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Corresponding Author:	Tiago L Duarte, PhD I3S: Universidade do Porto Instituto de Investigacao e Inovacao em Saude Porto, Porto PORTUGAL
Corresponding Author's Institution:	I3S: Universidade do Porto Instituto de Investigacao e Inovacao em Saude
Corresponding Author E-Mail:	tduarte@ibmc.up.pt
Order of Authors:	Tiago L Duarte, PhD
	João Neves
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TITLE:

Measurement of Tissue Non-Heme Iron Content Using a Bathophenanthroline-Based Colorimetric Assay

AUTHORS AND AFFILIATIONS:

Tiago L Duarte[1,2*], João V Neves[1,3,4*]

[1]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal

[2]Basic and Clinical Research on Iron Biology, IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, 4200-135 Porto, Portugal

[3]Iron and Innate Immunity, IBMC-Instituto de Biologia Molecular e Celular, Universidade do Porto, 4200-135 Porto, Portugal

[4]ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4050-313Porto, Portugal

*These authors contributed equally

Corresponding Authors:

Tiago L Duarte (tduarte@ibmc.up.pt)

João V Neves (jneves@ibmc.up.pt)

SUMMARY:

Here, a protocol for the measurement of the non-heme iron content in animal tissues is provided, using a simple, well-established colorimetric assay that can be easily implemented in most laboratories.

ABSTRACT:

Iron is an essential micronutrient. Both iron overload and deficiency are highly detrimental to humans, and tissue iron levels are finely regulated. The use of experimental animal models of iron overload or deficiency has been instrumental to advance knowledge of the mechanisms involved in the systemic and cellular regulation of iron homeostasis. The measurement of total iron levels in animal tissues is commonly performed with atomic absorption spectroscopy or with a colorimetric assay based on the reaction of non-heme iron with a bathophenanthroline reagent. For many years, the colorimetric assay has been used for the measurement of the non-heme iron content in a wide range of animal tissues. Unlike atomic absorption spectroscopy, it excludes the contribution of heme iron derived from hemoglobin contained in red blood cells. Moreover, it does not require sophisticated analytical skills or highly expensive equipment, and can thus be easily implemented in most laboratories. Finally, the colorimetric assay can be either cuvette-based or adapted to a microplate format, allowing higher sample throughput. The present work provides a well-established protocol that is suited for the detection of alterations in tissue iron levels in a variety of experimental animal models of iron overload or iron deficiency.

INTRODUCTION:

Iron is an essential micronutrient, required for the function of proteins involved in crucial biological processes such as oxygen transport, energy production, or DNA synthesis. Importantly, both iron excess and iron deficiency are highly detrimental to human health, and tissue iron levels are finely regulated. Abnormal dietary iron absorption, iron-deficient diets, repeated blood transfusions, and chronic inflammation are common causes of iron-associated disorders that affect billions of people worldwide[1–3].

Experimental animal models of iron overload or deficiency have been instrumental to advance our knowledge of the mechanisms involved in the systemic and cellular regulation of iron homeostasis[4]. Despite the substantial progress made during the last two decades, many key aspects remain elusive. In the coming years, the accurate measurement of total iron levels in animal tissues will remain a critical step to advance research in the iron biology field.

Most laboratories quantify tissue iron with either atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS), or a colorimetric assay based on the reaction of non-heme iron with a bathophenanthroline reagent. The latter is based on the original method described by Torrance and Bothwell over 50 years ago[5–6]. While a variation of this method was subsequently developed employing ferrozine as an alternative to bathophenanthroline[7], the latter remains the most widely cited chromogenic reagent in the literature.

The method of choice often depends on the available expertise and infrastructure. While AAS and ICP-MS are more sensitive, the colorimetric assay remains widely used because it presents the following important advantages: i) it excludes the contribution of heme iron derived from hemoglobin contained in red blood cells; ii) it does not require sophisticated analytical skills or highly expensive equipment; and iii) the original cuvette-based assay can be adapted to a microplate format, allowing higher sample throughput. The colorimetric approach presented in this work is routinely used to quantify alterations in tissue non-heme iron levels in a variety of experimental animal models of iron overload or iron deficiency, from rodents to fish and fruit fly. Here, a protocol for the measurement of the non-heme iron content in animal tissues is provided, using a simple, well-established, colorimetric assay that most laboratories should find easy to implement.

PROTOCOL:

C57BL/6 mice were commercially purchased and hepcidin-null (*Hamp1*$^{-/-}$) mice on a C57BL/6 background[8] were a kind gift from Sophie Vaulont (Institut Cochin, France). Animals were housed at the i3S animal facility under specific pathogen-free conditions, in a temperature- and light-controlled environment, with free access to standard rodent chow and water. European sea bass (*Dicentrarchus labrax*) were purchased from a commercial fish farm and housed at the ICBAS animal facility, in a temperature- and light-controlled environment, and fed daily *ad libitum* with standard sea bass feed. All procedures involving vertebrate animals were approved by the i3S Animal Ethics Committee and the national authority, Direção-Geral de Alimentação e Veterinária (DGAV). Information about commercial reagents, equipment, and animals is listed in the **Table of Materials**.

1. Solution preparation

NOTE: Handle and prepare all reagents and solutions with iron-free glassware or disposable plasticware. Do not allow metallic laboratory materials (e.g., stainless steel spatulas) to come in contact with any reagent or solution, due to the risk of iron contamination. Make sure any reusable glassware is iron-free. Wash the materials with appropriate laboratory detergent for 30–60 min, rinse with deionized water, soak overnight in a 37% nitric acid solution diluted 1:3 with deionized water, rinse again with deionized water, and allow to dry.

1.1. Acid mixture: Add 10 g of trichloroacetic acid to 82.2 mL of 37% hydrochloric acid in a glass bottle, thoroughly dissolve, and adjust the final volume to 100 mL with deionized water. Shake before use. Alternatively, use only 37% hydrochloric acid for tissue digestion.

NOTE: The solution is stable for at least 2 months when stored in dark brown glass reagent bottles.

CAUTION: Hydrochloric acid and trichloroacetic acid are corrosive, and concentrated forms release toxic acidic vapors. Wear protective garments, chemical-resistant gloves, and chemical splash goggles at all times when handling acids. Avoid breathing them in and always handle acids while under a fume hood.

