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Metabolic Profiling of Bioactive *Pancratium canariense* Extracts by GC-MS†

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ABSTRACT:

Introduction – *Pancratium canariense* Ker Gawler is a plant species belonging to family Amaryllidaceae. Plants from this family are known to synthesise a particular type of bioactive compounds, named Amaryllidaceae alkaloids, which have shown AChE inhibitory activity.

Objective – To perform the metabolite profiling of methanolic extracts from *P. canariense* in order to identify bioactive compounds.

Methodology – Methanolic extracts from bulbs, leaves and fruits were separated into alkaloid-free apolar and polar fractions, as well as alkaloid fractions, and subjected to AChE assay. Metabolite profiling of extracts and fractions of *P. canariense* was carried out by GC-EI-MS and LC-ESI-TOF-MS.

Results – AChE inhibitory activities of the alkaloid fractions at a concentration of 10 μg/mL were 29.80 ± 0.91, 40.93 ± 4.60 and 58.06 ± 1.18% for the bulbs, leaves and fruits, respectively. Seventy-six metabolites—mono-, di- and trisaccharides, fatty acids, amino acids, sterols as well as several Amaryllidaceae alkaloids—were detected. Further purification of the alkaloids from the methanolic extracts resulted in the detection of 31 compounds including several potent AChE inhibitors such as habranthine and galanthamine, and the structural elucidation of 3-*O*-acetylhabranthine, a new natural compound with potential AChE inhibitory activity.

Conclusion – The described method resulted in effective integration of both GC-EI-MS and LC-ESI-TOF-MS strategies, which permitted the identification of many metabolites, as well as the structural elucidation of new compounds with potential AChE inhibitory activity. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Metabolic profiling; GC-EI-MS; LC-ESI-TOF-MS; Amaryllidaceae alkaloids; acetylcholinesterase inhibitors

Introduction

Pancratium canariense Ker Gawler is an early autumn flowering bulbous plant species that grows spontaneously in the Canary Islands. The genus *Pancratium* (Amaryllidaceae) comprises about 20 species, extending from the Canary Islands through the Mediterranean region to tropical Asia, and southwards through West Africa to Namibia (Walters *et al*., 1986). Plants of the Amaryllidaceae family are known to synthesise a particular type of bioactive compounds, named Amaryllidaceae alkaloids (Bastida *et al*., 2006). A recent phytochemical study on *P. canariense* revealed 16 alkaloids, four of them new (Cedrón *et al*., 2009).

Acetylcholinesterase (AChE) inhibitors are used to treat Alzheimer's disease, a neurodegenerative disorder causing severe health problems in the aged population (Maelicke *et al*., 2001). Natural compounds from various structural classes such as alkaloids (Amaryllidaceae, isoquinoline, steroidal, indole) terpenoids, coumarines, etc. have shown AChE inhibitory activity (López *et al*., 2002; Houghton *et al*., 2006; Mukherjee *et al*., 2007). Galanthamine, an Amaryllidaceae alkaloid with long-acting, selective and reversible AChE inhibitory activity, is marketed as a hydrobromide salt for the treatment of Alzheimer's disease. The search for more effective AChE inhibitors from the inexhaustible natural sources has revealed other Amaryllidaceae alkaloids, such as sanguinine, habranthine and 1-*O*-acetyllycorine, which possess inhibitory activity similar to or stronger than galanthamine (López *et al*., 2002; Houghton *et al*., 2006).

Classical phytochemical isolation and structural elucidation is time- and solvent- consuming, and normally results in the isolation and identification of only 10–15 compounds. Bioactivity-guided isolation, also a time-consuming procedure, often results in the isolation of already known bioactive compounds. Exploring plant biochemical diversity by mass spectrometric techniques such as LC-MS (Schielmann *et al*., 2008) and GC-MS (Fiehn *et al*., 2000; Kopka *et al*., 2004) has proved to be a fast and reliable approach, allowing identification of a large number of

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compounds. In some cases, the GC-MS screening of plant samples has revealed compounds with unknown MS spectra, which has resulted in the isolation and spectroscopic identification of new natural bioactive molecules (Berkov *et al*., 2008b). The use of LC-ESI-MS is a rapidly developing approach for the analysis of secondary plant metabolites (Schielmann *et al*., 2008). In contrast to EI-MS, the application of ESI-MS used in LC to find or structurally elucidate unknown natural compounds is still limited due to the lack of spectral databases and the relatively poor structural information obtained. Recently, however, the use of time-of-flight (TOF) mass analysers, both in GC- and LC-MS, has provided the accurate mass of the molecule ion fragments, which converts it into a powerful tool for the structural elucidation of unknown compounds.

