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Recovery and Characterization of the Metal Polymeric Organic Fraction (Polymerin) from Olive Oil Mill Wastewaters

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A dark and complex metal polymeric organic mixture, named polymerin, was recovered from olive oil mill wastewaters (OMWW) and characterized by chemical analysis, diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS), and atomic absorption spectrometry (AAS). Polymerin proved to be composed of carbohydrates (52.40 mg 100^{-1}, w/w), melanin (26.14 mg 100^{-1}), and proteins (10.40 mg 100^{-1}), and the respective composition of monosaccharides, phenols, and amino acids was determined. It also contained metals (11.06 mg 100^{-1}), mainly K^+ and, to a lesser extent, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, and Cu^{2+}, which were naturally bound and chelated through carboxylate anions and other characteristic nucleophilic functional groups naturally occurring in polymerin. The distribution of polymerin relative molecular size was assessed to be approximately between 500.0 and 2.0 kDa by calibrated molecular weight gel filtration chromatography, indicating also that a fraction consisted of protein, melanin, and polysaccharide, strongly aggregated to each other in a supramolecular status by a combination of covalent and hydrogen bonds and CH/Π interactions, and another fraction of only free polysaccharide. Polymerin was transformed into a potassium salt deglycosylated derivative, named KSDpolymerin, which was also characterized by chemical analysis, DRIFTS, and AAS. KSDpolymerin consisted of carbohydrates (6.00 mg 100^{-1}), melanin (52.49 mg 100^{-1}), and proteins (35.40 mg 100^{-1}), and the composition of monosaccharides, phenols, and amino acids was determined. It also contained metals (6.11 mg 100^{-1}), mainly K^+ and to a lesser extent Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{3+}, bound as in polymerin. All the organic components were strongly linked in a supramolecular aggregate status and the relative average molecular size proved to be 6.3 kDa. Finally, we briefly discuss the possible use of such polymerins in agriculture as bioamendments and macro- and microelement biointegrators and as a biofilter for toxic metal removal, in light of their similarity with humic acids.

KEYWORDS: ***Olea europea*; olive oil mill wastewaters; polymers; supramolecular structure; pigments; metals; polysaccharides; proteins; melanin; phenols; chromatography; diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS); atomic absorption spectrometry (AAS)**

INTRODUCTION

Olive oil mill wastewaters (OMWW) are produced in large quantities in all countries throughout the world where olive oil is produced (*1*). They are rich in inorganic and organic compounds (*2*, *3*) and possess highly polluting properties documented by high chemical oxygen demand (COD) and biological oxygen demand (BOD) (*3*). This last is mainly due to their polyphenol content and synergies with other naturally occurring compounds (*4*).

However, OMWW may also be regarded as an inexpensive source of inorganic and organic compounds (*2*) to be recovered because of their potential economic interest or their ability to be transformed into products for use in agriculture, environmental biotechnology processes, and industry.

In this connection, several processes for their exploitation have been proposed (*5*−*10*). The main polyphenols have been isolated from OMWW and chemically and biologically characterized, together with some of their synthetic derivatives (*4*, *11*, *12*).

In a previous work (*3*), we found that the metal cations naturally occurring in OMWW, were mainly bound to the organic polymeric fraction with K^+ being the most abundant metal. In addition, this fraction showed much lower COD and BOD values in comparison with those of raw OMWW (*3*).

These findings prompted us to recover this biomaterial, which we named polymerin, with the aim of studying its recycling in agriculture and environmental biotechnology processes. This paper reports the procedure for recovering polymerin, as well as the chemical, physicochemical, and spectroscopic characterization of this metal polymeric organic fraction.

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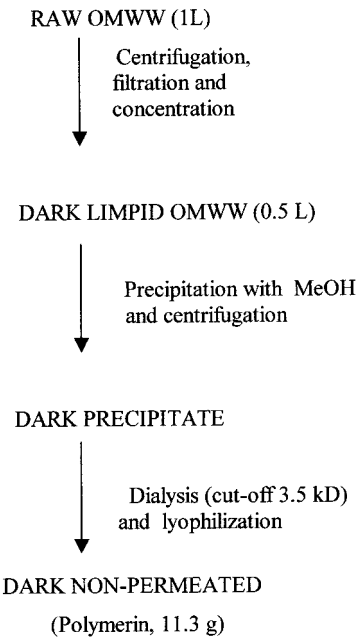
Scheme 1



Polymerin was also transformed into its potassium salt deglycosilated derivative, named KSDpolymerin, and into a methylester deglycosilated derivative, named CH_3Dpolymerin, both characterized in order to gain further knowledge of the chemical nature and the interactions of the polymerin components. Finally, the possible exploitation of such polymerins in agriculture and in environmental biotechnology processes is briefly discussed.

EXPERIMENTAL PROCEDURES

Materials. Samples of OMWW were supplied by a pressure processing plant located in Monteroduni (Isernia, Italy). Samples were kept refrigerated at −20 °C in tightly closed PVC vessels. Solvents were of HPLC grade and reagents were of analytical grade. High quality water (hqw), obtained using a MilliQ filtration system (Millipore), was used throughout.

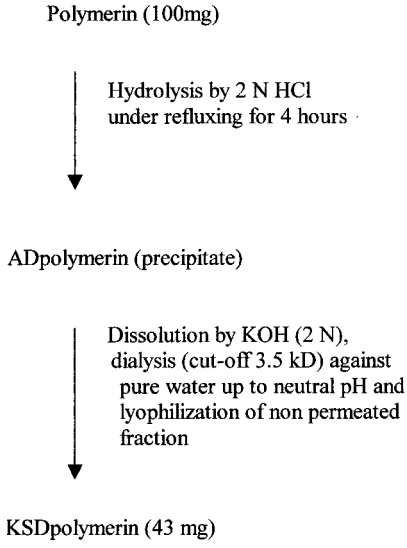
Safety and General Experimental Conditions for the Handling of OMWW Samples. Handling of the OMWW samples was carried out only when using disposable gloves. Dialysis was performed in the cold room at 10 °C mainly in the dark.

CH_2N_2 is a yellow toxic gas used as methylating agent in Etheral solution. Its preparation must be performed under a fume hood, and the preparer must wear disposable gloves and a gas mask.

Recovery of the Metal Polymeric Organic Fraction named Polymerin. Aliquots of 1 L of raw OMWW were first centrifuged at 11515*g* for 30 min and filtered through membranes of 1.2 and 0.2 μm (Millipore), obtaining a clear, dark solution, which was then concentrated to 0.5 L under reduced pressure. The solution thus obtained was treated with 1.5 L of cold methanol (−20 °C), and after 4 h a dark precipitate was obtained. This was separated by centrifugation at 11515*g* for 30 min and redissolved in a minimum volume of hqw, then dialyzed, using membranes (Spectrum Medical Industry, Houston, TX) with molecular weight cutoff of 3.5 kDa, against 5 sequential volumes (1:5) per time, at 4-h intervals. The nonpermeated dialyzed fraction was lyophilized, leaving a black residue of 11.3 g, named polymerin (**Scheme 1**).

Production of the Potassium Salt of Deglycosilated Polymerin, named KSDpolymerin. Aliquots of 100 mg of polymerin were hydrolyzed in 10 mL of 2 N HCl under refluxing for 4 h, obtaining a red-brown precipitate which was the acid derivative of deglycosilated polymerin, named ADpolymerin (**Scheme 2**). The heterogeneous mixture thus obtained was centrifuged at 11515*g* for 30 min. The supernatant was removed and the red-brown solid was redissolved in 2 N KOH. The solution was dialyzed, using membranes with molecular weight cutoff of 3.5 kDa, against hqw up to neutral pH of permeated water. The brown nonpermeated dialyzed solution was lyophilized, leaving a brown residue of 43 mg which was the potassium salt of deglycosyilated polymerin, named KSDpolymerin (**Scheme 2**).

Scheme 2



Preparation of the Methylester of Deglycosylated Polymerin, named CH_3Dpolymerin. Aliquots of 1 mL of 5 mg of KSDpolymerin suspended in MeOH were treated under stirring with an Etheral solution of CH_2N_2. The reaction was left overnight and additional CH_2N_2 Etheral solution was added to the reaction product, until the methylester of deglycosylated polymerin, named CH_3Dpolymerin, showed a constant diffuse reflectance infrared Fourier transform (DRIFT) spectrum.

