Rapid Quantitation of Furanocoumarins and Flavonoids in Grapefruit Juice using Ultra-Performance Liquid Chromatography

By: Karen M. VanderMolen, Nadja B. Cech, Mary F. Paine, Nicholas H. Oberlies

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Abstract:

Introduction

Grapefruit juice can increase or decrease the systemic exposure of myriad oral medications, leading to untoward effects or reduced efficacy. Furanocoumarins in grapefruit juice have been established as inhibitors of cytochrome P450 3A (CYP3A)-mediated metabolism and P-glycoprotein (P-gp)-mediated efflux, while flavonoids have been implicated as inhibitors of organic anion transporting polypeptide (OATP)-mediated absorptive uptake in the intestine. The potential for drug interactions with a food product necessitates an understanding of the expected concentrations of a suite of structurally diverse and potentially bioactive compounds.

Objective

Develop methods for the rapid quantitation of two furanocoumarins (bergamottin and 6′,7′-dihydroxybergamottin) and four flavonoids (naringin, naringenin, narirutin and hesperidin) in five grapefruit juice products using ultra-performance liquid chromatography (UPLC).

Methods

Grapefruit juice products were extracted with ethyl acetate; the concentrated extract was analysed by UPLC using acetonitrile:water gradients and a C_{18}-column. Analytes were detected using a photodiode array detector, set at 250 nm (furanocoumarins) and 310 nm (flavonoids). Intraday and interday precision and accuracy and limits of detection and quantitation were determined.

Results

Rapid (< 5.0 min) UPLC methods were developed to measure the aforementioned furanocoumarins and flavonoids. R^2 values for the calibration curves of all analytes were >0.999. Considerable between-juice variation in the concentrations of these compounds was observed,

and the quantities measured were in agreement with the concentrations published in HPLC studies.

Conclusion

These analytical methods provide an expedient means to quantitate key furanocoumarins and flavonoids in grapefruit juice and other foods used in dietary substance–drug interaction studies.

Keywords: UPLC | quantitation | furanocoumarin | flavonoid | grapefruit

Article:

Introduction

Grapefruit juice has been shown to increase the systemic exposure of a diverse array of oral medications that undergo extensive pre-systemic metabolism by cytochrome P450 3A (CYP3A) in the intestine, including felodipine, lovastatin and cyclosporine (Paine *et al*., 2006; Seden *et al*., 2010; Hanley *et al*., 2011; Won *et al*., 2012). The increase in systemic drug exposure can be sufficient to cause untoward effects, ranging from relatively mild (e.g. hypotension and dizziness with some calcium channel blockers) to potentially severe (e.g. nephrotoxicity with some immunosuppressants). The mechanism underlying these interactions is irreversible inhibition of intestinal CYP3A activity by grapefruit juice (Bailey *et al*., 1998; Paine *et al*., 2006; Paine and Oberlies, 2007; Hanley *et al*., 2011). Furanocoumarins, a class of compounds present in grapefruit juice, have been established as major CYP3A inhibitors in human volunteers (Paine *et al*.,2006). This effect may be augmented if the ‘victim’ drug also is a substrate for P-glycoprotein (P-gp), a transmembrane efflux transport protein located on the apical membranes of numerous cell types, including enterocytes (Paine and Oberlies, 2007; Hanley *et al*., 2011). P-glycoprotein demonstrates substrate specificity that overlaps with that of CYP3A substrates (Wacher *et al*., 1995), and several *in vivo* and*in vitro* studies have shown an inhibitory effect by grapefruit juice and/or furanocoumarins towards P-gp activity (Lown *et al*., 1997; Edwards *et al*., 1999; Ohnishi *et al*., 2000; De Castro *et al*., 2007; Paine *et al*., 2008; Dahan and Amidon, 2009). A more recently discovered mechanism underlying grapefruit juice–drug interactions is inhibition of intestinal organic anion transporting polypeptides (OATPs), which are uptake transporters located on the apical membranes of enterocytes and other cell types (Dresser and Bailey, 2003; Won *et al*., 2012). Opposite to P-gp, OATPs in the intestine act to facilitate drug absorption. Thus, inhibition of these transport proteins leads to a decrease in systemic exposure of drug substrates, including the anti-histamine fexofenadine (Dresser and Bailey, 2003; Dresser *et al*., 2005) and the anti-hypertensive agent aliskiren (Vanamala *et al*., 2005; Avula *et al*., 2007), with the consequent potential for therapeutic failure. Candidate OATP inhibitors in grapefruit juice include the flavonoids naringin and hesperidin (Bailey *et al*., 2007). The potential for drug interactions with a widely available food product necessitates an understanding of the expected concentrations of a suite of structurally diverse and potentially bioactive compounds.

