The Fe(III)-chelate of ethylenediamine-\(N,N'\)-bis(\(o\)-hydroxyphenylacetic) acid (\(o,o\)EDDHA) is generally considered as the most efficient and widespread Fe fertilizer for fruit crops and intensive horticulture. The determination of the xenobiotic chelating agent \(o,o\)EDDHA inside the plant is a key issue in the study of this fertilizer. Both the low concentrations of \(o,o\)EDDHA expected and the complexity of plant matrices have been important drawbacks in the development of analytical methods for the determination of \(o,o\)EDDHA in plant tissues. The determination of \(o,o\)EDDHA in plant materials has been tackled in this study by liquid chromatography coupled to mass spectrometry using several plant species and tissues. Two types of internal standards have been tested: Iron stable isotope labeled compounds and a structural analogue compound, the Fe(III) chelate of ethylenediamine-\(N,N'\)-bis(\(2\)-hydroxy-4-methylphenylacetic) acid (\(o,o\)EDDHMA). Iron stable isotope labeled internal standards did not appear to be suitable because of the occurrence of isobaric endogenous compounds and/or isotope exchange reactions between plant native Fe pools and the Fe stable isotope of the internal standard. However, the structural analogue Fe(III)-\(o,o\)EDDHMA is an adequate internal standard for the determination of both isomers of \(o,o\)EDDHA (\textit{racemic} and \textit{meso}) in plant tissues. The method was highly sensitive, with limits of detection and quantification in the range of 3–49 and 11–162 pmol g\(^{-1}\) fresh weight, respectively, and analyte recoveries were in the range of 74–116\%. Using this methodology, both \(o,o\)EDDHA isomers were found in all tissues of sugar beet and tomato plants treated with 90 \(\mu\)M Fe(III)-\(o,o\)EDDHA for 24 h, including leaves, roots and xylem sap. This methodology constitutes a useful tool for studies on \(o,o\)EDDHA plant uptake, transport and allocation.

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was extracted with water from tomato, pepper and lettuce leaves and roots, and tomato and pepper fruits. In that study, excess Fe(NO₃)₃ was added to the extracts, the pH was adjusted to neutrality to reconstitute the Fe(III)-o,oEDDHA chelate, and two Fe(III)-o,oEDDHA forms, the d,l-racemic mixture and the meso isomer, were separated by high-performance liquid chromatography (HPLC) and determined by spectrophotometric detection at 480 nm. The sensitivity of these methodologies was either quite poor (2.1 nmol g⁻¹ of fresh weight (FW))⁹ or not determined at all.⁷,⁸ It is important to remark that sensitivity and selectivity are important issues when determining xenobiotic compounds such as o,oEDDHA, because of the low concentrations expected and the complexity of the matrices analyzed.

Nowadays, reliable and sensitive detection of the Fe(III)-chelates of o,oEDDHA and other aminopolycarboxylate strong binding chelators could be accomplished, even in complex matrices, by using HPLC separation coupled to mass spectrometry (MS) detection (HPLC/MS).²⁰,²¹ These hyphenated techniques provide high selectivity, since they permit differentiation among analytes and co-eluting interfering compounds within a given chromatographic peak, by using exact molecular mass determination, isotopic distribution and/or fragmentation patterns.²² For example, o,oEDDHA was determined as Fe(III)-o,oEDDHA by HPLC/MS in commercial fertilizers²³,²⁴ and liquid agricultural matrices such as irrigation water, soil solution, nutrient solution and peach tree xylem sap.²⁵ In the case of the synthetic aminopolycarboxylate ligand ethylenediaminediaceitic acid (EDTA), Ni(II)-EDTA was determined by HPLC/MS in different plant materials such as roots, xylem sap, shoots and protoplasts of a metal hyperaccumulator plant²⁶ and Mn(II)-, Ni(II)-, Zn(II)- and Cd(II)-EDTA complexes were determined in xylem sap of barley.²⁷ HPLC/MS has been also used to determine natural metal chelates such those of nicotianamine (NA). For instance Ni(II)-NA was determined directly in xylem sap of Arabidopsis.²⁸

The high selectivity of current MS detectors enables the simplification of extraction procedures and chromatographic separation in HPLC/MS methods. However, the high variability in the ionization process can affect reproducibility, accuracy and sensitivity of the analysis. Changes in ionization efficiency during day-to-day operation are generally controlled by using internal standards (IS), compounds structurally related or similar to the analyte. Stable isotope labeled (SIL) compounds are generally chosen as IS because of the nearly identical chemical and physical properties compared to those of the target analyte.²⁹ Matrix effects are considered responsible for the high variability in the ionization process, especially in electrospray ionization (ESI)-MS analysis. A matrix effect could be defined as any change (suppression or enhancement) in ionization efficiency caused by the presence of co-eluting substances.³⁰ For instance, ion suppression would reduce the ion intensity of the analytes, thus affecting sensitivity.

Even when IS are used, co-eluting matrix components may cause different effects on the degree of ionization for analyte and IS, therefore affecting reproducibility and accuracy. Recent studies indicate that the use of IS, even if they are SIL analogues, does not always ensure a constant analyte/IS response ratio.²⁰–²⁴ This was found in studies using deuterium (D)-labeled compounds as IS, and attributed to the presence of different co-eluting interferences³²,³³ and different degrees of ionization suppression²⁴ as well as to the occurrence of isotope exchange reactions.²⁵ In these studies,¹³¹C-,¹⁵N- or ¹⁷O-labeled analogues were considered as better options than D-labeled compounds. Unfortunately, this type of SIL is not always available, due to the necessary labor and high cost associated with chemical synthesis.

