Antioxidant Activity and Phenolic Composition of Citrus Peel and Seed Extracts

Alessandra Bocco, Marie-Elisabeth Cuvelier,* Hubert Richard, and Claudette Berset

Laboratoire de Chimie des Substances Naturelles, Département Science de l'Aliment, Ecole Nationale Supérieure des Industries Agricoles et Alimentaires, 1 Avenue des Olympiades, 91744 Massy Cedex, France

A possible way to valorize citrus peels and seeds, which are byproducts of the juice extraction industry, is to use them as natural antioxidants. The antioxidant activity of several citrus peel and seed extracts obtained either by methanol extraction (free phenolic compounds) or by alkaline hydrolysis (bound phenolic compounds) was tested in a model system based on accelerated citronellal oxidation. Generally, seeds possessed greater antioxidant activity than peels. The composition of all tested samples was studied by HPLC: methanol extracts are rich in flavones and glycosylated flavanones, whereas hydrolyzed extracts contain mainly phenolic acids and flavonols. The phenolic composition of some citrus peels and seeds was described for the first time. No clear relationship could be shown between the antioxidant activity and the phenolic composition of the extracts.

Keywords: *Citrus fruits; byproducts; antioxidant activity; flavanones; phenolic acids*

INTRODUCTION

The world production of citrus fruits is near 80 million tonnes per year. The average percentage of fruits transformed into juices is 34%, but in the major producing countries (Brazil and the United States), this percentage reaches 96% (Anonymous, 1996). Since the juice yield of oranges and grapefruits is about half of the fruit weight (Bovill, 1996), very large amounts of byproducts are formed every year.

The peel and seed residue is the primary waste fraction. Peels are a source of molasses, pectin, cold-pressed oils, and limonene and can be used as cattle feed, mixed with dried pulps. Seeds are rich in unsaturated fatty acids, but the oil is not extracted commercially; however, seeds can be used to recover limonoids, which are typical citrus fruit triterpenoids, having an extremely bitter taste and, probably, anticarcinogenic/chemopreventive activities (Braddock, 1995).

Both peels and seeds are an interesting source of phenolic compounds, which include phenolic acids and flavonoids. Flavonoids are represented in citrus fruits by two very peculiar classes of compounds: the polymethoxylated flavones and the glycosylated flavanones. They are found only in citrus fruits, and their pattern is specific of each species, which makes them very good markers of adulteration in commercial juices (Marini and Balestrieri, 1995; Mouly et al., 1994; Ooghe and Detavernier, 1997). The citrus flavonoids have been found to have health-related properties, which include anticancer, antiviral, and antiinflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation (Huet, 1982; Benavente-Garcia et al., 1997). Some glycosylated flavanones can be easily transformed into the corresponding dihydrochalcones, which are potent natural sweeteners (Bör et al., 1990; Horowitz and Gentili, 1969).

Despite all of the possible uses listed above, citrus peels and seeds remain, for the major part, unutilized. Another way to valorize these byproducts could be their use as natural antioxidants in food, since the phenolic compounds they contain have shown antioxidant properties (Kroyer, 1986; Larson, 1988; Pratt and Hudson, 1990).

Several studies have already been realized on the antioxidant activity in food systems of several citrus fruits (sweet orange, lemon, grapefruit), used both directly (Piskur and Higgins, 1949; Williams and Harris, 1983) and as extracts (Kroyer, 1986; Pereira and Mancini-Filho, 1994; Sawamura et al., 1988; Ting and Newhall, 1965). The efficiency of many species (bergamot, lime, pummelo, mandarin), nevertheless, has not been investigated yet. Limited data are available on the phenolic composition of the peel and, especially, of the seed of citrus fruits.

The present investigation was undertaken to evaluate the antioxidant power of citrus peel and seed extracts and to identify and quantify their principal free and bound phenolic constituents.

