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Characterization and Quantitation of Antioxidant Constituents of Sweet Pepper (*Capsicum annuum* L.)

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Sweet peppers (*Capsicum annuum* L.) cv. Vergasa have been studied at four maturity stages (immature green, green, immature red, and red). The individual phenolics (hydroxycinnamic acids and flavonoids), vitamin C (ascorbic acid and dehydroascorbic acid), and individual carotenoids were characterized and quantified. Five hydroxycinnamic derivatives and 23 flavonoids were characterized and quantified from the pericarp of sweet pepper by high-performance liquid chromatography−diode array detection−electrospray ionization−mass spectrometry. Identification was carried out by their UV spectra, chromatographic comparisons with authentic markers, identification of hydrolysis products, and tandem mass spectrometry analysis. Hydroxycinnamic derivatives, *O*-glycosides of quercetin, luteolin, and chrysoeriol, and a large number of *C*-glycosyl flavones have been characterized. Some of these compounds were found for the first time in nature. Clear differences in the individual and total phenolic content were detected between the different maturity stages. Immature green pepper had a very high phenolic content while green, immature red, and red ripe peppers showed a 4−5-fold reduction. Ascorbic acid was the main form of vitamin C, and its content increased as the pepper reached maturity. The red ripe stage had a relevant impact on the carotenoids content. Thus, immature green peppers showed the highest content of polyphenols, while red ripe fruits had the highest content of vitamin C and provitamin A.

KEYWORDS: ***Capsicum annuum* L.; phenolics; hydroxycinnamates; flavones; ascorbic acid; dehydroascorbic acid; β-carotene; xanthophylls; HPLC-MS**

INTRODUCTION

In the past few years, there has been a renewed interest in studying and quantifying the antioxidant constituents of fruits and vegetables due to their health-promoting properties. These beneficial effects have been related to the presence of some vitamins (A, C, E, and folates), dietary fiber, and nonessential phytochemicals that are present in these plant food products. Among phytochemicals, polyphenols deserve a special mention due to their free radical scavenging properties and in vivo biological activities that are being investigated by many researchers. Numerous epidemiological studies have indicated a possible association between the dietary intake of polyphenols and the risk of coronary heart disease (*1*−*3*) and cancer (*4*). The consumption of fresh fruit and vegetables also provides important amounts of vitamin C, provitamin A, and other antioxidants (*5*−*8*). Ascorbic acid is a required human nutrient, and its biological functions are centered on its antioxidant properties in biological systems preventing common degenerative processes (*9*). Ascorbic acid is the principal biologically active form, but dehydroascorbic acid also exhibits biological activity since it can be easily converted into ascorbic acid in the human body. So, it is important to measure both ascorbic acid and dehydroascorbic acid. Vitamin C levels depend on several factors including cultivar, production practices, maturity at harvest, and storage conditions (*10*−*12*). The importance of the carotenoid compounds in the diet has been recognized, not only as precursors of vitamin A but also as antioxidants in cell protection and in the prevention of degenerative diseases (*13*).

In recent years, peppers have grown in popularity, and a wide number of varieties are now available in the grocery stores. Almost all peppers turn from green to yellow, orange, red, or purple when they are fully ripe. Green bell peppers are often harvested before they are ripe, and changes in the maturity may affect the content of phytonutrients, which play an important role in the diet antioxidant intake. Fresh pepper is one of the vegetables that has a higher content of vitamin C (*14*). The carotenoid pigments in fresh peppers have been widely studied to improve color retention during processing and storage (*15*−*17*). Peppers suffer a profound change during the course of ripening with the conversion of existing pigments. Thus, the green color of the fruit is principally due to the presence of chlorophyll and to the carotenoids typical of the chloroplast, such as oxygenated carotenoids or xanthophylls, violaxanthin,

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neoxanthin, and lutein, as well as β-carotene (*18*−*21*). Among the carotenoid pigments capsanthin, capsorubin and capsanthin 5,6-epoxide are almost exclusive to the genus *Capsicum* and are responsible for the final red color (*22*). Red peppers contain the highest amount of provitamin A (β-carotene and β-cryptoxanthin) (*15*). Regarding flavonoids, most of the studies on peppers have been concentrated only on flavonoid aglycones (quercetin and luteolin) obtained after hydrolysis (*23*, *24*). Materska et al. (*25*) have recently identified two new flavonoids in hot pepper pericarp, and other flavonoids were found for the first time in pepper fruits. Howard et al. (*24*) have studied the effects of pepper maturation on antioxidants content in different pepper types (*Capsicum annuum*, *Capsicum frutescens*, and *Capsicum chinense*). They found that the concentration of these antioxidant constituents increased as the peppers reach maturity (*24*).

There is a great interest in the nutritional value of foods to know what the contribution of an individual food product is to daily nutritional needs and how ripening and maturity affect nutritive composition. The aim of the present work was to determine the individual phenolic acids, flavonoids, vitamin C content (ascorbic acid and dehydroascorbic acid), and carotenoids of sweet peppers at different ripe and maturity stages. Immature green, green, immature red, and red peppers were evaluated to estimate the contribution that these antioxidants make to the nutritional value of its dietary intake depending on the maturity stage.

MATERIALS AND METHODS

Plant Material. Fruits of bell sweet pepper (*C. annuum* L.) cv. Vergasa were grown in an experimental field "Las Palmerillas" (El Ejido, Almeria, Spain) in a greenhouse under Mediterranean climate conditions. The peppers were harvested at the same time but at four successive maturity stages on the basis of their color as one ripening stage (named immature green) and three maturity stages (named green, immature red, and fully red ripe) (*26*). All peppers received similar water and fertilizer treatments. Immediately after harvest, the fruits were transported to the laboratory (75 km) where the peppers were carefully selected to ensure that fruits free of defects were chosen. The immature green fruit showed a mean weight of 135 ± 3 g, while for green, immature red, and red ripe fruits this mean was 188 ± 5 g. Three replicates of five peppers each were used for each maturity stage. The peppers were hand cut into small pieces and separated into different batches depending on the analysis (vitamin C, carotenoids, or polyphenols). The antioxidant constituents in fresh pepper were compared with those of the samples after freezing, after freezing and storage, or after freeze-drying to avoid underestimation of the constituents due to the influence of the sample preparation procedure. Thus, the vitamin C analysis was carried out with fresh samples, and for carotenoids, frozen samples stored at −80 °C for a period of less than 2 weeks were used. In the case of polyphenols, freeze-dried samples were employed. For phenolics identification, the pepper peel was separated and extracted separately to obtain a more concentrated extract.

