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Analytical Methods

A derivatization procedure for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates by GC–MS in mulberry (*Morus sp.*)

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ABSTRACT

Different derivatization procedures were assayed to simultaneously analyse iminosugars such as deoxynojirimycin (DNJ) or fagomine and other carbohydrates of low molecular weight by gas chromatography coupled to mass spectrometry (GC–MS) in *Morus sp*. Both oximation + trimethylsilylation and oximation + acetylation allowed the separation of target compounds, whereas trimethylsilyl (TMS) and acetylated derivatives showed several coelutions. Nevertheless, oximation + acetylation were discarded for giving inaccurate results for ketoses due to their incomplete derivatization. Different conditions for the conversion into trimethylsilyl oximes (TMSO) were assayed, the best results being achieved using hexamethyldisilazane with trifluoroacetic acid as silylation agent. Contents of iminosugars (DNJ, fagomine and pipecolic acid derivatives) and other carbohydrates such as mono and disaccharides, *myo*-inositol and galactinol isomers in mulberry extracts (fruits, leaves and branches) were determined by GC–MS using the TMSO procedure.

1. Introduction

Polyhydroxyalkaloids (also known as iminosugars) are hydroxylated derivatives of piperidine, pyrrolidine, indolizidine, pyrrolizidine and nortropane (Molyneux, Gardner, James, & Colegate, 2002), which have been detected in a high number of plant families (*Leguminosae, Moraceae, Hyacinthaceae, Campanulaceae,* etc.) and microorganism genus (*Streptomyces, Bacillus*, etc.) (Watson, Fleet, Asano, Molyneux, & Nash, 2001). Different pharmacological and ecological activities have been attributed to these compounds, the inhibitory effect against glycosidase being the most studied, which makes them potential drug candidates for the treatment of several diseases (i.e. diabetes, cancer, AIDS, and viral infections) (Yokoyama, Ejiri, Miyazawa, Yamaguchi, & Hirai, 2007).

Deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol; DNJ) is a polyhydroxyalkaloid typical of the six-membered ring piperidine group. It has been shown to be a potent α-glucosidase inhibitor, although DNJ does not inhibit α-amylases (Hughes & Rudge, 1994). Its presence has been described in leaves and roots of *Morus sp., Hyacinthus orientalis* bulbs and larvae of *Bombyx mori* (Watson et al., 2001) in which its content has been used as antihyperglycemic quality criterion (Kim et al., 2003).

Many reports have been published about the chemical synthesis of iminosugars (Ferreira, Botuha, Chemla, & Perez-Luna, 2009; Gupta & Vankar, 2009); however, there is a high interest in obtaining these compounds from natural sources. Different extraction procedures based on the use of polar solvents such as ethanol, methanol, hot water (Molyneux et al., 2002) or acidulated water (Kim et al., 2003) have been carried out. However, all these procedures are not selective for the target compounds and the extraction of interferences such as other low molecular weight carbohydrates takes also place, a purification step being necessary. Therefore, the use of analytical methods which allow the determination of iminosugars and interferences to control their extraction is of crucial importance.

HPLC has been used for the analysis of polyhydroxylated alkaloids; however, the poor resolution achieved by this technique and the extreme hydrophilicity and low solubility of these compounds in non-hydroxylic organic solvents make difficult the selection of the most suitable solvent system-column packing combination (Molyneux et al., 2002) and require the search of different alternatives. Moreover, the lack of chromophores in these chemical structures limits their detection. The use of HILIC systems coupled to evaporative light scattering detectors (Kimura et al., 2004) or amino columns coupled to MS detectors (Chen, George, Weir, & Leapheart, 1990; Nash, et al., 1988) have, in some measure, reduced these problems. Furthermore, different methods using direct ESI MS analysis have been proposed for rapid detection of polyhydroxyalkaloids (Egan, et al., 1999).

However, GC–MS combines the good resolution of GC and the structural information obtained by MS. Both trimethylsilyl (TMS),

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(Molyneux, 1993) and acetylated (Magalhães, Santos, Magalhães, & Nogueira, 2002) derivatives have been used to transform iminosugars into volatile compounds. Nevertheless, the appearance of multiple peaks for each reducing carbohydrate is one the main disadvantages (Molyneux, 1993), the development of new procedures to analyse iminosugars free from interferences being necessary.

Therefore, the aim of this work was to develop a derivatization procedure for the simultaneous analysis of iminosugars (mainly DNJ and fagomine) and other carbohydrates of low molecular weight, and validate its application to extracts of mulberry leaves, fruits and branches, which are known to be a natural source of these compounds.

