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Free and Bound Cinnamic Acid Derivatives in Corsica Sweet Blond Oranges

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Total determination of cinnamic acids (CA), including hydroxycinnamic acid derivatives is generally not accurate since, during hydrolysis, a possible degradation of dihydroxy CA such as caffeic acid could occur. Evaluations of CA (ferulic, *p*-coumaric, sinapic, cinnamic and caffeic acids) before and after hydrolysis have been undertaken using standards and either with or without addition of ascorbic acid and EDTA. The method was then applied to the determination of free and bound CA in five blond cultivars (Navelina, Washington navel, Pera, Salustiana and Valencia late) of sweet oranges [*Citrus sinensis* (L.) Osb.]. Four parts of the fruits (peel juice, flavedo, albedo and juice) have been investigated. Results show that CA are mainly bound (86% up to 92%) in the four fruit parts. The mean of total CA contents was found to be higher in peel juice (1.5 g kg^{-1}) in comparison with flavedo (0.7 g kg^{-1}), albedo (0.1 g kg^{-1}) and juice (0.6 g kg^{-1}). Free and bound ferulic acid represented 55-70% of CA in juices, followed by *p*-coumaric acid (20%), sinapic acid (10%) and caffeic acid (9%). Total contents of each CA in the four fruit parts are discussed and show the potential interest in orange peel wastes.

Keywords: HPLC, *Citrus sinensis*, orange fruit cultivars, hydroxycinnamic acids, caffeic acid, ferulic acid, sinapic acid, *p*-coumaric acid, polyphenols.

Citrus bioactive compounds, and in particular polyphenolics, have health-related actions, which are based on their antioxidant properties [1]. These properties include anticancer, antiviral, and anti-inflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation [1,2]. Although cinnamic acids (CA), such as *p*-coumaric, caffeic, ferulic and sinapic acids, have been determined in orange juices, little work has been reported on their concentrations in various parts of the orange fruit. Continuing our studies on Citrus phenolics [3], the purpose of this work was to analyze, using a rapid liquid chromatographic method, the different CA in various fruit parts (juice, albedo, flavedo and peel juice) of sweet orange cultivars that could potentially be used as added-value products by the fruit juice industry. Since CA could be found in both free and bound forms, we have determined them before and after hydrolysis for their complete determination. As it was shown that the experimental conditions commonly used to detect bound phenolic acids by alkaline hydrolysis resulted in loss of dihydroxy-CA, such as caffeic acid [4], we have compared our results using various methods commonly reported in the literature and with addition of ascorbic acid, a strong antioxidant and ethylene diamine tetracetic acid (EDTA).

The list of the five cultivars collected on the Experimental Domain of Corsica Island (EDC) is given in Table 1. These cultivars were chosen from among the seven hundred species and varieties of the EDC according to their industrial food importance and use. The Brix corrected from acidity varied from 9.5 to 11.9, showing a sugar ratio *r*, which could be correlated to maturity, of 12.7 for Washington navel and 10.9 for Salustiana cultivars.

Table 1: Blond orange cultivars investigated: Acidity, brix and ratio of juices.

Cultivar name	SRA[a]	Brix (° brix)	Acidity [b] (g/kg)	Brix corrected from acidity (° brix)
Navelina	312	10.8	16.0	11.1
Washington navel	203	10.1	8.0	10.2
Pera	399	9.6	15.2	9.9
Salustiana	484	11.7	11.0	11.9
Valencia late	17	8.9	30.0	9.5

[a] Agronomical Research Station number. [b] Expressed as anhydrous citric acid (ACA).

The five CA standards were easily separated by liquid chromatography, in our experimental conditions. As alkaline hydrolyses commonly used for bound phenolic acid detection generally lead to a loss of dihydroxy derivatives, such as caffeic acid [4], we have compared various alkaline hydrolysis procedures with a mixture of standards (Table 2) and with a Pera orange juice (Table 3). As can be seen from Table 2, the loss in CA was 100% for caffeic acid. The loss was 22% for sinapic acid (4-hydroxy-3, 5-dimethoxycinnamic acid) and less than 2% for the others. When ascorbic acid plus EDTA were added, the amount of recovered caffeic acid was 28 mM L^{-1}, showing a loss of only 4.8% for caffeic acid. A similar result was observed for sinapic acid, since 3.5% was lost. In the case of an orange juice (Table 3), the effect of EDTA and ascorbic acid on CA recovery after alkaline hydrolysis was compared. No significant change was observed either with or without EDTA. The addition of ascorbic acid to prevent sinapic acid degradation was not demonstrated and is low for caffeic acid. This is due to the natural occurrence of ascorbic acid in orange juice (433 mg L^{-1} in this experiment).