Protein Content Determination of Polymerin and KSDpolymerin. The protein concentration was estimated by the Bradford method (*13*) using bovine serum albumin as a standard.

Determination of Amino Acid Composition Protein Component of Polymerin and KSDpolymerin by an Automated Amino Acid Analyzer. Samples of polymerin and KSDpolymerin were hydrolyzed at 110 °C for 20 h with 6 N HCl in the presence of 0.1% phenol. Amino acid analyses were obtained with an amino acid analyzer (System Gold, Beckman Instruments, Inc.) equipped with the postcolumn ninhydrin detection system. Chemical and experimental conditions were as suggested by the manufacturer.

Carbohydrate Content Determination of Polymerin and KSDpolymerin. Carbohydrates were analyzed by means of an anthrone reagent (*14*) using D-mannose and D-galactose as standards.

Uronic Acid Content Determination of Polymerin and KSDpolymerin. Uronic acids were determined by means of the Blumenkrantz method (*15*) using glucuronic acid as standard.

Determination of Neutral and Acid Sugars Composition of Polysaccharide Component of Polymerin and KSDpolymerin by Anion Exchange with Pulsed Amperometric Detection (AE−PAD). Aliquots of polymerin and KSDpolymerin were hydrolyzed with 2 N HCl under refluxing for 20 h. Samples were purified by loading on a short Lichroprep RP-18 (Merck, Darmstadt, Germany, 25−40 μm) column (10 × 0.5 cm), which was eluted with hqw at low pressure (3 bar). A few milliliters of the filtrate was passed through a column (10 × 0.5 cm) packed with IRA-410-D Amberlite resin (Supelco, Inc., Bellefonte, PA). The column was first eluted with hqw to recover neutral sugars and then with 1 M NaOH for recovering acid sugars.

Sugars were determined by a Dionex model 2000 i/SP ion chromatograph, equipped with a Carbopac PA-10 (Dionex) column (4 × 250 mm, 10 μm thickness) and a Carbopac PA-10 (4 × 50 mm) guard column and revealed by pulsed amperometric detection. For neutral sugars eluents A (2 mM NaOH) and B (150 mM NaOH) were used at a flow rate of 1 mL min^{-1}. Mobile phase conditions were as follows: 0 min, eluent A/eluent B (100 + 0, start acquisition); 25 min, A/B (100 + 0, stop acquisition); 25.1 min, A/B (0 + 100, start clean up);

Table 1. Content (mg 100^{-1}) of Carbohydrates, Proteins, Melanin, and Metals in Polymerin and KSDpolymerin Determined by Chemical and Chromatographic Methods

	mg 100^{-1} [h] of organic and metal components			
	chemical analysis		chromatographic and spectrophotometric analysis	
organic and metal component	polymerin	KSDpolymerin	polymerin	KSDpolymerin
carbohydrates	52.40[a]	6.00[a]	55.00[d]	8.60[d]
proteins	10.40[b]	35.40[b]	16.60[e]	45.40[e]
melanin	26.14[c]	52.49[c]	17.34[f]	39.89[f]
(phenol + nonphenol aromatic units)	$(13.30^{c1} + 12.84^{c2})$[c]	$(27.00^{c1} + 25.49^{c2})$[c]		
metals	11.06[g]	6.11[g]	11.06[g]	6.11[g]
total	100	100	100	100

[a] Determined by anthrone reagent. [b] Determined by Bradford's reagent. [c] Determined by complement to 100 of the sum of the other components. [c1]Determined by Folin–Ciocalteu's reagent. [c2]Determined by difference between [c] and [c1]. [d] Determined by AE–PAD. [e] Determined by amino acid analyzer. [f] Amount of phenols obtained by the Perez method (*21*) and determined by complement to 100 of the sum of the amount of the other components. The qualitative identification was performed by HPLC. [g] Determined by atomic absorption spectroscopy (AAS). [h] The value 100 means the weight in mg of a sample of polymerin and KSDpolymerin.

40 min, A/B (0 + 100, stop clean up); 40.1 min, A/B (100 + 0, reequilibration); 50 min, A/B (100 + 0, stop reequilibration). Postcolumn addition of 300 mM NaOH at a flow rate of 0.6 mL min^{-1} optimized baseline stability and detector sensitivity.

Analysis of acid sugars, glucuronic and galacturonic acids, was performed using 100 mM NaOH and 150 mM NaOAc at a flow rate of 1 mL min^{-1}. Removal of carbon dioxide from the eluents was obtained by using hqw filtered through a 0.2-μm membrane filter and degassing by purging with helium for 20−30 min.

Polyphenol Content Determination of Polymerin and KSDpolymerin. Total phenol content was determined by the Folin−Ciocalteu method (*16*) using gallic acid as the reference standard and reading the absorbance at 760 nm. The phenol content determined by the Folin−Ciocalteu method does not provide the measure of nonphenol aromatic units which are also occurring in a melanin system (*17*−*20*), so the content of melanin was determined by complement to 100 of the sum of the amounts of the other chemical components of polymerin and KSDpolymerin (**Table 1**).

Determination of Phenol Composition of Melanin Component of Polymerin and KSDpolymerin. *Preparation of the Melanin Phenol Mixture Obtained by Oxidation with CuO.* Polymerin and KSDpolymerin were submitted to oxidation treatment according to the procedure reported by Perez et al. (*21*). Polymerin (50 mg) and KSDpolymerin (50 mg) were in separated experiments mixed with $Fe(NH_4)2SO_4)_2 \cdot 6H_2O$ (50 mg), CuO (250 mg), and NaOH 2 N (15 mL). The reaction was performed at 170 °C for 2 h. After cooling to room temperature, the supernatant was removed and the precipitate was resuspended in H_2O (10 mL) and centrifuged again. The two supernatants were collected, then acidified to pH 2 with HCl and left at room temperature for 1 h, and finally centrifuged at 11515*g* for 30 min. The resulting supernatant was extracted with ether (4 × 30 mL) for recovering phenols. The residue proved to be 3.2 mg for polymerin and 6 mg for KSDpolymerin.

Analysis of Melanin Phenols Mixture by High-Performance Liquid Chromatography (HPLC). All standard polyphenols (except hydroxytyrosol, which was obtained according to the procedure of Capasso et al. (*11*)), namely 3,5-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, catechol, tyrosol, *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde, syringic acid, 2,3 dihydroxybenzoic acid, vanillin, *p*-hydroxyphenylpropionic acid, methylcatechol, syringaldehyde, *p*-coumaric, ferulic acid, and salicylic acid were purchased from Sigma Chemical Co. and used at 1 mg/mL solutions in methanol for HPLC analysis. Sample volumes within the range of 1−5 μL were used.

A Perkin-Elmer 410 liquid chromatograph equipped with an LC 90 variable wavelength UV−visible spectrophotometric detector was used together with a Perkin-Elmer LC 100 integrator. The detector was set at 280 nm and the column was a Macherey and Nagel (Nucleosil RP 18; stainless steel 25 × 0.4 cm, i.d. 3 μm).

The following eluents were prepared: eluent A was a solution of a phosphate buffer at pH 2.0 with 10% acetonitrile; eluent B was a solution of acetonitrile with 30% of phosphate buffer at pH 2.0. The eluents were used and mixed according to the following program: 0 min, eluent A/eluent B (100 + 0, start acquisition); 20 min, A/B (100 + 0); 20.1 min, A/B (85 + 15); 40 min, A/B (85 +15); 40.1 min, A/B (70 + 30); 50 min, A/B (70 + 30); 50.1 min, A/B (0 + 100, start cleanup); 60 min, A/B (0 + 100, stop cleanup); 60.1 min, A/B (100 + 0, start reequilibration); 70 min, A/B (100 + 0, stop reequilibration).

In this case the phenol component content (reported later in **Table 5**) was also corrected by complement to 100 of the sum of the amounts of the other polymerin and KSDpolymerin chemical components, which were determined by chromatographic methods (**Table 1**). This was because of the complexity of the HPLC phenols chromatogram, which made a quantitative determination of the single phenol peak difficult.