Metabolic profiling of plant extracts provides information on their chemical composition. Therefore, it allows detection of chemically varied bioactive molecules and unknown compounds, as well as assessing the possibility of their isolation. There are many examples of general metabolic profiling (e.g. Grata *et al*., 2008), but only a few recent publications reporting the use of a metabolic profiling approach to search for bioactive molecules have been found (Bailey *et al*., 2004; Cardoso-Taketa *et al*., 2008; Mohn *et al*., 2009). A screening of methanolic extracts from *P. canariense* showed that AChE inhibitors were present, which encouraged us to perform a detailed mass spectral study of these extracts. However, in alkaloids previously reported for this plant species, the AChE inhibitory activity is known to be weak (Bastida *et al*., 2006; Houghton *et al*., 2006). In the present work, the metabolite profiling of bioactive methanolic extracts from *P. canariense* was performed for the first time by GC-EI-MS with quadrupole detector. It revealed more than 100 metabolites such as saccharides, fatty acids, amino acids, sterols and alkaloids. The combination of GC-EI-MS and LC-ESI-TOF-MS resulted in the identification of various known Amaryllidaceae alkaloids and a new potentially bioactive compound.

Experimental

Chemicals and reagents

Methanol (HPLC-grade), ethyl acetate, sulphuric acid and ammonium water (analytical grade) were purchased from SDS (Val de Reuil, France). *n*-Hydrocarbon mixture (C9–C36, Connecticut ETPH Calibration mixture) was supplied by Restek (Bellefonte, PA, USA). *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material

Plant materials (bulbs, leaves and fruits) of *Pancratium canariense* Ker Gawler (Amaryllidaceae) were collected in the post-flowering period in the Barranco del Sao (Agaete, NW Gran Canaria, Canary Islands, Spain), in December 2006. A voucher specimen (LPA015291) of the plant species has been deposited at the herbarium of the Jardín Botánico Canario 'Viera y Clavijo' (Las Palmas de Gran Canaria, Gran Canaria, Canary Islands, Spain).

Metabolite extraction

Fresh plant organs (bulbs, leaves and fruits) were separately macerated in methanol for 72 h. The solvent was evaporated *in vacuo* and a few milligrams of the dry residue were used for metabolite profiling. The alkaloid fractions were obtained from the methanolic extracts (ca. 100 mg), which were dissolved in 2% sulphuric acid (3 mL). The neutral compounds were removed with diethyl ether (3 × 5 mL). After basification of the solution with 25% ammonium water to pH 9–10, the alkaloids were extracted with ethyl acetate (3 × 5 mL). Ethyl acetate was evaporated from this extract, which was dissolved in 250 µL of methanol for further LC-ESI-TOF-MS and GC-MS analysis or dissolved in methanol at a concentration of 1 mg/mL for further AChE assays.

To obtain apolar fractions for the AChE assays, the methanolic extracts (ca. 100 mg) were dissolved in 3 mL of methanol–water (1:1) at pH 4–5 (adjusted with 2% sulphuric acid) and then extracted with 3 mL of chloroform. Polar fractions were obtained from the same amount of methanolic extract but dissolved in methanol–water at pH 8–9 (adjusted with 25% ammonium water) after elimination of the apolar compounds with chloroform. The solvents were evaporated under a stream of nitrogen and the fractions were dissolved in methanol at a concentration of 1 mg/mL for further AChE assays.

Derivatisation

Five millgrams of the dried methanolic extracts were dissolved in pyridine (50 µL) and derivatised for 90 min at 40°C with BSTFA (50 µL) for metabolite profiling following a variation of the protocol proposed by the method of Medeiros and Simoneit (2007). Derivatised extracts were evaporated to dryness with nitrogen gas and resolved in 250 µL of methanol before GC-MS injection.

GC-MS

The GC-MS analyses were carried out on Hewlett Packard 6890 coupled with MSD 5975 equipment (Hewlett Packard, Palo Alto, CA, USA) operating in EI mode at 70 eV. An HP-5 MS column (30 m × 0.25 mm i.d., film thickness 0.25 µm) was used. The temperature programme was: 100–180°C at 15°C/min, 180–300°C at 5°C/min and 10 min hold at 300°C. The injector temperature was 250°C. The flow-rate of carrier gas (helium) was 0.8 mL/min. A split ratio of 1:20 was used. A quantity of 1 µL of the solutions was injected.

LC-ESI-TOF-MS

The LC system consisted of an Agilent 1200 binary pump system (Waldbronn, Germany), equipped with an autosampler and a thermostatically controlled column compartment. The chromatographic separation of alkaloids was performed on a Luna C_{18} column (150 × 2.1 mm, 5 µm particle size; Phenomenex, Torrance, CA, USA) at a constant solvent flow rate of 400 µL/min. Gradient elution was performed with 0.05% aqueous CH_3COOH (v/v, solvent A) and methanol (solvent B). An increasing linear gradient (v/v) of solvent B was applied (min, %B): (0, 5), (15, 100), (25, 100), (25.5, 5) and (30, 5), and the injection volume was 15 µL.