EXPERIMENTAL PROCEDURES

Plant Material. Eight samples of seeds and three samples of peels were analyzed. The peels and seeds of lemon (*Citrus limon* Femminello Comune), bergamot (*C. bergamia* Fantastico), and sour orange (*C. aurantium,* unknown cultivar) and one sample of sweet orange (*C. sinensis* Biondo Comune) seed were kindly furnished by the Stazione Sperimentale per le Industrie delle Essenze e dei Derivati Agrumari (Reggio Calabria, Italy). The seeds of sweet orange (*C. sinensis* Valencia Late) (second sample), mandarin (*C. reticulata* Imperial Reticulate), pummelo (*C. grandis* Tahiti Pomelo), and lime (*C. limetta* West Indian) were obtained from Outspan International (Port Elizabeth, South Africa).

Peels and seeds were dried under a warm (40 °C) air stream until their water content was between 7 and 10%.

Preparation of Samples. *Extraction of the Free Phenolic Compounds.* Four grams of seed or peel was finely ground in an analysis blender IKA A10. The meal was extracted twice

* Author to whom correspondence should be addressed (telephone 0033-169935003; fax 0033-169935020; e-mail cuvelier@ensia.inra.fr).

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by 40 mL of methanol, under reflux, for 30 min periods. The methanol extract was filtered through Whatman No. 1 filter paper and washed three times with 40 mL of petroleum ether. It was then evaporated to dryness under vacuum at 40 °C. The residue was dissolved in 4 mL of dimethylformamide (DMF) and filtered on a 0.45 μm filter (Gelman GHP) for the determination of the antioxidant power and the identification and quantification of the free phenolic compounds.

The cake was used for the extraction of the bound phenolic compounds.

Extraction of the Bound Phenolic Compounds. The cake obtained from 4 g of peel or seed was hydrolyzed with 200 mL of 2 M NaOH, for 4 h, at room temperature and under nitrogen (Ribéreau-Gayon, 1968). The water phase was separated by filtration under vacuum and acidified with 6 N HCl at pH 1 and then extracted three times with 200 mL of ethyl acetate. The organic phase was evaporated to dryness under vacuum at 40 °C, and the residue was dissolved in 4 mL of DMF, filtered on a 0.45 μm filter (Gelman GHP), and used for the determination of the antioxidant power and the identification and quantification of the bound phenolic compounds.

Two extraction replicates were performed for each sample.

Antioxidant Activity Determination. The antioxidant activity was measured according to the method of Bocco et al. (1998), based on the accelerated oxidation of citronellal in chlorobenzene, under strong oxidizing conditions (80 °C, intensive oxygenation).

A small amount of DMF up to a ratio of 0.01 versus chlorobenzene was used to dissolve the dry residues of citrus peel and seed.

The disappearance of citronellal from the reaction medium (the initial citronellal concentration was 17 g/L) was monitored by gas chromatography. The analyses were performed on an HP 5890 gas chromatograph (Hewlett-Packard, Evry, France), equipped with an HP-5 capillary column (50 m $\times$ 0.32 mm i.d.) and a flame ionization detector. The oven temperature was programmed from 120 to 160 °C at 4 °C min^{-1} and then at 15 °C min^{-1} to a final value of 220 °C. The injector and the detector were maintained at 250 °C, and injection was in split mode (1/10).

The antioxidant activity was assessed by the percentage increase in the half-life time of citronellal, by comparison with a control test (without antioxidant). There is a linear relationship between the concentration and the activity of the antioxidants in the system. Therefore, we determined for each extract the concentration required to double the half-life time of the control: the lower it is, the stronger is the antioxidant. So, for reasons of clarity, we chose to speak in terms of antioxidant power (AOP) defined as the reciprocal of this concentration, which is proportional to the activity. AOP is then expressed in liters per gram of dry matter of peel or seed. The standard deviations were calculated from four values for each replicate sample.

Chromatography. *HPLC/MS Analysis.* Analyses were performed with a Trio 1000 quadrupole mass spectrometer (Fisons Instruments, Courtabœuf, France), using an atmospheric pressure chemical ionization (APCI) interface. The separation was carried out on an HPLC apparatus using a 600-MS pump (Waters, St. Quentin-en-Yvelines, France) and equipped with a 20 μL Rheodyne injector and a 250 $\times$ 4.6 mm i.d. Hypersyl ODS column (5 μm; Life Sciences International, Cergy-Pontoise, France).

The solvent systems used for the free phenolic compounds and for the bound phenolic compounds are reported in Table 1.