Quality Indices. Titratable acidity (TA), pH, and soluble solids content (SSC) were evaluated as quality indices. The TA was determined by titrating 10 mL of juice with 0.1 mol/L^{-1} NaOH to pH 8.1 (*27*). The pH values were measured using a pH meter and SSC with an Atago N1 handheld refractometer (Tokyo, Japan).

Extraction of Phenolic Compounds. The lyophilized pepper (10 g) was homogenized with an Ultra Turrax (Ika, Staufen, Germany) with 10 mL of extraction solution (methanol/water 7:3 v/v contaning 4 mM NaF to inactivate polyphenol oxidases and prevent phenolic degradation due to browning) for 1 min on ice. The homogenates were centrifuged at 10 500*g* in a model Sigma 1−13 Eppendorf centrifuge (B. Braun Biotech International, Osterode, Germany) for 10 min at 2−5 °C. The supernatant was recovered, filtered through a 0.45 μm Osmonics/MSI cameo Nylon filters (Fisher Scientific, Los Angeles, CA), and directly analyzed by high-performance liquid chromatography (HPLC). The results were expressed as mg per 100 g fresh weight.

Analysis of Phenolic Compounds by HPLC-Diode Array Detection (DAD) and HPLC-DAD-Tandem Mass Spectrometry (MS-MS). Samples of 50 μL of extracts were analyzed using an HPLC system (Merck Hitachi, Tokyo, Japan) equipped with a model L-7100 pump and a model L-7455 photodiode array UV/vis detector. The samples were injected by a model L7200 autosampler. The separations were achieved on a 250 mm × 4 mm i.d., 5 μm reversed phased LiChrocart C_{18} column (Merck, Darmstadt, Germany) with water/formic acid (95:5 v/v) (A) and methanol (B) as the mobile phases. The linear gradient started with 3% B in A to reach 25% B in A at 6 min, 35% B at 25 min, and 90% B at 35 min. The flow rate was 1 mL/min, and chromatograms were recorded at 340 nm. Hydroxycinnamic acid derivatives were quantified as chlorogenic acid (5-caffeoylquinic acid) (Sigma, St. Louis, MO), and flavonoids (*O*-glycosyl flavones and *C*-glycosyl flavones) were quantified as quercetin 3-rutinoside. The phenolic compound recovery was 88%, and the repeatability was ±4%.

The flavonoid identification in the pepper peel was carried out by means of their UV spectra, molecular weights, and their MS-MS fragments. Whenever possible, the compounds were identified by chromatographic comparisons with markers previously isolated and identified in our research group. The measurement conditions were the same as those used in the analytical HPLC-DAD explained above but using water with 0.1% formic acid (v/v) as the mobile phase.

The HPLC system equipped with a DAD detector and mass detector in series consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser, a G1315B photodiode array detector, and an ion trap mass spectrometer equipped with electrospray ionization (ESI) and operated in the negative ion mode controlled by software (v. 4.0.25) from Agilent Technologies (Waldbronn, Germany). The heated capillary and the voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS-MS) spectra were measured from *m*/*z* 100 to ∼1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative mode for all of the phenolic compounds.

Extraction and Analysis of Vitamin C. Ascorbic acid and dehydroascorbic acid contents were determined as described by Zapata and Dufour (*28*) with some modifications (*29*). Ten grams of frozen pepper was added to 10 mL of extraction medium (0.1 M citric acid, 0.05% w/v ethylenediaminetetraacetic acid disodium salt, 5% v/v methanol, and 4 mM NaF). The mixture was directly homogeneized for 30 s and filtered through a filter cloth. The filtrate was collected and centrifuged at 10 500*g* in an Eppendorf centrifuge for 5 min at 2−5 °C. The filtrate was flushed through an activated Sep-Pak C-18 cartridge (Waters, Milford, MA) and then filtered through a 0.45 μm filter. Then, 250 μL of 1,2-phenylenediamine dihydrochloride (OPDA) solution (35 mg/100 mL) was added to 750 μL of extract for dehydroascorbic acid derivatization into the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-b]quinoxaline-1-one (DFQ). After 37 min in darkness, the samples were analyzed by HPLC.

Ascorbic acid and dehydroascorbic acid were evaluated using an HPLC system (Merck Hitachi), equipped with a L-6000 pump and coupled to a D-2500 variable-wavelength UV detector. Twenty microliter samples were injected on a 250 mm × 4 mm i.d. 5 μm reversed phased Kromasil 100 C_{18} column (Tecnokroma, Barcelona, Spain) with an ODS guard C_{18} precolumn. The flow rate was kept at 0.9 mL/min. The detector wavelength was initially set at 348 nm, and after DFQ eluted, it was manually shifted to 261 for ascorbic acid detection. The vitamin C content was calculated by adding ascorbic acid and dehydroascorbic acid contents, and the results are expressed as mg per 100 g fresh weight. The coefficient of variation was less than 8%.

Extraction and Analysis of Carotenoids. The procedures used were as described by Minguez-Mosquera et al. (*15*). Samples of 10 g were extracted three or four times with 50 mL of acetone using a homogenizer until no color was extracted. The extracts were combined in a decanting funnel and treated with 100 mL of ethyl ether, shaken, and left to settle. Enough NaCl solution (10%) was added to separate the phases and to transfer the pigments to the ether. This solution was

treated several times with anhydrous Na_sSO_4 (2%) to remove the water. Then, the ether phase, containing the pigments in different states of esterification with fatty acids, was saponified with 100 mL of KOH−MeOH (20%) and left for 1 h with periodic shaking. The aqueous phase was removed while the organic phase was washed several times with distilled water until neutral, then filtered through a bed of anhydrous Na_2SO_4, and evaporated to dryness in the rotary evaporator at a temperature lower than 35 °C. The pigments were collected with acetone to a volume of 25 mL and kept refrigerated until their analysis by HPLC.

