Factors affecting galanthamine production in Narcissus

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Chapter III

Methods for the Analysis of Galanthamine and its Extraction from Laboratory to Industrial Scale.

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Abstract:

Galanthamine is a competitive acetylcholinesterase (AChE) inhibitor naturally produced by the plants of Amaryllidaceae family. It is used as a treatment against Alzheimer's disease (AD) and is an FDA approved drug. The content of this metabolite varies between different plant species as well as within the same species. To measure the galanthamine levels diverse extraction and pre-analytical methods at laboratory scale have been developed for chromatographic and spectroscopic analysis. Galanthamine identification and quantification methods are changing over time due to improvements in equipment. Development of new technologies also leads to new methods. At lab scale, there are various methods which can be used for the extraction of galanthamine from different sources. The final identification and quantitation of the alkaloid(s) can be achieved with different methods such as thin layer chromatography (TLC), liquid chromatography (HPLC, UPLC, LC-MS), GC(-MS) and nuclear magnetic resonance (NMR) spectroscopy. All these methods have been reviewed in this chapter. From different studies, it is evident that extraction time and method robustness are the basic parameters for selecting the best method. TLC, HPLC and GC-FID can be used for quick identification and quantitation, but for proper analysis standard reference compounds are required. Moreover, pre-analytical sample preparation is time consuming for LC-MS and particularly GC-MS. Considering these aspects 1HNMR spectroscopy is the fastest and best reproducible method, because compounds are identified and quantified based on their highly specific signals in the spectra of the extracts. For large scale extraction various methods (solvent extraction, microwave-assisted extraction, and supercritical carbon dioxide extraction) have been developed for galanthamine extraction and purification. But mostly classical organic solvent extraction is used in industrial production due to low cost and availability of large-scale extraction facilities. Galanthamine recovery rate is relatively low in this method. Recently, progress have been made towards developing better methods. Among these developments, super critical fluid (carbon dioxide) extraction has great interest because it is a more environmentally friendly (green) technology. Although the solvent extraction methods are commercially applied to date, there is still a possibility for improvement in the extraction and purification process of galanthamine from plants as based on the present yields, still only about 50% of galanthamine present is obtained from the plant material in the large-scale extraction process.

Keywords: *Narcissus*; Analytical Methods, TLC, HPLC, GC, NMR, Industrial Scale.

Introduction

Galanthamine is a tertiary isoquinoline alkaloid which can be found in plants of Amaryllidaceae family. It is a long-acting, selective, reversible and competitive acetylcholinesterase (AChE) inhibitor [1]. It was first isolated from snowdrops in 1952 [2] and its development as medicine was reviewed by Kadyrov and co-workers [3]. Later, it was reported that galanthamine could be isolated from a number of plant species [4]. Recently the yields of galanthamine from different plant sources have been reviewed [5]. Although many species have been reported as source of galanthamine, there are still efforts made to find new and better plant sources for galanthamine. This search of new and better plants sources is particularly focused on crops which are already under cultivation at large-scale commercially. The discovery of galanthamine attracted great attention due to its pharmacological properties. Its development as a medicin can be divided into three timelines. In the early times of galanthamine development, it was used to treat poliomyelitis in Eastern Europe. During the 1980s, it was found that it could penetrate the blood–brain barrier and more specifically can improve central cholinergic functions. This discovery made it a compound of high interest for the treatment of Alzheimer's disease (AD). This led to its clinical development as a medicin in the 1990s [6]. It was first marketed by Sanochemia Pharmazeutika (Austria) and Sopharma (Bulgaria) under the name of Nivalin® in 1960s for anesthesia. Later in the 1990s Shire Pharmaceuticals along with Janssen Pharmaceuticals started the natural as well as synthetic production and marketed it under the name Reminyl® for Alzheimer's disease.

Currently, galanthamine is widely used as a treatment for Alzheimer's disease (AD) due to its competitive acetylcholinesterase (AChE) inhibition ability. Alzheimer's is a form of dementia which is a progressively incurable neurodegenerative disease that mostly affects the elderly [7]. Currently, there are more than 30 million people which are affected by AD worldwide. Although this figure is expected to rise up to 42 million in 2020 [8]. According to some recently published reports, every sixth woman over 60 in the USA is affected by this disease in one or another way. Moreover, it is also the third leading cause of death in the USA after heart diseases and cancer. Although galanthamine (**Figure 3.1**) does not cure this disease fully but it can delay its progression. Moreover, it can relieve the symptoms by reversible and competitive AChE inhibition during the early to mild stages of the disease. It selectively affects those areas of the brain which have low acetylcholine [9].

Only in the USA, more than 100 billion dollars are being spent annually for the treatment of this disease, which includes personal care, drug treatment etc. Lyseng-Williamson and Plosker [10] reviewed cost effectiveness of galanthamine as medicine for use in AD. AD patients require a 16 to 24 mg dose of galanthamine daily to delay the symptoms of the disease.

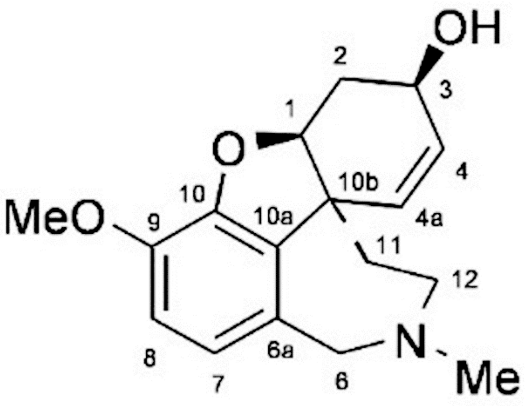

Figure 3.1. Chemical structure of galanthamine

Various methods have been applied to reduce the cost of galanthamine production. This includes cheaper production from natural sources and/or synthetic production. Moreover, cost can also be reduced by increasing the efficiency of production methods used for medicinal formulations. To further lower the costs of the active compound, there is a need for better extraction and purification methods at commercial level.

The aim of this study is to review all the methods used for extraction and analysis of galanthamine along with other related alkaloids in plant material. This includes the small-scale extraction by using different methods as well as analysis by using different chromatographic tools or NMR spectroscopy. Subsequently the large scale/industrial extraction and purification methods which have been reported by different groups are reviewed here. The main aim of this review process was to find out the best possible extraction and analysis method for targeted and non-targeted analysis of *Narcissus* metabolites.

Laboratory/ small-scale extraction and analytical methods

Different methods have been used for small-scale extraction and quantification of galanthamine. Thin layer chromatography (TLC) was used quite extensively in the earlier days of galanthamine development. Although TLC is still used in many laboratories particularly for screening, yet nowadays liquid chromatography (HPLC, UPLC and LC-MS), gas chromatography (GC) and nuclear magnetic resonance (NMR) are the most commonly applied methods for galanthamine quantification in plants. These different extraction techniques along with analytical methods are reviewed here chronologically.

Thin-layer chromatography:

Thin layer chromatography (TLC) is a technique in which a solid adsorbent such as silica gel, C_{18} bound to silica or alumina oxide coated on a thin sheet of glass or plastic is used. This method is generally used for screening purpose, but it can also be used for separation of total alkaloid extract to individual compounds.

In 1970 Volodina and Dobronravova [11] developed a chromatographic method for the quantitative analysis of galanthamine in chloroform extracts from raw epigeal (above ground) parts of plant *Ungernia victoris*. They used 10 g of air-dried plant material which was first moistened with 10 ml of 8% ammonia and then extracted exhaustively with chloroform in a Soxhlet apparatus. The extract was concentrated to 15-20 mL. From this extract, 0.5-1 mL was deposited at multiple spots on a non-bonded alumina oxide plate (13 x 18 cm) and developed with a mixture of chloroform–methanol (9.75:0.25) as a mobile phase for the separation of galanthamine from accompanying alkaloids. They obtained 0.04-0.06 mg/ml of galanthamine from the alkaloid abstract with 95-98% recovery rate.

Kalashnikov [12] studied the alkaloids extracted from *Galanthus nivalis* to explore other plant sources for galanthamine. The plant material was extracted with 2% H_2SO_4 and total alkaloids obtained were 0.38% in the extract of the below ground parts (dried bulbs with roots) and 0.41% total alkaloids from the above ground parts (leaves with stem and flowers). This total alkaloid extract was chromatographed on a silica gel plate and eluted with either ether-acetone-diethylamine (80:20:5); ether-methanol-diethylamine (90:5:5); *n*-hexane-chloroform-diethylamine (50:40:10) or benzene-chloroform-acetone-diethylamine (80:25:30:5) as solvent system. This solvent system leads to the detection of six alkaloids in the above ground parts and seven alkaloids in the below ground parts of the plant. A chloroform extract from the total alkaloid extracts was separated on an alumina oxide column and eluted with chloroform alone as well as chloroform with 5% methanol. The separation of the chloroform extract resulted in a yield of 0.059% of galanthamine from the above ground plant parts and 0.058% of galanthamine from the below ground plant parts on dry weight basis.

Volodina and coworkers [13] used *Ungernia severtzovii* and *Ungernia trisphera* as plant material for the extraction of lycorine. During this process, galanthamine was also detected with the help of TLC. Ten gram (10g) of air-dried leaves were moistened with 5% ammonia (10 mL) and exhaustively extracted with chloroform in a Soxhlet apparatus. The extract was separated with a chloroform–ethanol–acetone (8:1:4) solvent system on an alumina oxide TLC plate (activity class III). Dragendorff's reagent was sprayed at the end for detection of the alkaloids. Other alkaloids such as hippeastrine, ungminorine, tazettine, pancratine, and racemic narwedine were also found under these conditions.