Reported analytical methods for measuring furanocoumarins in fruit juices typically require separation times of 1 h or more (Fukuda *et al*.,2000; Ross *et al*., 2000; Manthey and Buslig, 2005; Vanamala *et al*., 2005; De Castro *et al*., 2006; Avula *et al*., 2007). For example, in 2005, a study of the distribution of furanocoumarins in grapefruit juice fractions utilised a 65 min high-performance liquid chromatography (HPLC) method (Manthey and Buslig, 2005). The same year, an exhaustive analysis of several furanocoumarins and furanocoumarin dimers in 58 juices employed a similar 65 min method (Widmer and Huan, 2005). The following year, a 45 min HPLC method was utilised to determine the concentrations of bergamottin and 6′,7′-dihydroxybergamottin in grapefruit juice (De Castro *et al*., 2006). In 2009, a shorter HPLC method was developed for the determination of five furanocoumarins (bergaptol, psoralen, bergapten, bergamottin and 6′,7′-dihydroxybergamottin) in citrus juices that required a run time of 23 min, which appears to be the most rapid method published to date (Lin*et al*., 2009).

As with the furanocoumarins, analytical procedures for measuring flavonoids often employ methods of 1 h or more. A survey of nine commercial grapefruit juices and associated flavonoid concentrations published in 2000 utilised a 60 min method (Ross *et al*., 2000). Six years later, a 65 min method in a similar survey of orange and grapefruit juices was published (Vanamala *et al*., 2005), followed the next year by another 60 min method for the simultaneous analysis of adrenergic amines and flavonoids in fruit jams and juices (Avula *et al*.,2007). De Castro *et al*. (2006) utilised a 70 min separation to determine the concentrations of naringin and naringenin. The most rapid HPLC analyses of flavonoids reported in the literature was a 45 min method published in 2008 (Fujita *et al*., 2008).

A handful of UPLC analyses of flavonoids in food, supplements and traditional Chinese medicines have appeared in the literature in the past few years as well. The majority of these studies (Baranowska and Magiera, 2011; Medina-Remon *et al*., 2011; Cao *et al*., 2012; Huang *et al*., 2012) utilise a C_{18}-column and UV detection, though one expansive study quantifying 39 phenolic compounds in apples (De Paepe *et al*., 2013) additionally used electrospray ionisation mass spectrometry (ESI-MS) to confirm the identity of the analytes. The most rapid of these UPLC studies quantified 11 flavonoids (including those listed in this paper) in three citrus fruit extracts, and had a run time of 5.5 min (Cao *et al*., 2012).

Ultra performance liquid chromatography offers significant advantages in sensitivity and speed compared with HPLC. After overcoming the difficulties presented by working with very high pressures (MacNair *et al*., 1997, 1999; Wu *et al*., 2001), early studies demonstrated enhanced resolution and sensitivity, reduced solvent consumption and rapid analyses (Gerber *et al*., 2004; Swartz, 2005; Kumar *et al*.,2012) with UPLC compared with that which can be achieved with HPLC. With greater integration in diverse research areas, UPLC offers the opportunity to streamline quantitative determinations, such as those described herein, reducing cost and expediting research.

