Recovery of Polymerin from Olive Oil Mill Waste Waters

Its Potential Utilization in Environmental Technologies and Industry

by Renato Capasso, Antonio De Martino, Massimo Pigna, Marianna Pucci, Filomena Sannino and Antonio Violante

Olive oil mill waste waters represent a very polluting vegetable biomass, which can also be regarded as an inexpensive source of organic compounds and metals to be recovered for use in agriculture, industry and environmental technologies. In the present review we describe the recovery and characterization of the salified polymeric organic fraction (polymerin) occurring in the waste waters and the results obtained by studying the adsorption of chrome, copper and zinc on the polymeric mixture.

Scheme 1

Olive oil mill waste waters (Omww) are vegetable biomass wastes produced in high amounts mainly in the Mediterranean basin, but also in all countries throughout the world where olive oil is produced [1]. These wastes 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 latter 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 [1] to be recovered because of their potential economic value and/or ability to be transformed into products for use in agriculture, environmental technology processes, and industry. In this connection, several processes for their exploitation have been proposed [5-7]. The main polyphenols have been isolated from Omww and chemically and biologically characterized, together with some of their synthetic derivatives [4, 8-10]. In our recent research, we recovered and characterized the Omww salified polymeric organic fraction, named polymerin [11], and investigated the potential utilization of this biomaterial in agriculture as a bio-amendment [12]. This use has been suggested because of the material's humic acid-like nature, the richness in macro- (K, Ca and Mg) and micro-nutrients (Fe and Zn), the scarce phytotoxicity, and the strongly reduced Cod and Bod compared with the raw Omww [3, 12].
Polymerin can be also regarded as a very promising bio-organic sorbent for remediation of waters contaminated with toxic metals. In fact, biosorption technologies in which vegetable biomass is used to accumulate heavy metals [13-16] is a method that can replace conventional processes for remediating metal pollution in waste waters. Conventional methods rely on relatively expensive mineral adsorbents or flocculating agents [17]. Among the more recent interesting papers on vegetable biomass biosorption, one of them examined the binding of Cr(III) to oat byproducts [18] and, another examined the remediation of waters contaminated with heavy metals, using as biosorbents two biomass byproducts that are commercially available in quantity and at low cost, namely *spillage*, a dried yeast, and a plant mixture from the production of ethanol from corn [19].
In particular, polymerin may be regarded as a bio-organic sorbent rich in negative binding sites and having many chelating functional groups [11], so that it would seem to be very suitable for the adsorption of heavy metal cations. Consequently, it was also transformed into its semisynthetic derivative, K-polymerin, which is richer in negative sites. This derivative was obtained by reaction of polymerin with KOH. With the prospect of employing these sorbents in waste water remediation processes, we have carried out a study on the adsorption on polymerin and K-polymerin of common heavy metal ions contaminating effluents, such as Cr(III), Cu(II) and Zn. The study was carried out on the metals dissolved individually and in binary and ternary combinations, thus simulating waste waters. The metals examined commonly originate from sources of pollution in-
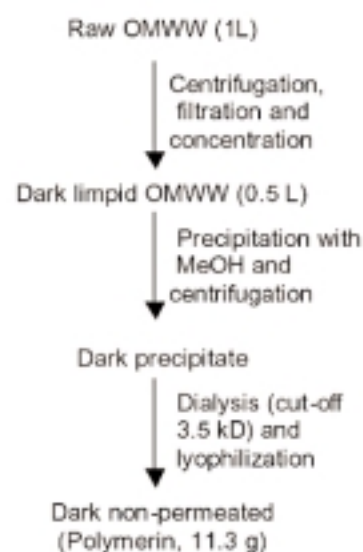
R. Capasso, A. De Martino, M. Pigna, M. Pucci, F. Sannino, A. Violante, Dipartimento di Scienze del Suolo, della Pianta e dell'Ambiente - Università di Napoli "Federico II". capasso@unina.it
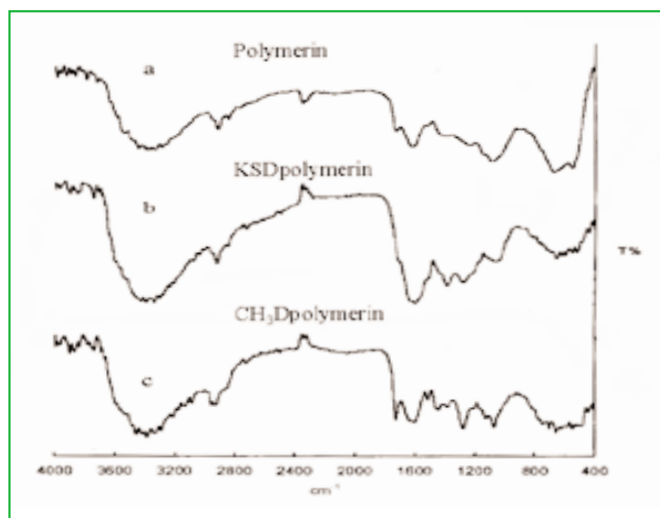
Figure 1 - Drift spectra of polymerin (a), KSDpolymerin(b) and CH₃Dpolymerin (c)