Gorbunova and Patudin [14] used a somewhat similar method to Volodina and Dobronravova [11]. During the extraction process, they used 15% ammonia instead of 10% ammonia. Afterwards, they extracted the material with chloroform and ethanol. Moreover, they used the shaker for 2 hours or soaking (steeping) for 18 hours instead of a Soxhlet apparatus. This altered method was used to determine galanthamine contents in bulbs and leaves of *Eucharis subentana, Vallota speciosa,* and *Galanthus nivalis* species. The extracts were dried and then dissolved in ethanol for analysis. These ethanol extracts were then chromatographed on a silica gel TLC plate and eluted with a chloroform–ethyl acetate–methanol (2:2:1) solvent system. For visualizing the alkaloids, the silica plates were exposed to iodine vapors and galanthamine band was scraped off the plate. The alkaloid was then eluted with a mixture of methanol and chloroform. The identity of the eluted alkaloid was confirmed by NMR. They found 0.10% of galanthamine in leaves and

0.05% in bulbs of *Eucharis subentana*; 0.05% in both leaves and bulbs of *Vallota speciosa* and 0.05% in leaves and 0.73% in bulbs of *Galanthus nivalis* based on the dry weight of raw plant material.

Wurst et al. [15] developed a TLC procedure for the quantitative determination of galanthamine in *Leucojum aestivum*. Three gram (3g) dried leaves were extracted by using ethanol (170 mL). The extract was then dried under reduced pressure at 40°C and alkaloids from this extract were separated by using acid-base extraction. The extracted alkaloids were eluted on a silica gel G60 plates with diethyl ether-methanol- diethylamine (80:15:5) as a solvent system. After elution, detection was carried out by spraying the TLC plates with Dragendorff's reagent.

Frederick and Murav'eva [16, 17] performed TLC on silica gel plates for the qualitative analysis of alkaloids extracted from *Pancratium trianthum* and *Hymanocallis littoralis* plants**.** They used chloroform without the addition of any base solution to extract alkaloids from 500 g of above and below ground parts of the plant separately. From this chloroform extract, they separated 0.25-0.40% total alkaloids in above ground parts and 0.40-0.50% total alkaloids in below ground parts by using an acid-base extraction method. The total alkaloid extracts were separated with benzene and stepwise benzene-methanol gradient (ratios from 99:01 to 90:10) on a KSK silica gel column. The yield of galanthamine was low (0.004%) in both plant parts, and numerous other alkaloids were also quantified.

Dobronravova and coworkers [18] used TLC to study the chromatographic behavior of alkaloid halides, including galanthamine hydrobromide. They used alumina plates and chloroform, acetone, and methanol in various proportions as the solvent system. Dragendorff's reagent was used for the detection of the alkaloids. They found that there were two different spots for the alkaloid salts. One spot for alkaloids in the free base form while the other spot about equal in size and shape at the site of application. The second spot might be an *N*-oxide of the alkaloids or otherwise the salt, which does not dissolve in the mobile phase.

Murav'eva and Mahbub Alam[19] used a method as previously reported for the extraction of alkaloids from 2 kg of above-ground parts of *Hippeastrum equestre***.** From this extract, they obtained 0.30 g of galanthamine by using a methanol-chloroform gradient on KSK silica gel column. Varying amounts of other alkaloids were also obtained.

Gorbunova and colleagues [20] also studied the leaves of Fortune variety from *Narcissus* in their quest for new plant sources of galanthamine. The chloroform extract from the dried plant material had 0.93% of total alkaloids including 0.15% galanthamine. The total alkaloid extract was separated on silica gel TLC plates impregnated with 2% sodium carbonate and eluted with chloroform–ethyl acetate–methanol (2:2:1) solvent system. Galanthamine was isolated from the plates and identified by IR, NMR and mass spectroscopy.

Bulb samples from *Lycoris guangxiensis* (Amaryllidaceae) were studied by Li and coworkers [21]. They isolated several alkaloids from the samples along with galanthamine. The chloroform extract of total alkaloids was eluted with chloroform–methanol (12:1 and 4:1) and chloroform–methanol–aqueous ammonia (12:1:0.05) solvent systems on silica gel HF_{254} plates. These plates were sprayed with Dragendorff's reagent and iodine for specific alkaloid detection. It showed the presence of 0.12% galanthamine on dry weight basis.

Tokhtabaeva [22] used leaves of *Narcissus* variety Fortune for the extraction of galanthamine. A combination of different solvent systems was used to separate galanthamine from the total alkaloid extract on KSK silica plates. From various solvent systems, the best separation was obtained with chloroform–ethyl acetate–methanol (2:2:1) solvent system. Alkaloids were detected with Dragendorff's reagent after elution.

Sadykov and Khodzhimatov [23] used a different method for the determination of alkaloids and specifically galanthamine in *Ungernia victoris* grown in Uzbekistan. They used alumina plates for the separation. The compounds were eluted by using chloroform–methanol (2:1) and chloroform–methanol–acetic acid (90:80:2) as solvent systems and spots were revealed by spraying Dragendorff's reagent or exposure to iodine vapors.

Tanahashi and coworkers [24] performed TLC of a synthetic galanthamine conjugate used for antiserum production. Galanthamine hydrobromide (180.2 mg, 0.49 mmol) and succinic anhydride (300 mg, 3.0 mmole) were dissolved in 3 ml of dry pyridine. The mixture was kept at 100 °C for 22 hours and stirred yielding galanthamine-2-0-

hemisuccinate (162.9 mg), as an amorphous powder after evaporation. TLC silica gel 60F$_{254}$ plates were used with chloroform–methanol–aqueous ammonia (90:9:1) as a solvent.

Saushkina and Popova [25] used TLC to detect impurities which are present in standard samples of galanthamine hydrobromide. They used two different types of plates with two different solvent systems. For alumina plates, they chromatographed the samples in a TLC chamber saturated with ammonia vapors, with the solvent chloroform–methanol (24:1), For KSK silica gel plates, elution was carried out with chloroform–ethyl acetate–methanol (2:2:1) solvent. Alkaloids were detected with Dragendorff's reagent.

Novikova and Tulaganov [26] developed a sensitive method specific for the identification of galanthamine and for the detection of possible impurities in the galanthamine hydrobromide salt. A 0.5% solution of galanthamine hydrobromide in a 50% aqueous ethyl alcohol was prepared for rapid application on the plate. For comparison, 0.5% solution (used for injections) and pure galanthamine 0.1% solution was also applied. Different solvent systems were checked and compared for best possible separation. A 20 × 20 cm Silufol UV-254 plate showed better separation than the other plates used in the experiment. For elution of alkaloids *n*-butanol–glacial acetic acid–water (BAW) in a ratio of (8:3:4, v/v) provided the most acceptable separation. For spotting the alkaloids UV light at 254 nm was the most sensitive (detection limit 0.5 µg) when TLC plates were sprayed with a 0.1% sodium eosinate solution.

Rhee et al. [27] combined TLC with bioactivity staining to detect acetylcholinesterase inhibitors in plants of the Amaryllidaceae family. Fifteen different species were tested with the help of this method. Ground and freeze-dried plant material were extracted with methanol (10 mL) and toluene (10 mL) at room temperature first for one day and then again with a fresh solvent for another week. After one week both extracts (1 day + 1 week respectively) were combined and evaporated under reduced pressure. These dried extracts were dissolved in methanol (10 mg/mL) and spotted (2.5 µL) on G60 F$_{254}$ Silica gel plates. Plates were developed with chloroform-methanol (8:2) solvent system. To detect the enzyme inhibitory activity, plates were sprayed with substrate (1mM DTNB and 1 mM ATCI in the buffer), and enzyme (acetylcholinesterase). The active compounds became visible after about five minutes as white or a yellow spot. These spots were observed and recorded within 15 minutes before their disappearance in 20-30 minutes. In another study Rhee et al. [28] developed a method to determine possible false positives. They tested various aldehydes and amines to determine whether the observed inhibition is due to a true enzyme inhibition or due to the inhibition of the reaction between thiocholine and 5, 5-dithiobis-(2-nitrobenzoic acid) in the Ellman's assay. After testing different compounds, they found that 4-dimethylaminobenzaldehyde, 3-ethoxy-4-hydroxybenzaldehyde, diethyl-amine, triethylamine, triethanolamine and tyramine showed real enzyme inhibition. Although these compounds were bioactive but their activity was about 10^3 times lower than what was shown by galanthamine. Heptanal, decanal, cinnamaldehyde, anisaldehyde, benzaldehyde, hexylamine and tryptamine appeared to show a non-specific chemical inhibition. In general, certain compounds may interact with the dye of the Ellman’s reagent, such as aldehydes and amines, giving false positive results. A control for this needs to be included in the TLC-assay. By using this method for chemical inhibition in the TLC assay, they checked the true enzyme inhibition in the toluene extract of *Nerine bowdenii* to find the active compounds.

A similar method was reported for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors from the bulbs and aerial parts of Amaryllidaceae and Papaveraceae family by Marston et al. [29]. Plant material (10 g fresh chopped/dried powder) was extracted by using 80% methanol (250 mL). After extraction, the solvent was evaporated, and material was freeze dried. The dried extract was dissolved in methanol (1 mg/mL) and spotted (15µL) on G60 F_{254} Silica gel TLC plates. The plates were developed with chloroform-methanol-water (65:35:5). After development, the plates were washed with acetone or isopropanol for solvent removal and air-dried. Then the enzyme dissolved in buffer was sprayed on these plates and incubated in a humid atmosphere at 37 °C for 20 minutes. After incubation plates were sprayed with naphtyl acetate and Fast Blue B salt solution for detection of compounds that inhibit the acetyl- or butyrylcholinesterase. The detection limit of galanthamine for inhibition activity was found to be 0.01 µg.

Kintsurashvili and Vachnadze [30] studied a number of plants of the Amaryllidaceae family grown in Georgia. They ground an aliquot of bulbs (1 kg) and alkalized these with a 12% aqueous ammonia solution. Then column chromatography was used for the isolation of different types of alkaloids. For determination of alkaloids, silica gel plates (LS 5/40) were used and alkaloids were eluted with chloroform–methanol–25% aqueous ammonia (86:14:1), chloroform–methanol (6:12) or chloroform–ethyl acetate–methanol (2:2:1) solvent systems. The alkaloids were detected with Dragendorff's reagent or by exposure to iodine vapor. For quantification of galanthamine specifically air-dried plant material was extracted with chloroform. This extract was treated with 10% aqueous sulfuric acid solution and alkalized with 25% aqueous ammonia solution. After alkalization, it was again extracted with chloroform and this extract after concentration was later analyzed by TLC using chloroform–methanol–25% aqueous ammonia (86:14:1) as the solvent system. Their results indicate that *Galanthus* and *Narcissus* species contain highest amount (0.22% of dried material) of galanthamine at the end of their growth cycle in underground parts while *Leucojum aestivum* contains the highest amount (0.17% of dried material) at the beginning of vegetation (bulbs).

