Talanta 83 (2011) 1455–1465

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and validation of a GC–MS method for rapid determination of galanthamine in *Leucojum aestivum* and *Narcissus* ssp.: A metabolomic approach

Strahil Berkov[a,b,*], Jaume Bastida[a], Francesc Viladomat[a], Carles Codina[a]

[a] *Departament de Productes Naturals, Biologia Vegetal i Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain*
[b] *AgroBioInstitute, 8 Dragan Tzankov Blvd., 1164 Sofia, Bulgaria*

ARTICLE INFO

Article history:
Received 8 June 2010
Received in revised form 8 November 2010
Accepted 14 November 2010
Available online 19 November 2010

Keywords:
Galanthamine
Narcissus
Leucojum
In vitro
Metabolomics
Method validation
GC–MS

ABSTRACT

Galanthamine, an acetylcholinesterase inhibitor marketed as a hydrobromide salt for the treatment of Alzheimer's disease, is obtained from some Amaryllidaceae plants. A new method was developed and validated for its quantification by GC–MS in different plant sources: bulbs and leaves from *Narcissus confusus*; bulbs from *N. pseudonarcissus* cv. Carlton; and leaves and *in vitro* cultures from *L. aestivum*. Samples (50 mg) were extracted with methanol (1 mL) for 2 h, then aliquots of the extracts were silylated and analyzed by GC–MS. The calibration line was linear over a range of 15–800 μg galanthamine/sample, ensuring an analysis of samples with a content of 0.03–1.54% analyte referred to dry weight. The recovery was generally more than 95%. Good inter- and intra assay precision was observed (RSD < 3%). Principal component analysis of GC–MS chromatograms allowed discrimination of the plant raw material with respect to species, organs and geographical regions. The analytical method developed in this study proved to be simple, sensitive and far more informative than the routine analytical methods (GC, HPLC, CE and NMR), so it may be useful for quality control of plant raw materials in the pharmaceutical industry.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Galanthamine is an acetylcholinesterase (AChE) inhibitor marketed as a hydrobromide salt (Razadyne®, Reminyl®) for the treatment of Alzheimer's disease [1]. Although its chemical synthesis has been achieved, plants remain an important source for the pharmaceutical industry. Currently, galanthamine is extracted from *Leucojum aestivum* and *Narcissus pseudonarcissus* cv. Carlton in Europe, and from *Lycoris radiata* and *Ungernia victoris* in Asia [2]. The search for new plant sources of galanthamine is of great importance for the competitiveness of companies producing this valuable natural product.

The most reported method for the quantification of galanthamine has been HPLC [3–7], but its capacity to separate complex alkaloid mixtures is generally limited to about 5–7 alkaloids [8], and the separation conditions need to be optimized according to the alkaloid composition (more than 100 alkaloids have been found in the genus *Narcissus*) [9]. GC–MS studies have shown that the alkaloid mixtures of amaryllidaceous plants include more than 10–15 compounds [10,11]. GC–MS, CE-UV [12,13] and HPTLC [14] methods have been also validated for analysis of galanthamine in *Narcissus* bulbs.

Due to the complex composition of plant extracts, before a chromatographic determination the alkaloids are usually fractionated by means of basic–acidic liquid–liquid extraction [13,15,16] or solid-phase extraction [7,17]. The methods described in the literature are time-consuming and include laborious sample preparation procedures. They require a relatively high volume of solvents and the use of prepacked columns to handle a high number of samples, which increases the cost of analysis. Direct quantitative determination of galanthamine in unpurified plant extracts by enzyme immunoassays, radioimmunoassays and NMR analysis has been reported [18–20]. The first two methods are very sensitive, but they involve raising antibodies or the use of radioactive substances, making the studies laborious and expensive. In addition, they do not provide any information about the other metabolites in the samples. The NMR method quantifies galanthamine directly in methanol/water extracts and can determine the origin of the samples by multivariate data analysis of their metabolite fingerprints. Its main disadvantages, however, are the high cost of the equipment (600 MHz NMR), relatively low sensitivity compared with other methods, occasional problems with overlapping signals and a lower number of compounds identified in the extracts.

Metabolomics, including both targeted and global metabolite profiling strategies, is rapidly becoming the approach of choice across a broad range of sciences including systems biology, drug

---

[*] Corresponding author at: Departament de Productes Naturals, Biologia Vegetal i Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Tel.: +34 934020268; fax: +34 934029043.
E-mail address: berkov_str@yahoo.com (S. Berkov).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.11.029

discovery, molecular and cell biology, and other medical and agricultural sciences [21]. Earlier metabolite profiling of potato extracts demonstrated that the GC–MS platform is a powerful tool for simultaneous detection and identification of a number of metabolites (amino acids, organic acids, mono-, di-, and trisaccharides, sugar alcohols, and aromatic amines) and also for quantification of selected targets in complex plant matrixes [22]. Multivariate data analysis of metabolite profiles allows discrimination between plant species and cultivars and can therefore be applied for quality control of plant raw materials [23,24].

The aim of the present work was to develop and validate a simple and rapid method combining the advantages of GC–MS metabolic profiling (resolution power, sensitivity, selectivity, analysis of a wide spectrum of compounds after derivatization, MS libraries) and multivariate data analysis (discrimination between samples) for direct quantification of galanthamine in extracts and determination of the origin of plant raw material.

2. Experimental

2.1. Chemicals

Galanthamine hydrobromide was supplied by Galen-N Ltd. (Bulgaria) and its purity and identity were checked by GC–MS and ^{1}H-NMR. Methanol (HPLC grade), chloroform, sulfuric acid and ammonia (analytical grade) were purchased from SDS (France). The hydrocarbon mixture (C9-C36, Restek, Cat no. 31614) was supplied by Teknokroma (Spain). *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Plant material

Dry leaves from *Leucojum aestivum* L. grown in the Netherlands and Bulgaria were provided by Ludwig & Co (Lisse, Netherlands) and Galen-N (Sofia, Bulgaria), respectively. *In vitro* plantlets from *L. aestivum* were provided by VitroFlora (Trzesacz, Poland) and propagated and maintained until the analysis as previously described [25]. Bulbs from *Narcissus pseudonarcissus* cv. Carlton were supplied by Ludwig & Co. Bulbs and leaves from *Narcissus confusus* (Pursley) were obtained from plants grown in the greenhouse of the Faculty of Pharmacy at the University of Barcelona, Spain. Voucher specimens were deposited at the herbarium of the University of Barcelona (No. 32936; BCN 71625 and BCN 71626).

The fresh plant material was cut into small pieces and dried at 60 °C until constant weight. The dried samples were powdered and stored in a chamber maintaining a constant humidity level of 20% until the analysis.

2.3. Methods of analysis

2.3.1. Sample preparation

50 mg of dried plant material was macerated in screw-top Ependorf tubes (1.5 mL of volume) with 1 mL of methanol adjusted to pH 8 with 25% of ammonia and containing 50 μg of codeine as an internal standard (IS). After 2 h of extraction at room temperature assisted by an ultrasonic bath for 15 min every 30 min, the samples were centrifuged at 10,000 rpm for 1 min. Then, 300 μL aliquots were transferred to glass vials and dried by heating at 45 °C. 100 μL pyridine and 100 μL of BSTFA were added to the dried samples and heated at 70 °C for 2 h. After cooling, 300 μL of chloroform were added and the samples were analyzed by GC–MS.

For GC–MS analysis of alkaloid profiles, 500 μL aliquots were transferred to other Eppendorf tubes and 500 μL of 2% sulfuric acid in distilled water was added. The neutral compounds were eliminated by duplicate extraction (vortexing) with 500 μL chloroform. The mixtures were basified with 200 μL 25% ammonia and the alkaloids extracted in triplicate with 500 μL chloroform. The organic solvent was evaporated and the dry extract dissolved in 300 μL chloroform for further GC–MS analysis without derivatization.

2.3.2. Chromatographic conditions

The GC–MS spectra were recorded on a Hewlett Packard 6890+ MSD 5975 (Hewlett Packard, Palo Alto, CA, USA) operating in EI mode at 70 eV. A DB-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100–180 °C at 15 °C min^{-1}, 1 min hold at 180 °C and 180–300 °C at 5 °C min^{-1} and 1 min hold at 300 °C. Injector temperature was 280 °C. The flow rate of carrier gas (Helium) was 0.8 mL min^{-1}. The split ratio was 1:15. Ions at *m*/*z* 287, 286 and 174 were used to collect SIM chromatograms in SIM mode. For analysis of underivatized alkaloid fractions, a splitless injection was used. 1 μL of solutions was injected.

2.3.3. Standard solution

12.8 mg galanthamine HBr (equivalent to 10 mg of galanthamine base) was accurately weighted into a volumetric measuring flask of 10 mL. 2–3 drops of 25% ammonia were added and then dissolved in methanol.