The aim of this work was to develop and validate a new procedure for the extraction and HPLC/ESI-MS time-of-flight (TOF) determination of o,oEDDHA in plant tissues, based on the methodology¹⁴ recently developed and validated for Fe(III)-o,oEDDHA in liquid agricultural matrices. Matrix effects have been assessed using as IS both SIL analogues such as⁵⁴Fe(III)-o,oEDDHA and ⁵⁷Fe(III)-o,oEDDHA and also a compound structurally similar to the analyte, Fe(III)-o,oEDDHMA. The design and validation of such methodologies will contribute to provide new insights on the traceability of o,oEDDHA in plant tissues.

**EXPERIMENTAL**

**Chemicals and reagents**

All eluents, buffers, and standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA, USA). Reagent-grade glacial acetic acid, hydrochloric acid (35%) and ammonium hydroxide (25%) were purchased from Panreac Química S.A. (Barcelona, Spain). Ammonium acetate (99.99%, Sigma), lithium hydroxide monohydrate (99.995%, Aldrich), formic acid (50%, Fluka), methanol and 2-propanol (both LC-MS grade, Riedel-de-Haën) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The chelating agents used were o,oEDDHA (98%, LCG Promochem, Barcelona, Spain) and ethylenediamine-N,N'-bis(2-hydroxy-4-methylphenylacetic) acid (o,oEDDHMA) (98%, LCG Promochem). Natural Fe(III) (thereafter called natFe, with isotopic abundances of 5.8%⁵⁴Fe, 91.7%⁵⁶Fe, 2.1%⁵⁷Fe and 0.2%⁵⁸Fe), Mn(II), Cu(II) and Zn(II) were Titrisol metal standards (1 g of metal in 15% HCl, Merck, Darmstadt, Germany). Nickel(II) was obtained as NiSO₄ (Panreac), and Co(II), Mg(II) and Ca(II) were obtained as CoCl₂, MgCl₂ and CaCl₂ from Sigma-Aldrich. Labeled Fe(II) (FeO₂⁺, 99.8%²⁶Fe) and ⁵⁷Fe oxides (FeO₂⁺, 95.06%⁵⁷Fe) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

**Standard solutions**

Stock solutions (1 mM) of o,oEDDHA complexes with natFe(III), ⁵⁴Fe(III), ⁵⁷Fe(III), ⁵⁷Fe(II), Ca(II), Mg(II), Cu(II), Mn(II), Ni(II), Co(II), Zn(II) and Fe(III)-EDDHMA were prepared by adding slowly inorganic solutions (in mM, 36 natFe, 9 ⁵⁴Fe, 8 ⁵⁷Fe, 78 Cu, 91 Mn, 76 Zn, 47 Ca, 50 Mg, 49 Ni and 50 Co in 15–30% HCl or water, in 5% excess over the molar amount of chelating agent) over high-pH solutions of chelating agents.³⁶ During the addition of the inorganic salt the solution pH was maintained in the range 5–8 by adding simultaneously NH₄OH. Then, solutions were adjusted to pH 7.0 with HCl, equilibrated overnight in the dark,
filtered through a 0.45 μm PVDF membrane and finally made up to volume with Milli-Q water. Stock solutions were stored in the dark at 4 °C. Standard solutions of metal-chelates with concentrations lower than 100 μM were prepared daily from the stocks.

**Plant materials**

_0.0EDEDDHA-free_ plant materials obtained from Fe-deficient and Fe-sufficient plants were used to develop the _0.0EDEDDHA_ extraction procedure. Also, Fe-deficient plants were treated with Fe(III)-_0.0EDEDDHA_ as described below to obtain tissues and plant fluids containing _0.0EDEDDHA_.

Sugar beet (_Beta vulgaris_ L. cv. ‘Orbis’) and tomato (_Lycopersicon esculentum_ L. cv. ‘Tres Cantos’) plants were grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 350 μmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation at leaf level, a photoperiod of 16 h light/8 h dark, a temperature of 23 °C during the day and 18 °C during the night, and 80% relative humidity. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for 2 more weeks in half-strength Hoagland nutrient solution with 45 μM Fe(III)-EDTA at pH 5.5 and then transplanted to plastic buckets containing half-strength Hoagland nutrient solution with either 0 (0.0EDEDDHA-free Fe-deficient plants) or 45 μM Fe(III)-EDTA (0.0EDEDDHA-free Fe-sufficient plants). Some Fe-deficient plants were treated with a Fe(III)-EDDHA commercial fertilizer (90 μM Fe(III)-0.0EDEDDHA) in the nutrient solution for 24 h. Nutrient solutions were buffered at pH values of approximately 7.0 for solutions without Fe and 5.5 for Fe-containing solutions. Leaves, roots and xylem sap were sampled from 42-day-old sugar beet and 35-day-old tomato plants by centrifugation of petioles and plant sap were sampled from 42-day-old sugar beet and 35-day-old tomato plants by centrifugation of petioles and then filtered through 0.45 μm PVDF membranes and stored at −20 °C until analysis. Plant tissues were washed with distilled water, frozen in liquid N$_2$ and stored at −80 °C until analysis.