1.2. Saturated sodium acetate: Add 228 g of anhydrous sodium acetate to 400 mL of deionized water in a glass bottle and agitate overnight at room temperature. Let the solution rest and precipitate for a day. If no precipitation occurs, continue adding small amounts of sodium acetate. Store the solution in a glass bottle.

1.3. Chromogen Reagent: To prepare 1 mL of chromogen reagent, add 1 mg of 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt to 500 µL of deionized water and 10 µL of concentrated (100%) thioglycolic acid, and thoroughly dissolve. Make up the final volume to 1 mL with deionized water.

NOTE: Prepare as much chromogen reagent as needed. The solution is stable for 1 month when protected from light.

1.4. Working Chromogen Reagent (WCR): Add 1 volume of the Chromogen Reagent to 5 volumes of saturated sodium acetate and 5 volumes of deionized water.

NOTE: This solution should be prepared freshly on the day of use.

1.5. Stock Iron Standard Solution: To prepare a 20 mM stock iron solution, place 111.5 mg of carbonyl iron powder in a 250 mL volumetric flask containing 5,480 µL of 37% hydrochloric acid. Leave to dissolve overnight at room temperature (or incubate in a boiling water bath). Then, make up the solution to a final volume of 100 mL with deionized water.

NOTE: The standard solution can be kept indefinitely when stored in a tightly sealed vessel.

1.6. Working Iron Standard Solution (WISS): Add 13.5 µL of 37% hydrochloric acid to 500 µL of deionized water. Add 10 µL of the Stock Iron Standard Solution and make up the final volume to 1 mL with deionized water (11.169 µg of Fe/mL, 200 µM; measured by AAS).

NOTE: The working solution should be prepared freshly on the day of use.

2. Sample drying

2.1. Cut a sample of tissue weighing 10–100 mg with a scalpel blade. Weigh it accurately in an analytical/precision balance over a small piece of parafilm (Fresh Weight).

2.2. Using plastic tweezers, place the piece of tissue in a 24-well plate (unlidded to allow for water evaporation) and let it dry on a standard incubator at 65 °C for 48 h.

2.3. Alternatively, use a laboratory microwave digestion oven for drying tissue samples. Using plastic tweezers, place the weighed piece of tissue in an iron-free Teflon cup and dry it in the microwave. Set the operating parameters according to the instrument's instructions manual.

NOTE: As a reference, operating parameters for drying liver samples using the specific digestion oven (see **Table of Materials**) are shown in **Table 1**.

2.4. Using plastic tweezers, place each dried piece of tissue over a small piece of parafilm inside an analytical/precision balance and weigh it accurately (Dry Weight).

3. Sample acidic digestion

3.1. Using plastic tweezers, transfer each dried piece of tissue into a 1.5 mL microcentrifuge tube.

3.2. Add 1 mL of the acid mixture and close the microcentrifuge tube. Prepare an acid blank in the same way, except that the tissue is omitted.

CAUTION: The acid mixture is corrosive and releases toxic vapors. Wear protective garments, chemical-resistant gloves, and chemical splash goggles when handling the acid mixture. Avoid breathing it in and always handle it while under a fume hood.

3.3. Digest the tissues by incubating the microcentrifuge tubes in an incubator at 65 °C for 20 h.

3.4. After cooling to room temperature, transfer 500 µL of the clear (yellow) acid extract (supernatant) into a new 1.5 mL microcentrifuge tube using a micropipette fitted with plastic tips. If it is not possible to obtain a clear supernatant, perform a short centrifugation spin.

CAUTION: The supernatant is highly acidic. Wear protective garments, chemical-resistant gloves, and chemical splash goggles when handling the supernatants. Always handle them while under a fume hood.

NOTE: At this point, the acid extracts can be immediately used for the colorimetric assay or frozen at -20 °C for later use. Completely thaw frozen samples to room temperature and vortex them prior to use.

4. Color development

4.1. Prepare chromogen reactions as indicated in **Table 2** in 1.5 mL microcentrifuge tubes or, for higher throughput, directly into the flat bottom, 96-well, clear, untreated polystyrene microplates. Prepare all reactions (acid blank, standard, and sample) at least in duplicate.

4.2. Incubate at room temperature for 15 min.

5. Absorbance reading

5.1. Measure sample absorbance in a spectrophotometer or plate reader at a wavelength of 535 nm against a deionized water reference. Plates can be read unlidded or lidded. In the lidded case, remove any condensation formed due to the release of acid vapors from the lid just prior to the measurement to avoid possible interference with the absorbance reading).

NOTE: The optical absorbance of the acid blank read against deionized water (reference) should be less than 0.015; the optical absorbance of the standard and samples should be between 0.100 and 1.000. For samples with very high or very low iron content, the volumes of acid extract (supernatant) and diH_20 may need to be adjusted (**Table 2**): if absorbance is greater than 1.0, use a smaller sample (supernatant) volume; when absorbance is lower than 0.1, use a higher volume of the supernatant. Sample volume (V_{smp}) is taken into account when calculating each sample's tissue iron content (see step 6).

6. Calculation of tissue iron content

6.1. Calculate non-heme tissue iron content with the following equation:

$$\text{Tissue iron (µg/g dry tissue)} = \frac{A_T - A_B}{A_S - A_B} \times \frac{Fe_s}{W} \times \frac{\frac{V_{rv}}{V_{smp}} \times V_f}{\frac{V_{rv}}{V_{std}}}$$

A_T = absorbance of test sample
A_B = absorbance of acid blank
A_S = absorbance of standard

Fe_s = iron concentration of WISS (µg Fe/mL)
W = weight of dry tissue (g)
V_{smp} = sample volume (variable volume of Supernatant in **Table 2** converted to mL)
V_f = final volume of acid mixture after overnight incubation at 65 °C (corresponding to acid volume plus dry tissue volume in mL; if sample weights do not differ significantly, assume a constant volume ≈ 1 mL)
V_{std} = iron standard volume (volume of WISS in **Table 2** converted to mL)
V_{rv} = final reaction volume (Total volume in **Table 2** converted to mL)

REPRESENTATIVE RESULTS:

Cuvette versus 96-well microplate comparison

The measurement of tissue non-heme iron by reaction with a bathophenanthroline reagent originally described by Torrance and Bothwell[5–6] relies on the use of a spectrophotometer for absorbance reading. Hence, the volumes employed in the chromogen reaction are compatible with the size of a regular spectrophotometer cuvette. The present work describes a method adaptation in which the chromogen reactions are prepared directly in a 96-well microplate for absorbance measurement in a microplate reader, requiring smaller reagent volumes and allowing higher throughput.

