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Evaluation of different hydrophilic stationary phases for the simultaneous determination of iminosugars and other low molecular weight carbohydrates in vegetable extracts by liquid chromatography tandem mass spectrometry

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ABSTRACT

Iminosugars are considered potential drug candidates for the treatment of several diseases, mainly as a result of their α-glycosidase inhibition properties. A method by hydrophilic interaction liquid chromatography tandem mass spectrometry has been optimized for the first time for the simultaneous determination of complex mixtures of bioactive iminosugars and other low molecular weight carbohydrates (LMWC) in vegetable extracts. Three hydrophilic stationary phases (sulfoalkylbetaine zwitterionic, polyhydroxyethyl aspartamide and ethylene bridge hybrid (BEH) with trifunctionally bonded amide) were compared under both basic and acidic conditions. The best sensitivity (limits of detection between 0.025 and 0.28 ng mL^{-1}) and overall chromatographic performance in terms of resolution, peak width and analysis time were obtained with the BEH amide column using 0.1% ammonium hydroxide as a mobile phase additive. The optimized method was applied to the analysis of extracts of hyacinth bulbs, buckwheat seeds and mulberry leaves. Iminosugar and other LMWC structures were tentatively assigned by their high resolution daughter ions mass spectra. Several iminosugars such as glycosyl-fagomine in mulberry extract were also described for the first time. Among the extracts analysed, mulberry showed the widest diversity of iminosugars, whereas the highest content of them was found in hyacinth bulb (2.5 mg g^{-1}) followed by mulberry (1.95 mg g^{-1}).

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1. Introduction

Iminosugars or iminocyclitols are low molecular weight carbohydrate (LMWC) analogues in which the endocyclic oxygen has been replaced by a nitrogen atom. They can be obtained by chemical synthesis [1–4], but can also be isolated from different plants (Leguminosae [5], Araceae [6], Moraceae [7], Campanulaceae [8], Polygonaceae [9], Hyacinthaceae [10]) and microorganisms such as *Streptomyces* and *Bacillus* [11,12]. Iminosugars have the capability of competitively inhibiting glycosidases because they are able to mimic the transition state of pyranosidic or furanosidic units of natural glycosidase substrates [13]. Due to the important role that glycosidases play in many essential life processes, iminosugars might have different potential therapeutic applications (antiviral, anticancer, antibiotic, etc.) [12]. Among them, those aimed to the reduction of the risk of developing insulin resistance and for overweight control have been the most reported [14–16].

Nojirimycin was the first iminosugar discovered in 1966 by Inouye et al. [17]. It was found to be a good inhibitor of both α- and β-glucosidases of different origins. Since then, several works have been focused on the study of other iminosugars, mainly, deoxynojirimycin (DNJ) and fagomine. DNJ is a highly potent intestinal α-glucosidase inhibitor present, among others, in mulberry (*Morus* sp.) leaves and in silkworms [18]. Fagomine was first isolated from buckwheat seeds and later found in different natural sources such as *Xanthocercis Zambesiaca* (Leguminosae) seeds, *Morus bombycis* and *Morus alba* (Moraceae) leaves and roots, respectively, *Lycium chinese* (Solanaceae) roots, etc [19]. Recent studies have attributed a double action to fagomine: lowering the postprandial blood glucose and modulating bacterial adhesion [20].

Analysis of iminosugars has been addressed by different techniques [21–23], and high performance liquid chromatography (HPLC) has become one of the most commonly applied for this purpose [24]. Several liquid chromatographic separation modes such as anion [25] and cation [26] exchange, hydrophilic interaction

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[18,27] and reversed-phase [28] have been applied for the analysis of iminosugars. Among them, the reported advantages of hydrophilic interaction liquid chromatography (HILIC) methods for the analysis of polar compounds and the possibility of direct coupling to mass spectrometry (MS) have contributed to extend the use of this HPLC mode for the analysis of complex mixtures of carbohydrates [29–31].

Regarding iminosugars detection, the lack of a chromophore or fluorophore group in their structures prevents the direct use of spectrophotometric detectors unless derivatization procedures are carried out [25,32,33]. Alternatively, other detectors such as evaporative light scattering and pulsed amperometric detectors have been used [28]. However, the structural information provided by MS makes this technique the most powerful tool for the characterization and elucidation of new or unidentified compounds.

On the other hand, most of the analytical methods developed so far have been focussed on the determination of DNJ [18,25,28,32] and/or fagomine [9,26] in different matrices, and the number of manuscripts devoted to the analysis of other iminosugars is more limited [7,34,35]. Moreover, iminosugars are generally present at low concentrations in natural sources together with other LMWC (monosaccharides, disaccharides, inositols, etc), which negatively contribute to the use of these extracts as bioactives [36]. The simultaneous analysis of all these compounds, scarcely considered in most previous papers, is therefore required, but it is not straightforward, considering the complexity of the mixtures and the similarity of carbohydrate structures and corresponding MS data.

A gas chromatography–mass spectrometry (GC–MS) method recently optimised in our laboratories has provided a good resolution among the variety of iminosugars and LMWC present in different plant extracts [36,37]. However, due to the low volatility and high polarity of carbohydrates, a previous derivatization step was required. Efficiency of the derivatization process and stability of the resulting derivatives were also crucial factors to be carefully controlled [36].

