JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 197−203 **197**

Characterization of Flavonoids and Pectins from Bergamot (*Citrus bergamia* Risso) Peel, a Major Byproduct of Essential Oil Extraction

GIUSEPPINA MANDALARI,[†] RICHARD N. BENNETT,[‡] GIUSEPPE BISIGNANO,[†] ANTONELLA SAIJA,[†] GIACOMO DUGO,[§] ROSARIO B. LO CURTO,[§] CRAIG B. FAULDS,*[,||] AND KEITH W. WALDRON[||]

Department of Pharmaco-Biology, and Department of Organic and Biological Chemistry, University of Messina, Vill. SS. Annunziata 98168 Messina, Italy, Phytochemicals and Health Program, and Sustainability of the Food Chain Exploitation Platform, Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, United Kingdom

Bergamot peel is an underutilized byproduct of the essential oil and juice-processing industry. As with other *Citrus* peels, it still contains exploitable components, such as pectins and flavonoids. Commercial glycoside hydrolases, specifically a combination of pectolytic and cellulolytic enzymes, solubilized a high percentage of the material (81.94%). The flavonoid profile of the peel consisted of characteristic *Citrus* species flavanone rutinosides and neohesperosides derived from naringenin, eriodictyol, and hesperetin. In addition, a number of minor flavanone and flavone glycosides, not found in orange and lemon peels, were identified. The majority of flavonoids were extracted in the two 70% v/v EtOH extractions. Processing this material clearly has economic potential leading to low environmental impact.

KEYWORDS: ***Citrus bergamia*; peel; byproducts; flavanones; flavones; psoralens; soluble sugars; cell wall structure; LC-MS; GC-MS**

INTRODUCTION

During the processing of *Citrus* fruits (orange, lemon, lime, grapefruit, etc.) for juice and essential oil extractions, peels represent between 50 and 65% of the total weight of the fruits and remain as the primary byproduct. If not processed further, it becomes waste and can give rise to serious environmental pollution. Therefore, the *Citrus*-processing industries are commercially interested in the valorization of these wastes, i.e., by recovering residual amounts of soluble solids after juice extraction. Peels are a good source of molasses, pectin, and cold-pressed oils; the dried peels can also be used in cattle feed (*1*). However, in Italy and in many Mediterranean countries, a major portion of the peels are not further processed due to the quantities produced and the small amounts able to be processed in the immediate locality. This surplus is not directly usable at the wet state, and drying is too expensive. An extensive understanding of the whole byproduct composition and the cell wall carbohydrate constituents is required to optimize feed utilization for dairy cattle and potentially for the generation of other valuable fractions.

Citrus peel wastes can be utilized in order to isolate pectin and flavonoids (*2*−*5*). Pectins are complex polysaccharides involved in the control of the cell wall ionic status, cell expansion, and separation (*6*). They are commercially extracted from *Citrus* peel and apple pomace. Because of their gelling capacity, they represent important additives in the food industry (*7*). *Citrus* flavonoids are a major class of secondary metabolites, and the highest concentrations of these compounds occur in the peel (*8*, *9*). Flavonoids have been found to have health-related properties, especially based on their antioxidant activity (*10*, *11*). Extensive *in vivo* and *in vitro* experiments employing these compounds showed beneficial health activities as protective agents against cancer and cardiovascular, inflammatory, allergic disorders, capillary fragility, and human platelet aggregation (*12*, *13*). In addition, they appear to have a natural defensive role in plants against invading pathogens, including bacteria, fungi, and viruses (*14*).

A less commercialized *Citrus* fruit, bergamot (*Citrus bergamia* Risso), is used mostly for the extraction of its essential oil, obtained by wash-scraping the fruit. The annual Italian production of bergamot amounts to 25000 tons (Consorzio del Bergamotto, personal communication). This oil is widely used in cosmetic, pharmaceutical, and food industries (*15*). Bergamot

* To whom correspondence should be addressed. Tel: +44(0)1603 255152. Fax: +44(0)1603 507723. E-mail: craig.faulds@bbsrc.ac.uk.
[†] Department of Pharmaco-Biology, University of Messina.
[‡] Phytochemicals and Health Program, Institute of Food Research.
[§] Department of Organic and Biological Chemistry, University of Messina.
[||] Sustainability of the Food Chain Exploitation Platform, Institute of Food Research.

10.1021/jf051847n CCC: $33.50
Published on Web 12/14/2005

peel, composed mainly of the inner part of the fruit (albedo, inner white layer of the peel) and the external part of the fruit (flavedo, outer colored part of the peel), is still rich in sugars, fibers, and other residual substances and, therefore, causes as many economic and environmental problems because of its fermentability as other *Citrus* fruits.

It is well-known that orange peel represents a rich commercial source of pectin (polygalacturonic acid) and flavonoids (*16*−*18*). The aim of our research was to evaluate the composition of bergamot peel in an attempt to determine if this source of byproduct could be more economically utilized. An enzymatic treatment was also developed to facilitate the solubilization of this byproduct, allowing possible further use as a feed component.

MATERIALS AND METHODS

Source of Bergamot Peel. Peel was obtained from Consorzio del Bergamotto, a bergamot-processing factory in Reggio Calabria (Southern Italy). Fresh peel, consisting of a mix of the three major commercial cultivars *Fantastico* (90%), *Femminello* (5%), and *Castagnaro* (5%), was divided into subsamples of approximately 500 g lots, stored at −18 °C, and lyophilized. Bergamot peel represented the mix of seeds, pulp, and deoiled flavedo after essential oil and juice extraction.

Chemicals. All solvents were high-performance liquid chromatography (HPLC) grade, and all water was ultrapure grade. All carbohydrate, flavonoid, and miscellaneous *Citrus* phytochemical standards [flavanone glycosides and aglycones, flavone glycosides, coumarins, and psoralens (furanocoumarins)] were obtained from either Sigma-Aldrich (Poole, United Kingdom) or Extrasynthese (Genay, France).