To compare the sensitivity of both approaches, a serial dilution of the working iron standard solution (WISS) was initially prepared, and the chromogen reactions were assembled either in 1.5 mL tubes or in 96-well plates, as indicated in **Table 2**. Absorbance was measured in a spectrophotometer (with cuvette) or in a microplate reader, respectively. Representative standard curves generated with the two approaches are depicted in **Figure 1A**. In both cases, linearity was very high (r^2 = 0.9996 and r^2 = 0.9997 for microplate and cuvette, respectively) across the iron concentrations tested.

Noteworthy, a WISS containing 11.169 µg of Fe/mL was used. The present data shows that lower iron concentrations may be used to prepare the standard. However, the use of a WISS with higher iron concentrations is not recommended, as this could lead to absorbance values that exceed the spectrophotometer's linear dynamic range of detection, causing standard curves to plateau.

To further compare the cuvette- and microplate-based assays, non-heme iron content was measured in a total of 55 mouse tissue samples (liver, spleen, heart, lung, bone marrow). A very high degree of correlation was observed between the two methodologies ($r = 0.999$, $p < 0.0001$), indicating that the microplate-based method is a valid alternative to the original cuvette-based method (**Figure 1B**).

Finally, non-heme iron levels in mouse tissues were quantified with the microplate-based method after acidic digestion of samples derived from the same tissues with either a mixture of hydrochloric acid and trichloroacetic acid (as per the original method description) or hydrochloric acid alone. A very high correlation was observed ($r = 0.999$, $p < 0.0001$, **Figure 1C**), showing that trichloroacetic acid can be omitted from the acid digestion.

The representative results included below were obtained by measuring sample absorbance in 96-well plates.

Measurement of tissue non-heme iron levels in a mouse model of genetic hemochromatosis

Using the bathophenanthroline-based colorimetric assay, non-heme iron content was determined in various tissues (liver, spleen, heart, and pancreas) from the commonly used mouse strain C57BL/6 and from hepcidin knockout (*Hamp1*$^{-/-}$) mice (in a C57BL/6 genetic background). Representative iron levels are depicted in **Figure 2**. Hepcidin is a key regulator of iron metabolism and its disruption leads to a hemochromatosis-like iron deposition phenotype, with severe hepatic, pancreatic, and cardiac iron accumulation, and splenic iron depletion[8].

Measurement of non-heme iron levels in sea bass liver after experimental iron modulation

Using the bathophenanthroline-based colorimetric assay, non-heme iron levels were determined in the liver of healthy (control), iron-treated (2 mg of iron dextran administered *via* the intraperitoneal route) and anemic (2% v/w of blood drawn from the caudal vessels) European sea bass (*Dicentrarchus labrax*). As expected, hepatic iron levels increased massively in iron-treated animals, whereas anemia caused a mild reduction in the hepatic iron stores (**Figure 3**).

Measurement of non-heme iron levels in whole *Drosophila melanogaster*

Although the current method is not appropriate to measure non-heme iron levels in individual *Drosophila* flies due to their small body mass (average weight being 0.6 mg and 0.8 mg for males and females, respectively)[9], it can be successfully used with pooled flies. **Figure 4** depicts representative non-heme iron levels for groups of 20 whole male flies, either wild-type Oregon-R or Malvolio (Mvl) knockout. Mvl is the homolog of the mammalian SLC11A2 gene, which encodes for a protein called divalent metal transporter 1 (DMT1), and its genetic disruption leads to iron deficiency[10]. As expected, Mlv flies presented a substantially lower body non-heme iron content compared to wild-type.

FIGURE AND TABLE LEGENDS:

Table 1: **Operating parameters for liver drying in a microwave digestion oven used here.**

Table 2: **Preparation of chromogen reactions in either 1.5 mL tubes or 96-well plates.**

Figure 1: **Determination of non-heme iron by the cuvette- or 96-well microplate-based colorimetric assays.** (**A**) standard curves for microplate- (open squares □) and cuvette-based (open triangles △) assays. (**B**) Correlation between cuvette-based and microplate-based non-heme iron levels in 55 tissue samples from 6-month-old male C57BL/6 mice, including liver (solid circles ●), spleen (open circles ○), heart (solid triangles ▲), lung (asterisks ✸), and bone marrow (diamonds ◇). (**C**) non-heme iron levels in a subset of tissues from 6-month-old male mice (liver, solid circles ●; spleen, open circles ○)measured with the 96-well microplate-based assay after acidic digestion of samples with a mixture of hydrochloric acid and trichloroacetic acid (HCl + $C_2HCl_3O_2$) or hydrochloric acid alone (HCl).

Figure 2: **Non-heme iron content determined in various tissues by bathophenanthroline-based**

colorimetric assay. Non-heme iron levels in the liver, spleen, heart, and pancreas of male C57BL/6 wild-type mice (open circles ○) and male hepcidin-deficient *Hamp1*$^{-/-}$ mice on C57BL/6 genetic background (open squares □) at the age of 8 weeks, measured with the bathophenanthroline-based colorimetric assay.

Figure 3: **Hepatic non-heme iron levels in juvenile female European sea bass following experimental iron modulation.** Iron overload was achieved by intraperitoneal administration of 2 mg of iron dextran, whereas anemia was induced by the withdrawal of 2% v/w of blood from the caudal vessels. Livers were collected at 4 days after iron treatment or blood collection. Control animals were healthy, untreated sea bass. Non-heme iron levels were measured with the bathophenanthroline-based colorimetric assay.

Figure 4: **Non-heme iron levels in 1-month old *Drosophila*, measured with the bathophenanthroline-based colorimetric assay.** Results show (**A**) the non-heme iron content in each pool of 20 flies or (**B**) the estimated iron content of each individual fly, considering an average weight of 0.64 mg/fly.

DISCUSSION:

A protocol for the measurement of the non-heme iron content in animal tissues is provided, using an adaptation of the bathophenanthroline-based colorimetric assay originally described by Torrance and Bothwell[5–6]. The critical steps of the method are tissue sample drying; protein denaturation and release of inorganic iron by acid hydrolysis; reduction of ferric (Fe^{3+}) iron to the ferrous state (Fe^{2+}) in the presence of the reducing agent thioglycolic acid, and its reaction with a bathophenanthroline reagent (chromogen reaction); absorbance reading of the resulting ferrous ion/bathophenanthroline pink complex; and calculation of tissue iron content.