cluding agricultural fertilization, manufacturing processes, smelting and refining, refuse and waste water treatment, and fuel combustion. The particular interest for this research was consistent with the performance of two potential future objectives: the recycling of Omww using polymerin as a bio-organic sorbent and the reuse as amendment, compost, or metal source of the material from the remediation process, thus eliminating completely the disposal problems of Omww. With the aim to completely remove the heavy metal cations from simulated waste waters, an investigation was also performed on their cyclic adsorption on polymerin renewed at each cycle.

Recovery and chemical characterization of polymerin

Polymerin was recovered from Omww by basically a three-step isolation procedure (Scheme 1). An amount of 11.3 g of dark and dry product was obtained from 1 L of Omww raw material [11]. Chemical analysis revealed polymerin to be a very complex metal polymeric organic mixture composed of polysaccharides, proteins, and melanins. Polymerin also showed a metal content consisting mainly of K and, in decreasing order, Ca, Na, Fe(III), Mg, Zn, and Cu(II) [11]. The main neutral sugars composing the polysaccharide component, determined by anion exchange with pulsed amperometric detection (AE-PAD), were, in decreasing order, arabinose, galactose, glucose, and rhamnose, and glucuronic and galacturonic acids [11]. The main amino acid of the protein component determined by an amino acid analyzer was glycine, followed by the amino acids glutamic and aspartic acid and smaller amounts of the other neutral amino acids. Lastly, the basic amino acids, arginine, histidine and lysine were present to a very low extent [11]. The phenols composing the melanin component, as determined by high performance liquid chromatography (Hplc) were catechol, vanillin, syringaldehyde, and 3,5-dihydroxybenzoic, p-hydroxybenzoic, vanillic, p-coumaric, ferulic, and salicylic acids [11]. The ultraviolet and visible absorptions at 270 and 470 nm were consistent with the presence of the chromophoric melanin and
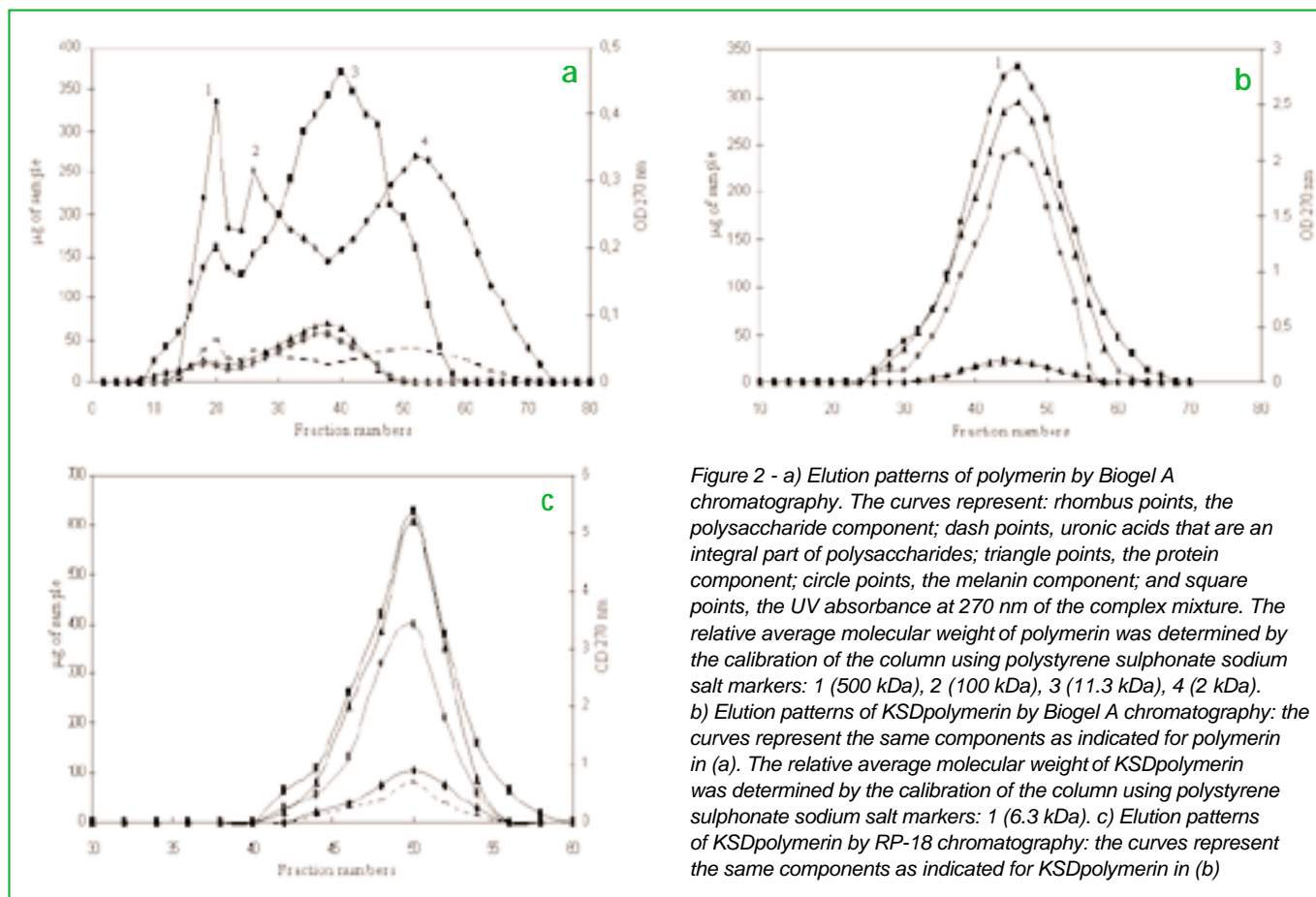

