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Analysis of Amaryllidaceae alkaloids from *Narcissus* by GC–MS and capillary electrophoresis

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Abstract

Amaryllidaceae are known as ornamental plants, furthermore some species of this family contain galanthamine, an acetylcholinesterase inhibitor approved for the treatment of Alzheimer's disease, and other alkaloids with interesting pharmacological activity. In the present work, the quali- and quantitative analysis of Amaryllidaceae-type alkaloids in the bulbs of *Narcissus* species is presented using different analytical approaches. Extracts of *Narcissus pseudonarcissus* cv. Carlton and *Narcissus jonquilla* Quail, were first examined by GC–MS using a Rtx®-5 MS (programmed temperature) and the major alkaloids were identified. Together with galanthamine, high contents of haemanthamine, were found. Galanthamine was reliably quantified by GC–MS, whereas haemanthamine partly decomposed under the GC conditions, thus alternative analytical methods were investigated. Firstly, reversed-phase HPLC–ESI-MS was applied to identify and isolate at semipreparative levels haemanthamine. The compound was fully characterized by MS/MS and ^{1}H NMR and then used as a reference substance. The quantitation of both galanthamine and haemanthamine was then accomplished by capillary electrophoresis with spectrophotometric detection. A non-aqueous (NACE) approach was selected in order to use a running buffer fully compatible with samples in organic solvent. In particular, a mixture methanol–acetonitrile (75:25, v/v) containing ammonium acetate (90 mM) was used as a background electrolyte. The same analytical sample was subjected to GC–MS and NACE analysis; the different selectivity displayed by these techniques allowed different separation profiles that can be useful in phytochemical characterization of the extracts. The GC–MS and NACE methods were validated and applied to the quantitation of galanthamine (GC–MS and NACE) and haemanthamine (NACE) in bulbs of *N. jonquilla*.

Keywords: Galanthamine; Haemanthamine; *Narcissus*; GC–MS; Non-aqueous capillary electrophoresis (NACE); Quantitation

1. Introduction

Amaryllidaceae is the name used to label both a family of bulbous plants and the group of alkaloids contained in their extracts; the chemical structures of these alkaloids are very variable as well as their pharmacological properties. In the recent years attention has been focused on the biological activity of several Amaryllidaceae alkaloids [1] which displayed antimalarial [2,3], antiviral [4,5] and antiproliferative activity [6]; the most important Amaryllidaceae alkaloid is undoubtedly galanthamine that is approved for the pharmacological treatment of Alzheimer's disease [7].

Several studies, mainly involving *Galanthus* and *Narcissus* species, have been published about the distribution of galanthamine in different subspecies and populations of these plants, as well as in their different organs and during different periods of harvesting. In these studies TLC [8,9], GC–MS [8–10] and HPLC [11,12] were successfully applied; in particular GC–MS was found to be suitable for the characterization of the alkaloids. The power of GC–MS has been exploited in these works, especially because the main Amaryllidaceae alkaloids can be easily subjected to direct GC analysis without any derivatization step. However, while galanthamine and other alkaloids can be reliably determined by this technique, haemanthamine, one of the major crinane-type Amaryllidaceae alkaloids, is reported to undergo thermal decomposition during the chromatographic separation [10]. As a consequence, doubts could be roused in identification of haemanthamine by retention time and mass spectrum as well as in its quantitation.

In the present paper capillary electrophoresis is proposed as a useful alternative to the existing HPLC method [11] for the determination of galanthamine and haemanthamine in

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Amaryllidaceae extracts; in particular, the association of CE with GC–MS was found to be well suitable for identification of the studied alkaloids in bulbs of two different species of ornamental *Narcissus*. The usefulness of the proposed approaches lies in the orthogonal selectivity of GC and CE; in fact different separation profiles are expected to be obtained for a given plant extract analysed with the two techniques. The availability of a double phytochemical "fingerprints" increases the confidence in the identification of the origin of extracts; furthermore, by CE analysis, the integrity of the thermally unstable haemanthamine is preserved, thus, allowing for reliable quantitation. In order to take advantage of the proposed GC–MS and CE off-line combination, a complete compatibility of the background electrolyte used in CE with the solvent of the samples prepared for GC analysis, should be useful. For this reason a fully non-aqueous CE method (NACE) was adopted; this approach often can allow improved separation compare to conventional CZE aqueous system because of the favorable effects of solvent in use on p*K* values and solvation of the analytes. These aspects have been considered in analysis of opium alkaloids in pharmaceuticals [13]; more recently applications of NACE to the analysis of different alkaloids in plant extracts have been reported [14–16] confirming the suitability of this technique in phytochemical analysis. At the best of our knowledge, however, this is the first study concerning NACE analysis of Amaryllidaceae alkaloids in extracts of *Narcissus*.