Healthy peach (_Prunus persica_ L. cv. ‘Andros’ and ‘Babygold7’ trees, not treated with Fe(III)-0.0EDEDDHA fertilizers in the full growing season, were sampled in the field in Zaragoza (Spain). Leaves were sampled 2–4 weeks after full bloom and fruits were harvested at commercial maturity dates. Leaves were washed with distilled water, frozen in liquid N$_2$ and stored at −20 °C until analysis. Fruits were peeled, a portion of the mesocarp was sampled from each opposite face and diced into 1 cm³ pieces, and a composite sample was built by mixing all of the small pieces from different peach fruits, immediately frozen in liquid N$_2$ and stored at −80 °C until analysis.

**Extraction of _0.0EDEDDHA_ from plant materials**

An extraction procedure suitable for subsequent HPLC/ESI-MS analysis was developed by modifying the procedure described by Bienfait et al.$^9$ An IS was always added (see below) to control possible systematic errors occurring during extraction, chromatography separation and ESI.

Frozen plant tissue (2.0–0.5 g FW) was ground with 1–2 mL of extraction solution (1 mM ammonium acetate, pH 6.0) containing the IS, in a ZrO$_2$ ball mill (MM301, Retsch, Haan, Germany) operating at a frequency of 30 rps until a good separation between pellet and supernatant. Then, the pellet was resuspended in 1 mL of extraction solution, centrifuged again and the supernatant was collected and combined with the previous one. The last step was repeated once. The combined final extract was filtered through 0.45 μm PVDF membranes and made up to volume with extraction solution.

**HPLC/ESI-MS(ToF) analysis**

_0.0EDEDDHA_ was determined as Fe(III)-0.0EDEDDHA using the HPLC/ESI-MS(ToF) method developed by Alvarez-Fernández et al.$^{14}$ Analyses were carried out with a Waters Alliance 2795 HPLC system (Waters, Milford, MA, USA) coupled to a MicrOTOF TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI source. The MicrOTOF was operated in negative ion mode with endplate and spray tip potentials of 0.5 and 3.0 kV, respectively. Nebulizer gas (N$_2$) pressure and drying gas (N$_2$) flow rate were kept at 1.6 bar and 8.0 L min$^{-1}$, respectively, orifice voltage was 120 V and drying gas temperature was 180 °C. Spectra were acquired in the 100–800 mass/charge ratio (m/z) range. The mass axis was calibrated using Li-formate adducts (10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol).

Samples were separated by HPLC using a Waters Symmetry C$_{18}$ column (5 μm particle size, 2.1 × 150 mm) protected by a Waters Symmetry C$_{18}$ guard column (3.5 μm particle size, 2.1 × 10 mm) and a gradient of methanol and Milli-Q water. pH was buffered in the chromatographic run with 5% of 20 mM ammonium acetate (pH 6.0). Injection volume and flow rate were 50 μL and 100 μL min$^{-1}$, respectively. Autosampler and column temperatures were 6 and 30 °C, respectively. The flow of the first 8 min of the chromatographic run was discarded by using a Reodhyne divert valve, in order to reduce ion suppression. The system was controlled with the software packages MicrOTOF Control version 2.2 (Bruker Daltonics) and HyStar version 3.2 (Bruker Daltonics). Data were processed with Data Analysis version 3.4 software (Bruker Daltonics). Concentrations of Fe(III)-0.0EDEDDHA were always quantified by external calibration with internal standardization.

**Internal standards**

Two types of internal standards (IS) were tested: (i) two Fe stable isotope labeled (SIL) Fe(III)-0.0EDEDDHA analogues, $^{54}$Fe(III)-0.0EDEDDHA and $^{57}$Fe(III)-0.0EDEDDHA, and (ii) a structural Fe(III)-0.0EDEDDHA analogue, Fe(III)-0.0EDEDDMA. Fe(III)-0.0EDEDDMA differs from Fe(III)-0.0EDEDDHA in the presence of two methyl groups located in both aromatic rings in para position (Figs. 1(A) and 1(D)).

Pre- and/or post-extraction IS addition techniques were applied as described by Taylor$^{21}$ using _0.0EDEDDHA-free_ plant material. Pure standard solutions used for spiking samples were analyzed throughout the process as indicated below for the tissue samples. _0.0EDEDDHA-free_ plant tissue extracts were first analyzed by HPLC/ESI-MS to ensure the absence of homogeneous extract was obtained (approximate grinding times were 0.5, 1 and 2 min for fruit, leaf and root materials, respectively). The suspension was centrifuged at 12000 g for 20 min at 4 °C and the supernatant was collected. These frequency and grinding time values were optimal to obtain a good separation between pellet and supernatant. Then, the pellet was resuspended in 1 mL of extraction solution, centrifuged again and the supernatant was collected and combined with the previous one. The last step was repeated once. The combined final extract was filtered through 0.45 μm PVDF membranes and made up to volume with extraction solution.
endogenous isobaric compounds co-eluting with the analyte and/or the IS that could cause analytical interferences. When using as SIL analogue \(^{56}\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA, two pre-extraction assays were carried out by spiking directly plant material before the extraction process with the analyte \(^{56}\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA and/or IS \(^{54}\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA. Plant tissue to final extract weight ratios (PTW/FEW ratios, \(\text{w/w}\)) were 160 mg g\(^{-1}\). Material before the extraction process with the analyte and IS concentrations were approximately 16 and 8 \(\mu\text{M}\), respectively. First, a post-extraction assay was carried out by spiking plant tissues after the extraction process with both the analyte and the IS. The extraction process was carried out at the optimum plant PTW/FEW ratio for each plant tissue as determined in the post-extraction assay. When using xylem sap from tomato and sugar beet, the sample was spiked directly with both the analyte and the IS just before the HPLC/ESI-MS analysis.