2.4. Method validation

The method was validated according to the International Conference on Harmonisation (ICH) guidelines [26] on the validation of analytical methods. All results were expressed as μg/g of dry weight (DW). For statistical analysis Excel 2000 (Microsoft Office) and GraphPad Prizm v. 3.00 were used. A 5% level of significance was selected.

2.4.1. Response function-calibration model

Eight concentration levels of galanthamine trimethylsilyl (TMS) were prepared ranging from 15 to 800 μg/reference solution (0.5 mL). Each reference solution contained 50 μg of codeine (IS). Each concentration was analysed twice. The ratios of the peak areas of selected ions in total ion current (TIC) mode of galanthamine TMS (*m*/*z* at 358) versus those of codeine (*m*/*z* at 371) were plotted against the corresponding concentration of galanthamine to obtain the calibration graph.

2.4.2. Precision

For intermediate precision four separate samples (100% or 50 mg) were analysed on day 1 and this was repeated on 3 consecutive days. Every sample was injected once. For repeatability at different concentration levels (linearity of the method) four samples with half the amount (50% or 25 mg) and four samples with twice the amount (200% or 100 mg) were analysed using the same method.

2.4.3. Accuracy

The accuracy of the method was investigated by means of a recovery experiment. To 50% of samples (25 mg), a standard solution of galanthamine base was added at three different concentration levels (50%, 100% and 120%) at the start of the analysis of *N. pseudonarcissus* cv. Carlton bulbs, *N. confusus* bulbs and leaves and *L. aestivum* leaves. Five different concentration levels (50%, 100%, 120%, 200% and 230%) were tested for samples (50% or 25 mg) of *in vitro* obtained cultures of *L. aestivum*. For each of the concentrations, four samples were analysed according to the developed method.

2.4.4. Specificity

To test the specificity of the method, the peak purity of galanthamine TMS was investigated by AMDIS 2.64 software (NIST, National Institute of Standardization and Technology, Gaithersburg, MD).

2.5. Data analysis

2.5.1. Identification of the metabolites

The compounds of the methanolic extracts were identified as TMS-derivatives with the help of the NIST 05 database (NIST Mass Spectral Database, PC-Version 5.0, 2005) and other plant-specific databases: the Golm Metabolome Database [27], lipid library [28] as well as literature data [29] on the basis of matching mass spectra and Kovats retention indexes (*RI*). The measured mass spectra were deconvoluted by AMDIS 2.64 before comparison with the databases. The spectra of individual components were transferred to the NIST Mass Spectral Search Program MS Search 2.0, where they were matched against reference compounds of the NIST Mass Spectral Library 2005 and the Golm Metabolome Database. The groups of unidentified compounds were determined on the basis of their specific mass spectral fragmentation and in comparison with the mass spectra of known metabolites.

RI values of the compounds were measured with a standard *n*-hydrocarbon calibration mixture (C9–C36) using AMDIS 2.64 software.

2.5.2. Principal component analysis

A target compound library was constructed from the analysed samples by AMDIS software including MS spectra, retention indexes and retention times. The samples were processed using retention index calibration data and the results were exported to an Excel format. The integrated values of the target compounds were normalized to the value of the internal standard (codeine). Principal component analysis (PCA) was performed with Unscrambler® (version 9.8, COMO software Inc.).

3. Results and discussion

In the present work samples from bulbs and leaves of *N. confusus*, bulbs of *N. pseudonarcissus* cv. Carlton, leaves from *L. aestivum* and *in vitro* cultures from *L. aestivum*, representing different matrixes, were used for the validation of galanthamine quantification. These plant raw materials have a broad range of galanthamine content. *N. confusus* is a galanthamine-rich plant species considered as a promising new source of this valuable alkaloid [17]. Plant biotechnology may also play an important role in galanthamine supply [30]. In the last 3–5 years studies of galanthamine biosynthesis have been mainly restricted to *in vitro* cultures of *L. aestivum* [30,31]. Therefore, a single validated method covering a broad range of concentration will be of use for the quality control of available and potentially new sources of galanthamine.

3.1. Method development

3.1.1. Sample preparation

Separation (clean-up) of a target compound or a group of structurally related compounds is a crucial procedure in method development and validation by HPLC, GC, CE, HPTLC, etc. In contrast, sample preparation procedures in GC–MS metabolic profiling aiming at the identification and (relative) quantification of the maximum number of metabolites are considerably more simple, including the separation of the total extract into polar and apolar fractions or when dealing with total extracts. In a former study on the extraction efficiency of various solvents, methanol followed by 1% tartaric acid methanolic solution was found to be the most effective for extracting galanthamine and other alkaloids [7]. We decided to work with the total methanol extracts, thus avoiding the separation step for polar and apolar metabolites.

Galanthamine is a tertiary amine with a pK_a of 8.2 [32] and therefore its base form has better solubility in organic solvents (including methanol) than its protonated (salt) form. We optimized the methanol extraction (using leaves of *L. aestivum*) with respect to pH and time, testing pH 7 and 8 at 1, 2, 4 and 6 h. The results, evaluated by means of an ANOVA single factor, showed that the galanthamine content extracted over 2 h with methanol at pH 7 was less (159.0 ± 1.7 μg/DW) than at pH 8 (164.3 ± 1.3 μg/DW), with longer extraction times producing no significant improvements. Therefore, the extraction of the final method was performed for 2 h with methanol adjusted to pH 8.

The preliminary observations indicated that sugars are the main group of compounds in the methanolic extracts. Aiming at reproducible derivatization and chromatograms, respectively, which can be further used for PCA analysis, we applied a derivatization procedure adapted for sugars [29]. The derivatization test (at 70 °C for 1, 2, 3, 4 and 6 h) indicated that after 2 h of reaction time there were no significant differences in the chromatograms. Derivatization of the standards from galanthamine and codeine for 2 h in the conditions described in Section 2.3.1 was complete. Representative chromatograms are shown in Fig. 1.

3.1.2. 3.1.2.Chromatographic conditions

Working with 300 μL of extract aliquots, a split ratio of 15:1 was found to be optimal with respect to sensitivity to galanthamine TMS and overloading of the column. The temperature ramp used was previously found to be suitable for separation of various groups of metabolites in other amaryllidaceous species [33]. Galanthamine TMS was well separated from the other metabolites in all tested samples. Codeine, an alkaloid structurally similar to galanthamine but not synthesized in the Amaryllidaceae family, was found to be suitable as the IS for quantification of galanthamine by HPLC and GC [16,34].

3.2. Validation

3.2.1. Response function-calibration model

The calibration was performed plotting the ratio of peak areas of selected ions of galanthamine TMS (m/z at 358) reference standard (15–800 μg) versus that of the internal standard codeine TMS (50 μg, m/z at 371). The regression line was constructed and tested on slope and intersept. In order to evaluate the lack-of-fit (LOF) of the linear model a LOF test was performed and the residuals were calculated and graphically examined (Fig. 2). The values of standard deviation of the residuals (0.078) and F_{LOF} (0.47, $F_{\text{crit}} = 4.89$) indicated a good linearity response on the selected range.

A wide range of the calibration model was chosen due to the broad variation of galanthamine content in plants. In *L. aestivum* leaves, for example, the amount of galanthamine ranged from 0.03 to 0.57% of DW (unpublished results). The proposed calibration model ensures quantification of galanthamine in plant samples (50 mg) ranging from 0.03 to 1.59% of DW, which covers the biological variability of the studied species. The *in vitro* cultures, especially undifferentiated callus cultures, may have a very low galanthamine content [31]. For quantification of lower concentrations, another calibration line and validation are required. In this case, the method may be modified with respect to the split, but the flexibility is limited due to a possibility of column overloading. A better option would be to work in SIM (selected ion monitoring) mode, where the sensitivity of the MS detector is significantly higher [16]. Such a validation was not performed because plant raw materials with a very low galanthamine content are not of practical interest.