2. Materials and methods

2.1. Standards

Analytical standards of fructose, glucose, galactose, ribose, sucrose, *myo*-inositol, galactinol (O-α-D-galactopyranosyl-(1-1)-L-*myo*-inositol), pipecolic acid (2-piperidinecarboxylic acid), nipecotic acid (3-piperidinecarboxylic acid), isonipecotic acid (4-piperidinecarboxylic acid), α-methyl-L-proline (2-methylpyrrolidine-2-carboxylic acid) and phenyl-β-D-glucoside were obtained from Sigma Chemical Co. (St. Louis, US). DNJ was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Glyceryl-β-galactoside was obtained by transglycosidation reaction of lactose treated with β-galactosidase (Cardelle-Cobas, Martínez-Villaluenga, Sanz, & Montilla, 2009). This product was kindly gifted by Dr. Montilla (CSIC, Spain).

2.2. Samples

Samples of *Morus alba* leaves, M1L and M2L were obtained from Tarancón (Cuenca, Spain) and from Madrid (Spain), respectively. Leaves (M3L), fruits (M3F) and branches (M3B) of *Morus alba* were collected from Hoyo de Pinares (Avila, Spain). Samples of leaves (M4L), fruits (M4F) and branches (M4B) from *Morus nigra* were obtained from Madrid (Spain).

2.3. Carbohydrate extraction

The extraction of carbohydrates from mulberry samples was carried out using acidulated water, previously suggested by other authors (Kim et al., 2003), as it has been described that the presence of acid may suppress the decomposition of some pyrrolidines (Molyneux et al., 2002). One gram of samples was ground before extraction in 10 mL of acidulated water with 0.1% HCl (v/v) for 24 h. Samples were filtrated through Whatman No. 4 paper and kept at −20 °C until analyses.

2.4. Carbohydrate analysis

2.4.1. Derivatization procedures

Except for those derivatization procedures specifically indicated, 1 mL of extracts were mixed with 0.5 mL of a 70% ethanolic solution of phenyl-β-L-glucoside (1 mg mL^{-1}) employed as internal standard and dried under vacuum at 38–40 °C.

2.4.1.1. Silylation. Trimethylsilyl (TMS) ethers were prepared according to Troyano, Olano, Fernández-Díaz, Sanz, and Martínez-Castro (1991). Hundred microliters of anhydrous pyridine, 100 μL of trimethylsilylimidazole (TMSI) and 100 μL of trimethylchlorosilane (TMCS) were added to the evaporated samples. Extraction of the TMS ethers was carried out using 100 μL of hexane and 200 μL of Milli-Q water. One microliter of the hexane upper layer was injected into the GC.

2.4.1.2. Acetylation. Acetylation was carried out following the procedure described by Magalhães et al. (2002). Samples (10 mg) were treated with acetic anhydride (0.7 mL) in pyridine (0.5 mL) for 24 h at room temperature. After reaction, samples were centrifuged at 8000 rpm for 10 min, and 1 μL of supernatants was injected into the GC injection port.

2.4.1.3. Oximation + Acetylation. Oximes were obtained by addition of 350 μL of a solution of 2.5% hydroxylamine chloride in pyridine after 30 min at 75 °C according to Brobst and Lott (1966). Then, they were acetylated with acetic anhydride (0.45 mL) for 5 h. After reaction, samples were centrifuged at 8000 rpm for 10 min, and 1 μL of supernatants was injected into the GC injection port.

2.4.1.4. Oximation + Silylation. Taking into account the problems found for iminosugar derivatization, different preparation procedures were followed for the formation of TMSO. Extracts were dried by two different ways:

(i) under vacuum, with and without acetone addition to help water removal and
(ii) freeze-drying: freeze dried samples were also treated by two different procedures: exposing them to atmospheric moisture prior to derivatization according to Kite and Hughes (1997) and in close vials without moisture exposition.

Oximes were formed as indicated above for acetylated oximes using a solution of 2.5% hydroxylamine chloride in pyridine, whereas TMS derivatives were obtained by four different procedures:

(i) 300 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and 3 μL of trimethylchlorosilane (TMCS) at 60 °C for 1 h (Molyneux et al., 2002);
(ii) 90 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10 μL of trimethylchlorosilane (TMCS) at room temperature for 1 h (Knapp, 1979);
(iii) 100 μL of trimethylsilylimidazole (TMSI) and 100 μL of trimethylchlorosilane (TMCS) at room temperature for 30 min (Troyano et al.,1991);
(iv) 350 μL of hexamethyldisylazane (HMDS) and 35 μL of trifluoroacetic acid (TFA) at 45 °C for 30 min (Brobst & Lott, 1966).

After reaction, samples were centrifuged at 8000 rpm for 10 min and kept at 4 °C for 24 h before analysis. 1 μL of supernatants was injected into the GC injection port.