2. Experimental

2.1. Materials

Galanthamine hydrobromide was from Tocris (UK), papaverine hydrochloride, ammonium acetate, acetic acid (99.9%) and the standard hydrocarbon mixture (C_8–C_{36}) for determination of Kovat's index (retention index) were from Sigma–Aldrich (Milan, Italy). Methanol, ethanol, acetonitrile, dichloromethane, *n*-hexane, sodium bicarbonate and sodium carbonate were all from Carlo Erba Reagenti (Milan, Italy).

Bulbs of ornamental *Narcissus pseudonarcissus* cv. Carlton and *Narcissus jonquilla* (Quail) were purchased in local market without any indication about period of harvesting.

2.2. Solutions

Stock solutions of galanthamine hydrobromide, papaverine hydrochloride and haemanthamine were prepared in methanol at a concentration of 2 mg/mL; these solutions were then properly diluted as requested in the specified experiments (optimization, calibration graphs, recovery studies).

Non-aqueous background electrolytes for NACE experiments were prepared by dissolving ammonium acetate at the selected concentration (range 50–90 mM) in the used solvent (mixtures of methanol–acetonitrile) in the presence of 0.5% acetic acid. All the solutions were filtered through a 0.45 μm filter.

2.3. Apparatus

2.3.1. GC–MS equipment and experimental conditions

A Trace GC 2000 Series (ThermoElectron Instruments, Austin, TX, USA) gas chromatograph equipped with a split–splitless injector (split ratios of 50:1) was used for the GC analysis. The column was an Rtx®-5 MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) consisting of Crossbond® (5% phenyl–95% dimethylpolysiloxane). Helium was the carrier gas at a flow rate of 0.8 mL/min and programmed temperature was applied to obtain the separation of the analytes; precisely the initial temperature was 80 °C then ramped at 10 °C/min to 280 °C (hold time 10 min). The gas chromatograph was interfaced with a GCQ Plus mass detector operating in the EI mode (70 eV) under autotune conditions. The temperatures of injector, transfer line and ionization source were 280, 225 and 285 °C, respectively. The mass spectra were recorded within 50–650 amu in full scan mode to collect the total ion current (TIC) chromatograms; single ion monitoring (SIM) chromatograms were reconstructed at the base peak of the studied analytes (galanthamine *m/z* 286 amu) and at the base peak of the internal standard papaverine (*m/z* 338 amu).

2.3.2. CE equipments and experimental conditions

Biofocus 2000 (BioRad, Hercules, CA, USA) Capillary Electrophoresis instrument was used for the analysis; the data were collected on a PC equipped with the Biofocus System Integration software. Fused-silica capillaries of 24 cm in length (19.5 effective length) × 50 μm i.d. (Composite Metal Service, Ilkley, UK), thermostated at 15 °C, were used. The voltage was maintained at 10 kV in the optimized conditions; the injections were performed hydrodynamically at 2 psi s and the detection wavelength was 280 nm. CE analysis were also performed using the HP^{3D}CE system by Agilent Technologies (Waldbronn, Germany) equipped with a diode-array detector. Fused silica capillaries were in this case of 32.5 cm in length (24 cm to the detector) × 50 μm i.d. by Composite Metal Service; the temperature was maintained constant at 15 °C and the voltage was at 15 kV. Hydrodynamic injections were performed at 50 mbar × 3 s; the on-line UV spectra were recorded in the range of 200–450 nm.

Before the first use the capillary was rinsed, in the order, with 1.0 and 0.1 M solutions of sodium hydroxide for 10 min to which followed a final rinse (10 min) with deionized water. Between consecutive injections the capillary was rinsed with sodium hydroxide 0.1 M (2 min), water (2 min), methanol (2 min) and separation buffer (2 min). In optimization experiments, the capillary was rinsed with water (10 min) before changing the running buffer composition. Running buffer electrolyte solutions were changed between every injection to achieve high reproducibility.