An adequate calibration of APCI parameters (needle potential, 4000 V; nebulizer heating, 500 °C; cone voltages, SKM 10 V, SMP 40 V) was realized to optimize sensitivity.

Quantification of the Phenolic Compounds. Before admission into the APCI interface, the eluates went through a 486 UV detector (Waters). The glycosylated flavanones were quantified at 284 nm using calibration curves of neoeriocitrin, eriocitrin, narirutin, naringin, neohesperidin, and hesperidin (Figure 1), obtained between 0.1 and 1.0 mg/mL DMF. The phenolic acids were quantified at 320 nm, using calibration

Flavanones
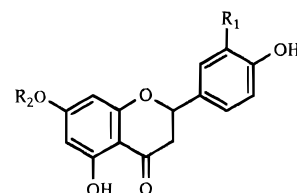
	R_1	R_2
eriodictyol	OH	H
eriocitrin	OH	rutinose
neoeriocitrin	OH	neohesperidose
hesperetin	OCH$_3$	H
hesperidin	OCH$_3$	rutinose
neohesperidin	OCH$_3$	neohesperidose
naringenin	H	H
narirutin	H	rutinose
naringin	H	neohesperidose

Phenolic acids
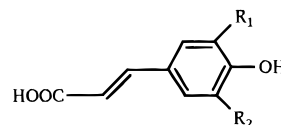
	R_1	R_2
caffeic acid	OH	H
ferulic acid	OCH$_3$	H
p-coumaric acid	H	H
sinapinic acid	OCH$_3$	OCH$_3$

Figure 1. Structures of the flavanones and the phenolic acids found in citrus extracts.

Table 1. HPLC Gradients To Separate (a) the Free Phenolic Compounds (Flavanones and Flavones) and (b) the Bound Phenolic Compounds (Phenolic Acids)

(a) free phenolic compounds			(b) bound phenolic compounds		
time (min)	water (%)	acetonitrile (%)	time (min)	1% acetic acid (%)	methanol (%)
0	83	17	0	77	23
2	83	17	40	77	23
20	80	20	55	0	100
30	55	45	65	0	100
40	0	100			
50	0	100			

curves of caffeic acid, ferulic acid, *p*-coumaric acid, and sinapinic acid (or sinapic acid, for 3,5-dimethoxy-4-hydroxy cinnamic acid) (Figure 1), obtained between 0.02 and 0.08 mg/mL DMF.

All of the standard compounds were purchased from Extrasynthèse (Genay, France).

Two HPLC analyses were realized for each replicate extract; the quantification data were therefore the average of four results.

UV Spectrophotometry. To collect spectral data of each separated component, citrus extracts were analyzed by HPLC with a 1040A photodiode array detector (Hewlett-Packard) under the chromatographic conditions described above.

RESULTS AND DISCUSSION

AOP of the Citrus Extracts Containing the Free Phenolic Compounds. The AOP of the seed extracts (Figure 2) varies in a notable way according to the species: the activity ratio of mandarin/sour orange is ≈5.

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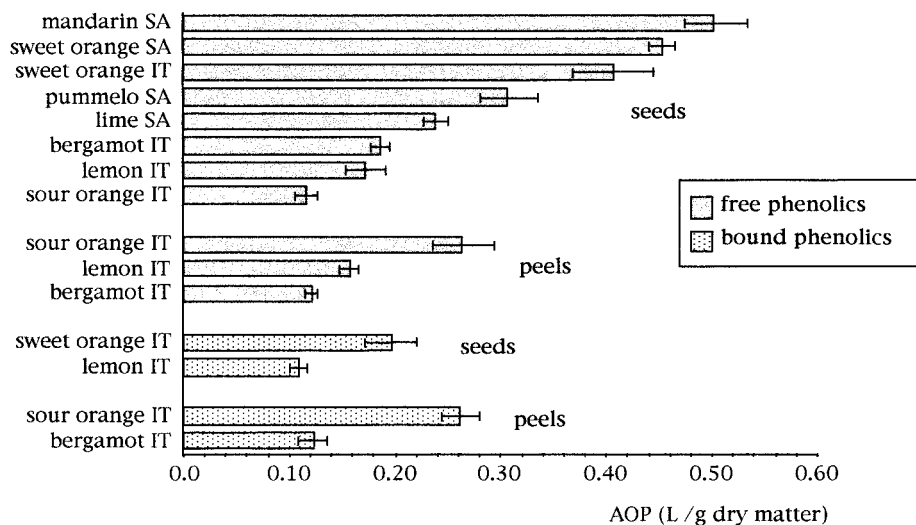
Figure 2. AOP of the seed and peel extracts containing the free and bound phenolic compounds (AOP = 1/concentration doubling the half-life of citronellal in accelerated oxidation conditions). SA, South Africa; IT, Italy.