A QSTAR Elite hybrid Quadrupole-Time of Flight (QToF) mass spectrometer (Applied Biosystems, PE Sciex, Concord, Ontario, Canada) coupled on-line to LC as described above was used. The instrument provided a typical resolution of 10000 (*m/z* 879.9723). All the acquisition and data analyses were controlled by Analyst QS version 2.0 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada). ToF MS data were recorded from *m/z* 70 to 700 with an accumulation time of 1 s and a pause between the mass range of 0.55 ms, operating in the positive mode. The Q1 transmission window was: 50.1% for *m/z* 80 and 49.9% for *m/z* 190. Reserpine (1 pmol/µL) in product ion scan mode of *m/z* 609 was used for

calibration of the mass spectrometer. The instrument parameter settings were the following: capillary voltage 4000 V, nebuliser gas (N_2) 40 (arbitrary units), curtain gas (N_2) 50 (arbitrary units), collision gas (N_2) 5 (arbitrary units), focusing potential 380 V, declustering potentials (DP) 80 V and (DP2) 10 V, drying gas (N_2) 70 (arbitrary units) heated to 400°C.

Identification of the metabolites

The compounds of the methanolic extracts were identified as TMS with the help of the NIST 05 database (NIST Mass Spectral Database, PC-Version 5.0, 2005, National Institute of Standardisation and Technology, Gaithersburg, MD, USA), and other plant-specific databases: the Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/home/gmd_sm.html) and the lipid library (http://www.lipidlibrary.co.uk/ms/ms01/index.htm), as well as literature data (Medeiros and Simoneit, 2007) on the basis of matching mass spectra and Kovats retention indexes (RI). The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS 2.64, NIST Gaithersburg, MD, USA) 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 many unidentified compounds were determined on the basis of the specific mass spectral fragmentation and in comparison with the mass spectra of the known metabolites. All unknown compounds, comprising more than 0.1% of total ion current (TIC), were placed in Table 1 and used to calculate the relative contribution of each metabolite group in Table 2.

The compounds in the alkaloid fraction were identified by comparing their GC-MS spectra and RI with those of authentic compounds previously isolated and identified by other spectrometric methods (NMR, UV, CD, MS) in our laboratory or by comparing their mass spectral fragmentation with standard reference spectra from the NIST 05 database, as indicated in Table 3.

RI values of the compounds were measured with standard *n*-hydrocarbon calibration mixture (C9–C36; Restek, Bellefonte, PA, USA, catalogue no. 31614, supplied by Teknokroma, Spain) using AMDIS 2.64 software.

The area of GC-MS peaks depends not only on the corresponding compounds but also on the intensity of their mass spectral fragmentation, so the data given in the tables are not true quantification. However, they can be used for comparing between the samples, which is the objective of this work.

Microplate AChE assay

Acetylcholinesterase (AChE) inhibitory activity of the plant methanolic extracts and their polar, apolar and alkaloid fractions at concentrations of 0.001, 0.01, 0.1, 1, 10 and 100 µg/mL were measured as described by López *et al*. (2002). The samples were measured in triplicate and the results are presented as a mean ± standard deviation. Galanthamine was used as a positive control in the assay.

Results and Discussion

The methanolic extracts of *P. canariense* bulbs inhibited AChE at a concentration of 100 µg/mL (54.38 ± 3.95%), thus indicating the presence of AChE inhibitors in this plant species. Methanolic extracts from bulbs, leaves and fruits were separated into alkaloid free apolar and polar fractions, as well as alkaloid fractions. As expected, the results showed that the alkaloid fractions of *P. canariense* possess bioactivity. AChE inhibitory activities of these fractions at a concentration of 10 µg/mL were 29.80 ± 0.91, 40.93 ± 4.60 and 58.06 ± 1.18% for the bulbs, leaves and fruits, respectively. Apolar fractions, containing lipids, showed no activity, whereas only the polar fraction of the fruits showed weak activity (17.27 ± 3.25%) at a concentration of 10 µg/mL.

Silylated methanolic extracts of bulbs, leaves and fruits of *P. canariense* were subjected to GC-MS analysis, which resulted in the detection of about 102 compounds including some Amaryllidaceae alkaloids (Tables 1 and 2, Fig. 1). The principal compounds were saccharides (mono-, di- and trisaccharides), followed by fatty acids, alkaloids and phosphates. With the exception of compound **58** (Table 1), all the other unidentified compounds showed fragmentation patterns characteristic for mono-, di- or trisaccharides (Medeiros and Simoneit, 2007). Their further identification, however, was hampered by the high number of isomers available in this group (Medeiros and Simoneit, 2007) and the lack of sufficient data on their RI.

Bulbs contained the highest content of sugars (*ca*. 90% of TIC), including the highest content of trisaccharides. The highest content (*ca*. 66%) of monosaccharides (mainly fructose and glucose) was found in the photosynthesising parts of the plants, such as leaves. The disaccharides (mainly sucrose) were accumulated in the fruits (Table 2), which also showed the highest amounts of alkaloids (7.3%), fatty acids (3.8%, mainly linoleic and palmitic acids), phosphates (3.3%) and organic acids (1%). The greater amounts of fatty acids and disaccharides in the fruits could be associated with their storage function, while the high content of alkaloids, particularly lycorine (**54**, 3.4%), is related to the defence functions of these compounds. Lycorine is known for its potent cytotoxic, antiviral and antifungal activities, among others (Bastida *et al*., 2006). Haemanthidine (**60**), a bioactive alkaloid, was detected in the leaf methanol extract as TMS derivative because the underivatised compound shows irregular and broad peak shape under GC conditions and its identification is problematic when it is in trace amounts or overlapped by other compounds. Sterols, mainly β-sitosterol (**72**), occurred in the extract with less than 1% of the TIC. Traces of amino acids were detected in the bulbs and leaves.

