74.01 mg/g | 100 | 300 | 8 | 300 | - | -Pre-wetting the material up to 32% moisture | (Tello *et al.*, 2011) |

**Table 4**. Details of selected SFE conditions for Alkaloids (*continued*)

| Extracted compounds | Matrix | Yield[a] | Extraction conditions | | | | Modifier/ treatment | Additional information | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | | Temp (°C) | Press (bar) | $CO_2$ flow[b] | Time[c] | | | |
| ***No addition of modifier*** | | | | | | | | | |
| Dictamnine, obacunone, and fraxinellone | Root bark of *Dictamnus dsycarpus* (Baixian-pi) | 1.63, 0.83, and 2.87 mg/g respectively for dictamnine, obacunone, and fraxinellone (after HSCCC separation) | 45 | 300 | 2 | 6.5 h | - | -1 h static time | (Palma *et al.*, 2000) |
| Pyrrolidine alkaloid | Leaves of *Piper amalago* | 5.11 mg/g | 40 | 125.5 | 2 | 90 | - | SFE | (Carrara *et al.*, 2011) |
| | | 2.20 mg/g | 40 | 150 | 2 | 65 | - | Compressed propane | |
| ***Other strategies*** | | | | | | | | | |
| dl-tetrahydropalmatine (THP) | Rhizome of *Corydalis yanhusuo* | 1.324 mg/g | 70 | 200 | 2 | 90 | 0.4 mL.min$^{-1}$ of 1,2-propanediol | -5 min static time<br>-dp <250 µm | (Liu *et al.*, 2008) |
| Chelidonine | Greater celadine (*Chelidonium majus*) | 2.6 mg/g | 38 | 250 | - | N.D | - | -fresh plant material<br>-1.8 g $CO_2$/g substrate | (Then *et al.*, 2000) |
| | | 0.7 mg/g | 38 | 250 | | N.D | 2.6 g Propylene glycol/g substrate | - first SFE residue plant material<br>-1.8 g $CO_2$/g substrate | |

[a]Yield based on dry weight of materials.
[b]Flow rate of $CO_2$ is in mL.min$^{-1}$ unless in different units.
[c]dynamic extraction time is in minutes unless specified otherwise.
[d]matrine (MT), oxysophocarpine (OSC), and oxymatrine (OMT)
[e]Methylephedrine (ME), norephedrine (NE), ephedrine (E), and pseudoephedrine (PE).

Hyoscyamine (HS), and scopolamine (SC) could not be produced from the non-treated aerial parts and roots of *Scopolia japonica* by pure SC-$CO_2$ (Choi *et al.*, 1999a) because HS and SC are present as salts in *Scopolia japonica* and only marginally soluble in SC-$CO_2$. HS and SC have a $pK_a$ of 11.7 and 7.75, respectively. Thus a strong basic modifier like diethylamine (DEA, $pK_a$ of 10.84) could be used to solubilize the alkaloids-salts, and in a mixture with MeOH an effective modifier was obtained for the SFE of *Scopolia japonica,* giving the same yields for HS and SC as compared with conventional extraction. A comparable result was the failing extraction of an ephedrine derivate from the aerial parts of *Ephedra sinica* by pure SC-$CO_2$ (Choi *et al.*, 1999b; Kim and Yoo, 2000). They also showed that the solubility of pseudoephedrine (PE) hydrochloride in SC-$CO_2$ was lower than its free base (at the same conditions of 80 °C, 340 bar). By applying a basic modifier, MeOH-DEA (80:20, v/v), the yield was improved to the same value as with organic extraction (Choi *et al.*, 1999b), see table 3. Due to pseudoephedrine being a strong base ($pK_a$ 10.3), the addition of DEA is needed. The addition of base was also successfully applied for extracting galanthamine from *Narcissus pseudonarcissus* cv. Carlton bulbs (Rachmaniah et al., 2014). Instead of using DEA, $NH_4OH$ addition was found to work out best. Moistening the plant material with $NH_4OH$ (25%, v/v), increased the yield to 0.303 mg/g; while moistening with DEA and $NaHCO_3$ (10%, w/w) only yielded 0.027 and 0.014 mg/g of galanthamine, respectively. The low yield was thought to be caused by hampered desorption of *N. pseudonarcissus* alkaloids from the plant material rather than the solubility of the alkaloids in the SC-$CO_2$.

SFE of cocaine from leaves of *Erythroxylum coca* var coca (Brachet *et al.*, 2000; Brachet *et al.*, 2002) with an addition of triethylamine (TEA, $pK_a$ 9.8) did not enhance the yield although the $pK_a$ of cocaine is 8.65. SFE of cocaine was optimized (839.90 kg/$m^3$ (40 °C, 200 bar)) by moistening the leaf material (Brachet *et al.*, 2000) as in the caffeine case. Cocaine also highly soluble in water (1.8 g/L at 22 °C). MeOH-$H_2O$-$CO_2$ (9:1:90, v/v/v) was the best combination for the extraction and yielded 600 mg/g of cocaine while hexane and toluene only gave ≤0.1% dry weight (Brachet *et al.*, 2000). Thus, solubility is the limiting step in the SFE extraction of cocaine instead of desorption step (Brachet *et al.*, 2002).

On the basis of the alkaloids extractability in SFE from the plant matrix, a combination between pre-destruction treatment and SFE was effective. Two-stage SFE of fresh *Chelidonium majus* produced 2.6 mg/g chelidonine in the first stage and in the last stage using propylene glycol 0.7 mg/g, which was only 3.3 mg/g in total of chelidonine. Water extraction coupled with microwave destruction (65 °C, 20 min) yielded 6.1 mg/g while water extraction (60 °C, 24 h), and pressing (i.e. maceration for 24 h in water followed by pressing) only yielded 5.9, and 1.9 mg/g respectively (Then *et al.*, 2000). Due to the higher degree of destruction of the plant material, the targeted compounds were easier released. A combination of SFE and HSCCC (high-speed counter-current chromatography) were successfully used to separate the alkaloids dictamnine, obacunone, and fraxinellone (Palma *et al.*, 2000). Here, SFE at low temperature was chosen as the first step instead of traditional column chromatography to avoid multiple

chromatographic steps and minimize the use of organic solvents in addition they are labile compounds. Dictamnine, obacunone, and fraxinellone were obtained at high purity of 99.2, 98.4 and 99.0% respectively in the two step process.

Thus, the physical properties of alkaloids, i.e. basicity (indicated by the $pK_a$ value), water solubility, vapour pressure, as well as their storage location in the plant greatly determine the pre-treatment steps. In addition, SFE of alkaloids require the use of modifiers, because of the polar and basic character of these compounds. The alkaloids need to be in the non-protonated form, which requires basification of the plant material prior to extraction or adding a base to the SC-$CO_2$. Consideration should be taken that the modifier might be persistent in the final extract.

The versatility of SFE is obvious. A great diversity of natural compounds has been extracted by SFE. At the same time, one must conclude that there is no universal solvent for all kind of compounds. Each material and targeted compound requires the development of a specific process. No a single standardized procedure of SFE that can be suitable for extraction of all secondary metabolites. This also allows a rather selective extraction which is particularly of interest for the isolation of pure compounds. On the other hand, SFE extracts of medicinal plants or of plants containing essential oils are different from the classical extracts or essential oils. Here, some parameters that should be considered in the development of a SFE procedure are: size of the matrix particles; pre-treatment of matrix, e.g. acid/base treatment, soaking; modifier addition to supercritical fluid; type of process: static, dynamic, or combination; ratio of plant material to solvent; density of supercritical fluid (pressure and temperature of the supercritical fluid); duration of extraction; flow rate of supercritical fluid; and fractionation conditions of extract; covering pre-extraction treatment step and extraction process of SFE.

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# CHAPTER 3

## Environmentally benign supercritical $CO_2$ extraction of galanthamine from floricultural crop waste of Narcissus pseudonarcissus

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**Abstract**

The influence of diverse factors on the supercritical fluid extraction (SFE) with supercritical $CO_2$ ($scCO_2$) of galanthamine from bulbs of Narcissus pseudonarcissus cv. Carlton was investigated. The parameters that were studied were $CO_2$ density (temperature and pressure), flow rate and plant material particle size and pre-treatment. The highest yield (303 µg/g) was achieved by extracting 53–1000 µm particle-size powdered dried bulb material moistened with $NH_4OH$ (25%, v/v) at 70◦C, 220 bar (690 kg/m$^3$) for 3 h. Other N. *pseudonarcissus* alkaloids such as O-methyllycorenine and haemanthamine were also obtained. N. *pseudonarcissus* alkaloids as free bases are highly soluble in $CO_2$ at a high pH as opposed to the slightly soluble salt form in which they are generally found in plants. Therefore, plant material pre-treatment with a base is an essential step for galanthamine extraction. Scanning electron microscope (SEM) results also revealed that the desorption of N. pseudonarcissus alkaloids from the plant material rather than the solubility of the alkaloids in the $scCO_2$ plays a major role in this $scCO_2$ extraction. This extraction method has a good potential for industrial application.

**Keywords**: alkaloid, daffodil, narcissus and flower bulb, supercritical fluid extraction

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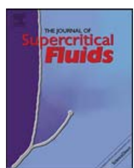

# Environmentally benign supercritical $CO_2$ extraction of galanthamine from floricultural crop waste of *Narcissus pseudonarcissus*

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ABSTRACT

The influence of diverse factors on the supercritical fluid extraction (SFE) with supercritical $CO_2$ ($scCO_2$) of galanthamine from bulbs of *Narcissus pseudonarcissus* cv. Carlton was investigated. The parameters that were studied were $CO_2$ density (temperature and pressure), flow rate and plant material particle size and pre-treatment. The highest yield (303 μg/g) was achieved by extracting 53–1000 μm particle-size powdered dried bulb material moistened with $NH_4OH$ (25%, v/v) at 70 °C, 220 bar (690 kg/m$^3$) for 3 h. Other *N. pseudonarcissus* alkaloids such as *O*-methyllycorenine and haemanthamine were also obtained. *N. pseudonarcissus* alkaloids as free bases are highly soluble in $CO_2$ at a high pH as opposed to the slightly soluble salt form in which they are generally found in plants. Therefore, plant material pre-treatment with a base is an essential step for galanthamine extraction. Scanning electron microscope (SEM) results also revealed that the desorption of *N. pseudonarcissus* alkaloids from the plant material rather than the solubility of the alkaloids in the $scCO_2$ plays a major role in this $scCO_2$ extraction. This extraction method has a good potential for industrial application.

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## 1. Introduction

The Netherlands is known as a major flowers exporting country and has been the center for worldwide flower bulbs trade for over 400 years [1]. A total land area of 3.4 million hectares (about 60% of total Netherlands area) is used for agricultural purposes more than 18,000 hectares (45,000 acres) of which are dedicated to bulb production [2]. More than half of all the flower bulb industry focuses on the production of cut flowers, pot plants and commercial flower bulbs [1,3] while the waste of this industry, though a potentially important source of novel products is not used. The major genera that contribute to the development of the bulb industry in the Netherlands are *Tulipa, Lilium, Narcissus, Gladiolus, Hyancinthus, Crocus*, and *Iris* [1].

*Narcissus* ranks as the third most popular genus for Dutch-grown flower production behind *Tulipa* and *Lilium* [1] and is presently an important ornamental bulb crop in the Western part of Europe [4]. It belongs to the *Amaryllidaceae* family that has long interested researchers due to the alkaloid and particularly galanthamine content of many of its members. Galanthamine is a tertiary amine and belongs to the isoquinoline alkaloids class that are derived from tyrosine [5]. It is an inhibitor of acetyl cholinesterase (AChE), an enzyme that hydrolyses the neurotransmitter acetylcholine (ACh). AChE inhibitors are used to treat Alzheimer's disease (AD) [6]. Finding new cheap natural sources of galanthamine and developing improved extraction and purification methods are of great interest because 1 out of 10 people above 65 years-old and almost half those older than 85 suffer from Alzheimer [6]. Today, galanthamine is mainly produced synthetically using a patented method that involves several steps and has a low yield [7]. Hille et al. [8] patented a liquid–liquid extraction method followed by subsequent acid–base purification steps with diethyl ether to obtain galanthamine. Another company, Agroceuticals Products (Wales, UK) claims that they can obtain natural galanthamine from daffodils but have not revealed their extraction process so far (www.agroceutical.com).

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Supercritical fluid extraction (SFE) is a modern technology with an increasing number of applications in the pharmaceutical and food processing industry. The principle of the process lies in the use of a supercritical fluid with physicochemical properties between those of a liquid and a gas. The supercritical condition occurs at a temperature and pressure that are above the vapor–liquid critical point, resulting in a supercritical fluid that possesses special properties such as a high rate of penetration into the materials to be extracted and a high rate of diffusion of the analyte into the supercritical fluids, low viscosity [9,10] and surface tension [10]. Today, $CO_2$ is the most frequently used fluid in SFE. The advantages of $CO_2$ are its abundant availability in nature, non-toxicity, non-flammability, environmental friendliness, low cost and moderate critical conditions ($T_c$ = 31.1 °C; $P_c$ = 72 bar). With these properties, $CO_2$ can be used to extract thermally labile compounds.

Considering the multistep and tedious synthesis of galanthamine, it would be useful to have an alternative, reliable, low-cost and environmentally safe process to provide the market with a cheaper, high quality product. Supercritical fluid extraction is an efficient and selective process suited to extract natural compounds from their matrix. To our knowledge, though a study on SFE of galanthamine from *Lycoris radiata* has been reported [11] there are no reports of the SFE of galanthamine from *Narcissus* bulbs. In the case of *L. radiata* extraction, the major focus was on the pre-treatment process of supercritical $CO_2$ ($scCO_2$) extraction with diminution, ultrasonic and basification methods. Galanthamine is a basic alkaloid that similarly to all alkaloids is present as a salt in plants. As described for *L. radiata*, it is necessary to convert alkaloids to their free bases in order to extract them successfully with SFE, so that a great amount of effort was put into finding the appropriate basic pre-treatment conditions since these vary according to the alkaloid. In this paper, we report a study on the $scCO_2$ extraction of galanthamine from *Narcissus pseudonarcissus* bulbs aimed at defining the best extraction conditions. The effects of particle size, $CO_2$ density (temperature and pressure), $CO_2$ flow rate, as well as plant material treatment were investigated. The alkaloid profile in the obtained extracts was also determined.

## 2. Materials and methods

### 2.1. Materials and chemicals

Fresh bulbs of *N. pseudonarcissus* cv. Carlton were supplied by Holland Biodiversity B.V. (Lisse, The Netherlands); the dried powdered bulbs and bulb pellets were supplied by Leenen B.V. (Sassenheim, The Netherlands). The bulb powder was ground, sieved and classified in three different particle sizes: >1000 μm, 53–1000 μm, and 25–53 μm. Galanthamine–HBr (Gal–HBr) reference compound was kindly donated by Tiofarma B.V. (Oud-Beijerland, The Netherlands) and palmitic acid and linoleic acid standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH), and trifluoroacetic acid (TFA) of HPLC grade and analytical grade diethylamine (DEA), acetic acid glacial (HAc), and concentrated ammonia 25% (v/v) ($NH_4OH$) were purchased from Sigma–Aldrich.

### 2.2. Supercritical $CO_2$ ($scCO_2$) extraction

#### 2.2.1. Preliminary $scCO_2$ extraction experiments

Freeze-dried fresh bulbs, pellets and powdered *N. pseudonarcissus* cv. Carlton bulbs were used in this stage. Before freeze-drying, the *N. pseudonarcissus* bulbs were rinsed with water to eliminate soil particles and the roots, bulbs and leaves were separated with a sharp blade. The basal plates were removed from the bulbs to aid grinding. Subsequently, the bulbs were frozen in liquid nitrogen and ground to a fine powder in a Waring laboratory blender (Waring Products Inc., Torrington, CT, USA). The ground plant material was then freeze-dried and kept at −80 °C until use. The water activity ($a_w$) test was determined by triplicate with a water activity instrument LabMaster aw (Novasina, Lanchen, Switzerland) at 20 °C.

**Table 1**
SFE parameters and values tested for galanthamine extraction.

| *P* (bar) | *T* (°C) | $CO_2$ density (kg/m$^3$)[a] | $CO_2$ flow rate (mL/min) |
|---|---|---|---|
| 150 | 40 | 780 | 1.25 |
| 200 | 40 | 840 | 1.65 |
| 220 | 40 | 856 | 1.65 |
| 150 | 50 | 700 | 1.25 |
| 200 | 50 | 784 | 1.65 |
| 220 | 50 | 804 | 1.65 |
| 150 | 70 | 515 | 1.25 |
| 200 | 70 | 659 | 1.25 |
| 220 | 70 | 690 | 1.25 |

[a] The density of $CO_2$ values were obtained from http://webbook.nist.gov.

The SITEC (FeyeCon B.V., Weesp, The Netherlands) extraction plant was used to perform preliminary experiments to optimize the $scCO_2$ extraction conditions (Section 3.1). The SITEC plant consisted of a $CO_2$ pump, condenser, and a 1 L extraction vessel. It was equipped with a sintered filter and a separator vessel. The temperature in the extraction vessel and separator were maintained constant with the aid of jackets with circulating oil from a thermostat-controlled bath. Other important components of the system included an equilibrium pump, a heat exchanger, a back-pressure regulator and a $CO_2$ flow meter. This plant was also equipped with a $CO_2$-recycle line, pressure release valve and a by-pass valve.

#### 2.2.2. ISCO supercritical extractor system

An SFX™-220 (ISCO) supercritical fluid extraction system with a 10 mL capacity was used to study the effects of the density of $CO_2$ (adjustable by tuning pressure and temperature of $CO_2$) and particle size on the yield of galanthamine because of the ease of handling (Section 3.2). The combination of different temperatures (40, 50, and 70 °C) and pressure (150, 200, and 220 bar) provided nine different densities. Different particle sizes (>1000 μm, 53–1000 μm, and 25–53 μm) of the plant material were also investigated in this study. The details of the pressure and temperature used in the study are displayed in Table 1.

The extractor was equipped with a restrictor, an extractor temperature controller (SFX™-200 controller), and a restrictor temperature controller (ISCO). It included a pump for $CO_2$ (ISCO syringe pump model 260D) and a chiller (cooling unit Hubbler-chiller control). The schematic diagram of the apparatus is shown in Fig. 1. Approximately 5 g of *N. pseudonarcissus* powder were loaded into the extraction vessel for each experiment and two replicates were made for each extraction. The extraction time was 3 h.

### 2.3. Plant material treatments and modifier

#### 2.3.1. Different plant material treatment

Before extraction, the plant material was treated with water and solutions of different basicity, i.e. 10% (w/w) $NaHCO_3$, DEA-$H_2O$ (20:80, v/v), DEA-MeOH (20:80, v/v), DEA, and $NH_4OH$ (25%, v/v). It was sprayed with the solution and left for 12 h in the refrigerator (in a closed plastic bag). The plant material was allowed to reach room temperature while in the plastic bag before loading it into the extractor vessel. In the case of DEA and $NH_4OH$ (25%, v/v), the samples were moistened with the solvent and left in a closed compartment until saturated which was indicated by constant weight (approx. 3 days).

**Table 2**
Recovery of galanthamine–HBr spiked in *Iris* × *hollandica* bulbs.

| Gal–HBr stock solution used | | | Number of analyzed extract | Gal–HBr in the extract | | | |
|---|---|---|---|---|---|---|---|
| μg/mL | $C_1$ [a] | $A_1$ [b] | | $C_s$ [c] | $A_2$ [b] | $C_2$ [d] | Average % recovery |
| 280.84 | 3.81 | 59828.30 | 4 | 1.01 | 15818.13 | 1.01 | 99.98 |
| 194.03 | 2.63 | 41334.90 | 4 | 0.90 | 14118.40 | 0.90 | 99.93 |

[a] nmol/5 mL injected.
[b] Peak area average (UV × min).
[c] nmol/5 mL spiked.
[d] nmol/5 mL analyzed (calculated by $C_2 = A_2/A_1 \times C_1$).

The pre-treatment of the plant material with water was also tested, by spraying 0%, 10%, 30%, or 40% water compared to the dry weight on the material which was subsequently left for 12 h in the refrigerator, in a closed plastic bag. The packed material was allowed to reach room temperature before loading it into the extractor vessel.

*2.3.2. Modifiers*

Methanol was used a modifier. Approximately 5 g of powdered material was loaded into the extractor (E), while MeOH was pumped into the line with a syringe pump (P1) through a tee inlet (T2) (Fig. 1). The fluid mixture of fluid MeOH and $scCO_2$ was continuously incorporated into the extractor (E) using an inlet tee (T1) at the selected ratio. The syringe pump was only connected to add the modifier.

## 2.4. Galanthamine analysis

Galanthamine content was determined using a HPLC-DAD (Varian 340). Extracts were dissolved in 90% methanol and 5 μL were injected onto a reversed phase analytical column Vydac C18, type 218MS54 (4.6 mm × 250 mm, 5 μm) 100 Å (Hesperia, CA). Samples were eluted isocratically with a mobile phase consisting of acetonitrile: 0.1%TFA in water (10:90, v/v) at a flow rate of 1.0 mL/min and detected at 210 nm, the wavelength of maximum absorption for galanthamine [12] with a total run time of 25 min.

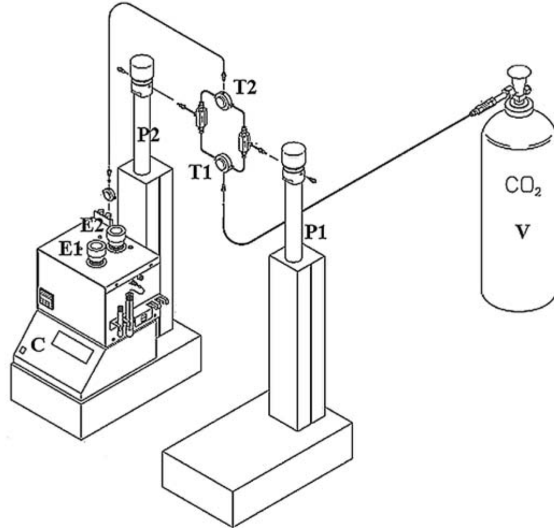



**Fig. 1.** Schematic diagram of the supercritical extractor system apparatus. Legends: V = $CO_2$ vessel, P = pump (ISCO syringe pump model 260D), T = inlet tee, E = extractor vessel, and C = temperature and pressure controller (SFX™-200 controller).

## 2.5. Analysis of galanthamine in raw materials

The galanthamine content in the raw material was first determined using a previously reported method [12,13] with slight modifications of sonication and vortexing times. The dried plant material (around 200 mg) was mixed with 10 mL of 0.1% TFA (v/v) in a falcon tube, vortexed (2500 rpm, 3 min) and then sonicated for 30 min. The clear supernatant was transferred to a 2 mL Eppendorf tube, centrifuged (13,000 rpm, 15 min) and 1 mL was collected for analysis. This method provided a high recovery (99.9%) of galanthamine (Table 2).

The recovery of galanthamine was determined by spiking ground bulbs of *Iris hollandica* cv. Blue Magic with known amounts of galanthamine–HBr standard since this species does not contain galanthamine. The same stock solution of galanthamine–HBr was used to prepare the calibration curve. Recovery was also carried out on samples prepared following the original method [12] to compare the result with our modified version (Table 2). The recovery tests showed that the method had an appropriate recovery.

## 2.6. GC analysis

*2.6.1. Fatty acids analysis*

Identification of fatty acids was carried out using gas chromatography coupled with mass spectrometry (GC/MS). A Varian 3800 gas chromatograph connected to a Varian Saturn 2000 mass EI detector was used for analysis. A DB-5 column (5% phenyl, 95% methyl polysiloxane), was employed with a 30 min temperature program of 60–300 °C at 10 °C/min followed by a 5 min hold at 300 °C; the flow rate of the carrier gas (He) was 1.2 mL/min; the split ratio was 1:20. Samples were dissolved in pyridine and derivatised with bis(trimethylsilyl)trifuoroacetamide (BSTFA) in *n*-hexane to convert non-volatile fatty acids to their TMS derivatives that give readily identifiable fragmentation patterns and mass ions. The mass spectra of TMS-derivatised fatty acids had one methyl group added to the parent molecule and served as a distinctive ion [14]. Galanthamine and fatty acids were identified by comparing the measured data with reference compounds (galanthamine, palmitic acid, linoleic acid) or with literature data [14].

*2.6.2. Alkaloid analysis*

Capillary GC/MS was employed to identify the alkaloids in the $scCO_2$ extract, using an Agilent 7890A GC system coupled to a 5975 C single quadruple Mass Spectrometric Detector and an Agilent 7693 Auto sampler (Agilent Technologies, Inc.). A DB-5 column was used with a two-step temperature program of 200–250 °C at 2.5 °C/min for 30 min followed by a ramp of 250–270 °C at 10 °C/min and an 8 min hold at 270 °C. The flow rate of the carrier gas (He) was 1.5 mL/min; the split ratio was 1:20. The identification was performed by comparing the measured mass spectral fragmentation data with those of authentic compounds (galanthamine) or with literature data as specified in the text [15,16] without the derivatisation step [17].

### 2.6.3. Alkaloids quantification

The alkaloids were quantified by gas chromatography-flame ionization detector (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 elimination of the solvent with a Speed Vac (Thermo Scientific, Waltham, MA, USA), 500 μL of the internal standard solution (papaverine–HCl at 21.2 μg/mL) and 150 μL of 0.05% (v/v) acetic acid in methanol were added to each vial. The sample preparation method was adopted from Gotti et al. [18]. The GC method was similar to that described previously (Section 2.6.2). The injection volume was 1 μL. The ratio of peak area of the analyte, i.e. galanthamine as its free base to the internal standard, papaverine, was plotted against the corresponding ratio of their weight to obtain the calibration curve. The linearity of the calibration graph was confirmed by a statistical method [19]. Assuming a similar detector response in GC-FID, the amount of other identified alkaloids besides galanthamine were expressed as μg galanthamine/g of dry weight of plant material.

## 2.7. Microscopy

Scanning electron microscope (SEM) was used to determine the structural changes of the plant material during the extraction process. For this, a JEOL JSM-840 scanning electron microscope (JEOL USA, Inc., Peabody, MA) was operated at 5 kV, 20 mm working distance. 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 discussions

## 3.1. Optimization of the $scCO_2$ extraction conditions

Preliminary experiments were done to find the best conditions for the $scCO_2$ extraction of galanthamine. The water content of plant material (reported as water activity, $a_w$) was taken into account since a high water content is considered to produce a mass transfer resistance for SFE [20].

The galanthamine content (11 mg/g) was determined in freeze-dried fresh bulbs since this was found to be the best way of preserving it. However, $scCO_2$ extraction of the freeze-dried bulbs gave a low yield of galanthamine, presumably due to a low content of water ($a_w$ = 0.16). A very low content of water causes the plant material to shrink, consequently hindering the release of the targeted compound from the plant matrix [10,20,21]. The galanthamine content of the powdered bulbs and pellets supplied by Leenen B.V. was determined to be 3 mg/g and 5 mg/g, which is quite below the fresh bulb content. However, both these processing methods have been adopted because they are the most suitable for handling and long-term storage of tons of bulb material. The pellet material itself is not suitable for the $scCO_2$ extraction as it is too compact and has a dense surface, but the powdered bulbs of *N. pseudonarcissus* proved to be adequate and was used for all further experiments in order to have comparable material and ensure the continuity of the bulb material supply. This material also had a suitable content of water ($a_w$ = 0.51–0.55) required for $scCO_2$ extraction.

Preliminary experiments of $scCO_2$ extraction were conducted using a SITEC (Section 2.2.1). The obtained SFE yields were compared with the yield of a classical ethanolic extraction method as reported by Sagdullaev [22]. With this last method, a yield of 246 μg/g of galanthamine was obtained from powdered *N. pseudonarcissus* bulbs. The obtained yields were reported as a ratio

**Table 3**
Supercritical $CO_2$ extraction conditions used in preliminary experiments.

| *P* (bar) | *T* (°C) | $CO_2$ flow rate (kg/h) | $CO_2$ density (kg/m$_3$) | Plant treatment | Ratio yield[a] |
|---|---|---|---|---|---|
| 120 | 60 | 7 | 532 | $H_2SO_4$[b] | 0.02 |
| 280 | 60 | 7 | 838 | $H_2SO_4$[b] | 0.25 |
| 120 | 70 | 7 | 347 | N.T[c] | –[d] |
| 120 | 60 | 7 | 532 | N.T[c] | 0.01 |
| 280 | 60 | 10 | 838 | N.T[c] | 0.34 |
| 280 | 70 | 7 | 769 | $NaHCO_3$[b] | 0.57 |

[a] The ratio between yields of the SFE and the yield of organic extraction by Sagdullaev [22].
[b] Aqueous $H_2SO_4$ (5%, v/v) was used for treating the plant material while for $NaHCO_3$ was 10% (w/w).
[c] N.T = No treatment.
[d] The obtained ratio yield was negligible ca. $2 \times 10^{-3}$.

yield and are shown in Table 3. In all tested conditions, the galanthamine yield was below that obtained with ethanol, even at a higher density of $CO_2$ ca. 838 kg/m$^3$ (280 bar, 60 °C). The highest ratio ca. 0.57 (corresponding with 140 μg/g) was obtained at 280 bar, 70 °C (769 kg/m$^3$) for 3 h, in the presence of bicarbonate, showing the importance of basic treatments for the extraction of galanthamine with SFE from its plant matrix. Acid treatment with $H_2SO_4$ (5%, v/v) did not increase the galanthamine solubility in $scCO_2$. Increasing the $CO_2$ flow rate appeared to enhance extraction slightly and a high liquid-like density of $CO_2$ was also preferable for these conditions. Thus, these parameters were chosen as the starting conditions of the $scCO_2$ extraction for further evaluation of the effects of particle size, $CO_2$ flow rate (pressure and temperature) and different plant material pre-treatments on the yield of galanthamine.

## 3.2. Effect of parameters on the yield of galanthamine

### 3.2.1. Effect of particle size

Three different particle sizes (>1000 μm, 53–1000 μm, and 25–53 μm) were tested to optimize this parameter. Additionally, a broader range of $CO_2$ density (515–856 kg/m$^3$) obtained by tuning the temperature and pressure of $CO_2$ was applied. Both temperature and pressure are important factors in supercritical extractions since they are directly correlated to the density of the supercritical fluids. Each target compound has its own optimal temperature and pressure for extraction since they affect external and internal mass transfer factors. Thus, the effect of three different temperatures (40, 50, and 70 °C) and three different pressures (150, 200, and 220 bar) were explored (Table 1). The corresponding results for all parameters are displayed in Fig. 2.

The best yield (≈8–24 μg/g) of galanthamine was obtained with 53–1000 μm (Fig. 2B); the bigger (>1000 μm) and smaller particle sizes (25–53 μm) yielded around 1–19 μg/g and around 6–19 μg/g, respectively (Fig. 2A and C). The dried bulb material was loaded into the extraction vessel as a fixed bed [23]. It is known that the rate of the extraction process can be improved by increasing the surface area or porosity of the materials. When particles differing in both size and density are used, segregation of the bed tends to occur during the extraction process resulting in the formation of two different beds of different densities which provide a better contact between the plant matrix and the $scCO_2$ [9,20,23]. Smaller particles reduce the path length of the solvent but if the particles are too small they may aggregate and clog the vessel or even be washed out with the solvent at high pressure [23]. This can also lead to a poor interaction between the $scCO_2$ and the plant matrix material because of channeling, dead zone/dead volume and flooding problems inside the extractor bed [20,24]. On the other hand, bigger particles can also cause inefficient interactions and it has also been reported to produce channeling of $scCO_2$ [25].