2.4.2. GC–MS analysis

GC–MS analyses of derivatised samples were carried out in a Hewlett–Packard 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using He at ~1 mL min^{-1} as carrier gas. A 30 m × 0.25 mm i.d. × 0.25 μm film thickness TRB-1 (crosslinked methyl silicone) from Teknokroma (Barcelona, Spain) was used. Oven temperature program was optimised, the best conditions being: from 100 °C to 200 °C at 15 °C min^{-1}, kept at this temperature for 15 min and finally programmed to 300 °C at 15 °C min^{-1} and kept at this temperature for 10 min. Injector temperature was 300 °C and injections were made in the split mode with a split ratio 1:20. Mass spectrometer was operating in electronic impact (EI) mode at 70 eV, scanning the 35–650 *m*/*z* range. Interface and source temperature were 280 °C and 230 °C, respectively. Acquisition was

done using a HP ChemStation software (Hewlett–Packard, Palo Alto, CA, USA).

Linear retention indices (I^T) were calculated from the retention times of TMSO derivatives and suitable *n*-alkanes.

Quantitative values were calculated using the internal standard method. Response factors were determined by injecting standards of different concentration containing internal standard (phenyl-β-D-glucoside) by triplicate. LOD was calculated as three times the S/N (signal-to-noise) ratio while LOQ was considered ten times this ratio according to Foley and Dorsey (1984). Accuracy was estimated by carrying out recovery assays by the addition of known amounts of glucose, DNJ and *myo*-inositol standards to a *Morus alba* extract; the three carbohydrates were measured in both unspiked and spiked samples and recoveries were calculated. Precision was calculated from the results obtained of a standard mixture and a *Morus alba* extract previously derivatised and analysed in five different days.

3. Results and discussion

3.1. Derivatization assays

Figs. 1A and B show the GC–MS profiles of acetyl and TMS derivatives of mulberry M1L extract. Derivatised sugars (ribose, glucose, fructose and sucrose), *myo*-inositol, galactinol and DNJ were identified by comparison of their retention times and mass spectra with the corresponding derivatised standards. Fagomine was identified by its characteristic *m*/*z* fragment ions according to Malgalhães et al., (2002) for acetylated derivatives (*m*/*z*: 43 (100), 98 (29), 140 (92), 182 (57), 196 (11), 242 (3)) and according to the fragmentation suggested by Molyneux, et al., (2002) for TMS derivatives of alkaloids (Table 1). However, up to five peaks can appear per reducing sugar (both methods keep the sugar ring intact and can afford a peak for each tautomer), leading to overlapped peaks. Although TMS derivatization has been the most common procedure used to analyse iminosugars (Molyneux, 1993), and DNJ could easily be quantified using this procedure in mulberry extract, fagomine coeluted with one of the five tautomeric peaks of ribose. In the acetylated sample, DNJ had the same retention time as glucose, making also its quantification difficult.

Therefore, a different derivatization procedure for the simultaneous analysis of sugars, iminosugars and inositols was assayed.

Fig. 1C shows the GC–MS profile of carbohydrates in mulberry M1L, previously submitted to the oximation + acetylation derivatization as indicated in materials and methods. By using this derivatization method reducing aldoses are converted into *per*-acetylated aldononitriles (PAAN), giving rise to a single peak. Non-reducing sugars, inositols and iminosugars gave rise to a single derivative corresponding to the *per*-acetylated compound. Reducing ketoses should form the *per*-acetylated oximes (with *syn* (*E*) and *anti* (*Z*) isomers) giving rise to two different peaks. However, derivatization of these carbohydrates was not complete, and up to five different peaks could appear for each ketose.

The effect of acetylation time of glucose, fructose, *myo*-inositol and DNJ, previously submitted to the oximation step, was evaluated by sampling the reaction mixture every hour by triplicate. Fig. 2 shows the behaviour of these standards during acetylation. Although previous studies suggested 24 h at 20 °C for carrying the acetylation of iminosugars out (Magalhães et al., 2002), incubation for 5 h was enough for the complete derivatization of glucose, *myo*-inositol and DNJ. Nevertheless, acetylation of fructose was variable during the 24 h period studied. Therefore, this derivatization procedure was discarded for mulberry samples, where fructose is one of the main sugars.

