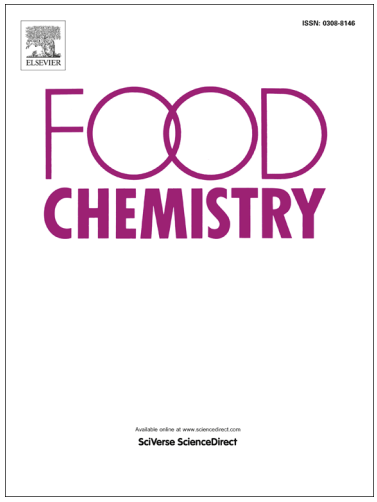

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Identification of the key excreted molecule by *Lactobacillus fermentum* related to host iron absorption

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Abstract

We have taken a vital step towards understanding why probiotic bacteria increase iron absorption in the gastrointestinal tract. We show here that *Lactobacillus fermentum*, one of the main probiotics of the microbiota, exhibits an extraordinary ferric-reducing activity. This activity is predominantly due to an excreted molecule: *p*-hydroxyphenyllactic acid (HPLA). Reduction of Fe(III) to Fe(II) is essential for iron absorption in the gastrointestinal tract. By reducing Fe(III), HPLA boosts Fe(II) absorption through the DMT1 channels of enterocytes. An *in vitro* experiment tested and confirmed this hypothesis. This discovery opens new avenues for the treatment of iron deficiency in humans, one of the most common and widespread nutritional disorders in the world.

Keywords. Iron metabolism, Probiotic bacteria, *Lactobacillus fermentum*, Iron supplement.

1. Introduction

Iron is an indispensable element for humans due to its involvement in essential biochemical processes, including energy production, biosynthesis, replication, and locomotion (Crichton, 2001; Silva & Faustino, 2015). Iron deficiency is the most common and widespread nutritional disorder in the world. Besides affecting many children and women in developing countries, it is the only nutrient deficiency prevalent in industrialized countries. The human body receives iron from food. Though the daily requirement is 20-25 mg, no more than 2 mg are really absorbed (Milto et al., 2016). Adequate iron levels in the blood are partially maintained by recycling the iron released upon the degradation of iron-containing proteins, especially hemoglobin.

Heme-iron, present in hemoglobin and myoglobin, has high bioavailability (Huch & Schaefer 2006); however, the majority of iron in food (>90%) is in non-heme forms, either as free iron or bound to low molecular-weight biomolecules (Crichton et al., 2008). Iron from food is mainly absorbed by the duodenum enterocytes (90%). The stomach does not play a significant role in the assimilation of iron, as its absorption is no more than 2% of the total (Shayeghi et al., 2005).

Iron transfer through the enterocyte membrane occurs by the combined activities of two proteins: DMT1 and DcytB. The coupled work of DcytB/DTM1 is required for iron absorption since iron enters the small intestine lumen mainly as Fe(III), the result of Fe(II) oxidation by gastric juice components. Free Fe(III) is not able to enter into the enterocyte and must be reduced (McKie, 2008; Lane et al., 2015). The iron-regulated ferrireductase protein, DcytB, is highly expressed at duodenal enterocytes and reduces Fe(III) to Fe(II). Upon reduction, Fe(II) is transferred across the apical membrane of enterocytes by divalent metal transporters, mainly DMT1. Once inside the enterocytes, Fe(II) can be used for synthesis of iron-containing proteins, transported to plasma by the membrane protein ferroportin, or stored inside ferritin for later use when needed by cells

(McKie, 2008; Lane et al., 2015).

Interestingly, the bioavailability of iron is influenced by a series of chemical species in the gastrointestinal tract. Whereas oxalates, phytates, phenolates and phosphates suppress iron absorption, ascorbic and citric acids, increase it (Conrad & Umbreit, 2002). For this reason commercial iron supplements are usually accompanied by ascorbic or citric acid. Strategies to increase the intake of iron-rich foods, as well as dietary factors that enhance iron absorption, are therefore extremely important for human health.

Probiotic bacteria constitute an important part of natural microbiota. Bacteria produce many different metabolic substances that can act as antioxidants, bioflocculants or even immune activators (Sommer & Bäckhed, 2013; Peccia & Kwan, 2016; Lin et al., 2014). They survive the harsh stomach conditions and nest in different areas of intestines. Though the European Food Safety Authority (EFSA) has recently concluded that there is insufficient evidence to claim that a probiotic can help boost iron absorption (EFSA, 2016), other studies have shown that the probiotic bacteria of the human gut microbiota facilitate iron absorption (Pérez-Conesa et al., 2007; Hoppe et al., 2015; Bering et al., 2006). For example, it has been observed that the amount of probiotic bacteria in gut flora is significantly lower in anemic patients than in healthy people.

The mechanisms of this influence are not entirely understood. As of yet no compound excreted by bacteria has been shown to be related to iron absorption. Some have proposed that bacterial fermentation in intestines decreases the pH due to the production of lactic acid, which can increase iron solubility, leading to higher iron absorption (Hoppe et al., 2015). No published study has evaluated the effect of the ferric-reducing activity of probiotic bacteria on iron absorption. We show that *Lactobacillus fermentum*, one of the main components of the microbiota, exhibits considerable extracellular ferric-reducing activity. We have isolated and identified the excreted

molecule that produces the ferric-reducing activity of *Lactobacillus fermentum*: *p*-hydroxyphenyllactic acid (HPLA). This molecule efficiently reduces Fe(III) to Fe(II) at acidic pH. From the results described here, we propose that the increase of iron absorption in humans, due to the presence of probiotic bacteria in gut flora, could be related, not only to the decrease of pH caused by these lactic acid bacteria (Hoppe et al., 2015), but also to the ferric-reducing activity of the excreted HPLA. We have performed an *in vitro* experiment on iron uptake by enterocytes to confirm that HPLA promotes iron absorption. These results shed light on the reason why probiotic bacteria increase iron absorption in humans and can lead to more efficient strategies to increase iron absorption in humans.

2. Materials and methods

2.1. Lactobacillus fermentum culture

L. fermentum CECT5716 was grown in anaerobic conditions in a synthetic medium at 37 ºC with orbital agitation for 24 h at an initial concentration of 1 mg of bacteria per ml of medium. The synthetic medium consisted of (g l^{-1}) Na_2HPO_4 – 5.0, KH_2PO_4 – 6.0, trisammonium citrate – 2.0, sucrose – 50.0, $MgSO_4$ – 1.0 and trace elements solution – 10 ml (consisting of (g l^{-1}): $MnSO_4$ – 2.0, $CoCl_2$ – 1.0, $ZnCl_2$ – 1.0 dissolved in 0.1 N HCl solution). The medium with an initial pH 6.7 was sterilized at 121 ºC. The final *L. fermentum* cell concentration was $3.3x10^8$ CFU ml^{-1}.

2.2. Ferric-reducing activity of L. fermentum

1 ml of bacterial culture was mixed with 3.6 µl of a 10 mM $Fe(NO_3)_3 \cdot 9H_2O$ water solution and 7 µl of a 70 mM 3-(2-pyridiyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (ferrozine, fz) water solution and the resulting mixture was incubated for 6 and 24 h and then centrifuged. The ferric-reducing capacity of *L. fermentum* was measured by UV-vis

spectroscopy through the apparition of a band centred at 562 nm due to the formation of the complex $[Fe^{II}(fz)_3]$ (ε^{562}= 27,900 M^{-1}). A control of the ferric-reducing activity of the culture media, without bacteria, showed no UV-vis band at 562 nm. Similar ferric-reducing activity was exhibited by the corresponding supernatant solution (1 ml) from the bacterial culture.

2.3. Isolation of excreted bacterial compound/s with ferric-reducing activity

Bacteria were removed by centrifugation at 3000 *g* for 10 min and then the supernatant was filtered, using EMD Millipore Steritop™ Sterile Vacuum Bottle-Top Filters. 2 l of the filtered supernatant solution were subjected to solid-phase extraction on a column packed with SP-207ss brominated polystyrenic resin (65 g to render a column bed of 3.5 cm diameter and 11 cm height) previously equilibrated with water. The column was washed with water (1 l) and afterwards eluted with 200 ml of methanol. The solvent was evaporated under a nitrogen stream and the resulting extract dissolved in 500 µl of DMSO and further purified by reversed phase semi-preparative HPLC (Agilent Zorbax SB-C8, 9.4 × 250 mm, 7 µm; 3.6 ml/min, UV detection at 210 and 280 nm) with a linear gradient of water-CH3CN (5% to 50%) over 40 min to yield 80 fractions of 1.8 ml. The ferric-reducing activity of each fraction was tested, using a 96-well microtitre plate containing 2 µl of each fraction diluted with 198 µl of water. 1.08 µl of a 10 mM $Fe(NO_3)_3$ stock solution and 2.1 µl of a 70 mM fz stock solution were added to each well. The presence of the complex $[Fe^{II}(fz)_3]$, determined by measuring the absorbance at 570 nm, using a microplate reader (Envision 2104 multilabel reader. Perkin Elmer), was detected only in fractions 8 to 11. These fractions were pooled, evaporated and then dissolved in 1 ml of distilled water. The resulting water solution was further purified by reversed phase semi-preparative HPLC (Waters Atlantis C18, 9.4 × 250 mm, 7 µm; 3.6 ml/min, UV detection at 210 and 280 nm) with a linear gradient of water-CH_3CN (0 to 25%) over 40 min to yield 80 fractions of 1.8 ml. The ferric-reducing activity

of these fractions was tested as before. Only fractions 28 and 29 exhibited ferric-reducing activity. These fractions were pooled, evaporated and analyzed by LC-ESI-TOFMS and NMR spectroscopy.