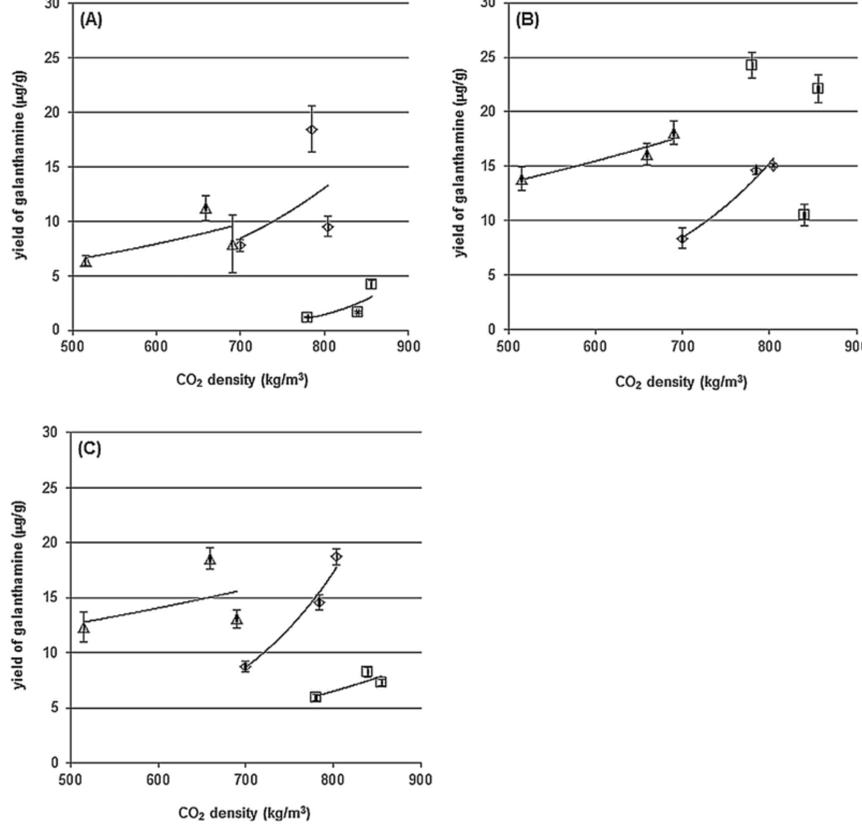



**Fig. 2.** Yield of galanthamine on SFE at different densities of $CO_2$ with a 3 h extraction time ((A) >1000 µm, (B) 53–1000 µm, (C) 25–53 µm; (□) 40 °C; (◊) 50 °C, and (△) 70 °C).

### 3.2.2. *Effects of $CO_2$ density (temperature and pressure of the extraction)*

The solvency power of supercritical fluids increases strongly and proportionally with its density [21]. Therefore, low (ca. 515–780 kg/m$^3$) and high density (ca. 784–856 kg/m$^3$) values of $CO_2$ were used for this study. The density itself cannot be adjusted but results from the temperature and pressure settings.

The highest solubility is achieved by increasing the extraction pressure (at a fixed temperature) to increase the density and thus the solvency power of the supercritical fluid. The yield of galanthamine increased from 11 µg/g at 40 °C, 200 bar (840 kg/m$^3$ $CO_2$ density) to 22 µg/g by increasing the pressure to 220 bar (856 kg/m$^3$ $CO_2$ density) (Table 4). This effect was observed for all particles sizes (Fig. 2).

When increasing the extraction temperature at a fixed high pressure, the yield increased, for example: at 50 °C, 220 bar (840 kg/m$^3$ of $CO_2$ density) the galanthamine yield was 15 µg/g while 18 µg/g were obtained at 70 °C, 220 bar (690 kg/m$^3$). A possible explanation to this behavior is the higher vapor pressure of the targeted compounds at higher temperatures. Kinetics might also play a role.

Assuming the different yields are mainly determined by the solubility differences and not by kinetics, this behavior corresponds with a crossover region, where the solubility decreases with an isobaric increase in temperature; while at a lower pressure the reversed trend is observed [27–29] as has been reported for SFE of several compounds [30–32].

### 3.2.3. *Effects of $CO_2$ flow rate*

Carbon dioxide flow rates of about 1.25 and 1.65 mL/min were observed at low (515–780 kg/m$^3$) and higher density (784–856 kg/m$^3$), respectively. This change in flow rates was the inevitable consequence of the changes in the pressure and temperature settings and cannot thus be set independently. This is due to the restrictor being outside the SFE oven [33]. As shown in Fig. 3, increasing the $CO_2$ flow rate at constant particle size and $CO_2$ density, slightly increased the yield of extracted galanthamine. Such effects have been explained by an increase in the amount of delivered and thus available $CO_2$ in the given extraction time resulting in an increase in the extraction of targeted compounds [34]. This was observed for two of the studied particle sizes, 25–53 µm and >1000 µm, (Fig. 3A and C). However, this did not always apply to all particle sizes and in the case of 53–1000 µm particles, the higher flow rate actually decreased the galanthamine yield (Fig. 3B). If the shorter residence time of the $CO_2$ were to cause a lower extraction rate per kg of $CO_2$, the total yield would be independent of the flow rate when reaching the high flow rate limits; the galanthamine would just be diluted in a larger amounts of $CO_2$. The observed lower yield must therefore result from hydrodynamic effects, for instance through a less favorable fluidization caused by the wider particle size distribution in the experiment of Fig. 3B. As reported previously, a wide particle size distribution tends to stimulate a segregation of the plant particles upon the extraction process [9,20,23] and generate a different behavior as compared to that of a uniform bed.

**Table 4**
Correlations between $CO_2$ density (pressure and temperature), $CO_2$ flow rate, residence time, and the galanthamine yield for 53–1000 μm size of plant materials.

| Extraction condition | $CO_2$ density[a] (kg/m$^3$) | $CO_2$ flow rate (mL/min) | Residence time (min)[b] | Galanthamine yield (μg/g) |
|---|---|---|---|---|
| 40 °C, 220 bar | 856 | 1.65 | 0.75 | 22.06 |
| 40 °C, 200 bar | 840 | 1.65 | 0.75 | 10.54 |
| 50 °C, 220 bar | 804 | 1.65 | 0.75 | 15.08 |
| 50 °C, 200 bar | 784 | 1.65 | 0.75 | 14.64 |
| 40 °C, 150 bar | 780 | 1.25 | 1.00 | 24.26 |
| 50 °C, 150 bar | 700 | 1.25 | 1.00 | 8.37 |
| 70 °C, 220 bar | 690 | 1.25 | 1.00 | 18.09 |
| 70 °C, 200 bar | 659 | 1.25 | 1.00 | 16.11 |

[a] The density of $CO_2$ values were obtained from http://webbook.nist.gov.
[b] Residence time was calculated based on the free space volume of the extractor per flow rate of $CO_2$. The bulk density of 53–1000 μm size of plant materials was 0.57 g/mL and was used to calculate the residence time.

The differences in yield between shorter and longer residence times are, however, insignificant. A shorter residence time of approximately 0.75 min yielded 11–22 μg/g of galanthamine while a longer residence time yielded 8–24 μg/g (Table 4). Hawthorne et al. [34] highlighted the significance of the supercritical fluid flow rate on the extraction yield of SFE and how it ultimately affects the recovery of the targeted compounds from matrices in some cases while in others it may only have a small effect because of the interconnection of all the extraction parameters.

### 3.2.4. Effects of plant material treatments and modifiers

*N. pseudonarcissus* alkaloids are moderately soluble in pure $scCO_2$ as free bases. However, they were scarcely detected in $CO_2$ extracts of plant material, coinciding with reports of the inefficiency of using pure $scCO_2$ to extract alkaloids from non-treated plant material [35–39]. This inconsistency was considered to be due to the fact that alkaloids are not majorly present in the plants as free bases but as salts that are insoluble in the lipophilic $CO_2$. Here, the plant material was treated with a basic modifier to convert the protonated (salt) form of galanthamine as stored in vacuoles [40] to their non-protonated free bases with the expectation of improving its solubility in $scCO_2$. This step could additionally improve the release of the target compounds from the plant matrix thus providing good accessibility of the $scCO_2$. Thus, plant material was treated with different basic aqueous solvents, i.e. $NaHCO_3$ (10%, w/w), DEA, $NH_4OH$ (25%, v/v) and pure water to test their efficiency.

Another way to improve the yield is by adding a polar modifier to increase the polarity of $CO_2$, since most alkaloids have a medium to high polarity. Methanol was chosen as a polar modifier due to its wide use as a modifier in SFE. Up to a 20% (v/v) of methanol is

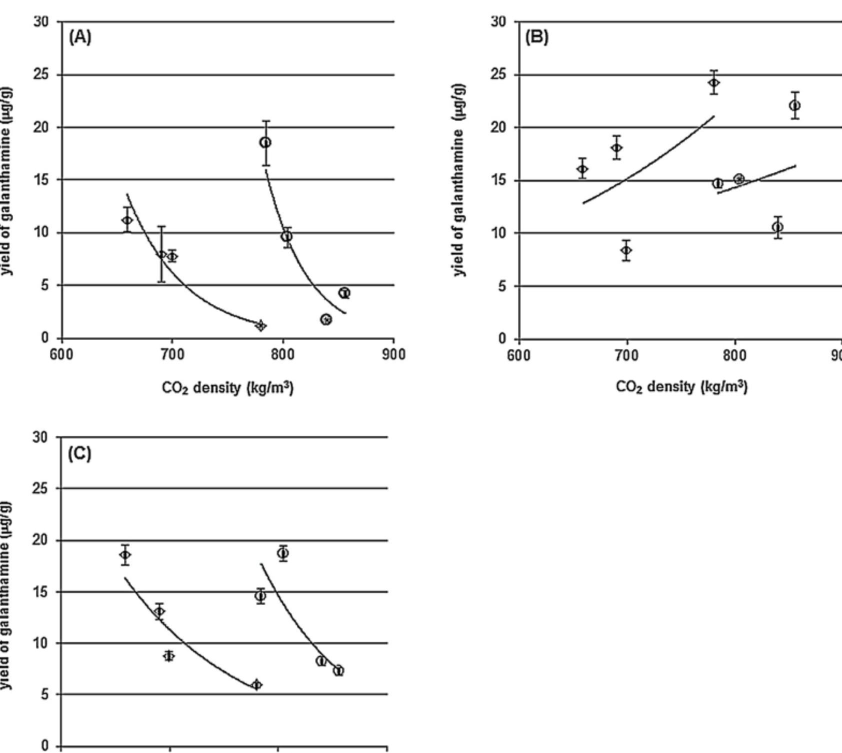



**Fig. 3.** Yield of galanthamine on SFE at different $CO_2$ flow rates with a 3 h extraction time ((A) >1000 μm, (B) 53–1000 μm, (C) 25–53 μm; (◇) 1.25 mL/min; (○) 1.65 mL/min).

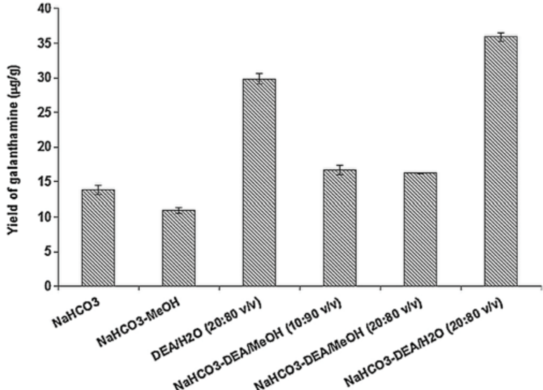



**Fig. 4.** The effect of different treatments on the galanthamine yield (all were added at a 10%-w ratio of dried plant material. SFE conditions were 70 °C, 220 bar and 3 h).

miscible with $CO_2$ [41]. Instead of adding it directly to the plant material, MeOH was mixed with the $scCO_2$. As expected the polarity of $scCO_2$ increased and was thus able to solubilize more galanthamine. The galanthamine yield (based on dry weight) is shown in Fig. 4. All of these experiments were conducted at 70 °C, 220 bar and 3 h.

*3.2.4.1. Plant material treatments.* The extractability of galanthamine increased to 14 µg/g when material was treated with 10% $NaHCO_3$ solution as compared to 0.12 µg/g without any treatment. With DEA-$H_2O$ (20:80, v/v), the galanthamine yield increased even more, to 30 µg/g (Fig. 4). Aqueous basic solutions ($NaHCO_3$-DEA/$H_2O$ 20:80, v/v) were more effective than mixtures of methanol with bases ($NaHCO_3$-DEA/MeOH 20:80, v/v) (Fig. 4).

This is in accordance with a previous report [35] of the $scCO_2$ extraction of tropane alkaloids, hyoscyamine and scopolamine. In particular, DEA may be more effective because having a low vapor pressure of 400 mmHg at 38 °C, it is therefore easy to vaporize and mix with the $scCO_2$ during the extraction process. Hence, the polarity of $scCO_2$ will be slightly modified increasing the galanthamine yield.

Considering the success of adding bases as pretreatments, double treatments, i.e., the combined used of different bases was attempted. However, none of the tested combinations improved the yield significantly as compared to the yield of each treatment independently. The mixture of $NaHCO_3$ and DEA/$H_2O$ 20:80 (v/v) yielded only 36 µg/g versus the 30 µg/g obtained with DEA-$H_2O$ (20:80, v/v) alone (Fig. 4). Thus, double treatments were not advised.

In view of the improvement observed with slightly basic treatments, stronger basic solutions were tested. For this, plant material was moistened with pure DEA and $NH_4OH$ (25%, v/v) with the purpose of suppressing ionization completely, ensuring that all alkaloids were converted to their non-protonated bases that are easily extracted in the $scCO_2$ as mentioned above. This proved to be successful and 27 and 303 µg/g of galanthamine were obtained with DEA and $NH_4OH$ (25%, v/v), respectively.

The pre-treatment of plant material with water was also attempted due to its matrix-swelling capability. Different ratios of plant material/water, i.e. 10%, 30%, and 40%-DW, were tested but the maximum yield was only 6 µg/g of galanthamine with 40% water. There were several other disadvantages of using water such as an acceleration of mold growth with more than 10% water and technical problems with the pump such as clogging. Due to the low molar solubility ($y$) of water in $scCO_2$ ($6900 \times 10^{-6}$ at 50 °C and 202 bar) [42], phase separation and ice formation in the tubing system resulted even at 60 °C and 340 bar [39].

Coalescence phenomena among the particles may occur when a high water content is present in the plant materials. This causes irregular extraction along the extraction bed increasing the mass transfer resistance [20]. On the other hand, the presence of water in the plant matrix expands the cell structure, facilitating mass transfer of solvent and solute through the solid matrix. The highest yields in supercritical fluid extraction have been achieved with a 3–10% water content in the plant material [43] so that the addition of 10%-DW of water was considered to be the most appropriate proportion.

*3.2.4.2. Methanol as modifier.* Methanol has been used to modify the $CO_2$ as it produces a dramatic increase in the solubility of the more polar compounds. While widely used as a modifier because of this, it is known to be selective in some cases, increasing the solubility for certain compounds without affecting others. A 10% volume of MeOH was added continually to the $scCO_2$ in line during the extraction at 70 °C, 220 bar, and 3 h. Surprisingly, while clear peaks of palmitic and linoleic methyl ester were observed, only a small galanthamine peak was detected (Fig. 5A) so that fatty acids were found to be the major compounds while the yield of galanthamine was only 3 µg/g. The GC-MS analysis of the extract allowed the identification of palmitic acid (C16:0), linoleic acid (C18:2) and a sterol by their molecular ions at $m/z$ 313, 337, and 396, respectively; the galanthamine ion was observed at $m/z$ 358. However, the presence of fatty acids in the $scCO_2$ extract was insignificant when plant material was treated with $NaHCO_3$ (Fig. 5B).

The formation of palmitic and linoleic methyl esters occurs due the presence of MeOH in the extraction process. Methanol is in its subcritical phase in these extraction conditions (70 °C, 220 bar) and can promote alkyl esterification of fatty acids. In a subcritical condition, both MeOH (polar molecule) and fatty acids (nonpolar molecules) become a single homogeneous phase [44] and this accelerates the esterification reaction. Moreover, MeOH acts both as a reactant and acid catalyst. Other researchers also reported simultaneous in situ extraction and derivatisation of fatty acids [45,46]. As noted previously, linoleic and palmitic acid are well solubilized in $scCO_2$ [47]. They occur in significant quantities as co-extractives in the extract. Therefore, fatty acids and their corresponding methyl esters are the major product when MeOH was as modifiers.

Fatty acids and their corresponding methyl esters are non-polar compounds that are have a higher solubility in $scCO_2$ than galanthamine that is considerably more polar. Besides that, fatty acids themselves are more accessible to $scCO_2$ because their solubility is higher than that other esterified fats such as triglycerides, diglycerides, sterols and monoglycerides. Their extractability is also increased because they are in the cell membranes which are easy to rupture and distort by mechanical forces [48]. Alkaloids, on the other hand, similarly to other secondary metabolites, are usually stored inside the vacuoles of plant cells [41] that are not readily accessible to $scCO_2$.

Palmitic acid [49–51] and linoleic acid [50,51] are the major fatty acids present in the bulbs. Linoleic acid represents nearly 70% of the total bulb fatty acids and the rest is composed of palmitic and stearic acid (about 20% and 10%, respectively) [51]. However, stearic acid was not extracted in these conditions (70 °C, 220 bar). This is probably because stearic acid is known to be the least soluble of these four fatty acids in $scCO_2$. Linoleic acid is the most soluble, followed by palmitic acid and stearic acid [47].

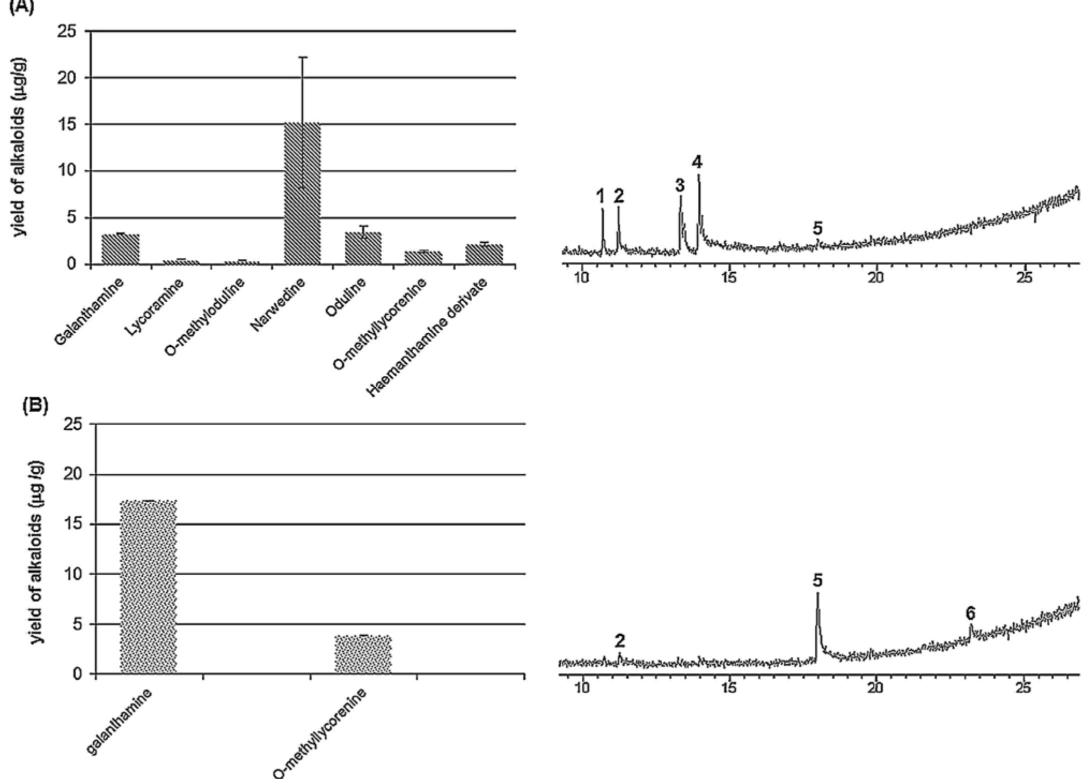



**Fig. 5.** Alkaloid profile of *Narcissus pseudonarcissus* along with their GC-MS chromatograms for fatty acid identification in the $scCO_2$ extract at 70 °C, 220 bar: (A) with 10% (v/v) MeOH as modifier and (B) treated with 10% (w/w) $NaHCO_3$. Legends: Palmitic methyl ester (**1**), palmitic acid (**2**), linoleic methyl ester (**3**), linoleic acid (**4**), galanthamine (**5**) and a sterol (**6**). (The bars represent the SD calculated from three replicates. Yield of alkaloid other than galanthamine are expressed as galanthamine.)

### 3.3. Effect of $scCO_2$ extraction on the cell destruction

The role of the plant matrix in the $scCO_2$ extraction process of galanthamine was also studied. Scanning Electron Microscope (SEM) was used in assessing the cell structures before and after the $scCO_2$ extractions. Based on previous results (Section 3.2), several factors were hypothesized to affect the extraction yield of galanthamine in the $scCO_2$ extraction. They are (1) penetration of $scCO_2$ in the plant matrix; (2) localization of galanthamine in the plant matrix; (3) solubility of galanthamine in the $scCO_2$. The mass transfer resistance will be discussed in the following subsection.

#### 3.3.1. Intact cell of the N. pseudonarcissus bulb

Successful extraction using supercritical fluids does not depend only on the solubility of the target compound and undesired compounds; mass transfer resistance due to the structure of the plant matrix and the localization of the compound(s) of interest including binding to the cells should also be taken into account. The *N. pseudonarcissus* bulbs were freeze-dried (**Frz**), in order to retain the intact cell structure of the bulbs; along with **Frz** material, powder (**Pwd**) material treated by 10%-w $NaHCO_3$ solution were observed to mimic the initial conditions of the plant material. The initial cell structure of both materials is shown in Fig. 6.

The globular granules observed are starch [52], in well-ordered shape, heavily packed (insert B of Fig. 6) and clustered. The well-ordered shape of granules was kept intact because of the freeze drying method, which preserves the structure of the plant without further damage. The granules condition is different when compared to the insert D of Fig. 6 that shows the **Pwd** material treated by 10% (w/w) $NaHCO_3$ solution. The, small, white, scattered spots – such as in insert C and insert D of Fig. 6 – are from the alkaline-modifier ($NaHCO_3$) that was added to the plant material at the treatment process and was deposited on the plant surface. Mechanical and shear forces developed during the grinding process result in deformation and fracture of the cell.

#### 3.3.2. Effect of $scCO_2$ density on the cell deformation

The explanation for the improvement in galanthamine extraction is not limited only to its solubility but also to desorption of the alkaloids from the plant matrix. Several researchers have mathematically modeled the phenomenology of the $scCO_2$ extraction of biological materials by means of mass transfer and diffusion [32,53–57].

Supercritical $CO_2$ as a fluid penetrates the plant matrix and dissolves the solute, i.e. targeted compounds. Solute-mass transfer from its plant matrix to its surface is fast when the surface matrix has been damaged or broken by mechanical treatment, i.e. grinding. Further transport to the fluid is also fast due to the primary properties of the supercritical fluid. Therefore, there is a hypothetical equilibrium which occurs between the surface layer of the plant matrix-solid phase and $scCO_2$-fluid phase. When the solute at the surface layer is transferred, diffusion of solute to matrix surface through the intact core matrix starts [53,57]. This diffusion to

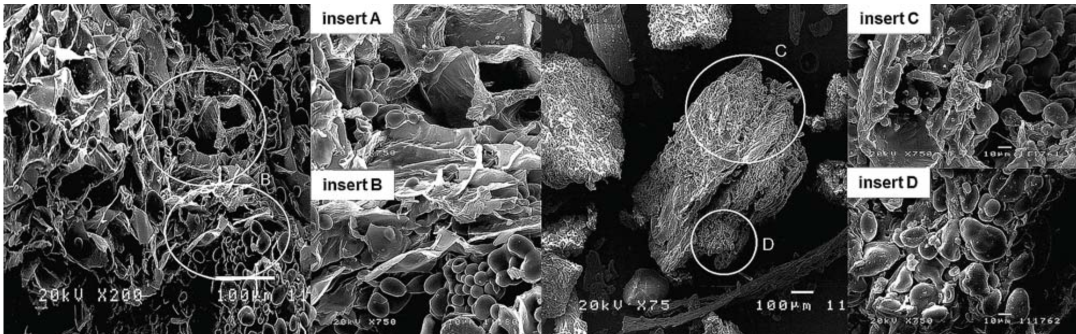



**Fig. 6.** SEM pictures of (A and B) **Frz** material with 200×, and (C and D) **Pwd** material treated with 10% (w/w) of $NaHCO_3$ solution (75×). Insert pictures are at 750×. One bar is equivalent to 100 µm for 75× and 200×, while 10 µm for 750×.

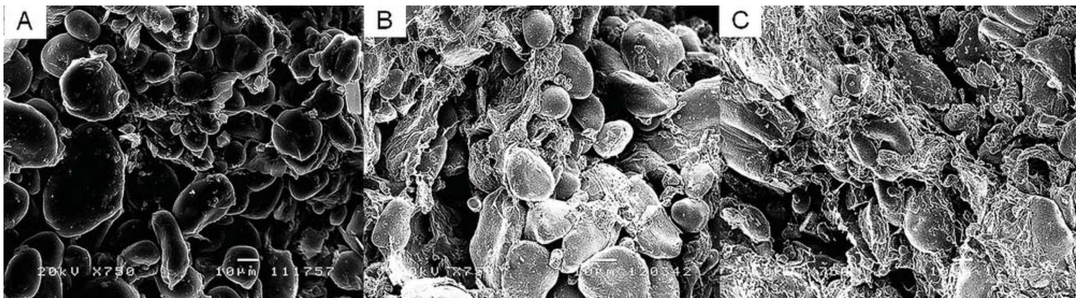



**Fig. 7.** SEM images of the residue of **Pwd** material treated with 10% (w/w) of $NaHCO_3$ solution after extraction with $scCO_2$ at (A) 70 °C, 220 bar, (B) 50 °C, 200 bar, and (C) 40 °C, 220 bar. All extractions lasted 3 h. The $CO_2$ densities were 690, 784, and 856 kg/m$^3$. Images are registered at 750×, one bar is equivalent to 10 µm.

matrix surface through the plant core is slower, because the mass transfer resistance of the intact cells is higher [58].

The approach model described above is called "broken intact cell" (BIC) [53,57]. Besides the broken surface, where the targeted compound easily diffuses to the matrix surface, there is the particle core containing intact cells. The mass transfer resistance of the intact cells is higher [58] resulting in slower extraction. Both types of mass transfer are hypothesized to be happening simultaneously. Regarding the low level of galanthamine in the dried bulbs powder of *N. pseudonarcissus* ca. 3 mg/g, the equilibrium between $scCO_2$ fluid phase and the plant matrix-solid phase is controlled by free solute and the galanthamine–plant matrix interaction [53,56].

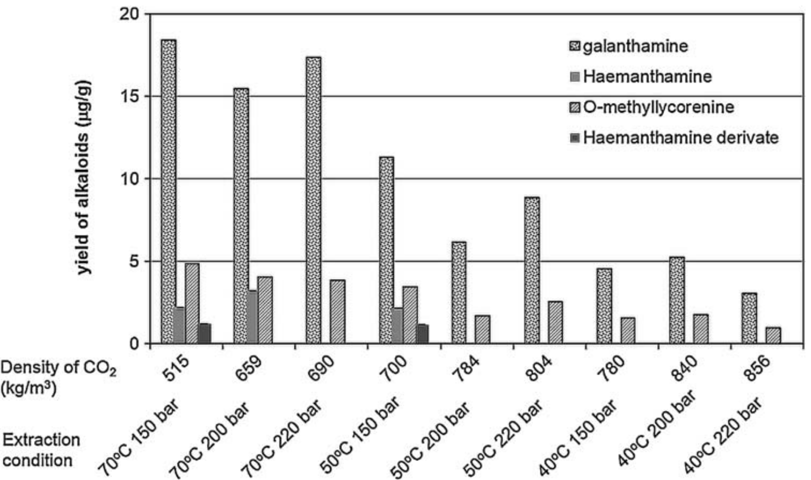
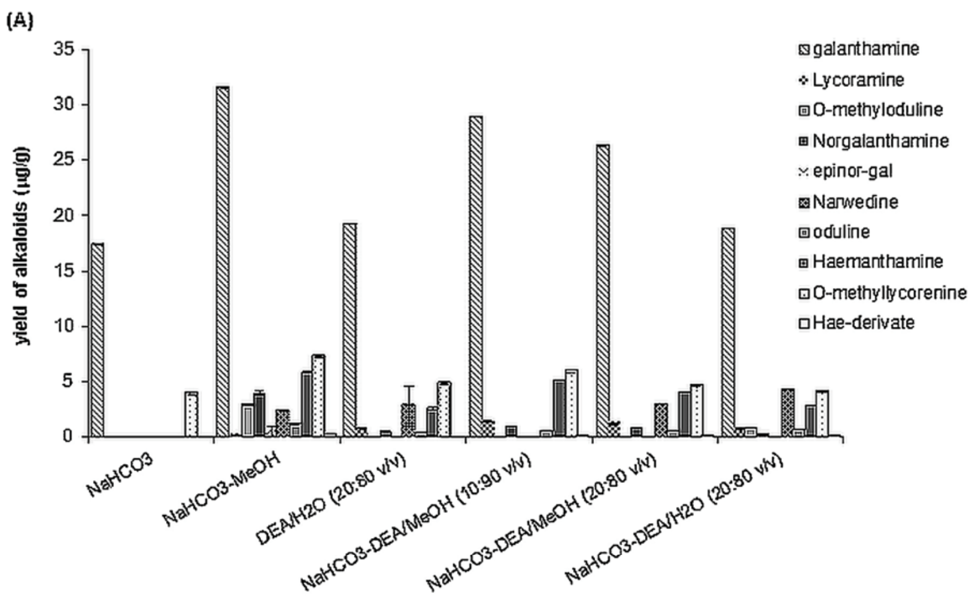



**Fig. 8.** Alkaloid profile of $scCO_2$ extract of *Narcissus pseudonarcissus* using 10% (w/w) $NaHCO_3$ as plant material treatment and $d_p$ > 1000 µm particle. (The bars represent the SD calculated from three replicates. Yield of alkaloid other than galanthamine are expressed as galanthamine.)



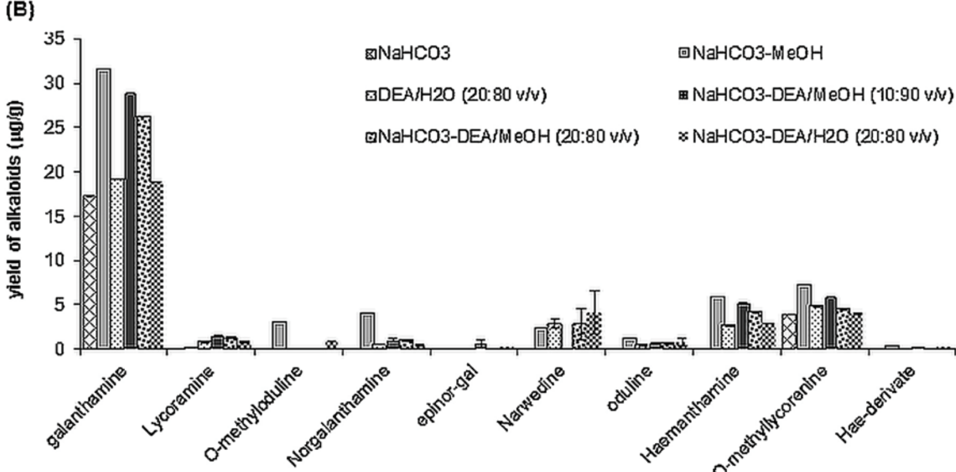




**Fig. 9.** Alkaloid profile of $scCO_2$ extract of *Narcissus pseudonarcissus* at 70 °C, 220 bar and 3 h with different plant treatments. (The bars represent the SD calculated from three replicates. Yield of alkaloid other than galanthamine are expressed as galanthamine.)

**Table 5**
*Narcissus pseudonarcissus* alkaloids identified $scCO_2$ extracts.

| Alkaloids | Retention time index[a] | Fragmentation ion $m/z$ (relative intensity, %)[b] | Ref. MS data |
|---|---|---|---|
| Galanthamine | 0.509 | 287 (84), 286 (100), 244 (24), 216 (33), 174 (30) | – |
| Lycoramine | 0.517 | 289 (59), 288 (100), 232 (7), 202 (13), 115 (21) | [18] |
| *O*-methyloduline | 0.522 | 315 (–), 284 (3), 175 (10), 110 (7), 109 (100), 108 (18) | [62] |
| Norgalanthamine | 0.541 | 273 (91), 272 (100), 256 (<1), 230 (28), 211 (1), 202 (27), 174 (3), 115 (20) | [63] |
| *epi*-norgalanthamine | 0.544 | 273 (100), 272 (57), 230 (31), 202 (29), 174 (10) | [64] |
| Narwedine | 0.566 | 285 (93), 284 (100), 216 (33), 174 (22) | [18] |
| Oduline | 0.578 | 301 (<1), 283 (<1), 175 (<1), 109 (100) | [17] |
| Haemanthamine[c] | 0.730 | 301 (7), 272 (100), 240 (13), 211 (13), 181 (40) | [17] |
| *O*-methyllycorenine | 0.867 | 331 (–), 191 (6), 110 (7), 109 (100), 108 (22), 94 (<1), 82 (3), 81 (3), 77 (2) | [65] |
| Haemanthamine derivate | 0.908 | 271 (100), 240 (6), 211 (14), 181 (53) | [17] |

[a] Retention time index was determined by dividing the retention time of alkaloid by the retention time of the internal standard. Papaverin was used as internal standard with retention time of 22.32 min.
[b] Percentages of intensity were compared to the base peak.
[c] Decomposition product of haemanthamine [17].