2.3.3. HPLC equipment and experimental conditions

HPLC–ESI-MS analysis was performed under reversed-phase conditions on a Waters Xterra C18, 3.5 μm (100 mm × 2.0 mm i.d.) column, using as the mobile phase a binary mixture of ammonium acetate (0.01 M; pH 6.5) buffer/acetonitrile 95:5 (v/v) at a flow rate of 0.2 mL/min. A loop valve of 20 μL volume

was used. The liquid chromatograph was by Jasco Corporation (Tokyo, Japan), model PU-1585, interfaced by means a T split valve with the LCQ Duo Mass Spectrometer (Thermo Electron, San Jose, CA, USA). The system was equipped with heated capillary interface and electrospray ionization (ESI) source and operated with both Ion Trap analyzer and Jasco UV-1575 detector (wavelength 280 nm). ESI system employed a 4.5 kV (positive polarity) spray voltage, heated capillary temperature and voltage of 220 °C and 43.93 V, respectively. The sheath gas and the auxiliary gas (nitrogen) flow rate were at 0.75 and 1.2 L/min, respectively. Electrospray ionization was optimized using galanthamine as reference compound. The mass chromatograms were acquired in total ion current (TIC) modality from 100 to 400 *m/z*, in MS/MS mode (relative collision energy 25%) on the ESI generated ions at *m/z* 302 for haemanthamine.

2.4. Calibration graphs

2.4.1. GC–MS method

Different volumes over the range 2–200 μL of the stock solution of galanthamine hydrobromide (about 2.0 mg/mL) were transferred in vials and evaporated under vacuum at ambient temperature, afterwards in each of the vials, 0.500 mL of papaverine (the internal standard; 0.1 mg/mL), and 0.150 mL of acetic acid (0.05%) in methanol, were added. After shaking, 1 μL of the resulting solution was injected into the GC–MS. The ratios of reconstructed peak areas (SIM) of galanthamine (*m/z* 286) versus those of internal standard papaverine (*m/z* 338) were plotted against the corresponding concentration of galanthamine hydrobromide (range 6.15–615 μg/mL) to obtain the calibration graphs.

2.4.2. CE method

Mixed calibration solutions for CE analysis of both galanthamine and haemanthamine were prepared similarly to those for GC–MS method; in this case, aliquots of haemanthamine free base solution (range 2–200 μL) were also transferred in the vials before evaporation to dryness under vacuum. After addition of the internal standard (0.500 mL of papaverine solution at 0.1 mg/mL) and 0.150 mL of acetic acid (0.05%) in methanol, the mixed solutions were injected into the CE apparatus. Ratio of the corrected peak area (area to migration time) of the analytes galanthamine and haemanthamine to those of internal standard, were plotted against the corresponding concentrations to obtain the calibration graphs.

2.5. Sample preparation

The bulbs were crushed and dried in oven at 60 °C for 24 h then they were subjected to extraction procedures either at semipreparative or analytical level as described below.

2.5.1. Analytical extraction

Aliquots of 100 mg of powdered bulb were extracted in a volumetric flask with 10.0 mL of methanol under ultrasonication at 50 °C for 5 h. After filtration (0.45 μm filter) a 5.0 mL volume of the methanol solution was evaporated under vacuum at ambient temperature. The residue was then dissolved with 0.500 mL of papaverine solution (internal standard) and 0.150 mL of acetic acid (0.05%) in methanol. After shaking and filtration (0.45 μm filter) CE and GC–MS analyses were performed.

2.5.2. Preparative extraction

A 5 g aliquot of powdered dry bulb was extracted by maceration with methanol (50 mL) for 12 h at ambient temperature and by 30 min sonication at intervals of 4 h. The extraction was repeated for three times and the filtered methanol solution was evaporated under vacuum; the residue was dissolved in 30 mL of acidified water (HCl 1%) and subjected to replicate extractions with *n*-hexane (3 × 50 mL) and dichloromethane (3 × 50 mL). The aqueous solution was alkalinized (pH 9.0) with a 0.1 M carbonate–bicarbonate buffer and the alkaloids were extracted with dichloromethane (5 × 50 mL). The collected organic phase was washed with water and evaporated to dryness affording about 22 mg of alkaloids. Methanol solutions of the extract were injected into the GC–MS and LC–ESI-MS for the identification of the alkaloids.

2.6. Identification and isolation of haemanthamine

Firstly, diluted bulb extracts were subjected to LC–MS analysis under the conditions described in Section 2.3.3. A peak strongly retained (retention time of about 50 min) exhibiting an ESI ion of *m/z* 302, was subjected to MS/MS experiments. The obtained MS/MS data, *m/z* (relative abundance) were: 302 (45) $[M+H]^+$, 270 (100) $[M-CH_3OH]^+$, 259 (24) $[M-CH_2CHOH]^+$, 211 (75) $[M-CH_2CHOH–OCH_2OH]^+$. These results are in agreement with the haemanthamine structure.