Berkov with coworkers [31] reported a fast TLC method for the determination of galanthamine in 12 plant species of the families Amaryllidaceae, Fumariaceae and Papaveraceae. According to this method dried and powdered plant material (3-5 g) was extracted three times with 10 mL of methanol each for 12 hours at room temperature. After evaporation of the organic solvent under reduced pressure, the extract was dissolved in 3 mL 2% H_2SO_4. Then 5 mL of diethyl ether was used three times for the removal of neutral compounds. The extract was then basified with 25% ammonium and alkaloids were extracted three times with 5 mL of ethyl acetate. After evaporation of the solvent, the extract was dissolved in methanol (10 mg/mL) for analysis. TLC was performed by using (10×20 cm) Silica-gel F_{254} plates. The alkaloids were eluted with ethyl acetate-methanol-25% aqueous ammonia (3:1:0.1 v/v/v) and n-hexane-ethyl acetate-methanol-25% aqueous ammonia (3:3:1:0.1 v/v/v/v). They identified several alkaloids and results indicated that bulbs of *Leucojum aestivum* contained the highest amount of galanthamine among the tested species.

Abou-Donia and coworkers [32] developed a high performance TLC method that requires a minimum sample clean-up. Ten gram (10g) of freshly chopped bulbs of *Narcissus* cv. Breath of Spring was exhaustively extracted with 2 L of methanol. After extraction, the solvent was evaporated under reduced pressure resulting in 0.1 g of crude extract. This crude extract was dissolved again in methanol (10ml), filtered, mixed thoroughly and spotted (2.0 µL) on Silica gel G60 F_{254} plates which were then developed in HPLTC with 25 mL of chloroform-methanol (9:1) as the mobile phase. With the help of this method, they found a level of 0.83 mg/mL of galanthamine in the crude extract (10 ml).

Mroczek and Mazurek [33] combined pressurized liquid extraction (PLE) with activity based HPTLC for quantification of Amaryllidaceae alkaloids from *Narcissus jonquilla* var. Pipit. They validated the solvents used for extraction as well as temperature for extraction. They also optimized the method for cation-exchange solid-phase extraction (CEx-SPE). They extracted 0.5 g of plant sample and used Silica gel G60 HPTLC plates for analysis which were developed with chloroform–methanol-25% ammonia water solution (9:0.5:0.5, v/v/v). According to their results, highest yields of galanthamine were obtained with pure methanol (0.086%) and 1% tartaric acid methanolic solution (0.089%). Concerning the temperature of extraction highest amount of galanthamine was obtained between 100-150 °C (0.085%) in HPTLC by using the above-mentioned solvents.

Ortiz and colleagues [34] used plant species of Amaryllidaceae family grown in Argentina to find new sources of galanthamine. Dried plant material (100 g for each plant) was extracted with 300 mL of methanol under reflux three times for 1 hour each. The combined extracts were dried under reduced pressure. The crude methanolic extract was dissolved in 2% H_2SO_4 and neutral material was removed with chloroform (200 mL). After basification with 25% NaOH up to pH 10-12, the alkaloids were extracted with 500 mL of chloroform (three times) and then concentrated by evaporation and used for spotting on Silica gel G60 F_{254} plates. These plates were developed with chloroform-methanol-ammonia (99:09:01) and sprayed with Dragendorff's reagent for detection.

Shawky et al. [35] used HPTLC method for determination and quantification of galanthamine in different plant parts of *Narcissus* papycareus and *Narcissus* tazetta. They chopped the plant material and exhaustively extracted with methanol. The solvent was then removed under pressure. The concentrated extract was then acidified with 5% tartaric acid until pH 2 was achieved. After that extract was defatted with petroleum ether, filtered and washed with diethyl ether. After washing the acidic aqueous phase was transformed to alkaline with aqueous Ammonium hydroxide to pH 10. Then basic aqueous solution was extracted successively with chloroform, ethyl acetate and n-butanol. Chloroform extract showed maximum alkaloids in a TLC screening test, so it was used for further study. For HPTLC analysis, 10 mg of chloroform extract was dissolved in 10 mL of methanol. From this extract, samples were applied on pre-coated silica gel 60GF254 aluminum sheets (6.3 20 cm) in 6 mm bands for analysis. These bands were eluted with chloroform-methanol (9:1) for 15 minutes in a double run. The air-dried plates were viewed in ultra-violet radiation to mid-day light. Zones were quantified by linear scanning at 254 nm with a Camag TLC Scanner 3.

Tallini et al. [36] used various analytical methods for alkaloid profiling in H. reticulatum. Bulbs and leaves were collected and macerated twice in methanol for four days each. Solvent was filtered and collected. Then solvent was removed under pressure. Crude extracts were then acidified to pH 3 with sulphuric acid. Then neutral compounds were removed with diethyl ether. Remaining crude extracts were extracted with ethyl acetate, n-hexane and ethyl acetate-methanol (3:1 v/v) for further processing. For TLC analysis, Silica gel 60F254 (20cm-20cm-0.25mm) were used. For elution, ethyl acetate-methanol (9:1 v/v) and ethyl acetate-methanol (8:2 v/v) were used in an ammonia saturated chamber.

Advances have been made over the years in the field of thin layer chromatography to detect and separate galanthamine from other alkaloids as well as other metabolites. Extraction of Amaryllidaceae alkaloids and galanthamine specifically, was mostly carried out by methanol or after basifying the plant material with e.g. ammonia and then extraction with chloroform. Also an acidic aqueous extract has been reported. As TLC is based on single use plates, rather “dirty” extracts can be used, if compared to HPLC and GC, where often an extra acid/base extraction or solid phase extraction step is applied.

Most TLC analyses were done on silica gel plates, though in the earlier times alumina was also used. Both neutral and basic eluents have been used. Because of less tailing the latter give better separations. Diethylamine and ammonia are the most common bases in the TLC eluents. The use of neutral eluents carries the risk of double spot formation in case of analyzing the alkaloids as salts, e.g. after acidic extraction [18]. The formation of N-oxides during the extraction should be avoided, particularly when using diethyl ether or chloroform. Also, the use of dichloromethane and chloroform as solvents [18] in the extraction carry the risk of the formation of various artefacts, e.g. N-oxides, and quarternary dichlorometho-derivatives of the amine group, particularly when using soxhlet extraction.

Concerning the solvent systems, good results were obtained with diethyl ether – methanol – DEA (90:5:5) and hexane-chloroform-diethylamine (90:5:5), both on silica. Chloroform-methanol in different ratios have been used on both alumina and silica plates. Detection method is most often Dragendorff's reagent.

Liquid chromatography (HPLC, UPLC and LC-MS):

Liquid chromatography (HPLC, UPLC and LC-MS) is another technique used to separate compounds from mixtures. It is a very important, versatile and one of the major technologies applied in metabolomics. HPLC is widely used in the analysis of secondary metabolites. Many different types of columns are available from which one must choose the most suited for certain metabolites. First HPLC methods for the analysis of galanthamine were reported in the 1980s although it was used before for the separation of other Amaryllidaceae alkaloids such as lycorine and ambelline along with crinine. First validated method for the analysis of galanthamine by HPLC was published in 1983 by Claessens and colleagues [37]. They analyzed biological samples (serum, urine, bile) for galanthamine after i.v. administration at 0.3 mg galanthamine/kg body weight. For extraction of galanthamine, they used 2 mL of serum and deproteinized it by adding 2 mL of 20% trichloroacetic acid in the test tube. After 10 seconds of vortexing, it was left at room temperature for 5 minutes to let the material settle down. Then it was centrifuged at 1600 g for 10 minutes. Three mL of supernatant was collected and alkalinized with 4 M NaOH (0.6 mL) and 2 mL of buffer (1 M Ammonia solution titrated with 2 M HCl to pH 9.0). The sample was extracted twice with dichloromethane (5 mL each) after 2-minute vortexing and 5-minute centrifuging at 1600 g every time. The alkaloids were back extracted two times by using 0.05 M H_2SO_4 with 2-minute vortexing and 5-minute centrifuge each. This extract was alkalinized with 4 M NaOH and buffer to pH 9.0 and extracted with dichloromethane with the above-mentioned method. The extract was then dissolved in the mobile phase (25 µL) and injected (10 µL) in HPLC. The HPLC with UV detector at 235 nm was used with normal phase (NP) and reverse-phase (RP) columns. The normal-phase column (10×0.46 cm) was packed with CP^{tm} Micro Spher Si3 (3 µm) while the reversed-phase column (15×0.46 cm) was packed with Polygosil 60-C_{18} (5 µm). Dichloromethane-*n*-hexane-ethanolamine (500:500:0.25, v/v) was used as mobile phase at a flow rate of 1 mL/min, which resulted in a 5 ng/mL detection limit for galanthamine and a retention time of 6 minutes with a total runtime of around 7 minutes for one sample.

Tencheva and colleagues [38] developed a reverse phase LC procedure using an RP-8 (5 µm Merck) column (125×4 mm) for the analysis of galanthamine and its metabolites in blood plasma and urine. The mobile phase was a methanol–water mixture (40:60) modified by a dibutylamine additive (5 mM, pH 7). This technique was appropriate for the quantitative determination of galanthamine and two main metabolites in human plasma and urine.

Pre-treatment of samples with preparative isotachophoresis (ITP) was tested as a more accurate and less time-consuming method than the HPLC previously reported by the same author for the analysis of galanthamine in biological samples. An improvement in the accuracy was found if compared to an HPLC method [39].