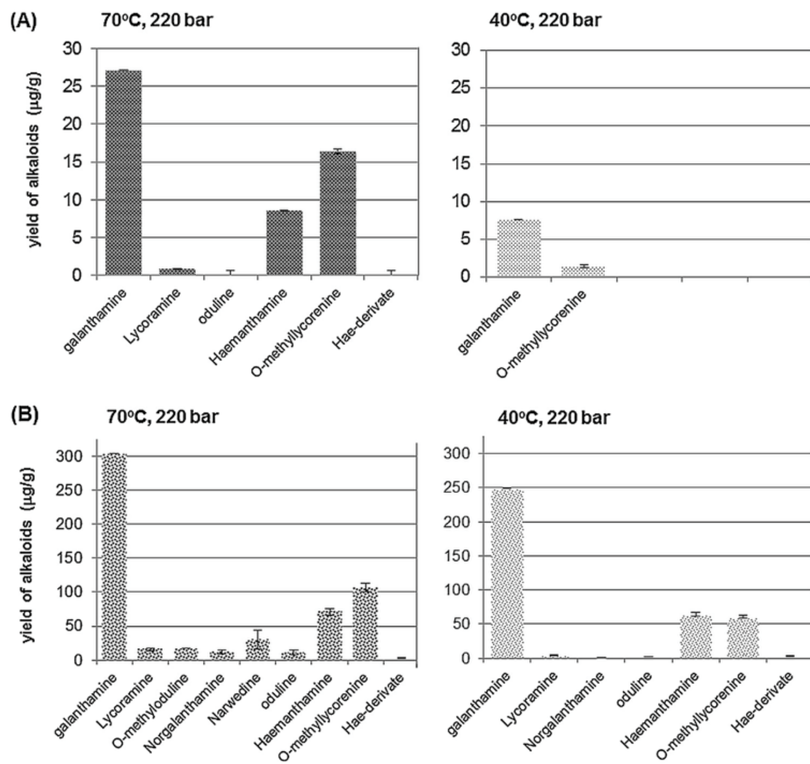



**Fig. 10.** Alkaloid profile of $scCO_2$ extract of *Narcissus pseudonarcissus* with (A) DEA-moistened plant material and (B) $NH_4OH$ (25%, v/v)-moistened plant material. All extractions lasted 3 h. (The bars represent the SD calculated from three replicates. Yield of alkaloid other than galanthamine are expressed as galanthamine.)

However, mathematical modeling of the experimental results is needed to mimic this and to have a better understanding of the mass transfer phenomena in SFE of galanthamine.

Fig. 7 shows the SEM pictures of the treated **Pwd** plant residue at different conditions of $scCO_2$ extraction. Different pressure and temperature of $scCO_2$ affects the density level of $CO_2$, i.e. 690, 784, and 856 kg/m$^3$ at 53–1000 µm of particle sizes. The density is proportional to the solvation power of the supercritical fluids and the liquid-like density achieved at high pressure will afford a high solvation power. Higher solvation power, however, implies lower selectivity. Granules were seen slightly detached from the structure (Fig. 8) but no further damage was observed in the cell structures or matrix swelling as the $CO_2$ density increased. Deposits of $NaHCO_3$ salt were also observed on the plant materials surface (Fig. 7).

These SEM pictures support our hypotheses; the ability to remove an analyte (targeted compound) from its matrix depends on analyte–matrix interaction and also analyte location within the plant matrix. Matrix swelling and structure damaging did not take place during $scCO_2$ extraction of galanthamine from dried bulb material. Even though $scCO_2$ penetrates adequately into the plant material, the release of galanthamine from its binding site is probably the limiting step.

### 3.4. Alkaloid profile of $scCO_2$ extracts

Supercritical $CO_2$ can selectively extract a specific compound from its biological matrix under optimized extraction conditions. Because of their polarity, alkaloids do not dissolve very well in $scCO_2$ and some sort of treatment or the addition of modifiers are required to convert them from their hydrophilic protonated form into the lipophilic free base or to increase the polarity of the $scCO_2$ [9,20]. Taking advantage of the different polarities of the individual alkaloids and especially of their different p$K_b$ values (p$K_b$ = −log $K_b$), it is possible to achieve the selective extraction of alkaloids. We thus studied the alkaloids profile of $scCO_2$ extracts. The profile is displayed in Fig. 8 and Fig. 9, while their identification based on retention times and fragmentation ions in GC-MS are summarized in Table 5.

#### 3.4.1. Effects of $NaHCO_3$ as plant treatment

Galanthamine, haemanthamine, and lycorenine-type alkaloids, i.e. *O*-methyllycorenine are the most frequently occurring and characteristic alkaloids of the genus *Narcissus* [59]. However, all of them, i.e., galanthamine, haemanthamine, *O*-methyllycorenine and a haemanthamine derivate have been obtained only at 515 and 700 kg/m$^3$ of $CO_2$ density [17]. Only galanthamine and *O*-methyllycorenine are found in all $scCO_2$ extracts (Fig. 8).

Haemanthamine (also known as its decomposition product [17]) was extracted at 515, 659, and 700 kg/m$^3$ $CO_2$ density but its derivate was only observed at 515 and 700 kg/m$^3$ $CO_2$ density. Highest selectivity of galanthamine was achieved at 690 kg/m$^3$ of $CO_2$ density (70 °C, 220 bar) ca. 52% with 12% of *O*-methyllycorenine (based on area of GC-FID chromatogram). Treating the plant material with 10% (w/w) of $NaHCO_3$ provides 27–52% purity of galanthamine. The lowest purity was obtained at 856 kg/m$^3$ of $CO_2$ density (40 °C, 220 bar) whilst the highest was at 690 kg/m$^3$ of $CO_2$ density 70 °C, 220 bar. A trace amount of *O*-methyloduline

(<0.60 μg/g) was only observed at 515 and 659 kg/m$^3$ of $CO_2$ density.

Haemanthamine was identified by its fragmentation pattern: *m/z* 301 (7), 272 (100), 240 (13), 211 (13) and 181 (40). The spectrum of haemanthamine did not always show the molecular ion (*m/z* 301, $[M]^+$). However the base peak of haemanthamine *m/z* 272 was always found. The peak, *m/z* 272, is presumed to be formed during decomposition on the GC column [17]. The haemanthamine derivate [17] was identified by *m/z* 271 (100), with 240 (6), 211 (4), and 181 (53) as fragmentation ions.

### 3.4.2. Effect of other treatments

There was a strong relationship between the extractability of alkaloids and the chemical pre-treatments of the sample material and the alkaloid profiles of their $scCO_2$ extracts of *N. pseudonarcissus* were different (Fig. 9). In sequence of ascending retention time of GC, the identified *N. pseudonarcissus* alkaloids were galanthamine, lycoramine, norgalanthamine, epi-norgalanthamine, narwedine, oduline, haemanthamine, and *O*-methyllycorenine.

A broader range of alkaloids was obtained using solely DEA/$H_2O$ and double treatments of $NaHCO_3$ either with DEA/$H_2O$ or DEA/MeOH. All the extracted alkaloids, except galanthamine, gave yields of 0.40–5.86 μg equivalent galanthamine/g. The haemanthamine derivate gave the lowest yields (0.17–0.21 μg equivalent galanthamine/g). Epinorgalanthamine, a galanthamine type alkaloid, was best extracted when plant material was double treated with $NaHCO_3$-DEA/MeOH (10:90, v/v) yielding 0.52 μg equivalent galanthamine/g (Fig. 9A). A minor amount of epinorgalanthamine was detected when plant material was treated with $NaHCO_3$-DEA/$H_2O$ (20:80, v/v). Only two *N. pseudonarcissus* alkaloids, galanthamine and *O*-methyllycorenine, were found when plant material was treated with aqueous $NaHCO_3$, but no other alkaloids were observed (Fig. 9). The most effective double treatment for galanthamine was $NaHCO_3$-DEA/$H_2O$ (20:80, v/v), but as mentioned above these treatments did not increase yields significantly (Fig. 4).

The profile of the alkaloids extracted with stronger basic media (higher pH) using DEA or ammonium hydroxide solution ($NH_4OH$ 25%, v/v) is displayed in Fig. 10. Extraction of plant materials moistened with DEA showed a higher selectivity for some alkaloids but a lower yield of galanthamine, while moistening with $NH_4OH$ 25% (v/v) showed high selectivity for galanthamine. Increasing the temperature increased the amount of alkaloids. Material moistened with DEA extracted 27 μg/g of galanthamine and trace amounts (<0.60 μg/g) of lycoramine, oduline, and the haemanthamine derivate and the yield of galanthamine was even higher (303 μg/g) when moistened with 25% $NH_4OH$ in the same extraction conditions (70 °C, 220 bar).

All GC-MS chromatograms of the $scCO_2$ extracts exhibited a peak with a lower retention time than haemanthamine. It showed only one single ion at *m/z* 109, which is typical of lycorene-type alkaloids, suggesting that it likely this type of alkaloid. [17]. The alkaloids of this type have been previously found in *N. pseudonarcissus* cv Carlton and are lycorenine and homolycorine [60,61]. Other lycorene type alkaloids, namely, *O*-methyloduline, oduline, and *O*-methyllycorenine were detected in our extracts. All these lycorenine-type alkaloids were identified by their *m/z* 109 base peak. Decomposition of lycorenine has been reported to occur during analysis [62].

## 4. Conclusion

Supercritical carbon dioxide is an excellent solvent for extracting galanthamine from the plant matrix. However, plant treatment using a basic modifier is essential in order to obtain highest yields. The highest amount of galanthamine (303 μg/g) was obtained at 70 °C, 220 bar, 3 h with 53–1000 μm particle size plant material moistened with $NH_4OH$ 25% (v/v). All other treatments i.e. with $NaHCO_3$, DEA, DEA/$H_2O$, DEA/MeOH and their combinations ($NaHCO_3$-DEA/$H_2O$, $NaHCO_3$-DEA/MeOH, and DEA-$H_2O$) gave lower yields. Fatty acids and their corresponding methyl esters became the major product when MeOH was used as a modifier. The highest selectivity for galanthamine, haemanthanmine, and *O*-methyllycorenine was obtained when plant material was treated with $NaHCO_3$, while other treatments gave a broader selectivity. The SEM analysis of the plant residue showed that increasing density of $CO_2$ neither destroys nor swells the structure of the plant material. Desorption of galanthamine from the plant material plays a more important role in these $scCO_2$ extraction rather than the galanthamine solubility in $scCO_2$ itself.

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# CHAPTER 4

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

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**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 coextracted, 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, 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

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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.

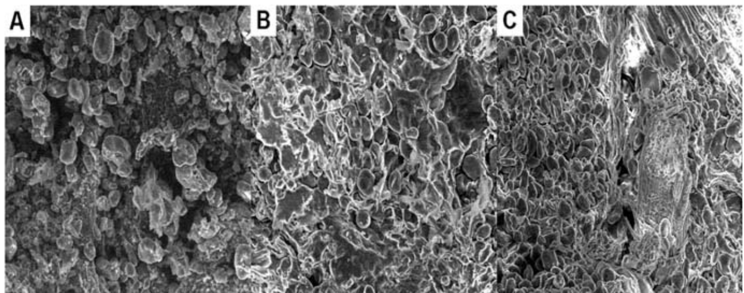


**Figure 1** SEM images of Narcissus pseudonarcissus residues of (A) HBr 1% (v/v) at 65 °C, (B) MeOH, and (C) pressurized water (PW) extractions (70 °C, 150 bar) of bulb powder of Narcissus pseudonarcissus. Images are registered at 700x magnification.

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.

## Acknowledgements

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# CHAPTER 5

## High Performance Liquid Chromatography Analysis of Galanthamine in Natural Deep Eutectic Solvent Extracts of *Narcissus* Bulbs

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**Abstract**

A new class of green solvents, natural deep eutectic solvents (NADES), are characterised by their great solubilizing power of a wide range of compounds, of which solubility is extremely low in conventional solvents like water. Galanthamine, an acetylcholinesterase inhibitor, has low solubility in water. However it showed high solubility in different types of NADES: choline chloride-citric acid (CCCA), malic acid-glucose (MAG), lactic acid-glucose (LAG), malic acid-sucrose (MAS), α-proline-sucrose (PrS), choline chloride-malic acid (CCMA), choline chloride-lactic acid (CCLA), and citric acid-sucrose (CAS). Amongst of them, an ionic liquid types of NADES, consist of a mixture of a base and an organic acid, showed the highest solubility of galanthamine. The analysis of the basic target compounds in these NADES is a challenging. The presence organic acids of the NADES components variated the pH of sample, thus galanthamine be partially ionized. Therefore, an isocratic HPLC system with C18 resin was developed for the quantitative analysis of the galanthamine in NADES extracts of *Narcissus pseudonarcissus* cv. Carlton bulbs. The system was optimized (type of stationary phase, concentrations of triethylamine, and percentages of methanol) to be able to handle the interference from NADES constituents. Samples were analysed using a wide range pH resistant C18 columns (100 x 4.6 mm, 3μ) at 280 nm. The mobile phase consisted of methanol-water (1:1, v/v) with 21.52 mM of trimethylamine (0.3%, v/v), a flow rate of 0.7 mL/min, and a run-time of 5 minutes. The system provides an accurate and reproducible (RSD ≤2%) separation of galanthamine from other compounds. The method was validated for linearity

(99.97%), recovery (99-100%), precision, repeatability, and specificity in the range of the analysed concentrations (3-23 μg/mL).

**Keywords**: Alkaline, Alkaloids, Galanthamine, High Performance Liquid Chromatography, extreme pH analysis, NADES, *Narcissus*.

## 1. Introduction

Natural deep eutectic solvents (NADES) are promising green solvents that consist of mixtures of certain molar ratios of two or more common primary metabolites such as organic acids and bases, sugars, alcohols, and amino acids (Dai *et al.*, 2013a). Compared to conventional synthetic deep eutectic solvents (DES) or ionic liquids (ILs), NADES have the advantage of their low cost and toxicity, and simple preparation method, e.g. prepared by mixing two solid components with gentle heat (usually around 70-80 °C), after which they remain in the liquid state at room temperature. Furthermore, NADES comply with all the requirements of green solvents since they are biodegradable, biocompatible, and sustainable (Paiva *et al.*, 2014)

The capacity of NADES to solubilise a wide range of molecules has been demonstrated by Dai *et al.* (2013a). This solubilising power extends over a wide range of polarity. Medium to highly polar metabolites that are weakly soluble in water such as rutin, quercetin, carthamin, cinnamic acid, taxol, ginkgolide B, as well as macromolecules such as gluten, starch, and DNA could be dissolved in different NADES. Dai et al. (2013b) also conducted a study on the efficacy of NADES as extraction solvent of phenolics from safflower (*Carthamus tinctorius*). These phenolics were better soluble (Dai *et al.*, 2013b) and more stable in sugar-based NADES than in water or aqueous ethanolic solutions (40%, v/v) (Dai *et al.*, 2014). Another type of application of NADES is as medium for enzymatically catalysed reactions as in the case of biodiesel production using *Candida antartica* lipase B (CALB) (Zhao *et al.,* 2013). The high activity of CALB for the enzymatic transesterification of triglycerides with ethanol could still be maintained, thus yielding a high reaction yield of biodiesel.

Galanthamine (Reminyl®) is one of the few cholinesterase inhibitors that has been approved by the Food and Drug Administration (FDA) of US for reducing the symptoms and progression of Alzheimer's disease (AD). To date, there are no drugs available in the market that can cure AD (Heinrich and Lee Teoh, 2004; Ponnayyan Sulochana *et al.*, 2014). Though natural galanthamine was used in the development as medicine, nowadays, galanthamine-HBr, the prescription form of galanthamine, is mainly produced by a patented synthesis. The patent of the medicine and the synthesis have run out, so a new market for generics has opened. For galanthamine, the option of extracting it from a natural source such *Narcissus* using "greener solvents" thus seems attractive. The present extraction method of galanthamine is a classical alkaloid extraction, including the use of large amount of organic solvents, acid and bases. As a green alternative, we studied extraction with NADES. Galanthamine proved to be very well soluble in some NADES (**Chapter 6**).

However, as well as the common extraction process, a separation method is needed to recover the galanthamine from the solvent. In case of NADES extraction, the low volatility of the solvent creates challenge for product separation and recovery. Supercritical $CO_2$ process seem to be more appropriate. The volatile SC-$CO_2$ is insoluble in the nonvolatile and polar NADES, and they form two-phase systems. The basic principle of this process is the solubility of the targeted compounds in $CO_2$. Unfortunately, none yet reported a recovery

process of SC-$CO_2$ of compounds from NADES. Therefore, analysis of complex mixtures of the *Narcissus* alkaloids in the NADES matrix is important. A pre-concentrated method by high performance liquid chromatography (HPLC) is first need to be developed.

The analysis of basic analytes such as alkaloids using C18-reversed phase-high performance liquid chromatography (RP-HPLC) can pose some difficulties such a tailing due to interaction with residual silanol groups. Moreover, the presence of organic acids such as choline chloride, organic acids, sugars, and amino acids as NADES components may further increase these difficulties. In particular, the presence of high concentrations of organic acids in NADES might generate problems with the HPLC analysis due to their interaction with the basic analytes. Marchand *et al.* (2008) observed that the presence of carboxylic acids in low pH mobile phases also contribute to peak tailing in the HPLC analysis of basic analytes due to the partially ionized of the anlytes. It was thus a challenge to obtain a good separation of galanthamine, our target alkaloid, with a symmetric peak shape and high reproducibility in the HPLC analysis of NADES extracts.

Here, we report the development of a simple, fast, and isocratic RP-HPLC method for quantification of galanthamine in *N. pseudonarcissus* cv. Carlton bulb NADES extracts. The separation was carried out using a mobile phase with methanol (MeOH), water, and as basic modifier - triethylamine - suppressing ionisation of the alkaloids at a pH around of 11-12; adjusting both of mobile phase and sample solution to pH around of 11-12, a shorter, consistent retention time, and sharper separation of galanthamine was achieved. The method was also validated to determine its linearity, reproducibility and robustness. The influence of the concentration of organic acids in the NADES extracts, is also discussed.

## 2. Materials and Methods

### 2.1. Chemicals

HPLC grade organic solvents: acetonitrile (Biosolve B.V., Valkenswaard, The Netherlands), methanol (MeOH), trifluoroacetic acid (TFA), and triethylamine (TEA) (Sigma, St. Louis, MO, USA) were used to prepare the mobile phase. Galanthamine–HBr (Gal-HBr, Selleckchem, Absource Diagnostics GmbH, Munich, Germany) was used both for an external reference compound and for recovery tests to evaluate matrix effect of NADES.

### 2.2. NADES preparation

All the NADES used in this study were prepared according to Dai *et al.* (2013a). Appropriate amounts of the two components of NADES constituents were mixed in a bottle with a magnetic stirrer, and heated gently in a water bath at 40 $^oC$ until a clear liquid was formed. In case of NADES with low ratio of water, the corresponding NADES need to dry in the freeze dryer, approximately 3 days, till a constant weight was reached.

### 2.3. HPLC analysis

Analyses were performed on an Agilent Technologies 1200 series HPLC system consisting of a G1310A pump and G1322A degasser, a G1329A auto sampler, a G1316A temperature controller, and a G1315D diode array detector (DAD). A Gemini NX-C18 comumn (C18, 100 x 4.6 mm, particle size 3.0 µm) purchased from Phenomenex (Torrance, CA, USA) equipped with a pre-guard column was used. The pH of all samples was measured prior to HPLC analysis to ensure that it was the same as that of the mobile phase (pH 11.6) and adjusted by dilution with the mobile phase if this were not the case. All samples were filtered through a 0.2 µm pore size Minisart regenerated cellulose (RC)-membrane syringe filter of 15 mm diameter (Sartorius Stedim, Goettingen, Germany). The injection volume was 5 µL. Galanthamine was quantified at 280 nm after identification based on retention time and UV-spectra compared with galanthamine-HBr (Gal–HBr) as a reference compound. Each sample was injected twice. The average of areas with RSD ≤2% was calculated and used for quantitation. A correction factor of molecular weight of Gal-HBr was also included to quantify galanthamine.

### 2.4. Validation

#### 2.4.1. Linearity

For the linearity test, a stock solution of Gal–HBr ca. 2.90 ± 0.01 mg was prepared and diluted in mobile phase (MeOH-$H_2O$ = 1:1 (v/v) with 21.52 mM of trimethylamine (TEA) (0.3%, v/v) to seven different concentrations between 3–23 µg/mL. These solutions were injected in triplicate and a curve of area vs concentration was plotted to calculate its linear fit.

#### 2.4.2. Accuracy

Accuracy was measured by evaluating the recovery of galanthamine from solutions choline chloride-citric acid (CCCA) (1:1 of mole ratio). This NADES ca. 298.27 ± 0.01 mg was accurately weighed and diluted to 5.0 mL in a volumetric flask with $H_2O$ (solution A). A stock solution of Gal-HBr was prepared by dissolving 4.49±0.01 mg in a 10.0 mL volumetric flask with MeOH-$H_2O$ (1:1, v/v) (solution B). The five sample points were prepared by mixing the appropriate volumes of solution A and B to obtain a 1.0 mL total volume. Subsequently, the mixed solutions were diluted to 10.0 mL with MeOH; after 3 h, the solution was filtered and 1.0 mL of this solution was diluted to 10.0 mL with MeOH-$H_2O$ (45:55, v/v) and TEA 0.34% (v/v). The final solution was thus MeOH-$H_2O$ (1:1, v/v) with 0.30% (v/v) (21.52 mM) of TEA which is similar to the mobile phase composition. The pH of all samples was measured prior to HPLC analysis to ensure that it was the same as that of the mobile phase (pH 11.6) and adjusted by dilution with the mobile phase if this were not the case. The final diluted sample was transferred to a 2 mL micro tube and subsequently centrifuged at 14,000 g for 15 min. All the samples were injected in duplicate. The preparation steps of this section are detailed in **Fig. 1**. A blank solution containing only CCCA was prepared and treated similarly with the samples.

#### 2.4.3. Recovery

##### 2.4.3.1. Recovery from choline chloride-citric acid (CCCA)

Recovery of Gal-HBr from CCCA test was calculated using the solution prepared in **section 2.4.2**. Duplicate standard solutions were prepared by accurately weighing approximately 2.50

± 0.01 mg of Gal–HBr reference compound. This was transferred to a 100.0 mL volumetric flask, dissolved with mobile phase and then taken to volume with the same solution. All the samples were injected in duplicate. The detected Gal–HBr was quantified using response factors.

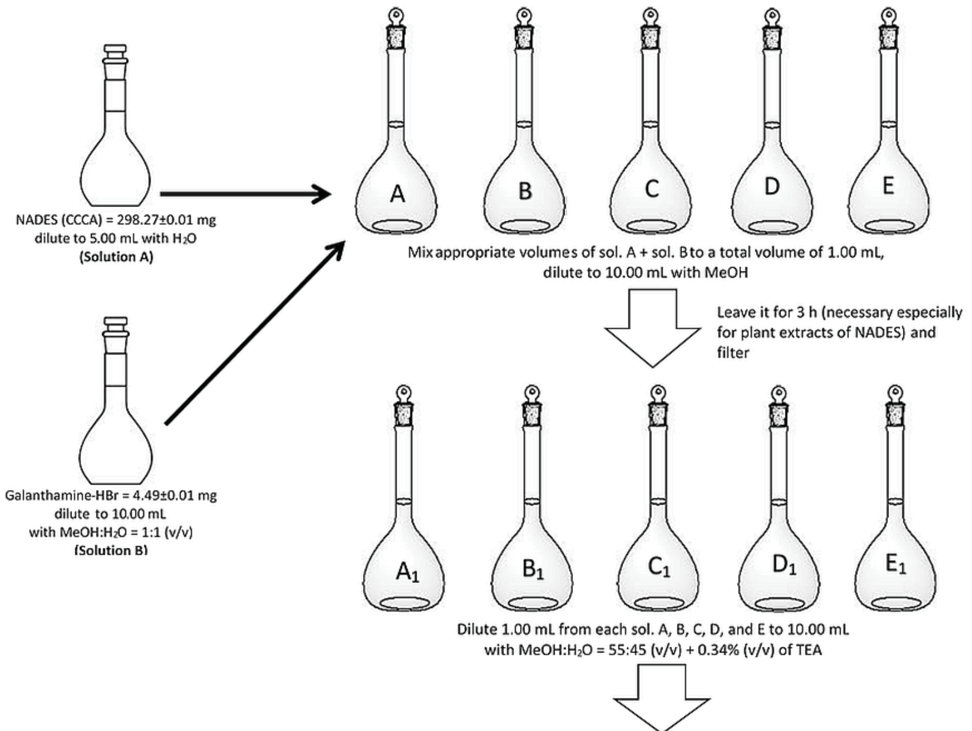


**Figure 1**. Sample preparation steps for accuracy (**section 2.4.2**) and recovery (**section 2.4.3**) tests.

**2.4.3.2. Recovery from different types of NADES**

Recovery of Gal-HBr of five different types of NADES was tested at one concentration level. Approximately 2.50 mg (±0.01) of Gal-HBr was added to approximately 1000 mg (±0.01) of NADES. Then 100 ±0.01 mg (approximately 100 µL) of a homogenous solution of Gal-HBr in NADES was diluted in a 5.0 mL volumetric flask with MeOH-$H_2O$ (1:1, v/v); after 3 h, the solution was filtered and this solution was diluted to 10.0 mL with MeOH:$H_2O$ (45:55, v/v) and TEA 0.34% (v/v). The pH of all samples was measured prior to HPLC analysis to ensure that it was the same as that of the mobile phase (pH 11.6) and adjusted by dilution with the mobile phase if this were not the case. The final diluted sample was transferred to a 2 mL micro tube and subsequently centrifuged at 14,000 g for 15 min. All the samples were injected in duplicate.

### 2.4.4. Precision

Five samples of CCCA NADES plant extract of *Narcissus pseudonarcissus* bulbs were analysed in duplicate. The NADES plant extract was diluted and prepared as described in **section 2.4.2** and then filtered through a 0.2 µm PVDF syringe filter (Whatman, Buckinghamshire, UK) to eliminate the *N. pseudonarcissus* lectins. The filtrate was analysed with HPLC-DAD at 280 nm.

## 3. Results and Discussion

Method of quantitative determination of *Narcissus* alkaloids, i.e. galanthamine, based on chromatography either with gas chromatography (GC) or high-performance liquid chromatography (HPLC) have been reported by researcher (Kreh *et al.*, 1995; Mustafa *et al.*, 2003; Gotti *et al.*, 2006; Ivanov *et al.*, 2009; Katoch *et al.*, 2012). Underivatized GC method was successfully separated mostly of *Amaryllidaceae* alkaloids, though haemanthamine and lycorenine were partly decomposed (Kreh *et al.*, 1995). The GC drawback, especially for haemanthamine analysis, is solved with non-aqueous capillary electrophoresis (NACE) and HPLC-ESI-MS method (Gotti *et al.*, 2006). However, analysis of complex mixture of *Amaryllidaceae* alkaloids was most often performed by HPLC; due to its advantages, ease to both quality standardize and controllable.

An isocratic reversed-phase method using trifluoroacetic acid (TFA) as an ion-pair reagent (pH 3.4-3.6) with C18-column has been applied to analyse galanthamine in *Crinum asiaticum* L., *Leucojum aestivum* L., *Narcissus* Jonquilla, and *Narcissus* Carlton extracts (Mustafa *et al.*, 2003). Ivanov *et al.* (2009) also applied this strategy to quantify galanthamine, lycorine, and norgalanthamine with a gradient elution of acetonitrile (ACN) (solvent A) and ammonium acetate buffer (1%, w/v, solvent B). Similarly, *Amarylidaceae* alkaloids of *Zephyranthes grandiflora* were quantified using formic acid (0.05%, v/v) and ACN-$H_2O$ as a mobile phase (Katoch *et al.*, 2012). All of the mentioned methods (Ivanov *et al.*, 2012; Katoch *et al.*, 2012; Mustafa *et al.,* 2003) allowed a good peak resolution and efficiency for galanthamine as a targeted compound with a low detection limit. However, none of them are dealing with NADES, consist of different constituent compounds form sugars, polyalcohol, organic acids, amino acids, and etc. Variances in matrix sample, more or less different in sample preparation prior the HPLC injection; minimizes the contents of sample are interfered the analysis.

### 3.1. HPLC method

In the case of chromatographic analysis of ionisable compounds, i.e. acids and bases, it is important to consider the pH of the mobile phase since it will greatly affect the separation. This is due to the ionisation of the compounds and their interaction with the stationary phase (Borges and Euerby, 2013). Basic compounds such as alkaloids will be totally ionised in the mobile phase at a pH below their $pK_a$, ca. pH = $pK_a$—1; while in a mobile phase with a pH above their $pK_a$, ca. pH = $pK_a$ + 1, they will be non-ionised. An intermediate pH is not recommended since a part of the weak basic (or acidic) analyte molecules (and silanol groups)

will be ionised. Thus, slight changes in pH can lead to significant changes in retention time of analytes resulting in non-reproducible analyse (Fornal *et al.*, 2006) or produce peak-tailing or even double peaks.

The non-ionised form will be more lipophilic (more hydrophobic), and thus be more strongly retained in a RP-system. However, the non-protonated binds easily to the weakly acidic residual silanol groups. The addition of an organic base like triethylamine (TEA) will solve this problem as it is much more abundant than the analyte; it will mask the silanol groups and thus reducing tailing. The better peak shape, will result in more reproducible results, both regarding retention time and peak area.

The use of extreme pH values of the mobile phase is limited in the case of chemically bonded silica stationary phases, and the use of mobile phases with a pH below 2 or above 8.5 leads to degeneration of the columns. The use of pre-columns which are regularly replaced can reduce the degeneration. Another possibility to reduce tailing of basic compounds is to work at an acidic pH and adding ion-pairing agents to the mobile phase (Mustafa *et al.*, 2003; Katoch *et al.*, 2012); that will form ion-pairs with the basic compounds in protonated form with no affinity to the silanol groups.

Despite its limited pH stability (pH 2-7), silica still remains the most common support for bonded-phase chromatographic stationary phases, due to its mechanical strength and efficiency. Shi *et al*. (2014) used a core-shell type of C18 column with slightly alkaline mixture of mobile phase for analysis of alkaloids from *Lycoridis radiatae* bulbs extract. Hence, no need to apply a special C18 column. To eliminate this drawback, hybrid silica particles have been developed, that provides a high range of pH resistance, ca. pH 1-12.

However, the use of acidic mobile phases with ion-pairing (TFA) (Mustafa *et al.,* 2003) did not work for the NADES plant extracts, as the retention time of galanthamine fluctuated for replicate injections as well as for different types of NADES (**Fig. 2**). A possible explanation is that at low pH values, galanthamine is totally ionised allowing it to form ion pairs with all available anions-in this case TFA from the mobile phase, and organic acids from the NADES, thus causing fluctuations in the retention behaviour. Furthermore, other interactions between TFA, the organic acids and other cations present in some of the NADES, resulted in considerable difficulties in obtaining stable retention times or even double peaks in some samples as compared to the reference solution as well as peak broadening and/or tailing.

In view of the above, a high pH strategy was chosen and TEA was used as the mobile phase modifier at the concentration necessary to provide a pH one unit above the pKa of galanthamine, i.e. pH of 10. However, it was necessary to use a stationary phase that could resist a high pH value. In this case we chose a hybrid silica reversed phase-column. A column with organo-slica layers grafted onto a pure silica core; hence, the stationary phase will have high resistance for low to high pH.

An optimum chromatographic separation of galanthamine in NADES was achieved using this high pH resistant stationary phase and the mobile phase consisting of MeOH-$H_2O$ (1:1, v/v) with the addition of 21.52 mM of TEA (0.3% v/v), resulting in pH = 11.6 (**Fig. 3**). Three

parameters of analysis, stationary phase, concentration of TEA, and percentages of MeOH, were evaluated for robustness and discussed below.