Because of the high number of compounds and low concentration of alkaloids, only a few alkaloids were detected in the methanolic extracts. Concentrated bioactive alkaloid fractions were directly subjected to GC-MS without any derivatisation step and as a result 31 compounds of galanthamine, haemanthamine, lycorine, homolycorine, tazettine and montanine types were detected (Tables 3 and 4, and Fig. 2). The haemanthamine and crinine series of Amaryllidaceae alkaloids only differ in the position of the 5,10b-ethano bridge, and they can only be distinguished by a circular dichroism spectrum (De Angelis and Wildman, 1969). We assigned the position of the 5,10b-ethano bridge for compounds **51**, **56** and **86** based on the results of a recent study (Cedrón *et al*., 2009). The identity of compounds **96**, **101** and **102**, for which we had no standards or reference spectra in the databases, was confirmed by LC-ESI-TOF-MS data in addition to their GC-EI-MS spectra. Compounds **84**, **89** and **99** were left unidentified. Considering their low concentration (<5% of TIC) and the complexity of alkaloid mixtures, their isolation and structural elucidation by other spectroscopic methods could be problematic.

Table 1. Metabolites identified in *P. canariense* methanolic extracts by GC-MS. Values are expressed as a percentage of the total ion current (TIC)

	Compound	Rt	Bulbs	Leaves	Fruits
1	Lactic acid[a,b]	3.61	—	—	tr
2	Alanine[a,b]	3.66	—	0.01	—
3	Glycolic acid[a,b]	3.72	—	0.02	0.05
4	Leucine[a,b]	4.55	0.01	—	—
5	β-Hydroxybutanoic acid[a]	4.58	tr	0.02	0.01
6	Isoleucine[a,b]	4.77	tr	—	—
7	Monomethylphosphate[a]	4.84	0.23	1.08	2.48
8	Serine[a,b]	5.62	tr	—	—
9	Glycerol[a,b]	5.76	0.02	0.01	0.01
10	Phosphoric acid[a,b]	5.81	0.04	0.94	0.86
11	Threonine[a,b]	5.98	tr	—	—
12	Succinic acid[a,b]	6.11	tr	0.05	0.86
13	Glyceric acid[a,b]	6.34	—	0.05	0.02
14	Fumaric acid[a,b]	6.42	—	0.01	0.03
15	3,4-Dihydro-2-furanone[a]	6.78	—	0.04	—
16	Pentonic acid 1,4-lactone[a]	9.45	—	0.04	0.04
17	Arabinose[a,c]	9.33	0.02	—	—
18	Gulonic acid[a,b]	9.41	—	0.06	0.01
19	Arabinose[a,c]	9.73	0.02	—	—
20	Xylitol[a,c]	10.43	tr	—	—
21	Xylose[a,c]	10.56	0.01	—	—
22	Arabitol[a,c]	10.75	0.04	—	—
23	UM	10.92	0.08	—	—
24	UM	11.11	4.97	6.73	5.38
25	UM	11.36	0.03	tr	5.11
26	UM	11.44	2.91	7.15	—
27	UM	11.52	—	1.56	—
28	UM	11.62	0.47	—	0.53
29	Altrose[a,c]	11.75	0.68	—	—
30	Mannose[a,c]	11.77	0.34	3.06	—
31	Fructose 1[a,c]	11.92	5.86	7.35	2.46
32	Fructose 2[a,c]	12.04	8.90	5.27	8.67
33	UM	12.16	8.42	3.78	8.60
34	Fructose 3[a]	12.42	1.35	2.86	—
35	Mannose[a,c]	12.53	2.05	4.30	0.62
36	UM	12.97	tr	0.78	—
37	Glucose[a,c]	13.31	6.45	9.03	0.84
38	UM	13.43	0.93	2.21	0.46
39	UM	13.63	—	—	1.10
40	UM	13.88	5.18	5.13	0.77
41	UM	13.94	1.29	2.31	—
42	UM	14.17	1.29	4.67	1.20
43	Glucose[a,c]	14.69	8.74	13.61	2.60
44	Palmitic acid[a,b]	15.17	0.03	0.13	1.16
45	Inositol[a–c]	16.53	0.53	0.13	0.20
46	Phytol[a,b]	17.26	—	0.07	1.57
47	Linoleic acid[a,b]	17.82	—	0.06	1.82
48	α-Linolenic acid[a,b]	17.92	0.06	0.02	0.72
49	Oleic acid[a,b]	18.01	0.01	—	—
50	Stearic acid[a,b]	18.26	—	—	0.12
51	Vittatine/crinine[d]	21.66	0.05	1.50	0.93
52	Uridine[a,b]	21.94	0.01	—	0.74
53	Habranthine[d]	22.56	—	—	0.73
54	Lycorine[d]	23.07	0.48	0.21	3.41
55	Heamathamine[d]	23.20	tr	0.11	1.35
56	11-Hydroxyvittatine[d]	23.41	0.07	0.30	0.88
57	UD	23.56	3.09	1.52	1.58

