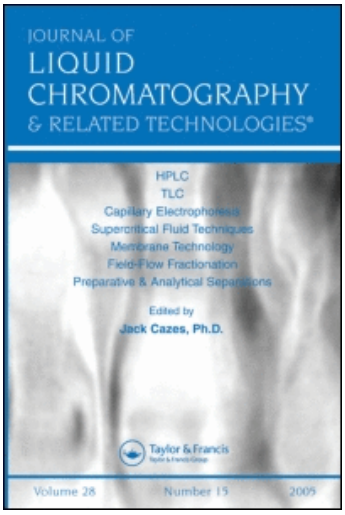


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# Rapid Method for Determination of Galanthamine in Amaryllidaceae Plants Using HPLC

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# Rapid Method for Determination of Galanthamine in Amaryllidaceae Plants Using HPLC

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## ABSTRACT

A method for rapid qualitative and quantitative determination of galanthamine in Amaryllidaceae plants using reversed phase high performance liquid chromatography has been developed. The use of 0.1% trifluoroacetic acid (TFA) in water as the extraction solvent provides an HPLC-ready sample, which makes this method simple and efficient. The separation was achieved by a system consisting of a reversed phase $C_{18}$ small pore (100 Å) column and TFA : water : acetonitrile (0.01 : 95 : 5 v/v/v) as the mobile phase. Ephedrine is a suitable internal standard.

*Key Words:* Galanthamine; Bulbs; Amaryllidaceae; *Narcissus* "Pipit" (Jonquilla); Acid extraction; HPLC-PDA; Quantitative analysis.

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## INTRODUCTION

Galanthamine (Fig. 1) is an isoquinoline alkaloid found in several species of the Amaryllidaceae family.[1,2] This compound, possessing acetylcholine esterase (AChE) inhibitor activity, is an important therapeutic agent for symptomatic treatment of mild and moderate cases of Alzheimer's disease (AD). Galanthamine is considered as one of the second generation AD-drugs, which is an alternative to the first generation AD-drug, tetrahydroaminoacridine (tacrine, Cognex®). Tacrine is known to have disadvantages such as hepatotoxic effects, low and highly variable oral bioavailability, limited efficacy, adverse cholinergic side effects in the periphery and narrow therapeutic ranges (reviewed in Refs.[3–5]). In some Eastern-European countries, galanthamine is available for clinical purposes as Nivalin® (Pharmachim, Sofia, Bulgaria) and Galanthamine (Medexport, Moscow, Russia).[6] In June 2001, the FDA approved it as Reminyl®.[7]

Although, the synthesis of galanthamine has been accomplished,[8] the production of galanthamine from a sustainable natural source is considered important. This resulted in studies on several aspects including: isolation; qualitative and quantitative analysis; biosynthesis; and biotechnological production of this compound. Methods for searching for new compounds having an AChE inhibitor activity in Amaryllidaceae species have been reported.[9,10] Besides Amaryllidaceae alkaloids and some *Narcissus* extracts, other plant species have also been searched for their AChE inhibitory activity,[11] in which for example a very potent AChE inhibitor, zeatin, has been isolated from *Fiatoua villosa*.[12]

The botanical sources of the Amaryllidaceae alkaloids have been reviewed.[2] This review shows that not all species of the known galanthamine-producing genera contain this compound. Galanthamine contents in some

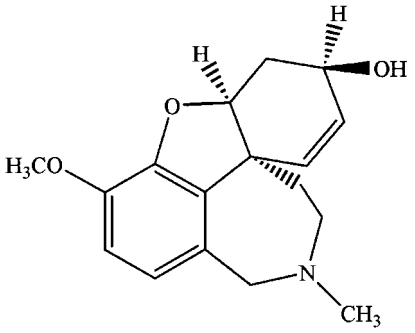


***Figure 1.*** The structure of galanthamine.





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Amaryllidaceae species, which have been determined by isolation, are for example: 0.0085% dry-weight (DW) in *Galanthus elwesii*,[13] 0.004% DW in *Haemanthus multiflorus*,[14] 0.00565% fresh weight (FW) in *Leucojum aestivum*,[15] 0.0045% FW in *Lycoris sanguinea*,[16] 0.1117% FW in *Narcissus confusus*,[17] 0.0095% FW in *Narcissus nivalis*,[18] 0.001% FW in *Narcissus obesus*,[19] and 0.005% FW in *Narcissus tazetta.*[20]