2.4. Structure elucidation of the excreted bacterial compound with ferric-reducing activity

For NMR analysis, the sample (pooled dry fractions or the HPLA standard) was dissolved in deuterated methanol, CD_3OD. NMR spectra, including ^1H, COSY, HSQC and HMBC, were recorded on a Bruker Avance III spectrometer (500 and 125 MHz for 1H and 13C NMR, respectively) equipped with a 1.7 mm TCI MicroCryoProbeTM, using the signal of the residual deuterated solvent as internal reference (δ_H 3.31 and δ_C 49.0 ppm). LC-DAD-ESI-HRMS analysis of an aliquot of the final pooled fractions was performed on an Agilent 1200 rapid resolution HPLC system hyphenated with a Bruker maXis QTOF mass spectrometer, using a Zorbax SB-C8 column (2.1 × 30 mm, 5 µm), maintained at 40 ºC and with a flow rate of 300 µl min^{-1}. Solvent A consisted of 10% acetronitrile and 90% water with 1.3 mM trifluoroacetic acid and ammonium formate, and solvent B was 90% acetronitrile and 10% water with 1.3 mM trifluoroacetic acid and ammonium formate. The gradient started at 10% B and went to 100% B in 6 minutes, kept at 100% B for 2 minutes and returned to 10 % B for 2 min to initialize the system. Full diode array UV scans from 100 to 900 nm were collected in 4 nm steps at 0.25 s/scan. The mass spectrometer was operated in positive ESI mode. The instrumental parameters were: 4 kV capillary voltage, drying gas flow of 11 l min^{-1} at 200 ºC, nebulizer pressure at 2.8 bars. TFA-Na cluster ions were used for mass calibration of the instrument prior to sample injection. Pre-run calibration was by infusion with the same TFA-Na calibrant. The structure of the target compound was established, using the NMR data. Its molecular formula was corroborated by the HRMS results. Further confirmation on the identity was obtained by direct comparison with the ^1H and HSQC NMR

spectra of an HPLA standard prepared in CD_3OD. The corresponding test of ferric-reducing activity of the HPLA standard unequivocally determined this molecule as the target excreted compound responsible for such reducing power displayed by the *L. fermentum* culture.

2.5. Ferric-reducing activity of HPLA vs pH

Racemic HPLA was acquired from Sigma-Aldrich. Solutions of HPLA (144 µM) were prepared in different buffers with pH ranging from 3.0 to 7.4 and mixed with solutions of 10 mM $Fe(NO_3)_3 \cdot 9H_2O$ and 70 mM of fz. Ferric-reducing activity of HPLA at these different pH values was measured by UV-vis spectroscopy through the appearance of a band centred at 562 nm, due the formation of the complex $[Fe^{II}(fz)_3]$ (ε^{562}= 27,900 M^{-1}). The results showed that HPLA exhibits high ferric-reducing activity only at low pH.

2.6. Ferric-reducing activity of L. fermentum after HPLA addition

The ferric-reducing capacity of *L. fermentum* was measured, as indicated above, after incubation for 6 and 24 h. HPLA (3.6 µM) was added to the supernatant obtained after centrifugation of the 6 h culture and the ferric-reducing activity of the resulting mixture was measured. The ferric-reducing activity of HPLA (3.6 µM) in the medium grown was used as control.

2.7. Ferric-reducing activity of HPLA versus lactic acid (LA), p-methylphenol (p-mPh) and the mixture p-methylphenol and lactic acid (LA+p-mPh)

Two different experimental conditions were assayed, excess and deficiency of Fe(III) with respect to the reducing molecules. The ferric-reducing activity was referred to the initial concentrations of Fe(III) or the chemical reductant, respectively.

A 50 mM stock solution of HPLA was prepared by dissolving 9.1 mg in 1 ml of Mili-Q water. Solutions of LA and *p*-mPh at the same 50 mM concentration were prepared, as well as a mixture solution of both (LA+*p*-mPh) containing 50 mM of each reducing molecule. Lactic acid and *p*-

methylphenol were also purchased from Sigma-Aldrich.

110 µl of each stock solution were added to 879 µl of Mili-Q water containing 3.6 µl of a 10 mM solution of $Fe(NO_3)_3 \cdot 9H_2O$ and 7 µl of a ferrozine solution (70 mM). The *p*-methylphenol and lactic acid mixture was preparing by adding 110 µl of the *p*-methylphenol stock solution and 110 µl of the lactic acid stock solution to 769 µl of Mili-Q water containing 3.6 µl of a 10 mM $Fe(NO_3)_3 \cdot 9H_2O$ solution and 7 l of a 70 mM fz solution. The final concentrations of the reducing molecules and Fe(III) were 5.5 mM and 36 µM. The ferric-reducing activity of each sample was determined at 0, 2, 4 and 24 h by monitoring the UV-vis band at 562 nm of the complex $[[Fe^{II}(fz)_3]$. In a second experiment a 25 µM concentration of each reducing molecule was used.

2.8. In vitro experiment on iron internalization in enterocyte cells

IEC6 epithelial rat small intestine cells were provided by the Scientific Instrumentation Centre (University of Granada, Spain). Cell line was grown adherently and maintained in DMEM + 2mM glutamine + 0.1 IU/ml of insulin + 5% of foetal bovine serum (FBS) at 37 °C in 5% CO_2. All experiments were performed in 12-well plates. Cells were seeded onto the plates at a density of 5 $\times 10^4$ cells per well and incubated for 48 h prior to the experiments.

The wells were filled up to a final volume of 1ml with a water solution of $Fe(NO_3)_3 \cdot 9H_2O$ (360 µM), the grown medium and HPLA, at two different concentrations, 360 and 36 µM. Two more wells with a blank sample containing only cells were also prepared. Plates were incubated at 37ºC for 0.5 or 5 h. Samples were filtered, using a 0.2 µm Minisart RC filter and the total iron concentration (Fe(II) and Fe(III)) measured by ICP-OES.

2.9. Statistical analysis

The ferric-reducing activity of HPLA versus LA, *p*-mPh and the mixture LA+*p*-mPh and the *in vitro* experiment on iron internalization in enterocyte cells were conducted in triplicates.

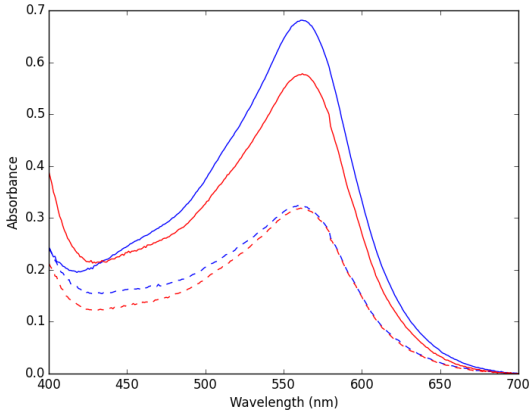
Descriptive error bars represent the standard deviation (SD) of the triplicates and it was calculated using the n-1 method.

3. Results and discussion

3.1. Lactobacillus fermentum shows a high ferric-reducing activity

The ability to reduce Fe(III) to Fe(II) is usually related to the antioxidant properties of a chemical species. In the context of iron metabolism, this redox reaction is essential for iron absorption since non-heme iron is usually in the form of Fe(III), which cannot be absorbed by enterocytes until it has been reduced to Fe(II).

The ferric-reducing activity of *L. fermentum* was measured through its incubation with Fe(III) and ferrozine by monitoring the formation of the Fe(II) complex with the chelator, $[Fe^{II}(fz)_3]$, through the appearance and increase of its characteristic UV-vis band at 562 nm. The supernatant liquid isolated after centrifugation of the bacteria exhibits activity similar to that of the bacterial broth after 6 and 24 h (Figure 1). This evidence indicates that the ferric-reducing ability of *L. fermentum* is due to the molecule(s) excreted by the bacteria, However, the patterns of ferric-reducing activity of the supernatant and the bacterial culture differ. Whereas the former is able to reduce a specific amount of Fe(III) and then is rendered inactive, the latter exhibits prolonged activity. The bacterial culture can reduce additional amounts of Fe(III) after reducing the same amount of Fe(III) as reduced by the supernatant. This could be explained if the bacterium continuously excreted or regenerated the molecule responsible for its ferric-reducing activity.



204

205 Figure 1. Ferric-reducing activity of *L. Fermentum* (red) and the supernatant liquid (blue) after 6
206 (dashed lines) and 24 h. Development of the UV-vis band centred at 562 nm due to $[Fe^{II}(fz)_3]$
207 shows the ferric-reducing activity.

208

209 The characterization of excreted active molecules by living organisms is of great interest in the
210 biomedical field. In fact, some of these molecules are in the pharmaceutical market for different
211 medical purposes. We addressed the challenge of isolating and characterizing the molecule
212 responsible for the ferric-reducing activity of *L. fermentum* because no such study has been thus
213 far done.