Figure 2 - a) Elution patterns of polymerin by Biogel A chromatography. The curves represent: rhombus points, the polysaccharide component; dash points, uronic acids that are an integral part of polysaccharides; triangle points, the protein component; circle points, the melanin component; and square points, the UV absorbance at 270 nm of the complex mixture. The relative average molecular weight of polymerin was determined by the calibration of the column using polystyrene sulphonate sodium salt markers: 1 (500 kDa), 2 (100 kDa), 3 (11.3 kDa), 4 (2 kDa). b) Elution patterns of KSDpolymerin by Biogel A chromatography: the curves represent the same components as indicated for polymerin in (a). The relative average molecular weight of KSDpolymerin was determined by the calibration of the column using polystyrene sulphonate sodium salt markers: 1 (6.3 kDa). c) Elution patterns of KSDpolymerin by RP-18 chromatography: the curves represent the same components as indicated for KSDpolymerin in (b)

protein therein [11]. The melanin nature of the polymerin phenol polymeric component was also strongly supported by the diffuse reflectance infrared Fourier transform (Drifit) spectra of polymerin and its two derivatives, the deglycosilated potassium salt, named KSDpolymerin, and the deglycosilated methylester, named CH$_3$Dpolymerin (Figures 1a-c) [11], which proved to be very similar to the infrared spectra described in the literature [20-23] for the melanin biomaterial. In fact, all the infrared bands of our complex polymer and those of the melanins reported in the literature occurred at very close frequency values in the 3350-3450 cm^{-1}, 1600-1630 cm^{-1}, 1700-1735 cm^{-1}, 1390-1450 cm^{-1}, 1280-1240 and 1050-1100 cm^{-1} regions. In some cases they were coincident, such as the bands at 2925 and 1240 cm^{-1}. These were characteristic of C-OH and C-O-C phenol and phenol ether stretching, respectively.

The combined chemical and chromatographic (gel-filtration and reverse phase chromatography) analyses carried out on polymerin and its derivative KSDpolymerin confirmed that polymerin was a complex mixture composed of an aggregated supramolecular portion and a polysaccharide-free portion and that its molecular size ranged approximately between values of 500 and 2 kDa [11]. In particular, polymerin showed in the gel filtration chromatographic elution patterns of Figure 2a four peaks corresponding to values of relative molecular sizes of 500.0, 100.0, 11.3 and 2.0 kDa.

The protein and melanin moieties were completely aggregated to each other, while the polysaccharide component was only in part aggregated, the rest was free. The gel filtration and RP-18 chromatographic elution patterns of KSDpolymerin (Figures 2b and 2c, respectively) confirmed that the carbohydrate, melanin and protein components, were strongly bound to each other in an aggregate form [11]. The metal moiety proved to be bound to both the aggregated supramolecular portion and the polysaccharide-free portion of polymerin through carboxylate anions and other nucleophile functional groups, also in chelating form, identified in the acidic amino acids, and uronic acids and in the melanin system. In addition, other functional groups, such as SH from cysteine, NH$_2$ from arginine, and OH from sugars and amino acids, could be involved in the chelating bonding of the metal cations [11]. The chemical bonds among the organic components of polymerin were highly likely to consist of covalent linkages and a combination of hydrogen bonds with CH/π interactions [24]. As regards the covalent bonding, we found evidence for the possibility of sulfide bridges between the protein moiety and the melanin component, in the form of an increased amount of cysteine residue in the protein moiety of KSDpolymerin in comparison with polymerin. In addition, we hypothesized the presence of ester linkages formed among the carboxy groups of the melanin component and the alcoholic group of the sugars and amino acids belonging, respectively, to the polysaccharide and protein components [11]. Hydrogen linkages and CH/π interactions should greatly contribute to stabilizing the aggregate status of polymerin. In fact, numerous hydrogen linkages could 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. Nishio *et al.* [24] provide an extensive