Various methods for quantitative analysis of galanthamine have been developed. HPLC was used to prefractionate the ethanol extract before analysis by radioimmunoassay (RIA).[6] This method, which utilized the specificity of high affinity antibodies, is sensitive, and allows a precise quantification of low galanthamine concentrations even in unpurified plant extracts. However, the use of this method is restricted to laboratories equipped for working with radioactivity.[21] A quantitative analysis of galanthamine using an enzyme immunoassay (EIA) has also been reported.[21] With this method a galanthamine content of 7.4% DW in *Phaedranassa megistophylla* was detected. A quantitative analysis of galanthamine in *Narcissus* cultivars has been performed using gas chromatography-mass spectrometry (GC-MS).[22] HPLC methods to quantify galanthamine in clinical samples[23,24] and in Nivalin® tablets[25] have been reported. An RP-HPLC method for quantitative determination of galanthamine and some other Amaryllidaceae alkaloids in plant- and tissue-culture extracts of *N. confusus* has been developed.[26] The same group reported an improved sample preparation method using solid-phase extraction (SPE) for *N. confusus* wild plants, galanthamine, and related alkaloids, which were further quantitatively analysed by RP-HPLC using a gradient system.[27] A normal phase HPLC method for analysis of galanthamine in plants has also been reported.[28]

Here, we report the development and validation of a reversed phase HPLC method for qualitative and quantitative determination of galanthamine in plants using a simple one step extraction method and an RP-HPLC system that, if desired, can be coupled with MS.

## EXPERIMENTAL

### Plant Material

The bulbs of *N. tazetta* L.; *Narcissus* "Sir Winston Churchill" (Double); *Crinum* x *powellii* Baker; *Hymenocallis* x *festalis* "Zwanenburg"; and the whole plant of *Nerine bowdenii* Will. Watson, were obtained from F. Leenen B.V., Sassenheim, The Netherlands. The bulbs of *Narcissus* "Pipit" (Jonquilla); *Narcissus* "Carlton" (Large cupped); *Narcissus* "Empress of Ireland" (Trumpet); *Crinum asiaticum* L.; *Galanthus nivalis* L.; *L. aestivum*





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L.; *Scadoxus multiflorus* (Martyn)Raf.; and the aerial root of *Clivia nobilis* Lindl., were obtained from the botanical garden of the Division of Pharmacognosy, Leiden University, Leiden, The Netherlands. They were identified by a botanist.

## Standard Compounds

Galanthamine-HBr, morphine-HCl, codeine-HCl, thebaine-HCl, arecoline-HBr, nicotine, nornicotine, ephedrine-HCl, theophylline, apomorphine-HCl, cocaine-HCl, hyoscyamine-HCl, hydrastine-HCl, strychnine-HCl, ajmalicine-HCl, and quinine-HBr were obtained from Sigma Chemical Co. (St. Louis, MO).



## Reagents

Water was purified using a Christ ultrapure water system model Ministil P-12, equipped with: a Cuno carbon filter model 1N1-FC; a Cuno filter model 1M1 with a diameter of 5 μm, and a filter with a diameter of 0.2 μm. HPLC-grade acetonitrile and methanol were obtained from Rathburn Chemicals Limited (Walkerburn, UK). Ammonium hydroxyde (25%) and dichloromethane were obtained from Baker Analyzed (Deventer, Holland). *ortho*-Phosphoric acid (85%) and ammonium acetate (>96%) were obtained from E. Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) (>99%) was obtained from Merck-Schuchardt (Munich, Germany).

## HPLC Equipment

The chromatographic system consisted of: a LKB Bromma 2150 HPLC pump (Sweden), a Gilson sample injector model 231 with a Gilson dilutor model 401 (Villiers Le Bel, France), a Waters photodiode array (PDA) detector type 990 (Milford, MA) connected to a personal computer, and a Waters 5200 printer plotter (Milford, MA). The reversed phase columns used were: a Lichrospher RP Select B, $C_{18}$ (Merck, Darmstadt, Germany), 150 × 4.6 mm (I.D.), 5 μm, 90 Å; a Vydac $C_{18}$, 214MS54, 250 × 4.6 mm (I.D.), 5 μm, 300 Å (large pore) equipped with a Vydac guard column (Vydac, Hesperia CA), and a Vydac $C_{18}$, 201SP54, 250 × 4.6 mm (I.D.), 5 μm, 100 Å (small pore) equipped with a Vydac Guard Kit 201GK54SP (Vydac, Hesperia CA). The eluents were filtered through a 0.45 μm NL 17 membrane filter (Schleicher & Schuell, Dassel, Germany) and degassed under vacuum in an ultrasonic bath.