The South African (SA) seed samples are more efficient than the Italian (IT) ones. Asking the South African producer for information about seed treatment, we knew that a fungicide (8-quinolinol sulfate) was used. The AOP of this compound measured with our test was very high (58.9×10^3 L mol^{-1}), but we never found it in the extracts. If the fungicide was present, it was in too small of a quantity to affect the efficiency of the extracts. The observed differences seem rather to be due to the species because the two samples of sweet orange seed, one treated (SA) and the other untreated (IT), show very similar activities.

Concerning the peel of the three studied species, the order of activity is exactly the opposite of that found for the seed.

In the literature, various methods have been used to study the AOP of citrus seed or peel extracts. As both the methods and the varieties are quite different, our results sometimes agree with the literature data (Tanizawa et al., 1992) and sometimes disagree (Ting and Newhall, 1965; Kroyer, 1986; Pereira and Mancini-Filho, 1994), so it is not worthwhile to make any comparison.

AOP of the Citrus Extracts Containing the Bound Phenolic Compounds. The AOP of the citrus extracts obtained by alkaline hydrolysis of the cakes and thus containing the bound phenolic compounds was studied for four Italian samples: lemon and sweet orange seeds and sour orange and bergamot peels (Figure 2).

Sweet orange seed is more active than lemon seed, and sour orange peel is more efficient than bergamot peel. This ranking is similar to that observed for the extracts containing the free phenolic compounds (Figure 2). The AOP due to the bound phenolic compounds is of the same order as that due to the free phenolics for the peels, while it is approximately half for the seeds.

Analysis of the Free Phenolic Compounds. The methanol seed and peel extracts show the same kind of HPLC profile, presented in Figure 3.

Two main classes of phenolic compounds are represented: flavanones and flavones. Flavanones are the most abundant compounds. In citrus they are usually present as diglycosides (Macheix et al., 1990). We identified six main molecules of this class in the citrus extracts. The mass spectra (MS) are exactly
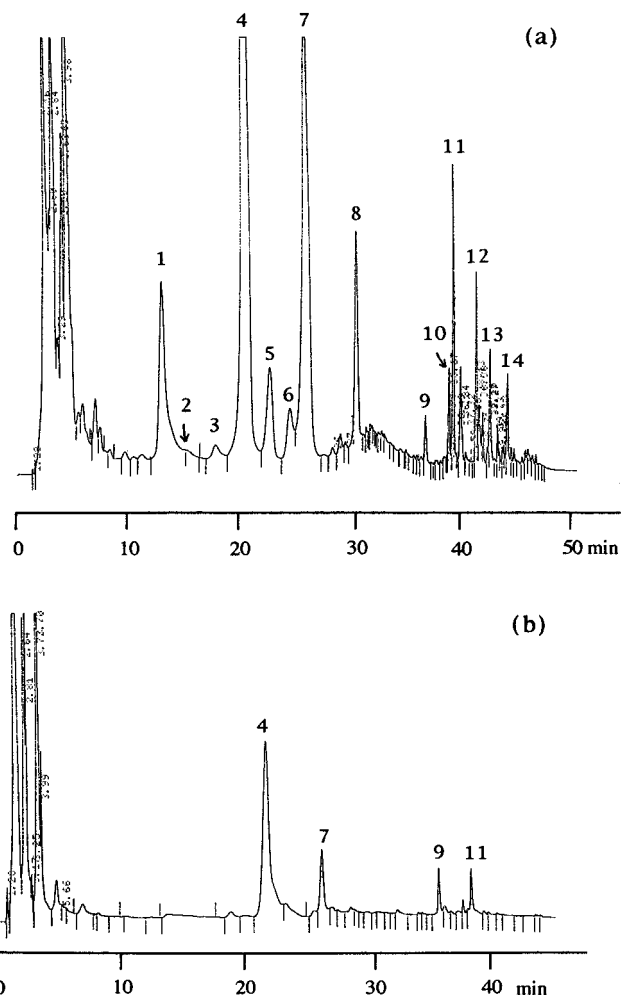