Fig. 1D shows the GC–MS profile of carbohydrates in mulberry M1 extract previously submitted to the oximation + trimethylsily-

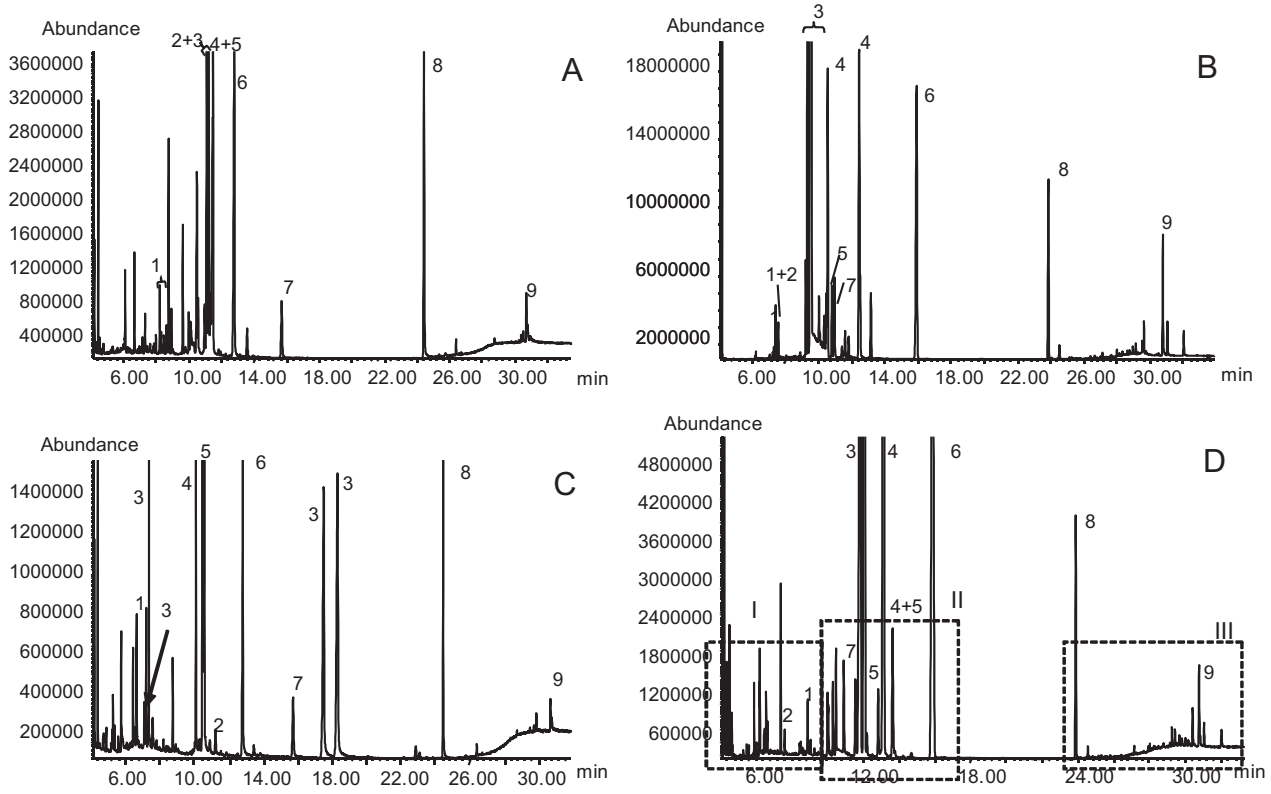
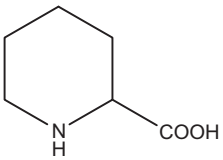
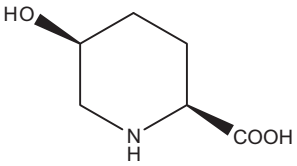
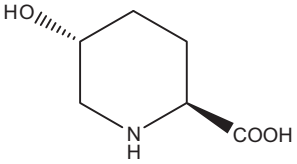
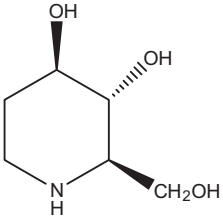
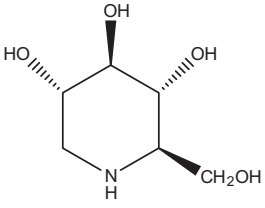


Fig. 1. GC profiles of mulberry leaves extract converted into their (A) acetylated, (B) trimethylsilyl, (C) acetylated oxime and (D) trimethylsilyl oxime derivatives. (1) Ribose, (2) fagomine, (3) fructose, (4) glucose, (5) galactose, (6) *myo*-inositol, (7) DNJ, (8) phenyl-β-glucopyranoside (i.s.), (9) galactinol.

Table 1
Linear retention indices (I^T) and MS data of TMS derivatives of polyhydroxy alkaloids found in mulberry.

