Improved sample preparation for fruits allowed histochemistry and X-ray microscopy to reveal conserved iron hotspots

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Running title: Extending histochemical and X-ray-based approaches to the mature fleshy fruits

Keywords: fruit, stain, X-ray, metal, mature, perls, microscopy, spatial, iron

Abstract

Mature fleshy fruits are the most challenging plant organs to study due to their high water and flavonoid content. We aim to develop a sample preparation protocol to analyze fleshy fruits with histochemical and X-ray based methods. By vacuum freeze drying and decolorization with fixatives, we were able to apply histochemical Perls staining to seamlessly localize iron in fruits. We screened the fruits to reveal possible conserved iron accumulation sites. This approach showed iron preferentially accumulated in fruit vascular tissues; at subcellular level, in the cell walls of vasculature and other tissues, and inside the epidermal cells of polyphenol-rich fruits. Furthermore, iron accummulated in the endosperm of tomato seeds, indicating a role in germination. X-ray microscopy of chemically untreated fruit slices showed similar iron distributions, indicating chemicals used in the protocol did not significantly mobilized the metals. This has been the first systematic study to extend histochemistry and X-ray fluorescence to obtain spatial information from mature fleshy fruits. This advancement in methodology may facilitate fruit research, potentially contributing to food security in the long run.

Introduction

Fruits constitute an indispensable component of a nutritious human diet, and to sustain the dietary needs of our burgeoning global population, fruit production must escalate from one billion tons to surpass two billion tons by 2050 [1]–[3]. Part of this challenge is to reduce the pre- and post-harvest losses, which can account for up to half of the production, putting fruits on the top of most wasted among all kinds of foods [4], [5]. One major factor leading to such

crop losses is metal deficiencies. Metal deficiencies lead to losses by limiting the quality and quantity of the fruits. These effects can be direct, as in apples, where insufficient calcium levels can reach the fruit, leading to the bitter pit disease Field [6]–[8]. The effects can also be indirect (i.e., systemic); for example, starving for a metal can induce remodeling of the metabolism, leading to lower quantity and quality of fruits [9]–[11]. Metal deficiencies may also reduce fruits' defense against several microbial diseases [12], [13], which are associated with a significant part of the post-harvest losses.

Despite the well-established importance of metals in fruit biology even the most basic protocols for their investigation are notably absent. Main methods to investigate spatial localization of metals in plant organs such as seeds, roots or leaves include X-ray based techniques [14], [15], histochemical stains and others [16]–[18] prove inconvenient for fleshy fruits due to fruits' very high water content. This water content prevents the use of many sample preparation techniques; fixatives may not effectively replace water, water loss may spoil the physical structure, and water-filled soft tissues might require expertise to cut to get uniform sections. In addition to being mostly water, fruits are enriched in colorful polyphenols, obscuring the color of simple histochemical stains. Therefore, spatial metal analysis of fruits is currently limited to non fleshy fruits -such as grains or pods- and does not extend to juicy and fleshy ones such as tomatoes or oranges- with only a few exceptions [19]. Consequently, the current practice of spatially localizing metals in fruits must be improved. Currently, investigators take samples from different parts of the fruit and compare the total metal concentration in them, providing extremely low spatial information [20]–[22].

Lacking suitable techniques for localizing metals in fleshy fruits may have consequences beyond the farmers' perspective. From the public's view, consumers sometimes choose one part of the food over other parts due to its nutritional value. They should know how metal nutrients are distributed among their everyday fruits, provided that consuming nutrient-rich parts can alleviate widespread micronutrient deficiency disorders in people [23]. To illustrate the importance of this choice, how people eat eggs can be given as an example. People doing exercise often choose to eat the protein-rich white part, while, vitamin-seekers choose the yellow, and many others choose to eat the membrane beneath the egg shells for therapeutic effects; consequently, each of these parts is commercialized and available seperately[24], [25]. From the plant physiologists' view, detecting preferential accumulation of metals is the first step to investigate their biological roles following the principle "form follows function." For example, the accumulation of metals may indicate the presence of enzymes that use these metals as cofactors and of metal transporter proteins mediating this accumulation.

We tested several tissue preparation protocols to allow spatial localization of metals by histochemical staining in fleshy fruits. We aimed to employ such a protocol to seamlessly detect metal distribution at tissue and subcellular levels. This approach allowed us to discover hot spots for metals, some surprisingly widely conserved.

Method

Fruits were sliced and freeze dried. These fruit slices then used for metal analyses either directly (for X-ray analyses) or after decolorization (Perls and Perls/DAB stainings).