3.2.2. Limits of detection and limits of quantification

The limits of detection and quantification were determined by analysis of samples with a known amount of analyte. The limit of

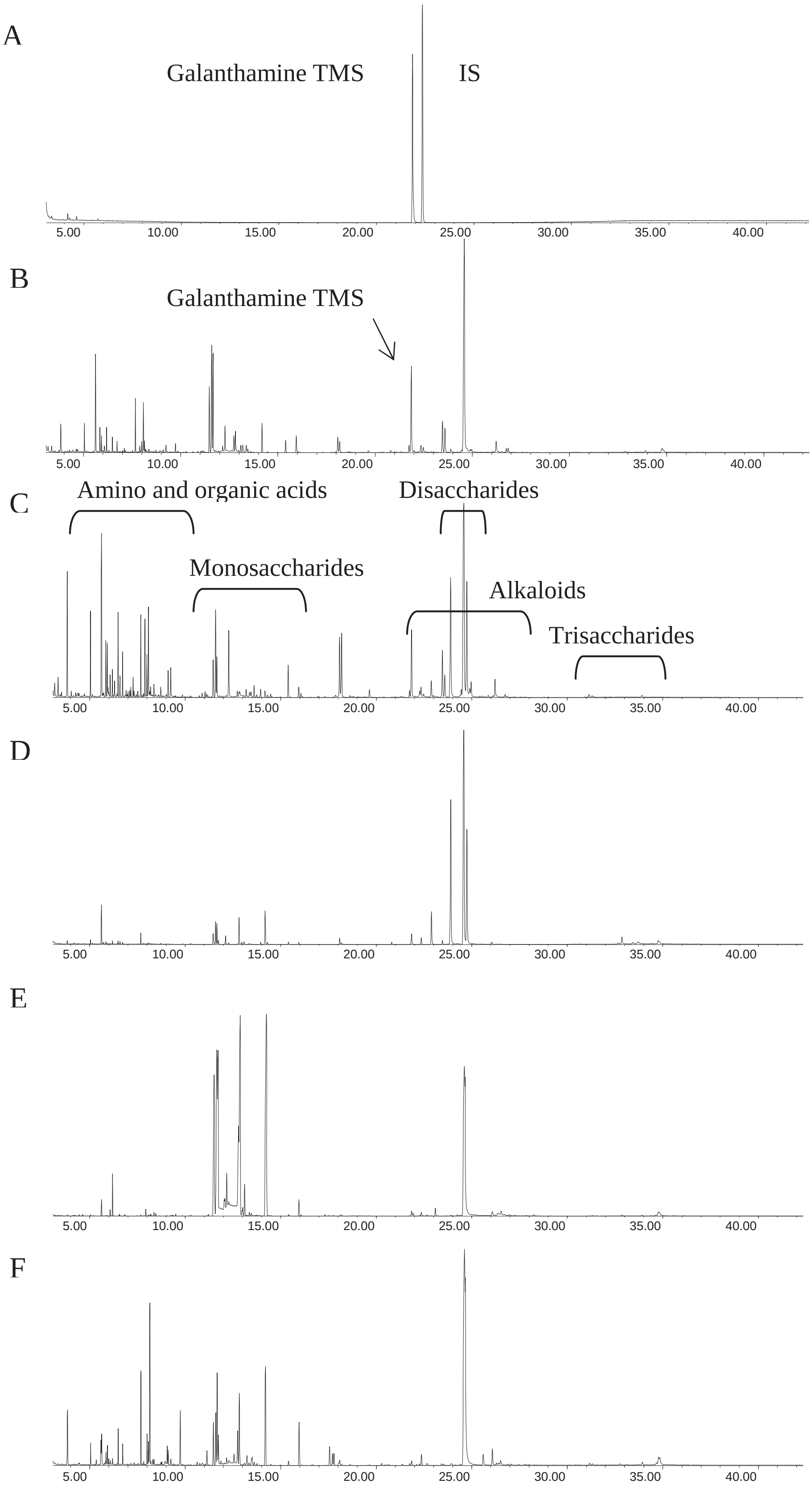

Fig. 1. GC–MS of silylated standards of galanthamine and codeine (A) and methanol extracts from *N. confusus* bulbs (B), *N. confusus* leaves (C), *N. pseudonarcissus* cv. Carlton bulbs (D), *L. aestivum* leaves (E) and *L. aestivum in vitro* shoot-clumps (F).

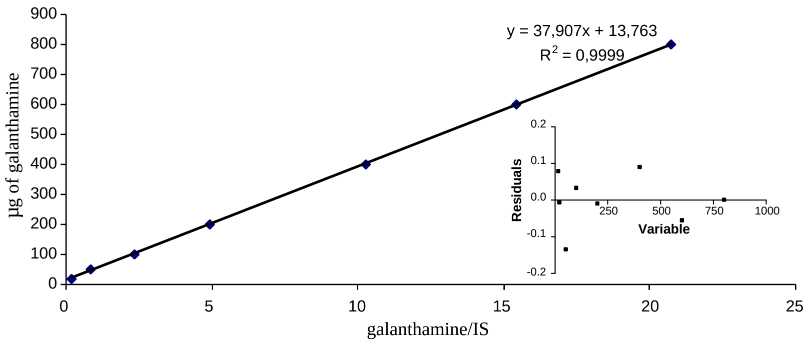

Fig. 2. Calibration curve and residual plot of galanthamine TMS.

Table 1
Validation data: precision.

	N. pseudonarcissus cv. Carlton bulbs	*N. confusus* bulbs	*N. confusus* leaves	*L. aestivum* leaves	*L. aestivum in vitro* shoot-clumps
Precision on different days ($n = 3$)					
Repeatability					
Mean (μg/g DW) ± SD (RSD%)					
Day 1	151 ± 6 (2.09)	1211 ± 21 (1.71)	708 ± 18 (2.52)	166 ± 2 (1.30)	68 ± 1 (1.59)
Day 2	149 ± 6 (3.69)	1183 ± 23 (1.93)	716 ± 11 (1.51)	164 ± 2 (0.98)	67 ± 2 (2.28)
Day 3	154 ± 3 (2.15)	1215 ± 14 (1.27)	710 ± 5 (0.65)	167 ± 5 (3.20)	68 ± 1 (0.77)
Intermediate precision					
Number of groups	3	3	3	3	3
Number of replicates	4	4	4	4	4
RSD (%) between groups/Horwitz[a]	2.72/3.53	1.93/2.61	1.78/2.82	1.89/3.52	1.95/4.08
Precision on concentration levels					
Repeatability					
Number of replicates	4	4	4	4	4
Mean (μg/g DW) ± SD (RSD%) 50%	164 ± 4 (2.08)	1233 ± 35 (2.86)	688 ± 14 (2.10)	166 ± 3 (1.64)	69 ± 2 (3.15)
Mean (μg/g DW) ± SD (RSD%) 200%	154 ± 1 (0.47)	1254 ± 56 (4.43)	672 ± 6 (0.78)	164 ± 2 (1.33)	58 ± 1 (1.41)

[a] 2/3 RSD% Horwitz.

detection was found to be 1 μg/sample (2 μg/mL) with a signal to noise ratio (S/N) of *ca.* 3:1. The limit of quantification was accepted as 5 μg/sample (10 μg/mL) showing S/N of 42:1 (ICH requires a value > 10:1) and good precision (RSD 2.29%, $n = 4$).

The sensitivity of the detector in SIM mode was also tested, indicating a detection limit of about 1 ng/sample (2 ng/mL, S/N of *ca.* 3:1) and limit of quantification of 5 ng/sample galanthamine (S/N of *ca.* 10:1). Thus, an amount of galanthamine as low as 0.000002% of DW can be detected in the samples (50 mg).

3.2.3. *Precision*

The precision was investigated at two levels: on different days, including repeatability (precision under the same conditions over a short period of time—one day) and intermediate precision (on different days), and precision at different concentrations. The mean, standard deviation and %RSD for each day and concentration level were calculated. The results indicated good intermediate precision (Table 1). With the exception of the values for the second day of the repeatability test for *N. pseudonarcissus* cv. Carlton, and those for the precision at concentration levels of *N. confusus* bulbs (RSD < 5%), within and between day and level RSDs were less than the limits set by the modified Horwitz equation [35]. ANOVA single factor analysis of the results showed that the mean values of the precision test for each type of plant material were not statistically different, with the exceptions of those for 50% level of bulbs from *N. pseudonarcissus* cv. Carlton and 200% levels of leaves from *N. confusus* and *in vitro* cultures from *L. aestivum*, showing deviations of +8%, −6% and −17%, respectively, which could be attributed to matrix effects.

3.2.4. *Accuracy*

The accuracy of the method was investigated by means of recovery experiments, adding a known amount of galanthamine to the samples at the start of the extraction. A mean recovery and %RSD were calculated. The accuracy was checked at 3 levels (50%, 100% and 120%) for the samples from the intact plants and at 5 levels (50%, 100%, 120%, 200% and 230%) for the *in vitro* samples considering the possible variation of galanthamine content due to further genetic or nutrient medium improvement. The results, presented in Table 2, showed acceptable recovery regarding the concentrations and the purpose of analysis [36].

Table 2
Validation data: repeatability and recovery by spiking of different amounts of galanthamine ($n = 3$).

