Trace Determination of Naringenin and Hesperitin by Tandem Mass Spectrometry

Keywords: *Citrus; flavanones; flavonoids; hesperidin; hesperitin; MS/MS; mass spectrometry; naringenin; naringin; tandem mass spectrometry*

INTRODUCTION

Flavanones are the most abundant of the flavonoids in the fruit of most citrus species. Citrus is the major dietary source of the flavanones naringin and hesperidin, with naringin in grapefruit and hesperidin in orange being the primary flavanones in these citrus fruits (Kühnau, 1976).

There is renewed interest in the pharmacology of citrus flavonoids, particularly those contained in grapefruit (Bailey et al., 1994). In the body, the rhamnoglucose moiety at position 7 is hydrolyzed, yielding the corresponding aglycons, naringenin and hesperitin. These compounds exert many physiologic effects, but their role in human nutrition is undefined (Kühnau, 1976) and their effect on xenobiotic metabolism is emerging (Bailey et al., 1994).

A sensitive and specific analytical method is needed for determining the presence of naringenin and hesperitin in the body after dietary ingestion of citrus. Tandem mass spectrometry (MS/MS) has been applied to problems involving trace analysis and metabolite identification in the biomedical field (Favretto and Traldi, 1993). In this paper, we evaluate the analytical potential of various modes of MS/MS in detecting trace levels of the citrus flavanones, naringenin and hesperitin, including their identification in human urine after oral ingestion of these flavanones.

MATERIALS AND METHODS

Materials. Naringenin (4′,5,7-trihydroxyflavanone) and hesperitin (3′,5,7-trihydroxy-4′-methoxyflavanone) standards (95% purity) and β-glucuronidase (from Helix Ponatia Mollusk; 420 000 activity units/g) were purchased from Sigma Chemical Co. (St. Louis, MO). Naringin (4′,5,7-trihydroxyflavanone 7-*O*-neohesperidoside) and hesperedin (3′,5,7-trihydroxy-4′-methoxyflavanone 7-*O*-β-D-rutinoside) were obtained from Lake Alfred Agricultural Research and Education Center, Lake Alfred, FL. Their purity, established by HPLC/UV analysis, was >95% for both naringin and hesperedin. For mass spectrometry, ultrahigh-purity methane (Matheson, Morrow, GA) was used for the reagent gas and zero grade nitrogen (Airco Industrial Gases, Research Triangle Park, NC) for the collision gas.

Instrumentation. Mass spectral data were collected with a triple quadrupole mass spectrometer (MS) (TSQ45; Finnigan MAT, San Jose, CA) equipped with a 4500 Series ion source, pulsed positive ion and negative ion chemical ionization, and an INCOS data system. Samples entered the ion source via a heatable direct insertion solids probe.

Sample Preparation. Biological samples consisted of blood and urine collected from subjects who ingested citrus products or naringin (Ameer et al., 1995). Blood samples were centrifuged, and plasma was separated from blood cells. Samples were split with one portion undergoing 30 h of incubation at 37 °C, pH 4.5, with β-glucuronidase (1000 units/mL of sample) and the other portion left unhydrolyzed. Samples were extracted, and their volume was reduced on a preconditioned C_{18} solid phase cartridge (Sep-Pak, Waters, Milford, MA) by elution with methanol.

Mass Spectrometry. Mass spectrometric analyses were performed on standards and biological samples using the following modes of MS operation: electron ionization (EI) (electron energy of 70 eV), positive chemical ionization (PCI; 100 eV), and negative chemical ionization (NCI; 100 eV). Some chemical ionization (CI) analyses employed a Townsend discharge (3.5 kV; 280 μA). Ion source temperatures were 100, 150, or 190 °C. Parent and daughter MS/MS experiments were performed by collisionally activated dissociation (CAD) of selected ions with a collision energy of 24 eV and a collision gas pressure of approximately 1.5 mTorr of N_2.