Fruits were purchased from local markets and bazaars in Turkey (Supplementary table 1). Fruits were freshly sliced longitudinally and cross-sectionally with a V-blade in 2-3mm thickness (Supplementary Figure 1). Prepared fruit slices were frozen in -80 °C for 24 hours. Frozen fruit samples were placed in a vacuum freeze dryer for 23 hours of the main drying phase at -54 °C, 0.024 millibar, and an hour of final drying phase at -60 °C, 0.011 millibars. These samples were used directly for X-ray analysis.

For X-Ray analysis, freeze-dried fruit samples were analyzed by an micro XRF analyzer (Horiba, Japan) with step size ranging from 120 to 200 µm with 15µm polycapillary beam.

For decolorization, freeze-dried samples were immersed in a fixing solution (Methanol: Chloroform: Glacial acetic acid; 6:3:1) in a glass petri dish and were shaken at 90 rpm until the fruit's natural color disappeared. The fixing solution was renewed several times as the fruit piece lost color. To stop the decolorization step, the fixative was removed by washing the samples three times with distilled water.

For the histochemical staining of Fe, Perls staining was used as described before [26]. Briefly, Perls stain solution was prepared fresh by mixing 4% K-ferrocyanide ($K_4Fe(Cn)_6$) and 4% HCl stock solutions in a 1:1 (v: v) proportion. Then, it was braught to 37 °C in a standard incubator. Decolorized samples were submerged in the solution and vacuum infiltrated for an hour at 500 millibars. Next, the staining solution was removed, and the samples were washed three times with distilled water for 1-2 min each by slightly shaking. The samples were stored in distilled water at 4°C. The stained samples were observed under a stereoscopic microscope.

To examine the subcellular localization of Fe, Perls/DAB staining was performed on thin sections. For this, freeze-dried fruit slices were embedded in paraffin, and 10 µm sections were obtained. Sections were then deparaffinized by incubation at 65°C for 15 minutes following a xylene treatment for 15 minutes and stained with Perls/DAB according to Roschzttardtz et al. (2009). Briefly, Perl's stain solution was prepared fresh by mixing 4% K-ferrocyanide ($K_4Fe(Cn)_6$) and 4% HCl stock solutions in a 1:1 (v: v) proportion. Then, the stained sections were incubated between 10 and 30 min in a 0.1 M phosphate buffer (pH 7.4) solution containing 0.025% (w/v) DAB (Sigma), 0.005% (v/v) H_2O_2, and 0.005% (w/v) $CoCl_2$. The reaction was stopped by rinsing samples with distilled water. Samples were imaged with a light microscope (Axioskop; Carl Zeiss, Jena, Germany).

RESULTS

For metal localization studies, arguably, the most widely used plant organ can be the seed. Generally, this is attributed to seeds' low (i.e., around %5-15) water content. We stained tomato fruit with Perls staining to examine how effective classical histochemical methods are in localizing metals in fruits. In literature, Perls turn to a bright blue in the presence of Fe and have been widely used for localizing Fe in various plant organs [27]. Being %95 water, tomato is one of the most prominent agricultural crop [28], making it a challenging target for metal localization studies. As expected, lentil seeds that were used as controls were heavily stained by Perls, producing more or less homogeneously distributed blue color, indicating the stain is working (Figure 1A). However, tomato fruits show very little or no coloration(Figure 1A). This indicated either the fruit lacked detectable Fe or Perls could not localize Fe in fresh tomato fruits. We hypothesized that the high water content of tomato inhibits Perls dye's action, and the bright red color of tomato fruit further obscures the presence of blue precipitates of Perls. To remove excess water, we first tried conventional drying methods. Oven drying spoiled the structure of the tomato(Figure 1B). However, when dried tomato slices were stained by Perls, we noticed more blue coloration (Figure 1B). Compared to the non-dried and stained tomato slices, indicating high water content was decreasing staining efficiency. We treated the tomato fruit with several solvents aiming to remove the colorful polyphenols while preventing the remobilization of Fe as much as possible. However, treating tomato fruits with solvents led to loss of rigidity, and fruit slices looked mushy (Figure 1B). We saw a similar trend with other fruits; when treated with solvents, they lost structural integrity, and developed brown discoloration (Supplementary Figure 2).