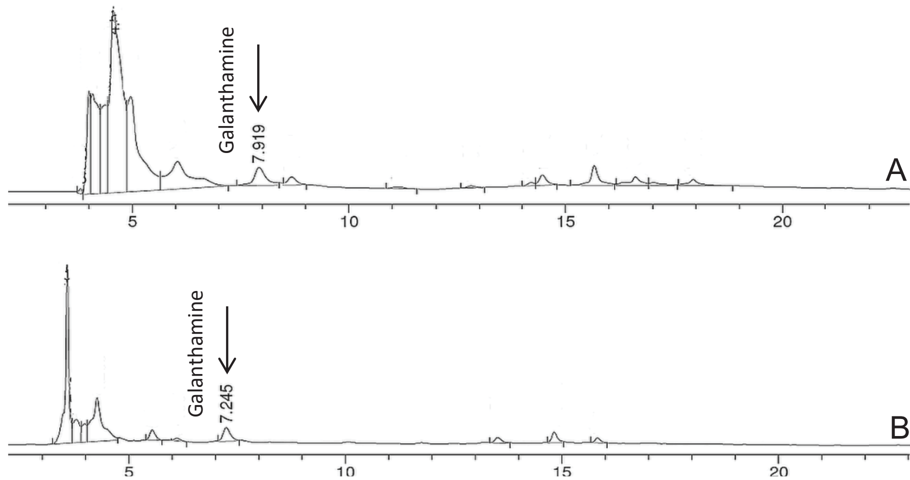


**Figure 2**. HPLC chromatogram of extracts of *Narcissus pseudonarcissus* cv. Carlton bulbs with NADES (A) Malic Acid-Glucose (MAG), and (B) β-Alanine-Malic Acid (βAMA) by previous method using a core-shell silica based column (Mustafa *et al.* 2003).

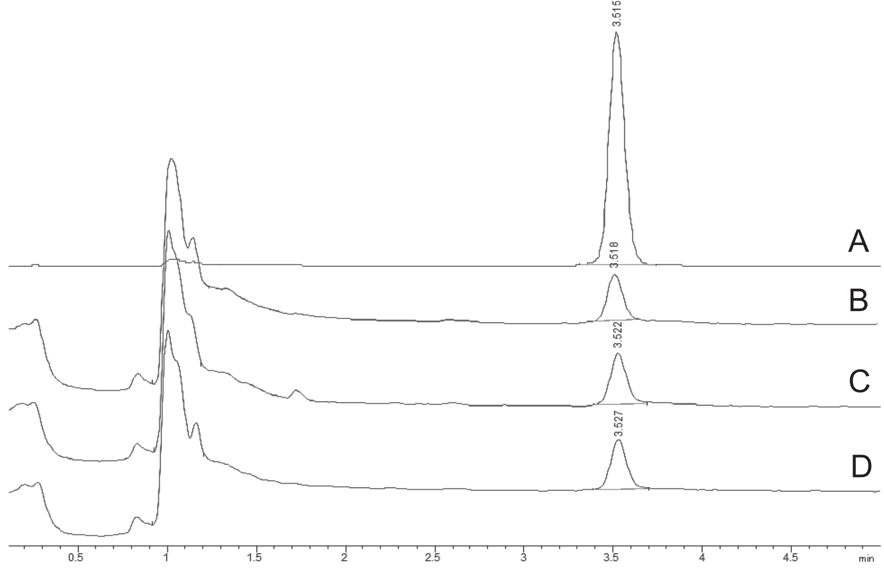


**Figure 3**. HPLC chromatogram of galanthamine-HBr dissolved in different types of NADES which were analysed using proposed method. (A) Galanthamine-HBr standard, (B) Choline chloride-malic Acid (CCMA), (C) Malic Acid-Glucose (MAG), and (D) β-Alanine-Malic Acid (βAMA).

### 3.2. Sample Preparation

A steep increase in the back pressure of the column was observed after a number of injections of NADES extracts. It might be due to the precipitation of polysaccharides or lectins which are present in *Narcissus* bulbs and could be well extracted with NADES. Opute and Osagie (1978) reported that flower bulbs as a storage organ accumulate polysaccharides rather than fats as a source of reserve energy. These are generally water soluble polysaccharides (WSPs) (Zhauynbaeva *et al.*, 2003a) as well as lectins (Van Damme *et al.*, 1988; Van Damme and Peumans, 1990). The WSPs, mostly glucomannans (MW 31,000), were obtained from the aqueous extract *of Narcissus tazetta* (Zhauynbaeva *et al.*, 2003a; Zhauynbaeva *et al.*, 2003b), while Malikova *et al.* (2002) reported WSPs of natively acetylated glucomannan (MW 32,000) from *Narcissus poeticus* bulbs as well as starch with an amylopectin-type structure (Rakhimov and Zhauynbaeva, 1997). Van Damme *et al.* (1988) and Van Damme and Peumans (1990) obtained mannose-specific lectins when extracting fresh bulbs of *N. pseudonarcissus* with 1 M of $(NH_4)_2SO_4$. Therefore, the utilization of NADES with their powerful solubilisation capacity for macromolecules (Dai *et al.*, 2013a), might also extract WSPs and lectins from the powder of *N. pseudonarcissus* bulbs.

This problem was minimized by simply diluting the NADES extracts with MeOH and filtering the resulting solution after 3 h with a 0.2 μm membrane before injection. Methanol was chosen to precipitate the WSPs and lectins following to the method of Zhauynbaeva *et al.* (2003a). It is important to bear in mind that NADES extracts of biological material may contain large amounts of such hydrophilic polymers.

### 3.3. Validation

Validation was carried out following the ICH Validation of Analytical Procedures Q2 (R1).

#### 3.3.1. Linearity

The response of galanthamine in the range of concentrations detected in the plant extracts of NADES was tested (3—23 μg/mL). The solutions were prepared in the mobile phase MeOH-$H_2O$ = 1:1 (v/v) with 21.52 mM of TEA (0.30%, v/v), giving a pH of 11.6. It showed a linear relation with an $R^2$ = 0.9997 as shown in **Fig. 4**.

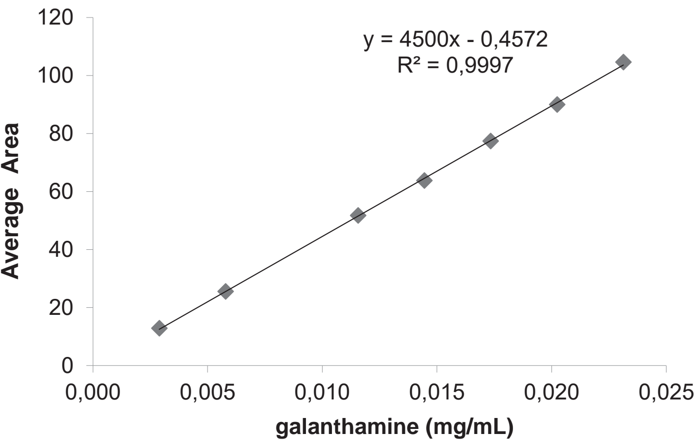


**Figure 4.** Linearity of galanthamine (3-23 μg/mL) tested at 7 levels of concentrations with trirplicates.

### 3.3.2. Accuracy

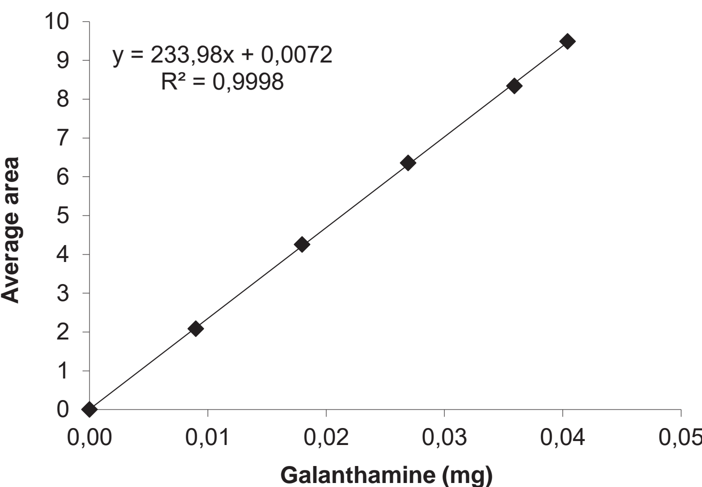


**Figure 5.** Accuracy of galanthamine (3-23 μg per injection) in Choline chloride-Citric Acid (CCCA)-NADES sample matrix tested at 5 levels of concentrations with duplicates.

### 3.3.3. Recovery and Robustness

The recovery test was done by spiking galanthamine-HBr in five different concentrations using CCCA as a NADES (**Table 1**) as described in **section 2.4.2**. This sample was prepared to mimic the concentration of galanthamine obtained with the NADES extraction of *N. pseudonarcissus* cv. Carlton bulbs.

**Table 1.** Recovery values for spiking galanthamine in NADES (CCCA) by using the proposed method (MeOH-$H_2O$ (1:1, v/v) + 21.52 mM of TEA (0.30%, v/v) as a mobile phase).

| NADES[a] (mg) | galanthamine (μg) | | Recovery (%) |
|---|---|---|---|
| | added | detected | |
| 0.513 | 40.41 | 40.55 | 100.34 |
| 1.027 | 36.92 | 35.65 | 99.26 |
| 2.053 | 26.94 | 27.17 | 100.84 |
| 3.080 | 17.96 | 18.17 | 101.16 |
| 4.107 | 8.98 | 8.89 | 99.02 |

[a]CCCA (Choline chloride-Citric Acid) = 1:1 mole ratio.

The recovery of galanthamine from other NADES was also tested. The results are shown in the **Table 2**, the corresponding chromatograms are in **Fig.5**.

**Table 2.** Recovery of galanthamine added to different types of NADES by using the proposed method (MeOH:$H_2O$ (1:1, v/v) + 21.52 mM of TEA (0.30%, v/v).

| NADES type[a] | NADES (mg) | galanthamine (μg) | | Recovery (%) |
|---|---|---|---|---|
| | | added | detected | |
| CCCA | 148.02 | 2,32 | 2,30 | 99,30 |
| CCMA | 120.16 | 2,61 | 2,55 | 97,55 |
| βAMA | 210.54 | 2,78 | 2,79 | 100,44 |
| GFS | 144.74 | 2,17 | 2,05 | 94,57 |
| CCF | 123.79 | 2,66 | 2,48 | 93,26 |

[a]CCCA (Choline chloride-Citric Acid) = 1:1, CCMA (Choline chloride-Malic Acid) = 1:1, βAMA (β-Alanine-Malic Acid) = 1:1, GFS (Glucose-Fructose-Sucrose) = 1:1:1, and CCF (Choline chloride- Fructose) = 5:2 (molar ratio's).

Both **Table 1** and **2**, clearly show a satisfactory recovery value. However, the pH of the sample proved to be crucial both for qualitative (**Fig. 6**) and quantitative analysis (**Table 1** and **2**). Thus it was necessary to ensure that all samples and standard solution had the same pH as the mobile phase

In all types of NADES extracts, with the exception of those containing organic acids, it was possible to obtain an acceptable chromatographic resolution below 0.3% (v/v) (21.52 mM) of TEA such 0.20, 0.25, and 0.27% (v/v) of TEA. However, in the case of NADES containing organic acids i.e. CCCA, CCMA, and βAMA, it is essential to work with 0.3% (v/v) (21.52 mM) to avoid double galanthamine peaks (**Fig. 6**). Hence, 0.3% (v/v) (21.52 mM) is the optimum concentration of TEA to have symmetrical and selective selectivity of galanthamine in NADES extracts without any interfere of NADES constituents.

**Figure 6(C)** shows a chromatogram obtained using 0.27% (v/v) (19.37 mM) of TEA in the mobile phase when NADES containing organic acids were analysed. The two peaks showed the same UV spectra as galanthamine but only the retention time of the second peak coincided with that of the reference compound solution. The sum of the areas of these two peaks coincided with the theoretical amount galanthamine present in the solution. A possible

explanation for this is that the organic anions such as citrate, malate, and lactate to different degrees may form ion-pairs with the ionised galanthamine and have a shorter retention time as they are more hydrophilic than the galanthamine base which elutes at a slightly different retention time than galanthamine; increasing the concentration of TEA suppresses the ionisation of galanthamine and also possibly pairs off the organic anions. A concentration of at least 0.3% (v/v) (21.52 mM) of TEA avoided these problems as can be observed in **Fig. 6(B)**. This, fits with the general experience that the used of pH controlling modifiers must be present at about 25 mM or more.

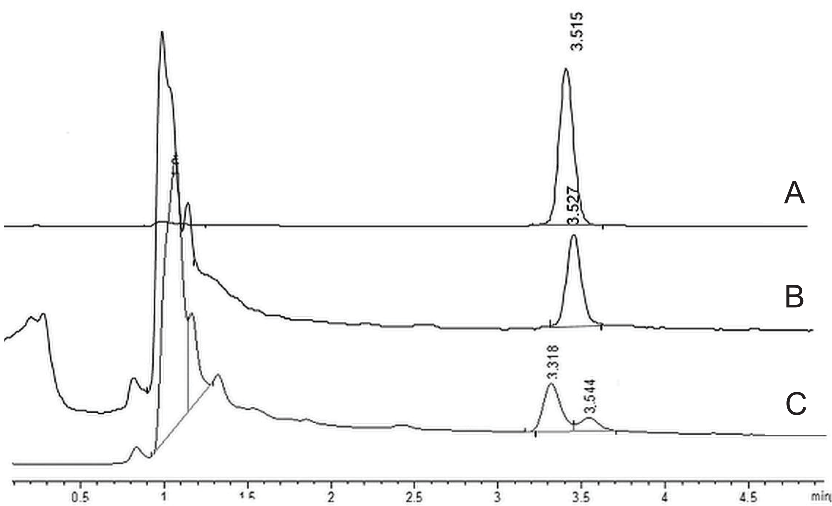


**Figure 6**. HPLC chromatogram of (**A**) Galanthamine-HBr standard, and (**B, C**) *Narcissus pseudonarcissus* cv. Carlton bulbs extracted with β-Alanine-Malic Acid (βAMA) of NADES. Chromatograms A and B were analysed using proposed method (MeOH-$H_2O$ (1:1, v/v) + 0.30% (v/v) (21.52 mM)) while chromatogram C analysed using MeOH-$H_2O$ (1:1, v/v) + 0.27% (v/v) (19.37 mM) of TEA. Both peaks have typical galanthamine UV spectra.

The impact of the change of three parameters was evaluated for robustness. They were the type of stationary phase, concentration of TEA, and percentages of MeOH.

In the case of the stationary phase being a core-shell type of silica, the result was satisfactory if pH of the mobile phase was kept at pH> 11. However, this type of stationary phase will not last approximately more than 150 injections, due to the rapid degeneration of the silica at high pH. Thus a stationary phase with high pH resistance such as those composed of hybrid particles must be used.

The concentration of TEA is a critical point as discussed above and any variation that resulted in a pH< 11 had a negative impact on the results especially when handling NADES containing organic acids.

In the case of the MeOH content of the mobile phase, up to ±10% change can still provide an acceptable resolution ($R_s$) and capacity factor (k) of the system for galanthamine. Above that, galanthamine can co-elute with earlier peaks while a lower MeOH content will result in an unacceptably high k value.

## 4. Conclusion

An isocratic HPLC method consisting of a mobile phase of MeOH-$H_2O$ (1:1, v/v) with triethylamine 0.30% (v/v) (21.52 mM) with a Gemini NX-C18 3.0µ column, 100 x 4.6 mm (Phenomenex, Torrance, CA, USA) and a flow rate 0.7 mL/min and detection at a wavelength of 280 nm allowed the fast analysis of galanthamine in NADES extracts of *Narcissus pseudonarcissus* cv. Carlton bulbs. The method was validated according to ICH Validation of Analytical Procedures: Text and Methodology Q2 (R1) proving to be accurate, reproducible, and it allowed the separation of galanthamine from other constituents of the extracts within 5 minutes. A pH of mobile phase and sample above 11 was crucial to avoid double peaks and variations in the retention time and area count of galanthamine peaks. An analogues HPLC method by means suppressing the ionization of target compound can be applied when analysing different kind of alkaloids in NADES matrix.


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# CHAPTER 6

## Pressurized Natural Deep Eutectic Solvent Extraction of Galanthamine and Related Alkaloids from Narcissus pseudonarcissus

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**Abstract**

The isolation of a compound from a natural source involves many organic and mostly toxic solvents for extraction and purification. Natural deep eutectic solvents have been shown to be efficient options for the extraction of natural products. They have the advantage of being composed of abundantly available common primary metabolites, being nontoxic and environmentally safe solvents. The aim of this study was to develop a natural deep eutectic solvent-based extraction method for galanthamine, an important therapeutic agent for the treatment of Alzheimer's disease. This alkaloid can be produced by synthesis or by extraction from Narcissus bulbs. To develop an efficient extraction method, a number of different natural deep eutectic solvents was first tested for their solubilization capacity of galanthamine bromide salt. Promising results were obtained for ionic liquids, as well as some amphoteric and acidic natural deep eutectic solvents. In a two-cycle extraction process, the best solvents were tested for the extraction of galanthamine from bulbs. The ionic liquids produced poor yields, and the best results were obtained with some acid and sugar mixtures, among which malic acid-sucrose-water (1: 1: 5) proved to be the best, showing similar yields to that of the exhaustive Soxhlet extraction with methanol. Furthermore, the natural deep eutectic solvent was more selective for galanthamine.

**Keywords**: alkaloids, extraction, eutectic, NADES, Narcissus pseudonarcissus, Amaryllidaceae, pressurized extraction, galanthamine

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# Pressurized Natural Deep Eutectic Solvent Extraction of Galanthamine and Related Alkaloids from *Narcissus pseudonarcissus*[#]

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**Key words**

alkaloids, extraction, eutectic, NADES, *Narcissus pseudonarcissus*, Amaryllidaceae, pressurized extraction, galanthamine

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**ABSTRACT**

The isolation of a compound from a natural source involves many organic and mostly toxic solvents for extraction and purification. Natural deep eutectic solvents have been shown to be efficient options for the extraction of natural products. They have the advantage of being composed of abundantly available common primary metabolites, being nontoxic and environmentally safe solvents. The aim of this study was to develop a natural deep eutectic solvent-based extraction method for galanthamine, an important therapeutic agent for the treatment of Alzheimer's disease. This alkaloid can be produced by synthesis or by extraction from *Narcissus* bulbs. To develop an efficient extraction method, a number of different natural deep eutectic solvents was first tested for their solubilization capacity of galanthamine bromide salt. Promising results were obtained for ionic liquids, as well as some amphoteric and acidic natural deep eutectic solvents. In a two-cycle extraction process, the best solvents were tested for the extraction of galanthamine from bulbs. The ionic liquids produced poor yields, and the best results were obtained with some acid and sugar mixtures, among which malic acid-sucrose-water (1:1:5) proved to be the best, showing similar yields to that of the exhaustive Soxhlet extraction with methanol. Furthermore, the natural deep eutectic solvent was more selective for galanthamine.

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## Introduction

Alkaloids are a major source of (novel) medicines from plants. In general, they are among the most active, and in some cases, toxic compounds found in nature, such as strychnine, aconitine, cocaine, morphine, atropine, nicotine, colchicine, and coniine. In the past decades, an alkaloid, galanthamine, was found to have pharmacologically significant acetylcholine esterase inhibition activity, thus becoming a potential candidate for the treatment Alzheimer's disease (AD) symptoms [1]. This potential was proved and galanthamine is now a major therapeutic tool in the treatment of AD patients [1]. Originally, this alkaloid was isolated from

[#] Dedicated to Professor Dr. A. Douglas Kinghorn on the occasion of his 75th birthday.

snowdrops and *Narcissus* bulbs, but later on, an efficient synthetic process was developed as well. Given the importance of the medical applications of galanthamine, its extraction from *Narcissus pseudonarcissus* L. cv. "Carlton" bulbs (family Amaryllidaceae), its main natural source [2], has been extensively studied [3–5]. The most efficient extraction methods involve the use of organic solvents.

The strong pharmacological activities of alkaloids are connected with the amine function that is typical of alkaloids. At basic pH values, amines behave as low to medium polar compounds, which are thus soluble in medium polar organic solvents such as diethyl ether and chloroform. In acidic conditions, amines are protonated, increasing their hydrophilicity to the point of being easily soluble in water at low pH values. This possibility of being hydro- or lipophilic according to the pH makes alkaloids ideal drugs, as they can be water soluble if needed but are lipophilic and basic at physiological pH levels, allowing them to pass the cell membranes and, in particular, the blood brain barrier easily. In fact, the majority of all medicines, both natural and (semi)synthetic, contain an amine function, precisely for their high bioavailability.

There are a number of examples of the extraction of alkaloids from diverse plant materials at industrial scale for their use as APIs (active pharmaceutical ingredient). These extraction methods respond to three different traditional approaches as follows [6]:

1. Extraction with water at acidic pH from plant material, followed by purification steps involving liquid-liquid partitioning with nonpolar organic solvents. The acidic water layer is extracted with the organic solvent (e.g., diethyl ether, ethyl acetate, chloroform, or dichloromethane) before and after adjusting the pH to about 8–9. The alkaloids will be found in the second organic extract, which can then be further purified to isolate the desired alkaloid and obtain it by crystallization from an organic solvent; alternatively, chromatography can be used to obtain the pure individual alkaloids.
2. Extraction of basified plant material with an organic solvent such as those mentioned before. The organic phase is then partitioned with an acidic aqueous phase. Preferred acids are sulfuric acid or phosphoric acid because counter ions like acetate and chloride form lipophilic ion pairs with alkaloids that can remain in the organic phase even at low pH values. Further purification steps similar to those described before are then implemented.
3. Extraction with methanol or ethanol of the defatted plant material after humidification with an acid. The extract can be taken to dryness and the crude alkaloid fraction subjected to the purification steps described previously.

These processes involve the use of enormous amounts of mostly toxic organic solvents that must be disposed of. Given the increasing awareness of the need to find greener and more environmentally friendly alternatives to such processes, a great deal of effort has been invested in the development of novel methods that could fulfill such requirements. Among these, supercritical extraction (SFE) with carbon dioxide has been successfully applied in some cases, but its efficiency is limited due to the low lipophilicity of most alkaloids, at least in their state in plant material. Rachmaniah et al. [7] optimized the SFE of galanthamine from bulbs of *N. pseudonarcissus*. The process required the moistening of the plant material with $NH_4OH$ 25% (v/v) prior to its extraction to obtain free bases of the alkaloids. However, the optimized conditions yielded only 0.303 g/g DW, far below the classical extraction methods yields.

Other alternative solvents considered were ionic liquids (ILs) and deep eutectic solvents (DESs); these have proved to be able to be tailored to the extraction of all kinds of compounds. While ILs are often toxic and non-environmentally friendly, DESs, introduced by Abbott and coworkers [8], are an advanced generation of eutectic solvents that are thought to be a much greener option. The mixture of choline chloride-urea is an example of these solvents [9]. In recent years, Choi et al. [10] discovered that combinations of certain molar ratios of primary metabolites such as sugars, sugar alcohols, polyalcohols, organic bases, organic acids, and amino acids could also form eutectic mixtures. These solvents were named natural deep eutectic solvents (NADES). They hypothesized that NADES occur in all living organisms allowing poorly fat- or water-soluble metabolites as well as macromolecules to solubilize in the cellular environment. The NADES ingredients are found in all living systems and in view of their great solubilizing power, could explain how medium polar and water-insoluble compounds can be biosynthesized, transported, and stored. Moreover, their existence could explain the survival of organisms (e.g., plants, lichen, insects, and prokaryotes) in extreme drought or cold conditions.

Most NADES have a moderate polarity, are highly stable, and are more hydrophilic than ILs, being miscible with water in general [11]. They consist of biodegradable components that are readily available, such as ubiquitous primary metabolites and cations and/or anions with low or even no toxicity at all [12]. An indication of their innocuity is that enzymes can be dissolved in a DES and preserve their conformation; while inactive in these conditions, they recover their activity after the addition of water [10, 13]. DESs are typically combinations of a hydrogen bond acceptor (HBA), like choline or betaine, both natural quaternary ammonium derivatives, with proline or other amino acids and hydrogen bond donors (HBDs) such as amides, e.g., urea [8], carboxylic acids, e.g., oxalic acid, malic acid, and citric acid, alcohols, e.g., glycerol and sugar alcohols [7, 10, 11], or sugars or sugar analogues [11, 14]. The properties of the NADES choline chloride-glycerol were also reported [15].

Dai et al. [11, 14, 16, 17] described and characterized a large number of NADES. The main advantages of NADES are that they are easy to make and consist of simple, readily available components. They are prepared by simply mixing the two compounds in certain molar ratios with gentle heating until they melt, yielding a liquid mixture at room temperature. No purification steps are needed. The eutectic condition results in a mixture with a lower melting point than that of any of its individual components. This was proved to be due to the generation of strong intermolecular hydrogen bonds.

The goal of this study was to select the NADES in which galanthamine HBr (hydrobromide) was best soluble, and then use these results to identify the solvent that provided the highest yield of galanthamine when applied to the extraction of bulb material.

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▶ **Table 1** Galanthamine-HBr solubility in different types of NADES. Color changes appearing after 3 months were recorded.

| | NADES[a] | Molar ratio | NADES classification | Color[b] | | Gal-HBr/NADES (mg/g ± SD) |
|---|---|---|---|---|---|---|
| | | | | NADES | NADES + Gal-HBr | Solubility[c] |
| 1 | FG-$H_2O$ | 1:1:2 | Neutral | – | – | 11.3 ± 0.1 |
| 2 | FS-$H_2O$ | 1:1:5 | Neutral | – | – | 11.3 ± 0.1 |
| 3 | FS-$H_2O$ | 2:1:6 | Neutral | – | – | 9.2 ± 0.0 |
| 4 | FS-$H_2O$ | 1:1:33 | Neutral | – | – | 3.7 ± 0.0 |
| 5 | CCMA-$H_2O$ | 1:1:1 | Ionic liquid | – | – | 18.0 ± 0.1 |
| 6 | CCCA-$H_2O$ | 1:1:9 | Ionic liquid | – | – | 62.2 ± 0.8 |
| 7 | CCLA | 1:1 | Ionic liquid | – | – | 16.6 ± 0.0 |
| 8 | BeMA-$H_2O$ | 1:1:3 | Ionic liquid | – | – | 7.7 ± 0.1 |
| 9 | PrS-$H_2O$ | 2:1:7 | Amphoteric | + | + | 23.1 ± 0.1 |
| 10 | PrS-$H_2O$ | 2:1:1 | Amphoteric | + | + | 24.8 ± 0.1 |
| 11 | PrCA-$H_2O$ | 1:1:5 | Amphoteric | + | + | 2.1 ± 0.0 |
| 12 | βAMA-$H_2O$ | 3:2:5 | Amphoteric | – | – | 4.9 ± 0.0 |
| 13 | CCS-$H_2O$ | 4:1:6 | Basic | – | – | 4.7 ± 0.0 |
| 14 | CCS-$H_2O$ | 4:1:4 | Basic | – | – | 2.4 ± 0.0 |
| 15 | CCG-$H_2O$ | 5:2:5 | Basic | – | – | 4.0 ± 0.0 |
| 16 | CCF-$H_2O$ | 5:2:5 | Basic | ++ | + | 3.9 ± 0.0 |
| 17 | CCGo-$H_2O$ | 2:1:1 | Basic | – | – | 8.5 ± 0.0 |
| 18 | CCGo-$H_2O$ | 2:2:1 | Basic | – | – | 12.4 ± 0.0 |
| 19 | MAG-$H_2O$ | 1:1:5 | Acidic | – | + | 35.7 ± 0.0 |
| 20 | MAF-$H_2O$ | 1:1:5 | Acidic | ++++ | ++++ | 7.8 ± 0.1 |
| 21 | MAS-$H_2O$ | 1:1:5 | Acidic | ++ | +++ | 26.4 ± 0.3 |
| 22 | CAG-$H_2O$ | 1:1:5 | Acidic | – | – | 2.0 ± 0.0 |
| 23 | CAF-$H_2O$ | 1:1:7 | Acidic | ++++ | ++++ | 2.4 ± 0.0 |
| 24 | CAS-$H_2O$ | 1:1:2 | Acidic | +++ | ++++ | 8.4 ± 0.0 |
| 25 | CAS-$H_2O$ | 1:1:9 | Acidic | +++ | ++++ | 10.1 ± 0.1 |
| 26 | LAG-$H_2O$ | 5:1:3 | Acidic | – | – | 32.9 ± 0.1 |
| 27 | MAGGo-$H_2O$ | 1:1:1:2 | Acidic | – | – | 1.3 ± 0.0 |
| 28 | MAGF-$H_2O$ | 1:1:1:2 | Acidic | +++ | +++ | 1.3 ± 0.0 |

## Results and Discussion

Galanthamine as a (protonated) salt in the plant has limited solubility in nonpolar organic solvents. Nevertheless, due to its basicity, galanthamine can be extracted with aqueous polar solvents containing dilute acids. The resulting extract contains other minor alkaloids and soluble impurities apart from galanthamine and thus has to be purified. An ideal solvent should extract galanthamine selectively, avoiding lengthy steps that can additionally reduce the yield. To study the potential of NADES for the selective extraction of galanthamine from bulbs, several were selected from the work of Dai et al. [10, 11, 14, 16, 17]. Some were modified by adding water to lower their viscosity for easier manipulation [18]. However, the amount of added water never exceeded 20% (w/w) to preserve the NADES properties. The solubility of galanthamine HBr (Gal-HBr) in various NADES is shown in ▶ **Table 1**.

▶ **Table 1** shows the results of the solubility experiments with 28 different NADES, representing the 5 different classes. The table is arranged according to the NADES constituents (in molar ratios). A non-supervised multivariate analysis of this data set was conducted using principal component analysis (PCA). The PCA score plot is a summary of the relationships among the observations (NADES), i.e., showing the maximum separation of all samples (▶ **Fig. 1 a**); the loadings plot is a summary of the predictors (NADES constituents) (▶ **Fig. 1 b**). The NADES with the highest solubility of Gal-HBr all cluster on the lower right side of the score



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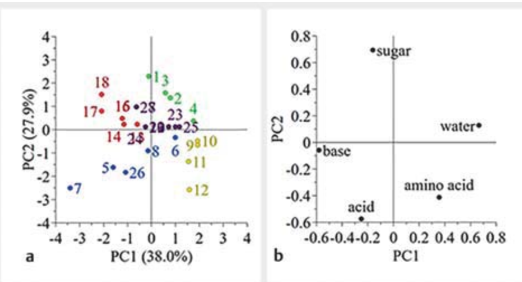


▶ **Fig. 1** **a** PCA score plot of the galanthamine-HBr data set and (**b**) loadings scatter plot. The composition of the NADES (sugar, base, acid, amino acid, and water), represented as the mole percentage, were used as predictors. NADES are numbered as in ▶ **Table 1**. Green are neutral NADES; blue are ionic liquids; yellow are amphoteric DESs; red are basic DESs; purple are acidic DESs.

plot (**6, 9, 10, 19, 21, 26**), though some NADES with poor solubility are also in that area. From the PCA, it can be deduced that Gal-HBr is neither soluble in basic (**13–18**) nor in neutral NADES (**1–3**). Supervised multivariate data analysis did not improve the separation obtained with PCA (data not shown).