Table 1. *(Continued)*

	Compound	Rt	Bulbs	Leaves	Fruits
58	UC	24.01	—	—	0.39
59	UD	24.51	4.52	1.96	1.51
60	Haemanthidine[d]	24.52	—	0.02	—
61	UD	24.80	2.31	0.91	1.95
62	Sucrose[a,c]	25.36	13.18	6.84	24.64
63	UD	25.41	0.61	0.90	2.22
64	UD	25.74	0.29	0.90	1.29
65	UD	26.08	0.84	0.71	2.03
66	UD	26.26	0.86	0.35	0.62
67	UD	26.65	—	—	1.40
68	Palatinose[c]	26.93	—	0.38	0.88
69	Campestrol[a,b]	32.62	0.02	0.06	0.06
70	Stigmasterol[a,b]	33.03	—	0.01	—
71	τ-Sitosterol[a]	33.33	—	0.09	—
72	β-Sitosterol[a,b]	33.81	0.23	0.38	0.39
73	UT	34.02	1.90	—	—
74	UT	34.95	2.32	tr	1.26
75	UT	35.68	2.31	0.11	1.25
76	UT	36.13	5.93	0.22	2.20

Identification: [a] NIST 05 Database; [b] Golm Database; [c] Literature data (Medeiros and Simoneit, 2007); [d] Standard compound.
UM, unidentified monosaccharide; UD, unidentified disaccharide; UT, unidentified trisaccharide; UC, unidentified compound; tr, traces; Rt, retention time.

Table 2. Distribution of the main metabolite groups in the different organs of *P. canariense*. Values are expressed as a percentage of TIC

Metabolite group	Bulbs	Leaves	Fruits
Organic acids	0.01	0.24	1.01
Amino acids	0.01	0.01	—
Phosphates	0.27	2.02	3.34
Carbohydrates	90.28	81.22	82.68
monosaccharides	51.24	66.18	35.74
disaccharides	26.01	14.58	42.03
trisaccharides	12.46	0.33	4.71
sugar alcohols	0.57	0.13	0.20
Free fatty acids	0.10	0.21	3.81
Diterpene alcohols	—	0.07	1.57
Alkaloids	0.60	2.13	7.31
Sterols	0.25	0.54	0.45

The alkaloid pattern of bulbs was dominated by lycorine (51% of TIC) and haemanthamine (16%) type compounds with lycorine (**54**, 38%) and the homolycorine type compound ungerine (**96**, 10%) being the major components. The alkaloid pattern of leaves was dominated by haemanthamine and lycorine type compounds (65 and 15%, respectively), vittatine (**51**, 56%) being the main component while the remaining compounds comprised less than 10% of the TIC. The alkaloid pattern of fruits was dominated by lycorine (37%), galanthamine (29%) and haemanthamine (27%) type compounds, and the major alkaloids in the fruits were lycorine (**54**, 33%), habranthine (**53**, 23%) and vittatine (**51**, 20%).

Compound A3 (**92**) showed ion fragments at the low mass range typical for galanthamine type alkaloids (at *m/z* 55, 65, 77, 91, 115, 128, 141 and 152). The presence of an ion at *m/z* 213 as well as the shifting of the base and the $[M]^+$ ions in habranthine from *m/z* 230 and 303, respectively, to *m/z* 272 and 345 (with 42 u more), indicated a compound similar to habranthine with a substitution of one of its hydroxyl groups by an acetyl moiety. The presumed GC-EI-MS fragmentation of compound A3 and habranthine is shown in Fig. 3. In contrast to the other galanthamine type alkaloids, the predominant process in habranthine mass fragmentation and compound A3, possessing a hydroxyl group at C-11, is the elimination of the N atom ($[M - 73]^+$) forming the base ions