	N. pseudonarcissus cv. Carlton bulbs	*N. confusus* bulbs	*N. confusus* leaves	*L. aestivum* leaves	*L. aestivum in vitro* shoot-clumps
50%: spiked (μg)/recovery (%)/RSD (%)	40/99.11/0.76	300/89.94/1.75	175/92.20/3.13	40/98.02/1.17	15/102.58/2.29
100%: spiked (μg)/recovery (%)/RSD (%)	80/93.09/2.48	600/93.63/2.11	350/94.60/1.78	80/97.85/2.42	30/101.29/2.52
120%: spiked (μg)/recovery (%)/RSD (%)	100/96.20/1.19	720/97.37/3.24	420/92.75/1.62	100/91.90/2.77	40/99.42/1.20
200%: spiked (μg)/recovery (%)/RSD (%)					60/95.60/2.19
230%: spiked (μg)/recovery (%)/RSD (%)					70/95.90/1.14

Table 3
Metabolites detected in the studied samples.

Compound	Rt	*N. pseudonarcissus* cv. Carlton bulbs	*N. confusus* bulbs	*N. confusus* leaves	*L. aestivum* leaves (BG)[a]	*L. aestivum* *in vitro* shoot-clumps	*L. aestivum* leaves (TR)[a]	*L. aestivum* leaves (NL)[a]
uc (**1**)	3.17	5 ± 1	30 ± 3	33 ± 0.5			<1	
Lactic acid (**2**)	3.37	2 ± 0.3	30 ± 4	84 ± 4	5 ± 1	1 ± 0.2	2 ± 1	2 ± 0.4
Glycolic acid (**3**)	3.55		5 ± 1	17 ± 1	7 ± 1	2 ± 0.4	1 ± 0.2	2 ± 0.3
L-Valine 1TMS (**4**)	3.75		6+4				1+1	1+1
L-Alanine (**5**)	3.84	10 ± 1	150 ± 25	531 ± 25	12 ± 1	197 ± 26	104 ± 2	117 ± 10
Glycine 2TMS (**6**)	4.04	<1	5 ± 1	24 ± 1				
uc (**7**)	4.15				6 ± 1		<1	1 ± 0.1
Pyruvic acid (**8**)	4.29		8 ± 1	13 ± 1	5 ± 0.2	1 ± 0.5	4 ± 1	4 ± 1
α-Hydroxybutyric acid (**9**)	4.46	>1	3 ± 0.4	12 ± 0.4	13 ± 1	5 ± 1	23 ± 1	3 ± 0.1
uc (**10**)	4.64	<1	11 ± 3	<1	16 ± 1	<1	6 ± 0.4	7 ± 1
L-Valine 2TMS (**11**)	5.06	13 ± 1	130 ± 21	278 ± 10	11 ± 2	63 ± 8	17 ± 0.3	31 ± 2
uc (**12**)	5.15	2 ± 0.4	5 ± 1	13 ± 1			<1	<1
4-Hydroxybutanoic acid (1**3**)	5.28	<1		<1		2 ± 1	<1	<1
Urea (**14**)	5.35		<1	1 ± 0.4		16 ± 3		<1
Serine-2TMS (**15**)	5.52	<1	4 ± 1	4 ± 0.4		2 ± 1		<1
uc (*N*-containing) (**16**)	5.60		3 ± 1		<1	44 ± 6	6 ± 1	5 ± 0.2
Glycerol (**17**)	5.62	101 ± 5	381 ± 28	557 ± 32	122 ± 6	89 ± 41	262 ± 8	196 ± 6
Phosphoric acid (**18**)	5.64	5 ± 0.3	119 ± 14	87 ± 20		18 ± 13		
uc (**19**)	5.74		2 ± 0.4	15 ± 2				
uc (**20**)	5.83		<1	<1	1 ± 0.2	4 ± 2	1 ± 0.3	
Isoleucine (**21**)	5.86	6 ± 0.3	99 ± 18	163 ± 5		30 ± 4	6 ± 0.2	14 ± 1
Proline (**22**)	5.93	2 ± 0.3	69 ± 15	196 ± 4	2 ± 1	55 ± 9	7 ± 1	11 ± 2
4-Aminobutyryc acid (**23**)	5.96			110 ± 5				
Glycine 3TMS (**24**)	6.01		5 ± 1	2 ± 2		15 ± 1	<1	<1
uc (**25**)	6.08	1 ± 0.3	9 ± 4		51 ± 12		14 ± 3	20 ± 1
Succinic acid (**26**)	6.09	1 ± 0.2	23 ± 1	62 ± 2		10 ± 1		
Glyceric acid (**27**)	6.20	7 ± 1	87 ± 6	78 ± 4	299 ± 13	21 ± 2	305 ± 21	312 ± 9
Fumaric acid (**28**)	6.44	1 ± 0.4	<1	9 ± 1		3 ± 1		
L-Serine 3TMS (**29**)	6.50	7 ± 5	63 ± 10	216 ± 10	1 ± 0.3	83 ± 11	2 ± 0.2	4 ± 1
uc (**30**)	6.57				13 ± 0.4		2 ± 3	6 ± 0.4
2-Pyperidinecarboxylic acid (**31**)	6.59	7 ± 5	3 ± 1	60 ± 2	1 ± 0.4		<1	<1
L-Threonine (**32**)	6.73	4 ± 3	44 ± 7	127 ± 6	1 ± 1	49 ± 7	2 ± 0.4	7 ± 1
uc (**33**)	6.85				10 ± 0.3		6 ± 2	8 ± 1
Thymine (**34**)	6.91		2 ± 1	17 ± 1				
2,4-Dihydroxybutanoic acid (**35**)	6.98		1 ± 0.1	6 ± 0.2	1 ± 0.4		2 ± 0.4	3 ± 0.2
3,4-Dihydroxybutanoic acid (**36**)	7.17		6 ± 0.5	26 ± 1	1 ± 0.1		<1	<1
β-Alanine 3TMS (**37**)	7.17					3 ± 1		
Homoserine (**38**)	7.33		1 ± 0.3	16 ± 0.4		3 ± 1		
uc (**39**)	7.46		1 ± 0.1	1 ± 0.2	3 ± 0.3		2 ± 0.2	2 ± 0.1
L-Aspartic acid (**40**)	7.54	1 ± 9.4	7 ± 1	7 ± 1	<1	3 ± 1	4 ± 1	4 ± 1
Malic acid (**41**)	7.68	27 ± 1	194 ± 13	242 ± 9	9 ± 1	256 ± 22	20 ± 1	16 ± 1
Erythritol (**42**)	7.83	2 ± 0.3	2 ± 1	9 ± 0.4	3 ± 0.5	8 ± 1	10 ± 0.2	7 ± 0.4
Parabanic acid (**43**)	7.90	2 ± 0.3	36 ± 9	263 ± 12				
2-Erythro-pentanoic acid (**44**)	7.94		3 ± 1		67 ± 3		2 ± 1	1 ± 0.3
L-Aspartic acid isomer (**45**)	8.01	2 ± 0.4	43 ± 6	119 ± 5		75 ± 9		<1
Pyroglutamic acid (**46**)	8.09	5 ± 1	232 ± 16	335 ± 14	12 ± 2	70 ± 10	9 ± 1	12 ± 1
4-Aminobutyric acid (**47**)	8.15	<1	51 ± 6	2 ± 1	3 ± 1	539 ± 42	16 ± 2	36 ± 3
Erythronic acid (**48**)	8.20		9 ± 2	28 ± 2	16 ± 1	7 ± 0.2	16 ± 1	12 ± 0.4
Norvaline (**49**)	8.31					13 ± 2		
Erytronic acid isomer (**50**)	8.37	1 ± 0.1	12 ± 1	42 ± 1	33 ± 2	14 ± 2	38 ± 3	49 ± 1
uc (**51**)	8.47		1 ± 2	<1	23 ± 2		<1	<1
2-Hydroxyglutaric acid (**52**)	8.58		<1	3 ± 0.2		2 ± 1		
4-Hydroxyphenylbutanol (**53**)	8.63	<1	4 ± 0.3	5 ± 0.1				
uc (organic acid) (**54**)	8.74	2 ± 1	9 ± 0.5	12 ± 1	<1	8 ± 1	2 ± 0.2	2 ± 0.3
2,3-Dihydroxybutanedioic acid (**55**)	8.79		1 ± 0.3	3 ± 1		7 ± 1	1 ± 0.2	
uc (organic acid) (**56**)	8.95	1 ± 1	17 ± 3	1 ± 2		21 ± 5		
Arabinose (**57**)	9.00				3 ± 0.4			
Ornithine (**58**)	9.06					43 ± 10		
uc (**59**)	9.07				4 ± 0.5	3 ± 0.1		<1
Glutamine (**60**)	9.12	3 ± 0.2	11 ± 2	113 ± 5		42 ± 4		1 ± 0.2
Xylonic acid (**61**)	9.23		2 ± 0.2	3 ± 0.2	7 ± 1		2 ± 0.4	3 ± 0.3
L-Phenylalanine (**62**)	9.26	<1	39 ± 5	122 ± 4		20 ± 4	<1	1 ± 0.2
Arabinoic acid (**63**)	9.32		6 ± 0.5	4 ± 3	11 ± 1	<1	<1	1 ± 0.2
Arabinose 2 (**64**)	9.37	<1	<1		5 ± 1		1 ± 1	1 ± 1
uc (organic acid) (**65**)	9.40			<1		3 ± 0.2	<1	
3,4,5-Trihydroxypentanoic acid (**66**)	9.52			3 ± 1	23 ± 2		1 ± 0.2	2 ± 0.4
p-Hydroxyphenylacetic acid (**67**)	9.42		1 ± 0.5	4 ± 0.2				
Asparagine (**68**)	9.75	<1	56 ± 10	11 ± 2		186 ± 33		
Arabinitol (**69**)	10.29	5 ± 2	6 ± 1	6 ± 0.5	11 ± 1	1 ± 0.3	1 ± 1	<1
2,3,4,5-Tetrahydroxypentanoic acid (**70**)	10.63			2 ± 1	1 ± 0.3			2 ± 0.1
Putrescine (**71**)	10.63					11 ± 2	1 ± 0.1	