At the beginning of the extraction process, β-apo-8′-carotenal was added (around 1 mg/ 10 g of peppers) as an internal standard since this pigment is absent in peppers and, under the proposed conditions, separates well from the other carotenoids.

trans-β-Carotene and β-apo-8′-carotenal were purchased from Sigma Chemical Co. Neoxanthin, violaxanthin, and lutein were obtained from a saponified extract of mint (*Mentha piperita*) by thin-layer chromatography (TLC) (*30*). Standards of capsanthin and capsorubin were isolated from a saponified extract of red pepper (*15*). For this purpose, 20 cm × 20 cm, 0.5 mm thick plates of silica gel 60 F_{254} (Merck) were used. Each compound was identified on the basis of its TLC R_f values, comparison with isolated standards and UV/vis spectra.

Carotenoids were evaluated using an HPLC system equipped with a model L-6200 pump (Merck-Hitachi) and SPD-M6A photodiode array UV/vis detector (Shimadzu, Japan). Separations were achieved on a 250 mm × 4 mm i.d., 5 μm LiChrocart C_{18} column (Merck) using a gradient program previously described (*15*). Elution was performed at a solvent flow rate of 1.5 mL/min with an injection volume of 20 μL and detection at 450 nm. Multicomponent mixtures were used for calibration. Once the pigments had been purified, solutions of each were prepared and the concentration was determined spectrophotometrically, using the corresponding values of ϵ_0 (*31*). The concentrations were calculated and expressed as mg per 100 mg fresh weight. The coefficient of variation for the main pigments was less than 3%.

RESULTS AND DISCUSSION

Elucidation of Phenolics. The HPLC-DAD analysis of the methanol extract obtained from the pepper pericarp showed that this fruit contained a very rich polyphenol pattern (**Figure 1**) contrasting with previous published reports that describe only quercetin and luteolin after acid hydrolysis (*23*, *24*). The polyphenol profile of the pepper extracts consisted of a combination of peaks with characteristic UV spectra of hydroxycinnamic acids (A−E) and other peaks with typical UV spectra of flavonols and flavones (1−23), and some of the spectra suggested the presence of *C*-glycosylflavones. In the study of pepper phenolics, the combination of DAD and ESI-MS detectors coupled to the HPLC equipment using reversed phase columns provided an accurate method for the identification and quantification of individual phenolics. The different flavonoids were then analyzed on the peel extract by HPLC coupled to an ESI interface and an ion trap MS to perform ion isolation and controlled fragmentation to help with the structure identification.

Hydroxycinnamic Acids. Compounds **A** and **C** showed characteristic UV spectra of caffeic acid derivatives, but their MS analyses did not provide enough information for compound identification. Compound **B** showed a UV spectrum characteristic of a *p*-coumaric derivative, and its ESI-MS analysis showed a deprotonated molecular ion at *m*/*z* 325 $[M - H]^-$, consistent with a *p*-coumaroyl-glucopyranoside, and the MS^2 of the isolated 325 ion gave the *p*-coumaroyl fragment at *m*/*z* 163. Compound **D** showed a UV spectrum characteristic of a dihydroxycinnamic acid derivative (caffeic or ferulic acid). Its ESI-MS analysis showed a deprotonated molecular ion at *m*/*z* 355 $[M - H]^-$, consistent with a feruloyl-glucopyranoside, and the MS^2 of the isolated 355 ion showed the feruloyl fragment at *m*/*z* 193.

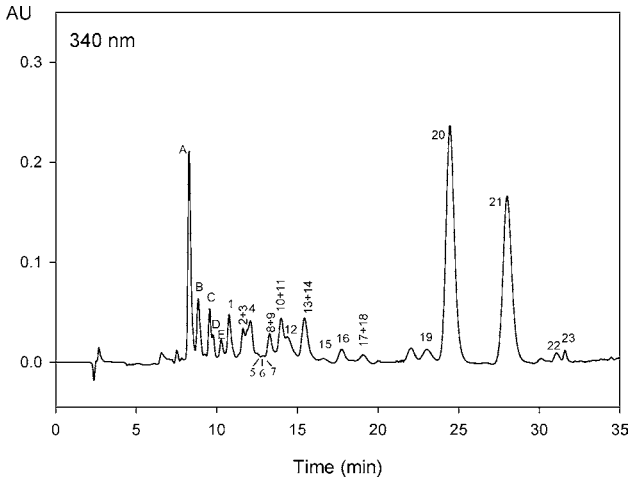

Figure 1. HPLC-DAD chromatogram of sweet pepper cv. Vergasa pericarp extract at the green maturity stage. (A) Caffeic acid derivative; (B) *trans*-*p*-coumaroyl-β-D-glucopyranoside; (C) caffeic acid derivative; (D) *trans*-feruloyl-β-D-glucopyranoside; (E) *trans*-synapoyl-β-D-glucopyranoside; (1) luteolin 6,8-di-*C*-hexoside; (2) apigenin 6,8-di-*C*-hexoside; (3) quercetin 3-*O*-rhamnoside-7-*O*-glucoside; (4) luteolin 6-*C*-hexoside-8-*C*-pentoside; (5) luteolin 6-*C*-hexoside-8-*C*-pentoside; (6) luteolin 6-*C*-pentoside-8-*C*-hexoside; (7) chrysoeriol 6,8-di-*C*-hexoside; (8) apigenin 6-*C*-pentoside-8-*C*-hexoside; (9) luteolin 6-*C*-(6-malonyl)hexoside-8-*C*-hexoside; (10) luteolin 6-*C*-pentoside-8-*C*-hexoside; (11) luteolin 8-*C*-hexoside; (12) chrysoeriol 6-*C*-hexoside-8-*C*-pentoside; (13) luteolin 6-*C*-hexoside-8-*C*-rhamnoside; (14) luteolin 6-*C*-hexoside; (15) luteolin 6-*C*-(6-malonyl)-hexoside-8-*C*-pentoside; (16) apigenin 6-*C*-hexoside-8-*C*-pentoside; (17) luteolin 6-*C*-rhamnoside-8-*C*-hexoside; (18) luteolin 7-*O*-(2-apiosyl)glucoside; (19) luteolin 7-*O*-(2-apiosyl-6-acetyl)glucoside; (20) quercetin 3-*O*-rhamnoside; (21) luteolin 7-*O*-(2-apiosyl-6-malonyl)glucoside; (22) chrysoeriol 7-*O*-(2-apiosyl-6-acetyl)glucoside; and (23) luteolin 7-*O*-(2-apiosyl-diacetyl)glucoside.