The goal of this study was to develop rapid (< 5.0 min) methods for the quantitation of two furanocoumarins (bergamottin and 6′,7′-dihydroxybergamottin) and four flavonoids (naringin, naringenin, narirutin, and hesperidin) in grapefruit juice using UPLC (Fig. 1). An additional goal was to apply these methods to determine the concentrations of the aforementioned analytes in five grapefruit juices used in previous clinical interaction studies.

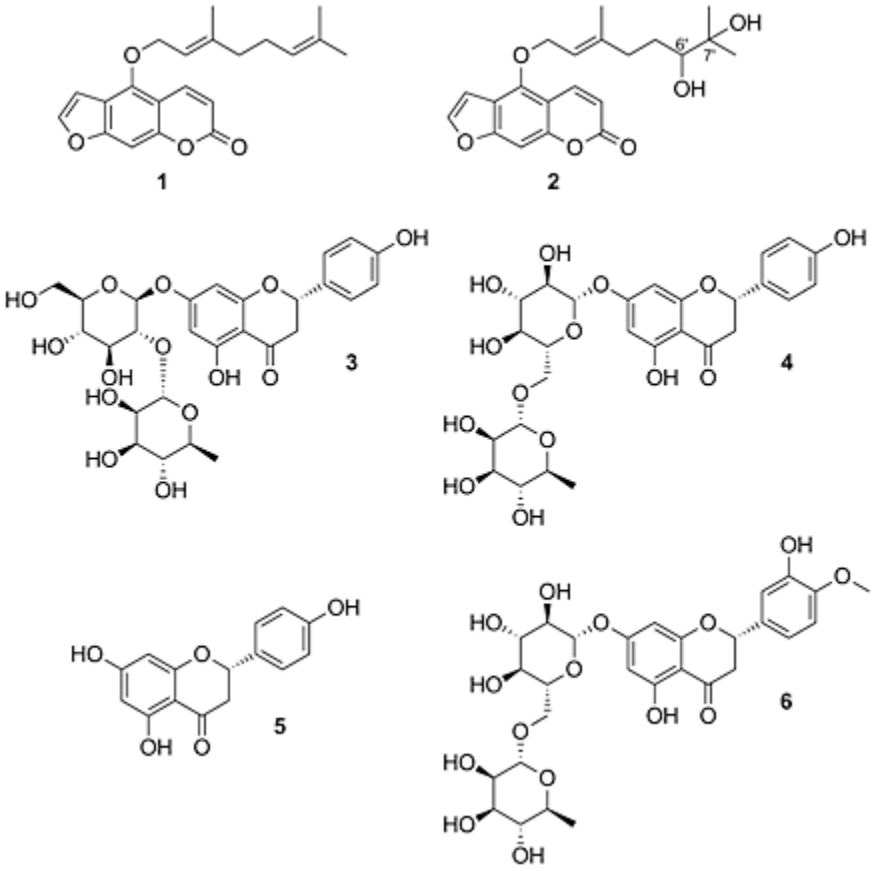

Figure 1. Structures of the furanocoumarins bergamottin (**1**) and 6′,7′-dihydroxybergamottin (**2**) and the flavonoids naringin (**3**), narirutin (**4**), naringenin (**5**) and hesperidin (**6**).

Experimental

Instrumentation

The UPLC analyses were performed using a Waters Acquity UPLC system (Milford, MA, USA) equipped with an autosampler, photodiode array detector (PDA), column manager and binary solvent manager. Data were collected and analysed using Empower software. A HSS C_{18}-column (50 mm × 2.1 mm i.d., 1.8 µm; Waters, Milford, MA, USA) was used for all chromatographic analyses.

Materials

Bergamottin (purity ≥ 96.9%) was purchased from ChromaDex (Irvine, CA, USA); narirutin (purity ≥ 99.0%) was purchased from Indofine (Hillsborough, NJ, USA); naringin (purity ≥ 96.8%), naringenin (purity ≥ 99.9%), hesperidin (purity ≥ 97.0%) and 6′,7′-dihydroxybegamottin (purity ≥ 97.2%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Purity of standards are reported as determined by HPLC by the manufacturers. Methanol, ethyl acetate and acetonitrile were purchased from Pharmco-Aaper (Shelbyville, KY, USA).