We hypothesized that the solvents we used were ineffective due to the high water content of the tomato fruit. To remove water while maintaining the structural integrity of tomato slices, we dried the slices using a vacuum freeze drier this time. Vacuum freeze drier kept the structure of tomato slices(Figure 2A). As expected, when treated with solvents, these dried tomato slices effectively lost most of their natural color. Among the solvents, we decided to use Methanol: Chloroform: Glacial acetic acid; 6:3:1 since it effectively removed colors from tomato fruit and at the same time had been succesfully used as a fixative in Perls staining [29], indicating this mixture should keep metal remobilization at minimal. When stained by Perls, freeze-dried and decolorized tomato slices showed intensive blue staining (Figure 2B-G). This blue staining was not just homogeneously distributed but preferentially localized in the region below the calyx (Figure 2C), in the vasculature (Figure 2D), and around the seeds (Figure 2E). Seeds of various species had been extensively investigated for metal distribution before [27], [30]; however, tomato seed remained elusive. To investigate whether Fe staining around the seed was in the outer or the inner tissues, we made 10 μm cross and longitudinal sections and stained with Perls. Results showed tomato seeds accumulate Fe in the embryo and in the endosperm. Embryo Fe was preferentially localized in the vasculature (Figure 2F), in agreement with the previous data [27]. However, Fe also observed in the endosperm (Figure 2F, G). Endosperm Fe localized preferentially in the region close to the germination site, including chalazal and micropyler, but not lateral endosperm. This novel observation may indicate Fe may be involved in the germination of tomato seeds.

Next, we assessed whether the protocol we developed was only effective for localizing metals in tomato fruits or could be used for other fruits as well. We employed the technique on several fruits and determined the Fe localizations (Figure 3 and 4). Results showed Fe accumulation

patterns successfully. Many fruits showed more or less homogeneous blue color (Figure 3). However, we noticed many fruits showed an intense blue color throughout their vasculature tissues, indicating a preferential Fe accumulatin in this tissue (Figure 3). We also noticed fruits that were known to accumulate extensive amounts of proanthocyanidins in the fruit skin showed preferential Fe accumulation in their skin (for example, blueberry in Figure 4H).

Perls staining can show Fe localization even in the subcellular level [26]. However, to reach subcellular resolution, thin sections are required. At this level, total Fe in the tissue is often insufficient to produce a signal. For such cases, Perls staining had been intensified by DAB [26]. To investigate Fe localization at the subcellular level, we made thin cross-sections of vacuum freeze-dried fruits slices. Perls staining following DAB intensification revealed the Fe distribution as black coloration. According to the results, most Fe resided outer parts of the cells, probably in organelles at the peripheries such as chloroplasts, chromoplasts, and mitochondria of the cell and in the cell wall (Figure 5). In contrast, Fe was found in inner regions, inside the vacuoles in the epidermis of blueberry (Figure 5A). In accordance with Perls staining of the slides, vascular tissues were heavily stained with Perls/DAB (Figure 5B,C).

Histochemical methods to localize metals have often been criticized for possible artifacts (i.e., remobilization of metals). To verify the Fe hotspots recently identified in the fruits with an independent technique, we used X-ray-based techniques where tissue preparation was minimal. Micro XRF mapping of vacuum freeze-dried slices revealed localizations of many metals in fruit slices cut horizontally or vertically (Figure 6). Next, we assessed whether Micro XRF can validate tissue level Fe hotspots detected by histochemical methods. Scanning of vacuum freeze-dried slices of fruits confirmed the Fe hotspots in the vascular tissues of selected fruits(Figure 7A, B) and in the skin of blueberries (Figure 7C).

Discussion

Fruits, especially the fleshy ones, are the fastest deteriorating part of plants upon harvest, posing a unique challenge to meet the ever increasing global food demand. Fruit spoilage should be highly related to metals, as metals activate key enzymatic processes such as those involved in fruit ripening/softening and pathogen defense. To examine metal-involved physiological processes in fruits, the very first step is to be able to localize the metals in fruit tissues, preferably, with low cost and fast methods offering high resolution at the same time. In this study we developed a tissue preparation method that can be applied to even most complex (i.e., very high water and colorful polyphenol content) fruits and showed that this allowed fruits to be analyzed by the common metal localization methods, including histochemical staining or micro X-Ray mapping.

Histochemical approach to mature fleshy fruits often face two main obstacles: too much of water content limiting penetration of chemicals and too much of colors obscuring the chemical

staining (Figure 1A). Although fruits are extensively dried in industry, such tecnologies have been rarely used for research. Among the drying technologies, vacuum freeze drying offers the golden standard for human consumption. It is used on freshly cut fruits to increase fruits' shelf life and simplify their transportation to the market. Freeze drying prevents shrinkage of the slices, minimizes remobilization of solutes in the tissues [31]. It relies on evaporation directly from the ice phase, skipping the water phase which is the most prone phase for chemical reactions and remobilization of solutes. After the evaporation porous tissue remains, perfect for penetration of chemicals for histochemical approach, despite these advantages, vacuum freeze drying almost never appears in histochemical methods in fruit research.