214 An assay-guided fractionation approach was implemented. First, an extract of the culture
215 supernatant was prepared. A brominated polystyrenic resin (SP207ss), with the capacity to retain
216 both polar and non-polar compounds, was used for the solid-phase. After confirming the ferric-
217 reducing activity of the extract obtained by elution from the resin with methanol, the extract was
218 subjected to semipreparative HPLC fractionation. After passing through a C8 reversed-phase
219 column, the fractions with the reducing ability were pooled and further fractionated with a C18
220 column. The two fractions showing ferric-reducing activity were pooled and analyzed by NMR

and LC-DAD-ESI-TOFMS. The ^{1}H NMR spectrum (Figure 2) revealed a mixture of a few compounds. The signals of a *para*-hydroxyphenyl group, corresponding to one of the major components, could easily be identified. Additional 2D NMR experiments, including COSY, HSQC and HMBC (supporting information S1-S8), were employed to elucidate the connectivity of the compound containing this structural moiety, which turned out to correspond to *p*-hydroxyphenyllactic acid (HPLA, Chart 1).

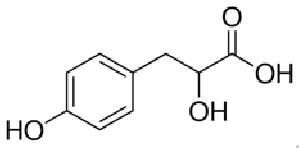


Chart 1. Chemical structure of *p*-hydroxyphenyllactic acid HPLA.

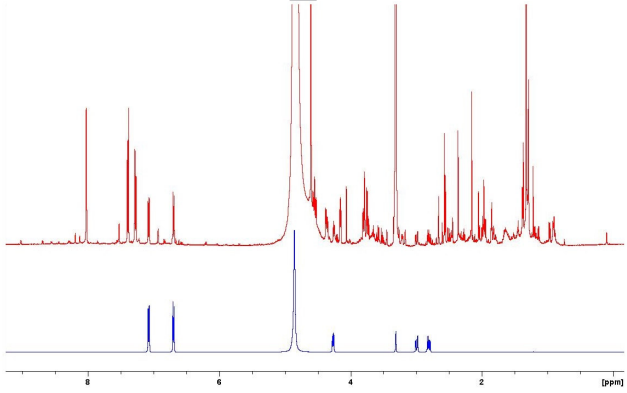


Figure 2. ^{1}H NMR spectra (500 MHz, CD_3OD) of the final pooled fractions displaying ferric-reducing activity (red) and p-hydroxyphenyllactic acid standard, HPLA (blue).

The molecular formula of HPLA, $C_9H_{10}O_4$, was a major component detected in the corresponding LC-HRMS analysis (Supporting Information S9).

Further confirmation of the identity of the compound was obtained by direct comparison of the

NMR spectra of the final pooled active fractions with the spectrum of a commercial HPLA standard (Figure 2). When the standard was evaluated for its ferric-reducing activity, it showed the same reducing power. Thus, HPLA is the compound excreted by *L. fermentum,* responsible for the bacterial ferric-reducing activity.

Interestingly, HPLA has already been identified as an antioxidant compound, in a radical-scavenging assay, produced by the sister species *Lactobacillus plantarum* (Suzuki et al., 2013). The production of HPLA by lactic acid bacteria (LAB) was first reported from *L. plantarum* 21B (Lavermicocca et al., 2000). This compound has shown antifungal activity and contributes to the biopreservative properties claimed for LAB (Crowley et al., 2013). HPLA has been isolated as its L- enantiomer in *L. plantarum* (Suzuki et al., 2013). The same chirality is expected for the HPLA produced by the *L. fermentum* strain employed in this work, assuming a common pathway for HPLA production among lactobacilli. The biosynthesis of HPLA in LAB likewise explains the apparent regeneration of the ferric-reducing activity observed for *L. fermentum* in reduction experiments with increasing amounts of Fe(III). In these bacteria, tyrosine is transformed by transamination into 4-hydroxyphenylpyruvate, which is further reduced to HPLA by a hydroxyacid dehydrogenase (Lavermicocca et al., 2000; Valerio et al., 2004; Li et al., 2007). Transamination is the rate-limiting step in such biosynthesis. It has been demonstrated that HPLA production dramatically increases in fermentations of *Lactobacillus sp. SK007* supplemented with 4-hydroxyphenylpyruvate (tyrosine as supplement renders just a moderate increase in production) (Mu et al., 2010). These results indicate that the bacterium is very efficient in reducing 4-hydroxyphenylpyruvate to HPLA. Such efficiency would help to recycle the HPLA consumed in the reduction of Fe(III). In this ferrireduction HPLA gets oxidized to 4-hydroxyphenylpyruvate which would get back to HPLA via the corresponding Lactobacillus hydroxyacid dehydrogenase,

that way explaining the apparent regeneration of the ferric-reducing activity already mentioned.

Once HPLA was identified as the molecule responsible for the ferric-reducing activity of *L. fermentum*, a study of its ability to reduce Fe(III) to Fe(II) as a function of pH was carried out. As shown in Figure 3, HPLA exhibits a ferric-reducing activity extremely dependent on pH. At pH above 6, HPLA hardly reduces Fe(III) to Fe(II) but, at pH 3.8, this reduction is practically complete.

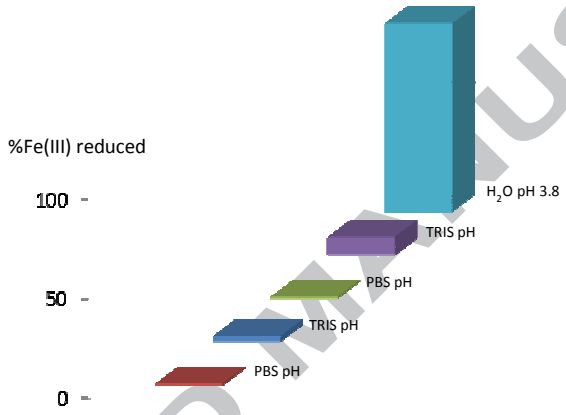


Figure 3. Ferric-reducing activity of HPLA at different pHs and buffers. The % of Fe(III) reduction was calculated as the ratio between concentrations of $[Fe^{II}(fz)_3]$ and the initial Fe(III).

To further verify that HPLA is the principal factor in the ferric-reducing activity of *L. fermentum*, we performed an experiment in which HPLA was directly added to a 6 h bacterial culture. The resulting ferric-reducing activity of the mixture was compared to that of the 24 h bacterial culture. As shown in Figure 4, the addition of HPLA to the culture media leads to an increase in the ferric-reducing activity (Figure 4, green bar). The activity of this mixture is close to the sum of that of *L. fermentum* (Figure 4, blue bar) and the HPLA control (Figure 4, red bar). Likewise, the addition of HPLA to the 6 h culture medium gives rise to a ferric-reducing activity close to that of the 24 h

culture medium, indicating that HPLA is continuously excreted during the bacterial growth from 6 to 24 h and that this molecule is responsible for the ferric-reducing activity of *L. fermentum*.

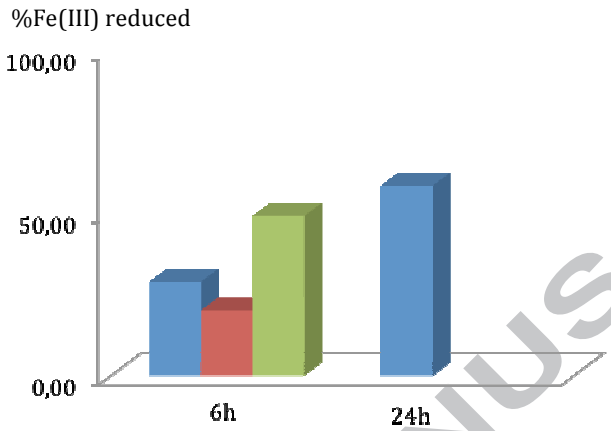


Figure 4. Ferric-reducing activity, as a percentage of Fe(III) reduction, of a 6 and 24 h culture of *L. fermentum* (blue), the addition of HPLA (3,6 µM) to the culture medium (green), and HPLA at the same concentration in the growth medium (red).

Interestingly, HPLA seems to be a specific reductant of Fe(III). It does not reduce other chemical species, such as Au(III) or the polyoxometalate $[P_2Mo^{VI}_{18}O_{62}]^{6-}$, which are quite reduced in the presence of *L. fermentum* (Carmona et al., 2014; Gonzalez et al., 2015). This result indicates that the chemical reducing mechanism of *L. fermentum* does not solely contain HPLA but also other reducing molecules. Our work has reached a milestone in unravelling such a reducing mechanism after having being able to identify one of the molecules excreted by *L. fermentum* and label its functionality for future applications.

The characterization of HPLA as the main agent of the pH-dependent ferric-reducing activity of *L. fermentum* and its strong reducing ability dependence on pH, provokes us to hypothesise that excreted HPLA could reduce Fe(III) under acid stomach conditions and facilitate Fe(II) absorption in the duodenum, as occurs for ascorbic and citric acids. Related studies report that

low molecular weight fractions exhibiting ferric-reducing activity in human milk enhance iron absorption in newborns (Pullakhandama et al., 2007).