The present work shows how the original cuvette-based protocol can be adapted to a 96-well-based format for higher throughput, without compromising the assay sensitivity. Notably, this adaptation allows for considerable time saving since: 1) a greater number of chromogen reactions can be prepared simultaneously in 96-well plates with the use of multichannel pipettes; and 2) absorbance readings are dramatically faster in a plate reader than when using a spectrophotometer. Another important advantage of the microplate-based assay is the adjustment of the chromogen reaction volumes, which substantially reduces the costs with reagents (in particular with the bathophenanthroline reagent). The whole protocol may be completed in 4 days. Sample drying (which may take up to 48 h when performed in an oven at 65 °C) and acidic digestion (which is conveniently performed overnight for 20 h) are the two most time-consuming steps. Sample drying can be accelerated using a microwave digestion oven designed for laboratory use in digesting, dissolving, hydrolyzing, or drying a wide range of materials. Since it is possible to dry most tissue samples in less than 2.5 h, one can perform the whole protocol in just 2 days. However, because the capacity of a microwave is limited to the number of teflon cups that it can fit, and because the cups need washing and decontamination after each use, users may find it more convenient to dry samples in 24-well plates at 65 °C when handling a high number of samples. The time required to dry the tissues may still be reduced by using temperatures above 65 °C; however, plastic material must be avoided due to the risk of

melting.

Previously, Grundy et al.[11] had developed an adaptation of the method in which the entire assay is carried out in 96-well plates, without including a sample drying step. However, the measurement of metals in tissues using wet weight instead of dry weight is significantly affected by the variable amount of weight loss through air-drying both in fresh and in frozen tissue samples[12]. Therefore, it is recommended to normalize the iron content against tissue dry weight. If tissue drying is not a viable option (e.g., adipose tissue), it is suggested that the assay is performed promptly upon sample collection, so that accurate determination of the fresh weight is not significantly affected by storage artifacts.

The composition of the acid solution was also addressed. According to the original protocol[5–6], tissues are digested with an acid mixture consisting of hydrochloric acid and trichloroacetic acid. However, the present work demonstrates that 37% hydrochloric acid alone is equally efficient, at least for digesting liver and spleen samples.

The stock iron standard solution is prepared using carbonyl iron powder, which is an inexpensive and highly pure iron powder. Following the approach described herein, a stock iron standard solution containing 1.1169 mg of Fe/mL (20 mM) was prepared, as subsequently determined by AAS. Other sources of iron (e.g., iron sulfate, iron nitrilotriacetate) or commercial standard solutions can be used to generate the stock iron standard solution, provided that the actual iron concentration is determined, and assay linearity is confirmed by performing a standard curve.

Using the current method, the non-heme iron content of a variety of animal tissues was successfully measured. Representative results obtained with rodent tissues (liver, spleen, heart, and pancreas), sea bass liver, and whole drosophila are included. The method does not require sophisticated analytical tools or expensive infrastructure, and can thus be easily implemented in most laboratories. In summary, the method described herein can be widely applied in studies of iron homeostasis and iron-related disorders employing experimental animal models of iron overload or deficiency.

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DISCLOSURES:
The authors have no conflicts of interest.

REFERENCES:
1. Muckenthaler, M. U., Rivella, S., Hentze, M. W., Galy, B. A red carpet for iron metabolism. *Cell*. **168**, 344–361 (2017).
2. Pagani, A., Nai, A., Silvestri, L., Camaschella, C. Hepcidin and anemia: A tight relationship. *Frontiers in Physiology*. **10**, 1294 (2019).
3. Weiss, G., Ganz, T., Goodnough, L. T. Anemia of inflammation. *Blood*. **133** (1), 40–50 (2019).

4. Altamura, S. et al. Regulation of iron homeostasis: Lessons from mouse models. *Molecular Aspects of Medicine*. **75**, 100872 (2020).
5. Torrance, J. D., Bothwell, T. H. A simple technique for measuring storage iron concentrations in formalinised liver samples. *South African Journal of Medical Sciences*. **33** (1), 9–11 (1968).
6. Torrence, J. D., Bothwell, T. H. Tissue iron stores. In: Cook, J. D., editor. *Methods in Haematology*. New York: Churchill Livingston Press. 104–109 (1980).
7. Rebouche, C. J, Wilcox, C. L., Widness, J. A. Microanalysis of non-heme iron in animal tissues. *Journal of Biochemical and Biophysical Methods*. **58** (3), 239–251 (2004).
8. Lesbordes-Brion, J. C. et al. Targeted disruption of the hepcidin 1 gene results in severe hemochromatosis. *Blood*. **108**, 1402–1405 (2006).
9. Jumbo-Lucioni, P. et al. Systems genetics analysis of body weight and energy metabolism traits in Drosophila melanogaster. *BMC Genomics*. **11**, 297 (2010).
10. Mandilaras, K., Pathmanathan, T., Missirlis, F. Iron Absorption in Drosophila melanogaster. *Nutrients*. **5**, 1622–1647 (2013).
11. Grundy, M. A., Gorman, N., Sinclair, P. R., Chorney, M. J., Gerhard, G. S. High-throughput non-heme iron assay for animal tissues. *Journal of Biochemical and Biophysical Methods*. **59**, 195–200 (2004).
12. Adrian, W. J., Stevens, M. L. Wet versus dry weights for heavy metal toxicity determinations in duck liver. *Journal of Wildlife Diseases*. **15**, 125–126 (1979).