The HPLC isolation at semipreparative level of haemanthamine, was carried out by slight modification of conditions given in Section 2.3.3. In particular using injection loop valve (100 μL), column size (150 mm × 4.6 mm i.d.) and flow rate (1 mL/min) as indicated, the fractions identified as haemanthamine (UV detection), were collected after several repeated injections and subjected to concentration by removing the volatile components under vacuum at room temperature. The aqueous residue was diluted with distilled water, alkalinized with carbonate–bicarbonate buffer (pH 9.0; 0.1 M) and finally extracted with dichloromethane as described in Section 2.5.2. The isolated haemanthamine (10 mg) was found to be pure by LC–MS analysis.

^{1}H NMR spectrum of haemanthamine was recorded in $CDCl_3$ on a Varian Gemini (200 MHz) NMR spectrometer, using TMS as the internal standard. The chemical shift is expressed in δ (ppm) and *J* in Hz: δ 3.38 (3H, s, $–OCH_3$), 3.72 (1H, d *J* = 16.8, *H*CHN), 4.35 (1H, d *J* = 16.8, HC*H*N), 5.92 (2H, s, OCH_2O), 6.38 (1H, dd *J* = 10.1; 4.8, –CH=*CH*–), 6.45 (1H, d *J* = 10.1, –*CH*=CH–), 6.50 (1H, s, Ar), 6.86 (1H, s, Ar). These data were in agreement with those reported in the literature for haemanthamine [17].

The compound was dried under vacuum overnight and used as standard reference substance for the CE analysis.

3. Results and discussion

In the present work, HPLC applications were restricted to LC–MS identification and LC-UV isolation of haemanthamine, as useful reference substance for analysis. The main objective was to develop a CE method, as an alternative/complementary approach to the chromatographic methods, suitable for reliable quali- and quantitative analysis of the major Amaryllidaceae alkaloids of biopharmaceutical importance. Therefore, the study involved the following aspects: (a) identification of the major Amaryllidaceae alkaloids in bulbs of *Narcissus*, (b) development of a non-aqueous capillary electrophoresis (NACE) method and (c) application of GC–MS and NACE–UV methods to the analysis of the alkaloids in *Narcissus* extracts.

3.1. Identification of Amaryllidaceae alkaloids

3.1.1. Identification by GC–MS

The GC–MS analysis of underivatized alkaloids from *Narcissus* extracts was performed using an Rtx®-5 MS column; in Table 1 are reported the GC–MS data and the structures of the major alkaloids identified in the extracts of *Narcissus* (*N. pseudonarcissus* cv. Carlton and *N. jonquilla*). The identification of alkaloids was obtained by comparison of mass spectra as well as of retention times and retention indices (RI) with those from literature data [9,10]. In Table 1 only the most important alkaloids are reported, however in *N. jonquilla* extracts *N*-demethyl galanthamine and *N*-demethyl lycoramine were also identified at significant levels (about 5 and 7%, respectively). In general the identified components corresponded to about 95–98% of the total peak area in TIC chromatograms. As expected, differences in the GC–MS phytochemical profiles of the two extracts were observed; in particular tazettine was found only in *N. jonquilla* providing a characteristic phytomarker for this species, whereas galanthamine was the principal alkaloid contained in both the extracts. Haemanthamine, the second major Amaryllidaceae alkaloid in *N. pseudonarcissus* cv. Carlton, is dominant in this family of plants and showed mass spectrum data according to those reported in recent literature [9]. Significant differences, however, were observed in comparison to mass spectrum reported in another paper [10] where the haemanthamine thermal decomposition was demonstrated under the GC conditions. In order to confirm the identity of haemanthamine, further investigations were thus performed.

3.1.2. Identification and isolation of haemanthamine by LC–MS/MS

Diluted extracts from *N. pseudonarcissus* cv. Carlton, were analysed under reversed phase LC condition using ESI-MS detection. A chromatographic peak with MS spectrum showing a pseudomolecular mass at *m/z* 302 was detected; experiments of MS/MS (specific fragments described in Section 2.6.) confirmed the haemanthamine structure for this peak.

By applying semipreparative LC conditions as described in Section 2, about 10 mg of haemanthamine was obtained, which was found to be chromatographically pure under LC–MS conditions (*m/z* 302) and fully identified by ^{1}H NMR (Section 2.6).