HPLA contains two chemical moieties with potential reducing Fe(III) activity: the terminal lactic acid and the phenol group. To get insight into the mechanism of the ferric-reducing activity of HPLA, we compared the HPLA ability to reduce Fe(III) at acidic pH with that of its pure chemical moieties, namely lactic acid (LA) and *p*-methylphenol (*p*-mPh), and the mixture of both (LA + *p*-mPh). Both an excess of reducing molecules with respect to Fe(III) and a slight excess of Fe(III) with respect to the active molecules were examined. The first was to understand the kinetics of Fe(III) reduction and the second was to evaluate the ferric-reducing activity of the individual chemical moieties.

The most interesting result of these experiments is that HPLA reduces Fe(III) to the same extent as the mixture of lactic acid and *p*-mPh, practically doubling the reducing capacity of separated LA and *p*-mPh (Figure 5). Thus, both HPLA chemical moieties are effective for Fe(III) reduction and exhibit ferric-reducing activity. When the experiment was done in excess of Fe(III) towards the reducing molecules, the ferric-reducing activity vs. time revealed that HPLA seems to reduce Fe(III), first by the LA moiety and then by *p*-mPh (Figure 5A). Fe(III) reduction versus time shows that the reduction by HPLA follows the same pattern as LA in the initial steps. The %Fe(III) reduction was about 20% by HPLA and 15% by LA after 2h, whereas that of *p*-mPh was 90%. This indicates that pure *p*-mPh has faster Fe(III) reduction mechanisms than LA and HPLA. However, over a prolonged period of time (24 h), the %Fe(III) reductions of HPLA, *p*-mPh, and the mixture, LA- *p*-mPh, are similar and close to 100%, whereas that of LA is about 80%.

In the second experiment, Fe(III) was in excess with respect to the reducing molecules. The results clearly indicate that the capacity of HPLA to reduce Fe(III) is double than that of LA and

of *p*-mPh and is close to the capacity of the mixture of both (Figure 5B). Therefore, the combination of both chemical moieties (lactic acid and phenol) makes HPLA an extraordinary Fe(III) reducing molecule.

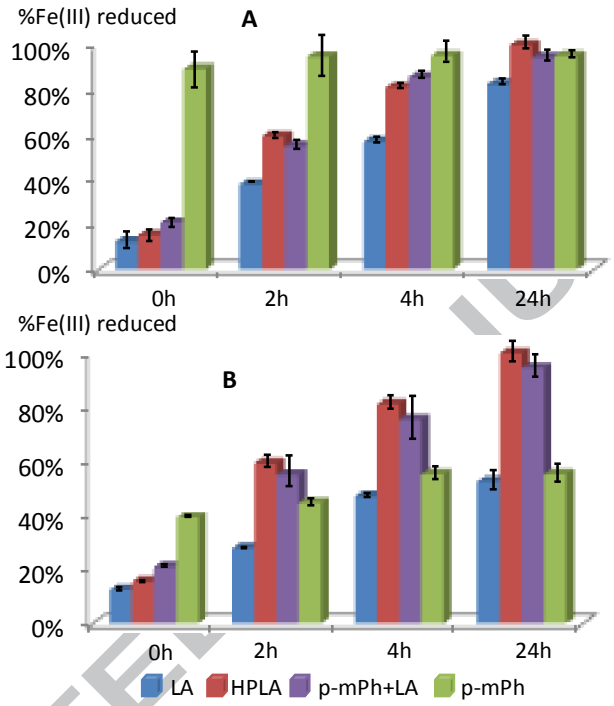


Figure 5. Ferric-reducing activity at pH 3.5 of HPLA, lactic acid (LA), *p*-methylphenol (*p*-mPh) and the mixture of LA and *p*-mPh at different times. A: Fe(III) is in default with respect to reducing molecules. B: Fe(III) is in excess with respect to the reducing molecules. The %Fe(III) reduction is referred to the concentration of Fe(III) (A) or the reducing molecules (B). Error bars, ±SD.

An *in vitro* experiment of iron uptake by enterocytes in the presence of HPLA was performed to test whether HPLA could be related to the effect on iron absorption by probiotic bacteria, (Figure

6). HPLA was incubated at two concentrations (36 and 360 µM) with enterocytes (IEC-6 cells) in the presence of Fe(III). *L. fermentum,* and its supernatant liquid obtained after bacterial centrifugation, were also incubated with IEC-6 enterocytes and Fe(III). The concentration of total iron (Fe(II) and Fe(III)) of the solutions was measured at two different times (30 and 300 min), by ICP-OES, to evaluate the amount of iron internalized into the cells.

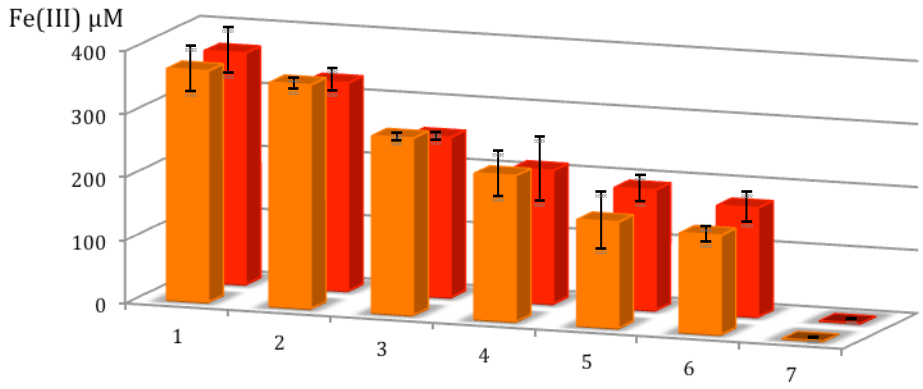


Figure 6. Fe(III) (367 µM) was incubated with enterocyte cells IEC-6 in the presence of HPLA at 36 µM (columns 3), 360 µM (columns 4), *L. fermentum* (columns 5) and the supernatant liquid obtained after centrifugation of *L. fermentum* (columns 6). Fe concentrations in the solutions were measured by ICP-OES after 30 (orange) and 300 min (red). Columns 1 correspond to the initial Fe(III) solutions; columns 2 correspond to Fe(III) after incubation with IEC-6 (control) and columns 7 data to the plates only containing cells (control). Error bars, ±SD.

The first conclusion drawn from these data is that *L. fermentum* and its supernatant liquid significantly increased iron absorption by enterocytes. Values for Fe(III) absorption after incubation with enterocytes are very close to initial values, meaning that enterocytes hardly absorb

Fe(III) (Figure 6). In contrast, both enterocytes with *L. fermentum* and enterocytes with the supernatant (the excreted molecules) reduce iron concentrations to less than half those of the controls. Thus, the bacteria and excreted molecules facilitate enterocyte iron uptake. Moreover, enterocyte iron absorption by bacteria and by supernatant liquid obtained after centrifugation are similar, which substantiates that the effect of probiotic bacteria on iron absorption is due to the molecules excreted by these bacteria.

HPLA is less effective in assisting enterocytes with iron absorption than the bacterial broth or its supernatant liquid. However, higher HPLA concentrations (360 μM) result in more absorption. Iron concentrations decrease from approximately 350 μM (Figure 6, columns 2) to 231 and 211 μM after 30 and 300 min of incubation, respectively. While the higher concentration of HPLA (360 μM) increases enterocyte iron uptake by about 40%, the increase for the lower HPLA concentration (36 μM) is only about 30%.

Though HPLA clearly increases enterocyte iron absorption, the higher quantities of enterocyte iron absorption by *L. fermentum* and its supernatant liquid with respect to HPLA suggest that other molecules excreted by the probiotic bacteria must also be involved in this complex process. Lactic acid is a likely candidate, since *L. fermentum* is defined as a lactic bacterium that excretes this molecule while proliferating.

Therefore, HPLA exhibits extraordinary ferric-reducing activity and increases enterocyte iron uptake. Taking into account enterocytes in the duodenum and the participation of the ferric-reducing protein DcytB (McKie, 2008; Lane et al., 2015), our results point to a mechanism of iron absorption in which HPLA would act similarly to DcytB by facilitating Fe(III) reduction in the stomach and ultimately promoting Fe(II) uptake by DMT1 channels.

4. Conclusions

L. fermentum, one of the main probiotics of the microbiota, exhibits an extraordinary ferric-reducing activity. This activity is mainly due to one of the molecules excreted by this bacterium: *p*-hydroxyphenyllactic acid (HPLA). HPLA effectively reduces Fe(III) to Fe(II) and in the gastroinstestinal tract can mimic the functionality of the DcytB ferric-reducing protein by promoting iron uptake by enterocytes. We have demostrated that the increase of iron absorption in the presence of probiotic bacteria is related to the ferric-reducing activity of excreted molecules. These results are a huge step towards solving the enigma of why probiotic bacteria increase iron absorption. This discovery opens new avenues for the treatment of human iron deficiency, one of the most common and widespread nutritional disorders in the world.

Supporting Information

Figure S1-S8. ^{1}H NMR spectrum (500 MHz, CD_3OD) of the final pooled fractions displaying ferric-reducing activity. Comparison of the ^{1}H NMR spectra of the final pooled fractions displaying ferric-reducing activity and the HPLA standard. COSY spectrum of the final pooled fractions displaying ferric-reducing activity showing the key correlations for HPLA. HSQC spectrum of the final pooled fractions displaying ferric-reducing activity. HSQC spectrum of the HPLA standard. HMBC spectrum of the final pooled fractions displaying ferric-reducing activity showing the key correlations for HPLA.