Juices A and B were used in clinical studies with felodipine (Paine *et al*., 2006), cyclosporine (Paine *et al*., 2008) and fexofenadine (Won *et al*., 2010). Juice A was a commercially available product. Juice B was the same juice, but the furanocoumarin fractions were removed using a series of food-grade solvents and absorption resins and column chromatography (Paine *et al*., 2006). Juice C was a commercially available concentrated grapefruit juice (Minute Maid Premium 100% Pure Frozen Concentrated Grapefruit Juice with added calcium). A dilution of Juice C was used in a clinical study examining the effect of grapefruit juice on the systemic exposure of loperamide and the CYP3A-mediated metabolite, desmethylloperamide (Wolf *et al*., 2011). Juice D (Florida's Natural Original Ruby Red 100% Pure Florida Grapefruit Juice) and Juice E (Simply Orange Juice Co. Simply Grapefruit 100% Pure Squeezed Grapefruit Juice) were commercially available not-from-concentrate juices and were purchased from a local grocery store.

Standards preparation

Bergamottin and 6′,7′-dihydroxybergamottin were dissolved in methanol to produce a 1.03 mm (bergamottin) and 1.00 mm (6′,7′-dihydroxybergamottin) multi-standard stock solution. This solution was used to prepare seven standard solutions at concentrations ranging from 10.3 to 659 μm (bergamottin) and from 10.0 to 640 μm (6′,7′-dihydroxybergamottin). Naringin, narirutin, naringenin and hesperidin were dissolved separately in methanol to produce stock solutions of 10.0 mm, 7.92 mm, 10.2 mm and 9.97 mm, respectively. These stock solutions were used to prepare standard solutions for each compound (six for naringin, five for each of the other flavonoids) at concentrations ranging from 0.649 to 5.00 mm (naringin), 0.198 to 3.17 mm (narirutin), 3.18 to 814 μm (naringenin) and 3.12 to 798 μm(hesperidin).

Sample preparation

Juice extracts were prepared by transferring 25.0 mL of each juice to a 50 mL conical polypropylene tube. After adding 20.0 mL of ethyl acetate, the contents were shaken vigorously and centrifuged (2500 × *g* for 30 min at 25°C). The resulting organic layer was transferred by Pasteur pipette to a 250 mL round-bottom flask. An additional 20.0 mL of ethyl acetate were added to the remaining contents of the tube, shaken and centrifuged, and the resultant organic layer was combined with the first organic layer. This extraction procedure was repeated a third time, and the combined organic layers were evaporated *in vacuo*. The residue in the round-bottom flask was transferred quantitatively to a 2 mL vial, using methanol as a rinse, and

evaporated to dryness under air. This material was resuspended with 5.00 mL of methanol, yielding a fivefold concentrated extract of each juice.

Chromatographic conditions

Quantitative analysis of furanocoumarins (bergamottin and 6′,7′-dihydroxybergamottin)

A 6 μL volume of each fivefold concentrated juice extract was injected and analysed at a wavelength of 250 nm. Chromatographic separations were carried out with a mobile phase consisting of HPLC-grade acetonitrile (solvent A) and nanopure water (solvent B) at a flow rate of 0.6 mL/min. The following linear gradient was used: 0.0 min, 30% A; 4.0 min, 100% A; 4.5 min, 100% A. The column temperature was 30°C.

Quantitative analysis of flavonoids (naringin, narirutin, naringenin and hesperidin)

A 6 μL volume of each fivefold concentrated juice extract was injected and analysed at a wavelength of 310 nm. The following linear gradient was used: 0.0 min, 10% A; 1.0 min, 20% A; 2.0 min, 20% A; 2.5 min, 40% A; 3.5 min, 100% A; 4.0 min, 100% A. Solvents A and B, the flow rate and column temperature were the same as described in the previous section.