We observed that once freed from water, the fruit slices behaved as regular tissue (Figure 2). After vacuum freeze drying, fixatives could decolorize the fruit (Figure 2 and Supplementary figure 2). Although part of the color was retained(Figure 2A), it did not interfere with the detection of our stain anymore. In literature, the most common getaround from the natural bright colors of fruits such as tomato has been to analyze the sample in earlier developmental stages before the fruit develops colors[32], [33]. This approach is in parallel with histochemistry applied to seeds. Seed coats develop a dark color due to accumulated proanthocyanins, forcing researchers to do histochemistry such as GUS staining before the fruit matures or the detection quality suffers [32], [33]. For fruits, we tested only one type of histochemical stain; however, the protocol potentially allowed other histochemical methods to be applied.

We observed the most prominent Fe accumulation in the vasculature, cell walls, then tissues around seeds, and sometimes in the skin of the fruits (Figure 4, 5). Regarding the vasculature, the physiological function of this Fe accumulation is unclear. The physiological functions of metals have often been studied by imposing their deficiency and investigating the developing phenotype. For example, Fe deficiency induces chlorosis in the leaves, indicating Fe may be involved in chlorophyll synthesis. However, Fe deficiency in fruit trees has never been reported to cause a disease specific to vasculature[9]. However, we cannot rule out a possible physiological function for this Fe reservoir. Recently, Fe oxidation was linked with the callose deposition [34], which may decrease xylem functionality in the developing fruits [35]. Another possibility is that the main vasculatures of fruits may serve as a sink to load Fe to the seeds. In this model, the vasculature may represent excess Fe reservoir left over from seed Fe filling, as this process is highly regulated not to induce Fe-mediated oxidative damage in the precious seeds [36]. The remaining Fe can then be absorbed by the cell walls, as shown cell walls can quickly absorb Fe(III) [37]. Our study identified vasculature as the main Fe-accumulating tissue; however, further investigation on which cells in the vasculature accumulate iron is yet to be investigated.

Fe accumulation in the cell walls has been well documented in other organs [37]–[39] but has not yet been discussed regarding the cell walls of fruits. We propose that the cell walls of fruits may act as a larger reservoir for metal ions such as Fe due to two main factors. First, among all the plant cell walls, the cell walls of fruits potentially have the highest concentration of pectin, the major component of the cell wall that binds Fe [38], [40]. Second, the cell walls of fruits may be less saturated with cations than other organs' cell walls. Demethylated pectins in cell walls

typically offer free carboxyl groups to absorb positively charged metals, notably calcium, a far more abundant element than Fe, Mn, and Zn. However, calcium is almost solely transported by the xylem, which is ineffective in fruits due to low transpiration [35]. Consequently, its low concentrations commonly cause diseases in fruits such as apples or tomatoes [41], [42]. On the other hand, all the rest of the cations are phloem mobile, meaning they are transported to the fruit continuously [43]. Positively charged molecules compete for binding the free carboxyl residues of pectins at least in the root; for example, in the field, sodium mobilizes Fe from the cell wall [44] or protons secreted from H+ ATPases mobilize the calcium [45]. We propose that in fruits, due to high pectin and low calcium concentration, cell walls may act as an unusually powerful absorbent for metal ions, explaining our observation of conserved Fe accumulation (Figure 4). Fe and maybe other metals unloaded/leaked from the phloem and xylem may immediately get stuck in the cell walls of helper cells in the vasculature.

We also detected iron inside the outer cell layers of blueberry or grapes and in the endosperm of tomato (Figure 5 and 7). These iron spots may represent tannins, as blueberry skin can often be rich in tannins that bind iron and may be stored in the vacuoles [46]–[48]. In literature, an increase in fruit polyphenols, including tannins was reported to be correlated with higher Fe concentrations [49]. Besides, we detected Fe in tomato seed endosperm (Figure 2). In seeds, main Fe storage is in the embryo, but exceptions such as seeds accumulating Fe in the endosperm have been reported [27]. However, Fe storage in tomato endosperm shows an evident preferential accumulation to the sides close to the germination (i.e., micropylar and chalazal regions). Therefore, we propose that this Fe storage may facilitate germination. For tomato seeds to germinate, endosperm should first soften to allow radical protrusion, a major process shown to include cell wall cutting hydroxyl radicals [50], [51]. A well-known source of hydroxyl radicals is Fe, which reacts with H_2O_2 through the Fenton reaction [52]. Therefore, this Fe storage in the endosperm may facilitate germination by generating a cell wall attacking hydroxyl radicals. Further studies should target whether manipulating Fe levels in seeds can change germination patterns.