The results (▶ **Table 1**) showed that the highest solubility of galanthamine correlated with the presence of acid components and water as NADES constituents. The solvent FS (**4**), a neutral type of NADES, consists of sugars and a high concentration of water. However, the solubility of 3.7 mg/g obtained with this NADES was relatively low compared to other neutral NADES with less water. Gal-HBr was not very soluble in basic NADES (**13–18**) whereas their solubility in ILs (**5–8**), which also contain a base, was much higher. In fact, the solubility of Gal-HBr in IL **6** is the highest among all tested NADES; this NADES contains 32.7% water. On the other hand, there is no clear trend regarding the effect of sugars. The NADES containing sugar and malic acid, in combination with fructose (**20**), showed a much lower solubility of Gal-HBr than in combination with glucose (**19**) or sucrose (**21**). Interestingly, acid NADES containing citric acid with sugars (**22–25**) had a lower solubilizing power than those containing malic acid. In contrast, ILs **5** and **6**, containing citric acid, scored better than those containing malic acid. The effects of the organic acids might be explained by the basic properties of galanthamine (pKa = 8.2) [3]. Gal-HBr is protonated in acidic conditions, so that an acid-containing NADES should solubilize Gal-HBr more efficiently. The hydrogen bonding properties of sugars and the presence of bases in a NADES is less significant and even detrimental for the solubilization of Gal-HBr. It is important to note that while the effect of a base on the solubility of alkaloids in an aqueous solution is totally predictable, little is known about the behavior of acids and bases in the predominantly nonaqueous environment of NADES. Following the solubility tests described before, 11 NADES were selected to compare their performance in the extraction of galanthamine from *N. pseudonarcissus* L. cultivar "Carlton" bulbs using pressurized extraction (▶ **Table 2**). This type of extraction was chosen as a simple reproducible and fast technique

▶ **Table 2** NADES candidates used in this study for the *N. pseudonarcissus* alkaloids extractability with a pressurized extraction method.

| No[a] | NADES[b] | Molar ratio[c] | NADES type |
|---|---|---|---|
| 1 | FG | 1 : 1 | Neutral |
| 2 | FS | 1 : 1 | Neutral |
| 5 | CCMA | 1 : 1 | Ionic liquids |
| 6 | CCCA | 1 : 1 | Ionic liquids |
| 9 | PrS | 2 : 1 | Amphoteric |
| 12 | βAMA | 1 : 1[d] | Amphoteric |
| 13 | CCS | 4 : 1 | Basic |
| 15 | CCG | 5 : 2 | Basic |
| 19 | MAG | 1 : 1 | Acidic |
| 21 | MAS | 1 : 1 | Acidic |
| 24 | CAS | 1 : 1 | Acidic |

[a]NADES number is based on ▶ **Table 1**; [b]G = glucose, F = Fructose, S = sucrose, CC = choline chloride, MA = malic acid, CA = citric acid, Pr = *α*-proline, *β*A = *β*-alanine; [c]Molar ratios, excluding the water mole ratio, were used due to the use of the solid form of NADES components; [d]Instead of using 3 : 2 as a molar ratio, a 1 : 1 molar ratio of *β*AMA was used in order to reduce the viscosity of this NADES and prevent the clogging problem during the extraction

Assuming that 200 mg of plant material, containing 4 mg/g galanthamine, is extracted with approximately 300 mg of NADES, a rough calculation of the saturation level of galanthamine in the NADES (▶ **Table 1**) shows that its solubility must not be lower than 2.7 mg/g solvent to avoid saturation of the solvent. Some of the NADES from ▶ **Table 1** exhibited a maximum solubility of galanthamine below this value. Thus, only NADES that solubilized between 23.1 and 62.2 mg/g of galanthamine were selected for these experiments (▶ **Table 2**)

Pressurized extraction is a simple, fast, and large-scale process involving the use of a pressurized extractor. The plant material was mixed with sand and the solid NADES and loaded into the extraction cells. This mixture was heated for 1 h at 50 °C during which the NADES is presumably formed by deep eutectic melting and the extraction process of the bulb material starts. The extraction column was then placed in the extractor and the pressure was increased to 50 bar at 50 °C after which the NADES extract was flushed out of the extraction vessel under pressure. This cycle was repeated twice, lasting a total of 8 min. It thus consisted of three steps: a heat up step (1 min), a hold step (2 min), and a discharge step. During the 2-min hold step, the void cell volume of the extractor is filled with water (approximately 42–44% of cell volume) at the same temperature and pressure for the second cycle.

Galanthamine amounts were extracted by pressurized extraction using different types of NADES as analyzed by HPLC. The NADES abbreviations refer to ▶ **Table 2**, while cy1 and cy2 refer to the first and second cycle, respectively. The third bar (green) is



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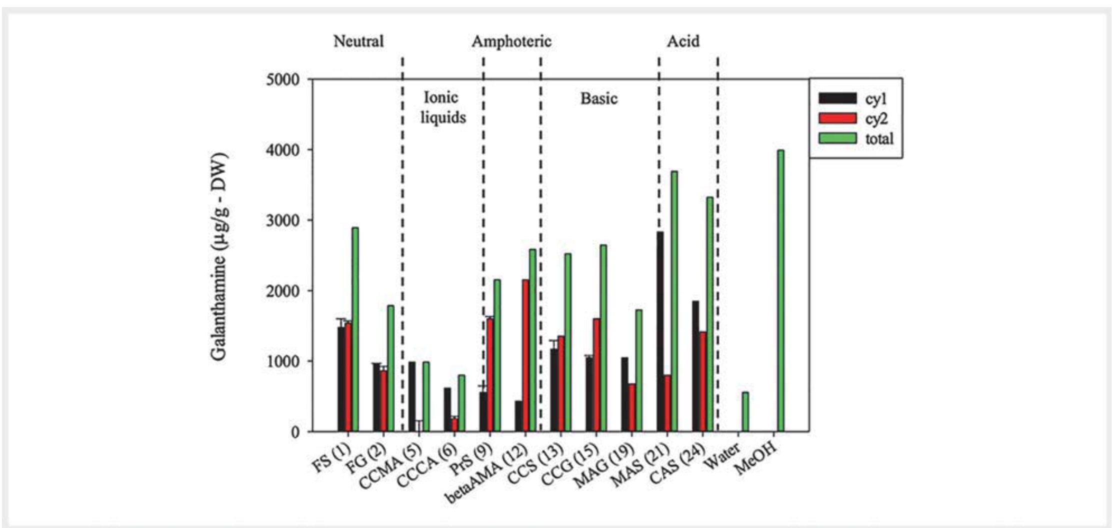


▸ **Fig. 2** Galanthamine amounts (as base) extracted by pressurized extraction using different types of NADES as analyzed by HPLC. The NADES abbreviations refer to ▸ **Table 2** while cy1 and cy2 refer to the first and second cycle, respectively. The third bar (green) is the total obtained with the two extraction cycles. Three replicates were made of all extractions.

the total obtained with the two extraction cycles. Three replicates were made of all extractions.

The extract obtained in the first cycle is considered to be the NADES extract. In the second cycle, water is forced through the extraction cell to flush out remaining soluble matter. ▸ **Fig. 2** shows the total galanthamine yield from both cycles obtained with the different NADES. The yield of the first and the second cycle are shown separately. Due to the large amount of water in the extract of the second cycle, it is much less viscous than the first NADES extract. The second cycle shows that pressing out the mixture of the leftover NADES with water still washes out quite a high amount of alkaloids from the extraction column. The alkaloid profiles are shown in ▸ **Table 3** and discussed below.

When analyzing the results, it is clear that the difference in the galanthamine yield in the first and the second extract depends on the type of NADES. In the case of amphoteric (**9, 12**) and basic (**15**) NADES, the second cycle significantly improved the galanthamine yield. Conversely, in the case of ILs (**5, 6**) and acid-type NADES (**19, 21, 24**), the highest yield was obtained in the first cycle (▸ **Fig. 2**). Neutral NADES consisting of sugars (**1, 2**) provided approximately equal yields of galanthamine in both cycles. This showed the need of using the two cycles to obtain the highest yields of galanthamine.

Acid-type NADES (**21, 24**) extracted the most galanthamine (3.7 and 3.3 mg/g DW, respectively) and the galanthamine contents of the first cycle were also the highest, ca. 2.8 and 1.8 mg/g DW, respectively. In the case of the IL types (**5, 6**), the yield was very poor, and the second cycle, with more water, hardly extracted any alkaloids at all. Thus, the high solubilities of Gal-HBr in the IL (▸ **Table 1**) did not translate to high extraction yields. Considering that the first extract is the pure NADES solvent, one would expect the solubility of Gal-HBr to be correlated to its extraction yield, but most of the high solubilizing solvents did not give very high yields of the alkaloids. In fact, MAS (**21**) was number 4 in the ranking of Gal-HBr solubility (▸ **Table 1**) but performed best in the extractor, whereas the best two (**6, 19**) did not perform very well in the extractor.

The importance of water as noted in the present experiment is illustrated by the high level of galanthamine in the second extract of the amphoteric NADES (**9, 12**) (▸ **Fig. 2**), though in the case of the ILs (**5, 6**) such an effect was not observed. Among the ILs, the solubility of Gal-HBr in **6**, which contained 32.7% water, was the highest (▸ **Table 1**). However, it produced the lowest extraction yield, practically the same as water. Possibly, the binding of the alkaloid to the plant matrix is too strong to allow it to solubilize readily in the solvents. This discrepancy between solubility and extractability has also been noticed in the case of vanillin extraction from vanilla pods. This is not entirely surprising, considering that in the case of the extraction of a compound from a biological matrix, apart from the solubility of the solute *per se*, the solute-matrix interaction must be considered. Actually, this solute-matrix interaction constitutes a barrier that has to be overcome to allow the release of the metabolite for its solubilization. It is thus a significant limiting factor in extraction efficiency [7, 19].

The high pressurized water and methanol extractions yielded 0.57 and 3.90 mg/g DW of galanthamine, respectively, under the same extraction conditions as those used for NADES (▸ **Fig. 2** and **Table 3**). The pressurized extraction using MAS (**21**) effectively extracted galanthamine with a yield comparable with the MeOH extraction. The galanthamine yield of an exhaustive Soxhlet extraction of *N. pseudonarcissus* with methanol was 3.5 mg/g (0.35%) DW (results not shown), which is consistent with reports by Lubbe



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**▶ Table 3** Alkaloid contents (as base) of pressurized extraction extracts of *N. pseudonarcissus* by different types of NADES.

| NADES[a] (No[b]) | | Alkaloid content[c] ± SD[d] | | | | | | | | Galanthamine/ Tot. minor alkaloids (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Alkaloids | Homolyco-rine | Galanthamine | Lycoramine | Norgalan-thamine | Haeman-thamine | Narwedine | Oduline | Un1 | |
| | Lipophilicity data (ACD/log P)[e] | 1.99 | 1.75 | 1.74 | 1.48 | 1.10 | 1.04 | – | – | |
| | Chemical formula (MW[f]) | $C_{18}H_{21}NO_4$ (315.36) | $C_{17}H_{21}NO_3$ (287.35) | $C_{17}H_{23}NO_3$ (289.37) | $C_{16}H_{19}NO_3$ (273.33) | $C_{17}H_{19}NO_4$ (301.34) | $C_{17}H_{19}NO_3$ (285.34) | $C_{17}H_{19}NO_4$ (301) | – | |
| FS (1) | cy1[g] | ND | 1385.6 ± 0.0 | 62.7 ± 2.5 | 861.9 ± 16.9 | 267.3 ± 7.3 | ND[h] | 100.7 ± 6.9 | ND | 52/48 |
| | cy2[g] | ND | 1524.2 ± 39.2 | 65.9 ± 11.0 | ND | 255.7 ± 32.7 | ND | 127.9 ± 2.1 | ND | 77/23 |
| FG (2) | cy1 | ND | 904.3 ± 0.0 | 27.2 ± 0.6 | 13.2 ± 4.3 | 179.5 ± 5.3 | ND | 72.4 ± 5.7 | ND | 76/24 |
| | cy2 | ND | 846.3 ± 38.6 | 25.9 ± 3.1 | ND | 158.6 ± 6.9 | ND | 64.1 ± 2.2 | ND | 77/23 |
| CCMA (5) | cy1 | ND | 986.6 ± 83.5 | 50.2 ± 7.7 | 106.8 ± 3.0 | 221.6 ± 5.9 | ND | 147.3 ± 6.3 | ND | 65/35 |
| | cy2[i] | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| CCCA (6) | cy1 | 24.0 ± 2.1 | 599.3 ± 13.6 | 27.1 ± 4.8 | 39.6 ± 3.1 | 108.5 ± 9.0 | 20.0 ± 0.0 | 98.3 ± 4.8 | ND | 65/35 |
| | cy2 | ND | 185.6 ± 1.0 | 4.2 ± 17.8 | ND | 39.2 ± 10.9 | ND | 123.8 ± 26.7 | ND | 53/47 |
| PrS (9) | cy1 | ND | 527.4 ± 60.8 | 13.7 ± 3.3 | 1.4 ± 1.3 | 66.7 ± 0.6 | ND | 16.4 ± 0.0 | ND | 84/16 |
| | cy2 | ND | 1646.8 ± 0.0 | 75.6 ± 14.9 | ND | 282.0 ± 21.1 | ND | 113.7 ± 2.7 | ND | 78/22 |
| *β*AMA (12) | cy1 | 23.9 ± 2.4 | 422.7 ± 61.8 | 14.5 ± 2.6 | 917.9 ± 10.0 | 45.3 ± 4.9 | ND | ND | 19.09 ± 1.6 | 29/71 |
| | cy2 | ND | 2167.4 ± 24.5 | 115.0 ± 2.2 | ND | 402.3 ± 24.8 | ND | 193.9 ± 13.2 | ND | 75/25 |
| CCS (13) | cy1 | ND | 1164.1 ± 0.0 | 50.0 ± 5.6 | 93.6 ± 0.0 | 214.5 ± 0.9 | 125.0 ± 0.0 | 81.3 ± 7.1 | ND | 67/33 |
| | cy2 | ND | 1375.8 ± 0.0 | 48.9 ± 3.1 | ND | 226.6 ± 12.4 | ND | 124.3 ± 4.8 | ND | 77/23 |
| CCG (15) | cy1 | ND | 1063.4 ± 0.0 | 44.1 ± 9.0 | 85.9 ± 10.0 | 160.8 ± 10.5 | ND | 71.0 ± 0.0 | ND | 75/25 |
| | cy2 | ND | 1595.4 ± 35.8 | 60.6 ± 10.7 | ND | 268.5 ± 13.8 | ND | 133.2 ± 18.2 | ND | 78/22 |
| MAG (19) | cy1 | ND | 1025.8 ± 16.7 | 42.4 ± 1.9 | 44.6 ± 0.7 | 181.7 ± 7.2 | ND | 102.0 ± 9.7 | ND | 73/27 |
| | cy2 | ND | 711.7 ± 29.8 | 29.3 ± 3.1 | ND | 130.5 ± 4.7 | ND | 83.4 ± 2.6 | ND | 75/25 |
| MAS (21) | cy1 | 121.5 ± 10.5 | 2797.9 ± 71.1 | 98.0 ± 8.0 | 60.8 ± 13.0 | 438.5 ± 21.9 | ND | 157.5 ± 12.2 | ND | 76/24 |
| | cy2 | ND | 849.4 ± 21.32 | 6.7 ± 9.9 | ND | 148.5 ± 13.3 | ND | 141.4 ± 5.3 | ND | 74/26 |

continued



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▶ **Table 3** *Continued*

| NADES[a] (No[b]) | | Alkaloid content[c] ± SD[d] | | | | | | | | Galanthamine/ Tot. minor alkaloids (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Alkaloids** | **Homolycorine** | **Galanthamine** | **Lycoramine** | **Norgalanthamine** | **Haemanthamine** | **Narwedine** | **Oduline** | **Un1** | |
| | **Lipophilicity data (ACD/log P)[e]** | **1.99** | **1.75** | **1.74** | **1.48** | **1.10** | **1.04** | **–** | **–** | |
| | **Chemical formula (MW[f])** | **$C_{18}H_{21}NO_4$ (315.36)** | **$C_{17}H_{21}NO_3$ (287.35)** | **$C_{17}H_{23}NO_3$ (289.37)** | **$C_{16}H_{19}NO_3$ (273.33)** | **$C_{17}H_{19}NO_4$ (301.34)** | **$C_{17}H_{19}NO_3$ (285.34)** | **$C_{17}H_{19}NO_4$ (301)** | **–** | |
| CAS (24) | cy1 | ND | 1832.5 ± 82.5 | 67.2 ± 23.4 | 155.9 ± 13.0 | ND | ND | 241.5 ± 45.4 | ND | 80/20 |
| | cy2 | ND | 1414.00 ± 37.7 | 46.9 ± 6.0 | ND | 244.2 ± 14.6 | ND | 186.9 ± 9.5 | ND | 75/25 |
| | $H_2O$[j] | ND | 569.1 ± 0.1 | 24.8 ± 6.4 | 39.7 ± 4.0 | 96.2 ± 6.2 | 60.0 ± 0.0 | 96.2 ± 6.2 | ND | 66/34 |
| | MeOH[j] | 299.5 ± 4.6 | 3902.3 ± 0.0 | 204.4 ± 31.2 | 171.4 ± 0.0 | 915.1 ± 40.4 | ND | ND | ND | 71/29 |

[a]Types of NADES, G = glucose, F = fructose, S = sucrose, CC = choline chloride, MA = malic acid, CA = citric acid, Pr = $\alpha$-proline, $\beta$A = $\beta$-alanine; [b]NADES number is as in ▶ **Table 1**; [c]Galanthamine concentration was determined by HPLC-DAD while the alkaloids lycoramine, norgalanthamine, narwedine, oduline, Un1, haemanthamine, and homolycorine were determined using GC-FID. The yield of alkaloids other than galanthamine are expressed as galanthamine equivalents (µg/g of dry weight), assuming a similar detector response for all alkaloids with GC-FID; [d]Standard deviation (SD) calculated from three replicates. [e]ACD/log P is the estimated lipophilicity value of the octanol-water partitioning coefficient based on the structure of the compound. Adapted from www.chemspider.com; [f]Chemical formula and molecular mass (MW) of all alkaloids were obtained from www.chemspider.com; oduline [4, 5]; [g]Cy1 and cy2 refer to extracts obtained from the first cycle and second cycle, respectively; [h]ND = below detection limit; [i]No extract of CCMA was obtained from second cycle; [j]Control solvents



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et al. [20] and Akram et al. [21, 22] of concentrations of galanthamine in the range of 0.2–0.4 % DW galanthamine in these bulbs.

Considering the large difference in the yield of galantamine obtained with the various types of NADES, the next point to explore was the selectivity of NADES and what this revealed about their interactions with galanthamine and solutes in general. More than 20 isoquinolinic alkaloids have been isolated from *N. pseudonarcissus* cv. Carlton [23]. These include the following types or classes: homolycorine type: homolycorine, massonine, hippeastrine, lycorenine, *O*-methyllycorenine, and oduline [24]; galanthamine type: galanthamine, narwedine, norgalanthamine, epinorgalanthamine, and lycoramine [25]; haemanthamine type: haemanthamine [25] and vittatine [4]; lycorine [5, 24]; crinine- [26] and narciclasine-type alkaloids) [27–29].

To learn more about the selectivity of the NADES extraction, gas chromatography-mass spectrometry (GC-MS) and gas chromatograph equipped with a flame ionization detector (GC-FID) were used to obtain the qualitative and quantitative profiles of the alkaloids in the extracts, respectively. The amount of other identified alkaloids besides galanthamine is expressed as µg of galanthamine equivalents in g of dry weight of plant material, assuming a similar detector (GC-FID) response for all alkaloids. Narwedine, norgalanthamine, galanthamine, lycoramine, oduline, homolycorine, and haemanthamine, i.e., representatives of the galanthamine-, homolycorine- and haemanthamine-type of alkaloids, were found in the NADES extracts. No lycorine-, crinine- and narciclasine-type of alkaloids were observed in the *N. pseudonarcissus* cv. Carlton bulb material. One unidentified (Un1) compound was observed in the extract with *β*AMA (**12**). This compound had an *m/z* 345 (M+) and a fragment ion of *m/z* 330 (M+ – 15), most likely due to the loss of a methyl (-CH3) radical from a methoxyl (R-O-CH3) group. However, the fragmentation pattern of Un1 does not show a fragmentation pattern similar to either haemanthamine- or homolycorine-type alkaloids, which are characterized by an ion with *m/z* 272 or *m/z* 109, respectively. Because of the lack of further spectral data, Un1 could not be identified and should be isolated for structural elucidation. A comparison of the alkaloid profiles of the NADES extracts of *N. pseudonarcissus* bulbs (▶ **Table 3**) revealed some selectivity for the abovementioned alkaloids. For example, narwedine was only extracted with NADES **6** and **13**, which are combinations of choline chloride with an acid and a sugar, respectively; homolycorine was only extracted with **6**, **12**, and **21**. Interestingly, **6** is also the NADES that solubilizes Gal-HBr best (▶ **Table 1**). Norgalanthamine was present in all types of NADES extracts, but significantly higher amounts were extracted with **1** and **12** (ca. 900 µg/g DW). In both cases, norgalanthamine was only found in the first extraction cycle.

An explanation for the selectivity for alkaloids of different types of NADES could lie in their degree of lipophilicity. An estimation of their lipophilicity based on the octanol-water partitioning coefficient obtained by applying the method described in www.chemspider.com (▶ **Table 3**) showed that, unsurprisingly, the higher the lipophilicity values of an alkaloid the higher their solubility in lipophilic solvents if compared to hydrophilic solvents including water. *N. pseudonarcissus* alkaloids (▶ **Table 3**) were arranged in descending order of lipophilicity, with *O*-methyllycorenine (not found in the extracts) being the most lipophilic amongst them (ACD/log P = 2.98) and narwedine the least (ACD/log P = 1.04). The hydrophilicity of narwedine is reflected in its presence in the water extract and absence even in the methanol extract, a behavior that is opposite to that of galanthamine, which has the highest yield by far in the methanol extract. Unfortunately, the lack of physicochemical data of both alkaloids and the NADES themselves do not allow more than speculations. The ratio of galanthamine to minor alkaloids (▶ **Table 3**) provides clear evidence of selectivity of the extraction. MAS (**21**) extracts, which are the richest in galanthamine, have relatively low levels of minor alkaloids. For certain minor alkaloids, it is possible to find a specific NADES that yields a relative high level of a particular alkaloid, e.g., norgalanthamine in the first cycle with the amphoteric NADES **12**, or haemanthamine with the neutral NADES FS **1**.

The yield of the extraction of galanthamine from *N. pseudonarcissus* bulbs with acidified water (1 % v/v of HBr, 65 °C, 3 h) and SFE ($CO_2$, 70 °C, 220 bar, 3 h, plant material moistened with NH4OH 25 %, v/v), was 2.7 mg/g DW and 303 µg/g DW, respectively [7], and the selectivity for galanthamine in both methods was below 70 %. Thus, NADES extraction with MAS **21** is more efficient both in terms of galanthamine yield and selectivity. The highest yield obtained with NADES extraction is similar to that of a methanolic extraction, but with a slightly higher selectivity for galanthamine (▶ **Table 3**).

Two papers published by Liu and coworkers [30, 31] reported the extraction of alkaloids with NADES. In the first paper, 43 NADES were tested for the extraction of different classes of compounds, including some quarternary isoquinoline alkaloids (berberine, palmatine, and jatrorrhizine). The best results were obtained with combinations of CC (choline chloride) (see ▶ **Table 3**) with different organic acids, among which levulinic acid (1 : 2) proved to be the most efficient. Combinations with sugars or betaine gave lower yields, similar to combinations with urea. Proline, sugars, or polyalcohol-containing NADES also gave low yields, though the combination of proline with organic acids was very efficient. All tested NADES contained 25 % water. In a second paper [31], 75 NADES were studied for the extraction of various alkaloids, including examples of the morphinan, protoberberine, bisbenzyl, indole, and quinolizidine type. The results are in line with our experience with Gal-HBr solubility, with ILs overall having the highest extraction yields of morphinan alkaloids, the type closest to galanthamine. Li and coworkers [32, 33] also explored the extraction of berberine and palmatine, obtaining similar results to those obtained by Liu and coworkers [30, 31]. The combination CCCA (choline chloride and citric acid) (see ▶ **Table 3**) (1 : 2) with 30 % water was shown to be the best for the extraction of the two alkaloids from the bark of *Phellodendron amurense* Rupr. [33]. For the extraction of these alkaloids from the rhizomes of *Coptis chinensis* Franch., betaine-based NADES were used [32]. In this case, the combination with organic acid and water (1 : 1 : 1) also performed best, particularly when using citric acid. On the other hand, the combinations of this acid with sucrose and urea extracted less than half that obtained with the acid-based NADES. Boldine, another isoquinoline alkaloid, gave the best results with proline-oxalic acid (1 : 1) with 20 % water. Yields were about 5- to 10-fold obtained with CC in combination with levulinic acid, among others [34].



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Si et al. [35] found CC-levulinic acid (1 : 2) and CC-oxalic acid (1 : 1) with 25 % water in both cases to be the most efficient NADES for the extraction of rutaecarpine, while betaine and CC in combination with glucose gave a poor yield. The combination of malic acid with betaine or CC yielded approximately half that obtained with levulinic acid. Takla et al. [36] compared several extraction methods for some isoquinoline alkaloids (crinine, lycorine, and crinamine) using diverse NADES that all contained 35 % water. The efficiency of the extraction of individual alkaloids differed considerably. The most universal was CCF (choline chloride and fructose) (see ▶ **Table 3**) with 35 % water, which extracted all three and showed the highest overall alkaloid yield. CCCA and CCLA (choline chloride and lactic acid) were also quite efficient, in accordance with our observations that the ILs are suited for alkaloid extraction. Some of the alkaloids were not extracted by neutral or acidic neutral types of NADES.

The poor performance of the tested ionic liquids as extraction solvents for galanthamine in our study is in contradiction with the results discussed in the previous paragraph for various other types of alkaloids [30–36], in which the ILs were actually the most efficient. In these studies, 25–30 % water in the NADES proved to increase yields. With this percentage of water, the NADES preserve characteristics that distinguish them from a simple aqueous solution of the NADES ingredients [18] The differences in the extractability of galanthamine in this study, if compared to the results obtained for other alkaloids, and the high solubility of Gal-HBr in ILs could reside in the release of the alkaloid from the matrix for which the ILs lack competence. Similar problems have already been observed with the SFE of galanthamine and with the extraction of vanillin from vanilla pods. In the case of galanthamine, the alkaloid must be extracted from bulbs, a sugar and lipid-rich matrix, which is quite different from leaves, roots, and stems.

Considering the aims of this study, the experiments afforded a great deal of information concerning the solubility of Gal-HBr as alternatives to organic solvents. Galanthamine hydrobromide proved to be the most soluble in ILS, amphoteric NADES, and NADES composed of acids with sugars and water in specific molar ratios. The results are, to some extent, similar to those obtained with other alkaloids, though there is a degree of selectivity that remains unexplained. Another conclusion is that the highest solubility is clearly achieved with NADES that contain 20–35 % water. These have the additional advantage of being much less viscous, and thus much easier to work with.

A second aim of the study was to develop an efficient extraction method for bulb material. By testing the NADES in which Gal-HBr were most soluble, it was found that this was not necessarily a good criterion for selecting the best solvent for galanthamine extraction from *Narcissus* bulbs. This was particularly notable in the case of ILS. Sugar-acid types of NADES proved to be the most efficient and malic acid-sugar-water (1 : 1 : 5) produced the highest yield, comparable to that of the exhaustive methanol Soxhlet extraction, with a slightly higher selectivity for galanthamine. The difference between the solubility of the pure compound and the extractability of galanthamine from the bulbs indicates that the interactions of the target compound and extraction solvent with the matrix might play an important role, as bulbs are very rich in sugars and lipids as well as special storage tissues that some NADES cannot penetrate easily.

## Materials and Methods

### Chemicals and materials

HPLC grade methanol (Biosolve B. V.) and triethylamine (Sigma-Aldrich) were used for the mobile phase. Gal-HBr for solubility studies was kindly donated by Tiofarma B. V. and the Gal-HBr reference compound was acquired from Selleckckem.com. Papaverine-HCl, used as an internal standard, and acetic acid were purchased from Sigma-Aldrich. Defatted sea sand was obtained from Büchi. All components of NADES (reagent grade) were purchased from Sigma-Aldrich. The powder of *N. pseudonarcissus* bulbs (family Amaryllidaceae) was kindly supplied by Leenen B. V.

### Galanthamine-HBr solubility experiment

The NADES used in this study were prepared according to Dai et al. [11]. The solubility test was conducted by gradually adding an accurately weighed amount of Gal-HBr to a sealed bottle. It was stirred at 40 °C on a multipoint stirring hot plate using a magnetic stirrer. The appearance of the samples was observed every 2 h. When the added amount of Gal-HBr dissolved completely in NADES yielding a transparent and colorless solution, more Gal-HBr was added. The experiment was interrupted either when saturation was reached or the solution changed color, e.g., into a yellowish or brownish color. The saturation point was defined as an opaque appearance of the solution.

### Extraction of bulbs with natural deep eutectic solvent

Extraction was performed in a Büchi pressurized extractor instrument E-916. The extraction was conducted using 2 cycles at 50 °C, 50 bar. Each cycle consisted of a heat up step (1 min, 50 °C, 50 bar), a hold step (2 min), and a discharge step (5 min). Before the extraction program, a preheating step was conducted at 50 °C for 1 h to allow the NADES constituents to melt, forming the liquid NADES. Each cycle of extraction lasted a total of 8 min.

Approximately 200 mg powdered bulbs of *N. pseudonarcissus* cv. "Carlton" (25–53 µm in particle size) were used for each extraction. The ratio of plant powder to NADES compounds (in solid state) was fixed at 40 % (plant : NADES, w/w). Before loading into the extraction cells, the plant material and NADES components were thoroughly mixed with 1.5 g defatted sand to obtain the desired void fraction. This is done to increase the contact between plant material and NADES, minimizing the dead volume in the extraction cell and preventing a blockage during the extraction process. Additionally, around 2.5 g of defatted sand was loaded at the bottom and top of the cells. The whole sample load occupied approximately 65 % of the total extraction cell volume. Finally, the metal frit of the extraction cell outlet was covered with a cellulose filter to prevent clogging.

Once the heating block was loaded with the extraction cells, the cells were automatically tightened when the extraction temperature was reached and the remaining cell volume, i.e., approximately 40 % of the total extraction cell volume, was filled with water and pressurized using nitrogen (an inert gas). After this,

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the first step of the first cycle was started. When the static extraction (hold step 1 min) was completed, the discharging step was immediately started by opening the pressure valve so that the extract flowed into the collection vials. The second cycle was started immediately after the discharge step of the first cycle was finished with no flushing or sample reloading between cycles. After the second cycle, the extraction cells were flushed with gas (3 min) at 50 mL/min and then water (1 min) at 10 mL/min. The extracts from each extraction cycle and the last flushing step were collected individually. Each extraction was done in triplicate. The yield of galanthamine of the pressurized extraction with NADES was calculated as the total yield of each cycle.

Apart from the NADES extraction, samples were also extracted with water and methanol in a speed extractor as classic solvent extractions. The conditions were similar to those used for NADES. The whole extraction process, including the sample preparations for analysis, is shown in **Fig. 1S**, Supporting Information.

## High-performance liquid chromatography determination of galanthamine

### Preparation of standard solution

A solution of Gal-HBr was prepared by dissolving an accurately weighed amount of approximately 2.50 mg (± 0.01 mg) in a 25-mL volumetric flask with the mobile phase. The resulting solution was filtered through Whatman 0.2 µm polyvinylidene difluoride (PVDF) syringe filters and injected twice. The standard solution was prepared in duplicate. All areas with an RSD (relative standard deviation) ≤ 2% were averaged.

### Preparation of sample solutions

Approximately 100 µL of the NADES solution were accurately weighed and diluted 50 times with methanol (MeOH). This solution was left to rest for 3–4 h to ensure the complete precipitation of sugar component NADES. The solution was then filtered with a 0.2-µm regenerated cellulose (RC) membrane syringe filter (Sartorius Stedim). An aliquot of 1.00 mL of this solution was diluted to 2.00 mL with TEA (triethylamine) (0.60%, v/v) in $H_2O$. The final solution was thus similar to the mobile phase MeOH-$H_2O$ (1 : 1, v/v) with 0.30% (v/v) (21.52 mM) of TEA. The pH of the sample was measured to ensure it was the same as that of the mobile phase (ca. pH 11.6) This solution was transferred to a 2-mL microtube and centrifuged at 14 000 *g* for 15 min. The suspension was filtered again through a Sartorius 0.2 µm RC-membrane syringe filter. The filtrate was analyzed with HPLC-DAD at 280 nm.

## High-performance liquid chromatography analysis

HPLC analysis was carried out to quantify Gal-HBr in the NADES solutions using an Agilent 200 series HPLC system consisting of a G1310A pump and G1322A degasser, a G1329A autosampler, a G1316A temperature controller, and a G1315D diode array detector (DAD) (Agilent). A Phenomenex Gemini NX-C18, 100 × 4.6 mm, particle size 3.0 µm column was used. A five-microliter sample was injected and measured at 280 nm. Samples were eluted with a mobile phase of methanol : $H_2O$ (1 : 1) with 0.30% (v/v) triethylamine at a 0.7 mL/min flow rate and 27 °C. The total run time was 5 min.

## Sample preparation for gas chromatography analysis

The NADES extracts were submitted to a clean-up for their GC analysis following the method described by Bastos et al. [37]. The obtained extracts (approximately 2.00 mg) were each diluted to 2.00 mL with water using a volumetric flask. The solutions were vortexed for 1 min and taken to a pH of 11 by the addition of a sufficient amount of 1 M NaOH. Three mL of chloroform were added to this solution and vortexed for 1 min. The two-layer mixture was centrifuged for 5 min at 4500 rpm, and the organic (lower) chloroform layer was collected. After passing the organic layer through a Pasteur pipet loaded with anhydrous sodium sulfate, the organic layer was evaporated under vacuum to dryness in a Savant SpeedVac. This purified alkaloid fraction was redissolved in 1.00 mL methanol containing 0.05% (v/v) acetic acid for both GC-MS and GC-FID analysis.