Polyhydroxyalkaloid	Structure	I^T	Mass spectrum
Pipecolic acid		1250[a] 1371[b]	56 (7)[*], 75 (7), 84 (100), 103 (4), 186 (6)[a] 73 (24), 156 (100), 147 (4), 230 (4) 258 (0.5)[b]
Methylpyrrolidine carboxylic acid	N.C.[**]	1458[a] 1506[b]	75 (28), 84 (100), 103 (3), 186 (7), 201 (0.3)[a] 73 (56), 147 (17), 156 (100), 230 (6), 258 (6)[b]
cis-5-Hydroxypipecolic acid		1532[a]	73 (27), 82 (100), 156 (5), 172 (80), 289 (0.1)[a]
Hydroxypipecolic acid with a methyl group	N.C.	1565[a]	73 (100), 142 (87), 147 (29), 186 (66), 288 (9), 303 (1)[a]
trans-5-Hydroxypipecolic acid		1578[a]	73 (88), 82 (100), 156 (4), 172 (82), 289 (0.3)[a]
Fagomine		1698[a]	73 (91), 129 (27), 144 (100), 170 (29), 260 (67), 348 (2)[a]
DNJ		1940[a] 1854[b]	73 (100), 144 (47), 217 (82), 258 (34), 348 (52), 436 (1)[a] 73 (100), 147 (26), 216 (32), 258 (6), 348 (8), 420 (87) 508 (0.7)[b]

[*] *m*/*z* Values (Relative abundances in brackets).
[**] N.C. not confirmed.
[a] Data corresponding to the compound with only hydroxyl groups derivatised.
[b] Data corresponding to the compound with both hydroxyl and imino groups derivatised.

lation procedure. Both reducing aldoses and ketoses showed two peaks from the *syn* (*E*) and *anti* (*Z*) isomers whereas non-reducing sugars and inositols gave rise to one peak. However, partial derivatization of iminosugars was the major drawback of this reaction: while the hydroxyl groups were readily silylated, the reaction for the amino group depended on the compound and on the derivatization conditions, resulting in one or two peaks for each iminosugar.

Therefore, different conditions were assayed trying to achieve a complete derivatization of iminosugars. Residual moisture and derivatization reagents appear to be important in controlling the extent of trimethylsilylation of these alkaloids.

Concerning residual moisture, previous studies have shown that a complete silylation of the hydroxyl groups of hydroxypipecolic acids occurs without silylation of the amino group when samples are first freeze-dried and then exposed to atmospheric moisture before derivatization (Kite & Hughes, 1997). When this behaviour was assayed in a triplicate analysis of pipecolic acid and DNJ, with and without exposing samples to atmospheric moisture after freeze-drying, no differences were shown between both treatments. Moreover, pipecolic acid and DNJ solutions were also evaporated under vacuum before derivatization treatment to compare the effect and no notable differences were observed. Similar results were also observed in those samples treated with and without acetone before the complete evaporation under vacuum (data not shown).

Regarding derivatization, four different approaches as indicated in materials and methods (Section 2.4.1.4) (MSTFA, BSTFA, TMSI/TMCS and HMDS) were followed after treatment with 2.5% hydroxylamine chloride in pyridine for pipecolic acid (Fig. 3A) and DNJ (Fig. 3B):

(i) MSTFA gave rise to the formation of two silyl derivatives for both pipecolic acid and DNJ differing in the presence of derivatization in the amino group. On the contrary, Molyneux et al. (2002) stated the formation of only one single derivative for polyhydroxy alkaloids (i.e.: swainsonine, castanospermine) when using MSTFA at 60 °C.
(ii) The use of BSTFA afforded a single peak corresponding to the non-derivatised imino group for DNJ. However, the extent of derivatization was non-reproducible for pipecolic acid giving randomly rise to one or two peaks; formation of these peaks was impossible to control.

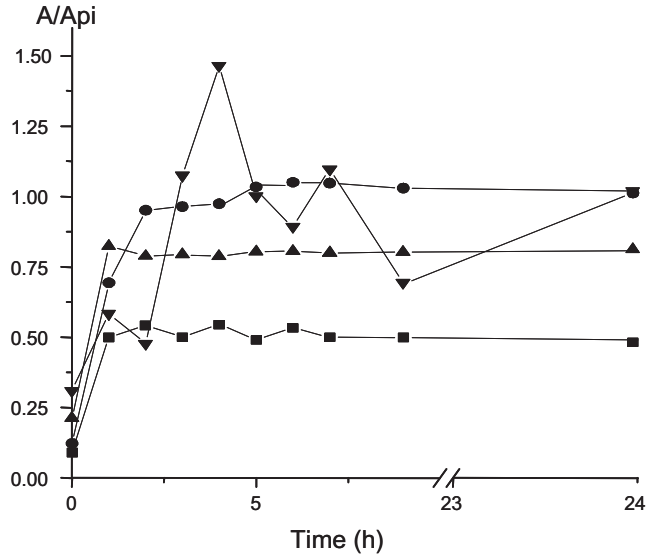


Fig. 2. Optimization of acetylation time of glucose (■), fructose (▼), *myo*-inositol (●) and DNJ (▲).