Increasing fruit production should target decreasing pre- and post-harvest losses. To reduce the loss, a better understanding of fruit biology is required. We developed a sample preparation technique for even the most difficult fruits. By using it, we systematically examined Fe localizations in the fruits. We determined some Fe hotspots which require further investigation for their potential physiological function. This study extended the basic and applied research to mature fleshy fruits, potentially facilitating research to prevent yield loss in the long run.

Figures and captions

Figure 1: The histochemical approach to fruits requires a new protocol for sample preparation. A, Effectiveness of direct staining of seeds compared with fleshy fruits. Lentil seed coat was removed, and lentil embryos were submerged in Perls staining solution for two hours. Cut tomato pieces were immersed directly in Perls solution overnight. B, Common obstacles for histochemical approach on fruits include high water content and bright colors. Left, a tomato slice was dried in an oven; simple drying led to structure loss. Middle, dried tomato ineffectively stained by Perls. The oven-dried tomato slice was incubated in Perls solution for two hours. Right, fixatives decolorized the tomato but led to structure loss. A freshly cut tomato slice was treated with fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1) overnight with continuous shaking. Blue coloration shows Fe. Bar 1cm unless otherwise indicated.

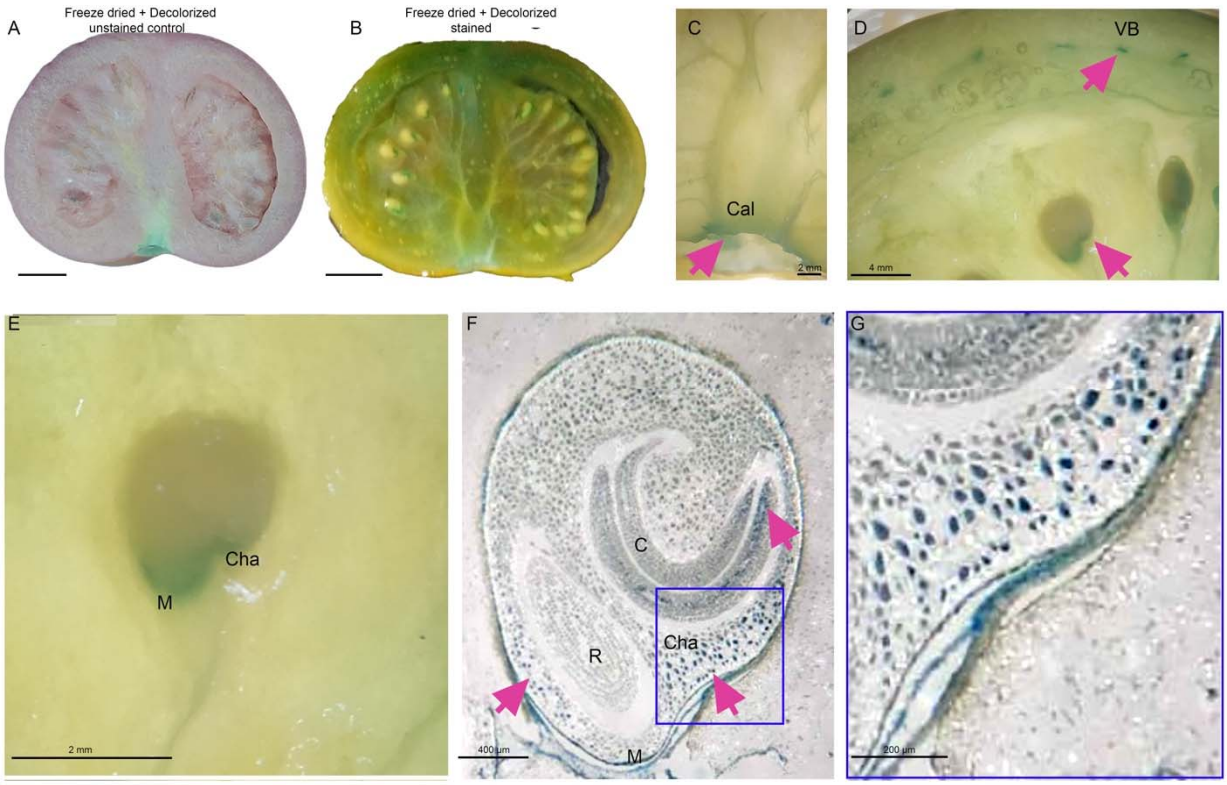


Figure 2: A newly developed protocol allowed histochemical methods to apply to tomatoes and revealed preferential Fe accumulation patterns. A, freeze-dried, decolorized tomato slice. B, freeze-dried, decolorized, and stained tomato slices. C-E, close-up pictures of B. F, G; stained thin cross sections of the tomato slice. For A, freshly cut tomato pieces were first freeze-dried and then decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). For B, after fixation, pieces were submerged in Perls solution for 2-4 hours. To see the staining inside the seeds, freshly cut tomato pieces were first freeze-dried and then decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). For F, G; after fixation, samples were embedded in wax, and 10 µm cuts were prepared on slides, following Perls staining. Blue coloration shows iron. Pink arrows point to blue coloration. Bar 1cm unless otherwise indicated. Cal: calyx detachment site, VB: vascular bundle, M: micropylar region, Cha: chalazal region, C: cotyledon, R: radicle