3.2. Non-aqueous capillary electrophoresis (NACE) in analysis of alkaloids from Narcissus

In order to provide an alternative approach to the chromatographic methods in the analysis of Amaryllidaceae alkaloids from *Narcissus* extracts, capillary electrophoresis was considered for its well known features such as the high resolution power and small sample and reagents consumption that make this technique suitable in the analysis of multicomponent samples available in small amounts. The CE analysis of alkaloids from plant extracts is often performed using non-aqueous solvents; in the present study, the influence on the separation of the different NACE parameters (nature of organic solvent, concentration of electrolyte, temperature of capillary and applied voltage), were investigated using a *N. pseudonarcissus* cv. Carlton extract as test solution.

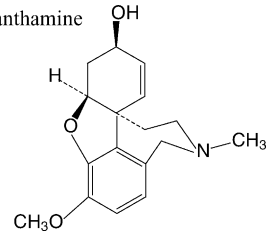
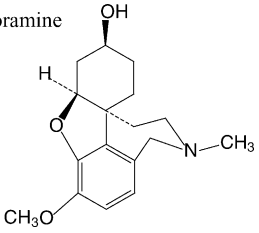
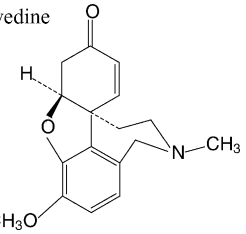
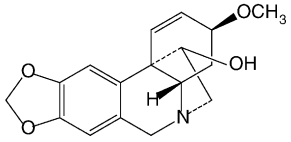
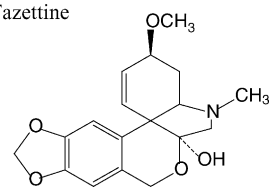
3.2.1. NACE optimization

In NACE the separation is strongly affected by the specific properties of the used organic solvents which can favorably and significantly change the migration of analytes and separation selectivity [18,19]. Methanol is often selected as the principal constituent of non-aqueous running buffer because it has favorable dielectric constant (33.7 at 20 °C), ability in intermolecular hydrogen bonds and autoprotolysis; selectivity tuning is then usually performed by adding acetonitrile, which can be classified as dipolar non-hydroxylic solvent [20,21]. Moreover, the low viscosity as well as low cut-off of these solvents allow for fast and sensitive spectrophotometric detection.

Effect of ammonium acetate concentration. Firstly, a mixture methanol–acetonitrile 85:15 (v/v) containing acetic acid 0.5% was used; under these conditions the alkaloids of interest resulted to be protonized. Ammonium acetate was added as the background electrolyte at different concentrations; using a capillary temperature of 25 °C and an applied voltage of 7 kV, the separation of galanthamine from haemanthamine was easily obtained at relatively low concentration of ammonium acetate; however, by increasing the ammonium acetate concentration enhanced resolution was observed. This effect can be ascribed to the electrostatic interactions of the protonized alkaloids with the acetate co-ion in non-aqueous environment [21]. Furthermore, the analysis time increased with the BGE concentration due to reduction of electroosmosis; in fact the increased ionic strength decreases the electric double layer thickness and the result is a diminished zeta potential [18–20]. The complete resolution of the two considered analytes from each of interferences present in actual samples, was obtained at a concentration of 90 mM in ammonium acetate (Fig. 1a).

Effect of solvent composition, capillary temperature and applied voltage. As widely reported in the literature, variations of methanol–acetonitrile composition significantly affect separation and migration time [18–21] because of the influence on the ratio dielectric constant to viscosity (ε/η). Precisely, the higher the acetonitrile percentages, the lower the analysis time, as a consequence of the enhanced ratio ε/η; using a mixture of 75/25 (v/v) methanol–acetonitrile as the solvent in the presence of ammonium acetate (90 mM) and acetic acid (0.5%)

Table 1
Structure and GC–MS data of underivatized alkaloids from *Narcissus* in the GC–MS conditions as in the text

	[M^+] and characteristic ions (%)	Carlton %GC[a]	Jonquilla %GC[a]	rt[b]	RI[c]
Galanthamine 	287(97);286(100);244(30);216(45);174(43)	51.95	55.12	20.5	2354
Lycoramine 	289(63);288(100);232(11);202(17);115(26)	3.79	11.36	20.7	2365
Narwedine 	285(95);284(100);216(30);174(52)	1.35	0.68	21.1	2414
Haemanthamine 	301(15);272(100);240(18);181(23)	37.50	4.04	22.5	2585
Tazettine 	331(30);298(22);247(100)	Not found	15.22	22.7	2590

[a] GC–MS peaks area (TIC) of total peak area (%) in the gaschromatogram.
[b] Retention time.
[c] Retention index.

relatively fast analysis time was achieved without loss in separation (Fig. 1b). Since the measured run current under the applied voltage (7 kV) was quite low, the electric field can be increased without generation of detrimental Joule heating. By using a combination of 10 kV and 15 °C, a significant improvement in peak symmetry was obtained and these conditions were considered as the optimum for quali- and quantitative analysis (Fig. 1c).

