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## ARTICLE

# Revalorization of a Two-Phase Olive Mill Waste Extract into a Micronutrient Fertilizer

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Micronutrient deficiencies in plants may be treated using metal complexes. A modified two-phase olive mill waste (OMWm) was characterized using FTIR spectroscopy. A study was also made of micronutrient (Fe, Zn, Mn and Cu) complexation and the stability of complexes. An evaluation was then made of the effectiveness of Fe(III)OMWm to supply Fe to soybean (*Glycine max.* cv Stine 0480) chlorotic plants through nutrient solution and foliar application. The OMWm presented structural similarities with the fulvic fraction of raw OMW containing abundant phenolic-hydroxyl and carboxyl groups able to form complexes. The OMWm could complex Fe, Zn, Mn and Cu, although the stabilities of the complexes under agronomic conditions were low. In dealing with Fe chlorosis, Fe(III)-OMWm improved the Fe nutritional status of soybean chlorotic plants when applied to the nutrient solution, while only regreening of leaves was observed in foliar applications. Hence, OMWm complexes constitute a promising eco-compatible and cheap alternative to synthetic chelates in dealing with micronutrient deficiencies when applied foliarly or to the nutrient solution, although further research is necessary to improve the stability and effectiveness of the complexes.

KEYWORDS: Complexes; fertilizer; iron chlorosis; micronutrient deficiencies; olive mill waste; soybean

## INTRODUCTION

Most olive oil production is located in countries within the Mediterranean basin. Spain is the world's major producer, with a mean output of 1 100 000 tons per year for the 2001−2007 period (International Olive Council database, 2008). Technical improvements in oil extraction systems in recent decades have favored the increase in production. Today, around 90% of olive mills in Spain use the so-called "continuous two-phase system", whose main disadvantage is the production of two-phase olive mill wastes (OMW), commonly known as "alperujos". OMW are solid and very wet byproducts, with acidic pH and low consistency, rich in organic matter, potassium and nitrogen, also containing fats, water-soluble carbohydrates, and phenols. It is estimated that 4 000 000 tons of OMW are generated yearly in Spain, and their disposal constitutes a technical, financial, and environmental concern (*1*). The application of OMW to soils as amendments or plant nutrient fertilizers (in most of cases after composting of the raw material) is one of their possible uses (*2, 3*).

Iron (Fe) chlorosis is a common and worldwide nutritional disorder, with high incidence in the Mediterranean region, occurring mainly in plants growing in calcareous and/or alkaline soils, where calcium carbonate buffers soil solution pH in the range 7.5−8.5 (*4*), high bicarbonate concentration is present, and Fe precipitates as Fe oxyhydroxides (*5*). In the case of zinc (Zn),

the high pH and the presence of active lime in the soil induces the retention of the cation, due the adsorption and the precipitation of Zn solid phases (*6−8*), reducing plant uptake. The solubility of manganese (Mn) is affected by pH, redox potential, and complexation, and its availability to plants is lower in alkaline soils due to the low solubility of Mn compounds under high pH conditions (*5*). Copper (Cu) deficiencies in plants appear mainly because copper availability is very low in several types of soils, including organic, peaty and calcareous soils, and some soils with high clay contents (*9*). All these deficiencies are very common in Mediterranean areas, where calcareous soils reduce the availability of Fe, Zn, Mn, and Cu, reducing the growth and yield of the affected crops.

Currently, different types of products are used to overcome micronutrient deficiencies. At present, Fe chelates formed with polyamino-carboxylates constitute the most efficient remedy for controlling Fe chlorosis (*10*). However, due to the high price and, in most of cases, scarce degradability of Fe chelates, alternative products such as complexes have been considered. Complexes are natural products that are cheaper than synthetic chelates, albeit with lower stability, so they are expected to deliver micronutrients to plants in mildly deficient conditions. Hence, their application to low value crops can be a profitable and environmentally friendly alternative. OMW are natural polymers containing carboxylic and phenolic groups, whereby they can complex micronutrients. Moreover, due to the low micronutrient concentrations applied as fertilizers, raw OMW could be employed to

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form metal complexes, without prior composting. Given the lower stability of complexes, the foliar application of this type of products to deficient plants, mostly in the form of Fe complexes, has been tested by several authors (*11−14*) as an alternative way of correcting crop nutritional disorders.

Although their composition means that OMW can complex micronutrients, several constraints impair their direct use as micronutrient fertilizers. The two-phase system OMW have an important solid fraction formed by the remaining parts of the olive (stone, pulp, etc.), with fats being a significant part of this organic matter (around 13% according to ref *1*). Accordingly, OMW have to be processed before they can be used to complex micronutrients. One such modification is that proposed by Canet-Benavent (2006) (*15*), which suggests a series of steps in which the liquid fraction of the original OMW was modified (by centrifugation, biological treatment, alcoholic fermentation, filtration and concentration) to produce a valued and multipurpose concentrate (OMWm).

The main objective of this work is to assess the ability of the OMWm extract to overcome micronutrient deficiencies. First, a study was made of the structural characteristics of the OMWm and its ability to complex Fe, Zn, Mn and Cu. The stability of the complexes at different pHs, their reactivity with soils and their perdurability under agronomic conditions were also tested. Two plant experiments were then conducted with soybean (*Glycine max.* cv Stine 0480) chlorotic plants grown in hydroponics to study the efficacy of Fe(III)OMWm complexes to cure Fe chlorosis when applied to the nutrient solution or through foliar sprays.

## MATERIAL AND METHODS

**Description of the OMWm.** The OMWm was kindly supplied by Ges-Biolives, and derived from fresh OMW subjected to a series of steps to obtain a valued byproduct (*15*). The valorized extract has a high water content (623 g·kg$^{-1}$ FW). It presents a total organic matter content of 884 g·kg$^{-1}$ DW with an important fulvic fraction (723 g·kg$^{-1}$). It is acidic pH (5.5), and the density was 1.17 kg·L$^{-1}$. Nutrient content (in dry weight) was C (385 g·kg$^{-1}$), N (11 g·kg$^{-1}$), H (54 g·kg$^{-1}$), S(1.3 g·kg$^{-1}$), P (27 g·kg$^{-1}$), and K (147 g·kg$^{-1}$) without significant amount of micronutrients. The mass spectra of the resulting OMWm consist largely of low molecular weight fractions, with the most abundant appearing in the range from M/z 160 to 460 g·mol$^{-1}$.