Method validation

The identities of analyte peaks in the juice extracts were confirmed by comparing the UV spectra of the peaks and their standards, as well as, in the case of the flavonoids, by co-injection of the standards with the juices (see online Supporting Information, Supplements 1 and 2).

Linearity of the calibration curves was assessed by least-squares analysis. Precision and accuracy were determined by calculating the relative standard deviation (RSD) and relative error (RE), defined as the percentage difference between the mean observed concentration and the nominal concentration, of three replicate analyses of the standards. All analyses were performed in triplicate in a single day. Interday RSD and RE were determined by analysing the standard solutions in triplicate on three separate days. The limit of detection (LOD) was defined as the concentration corresponding to the signal detection limit, which was defined as $b + 3s_y$, where b is the y-intercept of the calibration curve, and s_y is the standard deviation of the vertical deviations (Harris, 2007). The limit of quantitation (LOQ) was defined as $b + 10s_y$.

Results and discussion

Method validation

All standard curves exhibited coefficients of determination (r^2) greater than 0.999 (Table 1). Baseline resolution of all analytes (Fig. 1), both as standards and in the juice extracts, was achieved (Figs 2 and 3). Precision and accuracy data are summarised in Table 2. The RSD of the furanocoumarin standards (**1** and **2**) was below 0.33% (intraday) and 3.1 % (interday) for all standard concentrations. The intraday RSD values of analytes **3**, **4** and **5** were below 1.5%, 2.6%

and 2.9%, respectively, while the interday RSD averages for each analyte remained below 1.0%. For analyte **6**, the largest RSD value was 4.7% at the lowest concentration of that standard, while the interday RSD was 3.1%. For the furanocoumarins (**1** and **2**), intraday RE remained below 5.7% (**1**) and 2.8% (**2**). Interday RE values predominantly remained under 3.0%, except for the lowest concentrations of **1** and **2**, the highest variation being 21% for the lowest concentration of **1**. The RE values (both intraday and interday) of the flavonoids (**3–6**) were predominantly below 5%, and all below 15%. The only exception was the lowest of the standard concentrations of **5** (3.18 μm), which had much higher RE values (32%); thus, this concentration was below the LOQ as defined above. For each analyte measured, all standard concentrations above the LOQ resulted in an RE value of ≤ 15%. The LOD values for analytes **1**, **2**, **5** and **6** were all below 5 μm, whereas the LOD values for analytes **3** and **4** were higher (18 and 69 μm, respectively).

Table 1. Calibration curve data for analytes 1–6

Analyte	**Slope (±SD)**	r^2	**LOD (μm)**	**LOQ (μm)**
1. LOD, limit of detection; LOQ, Limit of quantitation.				
1	4.338×10^3 (±9)	1.000	0.78	2.6
2	3.784×10^3 (±5)	1.000	1.1	3.8
3	9.04×10^2 (±7)	0.999	69	230
4	9.98×10^2 (±7)	0.999	18	58
5	1.626×10^3 (±6)	1.000	2.5	8.4
6	8.95×10^2 (±5)	1.000	3.9	13