Cell Wall Fractionation. Alcohol insoluble residue (AIR) was prepared following the method of Waldron and Selvendran (*19*). Aliquots of lyophilized bergamot peel (100 g) were homogenized for 5 min using a Waring commercial blender in 1 L of 70% v/v ethanol (EtOH). The homogenates were boiled for 5 min, allowed to cool, and filtered through nylon mesh (100 μm). The residues were sequentially reextracted and rehomogenized (Ystrol D-79282 Ballrechten-Dottingen homogenizer) with a further 1 L of 70% EtOH v/v followed by two extractions with 100% EtOH v/v, creating four liquid fractions for each sequential fractionation of the peel: 70 E1, 70 E2, 100 E1, and 100 E2. The final residues were dried overnight after washing with acetone and then milled to a fine powder by using an analytical mill (Janke & Kunkel A10); this fraction was termed the AIR. Both the alcohol extracts and the AIR were analyzed for sugars, uronic acid, and phenolics (simple phenolics, *Citrus* flavonoids, and psoralens). AIR fractionation was performed by suspending 1 g of material in cold deionized water (100 mL), stirring for 2 h at 4 °C, and centrifuging (5000*g*, 30 min, 20 °C). The supernatant was filtered through a GF-C membrane. Hot water (100 mL) was added to the residue, stirred for 2 h with constant heating (80 °C), and refiltered through GF-C. Both filtrates were then lyophilized. The final residue was washed with acetone and dried overnight. Sugar composition, uronic acid, and phenolic content (residual, soluble and cell wall bound) were determined in all of the samples.

Neutral Sugar Composition. Sugars were released from the samples after hydrolysis with 72% H_2SO_4 for 3 h, followed by dilution to 1 M (Saeman hydrolysis). Hydrolyzed monosaccharides were analyzed as their alditol acetates by GLC on a cross-bonded 50% cyanopropyl methyl−50% phenyl methyl polysiloxane column (Thames Chromatography, Maidenhead, United Kingdom) using a flame ionization detector (*20*). The oven temperature program used was as follows: 140 °C (0 min), +2.5 °C min^{-1} (5 min), and 210 °C (45 min). A second set of samples was hydrolyzed with 1 M sulfuric acid only (100 °C) and otherwise treated the same as the first set; this second analysis excluded cellulose.

Uronic Acid Content. Total uronic acids were determined colorimetrically at 580 nm from a standard curve of galacturonic acid by the method of Blumenkranz and Asboe-Hansen (*21*). Distinction between galacturonic acid and glucuronic acid was not determined by this method.

Total Protein Assay. The total protein content of both bergamot starting material and AIR was determined by a micro-Kijeldahl according to the AOAC (*22*). Values were expressed as $N \times 6.25$.

Lipid Content. The total lipid content and fatty acids were determined using the method described by Mondello et al. (*23*).

Acetate Content. The total alkali-extractable acetate content of bergamot peel and AIR was determined by hydrolyzing with 2 M NaOH for 24 h at room temperature followed by centrifugation. Liberated acetic acid was determined with the Boehringer acetic acid test kit (Biopharma Enzymatic Bioanalysis/Food Analysis, United Kingdom).

Enzymatic Treatments. The following commercial pectolytic enzymes were used in this study: endo-polygalacturonase (Megazyme, Bray, Rep. of Ireland; 890 U/mg), depol 690 L (Biocatalysts Ltd, Cefn Coed, Wales, United Kingdom; 300 endogalacturonase U/g, 300 cellulase U/g), pectinase 444 L (Biocatalysts Ltd., 600 endogalacturonase U/g), pectinase 62 L (Biocatalysts Ltd., 2200 endogalacturonase U/g). Polygalacturonase (PGase) activity was determined by measuring the rate of D-galacturonic acid formation from 0.2% w/v orange polygalacturonic acid (Sigma Chemical Co., Dorset, United Kingdom) in 50 mM Na−acetate buffer, pH 5.0. One unit of enzymatic activity (U) is defined as the amount catalyzing the formation of 1 μmol of D-galacturonic acid per min at 30 °C and pH 5.0 (*24*). Enzyme solubilization of wall components was determined by incubating bergamot AIR (200 mg) with 10 U of PGase-equivalent activity in 50 mM Na−acetate buffer, pH 5.0, in a final volume of 20 mL for 24 h in a shaking incubator (37 °C, 100 rpm). Samples were then filtered through GF-C paper under vacuum (Millipore 1225 sampling manifold), and residues were washed with distilled water and dried (60 °C) to a constant weight before weighing. The amount of monosaccharide material left in the residues after 24 h hydrolysis was quantified against standard curves. All assays were prepared and analyzed in duplicate.

Extraction and Liquid Chromatography−Mass Spectrometry (LC-MS) Analysis of Phenolics, Flavonoids, and Psoralens in Whole Peel and Subfractions. Lyophilized bergamot peel aliquots (3 × 40 mg) were weighed into 2 mL screw-top microtubes and extracted with 950 μL of 70% v/v methanol in ultrapure water at 70 °C for 20 min with 50 μL of 0.1 mg/mL quercetin 3-*O*-rhamnoside (quercitrin) added as an internal standard. Samples were vortex mixed every 5 min to improve extraction efficiency. After extraction, samples were centrifuged (17000*g*, 4 °C, 20 min), and the supernatants were filtered (0.2 μm PTFE, Chromos Express, Macclesfield, United Kingdom) into 1.5 mL HPLC vials. Recovery of the internal standard was consistently 90−95%. Ethanol extracts generated from the fractionation of the peel were simply filtered (0.2 μm PTFE) prior to LC-MS analyses.