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## Sample Preparation

The alkaloid extraction was performed using an acid–base extraction method (method A) or using an acid extraction method (method B).[29] Both of the methods were used during the work in the optimization of the separation, and method B was found to be best suited for the quantitative analysis. The presence of galanthamine in the extract was confirmed by the comparison of its retention time and UV-spectra, with those of galanthamine (HBr) reference compound analysed under the same conditions. The quantitative measurements were performed at $\lambda$ 210 nm.

### Method A

The bulbs of *N. tazetta* and *Narcissus* "Sir Winston Churchill," stored at −20°C, were thawed at room temperature for 24 hours and subsequently chopped and weighed. The chopped material (10 g), with 45 mL of 0.1 M $H_3PO_4$, was ground using an ultra turrax (IKA, Germany, 8000 rpm, 8 min) and sonicated in an ultrasonic bath for 10 min. After the separation from the solid material by centrifugation (4500 rpm, 10 min) using a Minifuge GL (Heraeus-Christ Gmbh, Osterode, Germany), the acid extract was made basic with concentrated $NH_4OH$ until pH 9 and extracted with dichloromethane ($2 \times 50$ mL). The organic phases were collected and evaporated under vacuum until dryness. The dried extract was dissolved in 3 mL MeOH and centrifuged (13,000 rpm, 15 min) using a BHG HermLe Z 231 M centrifuge (B. HermLe Gmbh & Co., Gosheim, Germany), before injection (20 µL) into the HPLC.

### Method B

The bulbs, stored at −20°C, were thawed at room temperature for 24 hours. These thawed bulbs were chopped and subsequently blended in the presence of liquid nitrogen. For the quantitative determination, 109, 218, 327, and 436 mg of the blended material of *Narcissus* "Pipit" were extracted with 3 mL of the solvent (0.1% TFA in water), with ephedrine-HCl spiked as the internal standard. The extraction process was performed at room temperature using a Vibrofix VF1 (IKA, Germany) electronic vortex (2500 rpm, 3 min), followed by sonication in an ultrasonic bath for 20 min. Subsequently, the sediment (most of the solid material) was separated by pouring off the extract, which was then centrifuged (13,000 rpm, 15 min) using a BHG HermLe Z 231 M centrifuge. Before analysis, the extract was diluted to achieve the same concentration of the extract as that of the 109 mg bulb extract. The dilution of each extract was done in duplo, and each sample was injected (20 µL) at least





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in duplo, into the HPLC. In addition to these samples, the extract of 436 mg bulb in 12 mL solvent was also tested. For the qualitative determination, the amounts of plant material extracted can be increased to improve sensitivity.

### Calibration Curves

For the construction of the calibration curves of galanthamine and ephedrine, different amounts of these reference compounds were weighed and dissolved in the same amount of the acid solvent (0.1% TFA in water). Subsequently, via dilution (in duplo), a range of concentrations was obtained and injected in the HPLC system (in triplo). The mean of the resulting peak areas was used for the regression equation.

### Detection Limit and Galanthamine Recovery

The recovery of galanthamine was determined by adding galanthamine-HBr to ground bulbs of *N. tazetta* and following the sample preparation method B. Ephedrine was also spiked for comparison. To determine the detection limit, one of these extracts was diluted in order to obtain a series of concentrations. *Narcissus tazetta* used in our experiment did not contain galanthamine. Here, the same stock solutions of galanthamine-HBr and ephedrine-HCl as those for making the calibration curves, were used.