(iii) After derivatization with TMSI and TMCS, DNJ with the non-derivatised imino group was the only peak formed. However, pipecolic acid was not derivatised under these conditions (data not shown).

(iv) Best results were obtained using HMDS and TFA: for both DNJ and pipecolic acid all the hydroxyl groups being derivatised, whereas for pipecolic acid only traces of the derivatised imino group were detected. These derivatives are sufficiently volatile for analysis (Molyneux et al., 2002) and the formation of a single peak is more appropriate to avoid coelutions and quantification problems. Therefore, the use of HMDS and TFA was selected for further analyses.

Derivatised samples were kept at 4 °C at least for 24 h till their analyses, this period being of time also critical. Their stability was also controlled.

3.2. Qualitative analysis

Fig. 4 shows close-up views of the GC profile of the mulberry extract M1L previously submitted to the oximation + silylation derivatization procedure. No coelution was observed for DNJ and

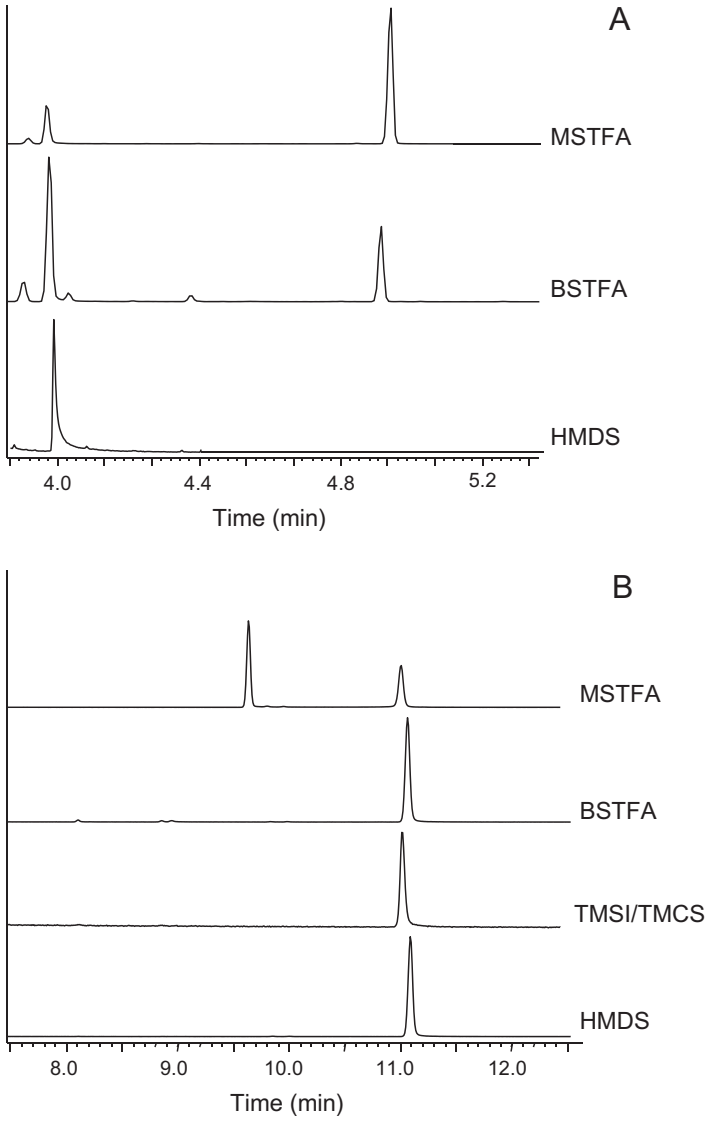


Fig. 3. GC–MS profiles of TMSO of pipecolic acid (A) and DNJ (B) obtained using MSTFA, BSTFA, TMSI/TMCS and HMDS. (1) monosilylated derivatives, (2) disilylated derivatives.