A multipurpose HPLC method was developed for the simultaneous separation of simple phenolics, many classes of flavonoids, and psoralens. The samples were analyzed using an Agilent HP1100 system (photodiode array and binary pump) coupled with an Agilent SL MSD. A linear gradient was used where solvent A = 0.1% v/v trifluoracetic in ultrapure water and solvent B = 0.1% trifluoroacetic acid in HPLC grade MeOH; T = 0 (100% A), 5 (100% A), 15 (83% A, 17% B), 17 (83% A, 17% B), 22 (75% A, 25% B), 30 (65% A, 35% B), 35 (50% A, 50% B), 40 (100% B), 50 100% (A), 55 (100% A), and 65 min (100% A). The flow rate was 1 mL/min, and the thermostatically controlled autosampler and column oven were set at 10 and 30 °C, respectively. Specific UV−visible data were collected at 220, 270, 325, 370, and 520 nm (with overall data collected between 200 and 600 nm). Full-scan data were collected with the MSD, using an electrospray source, in both negative ion (ES−) and positive ion (ES+) modes with the following settings: nitrogen gas; gas temperature, 350 °C; drying gas flow rate, 13 L/min; nebulizer pressure, 50 psig; capillary voltage, 4000 V (ES+ mode) and 3000 V (ES− mode); mass range, 50−1600 amu; fragmentor set at 100; gain, 1.0 EMV; threshold, 150; and step size, 0.10. Commercial standards of previously identified bergamot and *Citrus* phytochemicals were also run using the following: eriocitrin, neoeriocitrin, naringin, narirutin, hesperidin, neohesperidin, 7-hydroxyflavone, scutellarein tetra-methyl ether, sinensetin, citropten, bergapten, 5-geranoxy-7-methoxy-coumarin, and bergamottin. In addition, mixtures

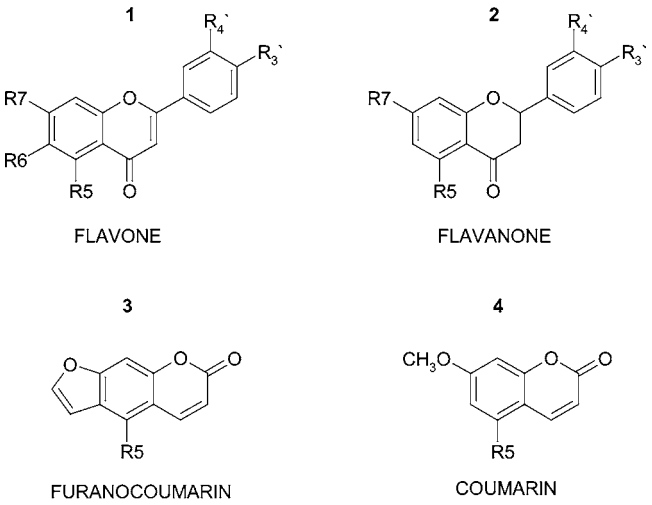

Figure 1. Chemical structures of previously identified bergamot phenolics (for the flavonoids, only the core aglycone structures are defined; see table of bergamot composition for details of sugar moieties and substitution positions). Structure 1: apigenin (R6 = R3′ = H; R5 = R7 = R4′ = OH), luteolin (R6 = H; R5 = R7 = R3′ = R4′ = OH), tetramethylscutellarein (R3′ = H; R5 = R6 = R7 = R4′ = OCH_3), sinensetin (R5 = R6 = R7 = R3′ = R4′ = OCH_3), and 7-hydroxyflavone (R5 = R6 = R3′ = R4′ = H; R7 = OH). Structure 2: naringenin (R3′ = H; R7 = R4′ = OH), eriodictyol (R7 = R3′ = R4′ = OH), and hesperetin (R7 = R3′ = OH; R4′ = OCH_3). Structure 3: bergapten (5-methoxy-psoralen; R5 = OCH_3) and bergamottin (5-geranyloxy-psoralen; R5 = O–CH_2–CH=C[CH_3]–CH_2–CH_2–CH=C[CH_3]$_2$). Structure 4: citropten (R5 = OCH_3) and 5-geranyloxy-7-methoxycoumarin (R5 = O–CH_2–CH=C[CH_3]–CH_2–CH_2–CH=C[CH_3]$_2$).

of other common plant phenolics (benzoic, hydroxycinnamic, and phenylacetic acids) and various flavonoids (flavones and flavonols) were also run.

RESULTS AND DISCUSSION

Phenolic Acids, Flavonoids, and Psoralens in Whole Peel and Subfractions. The chemical structures of previously reported bergamot phytochemicals are shown in **Figure 1**. In addition to the previously reported bergamot flavanones, a number of additional minor flavones (apigenin, luteolin, and diosmetin-derived) and flavanones (eriodictyol, naringenin, and hesperetin-derived) were detected in the 70% MeOH and EtOH fractions of the bergamot peel (**Table 1**). The recently reported di-*C*-glucosides of apigenin and diosmetin were also identified in the bergamot peel (*27*). No significant concentrations of simple phenolic acids were found in the peel samples or EtOH fractions. In addition, the levels of psoralens and other lipophilic phenolics were also low in the peel. This was not unexpected since other researchers had shown that high levels of these compounds are found in the oil prepared from bergamot, indicating that the peel waste was essentially free of most of the oil-soluble components (*28*). The predominant sugars of the flavonoids were rhamnose (Rha) and glucose (Glc); mono-glycosides (mono-Rha or mono-Glc), di-glucosides (mono-Glc/mono-Rha), rutinosides (-Glc [6→1]Rha), or neohesperosides (-Glc [4→1]Rha). The flavonoids contributed a small percentage to the total amounts of Glc and Rha in the various fractions (**Table 2**). LC-MS and UV−visible spectral data for all of the phenolic phytochemicals found in the peel can be found in the Supporting Information. **Figure 2** shows example chromatograms (270 nm) for the starting peel material and mixtures of various commercial standards. No significant peaks were detected before 20 min in the peel sample.