## RESULTS AND DISCUSSION

### Development of the HPLC System

To develop an isocratic HPLC system for the analysis of galanthamine in plant material, we started our experiment using a system that consisted of a Lichrospher RP select B $C_{18}$ column and 0.01 M ammonium acetate in methanol : water 30 : 70 v/v as the mobile phase.[9] With a flow rate of 1.5 mL/min, galanthamine (reference compound) eluted within 8 min. The *N. tazetta* extract tested did not show any galanthamine peak in the chromatogram. Using this extract spiked with galanthamine, some alkaloids (codeine, ajmalicine and yohimbine) were tested in this system as internal standard, but they were not suitable because of long retention times or overlapping peaks. Since we could not find an internal standard suitable for the quantitative analysis and we also wanted to improve the peak shape, we tested another system,[29] which was developed for the analysis of indole alkaloids from *Catharanthus roseus* leaves. Using this system, we found that





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the affinity of galanthamine was higher for the mobile phase than for the stationary phase. By increasing the amount of water to the composition of acetonitrile : water 3 : 97 v/v, a retention time was achieved within 9 min. However, the *Narcissus* "Sir Winston Churchill" extract contained a lot of compounds around the same retention time as galanthamine.

By changing to a Vydac $C_{18}$ column with smaller porosity, the problem could be solved. Several concentrations of acetonitrile in water were tested to obtain sufficient resolution and suitable retention of galanthamine and other compounds extracted from the plant material. Addition of 0.01% TFA in the mobile phase giving a pH of 3.4–3.6, provided good peak shape and seemed sufficient as ion pairing agent. Increasing the TFA concentration to 0.02% (pH 3.2–3.3) did not influence the peak shape but caused an increased-galanthamine retention. A baseline separation of the galanthamine peak was achieved in *Narcissus* "Pipit" extracts using the solvent system acetonitrile : water 5 : 95 v/v in the presence of 0.01% TFA, pumped at a flow rate of 1 mL/min. Galanthamine was eluted within 15 min. This solvent system was used for the next experiments. The first experiment was to apply this system for the qualitative screening of a series of Amaryllidaceae plants, for the presence of galanthamine.



## Screening for Galanthamine in Some Amaryllidaceae Species

The results are shown in Table 1. The system proved to be suitable for the retention of galanthamine. No problem of interfering peaks was observed. The next step was to further develop the system for quantitative analysis.

## The Search for a Suitable Internal Standard

Several alkaloids expected not to be produced by Amaryllidaceae species were selected in order to find an internal standard with a suitable retention time and similar extraction properties.

By considering their structural similarity with galanthamine, three morphinan alkaloids (morphine, codeine, and thebaine) were tested. Although, morphine eluted with a suitable retention time, it overlapped with some major peaks from the plant extract. Codeine and thebaine were excessively retained. Subsequently, a series of other alkaloids were tested (Table 2). Some of these were too strongly retained, whereas others overlapped with major peaks in the *Narcissus* "Pipit" extract. Finally, ephedrine was found to be most suited (Fig. 2).



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***Table 1.*** Occurrence of galanthamine in some Amaryllidaceae plant materials.

| Species | Material | Presence of galanthamine |
|---|---|---|
| *Clivia nobilis* Lindl. | Aerial root | − |
| *Crinum asiaticum* L. | Bulb | + |
| *Crinum* x *powellii* Baker | Bulb | − |
| *Galanthus nivalis* L. | Bulb | − |
| *Hymenocallis* x *festalis* "Zwanenburg" | Bulb | + |
| *Leucojum aestivum* L. | Bulb | + |
| *Narcissus tazetta* L. | Bulb | − |
| *Narcissus* "Pipit" (Jonquilla) | Bulb | + |
| *Narcissus* "Sir Winston Churchill' (Double) | Bulb | − |
| *Narcissus* "Carlton" (Large cupped) | Bulb | + |
| *Narcissus* "Empress of Ireland" (Trumpet) | Bulb | − |
| *Nerine bowdenii* Will. Watson | Whole plant | − |
| *Scadoxus multiflorus* (Martyn)Raf. | Bulb | − |

*Note:* The system consisted of, a Vydac $C_{18}$ small pore column (100 Å) and the mobile phase of TFA : acetonitrile : water (0.01 : 5 : 95, v/v/v), a flow rate 1 mL/min.
*Key:* −, Not present; +, present.

The effect of acetonitrile concentration in the mobile phase on retention of galanthamine and ephedrine is different (Fig. 3). To avoid interference with other peaks, we found 5% acetonitrile to be optimal.