## Alkaloid identification by gas chromatography-mass spectrometry

All the alkaloids of the obtained extracts were identified by GC-MS with no derivatization step [16, 24]. The GC-MS analysis was carried out on an Agilent 7890A GC system with a 5975C single quadrupole mass spectrometric detector and an Agilent 7693 autosampler (Agilent Technologies, Inc.). Samples were injected into a DB-5 column (30 m × 0.25 mm i. d., 0.25 µm; JW Scientific, Agilent Technologies, Inc.) and eluted with a 30 min temperature gradient of 200–250 °C at 2.5 °C/min, then 250–270 °C at 10 °C/min followed by an 8-min hold at 270 °C. The injector and detector temperatures were 250 and 270 °C, respectively. The injection volume was 1 µL and the flow rate of the carrier gas (helium) was 1.5 mL/min with a split ratio of 1 : 20. The MS analysis was done in scan mode (*m/z* 50–350) using electron ionization at 70 eV. Galanthamine identification was accomplished by comparison of the *m/z* data with that of a reference compound. Other alkaloids present in *N. pseudonarcissus* extracts were identified by comparing their corresponding mass spectral fragmentation data to literature data [4, 5, 24, 25, 38–40].

## Alkaloids quantification by gas chromatography

The alkaloids were quantified using GC-FID. The preparation of the calibration curve was done following the method published by Gotti et al. [25]. Different volumes (75, 100, 125, 150, and 175 µL) of a Gal-HBr stock solution (78.4 µg/mL) were transferred to vials. These were then vacuum-dried and 500 µL of an internal standard solution consisting of 88.8 µg/mL of papaverine-HCl and 150 µL of 0.05% acetic acid in methanol were added to each vial. One µL of the resulting solution was injected into the GC-FID. All chromatographic conditions were identical to those described above. The ratio of the peak area of Gal-HBr to the internal standard, papaverine-HCl, was plotted against their corresponding weight ratios to obtain the calibration plot. The amount of other identified alkaloids besides galanthamine is expressed as galanthamine equivalents (µg/g of dry weight of plant material), assuming a similar detector response for all alkaloids in GC-FID.

## Data processing

NADES constituents such as sugars, bases, acids, amino acids, and water in their molar ratio were used as predictors, while galanth-



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amine solubility was used as the response. The data set is shown at ▸ **Table 2**. Correlation of NADES constituents to the solubility of Gal-HBr was interpreted by PCA, SIMCA-P software (version 13.0 Umetrics) [41], using unit variance scaling.

## Data availability statement

The manuscript contains all the generated data used for this project. The raw data will be available online when the PhD thesis that is the basis for this study is finished and approved and placed in the university PhD thesis repository.


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## Contributors' Statement

Conceptualization: O.R., Y.H.C., and R.V.; methodology: Y.H.C. and E.G.W.; software: Y.H.C.; validation: Y.H.C., R.V., and G.J.W.; analytical analysis: E.G.W.; data curation: Y.H.C.; writing–original draft preparation: O.R.; writing–review and editing: O.R.; visualization: O.R.; supervision: R.V., Y.H.C., and G.J.W.; funding acquisition: O.R. All authors have read and agreed to the published version of the manuscript.


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## Conflict of Interest

The authors declare that they have no conflict of interest.

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# CHAPTER 7

## Economic and Environmental Evaluation of The Sustainable Production of Galanthamine from *Narcissus pseudonarcissus*


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**Abstract**

An economical and environmental evaluation of the production process of galanthamine-HBr from plant material of *Narcissus pseudonarcissus* was done by using the volatile organic compound (VOC), diethyl ether. It was compared with an alternative process using supercritical $CO_2$ ($scCO_2$). The alternative $scCO_2$ process has 17% lower production costs of galanthamine-HBr (€/kg) compared to the conventional extraction process using diethyl ether followed with an acid-base alkaloids purification step (at 22 tonnes annual production). From the environmental point of view, the alternative process is more sustainable as it consumes 99% less of energy and generates 46% less of waste. In addition, approximately 1550 kg/kg of mass intensity as well as 60 kg/kg solvent intensity were calculated for the alternative process which are 37% and 94% lower, respectively, than for the conventional process. Thus, the $scCO_2$ process is preferable both from the economic and environmental point of view. For maximising the benefit of the $scCO_2$ process and winning the galanthamine-HBr market, an integrated process of $scCO_2$ using a multi-products plant producing also other high purity *N. psudonarcissus* alkaloids as by-products should be considered.



**Keywords**: economy analysis; estimation; rough calculation

# 1 Introduction

Razadyne® (formerly Reminyl®), a medicine containing a galanthamine-HBr as an acetylcholine esterase inhibitors (AChEIs), is at present the most successful amongst other AChEIs based medicines such Cognex® (containing tacrine), Aricept® (containing donepezil), and Exelon® (containing Rivastigmine) in treating mild-to-moderate Alzheimer's Disease (AD) (Sabbagh, 2009).

At present, there is no preventative or curative treatment available for AD. Thus, the AChEIs therapy is the only approved therapeutic options (Heinrich and Lee Teoh, 2004). Galanthamine was first marketed in Bulgaria at late 1950s, with a commercial name of Nivalin® (Cronnin, 2001). It is approved in US, many European and some Asian countries (Heinrich and Lee Teoh, 2004).

After its initial launch, the galanthamine price reached $40,000/kg (Heinrich and Lee Teoh, 2004). The commercial availability was critical due to the limited amount of the natural raw material *Galanthus nivalis* (snowdrop) (Cronnin, 2001). However, the price went down after the synthetically produced galanthamine was introduced into the market. The first synthetis patented of galanthamine was reported by Sanochemia Pharmazeutika in 1996 (Heinrich and Lee Teoh, 2004). Nowadays, galanthamine-HBr is mainly produced by total synthesis. The synthetic routes of galanthamine-HBr were reviewed by Bulavka and Tolkachev (2002) including the patents (Bolugoddu *et al.*, 2006; Koilpillai *et al.*, 2012; Lahiri *et al.*, 2006). The galanthamine purification via the galanthamine-HBr salt was patented by Gabetta and Mercalli (2011).

For non-patent drugs, the generic manufacturers are looking for the lowest priced API. A production process selection yielding a high purity API without damaging the environment and lowering the health and safety issues is critical. This can be reached by reducing and minimising the use of volatile organic compounds (VOCs) in the process production. A minimised solvent use leads to lower costs for disposal, legal liabilities and may overcome regulatory constraints.

In this study, two different processes for galanthamine-HBr production are compared. One is an extraction process by means of supercritical carbon dioxide ($scCO_2$), and the other a US patented conventional extraction process (Hille *et al.*, 2002). For this purpose, bulbs of *Narcissus pseudonarcissus* cv. Carlton were chosen as the raw material. Both economic and environmental aspects (waste production) will be taken into consideration for a comparative the two processes. Calculations of the economy of the processes were also applied.

The results will enable the stake holders to make a rational decision on the suitability and sustainability of the evaluated production processes of galanthamine-HBr.

## 2 Market size of galanthamine

There will be an estimated 65.7 million people with AD in 2030, and 115.4 million in 2050 (Anonimous, 2013; Prince *et al.*, 2013). This estimation includes both men and women, in 5-year age bands from 60 to 84 years, and for those aged 85 years and older (Ferri *et al.*, 2005; Prince *et al.*, 2013). The rate of increase is higher in the developing countries than in developed countries. It is forecasted to increase by 100% between 2001 and 2040 in developed countries. Whereas a scary number of an increase rate of ca. 300% is estimated for India, China, and their South Asian and Western Pacific neighbours (Ferri *et al.*, 2005).

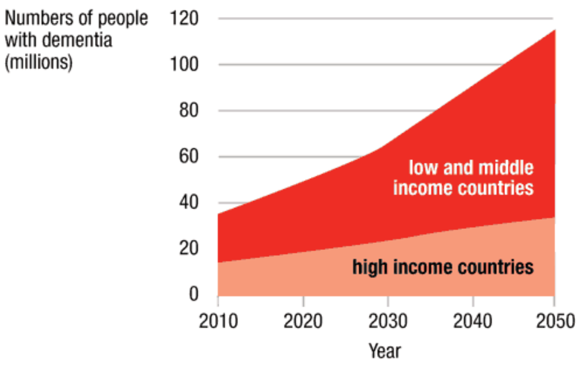



**Figure 1**. Statistics data of people with AD (Anonimous, 2013).

The costs of the huge number of AD patients in 2010 were worldwide US$ 604 billion, of which approx. 70% in Western Europe and North America (Wimo *et al.*, 2013). This includes direct costs (medical and social care) and indirect costs (unpaid caregiving by families and friends). The United States spent US$ 216 billion/year only for paying professional caregivers. Thus, this disease is affecting every health system in the world. AD patients live ca. 11.2% years longer than patients with stroke, cardiovascular disease, or cancer (Ballard *et al.*, 2011). The dependency of AD patients on caregivers and the inherent high costs urge governments to collaborate with social communities, e.g. families, neighbourhoods, and health and social care systems, and raise awareness to be better prepared for the future. Obviously, medication might offer relieve for the caretaker with galanthamine as only medicine that has some effects on the symptoms and consequently there will be a growing demand for this compound.

A clinical study on galanthamine-HBr showed that clinical doses of 8, 16, or 24 mg/day/patient for 5 months treatment give a minimum of adverse effects (Heinrich and Lee Teoh, 2004). Assuming 16 mg/day/patient as a reference; a single patient will need around 2.4 g of galanthamine per year within an assumption of 5 months of medication, thus minimising the adverse effects. It is assumed, that the current product will only take a 30% market share, a possible standard goal for pharmaceutical companies to invest into a new product (Rita, 2002); by using the predicted statistical data (**Fig. 1**), there will be ca.

50 million people with AD at 2025. Thus, a process size of 36 metric tonne of galanthamine-HBr would be necessary if 30% of all patients are treated annually.

Taking a $3.18/tablet of 4 mg of galanthamine-HBr (www.drugbank.ca) within the mentioned assumptions, a medical expenses around $2000 should be spent by a single patient only for 5 months treatment of single drug, i.e. galanthamine-HBr, consumption. Thus, indicating that a lower price is needed to benefit the patients, especially in the developing countries.

## 3 The potential of Narcissus pseudonarcissus as a raw material for galanthamine production

*Narcissus pseudonarcissus* cv. Carlton is cultivated since many years in large amounts. It is the most cultivated *Narcissus* cultivar in The Netherlands. *Narcissus* ranked third position for the popularity of common planted flower in The Netherlands (Anonimous, 2008). Its high rate of propagation, flexibility to diseases, and climate makes Carlton superior amongst the *Narcissus* cultivars (Kreh, 2002; Rossing *et al.*, 1997). The commercial availability of large quantities in the market makes it interesting for cultivation for medicinal purposes.

Moreover, the well-established cultivation system which is supported by a robust management system will help to monitor and maintain the quality products with botanical source assurance and assure a stable market supply both for ornamental flowers and medicinal purposes.

## 4 The potential of *Narcissus pseudonarcissus* alkaloids other than galanthamine

The research described in this thesis show that haemanthamine can be obtained from $scCO_2$, water, and NADES extracts (**Chapter 3, 4, and 7**). *O*-methyllycorenine was found in $scCO_2$ and water extracts (**Chapter 3** and **4**), *O*-methyloduline was present in water extracts including the pressurised extraction method (**Chapter 4**), while norgalanthamine was selectively extracted with the fructose-sucrose (FS) NADES, and oduline with malic acid-sucrose (MAS), malic acid-glucose (MAG), and citric acid-sucrose (CAS) NADES (**Chapter 7**). One should take advantage of the selectivity of the different solvents for producing high added value by-products and thus gain more benefit from the extraction process.

Galanthamine, lycorine, narciclasine, and pretazettine are the most studied *Narcissus* alkaloids due to their multiple bioactivities (Bastida *et al.*, 2006). *Narcissus* is found to contain a high level of galanthamine especially in the bulbs (Lubbe *et al.*, 2013; Rita, 2002); with haemanthamine at the second important product, followed by the acid alkaloid, narciclasine (Lubbe *et al.*, 2013). Haemanthamine is abundant in the *Narcissus* leaves, where galanthamine and narciclasine are at the same level. However, narciclasine is not found in the roots. The most abundant alkaloid in bulbs is galanthamine throughout

the whole growing season, whereas haemanthamine and narciclasine concentrations are relatively low. However, haemanthamine and narciclasine production can be optimised as to obtain valuable by-products from the *N. pseudonarcissus* cv. Carlton bulbs extraction.

Like galanthamine, haemanthamine and narciclasine have interesting properties for medicinal purposes. Antimalarial activity was detected for haemanthamine (Bastida *et al.*, 2006) while an anti-tumour effect was found at micromolar concentration (Jimenez *et al.*, 1976; McNulty *et al.*, 2007). Its effect against various cancer cells was also reported by Bastida and Viladomat (2002). Narciclasine and its semi-synthetic derivatives are also promising candidates for cancer drugs. Hence, *N. pseudonarcissus* bulbs was chosen as the plant material for industrial scale production of galanthamine. It is well supported by a well-established cultivation system, and extensive research and development.

In addition, *O*-methyloduline, norgalanthamine, oduline, and *O*-methyllycorenine are further bioactive compounds of *Narcissus*. Norgalanthamine has a cytotoxic effect against fibroblastic cell lines, as well as acetylcholine esterase activity though weaker than galanthamine. Cytotoxic activity was also observed for *O*-methyllycorenine (Bastida and Viladomat, 2002).

## 5 Process descriptions

### 5.1 Existing medium-scale process of galanthamine extraction

As earlier mentioned, galanthamine-HBr is presently mainly produced by a total synthetic process (Heinrich and Lee Teoh, 2004). Although for commercial purposes, a galanthamine production from plant material seems a feasible alternative process. Some published medium- to large-scale production processes of galanthamine from plant material such as bulbs and leaves are summarized in **Table 1**.

Organic solvent extraction and acidified water extraction followed by subsequent acid-base liquid-liquid extraction (LLE) step is generally used for the purification of galanthamine. This is similar to the common laboratory scale preparation of alkaloids from plant matrices (Bastos *et al.*, 1996; Berkov *et al.*, 2008; Ptak *et al.*, 2008), where organic solvent extraction using alcohol (MeOH or EtOH) and acidified water extraction are the most widely use extraction methods.

Block diagrams of some production processes of galanthamine as mentioned in **Table 1** are shown in **Figure 2**, and **3**. In general, they have common process units such as: organic or acidified water extraction, followed by purification through of liquid-liquid partition extraction (LLE) and finally crystallisation. Cvak *et al.* (2008) applied adsorption processes, i.e. ion exchange and column chromatography, to increase the purity of galanthamine-HBr. The acid-base separation principle is applied in LLE with volatile organic solvents such as chloroform, diethyl ether, methyl isobutyl ketone, and gasoline but also using ion exchange columns. Crystallisation is applied at the end of the process

both for increasing the purity and obtaining galanthamine as a water-soluble salt, i.e. galanthamine-HBr or galanthamine-HCl.

Plant matrices as raw material for the industrial production of galanthamine has several drawbacks in processing (Cvak *et al.*, 2008):

(1) The lack of robustness and scalability to a large-scale isolation process. The process steps are usually tailor made for a specific plant material. The developed process for small-laboratory scale is hard to be implemented in a larger-industrial process.
(2) The volatile organic compounds (VOCs) used as solvents which are toxic and not environmentally friendly are not suitable for an industrial scale process.
(3) The problem of the formation of emulsions in LLE.

### 5.2 The proposed alternative method of the galanthamine extraction

Several medium size processes of galanthamine isolation from plant material were reported (**Table 1**). For the purposes of economic evaluation, a protocol of conventional extraction process of galanthamine-HBr production as described by Hille *et al.* (2002) was chosen as it represents an alternative for the common alkaloid extraction methods from plant matrices. A combination of ion exchange and liquid-liquid extraction as reported by Cvak *et al.* (2008) as a purification method requires ion exchange column chromatography which is a cost increasing factor.

Considering the main message of the 12 principles of the green chemistry, reducing or eliminating the use or the generation of hazardous substances in the design, manufacture, and chemical products (Deetlefs and Seddon, 2010), this classical method does not score high. Another disadvantage of the acidified water extraction as patented by Cvak *et al.* (2008) is the formation of stable emulsions, which was also observed in **Chapter 4**. This makes the method less robust. Moreover it, requires more organic solvents, and must be followed by another separation process (Cvak *et al.*, 2008). While the sc$CO_2$ extraction process (**Chapter 3**), as a closed system does not lose any solvent, and possibly consumes less energy.

A pre-treatment step, moistening with $NH_4OH$ (25%, v/v), is included in the sc$CO_2$ extraction to increase the solubility of galanthamine. This pre-treatment process followed by sc$CO_2$ extraction and another purification step followed by crystallization step is here considered as an alternative greener process.

Thus, an assessment of the performance of these two different methods was conducted, i.e. a conventional and a sc$CO_2$ extraction process. This will enable a rational choice based on the suitability and sustainability of a method to produce galanthamine.

Comparison of technologies based on costs is the normal approach for the economic assessment of processes. There are capital costs associated with the purchase of specific equipment for high pressurised process such as sc$CO_2$ extraction, and there are operational costs. Increasing energy costs and regulations to replace existing production processes with environmentally more benign processes (e.g. reducing waste generation, solvent emission, and energy consumption) are the driving forces behind the

development of novel extraction processes. When comparing mature-conventional processes with novel emerging technologies, like $scCO_2$ extraction, there are several uncertainties, like low accuracy of estimated costs, and lack of the optimal performance data for the emerging technologies.

Curzons *et al.* (2001) proposed relatively simple parameters for assessing the greenness of the process, such as total solvent consumption per mass of product, number of solvents used, and energy for solvent recovery (MJ/kg).

**Table 1.** Some published reports of medium-scale galanthamine production

| Raw materials | Processed materials | Pre-treatment Method | Extraction Method | Purification Method | Yield | Ref. |
|---|---|---|---|---|---|---|
| Bulbs of *Narcissus pseudonarcissus* cv Carlton[α] | 100 kg | mixed with $Na_2CO_3$ (4%, w/w) | Organic extraction (special boiling point gasoline 80/100) | ○ Acid-base purification step using diethyl ether ($Et_2O$)<br>○ Crystallisation using iso-propanol | 0.1 g/kg[ϕ] | (Hille *et al.*, 2002) |
| Leaves of *Ungernia victoris* Vved[α] | 80 kg | None | Organic extraction ($H_2O$: EtOH 80% = 1:4.2, v/v) | ○ Acid-base purification step using chloroform ($CHCl_3$)<br>○ Crystallisation using ethanol (EtOH) | 0.5 g/kg[ϕ] | (Sagdullaev, 2005) |
| Bulbs of *Narcissus pseudonarcissus* cv Carlton[α, β] | 75 kg | None | Acidified water ($H_3PO_4$ 0.1%, w/w) | ○ Resin column (non-ionic SP-825L)<br>○ Resin column (cation exchange SK 104)<br>○ Methyl isobutyl ketone (MIK) extraction after basification<br>○ Column chromatography with basic alumina and MIK as mobile phase<br>○ Crystallisation using MIK | 1.0 g/kg[ϕ] | (Cvak *et al.*, 2008) |
| Dried leaves of *Leucojum aestivum* L[α, γ] | 40 kg | None | Acidified water ($H_3PO_4$ 0.1%, w/w) | ○ Resin column (non-ionic SP-825L)<br>○ Methyl isobutyl ketone (MIK) extraction after basification<br>○ Column chromatography with basic alumina and MIK as mobile phase<br>○ Crystallisation using tert-butyl methyl ether | 2.1 g/kg[ϕ] | (Cvak *et al.*, 2008) |

[α] Dried raw materials.
[β] Bulbs of *Narcissus pseudonarcissus* cv Carlton contained 0.12%-DW of galanthamine (Cvak *et al.*, 2008).
[γ] Dried leaves of *Leucojum aestivum* L. contained 0.26%-DW of galanthamine (Cvak *et al.*, 2008).
[ϕ] The yield was calculated by the obtained galanthamine in the end of the process (after crystallisation process) per dry weight of the used raw material (g/kg).

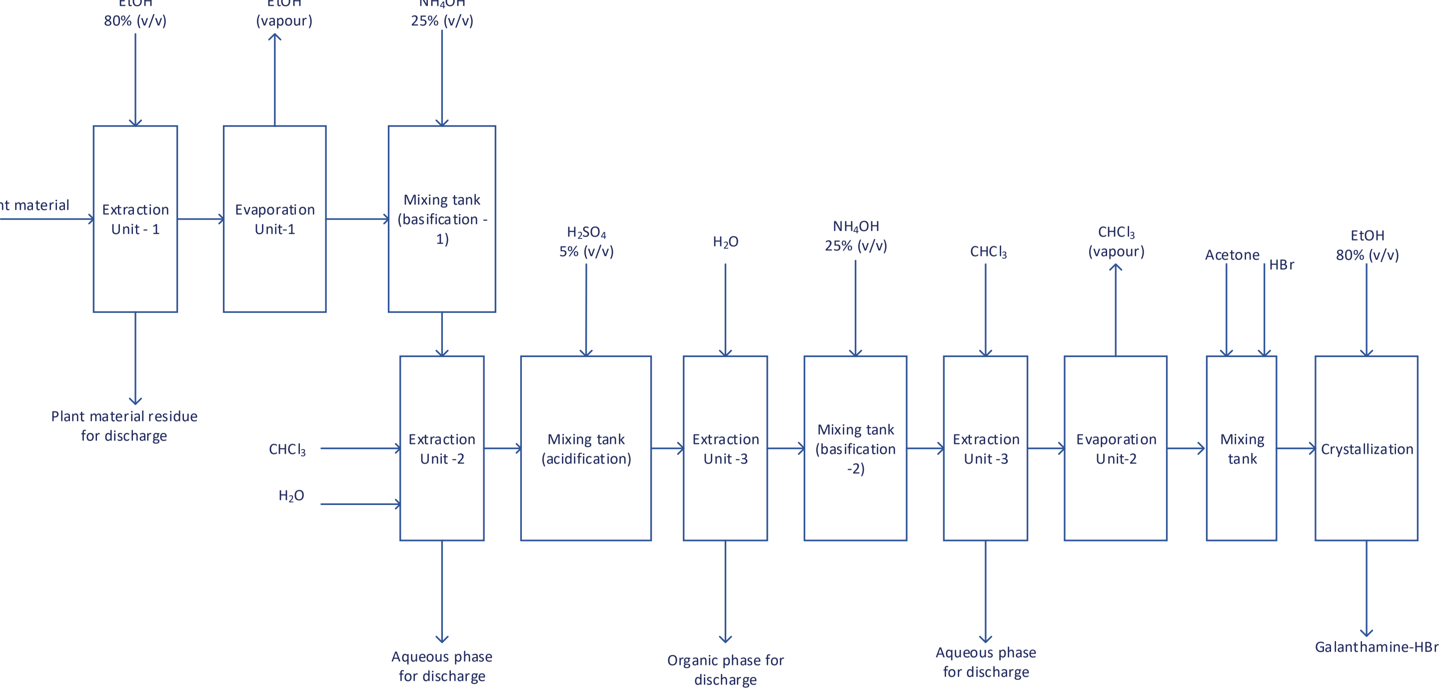



**Figure 2**. Block diagram of galanthamine production from Leaves of *Ungernia victoris* Vved adopted from Sagdullaev (2005).

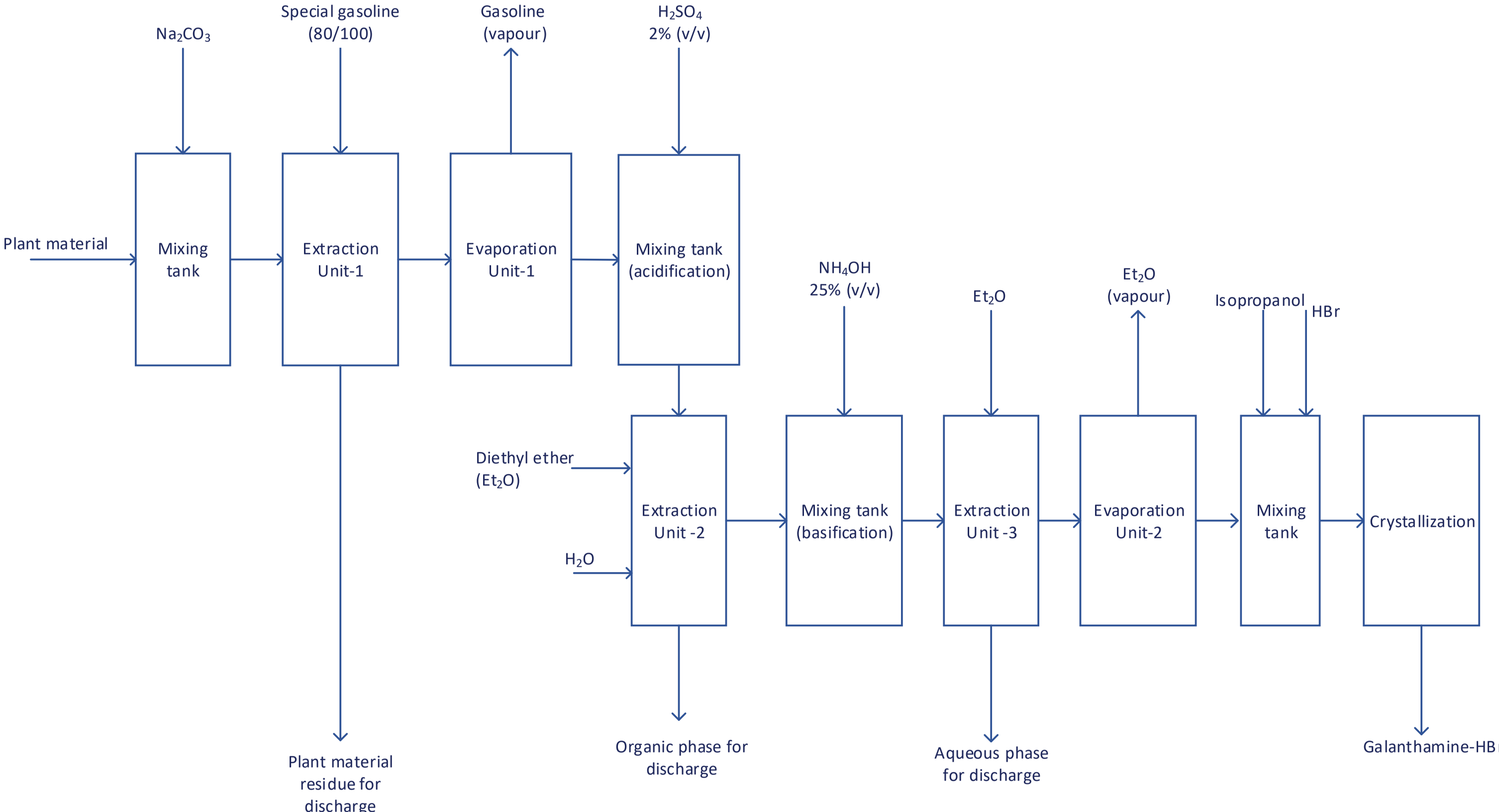



**Figure 3**. Schematic diagram of galanthamine production from Bulbs of *Narcissus pseudonarcissus* adopted from US 2002/0028802 A1 patent application (Hille *et al.*, 2002).

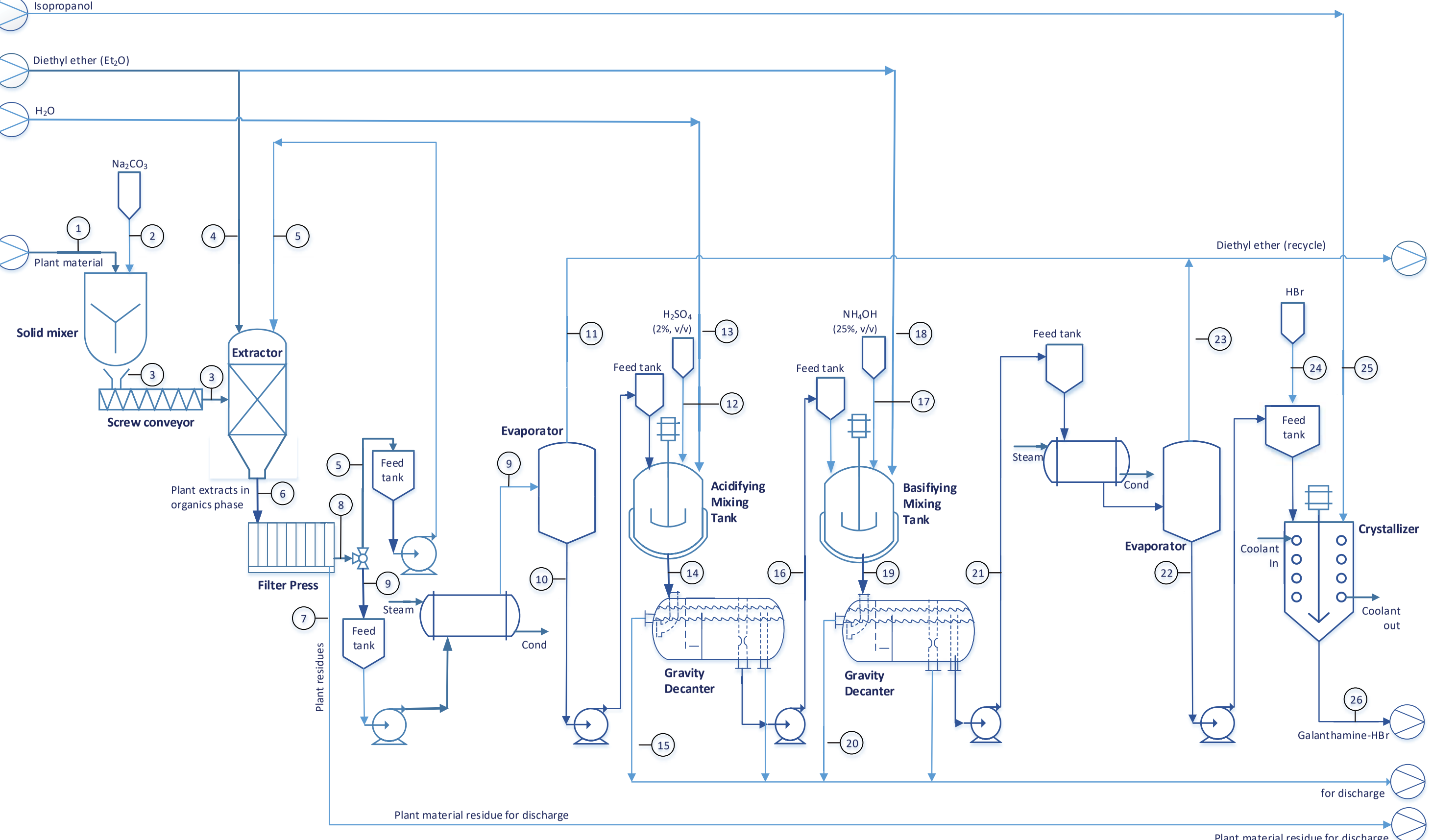



**Figure 4**. Pictorial diagram of galanthamine extraction from its biological matrix using conventional method (the drawing was made based on the process by Hille *et al*. (2002). The main streams with indicated numbers are listed in the appendix.