review of this kind of bonding between CH groups and polyaromatic systems. Based on a careful examination of all these data, we hypothesize the structure of the core of the aggregated part of polymerin to be that shown in Figure 3.

The aggregated form that contains the melanin polymer represents the complex melanin pigment that 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 and polyelectrolyte characteristics. The melanins alone are known to be very insoluble biomaterial [25,
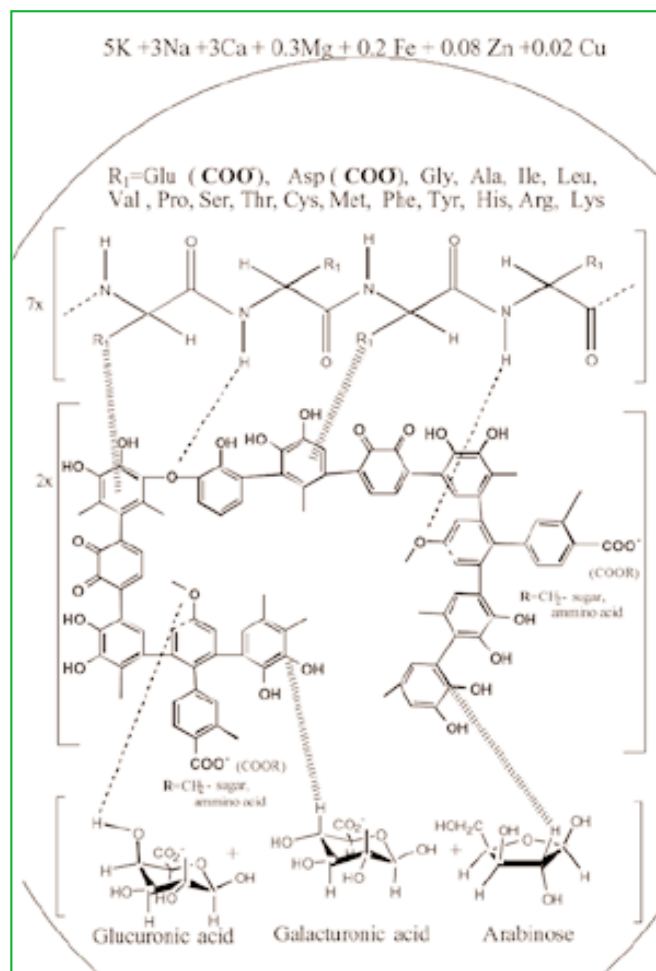

Figure 3 - Hypothetical structure of the core
of polymerin aggregate

26]. The presence of proteins, sugars, and melanins, because they are a source of inorganic macronutrients such as oxygen, carbon, and nitrogen and of macro- and micronutrient metals, makes polymerin a very promising biomaterial for use in agriculture as a bioamendment [12]. In particular, polymerin is rich in K, compared with the other macronutrient (Ca and Mg) and micronutrient metals [Zn, Mn, Fe(III) and Cu(II)]. In addition, the melanins are humic acid-type polymers that are involved in soil fertility. Paim *et al.* [20], Biliska [23] and Linhares and Martin [27] have previously demonstrated this similarity. As confirmation, we found that our Drifit spectra of KSDpolymerin (see Figure 1b) and its metal derivatives Na- and CuSDpolymerin [12] were very similar to the Drifit spectra of Na- and Cu-humate, published by Piccolo and Conte [28].