## Detection Limit and Calibration Curves of Galanthamine and Ephedrine

The detection limits (the ratio of signal to noise was more than 3) of galanthamine and ephedrine were 0.02 and 0.06 nmol, respectively.

The calibration curves of both compounds were made and used to extrapolate the peak areas of galanthamine and ephedrine provided by each assay (Table 3).

## Sample Preparation, Recovery, Linearity of the Method, and Galanthamine Content

The extraction of the compounds to be analysed is an important part of a quantitative assay. The presence of the desired compound(s) in a measurable amount, a relatively pure extract with a minimum amount of undesirable





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***Table 2.*** The capacity factor $k'$ for galanthamine and some alkaloids tested as internal standard.

| Alkaloid | Capacity factor $k'$ |
|---|---|
| Galanthamine | 4.78 |
| Morphine | 2.61 |
| Codeine | 8.83 |
| Thebaine | >10 |
| Arecoline | 0.81 |
| Nornicotine | 0.44 |
| Nicotine | 0.98 |
| Ephedrine | 4.19 |
| Theophylline | 7.93 |
| Apomorphine | >10 |
| Cocaine | >10 |
| Hyoscyamine | >10 |
| Hydrastine | >10 |
| Strychnine | >10 |
| Ajmalicine | >10 |
| Quinine | >10 |

*Note:* The system consisted of, a Vydac $C_{18}$ small pore column (100 Å) and the mobile phase of TFA : acetonitrile : water (0.01 : 5 : 95, v/v/v), a flow rate 1 mL/min.



compounds, good reproducibility, and simplicity of the method, are the major goals in developing a sample preparation method.

Common methods for the extraction of galanthamine and other related alkaloids are based on an acid–base extraction method. In this experiment, the acid solvent (0.1% TFA in water) was used as the extraction solvent, similar to the extraction of indole alkaloids from *C. roseus* leaves.[29] Based on the property of alkaloids that can form salts with strong acids, 0.1% TFA in water was used to separate alkaloids from other neutral non-polar compounds. Such an extraction results in a relatively pure extract. Compared to the conventional acid–base extraction methods, which are time-consuming and laborious, this extraction method consists of fewer steps. TFA is a relatively strong acid, but in a reversed phase column, a low concentration of this acid can act as an ion-pairing agent for ionic compounds like alkaloids, which retards the compounds to the column. Combining the functions of the TFA as an acid extraction solvent and as an ion-pairing agent, has shown to be a viable approach that makes the present method simple and efficient. The HPLC system we developed makes such an extraction procedure possible.



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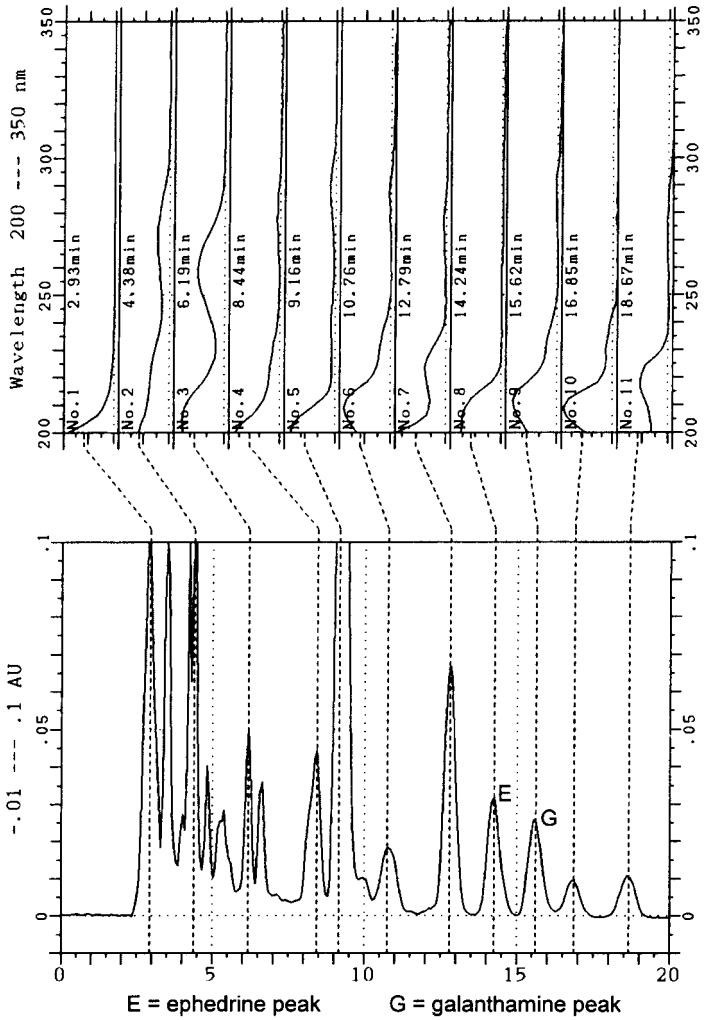