Compound **E** showed a UV spectrum characteristic of a sinapoyl derivative, and its ESI-MS analysis showed a deprotonated molecular ion at *m*/*z* 385 $[M - H]^-$ consistent with a sinapoyl-glucopyranoside. The MS^2 of the isolated 385 ion showed the sinapoyl fragment at *m*/*z* 223. Compounds **D** and **E** have been recently reported in hot peppers (*25*), but this is the first time that they are reported in sweet peppers.

Flavonoid O-Glycosides. Compound **3** was identified as a quercetin derivative (aglycone fragment at *m*/*z* 301) glycosylated with a desoxyhexose and a hexose. Both sugars should be linked to different phenolic hydroxyls of the quercetin molecule as both (M − H − 146) and (M − H − 162) fragments were observed (**Table 1**). This compound could not be a quercetin rutinoside or neohesperidose as the fragmentation did not correspond with that of a rutinoside (*32*) nor with a neohesperidoside, and the fragment at (M − H − 162) was also observed, showing a direct loss of the hexosyl residue leaving the deoxy-hexosyl residue linked to the quercetin molecule. Thus, this compound is tentatively identified as quercetin 3-*O*-rhamnoside-7-*O*-glucoside, recently reported in hot pepper (*25*).

Compound **18** shows a negative ion MS fragmentation indicating that this is a tetrahydroxyflavone glycoside (aglycone ion at *m*/*z* 285). Its UV spectrum suggested that this is a luteolin derivative. The molecular ion corresponds to a pentosyl-hexoside of luteolin, and after MS^2, the first fragment lost was the pentosyl residue showing that the hexosyl residue must be directly linked to the aglycone (**Table 1**). The fragment at *m*/*z* 447 with a relative abundance of 37% suggests that the interglycosidic linkage is (1 → 2) (*32*); therefore, compound

Table 1. HPLC-DAD-ESI-MS Analysis of *O*-Glycosyl Flavones in Sweet Peppers[a]

O-glycosyl flavones

peak no.	R_t (min)	UV (nm)	[M − H]$^-$	−MS^2[(M − H]$^-$ *m/z* (%)							
				−42	−44	−(42 + 18)	−132	−146	−162	−(132 + 42)	[Agl − H]$^-$
3	11.6	*b*	609					463 (59)	447 (74)		301 (100)
18	19.1	255, 267sh, 350	579				447 (37)				285 (100)
20	24.5	256, 268sh, 299sh, 353	447					301 (100)			301 (100)

O-glycosyl acetyl flavones

peak no.	R_t (min)	UV (nm)	[M − H]$^-$	−42	−44	−(42 + 18)	−132	−146	−162	−(132 + 42)	[Agl − H]$^-$	−MS^3[(M − H) → −42]$^-$	
												−132	[Agl − H]$^-$
19	23.0	255, 267sh, 350	621	579 (86)		561 (100)	489 (19)			447 (7)	285 (39)	447 (38)	285 (100)
22	31.1	250sh, 255, 268, 349	635	593 (55)		575 (21)	503 (32)			461 (11)	299 (100)	461 (40)	299 (100)
23	31.6	255, 268sh, 350	663	621 (100)		603 (98)	531 (3)			489 (3)	285 (9)	489 (57)	285 (100)

O-glycosyl acetyl flavones

peak no.	R_t (min)	UV (nm)	[M − H]$^-$	−42	−44	−(42 + 18)	−132	−146	−162	−MS^3[(M − H) → −44]$^-$			−MS^4[(M − H) → −44 → −42]$^-$	
										−42	−132	[Agl − H]$^-$	−132	[Agl − H]$^-$
21	28.0	256, 268sh, 349	665		621 (100)					579 (33)	489 (89)	285 (100)	447 (13)	285 (100)

[a] MS^2, MS^3, and MS^4 fragments are shown; see Figure 1 for flavonoid structures. [b] An accurate UV spectrum was not recorded due to coelution with other compounds. Agl, aglycone.

18 was tentatively identified as luteolin-7-*O*-(2″-apiosyl)-glucoside reported in hot pepper (*25*).

Compound **20** is one of the main compounds in pepper, and its MS-MS analysis (**Table 1**) clearly showed that this is a quercetin deoxyhexoside. Its UV spectrum showed that the hydroxyl at the 3-position is blocked, and this was identified as quercetin 3-*O*-rhamnoside, one of the main flavonoids also identified in hot pepper (*25*).

Compound **19** is an acetylated derivative of **18**. In the MS^2, analysis of the [M − H]$^-$ ion, the ions (M − H − 42) and (M − H − 42 − 18) were observed and these are characteristic of the loss of an acetyl residue. The acetylation must be located on the glucose residue of compound **18** as the ion produced by the loss of the pentosyl residue (*m/z* 489) was observed with a relative intensity of 19% (**Table 1**), in which the acetyl residue remains attached to the glucosyl residue (*m/z* 489 = 285 + 162 + 42), suggesting that the most feasible position linkage for the acetyl is the hydroxyl at the 6-position of the glucose as the apiosyl residue must be linked at position 2 in accordance with previously published data (*25*). Thus, this compound is tentatively identified as luteolin-7-*O*-(2-apiosyl-6-acetyl)glucoside, a new naturally occurring compound.

Compound **22** shows a MS^2 [M − H]$^-$ fragmentation similar to that of **19** (**Table 1**), differing mainly in the aglycone that in this case is a trihydroxy-methoxyflavone. The UV spectrum showed a band I with a maximum at 349 nm that is consistent with chrysoeriol (luteolin 3′-methyl ether) rather than diosmetin (luteolin 4′-methyl ether), and this compound was tentatively identified as chrysoeriol 7-*O*-(2-apiosyl-6-acetyl)glucoside. Compound **23** is an acylated derivative of **18** with a mass consistent with its diacetylated derivative. The MS^2 [M − H]$^-$ fragmentation shows the loss of an acetyl residue (−42) (*m/z* 621, 100%) and the loss of acetyl + H_2O (*m/z* 603, 98%). In addition, a fragment consistent with the loss of two acetyl residues is also observed (*m/z* 579, 2% data not shown in **Table 1**). The ion produced by the loss of the apiosyl residue from the (M − H − 42) fragment was clearly observed (−MS^3 [(M − H) → −42]$^-$), at *m/z* 489 (57%) (**Table 1**) and is similar to the behavior observed for **19**, showing that the acetyl residue was linked to the glucosyl residue. Thus, **23** was tentatively identified as luteolin-7-*O*-(2-apiosyl-di-acetyl)glucoside.