Sugar Composition. Sugar compositions of the bergamot peel, the AIR, and the ethanol extracts are reported in **Table 3**. Sixty-one percent of the dry weight of the AIR was carbohydrate. Glucose and galacturonic acid were the major sugars of the peel and the AIR, while arabinose, galactose, mannose, xylose, and rhamnose were present in small amounts. Only traces of fucose were detected. The percentages of different monomeric sugars (of total sugar content) including galacturonic acid, glucose, arabinose, and galactose were 31.83, 34.64, 12.95, and 7.68%, respectively. Approximately 20% of the glucose of AIR could be released by hydrolysis results with 1 M sulfuric acid alone (results not shown), indicating that the bulk of the glucose was cellulosic in origin. The sum of the percentages of arabinose, rhamnose, galactose, and galacturonic acid in the 1 M sulfuric acid hydrolysis fraction accounted for 75% of the total sugar content. These results indicated that the bergamot peel AIR was mainly composed of pectic substances, encasing the cellulose microfibrils. In lime pectins, only short regions of rhamnogalacturonan I have been reported (*29, 30*). However, the small amounts of noncellulose glucose and xylose indicated that hemicelluloses such as xyloglucan and α-glucans were minor components. These data are consistent with the earlier results obtained from Miron et al. (*31*), where the cell walls of dicotyledonous species, such as those from dried *Citrus* peels, were higher in glucose and lower in xylose as compared to cell walls of monocotyledonous species. Moreover, in the dried *Citrus* peels, α-glucans, pectin, fructose, plus soluble arabinose and galactose contributed together up to 98% of carbohydrates. In our study, the composition of bergamot peel is similar to that identified in the orange peel (*Citrus sinensis* L. Cv. Liucheng), which was also mainly composed of pectic substances, followed by cellulose (*32*).

The neutral sugar composition of the EtOH fractions showed the presence of very high amounts of glucose (70% mol %) and small amounts of all of the other monosaccharides (**Table 3**). Approximately 75% of the glucose in the ethanol extracts could be released by hydrolysis with 1 M sulfuric acid, showing that the bulk of glucose was, as expected, not cellulosic in origin. In addition, an iodine test revealed that starch was not present in the extracts. A small percentage of the glucose and rhamnose in the EtOH fractions was derived from the flavonoids glycosides (X-Glc-Rha and X-Rha) (**Table 2**).

To provide information on the nature of the polysaccharides present and to solubilize the major part of noncellulosic polysaccharides, AIR was sequentially extracted with cold water and hot water. The yields of the fractions and their sugar composition are given in **Table 3**. Only 35% of the bergamot AIR was solubilized by water extraction; the majority was recovered in hot water. Only small amounts of glucose were detected in both cold water (CWE) and hot water extracts (HWE) showing that the glucosic polymer of AIR remains water insoluble. In CWE, the neutral monosaccharides arabinose, galactose, and galacturonic acid represented the 34.32, 13.89, and 23.19% of the total sugars, respectively, showing that some pectic solubilization occurred in this step. Higher amounts of these sugars were present in the HWE, demonstrating a better solubilization of pectic polysaccharides in this material.

Other Components. A 5.71% amount of the dry weight of bergamot peel is protein, while in the AIR the percentage decreased to 5.53. This result is similar to previous reports on orange peel, where the protein content ranged from 4.9 to 6.0

Table 1. Flavonoids and Psoralens in Bergamot Peel and Ethanolic Fractions[a]

bergamot phytochemical (peak ID in **Figure 1**)	100 g of peel	sequential fractions of 100 g of peel				
		70 E1	70 E2	100 E1	100 E2	AIR
		A. Flavanones				
eriocitrin (eriodictyol 7-*O*-rutinoside) (C3)	31.2	15.1 (48.4%)	4.0 (12.8%)	0	0	11.5 (36.8%)
neoeriocitrin (eriodictyol 7-*O*-neohesperoside) (C4)	953.9	594.4 (62.3%)	119.8 (12.6%)	11.8 (1.2%)	3.2 (0.3%)	211.8 (22.2%)
narirutin (naringenin 7-*O*-rutinoside) (C8)	66.8	53.3 (79.8%)	8.3 (12.4%)	0	0	5.1 (7.6%))
naringin (naringenin 7-*O*-neohesperoside) (C9)	1104.6	779.8 (70.6%)	153.1 (13.9%)	19 (1.7%)	4.6 (0.4%)	153.1 (13.9%)
eriodictyol mono-rhamnoside (most likely 7-*O*-substituted) (**C11**)	137.9	63.7 (46.2%)	12.9 (9.4%)	1.2 (0.9%)	0.3 (0.2%)	35.4 (25.7%)
neohesperidin (**hesperetin 7-*O*-neohepseroside**) (**C13**)	919.6	396.1 (43.1%)	73 (7.9%)	8 (0.9%)	1.9 (0.2%)	298.8 (32.5%)
naringenin mono-rhamnoside (most likely 7-*O*-substituted) (**C14**)	260.5	142.1 (54.5%)	24.4 (9.4%)	3.7 (1.4%)	0.5 (0.2%)	60.3 (23.1%)
hesperetin mono-rhamnoside (most likely 7-*O*-substituted) (**C15**)	455.3	268.6 (59.0%)	47.5 (10.4%)	6.6 (1.4%)	1.1 (0.2%)	95.1 (20.9%)
		B. Flavones				
apigenin 6,8-di-*C*-glucoside (C1)	50.4	25.5 (50.6%)	4.3 (8.5%)	0.4 (0.8%)	0.1 (0.2%)	15.5 (30.8%)
diosmetin 6,8-di-*C*-glucoside (C2)	32.2	16.5 (51.2%)	2.6 (8.1%)	0.3 (0.9%)	0	10.1 (31.4%)
luteolin mono-glucoside/mono-rhamnoside (**C5**)	63.1	39.8 (63.1%)	7.1 (11.3%)	0.6 (1.0%)	1 (1.6%)	13.9 (22.0%)
diosmetin mono-glucoside isomer 1 (**C6**)	14.6	8.6 (58.8%)	1.4 (9.6%)	0.3 (2.1%)	0	2.2 (15.1%)
diosmetin mono-rhamnoside (**C7**)	25.4	15.3 (60.3%)	2.5 (9.8%)	0.6 (2.4%)	0	3.2 (12.6%)
apigenin mono-glucoside/mono-rhamnoside (**C10**)	112.2	64.3 (57.3%)	11.3 (10.1%)	1.3 (1.2%)	0.3 (0.3%)	27.3 (24.3%)
diosmetin mono-glucoside isomer 2 (**C12**)	41.2	28.3 (68.7%)	4.9 (11.9%)	0.7 (1.7%)	0	6.7 (16.3%)
		C. Psoralens				
bergapten (C16)	36.7	20.5 (55.8%)	3.3 (9.0%)	0.5 (1.4%)	0.1 (0.3%)	0
bergamottin (C17)	22.9	19.0 (83.0%)	3.2 (14.0%)	0.6 (2.6%)	0.3 (1.3%)	0