Analyses of Biological Samples. Preliminary screening experiments were conducted in EI, PCI, and NCI full scan normal MS mode. These experiments were followed by confirmatory analyses with both PCI-CAD daughter scanning and EI-CAD selective reaction monitoring (SRM). On the basis of the findings from the above analyses, subsequent analyses of samples were performed with PCI-CAD daughter scans of the detected $(M + H)^+$ ions and PCI-SRM of a daughter ion found to be common to the flavanones of interest (see Results and Discussion).

For MS analyses, 5 μL of sample extract concentrate was placed in a 6 μL glass vial and allowed to air-dry. The vial was then placed on a heatable direct insertion solids probe for introduction into the ion source. Sample vaporization was computer controlled. The probe temperature for initial experiments increased ballistically from ambient to 380–400 °C in about 3 min. In subsequent studies, the temperature began at 50 °C for 0.2 min and then increased at a rate of 100 °C/min up to 375 °C, where the temperature was held.

RESULTS AND DISCUSSION

Standards. Our preliminary experiments evaluated citrus flavanone fragmentation patterns under methane PCI, NCI, and EI conditions, the latter EI approach having been used in early structural studies of flavonoids (Grayer, 1989). In the MS/MS analyses of naringenin and hesperitin standards, CAD of the EI-generated $(M^{\bullet+})$ ions at m/z 272 and 302 yielded many daughter ions but did not yield a common daughter ion in any appreciable amount. In contrast, PCI-CAD of the $(M + H)^+$ ions yielded m/z 153 as a major ion for both of these flavanones (supporting information). This ion probably results from a retro-Diels–Alder reaction, i.e., pyrone ring fragmentation of the aglycon flavanone (Grayer, 1989). The m/z 153 daughter ion was useful as a diagnostic ion in searching for naringenin and hesperitin. In the daughter ion and parent ion MS/MS modes, the m/z 153 ion along with corresponding $(M + H)^+$ ions was used to search for closely related compounds or metabolites in biological samples. For this reason, PCI was judged to be superior to EI in MS/MS analysis for identification of these citrus flavonoids, particularly in complex mixtures such as biological fluids. The assignment of a structure to the m/z 153 ion and to other major PCI-CAD daughter ions of the $(M + H)^+$ ions of naringenin and hesperitin are presented in Table 1.

While characterization of the citrus flavanones was successful with MS/MS involving CAD of either EI-generated $M^{\bullet+}$ ions or methane PCI-generated $(M + H)^+$ ions, attempts to fragment the NCI-generated $M^{\bullet-}$ ions via CAD were not successful. The failure of NCI was probably due to autoejection of an electron upon collisional activation.

The mass spectra of the glycosides naringin and hesperidin were dominated by the same ions as the

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Table 1. Proposed Structures of Daughter Ions in PCI-CAD Mass Spectra of Standards

Naringenin			Hesperitin		
m/z	formula	proposed structure	*m/z*	formula	proposed structure
273	$C_{14}H_{12}O_5$		303	$C_{16}H_{14}O_6$	
153 [a]	$C_7H_5O_4$		153	$C_7H_5O_4$	
147	$C_9H_7O_2$		177	$C_{10}H_9O_3$	
			137	$C_8H_9O_2$	

[a] *m/z* 153 is the common daughter ion.

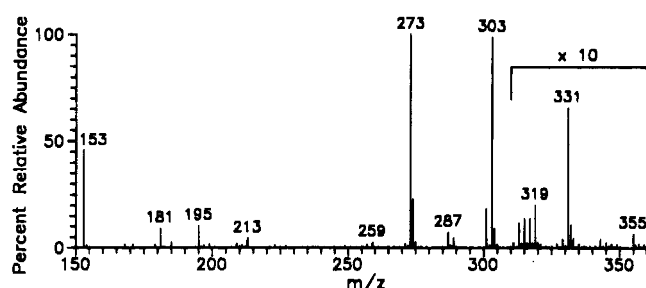

Figure 1. Parents of *m/z* 153 ion PCI-CAD mass spectrum of β-glucuronidase-hydrolyzed urine collected 0–6 h after a single, combined ingestion of orange and grapefruit juices.

corresponding aglycons, naringenin and hesperitin. This finding suggests that the glycosides are labile under experimental conditions, probably during ionization. Minimal or absent molecular ions of EI or CI-MS of diglycoside flavonoids have been reported (Itokawa et al., 1982).