Compound **21** is a malonated derivative of **18**. In the MS^2 [M − H]$^-$ fragmentation, only the COO (−44) loss was observed (**Table 1**). The −MS^3 [(M − H) → −44]$^-$ showed an ion produced by the loss of the terminal apiosyl residue (*m/z* 489, 89%) that retains the rest of the malonyl residue after the decarboxylation linked to the glucose and that shows, as in the case of the previously mentioned flavonoids, the position of linkage of the acyl residue. Thus, this is luteolin 7-*O*-(2-apiosyl-6-malonyl)glucoside. The luteolin 7-*O*-(2-apiosyl-4-glucosyl-6-malonyl)glucoside that was recently reported in hot pepper (*25*) was not detected in the analyzed sweet pepper.

Flavonoid C-Glycosides. Sweet pepper contains a good number of flavone *C*-glycosides (**Table 2**). Their study by HPLC-MS was achieved on the basis of previous reports by Waridel et al. (*33*) and Ferreres et al. (*34*) in which the locations of sugars at *C*-6 and *C*-8 are related to the preferential fragmentation of the sugar linked at C-6, and the nature of the sugar is assigned by the loss sequence of hexose residues (−120, −90), the pentosyl residues (−90 and −60), and the rhamnosyl residues (−104 and −74) giving rise, respectively, to the ions $^{0,2}X^-$ and $^{0,3}X^-$ (*35*). The fragment ions $^{k,l}X^j$ represent the ions still containing the aglycone, where *j* is the number of bonds to the aglycone and *k* and *l* denote the cleavage within the carbohydrate rings (**Figure 3**).

Compounds **1**, **2**, and **7** are di-*C*-hexosyl derivatives (most probably di-*C*-glucosyl derivatives) of luteolin, apigenin, and chrysoeriol, respectively, with a characteristic fragmentation of these compounds (*34*). Compounds **4** and **5** are two luteolin 6-*C*-hexoside-8-*C*-pentoside derivatives, and **6** and **10** are two

Table 2. HPLC-DAD-ESI-MS Analysis of *C*-Glycosyl Flavones in of Sweet Peppers[a]

peak no.	R_t (min)	UV (nm)	$[M-H]^-$	$-MS^2[(M-H]^-$, *m/z* (%)							
				–18	–60	–74	–90	–104	–120	Agl + 113	Agl + 83
				6,8-di-*C*-symmetric glycosyl flavones							
1	10.8	256, 272, 349	609	591 (3)	549 (1)		519 (18)		489 (100)	399 (46)	369 (38)
2	11.6	*b*	593	575 (4)	533 (2)		503 (23)		473 (100)	383 (30)	353 (83)
7	12.8	250sh, 257, 272, 349	623	605 (2)	563 (2)		533 (14)		503 (100)	413 (26)	383 (63)
				6,8-di-*C*-asymmetric glycosyl flavones							
4	12.1	255, 272, 349	579	561 (7)	519 (5)		589 (28)		459 (100)	399 (85)	369 (55)
5	12.9	256, 272, 349	579	561 (4)	519 (6)		589 (32)		459 (100)	399 (73)	369 (38)
6	12.9	256, 272, 350	579	561 (9)	519 (14)		589 (100)		459 (12)	399 (36)	369 (40)
10	14.0	*b*	579	561 (5)	519 (14)		589 (100)		459 (7)	399 (58)	369 (50)
8	13.3	272, 335	563	545 (21)	503 (65)		473 (100)		443 (55)	383 (99)	353 (100)
16	17.8	272, 335	563	545 (10)	503 (2)		473 (90)		443 (100)	383 (35)	353 (65)
12	14.4	250sh, 257, 270, 349	593	575 (11)	533 (21)		503 (34)		473 (82)	413 (55)	383 (100)
13	15.4	*b*	593	575 (15)		519 (5)	503 (8)	489 (7)	473 (100)	399 (44)	369 (44)
17	19.1	*b*	593	575 (30)		519 (5)	503 (38)	489 (100)	473 (30)	399 (70)	369 (64)
				6- and 8-mono-*C*-glycosyl flavones							
11	14.0	*b*	447				357 (35)		327 (100)		
14	15.4	*b*	447	429 (17)			357 (100)		327 (71)		

C-glycosyl acyl flavones

peak no.	R_t (min)	UV (nm)	$[M-H]^-$	$-MS^2[(M-H]^-$, *m/z* (%)		$-MS^3[(M-H) \rightarrow -44]^-$, *m/z* (%)							
				–44	–86	–18	–60	–90	–120	–(90 + 42)	–(120 + 42)	Agl + 113	Agl + 83
9	13.3	*b*	695	651 (100)	609 (12)	633 (14)		561 (20)	531 (50)	519 (11)	489 (100)	399 (57)	369 (41)
15	16.6	256, 272, 349	665	621 (100)		603 (34)	561 (46)	531 (12)		489 (10)	459 (80)	399 (100)	369 (71)

[a] MS^2 and MS^3 fragments are shown; see Figure 1 for flavonoid identification. [b] An accurate UV spectrum was not recorded due to coelution with other compounds; Agl, aglycone.