Metal Determination of Polymerin and KSDpolymerin by Atomic Absorption Spectrometry (AAS). Metal cations were determined by a Perkin-Elmer model 3030 B atomic absorption spectrometer equipped with deuterium-arc background correction. Either air−acetylene or nitrous oxide−acetylene flames were used as an atomization source. Measurements were performed using the manufacturer's recommended operating parameters. Moreover, the spectrometer was controlled by AA Winlab software (Perkin-Elmer, Norwalk, CT). Background correction (AA−BG) was used for lower wavelength elements (Zn, Mn, Fe, and Cu) which could be more susceptible to interferences from molecular absorbance. The average and standard deviation of three absorption measurements were recorded for each sample.

All glassware, plasticware, and storage bottles used were previously immersed for several h in a solution containing 10% v/v concentrated HCl and 20% w/v concentrated HNO_3 (Carlo Erba, Milan, Italy) to avoid any kind of contamination, and they were air-dried before use. Stock standard solution of cations (1 g L^{-1}) was obtained from BDH Reagents (Poole, UK) and diluted in water containing the same amount of acids as the samples to obtain working standards.

Samples of 500 mg of polymerin and KSDpolymerin were digested with 8 mL of nitric acid (65% w/v) and 2 mL of perchloric acid (72% w/v) on a heating sand bath at 80 °C. Once dried, samples were added to 5% HCl solution, filtered through no. 40 Whatman paper filter, ∅ 12.5 cm, and raised to a final volume of 50 mL with 5% HCl.

UV Spectroscopy of Polymerin and KSDpolymerin. A Perkin-Elmer Lambda 3B UV/VIS spectrophotometer was used to analyze the total phenol, protein, and carbohydrate contents and to analyze the absorption of the two polymerins dissolved in hqw.

Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) of Polymerin and KSDpolymerin. Sample preparation for DRIFTS determinations was as follows: 0.2 mg of sample was mixed with 200 mg of KBr (FTIR grade, Aldrich Chemical Co., Milwaukee, WI). The mixture was finally ground in an agate mortar, then transferred to a sample holder, its surface was smoothed with a microscope glass slide, and DRIFT spectra were recorded. The DRIFT spectra were obtained using a Perkin-Elmer 1720 X FT−IR spectrophotometer and a diffuse-reflectance attachment (Perkin-Elmer). The diffuse-reflectance cell containing the samples was flushed with N_2 gas for 10 min before scanning to remove atmospheric water vapor and CO_2 from the

Table 2. Metal Composition (mg and mmol 100^{-1}) of Polymerin and KSDpolymerin Metal Component Determined by AAS

	mg 100^{-1} [a]		mmol 100^{-1} [a]	
metal	polymerin	KSDpolymerin	polymerin	KSDpolymerin
K^+	8.26	2.88	0.21	0.074
Na^+	0.82	1.21	0.036	0.053
Ca^{2+}	0.85	1.69	0.021	0.042
Mg^{2+}	0.46	0.10	0.019	0.0041
Fe^{3+}	0.54	0.15	0.0097	0.0027
Zn^{2+}	0.11	0.080	0.0017	0.0012
Cu^{2+}	0.02	0.00	0.00031	0.00
total	11.06	6.11	0.30	0.18

[a] The value 100 means the weight in mg of a sample of polymerin and KSDpolymerin.

spectrophotometer. The instrument had a special resolution of 1 cm^{-1}, which was used in all spectra determinations.

pH Determination and Automatic Potentiometric Titration of Polymerin and KSDpolymerin. pH was determined using an Orion expandable ion analyzer EA 940. Dry samples (50 mg) were dissolved in 20 mL od hqw, brought to pH 12.0 with 2 M NaOH, and brought to pH 2.0 with 0.05 M HCl solution by an automatic titrator (VIT 909 Videotritator, Copenhagen) under N_2 stream.

Relative Average Molecular Weight Determination of Polymerin and KSDpolymerin. The relative average molecular weights of polymerin and KSDpolymerin were separately determined by eluting these bioaggregates (5 mg) on a calibrated Biogel A (Pharmacia) chromatography column (60 × 1.5 cm), using a 0.5 M NaCl at native pH (6.5). Fractions of 1.5 mL were obtained and were monitored by means of the UV absorption at 270 nm and protein, carbohydrate, uronic acid, and phenol content. The calibration was obtained with the markers of sodium polystyrene sulfonate (Chemtek), with the following average molecular weight: 990, 130, 48.6, and 8.6 kDa, using the Whitaker method (*22*).

RESULTS AND DISCUSSION

The metal polymeric organic fraction was recovered from OMWW by substantially a three-step purification procedure (**Scheme 1**). In the first step, a dark, muddy sample of OMWW (1 L) was centrifuged and filtered, obtaining a dark, clear solution, which was concentrated to 0.5 L. In the second step, the concentrated sample was precipitated with cold methanol and centrifuged. In the third step, the dark precipitate was redissolved in ultrapure water, and dialyzed, and the nonpermeated fraction was lyophilized, leaving a brown residue of 11.3 g, which we named polymerin.

Chemical analysis revealed polymerin to be a very complex metal polymeric organic mixture. In fact, as shown in **Table 1**, it had a carbohydrate content of 52.40 mg 100^{-1} (w/w), a protein content of 10.40 mg 100^{-1}, and a melanin content of 26.14 mg 100^{-1}. Polymerin also showed a metal content of 11.06 mg 100^{-1} (**Table 1**), which consisted mainly of K^+ (8.26 mg 100^{-1}) and, in decreasing order of percentage weight, of Ca^{2+} (0.85 mg 100^{-1}), Na^+ (0.82 mg 100^{-1}), Fe^{3+} (0.54 mg 100^{-1}), Mg^{2+} (0.46 mg 100^{-1}), Zn^{2+} (0.11 mg 100^{-1}), and Cu^{2+} (0.02 mg 100^{-1}) (**Table 2**). In terms of mmolar percentage, K^+ proved to be 0.21 mmol 100^{-1} and, in decreasing order, Na^+ 0.036 mmol 100^{-1}, Ca^{2+} 0.021 mmol 100^{-1}, Mg^{2+} 0.019 mmol 100^{-1}, Fe^{2++} 0.0097 mmol 100^{-1}, Zn^{2+} 0.0017 mmol 100^{-1}, and Cu^{2+} 0.00031 mmol 100^{-1}, for a total amount of 0.30 mmol 100^{-1} (**Table 2**).

The hydrolysis of polymerin released an amount of 55 mg/100 (w/w) (0.32 mmol 100^{-1}) of carbohydrates (**Table 3**), corresponding to the polysaccharide component, which were determined by AE−PAD, and the corresponding monosaccharide composition is reported in **Table 3**. The main neutral sugars were arabinose (20.90 mg 100^{-1}, 0.13 mmol 100^{-1}), followed, in decreasing order, by galactose (10.50 mg 100^{-1}, 0.058 mmol 100^{-1}), glucose (9.90 mg 100^{-1}, 0.055 mmol 100^{-1}), and rhamnose (5.50 mg 100^{-1}, 0.034 mmol 100^{-1}). Glucuronic (4.40 mg 100^{-1}, 0.020 mmol 100^{-1}) and galacturonic (3.85 mg 100^{-1}, 0.020 mmol 100^{-1}) acids were also determined.

Table 3. Monosaccharide Composition (mg and mmol 100^{-1}) of Polymerin and KSDpolymerin Polysaccharide Component Determined by AE−PAD

	mg 100^{-1} [a]		mmol 100^{-1} [a]	
monosaccharide	polymerin polysaccharide	KSDpolymerin oligosaccharide	polymerin polysaccharide	KSDpolymerin oligosaccharide
arabinose	20.90	2.40	0.13	0.014
galactose	10.45	absent	0.058	absent
glucose	9.90	absent	0.055	absent
rhamnose	5.50	absent	0.034	absent
glucuronic acid	4.40	3.10	0.023	0.016
galacturonic acid	3.85	3.10	0.020	0.016
total	55.00	8.60	0.32	0.046

[a] The value 100 means the weight in mg of a sample of polymerin and KSDpolymerin.