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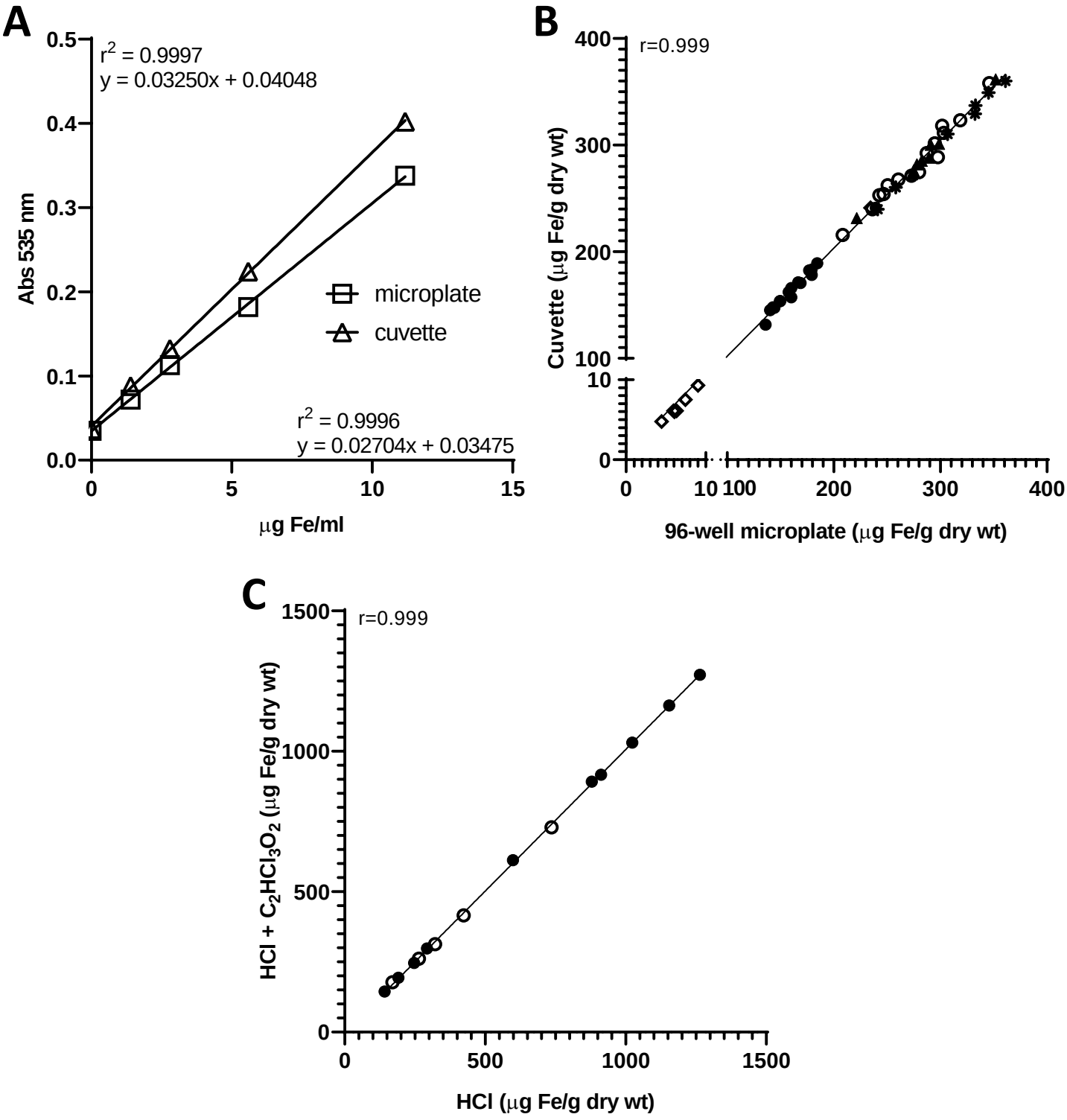

Figure 2

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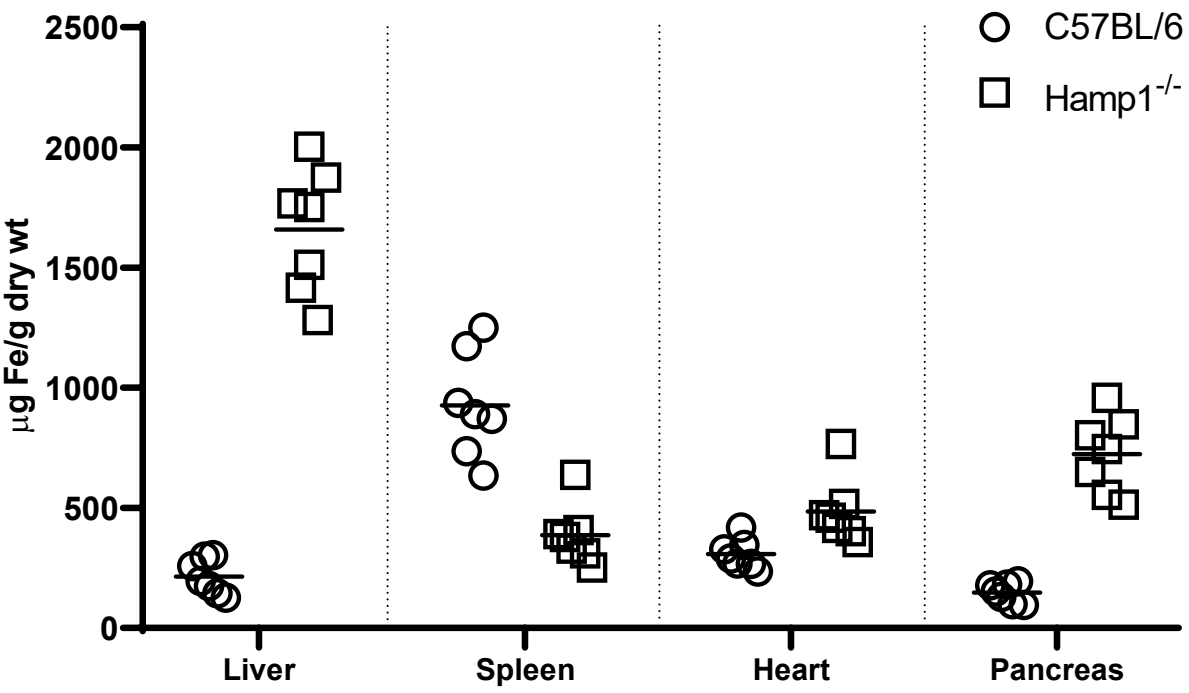