The loss of CA without EDTA and ascorbic acid addition during alkaline hydrolysis depends on the natural ascorbic acid present in the orange juice. As in the case of albedo, flavedo and peel juice, in which the ascorbic acid content ranged from 8 to 954 mg.kg[1], the addition of this compound was necessary to obtain accurate CA determination in albedo and flavedo, as shown in Table 3. Chromatographic profiles at 300 nm of the various fruit parts, before and after hydrolysis, are given in Figure 1. As can be seen, cinnamic acid, which was previously identified in a blood orange juice variety [5], was not detected in this blond orange cultivar. Before quantitative determinations, repeatability of the method was checked on Pera cultivar, as shown in Table 4.

The free CA contents in the various parts of sweet blond orange fruits are low (Table 5), particularly in the juice (4-9 mg kg^{-1}) compared with peel juice (98-215 mg kg^{-1}). If the albedo content of CA is about 2-2.5 times higher than in the juice, we can note an increasing content of these free CA in flavedo, but the contents remain under those of peel juice. Free sinapic, *p*-coumaric and ferulic acids are the main CA in peel

Table 2: Comparison of cinnamic acid standard recoveries after alkaline hydrolysis

Phenolic acid	Before hydrolysis[a]	Recovery after hydrolysis.[a]			
		NaOH[b]	% loss	NaOH +EDTA[c]	% loss
Sinapic	25.7	20.0	22.2	24.8	3.5
Caffeic	29.4	0.00	100	28.0	4.8
Ferulic	30.3	29.8	1.6	29.9	1.3
p-Coumaric	32.0	31.8	0.6	32.0	0.0
Cinnamic	35.8	35.1	1.9	34.4	3.9

[a] mM L^{-1}. [b] 2 mol. L^{-1}. [c] NaOH 2 mol. L^{-1} + ascorbic acid, 10 g L^{-1} + EDTA, 0.01 mol. L^{-1}

Table 3: Hydroxy cinnamic acid recoveries from Pera orange fruits after alkaline hydrolysis.

Fruit part	Ascorbic acid [a]		CA [b]			
	Present in fruit	Added	Sinapic	Caffeic	Ferulic	*p*-Coumaric
Peel juice	954	0	96	46	916	485
		1000	96	46	918	487
		10000	99	47	920	490
Flavedo	10 [b]	0	74	22	750	338
		1000	81	35	754	340
		10000	83	39	760	345
Albedo	8 [b]	0	5	0.6	11	6
		1000	8	1.4	23	7
		10000	9	1.3	23	7
Juice	433	0	5	5	35	7
		1000	5	5	34	7
		10000	5	5	35	7

[a] mg L^{-1}. [b] mg kg^{-1} of fruit part

Table 4: Repeatability, in orange fruit parts of Pera variety, after hydroxycinnamic acid hydrolysis**.**

Acid [a]	Sinapic				Caffeic				Ferulic				*p*-Coumaric			
Fruit part [b]	P.J.	Flav.	Alb.	Juice	P.J.	Flav.	Alb.	Juice	P.J.	Flav.	Alb.	Juice	P.J.	Flav.	Alb.	Juice
min.[c]	150	19.0	15.0	5.2	4.9	4.8	1.5	1.3	1245	980	38.5	39.7	415	358	22.5	11.2
max.[c]	158	20.7	15.9	5.7	5.3	5.1	1.7	1.6	1270	998	39.5	42.0	427	366	23.6	11.7
mean[c]	153	19.6	15.4	5.5	5.1	5.0	1.6	1.5	1261	991	39.0	41.6	423	362	23.3	11.5
c.v.	8.2	0.4	0.10	0.03	0.02	0.01	0.01	0.01	94.8	49.4	0.1	0.8	21.9	9.8	0.2	0.03
s.d.	2.9	0.6	0.3	0.2	0.1	0.1	0.08	0.1	9.74	7.03	0.4	0.9	4.7	3.1	0.4	0.2

[a] mg kg^{-1} of fruit part; sinapic acid, retention time (min) in HPLC, 17.07; caffeic acid, 21.27; ferulic acid, 24.70; *p*-coumaric acid, 29.60.
[b] P.J., Peel juice; Flav., Flavedo; Alb., Albedo**.** [c] mean of 6 determinations at 300 nm

Table 5: Free hydroxycinnamic acid contents in various orange fruit parts (mg kg^{-1}).