**Structural Characterization of the OMWm.** The OMWm was freeze-dried and analyzed by Fourier transform infrared (FTIR) spectroscopy. FTIR spectra were obtained on a FTIR Bruker IFS60v spectrophotometer with a MTC detector and diffuse reflectance (DRIFT) accessory. Spectra of OMWm samples were recorded in the 4000−400 cm$^{-1}$ region at a resolution of 4 cm$^{-1}$ in the diffuse reflectance mode.

**Complexing Ability of Fe, Zn, Cu, and Mn and Preparation of Metal−OMWm Complexes.** The ability of the OMWm to complex micronutrients was determined as described by Villén et al. (*16*), by adding increasing volumes of 200 g·L$^{-1}$ solutions of FeSO$_4$·7H$_2$O (the source of Fe commonly used in the production of Fe fertilizers), FeCl$_3$·6H$_2$O (as an alternative to FeSO$_4$), CuSO$_4$·5H$_2$O, and 100 g·L$^{-1}$ ZnSO$_4$·H$_2$O and MnSO$_4$·H$_2$O over 20 mL of the OMWm. The complexed elements were determined by atomic absorption spectroscopy (AAS, Perkin-Elmer Analyst 800 spectrophotometer) after removal of the organic compounds by digestion and the addition of a matrix modifier.

After determining the highest complexing ability of the OMWm for each metal, OMWm−metal complexes were formed by adding the appropriate amount of metal solution (200 g·L$^{-1}$ FeSO$_4$·7H$_2$O, FeCl$_3$·6H$_2$O, CuSO$_4$·5H$_2$O, and 100 g·L$^{-1}$ ZnSO$_4$·H$_2$O and MnSO$_4$·H$_2$O) to the OMWm. To ensure that the whole amount of metal is complexed by the OMWm, an excess of complexing agent was used in the formation of the complexes, with the metal/OMWm ratio being 1:1.1. Soluble and complexed elements were determined. The precipitation method of Villén et al. (*16*) was used for the complexed elements. The soluble element

**Table 1.** Chemical and Physical Characteristics of the Soils Used in the Interaction Assays

|  | S1 | S2 |
|---|---|---|
| sand (g·kg$^{-1}$) | 650 | 460 |
| silt (g·kg$^{-1}$) | 120 | 280 |
| clay (g·kg$^{-1}$) | 230 | 260 |
| texture | sandy loam | sandy clay loam |
| pH (H$_2$O) | 7.69 | 7.82 |
| pH (KCl) | 7.15 | 7.23 |
| EC, extract, 1:5 (dS·m$^{-1}$) | 0.235 | 0.188 |
| OM oxidizable (g·kg$^{-1}$) | 8.0 | 24.0 |
| [N], Kjeldahl (g·kg$^{-1}$) | 0.79 | 1.40 |
| C/N | 10.5 | 9.9 |
| CaCO$_3$ (g·kg$^{-1}$) | 150 | 179 |
| Active lime (g·kg$^{-1}$) | 40 | 52 |
| *Macronutrient (Soltanpour, cmol$_c$·kg$^{-1}$)* | | |
| Ca | 1.76 | 1.55 |
| Mg | 1.72 | 0.66 |
| K | 1.26 | 1.02 |
| Na | 0.150 | 0.047 |
| *Micronutrient (Soltanpour, mg·kg$^{-1}$)* | | |
| Fe | 14.3 | 27.0 |
| Mn | 13.0 | 5.4 |
| Cu | 1.46 | 47.1 |
| Zn | 1.08 | 4.3 |

content was measured according to official EC methods 9.2 and 9.4 (*17*).

**Stability of OMWm Complexes in Agronomic Conditions.** The stability of the metal−OMWm complexes was tested in three independent experiments in order to evaluate the effect of pH, their stability in interaction with soils at a fixed alkaline pH, and their perdurability in agronomic conditions. Fe(II)OMWm, Fe(III)OMWm, Zn(II)OMWm, Cu(II)OMWm, and Mn(II)OMWm complexes were studied in all the cases, and three replicates per complex were performed in each experiment. Interactions were made in the dark to avoid the possible photodecomposition of the complexes.

*Stability of OMWm Complexes in Solution. Effect of pH.* The amount of complexed metal remaining in solution at different pH values was studied following the methodology described by Álvarez-Fernández et al. (*18*). In brief, for each micronutrient studied, 1 mL of 10$^{-2}$ M solutions of the metal−OMWm complexes was added to 25 mL of Type I water and 5 mL of a solution containing 1.0 × 10$^{-1}$ M CaCl$_2$ and 1.0 × 10$^{-2}$ M biological buffer (MES, HEPES, CAPS, and AMPSO). The pH was then adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, or 11.0 with NaOH or HCl, and the volume was increased to 50 mL. Samples were placed in a shaker bath at 25 °C and 56 min$^{-1}$ for 3 days.

*Interaction of OMWm Complexes with Soils (Soil Experiment I).* The interaction experiments were performed according to Álvarez-Fernández et al. (*18*). In brief, 2 g of two calcareous soils (S1 and S2) collected from different Spanish regions (S1 from Valencia and S2 from Lérida) were allowed to interact with 5 mL of 4.0 × 10$^{-4}$ M metal−OMWm solution, 5 mL of a solution containing 2.0 × 10$^{-2}$ M CaCl$_2$ and 2.0 × 10$^{-3}$ M HEPES (pH 8) in 60 mL sterile polyethylene flasks. The soils had already been characterized elsewhere, (*18*) and their main characteristics are reported in **Table 1**. In addition, blanks for both the complexes and the soils were provided and prepared in a similar manner, albeit without the addition of the soil or the complexes, respectively. The flasks were shaken for 1 h at 25 °C and 56 min$^{-1}$ and then allowed to stand for 3 days in a thermostatized incubator at 25 °C.