***Figure 2.*** The separation of *Narcissus* "Pipit" fresh bulb extract (0.1% TFA in water), spiked with ephedrine-HCl.

Using the TFA extraction method, the quantitative determination of galanthamine in *Narcissus* "Pipit" bulb was performed. Homogenous blended bulbs of *Narcissus* "Pipit" were spiked with ephedrine-HCl and were assayed to determine the recovery of ephedrine-HCl, the galanthamine content, and the linearity of the method. The results are shown in Table 4.

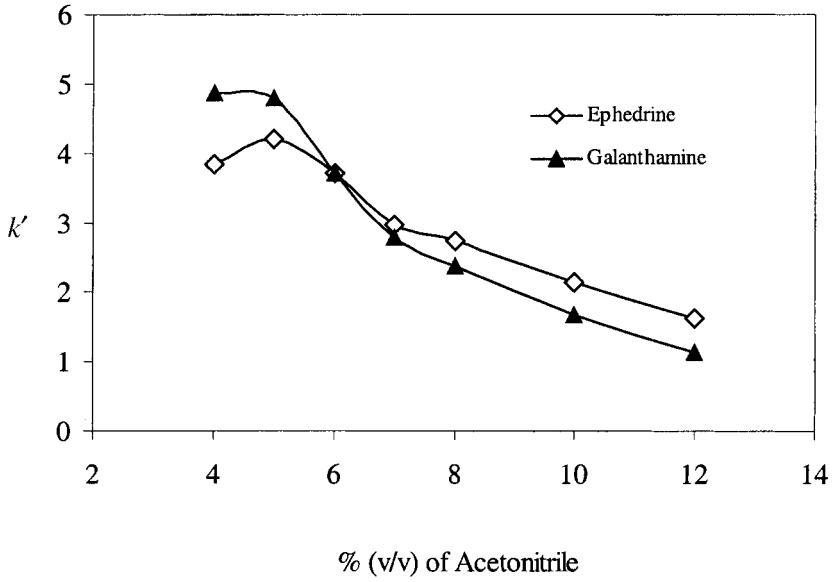


***Figure 3.*** The capacity factor $k'$ of ephedrine and galanthamine as function of acetonitrile % (v/v) in the mobile phase.

The galanthamine content in the bulb was 0.025%, as calculated after correction for the internal standard recovery, and showed good reproducibility. Spiking ephedrine in *N. tazetta* bulb material gave reproducible recoveries (Table 5). Also, recovery of galanthamine showed good reproducibility from this plant material, after spiking of these bulbs that do not contain endogenous galanthamine (Table 6).

***Table 3.*** The regression equations of galanthamine-HBr and ephedrine-HCl.

| Standard compound | Regression equation | $\gamma^2$ | Number of concentrations analyzed ($n$) |
|---|---|---|---|
| Galanthamine-HBr | $Y = 28333x - 5439$ | 0.9679 | 7 |
| Ephedrine-HCl | $Y = 33297x - 1031.8$ | 0.9996 | 6 |





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***Table 4.*** The recovery of ephedrine, extraction profile, and galanthamine content in *Narcissus* "Pipit" bulb.

