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Ultra(high)-pressure liquid chromatography–electrospray ionization-time-of-flight-ion mobility-high definition mass spectrometry for the rapid identification and structural characterization of flavonoid glycosides from cauliflower waste

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ABSTRACT

In this paper, a strategy for the detection and structural elucidation of flavonoid glycosides from a complex matrix in a single chromatographic run using U(H)PLC–ESI-IMS-HDMS/MS^E is presented. This system operates using alternative low and high energy voltages that is able to perform the task of conventional MS/MS in a data-independent way without re-injection of the sample, which saves analytical time. Also, ion mobility separation (IMS) was employed as an additional separation technique for compounds that are co-eluting after U(H)PLC separation. First, the fragmentation of flavonoid standards were analyzed and criteria was set for structural elucidation of flavonoids in a plant extract. Based on retention times, UV spectra, exact mass, and MS fragment characteristics, such as abundances of daughter ions and the presence of radical ions ($[Y_0-H]^{\bullet -}$), a total 19 flavonoid glycosides, of which 8 non-acylated and 11 acylated, were detected and structurally characterized in a cauliflower waste extract. Kaempferol and quercetin were the main aglycones detected while sinapic and ferulic acid were the main phenolic acids. *C*-glycosides were also found although their structure could not be elucidated. The proposed method can be used as a rapid screening test for flavonoid identification and for routine analysis of plant extracts, such as these derived from cauliflower waste. The study also confirms that agroindustrial wastes, such as cauliflower leaves, could be seen as a valuable source of different bioactive phenolic compounds.

1. Introduction

Flavonoids are the most widely spread and diverse group of polyphenols, which belong to a large family of secondary plant metabolites [1]. To date, more than 8000 different flavonoids have been identified, where the most abundant aglycones are quercetin and kaempferol [2]. These often occur as complex conjugates with glycosides and acyl groups [2,3] Recently, much attention has been given to these metabolites due to their biological activity, which include, among others, anti-oxidant activity [3,4], angiotensin-converting enzyme inhibitory activity [5,6], anti-obesity activity [7], among others. The biological effects of flavonoids on mammalian cells has already been extensively reviewed elsewhere [8].

LC coupled to MS/MS has been the method of choice for many chemists for the detection and structural identification of flavonoid glycosides since producing isolated components in sufficient quantities for NMR analysis is difficult [9]. These techniques (especially those using ion traps and orbitraps), although sensitive, are limited to certain transitions that are monitored, require multiple injections and involve manual identification of parent compounds, which are then subjected to succeeding MS experiments (MS^n) [10]. This process is therefore time-consuming, which makes it unsuitable for routine analysis and screening of a wide array of samples. Recently, the use of U(H)PLC–MS^E has been used as a rapid, data-independent strategy that collects both precursor and product ion information in one chromatographic run. This method uses parallel alternative scans of low and high energy which provides precursor ion, accurate mass fragments, and neutral loss information "all-in-one" [11–14]. This therefore eliminates the need for manual parent ion selection in MS^n experiments that require multiple injections, thus cutting analysis time. However, as complex samples, such as

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plant extracts, contain a wide array of metabolites and other components, U(H)PLC analysis is sometimes not enough to separate all these to obtain a single component that is passed onto the MS in an LC–MS system. Once compounds are convoluted, they enter into the MS at the same time, which makes structural analysis using MS^E rather difficult and ambiguous [15]. To address this, a further separation step is essential.

Ions generated after ionization (ESI, MALDI, etc.) may be further separated by exploiting their different behaviors across a tube filled with an inert gas. Ion mobility refers to the velocity of an ion across a drift tube filled with a neutral gas, often nitrogen, under the influence of a weak electric field. In a drift tube, smaller molecules experience lesser collisions with the gas and thus travel faster than larger molecules [16–18]. This therefore provides an additional separation of compounds found in complex matrices. This added orthogonal separation technique can therefore make structural elucidation of molecules from plant extracts more efficient [16]. By combining ion mobility to LC–MS^E, a system composed of LC separation–ionization–ion mobility separation (IMS)-ion detection and mass analysis is created, which can serve as an alternative to traditional tandem MS.

Vegetables belonging to the *Brassica* group have been found to be an excellent source of polyphenols, especially flavonoid glycosides mainly composed of quercetin and kaempferol [2,3,19–21]. Considering that vegetables belonging to this group are one of the most consumed [22], a wide variety of *Brassica* species and cultivars have been analyzed for their phenolic contents [21,23,24].However, most studies have mainly focused on the edible parts of the vegetables. To the best of our knowledge, very limited researches have been done on the waste stream of *Brassica*. Selected *Brassica* waste streams have been previously analyzed for their antioxidative effects, which were attributed to the content of phenolic compounds [23,25]. Llorach et al. [26] studied the flavonoid composition of cauliflower agroindustrial products (mainly leaves). They used LC–MS^n after isolation of individual compounds, where 23 flavonoid glycosides, mainly kaempferol and quercetin conjugates with 1-4 glucose units and hydroxycinnamic acids, free kaempferol and 4 hydroxycinnamic acid derivatives were detected [26]. Studying the flavonoid content of these waste products is an initial step into the valorization of agricultural waste into high value added products.