3.3. GC–MS and NACE quantitation of alkaloids in Narcissus

The quantitative determination of pharmacologically active galanthamine and haemanthamine in the bulbs of *Narcissus* can be a useful way in controlling the variability of secondary metabolism of Amaryllidaceae plants [8,9]. The availability of a suitable GC–MS method optimized for identification of alkaloids, prompted us to evaluate its feasibility in quantitative analysis. As already discussed under GC conditions, haemanthamine cannot be reliably determined, thus, only galanthamine was considered as analyte in this approach. Differently, NACE can be potentially useful for the simultaneous analysis of both the considered alkaloids.

3.3.1. Extraction procedure

The evaluation of the content of alkaloids in the bulbs first involved the optimization of the extraction procedure at analytical level. The extraction was carried out on aliquots of 100 mg of dried powdered bulbs; preliminary experiments suggested

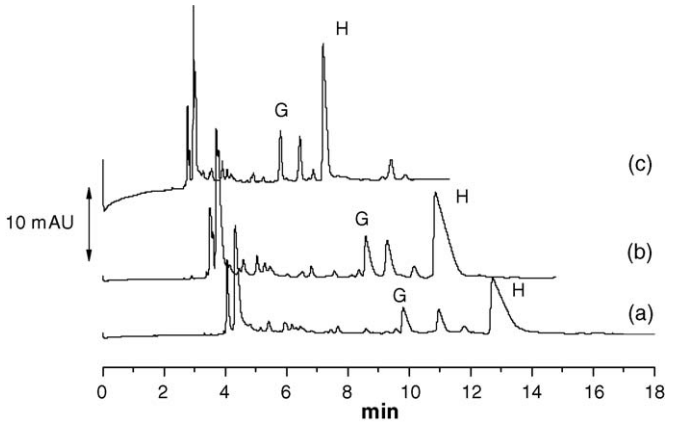


Fig. 1. Electropherograms of an extract from *N. pseudonarcissus* cv. Carlton using NACE in the presence of ammonium acetate 90 mM and acetic acid 0.5% in mixtures of methanol–acetonitrile. Other conditions: fused silica capillary of 19.5 cm effective length (24 cm total length; 50 μm i.d.); hydrodynamic injection at 2 psi s; detection at 280 nm. (a) Solvent: methanol–acetonitrile (85:15, v/v); voltage 7 kV; capillary temperature 25 °C. (b) Solvent: methanol–acetonitrile (75:25, v/v); voltage 7 kV; capillary temperature 25 °C. (c) As in (b) using a voltage of 10 kV and capillary temperature of 15 °C. Symbols: (G) galanthamine; (H) haemanthamine.

that combined use of heat and ultrasonication was effective in improving extraction from plant material. This has been reported to be mainly due to mechanical action of acoustic cavitation, which enhances both solvent penetration into the plant material and the intracellular product release by disrupting the cell walls [22]. In the present work the extraction optimization was carried out by evaluating the yield of galanthamine by GC–MS analysis performed in the presence of papaverine as internal standard. Using a ultrasonic bath at 50 °C, different solvents, suitable for direct GC analysis were tested, such as acetonitrile, methanol and ethanol; among them, methanol provided the best results likely due to the high dielectric constant and ability in hydrogen bonding that allow for a good solubility of alkaloids. Experiments performed using the standard compounds, showed that under the described conditions, galanthamine was stable. The extraction yield was found to be maximum after 5 h.

3.3.2. Selectivity

Selectivity of GC–MS method was evaluated by comparison of the migration time and mass spectrum of standard reference galanthamine with those of the peak obtained in the analysis of real extracts from the bulbs. Similarly, in NACE analysis the identification of galanthamine and haemanthamine peaks was performed by comparing the retention times of standard references with those of the peaks in electropherograms from the extracts. In Fig. 2 a representative electropherogram of a real extract from *N. jonquilla* in optimized NACE conditions and in the presence of papaverine as internal standard, is reported. Further confirmations of the peak identity were carried out by spiking experiments and comparison of the analyte UV spectra obtained using the HP^{3D}CE instrument equipped with a diode-array detector. The peak purity of galanthamine and haemanthamine was evaluated by ^{3D}CE-Chemstation software version A 09 (Agilent Technologies), using the "similarity curve" technique. In this approach a selected spectrum is compared with all the other spectra taken as the peak eluted; the degree of similarity of these spectra is plotted over the time during elution. By considering that ideal similarity factor is correspondent to a match factor of 1000, the similarity factor obtained for the peaks of galanthamine and haemanthamine were found to be higher than 990 thus confirming good peaks purity and high selectivity of NACE conditions. In the inset of Fig. 2 are reported the UV spectra of galanthamine and haemanthamine.

