Journal of Chromatography A, 1447 (2016) 64–71

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Differentiation of flavonol glucoside and galactoside isomers combining chemical isopropylidenation with liquid chromatography–mass spectrometry analysis

Lauro M. de Souza[a,b,*], Nessana Dartora[b], Camila T. Scoparo[b], Philip A.J. Gorin[b,1], Marcello Iacomini[b], Guilherme L. Sassaki[b]

[a] Instituto de Pesquisa Pelé Pequeno Príncipe, Faculdades Pequeno Príncipe, 80250-200 Curitiba, Paraná, Brazil
[b] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, 81531-980 Curitiba, Paraná, Brazil

ARTICLE INFO

Article history:
Received 6 December 2015
Received in revised form 21 March 2016
Accepted 23 March 2016
Available online 26 March 2016

Keywords:
Glycoside isomers
UHPLC–MS
Isopropylidene
Multiple-stage mass spectrometry
Maytenus ilicifolia

ABSTRACT

Flavonol glycosides are important components of leaves from vascular plants. A lot of isomers of these compounds are produced by plants, making their analysis very difficult and causing many structural misinterpretations. Galactosides and glucosides as mono- or oligosaccharides yield many diastereoisomers, hindering the analysis by mass spectrometry. In order to enable the mass spectrometric distinctions of these isomers, in this work we combine an isopropylidene based chemical derivatization with liquid chromatography with multiple-stage mass spectrometry (LC–MS^n) analysis. The isomers of flavonol triglycosides, after the reaction, yielded products with different molecular weight, therefore, they were no longer isomers, allowing their identification by MS^1 analysis. However, to the 4 isomers of flavonol diglycosides, only one yielded, after isopropylidenation, a product with different molecular weight. To the other 3 species, the incorporation of 2 isopropylidene groups retained them in the isomeric form. For such species, chromatographic separation and MS^n detection targeting the lithium adducts of 3,4-*O*-isopropylidene-galactosyl or 4,6-*O*-isopropylidene-glucosyl residues (*m*/*z* 209.099) provided specific MS profile.

1. Introduction

Flavonoid is an important class of biologically active natural compounds found in leaves and fruits of many species of plants used for human consumption. The impacts of these compounds on human health are of interest since they can act as chemoprotective adjuvants. Their properties include the well-known antioxidant activity and free radicals scavenger, but many other pharmacological properties were reported [1–7]. Flavonoids are formed by a combination of three rings containing hydroxyl and/or carbonyl groups. Mono- and/or oligosaccharides are, also, frequently found attached to flavonoids, yielding many different structures, which can play different biological activities. Accordingly, the development of analytical methods to describe the structural details of these compounds is still a challenge.

Flavonols (3-hydroxyflavones) are common flavonoids found in plants, being kaempferol, quercetin and myricetin the main representatives. Frequently this kind of flavonoid is found linked to glycans, yielding many different structures with several isomers. Some of these isomers are formed with different constituents, such as quercetin-rhamnoside and kaempferol-galactoside, which can be differentiated by tandem mass spectrometry (MS), since they generate different fragment-ions, notably from the aglycone, which has different degree of hydroxyl substitution [8,9].

The glycans can be attached to flavonoid at different positions and these positional isomers are frequently found in flavonoids. From isolated molecules, the glycosylation sites can be assessed by 2D nuclear magnetic resonance (NMR) experiments, being not suitable for complex mixtures. Many works had developed strategies to differentiate the glycosylation sites of flavonoids. Indirect determination was provided by UV-spectral shift data, obtained by liquid chromatography combining a post-column derivatization with specific shifting reagents and photodiode array (PDA) detection [10–12]. Depending on the glycosylation site, different complexes with the reagents were produced, yielding characteristic spectral shifts.

* Corresponding author at: Av. Silva Jardim, 1632, CEP 80250-200 Curitiba, Paraná, Brazil.
E-mail address: laurosouza@hotmail.com (L.M. de Souza).
[1] In memoriam.

http://dx.doi.org/10.1016/j.chroma.2016.03.069
0021-9673/

Metal complexation, followed by tandem-MS, was also employed in the characterization flavonoid glycoside isomers. However, the basis for such differentiation was the positional isomerism, related to the glycosylation sites, but some differences in the interglycosidic linkages, as in rutinosides [α-L-Rha*p*-(1 → 6)-β-D-Glc*p*] and neohesperidosides [α-L-Rha*p*-(1 → 2)-β-D-Glc*p*], were distinguished by different fragmentation patterns due to the different complexes with metals [13–15]. The identification of glycosylation sites was also provided by Cuyckens and Claeys [16] after the observation of Hvattum and Ekeberg [17] that the flavonoid glycosides generated a radical ion when subjected to collision induced dissociation-mass spectrometry (CID-MS). This ion resulted from a homolytic cleavage between the carbohydrates and flavonoids, and its formation and intensity were directly related to the glycosylation sites [9,16].

Nevertheless, the identification of flavonol glycoside diastereoisomers in complex mixtures remains a challenge, considering that differences are not directly observed by mass spectrometry. If purified, NMR can provide an accurate identification or, after hydrolysis, the monosaccharide composition can be assessed by chromatographic analysis. However, the purification steps are usually laborious, time-consuming and, frequently, only the most abundant components are obtained. Some different MS patterns were provided by Davis et al. [18], that studying the behavior of flavonoid monoglycosides complexed with manganese were able to distinguish between glucopyranosides and galactopyranosides and, also, between arabinofuranosides and xylopyranosides. However, glycosides with a different degree of glycosylation have not been explored, perhaps because of the different affinity of the metal for the oligosaccharides would hinder the analysis.