Bickel et al. [40] used HPLC to determine the pharmacokinetics of galanthamine and its metabolites by measuring them in mice brain and blood. Two hundred (200 µL) of plasma, homogenized tissues or haemolyzed erythrocytes were mixed with 100 µL of a water solution containing codeine (1 µg) as an internal standard. This solution was diluted with 1 mL of 0.9% NaCl and precipitated with 1 mL of 20% trichloroacetic acid, vortexed for 30 sec and centrifuged at 6000 g for 10 min, after removing 2 mL of supernatant, it was alkalinized by adding 5 M NaOH (220 µL). To maintain pH (11-11.5) 0.5 M phosphate buffer (1 mL) was added to the mixture before extraction with 5 mL of chloroform (15 minutes vortexing). Chloroform was then dried by using Na_2SO_4 and finally the solvent was evaporated on a water bath at 50 °C. Dried extract was dissolved in 100 µL of mobile phase and 50 µL was injected for analysis. Alkaloid separation was carried out on Intersil-C8 (5 µm) silica packed reverse-phase column (250×4 mm) with acetonitrile-tetrahydrofuran-water-di-*n*-butylamine (120:30:850:0.85) as mobile phase at 0.7 mL/min flow rate. The method had a 1 ng/200 µL detection limit with 75% extraction recovery.

Sulochana and co-workers [58] reviewed the extraction methods as well as the determination methods for galanthamine and other Alzheimer's disease drugs with HPLC and LC-MS in mammalian biological samples.

Tanahashi with co-workers [24] quantified galanthamine in species of the Amaryllidaceae family by using HPLC and a radioimmunoassay (RIA). Samples of plant material were extracted twice by boiling them with 80% ethanol for 15 minutes each. After filtration, the combined extracts were used directly in HPLC and fractions were collected. This method used a Hyperchrome, Synchropak RP8 column (250 × 3.2 mm) and A) methanol-water (5:95), B) methanol-water (90:10) gradient solvent system with a 1 mL/minute flow rate. Fractions were collected from the HPLC separation and used in RIA for quantification of galanthamine. For detection of Amaryllidaceae alkaloids from natural sources, reversed-phase (RP) HPLC was combined with automated multiple developments (AMD) TLC on normal phase silica. At first, conditions such as stationary phase, solvent, modifier, buffer (pH and concentration), gradient profile, flow rate, temperature and sample size, and solvent were optimized to achieve the best HPLC resolution for 20 references Amaryllidaceae alkaloids [41]. Under optimum HPLC conditions, the eluent from the HPLC column was collected in fractions and applied on silica TLC plates and subjected to AMD. Compounds on the TLC plate were detected and quantified by different spectroscopic and chemical methods. The UV spectra of individual bands were used in combination with retention data to confirm the identity of each compound. The authors applied the method to re-investigate the alkaloid extract of *Amaryllis belladona* by using a supercritical fluid extraction method which led to the identification of nine alkaloids, in addition to five other alkaloids previously known from this species [42].

Bergoñón and co-workers [43] developed an HPLC method for the quantification of galanthamine from *Narcissus* shoot cultures. Alkaloids were extracted from dried and crushed shoot clumps which were ultrasonicated for 30 minutes with methanol (5 mL) at 25 °C followed by centrifugation for 10 minutes 4000 g (-5 °C). This complete process was repeated 5 times and the extracts from all the processes were combined and concentrated to 1 mL after concentration. Then the concentrate (1 mL) was re-dissolved in 5% acetic acid (10 mL) and washed with petroleum ether (10 mL) three times. Ammonium hydroxide (NH_4OH) was added to obtain a pH which is higher than 8 that was followed by extraction with dichloromethane (10 mL three times). After evaporation of dichloromethane, the residue was dissolved in 10 mL of 2.5% acetic acid-acetonitrile (2:3 v/v) solution that was used for analysis. Galanthamine and other alkaloids were quantified with HPLC using Spherisorb-ODS column type 2 C_{18} (5 mm) with the gradient mobile phase of acetonitrile and 1% (w/v) ammonium acetate buffer that was maintained at pH 5.8 with acetic acid. Twenty (20) µL of the extract was injected for analysis and flow rate of the mobile phase was set at 1.2 mL/min with the UV detector at 280 nm. From the total alkaloids extracted which were extracted from the shoots/ green parts of the plant, there was 13 mg/g of galanthamine from the total extract (18 mg/g) on dry weight basis. This analytical method was used for the quantification of galanthamine in several other studies due to its effectiveness [44, 45] .

HPLC was used to separate and quantify galanthamine among other alkaloids from *Narcissus confusus*. An alkaline extract was used for this purpose and four alkaloids were quantified [46]. In another study, medium pressure liquid chromatography (MPLC) was used with a LiChroprep silica gel column to separate galanthamine and other alkaloids from the flowers of *Pancratium maximum* total extract. Fresh flowers (1.5 kg) were macerated three times with ethanol after crushing. Extracts were combined and then evaporated under reduced pressure. The dried brown residue (13 g) was dissolved in methanol (100 mL) which was diluted with 100 mL of water and extracted with n-hexane. This hexane extract was then subjected to vacuum liquid chromatography (VLC) for fractionation. Fractions were monitored by TLC and subjected to further MPLC separation. Chloroform–methanol (8:2) was used as a mobile phase for MPLC at a flow rate of 1 mL/minute [47].

Ingkaninan and coworkers [48] combined HPLC with MS and a UV-online biochemical detector based on the Ellman’s assay for the identification of acetylcholinesterase (AChE) inhibitors from natural sources (**Figure 3.2**). They used bulbs of *Narcissus pseudonarcissus* var. Carlton as a model to validate this method. Compounds including galanthamine were extracted by using methanol which was then fractionated by using centrifugal partition chromatography (CPC). A Lichrospher RP SelectB column (I.D.125×4.6 mm) was used with methanol-water-diethylamine (40:60:0.05 v/v/v) as mobile phase at a flow rate of 1.2 mL/min. The HPLC was coupled to the UV-biochemical (UV-Vis) detector. The mobile phase was methanol-water (pH 7.0) with 0.01 M ammonium acetate (30:70 v/v) when HPLC was coupled with UV and electrospray ionization (EI) mass spectroscopy [34]. The same method was used for another variety of *Narcissus* (Sir Winston Churchill) where high performance centrifugal partition chromatography (HPCPC) was applied for the purification of compounds [49].

De Jong et al. [50] further developed an online bioassay method for acetylcholinesterase inhibitors by using fluorescent assays. They reduced the risks of false positives and also made the method more sensitive. A solid phase extraction (SPE) C_{18} column (2×4 mm, I.D.; 5 mm) was used for trapping the analytes before the analysis. A Luna C_{18}(2) column (1×50 mm, I.D.; 3 mm) was used with a linear gradient mobile phase at 20 µL/min flow rate. Mobile phase was a gradient made by mixing solvent A) 97.5% 10 m*M* aqueous ammonium bicarbonate (pH 7.8) with 2.5% methanol and solvent B) 97.5% methanol with 2.5% of 10 m*M* ammonium bicarbonate (pH 7.8). A Q-ToF2 mass spectrometer with electrospray source was operated in positive ion mode for detection. Analysis of *Narcissus* extracts showed that galanthamine is responsible for the inhibition of acetylcholinesterase. Although this method can be used to detect AChE activity, the sensitivity is reduced due to the connection of the bioassay continuous flow system. Ion suppression was caused by the presence of high concentrations of some compounds in the extract.

Lopez et al. [51] used a solid phase extraction method to concentrate the alkaloids before analysis by HPLC. Bulbs of *Narcissus confusus* were cut and dried at 40 °C before grinding them to powder. Fifty (50) mg of this powder was macerated with methanol (10 mL) for 24 hours which was sonicated for 30 minutes after every 8 hours. Then the extract was filtered after 24 hours and centrifuged for 10 minutes at 8500 g. After adjusting the volume of the supernatant to 10 mL, the extract was subjected to SPE with Snap-Cap C_{18}

cartridges. These elutes collected from the SPE cartridges were used for HPLC analysis. HPLC with diode array detector operating at 280 nm was used with a Kromasil 100 C_{18} column (150×4 mm i.d.; 5 µm) which was protected by a C_{18} guard column (20×3.9 mm i.d.; 5 µm). Mobile phase for this method was constituted with two solvents. Solvent A as mobile phase was made of water-acetonitrile (33:67) with 7 mM sodium dodecyl sulfate, 25 mM sodium phosphate, and 1 m*M* ammonium acetate whereas methanol was used as solvent B for gradient elution at a flow rate of 0.7-0.9 mL/min. With this method, they were able to achieve 0.6 µg/mL limit of detection and 2 µg/mL limit of quantification for galanthamine among other alkaloids.

Mustafa and colleagues [52] developed and validated an HPLC method for rapid determination of galanthamine in Amaryllidaceae plants. Two different extraction methods (acid-base extraction method and acid extraction method) were tested as well as a number of columns and number of internal standards to find out the best combination. Best extraction was with 0.1% TFA in water. The method was validated by determining reproducibility, limit of detection, limit of quantification, the linearity of the method and recovery. A Vydac 201SP54 C_{18} column (250×4.6 mm, I.D.; 5 mm) with TFA-water-acetonitrile (0.01:95:5) was suitable for quantitative analysis. They reported a 90% recovery with this method. This method was used later as a reference for the development of new methods using different instruments such as HPTLC and NMR [32, 53].

To determine galanthamine contents in the bulbs of *Lycoris radiate*, a method was established by Wu et al. [54]. An ODS column in RP-HPLC was used with phosphate buffer (pH 3-4)-methanol (93:7) as mobile phase at a flow rate of 1 mL/min and the UV detector was set at 289 nm. A 99% recovery was reported with this method with a detection limit of 0.3 ng.

Mroczek and Mazurek [33] developed a solid phase extraction (SPE) for HPLC analysis of alkaloids of *Narcissus* varieties. This method was optimized for the temperature of extraction and extraction solvent as well as solid phase extraction. Galanthamine in the extract was quantified it by using a 3.5 µm XTerra C_{18} analytical column (150×4.6 mm) at 25 °C with a flow rate of 0.3 ml/min of acetonitrile and 15mM aqueous ammonia solution as a gradient mobile phase. Photodiode array detector was used for detection.