*Perdurability of OMWm Complexes in Solution under Agronomic Conditions (Soil Experiment II).* The perdurability of the complexes over time was determined after their interaction with a soil (S1). A 5 mL aliquot of 4.0 × 10$^{-4}$ M metal−OMWm solutions was added to 60 mL sterile polyethylene flasks containing 2 g of S1 and 5 mL of Type I water. Samples were placed in a shaker bath at 25 °C and 56 min$^{-1}$ for 1, 3, 7, 14 h and 1, 3, 7 and 14 days. In addition, blanks for both the complexes and the soils

**Table 2.** OMWm Complexing Ability and Formation of Complexes with Fe, Zn, Mn, and Cu

| | OMWm complexing ability | | metal−OMWm complexes | | |
|---|---|---|---|---|---|
| sample | mol of element · kg$^{-1}$ of FW OMWm[a] | % recovered element/ added element[b] | mol of complexed element · kg$^{-1}$ of FW complex[a] (%)[c] | mol of soluble element · kg$^{-1}$ of FW complex (%)[d] | % complexed element/ soluble element |
| Fe(II)OMWm | 0.43 | 60.4 | 0.28 | 0.50 | 57.2 |
| Fe(III)OMWm | 0.56 | 89.6 | 0.54 | 0.77 | 70.1 |
| Zn(II)OMWm | 0.52 | 50.2 | 0.45 | 0.62 | 72.5 |
| Cu(II)OMWm | 0.85 | 83.6 | 0.81 | 0.98 | 82.5 |
| Mn(II)OMWm | 0.51 | 42.3 | 0.25 | 0.50 | 50.9 |

[a] Determined according to the titration methodology described in ref 16. FW: fresh weight. [b] Obtained from the intersection point of the two segments obtained in the titration curves, as presented in **Figure 2**. [c] Determined according to the AOAC modified methodology described in ref 16. [d] Official EC methods 9.2 and 9.4 (ref 17).

were provided and prepared in a similar manner, albeit without the addition of the soil or the complexes, respectively. The solutions were filtered through 0.45 μm Millipore membranes at the end of the corresponding interaction time (three days in the first and second experiments) and at timed intervals in the third one, and the pH was measured using an Orion Research Ion Analyzer (EA920). After acidification, the total element in the filtrate (Fe, Zn, Mn, and Cu) was quantified by AAS.

**Biological Experiments.** Two biological experiments were performed in hydroponics to test the ability of Fe(III)OMWm complex to provide Fe to soybean chlorotic plants, which are Fe-susceptible, when this complex was applied to the nutrient solution (Biological Experiment I) or through foliar sprays (Biological Experiment II).

Its effectiveness was compared with two commercial products used to correct micronutrient deficiencies: a synthetic chelate commonly used to treat Fe chlorosis (Fe(III)EDTA), and a commercial lignosulfonate (pulp paper natural polymer obtained during the sulfite pulping process when original lignin is broken down, Fe(III)LS) (19). In both cases, the soluble element was measured according to official EC methods 9.2 and 9.4 (17). The percentage of Fe complexed by the LS (6.9%) was determined following the precipitation methodology of Villén et al. (16), while the method proposed by Lucena et al. (20) was used for Fe chelated by EDTA (12.9%). For both products, the complexed vs soluble Fe was 100%. Furthermore, one control treatment without Fe addition was fixed (Control −Fe).

Soybean plants (*Glycine max* L. cv Stine 0480) were used in the biological experiments. Soybean seeds were kindly supplied by Prof. R. J. Goos (North Dakota State University, Fargo, ND). The seeds were washed with distilled water for 30 min and then placed in trays on filter paper moistened with distilled water with another filter paper placed over them. A 30 mL aliquot of distilled water and 20 mL of CaSO$_4$ $1.0 \times 10^{-3}$ M were added. The trays were placed in a thermostatized stove, in darkness, at 28 °C, for 3 days. After this time, seedlings were transferred to a growth chamber, where they were grown under controlled climatic conditions: day/night photoperiod, 16/8 h; temperature (day/night), 30/25 °C; R.H. (day/night), 50/70%. The seedlings of similar development were placed on a perforated plate, floating over containers with continuously aerated $^1/_5$ diluted nutrient (with 5 μM Fe(III)EDTA) solution for 5 days.

The diluted nutrient solution was then replaced by a full-strength solution, with the following composition (macronutrients in M): $1.0 \times 10^{-3}$ Ca(NO$_3$)$_2$, $9.0 \times 10^{-4}$ KNO$_3$, $3.0 \times 10^{-4}$ MgSO$_4$, $1.0 \times 10^{-4}$ KH$_2$PO$_4$; (cationic micronutrients in M, as EDTA buffered micronutrient solution) $2.5 \times 10^{-6}$ MnSO$_4$, $1.0 \times 10^{-6}$ CuSO$_4$, $10.0 \times 10^{-6}$ ZnSO$_4$, $1.0 \times 10^{-6}$ NiCl$_2$, $1.0 \times 10^{-6}$ CoCl$_2$, $115.5 \times 10^{-6}$ EDTANa$_2$, $2.31 \times 10^{-4}$ M KOH; (anionic micronutrients, in M) $35.0 \times 10^{-6}$ NaCl, $10.0 \times 10^{-6}$ H$_3$BO$_3$, $5.0 \times 10^{-8}$ Na$_2$MoO$_4$. The pH was adjusted to 7.5 with KOH 1.0 M, and buffered with HEPES $1.0 \times 10^{-4}$ M. The seedlings were kept in this solution for 6 days, until chlorotic symptoms were observed.