Figure 3. HPLC profiles at 284 nm of the methanol extracts of sour orange peel (a) and seed (b). Peaks: 1, neoeriocitrin; 2, glycosylated luteolin; 3, narirutin; 4, naringin; 5, glycosylated apigenin; 6, hesperidin; 7, neohesperidin; 8, glycosylated diosmin; 9–14, flavones.

identical for pairs of them. Their retention times, UV maxima, and mass spectra are shown in Table 2. Each flavanone was identified by its retention time and MS. The technique we chose (APCI) is characterized by a limited fragmentation of the molecular ion. Three main

Table 2. Spectral Characteristics of the Glycosylated Flavanones Identified in the Methanol Citrus Extracts (Spectra Collected during the HPLC Elution)

compound	retention time (min)	max abs (nm)	mass spectrum	
			MH⁺	fragments
eriocitrin	10.8	284, 326 (sh)[a]	597	451, 289
neoeriocitrin	11.8	282, 324 (sh)	597	451, 289
narirutin	17.5	282, 328 (sh)	581	435, 273
naringin	20.3	286, 328 (sh)	581	435, 273
hesperidin	22.7	284, 326 (sh)	611	465, 303
neohesperidin	25.5	284, 324 (sh)	611	465, 303

[a] sh, shoulder.
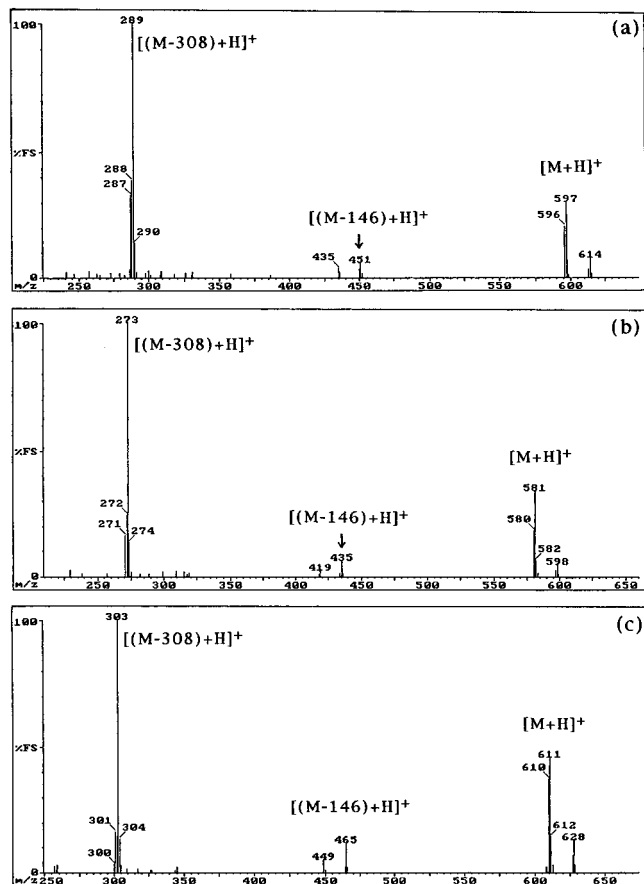
Figure 4. APCI-MS spectra of glycosylated eriodictyol (a), naringenin (b), and hesperetin (c).

fragments are visible for every flavanone (Figure 4): the first corresponds to the protonated molecular ion $[M + H]^+$, that is the diglucoside, the second to the monoglu-coside $[(M - 146) + H]^+$, and the third to the aglycon $[(M - 308) + H]^+$. These results are in perfect agreement with those of Robards et al. (1997). The three pairs of compounds could then be identified as the glycosylated forms of eriodictyol, naringenin, and hesperetin. Two forms of glycosides exist, in fact, the rutinosides and the neohesperidosides (Macheix et al., 1990): they have the same mass and the same spectrum and could be distinguished only by their retention times.