Table 4. Amino Acid Composition (mg and mmol 100^{-1}) of Polymerin and KSDpolymerin Protein Component Determined by an Automated Amino Acid Analyzer

	mg 100^{-1} [a]		mmol 100^{-1} [a]	
amino acid	polymerin protein component	KSDpolymerin protein component	polymerin protein component	KSDpolymerin protein component
Asp + Asn	1.95	4.62	0.015	0.035
Glu + Gln	2.02	6.80	0.014	0.046
Cys	0.10	1.70	0.00083	0.014
Thr	0.87	2.10	0.0073	0.018
Ser	1.37	3.10	0.013	0.030
Pro	1.30	6.10	0.011	0.053
Gly	2.29	7.50	0.031	0.10
Ala	1.36	3.25	0.015	0.037
Val	1.35	1.20	0.012	0.010
Met	0.37	0.90	0.0024	0.0060
Ile	0.60	1.60	0.0046	0.012
Leu	1.15	3.00	0.0088	0.018
Tyr	0.55	1.00	0.0030	0.0055
Phe	0.54	1.33	0.0033	0.0081
Lys	0.054	0.00	0.00037	0.00
His	0.088	0.20	0.00057	0.0013
Arg	0.64	1.00	0.0037	0.0057
total	16.60	45.40	0.15	0.41

[a] The value 100 means the weight in mg of a sample of polymerin and KSDpolymerin.

The amino acid composition of the polymerin protein component is reported in **Table 4**. The main amino acids were Glu and Asp (about 4.00 mg 100^{-1}, overall), followed, in decreasing order of percentage weight, by the neutral amino acids Gly (2.29 mg 100^{-1}), Ala, Val, and Ser (ca. 1.40 mg 100^{-1}), Pro (1.30 mg 100^{-1}), Leu (1.15 mg 100^{-1}), Thr (0.87 mg 100^{-1}), Ile, Phe, and Tyr (ca. 0.60 mg 100^{-1}), Cys (0.10 mg 100^{-1}), and, finally, by the basic amino acids Arg, His, and Lys (0.64, 0.088, and 0.054 mg 100^{-1}, respectively) for a total amount of 16.60 mg/100.

As regards the amino acid mmolar percentage composition (**Table 4**), the main amino acid was Gly (0.031 mmol/100), followed by the acid amino acids Glx and Asx, at similar amounts (0.029 mmol/100, overall); a smaller content was observed for the other neutral amino acids; last, with regard to

Table 5. Phenol Composition of Polymerin and KSDpolymerin Melanin Component Determined by CuO Oxidation and HPLC and Compared with that of Perez et al. (*21*) Determined on the Dark Polymeric Fraction of OMWW

phenols	polymerin and KSDpolymerin	Perez et al. (*21*)
hydroxytyrosol (5.19)	-	-
3,5-dihydroxybenzoic acid (5.29)	+	-
3,4-dihydroxy phenylacetic acid (6.01)	-	-
catechol (7.84)	+	-
p-tyrosol (8.64)	-	+
p-hydroxybenzoic acid (9.68)	+	+
vanillic acid (11.39)	+	+
p-hydroxybenzaldehyde (12.41)	+	+
syringic acid (14.40)	-	+
2,3-dihydroxybenzoic acid (14.55)	-	-
vanillin (16.83)	+	+
p-hydroxyphenylpropionic acid (17.24)	-	+
methylcatechol (17.91)	-	-
syringaldehyde (21.46)	+	+
p-coumaric acid (31.59)	+	+
ferulic acid (35.77)	+	+
salicylic acid (40.26)	+	-

[a] Retention time values of the phenol standards are reported in parentheses.

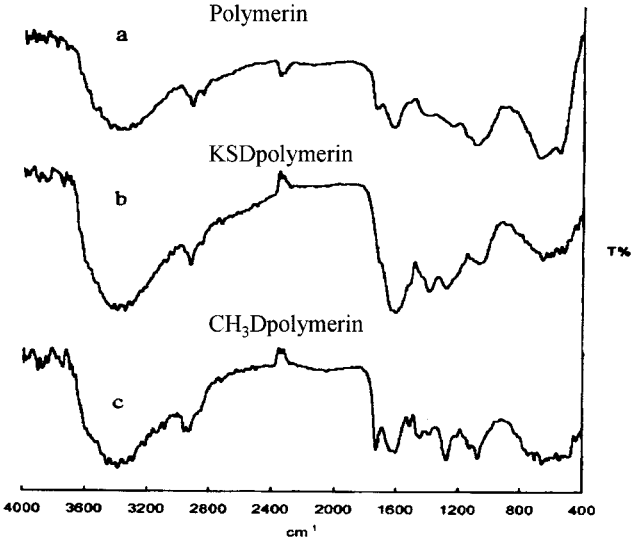


Figure 1. DRIFT spectra of (a) polymerin, (b) KSDpolymerin, and (c) CH_3Dpolymerin. The absorption values (cm^{-1}) are reported in detail in **Table 6**.

the basic amino acids, Arg, His, and Lys were 0.0037, 0.00057, and 0.00037 mmol/100, respectively. The total amount was 0.15 mmol/100.

Importantly, the acid amino acids present in polymerin and KSDpolymerin (see below) are likely present in both acid (Asp and Glu) and amide form (Asn and Gln). This means that the ammidate and acid forms of acid amino acids are generally equivalent in the proteins according to Dayhoff (*23*). This appears confirmed by the DRIFT spectrum of CH_2N_2-treated KSDpolymerin, named CH_3Dpolymerin, where the band of $CONH_2$ at 1441 cm^{-1} can be attributed to the Asn and Gln (shown later in **Figure 2c** and **Table 6**).

The qualitative phenol composition of the melanin component was determined by HPLC analysis of a sample obtained after a strongly oxidative treatment of this polymeric mixture, according to the procedure described previously by Perez et al. (*21*). The phenols thus identified are reported in **Table 5**. In addition to those identified by Perez et al. (*21*), we detected catechol, *p*-hydroxybenzoic acid, salicylic acid, and 3,5-dihydroxybenzoic acid, whereas we did not find tyrosol, *p*-hydroxyphenylacetic acid, or syringic acid. Catechol and salicylic acid were also identified by Piattelli et al. (*17*, *18*) in a fungal melanin system. Vas et al. (*20*) found in the degradation products of some melanins of different sources, in addition to phenol, benzoic acid phenyl ester, methyl phenol, benzaldehyde, and ethenylbenzene, which correspond to the base structure of the benzene derivatives (**Table 5**) identified in the polymerin melanin component.

The total amount of phenols was calculated to be 17.34 mg/100 (see **Table 1** and the experimental procedure section).

In addition to the characteristic phenol system and other substituted aromatic rings, we also found evidence of the presence of carboxylic groups, which were detected by the automated acid−base titration. Two flex points were monitored at pH = 10 (pK_a of phenol) and at pH 4.50 (pK_a of carboxylic group). In addition, the treatment with CH_2N_2 of a sample of the polymerin derivative KSDpolymerin (see below) revealed the formation of carboxymethyl groups detected by DRIFTS. All these data are in accordance with those shown by the analysis of melanins performed by Piattelli et al. (*17*, *18*) and more recently by Paim et al. (*19*). In our case, the contribution for the carboxylic groups derives also from the uronic acids and acid amino acids from polysaccharide and melanin components, respectively (**Tables 3** and **4**).

However, the melanin nature of the polymerin phenol polymeric component is strongly supported by the DRIFT spectra of polymerin and its derivatives KSDpolymerin and CH_3Dpolymerin (see below for a detailed description of the spectra and **Figure 1** and **Table 6**), which proved to be very similar to the infrared spectra described in the literature (*19*, *24*−*26*) for the melanin biomaterial. In fact, all the infrared bands of our complex polymers and those of the melanins reported in the literature occur at very close frequency values in the 3350−3450, 1600−1630, 1700−1735, 1390−1450, 1280−1240, and 1050−1100 cm^{-1} regions, and in some cases they are coincident, like the bands at 2925 and 1240 cm^{-1}. The latter is characteristic of C−OH and C−O−C phenol and phenol ether stretching.