After this time, the plants were transferred to 2 L polyethylene pots containing 2 L of full-strength nutrient solution. The stems of two plants were wrapped together with foam and placed in the pots (three holes in the lid, three pairs of plants per pot). This nutrient solution had the same composition as the initial one, except in the cationic micronutrients content (not buffered micronutrient solution, in M): $1.0 \cdot 10^{-6}$ MnSO$_4$, $5.0 \cdot 10^{-7}$ CuSO$_4$, $5.0 \cdot 10^{-7}$ ZnSO$_4$, $1.0 \cdot 10^{-7}$ NiCl$_2$, $1.0 \cdot 10^{-7}$ CoCl$_2$. The pH was adjusted to 7.5 with KOH 1.0 M, and buffered with HEPES $1.0 \cdot 10^{-4}$ M, and $0.2$ g·L$^{-1}$ of solid CaCO$_3$ per pot were added to simulate conditions in calcareous soils. Water was added every two days, and the

solution was renewed every week. At this point, Fe treatments were applied, being repeated 8 and 15 days after transplanting in both experiments.

*Biological Experiment I* (*Treatments Applied to the Nutrient Solution*). First, Fe was added in a small dose of 5.0 μM. The concentration for each treatment was calculated according to the soluble Fe content of the products (**Table 2**). As plants did not recover from chlorosis after a week, the dose was increased to 10.0 μM in the second and third applications. For Control −Fe, Fe was not added to the solution.

*Biological Experiment II* (*Application through Foliar Sprays*). Each pair of plants was sprayed with 2 mL of the products, with Fe concentration of 5.0 mM. The concentration for each treatment was calculated according to the water-soluble Fe content of the products (**Table 2**). Leaf sprays were applied both on the adaxial and abaxial leaf surface using a nebulizer system. All solutions were adjusted to pH 5.0 to avoid altering the ion exchange properties of the cuticle (21). Tween 80 (nonionic surfactant, PROBUS) was added to the foliar solutions just before leaf spraying at a rate of 0.1% (v/v). For Control −Fe, leaves were sprayed with 2 mL of distilled water containing Tween 80 at the same rate as the treatments.

In both assays, three replicates per treatment were performed. During the experiments, SPAD readings were regularly taken with a chlorophyll meter (Minolta SPAD-502) for all the leaf stages (average of three readings per leaf). Whole plants were sampled after transplanting at 8 days (two pairs of plants) and 22 days (one pair of plants). The sampled roots, stems, and leaves were separated and washed (22), weighed and dried at 65 °C for 3 days. Micronutrients in the leaves were determined by AAS after dry digestion procedure.

**Statistical Analysis.** Data were statistically evaluated using analysis of variance (ANOVA) with SPSS 15.0 software to assess the significance of the main factors and interactions. Means were also compared using Duncan's test at $P \leq 0.05$ in order to find significant differences between treatments.

## RESULTS AND DISCUSSION

**Structural Characterization of the OMWm.** The FTIR spectrum for the OMWm is presented in **Figure 1**. Infrared bands were interpreted according to Plaza et al. (3) and Ait Baddi et al. (23), and its main features were (1) a broadband at 3409 cm$^{-1}$ due to O−H stretching of phenol, alcohol, or carboxyl OH and also to amide and amine N−H stretching; (2) bands at 2970−2935 cm$^{-1}$ and at 2885 cm$^{-1}$ due to aliphatic C−H stretching; (3) a band at 1720 cm$^{-1}$ due to C=O stretching of COOH, and also ascribed to other carbonyl groups such as ketones and aldehydes; (4) a band at 1595 cm$^{-1}$ that could be considered a group of unresolved absorptions mainly due to aromatic C=C, C=O stretching of amide groups, quinonic C=O and/or C=O of H-bonded conjugated ketones; (5) a band at 1400 cm$^{-1}$ attributed to stretching of antisymmetric COO$^-$, aliphatic C−H bending, O−H bending, and C=O stretching of phenolic groups; (6) a band at 1260 cm$^{-1}$ due to C−O stretching of aryl ethers and phenols and C−O stretching and O−H deformation of carboxylic groups; (7) a band at 1120 cm$^{-1}$ due to alcoholic group vibrations; (8) a band at 1045 cm$^{-1}$ due to C−O stretching of carbohydrates and
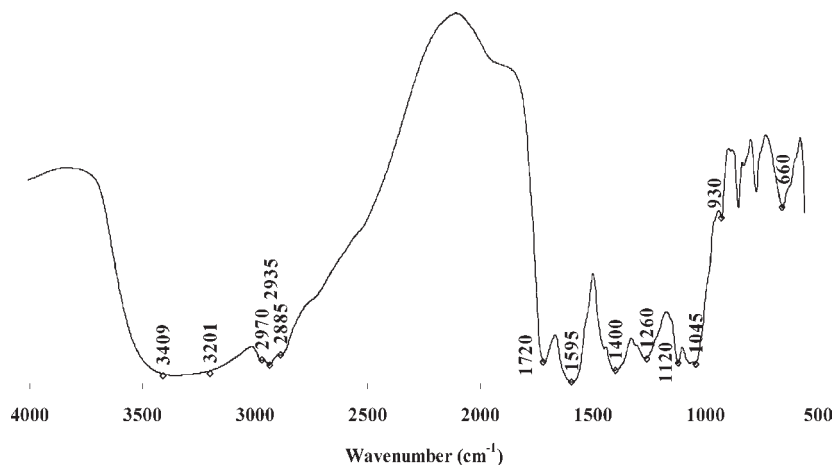
**Figure 1.** FTIR spectrum of the OMWm.

polysaccharides; and (9) peaks at 930–660 cm$^{-1}$ attributed to C–H bending of substituted aromatic groups. The FTIR study indicates that the complexing groups able to complex micronutrients, mostly phenolic and carboxylic, were abundant. These structures would be included in the low molecular fractions shown by the mass spectra, and could be attributed to organic acids and natural chelating agents, such as siderophores and phytosiderophores. On the other hand, the major bands observed in the spectra are the same as those observed in the fulvic fraction acids extracted from OMW in the first stage of the composting process (23), suggesting that the fulvic fraction is very important in the OMWm studied here, and supports its potential ability to form metal complexes (24). The abundance of carboxylic and phenolic groups capable of complexing metals also supports this hypothesis.