[a] Values are expressed as mg/100 g in peel and percentage in fractions relative to peel (100 g of peel extracted yielding 970 mL of 70 E1, 1000 mL of 70 E2, 980 mL of 100 E1, and 970 mL of 100 E2). All known compounds were measured directly using standard curves. Unidentified flavones: apigenin derivatives expressed as apigenin 7-*O*-glucoside equivalents; luteolin derivatives expressed as luteolin 7-*O*-glucoside equivalents; and diosmetin glycosides expressed as diosmetin 7-*O*-rutinoside equivalents. Mean data presented; SDs for triplicate extractions and fractionations were <1% for every sample. Flavonoids in bold have not previously been reported in bergamot.

Table 2. Comparison of Monosaccharide Content of Ethanolic Fractions[a]

monosaccharide	70 E1		70 E2		100 E1		100 E2	
	total	flavonoid-derived (%)	total	flavonoid-derived (%)	total	flavonoid-derived (%)	total	flavonoid-derived (%)
rhamnose	600.7	72.2 (12.01%)	137.5	8.8 (6.41%)	19.3	1.31 (6.77%)	7.2	0.31 (4.28%)
fucose	3.9	0.00	7.2	0.00	2.1	0.00	1.6	0.00
arabinose	720.3	0.00	214.5	0.00	17.9	0.00	15.9	0.00
xylose	46.8	0.00	16.3	0.00	5.3	0.00	6.0	0.00
mannose	483.0	0.00	97.3	0.00	12.7	0.00	6.8	0.00
galactose	77.5	0.00	32.5	0.00	12.3	0.00	13.5	0.00
glucose	5421.3	61.8 (1.14%)	1285.2	11.6 (0.90%)	180.4	1.28 (0.71%)	78.1	0.32 (0.41%)
GalA	316.6	0.00	100.6	0.00	29.2	0.00	45.5	0.00

[a] Values represent the percentage of sugars derived from flavonoids and are expressed as μg/mL EtOH extract.

Table 3. Monosaccharide Composition of the Bergamot Peel and Extracts[a]

material	yield (%)	rhamnose	fucose	arabinose	xylose	mannose	galactose	glucose	galacturonic acid
peel	100.0	24.6 (±0.02)	5.1 (±0.09)	64.5 (±1.41)	15.7 (±0.70)	24.5 (±0.19)	47.7 (±0.15)	244.7 (±2.20)	256.2 (±5.60)
70 E1	29.8	36.4 (±1.42)	2.2 (±0.01)	43.7 (±1.91)	2.8 (±0.01)	24.3 (±1.32)	4.7 (±0.48)	328.6 (±6.30)	19.2 (±1.89)
70 E2	9.7	33.1 (±0.78)	2.7 (±0.02)	51.6 (±0.26)	3.9 (±0.07)	23.4 (±1.96)	7.8 (±0.39)	308.9 (±2.63)	24.2 (±0.96)
100 E1	2.98	29.5 (±1.24)	3.3 (±0.05)	51.3 (±1.85)	8.1 (±0.08)	22.2 (±0.28)	18.8 (±0.25)	275.4 (±4.20)	44.5 (±2.29)
100 E2	1.36	23.8 (±1.79)	3.2 (±0.09)	52.3 (±1.88)	11.4 (±0.05)	23.3 (±0.22)	44.4 (±0.99)	256.8 (±8.02)	149.7 (±6.47)
AIR	45.2	7.8 (±1.13)	5.1 (±0.46)	66.2 (±2.31)	29.7 (±1.90)	24.6 (±1.45)	48.1 (±0.88)	217.1 (±6.88)	216.6 (±5.39)
CWE	[6.9]	6.5 (±0.93)	2.3 (±0.13)	62.2 (±2.99)	7.1 (±2.30)	11.1 (±0.92)	33.2 (±1.21)	34.8 (±1.02)	54.4 (±2.22)
HWE	[14.8]	6.6 (±0.95)	5.2 (±0.12)	103.4 (±4.08)	15.6 (±1.12)	28.9 (±0.96)	72.9 (±1.09)	55.4 (±1.09)	186.3 (±1.92)
residue	[68.9]	6.5 (±0.71)	7.0 (±0.73)	49.1 (±0.58)	36.8 (±0.44)	23.5 (±0.03)	42.9 (±1.90)	252.6 (±1.97)	106.7 (±2.09)

[a] Composition is expressed as μg/mg material. The yield is calculated as the dry weight recovered from the extraction volume as a percentage of the initial volume. The percentage yield from AIR is shown in [brackets].