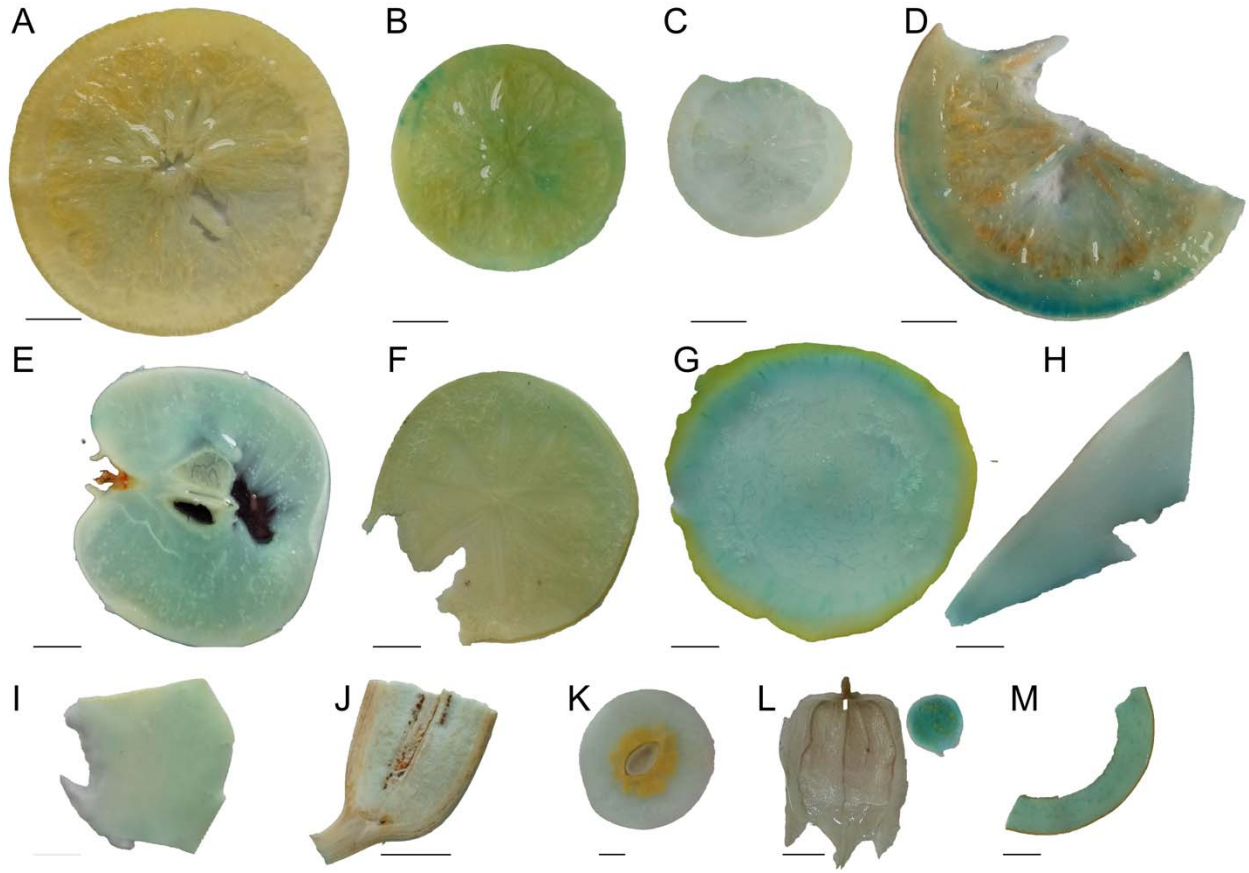


Figure 3: **The newly developed protocol revealed Fe accumulation in various fruits.** Fruits were either cut cross-sectional or longitudinal with a V- blade (For the V-blade, refer to supplementary figure 1). These pieces were then freeze-dried and decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). After fixation, pieces were submerged in Perls' staining solution between 2-16 hours. From A to M; orange, mandarin, lemon, grapefruit, apple, persimmon, honey melon, mango, pumpkin, plantain, nectarin, cape gooseberry, avakado. Note that despite the differences in fruit types, the optimized protocol preserved the structure and decolorized the tissues efficiently. For the complete list of fruits successfully stained, refer to supplementary table 1. Blue coloration shows Fe. Bar 1cm unless otherwise indicated.