Table 3 (*Continued*)

Compound	Rt	*N. pseudonarcissus* cv. Carlton bulbs	*N. confusus* bulbs	*N. confusus* leaves	*L. aestivum* leaves (BG)[a]	*L. aestivum in vitro* shoot-clumps	*L. aestivum* leaves (TR)[a]	*L. aestivum* leaves (NL)[a]
uc (**72**)	10.77					7 ± 1		
Ribonic acid (**73**)	10.75			8 ± 0.3	<1		1 ± 0.2	1 ± 0.1
2-Keto-L-gluconic acid (**74**)	10.90		8 ± 1	24 ± 1		7 ± 1		
Glycerophosphoric acid (**75**)	10.91	<1	<1	2 ± 0.1		1 ± 0.4		6 ± 2
Ribonic acid isomer (**76**)	11.04		8 ± 1	27 ± 1				
2-Ketogluconic acid (**77**)	11.05				2 ± 1		<1	1 ± 0.2
Ribonic acid isomer?(**78**)	11.14		8 ± 2	15 ± 13	4 ± 1			1 ± 0.2
Glutamine (**79**)	11.15			41 ± 4		52 ± 7		
uc (**80**)	11.19		8 ± 0.4	8 ± 0.5				
uc (**81**)	11.20	<1			28 ± 6		26 ± 1	30 ± 5
uc (**82**)	11.23		4 ± 3			1 ± 0.1		
Fructosa-1 (**83**)	11.51	29 ± 6	360 ± 9	196 ± 66	2350 ± 214	167 ± 9	1289 ± 84	1390 ± 28
Fructose-2 (**84**)	11.65	85 ± 5	611 ± 9	159 ± 107	2006 ± 657	196 ± 23	1095 ± 604	1235 ± 446
Fructose-3 (**85**)	11.73	68 ± 3	622 ± 57	93 ± 31	241 ± 456	342 ± 45	1245 ± 100	1666 ± 162
Isocitric acid (**86**)	11.74	22 ± 1	3 ± 1	1 ± 0.3		92 ± 30		
uc (**87**)	11.96		5 ± 1	13 ± 1				
Altrose (**88**)	12.08	<1	<1		52 ± 19	2 ± 0.4	27 ± 18	36 ± 16
uc (**89**)	12.16			5 ± 2				<1
Fructose-4 (**90**)	12.18	1 ± 1	36 ± 4	4 ± 1	325 ± 41	22 ± 4	82 ± 4	139 ± 6
uc (**91**)	12.10	55 ± 3						
Quinic acid (**92**)	12.29	5 ± 0.2	145 ± 8	349 ± 17		8 ± 1		
uc (organic acid) (**93**)	12.56			1 ± 0.3		29 ± 4		
uc (organic acid) (**94**)	12.80	2 ± 0.3	95 ± 11	14 ± 3	1197 ± 25	129 ± 12	342 ± 40	694 ± 52
uc (monosaccharide) (**95**)	12.87	85 ± 15	121 ± 9	6 ± 2	3965 ± 577	298 ± 28	1757 ± 70	2126 ± 135
Tyrosine 2TMS (**96**)	12.86	1 ± 0.5	29 ± 2	34 ± 9			<1	
Glucose 1 (**97**)	13.01	8 ± 6	11 ± 1	7 ± 1	105 ± 4	2 ± 0.2	84 ± 0.4	22 ± 4
nc (**98**)	13.11	5 ± 2	55 ± 1	2 ± 1	464 ± 89	10 ± 1	1266 ± 104	1295 ± 163
3,4-Dihydrophenylethylamine (**99**)	13.24		16 ± 4			44 ± 4		3 ± 0.4
Lysine (**100**)	13.34					9 ± 2		
Glucitol (**101**)	13.36	4 ± 1	5 ± 5	19 ± 14	50 ± 8	21 ± 3	2 ± 1	2 ± 1
uc (phosphate) (**102**)	13.38		6 ± 12	25 ± 4				
Glucosamine (**103**)	13.38		36 ± 1		5 ± 1		1 ± 1	1 ± 2
uc (monosaccharide) (**104**)	13.51					25 ± 2		
L-Tyrosine 3TMS (**105**)	13.62		11 ± 5	77 ± 4		13 ± 3		
uc (monosaccharide) (**106**)	13.76		1 ± 0.4		7 ± 2	6 ± 1	6 ± 1	
uc (monosaccharide) (**107**)	13.95	9 ± 1					4 ± 2	2 ± 1
Glucose 2 (**108**)	14.20	103 ± 15	180 ± 12	13 ± 5	3289 ± 186	408 ± 47	2093 ± 113	2098 ± 561
nc (**109**)	14.31	11 ± 1	<1	41 ± 5				
Gluconic acid (**110**)	14.51		7 ± 1	13 ± 6	3 ± 1	3 ± 1		
Hexadecanoic acid (**111**)	15.54	20 ± 3	86 ± 7	181 ± 4	25 ± 4	16 ± 8	58 ± 3	37 ± 1
Mioinositol (**112**)	15.96	5 ± 1	114 ± 4	14 ± 2	199 ± 8	203 ± 25	439 ± 5	491 ± 22
nc (**113**)	16.06		6 ± 1	19 ± 3	15 ± 1			4 ± 1
nc (**114**)	17.32		3 ± 0.4	4 ± 1	22 ± 2		<1	3 ± 0.4
uc (monosaccharide) (**115**)	17.56				2 ± 2	95 ± 10	6 ± 1	
uc (monosaccharide) (**116**)	17.71		<1		<1	52 ± 5	1 ± 1	
nc (**117**)	17.78		2 ± 1	1 ± 1	8 ± 1			
Tryptophan (**118**)	17.87		2 ± 2	21 ± 2				
nc (phosphomonosaccharide) (**119**)	18.04				3 ± 1	15 ± 2	10 ± 1	19 ± 2
9,12-Octadecadienoic acid (**120**)	18.10	50 ± 5	114 ± 12	376 ± 15	3 ± 1	25 ± 5	26 ± 3	36 ± 3
nc (**121**)	18.15				24 ± 4			
Linoleic acid (**122**)	18.19		89 ± 5	373 ± 34			157 ± 6	131 ± 5
9-Octadecaenoic acid (**123**)	18.20	13 ± 2	<1	2 ± 1	3 ± 3	4 ± 1		
Octadecanoic acid (**124**)	18.64	2 ± 1	7 ± 1	11 ± 1	3 ± 2	2 ± 1	5 ± 1	5 ± 0.3
uc (disaccharide) (**125**)	19.65	<1	14 ± 1	47 ± 2	4 ± 0.5		49 ± 1	86 ± 1
uc (disacharide) (**126**)	20.29				1 ± 0.2	8 ± 1	1 ± 0.3	1 ± 0.1
uc (**127**)	20.80	26 ± 4						
uc (disaccharide) (**128**)	21.37					7 ± 2		1 ± 1
Uridine (**129**)	21.75		52 ± 3	41 ± 3	7 ± 1		2 ± 0.4	1 ± 0.1
Galanthamine (**130**)	21.86	75 ± 6	671 ± 21	433 ± 20	83 ± 5	23 ± 4	34 ± 4	19 ± 1
nc (**131**)	21.93				37 ± 2			
uc (disaccharide) (**132**)	22.27					7 ± 1		
uc (disaccharide) (**133**)	22.28		3 ± 2	23 ± 4	9 ± 2			
N-Demethylgalanthamine (**134**)	22.48		46 ± 3	21 ± 0.4	<1			
nc (**135**)	22.66				18 ± 2	10 ± 1	82 ± 3	25 ± 1
uc (disaccharide) (**136**)	22.89	411 ± 101	4 ± 1	732 ± 144	2 ± 1		6 ± 0.3	2 ± 1
uc (alkaloid) (**137**)	23.00		3 ± 1	10 ± 1				
Lycorine (**138**)	23.10				112 ± 7		86+4	266+9
Haemanthamine (**139**)	23.47	27 ± 2	243 ± 7	287 ± 12				
Pretazettine (**140**)	23.59		222 ± 16	67 ± 14				
11-Hydroxyvittatine (**141**)	23.63		6 ± 1	5 ± 2		1 ± 0.3		
uc (disaccharide) (**142**)	23.90	974 ± 67	27 ± 2	1185 ± 85	7 ± 1		34 ± 10	19 ± 3
1-Hexadecanoyl glycerol (**143**)	23.98		1 ± 1	1 ± 0.1	2 ± 0.5	4 ± 1	4 ± 1	2 ± 1
Haemanthidine (**144**)	24.46		14 ± 4	34 ± 2				
Haemanthidine isomer (**145**)	24.53		26 ± 2	34 ± 4				