SRA number [a]	Fruit part [b]	Sinapic acid	Caffeic acid	Ferulic acid	*p*-Coumaric acid	TOTAL FCA[c]
312	P.J.[d]	2.3 [e]	9.2	46.5	46.0	104
	Flavedo	9.2	0.0	15.7	34.5	59.4
	Albedo	5.0	1.4	10.2	3.5	20.1
	Juice	2.3	0.9	1.1	0.2	4.6
203	P.J.	17.7	11.5	40.3	51.8	121
	Flavedo	16.9	3.5	28.8	34.5	83.7
	Albedo	4.6	1.2	14.8	5.9	26.5
	Juice	3.0	1.2	3.9	1.1	9.2
399	P.J.	2.9	1.5	46.0	133.4	184
	Flavedo	12.1	1.5	25.0	9.5	48.1
	Albedo	3.5	0.0	10.6	6.4	20.5
	Juice	0.0	0.0	3.3	0.7	4.0
484	P.J.	2.3	2.6	47.2	46.0	98.2
	Flavedo	4.9	1.7	46.0	40.3	92.9
	Albedo	1.0	0.0	9.2	2.3	12.5
	Juice	1.1	1.5	4.4	1.7	8.7
17	P.J.	4.6	5.9	43.7	160.3	215
	Flavedo	6.9	0.5	46.0	135	188
	Albedo	0.3	0.2	7.9	1.3	9.7
	Juice	0.2	0.5	6.5	1.8	9.1

[a] Agronomical Research number, see Table 1 for orange cultivar name. [b] 1 to 2 kg of fruits (6 up to 8 fruits) investigated.
[c] free cinnamic acids. [d] peel juice. [e] mg kg^{-1} of fruit part

Table 6: Total hydroxycinnamic acid (bound and free) contents in various orange fruit parts (mg kg^{-1}) investigated

SRA number [a]	Fruit part [b]	Sinapic acid	Caffeic acid	Ferulic acid	*p*-Coumaric acid	TOTAL BFCA[c]
312	P.J.	90.1 [e]	12.4	675	288	1066
	Flavedo	13.4	4.6	52.5	67.9	138
	Albedo	7.7	2.8	15.0	4.6	30.1
	Juice	5.3	4.0	39.0	11.2	59.5
203	P.J.	118	34.7	890	491	1534
	Flavedo	74.6	14.2	606	311	1006
	Albedo	16.8	1.6	49.5	27.6	95.5
	Juice	5.4	4.0	32.4	9.9	51.7
399	P.J.	158	5.3	1269	427	1859
	Flavedo	20.7	5.0	998	366	1390
	Albedo	15.9	1.7	40.0	23.5	81.1
	Juice	5.7	1.5	42.0	11.7	60.9
484	P.J.	124	17.7	848	380	1370
	Flavedo	34.0	9.2	138	194	375
	Albedo	49.5	4.0	94.0	20.7	168
	Juice	6.1	2.6	37.6	11.2	57.5
17	P.J.	108	17.3	1264	682	2072
	Flavedo	80.5	8.3	966	501	1556
	Albedo	16.0	1.4	55.8	14.2	87.2
	Juice	3.5	2.0	65.3	6.9	77.7

[a] Agronomical Research number, see Table 1 for orange cultivar name. [b] 1 to 2 kg of fruits (6 up to 8 fruits) investigated.[c] Sum of the free and bound cinnamic acid derivatives. [d] peel juice. [e] mg kg^{-1} of fruit part. [f] no fruits collected. [g] no HPLC detection (< 0.01 %)