Table S1. NMR spectroscopic data (500 MHz, CD_3OD, at 24 ºC) for HPLA.

Figure S9. LC-UV-HRMS analysis of the final pooled fractions displaying ferric-reducing activity showing detection of a major component matching the molecular formula of HPLA.

Acknowledgements

This work was funded by MINECO and FEDER (project CTQ2015-64538-R) and Biosearch S.A. (CARMENTA Project-FEDER INTERCONECTA). The HPLC and NMR equipment used in this work were acquired via grants for scientific and technological infrastructures from the Ministerio de Ciencia e Innovación [Grants No. PCT-010000-2010-4 (NMR) and INP-2011-0016-PCT-010000-ACT6 (HPLC)].

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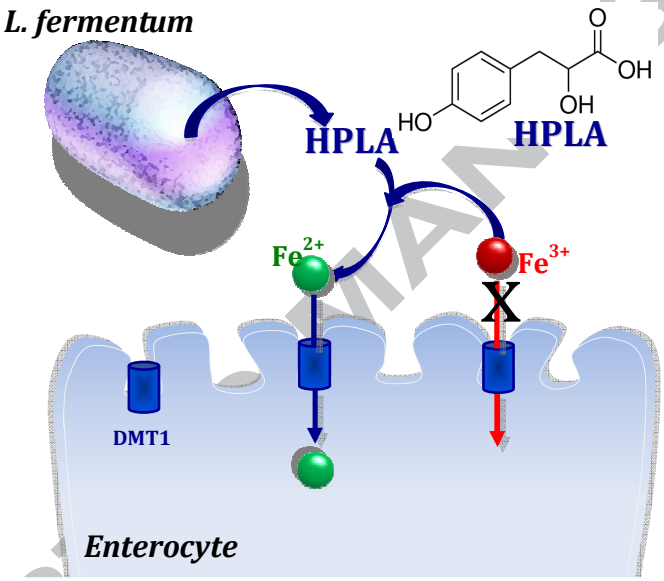
Graphical Contents

Identification of the Key Excreted Molecule Related to Host Iron Absorption

Ana González, Natividad Gálvez, Jesús Martín, Fernando Reyes, Ignacio Pérez-Victoria, Jose M. Dominguez-Vera

Probiotic bacteria increase iron absorption in humans due to their ferric-reducing activity. One of these bacteria, *Lactobacillus fermentum* excretes HPLA, which reduces Fe(III) to Fe(II). This Fe(II) is subsequently internalized into enterocytes by DMT1.



Iron deficiency is one of the most common and widespread nutritional disorders in the world. Two billion people, over 30% of the world's population, are anemic, mainly due to iron deficiency.

Probiotic bacteria have a positive effect on iron absorption. However, the chemical mechanisms of this influence are not understood. In fact, different international organizations have recently concluded that there is insufficient evidence to claim a probiotic can help boost iron absorption.

We demonstrate how and why probiotic bacteria increase iron absorption. We demonstrate that *L. fermentum*, one of the main probiotics of the microbiota, exhibits an extraordinary ferric-reducing activity and that this activity is mainly due to one of the molecules excreted by this bacterium: p-hydroxyphenyllactic acid (HPLA). Because reduction of Fe(III) to Fe(II) is an essential step for iron absorption in the gastrointestinal tract, HPLA reduction of Fe(III) can boost Fe(II) absorption through the DMT1 channels of enterocytes. An in vitro experiment has been done to confirm this hypothesis.

This discovery opens new avenues for the treatment of human iron deficiency, one of the most common and widespread nutritional disorders in the world.

Identification of the Key Excreted Molecule by *Lactobacillus fermentum* Related to Host Iron Absorption

Ana González[1], Natividad Gálvez[1], Jesús Martín[2], Fernando Reyes[2], Ignacio Pérez-Victoria[2,*], Jose M. Dominguez-Vera[1,*]

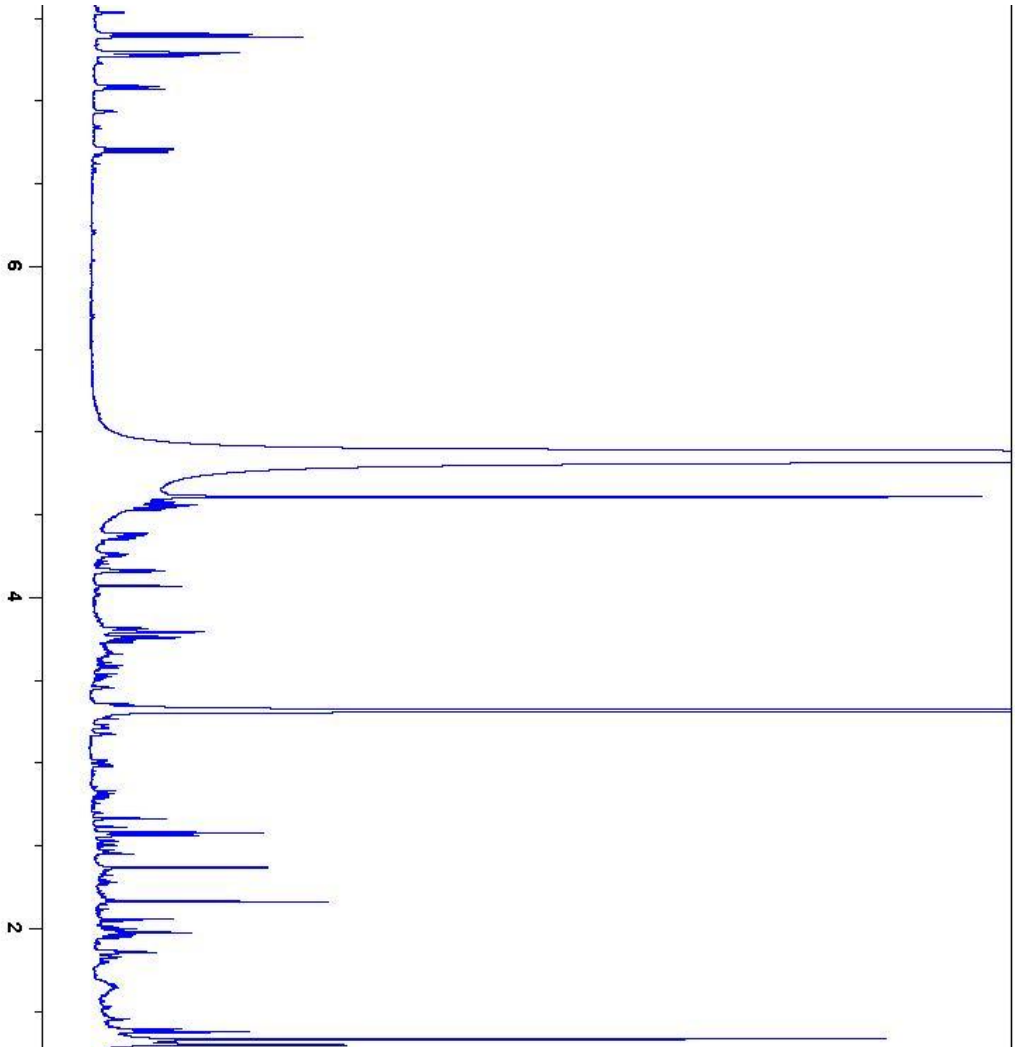


Figure S1. ^{1}H NMR spectrum (500 MHz, CD_3OD) of the final pooled fractions displaying ferric-reducing activity.