Table 3 (*Continued*)

Compound	Rt	*N. pseudonarcissus* cv. Carlton bulbs	*N. confusus* bulbs	*N. confusus* leaves	*L. aestivum* leaves (BG)[a]	*L. aestivum* *in vitro* shoot-clumps	*L. aestivum* leaves (TR)[a]	*L. aestivum* leaves (NL)[a]
Sucrose (**146**)	24.60	1230 ± 143	2331 ± 53	1129 ± 122	3250 ± 260	1990 ± 194	3754 ± 280	2968 ± 1365
uc (disaccharide) (**147**)	24.74	639 ± 34		1019 ± 106				
uc (alkaloid) (**148**)	24.87			15 ± 9				
uc (**149**)	24.90		11 ± 1		<1			
uc (disaccharide) (**150**)	24.97		19 ± 1	69 ± 7				
uc (disaccharide) (**151**)	25.60					66 ± 6	1 ± 2	
uc (**152**)	26.04	18 ± 2						
uc (disaccharide) (**153**)	26.07	2 ± 0.3	10 ± 1		72 ± 6	91 ± 9	33 ± 2	70 ± 3
Homolycorine (**154**)	26.23	2 ± 0.3	117 ± 8	125 ± 9				
uc (disaccharide) (**155**)	26.35				61 ± 10		17 ± 5	33 ± 13
uc (disaccharide) (**156**)	26.51		5 ± 3			23 ± 4		
uc (disaccharide) (**157**)	26.54				59 ± 5		26 ± 2	23 ± 2
8-*O*-Demethylhomolycorine (**158**)	26.75		32 ± 2	16 ± 3				
Monostearin (**159**)	26.76	1 ± 1					1 ± 2	
uc (**160**)	26.85		36 ± 4					
uc (disaccharide) (**161**)	26.97				16 ± 1		5 ± 0.3	1 ± 1
Squalene (**162**)	27.23			3 ± 1				
uc (disaccharide) (**163**)	27.25				7 ± 0.4		3 ± 2	<1
Tetracosanoic acid (**164**)	27.57		1 ± 0.3	3 ± 1				
uc (disaccharide) (**165**)	28.23			<1	21 ± 0.3		33 ± 3	18 ± 3
O-Methylleucotamine (**166**)	28.79		<1	1 ± 1				96 ± 10
uc (**167**)	29.10				2 ± 0.2		30 ± 2	5 ± 0.2
uc (**168**)	31.03				2 ± 0.2			22 ± 1
uc (hydrocarbone) (**169**)	31.06		2 ± 0.4	7 ± 0.4	1 ± 1		7 ± 0.2	10 ± 2
uc (**170**)	31.14		1 ± 0.1	21 ± 3				
uc (**171**)	31.18		<1		1 ± 1	10 ± 3	9 ± 1	4 ± 0.5
nc (**172**)	31.29						177 ± 4	100 ± 1
Digalactosylglycerol (**173**)	31.30			11 ± 1	7 ± 1	6 ± 1		
uc (**174**)	31.39				<1		29 ± 2	11 ± 1
Octacosanol (**175**)	31.54				1 ± 1		11 ± 1	19 ± 1
uc (**176**)	32.72	5 ± 2						
uc (trisaccharide) (**177**)	32.76					7 ± 2		
uc (trisaccharide) (**178**)	32.85	71 ± 5	9 ± 2	1 ± 0.5	16 ± 2	10 ± 11	4 ± 2	2 ± 1
uc (trisaccharide) (**179**)	33.43	17 ± 0.5						
uc (trisaccharide) (**180**)	33.68	11 ± 1						
β-Sitosterol (**181**)	33.92	3 ± 0.2	15 ± 1	14 ± 1	16 ± 2	18 ± 2	16 ± 2	22 ± 1
uc (trisacharide) (**182**)	34.67				15 ± 4	15 ± 4	52 ± 5	82 ± 3
uc (trisaccharide) (**183**)	34.77	33 ± 5	50 ± 8	<1	67 ± 16	46 ± 9	1 ± 0.2	24 ± 2
uc (trisaccharide) (**184**)	34.83	9 ± 3	21 ± 3		66 ± 5	51 ± 10	10 ± 3	11 ± 8
Total ± SD		4362 ± 227	8856 ± 386	11603 ± 529	21420 ± 2287	6750 ± 687	15437 ± 614	16324 ± 2256

uc—Unknown compound. Results represent the means ± SE of response ratios of measurements in 4 samples (50 mg). Response ratio represents peak area ratio using codeine (50 μg) as a quantitative internal standard.

[a] Origin: BG—Bulgaria; TR—Turkey; NL—Netherlands.

3.2.5. *Specificity*

The peak purity of galanthamine TMS was checked by mass spectral deconvoluting software -AMDIS 2.64. In the samples, the extracted spectrum, retention time and retention index of galanthamine TMS were identical with those obtained from the standard compound.

3.3. *GC–MS analysis of plant samples*

Along with the samples used for method validation, two samples of *L. aestivum* leaves from plants of different geographical origin (Turkey and Netherlands) growing in the Netherlands were analyzed for their alkaloid content by means of the proposed method. The galanthamine content of the sample from Turkey was found to be 0.059% DW and that of plants from the Dutch market was 0.079% DW. Both contents of galanthamine were considerably lower than in *L. aestivum* plants growing in Bulgaria (0.166% DW).

The methanolic extracts showed metabolite profiles characteristic for each species and plant organ. Organic, amino, and fatty acids, sterols, mono-, disaccharides and alkaloids, showing specific time clustering, were detected (Fig. 1). About one hundred metabolites were identified (Table 3).

The amount of total extract (extractable compounds, estimated on basis of the response ratio), alkaloid fraction, and proportion of galanthamine in the alkaloid fraction are important characteristics of plant raw material, determining the technology for galanthamine isolation and purification. The amount of the total extract from *N. pseudonarcissus* cv. Carlton was about 2-times less than from the bulbs of *N. confusus*. The highest amount of total extract was obtained from the leaves of Bulgarian *L. aestivum*, which was significantly higher than those of samples grown in the Netherlands (Table 3). The alkaloid fraction varied between 0.35% and 15.86% of total extract in the *in vitro* shoot-clumps from *L. aestivum* and *N. confusus* bulbs, respectively (Table 4). Several alkaloids were detected as TMS derivatives in the methanolic extracts. To our knowledge, there is no MS data on TMS-derivatives for amaryllidceous alkaloids in the related literature and databases. In Fig. 3, we present MS spectra of the alkaloid TMS derivatives found in the samples. Homolycorine was detected as a non-derivatized compound due to the lack of a hydroxyl group.

Information on the galanthamine proportion in the alkaloid fractions was difficult to obtain from the total methanol extracts due to the low abundance of the minor alkaloids as well as co-elution with other more abundant metabolites. In addition, identification of the Amaryllidaceae alkaloids as TMS derivatives was hampered by lack of reference MS spectra. For that reason, we fractionated the alkaloids and detected 30 compounds without derivatization (Table 5). The results indicated that the galanthamine proportion was higher

Table 4
Main groups of compounds in the methanolic extracts.