Precision, limit of detection, limit of quantification, inter-day and intra-day variations were determined.

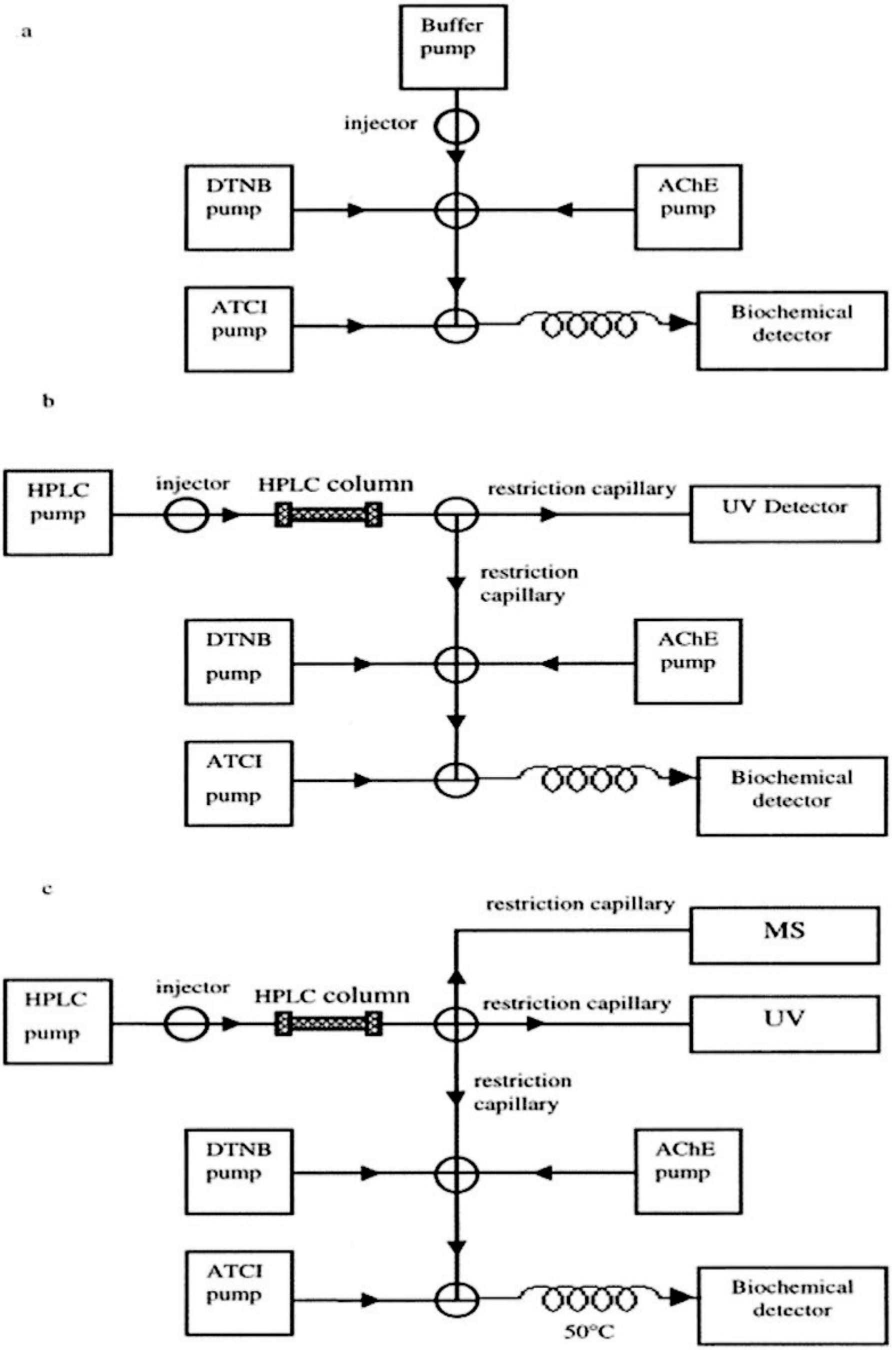

Figure 3.2. The combination of CPC-HPLC-MS-UV used for the quick determination of galanthamine/acetylcholinesterase inhibitors in plants.

This method achieved 0.018 ng/µL limit of detection and 0.059 ng/µL limit of quantification for galanthamine. In another method to determine galanthamine in *Lycoris radiate,* an HPLC method was established using an Eclipse XDB-C_{18} column and acetonitrile - 0.2% H_3PO_4 solution as mobile phase at 1 mL/min flow rate. This method proved to be efficient and effective for galanthamine determination in these plants [55].

HPLC-DAD was used for the determination of galanthamine and lycorine in *Galanthus woronowii* and *Galanthus elwesii*. Plant material (200 mg) was extracted by maceration in 2% HCl (5 mL) for 5 hours at 40°C. After adding 1 mL of 26% aqueous ammonia solution, the volume was adjusted to 10 mL and centrifuged. The extract was loaded on an Extrelut® column which was eluted with chloroform (5 mL×3) and combined extract from these elutions were taken to dryness. The dry extract was dissolved in 0.1% TFA and analyzed by using HPLC with a Hichrom C_{18} column (250 x 4.6mm, i.d.; 5µm) and TFA-water-acetonitrile (0.01:90:10) as a mobile phase which was kept at a flow rate of 1mL/min. With the help of this method, they were able to quantify galanthamine which was present at levels up to 0.506% in total. Moreover, along with galanthamine they have also found lycorine in the extract [56, 57].

Nagamallika and Arunadevi [58] developed a method for the rapid determination of galanthamine in pharmaceutical formulations. They used RP-HPLC with an ODS C_{18} column (100x4.6mm, i.d.; 5mm) and acetonitrile-phosphate buffer (40:60) as mobile phase at 1 mL/min flow rate. A photodiode array detector was used for detection of galanthamine, the method was sensitive, accurate, reproducible and advantageous for rapid routine quality control.

Petruczynek et al. [59] compared different extraction steps and HPLC systems for galanthamine and lycorine analysis from Amaryllidaceae family. For this purpose, 5 g of plant material from different organs was macerated in methanol (100 mL) for 72 hours. Secondly, continuous extraction was carried out for 5 hours in a sonication bath. Extracts were filtered, and solvent was removed under vacuum. Crude extract was dissolved in 30% sulphuric acid. After dissolving, samples were defatted with diethyl ether. After that, the aqueous phase was collected and its pH was set at 9.5-10 with 25% ammonia hydroxide. These defatted extracts were then extracted with chloroform to yield the alkaloid extract used in the further analysis. The solvent was then dried and re-dissolved in methanol. For HPLC system comparison, three columns (octadecyl silica, SMC18, and strong cation-exchange (SCX)) were tested with various mobile phases. These different mobile phases includes a mixture of 5% acetonitrile, 20% acetate buffer at pH 3.5 and 0.025 Moles per liter diethylamine as a mobile phase for C18 and SM C18 column. While for the SCX column mobile phase includes 8% acetonitrile and phosphate buffer at pH 2.5. From the results, it was clear that SCX column with the mentioned mobile phase gave the best results for galanthamine analysis. As for the extraction method, maceration followed by sonication proved to be better than other methods. From the different plant species, *Leucojum vernum* was highlighted as a plant with highest level of total alkaloids, and highest level of galanthamine.

Benedec et al. [60] investigated various Romanian Amaryllidaceae species for developing new medicinal product with help of HPLC-MS coupled with UV detector. For this purpose, they extracted powder plant material with 20 mL of 70% ethanol in an ultrasonication bath at 60C for 30 minutes. The extracts were then filtered and centrifuged at 4500 rpm. The resulting supernatant was collected for further processing. Separation of compounds was carried out on Zorbax SB C18 column while column temperature was 48C. Methanol and acetic acid 0.1% (v/v) was used as a mobile phase in a binary gradient at a flow rate of 1 mL/min. Detection was carried out at UV and MS detector simultaneously.

For HPLC analysis, most of the methods used reversed phase C_{18} columns, though one needs to consider that different commercial columns give slightly different separations of the alkaloids. To avoid the problem of tailing of alkaloids due to residual acidic silanol groups in reversed phase columns, specific columns for basic compounds are required or an ion pairing agents can be added to the eluent. For human biological samples, the method developed by Claessens and colleagues [37] in 1983 is considered the standard method which can be used for the comparison and analysis of galanthamine. In the case of plants, a number of extraction methods have been developed over time by using different solvents. Although Amaryllidaceae alkaloids can be easily quantified by a UV or fluorescent detector hyphenated to HPLC, it (HPLC) is not always suitable for these types of analyses due to the presence of a complex mixture of compounds in the extract. The resolution and peak shape for a mixture of standards also appears to vary widely under seemingly identical experimental conditions. The detection of galanthamine by UV is not very sensitive due to a relatively weak chromophore. Mass spectroscopy seems to be more sensitive. Some alkaloids do have strong fluorescence making this an option though not all alkaloids will be observed. These methods have some selectivity, but MS has the advantage of adding a further dimension to the chromatogram, for example in the case of overlapping peaks, different molecular weights will be observed in the MS. Thus, the combination of DAD and MS gives a fairly strong evidence for the identification of alkaloids.

The main disadvantage of the common HPLC detection methods with UV and MS is that each compound has a specific detection response, which means that for absolute quantification for each compound a calibration curve is required.

Gas chromatography:

Gas chromatography is quite popular and widely applied method in metabolomics field. This is due to the robustness of detection and separation of a large number of metabolites as well as the availability of some excellent libraries for identification of metabolites. This method combines high sensitivity and resolution with a specific mass spectroscopy (MS) fragmentation pattern of the separated molecules. However, this technique is limited to the separation and detection of volatile compounds. Some non-volatile compounds may be converted to volatiles by chemical derivatization. The possibility of using gas chromatography (GC) for the separation of galanthamine and its analogs has been studied for quite some years and the early methods have been reviewed [61]. There are also studies about combining gas chromatography with column chromatography and capillary electrophoresis which were reviewed along with the reported methods for the determination and quantification of galanthamine specifically and Amaryllidaceae alkaloids in general till the end of 2000 [62].