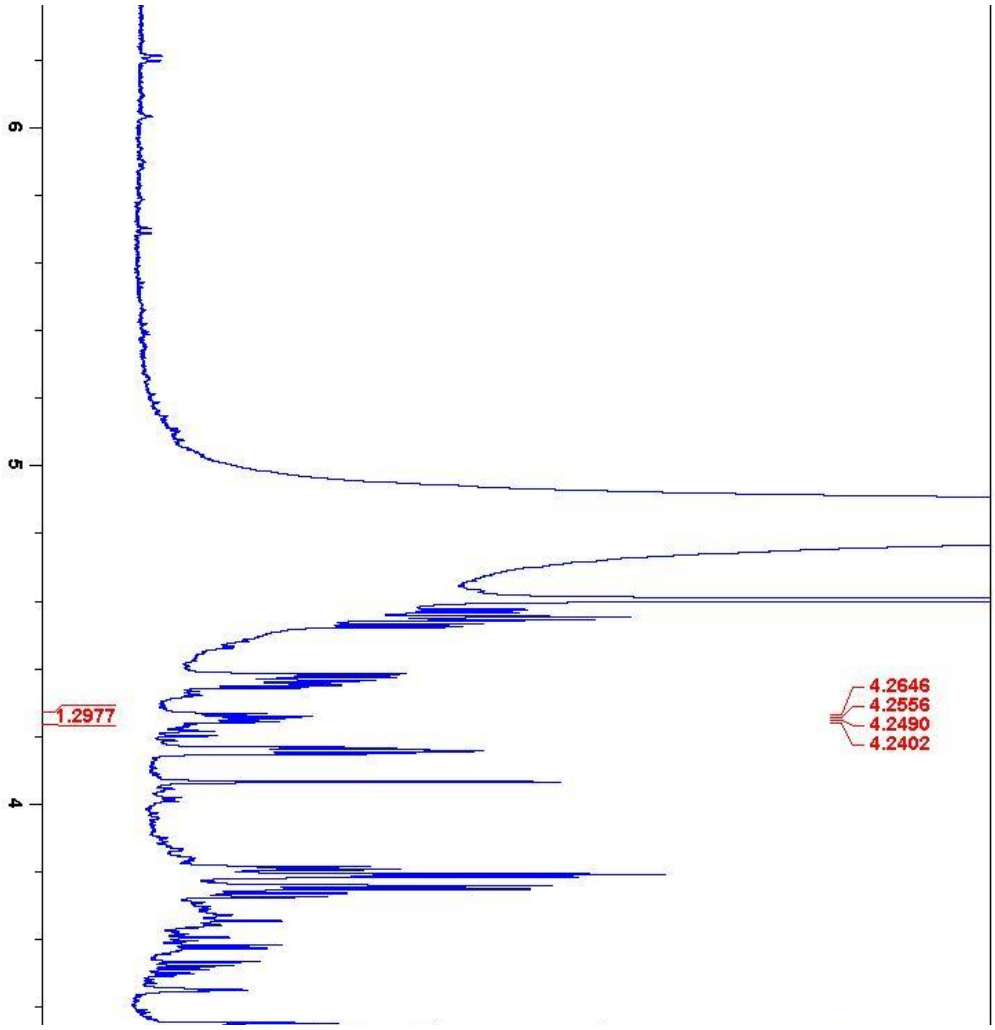


Figure S2. Expansion of the ^{1}H NMR spectrum of the final pooled fractions displaying ferric-reducing activity, highlighting the signals of HPLA.

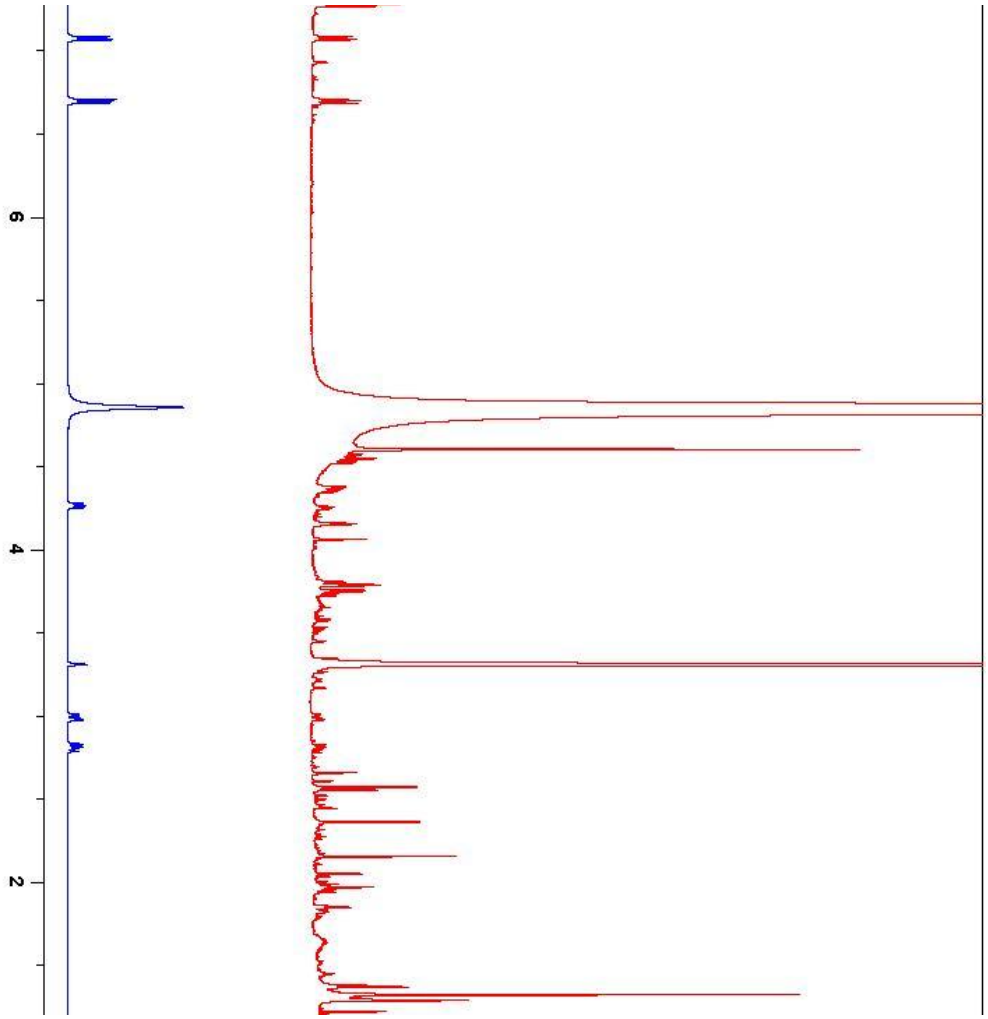


Figure S3. Comparison of the ^{1}H NMR spectra of the final pooled fractions displaying ferric-reducing activity (red) and the HPLA standard (blue).

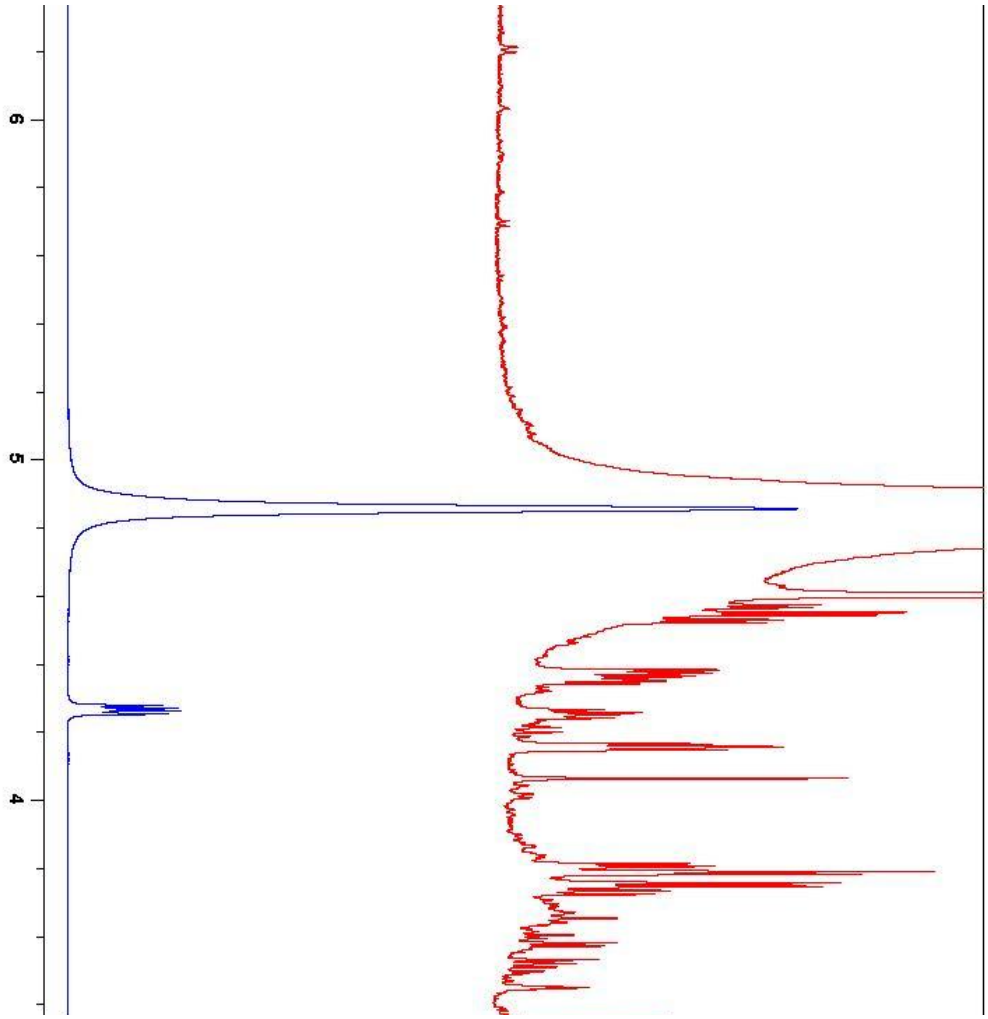


Figure S4. Expansion of the ^{1}H NMR spectra of the final pooled fractions displaying ferric-reducing activity (red) and the HPLA standard (blue).