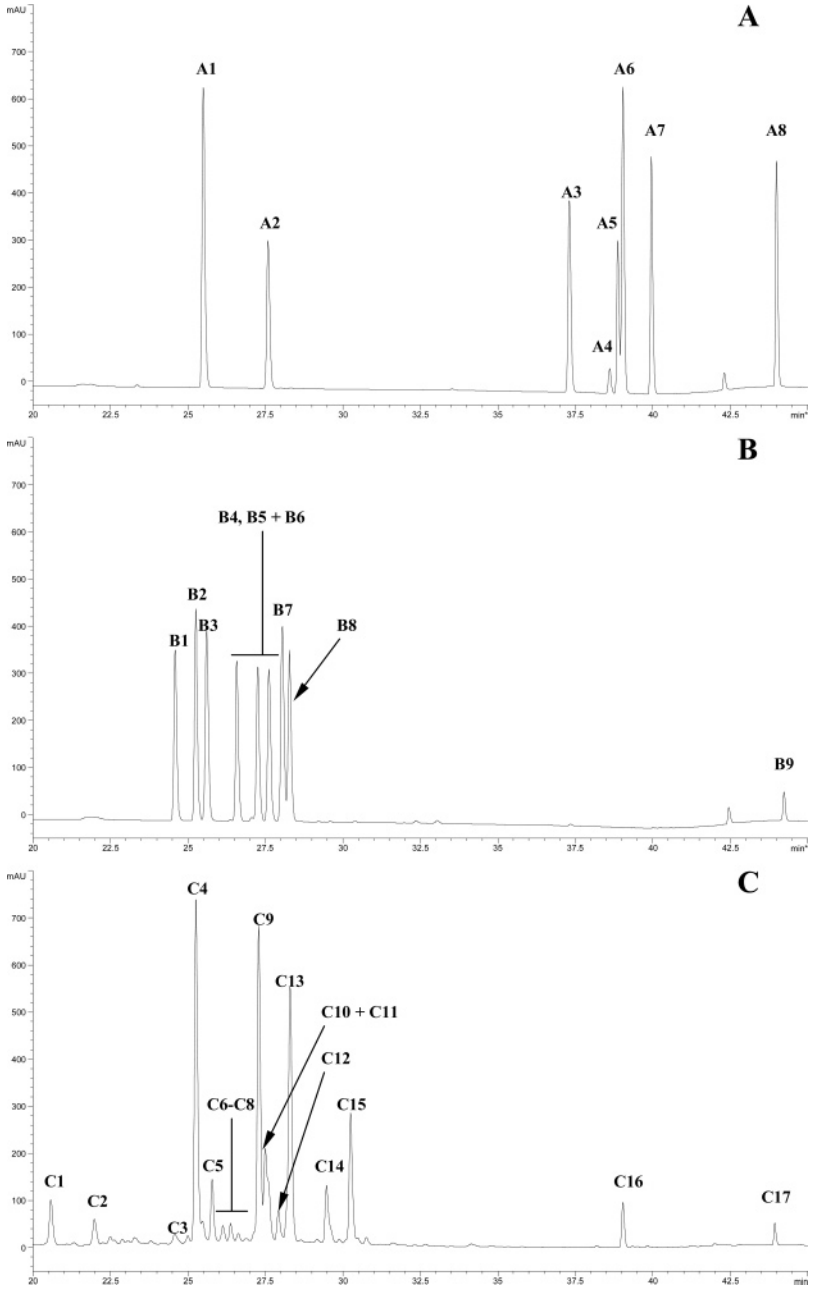

Figure 2. Example HPLC data (270 nm) for bergamot peel 70% v/v MeOH extract (**A**) and mixes of standards (**B** and **C**). Peak ID chromatogram **A**: A1, luteolin 7-O-Glc; A2, apigenin 7-O-Glc; A3, 7-hydroxyflavone; A4, citropten, A5, sinensetin; A6, bergapten; A7, scutellarein tetramethyl ether; and A8, bergamottin. Peak ID chromatogram **B**: B1, eriocitrin; B2, neoeriocitrin; B3, eriodictyol 7-O-Glc; B4, narirutin; B5, naringin; B6, hesperidin; B7, naringenin 7-O-Glc; B8, neohesperidin; and B9, 5-geranoxy-7-methoxycoumarin. Peak ID chromatogram **C**: C1, apigenin 6,8-di-*C*-glucoside; C2, diosmetin 6,8-di-*C*-glucoside; C3, ericocitrin (B1); C4, neoeriocitrin (B2); C5, luteolin-mono-glucoside/mono-rhamnoside (isomer 1); C6, diosmetin mono-glucoside (isomer 1); C7, diosmetin mono-rhamnoside; C8, naringenin-mono-glucoside/mono-rhamnoside; C9, naringin (B5); C10, apigenin-mono-glucoside/mono-rhamnoside; C11, eriodictyol-mono-rhamnoside; C12, diosmetin mono-glucoside (isomer 2); C13, neohesperidin (B9); C14, narigenin mono-rhamnoside; C15, hesperetin mono-rhamnoside; C16, bergapten; and C17, bergamottin (A8) with a minor amount of 5-geranoxy-7-methoxycoumarin (B8).

(*33*). The lipid content of the peel was 1.61%, with only 0.17% in the AIR. The fatty acid profile of the peel (starting material) was shown to be as follows (expressed as percent total fatty acids): myristic (0.18), palmitic (17.88), palmitoleic (0.64), heptadodecanoic (0.21), heptadodecenoic (0.24), stearic (3.51), oleic (25.08), linoleic (37.15), linolenic (12.70), arachidonic (2.27), eicosenoic (0.09), behenic (0.02), and lignoceric (0.02). The very low lipid content of the AIR meant that is was not possible to measure the individual fatty acids. It is believed that most of the peel lipids originate from the seeds. The acetate contents were 0.41 and 0.19% in the bergamot peel and in the AIR, respectively.