Table 2
Repeatability of retention time and migration time for galanthamine and haemanthamine in GC–MS and NACE

Analyte	Time[a] (R.S.D.%)	
	Intra-day ($n=3$)	Inter-day ($n=9$)
Galanthamine$^{GC–MS}$	20.51 (0.03)	20.54 (0.80)
Galanthamine^{NACE}	5.72 (0.62)	5.43 (1.92)
Haemanthamine^{NACE}	7.10 (0.81)	6.92 (2.23)

NACE conditions as in Fig. 1c; GC–MS conditions as in the text.
[a] Retention time (GC–MS) and migration time (NACE) in min.

3.3.3. Repeatability

Intra-day and inter-day precision of migration times was evaluated by replicated analysis of standard solutions (methanol) at a concentration level of about 0.05 mg/mL for each of the analytes. The results are reported in Table 2; an expected higher reproducibility was obtained in GC–MS, however, adequate R.S.D.% values (<2.5%) were obtained under NACE conditions.

3.3.4. Linearity and sensitivity

The linearity of response to UV detector (280 nm) in NACE was evaluated simultaneously for galanthamine and haemanthamine in the presence of papaverine as internal standard. The corrected peak area (area/migration time) ratios (analyte to internal standard, response y) of both the analytes were plotted against the related concentrations (c) to obtain the calibration graphs.

The same calibration solutions were analysed by GC–MS; the linearity of response to MS detector in GC analysis was evaluated only for galanthamine. The SIM area ratios (galanthamine versus internal standard; response y) were plotted against the related concentration of galanthamine (c) to obtain the calibration graph. The results of linear regression analysis are reported in Table 3; as it can be seen, the two techniques performed similarly in terms of linearity, with excellent correlation coefficients, and sensitivity. A reasonable estimation of LOQ can be provided by the lowest calibration point (about 6 μg/mL). The obtained sensitivity can be considered adequate to the determination of alkaloids from real samples; however, as shown by the UV spectra reported in Fig. 2, under NACE conditions, enhanced detectability could be achieved at the wavelength of 220 nm.

3.3.5. Applications

Analysis of real samples was performed on dried powdered bulbs (100 mg) of *N. jonquilla* extracted with methanol (10 mL) under the optimized conditions. Aliquots of 5.0 mL of the obtained methanol extract were evaporated to dry under

Table 3
Regression curves and sensitivity data for the studied analytes by GC–MS and NACE

	Concentration range (mg/mL)	m	q	r	LOD (μg/mL)
Galanthamine$^{GC–MS}$	0.00615–0.615	5.78	0.043	0.999	1.8
Galanthamine^{NACE}	0.00615–0.615	10.18	−0.055	0.999	2.0
Haemanthamine^{NACE}	0.00615–0.615	8.07	−0.031	0.999	2.0

Regression curve data for five calibration points. $y = mc + q$, where y is the ratio between the corrected peak area (area/migration time) of analyte to the internal standard (NACE) or SIM area ratios (galanthamine vs. internal standard), c the concentration, m the slope, q the intercept and r is the correlation coefficient. Conditions as in Table 2.

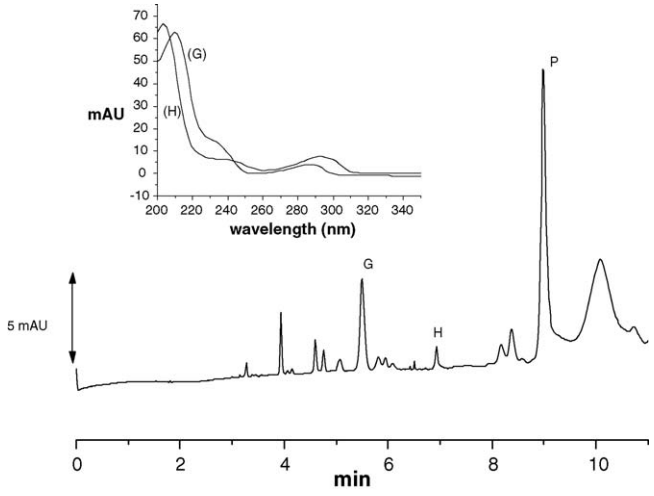


Fig. 2. NACE analysis of *N. jonquilla* extract in the presence of the internal standard papaverine (P). The inset shows the UV spectra of galanthamine (G) and haemanthamine (H) obtained using a diode-array detector. Conditions and symbols as in Fig. 1c.