**Complexing Ability of Fe, Zn, Cu, and Mn and Preparation of Metal–OMWm Complexes.** The ability of OMWm to complex Fe, Zn, Cu, and Mn was evaluated. In the case of Fe complexes, they are usually prepared with Fe(II) (from FeSO$_4$) when commercial complexes are manufactured and its natural oxidation to Fe(III) is expected to occur. In this experiment, the complexing ability of OMWm with both forms of Fe and the stability of the Fe complexes were studied in order to evaluate the influence of the initial status of Fe in the behavior of the complex.

**Figure 2** shows the measured versus added element when the complexing ability was studied. The curves obtained are consistent with those described by Villén et al. (16), having a rising segment that corresponds with the complexing process, followed by another decreasing one that implies the coagulation of the material through the excess of metal. In addition, for each metal **Table 2** presents the percentage of element that remains in solution with regard to the added element (obtained from the intersection point of the two lines in **Figure 2**). As elucidated from the FTIR spectra, all the elements studied could be complexed by the OMWm, especially in the case of Fe(III) and Cu, although a fraction of the added element flocculated and/or precipitated as from the beginning of the addition of the metal solutions to the OMWm (the measured element was always lower than the added element), especially in the case of Zn and Mn. This indicates that both the strong and the weak complexing sites produce coagulation of the complex or precipitation of the metal. In addition, different complexation abilities were measured for Fe(II) and Fe(III). The different behavior for both Fe forms suggests that the expected Fe(II) oxidation did not occur in the experimental period and that less stable Fe complexes were formed with divalent than with trivalent Fe. Hence, the formation of Fe-OMWm complexes with Fe(III) would be recommended.
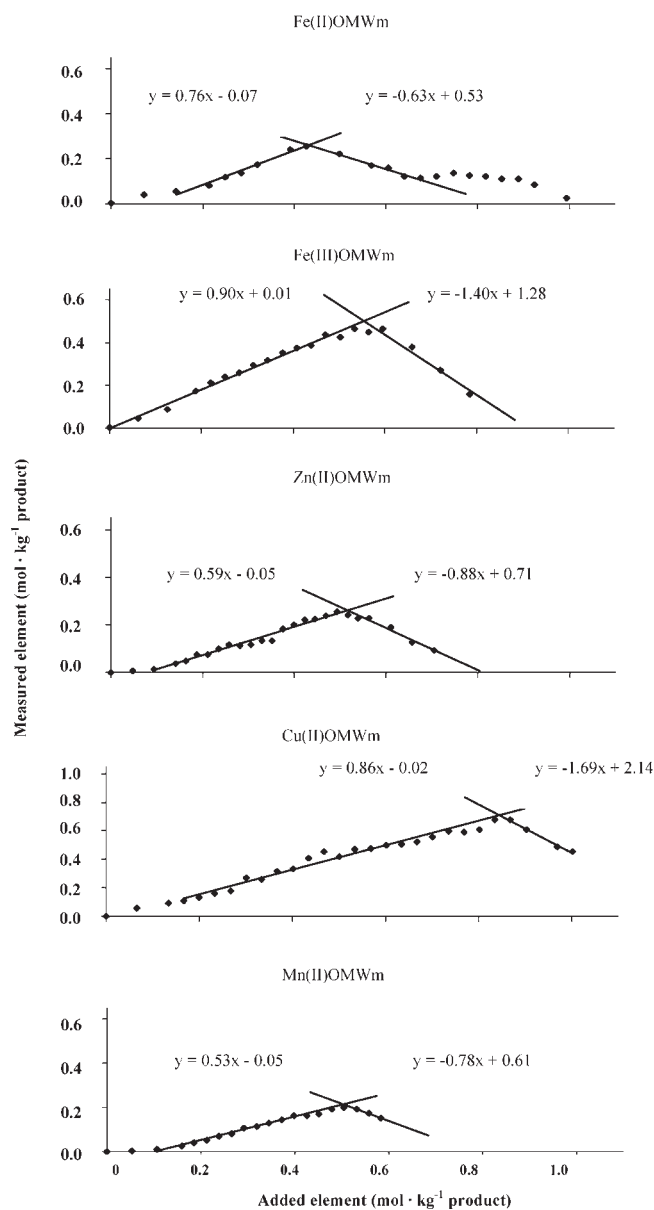


**Figure 2.** Complexing ability for OMWm with Fe (divalent and trivalent), Zn, Cu, and Mn.

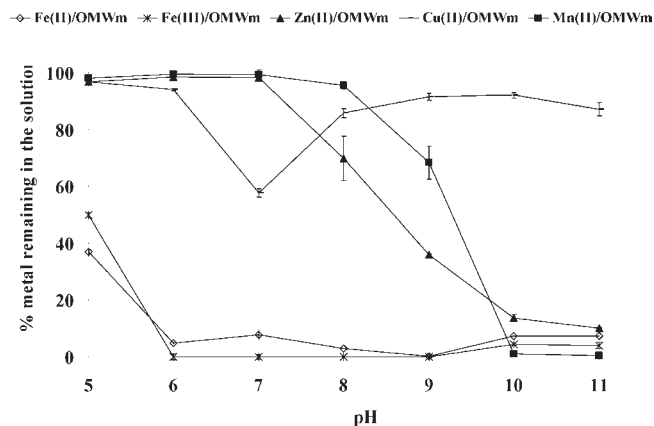Once the complexing ability was known, the metal–OMWm complexes were formed and characterized. The highest

**Figure 3.** Percentage of Fe(II), Fe(III), Zn, Cu, and Mn remaining in a $1.0 \times 10^{-1}$ M $CaCl_2$ solution with regard to the total element added ($1.0 \times 10^{-2}$ M) for the different OMWm complexes at different pH values after 3 days of interaction.
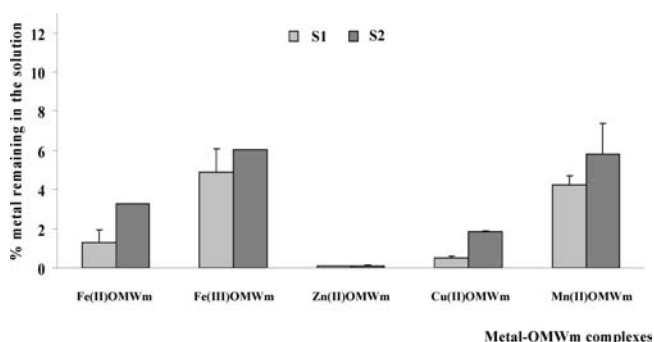


**Figure 4.** Percentage of metal remaining in a $2.0 \times 10^{-2}$ M $CaCl_2$ and $2.0 \times 10^{-3}$ M HEPES (pH 8) solution with regard to the total element added ($4 \times 10^{-4}$ M) for the different OMWm complexes after 3 days of interaction with the two soils studied (Soil Experiment I).

percentage of complexed element with regard to soluble element corresponded to Cu, Zn, and Fe(III). In any case, for all the elements the amount of metal complexed and the degree of complexation exceeded the requirements of Spanish Regulation Order APA/863/2008, so they can be commercialized.