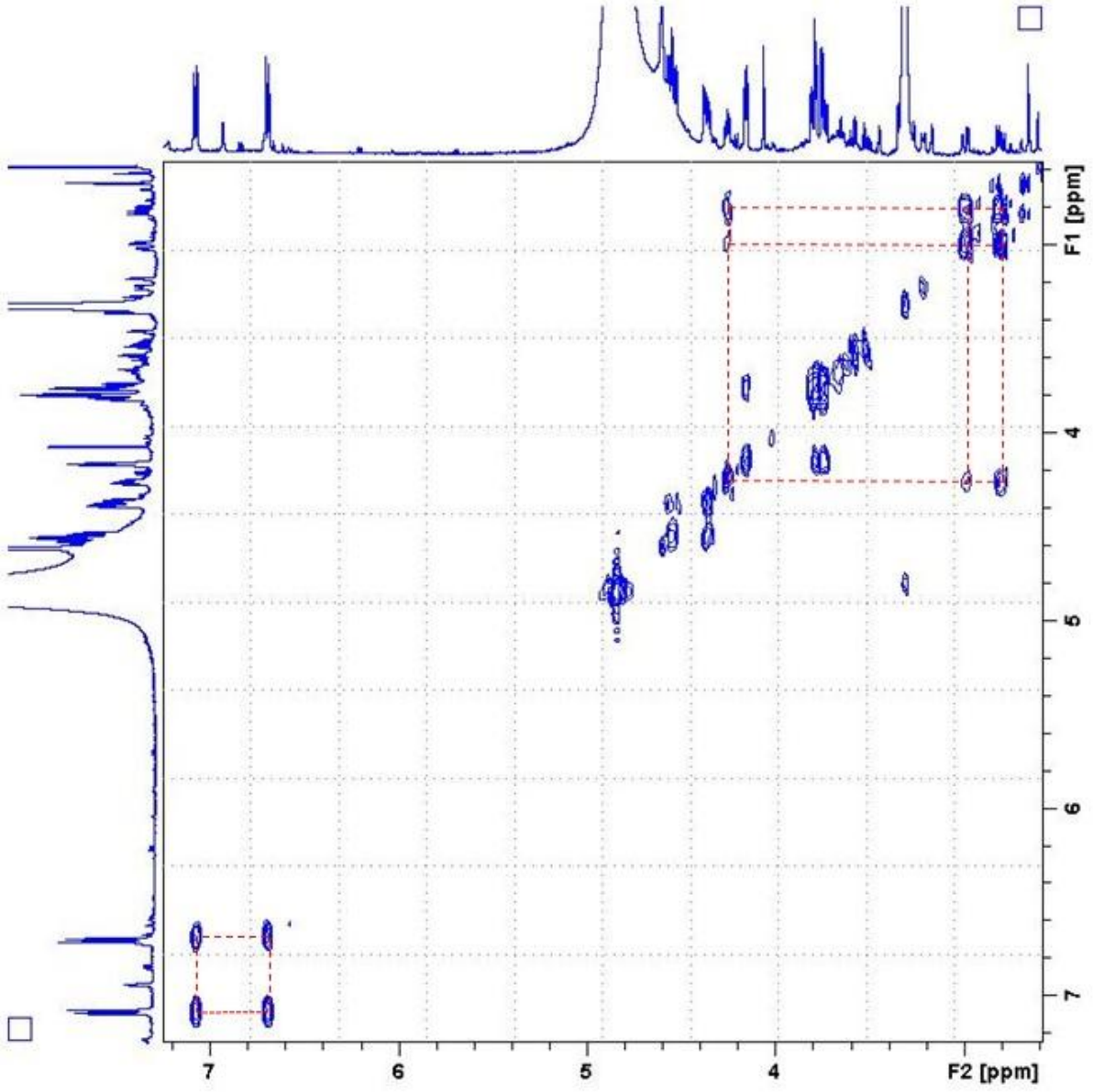


Figure S5. Expansion of the COSY spectrum of the final pooled fractions displaying ferric-reducing activity showing the key correlations for HPLA.

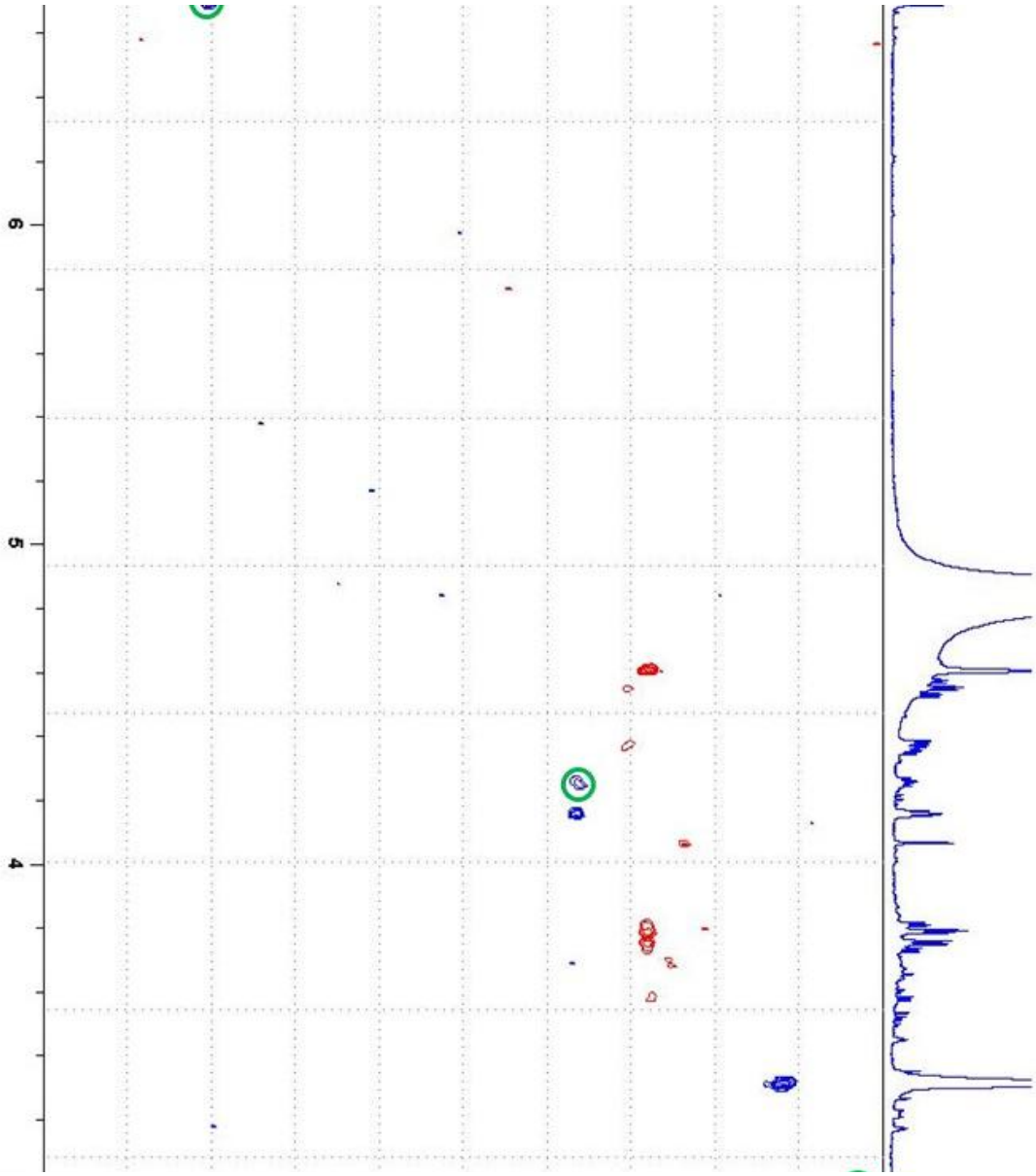


Figure S6. Expansion of the HSQC spectrum of the final pooled fractions displaying ferric-reducing activity showing the cross peaks of HPLA.