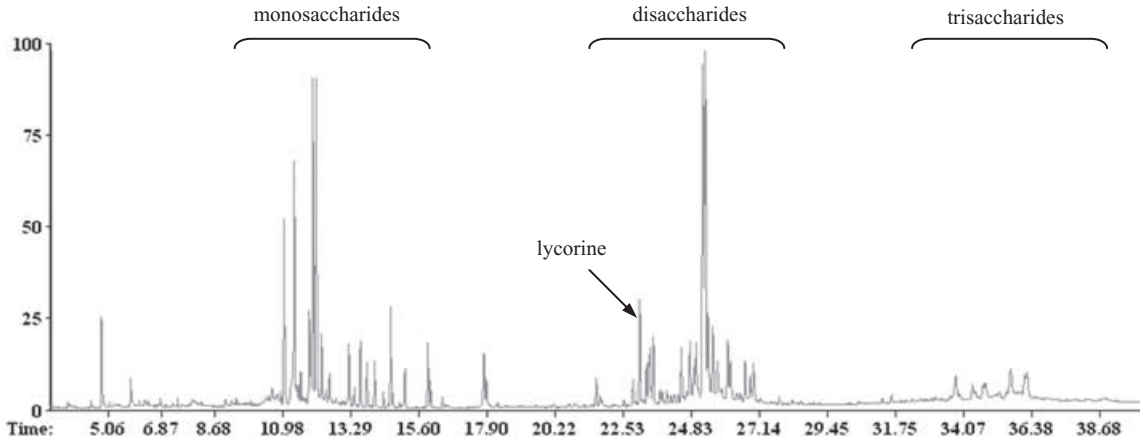


Figure 1. GC-MS chromatogram of the silylated methanolic extract of *Pancratium canariense* fruits.

Table 3. Alkaloids found in *P. canariense*. Values are expressed as a percentage of TIC

Alkaloid	Rt	$[M]^+$	Bulbs	Leaves	Fruits
Ismine (**77**)[a]	18.61	257	1.06	0.17	0.34
Trisphaeridine (**78**)[a]	18.63	223	0.08	0.03	0.17
Galanthamine (**79**)[a]	20.56	287	2.07	0.38	2.59
Buphanisine (**80**)[b]	20.86	285	tr	0.20	—
N-Demethylgalanthamine (**81**)[a]	21.19	273	3.31	2.41	2.91
Vittatine (**51**)[a]	21.65	271	6.99	55.61	20.32
Narwedine (**82**)[a]	21.77	285	0.01	—	—
Anhydrolycorine (**83**)[b]	22.03	251	0.63	0.14	0.62
A1 (**84**)	22.19	281	3.24	1.05	1.64
Deoxytazettine (**85**)[b]	22.66	315	0.06	0.01	—
Habranthine (**53**)[a]	22.78	303	7.97	4.06	22.88
Vittatine acetate (**86**)[b]	23.08	313	—	0.09	—
Cheriline (**87**)[b]	23.2	313	tr	—	—
Pancratinine C (**88**)[c1]	23.33	287	2.51	3.48	0.24
A2 (**89**)	23.61	249	1.31	0.54	0.98
Haemanthamine (**55**)[a]	24.02	301	1.24	4.02	0.27
Tazettine (**90**)[a]	24.26	331	2.63	2.13	1.83
Hippamine (**91**)[b]	24.59	301	0.93	0.22	—
A3, 3-*O*-Acetylhabranthine (**92**)	24.82	345	—	0.76	0.13
Pancracine (**93**)[b]	25.05	287	0.38	2.50	2.52
Galanthine (**94**)[b]	25.18	317	4.43	3.20	1.05
11-Hydroxyvittatine (**56**)[a]	25.27	287	5.13	1.24	6.43
Lycorine (**54**)[a]	25.95	276	37.53	6.50	32.74
Incartine (**95**)[a]	25.86	333	tr	3.08	0.58
Ungerine (**96**)[c2]	26.28	329	10.44	1.08	0.37
N-Formylnorgalanthamine (**97**)[a]	26.61	301	—	0.01	0.30
Pseudolycorine (**98**)[a]	26.84	289	—	0.01	—
A4 (**99**)	27.29	373	1.17	0.06	0.12
Hyppeastrine (**100**)[a]	27.54	315	0.28	6.19	0.38
Ungiminorine acetate (**101**)[c3]	28.06	359	5.69	0.59	0.53
Narcissidine acetate (**102**)[c4]	28.35	375	0.93	0.24	0.05

Identification: [a] standard compounds; [b] NIST 05 Database; [c] recursive procedure, HR-MS and literature data—[c1] Cedrón *et al*., 2009; [c2] Razakov *et al*., 1967; [c3] Kobayashi *et al*., 1985; Suau *et al*., 1988; [c4] Kihara *et al*., 1995.

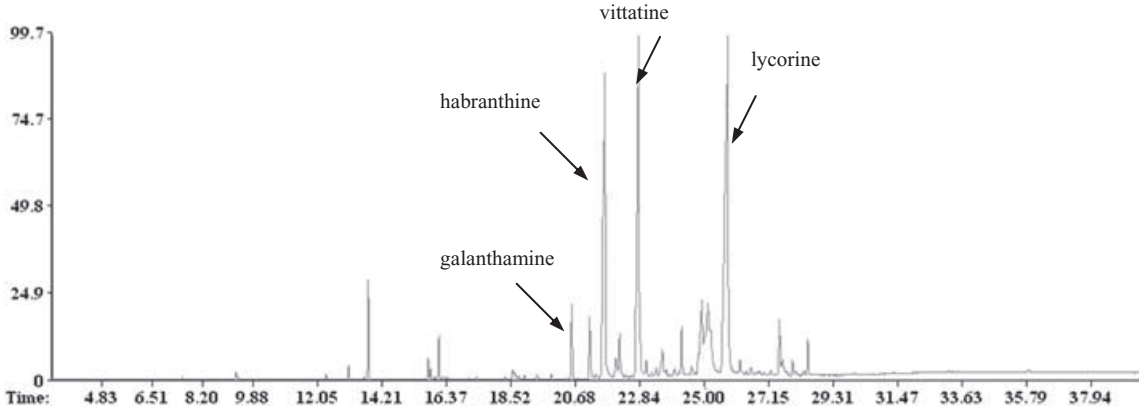


Figure 2. GC-MS of the alkaloid fraction (not silylated) of *Pancratium canariense* fruits.