Figure 3

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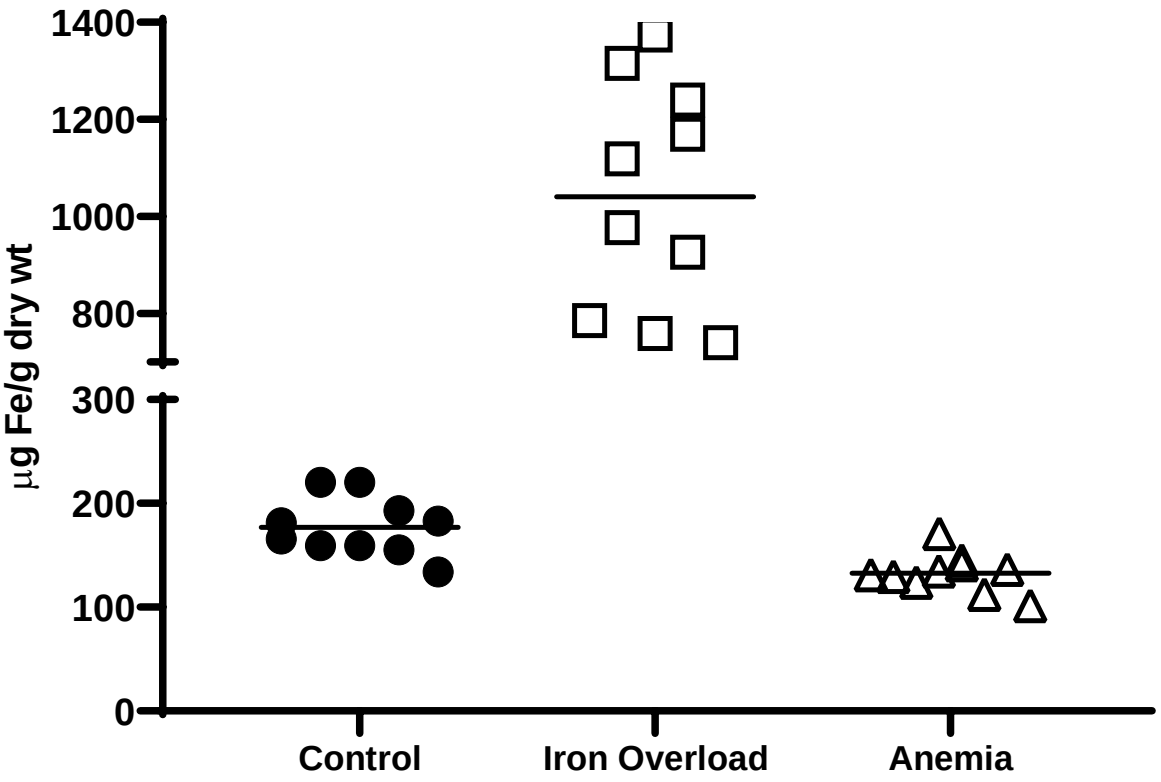

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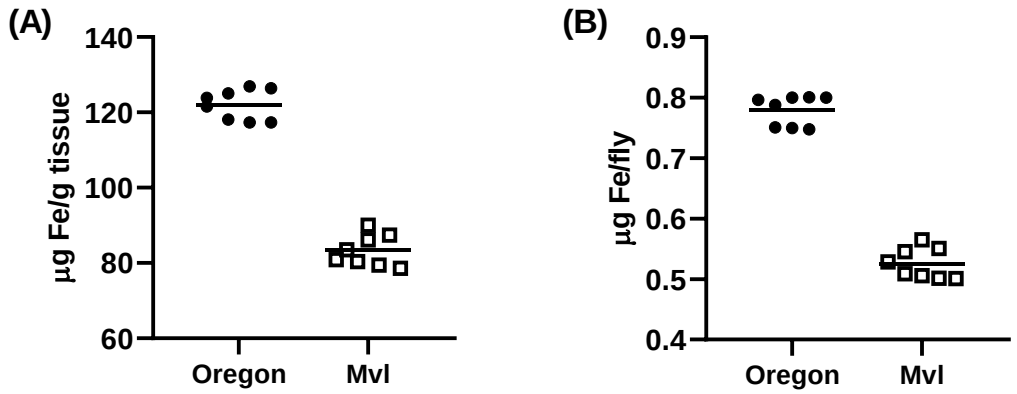

Table 1

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Power 650W (%):	10	15	20	25	30
Pressure (PSI):	0	0	0	0	0
Time (min):	10	15	30	30	40
Time at pressure (TAP):	0	0	0	0	0
FAN:	50	50	50	50	50

Table 2

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		WCR (µL)	Acid mixture (µL)	Supernatant (µL)	WISS (µL)	diH_2O (µL)	Total volume (µL)
1.5 mL tubes	Acid Blank	1000	150			150	1300
	Standard	1000	150		150		1300
	Sample	1000		Variable		Variable	1300
96-well microplate	Acid Blank	150	22.5			22.5	195
	Standard	150	22.5		22.5		195
	Sample	150		Variable		Variable	195

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We thank you for the opportunity to revise our manuscript. We also thank the 3 Reviewers for their careful reading of the manuscript and for their comments. Please find below our replies to the Editorial and Reviewers' comments. We hope that all the issues were satisfactorily addressed and that the manuscript is now ready for publication in JoVE.

Yours sincerely,

Tiago Duarte

Instituto de Investigação e Inovação em Saúde, Universidade do Porto
Rua Alfredo Allen, 208 | 4200-135 Porto, Portugal
E-mail: tduarte@ibmc.up.pt

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R: We have revised the text, as requested.

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3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Neodisher, etc.

R: We have revised the text, as requested.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

R: We have revised the text, as requested.

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

R: We have revised the text, as requested.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

R: We have revised the text, as requested.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

R: We have revised the text, as requested.

8. Please ensure to include details regarding the test animals involved in the study. Please mention their age, sex, etc. This can be included along with the ethical statement at the beginning of the protocol.

R: Details on test animals’ origin and maintenance conditions have been added to the protocol along with the ethical statement. Further details (species name, genetic background, genotype, gender and age) are included in the respective figure legends.

9. Line 131: What is the sample tissue here?

R: We’re not specifying the tissue intentionally because the protocol works with a variety of animal tissues.

10. Line 150: For what weight of the tissue is 1 mL of acid mixture added?

R: That’s for a piece of tissue weighing 10-100 mg, as described in Protocol Step 2.1.

11. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

R: Highlighted in yellow as requested.

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

R: The Discussion was revised to include the critical steps within the protocol and a discussion of modifications/troubleshooting, as requested.

13. Please ensure that the Table of Materials includes all the supplies (reagents, chemicals, instruments, equipment, software, etc.) used in the study.

R: We believe all relevant supplies are included.