We therefore believe that the phenol polymeric component of polymerin is a melanin pigment, which confers the characteristic brown color to the very soluble polymerin and consequently to OMWW. In fact, the solubility of this melanin pigment is a peculiar property of polymerin, due to the polysaccharide and protein aggregation, whereas the melanins alone are known to be very insoluble biomaterial.

The content of melanin in polymerin proved to be 26.14 mg 100^{-1} (**Table 1**).

The ultraviolet and visible absorptions at 270 and 470 nm, shown by the considered polymeric mixture, are consistent with the presence of the chromophoric melanin and protein therein.

The DRIFT spectrum (**Figure 1a** and **Table 6**) of polymerin revealed a very strong, broad band centered at 3387 cm^{-1}, which is consistent with the overlapping of the absorptions of the OH groups of the polysaccharide component, the melanin system, and the peptide NH group. Moreover, these groups were also detected by chemical, physicochemical (automated titration of phenolic OH and COOH), and chromatographic analyses, as reported above.

A sharp and medium band appears at 2922 cm^{-1}, arising from the stretching of CH groups of the polysaccharide and the side chains of protein. A characteristic strong absorption band appears at 1097 cm^{-1}, due mainly to alcoholic bonding of the polysaccharide component and, to a lesser extent, to serine and threonine C−OH and C−NH_2 stretching of basic amino acids.

Table 6. Physicochemical Analysis and Spectroscopic Data of Polymerin, KSDpolymerin, and CH_3Dpolymerin[a]

	polymerin	KSDpolymerin	CH_3Dpolymerin
titration	1st flex point pH 10.0 = pK_a OH (phenol) 2nd flex point pH 4.5 = pK_a COOH (carboxylic acid)	1st flex point pH 10.0 = pK_a OH (phenol) 2nd flex point pH 4.5 = pK_a COOH (carboxylic acid)	
UV: λ_{max}^{MeOH} nm	270 (shoulder) 470 (peak)	270 (shoulder) 470 (peak)	
DRIFTS: ν_{max}^{KBr} cm^{-1}	3387 (s) OH, NH peptide 2922 (m) C–H 1735 (m) C═O ester	3394 (s) OH, NH peptide 2926 (m) C–H 1603 (s) C═C aromatic nucleus + C═O peptide + COO^-	3387 (s) OH e NH peptide 2961 (m) C–H 1725 (s) C═O ester
	1626 (s) C═C aromatic nucleus + C═O peptide + COO^-	1395 (m) COO^-	1601 (s) C═C aromatic nucleus + C═O peptide+ COO^-
	1400 (w) COO^-	1279 (m) C–O–C aromatic nucleus + C–OH phenol + OC–O–CH_3 ester	1512 (m) aromatic nucleus
	1240 (w) C–OH phenol + C–O–C aromatic nucleus + OC–O–CH_3 ester	1072 (w) C–O–C ether e C–OH sugars + NH_2 basic amino acid	1441 (m) $CONH_2$
	1097 (s) C–OH sugars and amino acid + NH_2 basic amino acid 678 (s) aromatic nucleus	658 (w) aromatic nucleus	1273 (s) C–OH phenols + C–O–C aromatic nucleus + COC ester 1073 (m) C–OH amino acid and sugars + NH_2 basic amino acid 678 (w) aromatic nucleus
average relative mw (kDa)	1st peak 500.0 2nd peak 100.0 3rd peak 11.3 4th peak 2.0	1st peak 6.3	

[a] s = strong; m = medium; w = weak.

A weaker band is observed in the spectrum at 1240 cm^{-1}, due to the absorption of C−OH phenolic bonding, which overlaps with the absorption of the C−O−C ether bond of the polymerin melanin component. The latter system also possesses a correlated absorption at 1626 (aromatic C═C stretching) and 678 cm^{-1} (bending of aromatic nucleus).

The band at 1626 cm^{-1} is also due to the absorption of a C═O peptide group, to which the absorption of an NH peptide group with the band centered at 3387 cm^{-1} is also correlated.

A further characteristic, albeit weak, band, appears at 1735 cm^{-1}, arising, in all likelihood, from the absorption of the C═O methylester group. Its correlated absorption, due to the stretching of OC−CH_3 bond, should be included in the band at 1240 cm^{-1}. These data were also previously found by Drake et al. (*27*) in DRIFTS analysis of naturally occurring plant biomasses and were attributed to a methylester group.

Finally, a weak band at 1400 cm^{-1} is to be attributed to the COO^- asymmetric stretching vibrations, whose symmetric stretching vibrations are likely included in the strong band at 1626 cm^{-1}. These groups were also detected by the automated acid−base titration (see above)

Investigation of the molecular size of polymerin was also performed (see experimental procedure section).

Figure 2a shows that the polymerin molecular size ranges approximately between values of 500 and 2 kDa. In particular, the curves relative to the melanin component (circle points), that relative to the protein component (triangle points), and the absorption curve at 270 nm (square points), appear approximately distributed in the range of 500.0 (point 1) and 2.0 kDa (point 4), with two peaks at 500.0 and 11.3 kDa (point 3). These curves appear all coeluted with the same profile, indicating that the protein and melanin components are, in all likelihood, strongly aggregated.

The curve (rhombus points) relative to the polymerin polysaccharide component, which includes the curve (dash points) of uronic acids as an integral part, appears distributed in a range of molecular size wider than that of the other two polymerin organic components, lying approximately between 500.0 and 2.0 kDa (points 1 and 4 in **Figure 2a**): the curve in question includes three maximum points corresponding to 500.0 (point 1), 100.0 (point 2), and 2.0 kDa (point 4), and a minimum point at 11.3 kDa (corresponding to point 3). The chromatographic behavior of the relative polysaccharide component indicates that this is only in part in aggregated form, i.e., those parts corresponding to 500.0 (point 1) and 11.3 kDa (point 3) (see also below the dicussion on the molecular size of KSDpolymerin). The remaining part is in free form, ranging approximately between 100.0 (point 2) and 2 kDa (point 4). In fact, this part corresponds to two points (2 and 4) of the curve with an elution profile substantially dephased with respect to the curve of protein (triangle points) and melanin (circle points) components (**Figure 2a**).

Summing up, polymerin shows in the elution patterns of **Figure 2a** four peaks corresponding to values of relative molecular size of 500.0, 100.0, 11.3, and 2.0 kDa. The protein and melanin moieties are completely aggregated to one another, whereas the polysaccharide component is only in part aggregated, the remainder being free.

The chemical, chromatographic, acid−base titration, and spectroscopic data herein described indicate that K^+ and the all other metal cations are bound to the carboxylate anions identified in the acid amino acids, uronic acids, and the melanin system. In addition, other functional groups, such as SH from Cys, NH_2 from Arg, and alcohol OH from sugars and amino acids, could be involved in the chelating bonding of the metal cations. The presence of bonding between metals and both the aggregated and free forms of polysaccharide may also be inferred from the curve of the uronic acids (dash points), occurring along the entire elution patterns of **Figure 2a** and representing the integral part of the polysaccharide aggregate