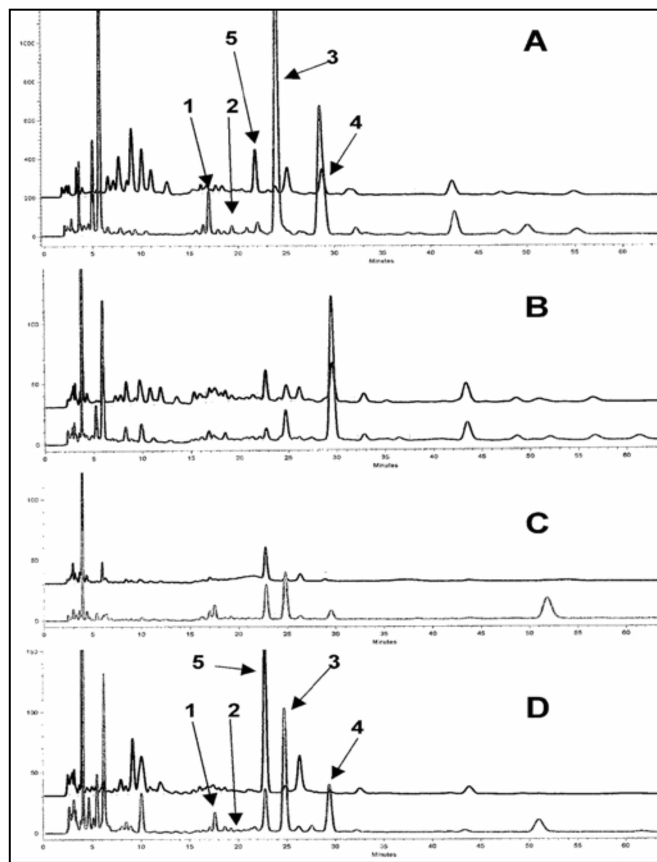

Figure 1: Chromatographic profiles of peel juice (A), aqueous flavedo (B) and albedo (C) extracts and juice (D) of blond orange Pera cultivar. Upper trace, before hydrolysis, lower trace, after hydrolysis using NaOH 2N + ascorbic acid. For chromatographic conditions, see Experimental. Peak identifications: 1, sinapic acid; 2, caffeic acid; 3, ferulic acid; 4, *p*-coumaric acid; 5, hesperidin.

juice and flavedo. Ferulic acid is, in most cases, the main CA, followed by *p*-coumaric acid. If caffeic acid is relatively abundant in peel juice of Navelina and Washington navel cultivars, their content decreased dramatically in other cultivars and parts of the fruits. Sinapic acid is found in a significative amount in these two cultivars, in particular in albedo and juice.

The sum of free and bound CA contents determined after alkaline hydrolysis is given in Table 6. Unsubstituted cinnamic acid was not detected in those cultivars investigated. The content ranges from 1066 to 2072 $mg.kg^{-1}$ for peel juice, 138 to 1556 $mg.kg^{-1}$ for flavedo, 30 to 168 mg kg^{-1} for albedo and 52 up 78 $mg.kg^{-1}$ for juice. If we consider each CA, we can observe that the sum of free and bound ferulic acid was the main acid, whatever the fruit part, representing 50-80% of the overall CA, followed by *p*-coumaric acid (15-30%). In only one case, for the Salustiana cultivar (SRA 484), the content of *p*-coumaric acid was slightly higher compared with those of ferulic acid (194 *versus* 138 mg kg^{-1}). The caffeic acid content is the lowest of the investigated acids with 0.5-5%, in agreement with hydrolysed citrus extracts [6].

The bound CA represents 86-92% of the overall CA. The free and bound CA contents decreased from the external to the internal parts of the fruit, taking into account the four fruit parts of the cultivars investigated. Peel juice, flavedo and albedo, which are considered as wastes, show a great variability of phenolic compounds and contain from 20 to 30 times more CA than juice. These by-products should be better considered by industrial juice producers since they contain health-promoting phytochemicals.

Experimental

Cultivars investigated and fruit maturity control: One to 2 kg of fruits from each cultivar (corresponding to from 6 to 8 fruits) were collected from trees growing in the citrus germplasm of the Experimental Domain of Corsica (EDC), San Giuliano, Agronomic Research Station (SRA, Corsica Island, France). All trees were healthy, over 10 years old and grafted onto 2 different rootstocks depending on their genetic compatibility. Orange trees were grafted on either *Poncirus trifoliata* (L.) Raf. Pomeroy or on citranges Carrizo, which is a hybrid between *P. trifoliata* (L.) Raf. and *Citrus sinensis* (L.) Osb. Sweet blond orange varieties (Navelina, Washington navel, Pera, Salustiana and Valencia late) were obtained by vegetative propagation. The orange cultivars investigated are given in Table 1 with their SRA numbers, which identify the variety in the germplasm field and certify the good controlled sanitary status of the trees.

Fruit maturity was determined using the sugar ratio *r* value, which is the Brix corrected from acidity divided by the percentage of acidity expressed versus anhydrous citric acid. The sugar determination was achieved using the method of the Fédération Internationale des Producteurs de Jus de Fruits [7]. Each species and fruit variety was cut into two pieces. Then, each part was hand-squeezed, carefully with a juice squeezer, avoiding contact with albedo, without using strong pressure. The juice was, therefore, not completely extracted. The total soluble solid content as ° Brix was measured with a RFM-91 refractometer (Bellingham and Stanley Ltd, England) for raw juices. Then, the titratable acidity at pH 8.1 was determined [8]. The raw juices were immediately frozen at -20°C.