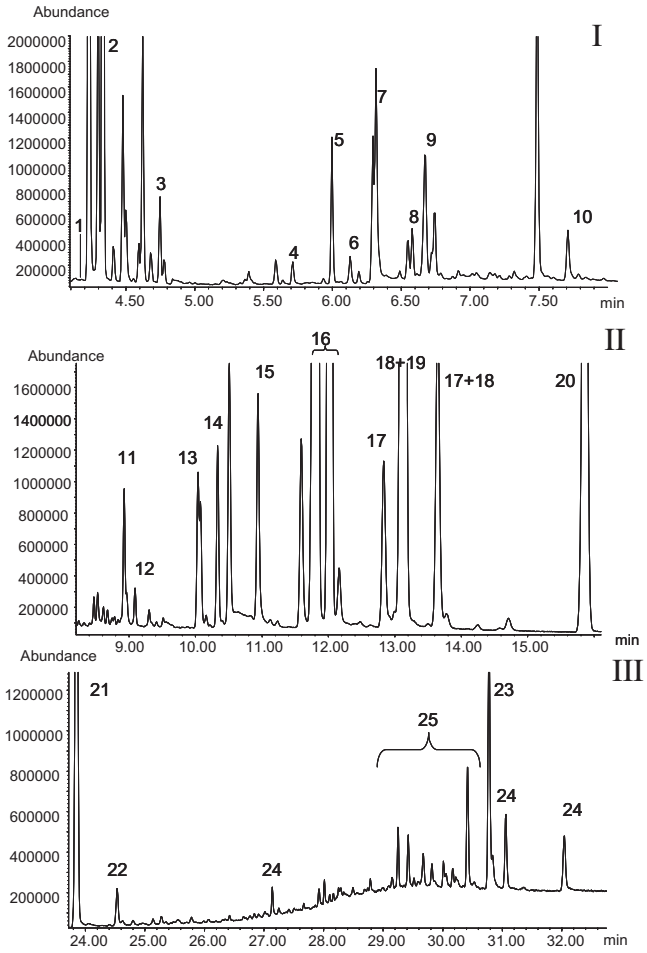


Fig. 4. Close-up views of GC profile of mulberry leaves (Fig. 1D) previously converted into their trimethylsilyl oximes under optimised conditions. I, II and III: regions from Fig. 1D. (1) Pipecolic acid, (2) glycerol, (3) glyceric acid, (**4**) monosilylated methyl-pyrrolidinecarboxylic acid, (5) malic acid, (**6**) disilylated methyl-pyrrolidinecarboxylic acid, (**7**) *cis* 5-hydroxypipecolic acid, (8) hydroxypipecolic acid with a methyl group, (**9**) *trans* 5-hydroxypipecolic acid, (10) fagomine, (11) ribose, (12) citric acid, (13) quinic acid, (14) gluconic acid (lactone), (15) DNJ, (16) fructose, (17) galactose, (18) glucose, (19) gluconic acid, (20) *myo*-inositol, (**21**) phenyl-β-glucopyranoside (i.s.), (22) glyceryl-galactoside, (23) galactinol, (24) isomers of galactinol, (**25**) disaccharides.

fagomine; other alkaloids were also detected. Pipecolic acid (I^T = 1250; peak 1) was observed at trace levels. Two peaks eluting at I^T = 1458 (peak 4) and I^T = 1506 (peak 6) were also observed. Their mass spectra were similar to that of monosilylated pipecolic acid and disilylated pipecolic acid (see Table 1). Their GC retention times were compared with those of available standard compounds having a similar structure to that of pipecolic acid (methylproline, nipecotic and isonipecotic acids) with negative results. Taking into account the similarity among their mass spectra, these peaks could correspond to a methylated pyrrolidine carboxylic isomer.

Two peaks with mass spectra corresponding to 5-hydroxypipecolic acid (Table 1) with I^T of 1532 (peak 7) and 1578 (peak 9) could be assigned to *cis* and *trans* isomers, respectively, according to Kite and Hughes (1997). This compound had been previously described in mulberry roots (Suzuki & Kohno, 1987). A compound with a I^T of 1565 (peak 8) was also identified as a derivative of pipecolic acid: although its chemical structure could not be confirmed, its mass spectrum (Table 1) is compatible with a hydroxypipecolic acid structure with an additional methyl group.

Different acids such as glyceric (I^T = 1333, peak 3), malic (I^T = 1490, peak 5), citric (I^T = 1808, peak 12), quinic (I^T = 1877, peak 13), gluconic (I^T = 2115, peak 19) acids and the gluconic lactone (I^T = 1899, peak 14), were also detected. Moreover, a peak with I^T = 2784, and a characteristic *m/z* ion at 337 (peak 22) was identified as a glyceryl-β-galactoside by comparison with previous data (Cardelle-Cobas et al., 2009).

Galactinol (I^T = 3907, peak 23) was identified by comparing with the commercial standard. Other peaks (peaks 24 of Fig. 4C) which showed a similar mass spectrum were identified as isomers of galactinol, whereas some disaccharides were also detected in the samples (peaks 25 of Fig. 4C).

3.3. Validation of the method

The range of linearity of MS response was checked by employing calibration curves in the range from 0.013 to 1.3 mg mL^{-1} of standards previously converted to their TMSO. Response factors relative to phenyl-β-D-glucoside varied from 0.75 of DNJ to 2.1 of *myo*-inositol and galactinol.