Enzymatic Solubilization of Bergamot Peel Components. When incubated with 10 U polygalacturonase equivalent activity/g cell wall material, high levels of biomass were solubilized by using most of the enzymes utilized in this study. The best enzyme preparation, in terms of biomass solubilization, was Depol 690L (81.94%), while the polygalacturonase (PGase) from Megazyme solubilized only 28.34% of the starting material. The percentage of solubilization was 66.99 and 50.67 by using Pectinase 62L and Pectinase 444L, respectively. The high percentages of solubilization obtained by using the commercial preparations could be explained by the fact they contain a combination of different activities. Depol 690L

Table 4. Monosaccharide Analyses of AIR Residues Posttreatment with Glycoside Hydrolase Preparations[a]

	AIR residue after treatment with			
monosaccharide	depol 690L	pectinase 62L	pectinase 444L	polygalacturonase
rhamnose	4.4 (±0.20)	2.8 (±0.26)	4.1 (±0.38)	8.0 (±0.60)
fucose	5.4 (±0.20)	7.4 (±0.28)	8.7 (±0.02)	8.6 (±0.54)
arabinose	19.1 (±0.98)	19.2 (±0.83)	25.5 (±0.01)	71.8 (±3.40)
xylose	48.7 (±2.44)	63.5 (±0.52)	61.1 (±1.79)	51.6 (±2.61)
mannose	24.0 (±1.17)	37.0 (±1.29)	38.1 (±0.74)	36.6 (±0.57)
galactose	28.5 (±0.29)	34.6 (±1.36)	39.8 (±0.82)	54.8 (±2.22)
glucose	250.5 (±9.48)	421.8 (±8.22)	334.1 (±5.02)	346.6 (±5.05)
galacturonic acid	77.8 (±2.43)	86.2 (±1.45)	124.5 (±1.45)	160.8 (±0.78)

[a] Values are expressed a μg/mg AIR.

contains a high PGase to pectin lyase (PL) ratio ideal for the peeling of *Citrus* fruit. Pectinase 62L and Pectinase 444L have both PGase and PL activity, as well as arabinanase activity, which will hydrolyze arabinan side groups and rhamnogalacturonan chains. PGase alone is not sufficient to significantly reduce the peel biomass.

The enzyme-resistant residues after 24 h incubations were analyzed to determine the composition of the polysaccharides remaining (**Table 4**). The main sugar present in the residue was glucose, showing that the enzyme preparations lack cellulolytic activities, with the preferential solubilization of pectic polysaccharides. In particular, the residues after digestion with Depol 690L and Pectinase 62L showed the lowest amounts of galacturonic acid remaining, corresponding to the highest percentages of biomass solubilization. The amount of arabinose decreased in the residues after treatment with Depol 690L and Pectinases 62L and 444L. This could be due to arabinanase and α-L-arabinofuranosidase activities contained in these preparations. A minor decrease of galactose was observed in the residues after enzyme treatment, suggesting galactanase activity on galactose side chains. No significant differences were shown in the amounts of all of the other sugars.

The present study demonstrated that the peel and initial EtOH extracts of bergamot fruit contain a significant amount of flavanone glycosides and lower amounts of flavone di-*C*-glucosides and flavone mono- and di-*O*-glucosides. There is clearly scope to process this peel to generate not only useful animal feed and bioreactor substrates but also a potential source of *Citrus* flavonoids (found in lower levels in other *Citrus* peels) for phytopharmaceutical applications and as natural additives for functional foods. A further advantage of using the peel waste is the very low psoralen content as compared with fresh/pre-oil removal material. Psoralens are known to exert both positive and negative effects on health; one major negative effect is their ability to act as photosensitizers and initiate procarcinogenic events. Both the carbohydrate-enriched and the flavonoid-enriched fractions are quickly and easily generated from the original peel waste using processes and solvents that have a negligible impact on the environment as compared with the current methods of bergamot peel waste disposal, i.e., landfill or burning.

ACKNOWLEDGMENT

We gratefully acknowledge the help provided by John Eagles (IFR) for help with the GC-MS analyses. We thank Biocatalysts Limited for providing pectinolytic enzyme samples.

Supporting Information Available: LC-MS and UV−visible spectral data for all of the phenolic phytochemicals found in the peel. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Bocco, A.; Cuvelier, M. E.; Richrad, H.; Berset, C. Antioxidant activity and phenolic composition of citrus peel and seed extracts. *J. Agric. Food Chem.* **1998**, *46*, 2123−2129.

(2) El-Nawawi, S. A.; Heikal, Y. A. Production of a low ester pectin by deesterification of high ester citrus pectin. *Carbohydr. Polym.* **1995**, *27*, 191−195.

(3) Graham, R. P.; Shepherd, A. D. Pectin production-pilot plant production of low-methoxyl pectin from citrus peel. *J. Agric. Food Chem.* **1953**, *1* (16), 993−1001.

(4) Leuzzi, U.; Caristi, C.; Panzera, V.; Licandro, G. Flavonoids in pigmented orange juice and second-pressure extracts. *J. Agric. Food Chem.* **2000**, *48*, 5501−5506.

(5) Manthey, J. A.; Grohmann, K. Concentration of hesperidin and other orange peel flavonoids in citrus processing byproducts. *J. Agric. Food Chem.* **1996**, *44*, 811−814.

(6) Willats, W. G. T.; Steele-King, C. G.; McCartney, L.; Orfila, C.; Marcus, S. E.; Knox, J. P. Making and using antibody probes to study plant cell walls. *Plant Physiol. Biochem.* **2000**, *38*, 27−36.

(7) May, C. D. Industrial pectins: Sources, production and applications. *Carbohydr. Polym.* **1990**, *12*, 79−99.

(8) Maurer, R. H.; Burdick, E. M.; Waibel, C. W. Distribution of naringin in Texas grapefruit. *Proc. Rio Grande Valley Hortic. Inst.* **1950**, 147−181.