The flavones present in the extracts can be divided into two groups: those that are eluted together with the glycosylated flavanones and those eluted later (between 35 and 45 min). The first group is formed by glycosylated flavones (luteolin, apigenin, and diosmin glucosides), according to their retention times and their MS. The second group consists of polymethoxylated flavones, which are much less polar and then eluted further. The identification of the polymethoxylated flavones was not realized because, due to their chemical structure, we consider that they must have a very low antioxidant activity.

The quantification of the glycosylated flavanones was carried out using standard curves (Table 3). Peels are much richer than seeds (Barthe et al., 1988; Yusof et al., 1990). The composition of seeds and peels is not always the same for a determined species. In lemon, for instance, the seed contains principally eriocitrin and hesperidin, whereas the peel is rich in neoeriocitrin, naringin, and neohesperidin. The ratios of the concentrations of the glycosylated flavanones are also different: neoeriocitrin and naringin have similar concentrations in the peel, whereas in the seed eriocitrin is 40 times more abundant than naringin.
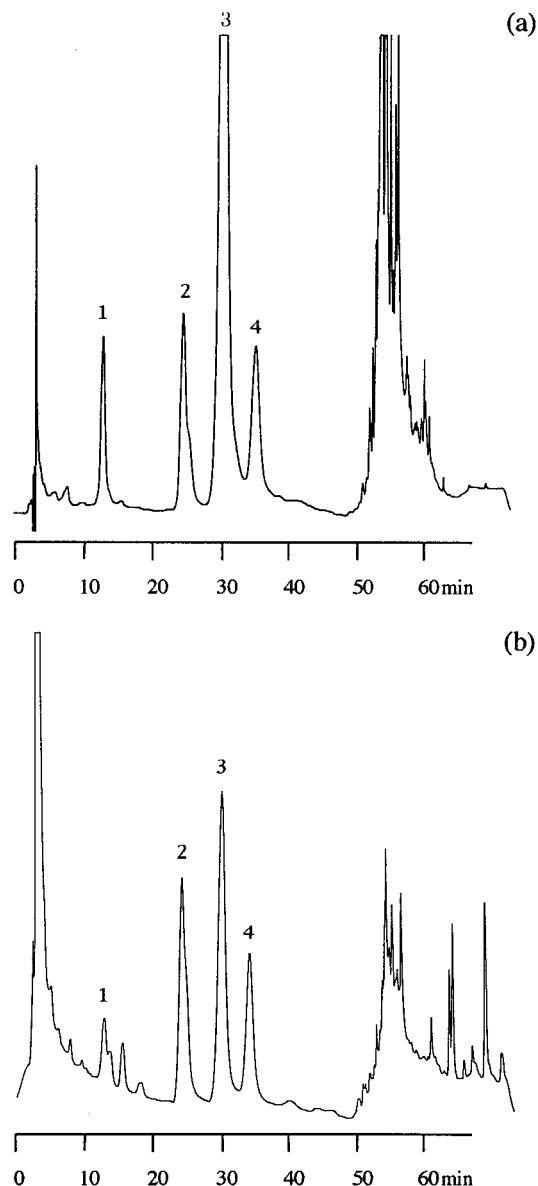
The yield of neoeriocitrin, naringin, and neohesperidin in peels is very high. Sour orange, in particular, is a very interesting source of naringin and neohesperidin, which can be used for the production of sweeteners. No data are available in the literature to make comparisons with our results, except those of naringin in bergamot peel: Calvarano et al. in 1996 measured 2.33–2.94 mg/g, whereas we found 4.55 mg/g. Since the plant material used was the same in both studies, our method of extraction seems more efficient for naringin than the 60 min boiling water extraction used by Calvarano et al.