**Stability of OMWm Complexes in Agronomic Conditions.** The effect of pH on the amount of element remaining in solution after 3 days is reported in **Figure 3**. For Fe(III), the recovery was negligible in the alkaline pH range. In the case of Cu, the solubility of the complex was high in the entire pH interval studied, decreasing only between pH 6 and pH 8. This behavior was ascribed to the solubility of inorganic Cu species below pH 6 and to the strong complexation processes occurring over pH 8. Mn and Zn complexes readily maintained a high amount of the element in the pH range corresponding to alkaline soils, with the complexed element decreasing sharply when the pH was above 9.

**Figure 4** reports the amount on Fe, Zn, Cu, or Mn remaining in solution with regard to the total amount of element added in the interaction of the complexes with both calcareous soils (Soil Experiment I). Soil blanks showed that the contribution of the soils to the amount of metal found in solution was negligible (data not shown). After 3 days of interaction, the percentage of metal recovered was very low, especially for Zn(II)OMWm. The highest values corresponded to the complexes formed with trivalent Fe and Mn, although in both cases the metal remaining in solution was below 8%. Two-way ANOVA indicated that there were no statistical differences in the percentage of recoverable metal
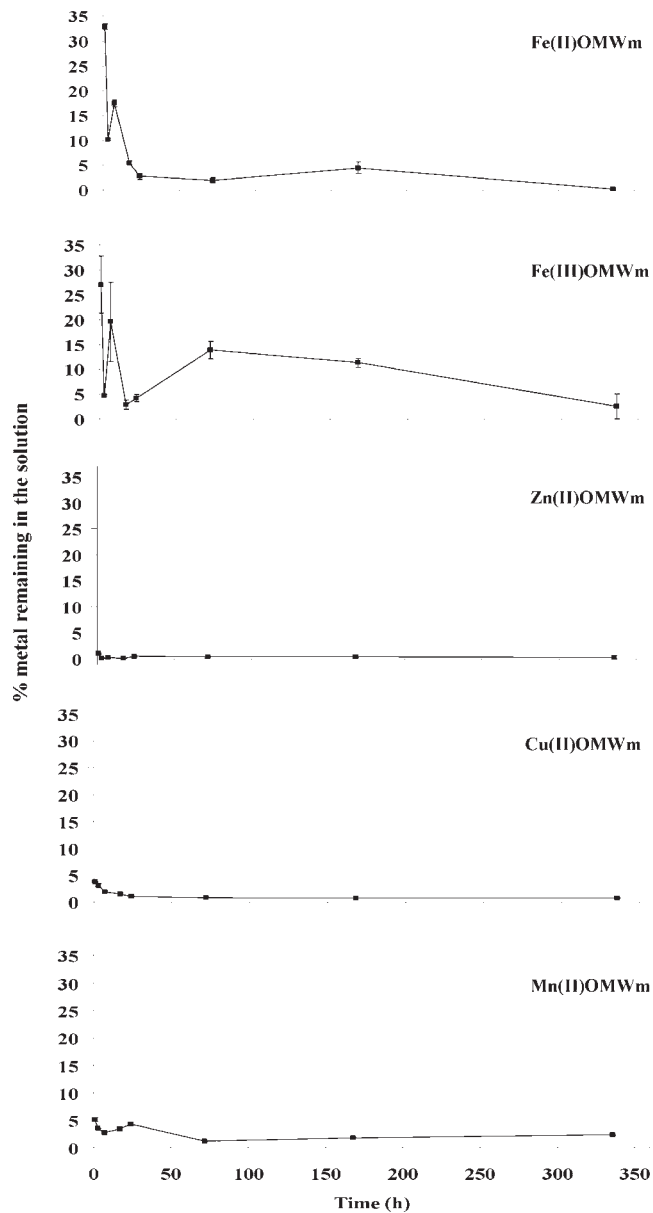


**Figure 5.** Percentage of metal remaining in the solutions at different sampling times ($t$ = 1, 3, 7 , and 14 h and 1, 3, 7, and 14 d) with regard to the total element added ($4.0 \times 10^{-4}$ M) for the different OMWm complexes interacting with the S1 (Soil Experiment II).

according to the type of soil used or to the metal interacting with the soil (data not shown).

To evaluate the perdurability of the complexes in agronomic conditions, Fe, Zn, Cu, and Mn complexes were allowed to interact with a calcareous soil (S1, **Figure 5**) at different periods (Soil Experiment II). For all the complexes, less than 5% of the initial amount of element remained in solution after 24 h of interaction. Fe complexes recorded a slightly better behavior than the rest, especially in the case of trivalent Fe.