Reviewer #1:

Manuscript Summary: The authors describe an accessible method for measuring tissue non-heme iron, based on a colorimetric reaction with a bathophenanthroline reagent as previously established by others. Grundy et al (2004) had published a method to convert a previous cuvette-based version of the assay to a microplate format. While the current protocol therefore isn't entirely novel, it provides its own set of options and technical suggestions to achieve the same ends and would be useful to people new to the field.

Major Concerns:

1. 1.5 Stock Iron Standard Solution and 1.6 Working Iron Standard Solution. It would help the viewer/reader to give suggested final concentrations of iron in the working solution at this point. It may also be helpful to express concentrations in both weights per ml and as molar concentrations.

R: As suggested, we have added the final concentrations for both iron standard solutions in weight per ml and in molar concentrations.

2. Sample drying and acid digestion - do the authors add 1 ml of acid solution regardless of tissue wet weight? This seems like it would generate a wide range of concentrations. Please give suggested tissue amounts and comment on whether this volume could or should be scaled down for small samples.

R: In Protocol step 2.1, we provide a range of tissue amounts (10-100 mg). The Reviewer is right when she/he says that using 1 ml of acid solution regardless of tissue wet weight generates a wide range of concentrations. However, this is taken into consideration in the equation used to calculate tissue iron concentration. We know from our experience that 1 ml of acid is sufficient to digest up to 100 mg of tissue. In samples with very low weight (and low iron content), it is possible that the absorbance of the chromogen reaction is below the spectrophotometer's linear dynamic range of detection. In these situations, we could indeed reduce the volume of acid used in sample digestion (and subsequently adjust the volume of acid mixture in the final equation), or we can increase the sample volume when preparing the chromogen reaction (and adjust the sample volume in the final equation), as suggested in Protocol Step 5. The final result is the same, hence the choice is a matter of individual preference. We find it easier to add the same volume of acid to all samples and adjust the sample volume used in the chromogen reaction in smaller weight samples (especially when dealing with hundreds of samples).

3. I like the simplicity of drying the tissue samples rather than homogenizing in another buffer prior to the acid digestion step. Have the authors compared methods (drying versus homogenizing)? A question in my mind about using whole blocks of desiccated tissue rather than a method that mechanically disrupts the tissue is whether the acid extraction step is as effective. If the authors have experience with this it would be good to comment.

R: Unfortunately, we have not tested this method with homogenized tissues. We have, however, compared data obtained using this methodology and atomic absorption, and the final concentrations obtained were very similar. We are thus confident that the acidic digestion is effective. Moreover, visual inspection suggests that samples are fully digested at the end of the 20-hour period of acidic digestion.

4. The final concentrations of iron are normalized to dry weight in the current manuscript. The authors have a good rationale for doing it this way rather than normalizing to wet weight. Accurately measuring dry weights of small pieces of tissue that need to be removed from a 24-well plate prior to weighing isn't trivial either. If the authors have tips for how to do this or whether it poses a problem, this should be briefly included. Results in the existing literature are often expressed per g wet weight. The authors could simply make a comment about using the accurate fresh weight to normalize data should future users want to compare their data to literature values.

R: We thank the Reviewer for the suggestions. We weigh small pieces of tissue in an analytical balance by placing them over a small piece of parafilm. We use plastic tweezers (or alternatively, stainless steel tweezers protected with parafilm) to pick the tissues. We have added this information to the Protocol. Also, we have added the following recommendation to the Discussion: When measuring iron content in tissues for which drying is not a viable option (e.g. adipose tissue), it is suggested that the assay is performed promptly upon sample collection, so that accurate determination of the fresh weight is not significantly affected by storage artifacts.

5. The calculation is a little complicated. Can the authors identify the volumes in the text as the method is described to better help the reader understand which numbers they need to incorporate into their calculations? Including a worked example would be helpful.

R: When presenting the different volumes in Protocol section 6, we have now referred to Table 2, which provides examples of volumes. We hope this helps readers interpret the Equation.

6. Researchers using the microplate format may find it more convenient to generate a standard curve and have their plate reader calculate concentrations for them, followed by a dilution factor to account for the tissue processing steps. If there is a way to show the calculation using such a method as well?

R: We appreciate that it would be useful for users to have their plate reader software calculate concentrations for them based on absorbance values and a standard curve. However, as the Reviewer acknowledges, the plate reader software would have to take into account the tissue weight and the supernatant volumes in the final calculations. We believe that the present equation can be easily computed in a spreadsheet program like Excel.

Minor Concerns:

7. Minor typos: trichloroacetic acid is spelled incorrectly (thrichloroacetic acid) (e.g. lines 89-90).

R: We thank the Reviewer for detecting this typo. It has been corrected.

Line 91 says to adjust the final volume to 1ml but 100ml is what this should read as.

R: We thank the Reviewer for detecting this typo. It has been corrected.

Line 116 - flask not flash.

R: We thank the Reviewer for detecting this typo. It has been corrected.

Reviewer #2:

Manuscript Summary: This manuscript reports one version of a protocol for measuring tissue non-heme iron using 96-well plates. The protocol is relatively simple and useful.

Major Concerns:

Line 25: "…which is why…" ascribes purpose to biological phenomena. This is very controversial teleological reasoning and not necessary to explain biological phenomena.

R: At the Reviewer's request, we have removed the expression "… which is why".

Lines 43 and 44: see above

R: At the Reviewer's request, we have replaced "For this reason, tissue iron levels need to be tightly regulated" with just "Tissue iron levels are finely regulated".

Line 44: "Abnormal dietary iron absorption" is not the main reason for iron deficiency in the world. Many diets simply do not contain enough iron, and increased blood loss (parasites, multiple child birth) may also contribute.

R: We have not stated that abnormal dietary iron absorption is the main reason for iron deficiency. What we wrote is that, among other reasons like repeated blood transfusions and chronic inflammation, abnormal dietary iron absorption is a common cause of iron-associated disorders. It is worth noting that the latter include iron deficiency as well as iron overload conditions (not just iron deficiency). Anyway, we followed the Reviewer's suggestion and included iron deficient diets in the examples of common causes of iron disorders.

Line 58: To this reviewer's knowledge, the exclusion of the heme component of iron depends on the acid precipitation/extraction step so it is applicable to any method that detects iron, including ICP-MS and AAS.