The most interesting sources of glycosylated flavanones among the seeds are bergamot, rich in naringin and neohesperidin; lemon, rich in eriocitrin and hesperidin; and sour orange, rich in naringin. All of the other species contain very small quantities of glycosylated

Table 3. Glycosylated Flavanone Content (Milligrams per Gram of Dry Matter) of the Methanol Citrus Extracts[a]

extract	ERI	NER	NAT	NAR	HES	NEH	total
seeds							
mandarin SA		0.07 ± 0.001	0.04 ± 0.01	0.02 ± 0.005	0.13 ± 0.02		0.26
sweet orange IT			0.13 ± 0.02		0.28 ± 0.04		0.41
sweet orange SA			0.07 ± 0.01	0.01 ± 0.000	0.22 ± 0.02		0.30
pummelo SA			0.12 ± 0.01	0.29 ± 0.04	tr	0.04 ± 0.000	0.45
lime SA			tr	0.02 ± 0.000	0.02 ± 0.002		0.04
bergamot IT	0.23 ± 0.01	0.51 ± 0.02		1.43 ± 0.05		1.11 ± 0.09	3.28
lemon IT	1.61 ± 0.19			0.04 ± 0.006	0.50 ± 0.03		2.15
sour orange IT				0.77 ± 0.11		0.25 ± 0.01	1.02
peels							
sour orange IT		3.80 ± 0.27	0.25 ± 0.05	10.97 ± 0.38	0.66 ± 0.11	6.62 ± 0.54	22.30
lemon IT	6.12 ± 0.07			6.06 ± 0.14		4.37 ± 0.22	16.55
bergamote IT	tr	4.98 ± 0.46		4.55 ± 0.33		3.92 ± 0.37	13.45

[a] ERI, eriocitrin; HES, hesperidin; NAR, naringin; NAT, narirutin; NEH, neohesperidin; NER, neoeriocitrin IT, Italy; SA, South Africa; tr, traces.

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Figure 5. HPLC profiles at 320 nm of the hydrolyzed cake of sour orange peel (a) and seed (b). Peaks: 1, caffeic acid; 2, *p*-coumaric acid; 3, ferulic acid; 4, sinapinic acid.

Table 4. Spectral Characteristics of the Phenolic Acids Identified in the Hydrolyzed Citrus Extracts

compound	retention time (min)	λ_{max} (nm)
caffeic acid	13.0	295.5, 323.0
p-coumaric acid	24.5	298.5 (sh),[a] 308.5
ferulic acid	30.5	298.5 (sh), 322.5
sinapinic acid	35.0	310.5

[a] sh, shoulder.

flavanones. The yields we measured are slightly smaller than the yields reported by other authors: hesperidin in sweet orange varies between 0.22 and 0.28 mg/g, whereas Barthe et al. (1988) found 0.35 mg/g; the

naringin content in lime is for us 0.19 mg/g, whereas Yusof et al. (1990) had 0.29 mg/g. These differences can be due to the variety and the origin of the fruits used.

The glycosylated flavanone composition of peels and seeds is different from that of juices. In lemon peel and seed and in mandarin seed, we found naringin, which is not normally reported in the juices of these fruits (Mouly et al., 1996; Ooghe and Detavernier, 1997). On the other hand, we did not find compounds generally present in juices (Mouly et al., 1995, 1996): eriocitrin in lime, pummelo in sweet orange, and neoeriocitrin in lime.

In the SA sample of sweet orange seed we found traces (0.01 mg/g) of naringin. In sweet orange juices, this glycosylated flavanone is never present and is used as a marker of adulteration (Mouly et al., 1994; Rouseff et al., 1987). Since we could not detect this compound in the Italian sweet orange seed, we think that naringin can really be present in a very small concentration in seed, depending on the sample variety and its geographical origins. The study of a larger range of sweet orange seeds would be necessary to prove this hypothesis.

Analysis of the Bound Phenolic Compounds. The HPLC profile at 320 nm of the four peel and seed hydrolyzed extracts that we obtained shows several peaks (Figure 5). Compounds **1**−**4** were identified as phenolic acids. Their spectral characteristics (UV maxima and MS) and their retention times correspond to those of, respectively, caffeic, *p*-coumaric, ferulic, and sinapinic acids (Table 4). In the sweet orange seed extract two other phenolic acids are present, between caffeic and *p*-coumaric acid, that we could not identify. In lemon seed and bergamot peel, the *p*-coumaric acid peak coeluted with another peak that has its maximun of absorbance at 296 nm. The retention time and the UV spectrum correspond to those of *cis-p*-coumaric acid identified in barley extracts (Maillard, 1996).