In studies of this nature, a decrease in the metal concentration remaining in solution can be due to complex sorption, element displacement from the complex and precipitation, or complex degradation. In the case of Fe complexes, whether this decrease is a consequence of complex retention or Fe displacement cannot be elucidated from our data. The study of complexing ability indicated that OMWm formed stable complexes with Fe, especially with Fe(III), under the conditions described in the methodology used (*16*), after 24 h of interaction at pH 9. Since the

experiment testing the stability of the complexes at different pHs showed that soluble Fe did not remain in the solution in the alkaline pH range, it can be concluded that Fe−OMWm complexes were not stable after 3 days in the presence of $Ca^{2+}$. However, in the experiments with calcareous soils (Soil Experiments I and II) significant amounts of soluble Fe were recovered after 3 days of interaction. This different behavior might be ascribed to the lower Fe−OMWm concentration used in the pH assay with regard to both soil experiments. In the case of LS polymers, their structural swelling at high alkaline pH values, due to the ionic groups inside the molecule and to its branched structure in aqueous solution, has been described (*25*). This phenomenon would favor $Ca^{2+}$ complexation because the polymer "opens" and more complexing sites would be available to form complexes. Although a similar study has not been performed for OMWm, the similarities between both complexing agents with regard to the presence of functional groups able to complex metals (mostly carboxylic and phenolic hydroxyl radicals) suggest that swelling and expansion could also occur in the case of OMWm at alkaline pH levels, favoring complexation. Moreover, the lower concentrations used in both soil experiments would have promoted swelling to a higher extent, facilitating the contact between the Fe and the complexing sites and, consequently, the complexation process. For soil experiments (Soil Experiment I and II), the percentage of recoverable Fe was higher when the pH of the initial metal−OMWm solution was not fixed and $Ca^{2+}$ was not added to the solution (Soil Experiment II). Both the initial pH and $Ca^{2+}$ concentrations might have decreased when they were not fixed favoring the presence of soluble Fe. Theoretical modeling using Visual Minteq ver.2.52 confirmed this hypothesis.

For Zn, Mn, and Cu complexes, more than 60% of the initial amount of metal remained in the solution at alkaline pH when the complexes did not interact with soils (**Figure 3**). The low concentration of element remaining after interaction with soils (**Figures 4** and **5**) suggests that in this case the sorption of the complex or the displacement of the metal occurred. This supposition is confirmed by the results of the experiment of perdurability under agronomic conditions.

**Biological Experiments.** Given the high incidence of Fe deficiencies in the Mediterranean region and the elevated costs associated to its correction with synthetic chelates, the capacity of Fe(III)OMWm to solve these deficiencies when applied to the nutrient solution or through foliar sprays was tested.

*Nutrient Solution Application.* In general, all the treatments recorded a low ability to recover plant Fe deficiencies. Since Fe(III)OMWm complexes performed better than Fe(II)OMWm with regard to their complexing ability and in the stability experiments, trivalent Fe was used to form the Fe-containing compounds used in the biological experiments. The trend in the SPAD Index from the beginning of the experiment showed that the highest regreening values corresponded to the Fe(III)EDTA treatment, followed by Fe(III)OMWm (without statistical differences with Fe(III)EDTA), Fe(III)LS, and Control −Fe. The average values in the second level of leaves were $12.9 \pm 1.1$(a), $10.0 \pm 0.6$(a), $5.4 \pm 0.7$(b) and $5.7 \pm 1.1$(b), respectively, with different letters denoting statistical differences between treatments.

Two-way ANOVA of plant dry weight, leaf Fe concentration, and Fe/Mn ratio in leaves was performed, using the treatments and sampling time as factors (**Table 3**). The analysis revealed that statistical differences due to the interaction between both factors occurred for plant dry weight and leaf Fe concentration.

**Table 4** reports biometric data for all treatments at both sampling times. For the first sampling time there were no

**Table 3.** Two-Way ANOVA Analysis of Data Corresponding to Plant Dry Weight, Leaf Fe Concentration, and Leaf Fe/Mn Ratio after Application of the Fe Compounds to the Nutrient Solution[a]

|  | dry weight | Fe | Fe/Mn |
|---|---|---|---|
| treatment | *** | *** | * |
| time (days since the 1st application of the treatments) | *** | ns | ** |
| treatment* time | ** | ** | ns |

[a] (∗) Significant at $P \leq 0.05$; (∗∗) significant at $P \leq 0.01$; (∗∗∗) significant at $P \leq 0.001$; ns, non-significant.

**Table 4.** Effect of the Different Fe-Containing Compounds Applied to the Nutrient Solution on the Dry Weight and Leaf Fe Concentration and Fe/Mn Ratio in Soybean Plants Grown in Hydroponics after 8 and 22 Days[a]

|  | dry weight (g·plant$^{-1}$ DW) | Fe concentration ($\mu$g·g$^{-1}$ DW) | Fe/Mn |
|---|---|---|---|
| **8 Days after the 1st Application of the Treatments** | | | |
| Fe(III)OMWm | $0.94 \pm 0.02$ ns | $48.5 \pm 5.8$ a | $0.49 \pm 0.14$ ns |
| Fe(III)LS | $0.79 \pm 0.09$ | $40.1 \pm 5.7$ ab | $0.29 \pm 0.06$ |
| Fe(III)EDTA | $1.01 \pm 0.15$ | $44.5 \pm 3.9$ ab | $0.38 \pm 0.05$ |
| Control −Fe | $0.72 \pm 0.05$ | $31.5 \pm 0.4$ b | $0.22 \pm 0.01$ |
| **22 Days after the 1st Application of the Treatments** | | | |
| Fe(III)OMWm | $1.46 \pm 0.12$ b | $37.4 \pm 0.3$ b | $0.19 \pm 0.03$ b |
| Fe(III)LS | $1.17 \pm 0.09$ b | $31.7 \pm 2.5$ bc | $0.11 \pm 0.01$ c |
| Fe(III)EDTA | $2.61 \pm 0.31$ a | $65.9 \pm 2.2$ a | $0.38 \pm 0.02$ a |
| Control −Fe | $0.96 \pm 0.06$ b | $26.9 \pm 1.3$ c | $0.16 \pm 0.01$ bc |

[a] Data are means $\pm$ SE. Different letters within the same column denote significant differences between the treatments ($P < 0.05$); ns, nonsignficant; DW, dry weight basis.

statistical differences between the treatments tested with regard to plant dry weight, but at the end of the assay Fe(III)EDTA treated plants recorded higher growth than the rest. The same behavior was observed for Fe concentration and Fe/Mn ratio in leaves. Statistical differences between treatments were observed only at the end of the assay, with Fe(III)EDTA treated plants recording the highest values. After this chelate, Fe(III)OMWm was the most efficient compound, while Fe(III)LS was not statistically different to the Control −Fe.