**Recovery assays and limits of detection and quantification**

Two recovery assays were also carried out with \(\sigma_\sigma\)EDDHA-free Fe-sufficient plant materials (leaves and roots from sugar beet and tomato plants and leaves and fruits from peach trees). This was carried out by adding, before the extraction process, known amounts of either a mixture of \(\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA plus the IS \(\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA or a combined solution of different metal-\(\sigma_\sigma\)EDDHA chelates (Ni(II)-, Co(II)-, Zn(II)-, Mn(II)-, Mg(II)-, Ca- and Cu(II)-\(\sigma_\sigma\)EDDHA) plus the IS \(\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA. An aliquot of the extract obtained as described above was treated with \(\text{NH}_4\text{OH}\) until pH 11–12 to dissociate the metal-\(\sigma_\sigma\)EDDHA complexes, an excess of \(\text{FeCl}_3\) was slowly added and the pH was adjusted to 6.0–7.0 with \(\text{HCl}\) to form \(\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA. Then, the solution was filtered through 0.45 \(\mu\text{m}\) PVDF membranes and made up to volume with extraction solution at the final PTW/FEW ratios found optimal for each plant tissue (see Table 1). Final concentrations of the analyte and IS were 20 and 10 \(\mu\text{M}\), respectively. For xylem sap, the recovery assays were carried out only with tomato, by directly spiking both the analyte and the IS just before the extraction process. The extraction process was carried out at the optimum plant PTW/FEW ratio for each plant tissue as determined in the post-extraction assay. When using xylem sap from tomato and sugar beet, the sample was spiked directly with both the analyte and the IS just before the HPLC/ESI-MS analysis.

**Analysis of plant materials treated with \(\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA**

Samples of leaves (1 g FW), roots (2 g FW) and xylem sap (0.3 g FW, approximately 300 \(\mu\text{L}\)) from sugar beet and tomato plants treated with \(\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA were spiked directly with the IS \(\text{Fe(III)}\)-\(\sigma_\sigma\)EDDHA. Tissues were extracted
Table 1. Recoveries (in %) of racemic and meso Fe(III)-o,oEDDHA in the post-extraction assay, using Fe(III)-o,oEDDHA as IS. Six different plant tissues were used at different plant tissue/final extract weight ratios (PTW/FEW, w/w). Data are means ± SE (n = 3)

<table>
<thead>
<tr>
<th>Plant tissue (PTW/FEW, mg g⁻¹ FW)</th>
<th>racemic</th>
<th>meso</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar beet leaves</td>
<td>160</td>
<td>110±4</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>111±2</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>103±2</td>
</tr>
<tr>
<td>Sugar beet roots</td>
<td>250</td>
<td>117±4</td>
</tr>
<tr>
<td></td>
<td>160’</td>
<td>102±2</td>
</tr>
<tr>
<td>Tomato leaves</td>
<td>160</td>
<td>114±4</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>110±1</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>103±2</td>
</tr>
<tr>
<td>Tomato roots</td>
<td>250</td>
<td>114±4</td>
</tr>
<tr>
<td></td>
<td>160’</td>
<td>102±2</td>
</tr>
<tr>
<td>Peach leaves</td>
<td>160</td>
<td>109±2</td>
</tr>
<tr>
<td></td>
<td>120’</td>
<td>99±2</td>
</tr>
<tr>
<td>Peach fruits</td>
<td>160</td>
<td>117±2</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>119±2</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>113±1</td>
</tr>
<tr>
<td></td>
<td>60’</td>
<td>104±2</td>
</tr>
</tbody>
</table>

*Optimal PTW/FEW ratios.

RESULTS AND DISCUSSION

Matrix effects for both Fe(III)-o,oEDDHA isomers (racemic and meso) were evaluated for all plant tissues at different plant tissue to final extract weight ratios (PTW/FEW). Strong matrix effects during the ionization of the analyte were found, with ion suppression occurring in most cases, although tomato tissues were an exception. Ion suppression accounted for approximately between 10 and 40% of the analyte signal, the largest values being found for peach tissues. Matrix effects were very similar for both racemic and meso isomers and were less marked when PTW/FEW values were lower (data not shown). In some materials such as tomato tissues, matrix effects were only minor. Since the use of an IS is considered the most appropriate method for controlling matrix effects during LC/ESI-MS experiments, several IS were tested in subsequent experiments.

Internal standards

Using Fe stable isotope labeled Fe(III)-o,oEDDHA as internal standards

LC/ESI-MS(TOF) ion chromatograms of the analyte and the IS in these solutions was 1 molecular ions (412.0 for ⁵⁶Fe(III)-o,oEDDHA, 413.0 for ⁵⁷Fe(III)-o,oEDDHA and 410.0 for ⁵⁴Fe(III)-o,oEDDHA), with a precision of ±0.02 m/z (Figs. 1(A)–1(C)). Fe(III)-o,oEDDHA geometric isomers were separated by HPLC, with elution times for the racemic and meso isomers of 14.8 and 16.5 min, respectively.