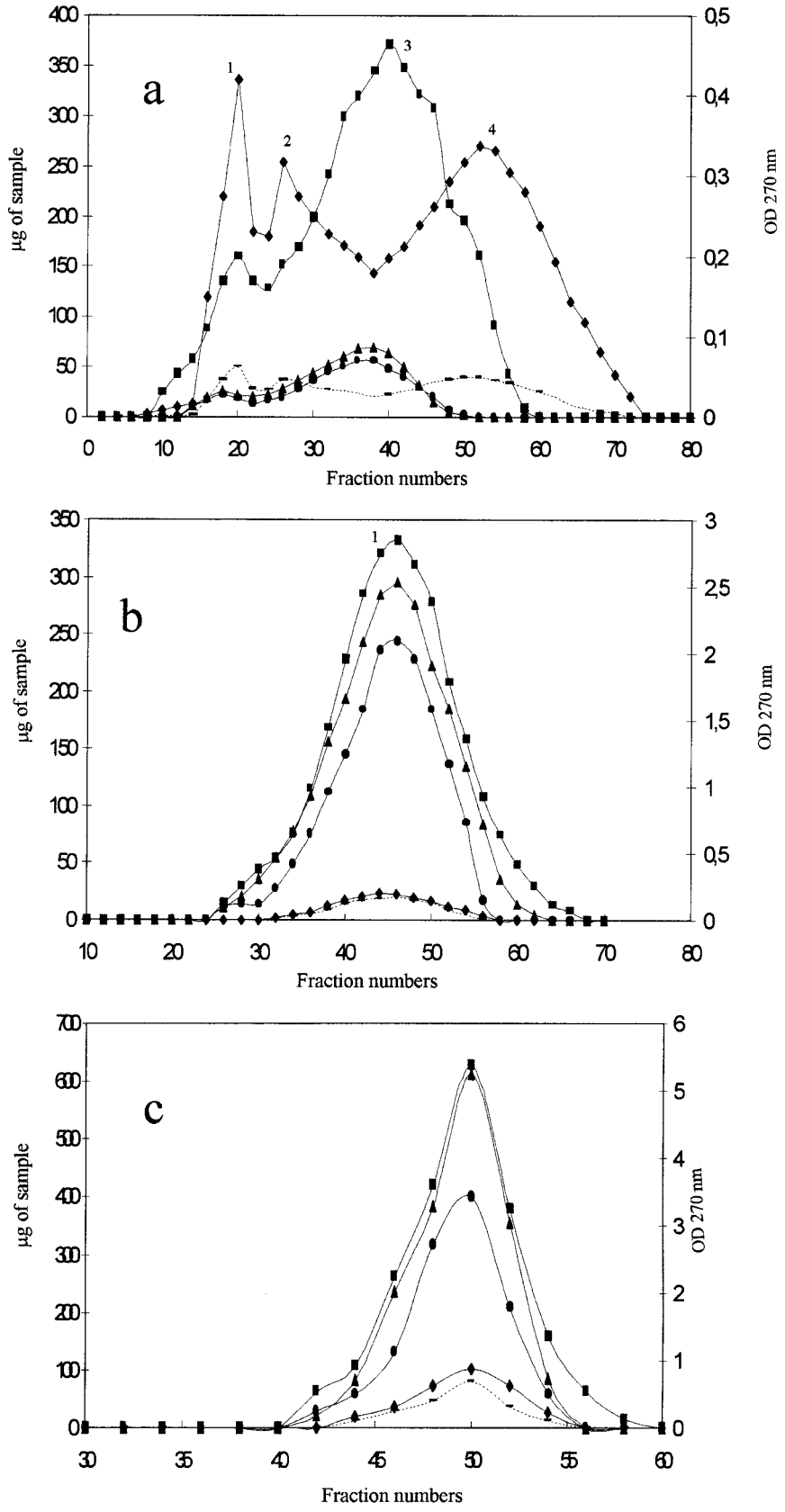


Figure 2. (a) Elution patterns of polymerin by Biogel A chromatography. The curves represent: rhombus points, the polysaccharide component; dash points, uronic acids which are an integral part of polysaccharides; triangle points, the protein component; circle points, the melanin component; last, square points, the UV absorbance at 270 nm of the complex mixture; 1 (500 kDa), 2 (100 kDa), 3 (11.3 kDa), and 4 (2 kDa). (b) Elution patterns of KSDpolymerin by Biogel A chromatography: the curves represent the same kind of component indicated for polymerin in (a); 1 (6.3 kDa). (c) Elution patterns of KSDpolymerin by RP-18 chromatography: the curves represent the same kind of components indicated for KSDpolymerin in (b).

and free portion of polymerin; the uronic acids are the counteranions of the metals considered above. Moreover, Arienzo and Capasso (*3*) showed that only a small amount of K^+ was free (ca. 30%) in OMWW solution, with the remainder being bound to the polymeric organic fraction, and all the other metals, including Na^+, were bound to it for over 90%.

Polymerin was transformed into a potassium salified deglycosylated derivative (see **Scheme 2** and experimental procedure section) termed KSDpolymerin, which was characterized in order to confirm the chemical nature of the polymeric organic components of polymerin, their aggregate status, at least partially for the polysaccharide part, and the bonding of the metal component with the polymeric organic moieties through carboxylate anions.

In particular, the acid hydrolysis of polymerin formed a dark precipitate, termed ADpolymerin, which represents an acid deglycosylated intermediate biomaterial for the production of KSDpolymerin. In fact, ADpolymerin was transformed into KSDpolymerin by dissolution using 2 N KOH. This phase was dialyzed to neutralize the dark nonpermeated soluble fraction, which left after lyophilization a residue of 43 mg of KSDpolymerin from 100 mg of polymerin (**Scheme 2**).

Chemical analysis of KSDpolymerin, compared with that of polymerin (**Table 1**), shows a substantial loss of the polysaccharide moiety, from 52.40 to 6.00 mg 100^{-1} (w/w), and an increase in the protein and melanin moieties, from 10.4 to 35.40 mg 100^{-1} and from 26.14 to 52.49 mg 100^{-1}, respectively. We also observed a decrease in the metal component, from 11.06 to 6.11 mg 100^{-1}.

The residual oligosaccharide moiety of KSDpolymerin was removed by a drastic acid hydrolysis, and the resulting sugars were determined by AE−PAD. As shown in **Table 3**, they proved to be glucuronic acid (3.10 mg 100^{-1}), galacturonic acid (3.10 mg 100^{-1}), and arabinose (2.4 mg 100^{-1}), for a total amount of 8.60 mg 100^{-1}. In terms of mmolar percentage, the decrease in monosaccharides from 0.32 to 0.046 mmol 100^{-1}, observed in KSDpolymerin compared with that of polymerin, was due especially to the marked decrease in arabinose (from 0.13 to 0.014 mmol 100^{-1}) and the complete loss of galactose, glucose, and rhamnose (**Table 3**).

The amino acid composition, relative to the protein component of KSDpolymerin, which was determined in a sample obtained by a strong acid hydrolysis of polymerin (experimental section), is illustrated in **Table 4**. As shown in the third column, the acid amino acids are prevalent (11.42 mg 100^{-1}, overall), as in polymerin. As regards the neutral amino acids, we observed the following in decreasing amounts: Gly (7.50 mg 100^{-1}), Pro (6.10 mg 100^{-1}), Ala, Ser, and Leu (3.25, 3.10, and 3.00 mg 100^{-1}, respectively), Thr (2.10 mg 100^{-1}), Cys, Phe, and Val (1.70, 1.33, and 1.20 mg 100^{-1}, respectively), and Met (0.9 mg 100^{-1}). Arg (1.00 mg 100^{-1}) and His (0.20 mg 100^{-1}) were detected only under the basic amino acids. The total amount was 45.40 mg 100^{-1}. In relation to the mmolar percentage composition (**Table 4**), such as shown in polymerin, the main amino acid was Gly (0.10 mmol 100^{-1}), followed by the acid amino acids Glx and Asx, in similar amounts (0.081 mmol 100^{-1} overall) and, in all likelihood, in both amide and acid form (*23*). As regards the other amino acids, the only considerable difference, with respect to polymerin, was the decrease in Val and Hys and the loss of Lys, which can be attributed to the fact that these three amino acids could be in terminal positions in polymerin, hence more easily removable. In addition, we detected a sharp increase in Cys (from 0.00083 to 0.014 mmol 100^{-1} mg), which can be attributed to the break of the disulfide and/or sulfide bridges between cysteins in the protein and/or cysteins and melanin component of polymerin, as also previously proposed by Bell and Wheeler (*28*), regarding the fungal melanins. The total mmolar percentage amount was 0.41 mmol 100^{-1} (**Table 4**).

The polymeric phenol component of KSDpolymerin confirms the melanin nature detected in polymerin. Indeed, HPLC analysis of the mixture obtained by treatment of samples of KSDpolymerin with CuO provided the same qualitative phenol composition shown by polymerin (**Table 5**). The total weight percentage proved to be 39.89 mg 100^{-1}. The titration of the polymeric mixture also revealed in this case phenol ($pK_a = 10$) and carboxylic groups ($pK_a = 4.50$), and its DRIFTS data (see below for a detailed description) confirm the high similarity with the infrared data of melanins (*19*, *20*, *24*−*26*), in accordance with that discussed above for polymerin.