Reagents and preparation of standards: All reagents used were of HPLC grade: methanol Hipersolv (VWR, France), THF, 2N HCl, 2N NaOH (Sigma Aldrich, France), and dry acetic acid (Carlo Erba, France). Cinnamic standards (cinnamic, sinapic, caffeic, ferulic

and *p*-coumaric acids) used for retention time determinations, spectral identifications and recovery after hydrolysis of bound derivatives, were purchased from Sigma-Aldrich. All standards were diluted in methanol (50 mg/50 mL methanol), then in water to give a final concentration of 5 mg/L, 50 mg/L and 500 mg/L, for HPLC calibration lines.

Processing techniques of citrus fruits and sample preparations: Each orange fruit was peeled, separating carefully the flavedo from the albedo; the two parts were recovered and weighted. The remainder of the orange was squeezed carefully with a squeezer (Seb, model 830802, France) in order to obtain the juice without reaching the albedo and then frozen at –20°C with the two other parts, until analysis. After defrosting, the juice was centrifuged at 4000*g* for 10 min and supernatant phase was recovered for analysis. The albedo was cut into small pieces, diluted (10 fold, w/w) in deionized water (0.01% HCl), mixed with a homogenizer (Ultra-Turrax T25, Janke & Kunkel, Germany) at 9500 *g* for 2 min, and then centrifuged to recover the supernatant juice for analysis. From the supernatant liquid, 3 mL was taken for free CA determination and 10 mL for free and bound CA determination after alkaline hydrolysis. The flavedo was separated into 2 parts using a juice extractor (Moulinex, model AY364E). The liquid phase was centrifuged for clarification, as described before. The peel juice was directly analyzed after 5 times dilution in eluent A (acetic acid, methanol, water; 15/20/65 v/v/v) for free CA determination. The remaining solid part was extracted, as described for albedo. For free CA determination, 3 mL of each supernatant was absorbed on a SPE cartridge (VWR, Bond Elut Jr, 6 mL/g), previously conditioned with methanol (1% HCl v/v) and water (0.01% HCl). The cartridge was dried under a stream of nitrogen and CA were removed with methanol (1% HCl). The solvent was evaporated under a stream of nitrogen at 40°C. The resulting dried matter was dissolved in 5 mL of eluent A and then injected into a 20 µL sample loop for HPLC analysis. To perform the alkaline hydrolysis of CA esters, 10 mL of peel juice, flavedo extract, albedo or juice were added to 10 mL of either 2N NaOH or 2N NaOH containing ascorbic acid (Sigma-Aldrich) at various concentrations and ethylene diamine tetraacetic acid (EDTA) di-sodium salt dehydrate (Calbiochem). The reaction was carried out at room temperature for 4 h, in the dark. The samples were then acidified with 10 mL 2N HCl and centrifuged. The supernatant solution (10 mL) was absorbed onto a SPE tube and the CA were extracted with methanol (1% HCl) and diluted with eluent A for HPLC analyses.

High performance liquid chromatography determinations: Analyses were performed with a Beckman System Gold instrument equipped with a Photo Diode Array Detector. The samples (20 µL) were injected into a RP18 VWRd Purospher Star column (250 x 4 mm i.d., 5 µm), using a column temperature controller (30°C). Analyses were acquired from 190 to 400 nm, with integration at 300 nm, using a gradient elution of solvent A (THF/water/acetic acid, 18/80/2 v/v/v) and water at a constant flow rate of 0.8 mL min^{-1}. The linear gradient was programmed from 70% of solvent A and 30 % of water for 10 min, then 100 % solvent A for 52 min, and finally a return to the initial conditions for 1 min. The eluent was routinely monitored by UV at 300 nm. All solutions were filtered through a 0.22 µm filter before use. The peak assignments were based on the retention times of standards. Retention times and λ_{max} of acids were [min, λ_{max} (nm): sinapic 17.07, 324; caffeic, 21.27, 326; ferulic, 24.70, 324; *p*-coumaric, 29.60, 311; cinnamic, 58.4, 277]. The concentrations of acids were estimated from experimental peak areas by analytical interpolation of standard calibration lines. Extraction and alkaline hydrolysis were carried out in duplicate for each cultivar. To evaluate the repeatability of the method, in the case of Pera cultivar, 6 experiments were conducted for the 4 fruit parts.

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