Plant tissue samples free of o,oEDDHA were also analyzed by HPLC/ESI-MS to look for possible isobaric compounds co-eluting with the analytes and/or the IS that may interfere with the determination. An endogenous isobaric interference at m/z 413.0 was found to co-elute partially with meso ⁵⁷Fe(III)-o,oEDDHA in sugar beet materials, whereas no isobaric interferences were found in all other cases (data not shown). Therefore, ⁵⁷Fe(III)-o,oEDDHA was discarded as a possible IS internal standard, and only ⁵⁴Fe(III)-o,oEDDHA was used as SIL internal standard in further experiments.

A pre-extraction assay was carried out by spiking a combined natFe(III)-o,oEDDHA plus ⁵⁴Fe(III)-o,oEDDHA solution into sugar beet and tomato plant tissues before the extraction process, and racemic and meso Fe(III)-o,oEDDHA were quantified by HPLC/ESI-MS. Recoveries were calculated for each isomer by dividing the concentration found in the extract by the concentration found in the pure combined standard solution used to spike the samples. Recoveries found were in the range of 150–180% for all plant materials (data not shown), indicating that the use of the IS ⁵⁴Fe(III)-o,oEDDHA did not give satisfactory results. Problems are unlikely to occur during HPLC/ESI-MS analysis, since natFe(III)-o,oEDDHA and ⁵⁴Fe(III)-o,oEDDHA co-elute in pure solutions (Fig. 1) and plant extracts (not shown). A likely cause for the >150% recovery values found in the spiked plant materials is the occurrence of Fe isotope exchange reactions between the ⁵⁴Fe originally bound to o,oEDDHA and the added natFe(III)-o,oEDDHA and native Fe present in plant materials, that could result in the de novo formation of natFe(III)-o,oEDDHA, the breakdown of ⁵⁴Fe(III)-o,oEDDHA, or both (Eqn. (1)). The equilibrium could be shifted towards the formation of natFe(III)-o,oEDDHA because of the higher concentration of natFe compounds present in plant materials compared with ⁵⁴Fe bound to o,oEDDHA.

\[
\begin{align*}
\text{Fe(III)} + \text{o,oEDDHA} &\leftrightarrow \text{Fe(III)} + \text{o,oEDDHA} + \text{Fe} \\
\text{Fe(III)} + \text{o,oEDDHA} + \text{Fe} &\leftrightarrow \text{Fe(III)} + \text{o,oEDDHA} + \text{Fe} \\
\end{align*}
\]
are means (leaves and roots) or the spiking process (xylem sap). Data o,o
o,o sap (E, F). Plant tissues were spiked only with 54Fe(III)-
# Copyright and roots, only the less stable isomer (meso
0.5) and roots (6.0–8.0) were significantly higher (at
p ≤ 0.05) for both isomers (Figs. 2(A)–2(D)). No
dependence of the As/AIS ratio with the time elapsed
between extraction and analysis was observed, indicating
that isotope exchange reactions occurred mainly during the
extraction process. The plant Fe nutritional status also
affected markedly the extent of isotope exchange reactions,
since the As/AIS ratios obtained for Fe-sufficient leaves (0.3–
0.5) and roots (6.0–8.0) were significantly higher (at p < 0.05)
than those corresponding to Fe-deficient tissues (values of
0.1–0.3 for leaves and 0.1–0.3 for roots). Iron concentrations in
Fe-sufficient roots were 8-fold higher than those of Fe-
deficient ones, whereas Fe-sufficient leaves had Fe concen-
trations only 3-fold higher than those of Fe-deficient ones.

In xylem sap, and in contrast to what happened in leaves
and roots, only the less stable isomer (meso 54Fe(III)–
oneEDDHA; log K 34.15)30 appeared to participate in Fe
isotope exchange reactions, whereas the most stable isomer
(racemic 54Fe(III)–o,oneEDDHA; log K 35.86)30 did not. This
could be due to an effect of the different endogenous Fe/
spiked 54Fe ratios occurring in leaf and root extracts and
xylem sap samples, which were in the ranges of 1.2–1.5 in leaf
and root extracts, and 0.2–0.5 in xylem sap. Therefore, the
amount of native Fe in xylem sap was likely limiting for isotope
exchange reactions, favoring isotopic exchange only
with the less stable isomer. Time-dependence of the Fe
isotope exchange reactions of meso 54Fe(III)–o,oneEDDHA was
observed in the xylem sap of both Fe-deficient and -sufficient
plants (Figs. 2(E) and 2(F)), since the As/AIS ratio increased
with time. At time 0 h (analyzed immediately after spiking)
very little, if any, isotope exchange reactions had occurred,
whereas significant increases in the As/AIS ratio occur
mainly before 6 and 12 h in Fe-deficient and Fe-sufficient
samples, respectively. Isotope exchange reactions occurring
in xylem sap were favored in Fe-deficient extracts compared
with Fe-sufficient ones, in spite of the fact that Fe
concentrations were lower in Fe-deficient than in Fe-
sufficient xylem sap (6 and 10 μM Fe, respectively). This
suggests that endogenous Fe compounds present in Fe-
deficient xylem sap undergo Fe isotope exchange reactions
more easily than those present in Fe-sufficient xylem, most
likely due to the significant differences occurring in chemical
composition.27