Berkov and co-workers [63] used GC-MS due to its rapid quantitative and identification qualities of complex alkaloid fractions. Alkaloids of *Leucojum aestivum* were extracted from fresh samples by cutting them into small pieces and using ethanol (1:10 w/v) three times for 48 hours each. The combined extracts were concentrated in *vacuo* and acidified with 3% H_2SO_4 to pH 1-2. Then the extract was defatted three times with chloroform and basified to pH 10-11 with 25% aqueous ammonia. After basification, the alkaloids were extracted with chloroform (3 times) and after evaporation, the extract was dried over anhydrous sodium sulfate. A small amount was used for GC-MS analysis. Samples were run on GC-MS operating in the EI mode with HP-5 MS column (30 m × 0.25 mm i.d.; 0.25 μm) and a temperature program of 80-280 °C for 30 minutes at a gas flow rate of 0.8 mL/min. A number of alkaloids were identified by using this method including galanthamine. However, lycorine could not be detected because it has a very low intensity of its M^+ ion.

Narcissus species have been studied for alkaloids with the help of both GC-MS and CE. Samples (5 g) were extracted with methanol (50 mL) for 12 hours at room temperature with 30 minutes sonication period after every 4 hours. This whole process was repeated three times and methanol was evaporated after filtration under *vacuum.* The residue of the extract after evaporation was dissolved in 1% HCl. The aqueous solution was extracted with *n*-hexane (3×50 mL) first and then with dichloromethane (3×50 mL). The solution was then basified (pH 9.0) with carbonate-bicarbonate buffer, and then alkaloids were extracted with dichloromethane (5×50 mL). This solution was then washed with water and dried. The dried extract was dissolved in methanol for analysis. Gas chromatograph equipped with split-splitless injector with an Rtx®-5 MS column (30 m × 0.25 mm i.d.; 0.25 μm) was used. Helium was the carrier gas at a flow rate of 0.8 mL/min. The temperature gradient program was 80-280 °C in 30 minutes. The mass detector used was operating in EI mode (70 eV). The method was validated according to the usual validation parameters and galanthamine was quantified (2.17 mg/g DW) using this method for *Narcissus* species. A method for non-aqueous capillary electrophoresis along with GC-MS was also validated and showed similar (2.20 mg/g) results as GC-MS [64].

Berkov et al. [65] developed a microextraction method for alkaloids in plants. Plant material (200 mg) was extracted with 5 mL of 2% H_2SO_4 for 6 hours at 40 °C. The solution was then basified with 20% ammonia (1 mL) and the volume was adjusted to 10 mL with distilled water. After centrifugation an aliquot (3 mL) was applied on Isolute HM-N columns and eluted with ethyl acetate (15 mL), the collected extract was evaporated under a nitrogen stream and dry residue was dissolved in methanol (250 µL). For micro-extraction, they used 5 mg of sample and a smaller amount of above-mentioned solvents (200 µL) for a shorter time (1/3 of normal). A HP5-MS column (30 m × 0.25 mm i.d.; 0.25 µm) was used for GC-MS analysis. The temperature program ranged from 100-300 °C in 25 min. A number of alkaloids (sanguinine, chlidanthine, lycoramine, epinorgalanthamine) along with galanthamine were quantified with this method. Galanthamine concentration found in *Galanthus elwesii* was 0.213% and in *Zephyranthes concolor* 0.098% on dry weight basis. According to their report, there was no galanthamine in *Leucojum aestivum* grown under greenhouse conditions but other galanthamine derivatives were found in these plants [66]. The same GC-MS conditions were used for the quantification of galanthamine in *Pancratium canariense* though with a different extraction method. Plant material was extracted by maceration for 72 hours and solvent (methanol) was evaporated. The dry residue was dissolved in 2% H_2SO_4 (3 mL). Neutral compounds were removed by using diethyl ether (3×5 mL) and the resulting solution was basified to pH 9-10 by using 25% aqueous ammonia. From this extract, alkaloids were extracted with ethyl acetate (3×5 mL) which was evaporated later and dissolved in methanol for analysis.

Berkov and co-workers [67] developed and validated a GC-MS metabolomics method with the aim of combining advantages of GC such as resolution power, sensitivity, selectivity and analysis of a wide spectrum of compounds by derivatization of the samples. Metabolites were extracted from 50 mg of dried plant material by maceration with 1 mL of methanol. The pH of the sample was adjusted to 8 by using 25% aqueous ammonia and codeine (50 µg) was added as internal standard. The extraction lasted for two hours with a 15 minutes ultrasonic bath treatment after every 30 minutes. The samples were centrifuged for 1 min and an aliquot (300 µL) was collected and dried at 45 °C. The samples were derivatized by adding pyridine (100 µL) and BSTFA (100 µL) before GC-MS analysis. The method was validated following the ICH guidelines and it was used for identification of different metabolites including galanthamine. Principal component analysis was used for the combined analysis of the metabolomics data. It is also possible to quantify the alkaloids individually with the help of this method. The conclusion at the end of this study was that the above-mentioned method is very simple, practical and sensitive. This same method was used in the later studies for determination of galanthamine in plants [34, 68].

Shawky et al. [35] also used a GC-MS method for determination and quantification of galanthamine in different plant parts of *Narcissus* papycareus and *Narcissus* tazetta. They chopped the plant material and exhaustively extracted with methanol. The solvent was then removed under pressure. The concentrated extract was then acidified with 5% tartaric acid until pH 2 was achieved. After that extract was defatted with petroleum ether, filtered and washed with diethyl ether. After washing the acidic aqueous phase was transformed to alkaline with aqueous Ammonium hydroxide to pH 10. Then basic aqueous solution was extracted successively with chloroform, ethyl acetate and n-butanol. Chloroform extract showed maximum alkaloids in a TLC screening test, so it was used for further study. For GC-MS analysis, a sapiens-X5 MS column was used operating the GC in EI mode at 70 eV. The temp. program was: 100– 180 °C at 15 °C per minute, 1 min hold at 180 °C, 180–300 °C at 5 °C per minute and1 min hold at 300 °C. The injector temperature was 280 °C. The flow rate of Helium carrier gas was 0.8 ml per minute. In most cases, the split ratio was 1 :20, but with more diluted samples, a split ratio of 1 :5 was applied.

Breiterova et al [69] used GC-MS for the alkaloid analysis from daffodils. Fresh bulbs were extracted 3 times with methanol at room temperature for 24 hours. The solvent was collected and evaporated under reduced pressure. The resulting crude extract was dissolved in 10 mL of 2% sulfuric acid. Neutral compounds were removed with diethyl ether. After that solution was basified with 25% ammonia. Alkaloids were extracted with ethyl acetate and dried for further processing. GC-MS was performed in EI mode at 70 eV. Separation was carried out on DP-5 MS column with a time program of 100-180 °C at 15 °C per minute, 1minute hold at 180 °C, 180-300 °C at 5 °C per minute and 35-minute hold at 300 °C with an injection of 1µL.

GC-MS is quite popular and widely applied for metabolite identification in general, i.e. as a tool in metabolomics and specifically for alkaloids. This is due to its robustness for both separation and detection as well as the availability of databases for the identification of metabolites from mixtures. It combines high sensitivity and resolution to produce better results and where resolution is not sufficient MS offers a third dimension to resolve overlapping peaks. A limiting factor in this technique is the pre-analytical processing, as GC is limited to volatile metabolites. For the analysis of non-volatile compounds, such as *Narcissus* metabolites, derivatization is carried out but sometimes derivatization is incomplete. Derivatization and/or thermal instability can cause problems of multiple peaks for the same compound. In the case of galanthamine or alkaloids mostly an acid base extraction is the standard method used for extraction. It is due to the requirement that the final sample must contain the alkaloids in the non-protonated form. The detection in GC is usually carried out by FID or MS. Between these detection methods, MS has a distinct advantage of adding an extra dimension to the separation as the molecular weight and fragmentation are strong supporting evidence for the identification of a peak. However, every compound has a different detector response, which means that for absolute quantification of each compound a calibration curve is required. The FID detection has a distinct advantage here. Although it does not give extra information on the identity of the peak, the intensity of the peak is directly related to the amount present which means that peak height or surface area of different compounds can be compared directly in the peak pattern. GC-FID thus gives a reasonable impression about the absolute quantities of all compounds present.

Nuclear magnetic resonance spectroscopy:

Nuclear magnetic resonance spectroscopy (NMR) techniques have been employed for solving various problems encountered in the pharmaceutical analysis due to its wide range of detection. This wide range of detection is due to the reason that any molecule with non-zero magnetic moments can be detected such as ^{1}H, ^{13}C, ^{15}N, and ^{31}P molecules. NMR has been widely applied in plant metabolomics and is mostly the first choice for medical metabonomics of body fluids. Owing to the highly informative character of the NMR spectra, they are the basis of identification of known compounds and structure elucidation of novel compounds. A major advantage of NMR is that signal intensity is only determined by the molar concentrations. That means all compounds can be compared and quantitation of all compounds can be done by comparison with a single internal standard. All the previous studies which were mostly for identification of galanthamine and its derivatives have been reviewed [26]. Recently a method was developed for the identification and direct quantification of galanthamine in *Narcissus* bulb extracts by means of NMR. Fifty (50) mg of freeze dried bulb material was used for extraction of metabolites. Aqueous phosphate buffer- methanol-d_4 (1:1) was added to the plant material (1.5 mL) and vortexed for 30 seconds at room temperature, then the sample was ultrasonicated for 30 minutes and centrifuged for 10 minutes at 13000 rpm. After centrifugation, an aliquot (1 mL) was collected and transferred into an NMR tube for analysis. The ^{1}H NMR spectra were recorded using a Bruker AV 600 spectrometer. In the plant material (bulbs) 3.35 mg/g of galanthamine on the dry weight basis was found to be present by using this method [53]. This NMR-based metabolomics method was used later on in different studies to determine galanthamine production in *Narcissus* plants grown under different conditions such as the effect of fertilizer, pesticides and seasonal accumulation of metabolites [70-72]. In comparison with the other techniques, NMR is better in terms of overall analysis of compounds in a short time. While for galanthamine specific determination, it also shows better results in terms of quantification when compared with HPLC. These results also clearly indicate that with the help of NMR overall metabolite comparison under different conditions can be easily made and conclusions can be drawn from these comparisons to pinpoint the effect of different changes in the nutrient availability and growth cycle of *Narcissus*. Although an overview of metabolic changes in *Narcissus* is also possible with HPLC, LC-MS and GC-MS, these methods require calibration curves for every compound that needs to be quantified. Otherwise, only relative change for a compound can be determined by comparison of the peak intensity of the particular compound in different chromatograms. With NMR absolute quantitation is possible from a single spectra.