| | | Ephedrine-HCl | | | Galanthamine | | | |
|---|---|---|---|---|---|---|---|---|
| Bulb extract (mg/mL) | Number of extract analyzed | Spiked (μg) | $M_1$[a] ± SD (μg) | Recovery (%)[b] ±SD | $M_2$[c] ± SD (μg) | $M_{2*}$[d] ± SD (μg) | w/w[b] (%) | $M_2/M_1$[b] |
| 109/3 | 7 | 73.75 | 68.52 ± 7.20 | 93.14 ± 9.89 | 25.16 ± 2.18 | 27.06 ± 0.74 | 0.025 | 0.37 |
| 218/3 | 3 | 147.50 | 141.96 ± 16.27 | 96.13 ± 11.21 | 53.91 ± 6.11 | 56.09 ± 0.32 | 0.026 | 0.38 |
| 327/3 | 3 | 221.25 | 202.53 ± 7.88 | 91.44 ± 3.66 | 76.67 ± 2.23 | 83.89 ± 1.89 | 0.025 | 0.38 |
| 436/3 | 3 | 295.00 | 281.87 ± 18.35 | 95.55 ± 6.22 | 103.47 ± 4.75 | 108.38 ± 2.2 | 0.025 | 0.37 |
| 436/12 | 3 | 295.00 | 290.02 ± 5.04 | 98.31 ± 1.71 | 110.99 ± 2.76 | 112.89 ± 1.02 | 0.026 | 0.38 |

*Note:* $M_2/M_1$, the absolute weight ratio of galanthamine and ephedrine (the small variation indicates a good consistency of the extraction profiles of both compounds); SD, standard deviation.
*Key:* % w/w, % weight/weight of galanthamine in bulb.
[a]$M_1$ is the absolute weight average of ephedrine-HCl analyzed.
[b]Average.
[c]$M_2$ is the absolute weight average of galanthamine analyzed.
[d]$M_{2*}$ is the average of galanthamine weight after correction with recovery of internal standard (ephedrine-HCl).

***Table 5.*** The recoveries of ephedrine-HCl from *N. tazetta* bulb material after spiking.

| Ephedrine-HCl stock solution used | | | Ephedrine-HCl in the extract | | | | |
|---|---|---|---|---|---|---|---|
| μg/mL | $C_1$[a] | $A_1$[b] | Number of extract analyzed | $C_s$[c] | $A_2$[b] | $C_2$[d] | Recovery average (%) |
| 2,950 | 2.925 | 18,212 | 4 | 2.438 | 13,437 | 2.158 | 88.52 |
| 4,056 | 2.413 | 15,281 | 3 | 2.413 | 13,975 | 2.207 | 91.45 |

*Note:* The recovery average, standard deviation and coefficient of variation was 89.78%, 2.33%, and 2.59% respectively.
[a]nmol/20 μL injected.
[b]Peak area average (AU∗min).
[c]nmol/20 μL spiked.
[d]nmol/20 μL analyzed (calculated by $C_2 = A_2/A_1 \times C_1$).

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***Table 6.*** The recoveries of galanthamine-HBr from *N. tazetta* bulb material after spiking.

| Galanthamine-HBr stock solution used | | | Galanthamine-HBr in the extract | | | | |
|---|---|---|---|---|---|---|---|
| µg/mL | $C_1$[a] | $A_1$[b] | Number of extract analyzed | $C_s$[c] | $A_2$[b] | $C_2$[d] | Recovery average (%) |
| 564 | 3.063 | 75,088 | 2 | 0.663 | 15,163 | 0.619 | 93.29 |
| 404 | 2.194 | 61,509 | 2 | 0.695 | 18,104 | 0.646 | 92.92 |
| 416 | 2.259 | 60,453 | 3 | 0.693 | 17,054 | 0.637 | 91.96 |

*Note:* The recovery average, standard deviation and coefficient of variation was 92.61%, 2.32%, and 2.50% respectively.
[a]nmol/20 µL injected.
[b]Peak area average (AU*min).
[c]nmol/20 µL spiked.
[d]nmol/20 µL analyzed (calculated by $C_2 = A_2/A_1 \times C_1$).

## CONCLUSION

An isocratic HPLC system of TFA : water : acetonitrile (0.01 : 95 : 5, v/v/v) combined with a small pore $C_{18}$ column such as the Vydac 201SP54, can be used for routine quantitative analysis of galanthamine from plant material extracted with 0.1% TFA in water. Ephedrine-HCl is suitable as internal standard. Compared to the acid–base extraction method, the alkaloid extraction with TFA contains fewer steps. The HPLC system we developed—which enables to analyse the extract directly—provides a simple and efficient analysis method. This rapid method has a good reproducibility.

*Narcissus* "Pipit" (Jonquilla) could be a good source for the galanthamine production.

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