These results support that Fe SIL compounds are not
suitable as IS for the quantification of Fe(III)–o,oneEDDHA in
many plant materials. However, they could be adequate for
the quantification of different synthetic Fe(III)-chelates
(including Fe(III)–o,oneEDDHA) in liquid agricultural matrices
such as soil solutions, irrigation waters, nutrient solutions
and even in some plant samples such as peach xylem sap.14
Although SIL compounds are generally supposed to be the
best IS, the results of the present study indicate that they
must be tested for each specific matrix. Isotope exchange
reactions between SIL compounds used as IS have been also
described in previous studies. Chavez et al. found isotope
exchange reactions between the D atoms of the rofecobix SIL
13CD3rofecoxib and the 1H atoms of the water-containing LC
solvents when they were analyzed by HPLC/MS, but not
when non-polar solvents were used in GC/MS analysis.25 In
that study, a 13C-labeled compound, 13C7rofecoxib, was
successfully used, concluding that 13C-, 15N- or 17O- SIL
analogues may better choices than D-labeled compounds.
However, these SIL compounds are not always commercially
available or they are very expensive, as occurs in the case of
o,oneEDDHA (approximately 3000 euros per mg). In these
cases, structural analogues can be an alternative to SIL
internal standards, taking into consideration structural
similarities between the IS and the analyte.22,31

Using the structural analogue Fe(III)–o,oneEDDHA as
internal standard
In our case, the compound Fe(III)–o,oneEDDHA was
considered as the best option for a IS structural analogue
of Fe(III)–o,oneEDDHA, because of the high chemical similarity,
the only differences being the two methyl groups located
in para position (Figs. 1(A) and 1(D)). The compound
Fe(III)–o,oneEDDHA is also accepted by EU legislation as a

Figure 2. Time dependence of the 56Fe(III)–o,oneEDDHA/54-
Fe(III)–o,oneEDDHA ratios in a pre-extraction addition assay
carried out with extracts of Fe-deficient (-Fe) and Fe-sufficient
(+Fe) sugar beet leaves (A, B) and roots (C, D) and with xylem
sap (E, F). Plant tissues were spiked only with 54Fe(III)–
oneEDDHA and analyzed at different times after the extraction
(leaves and roots) or the spiking process (xylem sap). Data
are means ± SE (n = 3).

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commercial fertilizer, but its use is much less frequent than that of Fe(III)-o,o-EDDHA (3 and 72% of the commercial products in Spain in 2007, respectively).32 Therefore, it would be necessary to ascertain that Fe(III)-o,o-EDDHA is not present in plant samples prior to application of the proposed o,o-EDDHA analysis method. An LC/ESI-MS/TOF ion chromatogram of Fe(III)-o,o-EDDHA is shown in Fig. 1(D). The ion chromatogram was extracted at the corresponding 56Fe isotope signal of the [M–H]– molecular ion at an m/z value of 440.0. Retention times were 16.5 and 18.6 min for isomer 1 and isomer 2 of Fe(III)-o,o-EDDHA, respectively (Fig. 1(D)). Isomer 1 of Fe(III)-o,o-EDDHA co-eluted with the meso isomer of the analyte, and therefore it was chosen to be tested as IS. Since the racemic Fe(III)-o,o-EDDHA isomer eluted at a different time than the IS, the possibility that matrix effects associated with the presence of other co-eluting compounds may occur should be also assessed.

The post-extraction assay previously used by other authors12,33–35 was carried out by spiking a combined NaFe(III)-o,o-EDDHA plus NaFe(III)-o,o-EDDHA solution into plant extracts before the HPLC/ESI-MS analysis. Different PTW/FEW ratios were tested for each plant material, including sugar beet and tomato leaves and roots and peach leaves and fruits. PTW/FEW ratios tested were similar to or lower than those used by Bienfait et al.9 Recoveries were calculated as described above (Table 1). For all plant materials, recoveries of racemic Fe(III)-o,o-EDDHA were affected by the PTW/FEW ratio (Table 1). For high PTW/FEW ratios, recoveries were in the range of 119–109%, which indicated a slight increase of the analyte/IS response in comparison with the pure standard solution. Therefore, the co-eluting matrix components caused either an enhancement of the analyte ionization or a suppression of the IS ionization. With the lowest PTW/FEW ratios used with each material, recovery values were good, in the range of 96–104%, indicating that matrix effects affected in a similar way analyte and IS. Optimal PTW/FEW ratio values (in mg g−1) were 80 for sugar beet and tomato leaves, 120 for peach leaves, 160 for roots and 60 for peach fruits.

Using these optimal PTW/FEW ratios, a pre-extraction assay was carried out to test the suitability of the structural analogue Fe(III)-o,o-EDDHA as IS in the whole analytical method, including the extraction process, by spiking directly plant materials before the extraction process with a combined Fe(III)-o,o-EDDHA plus Fe(III)-o,o-EDDHA solution. Good recoveries, in the range of 93–106% for racemic Fe(III)-o,o-EDDHA and 96–105% for meso Fe(III)-o,o-EDDHA, were obtained for all plant materials (Table 2). In summary, the structural analogue Fe(III)-o,o-EDDHA could be considered as a suitable IS for the determination of Fe(III)-o,o-EDDHA in plant tissue extracts.