Hence, it would be of great interest the development of a sensitive and high resolution HILIC-MS method, which allows the simultaneous determination of the complex mixtures of iminosugars and LMWC present in extracts from natural sources without a prior derivatization process. To that aim, three hydrophilic stationary phases (amide, aspartamide and zwitterionic) have been evaluated in this paper in terms of resolution, time of analysis, peak width and peak symmetry. As an application example, selected phase and conditions have been used for the analysis of these compounds in three different vegetable (*Morus alba* leaves, *Fagopyrum esculentum* seeds and *Hyacinthus orientalis* bulbs) extracts. Tandem MS data have also been used to confirm the identity of iminocyclitols or for elucidation of new structures.

2. Materials and methods

2.1. Standards

2,5-dideoxy-2,5-imino-D-mannitol (DMDP) and α-homonojirimycin (α-HNJ) were purchased from Dextra Laboratories (Reading, UK), whereas fructose, glucose, *myo*-inositol, *chiro*-inositol, galactinol (*O*-α-D-galactopyranosil-(1→1)-D-*myo*-inositol), sucrose, 1-deoxynojirimycin hydrochloride (DNJ), 1-deoxymannojirimycin hydrochloride (DMJ), *N*-(2-hydroxyethyl)-1-deoxynojirimycin (miglitol), and *N*-methyl-1-deoxynojirimycin (*N*-methyl-DNJ) were obtained from Sigma (St. Louis, USA).

Standard solutions (0.01–0.02 mg mL^{-1}) in CH_3CN:water (80:20, v:v) were filtered through nylon FH membranes (0.22 μm) (Millipore, Bedford, MA, USA) before injection.

2.2. Samples

Mulberry (*Morus alba*) leaves were collected in Madrid (Spain). Samples of hyacinth (*Hyacinthus orientalis)* bulbs and buckwheat (*Fagopyrum esculentum*) seeds were acquired in local markets in Madrid. All the samples were air-dried, ground in a domestic mill (Moulinex) and sieved (<500 μm) before carbohydrate extraction.

2.3. Carbohydrate extraction

Sample extracts were obtained by pressurized liquid extraction (PLE) according to the method optimised by Rodríguez-Sánchez et al. [37]. In brief, samples (0.05 g) were extracted with 0.5 mL of water at 10 MPa and 50 °C for 5 min. Extracts were diluted with acetonitrile to achieve a final 80:20 (v:v) CH_3CN:water ratio.

2.4. LC–MS analysis

Two LC–MS instruments (both from Agilent Technologies, Santa Clara, CA, USA) were used in this study. The first one was a 1200 Series HPLC system, provided with a binary pump, a Rheodyne 7125 injection valve and an oven (Kariba Instruments) to control column temperature, coupled via an electrospray ionization (ESI) interface working under positive polarity to a single quadrupole MSD 1100 mass spectrometer. The electrospray source parameters were adjusted as follows: spray voltage, 4 kV; drying gas (N_2, 99.5% purity) temperature, 300 °C; drying gas flow, 12 L min^{-1}; nebulizer (N_2, 99.5% purity) pressure, 276 kPa; and fragmentor voltage, 80–100 V. Optimization of ion transmission into the analyzer was performed by infusing the default test mixture. Molecular ion adducts for sugars and iminosugars were recorded in the selected ion monitoring (SIM) mode. Data acquisition and processing were performed using HP Chemstation Rev. A.07.01 software.

The second instrument was an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler, and a column oven) coupled to a 6520 quadrupole-time of flight (QTOF) mass spectrometer using an ESI interface working in the positive-ion mode. The electrospray voltage was set at 4.5 kV, the fragmentor voltage at 150 V and the drying gas temperature at 300 °C. Nitrogen (99.5% purity) was used as nebulizer (207 kPa) and drying gas (6 L min^{-1}), while nitrogen of higher purity (99.999%) was used as the collision gas. Optimization of ion transmission into the analyzer was performed by infusing the default test mixture. Full scan mass spectra were recorded in the 130–2000 m/z range. Tandem mass spectra were obtained by collision induced dissociation (CID), applying collision energies between 10 and 37 eV to the selected precursor ions ($[M+H]^+$ for iminosugars and $[M+NH_4]^+$ for other LMWC). These MS^2 spectrum data were used to confirm the identity of iminosugars present in natural extracts and for characterization of unknowns. Data acquisition and processing were performed using Agilent Mass Hunter Workstation Acquisition Rev. B.02.00 software.

The LC experiments were carried out on three different columns: (i) PolyHydroxyethyl-aspartamide stationary phase (PolyHydroxyethyl-A column (PHEA); 100 mm × 2.1 mm, 3 μm, 300 Å pore size; The Nest Group, Inc., Southborough, MA, USA); (ii) sulfoalkylbetaine zwitterionic stationary phase (ZIC®-HILIC column (ZIC); 150 mm × 2.1 mm, 3.5 μm, 200 Å pore size; SeQuant™, Umea, Sweden) and (iii) ethylene bridge hybrid with trifunctionally bonded amide phase (XBridge column (BEH); 150 mm × 4.6 mm, 3.5 μm, 135 Å pore size, Waters, Hertfordshire, UK). Injection volume was 5 μL, and the column temperature was maintained at 25 °C.

Different binary (CH_3CN:water) gradients and additives (0.1% acetic acid or 0.1% ammonium hydroxide) were assayed for each of the columns tested.

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