R: To the best of our knowledge, even after acid digestion, AAS or ICP-MS will measure all elementary iron in a sample, including that contained in heme (Van Deursen C et al. 1988 Accumulation of Iron and Iron Compounds in Liver Tissue. A Comparative Study of the Histological and Chemical Estimation of Liver Iron. Clinical Chemistry and Laboratory Medicine, 26(10); Rodgerson DO & Helfer RE 1966 Determination of Iron in Serum or Plasma by Atomic Absorption Spectrophotometry. Clinical Chemistry, 12(6), 338–349). To separate the heme iron 'compartment', one would need to perform some kind of ultrafiltration or selective extraction (e.g. Vácha J et al. 1978 Determination of heme and non-heme iron content of mouse erythropoietic organs. Exp Hematol, 6(9):718-24). In contrast, the current method uses milder conditions (temperature/acid pH) that denature proteins such as ferritin or transferrin, without breaking the heme molecule. This way, only the non-heme iron released from proteins will bind the chromogenic reagent (bathophenanthroline), with no need for additional sample processing.

Line 92: The proposed omission of trichloroacetic acid (misspelled) needs to be validated or a reference to validation provided.

R: At the Reviewer's request, we have included in Figure 1 a new graph showing excellent agreement between the non-heme iron levels in mouse tissues after acidic digestion of samples derived from the same tissues with a mixture of hydrochloric acid and trichloroacetic acid or hydrochloric acid alone. Also, we thank the Reviewer for detecting the typo. It has been corrected.

Line 129: Is tissue drying necessary? Many labs skip this step.
It would be helpful to explain the principle of the method, the need for the specific steps and what they do.

R: The reason for drying tissues was explained in Discussion: “the measurement of metals in tissues using wet weight instead of dry weight is significantly affected by the variable amount of weight loss through air-drying both in fresh and in frozen tissue samples11”. We have now briefly mentioned the critical steps of the method in the first paragraph of Discussion.

Additional references that validate the method should be provided. I would suggest: C.J. Rebouche et al. / J. Biochem. Biophys. Methods 58 (2004) 239-251

R: We thank the Reviewer for bringing the paper of Rebouche et al. to our attention. In their work, Rebouche et al. described the measurement of non-heme iron in animal tissues using ferrozine (not bathophenathroline) as the chromogen reagent. So, we don’t really think we could use it to validate the present method. Other significant differences include the use of fresh tissue homogenates (versus dry tissue samples) and absorbance reading on microcuvette (versus microplate). Nevertheless, we have cited this work in the Introduction.

Reviewer #3:

Manuscript Summary: In this work, Duarte et al describe the protocol to measure tissue non-heme iron content using a colorimetric assay. This protocol is of interest for the scientific community, in particular given that the original paper from "Torrance and Bothwell" is not easy to be acquired (Torrance JD, Bothwell TH. A simple technique for measuring storage iron concentrations in formalinised liver samples. S Afr J Med Sci. 1968;33(1):9-11.) The protocol is well written and easy to be followed. However I have a few points that would improve it.

Minor Concerns:

- The authors should mention that an alternative to the normalization against dry tissue can be the use of wet tissue. This is particularly useful when measuring the iron content of adipose tissue (e.g. white or brown adipose tissue for example in the db/db or ob/ob mouse models of type 2 diabetes.

R: We appreciate that in some cases drying tissues may not be practical. While we favor this approach, we have added the following to Discussion: When measuring iron content in tissues for which drying is not a viable option (e.g. adipose tissue), it is suggested that the assay is performed promptly upon sample collection, so that accurate determination of the fresh weight is not significantly affected by storage artifacts.

- The authors should use a better way to describe the concentraiont of the solutions. For example, 228 sodium acetate in 400ml of deionized water should be converted in molarity. This is also because sodium acetate can exist in different hydration forms.

R: We thank the Reviewer for this comment. We have corrected the reagent designation to anhydrous sodium acetate. We have provided the molarity of the iron standard solutions. However, in the case of sodium acetate, we cannot provide its exact concentration as this is a saturated solution. In our hands, solution may or may not precipitate, in which case a bit more sodium acetate must be added until precipitation occurs. We're not sure why this happens, since over the years we have noticed batch to batch differences when using different lots of the exact same reagent (i.e. same supplier and catalog number)

- I do not particularly like the way the authors prepare the standard soluion. This is something quite important in particular for reproducibility among different laboratories. Using a weight of 22.3mg of iron in a so precise volume of HCl (1096ul) is quite optimistic to be reached, in particular if then the authors state that the concentration will be exactly 1.1169mg Fe/ml. Sigma aldrich and other companies are selling iron standards already validated and I would strongly suggest to indicate that as preferential standard source.

R: The Reviewer is right in saying that it is hard to weigh 22.3 mg iron in 1096 ul acid, for a final volume of 20 mL. We have corrected this to suggest that 111.5 mg iron are weighed in 5480 ul acid for a final volume of 100 mL. This should be more doable. Regarding the final concentration, please note that 1.1169 mg Fe/ml is just the concentration of iron in our standard solution as measured by AAS. As long as researchers determine the concentration of iron in their own standard solutions, they only need to change the value in the final equation accordingly. As suggested by the Reviewer, we have also mentioned the option to use commercial standard solutions in the Discussion.

- The authors use 1ml of acid mixture independently of the amount of dry tissue used (10-100mg). Is the iron extracting capacity of the acidic solution preserved if you use 10 times more tissue? Wouldn't it be better to use instead an amount o f acidic mixture relaive to the weight of the tissue? In this way the ratio solvent/dry tissue would be preserved.

R: The current protocol is optimized for processing 10-100 mg of animal tissue. We know from our experience that 1 ml of acid is sufficient to digest up to 100 mg of tissue. Regarding the ratio solvent/dry tissue, the Reviewer is right when he/she says that by using 1 ml of acid solution regardless of tissue wet weight, the ratio is not preserved. However, this is taken into consideration in the equation used to calculate tissue iron concentration. In samples with very low weight (and low iron content), it is possible that the absorbance of the chromogen reaction is below the spectrophotometer's linear dynamic range of detection. In these situations, we could indeed reduce the volume of acid used in sample digestion (and subsequently adjust the volume of acid mixture in the final equation), or we can increase the sample volume when preparing the chromogen reaction (and adjust the sample volume in the final equation), as suggested in Protocol Step 5. The final result is the same, hence the choice is a matter of individual preference. We find it easier to add the same volume of acid to all samples and adjust the sample volume used in the chromogen reaction in smaller weight samples (especially when dealing with hundreds of samples).