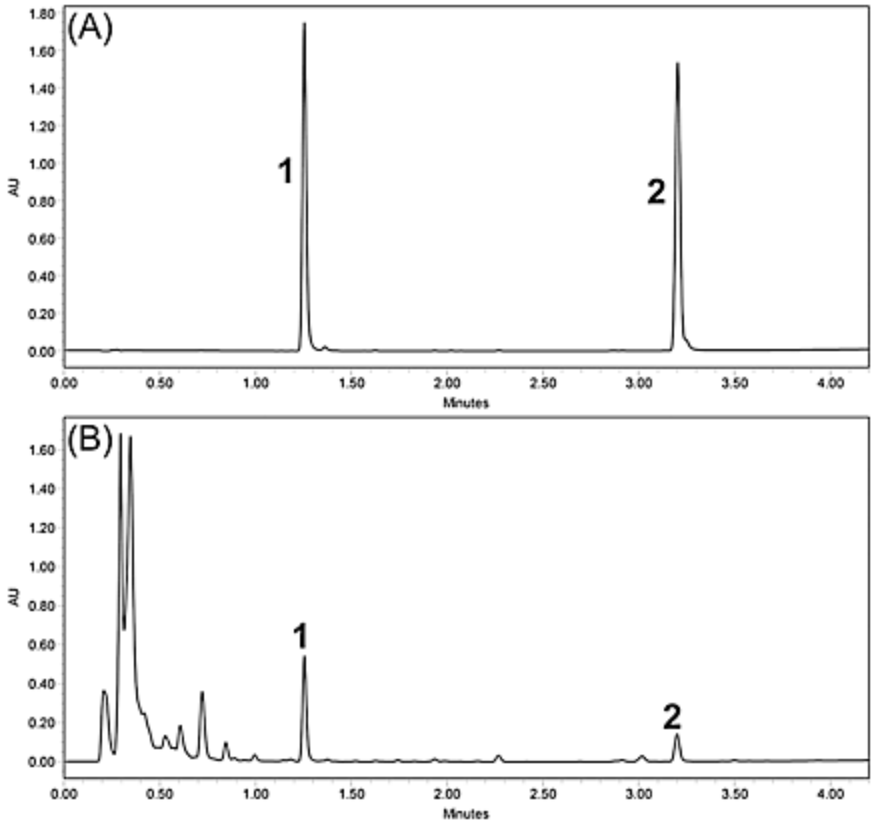

Figure 2. The UPLC separation of furanocoumarins. Numbered peaks correspond to the compounds in Fig. 1: (A) furanocoumarin standards (659 and 640 μm, respectively) and (B) Juice A.