Table 1 - Content (%, w:w) of polysaccharides, proteins, melanins and metals and relative molecular weight (rmw) of polymerin

Component	% (w:w)
Polysaccharide	47.4
Protein	20.4
Melanin	30.2[a]
(phenol + non phenol aromatic units)	(15.0[a1]+15.2[a2])[a]
Metals	2.0 [Na (0.8), K (0.3), Ca (0.4), Mg (0.3), Cu (<<0.1); Zn (<0.1), Fe (0.2)]
rmw, Da	1st peak: 3,500<rmw<10,000; 2nd peak : 45,000

[a]Determined by complement to 100 of the sum of the other components; [a1] Determined by chemical analysis; [a2]Determined by difference between [a] and [a1]

Table 2 - Physico-chemical data of polymerin

	Polymerin
Titration	1th flex point pH 10.0=pKa OH (phenol)
	2th flex point pH 4.5=pKa COOH (carboxylic acid)
UV: λ_{max}^{MeOH} nm	270 (shoulder)
	470 (peak)
Drifts:	3387 (s) OH, NH peptide
$_{max}^{KBr}$ ν cm^{-1}	2922 (m) C-H
	1735 (m) C=O ester
	1626 (s) C=C aromatic nucleus +
	C=O peptide + COO⁻
	1400 (w) COO⁻
	1240 (w) C-OH phenol +
	C-O-C aromatic nucleus + OC-O-CH$_3$ ester
	1097 (s) C-OH sugars and amino-acid
	+ NH$_2$ basic amino-acid
	678 (s) aromatic nucleus

s= strong; m= medium; w=weak

Table 3 - Content (%, w:w) of polysaccharides, proteins, melanins and metals and relative molecular weight (rmw) data regarding K-polymerin

Component	% (w:w)
Polysaccharide	44.0
Protein	23.8
Melanin	26.2[a]
(phenol +non phenol aromatic units)	(13.1[a1]+13.1[a2])[a]
Metals	6.0 [Na (1.0), K (4.0), Ca (0.5), Mg (0.3), Cu (<<0.1); Zn (<0.1), Fe (0.2)]
rmw, Da	only an individual peak peak: 3,500<rmw<10,000

[a]Determined by complement to 100 of the sum of the other components; [a1] Determined by chemical analysis; [a2]Determined by difference between [a] and [a1]
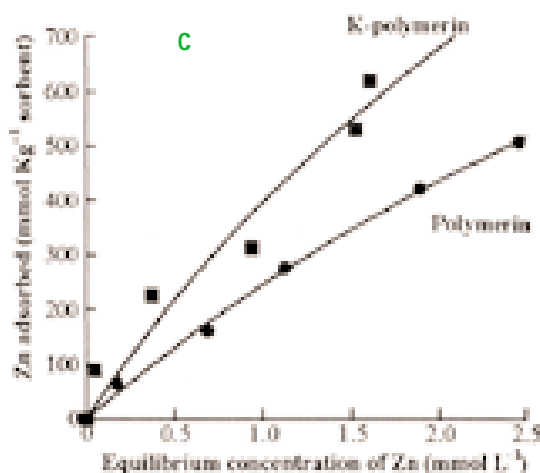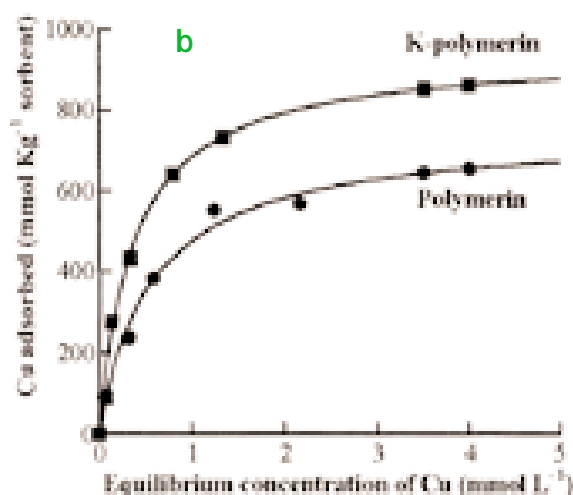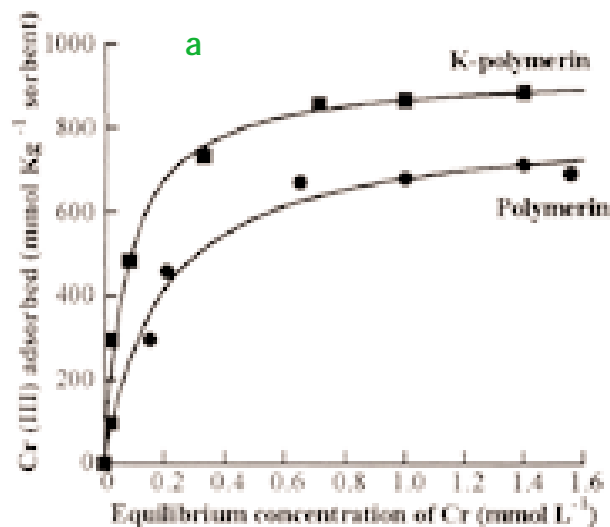

Figure 4 - Adsorption isotherms on polymerin and K-polymerin at pH 4.0 of Cr(III) (a), Cu (b) and Zn (c)

The use of raw Omww in trials to increase fertility in the soil is well known, so the utilization of polymerin, which is a more refined product than Omww, could be of major interest for use in agriculture. To investigate the potential of polymerin as a bio-organic sorbent for the removal of toxic metals from contaminated effluents, an aliquot of Omww (year 2000) from a pressure processing system, located in the Monteroduni plant, was transformed into the complex polymeric mixture, whose chemical composition, molecular size and Drift data are reported in Tables 1 and 2. The data confirm the complex melanin nature and the polyelectrolyte characteristics of the polymeric biomaterial.