Table 4. Distribution of the main alkaloid groups in the organs of *P. canariense*. Values are expressed as a percentage of TIC

Alkaloid	Bulbs	Leaves	Fruits
Miscellaneous	1.13	0.2	0.51
Galanthamine	14.54	7.68	28.93
Haemanthamine	15.87	64.64	27.26
Lycorine	51.45	14.52	36.53
Homolycorine	10.71	7.27	0.76
Montanine	0.38	2.5	2.52
Tazettine	2.68	2.14	1.83
Unknown compounds	3.24	1.05	1.64

at *m/z* 230 for habranthine and *m/z* 272 for alkaloid A3 (Fig. 3). Another characteristic loss for the galanthamine type compounds is the elimination of the substituent at C-3 resulting in an ion fragment with low abundance when there is a hydroxyl group as in galanthamine (at *m/z* 270) and habranthine (at *m/z* 286), or in an intense ion when the substituent is an acetyl group as in 3-*O*-acetylsanguinine (Hesse and Berhard, 1975; Berkov *et al.*, 2008a) and compound A3 (at *m/z* 286). The loss of ring C forming an ion at *m/z* 232 as well as the loss of the substituent at C-3 (*m/z* 213) from the base ion fragments of habranthine and compound A3 confirms the allocation of the acetyl group at C-3 (Fig. 3).

The concentration of compound A3 was less than 1 % of TIC, and therefore, its isolation was also problematic. In order to have more reliable identification data for this compound, the alkaloid fraction was subjected to LC-ESI-TOF-MS analysis, resulting in the detection of a compound with a $[M + H]^+$ of *m/z* 346.1666 (calculated for A3 $[M + H]^+$ 346.1654, $C_{19}H_{24}NO_5$, ppm error 4.3). On the basis of EI-MS and HR-MS spectral data, compound A3 was identified as 3-*O*-acetylhabranthine, a new natural compound.

Ungerine (**96**), as a homolycorine alkaloid with a $\Delta^{3,4}$ bond, displayed an $[M]^+$ with very low intensity by EI-MS (Schnoes *et al.*, 1968). The intense ions at *m/z* 124 and 139 of compound **96** indicated a methoxyl group at C-2 while ions at *m/z* 162 and 190 were congruent with a lactone alkaloid with a methylenedioxy ring at C-8/C-9 (Schnoes *et al.*, 1968). Its mass spectrum is congruent with those reported in the literature (Razakov *et al.*, 1967). Furthermore, LC-TOF-MS analysis displayed an $[M + H]^+$ at *m/z* 330.1340 ($C_{18}H_{20}NO_5$, ppm error 0.5)

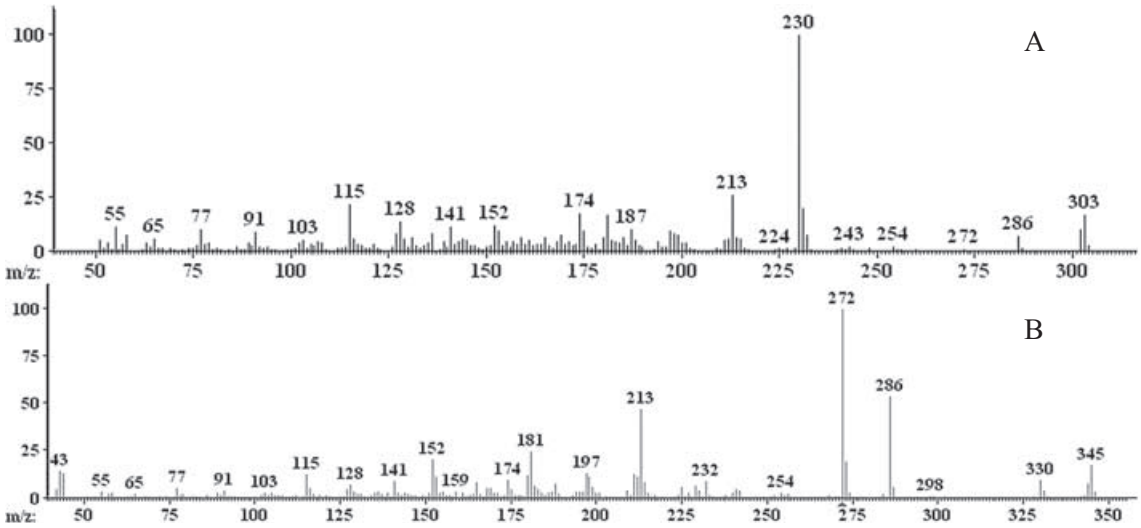
Ungiminorine acetate (**101**) and narcissidine acetate (**102**) were identified by comparing their spectra with those of ungiminorine and narcissidine literature data (Kovayashi *et al.*, 1985; Suau *et al.*, 1988; Kihara *et al.*, 1995) as well as by LC-ToF-MS analysis, displaying an $[M + H]^+$ at *m/z* at 360.1461 ($C_{19}H_{22}NO_6$, ppm error 4.5) and 376.1774 ($C_{20}H_{26}NO_6$, ppm error 3.7), respectively.