These data indicate that Fe(III)EDTA and Fe(III)OMWm improved plant nutritional status, though recovery was scarce in all cases. Fe(III)EDTA was clearly the most efficient treatment, which may be explained by the percentage of chelated Fe, which was higher than the amount of element complexed by Fe-(III)OMWm and Fe(III)LS. In other experiments, we have shown that synthetic chelates were more efficient than complexes at recovering Fe chlorotic plants (*19*). Regarding the effectiveness of Fe complexes, Fe(III)LS was less efficient than Fe(III)OMWm in spite of the higher complexing capacity of the amount of Fe complexed (6.9% and 3.0%, respectively), although the stability of Fe(III)OMWm at alkaline pHs was lower than that described for Fe(III)LS in a previous paper (*26*). In this case, complexation and plant development were not correlated. Stability constants may explain the lower effectiveness of Fe(III)LS. As could be elucidated from the FTIR spectra (**Figure 1**), the OMWm studied was rich in functional groups able to complex metals, and the results reported here suggest that the OMWm molecule could release Fe to the plant more easily that the LS. Stability constants are usually used to explain this behavior. However, stability constants in this type of polymers correspond to a range of values that are highly dependent on several factors, such as pH, pe + pH and others, so comparison is difficult. The Fe/ligand ratio also has to be considered to explain the effectiveness of complexes,

**Table 5.** Effect of the Different Fe-Containing Compounds Applied through Foliar Sprays on the Dry Weight and Root Fe Concentration and Fe/Mn Ratio in Soybean Plants Grown in Hydroponics after 22 Days[a]

| | dry weight (g · plant$^{-1}$, DW) | Fe concentration ($\mu$g · g$^{-1}$ DW) | Fe/Mn |
|---|---|---|---|
| Fe(III)OMWm | 0.74 ± 0.06 ns | 65.1 ± 9.1 b | 0.18 ± 0.04 b |
| Fe(III)LS | 0.97 ± 0.15 | 129.6 ± 16.5 a | 0.38 ± 0.06 a |
| Fe(III)EDTA | 0.96 ± 0.23 | 65.7 ± 6.5 b | 0.15 ± 0.02 b |
| Control -Fe | 0.82 ± 0.19 | 54.1 ± 1.7 b | 0.17 ± 0.02 b |

[a] Data are means ± SE. Different letters within the same column denote significant differences between the treatments ($P < 0.05$); ns, nonsignificant; DW, dry weight basis.

since low stability ones have a high ratio (*24*). When weakly bound Fe is released, the remaining Fe would be strongly bonded and may not be available for use by the plants. Thus, higher concentrations should be tested in order to improve the effectiveness of complexes with regard to synthetic chelates.

*Foliar Application.* The same Fe compounds applied to the nutrient solution were tested when applied through foliar sprays. None of the treatments was particularly effective at recovering plants suffering from severe chlorosis. One-way ANOVA revealed that the SPAD Index recorded statistical differences between treatments. The trend in the SPAD Index during the assay showed that Fe(III)EDTA favored regreening of leaves to a higher extent than the other treatments. Fe(III)OMWm was also effective at increasing SPAD values, while regreening was lower for Fe(III)LS. The average values in the second level of leaves were 20.3 ± 3.0(a), 15.9 ± 3.1(ab), 10.5 ± 1.4(b), and 5.2 ± 1.0(c) for Fe(III)EDTA, Fe(III)OMWm, Fe(III)LS, and Control −Fe, respectively, with different letters denoting statistical differences among treatments.

Two samplings were made in order to determine plant dry weight. For this parameter, statistical differences between treatments and for sampling time were studied using two-way ANOVA, as well as for the interaction between both factors, which revealed no interaction between them. The dry weight of the plants was influenced solely by the time elapsed after the application of the foliar sprays (at $P \leq 0.001$), while no statistical differences between treatments were observed. Since contamination of the treated leaves due to the foliar sprays has been observed previously (*19*), Fe concentration and Fe/Mn ratio were measured only in roots at the end of the experiment. Statistical differences between treatments were observed for both parameters (at $P \leq 0.01$ and $P \leq 0.05$ for leaf Fe concentration and Fe/Mn ratio, respectively).

Fe concentration and Fe/Mn ratio in roots are reported in **Table 5** and indicate that significant redistribution of Fe foliarly applied from leaves to roots occurred only for Fe(III)LS. The factors that favored the high translocation of Fe to roots for Fe(III)LS cannot be elucidated from our data, but in any case this did not improve the general nutritional status of the plants nor promote leaf regreening. The redistribution to roots of Fe applied through foliar sprays has been observed previously, working with labeled Fe and mildly chlorotic plants complexed by humic substances and LS (*11, 14*). The foliar application of Fe is usually performed in plants with an additional supply of Fe through the roots (*12−14*). Thus, given the low mobility of Fe through the phloem, the performance of Fe(III)OMWm should be considered when applied to mildly chlorotic plants receiving an additional supply of Fe through the roots.

**Conclusions.** The OMWm extracts tested in the work are valorized byproduct capable of complexing Fe, Zn, Cu, and Mn. The OMWm formed micronutrient complexes suitable for commercialization according to Spanish legislation, although

given their low stability under alkaline soil conditions their use is recommended solely for application to the nutrient solution in hydroponic systems or when applied through foliar sprays. Because of the low concentrations of complexes applied in micronutrient fertilization, the composting of the extract can be omitted and raw materials could be used. Further research is necessary to elucidate the structural characteristics that may improve the stability of the complexes, and FTIR analysis may be a useful tool in the structural analysis of modified OMWm. Hence, Fe-OMWm complexes constitute a promising eco-compatible and cheap alternative to synthetic chelates for dealing with micronutrient deficiencies when applied foliarly or to the nutrient solution of hydroponically grown plants.

## ABBREVIATIONS USED

OMW, olive mill waste; EDTA, ethylenediaminetetraacetic acid; LS, lignosulfonate.

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