Biological Extract. The presence of flavanones in juice and in human urine after ingestion of citrus was initially indicated in the solids probe chromatogram. It displayed "peaks" corresponding to the $M^{\bullet+}$, $(M + H)^+$, and $M^{\bullet-}$ ions of naringenin and hesperitin, created under their respective ionization methods in normal MS mode. The presence of naringenin and hesperitin was subsequently confirmed by CAD (with either a wide mass range daughter scan or selected reaction monitoring) of the detected $M^{\bullet+}$ or $(M + H)^+$ ions to form spectra matching the corresponding spectra for naringenin and hesperitin standards. PCI-CAD parent ion experiment mass spectrum of the diagnostic *m/z* 153 ion in human urine showed $(M + H)^+$ ions at *m/z* 273 (naringenin) and 303 (hesperitin) as well as other ions (Figure 1). In juice extracts and in human urine after citrus flavonoid ingestion, ions detected at *m/z* M + 29 and M + 41 were presumed to be the commonly observed methane adduct ions (Table 2). The presence of the methane adduct ions in the parents of *m/z 153* spectra indicated the presence of naringenin and hesperitin and suggested the presence of other $(M + H)^+$ ions. Methane adduct ions at *m/z* M + 29 and M + 41 were detected in the PCI normal mass spectra of naringenin and hesperitin standards (data not shown). PCI-CAD daughter spectra of some of the ions thought to be related to naringenin or hesperitin [i.e., suspected $(M + H)^+$ ions] contained fragmentation patterns similar to patterns observed for these flavanones (Table 3). These ions were not detected in spectra of urine collected during a control period of no dietary citrus intake (data not shown).

Table 2. Adduct Ions[a] in PCI-CAD Mass Spectra of Analytes Found in Human Urine and in Orange and Grapefruit Juices

	adduct ions[a]		
mol wt (M)	M + 1	M + 29	M + 41
272	273	301	313
286	287	315	327
300	301	329	341
302	303	331	343
316	317	345	357

[a] M + 1 = (M + H); M + 29 = $(M + C_2H_5)$; M + 41 = $(M + C_3H_7)$.

Table 3. Major Daughter Ions of Analytes Found by PCI-CAD MS/MS in Orange and Grapefruit Juices

parent (M + H)	mass data of daughter ions, *m/z* (intensity as % of BP)
331 (100)	317 (17), 303 (8), 273 (3), 181 (40), 177 (8), 153 (17)
317 (100)	302 (14), 227 (7), 257 (7), 153 (8)
303 (100)	257 (7), 179 (4), 177 (15), 153 (44), 137 (4)
301 (100)	273 (21), 286 (5), 181 (54), 153 (25), 147 (7)
287 (100)	259 (4), 167 (8), 161 (16), 153 (43), 147 (6)
273 (100)	227 (2), 179 (5), 153 (61), 147 (18)

Little has been published on the absorption of flavonoids in general and of flavanones in particular (Scheline, 1978). In the current study, the triple quadrupole MS separation/identification system provided sufficiently selective analysis of trace flavanone components in the complex matrix of extracted urine to permit the evaluation of flavanone absorption from orange and grapefruit. We used MS/MS along with HPLC techniques to evaluate citrus flavanone oral absorption and disposition in humans and will report the results elsewhere.

Supporting Information Available: Comparison of mass spectra of naringenin standard by (A) EI-CAD and (B) PCI-CAD and of hesperitin standard by (C) EI-CAD and (D) PCI-CAD (2 pages). Ordering information is given on any current masthead page.

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