Industrial/Large Scale Extraction Methods

Metabolites are produced in small quantities by plants to counter different physiological stresses at different developmental stages. Due to their wide range of biological activities, they play an important and crucial role in the development of new medicines. For example, 60-70% of the approved drugs for cancer and other infectious diseases in the last 25 years were derived from natural bioactive compounds from different sources, including plants [73]. Still, about 70% of the world population relies on the medicinal plants for primary health care in different capacities. Sometimes after identification of the active constituents, these compounds can be produced by a synthetic process in the laboratory. However, in many cases, the natural sources are the compulsory requirement as the complex structures do not allow economically feasible synthesis. In terms of chemical synthesis of galanthamine, Shieh and Calson [74] reviewed all the previous methods and described a new method for the production of (-)-galanthamine. Galanthamine produced with the help of this method was without contamination of other isomers. Due to this reason, it was used as the basis for the commercial synthetic production.

In the case of galanthamine besides synthesis, isolation from plants is also performed on a large scale. *Narcissus* species and *Leucojum aestivum* are already in production on a large scale for other purposes and can be used as source material for the extraction of galanthamine. In the large-scale production, the extraction from the plant material is the most crucial step. Several aspects need to be considered before extraction such as drying of the plant material, a method of grinding, choice of extraction solvent, type of extraction, temperature and pressure for extraction as well as the use of ultrasound and microwaves to increase the availability of compounds.

The isolation of galanthamine usually involves a liquid-liquid extraction to obtain an enriched alkaloid fraction. Basically, large-scale extraction of galanthamine is achieved by using two approaches. The first approach starts with acidic aqueous extraction of metabolites from the plant material which contains galanthamine along with medium to highly polar neutral compounds. These compounds can be later removed by washing with organic solvents. Then the aqueous extract is basified by adding a base to obtain a pH higher than 9 after which alkaloids are extracted with an organic solvent followed by a fixed purification step through crystallization.

The second approach is to extract the alkaloids in the base form making a pH higher around 9 in the plant material with the help of ammonia or $NaHCO_3$ followed by extraction with a non-polar to medium polar solvent e.g. ethanol. After evaporation of the solvent, the alkaloids can be dissolved in acidic water and acid-base liquid-liquid extraction can be used for further purification of the alkaloid fraction.

Earliest report that can be found about galanthamine production on a large scale was from Russia. According to that report, galanthamine was extracted from the dried leaves of *Ungernia vitoris*, using chloroform as a solvent by Moscow and Tashkent Pharmaceutical Chemistry. A small scale method was described for the extraction using as an aqueous solution of mineral acids where desorption was carried out by a mixture of 96% alcohol-chloroform (1:4), containing 1.5-2% ammonia and 2 L/kg eluent was consumed in this method. Shakirov et al. [75] tested different 1% acidic solutions (Acetic acid, hydrochloric acid, sulphuric acid) using previously reported conditions i.e. 1 kg sample was decanted 8 times with 3 liters of solvent. The results showed that the maximum amount of galanthamine (0.10% of DW) was obtained with 1% hydrochloric acid while the lowest amount was obtained with water. After optimization of the extraction solvent (1% HCl), a number of ion exchange resins for binding the alkaloids were tested and KU-1 was found best which was followed by the optimization of the eluents (methanol-ammonia-water, ethanol-ammonia-water) but these did not show any difference. After optimization of all the parameters, this method was used on a large scale with 100 kg of air-dried *Ungernia victoris* (galanthamine raw content 0.12%). The yield obtained was 90% and the total amount of 96 g galanthamine was obtained with this method.

Heliang et al. [76] applied a cation exchange method for the extraction of galanthamine from Amaryllidaceae plants. They tested a number of cation exchange resins with varying amount of alkaloids. They used 10 kg plant material that was extracted with 1% HCl. The solution was run over a cation exchange column with STREAMLINE SP exchange resin (2 L). The column was then washed with water to neutral, with 1 M aqueous sodium chloride. The fractions were screened for galanthamine with TLC. The galanthamine fraction was basified to pH 9.2 by adding sodium carbonate solution and extracted with ethyl acetate (5×2.25 L). Ethyl acetate was dried over anhydrous sodium sulfate and evaporated under reduced pressure which resulted in 13.1 g of galanthamine. This galanthamine was further purified by recrystallizing it with acetone to get 99% pure free galanthamine crystals (8.0 g). Amberlite CG-120, Amberlite IRC-50 and Amberlite IRC-76 cation exchange did not give as much yield, but the difference was not very high. This invention was claimed to provide the alkaloid extraction from plants with an emerging method of cation exchange made from acidic clay by adsorption and provides highly pure (99%) galanthamine crystals.

Cvak et al. [77] developed a robust method for the extraction of galanthamine from plant material obtaining a higher amount of purified end product and also eliminating the use of diethyl ether from previous reports of galanthamine extraction. Bulbs of *Narcissus pseudonarcissus* var. Carlton which contained 0.12% galanthamine, were used for the extraction process. Bulbs (75 kg) were filled in one extractor of a pilot plant battery of percolators (4×100). Extraction was carried out with 0.1% (w/w) aqueous solution of phosphoric acid in the counter current way. The resulting primary extract (125 L/extractor) was basified with 10% ammonia to pH 9-10 and loaded into 60 L column filled with non-ionic resin SP-825L. Which was washed with 100 L of water and organic compounds were eluted with 60% (v/v) aqueous ethanol, resulting in 220 L of alkaloid extract. This extract was loaded on a 3 L column with the cation exchange resin SK-104 on which the alkaloids were bound. The column was washed with water and alkaloids were eluted with 0.5% (w/w) aqueous ammonia, resulting in 30 L of aqueous alkaloid extract.

This solution was extracted with methyl isobutyl ketone and the extract was evaporated to 1 L of crude extract. This extract containing 45.8% of galanthamine on a dry basis, was subjected to flash chromatography in a column containing 2 kg of basic alumina using methyl isobutyl ketone as mobile phase and the resulting fractions were tested with TLC. The fractions containing galanthamine were combined and the solvent was dried which resulted in 134 g dry residue of purified galanthamine. To obtain galanthamine hydrochloride, dried extract was dissolved in 450 mL of ethanol and pH was adjusted to 4 by adding concentrated HCl. The suspension was cooled in a refrigerator and crystals were filtered off, resulting in 106 g of galanthamine hydrochloride. After crystallization, 99% pure galanthamine hydrochloride was obtained. The above-mentioned method proved that galanthamine can be extracted with an aqueous solution of suitable organic or inorganic acids to get a primary extract and then purifying it later.

Jiqian and co-workers [78] reported a method for the extraction of galanthamine with methanol from *Lycoris aurea* with the objectives of yield improvement, reduce costs of extraction and suitability for an industrial scale production. They separated galanthamine with a cation exchange method after pretreatment of the raw material and heat reflux extraction. The extract was concentrated and extracted with 2% acetic acid where the resin was used for desorption. Hydrogen bromide was then introduced in the form of gas to convert galanthamine into galanthamine hydrobromide. According to the results, this simplified extraction process improved the yield by reducing the loss of alkaloids and reduce the cost of extraction as compared to the other methods.

Hille and co-workers [79] modified a previously reported extraction method [80] which gave some toxic side products by developing a method for the isolation of galanthamine from plants by using toxicologically safe organic compounds. They followed the second approach and mixed the plant material with a basic compound to obtain the alkaloids in the non-protonated base form. First, they tested and compared the previously reported methods by using 8% ammonia to basify the plant material which resulted in an unbreakable emulsion that cannot be used further. So, they modified the method and instead of ammonia added sodium bicarbonate (400 g in 10 kg plant material) and extracted with dichloroethane (23 L), galanthamine was further purified by using acid-base liquid-liquid extraction. The resulting product was very low in yield (1.3 g) and also contaminated so it could not be used further. To obtain a better-quality product, the method was further modified by using sodium bicarbonate (4 kg in 100 kg plant material) and extraction was carried out with gasoline at boiling point (15 L). After drying, acid-base extraction was performed yielding a higher quantity of more pure galanthamine (10 g). According to the inventors, this method is better, toxicologically safe and more efficient than the previously reported methods.

Sagdullaev [81] proposed that it is more economical to extract galanthamine from plant material by using aqueous alcoholic solvents and developed a method for this purpose. He used 80 kg of *Ungernia victoris* ground leaves and extracted six times with 80% aqueous ethanol (1:4.2 w/v). The combined extracts were then concentrated to 65 L in total and basified with 25% ammonium hydroxide to pH 9-10. From this solution, alkaloids were extracted by using chloroform (3×20 L) and the combined extracts were extracted with 5% H_2SO_4 (3×10 L) which was then washed with chloroform (2×10 L) to remove impurities. The solution was again basified to pH 9-10 and alkaloids were extracted with chloroform which after evaporation resulted in 115 g of total alkaloids. This total alkaloid extract was then dissolved in acetone (0.230 L) chilled, stirred and treated with concentrated HBr which resulted in the precipitation of galanthamine hydrobromide crystals. This precipitate was collected and recrystallized from ethanol (55%) to obtain 98-100% pure galanthamine (0.05% on DW basis).

Gabetta and Mercalli [82] reported a method for the preparation of galanthamine hydrobromide where galanthamine base was extracted from the plant source. They used bulbs of *Narcissus pseudonarcissus* var. Carlton as a plant source. Ground plant material (500 kg) was mixed with 10% aqueous sodium carbonate (850 L) and extraction was carried out by toluene (7×1000 L) at 65-70°C. The combined extract was then subjected to acid-base extraction method and 2.2 kg of 88% pure galanthamine hydrobromide was obtained by using toluene as solvent. To increase the purity of galanthamine, the dry crystals were further processed with a detailed recrystallization procedure which resulted in 99% pure galanthamine hydrobromide (1.4 kg).