Isopropylidenation is a common derivatization used to block specific hydroxyl groups of monosaccharides, used in oligosaccharide synthesis [19]. In an analytical approach, isopropylidenation was used to improve the identification of saponins by mass spectrometry with different glycosylation patterns [20]. Isopropylidenation was also crucial to the correct identification of isomeric dicaffeoylquinic acids with potent antiulcer activity [21]. Thus, the goal of the present work was to explore whether the application of isopropylidenation could allow the identification of various flavonol glycoside diastereomers via multiple fragmentation cycles (MS^n), to provide a differential MS/MS pattern from galactopyranosides and glucopyranosides present as inner moieties of different oligosaccharides.

2. Experimental

2.1. Chemicals

Standards of rutin [α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-quercetin], quercetin-3-*O*-β-glucopyranoside (Quer-Glc), quercetin-3-*O*-β-galactopyranoside (Quer-Gal) and the reagent $CuSO_4{\cdot}5H_2O$ were purchased from Sigma-Aldrich. The HPLC-grade acetone, methanol and formic acid were purchased from Tedia and the ultrapure water was from Milli-Q (Millipore, USA). Prior to the reactions, the copper(II) sulfate crystals were powdered in a mortar and held 1 h at 110 °C.

2.2. Extraction the flavonol glycosides

Leaves of *Maytenus ilicifolia*, known to contain several flavonol glycoside isomers [8,9], were purchased in the local market. After dried and ground, the leaves (100 g) were twice extracted with 300 mL of ethanol-water (7:3, v/v) under reflux, yielding the crude extract, which was evaporated to dryness under reduced pressure. A portion of the extract (1 g) was fractionated by liquid–liquid partition in water/chloroform then water/*n*-butanol, each solvent at 100 mL. The butanolic layer was separated, evaporated and analyzed by ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS) to confirm the presence of flavonol glycosides, being further used in all experiments.

2.3. Isopropylidenation

Many procedures for the isopropylidenation were evaluated, varying the catalyzer such as H_2SO_4, *p*-toluenesulfonic acid, HCl, $AlCl_3$, as well as the solvent (pure acetone or with small amounts of methanol), reaction time and temperature. Some conditions promoted glycoside hydrolysis and, in other, the samples were not completely dissolved. Thus, the standards rutin, Quer-Glc and Quer-Gal (1 mg) were each dissolved in 100 μL of methanol then mixed with dry acetone (2 mL), used as solvent and reagent, and 100 mg of powdered $CuSO_4$. The mixtures were stirred at room temperature (~22 °C) for 14 h and, then, centrifuged at 10,000 rpm, for 5 min in Eppendorf tubes. An aliquot of 200 μL from each soluble fraction, containing the isopropylidene (IPP) derivatives, was mixed with 300 μL of MeOH-H_2O (1:1, v/v) and submitted to LC–MS analysis. Another aliquot of 200 μL of each sample was dried under N_2 stream, then hydrolyzed in MeOH/HCl (0.5 N) for 3 h at 100 °C. The resulting material was dried under N_2 stream for further LC–UV analysis. The remaining samples were separated from insoluble and stored at −20 °C. The butanolic fraction of *M. ilicifolia*, containing the targeted flavonol isomers, was similarly derivatized.

2.4. Analytical procedures

2.4.1. Liquid chromatography (LC)

The chromatographic separation was developed on Acquity-UPLC™ system (Waters, MA, USA), composed of a binary pump, sample manager and column oven. The samples were held at room temperature (22 °C) and column oven at 60 °C. The separations were developed on Waters HSS T3C18 column, with 100 mm × 2.1 mm i.d. and 1.7 μm of particle size, using water (solvent A) and methanol (solvent B), both containing 0.1% formic acid.

The injection volume was 5 μL and the chromatography was developed at a flow rate of 400 μL/min, with a linear gradient of the solvent B, from 0 to 40% in 13 min, in the further 2 min the solvent B was increased to 80%, backing to initial condition (0% B) in 1 min. Additional 3 min were held to re-equilibrate the system. A solution of 10 mM LiCl was co-injected at 1 μL/min and post-column mixed with samples in the ion source.

2.4.2. High-resolution multiple stages mass spectrometry ($HR\text{-}MS^n$)

From the mass spectrometry analysis, 1 mM LiCl was added to the sample and standards (IPP-derivatives) which were directly infused into electrospray source at a flow rate of 2 μL/min. HR-MS analyzes were performed on LTQ-Orbitrap XL (Thermo Scientific) at atmospheric pressure ionization (API) using nitrogen for sample desolvation and helium 6.0 as damping gas. The source temperature was 350 °C and N_2 was used in sheath gas at a flow rate of 8 arbitrary units (a.u.) and auxiliary gas of 2 a.u. in offline mode, or 60 and 20 a.u., respectively, in LC–MS. The spray voltage was set at 4.5 kV, the capillary at 30 V and tube lens at 100 V in the positive ionization mode, and 3.5 kV, 40 V, 120 V, respectively, for the negative ionization. MS^n experiments were obtained in the linear trap by collision induced dissociation-mass spectrometry (CID-MS), using helium as collision gas, applying energies of 20–25 (normalized collision energy). Considering that the ion trap mass range is limited in CID-MS mode, in order to obtain the fragments with lower *m*/*z*