**Recovery assays**

It is assumed that the chelating agent o,o-EDDHA could occur inside the plant in chemical forms other than Fe(III)-o,o-EDDHA.36 Therefore, to ensure the total o,o-EDDHA determination in plant materials, recovery assays were also carried out by adding an excess of Fe(III) to the plant extracts to facilitate the chelation of all o,o-EDDHA chemical forms with Fe(III), and its subsequent determination as Fe(III)-o,o-EDDHA by HPLC/ESI-MS. The pH of plant extracts were adjusted to pH 11–12 before the addition of Fe excess to facilitate the existence of free, soluble o,o-EDDHA forms, and then the pH was slowly adjusted to neutral values, where Fe(III)-o,o-EDDHA is known to be stable.30

Two different pre-extraction recovery assays were carried out by spiking plant tissues with either Fe(III)-o,o-EDDHA plus the IS Fe(III)-o,o-EDDHA or a combined standard solution of other metal-o,o-EDDHA complexes plus the IS Fe(III)-o,o-EDDHA. For both assays, recoveries were similar and acceptable (in the range of 74–118%) independently of the metal-o,o-EDDHA complex spiked into the plant material (Table 2). Differences between isomers were observed, with meso recovery values being better (average of 104%) than those of racemic (average of 90%), as also observed in the previous assays (Tables 1 and 2). The reason for that is likely due to the fact that the IS co-eluted with the meso Fe(III)-o,o-EDDHA isomer, whereas the racemic Fe(III)-o,o-EDDHA isomer had a different retention time. When comparing plant materials, the lowest recoveries were obtained for the racemic isomer in tomato leaves (average 79%) and roots (average 77%). Since the protocol of these assays was more complex than those used in previous assays (Tables 1 and 2), involving several steps and including pH changes, higher standard errors were obtained (Table 2).

**Limits of detection and quantification**

LODs and LOQs for racemic and meso o,o-EDDHA in different plant materials are shown in Table 4. Overall sensitivity was high, with LOQs between 11 and 162 pmol g−1 FW for plant tissues and between 60 and 151 pmol mL−1 for xylem sap. These values are at least an order of magnitude better than the only LOQ reported so far, that was 2140 pmol g−1 FW for tomato, lettuce and pepper using HPLC/UV-VIS.9 In other studies with tobacco and tomato, LOQs were not reported but the minimum amount of Fe(III)-o,o-EDDHA determined was also very high, 3500 pmol Fe(III)-o,o-EDDHA g−1 FW.8

**Analysis of o,o-EDDHA in plant materials**

Fe-deficient sugar beet and tomato plants were treated with a Fe(III)-EDDHA commercial fertilizer (90 μM Fe(III)-o,o-EDDHA) for 24 h. Leaves, roots and xylem sap were sampled and submitted to the extraction method developed, using as IS Fe(III)-o,o-EDDHA. HPLC/ESI-MS(TOF) analyses of the

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**Table 2. Recoveries (in %) of racemic and meso Fe(III)-o,o-EDDHA in the pre-extraction assay, using Fe(III)-o,o-EDDHA as IS. Eight different plant tissues were used at the optimal PTW/FEW ratios found in the post-extraction assay (see Table 1). Data are means ± SE (n = 5)**

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Recovery (°)</th>
<th>Racemic</th>
<th>Meso</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar beet</td>
<td>Leaves</td>
<td>95 ± 3</td>
<td>100 ± 1</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>102 ± 2</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>Tomato</td>
<td>Leaves</td>
<td>93 ± 4</td>
<td>100 ± 1</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>101 ± 2</td>
<td>103 ± 2</td>
</tr>
<tr>
<td></td>
<td>Xylem sap</td>
<td>95 ± 2</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>Peach</td>
<td>Leaves</td>
<td>100 ± 4</td>
<td>105 ± 2</td>
</tr>
<tr>
<td></td>
<td>Fruits</td>
<td>106 ± 2</td>
<td>101 ± 1</td>
</tr>
</tbody>
</table>
Table 3. Recoveries (in %) of racemic and meso o,o-EDDHA in a pre-extraction assay in the presence of an Fe excess, using Fe(III)-o,o-EDDHA as IS, after spiking plant tissues with Fe(III)-o,o-EDDHA (assay 1) or a combined solution of Ca(II)-, Mg(II)-, Cu(II)-, Mn(II)-, Ni(II)-, Co(II)-, Zn(II)-o,o-EDDHA (assay 2). Data are means ± SE (n = 5)

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>racemic</td>
<td>meso</td>
</tr>
<tr>
<td>Sugar beet</td>
<td>110 ± 4</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>Leaves</td>
<td>94 ± 6</td>
<td>106 ± 8</td>
</tr>
<tr>
<td>Roots</td>
<td>83 ± 3</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>Tomato</td>
<td>80 ± 1</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>Xylem sap</td>
<td>103 ± 7</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>Leaves</td>
<td>93 ± 6</td>
<td>116 ± 10</td>
</tr>
<tr>
<td>Fruits</td>
<td>93 ± 2</td>
<td>102 ± 1</td>
</tr>
</tbody>
</table>

extracts were performed before and after the addition of an Fe excess to determine the o,o-EDDHA bound to Fe(III) and the sum of all o,o-EDDHA chemical forms occurring in the extracts, respectively. Ion chromatograms showing the occurrence of Fe(III)-o,o-EDDHA in tomato leaf and root extracts and xylem samples are shown in Fig. 3. Racemic and meso Fe(III)-o,o-EDDHA were found at 14.8 and 16.5 min, respectively; a third peak was found at 14.8 min and was identified as the Fe(III)-o,p-EDDHA isomer.14 That is also present in the commercial Fe(III)-EDDHA fertilizer used (this product contains approximately 4.3 and 0.9 g of Fe(III)-o,o-EDDHA and Fe(III)-o,p-EDDHA per 100 g of product, respectively).14 Both o,o-EDDHA and Fe(III)-o,p-EDDHA were found in all plant materials in both plant species (Table 5). o,o-EDDHA was mainly in the Fe(III)-chelate form in leaves, roots (approximately 67% in both cases) and in tomato xylem sap (approximately 90%).