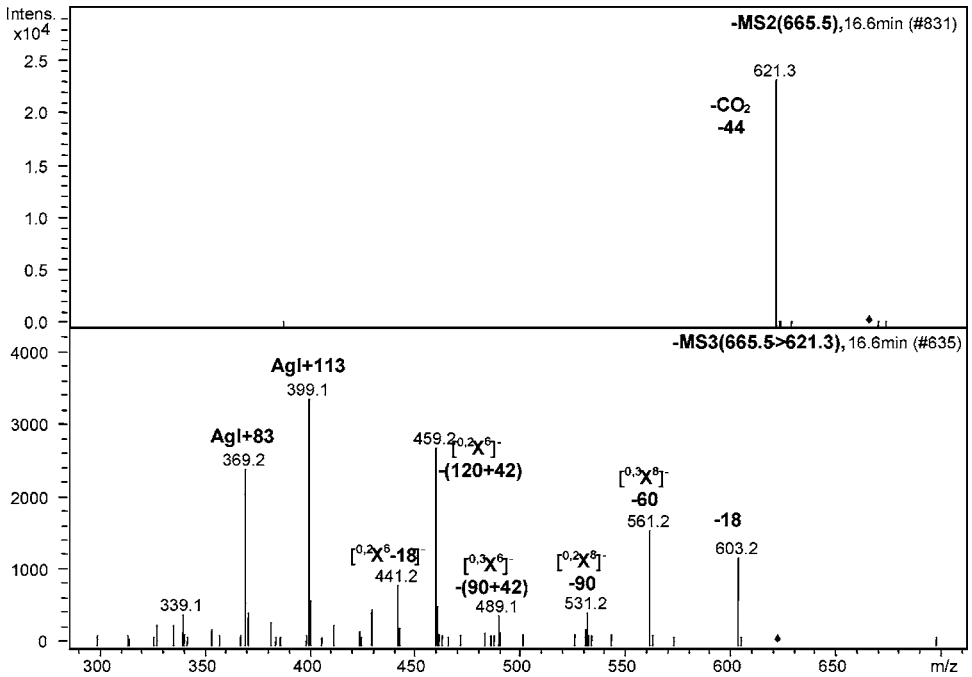

Figure 2. $-MS^2$ $[M-H]^-$ and $-MS^3$ $[(M-H) \rightarrow -44]^-$ of luteolin-6-*C*-(6-malonyl)hexoside-8-*C*-pentoside.

luteolin 6-*C*-pentoside-8-*C*-hexoside derivatives. Compound **4** coincides most probably with luteolin 6-*C*-glucoside-8-*C*-arabinoside recently reported in *Capsicum* (*25*). Compounds **8** and **16** are, respectively, apigenin 6-*C*-pentoside-8-*C*-hexoside and its isomer apigenin 6-*C*-hexoside-8-*C*-pentoside (**Table 2**). Compound **16** may correspond to apigenin 6-*C*-glucoside-8-*C*-arabinoside recently reported in *Capsicum* (*25*). Compound **12** showed a mass spectrum as a trihydroxymonomethoxyflavone (**Table 2**), and its UV spectrum showed that this is a chrysoeriol derivative (*36*). Its MS spectrum suggested that this is chryso-

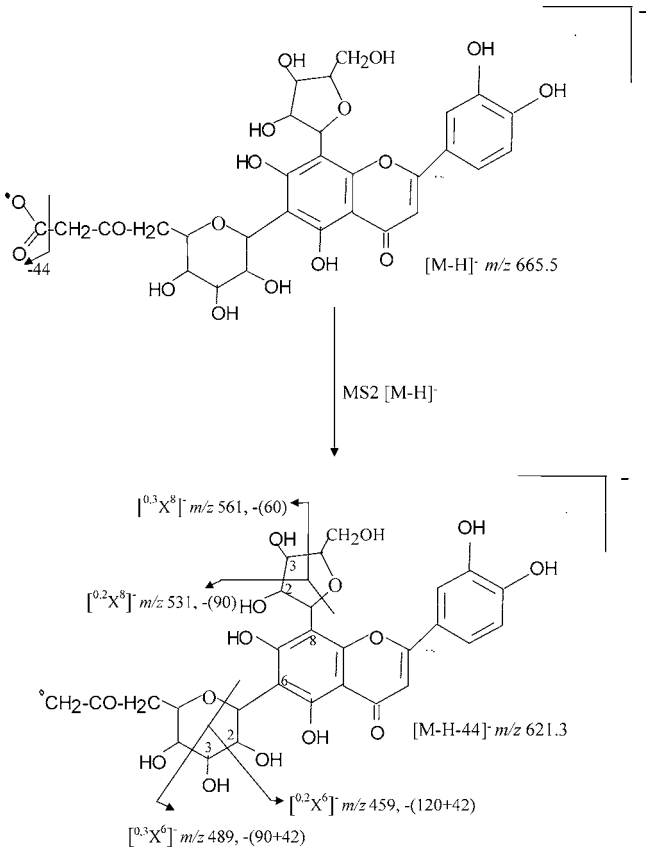

Figure 3. Fragmentation pathway of luteolin-6-*C*-(6-malonyl)hexoside-8-*C*-pentoside. The nomenclature is according to Domon and Costello (*35*).

eriol 6-*C*-hexoside-8-*C*-pentoside. Compound **13** is luteolin 6-*C*-hexoside-8-*C*-rhamnoside, and its isomer is compound **17** (luteolin 6-*C*-rhamnoside-8-*C*-hexoside) (**Table 2**). As far as we know, **17** has not been reported in nature so far. Compounds **11** and **14** are luteolin 8-*C*-hexoside derivatives.

Pepper also contains acylated *C*-glycosyl-flavones. Compounds **9** and **15** show MS spectra characteristic of luteolin di-*C*-glycosides acylated with dicarboxylic acids, as an ion produced by decarboxylation is observed (M − 44) in the MS^2 [M − H]- analysis (**Table 2**). Compound **9** showed an $[M - H]^-$ ion at *m*/*z* 695 that coincides with a luteolin 6,8-di-*C*-hexoside acylated with malonic acid. In the MS^3 $[(M - H) \rightarrow -44]^-$, the ions $^{0,2}X^-$ (−120) and $^{0,3}X^-$ (−90) are observed, but the base peak was that at *m*/*z* 489 (−120 − 42) that clearly shows that the malonyl residue (42) is linked to the hexosyl at *C*-6, as it remains attached to this sugar after the first decarboxylation, and this ion is $[^{0,2}X^6]^-$. On the other hand, the occurrence of this ion and that of $[^{0,3}X^6]^-$ at *m*/*z* 519 (−90 − 42) showed that the 42 residue is not linked to positions 2 or 3 of the hexose, as these carbons are not involved in these losses; therefore, the acylation should be at either positions 4 or 6 in the sugar residue. Position 6 is much more frequent in nature, and we tentatively identify compound **9** as luteolin 6-*C*-(6-malonyl)-hexoside-8-*C*-hexoside.