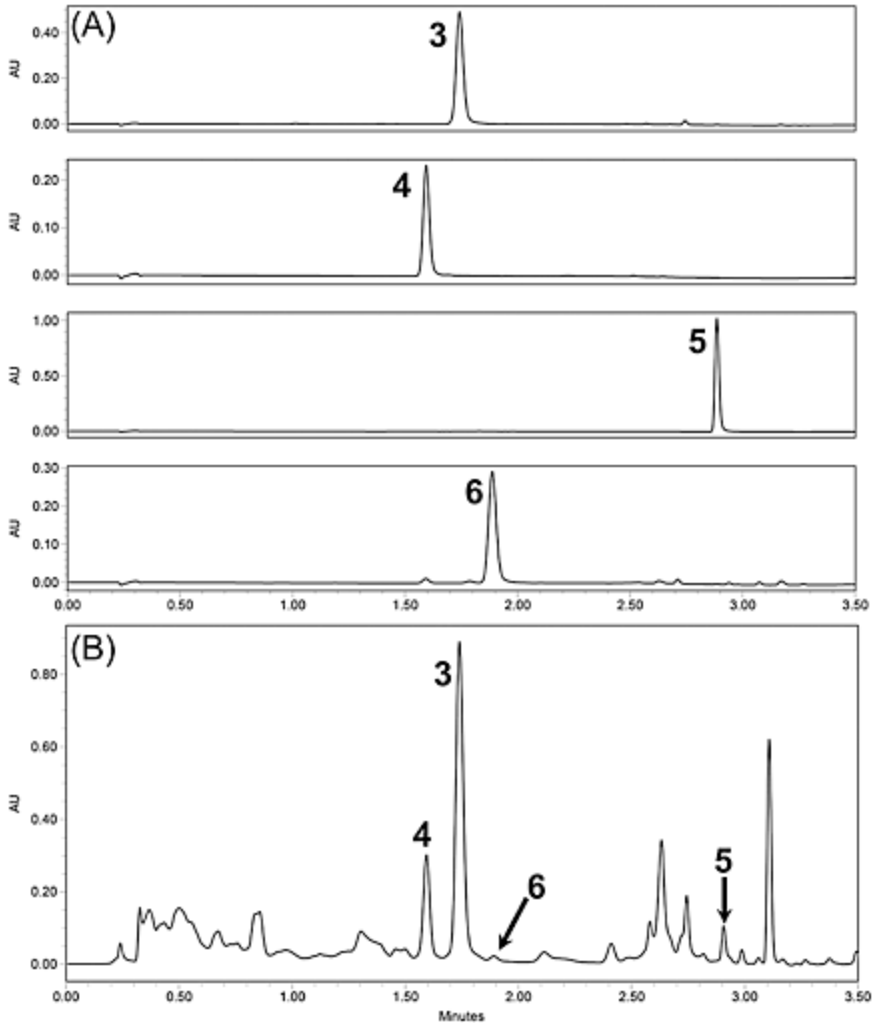

Figure 3. The UPLC separation of flavonoids. Numbered peaks correspond to the compounds in Fig. 1: (A) flavonoid standards (1080, 500, 800 and 800 μm, respectively) and (B) Juice A.

Table 2. Precision (RSD) and accuracy (RE) for analysis of analytes 1–6

Analytes	Concentration of standard solution injected (μm)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	10.3	0.33	5.7	3.1	21.
1	20.6	0.27	4.4	0.48	8.5
	41.2	0.091	1.6	0.31	2.4

Analytes	Concentration of standard solution injected (µm)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	82.4	0.19	0.21	0.53	1.1
	165	0.16	0.03	0.26	2.1
	330	0.29	1.2	0.16	0.83
	659	0.32	0.29	0.32	0.33
	10	0.15	2.8	1.2	9.2
	20	0.11	1.1	0.67	2.7
	40	0.017	0.94	0.62	0.69
2	80	0.21	0.10	0.56	2.1
	160	0.16	0.64	0.59	2.5
	320	0.26	0.70	0.61	2.1
	640	0.31	0.13	0.53	1.4
	649	1.5	15.	0.84	17.
	1081	1.3	4.6	0.14	5.7
	1802	1.1	0.81	0.38	0.79
3	3003	0.36	3.3	0.45	3.0
	5005	0.72	3.6	0.29	2.1
	10010	0.99	1.0	0.28	1.9

Analytes	Concentration of standard solution injected (μm)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	198	1.6	13.	0.67	7.7
	396	2.6	1.4	0.72	2.1
4	792	1.5	1.6	0.43	1.3
	1584	1.3	2.5	0.26	2.0
	3168	0.72	0.66	0.78	0.55
	3.18	1.4	32.	0.03	32.
	12.7	2.9	9.7	0.32	5.3
5	50.9	1.4	0.4	0.23	1.4
	203	0.23	1.6	0.35	0.66
	814	1.2	0.094	0.18	0.39
	3.12	4.7	2.4	3.1	3.7
	12.5	0.64	2.8	0.96	8.3
6	49.9	0.93	3.5	0.69	9.0
	199	0.71	4.1	0.47	8.1
	798	1.6	3.0	0.72	1.4

Rapidity of method

Compared with HPLC analyses of furanocoumarins and flavonoids in the literature, the UPLC method described above markedly reduces the time required for the analyses of these compounds

in grapefruit juice. A run time of 4.5 min represents an 80% reduction in the time required to analyse furanocoumarin content when compared with the most rapid published method (Lin *et al*., 2009), whereas a run time of 4.0 min represents an order of magnitude less time required for the analysis of flavonoids (Fujita *et al*., 2008). Such methods facilitate the determination of furanocoumarin and flavonoid concentrations in several grapefruit juices in a single day, reducing costs associated with both labour and materials. Moreover, the time saved expedites the characterisation of juices used in *in vitro* or *in vivo* studies, thus accelerating the evaluation and determination of active constituents.