The presence of o,o-EDDHA in plants was first demonstrated in leaves8 by direct VIS spectrophotometry of purified extracts. More recently, o,o-EDDHA was found in leaves and roots by HPLC/VIS.9 In our study, o,o-EDDHA concentrations in the range of 12–63 nmol g⁻¹ FW with a short exposure time to Fe(III)-o,o-EDDHA, whereas in previous studies,8,9 carried out in many cases with longer exposure times, concentrations were in the range 7–30 nmol g⁻¹ FW. In the present study, o,o-EDDHA concentrations in leaves were always 2–4-fold higher than those of roots in sugar beet and tomato, whereas previous studies reported similar values in roots and leaves.9 Both isomers occurred in similar concentrations in all tissues and in both species, with racemic/meso ratios in the range of 0.9–1.2. However, lower values for that ratio were found in pepper and tomato leaves (0.7–1.0), whereas in roots ratios were of approximately 1, and this was attributed to differences in translocation from roots to shoots or and in degradation in leaves between isomers.9 Prolonged treatments such as those

Table 4. Limits of detection (LOD) and quantification (LOQ) for racemic and meso Fe(III)-o,o-EDDHA in eight different plant tissues. LOD and LOQ values are in pmol g⁻¹ FW for plant tissues and in pmol mL⁻¹ for xylem sap

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>LOD (pmol g⁻¹ FW)</th>
<th>LOQ (pmol g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>racemic</td>
<td>meso</td>
</tr>
<tr>
<td>Sugar beet</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>Leaves</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Roots</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td>Tomato</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Leaxes</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Xylem sap</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td>Peach</td>
<td>49</td>
<td>29</td>
</tr>
<tr>
<td>Fruits</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Figure 3. Ion chromatograms (at m/z 412.0) for extracts of leaves (A) and roots (B), and xylem sap (C) from tomato plants treated with a Fe(III)-EDDHA commercial fertilizer for 24 h. Racemic and meso Fe(III)-o,o-EDDHA were found at 14.2 and 16.0 min, respectively. The peak at retention time 14.8 min corresponded to Fe(III)-o,p-EDDHA.

Table 5. Fe(III)-o,o-EDDHA and o,o-EDDHA concentrations (in nmol g⁻¹ FW for plant tissues and in nmol mL⁻¹ for xylem sap) in plants treated with a Fe(III)-EDDHA commercial fertilizer (90 μM Fe(III)-o,o-EDDHA) for 24 h. Data are means ± SE (n = 3)

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Fe(III)-o,o-EDDHA Concentrations</th>
<th>o,o-EDDHA Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>racemic</td>
<td>meso</td>
</tr>
<tr>
<td>Sugar beet</td>
<td>Leaves</td>
<td>8.5 ± 0.5</td>
</tr>
<tr>
<td>Roots</td>
<td>4.3 ± 0.4</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Xylem sap</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Tomato</td>
<td>Leaves</td>
<td>19.9 ± 0.8</td>
</tr>
<tr>
<td>Roots</td>
<td>6.4 ± 1.2</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>Xylem sap</td>
<td>0.5 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

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used by Bienfait et al. apparently seem to facilitate a preferential occurrence of the meso isomer.9

Results show for the first time that Fe(III)-o,pEDDHA is also present in leaves, roots and xylem sap of plants treated with commercial Fe(III)-EDDHA products (Fig. 3). However, quantification was not possible because of the lack of commercially available standards. Further research is needed to design and validate an appropriate methodology to determine this compound.

CONCLUSIONS

The method developed permits the determination by HPLC/ESI-MS of the xenobiotic o,oEDDHA chelating agent used in Fe fertilizers, with extreme selectivity, high sensitivity and sufficient accuracy and reproducibility, in a wide range of species and plant tissues. Samples tested include sugar beet leaves and roots, tomato leaves and roots and peach leaves and fruits. The results presented in this paper demonstrate the need for a careful evaluation and proper choice of the internal standard (IS) used for quantification in complex matrices such as plant materials, when using HPLC/ESI-MS-based methods. Iron stable isotope labeled Fe-o,oEDDHA does not appear to be a suitable IS, mainly because of the occurrence of isotope exchange reactions during extraction and/or sample treatment. An adequate IS would probably be any $^{13}$C-, $^{15}$N- or $^{17}$O-stable isotope labeled chelating agent (o,oEDDHA), but they are not commercially available. A structural analogue, one of the Fe(III)-o,oEDDHA isomers, has been confirmed to be an adequate IS for o,oEDDHA determination in plant tissues by HPLC/ESI-MS, therefore constituting a useful tool for studies on o,oEDDHA plant uptake, transport and allocation. o,oEDDHA was found in all plant tissues tested in tomato and sugar beet plants treated with moderate (90 μM) Fe(III)-o,oEDDHA doses for only one day.

Acknowledgements

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