Compound **15** is an analogue of **9** in which the sugar at position *C*-8 is a pentose instead of a hexose, and the ions from $-MS^3$ $[(M - H) \rightarrow -44]$, $[^{0,2}X^8]^-$ (−90) and $[^{0,3}X^8]^-$ (−60) corresponding to the fragments loss from a pentose at *C*-8 were observed (**Figure 2**). The (−120) loss was not observed, as there is no free hexose, since the hexose is acylated with malonic acid. The fragments obtained by the loss (−90 − 42) $[^{0,3}X^6]^-$ (*m*/*z* 489, 10%) and (−120 − 42) $[^{0,2}X^6]^-$ (*m*/*z* 459, 80%) were however observed (**Figure 3**), showing as in the case of compound **9** that the acylation should be located at the hexose at the *C*-6 position. This was identified as luteolin-6-*C*-(6-malonyl)hexoside-8-*C*-pentoside. To our knowledge, compounds **9** and **15** are found in foods for the first time.

Table 3. Hydroxycinnamic Derivatives and Flavonoids Content of Sweet Peppers (*C. anuum* L.) cv. Vergasa at Different Maturity Stages[a]

phenolics	immature green	green	immature red	red
	hydroxycinnamic derivatives			
A	1.37 (0.03)			
B	0.10 (0.01)	0.09 (0.01)	0.06 (0.01)	0.05 (0.01)
C	0.52 (0.04)			
D		0.10 (0.01)	0.10 (0.01)	0.09 (0.01)
E		0.14 (0.01)	0.29 (0.01)	0.30 (0.05)
total hydroxycinnamics	1.99 (0.08)	0.33 (0.03)	0.45 (0.03)	0.44 (0.07)
	flavonoids			
20	4.22 (0.21)	0.57 (0.06)	0.32 (0.01)	0.31 (0.02)
21	4.14 (0.12)	0.73 (0.02)	0.44 (0.01)	0.39 (0.03)
total *O*-glycosylflavones	9.43 (0.11)	1.54 (0.18)	1.20 (0.10)	1.00 (0.07)
total *C*-glycosylflavones	2.45 (0.27)	1.14 (0.11)	1.20 (0.14)	0.84 (0.03)
total flavonoids	20.24 (0.71)	3.98 (0.37)	3.16 (0.26)	2.54 (0.15)

[a] Means in mg 100 g^{-1} fresh weight. Standard deviations ($n = 3$) in parentheses. Total flavonoids are the addition of the different flavonoid groups.

Changes in Phenolic Content during Ripening and Maturity. The studies available in the literature on the flavonoid analyses of sweet bell pepper have been based on the quantification of flavonoid aglycones (quercetin and luteolin) obtained after acid hydrolysis (*23*, *24*). Lee et al. (*23*) described the presence of quercetin and luteolin conjugates in the pepper extracts and that some of them were resistant to acid hydrolysis. The accurate quantification of sweet pepper polyphenols has been very difficult as many of these conjugates are *C*-glycosides that are not hydrolyzed under acidic conditions, and more sophisticated equipment, such as HPLC-MS, is needed for the identification of these compounds in crude extracts. The recent study of hot pepper by Materska et al. (*25*) and our results reveal the presence of a large number of individual polyphenols. Five hydroxycinnamic acid derivatives and six groups of flavonoids have been quantified in sweet pepper at different maturity stages (**Table 3**). As hydroxycinnamic acid derivatives and flavonoids were mainly located in the peel, freeze-dried samples were used to minimize quantification errors and obtain a more representative sample.

The immature green peppers showed 1.99 mg of hydroxycinnamates per 100 g fw while green, immature red, and red ripe fruits contained 0.33−0.45 mg/100 g (**Table 3**).

The content of flavonoids in the immature green pepper was generally 4−5 times higher than that detected for other maturity stages. A marked decrease was observed during ripening from the immature green stage to the green one. The flavonoid content gradually decreased from the green to the immature red and red ripe stages. The amount of *O*-glycosylflavones in pepper was larger than that of other flavonoids for all of the maturity stages. The *O*-glycosylflavanones content in immature green pepper decreased by more than 85% of the content in green fruits. This decrease was not only due to the fruit size increase with advancing maturity but also to degradation of flavonoids since the size of immature green fruit was only slightly smaller than that of more mature fruit (135 vs 188 g). Maturity is one

Table 4. Ascorbic Acid, Dehydroascorbic Acid, and Total Vitamin C of Sweet Peppers (*C. anuum* L.) cv. Vergasa at Different Maturity Stages[a]

maturity stage	ascorbic acid	dehydroascorbic acid	vitamin C
immature green	31.3 (0.1)	11.0 (0.8)	42.3 (0.9)
green	51.3 (3.7)	3.0 (0.3)	54.3 (4.0)
immature red	61.0 (2.8)	2.0 (0.1)	63.0 (2.9)
red	90.7 (9.0)	2.3 (0.2)	93.0 (9.2)

[a] Means in mg 100 g^{-1} fresh weight. Standard deviations ($n = 3$) in parentheses.

of the major factors that determine the content of phenolics in fruit and vegetables. Howard et al. (*24*) have reported an increase in the concentration of total soluble phenolics as the peppers reached maturity for different pepper cultivars. Those results are difficult to compare because the cultivar used in this study was a red bell pepper and in the previous report other different *C. annuum* cultivars were studied. The phenolic changes during maturation in red bell pepper have not been described so far. In general, immature fruits contained the highest concentration of phenolics while ripe fruits contained the lowest. In our case, the total phenolic content decreased during fruit maturation from 20.24 to 2.54 mg/100 g fw and this was not due to the fruit size increase with advancing maturity since similar sizes were used for all maturity stages (**Table 3**).

Quercetin-3-*O*-rhamnoside (compound **20**) and luteolin 7-*O*-(2-apiosyl-6-malonyl)glucoside (compound **21**) were the major phenolic compounds found in sweet pepper cv. Vergasa, which represented 41% of total flavonoids (**Table 3**). In hot pepper, sinapic and ferulic acids have been reported to be 60% of the dry mass while luteolin apiosylglucoside and quercetin rhamnoside were less relevant (35%) (*25*).