Detection limits (LOD) and quantification limits (LOQ) of 54 ng g^{-1} of product and 180 ng g^{-1} of product respectively, were obtained. The average recovery values ranged from 91% to 94%, whereas precision of the method ranged from 5.9% to 7.0% for all the target compounds which can be considered adequate for quantitative analysis. However, as indicated before, derivatization procedure and preservation of samples should be very carefully controlled to obtain this reproducibility values.

Table 2
Content (mg g^{-1} of product) of iminosugars, sugars and inositols in mulberry samples.

	M1L	M2L	M3L	M3F	M3B	M4L	M4F	M4B
Pipecolic acid	tr*	tr	tr	tr	tr	tr	tr	tr
Methylpyrrolidine carboxylic acid	0.68 (0.05)**	0.34 (0.02)	0.55 (0.06)	tr	0.14 (0.06)	0.05 (0.00)	0.36 (0.03)	tr
cis-5-Hydroxypipecolic acid	5.98 (0.27)	0.52 (0.10)	0.11 (0.02)	tr	tr	0.02 (0.00)	0.31 (0.04)	tr
Hydroxypipecolic acid with a methyl group	1.39 (0.09)	0.85 (0.00)	0.70 (0.01)	0.13 (0.03)	0.08 (0.00)	0.16 (0.03)	0.58 (0.02)	tr
trans-5-Hydroxypipecolic acid	1.53 (0.35)	0.31 (0.11)	0.25 (0.03)	0.11 (0.02)	tr	0.30 (0.04)	0.54 (0.06)	tr
Fagomine	0.12 (0.01)	0.66 (0.09)	0.05 (0.00)	0.10 (0.04)	tr	0.05 (0.00)	0.22 (0.10)	0.05 (0.01)
DNJ	2.68 (0.52)	1.23 (0.03)	0.04 (0.00)	0.11 (0.01)	4.75 (1.48)	0.22 (0.03)	2.08 (0.05)	0.27 (0.08)
Ribose	1.76 (0.20)	1.08 (0.04)	0.34 (0.01)	0.39 (0.01)	0.28 (0.10)	0.32 (0.10)	0.41 (0.02)	0.13 (0.06)
Fructose	72.88 (0.32)	57.40 (0.18)	12.84 (0.19)	14.39 (0.88)	8.91 (0.54)	38.08 (1.69)	31.71 (1.30)	6.24 (0.38)
Galactose	2.67 (0.16)	2.07 (0.06)	0.93 (0.00)	0.35 (0.08)	1.72 (0.66)	5.66 (0.85)	1.34 (0.43)	0.86 (0.03)
Glucose	16.50 (1.07)	20.72 (0.40)	12.16 (0.14)	25.92 (0.81)	10.44 (3.53)	40.51 (4.51)	29.40 (4.11)	1.98 (0.16)
Myo-inositol	11.17 (0.16)	6.59 (0.14)	3.09 (0.01)	4.37 (0.77)	6.91 (2.61)	7.12 (0.37)	12.34 (2.94)	0.75 (0.09)
Galactinols	2.04 (0.33)	0.41 (0.08)	0.28 (0.01)	0.03 (0.00)	0.06 (0.00)	3.37 (0.37)	0.27 (0.01)	0.01 (0.00)
Disaccharides	1.57 (0.14)	0.27 (0.07)	0.36 (0.02)	0.12 (0.00)	0.15 (0.04)	2.74 (0.13)	0.34 (0.12)	0.06 (0.03)

* tr: Traces.
** Standard deviation in brackets.

3.4. Sample analysis

Table 2 shows the sugar, iminosugar and inositol contents of leaves of mulberries M1L and M2L and leaves, fruit and branches of mulberries M3 and M4. The highest values of DNJ were detected in samples M3B, M1L and M4F (4.75, 2.68, and 2.08 mg g^{-1} of product, respectively). However, among all samples under study, mulberry M2L showed the highest concentration of fagomine (0.66 mg g^{-1} of product). In general, carboxylic acid derivatives were higher for leave samples, showing the highest values for M1L; only minor amounts of these compounds were observed in bark samples (M3B and M4B).

According to sugars, both M1L and M2L leaves samples showed the highest fructose and ribose concentrations; however, glucose and galactose was the most abundant in M4L. Galactinol and its isomers also showed the highest values in mulberry M4L.

4. Conclusions

Iminosugars are frequently present in natural products together with other low molecular weight carbohydrates. GC–MS is a powerful tool for their analysis, but it requires a previous derivatization step, which can lead to problems in both qualitative and quantitative determination. The proposed procedure for derivatization to TMSO appears to be the most appropriate method to simultaneously quantify sugars, inositols and iminosugars in mulberry extracts, although for the last compounds, control of derivatization conditions is of crucial importance. Derivatization of complex samples containing other different iminosugars will possibly require a similar study of the effect of the silylation procedures and their conditions.

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