In this paper, we describe a strategy for the detection and structural elucidation of flavonoids and flavonoid glycosides in a single chromatographic run using U(H)PLC-DAD–ESI-IMS-HDMS/MS^E. Initially, fragmentation behavior of flavonoid standards under MS^E conditions was studied and separation by IMS was optimized. Information on their fragmentation behavior was then used as basis for the structural characterization of phenolics from a sample matrix. With this, a method for the simultaneous detection, identification, and structural characterization of polyphenols in a single chromatographic run was developed, which could be used for routine analysis of complex samples, such as cauliflower waste.

2. Materials and methods

2.1. Reagents

Rutin, baicalin, hesperidin, quercetin-3-*O*-glucoside, kaempferol, quercetin, hesperitin, myricetin, isorhamnetin, *p*-coumaric, sinapic, and ferulic acids were purchased from Sigma (St. Louis, MO) while robinin was from Phytolab (Vestenbergsgreuth, Germany). U(H)PLC–MS grade methanol and formic acid were bought from Biosolve (Valkenswaard, the Netherlands). Analytical grade methanol used for extraction, HCl and NaOH were purchased from VWR International (Leuven, Belgium). LEU-ENK was purchased from Waters (Milford, MA, USA).

2.2. Fragmentation behavior of flavonoid standards

Solutions (ca. 10 ppm) of 9 flavonoid standards, robinin, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside (rutin), hesperitin-7-*O*-rutinoside (hesperidin), baicalein 7-*O*-glucuronide (baicalin), kaempferol, quercetin, isorhamnetin, and myricetin, were prepared in 50% (v/v) methanol + 0.1% (v/v) formic acid and directly injected to the Waters Synapt G1 HDMS (Waters Corp., Milford, MA, USA) equipped with an ESI source via direct injection at a flow rate of 5 μL/min. Solutions of (ca. 10 ppm) of ferulic, sinapic, coumaric, benzoic, gallic acids were also prepared and analyzed in the same way. Data were acquired in continuum negative ionization V-mode for 2 min. Source parameters for the MS were set as follows: capillary voltage, 2 kV; sampling cone voltage, 40 V; extraction cone voltage, 4 V; source temperature 150 °C; desolvation temperature, 350 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 550 L/h. Trap and transfer collision energies were set at 6 volts for the low energy and 45 V for high energy. Mass range was set at 100–1500 Da with a scan speed of 0.2 s per scan using the MassLynx software 4.1 (Waters Corp., Milford, MA, USA).

2.3. T-wave ion mobility separation (IMS)

IMS was optimized by injecting a sample extract spiked with various flavonoid aglycones and glycosides directly into the MS in IMS mode using a 250 μL analyte syringe at a flow of 5 mL/min. IMS parameters were then optimized to ensure that the components are separated over the entire drift time range. Nitrogen gas was used with a flow rate of 30 ml/min. Bias voltage was set to 40 V. IMS T-wave velocity and height were 850 m/s and 8 V, respectively. Transfer T-wave velocity and height were adjusted to 60 m/s and 30 V, respectively. Mobility trapping was set at maximum value of 30 V. In this approach, intact ions are allowed to pass through the trap cell and separated in the drift tube. Then high energy CID (45 V) was applied after the ions have been separated, which makes it possible to associate the product ions to their respective precursor ions (time aligned). Time aligned parallel (TAP) chromatograms were obtained using Waters MS^E data viewer software.

2.4. Extraction of phenolic content

Cauliflower (*Brassica oleracea* L. var. *botrytis*) byproducts (outer leaves) were obtained in a field in Ieper (Belgium) during harvest (July 2012) and stored at −20 °C until processing. Frozen leaves were ground using liquid nitrogen and homogenized using a stirrer. Extraction was based on the method of Olsen et al. [27]. Briefly, approximately 5 g of leaves were placed in 50 mL tubes and homogenized with 15 mL methanol at 10,000 rpm using an ultraturrax for 45 s. The tubes were then placed on ice for 15 s. The mixture was centrifuged at 13,000 × *g* for 10 min at 4 °C. Supernatant was collected. The residue was re-extracted with 80% MeOH using the same procedure. All supernatants were pooled and the volume was adjusted to 50 mL using methanol. The extract was stored in a −20 °C freezer until further use. Extractions and analyses were done in triplicate.

2.5. Acid and alkaline hydrolysis

Acid hydrolysis was performed by adding equal amounts of 4 M HCl to the methanolic extract (1 mL:1 mL) for 1 h at 100 °C. Alkaline hydrolysis on the other hand was done by adding 4 M NaOH to the methanolic extract (1 mL:1 mL) at room temperature overnight.