The composition of the KSDpolymerin metal component with respect to that of polymerin (**Table 2**) reveals a significant decrease in K^+, from 8.06 to 2.88 mg 100^{-1} (from 0.21 to 0.074 mmol 100^{-1}), in Mg^{2+}, from 0.46 to 0.10 mg 100^{-1} (from 0.019 to 0.0041 mmol 100^{-1}), Fe^{3+} from 0.54 to 0.15 mg 100^{-1} (from 0.0097 to 0.0027 mmol100^{-1}), and of Zn^{2+} from 0.11 to 0.080 mg 100^{-1} (from 0.0017 to 0.0012 mmol 100^{-1}); Cu^{2+} disappears, whereas Na^+ and Ca^{2+} increase from 0.82 to 1.21 mg 100^{-1} (from 0.036 to 0.053 mmol 100^{-1}) and from 0.85 to 1.69 mg 100^{-1} (from 0.021 to 0.042 mmol 100^{-1}), respectively.

These data indicate that K^+, Mg^{2+}, Zn^{2+}, Fe^{3+}, and Cu^{2+} were bound and chelated in polymerin mainly to its entire polysaccharide component (i.e., both the free and aggregate forms), whereas Ca^{2+} and Na^+ were in prevalence bound in the polymerin to the protein, melanin, and the residual oligosaccharide component of KSDpolymerin.

The results of KSDpolymerin analysis indicate a reduction of metal and polysaccharide components, and the increase in the protein and melanin moieties relative to polymerin, but the melanin keeps substantially the same molecular composition and the protein keeps a very similar one. The lack of a complete identity of the protein moiety arises from the loss of the more exposed amino acids and the breaking of sulfide and /or disulfide bridges during the hydrolysis of polymerin. In addition, the UV−Vis KSDpolymerin spectrum confirms the same absorptions at 270 nm and at 470 nm observed in polymerin, attributed to the protein and melanin mixture (**Table 6**).

The DRIFT spectrum (**Figure 1b** and **Table 6**) of KSDpolymerin shows, with respect to polymerin, the shift of the COO^- asymmetric stretching band from 1400 to 1395 cm^{-1}, with a stronger and more resolved band than that of polymerin, while the symmetric stretching band is associated to those of the other functional groups at 1603 cm^{-1}. The higher intensity and resolution of the band at 1395 cm^{-1} in KSDpolymerin may originate from the acid hydrolysis of the carboxymethyl group, which is transformed into COO^-.

The severe decrease in the band of the C−OH linkage at 1072 cm^{-1} in KSDpolymerin has to be attributed to the loss of most of the polysaccharide component of polymerin after acid hydrolysis. Finally, the band of the stretching of the C−OH linkage of the phenol group shifts from 1240 cm^{-1} in polymerin to 1279 cm^{-1} in KSDpolymerin.

This latter was transformed into its methylester deglycosylated derivative, named CH_3Dpolymerin, by CH_2N_2 and analyzed by DRIFTS (**Figure 1c** and **Table 6**).

This treatment caused the appearance of two strong bands at 1725 and 1273 cm^{-1}, which are characteristic of the stretching of CO and CO−O−CH_3, respectively, of the carboxymethylester

group, formed by the reaction of COO^- with CH_2N_2. These bands appear much more increased and resolved with respect to those corresponding to the KSDpolymerin spectrum. This is caused by the transformation of the carboxylate anions by CH_2N_2 and the achievement of a higher vibrational freedom of the carboxymethylester group, no longer involved in the bonding with the metals.

Interestingly, all the signals shown by CH_3Dpolymerin DRIFT spectrum appear much more resolved than those of KSDpolymerin (**Figure 1b,c**), because of the loss of bonding between the metals and COO^- and other functional groups (C−OH of sugars, Ser and Thr, SH of Cys, involved, in all likelihood, together with the same COO^-, in a chelating bond), with the consequent high vibrational freedom of the considered groups. Therefore, these data confirm that the metal cations present in polymerin and in KSDpolymerin are bound through carboxylate anions coming out from the acid amino acids, the uronic acids, and the melanin system.

It is to be noted that a band also appears at 1441 cm^{-1} in the CH_3Dpolymerin spectrum, which can be attributed to the stretching of the $CONH_2$ group of Asn and Gln. This band most probably results from the relative decrease in the absorption of the COO^- at 1386 cm^{-1}, due its conversion into a methyl ester group.

The relative molecular size of KSDpolymerin was assessed like that of polymerin (experimental procedures section). The chromatogram shown in **Figure 2b** consists of a single group of five peaks coeluted with a very similar profile and corresponding to a relative molecular weight of 6.3 kDa (**Figure 2b** and **Table 6**). The curve marked with square points, corresponding to the absorption at 270 nm, is due to the protein and melanin moieties, whereas the other three curves are to be attributed to the protein (triangle points), the melanin (circles points), and the oligosaccharide (rhombus points) moiety. The fifth peak (dash points) is relative to the uronic acids (glucuronic and galacturonic acids), which are an integral part of the oligosaccharide component.

Summing up, the elution patterns of **Figure 2b** confirm that the carbohydrate content in KSDpolymerin is much smaller than that of the other two organic components and indicates that these components are strongly bound to each other in an aggregate form. Obviously, the metal component is bound to this system by the carboxylate anions.

To confirm the aggregate status of KSDpolymerin, a sample of the mixture was also chromatographed through an RP-18 column eluted stepwise under low pressure with water and a mixture of water and acetonitrile (50:50), obtaining the elution patterns reported in **Figure 2c**. KSDpolymerin shows also in these elution patterns the same behavior observed in the elution patterns of **Figure 2b**. A single group of five peaks coeluted with the same profile appears in the elution patterns of **Figure 2c** obtained by eluting KSDpolymerin with the water/acetonitrile mixture (50:50). Such behavior can originate only from a strong bonding among the organic components of the mixture, confirming an aggregate status of these latter.

Regarding the nature of chemical bonds among the organic components of KSDpolymerin, they are highly likely to consist of covalent linkages and a combination of hydrogen bonds with CH/Π interactions. As regards the covalent bonding, we only found evidence for the possibility of sulfide bridges between the protein moiety and the melanin component, as we found an increased amount of cystein residue in the protein moiety of KSDpolymerin in comparison with that found in polymerin (**Table 4**). However, such a kind of bonding has been previously described in fungal melanins (*28*).

Hydrogen linkages and CH/Π interactions should greatly contribute to stabilizing the aggregate status of KSDpolymerin. In fact, numerous hydrogen linkages can be formed between the protein moiety functional groups such as peptide NH, NH_2, and OH, arising from the side chain of the protein primary structure, and the ether oxygen bridges of melanin polymer, such as between the latter and OH groups of the oligosaccharide moiety. In the same way, many CH/Π interactions can be formed between the CH groups of sugars and proteins with the molecular Π orbitals of the benzene ring of the melanin system. This kind of bonding between CH groups and polyaromatic systems is extensively reviewed by Nishio et al. (*30*).

In conclusion, KSDpolymerin is a metal polymeric organic aggregate composed of protein, oligosaccharide, and melanin components, and a metal moiety bound to the organic system through carboxylate anions and other nucleophilic functional groups, also in chelating form, forming overall a natural supramolecular structure. Polymerin is a more complex and greater size mixture composed of an aggregate supramolecular portion and a polysaccharide free portion, both bonding the above considered metals, of which K^+ is prevalent (**Table 2**). The aggregate form which contains the melanin polymer represents the characteristic dark polymeric pigment of OMWW.

The presence of proteins, sugars, and melanin, as they are sources of inorganic macronutrients such as oxygen, carbon, and nitrogen, and of macro- and micronutrient metals, make both polymerin and KSDpolymerin very promising biomaterials to be used in agriculture as metal biointegrators and bioamendments. In particular, polymerin is rich in K^+, which is the more prevalent macronutrient compared with the other macro- (Ca^{2+} and Mg^{2+}) and micronutrient metals (Zn^{2+}, Mn^{2+}, Fe^{3+}, and Cu^{2+}) (**Table 2**). In addition, the melanins are humic acid-type polymers which are involved in soil fertility. This similarity has been previously demonstrated by Paim et al. (*19*), Biliska (*26*), and Linhares and Martin (*29*). As confirmation, we found that our DRIFT spectra of KSDpolymerin were very similar to the DRIFT spectra of Na- and Cu-humate, published by Piccolo and Conte (*31*).