Changes in Vitamin C Content during Ripening and Maturity. An increase in the ascorbic acid content was observed for fresh peppers as fruit maturity advanced (**Table 4**). These results are in agreement with previous studies that reported increases in ascorbic acid during pepper ripening and maturation (*24*, *37*, *38*). The role of ascorbate content as a photoprotector has been reported for leaves acclimated to high light, which have a higher ascorbate concentration than leaves grown at low intensity (*39*). The amount and intensity of light during the growing season have also been described to have a definite influence increasing the ascorbic acid formed (*10*). The light exposure could explain the increase in ascorbic acid content of red fruits as compared to the green ones. However, some contradictory results were observed in bell pepper fruits where an increase during development was described with a maximum of 136.1 mg/100 g at 51 days from fruit set and then decreased suddenly to a minimum of 65.5 mg/100 g at 64 days (*40*).

The cultivar studied in this work contained a lower vitamin C content than other reported pepper cultivars, which ranged from 63 to 243 (mg/100 g) (*23*, *37*). In a review by Lee and Kader (*10*), red peppers are described as the vegetable with the highest vitamin C content among other important plant foods including broccoli and spinach (*25*, *41*). Ascorbic acid was present in much greater amounts than dehydroascorbic acid (**Table 4**). In this case, dehydroascorbic acid tended to decrease during pepper maturation. As peppers mature, pH values become more adequate for ascorbic acid stability (6.23 in immature fruits and 5.08 in red ripe peppers). The total vitamin C of red pepper cv. Vergasa was about 45% higher than that of immature green pepper. However, Yahia et al. (*40*) have described a decrease in total ascorbic acid content that coincided with the initiation of ripening from 136 mg to 65.5 mg/100 g. Fruit senescence could occur if harvest is delayed or if fruits are subjected to inadequate handling practices. In other fruits such as tomato, a rather large vitamin C increase (88%) was observed up to the red stage with a subsequent decrease as the fruit overmatured (*40*).

Table 5. Carotenoid Pigment Composition of Sweet Peppers (*C. anuum* L.) cv. Vergasa at Different Maturity Stages[a]

pigment	immature green	green	immature red	red
neoxanthin	0.20 (0.03)	0.23 (0.05)	0.22 (0.02)	
lutein	2.28 (0.06)	1.36 (0.03)		
cis-lutein	0.10 (0.01)	0.09 (0.01)		
violaxanthin	0.65 (0.14)	1.01 (0.05)	1.35 (0.02)	2.29 (0.16)
β-cryptoxanthin	0.07 (0.02)	0.08 (0.01)	0.47 (0.11)	1.49 (0.12)
β-carotene	1.71 (0.10)	2.09 (0.21)	1.94 (0.02)	4.29 (0.13)
capsorubin				3.00 (0.28)
capsanthin 5-6, epoxide			0.20 (0.01)	0.91 (0.13)
capsanthin			1.11 (0.13)	19.89 (0.21)
antheraxanthin			0.86 (0.01)	1.06 (0.16)
cis-capsanthin				6.30 (0.15)
cucurbitaxanthin A			0.41 (0.010)	2.35 (0.14)
zeaxanthin			2.94 (0.010)	3.66 (0.19)
cis-zeaxanthin				0.35 (0.01)
total pigments	5.07	4.86	9.52	45.59
provitamin A[b]	295.71	355.15	362.79	838.80

[a] Means in mg 100 g^{-1} fresh weight. Standard deviations ($n = 3$) in parentheses. [b] IU of provitamin A/mg fresh fruit = (166.7 × mg of β-carotene + 83.3 × mg of β-cryptoxanthin)/100 g fresh fruit.

Changes in Carotenoid Content during Ripening and Maturity. During pepper ripening, the chloroplast pigments (chlorophylls and carotenoids such as lutein and neoxanthin) disappear, while exclusively carotenoid chromoplast pigments are synthesized and esterified by fatty acids (*18*−*21*). In our study, preliminary work determined that the gradient HPLC in the saponified extracts was the optimal protocol for separation and quantification of carotenoid pigments (*15*, *42*−*44*). Our results for carotenoid analysis were consistent with other studies where pepper carotenoids were quantified during maturation (*18*, *20*, *21*). Lutein was the predominant carotenoid for immature green pepper while β-carotene was the predominant pigment for green peppers (**Table 5**). Lutein and *cis*-lutein contents decreased during maturation to nondetectable values in immature red and red ripe stages. These pigments have been described as peculiar to green peppers (*15*). The neoxanthin content was similar for immature green, green, and immature red fruits but was absent in the red ripe fruits. The concentrations of xanthophylls such as capsorubin, *cis*-capsanthin, and *cis*-zeaxanthin appeared only when the peppers reached the red stage. The total carotenoid pigments increased four times for red ripe fruits; capsanthin was the major pigment among them. The red stage also has a high content of provitamin A due to the high concentrations of β-carotene and β-crytoxanthin.

In the present work, it was shown that the phenolic compounds in sweet pepper were mainly located in the peel. Two *O*-glycosides of quercetin (quercetin 3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside-7-*O*-glucoside), luteolin 7-*O*-(2-apiosyl)-glucoside, three acylated luteolin derivatives as luteolin-7-*O*-(2-apiosyl-6-acetyl)glucoside, luteolin-7-*O*-(2-apiosyl-diacetyl)-glucoside, and luteolin-7-(2-apiosyl-6-malonyl)glucoside, and also chrysoeriol-7-*O*-(2-apiosyl-6-acetyl)glucoside were identified. A large number of *C*-glycosyl flavones have been characterized as 6,8-di-*C*-hexosyl and 6-*C*-pentosyl-8-*C*-hexosyl of luteolin, apigenin, and chysoeriol; 6-*C*-hexosyl-8-*C*-pentosyl of luteolin, apigenin, and chysoeriol; luteolin 6-*C*-hexosyl-8-

C-rhamnosyl and luteolin 6-*C*-rhamnosyl-8-*C*-hexosyl; and two acylated luteolin derivates, 6-*C*-(6-malonyl)hexosyl-8-*C*-hexoside and 6-*C*-(6-malonyl)hexosyl-8-*C*-pentoside. The optimum maturity stage for pepper harvest based on vitamin C and carotenoids content was the red ripe stage. If pepper fruits are harvested in an immature stage, they have a considerably higher content of polyphenols (hydroxycinnamates and flavonoids).

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