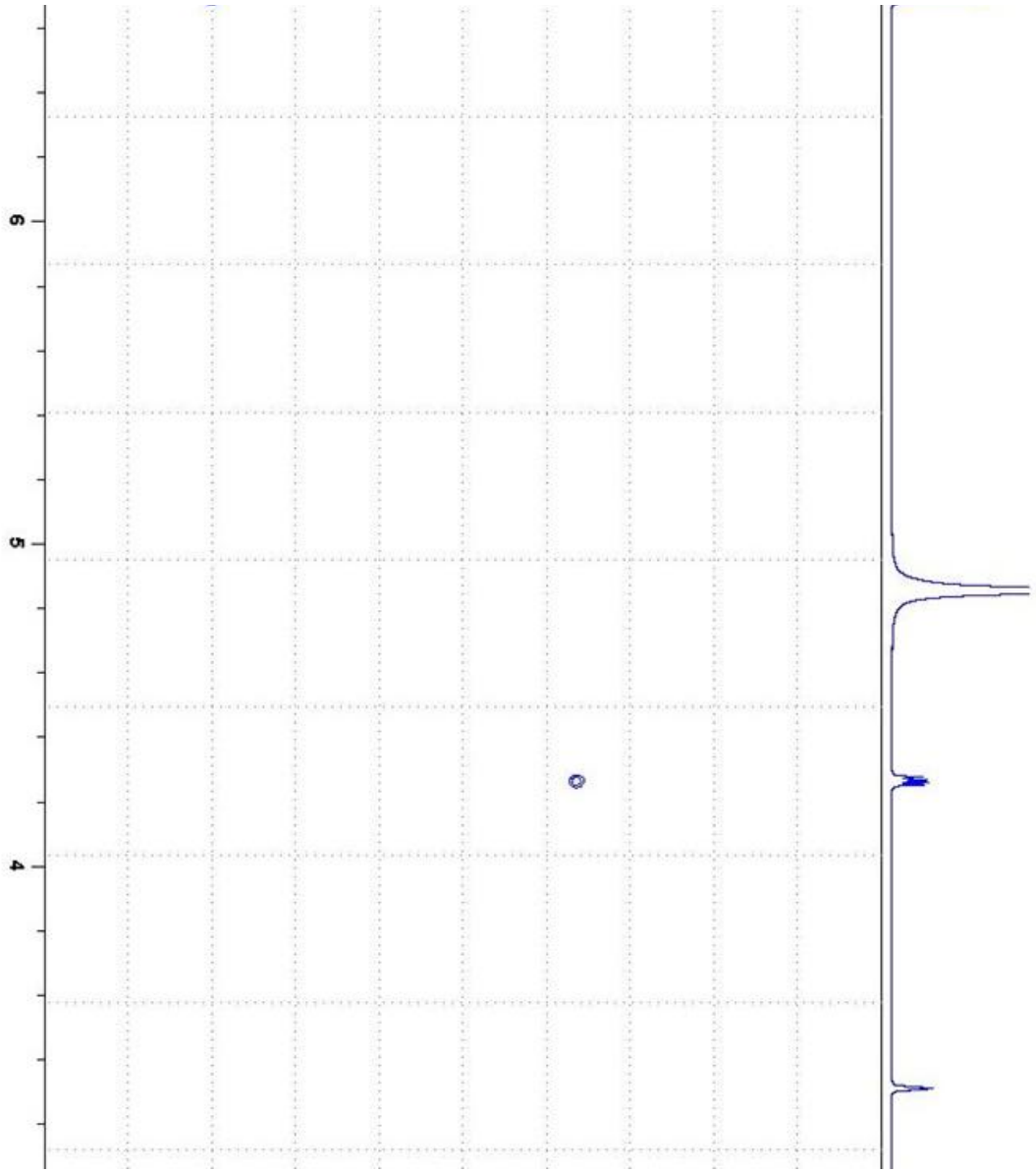


Figure S7. HSQC spectrum of the HPLA standard.

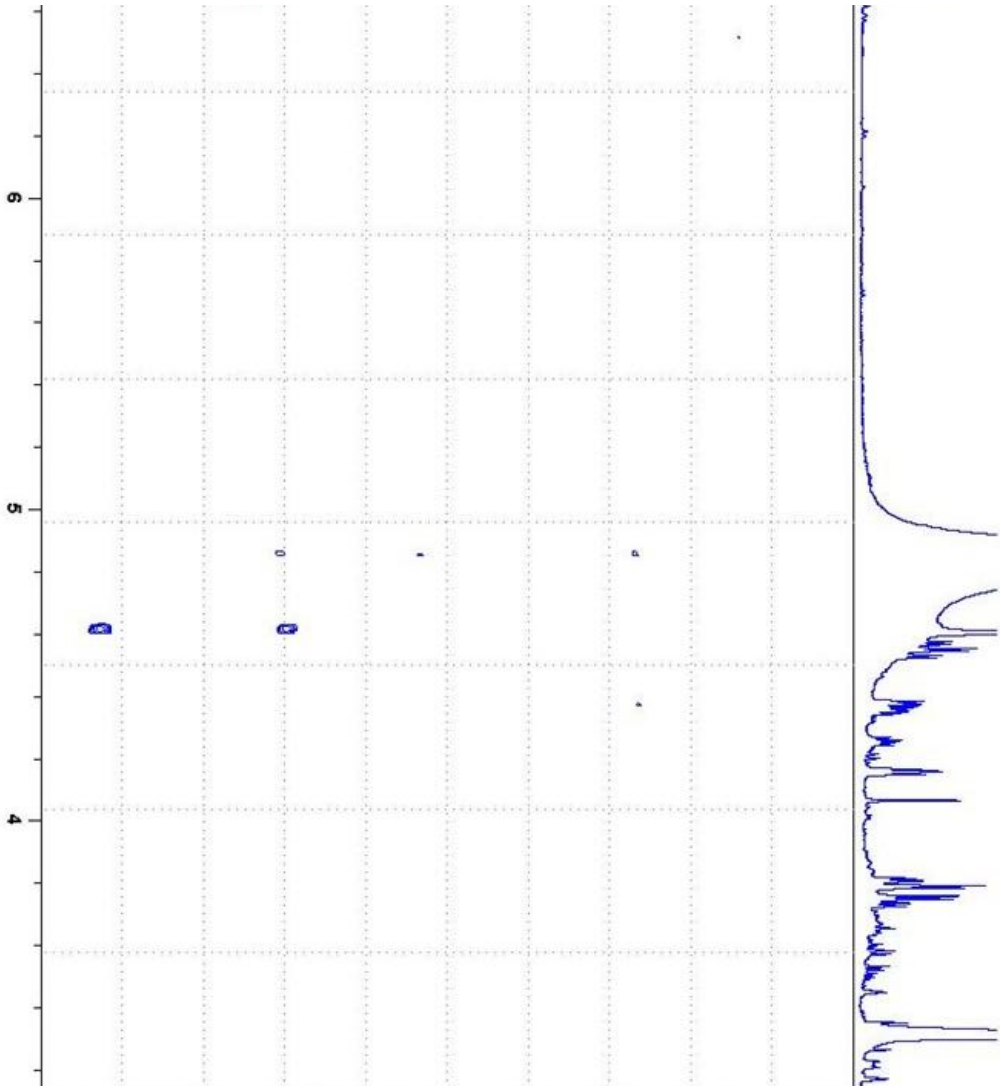
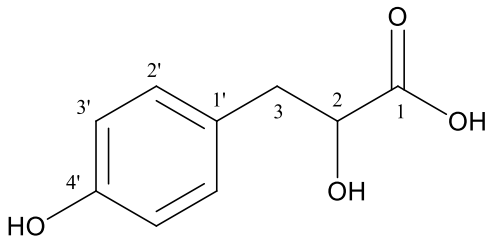


Figure S8. Expansion of the HMBC spectrum of the final pooled fractions displaying ferric-reducing activity showing key correlations for HPLA.

Table S1. NMR Spectroscopic Data (500 MHz, CD_3OD, at 24 ºC) for HPLA

position	δ_H (*J* in Hz)	δ_C , type
1	-	n. d. , C
2	4.25, dd (7.7, 4.5)	72.7, CH
3	a 2.99, dd (14.0, 4.4) b 2.80, dd (14.0, 7.8)	40.4, CH_2
1’	-	129.8, C
2’	7.07, d (8.5)	131.3, CH
3’	6.69, d (8.5)	115.7, CH
4’	-	156.8, C



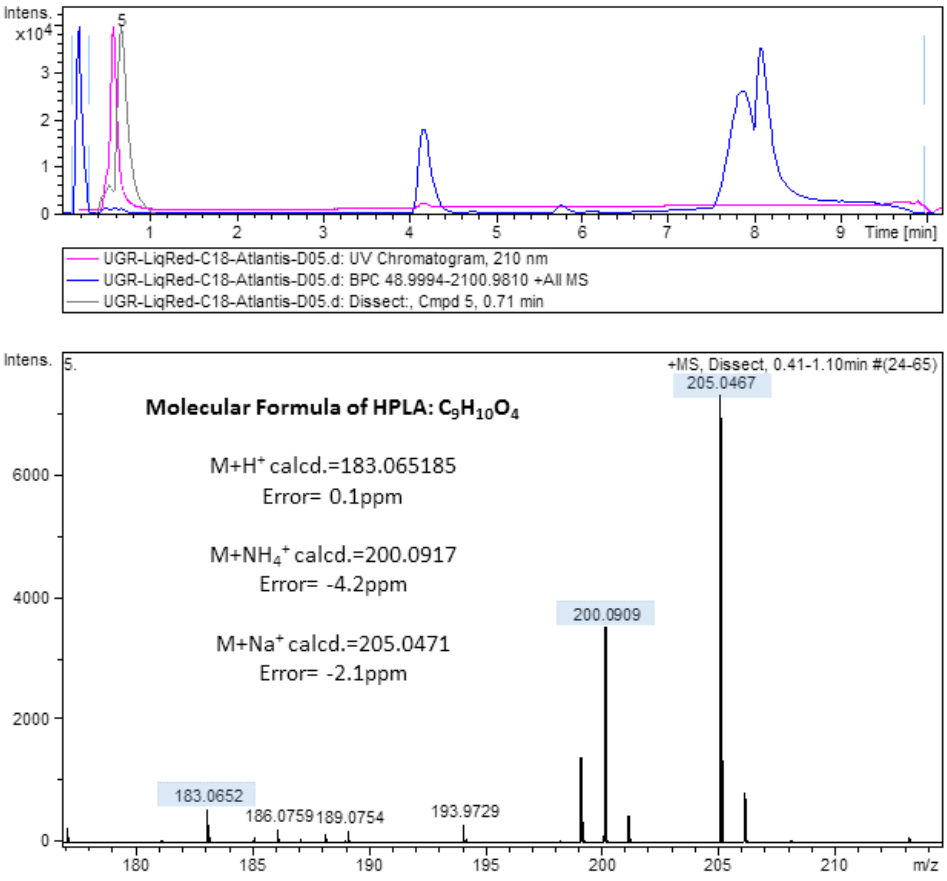


Figure S8. LC-UV-HRMS analysis of the final pooled fractions displaying ferric-reducing activity showing detection of a major component matching the molecular formula of HPLA.