(9) Kanes, K.; Tisserat, B.; Berhow, M.; Vandercook, C. Phenolic composition of various tissues of Rutaceae species. *Phytochemistry* **1993**, *32*, 967−974.

(10) Subra, P.; Vega, A. Retention of some components in supercritical fluid chromatography to bergamot peel oil fractionation. *J. Chromatogr. A* **1997**, *771*, 241−250.

(11) Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. Effect of citrus flavonoids on HL-60 cell differentiation. *Anticancer Res.* **1999**, *19*, 1261−1269.

(12) Huet, R. Constituants des agrumes à effet pharmacodynamique. Les citroflavonoïdes (Constituents of citrus fruits with pharmacodynamic effect: Citroflavonoids). *Fruits* **1982**, *37*, 267−271.

(13) Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Ortuno, A.; Del Rio, J. A. Uses and properties of *Citrus* flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4505−4515.

(14) Sohn, H. Y.; Son, K. H.; Know, C. S.; Kang, S. S. Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: Morus alba L., Morus mongolica Schneider, Broussnetia papyrifera (L.) Vent, Sophora flavescens Ait and Echinosophora koreensis Nakai. *Phytomedicine* **2004**, *11*, 666−672.

(15) Verzera, A.; Trozzi, A.; Gazea, F.; Cicciarello, G.; Cotroneo, A. Effects of rootstock on the composition of bergamot (*Citrus bergamia* Risso et Poiteau) essential oil. *J. Agric. Food Chem.* **2003**, *51*, 206−210.

(16) Savary, B. A.; Hotchkiss, A. T.; Cameron, R. G. Characterization of a salt-independent pectin methylesterase purified from valencia orange peel. *J. Agric. Food Chem.* **2002**, *50*, 3553−3558.
(17) Manthey, J. A. Fractionation of orange peel phenols in ultrafiltered molasses and mass balance studies of their antioxidant levels. *J. Agric. Food Chem.* **2004**, *52*, 7586−7592.
(18) Anagnostopoulou, M. A.; Kefalas, P.; Kokkalou, E.; Assimopoulou, A. N.; Papageorgiou, V. P. Analysis of antioxidant compounds in sweet orange peel by HPLC-diode array detection-electrospray ionization mass spectrometry. *Biomed. Chromatogr.* **2005**, *19*, 138−148.
(19) Waldron, K. W.; Selvendran, R. R. Composition of the cell walls of different asparagus (*Asparagus* officinalis) tissues. *Physiol. Planta* **1990**, *80*, 568−575.
(20) Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res*. **1983**, *113*, 291−299.
(21) Blumenkrants, N.; Asboe-Hansen, G. New method for qualitative determination of uronic acids. *Anal. Biochem*. **1973**, *54*, 484−489.
(22) AOAC. In *Official Methods of Analysis of AOAC International*, 17th ed.; AOAC: Arlington, VA, 2000; Chapter 4.
(23) Mondello, L.; Zappia, G.; Bonaccorsi, I.; Dugo, G.; Dugo, G.; McNair, H. M. Fast GC for the analysis of natural matrixes. Preliminary note: The determination of fatty acid methyl esters in natural fats. *J. Microcolumn Sep.* **2000**, *12*, 41−47.
(24) Bailey, M. J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol*. **1992**, *23*, 257−270.
(25) Moufida, S.; Marzouk, B. Biochemical characterisation of blood orange, sweet orange, lemon, bergamot and bitter orange. *Phytochemistry* **2003**, *62*, 1283−1289.
(26) Mondello, L.; Casilli, A.; Tranchida, P. Q.; Cicero, L.; Dugo, P.; Dugo, G. Comparison of fast and conventional GC analysis for Citrus essential oils. *J. Agric. Food Chem.* **2003**, *51*, 5602−5606.
(27) Caristi, C.; Bellocco, E.; Gargiulli, C.; Toscano, G.; Leuzzi, U. Flavone-di-C-glycosides in *citrus* juices from Southern Italy. *Food Chem.* **2006**, *95*, 431−437.
(28) Dugo, P.; Mondello, L.; Dugo, L.; Stancanelli, R.; Dugo, G. LC-MS for the identification of oxygen heterocyclic compounds in citrus essential oils. *J. Pharm. Biomed. Anal*. **2000**, *24*, 147−154.
(29) Bonnin, E.; Dolo, E.; Le Goff, A.; Thibault, J.-F. Characterization of pectin subunits released by an optimised combination of enzymes. *Carbohydr. Res.* **2002**, *337*, 1687−1696.
(30) Hellin, E.; Ralet, M.-C.; Bonnin, E.; Thibault, J.-F. Homogalacturonans from lime pectins exhibit homogeneous charge density and molar mass distribution. *Carbohydr. Polym.* **2005**, *60*, 307−317.
(31) Miron, J.; Yosef, E.; Ben-Ghedalia, D. Composition and in vitro digestibility of monosaccharide constituents of selected byproduct feeds. *J. Agric. Food Chem.* **2001**, *49*, 2322−2326.
(32) Chau, C.-F.; Huang, Y.-L. Comparison of the chemical composition and physicochemical properties of different fibers prepared from the peel of *Citrus sinensis* L. Cv. Liucheng. *J. Agric. Food Chem.* **2003**, *51*, 2615−2618.
(33) Grohmann, K.; Manthey, J. A.; Cameron, R. G.; Buslig, B. S. Purification of citrus peel juice and molasses. *J. Agric. Food Chem.* **1999**, *47*, 4859−4867.

Received for review July 28, 2005. Revised manuscript received November 2, 2005. Accepted November 6, 2005. This research was funded by the Biotechnology and Biological Research Sciences Council (BBSRC, United Kingdom) and by Ministero dell'Universita e della Ricerca (MIUR), Italy (project no. 12930).

JF051847N