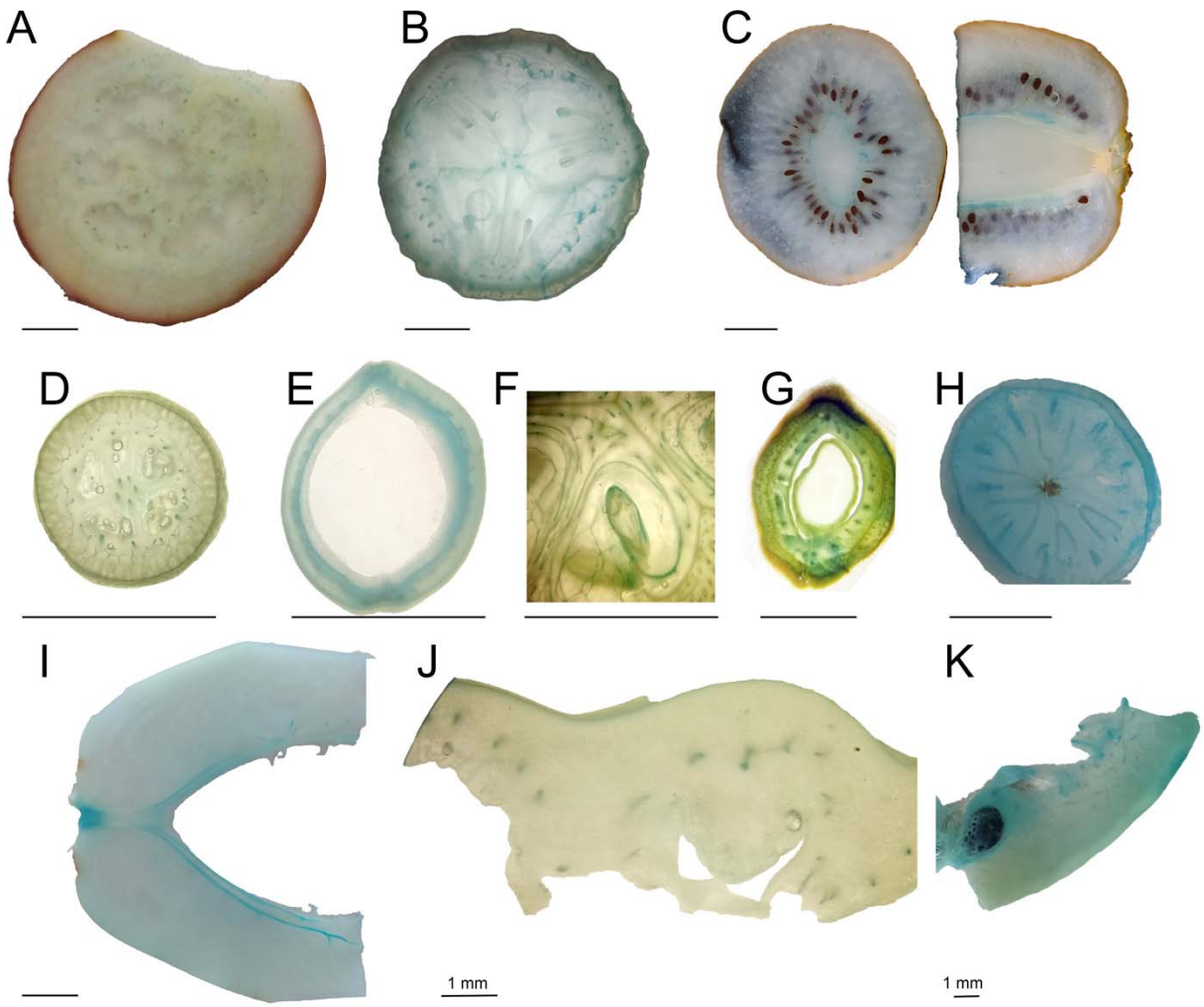


Figure 4: Fe accumulation was detected in the main vasculatures of fruits. Fruits were either cut cross-sectional or longitudinal with a V- blade(For the V-blade, refer to supplementary figure 1). These pieces were then freeze-dried and decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). After fixation, pieces were submerged in Perls' staining solution between 2-16 hours. For this figure, fruit pictures with apparent Fe accumulation in the vascular bundles were chosen. From A to K; eggplant, cucumber, kiwi, kumquat, haricot, squash, green almond, blueberry, papaya, pepper, passion fruit. Bar 1cm, unless otherwise indicated.