Even though KSDpolymerin is analogous to polymerin and richer in Ca^{2+} (**Table 2**), its lower content of other metals and sugars limits large scale industrial applications. These are also hindered by the more complex and time-consuming extraction procedure.

The use of raw OMWW in trials for increasing fertility in soil is well-known, so the utilization of polymerin and its derivative KSDpolymerin, which are more refined products than OMWW, could be of major interest for use in agriculture.

Finally, the characteristics of polyelecrolyte of polymerin and its relatively high content of the very removable K^+ suggest this biomaterial may be used as a potential biofilter for solutions contaminated by heavy and/or unwanted metals. In fact, the potential use of plant biomasses for decontamination of wastewaters by biosorption of pollutants is of great importance and interest because it allows the resolution of both the problem of their disposal and the decontamination of wastewaters by environmentally clean processes (*32*).

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LITERATURE CITED

(1) Demicheli, M.; Bontoux, L. *Survey on Current Activity on the Valorization of Byproducts from Olive Oil Industry*. European Commission, Joint Research Centre: Brussels, 1997; pp 4−24.

(2) Pacifico, A. Acque di vegetazione. In Agricoltura ed Innovazione. *Notiziario dell'ENEA e di RENAGRI*, July-September, **1989**, *11*, 33−37.

(3) Arienzo, M.; Capasso, R. Analysis of metal cations and inorganic anions in olive oil mill wastewaters by atomic absorption spectroscopy and ion chromatography. Detection of metals bound to the organic polymeric fraction *J. Agric. Food Chem*. **2000**, *48*, 1405−1410.

(4) Capasso, R. The chemistry, biotechnology and ecotoxicology of the polyphenols naturally occurring in vegetable wastes. In *Current Topics in Phytochemistry*; Asakawa, J., Gottlieb, O. R., Towers, G. H. N., Wagner, H. Waterman, P. G., Eds.; Research Trends: Trivandrum, India, 1997; vol. 1, pp 145−156.

(5) Sanjut, E.; Pompei, R.; Resiqno, A.; Rinaldi, A.; Ballero, M. Olive milling wastewater as a medium for growth of four *Pleurotus* species. *Appl. Biochem. Biotechnol*. **1991**, *31*, 223−235.

(6) Garcia-Barrionuevo, A.; Moreno, E.; Quevedo-Sarmiento, J.; Gonzalez-Lopez, J.; Ramos Cormenzana, A. Effect of wastewaters from olive oil mills (alpechin) on *Azotobacter* nitrogen fixation in soil. *Soil Biol. Biochem*. **1992**, *24*, 281−283.

(7) Sayadi, S.; Ellouz, R. Decolourization of olive mill wastewaters by the white-rot fungus, *Phanerochaete chrysosporium*: involvement of the lignin-degrading system. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 813−817.

(8) Vlyssides, A. G.; Bouranis, D. L.; Loizidou, M.; Karvouni, G. Study of a demonstration plant of the co-composting of olive-oil-processing wastewater and solid residue. *Bioresour. Technol.* **1996**, *56*, 187−193.

(9) Paredes, C.; Cegarra, J.; Roig, A.; Sánchez-Monedero, M. A.; Bernal, M. P. Characterization of olive mill wastewater (alpechin) and its sludge for agricultural purposes. *Bioresour. Technol*. **1999**, *67*, 111−115.

(10) Sayadi, S.; Allouche, N.; Jaoua, M.; Aloui, F. Detrimental effects of high molecular-mass polyphenols on olive mill wastewater biotreatment. *Process Biochem.* **2000**, *35*, 725−735.

(11) Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural wastewaters. *J. Agric. Food Chem*. **1999**, *47*, 1745−1748.

(12) Chimatsu, Y.; Ando, H.; Yamamoto, A.; Kyo, S.; Yamashita, K.; Dojo, K. Hydroxytyrosol as melanin formation inhibitor and lipid peroxide formation inhibitor and its application to topical preparations and bath preparations. Japanese Patent n. 8, 119825, 1−10, 1996.

(13) Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.

(14) Fung, F.; Scheffer, F. L.; Kirk, P. K. The ultramicro-determination of glycogen in liver. A comparison of anthrone and reducing sugars methods. *Arch. Biochim. Biophys. Acta* **1953**, *71*, 319−326.

(15) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484−489.

(16) Jenning, A. C. The determination of dihydroxy phenolic compounds in extracts of plant tissues. *Anal. Biochem*. **1981**, *118*, 396−398.

(17) Piattelli, M.; Fattorusso, E.; Magno, S.; Nicolaus, A. Ustilago melanin, a naturally occurring catecholmelanin. *Tetrahedron Lett.* **1963**, *15*, 997−998.

(18) Piattelli, M.; Fattorusso, E.; Nicolaus, A.; Magno, S. The structure of melanins and melanogenesis. *Tetrahedron* **1965**, *21*, 3229−3236.

(19) Paim, S.; Linhares, L. F.; Mangrich, A. S.; Martin, J. P. Characterization of fungal melanins and soil humic acids by chemical analysis and infrared spectroscopy. *Biol. Fertil. Soils* **1990**, *10*, 72−76.

(20) Vas, G.; Vekey, K.; Czira, G.; Tamas, J.; Favretto, D.; Traldi, P.; Bertazzo, A.; Costa, C.; Allegri, G. Chracterization of melanins by pyrolysis/gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 870−873.

(21) Perez, I.; Hernandez, M. T.; Ramos Cormenzana, A. S.; Martinez, S. Caracterización de fenoles del pigmento del alphecin y transformación por *Phanerochaete chrysosporium.* Grasas Aceites **1987**, *38*, 367−371.

(22) Whitaker, J. R. Determination of molecular weights of protein by gel filtration on Sephadex. *Anal. Chem.* **1963**, *35*, 1950−1953.

(23) Dayhoff, M. O. *Atlas of Protein Sequences and Structure*, 4th ed.; National Biomedical Research Foundation: Silver Spring, MD, 1978, p 363.

(24) Russel, J. D.; Jones, D.; Vaughan, D.; Fraser, A. R. A preliminary study of fungal melanin by infrared spectroscopy. *Geoderma* **1980**, *24*, 207−213.

(25) Pierce, J. A.; Rast, M. D. Comparison of native and synthetic mushroom melanins by Fourier Transform Infrared Spectroscopy. *Phytochemistry* **1995**, *39*, 49−55.

(26) Biliska, B. Progress of infrared investigations of melanin structures. *Spectrochim. Acta Part A: Mol. Spectrosc.* **1996**, *52*, 1157−1162.

(27) Drake, L. R.; Shan, L.; Rayson, G. D.; Jackson, P. J. Chemical modification and metal binding studies of *Datura Innoxia*. *Environ. Sci. Technol.* **1996**, *30*, 110−114.

(28) Bell, A. A.; Wheeler, M. H. Biosynthesis and functions of fungal melanins. *Annu. Rev. Phytopathol*. **1986**, *24*, 411−451.

(29) Linhares, L. F.; Martin J. P. Carbohydrate content of fungal humic acid-type polymers (melanin). *Soil Sci. Soc. Am. J.* **1979**, *43*, 313−318.

(30) Piccolo, A.; Conte, P. Advances in Nuclear Magnetic Resonance and Infrared Spectroscopy of Soil Organic Particles. In *Structure and Surface Reactions Soil Particles*, Huang, P. M., Senesi, N., Buffle, J., Eds.; J. Wiley and Sons: Chichester (England) **1998**; pp 183−250.

(31) Nishio, M.; Hirota, M. H.; Umezawa, Y. T*he CH-*Π *Interactions: Evidence, Nature and Consequences*; Wiley: New York, 1998; pp 1−217.

(32) Schneegurt, M. A.; Jain, J. C.; Menicucci Jr., J. A.; Brown, S. A.; Kemner, M. K.; Garofalo; D. F.; Quallick, M. R.; Neal, C. R.; Kulpa,C. F., Jr. Biomass byproducts for the remediation of wastewaters contaminated with toxic metals. *Environ. Sci Technol.* **2001**, *35*, 3786−3791.

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