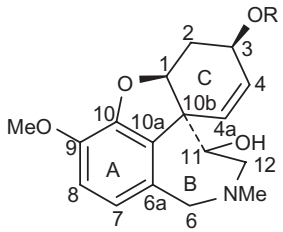
It is worth mentioning that, in contrast to recent data (Cedrón *et al.*, 2009), we found relatively high levels of galanthamine-type alkaloids in the alkaloid mixtures (up to 29% from fruits). These biochemical variations could be due to the different phenological stages of the studied plants and/or the different locations of their collection (Tenerife and Gran Canaria islands, Spain).

The AChE inhibitory activity among the Amaryllidaceae alkaloids is ascribed mainly to the lycorine and galantha-mine type compounds (López *et al.*, 2002; Houghton *et al.*, 2006). Lycorine-type compounds are less active inhibitors than the galanthamine-type compounds and their activity is associated with a substitution at positions C-1 and C-2 (Houghton *et al.*, 2006). Lycorine (**54**), incartine (**95**) and pseudolycorine (**98**) possess weak AChE inhibitory activity (López *et al.*, 2002; Houghton *et al.*, 2006; Berkov *et al.*, 2007). There is no information about the AChE inhibitory activity for the other lycorine type compounds found in this plant species.

The galanthamine-type compounds galanthamine (**79**), *N*-demethylgalanthamine (**81**), narwedine (**82**), habranthine (**53**) and *N*-formylnorgalanthamine (**97**) are known to be potent AChE inhibitors. Habranthine is even 10 times more potent than the approved drug galanthamine (López *et al.*, 2002). Therefore, the presence of the above-mentioned compounds, and habranthine in particular, could explain the AChE inhibitory activity of the extracts from *P. canariense*. The isolation or synthesis of the new galanthamine type alkaloid 3-*O*-acetylhabranthine (**92**) is of interest due to its potential activity as an AChE inhibitor.

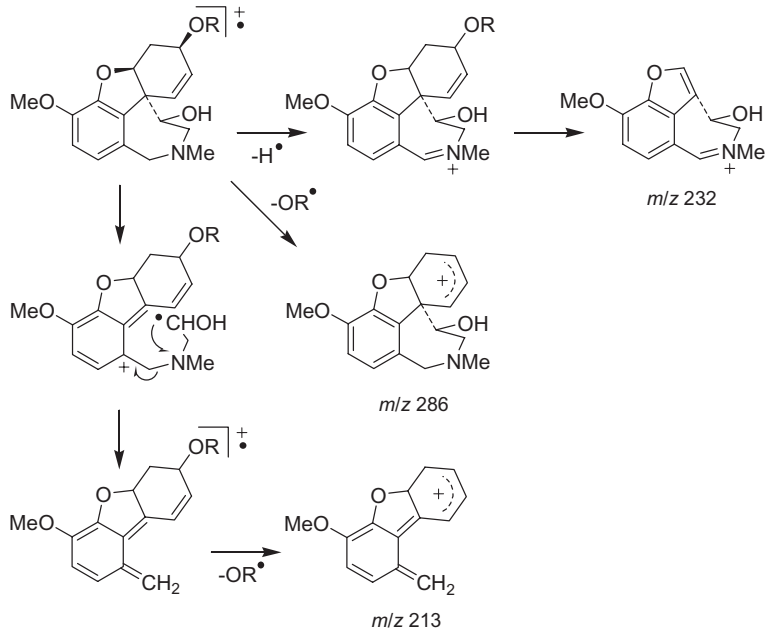
Despite the advantages of GC-MS, namely a high resolution allowing separation of a number of compounds and fast identification of metabolites due to the structural information provided by EI-MS, this technique is limited to volatile compounds and does not permit on-line detection of AChE inhibitors as is possible with HPLC-MS (Ingkaninan *et al.*, 2000).





53: Habranthine, R=H

92: 3-*O*-Acetylhabranthine, R=Ac



53: R=H *m/z* 230

92: R=Ac *m/z* 272

Figure 3. GC-EI-MS spectra and tentative fragmentation (after Hesse and Bernhard, 1975) of habranthine (A) and 3-*O*-acetylhabranthine (B).

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