The part of the chromatogram between 50 and 60 min shows a peak clump. Most of these peaks present UV spectra very similar to those of flavonols, with two maxima between 250 and 380 nm. To our knowledge, no data on the existence of flavonols bound to plant cell walls are available in the literature. To date, their presence has not been reported perhaps because HPLC procedures generally stop before their elution (Maillard and Berset, 1995; Peleg et al., 1991). To identify them, it would be necessary to improve the chromatographic conditions.

We quantified only the four main phenolic acids (Table 5). Sour orange peel is the richest sample, especially in ferulic and sinapinic acids. The other three extracts contain only about $^1/_{20}$ of the phenolic acids found in sour orange. Unfortunately, in all samples, caffeic acid is the least abundant compound, whereas it has the highest AOP value (Bocco et al., 1998).

The only results available from other authors are those by Peleg et al. (1991) concerning the peels of sweet orange and grapefruit, which contain the same four phenolic acids as our samples. The order of concentra-

Table 5. Phenolic Acid Content (Milligrams per Gram of Dry Matter) of the Hydrolyzed Citrus Extracts

extract	caffeic acid	*p*-coumaric acid (*cis* and *trans*)	ferulic acid	sinapinic acid	total
lemon seed	0.019 ± 0.002	0.072 ± 0.006	0.045 ± 0.007	0.047 ± 0.009	0.183
sweet orange seed	0.011 ± 0.002	0.018 ± 0.002	0.046 ± 0.007	0.069 ± 0.008	0.144
sour orange peel	0.229 ± 0.021	0.193 ± 0.011	1.580 ± 0.132	0.954 ± 0.027	2.956
bergamot peel	0.006 ± 0.000	0.071 ± 0.006	0.036 ± 0.008	0.030 ± 0.007	0.143

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tion observed is ferulic > sinapinic > *p*-coumaric > caffeic acids. Our sour orange peel extract contains ~10 times more ferulic and sinapinic acids and ~5 times more caffeic and *p*-coumaric acids than the sweet orange peel extract from Peleg et al. (1991).

AOP and Phenolic Composition. Comparison of Figure 2 with Table 3 does not show any clear relationship between the AOP and the glycosylated flavanone concentration of an extract, but it is known that flavanones do not belong to the best antioxidant family.

Seeds are, on the average, more antioxidant than peels, but their flavanone content is lower. Moreover, the extracts that contain eriocitrin and neoeriocitrin, which are the most efficient glycosylated flavanones (Bocco et al., 1998), are not more active than those in which these two compounds are not present.

The calculation of the expected AOP of the extracts, related to the activity of each flavanone (Bocco et al., 1998) and to its concentration in the extract, shows that flavanones can explain only from 1 to 20% of the activity of the seed extracts and from 36 to 83% of the activity of the peels. It is then clear that, especially in the seeds, even if synergistic effects exist between the glycosylated flavanones, the antioxidant activity is mainly due to other compounds, such as tocopherols, ascorbic acid, limonoids, and other nonidentified substances.

Concerning the bound phenolic compounds, the results are similar to those of the free phenolics (Figure 2 and Table 5). The AOP is not always proportional to the phenolic acid yields, and these compounds can explain only from 2 to 22% of the activity in the extracts studied. In this case, too, other substances (such as the flavonols) are surely responsible for the majority of the activity of the extracts.

Conclusion. Citrus peels and seeds have an interesting antioxidant activity with regard to citronellal. Perhaps their extracts could well be useful to prevent oxidation in fruit juices and essential oils. The methanolic extracts of mandarin and sweet orange seeds have the best antioxidant properties, while bergamot peels are an interesting source of free phenolic compounds.

However, the lack of correlation observed between the antioxidant activity and the identified phenolic compounds of the extracts, especially in the case of seeds, shows that other substances must be responsible for the major part of the efficiency of the extracts. Only sour orange peel shows a high efficiency, considering both the methanolic and the hydrolyzed extracts, in relation with a high content of glycosylated flavanones and phenolic acids.

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