	N. pseudonarcissus cv. Carlton bulbs	*N. confusus* bulbs	*N. confusus* leaves	*L. aestivum* leaves (BG)[a]	*L. aestivum in vitro* shoot-clumps	*L. aestivum* leaves (TR)[a]	*L. aestivum* leaves (NL)[a]
Organic acids	74 ± 2	504 ± 36	1042 ± 38	508 ± 23	574 ± 76	423 ± 28	428 ± 9
Amino acids	52 ± 3	1011 ± 132	2312 ± 70	48 ± 9	1532 ± 172	170 ± 5	241 ± 17
N-Containing compounds	–	69 ± 7	41 ± 2	7 ± 1	71 ± 8	3 ± 0.5	4 ± 0.3
Sugar alcohols	120 ± 9	606 ± 26	625 ± 36	1582 ± 29	455 ± 78	1057 ± 45	1390 ± 74
Monosaccharides	443 ± 34	1943 ± 77	479 ± 149	14590+1903	1615 ± 159	7691 ± 446	8716 ± 1129
Disaccharides	3300 ± 171	2425 ± 53	4155 ± 252	3510 ± 277	2169 ± 202	3963 ± 285	3221 ± 1378
Trisacharides	140 ± 8	79 ± 8	traces	165 ± 20	129 ± 31	67 ± 5	118 ± 7
Fatty acids	87 ± 10	304 ± 18	963 ± 51	48 ± 8	58 ± 11	271 ± 10	240 ± 12
Alkaloids	104 ± 8 (2.39%)	1391 ± 53 (15.86%)	1050 ± 37 (9.19%)	197 ± 11 (0.92%)	24 ± 4 (0.35%)	120 ± 7 (0.78%)	403 ± 18 (2.74%)
Sterols	5 ± 0.3	15 ± 1	14 ± 1	16 ± 2	18 ± 13	16 ± 2	22 ± 1
Phosphates	5 ± 0.3	126 ± 15	112 ± 20	3 ± 1	33 ± 15	10 ± 1	19 ± 2
Unkown compounds	32 ± 2	381 ± 37	830 ± 44	742 ± 120	88 ± 14	1628 ± 101	1534 ± 167

Results represent the means ± SE of response ratios and% of TIC (total ion current) of measurements in 4 samples (50 mg). Response ratio represents peak area ratio using codeine (50 μg) as a quantitative internal standard.

[a] Origin: BG—Bulgaria; TR—Turkey; NL—Netherlands.

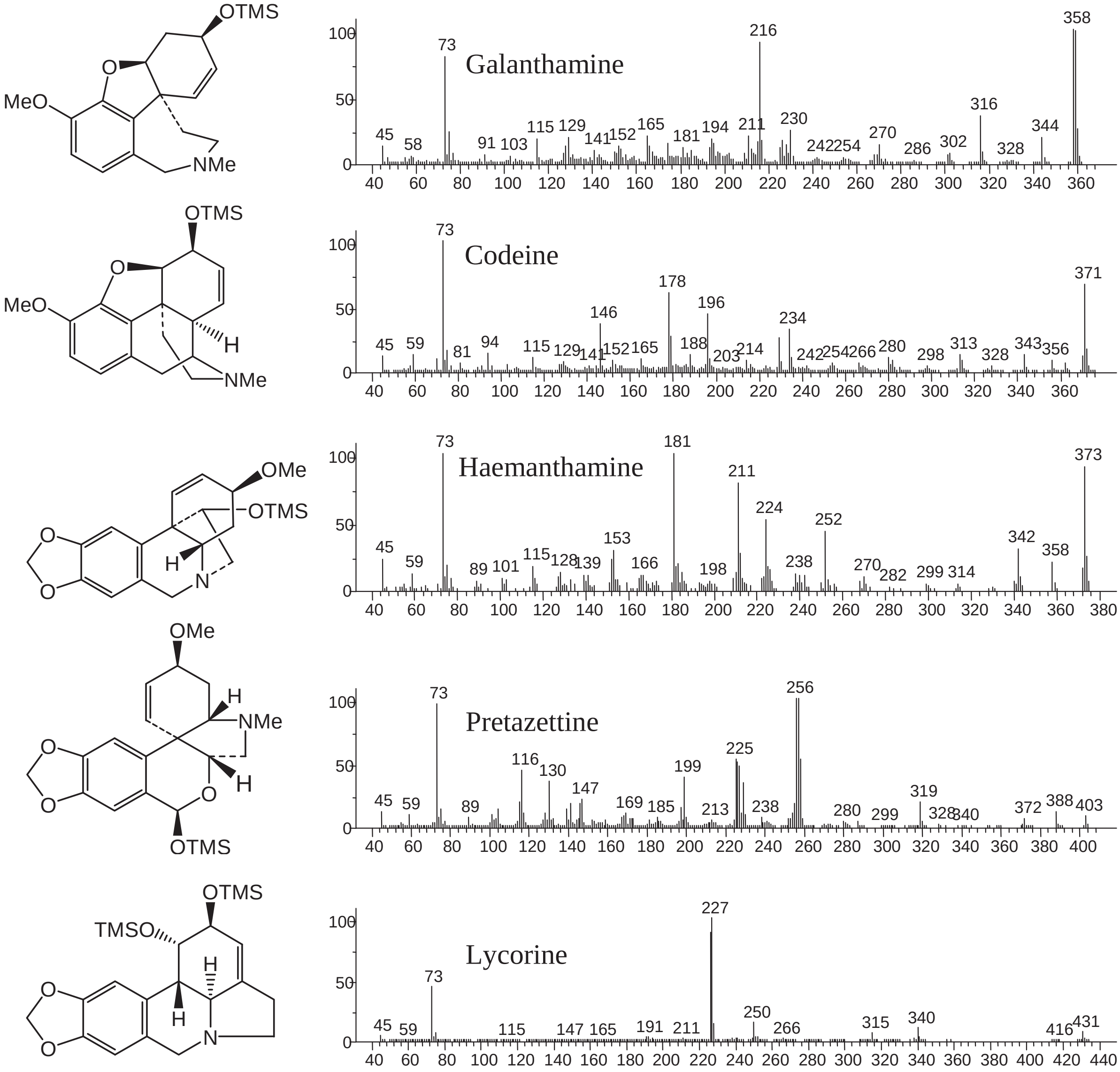

Fig. 3. GC–MS spectra of the main alkaloids (TMS-derivatives) in the samples.

Fig. 4. Score plot of principal component analysis using GC–MS chromatograms (PC1 vs PC4). NL—*N. confusus* leaves; NB—*N. confusus* bulbs; NC—*N. pseudonarcissus* cv. Carlton bulbs; IV—*In vitro* cultures of *L. aestivum*; LL—*L. aestivum* plants grown in Bulgaria; L—*L. aestivum* plants grown in the Netherlands (samples 531, 532, 533 and 534—Turkey, 561, 562, 563 and 564—Dutch market).

in the samples from *L. aestivum in vitro* cultures (74% of total ion current, TIC) and *N. pseudonarcissus* cv. Carlton bulbs (63% of TIC). Three to four major (>10% of TIC) accompanying compounds were found in the *N. confusus* samples, with only two major compounds observed in the others.

3.4. PCA analysis

The GC–MS chromatograms of the samples from different plant species, including those of *L. aestivum* grown at different geographical regions were analysed by PCA. Good separation was

Table 5
Alkaloids identified in the alkaloid fractions.

Alkaloid	Rt	M⁺	*N. pseudonarcissus* cv. Carlton bulbs	*N. confusus* bulbs	*N. confusus* leaves	*L. aestivum* leaves	*L. aestivum in vitro* shoot-clumps
Tyramine (**1**)[1]	7.30	137					4.44
Anhydrogalanthamine (**2**)[2–a]	18.96	269	2.56			0.01	5.24
Ismine (**3**)[3]	19.61	257	0.43	0.58	0.47		
Trisphaeridine (**4**)[3]	19.81	223	0.20	0.27	0.36	0.03	1.35
Apogalanthamine-isomer (**5**)[2–a]	20.18	269	0.17			0.17	0.31
A1 (**6**)	20.52	239	0.20	0.22	0.18	0.33	
Galanthamine (**7**)[3]	21.71	287	62.55*	49.99*	39.98*	51.24*	73.83*
Lycoramine (**8**)[3]	21.87	289	3.02				
N-Demethylgalanthamine (**9**)[3]	22.21	273	0.64	1.46	0.27	0.43	0.80*
A2 (**10**)	22.60	301		0.30			
Narwedine (**11**)[3]	22.78	285	0.66	1.10	3.07	1.63	0.49
Vittatine (**12**)[3]	22.83	271		0.40*			
6-*O*-Methylycorenine (**13**)[3]	22.88	331	1.70	0.44		0.06	
A3 (**14**)	23.15	281		3.44	2.51		
Anhydrolycorine (**15**)[1]	23.21	251				2.07	
8-*O*-Demethylmaritidine (**16**)[3]	23.22	273					23.03
3-*O*-Acetylgalanthamine (**17**)[3]	23.66	329		0.04	0.03		
6-*O*-Methylpretazettine (**18**)[3]	24.67	345		0.26	0.22		
11,12-Dehydroanhydrolycorine (**19**)[2–b]	24.72	249	0.36	0.19	0.25	0.76	0.19
Haemanthamine (**20**)[3]	25.25	301	24.03*	17.72*	25.72*		
Tazettine (**21**)[3]**	25.42	331		10.85*	11.27*	0.05	
A4 (**22**)	25.99	331		0.70	1.01		
11-Hydroxyvittatine (**23**)[3]	26.18	287		0.13			
Haemanthidine (**24**)[3]	26.43	317		0.04	0.14		
Lycorine (**25**)[3]	26.79	287				43.06*	
Homolycorine (**26**)[3]	26.81	301	6.04*	8.66*	12.08*		
N-Formylnorgalanthamine (**27**)[3]	27.48	301		0.04		0.16	
8-*O*-Demethylhomolycorine (**28**)[3]	27.54	315		2.73*	1.76*		
Epimacronine (**29**)[3]	27.67	329		0.35	0.38		
O-Methylleucotamine (**30**)[2]	28.74	373		0.08	0.29		

The values represent the% of TIC from the total alkaloid mixture.
Identification: 1) NIST database; 2) literature data: a [37], b [38], [39]; 3) standard.