vacuum and the residue was reconstituted with the solution of internal standard (papaverine in methanol). The same analytical sample was subjected to NACE and GC–MS analyses; representative separation profile of *N. jonquilla* extracts using both the techniques are shown in Figs. 2 and 3, respectively. The quantitation of the alkaloids was performed using the

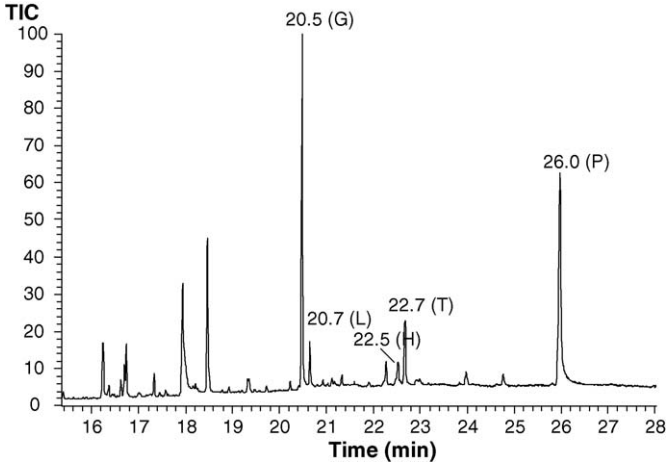


Fig. 3. GC–MS of *N. jonquilla* extract under optimized conditions and in the presence of internal standard papaverine (P). Conditions as in the text. Other symbols: (G) galanthamine; (H) haemanthamine; (L) lycoramine; (T) tazettine.

Table 4
Recovery data for galanthamine in *N. jonquilla* by GC–MS

Added amount (μg)[a]	Found (μg)	Recovery (%)	R.S.D.%	
			Intra-day ($n = 3$)	Inter-day ($n = 9$)
52	44.3	85.2	2.3	3.6
104	88.7	85.3	1.4	4.3
156	134.2	86.0	1.1	3.5

[a] Amount added to 100 mg of powdered bulb.

external standard method either in NACE and GC–MS; a content of 217.1 mg/100 g (dried bulb) of galanthamine was found (R.S.D.% 2.6; $n = 3$) by GC–MS analysis and 220.6 mg/100 g (R.S.D.% 5.2; $n = 3$) by NACE. The results obtained by the two techniques were comparable as it was estimated by the Student's t-test at 95% confidence level which indicated no significant difference between the methods. The content of haemanthamine determined by NACE was of 31.8 mg/100 g (R.S.D.% 4.0; $n = 3$).

The accuracy of the GC–MS method was evaluated by recovery studies performed on galanthamine at three levels (spiked at about 20–70% level of the target amount); the results are reported in Table 4. In NACE method, the recovery was evaluated for both the studied alkaloids (each spiked at 50% level of the target); the recoveries were 83.3% (R.S.D.% 1.8, $n = 3$) and 81.3% (R.S.D.% 1.5, $n = 3$) for galanthamine and haemanthamine, respectively. In general, the mean recovery was higher than 80% and the obtained R.S.D.% values (<4.5%) demonstrated the good precision of the whole analytical method.

4. Conclusion

The analysis of Amaryllidaceae alkaloids in *Narcissus* extracts was performed by NACE and GC–MS. The use of non-aqueous running buffers made capillary electrophoresis a fully compatible tool respect to GC and the two techniques were applied on the same extracts obtained with a minimal sample preparation. GC–MS demonstrated to be superior in comparison with NACE in terms of efficiency and opportunities in identification of analytes, however, drawbacks in direct GC determination due to thermo decomposition of analytes such as haemanthamine can limit its applicability. After identification in actual extracts, haemanthamine was isolated at semipreparative level by RP-HPLC and it was fully characterized by ^{1}H NMR before the use as reference substance. Quantitation by NACE was reliably carried out on haemanthamine and galanthamine in bulbs

of *N. jonquilla*, whereas GC–MS was applied only in determination of the latter. The results obtained in quantitation of galanthamine by GC–MS and NACE were in good agreement, but the NACE method offered the opportunity for more rapid analysis. The obtained validation parameters demonstrated that NACE is potentially useful in analysis of the other Amaryllidaceae alkaloids as an alternative method to the existing HPLC approaches.

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