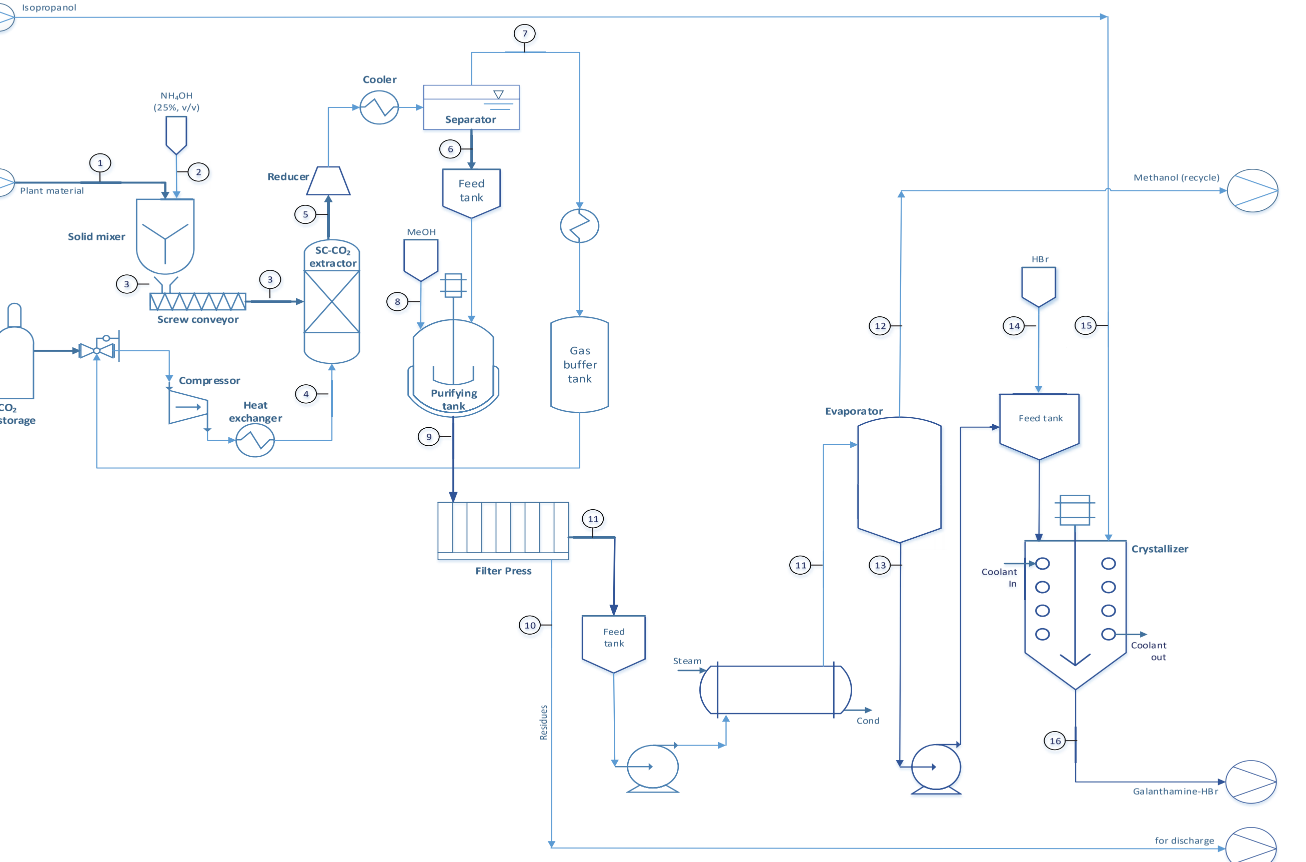



**Figure 5**. Flow sheet of galanthamine extraction from its biological matrix using $scCO_2$ extraction method (the drawing was made based on the proposed method described in **Chapter 3**). The main streams with indicated numbers are listed in the appendix.

## 6 Economic evaluation

### 6.1 Mass balance and the used data for calculation

Mass balance calculation was performed; covering the initial stage of the pre-treatment step until the final stage-crystallization step as the process production of the system. The raw material which was used in the calculation is dried bulbs of *Narcissus pseudonarcissus* cv Carlton containing 3 mg/g of galanthamine (Rachmaniah *et al.*, 2014). The drying process as well as their cultivation process were not included both in the scope of mass balance and economic calculation. Mass balance was calculated assuming 25 working days per month, giving 300 of working days per year. Thus, with an assumed annual production capacity of 36 tonne of galanthamine-HBr (see **Section 2**), approximately 120 kg of galanthamine-HBr will be produced per day.

In addition, data used for the calculations are obtained either from experiments or references. Primary data were obtained from the experiment results of this thesis, i.e. pre-treatment conditions of plant material, parameters of the extraction process, yield of alkaloid in the extraction process, and the selectivity for alkaloids. All these paramaters are needed for calculating a mass balance of the sc$CO_2$ extraction process (**Chapter 3**). Other parameters were obtained from literature, we consider these as secondary data, which were mostly needed for calculating the mass balance of the conventional process and making its economic evaluation.

A mass balance of the conventional extraction process was calculated based on the protocols of Hille *et al.* (2002), using diethyl ether ($Et_2O$) as a solvent. Data which was not provided in Hille *et al.* (2002) were obtained from other literature sources. Due to the lack of reliable extraction yield data, the yield of an experimental exhaustive dichloromethane-soxhlet extraction of basified plant material was used for calculating the mass balance of the conventional process.

The physical properties of chemicals were obtained from the Sigma-Aldrich catalogue (Zwijndrecht, The Netherlands), the chemical properties came from www.chemspider.com. The price of chemicals and equipment were obtained from the global trade platform (www.alibaba.com), while $CO_2$ price was provided by Linde-Gas-Benelux (Schiedam, The Netherlands). The chemical prices were for a pharmaceutical grade quality for industrial uses. The price of fresh bulbs of *N. pseudonarcissus* cv. Carlton in bulk quantity was from a quote from Holland Biodiversity B.V. (Lisse, The Netherlands), assuming, that the dried-powder of bulbs has a price three times higher than fresh bulbs. Details on both of experimental data and assumptions which have been applied for the mass balance calculations are shown in **Table 2**.

**Table 2**. Assumptions used in mass balance calculations

| | A – conventional process | | B – $scCO_2$ process extraction |
|---|---|---|---|
| **Pre-treatment step** | $Na_2CO_3$ (4%, w/w) was used for pre-treatment (Hille *et al.*, 2002). | **Pre-treatment step** | $NH_4OH$ 10% (w/w) was used for pre-treatment (**Chap. 3**). |
| **Extraction step** | - Ratio of plant material to solvent used was based on Hille *et al.* (2002) as well as the amount of diethyl ether (Et2O) needs for liquid-liquid extraction (LLE).<br>- While, Alkaloids selectivity was based on the dichloromethane-Soxhlet extraction results. | **Extraction step** | - Extraction parameters based on experiment results (**Chap. 3**), such alkaloids selectivity, $CO_2$ flow rate, $CO_2$ density (at 70 °C, 220 bar).<br><br>#plant materials residue was discharged (bio-material waste) |
| **Purification step** – ***Filter press*** | - No solid plant particle dissolved in filtrate, thus all $Na_2CO_3$ and solids will discharge as cake<br>- Only 5% filtrate was carried over in the cake<br>- 10% (w/w) of is recycled into the extraction vessel (stream no. 5)<br><br>#Cake consisted of plant materials residue and impurities was discharged (bio-material waste) | **Purification step** – ***Condenser*** | - All the extracted alkaloids including the impurities were condensed and flowed into purifying tank (stream no. 6) |
| ***Evaporator-1*** | Evaporated 60% of diethyl ether ($Et_2O$) | ***Purifying tank*** | - It was assumed that alkaloid concentration was 40% (w/w) in methanol solution. |
| ***Acidifying mixing tank*** | - Density of concentrated alkaloid solution (stream no.10) was assumed has similar density of $Et_2O$ (0.714 kg/L).<br>- pH of solution was adjusted into approximately pH of 4 with an $H_2SO_4$ (Hille *et al.*, 2002).<br>- Fresh $H_2O$ (125% (v/v) of treated volume) was added, obtaining immiscible layers. | ***Filter press*** | - It was assumed that all impurities were discharged as a cake<br>- Only 5% filtrate was carried over in the cake |
| ***centrifugal separation-1*** | - 5% (w/w) of alkaloids in heavy phase (water layer) was carried over in light phase ($Et_2O$ layer) | ***Evaporator*** | Evaporated 60% of methanol |

| | | | |
|---|---|---|---|
| | - While only 5% (w/w) of $Et_2O$ was carried over in heavy phase | | |
| ***Basifying mixing tank*** | - Density of solution (stream no.16) was assumed to have similar density as $H_2O$.<br>- pH of solution (stream 16) was adjusted with $NH_4OH$ to about pH of 9 (Hille *et al.*, 2002).<br>- Fresh $Et_2O$ (125% (v/v) of treated volume) was added, obtaining immiscible layers. | | |
| ***Centrifugal separation-2*** | - 5% (w/w) of alkaloids in light phase ($Et_2O$ layer) was carried over in heavy phase ($H_2O$ layer)<br>- While only 5% (w/w) of $Et_2O$ was carried over in heavy phase | | |
| ***Evaporator-2*** | Evaporated 90% of diethyl ether ($Et_2O$) | | |
| **Crystallization step** | - Crystallization was conducted at pH 2-4, which was adjusted with HBr (Lahiri *et al.*, 2006).<br>- Galanthamine was assumed to crystallise from isopropanol (55%, v/v); Sagdullaev (2005) crystallised galanthamine from ethanol (55%, v/v).<br>- The solubility of free-base galanthamine in isopropanol was assumed to be the same as its solubility in acetone ca. 0.632 g galanthamine/g acetone (Sagdullaev, 2005). | **Crystallization step** | - Crystallization was conducted at pH 2-4, which was adjusted with HBr (Lahiri *et al.*, 2006).<br>- Galanthamine was assumed to crystallise from isopropanol (55%, v/v); Sagdullaev (2005) crystallised galanthamine from ethanol (55%, v/v).<br>- The solubility of free-base galanthamine in isopropanol was assumed to be the same as its solubility in acetone ca. 0.632 g galanthamine/g acetone (Sagdullaev, 2005). |

## 6.2 Raw material costs

Raw material costs (**Table 3**) were calculated using the mass balance of the two proposed process (**Fig. 4** and **5**, respectively, for a conventional and an alternative process). Simplifying the calculation, 1000 kg of galanthamine-HBr production was used as basis of calculation.

**Table 3.** Price estimation of material used for producing 1000 kg Galanthamine-HBr (Gal-HBr).

| **Material components (*stream number*)[α]** | **Price** | **Mass needed with different production process[β] (kg)** | | **Total price (€)** | |
|---|---|---|---|---|---|
| | €/kg | A | B | A | B |
| Powder of *N.pseudonarcissus* bulbs (*1*)[A, B] | 4.71 | 912,500 | 1,353,450 | 4,297,875 | 6,374,750 |
| $Na_2CO_3$ (*2*)[A] | 0.15 | 36,500 | 0 | 5,649 | 0 |
| Diethyl ether (*4 + 18*)[A] | 2.00 | 1,493,092 | 0 | 2,986,184 | 0 |
| Isopropanol (*25*)[A] / (*15*)[B] | 1.31 | 1,791 | 1,234 | 2,345 | 1,616 |
| HBr (*24*)[A] / (*14*)[B] | 2.38 | 450 | 220 | 1,071 | 524 |
| $H_2SO_4$ (95%, v/v) (*12*)[A] | 0.18 | 5 | 0 | 1 | 0 |
| $NH_4OH$ (28%, v/v) (17)[A] / (2)[B] | 0.20 | 6 | 135,345 | 1 | 26,854 |
| $CO_2$ (*4*)[B] | 0.11 | 0 | 56,037 | 0 | 6,164 |
| MeOH (*8*)[B] | 0.35 | 0 | 2,550 | 0 | 880 |
| **Total chemicals costs (€/1000 kg Gal-HBr)** | | | | 7,293,124 | 6,410,788 |
| **Total chemicals costs (€/kg Gal-HBr)** | | | | **7,293** | **6,411** |

[α] Stream number on pictorial diagrams (Fig. 4 and 5, respectively, for conventional and alternative process).
[β] A – Conventional process followed the extraction protocols of Hille *et al.* (2002); B – Alternative process (proposed $scCO_2$ extraction method in Chapter 3).

Costs of chemicals for the conventional extraction process are about 1.2 times higher than the alternative process via $scCO_2$ extraction (**Table 3**). This is due to the high consumption of $Et_2O$ as an extraction solvent, which stands for about 41% of the total costs of chemicals. This amount is needed both to extract and to do the LLE separation, but also to solve the problem of the formation of emulsions in the LLE process. Therefore, a recycle stream for $Et_2O$ needs to be considered in the process flow which is important for the economic evaluation. Recycling 50% of $Et_2O$, will reduce the total costs of chemicals for the conventional process to € 5,800/kg Gal-HBr; thus a 21% decrease.

In case of chloroform, the most common solvent for separating alkaloids as immiscible VOCs instead of $Et_2O$, the costs of the solvent will be decreasing to about 30%, giving a total costst of chemicals of € 5,136/kg Gal-HBr. However, considering its risks properties such carcinogenic, and its classification as a Hazardous Air Pollutants (HAPs), the use in pharmaceutical production is minimised.

Dichloromethane (DCM) as a replacement solvent was proposed. It is the best choice as an alternative solvent of the four chlorinated solvents, chloroform, dichloroethane, and carbon tetrachloride (Alfonsi *et al.*, 2008). The costs of chemicals were estimated to be € 1,128,778 when DCM is used as a solvent. Combining the use of DCM as solvent and recycling 50% of DCM in the process, a total costs of chemicals of € 4,871/kg Gal-HBr was calculated. Thus, about 33% cheaper than using $Et_2O$ and also cheaper than the alternative $scCO_2$ process. However, $scCO_2$ extraction gave a cleaner product free from solvent residues, due to minimal use of VOC and $CO_2$ is easily to evaporate at ambient temperature and pressure. Leaving an extract-product without any trace of $CO_2$-solvent. Therefore, other parameters to assess the greenness of the processes will be discussed.

Alfonsi *et al.* (2008) also proposed ethyl acetate (EtOAc) as a replacement for DCM in the extraction process. However, no alkaloids were detected when the LLE process was performed using EtOAc as a solvent. Details of the total costs of chemicals for various VOCs are shown in **Table 4**.

**Table 4.** Estimation of the total costs of chemicals for various Volatile Organic Solvents (VOCs)$^{\alpha}$.

| Solvent (kg)$^{\beta}$ | Price (€/kg) | Mass (kg) | Price (€) | Total chemicals costs (€/kg Gal-HBr)$^{\gamma}$ |
|---|---|---|---|---|
| Diethyl ether ($Et_2O$) | 2.00 | 1,493,092 | 2,986,184 | 7,293 |
| Diethyl ether ($Et_2O$) + 50% of recycle | 2.00 | 746,546 | 1,493,092 | 5,800 |
| Chloroform (CHL) | 0.56 | 1,493,092 | 829,496 | 5,136 |
| Dichloromethane (DCM) | 0.76 | 1,493,092 | 1,128,778 | 5,435 |
| Dichloromethane (DCM) + 50% of recycle | 0.76 | 746,546 | 746,546 | 4,871 |

$^{\alpha}$ The estimation was calculated based on the production process of 1000 kg of Galanthamine-HBr (Gal-HBr) with a conventional process following the extraction protocols of Hille *et al.* (2002).

$^{\beta}$ The total mass of solvent needed for streams no 4 and 18 (see **Fig. 4).**

$^{\gamma}$ Price of other chemicals referred to **Table 3.**

The total yield of the process, i.e. the amount of Gal-HBr after crystallization per kg dry-weight of plant material (kg/kg), is 0.11% (1.1 g/kg) and 0.07% (0.7 g/kg) respectively for the conventional and alternative process. That means they are more or less similar, considering all uncertainties in our assumptions. Meanwhile, the theoretical yields are 37% and 25% respectively for the conventional and alternative process, assuming that bulbs contain 3 mg/g of galanthamine (Rachmaniah *et al.*, 2014). In the the conventional process the yield of an exhausted dichloromethane-Soxhlet extraction was used as benchmark, while in case of $scCO_2$. An extraction process a high yield is important, as it determines the cost of galanthamine per kg. (**Table 6**).

Both calculated yields were low, about 0.10%;, though they are higher than to the processes described by Hille *et al*. (2002) and Sagdullaev (2005), who reported a total yield of Gal-HBr of 0.1 g/kg and 0.5 g/kg of Gal-HBr respectively.. A possible explanation could be in lower levels of galanthamine in the plant material used.

## 6.3 Capital costs estimation

Capital costs were estimated using the factorial method proposed by Lang (1948) (Sinnott, 2005). This estimation method is based on the purchase costs of the major equipment required for the process, while the other costs are estimated as specific factors for each of the various types of equipment.

The Lang's approach is simple, using factors that varies only with the type of process such as for predominantly solids, predominantly fluids, or for a mixed fluids-solids processing plant (Sinnott, 2005). The fixed capital costs of the project are estimated by a function of the total purchase of major equipment costs by the equation (Sinnott, 2005):

$$C_f = f_L C_e$$

Where $C_f$ = fixed capital costs

$C_e$ = the total delivered costs of all major equipment

$f_L$ = the 'Lang factor' (which depends on the type of process)

As both the conventional and $scCO_2$ process discussed in this chapter involve a mixed process of fluids-solids, a Lang factor 3.6 is applied.

This method roughly estimates the capital costs (accuracy with a typically ±30% margin of error). It gives an approximate order of magnitude for the purpose of comparing different processes (Rostagno and Prado, 2013) when 'go' or 'not go' decisions are involved. Thus, it can be used in an initial feasibility study.

To make a more accurate estimation of capital costs, the direct and indirect-costs items are added. In case of a mixed fluids-solids processing plant the typical factors for the components of capital costs are (Sinnott, 2005):

| Major equipment, total purchase costs (PCE) | PCE/ $C_f$ |
|---|---|
| **1. Direct-costs items** | |
| $f_1$ equipment erection | 0.45 |
| $f_2$ piping | 0.45 |
| $f_3$ instrumentation | 0.15 |
| $f_4$ electrical | 0.10 |
| $f_5$ buildings, process | 0.10 |
| $f_6$ utilities | 0.45 |

$f_7$ storage 0.20

$f_8$ site development 0.05

$f_9$ ancillary buildings 0.20

Total Lang factors of direct-costs 3.15

Total physical plant costs (PPC), PPC = PCE (1 + $f_1$ + … + $f_9$ ) = PCE x 3.15

**2. Indirect-costs items**

$f_{10}$ design and engineering 0.25

$f_{11}$ contractor's fee 0.05

$f_{12}$ contingency 0.10

Total Lang factors of indirect-costs 1.40

Fixed capital costs = PPC (1 + $f_{10}$ + $f_{11}$ + $f_{12}$ ) = PPC x 1.40

Thus, a quick estimation of capital costs can be made in the early stage of the project design, when the preliminary flow-sheets have been drawn and the size of the major equipment is set.

Adopting the Free On Board (FOB) of equipment price from www.alibaba.com, a global e-commerce trading platform, the estimated capital costs using the Lang's approach are displayed in **Table 5**.

**Table 5**. Summary of capital costs estimation applying the Lang factor for a 22-tonne annual production capacity of Gal-HBr (approximately 74 kg/day)

| | **Conventional** | **Alternative $scCO_2$** |
|---|---|---|
| Tot. equipment prices for specified Gal-HBr capacity ($C_E$) | € 290,393 | € 264,552 |
| PCE/$C_f$ ($C_f = F_L C_e$) | € 1,045,415 | € 952,386 |
| PPC (Lang factor calculations) = PCE x 3.15 | € 3,293,057 | € 3,000,015 |
| Fixed capital costs (Lang factor calculations) = PPE x 1.40 | € 4,610,279 | € 4,200,021 |

The capital costs of the extraction process following the protocols of Hille *et al.* (2002) would be € 4,610,279 (5 M€), and for the alternative $scCO_2$ process € 4,200,021 (4.2 M€). In the conventional process a vessel-mixer for LLE separation (ca. 35%), and an evaporator to deal with the huge amount of $Et_2O$ (ca. 61%) are the major equipment costs. While in the $scCO_2$ extraction method, 91% of equipment costs were for high-pressure equipment of $scCO_2$ extraction.

### 6.4 Production costs estimation

To estimate the production costs, the data inTable 6.6, page 267, in Sinnott (2005) were used. The fixed capital costs were estimated in **section 6.3**.

The following assumptions were made for estimating the production costs (Sinnott, 2005):

- The miscellaneous materials costs were 10% of maintenance costs

- The maintenance costs were 5% of fixed costs
- Operating labour costs were 15% of operating costs
- Plant overheads costs were 50% of operating labour costs
- Laboratory costs were 30% of operating labour costs
- Capital charge costs were 6% of capital costs
- Insurance costs were 1% of capital costs as well as royalties costs, and
- Local taxes was 2% of capital costs

Hence, by applying these assumptions, production costs (€/kg of Gal-HBr) for both conventional and alternative process was obtained (**Table 6**).

**Table 6**. Summary of production costs with 22 tonne annual capacity of Gal-HBr (approximately 74 kg/day).

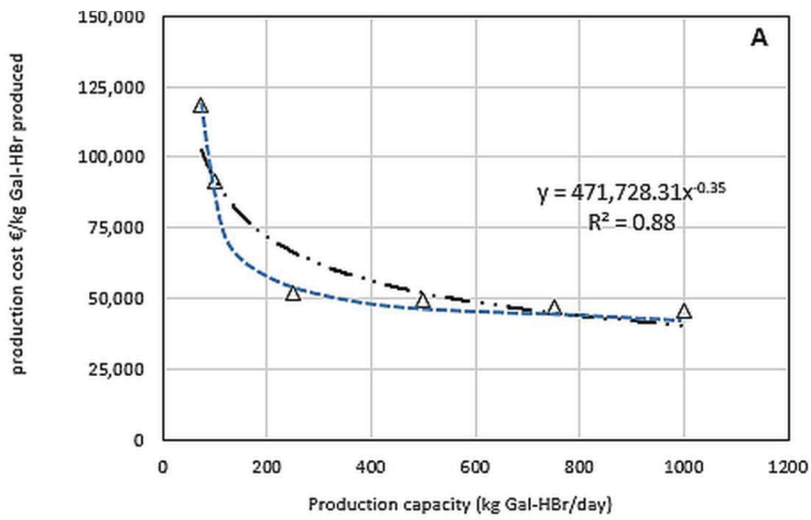
| Costs items* | Production Process | |
|---|---|---|
| | Conventional | Alternative $scCO_2$ |
| ***Fixed Capital Costs*** **(A)** | € 4,610,279 | € 4,200.021 |
| **SUB TOTAL A** | **€ 4,610,279** | **€ 4,200,021** |
| ***Variable costs*** **(B)** | | |
| 1. Raw plant & chemical materials | € 1,085,595 | € 474,847 |
| 2. Miscellaneous materials | € 23,051 | € 21,000 |
| 3. Shipping and packaging (negligible) | 0 | 0 |
| **SUB TOTAL B** | **€ 1,108,647** | **€ 495,847** |
| ***Fixed costs*** **(C)** | | |
| 4. Maintenance | € 230,514 | € 210,001 |
| 5. Operating labour (15% of item D) | 15%D | 15%D |
| 6. Laboratory costs (30% of item 5) | 4.5%D | 4.5%D |
| 7. Plant overheads (50% of item 6) | 7.5%D | 7.5%D |
| 8. Capital charges (6% of item A) | € 276,617 | € 252,001 |
| 9. Insurance (1% of item A) | € 46,103 | € 42,000 |
| 10. Local taxes (2% of item A) | € 92,206 | € 84,000 |
| 11. Royalties (1% of item A) | € 46,103 | € 42,000 |
| **SUB TOTAL C** | **€ 691,542 + 27%D** | **€ 630,003 + 27%D** |
| ***Direct production costs*** **(B + C)** | € 1,800,189 **+ 27%D** | € 1,125,850 **+ 27%D** |
| ***Operating costs*** **(A + B + C = D)** | € 6,410,468 **+ 27%D** | € 5,325,872 **+ 27%D** |
| ***Operating costs*** **(D)** | € 8,781,463 | € 7,295,714 |
| Plant capacity of Gal-HBr (kg/day) | **74.07** | **74.07** |
| **Production costs €/kg** | € 118,556 | € 98,498 |

*Assumptions for the calculations were adapted from Table 6.6. (Page 267, Sinnott, 2005).

By applying a 36-tonne annual capacity, both processes did not give a competitive price of galanthamine-HBr compared to the existing price in the commercial market. A conventional process results in higher production costs of galanthamine-HBr, mainly due to solvent costs in the variable costs items; while

equipment costs, fixed capital costs, are the major items influencing the production costs in the alternative process (**Table 6**).

Currently, the price of galanthamine-HBr produced from *Leucojum aestivum* is 20,000 €/kg (Codina, 2011). This indicates that our proposed method for 36 tonne annual production does not meet the expectation of providing a lower price for galanthamine-HBr. Therefore, a simulation of production capacity was performed in order to find the minimal production capacity; producing a lower costs (€/kg) of galanthamine-HBr than the existing market price.



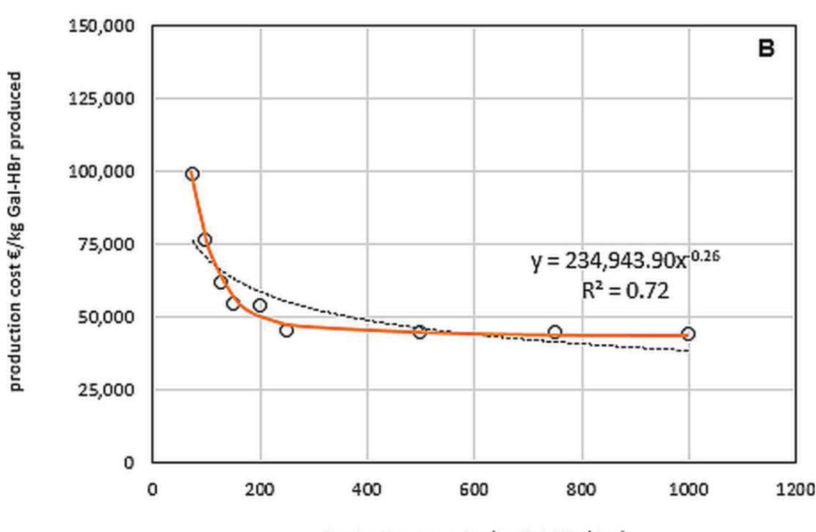



**Figure 6**. The estimated production costs of galanthamine-HBr (€/kg) at different production capacity levels. (**A**) a conventional production process following protocols of Hille *et al.* (2002), and (**B**) an alternative $scCO_2$ process.

By varying the production capacity, the variable costs (i.e. raw plant materials, chemicals, as well as miscellaneous materials) and fixed capital costs for the equipment will change, and thus change the production costs. The existing market price for natural galanthamine-HBr (ca. 20,000 €/kg) can be achieved at 2,500 and 3,900 tonne production capacity, respectively, for conventional and $scCO_2$ process (**Fig. 6**). Meaning, a plant should be operated at that minimum capacity of process in order to reach the existing price; entering the supply chain of galanthamine-HBr.

Codina (2011) claimed that the combination of *in vitro* production with an airlift-bioreactor for large scale cultivation of *Narcissus confuses*, can produce galanthamine-HBr at a price of ca. 12,000 €/kg. However, their extraction and purification protocols were not revealed. To produce for this price with our $scCO_2$ process, the productions should be 11,000 tonnes annually, whereas the conventional process would reach this goal at an annual production of 28,000 €/kg.

Entering the existing supply chain of galanthamine-HBr market with this strategy is not interesting due to the low price of commercial galanthamine-HBr synthetic. Therefore, a new strategy of developing process for the production of high quality galanthamine-HBr at lower price is necessary. Different approaches can be thought of instead of using the $scCO_2$ process as proposed (**Fig. 5**), coupling the $scCO_2$ extraction with another method of purification or fractionation as well as producing multi-products from the same biomass, such as *O*-methyloduline, norgalanthamine, oduline, haemanthamine, and *O*-methyllycorenine is preferred. Thus maximising the potential of other N*. pseudonarcissus* alkaloids. This seems to be the best way for entering the market of galanthamine-HBr.

### 6.5 Analysis of sensitivity

The effects of the existing costs such as fixed capital costs, variable costs (i.e. raw plant material and chemicals), and fixed costs on the production costs of galanthamine-HBr was studied further in more detail.

Fixed capital costs (see **section 6.4**) and the raw material costs were estimated by varying the production capacity (**Fig. 7**), while fixed costs will be more or less constant throughout capacity variations. Obviously raw material and chemicals costs increase as the production capacity increases. Higher production capacity will directly increase the consumption of bulbs material as well as other chemicals. Thus, the raw materials costs increases. In case of a conventional process, $Et_2O$ as the extraction solvent dominated the chemicals costs at 71%, followed with plant material at 29%. However, 99% of materials costs were

dominated by plant material in the $scCO_2$ process, due to a lower yield of galanthamine. Galanthamine yield was a 303.4 µg/g DW of bulbs (Rachmaniah *et al.*, 2014) in $scCO_2$ extraction compared with a 1.05 mg/g in the conventional extraction-using VOC-$Et_2O$ as a solvent (**Table 2**).

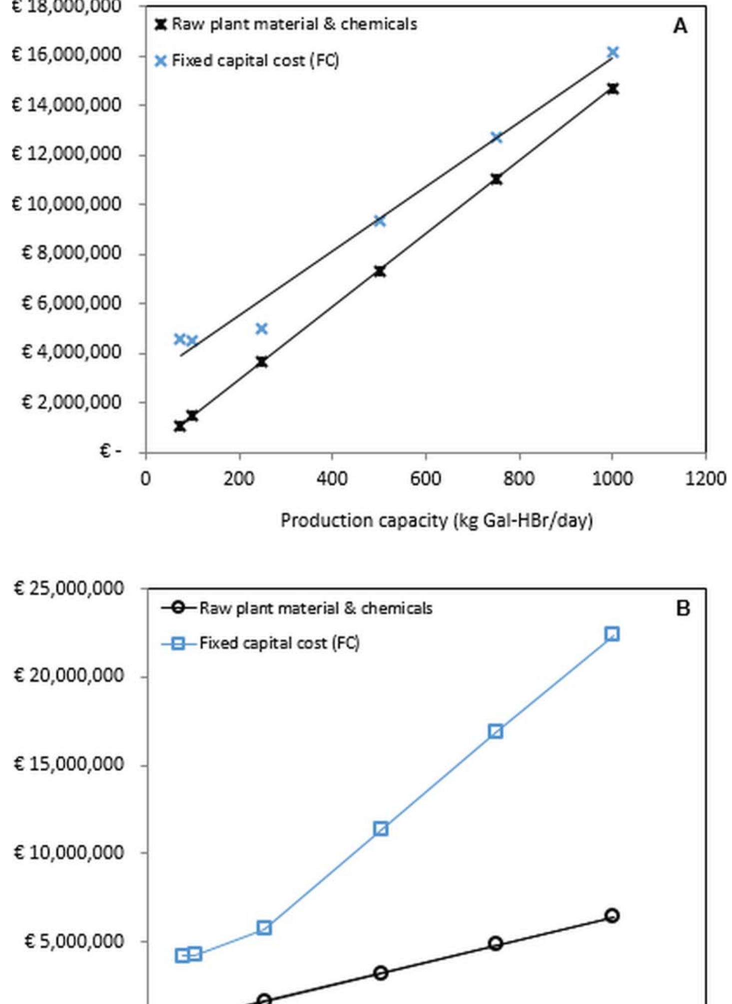



**Figure 7**. Raw material and fixed capital costs at different production capacity of galanthamine-HBr. (**A**) a conventional production process following protocols of Hille *et al.* (2002), and (**B**) an alternative $scCO_2$ process.

The trends as shown in **Fig.7**, make clear that jus upscaling is not the way to go. To reach to the current market price the scale needed is larger than the total

global market. So novel approaches should focus on higher levels of alkaloids in the plant material, higher efficiency of liberating alkaloids from the plant matrix, and coming to an integrated process with multiple products leading to cheaper and high purity galanthamine-HBr and some other alkaloids. This alternative strategy will be further discussed at **section 8**. But first also an assessment of the greenness will be made, as there might be quite some hidden environmental costs in the processes.

# 7 Assessment of the greenness

## 7.1 Waste production

Waste generation originates either from non-recycled solvent (initial assumption) as well as biomass waste. In case of the conventional process, principally, there are two generated wastes: non-recycled $Et_2O$ (from evaporation process) and plant material residues including the liquid waste of aqueous and organic phase of LLE.

None of the organic solvent, i.e. $Et_2O$, is recycled as described at the protocols of Hille *et al.* (2002). In addition, extra processes are necessary to remove the organic solvent from the plant matrix and liquid waste prior to discharge into the environment or re-use as compost. **Table 4** shows that recycling of 50% of solvent will significantly reduce the total chemicals costs. However, a mass balance calculation with recycle stream of solvent was not further performed, thus an initial assumption of the costs calculation (no recycled solvent) was used. Recycling the evaporated $Et_2O$ and MeOH (from evaporator) can be a way for minimising the generated of VOC waste as well as reducing the chemical expenses. However, a purge stream should be managed in order to avoid an accumulation of impurities which might otherwise build up in the recirculation system.