Concentrations of analytes in grapefruit juices

Five different grapefruit juices, four of which were commercially available products, were analysed to measure the concentrations of selected furanocoumarins and flavonoids (Fig. 1). Samples were analysed in the same run with the standards to eliminate concerns of systematic error introduced by day-to-day variability in instrument response. Each juice was analysed using a fivefold concentrated extract, and the reported values (Table 3) have been adjusted to reflect the concentration in the original juices. Considerable variation in the concentration of each compound was observed. Regarding the furanocoumarins, the concentrations of **1** and **2** in four of the juices ranged from 7.48 (±0.17) to 24.73 (±0.16) μm and 7.65 (±0.17) to 89.03 (±0.17) μm, respectively. These values were consistent with those reported in the literature using HPLC (Widmer and Huan, 2005; De Castro *et al*., 2006). The highest concentration of **2** (Juice C) was slightly higher than the ranges reported by De Castro *et al*. (2006), though this was to be expected, as Juice C was a juice concentrate. Regarding the flavonoids, the concentrations of **3** and **4** ranged from 309 (±15) to 1182 (±16) μm and 73.4 (±4.8) to 286.5 (±3.8) μm, respectively, which again were consistent with literature values using HPLC (Ross *et al*., 2000; De Castro *et al*., 2006). The concentration of **6** ranged from below LOQ (although detected in all juices) to 39.48 (± 0.83) μm. The concentration of **5** ranged from below LOQ to 34.72 (±0.55) μm, which agrees with literature values (Wilson *et al*., 2000; Wanwimolruk and Marquez, 2006). As with the furanocoumarins, the highest concentrations of the flavonoids were in Juice C. The concentrations of **1–4** in Juices A and B were measured previously using an HPLC method (Paine *et al*., 2006; Won *et al*., 2010). The concentration of the furanocoumarins (**1** and **2**) were consistent between methods (within 30 and 7%, respectively), whereas the concentrations of the flavonoids (**3** and **4**) by HPLC were approximately twofold higher than those by UPLC. Reasons for this difference remain unexplored, as a between-laboratory validated study was beyond the scope of this work. The extracts used in this study were analysed at a fivefold concentration. In the future, the accuracy of the measurements for analytes present in very low concentrations could be improved by using a more concentrated extract.

Table 3. Concentrations of analytes 1–6 in grapefruit juices

Juice	Concentration[a] (μm) ± SD

	Bergamottin (1)	DHB[b] (2)	Naringin (3)	Narirutin (4)	Naringenin (5)	Hesperidin (6)
A	11.76 ± 0.16	41.31 ± 0.16	412 ± 15	100.5 ± 4.5	18.89 ± 0.66	NQ
B	ND	ND	309 ± 15	73.4 ± 4.8	ND	NQ
C	24.73 ± 0.16	89.03 ± 0.17	1182 ± 16	286.5 ± 3.8	34.72 ± 0.55	39.48 ± 0.83
D	7.48 ± 0.17	7.65 ± 0.17	371 ± 15	85.2 ± 4.7	NQ	NQ
E	12.26 ± 0.16	11.58 ± 0.16	381 ± 15	103.5 ± 4.5	5.60 ± 0.79	NQ

ND, not detected; NQ, the analyte was detected but was below the limit of quantitation.

a Each juice was analysed in triplicate using a fivefold concentrated extract, and the values reported have been adjusted to reflect the concentration in the juices, not in the extract.

b 6′,7′-dihydroxybergamottin.

Wide between-juice variation in the concentrations of each grapefruit juice constituent was observed, as would be expected for natural products, and the concentrations agreed well with values reported in the literature using HPLC. This marked variation between commercially available brands undoubtedly contributes to the large between-study differences in effect size (i.e., change in drug area under the curve [AUC]) associated with clinical grapefruit juice–drug interaction studies (Won *et al*., 2012). Quantitation of one or more constituents in a given juice would provide a means for between-study comparisons of clinical, as well as *in vitro*, data. The UPLC methods developed in the current work offer a rapid means for the quantitation of representative constituents. While the methods were applied to grapefruit juices, they should be applicable to other foods, including other fruit juices.

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