* Detected in the silyated samples.

** Tazettine is an extraction artifact of pretazettine [9].

observed between the samples by the principal components PC1 and PC4 (Fig. 4). Using this unsupervised multivariate data analysis technique, major principal components clearly distinguished between different species. Furthermore, PCA analysis allowed *L. aestivum* samples from different geographical regions to be distinguished, although it could not clearly separate those from the Dutch market and Turkey, both growing in the same climatic conditions (in the Netherlands). However, a comparison of the chromatograms revealed that both samples of *L. aestivum* plants grown in the Netherlands could be distinguished by several minor metabolites, whose presence and abundance is characteristic and indicate genetic differences. The sample of Dutch origin contained *O*-methylleucotamine (**30**) and significantly higher amounts of both alkaloid fractions and lycorine (**25**) than the sample of Turkish origin.

Thus, the PCA analysis of the metabolite patterns allowed an effective control of the plant raw material (regarding species, plant organ and geographic region) used for galanthamine extraction.

4. Conclusion

The analytical method developed in this study, using GC–MS to determine galanthamine in plant sources, proved to be simple, practical and sensitive. The method is very informative compared with the routine analytical methods (GC, HPLC, CE and NMR) and may be useful for the quality control of plant raw materials used in the pharmaceutical industry, providing information on galanthamine content, alkaloid profiles, plant species, the plant organ and geographic region. Due to the low mass of plant material used for extraction, the method may be applied for metabolic analysis in biotechnological or agrochemical experiments.

Acknowledgments

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 219795, and from the Generalitat de Catalunya (ref. 2009 SGR 1060). The authors thank Ludwig &Co (Netherlands), VitroFlora (Poland) and Galen-N (Bulgaria) for the provided plant material.

References

[1] A. Maelicke, M. Samochocki, R. Jostok, A. Feherbacher, J. Ludwig, E.X. Albuquerque, M. Zerlin, Biol. Psychiatry 26 (2001) 279.
[2] S. Berkov, L. Georgieva, V. Kondakova, A. Atanassov, F. Viladomat, J. Bastida, C. Codina, Biotechnol. Biotechnol. Equip. 23 (2009) 1170.
[3] J. Tencheva, I. Jamboliev, Ts. Zhivkova, J. Chromatogr. 421 (1987) 396.
[4] G.S.J. Mannens, C.A.W. Snel, J. Hendrickx, T. Verhaeghe, L. le Jeune, W. Bode, L. van Beijsterveldt, K. Lavrijsen, J. Leempoels, N. van Osselaer, A. van Peer, W. Meuldermans, Drug Metab. Dispos. 30 (2002) 553.
[5] T. Verhaeghe, L. Diels, R. de Vries, M. de Meulder, J. de Jong, J. Chromatogr. B 789 (2003) 337.
[6] N. Mustafa, I. Rhee, R. Verpoorte, J. Liq. Chromatogr. Relat. Technol. 26 (2003) 3217.
[7] T. Mroczek, J. Mazurek, Anal. Chim. Acta 633 (2009) 188.
[8] N.P.D. Nanayakkara, J.K. Bastos, in: G. Hanks (Ed.), Extraction and Quantitative Analysis of Amaryllidaceae Alkaloids. *Narcissus* and Daffodil, the Genus *Narcissus*, (Medicinal and Aromatic Plants—Industrial Profiles, vol. 21), Taylor and Francis, London and New York, 2002, pp. 286–303.
[9] J. Bastida, R. Lavilla, F. Viladomat, in: G.A. Cordell (Ed.), Chemical and Biological Aspects of *Narcissus* Alkaloids. The Alkaloids, vol. 63, Elsevier Scientific Publishing, Amsterdam, 2006, pp. 87–179.
[10] M. Kreh, R. Matusch, L. Witte, Phytochemistry 38 (1995) 773.
[11] S. Berkov, J. Bastida, B. Sidjimova, F. Viladomat, C. Codina, Biochem. Syst. Ecol. 36 (2008) 638.
[12] A. Rizzi, R. Schuh, A. Brüchner, B. Cvitkovich, L. Kremser, U. Jordis, J. Fröhlich, B. Küenburg, L. Czollner, J. Chromatogr. B 730 (1999) 167.
[13] R. Gotti, J. Fiori, M. Bartolini, V. Cavarini, J. Pharm. Biomed. Anal. 42 (2006) 17.
[14] Abou-Donia, S. Toaima, H. Hammoda, E. Shawky, Phytochem. Anal. 19 (2008) 353.
[15] J. Bastos, L. Xu, D. Nanayakkara, C. Burandt, R. Moraes-Cerdeira, J. McChesney, J. Nat. Prod. 59 (1996) 638.
[16] S. Berkov, J. Bastida, F. Viladomat, C. Codina, Phytochem. Anal. 19 (2008) 285.
[17] S. López, J. Bastida, F. Viladomat, C. Codina, Phytochem. Anal. 13 (2002) 311.
[18] A. Poulev, B.D. Neumann, M.H. Zenk, Planta Med. 59 (1993) 442.
[19] T. Tanahashi, A. Poulev, M.H. Zenk, Planta Med. 56 (1990) 77.
[20] A. Lubbe, B. Pomahačová, Y. Choi, R. Verpoorte, Phytochem. Anal. 21 (2009) 66.
[21] L.-F. Shyur, N.-S. Yang, Curr. Opin. Chem. Biol. 12 (2008) 66.
[22] U. Roessner, C. Wagner, J. Kopka, R. Trethewey, L. Willmitzer, Plant J. 23 (2000) 131.
[23] Y.H. Choi, H.K. Kim, A. Hazekamp, C. Erkelens, A.W.M. Lefeber, R. Verpoorte, J. Nat. Prod. 67 (2004) 953.
[24] Y.H. Choi, S. Sertic, H.K. Kim, E.G. Wilson, F. Michopoulos, A.W.M. Lefeber, C. Erkelens, S.D.P. Kricun, R. Verpoorte, J. Agric. Food Chem. 53 (2005) 1237.
[25] S. Berkov, A. Pavlov, M. Ilieva, M. Burrus, S. Popov, M. Stanilova, Phytochem. Anal. 16 (2005) 98.
[26] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073384.pdf (accessed February 2010).
[27] http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/home/gmd_sm.html (accessed February 2010).
[28] http://www.lipidlibrary.co.uk/ms/ms01/index.htm (accessed February 2010).
[29] P.M. Medeiros, B.R.T. Simoneit, J. Chromatogr. A 1141 (2007) 271.
[30] A. Pavlov, S. Berkov, E. Courot, T. Gocheva, D. Tuneva, B. Pandova, M. Georgiev, V. Georgiev, S. Yanev, M. Burrus, M. Ilieva, Process Biochem. 42 (2007) 734.
[31] S. Berkov, A. Pavlov, V. Georgiev, J. Bastida, M. Burrus, M. Ilieva, C. Codina, Nat. Prod. Commun. 4 (2009) 359.
[32] J. Sramek, E. Frackiewicz, N. Culter, Exp. Opin. Invest. Drugs 9 (2000) 2393.
[33] L. Torras-Claveria, S. Berkov, O. Jáuregui, J. Caujapé, F. Viladomat, C. Codina, J. Bastida, Phytochem. Anal. 21 (2010) 80.
[34] J. Malakova, M. Nobilis, Z. Svoboda, M. Lısa, M. Holcapek, J. Kvetina, J. Klimes, V. Palicka, J. Chromatogr. B 853 (2007) 265.
[35] R. Albert, W. Horwitz, Anal. Chem. 69 (1997) 789.
[36] http://www.aoac.org/Official_Methods/slv_guidelines.pdf (accessed February 2010).
[37] T. Kametani, K. Yamaki, S. Shibuya, K. Fukurnoto, K. Kigasawa, F. Satoh, M. Hiiragi, T. Hayasaka, J. Chem. Soc. (1971) 590.
[38] S. Berkov, J. Bastida, R. Tsvetkova, F. Viladomat, C. Codina, Z. Naturforsch. 64 c (2009) 311.
[39] A. Latvala, M. Önür, T. Gözler, A. Linden, B. Kivçak, M. Hesse, Phytochemistry 39 (1995) 1229.