**Table 7**. The estimation of waste generation for producing 1000 kg galanthamine-HBr (Gal-HBr).

| Generated waste (streams number)$^{\alpha}$ | Mass for different production process$^{\beta}$ (kg) | |
|---|---|---|
| | **A** | **B** |
| Plant material residue (*7*)$^{A}$ / (*5*)$^{B}$ | 1,038,408 | 1,487,454 |
| $Et_2O$ (from evaporation) (*11 + 23*)$^{A}$ | 962,291 | 0 |
| Liquid wastes (from LLE) (*15 + 20*)$^{A}$ | 777,273 | 0 |
| $CO_2$ (gas) (*7*)$^{B}$ | 0 | 56,037 |
| MeOH (from evaporation) (*12*)$^{B}$ | 0 | 2,180 |
| **Total generated waste (kg/1000 kg Gal-HBr)** | 2,777,972 | 1,545,671 |
| **Total generated waste (kg/kg Gal-HBr)** | **2,778** | **1,546** |

$^{\alpha}$ Stream number on pictorial diagrams (**Fig. 4** and 5, respectively, for conventional and alternative process).

$^{\beta}$ A – Conventional process following Hille *et al.* (2002); B – Alternative process (proposed $scCO_2$ extraction method in Chapter 3).

While in the $scCO_2$ process, the plant material residue, $CO_2$, and MeOH (from evaporation process) are generated as wastes. An advantage of this alternative process using $scCO_2$ is the fact that $CO_2$ can be directly vented into surrounding without any fears for polluting the environment. This is also applied to the plant matrix residue, it is clean from any organic solvent residue and could be recycled as biomass, like for manure. However, due to the use of $NH_4OH$ for moistening the plant material in the pre-treatment step, it should be observed that there is no $NH_4OH$ left in the waste or at least has an allowable level of chemicals in the biomass waste. Clearly, the alternative process drastically reduces the generated waste (**Table 7**).

### 7.2 Energy consumption

In case of the conventional process, the main energy consumption is for the evaporation of organic solvents, i.e. $Et_2O$. In the alternative process energy for pressurising the $CO_2$ is the major energy requiring part of the process, also some energy energy goes to the evaporation of MeOH.

The amount of energy needed to evaporate a certain amount of organic solvent consist of the amount of energy to heat the solvent from room temperature (20 °C) to their boiling point ($T_b$) and the heat of evaporation. The calculation follows the equation below:

$$\Delta H = C_{p,l}\Delta T + \Delta_{vap}H$$

Where $\Delta H$ = amount of energy (kJ/kg)
$C_{p,l}$ = specific heat of liquid (kJ/kg.K)
$\Delta T$ = temperature difference (K)
$\Delta_{vap}H$ = enthalpy of evaporation (kJ/kg)

While the amount of energy to pressure $CO_2$ is estimated in the following way:

$$W = \frac{1}{\eta} x \int \frac{1}{\rho} dP \approx \frac{\Delta P}{\eta \rho_{av}}$$

Where W = work (J/kg)
η = pump efficiency
ρ = density (kg/m$^3$)
ΔP = pressure difference (Pa = J/m$^3$)

Thus, assuming a 75% of pump efficiency, an average density of $CO_2$ 350.93 kg/m$^3$ in the range of 6-22 MPa (at 343 K) which has 16 MPa of pressure difference. The amount of energy to pressurise $CO_2$ from 6 to 22 MPa and the calculated energy

consumption for evaporating solvents (based on 1000 kg production of galanthamine-HBr) are shown in Table **9**.

**Table 8**. Properties of chemicals to be evaporated.

| Process production | Chemical | $C_{p,l}$ (kJ/kg.K) | $T_b$ (K) | $\Delta_{vap} H$ (kJ/kg) | $\Delta H$ (kJ/kg) |
|---|---|---|---|---|---|
| Conventional | $Et_2O$ | 2.33 | 308 | 357.79 | 1,664.92 |
| $scCO_2$ | MeOH | 2.53 | 338 | 1,099 | 2,594.23 |

In terms of total energy consumption  the alternative $scCO_2$ process needs much less energy than the conventional process. Besides these major energy requiring steps, there is still some energy needed heating the extraction vessel (in case of $scCO_2$ process), and to crystallis the galanthamine at low temperature (-15 to 10 $^{o}$C). Due to lack of information, a more detailed and fair comparison is not possible at this stage.

**Table 9**. Estimated energy consumption for the evaluated processes for producing 1000 kg galanthamine-HBr (Gal-HBr).

| Process production | Energy for evaporating solvent ($\Delta H$, kJ) | Energy for pressurising $CO_2$ ($W$, kJ) | Total energy consume (kJ) |
|---|---|---|---|
| Conventional | 1,602,137,439 | - | 1,602,137,439 |
| $scCO_2$ | 5,655,975 | 3,406,530 | 9,062,505 |

By comparing the generated waste and the consumed energy between the two processes as well as the proposed parameters by Curzons *et al.* (2001) for assessing the greenness of the process (**Table 10**) it is clear that $scCO_2$ as alternative process for producing Gal-HBr comes out best. Though, more volume of solvent was used in the $scCO_2$ process, $CO_2$, MeOH, and isopropanol (IPA), compared to conventional, the amount of $scCO_2$ mass intensity (kg mass of solvent/kg mass of product) as well as the energy needed for the solvent recovery are much lower.

**Table 10.** Selected 'green' metrics$^{\alpha}$

| Green metrics term category$^{\beta}$ | Units | Process$^{\gamma}$ A | Process$^{\gamma}$ B |
|---|---|---|---|
| ***Mass*** | | | |
| $\frac{\text{Total mass (kg)}}{\text{Mass of product (kg)}}$ | kg/kg | 2,444 | 1,549 |
| $\frac{\text{Total mass solvent (gross)(kg)}}{\text{Mass of product (kg)}}$ | kg/kg | 1,495 | 60 |
| ***Energy*** | | | |
| $\frac{\text{Total solvent recovery energy (MJ)}}{\text{Mass of product (kg)}}$ | MJ/kg | 1,602 | 6 |
| ***Solvent*** | | | |
| Number of different process | - | 2 | 3 |

$^{\alpha}$ The parameters was selected based on Curzons *et al.* (2001).

[β] The term was calculated based on mass balance calculation to produce 1000 kg of galanthamine-HBr (**Table 4**).
[γ] A – Conventional process following the protocol of Hille *et al.* (2002); B – Alternative process (proposed $scCO_2$ extraction method, Chapter 3).

In addition, at low production capacity of galanthamine-HBr such 22 tonnes annually (74 kg/day), the energy required to recovery the solvent can be negligible. Due to a small flow rate of MeOH to be evaporated (ca. 161 kg/day) and a low energy needed to evaporate MeOH (ca. 2600 kJ/kg of MeOH). It can be compensated by a waste of heat generated by pump.

Conclusion of all these analyses is that, in general, the alternative-$scCO_2$ extraction process is better than the conventional process such as in term of total costs of chemicals and production costs at the same production capacity (**Table 10).** The alternative process is thus not only the cheapest, but also the greenest. It is thus the best candidate for developing an integrated multi-product process.

## 8 Integrated process – NADES extraction coupled with $scCO_2$ fractionation

From the NADES experiments we learned that these solvents are significantly increasing the extractability of alkaloids (Chapter 7). That means it could be a very useful first step at the $scCO_2$ suffers from a very low extractability. Therefore we propose nn integrated process of NADES extraction-$scCO_2$ fractionation with malic acid-sucrose (MAS = 1:1) as solvent using pressurised extraction in fixed bed-type of extractor (**Fig. 8**). This NADES gave highest yield of galanthaminein addition to lycoramine, norgalanthamine, narwedine, oduline, haemanthamine, and homolycorine (**Chapter 7**).

The NADES extract flow goes into a $scCO_2$ extractor, the pressure is adjusted according to the process conditions of the $scCO_2$ extraction (Chapter 3). The non-volatile and more polar MAS will form a two-phase system with the volatile and non-polar $scCO_2$.

In order to obtain a multi-products process a $scCO_2$ fractionation process is proposed as the last step in the process (**Fig. 8**). This maximises the benefits of supercritical fluid as well in yielding high purity galanthamine and some further pure alkaloids of *Narcissus*. This will make the production process of galanthamine-HBr more economically competitive.

Two methods are possible for fractionation in $scCO_2$ extraction (Smith *et al.*, 2013): (1) fractional extraction, the extraction pressure is increased in steps for given periods of time while separator pressure is held constant; (2) fractional separation, the extraction pressure is held constant but separator pressure is decreased in steps. Once the solutes have been solubilised in $scCO_2$, it is possible to fractionally separate them by reducing the pressure in stages or by stepwise changing other conditions such as temperature or flow rate.

The crystallisation process is the next unit in the purification process, producing high purity of alkaloids-salts (**Fig.8**).

**Table 11**. *Narcissus* alkaloids extracted by Malic acid-sucrose (MAS) of NADES (**Chapter 7**)

| Alkaloid Name | Structure[a] | Molecular weight[a] |
|---|---|---|
| ***Galanthamine Type*** | | |
| Galanthamine ($C_{17}H_{21}NO_3$)[a] | | 287 |
| Norgalanthamine ($C_{16}H_{19}NO_3$) | | 273 |
| Narwedine ($C_{17}H_{19}NO_3$) | | 285 |
| ***Homolycorine Type*** | | |
| Homolycorine ($C_{18}H_{21}NO_4$) | | 315 |
| Oduline ($C_{17}H_{19}NO_4$) | | 301 |
| ***Haemanthamine Type*** | | |
| Haemanthamine ($C_{17}H_{19}NO_4$) | | 301 |
| ***Lycoramine Type*** | | |
| Lycoramine ($C_{17}H_{23}NO_3$) | | 289 |

[a]Structure, molecular weight, as well as molecular formula adopted from Bastida and Viladomat (2002)

Due to a lack of knowledge of physical-chemical properties of *Narcissus* alkaloids such as boiling point, vapour pressure, melting point, solubility in $scCO_2$, as well as unavailability of experimental data, precise mass balance calculations of the process **Fig. 8** cannot be conducted. Hence, the separation in the separators were approached using the similarity of the chemical structures (**Table 11**), which can be classified as groups: Galanthamine, norgalanthamine and narwedine galanthamine-type of *Narcissus* alkaloids; homolycorine and oduline, are

homolycorine-type of *Narcissus* alkaloids; haemanthamine and homolycoramine are each a separate class. One may expect that the fractionation will separate at least at the level of these groups. Considering the retention time in gas chromatography, galanthamine type of alkaloids will be obtained as the first fraction, followed by homolycorine type of alkaloids in the second fraction, while the third and fourth fraction will be haemanthamine and homolycorine. But more data are needed to conduct the mass balance calculation for all products.

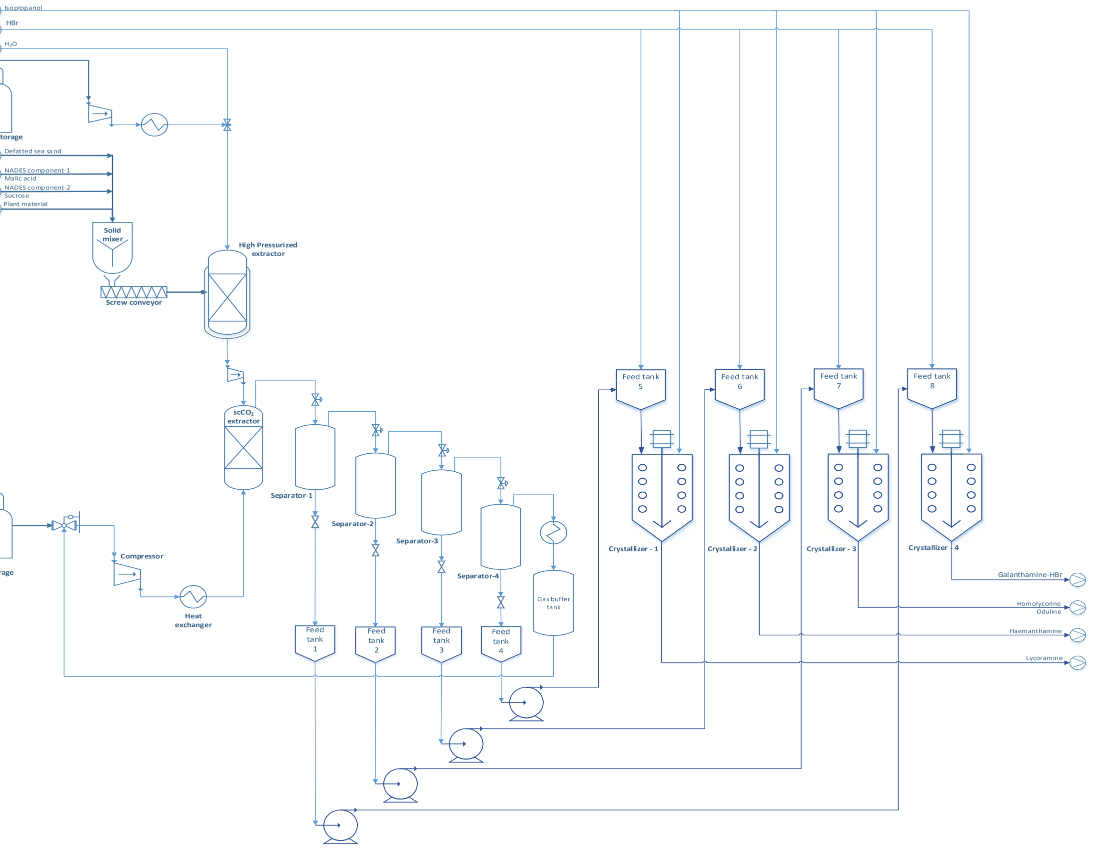



**Figure 8**. Proposed integrated process between NADES extraction with high pressurised system and $scCO_2$ extraction equipped with multiple separators.

## 9 Conclusion

The proposed $scCO_2$ extraction process (Chapter 3) presents many advantages compared to the conventional process following the protocols of Hille *et al.* (2002). The advantages concern both economical and environmental aspects, such as:

- The number of functional units in the process is reduced from 6 to 4, leading to a capital costs reduction.
- The costs of chemicals of the $scCO_2$ process is 57% lower than of the conventional process
- The wastes production is lower in the $scCO_2$ proces.
- The productioncosts of galanthamine-HBr at 36 tonnes annual capacity is 17% lower than the conventional process.

However, to maximize the potential of $scCO_2$ (high purity of product) as well as the greenness of the process, an integrated process with multi-end products should be applied. Compensating the high expenditure on the equipment costs. The development of an integrated process of NADES extraction coupled with $scCO_2$ fractionations iss proposed, in which the greenness is further improved and particularly the extractability of the alkaloids is expected to be improved.

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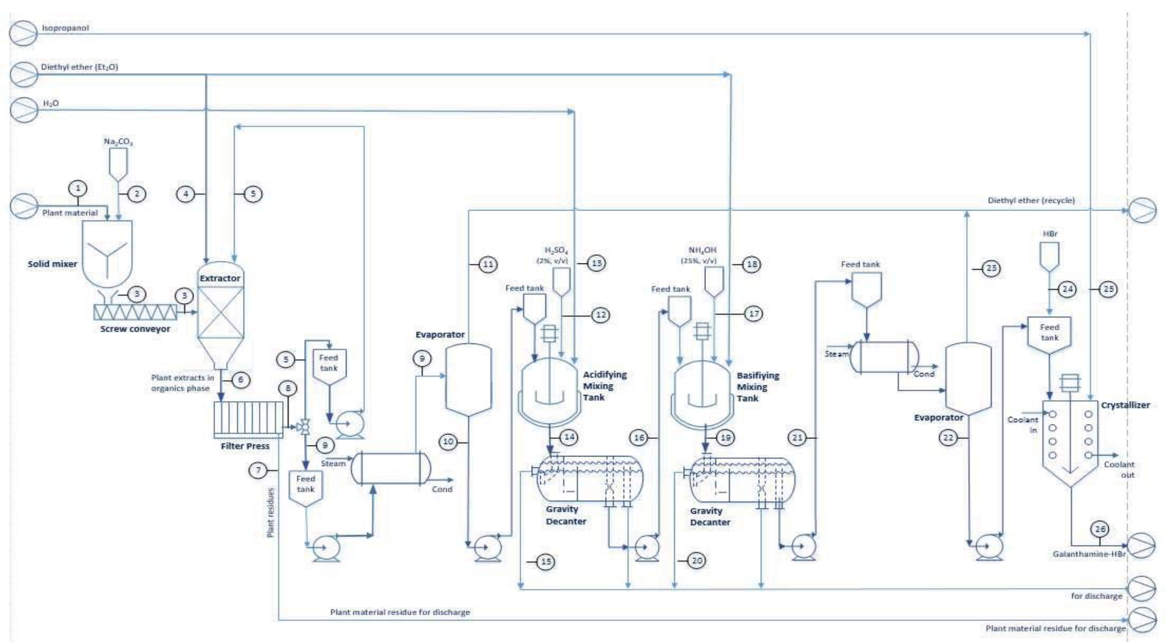
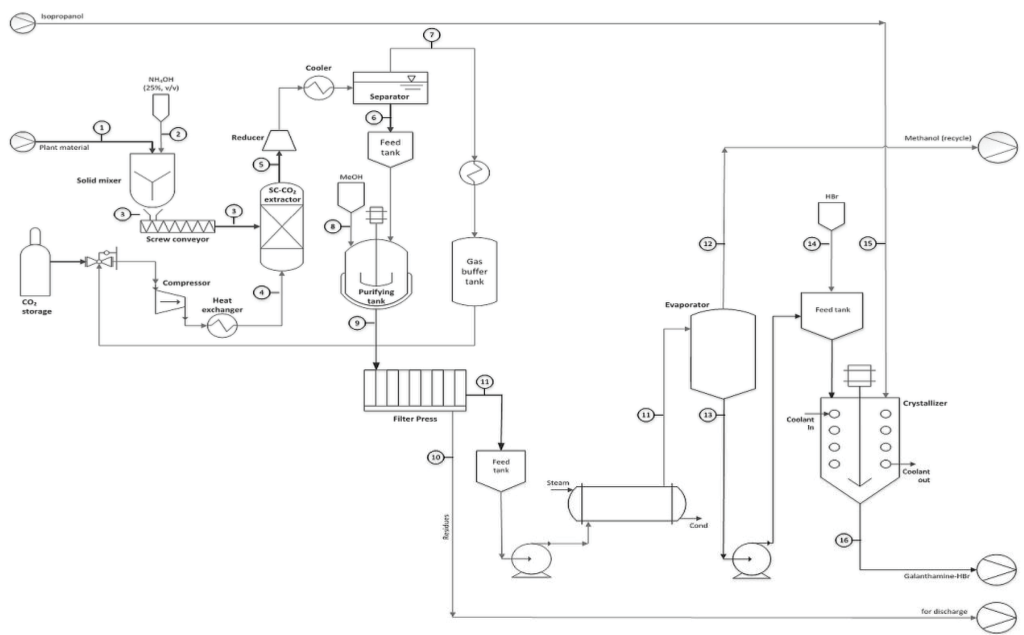
**Appendix:** Process flow diagrams and indicative main stream sizes and compositions for processes based on Hille (2002) , and the scCO2 process. Note: the streams are indicative only, as the mass balances are not complete .




Mass Balance for overall system (Conventional Process based on Hille et al. (2000))
Production capacity = 1000 kg Gal-HBr/day
Basis calculation: per day

| Components | Mass in (kg) | | | | | | | | | Mass out (kg) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Streams Number | plant (1) | $Na_2CO_3$ (2) | $Et_2O$ (4) | $Et_2O$ (18) | $H_2O$ (13) | $H_2SO_4$ (12) | $NH_4OH$ (17) | HBr (24) | i-propanol (25) | plant residue (7) | (15) | (20) | (23) | (11) | Gal-HBr (26) | mother liquor (27) |
| **plant (bulbs powder)** | 911.856 | | | | | | | | | | | | | | | |
| galanthamine | | | | | | | | | | | 43 | 41 | | | | |
| nor-gal | | | | | | | | | | | 15 | 14 | | | | 265 |
| Haemanthamine | | | | | | | | | | | 28 | 26 | | | | 497 |
| O-methyllycorenine | | | | | | | | | | | 5 | 5 | | | | 98 |
| **plant residue (cake)** | | | | | | | | | | 909.838 | | | | | | |
| **$Na_2CO_3$** | | 36.474 | | | | | | | | 36.474 | | | | | | |
| **$Et_2O$** | | | 878.938 | 613.100 | | | | | | 91.363 | 269.351 | 31.364 | 536.322 | 425.290 | | 59.591 |
| **$H_2O$** | | | | | 499.551 | 0,26 | 2 | | 555 | | 24.978 | 450.849 | | | | 24.284 |
| **$H_2SO_4$** | | | | | | 4,85 | 0 | | | | 0,24 | | | | | |
| **$NH_4OH$** | | | | | | | 3 | | | | | 1,65,E-05 | | | | |
| **$Na_2SO_4$** | | | | | | | | | | | | 5,89 | | | | 0 |
| **HBr** | | | | | | | | 230 | | | | | | | 220 | 10 |
| **Isopropanol** | | | | | | | | | 1.234 | | | | | | | 1.234 |
| **Gal-HBr** | | | | | | | | | | | | | | | 780 | |
| **Mother liquor** | | | | | | | | | | | | | | | | |
| **mass (kg)** | 911.856 | 36.474 | 878.938 | 613.100 | 499.551 | 5 | 6 | 230 | 1.789 | 1.037.675 | 294.420 | 482.305 | 536.322 | 425.290 | 1.000 | 85.979 |
| **mass overall (kg)** | **2.941.949** | | | | | | | | | **2.862.991** | | | | | | |




Mass Balance for overall system (sc$CO_2$ proposed Process)
Production capacity = 1000 kg Gal-HBr/day
Basis calculation: per day

| Components | Mass in (kg) | | | | | | Mass out (kg) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Streams Number | plant (1) | $NH_4OH$ (2) | $CO_2$ (4) | MeOH (8) | HBr (14) | i-propanol (15) | cake from filter (10) | Gal-HBr (16) | MeOH recycle (12) | mother liquor (17) | plant residue from extractor |
| **plant (bulbs powder)** | 1.353.463 | | | | | | | | | | 1.351.763 |
| galanthamine | | | | | | | | | | | |
| nor-gal | | | | | | | | | | | |
| Haemanthamine | | | | | | | | | | | |
| O-methyllycorenine | | | | | | | | | | | |
| **plant residue (cake)** | | | | | | | 232 | | | | |
| **$H_2O$** | | 101.510 | | | | | | | | | 101.510 |
| **$CO_2$** | | | 56.037 | | | | | | | | |
| **MeOH** | | | | 2.550 | | | 127 | | 2.180 | 242 | |
| **$NH_4OH$** | | 33.837 | | | | | | | | | 33.837 |
| **$Na_2SO_4$** | | | | | | | | | | | |
| **HBr** | | | | | 220 | | | 220 | | 0 | |
| **Isopropanol** | | | | | | 1.234 | | | | 1.234 | |
| **Gal-HBr** | | | | | | | | 780 | | | |
| **Mother liquor** | | | | | | | | | | 688 | |
| **mass (kg)** | 1.353.463 | 135.346 | 56.037 | 2.550 | 220 | 1.234 | 359 | 1.000 | 2.180 | 2.165 | 1.487.110 |
| **mass overall (kg)** | 1.548.851 | | | | | | 1.492.814 | | | | |

# CHAPTER 8

## Conclusions and Perspectives

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### 1. Conclusions

The extraction of galanthamine, a medium polar alkaloid of *Narcissus*, using green solvents was evaluated in this thesis. Galanthamine can be obtained both by using supercritical $CO_2$ ($scCO_2$) and natural deep eutectic solvents (NADES) from its biological matrix as alternatives to aqueous acids and VOCs. In terms of environmental and economic benefits, as solvents, the first have some advantages: they are readily available, safe, sustainable, cheap, and have a low toxicity. Moreover, NADES made by simple "mixing" are non-volatile and biodegradable.

In case of $scCO_2$ extraction, galanthamine was successfully extracted from *Narcissus* bulbs. The extraction was found to be quite selective, providing galanthamine as the major compound followed with haemanthamine, and *O*-methyllycorenine as minor products (**Chapter 3**). Pre-treatment with bases to provide an alkaline medium in the plant matrix is necessary, suggesting that free bases of *Narcissus* alkaloids are well soluble in $scCO_2$. However, desorption of galanthamine from its matrix rather than its solubility in the $scCO_2$, plays a major limiting role in the extraction. A comparison was conducted with a classical extraction method of alkaloids as well as Soxhlet extraction. The classical extraction method that uses acidified water to obtain a crude extract followed by acid-base purification steps of alkaloids gave higher yield of galanthamine than the $scCO_2$ extraction (**Chapter 4**). Selectivity for alkaloids was observed in all extraction methods. Though the strong interaction between galanthamine and the matrix can be eliminated by classical-acid extraction methods as well as using hot pressurised water extraction (HPW), unfortunately, a persistent emulsion problem hampers the subsequent liquid/liquid purification step. A benchmark extraction method from solid material was performed by using methanolic-Soxhlet extraction. A similar yield of galanthamine was obtained with the classical-water extraction using hydrobromic acid (1%, v/v) and the methanolic-Soxhlet extraction.

As green solvents, NADES were tested for the selective extraction of galanthamine. A high-performance liquid chromatography (HPLC) method was developed to analyse the extracts (**Chapter 5**). However, the presence of high amounts of organic acids as constituents

of NADES such as citric acid, malic acid, and lactic acid was found to influence the galanthamine analysis. Unreproducible chromatograms as well as peak broadening and tailing were obtained when NADES containing an organic acid were analysed in a low pH value mobile phase following the method of Mustafa *et al.* (2003). This was related to the fact that at low pH values (pH <$pK_a$ of target compound) galanthamine will be totally ionised and ion-pair formation with the acids (i.e. TFA) may affect the separation. To avoid this, a basic modifier was added to the mobile phase. An optimum chromatographic separation was thus achieved using a high pH resistant stationary reverse phase column with the addition of 0.3% (v/v) of triethylamine (TEA) in the mobile phase. It was also necessary to eliminate some polysaccharides and lectins that co-extract using NADES. These compounds precipitated when in contact with a mobile phase containing MeOH, causing a sharp increase in back-pressure of the system after a series injection of NADES extracts. This problem could be simply overcome by adding methanol to the NADES extract and after 3 hours of contact, filtering over a 0.2 μm membrane before HPLC injection. Following the study of the extraction of galanthamine from the plant material, a solubility test of galanthamine-HBr (Gal-HBr) in different kinds of NADES was performed (**Chapter 6**). Multivariate data analysis (MVDA) of the results (principal component analysis method) clearly shows that the solubility of galanthamine-HBr is positively correlated with the acid and water constituents of NADES. Moreover, sugars and basic compounds have a clear lowering effect on the solubility of Gal-HBr. Thus, ionic liquids and acids classes of NADES have a high solubilisation capacity, while none from neutral and basic class of NADES showed high solubility of Gal-HBr.

A part of the solubility study of galanthamine, an application of extraction with pressurised method was used to extract the alkaloids from *N. pseudonarcissus* bulbs. This proved to be very successful as the obtained NADES extracts showed the highest galanthamine yields as compared with other extraction methods, i.e., $scCO_2$, water extraction and HPW extraction. Similar alkaloids profiles were found for water and all NADES extracts - with the exception of the β-Alanine-malic acid (βAMA) extract- but they were quite different to that of the methanolic extract. While the selectivity of NADES differs according to the kind of NADES, galanthamine, lycoramine, oduline, and haemanthamine were observed in all NADES extracts. In conclusion, NADES are promising for selective *Narcissus* alkaloid extraction.

The economic evaluation of the $scCO_2$ extraction process of galanthamine was conducted as a complement and compared with a conventional extraction method using diethyl ether. Lang factor approach was used to estimate the capital costs, this gives accuracy with a margin error of approximately ±30% of the actual production costs (€/kg of galanthamine). This estimation showed that production costs of galanthamine are lower by applying a $scCO_2$ extraction process than the conventional process; less energy is consumed and there is less generated waste. Thus, it was concluded that this greener $scCO_2$ extraction process is promising for the galanthamine production. However, an integrated production process yielding multiple products should be considered to maximise the benefits of $scCO_2$ process,

## 2. Perspectives

Both $scCO_2$ and NADES are considered to be green solvents that fit very well with the trend in scientific research and process technology in designing environmentally sustainable methods or processes. Despite its drawbacks, i.e. its limitation to solubilize the medium to polar compounds, $scCO_2$ has a great advantage if compared to other solvents in terms of selectivity, especially considering that it can be modified easily by changing extraction variables such as pressure and temperature. However, taking full advantage of the benefits is challenging. In the case of $scCO_2$ extraction from plant matrices, different strategies can be applied depending on the target compounds and type of plant matrix. By combining $scCO_2$ extraction with either a reaction process (Akgün, 2011) or another separation process, i.e. designing an integrated process, it may be possible to achieve higher yields (than solely single procedures of $scCO_2$ extraction) while maintaining and even maybe increasing selectivity. In fact, the full potential of supercritical fluid (SCF) can only be optimally achieved by using such an integrated approach.

The presence of NADES in living organisms might be involved in storage and transport of various non-water-soluble metabolites in cells. Our studies showed clear advantages of NADES as medium polar solvents with good selectivity for *Narcissus* alkaloids. The abundance of primary metabolites, such as sucrose, glucose, choline, alanine, citric acid, and malic acid, in *Narcissus pseudonarcissus* bulbs as observed by $^1H$-NMR in methanol-$d_4$-$KH_2PO_4$ in $D_2O$ (pH 6) extracts (Lubbe *et al.*, 2013) are in accordance with the hypothesis about the presence of NADES in living organisms. However, as a newly discovered solvent, their properties and behaviour are still largely unknown and thus need further in-depth studies before a rational development of applications is possible.

More research on the physical-chemical properties of NADES, such as viscosity, conductivity, density, surface tension, and polarity on a broad range of NADES is necessary. A deeper fundamental study would be required to understand the possible intermolecular interactions between the molecules of NADES mixtures and various solutes, including the role of hydrogen bonding. This should lead to a theory that could predict the solubility of a target compound and thus allow rational engineering of NADES for various applications. Moreover, insight in terms of toxicity of the individual NADES compounds and the mixture as well as the potential chemical interactions with solutes should be studied, including the toxicity of any artefact formed. A direct use of NADES extract would then be possible.

One of the challenges to meet is the recovery of the extracted compounds from the non-volatile NADES. A back-distillation method may be used to recover volatile target compounds from NADES. However, great attention should be paid to sugar based NADES considering the browning/caramelisation of sugars at high temperatures. In the case of non-volatile or thermo-sensitive targeted medium polar compounds, it may be promising to combine the NADES extractions with $scCO_2$ extraction or fractionation as an integrated process. It could offer environmentally benign alternatives to the conventional extraction methods strategies. Implementing this integration strategy of processes would allow a full exploration of the potential of *Narcissus pseudonarcissus* bulbs, yielding a multi-product

extract with different alkaloids. Such a strategy would allow the elimination of undesirable compounds (i.e. impurities), at the same time also increasing the yield, and improving the purity of targeted compounds.

NADES has a negligible vapour pressure (non-volatile) and polar properties so that a two-phase system will be formed with the volatile and non-polar $scCO_2$. Hence, the recovery principle is based on the solubility of the extracted compounds in $scCO_2$. High volatility and low polarity of compounds will favour their solubility in $scCO_2$, yielding the targeted compounds-rich $scCO_2$ phase while the insoluble compounds would remain in the NADES phase. Moreover, the presence of NADES may increase the selectivity of $scCO_2$ for the components of interest. It will allow the performance of different extraction processes while keeping the benefits of both ecological and economics aspects; this offers a great potential for various applications.

Fusing the strengths of both processes, the selectively and the great ability of NADES to extract medium polar compounds and its safety, with the advantage of the absence of residual solvents in the final product of an $scCO_2$ extraction or fractionation, make this an ideal integrated extraction and purification process of natural products, e.g. for pharmaceutical, food, and cosmetic purposes. However, some practical issues require more attention, among them the high viscosity of NADES extracts, and the effect of the water content as well as the solubility of the targeted compounds (in this case the *Narcissus* alkaloids) in $scCO_2$, the phase behaviour of binary systems of NADES/$scCO_2$ and ternary systems of NADES/$scCO_2$/solutes (*Narcissus* alkaloids). The solubility of different *Narcissus* alkaloids in $scCO_2$ should also be considered to design selective fractionation conditions. Thus, all these issues require further research in a multidisciplinary approach, bringing together the biology, natural products chemistry, and process technology.

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