Polymerin (2 g)

> Reaction with KOH
> Dialysis (cut-off
> 1000 Da)
> Lyophilization

K-Polymerin (1 g)
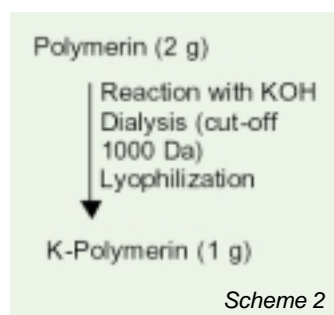
Scheme 2

Semisynthetic production of K-polymerin

Polymerin was transformed into its derivative saturated with potassium (K-polymerin) according to the procedure described in Scheme 2. This conversion was performed with the aim to produce a sorbent with increased metal cation adsorption capacity with respect to polymerin. In fact, the percentage composition of metal in K-polymerin was higher than that in polymerin (compare the data of Tables 1 and 3), particularly with regard to potassium, as a consequence of the hydrolysis of the ester linkages present in polymerin [11]. Therefore K-polymerin released further carboxylate anions, corresponding to the production of further negative adsorption sites.

Adsorption of Zn, Cu, and Cr(III) on polymerin and K-polymerin

K-polymerin adsorbed greater amounts of Cr(III), Cu and Zn than polymerin (Figure 4a-c). This behavior is not surprising because K-polymerin showed more adsorption sites than polymerin (Tables 1 and 3). Both the sorbents fixed greater quantities of Cr(III) and Cu(II) than Zn. In fact, at an equilibrium concentration of each metal of 1.6 mmol L^{-1}, K-polymerin and polymerin adsorbed 890 and 725 mmol Cr Kg^{-1}, (Figure 4a), 778 and 552 mmol Cu Kg^{-1} (Figure 4b) and 577 and 366 mmol Zn Kg^{-1} (Figure 4c), respectively. However, Zn showed a lower affinity for both the sorbents than the other two metals (Figure 4a-c). The adsorption of the heavy metals on polymerin promoted the release of K, Ca and Mg natively present on the bio-sorbent (data not shown).

Adsorption of binary and ternary mixtures of Zn, Cu, and Cr(III) on polymerin

The high individual adsorbing capacity of Cr(III), Cu and Zn on polymerin prompted a study on their adsorption in binary and ternary combinations, thus simulating contaminated waste waters. Experimentation on K-polymerin was avoided, in spite of its good adsorbing capacity, because of its more elaborate preparation and lower yield production than polymerin. Results showed that the adsorption of Zn on the bio-sorbent strongly decreased when mixed with Cu, whereas the adsorption of Cu decreased only slightly when mixed with Zn (Figure 5a). However, the overall adsorbing capacity of the mixture of two metals on
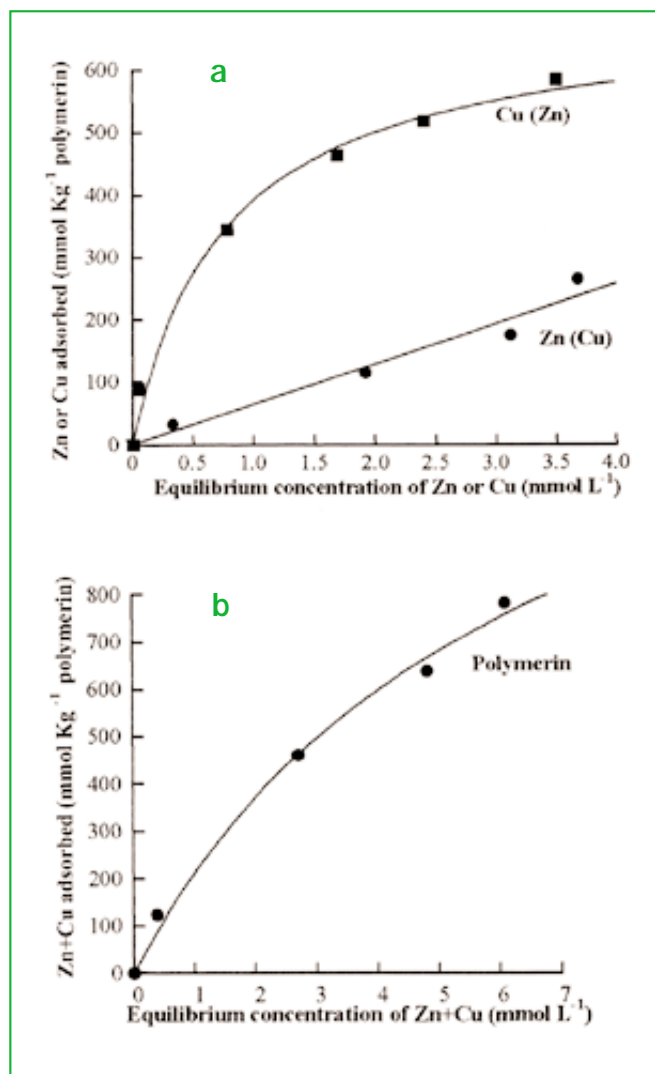