Zhimin and Cong [83] developed a method for the extraction of galanthamine hydrobromide form the alkaloid extract of the bulbs of *Lycoris radiata.* The alkaloids were extracted from the bulbs (8 kg) through overnight maceration with 2% HCl. After adjusting the pH of the combined extract to 7, it was filtered to get a total alkaloids precipitate (2 kg). The alkaloid extract was mixed with dibenzoyl-l-tartaric acid in 75-95% alcohol as solvent at 70-100 °C obtaining a clear solution. The clear solution was chilled to 10-25°C till crystals were separated out which were filtered and dried. This crude product was dissolved in alkaline solvent and pH was adjusted between7-9. Extraction was carried out with chloroform which was then evaporated and the residue was dissolved in acetone. The acetone solution was mixed with aqueous solution of hydrobromic acid and the precipitate was filtered to obtain galanthamine hydrobromide. With the help of this method, 90-99% pure galanthamine hydrobromide can be obtained with a yield of 80-90%. The method has good reproducibility and low implementation cost as compared to other methods of galanthamine hydrobromide purification

Another extraction method on the medium scale was published in a thesis on galanthamine purification with CPC pH-zone refining. According to this method, 1.6 kg of dried and powdered plant material was moistened with 10% ammonia (1 L) and macerated for 18 hours in ethyl acetate (30 L). Then the solvent was evaporated after filtration and extracted with 3% sulphuric acid for six times (3×3 L, 3×2 L). This combined extract was then basified with 20% ammonia to pH 9-10 and alkaloids were extracted three times (2×3 L, 1×2 L) with chloroform. This chloroform extract was then washed with water until neutral pH and dried over sodium sulfate under reduced pressure. The extract was used for purification of galanthamine by centrifugal partition chromatography [84].

Renault and co-workers [85] extracted alkaloids from *Leucojum aestivum* and then subjected the extract to FCPC using toluene-heptane-acetone-water (28:8:10:34 v/v) as a biphasic solvent system.

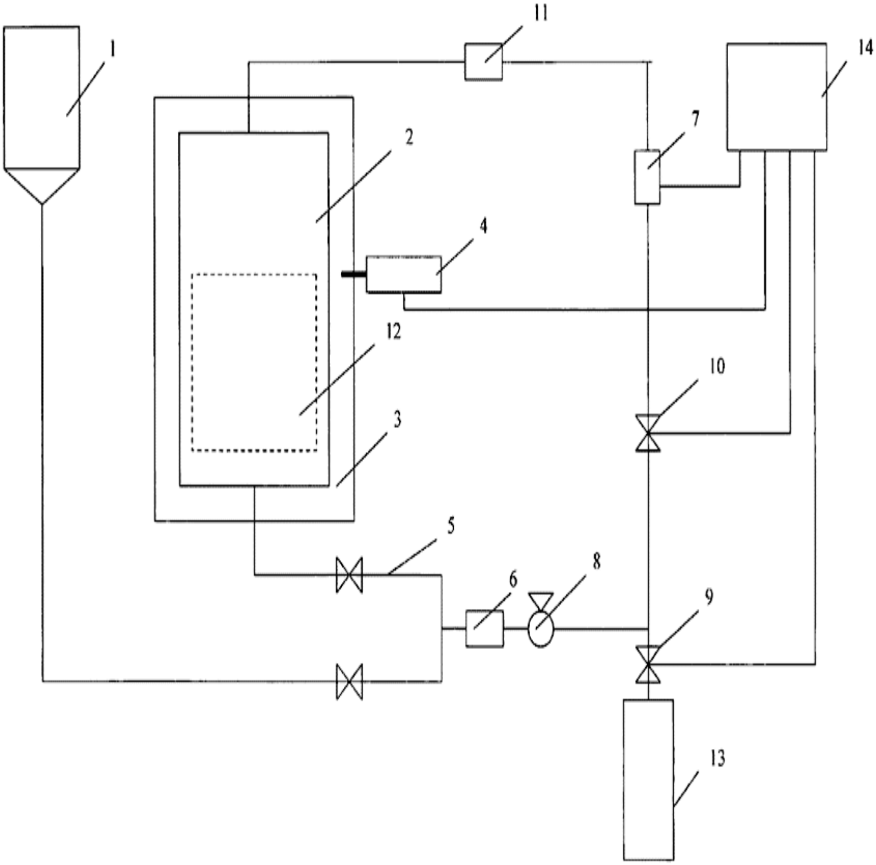

Figure 3.3. Microwave assisted extraction plant as described by Lubenova [86] where 1) solvent container, 2) extractor, 3) placed in a chamber's wall, 4) microwave source-magnetron, 5) circulation contour, 6) filter, 7) temperature sensor, 8) pump, 9) drain

valve, 10) circulation valve, 11) pH meter, 12) inner perforated wall container, 13) extract collector, 14) control unit for temperature.

The aqueous phase was acidified by 10 mM of methane sulfonic acid and the organic phase was alkalinized by trimethylamine where aqueous phase was used as stationary phase and the organic phase was used as mobile phase. To ionize the alkaloids, the extract was dissolved in methanol and acidified with methane sulfonic acid. The solution was evaporated under reduced pressure and the syrupy residue was dissolved in 20 mL of the aqueous phase and 1 mL of the organic phase and injected into CPC. Fractions were collected and combined on the basis of TLC analysis. Pure galanthamine (1.219 g) was obtained by using this method while CPC was operating in ascending mode and 1.142 g of galanthamine when using the descending mode. For *Narcissus pseudonarcissus* var. Carlton extraction method was the same as mentioned above but the biphasic system was different. For *Narcissus pseudonarcissus* var. Carlton diethylether-acetonitrile-water (4:1:5 v/v) was used as a biphasic solvent system. By using this system, from 5g of alkaloid extract, 2.031 g of pure galanthamine was obtained in the ascending mode and 2.650 g in the descending mode. Without methane sulfonic acid (a retaining agent) in the ascending mode, the amount of galanthamine was reduced to 1.660 g. The purity of galanthamine obtained by CPC is above 98%. This method can be used to separate galanthamine and its derivatives where starting concentration of galanthamine in the solution is 20% and above both from synthesized mixtures and from biological samples.

Wang and Qingbin [87] used supercritical CO_2 extraction method for the extraction of galanthamine from the powdered bulbs of *Lycoris aurea* (10 kg) which were soaked overnight with trimethylamine before extraction. The extract was then dried and placed in a supercritical extraction unit. CO_2 2 mL/min/g was used and aqueous ethanol (90:10) at the flow rate of 0.4 mL/min/g as modifier was used. The extraction lasted 2 hours at 60°C and 30 MPa pressure, the extract was then dissolved in 2% aqueous HCl, filtered and extracted with chloroform. The chloroform extract was passed through an alumina column with chloroform-methanol-water (5:3:1) as eluent. Fractions containing galanthamine were collected, combined and evaporated. The extract was re-dissolved in acetone-ethanol (1:1) and crystallized to get 213 mg of galanthamine with purity of 94.5%. They had also tested ammonia as soaking solvent at the start of the process but it gave lower yields than triethylamine. Flow rate, pressure and temperature for the extraction of galanthamine were optimized with the maximum amount of galanthamine (23.5 mg/kg dry weight basis) resulting from CO_2 flow rate 5 mL/min/g and 0.8 mL/min/g for the modifier at a temperature 80°C and pressure of 50 MPa. This method can be used to obtain galanthamine with high purity in supercritical CO_2 extraction. Rachmaniah et al. [88] optimized the conditions for the extraction of galanthamine from plant material. The size of the dried and ground particles of the plant material, as well as the extraction conditions were optimized. Highest yield of galanthamine (303 mg/kg) was obtained at 70 °C, 220 bar for 3 h extraction time with 53–1000 µm particle size. The desorption of the alkaloid from the plant matrix seemed to be the major limiting step in the process.

Lubenova [86] developed a microwave assisted extraction method for the extraction of galanthamine from plant material of *Leucojum aestivum*. Three kg of dried and milled plant material of aerial parts of the plant were fed into the extractor (2) by means of perforated wall container (12). An aqueous solution of calcium carbonate was added in the extractor and the microwave radiation source (4) was turned on, from which radiation at 2450 MHz with a power of 2.55 kW was delivered for 30 minutes. The solution recirculated every 10 minutes for 3 minutes in the circulation contour (5) while the temperature is maintained at 32°C. The extract is obtained through the filter (6) by means of a pump (8) and collected in the collector (13) at the completion of extraction. Extraction was carried out for a second time at the same conditions. The combined extracts were checked for alkaloid content (0.05%) of which galanthamine contents were more than 80%. It was claimed in this study that up to 99% yield of alkaloids can be obtained with this method.

The maximum amount of galanthamine extracted from different plant materials by using these methods varies from 0.1-0.2% of the total dry weight of plant material. It cannot be considered very high as galanthamine concentration determined with the help of small-scale quantitative methods ranges from 0.3-0.5% on a total dry weight basis. So it can be said with certainty that these large-scale methods need a definite improvement to get better yields from the same plant material.

Conclusion:

The extraction methods for the analysis of galanthamine have improved since galanthamine was identified for the first time. However, these microscale methods are not suited for large-scale industrial production. There is thus a need for optimized large-scale extraction methods to make galanthamine production from plants economically feasible. Particularly the high cost of waste disposal of the classical liquid-liquid extraction methods is an important area for improvement. That means a greener extraction process. Further improvements can be made in the production of galanthamine by plant breeding and change the agronomic conditions of plant growth in the field, but improvements of the extraction methods would be a faster and easier way to reach the goal of doubling the yield of galanthamine from natural sources as breeding good high producing varieties of bulbs would take 10-15 years. Efforts to improve yield by means of agricultural practices so far only resulted in levels that are marginally better than the current cultivation practices.

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