Figure 5 - Competitive adsorption of Cu and Zn on polymerin at pH 4.0 (a) and overall adsorption of Cu+Zn mixture on the biosorbent at pH 4 (b)

polymerin was also determined and was demonstrated to be considerable (Figure 5b), and therefore of high interest for a potential application in remediation technologies for contaminated effluents. The adsorption capacity of the equimolar ternary mixture of Cr, Cu and Zn on polymerin, showed the following decreasing order: Cr>Cu>Zn (Figure 6a), according to the respective adsorption isotherms. The overall adsorbing capacity of the mixture on the biosorbent was also considerable (Figure 6b).

Cyclic adsorption of binary and ternary mixtures of Zn, Cu, and Cr(III) on polymerin

The results described above on heavy metals adsorption by polymerin prompted the investigation of cyclic adsorption by this sorbent, with renewal it after each cycle. The goal was the total removal of the above heavy metals from simulated waste waters. Figure 7a shows that the total removal of Cu and Zn from an equimolar solution was performed after three cycles. The removal of Cu and Cr(III) from a ternary equimolar solution of Cu, Cr and Zn was complete in three cycles, whereas residual Zn (25% of that initially present) remained even after five adsorption
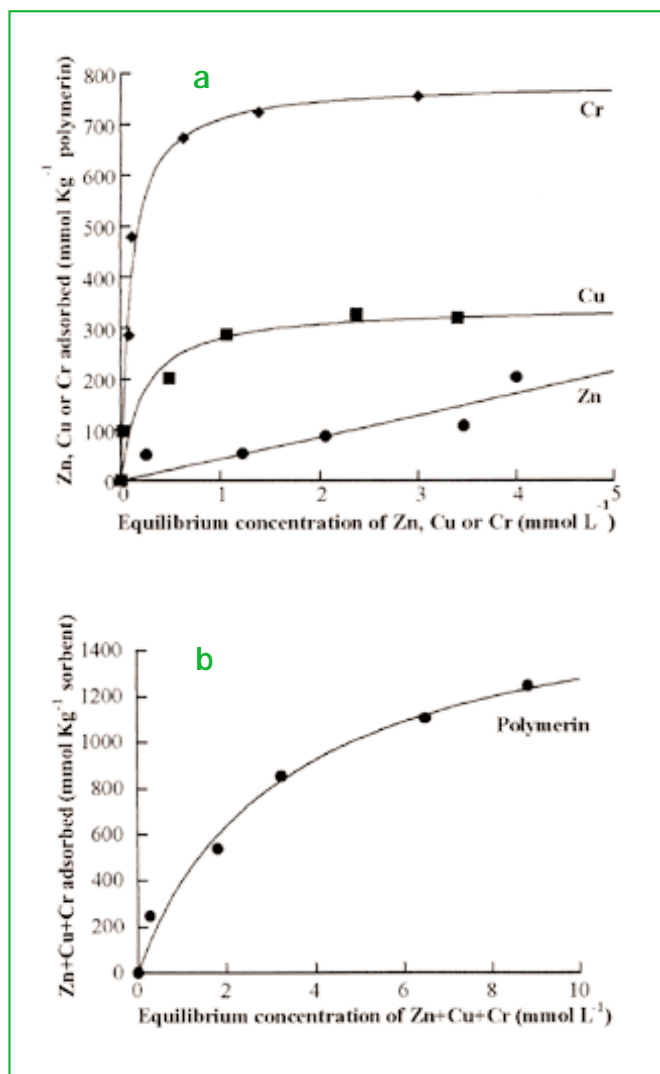
Figure 6 - Competitive adsorption of Cr(III),
Cu and Zn on polymerin (a) and overall adsorption
of Cr(III)+Cu+Zn mixture on the biosorbent at pH 4 (b)

Figure 7 - Removal by cyclic adsorption
on polymerin from equimolar solutions of Zn+Cu (a)
and Cr(III)+Cu+Zn (b)

cycles (Figure 7b). Regarding an environmental technology application, the latter findings suggest the use of greater amounts of polymerin to reduce the number of cycles and render the remediation process more efficient. Furthermore, the cyclic remediation of the simulated waste waters caused the release of Ca, Mg, Fe and K from polymerin, as a consequence of their replacement by the heavy metals (data not shown).

Potential exploitation of polymerin in environmental technology processes and the biotechnological industry

The possible future exploitation of polymerin should provide environmental and industrial advantages. First, it is the main byproduct originating from the recycling of Omww, and it has a low phytotoxicity and very low Bod and Cod values [12]. Therefore, its use could contribute to the resolution of the problem of disposing of Omww, which is a very polluting biomass. The adsorbing capacity of the considered heavy metals on this bio-organic material is very high and not lower than other biomass by-products used as metal bio-sorbents, which are widely described in the literature [13-18]. In addition, the employment of
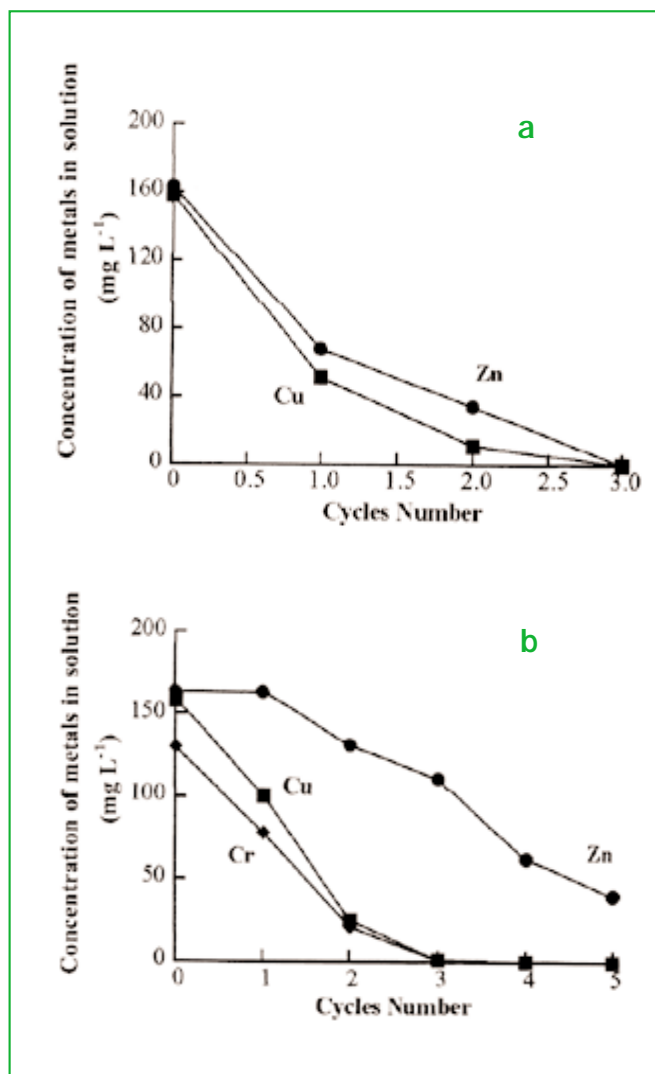
polymerin in waste waters remediation processes should not only decontaminate the waters of heavy metals, but also enrich them in biologically usefull metals as Ca, Mg and K, which are released from polymerin during the clean up process. Furthermore, polymerin could be utilized also as potential biofilter for the remediation of waste waters contaminated with pesticides, originating from contamination point sources [29]. Second, polymerin can also be regarded as a bio-organic sorbent with a low cost, simple preparation, and good adsorbing capacity. In addition, the material obtained after the waste waters clean-up process could be potentially reused as an amendment or compost, considering its humic acid-like nature, or a potential source of metals after desorption or incineration processes.
For the biotechnology industry, polymerin could be utilized as a natural antioxidant for the pharmaceutical and food industry because of its demonstrated significant antioxidant activity [30].

Conclusions

Polymerin proves to be a very promising vegetable biomass to be exploited in environmental technology processes and in the

agriculture and biotechnology industries. The beneficial use of polymerin also contributes to the resolution of the disposal problems for Omww. Polymerin's semisynthetic derivative K-polymerin showed adsorbing capacities of heavy metal ions higher than polymerin, but its production is more elaborated and has a yield of only 50%. The convenience of its exploitation compared with polymerin and other bio-adsorbents should be carefully evaluated.

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