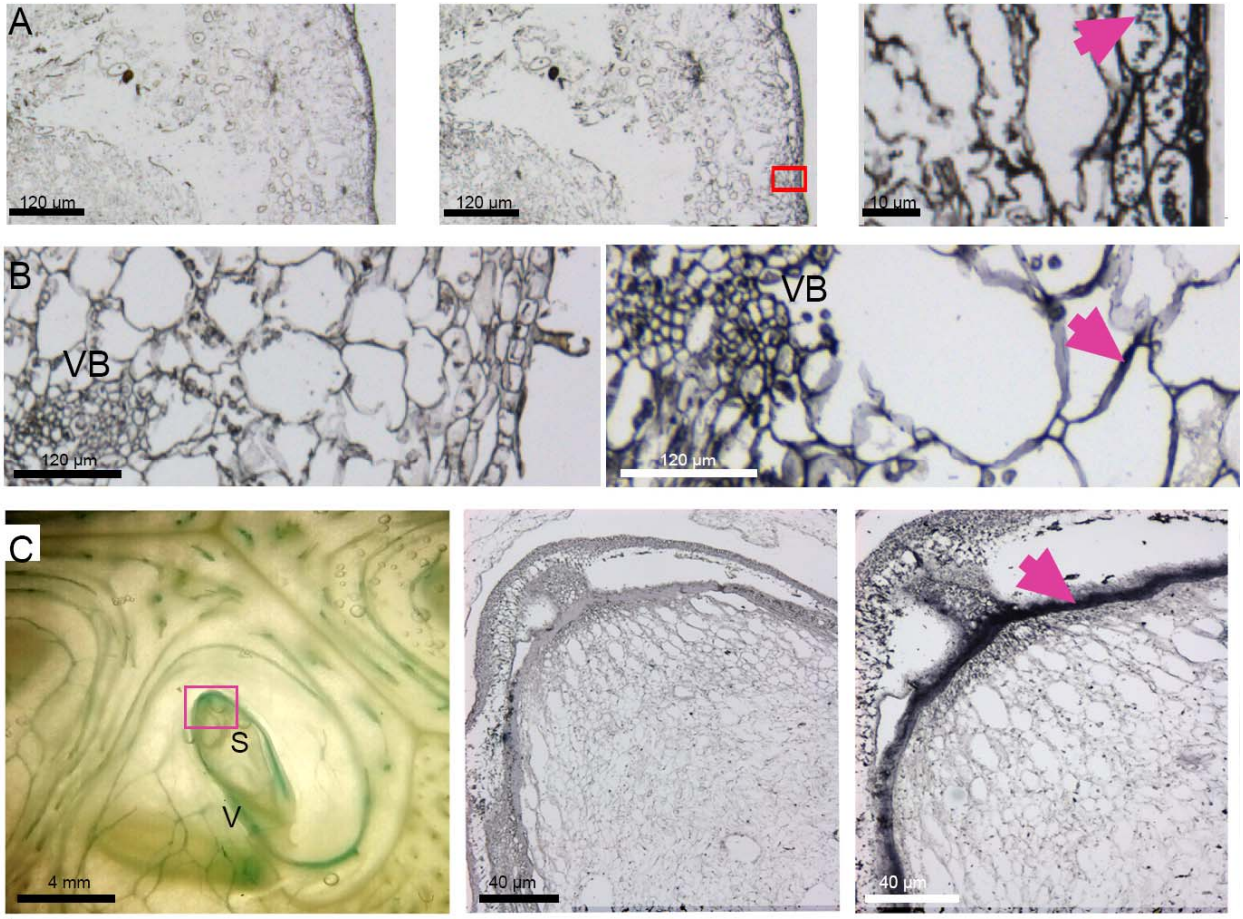


Figure 5: Fe accumulation in fruits could be detected at the subcellular level. A, Fe accumulated inside the epidermal cells of blueberry, most probably bound to polyphenols in the vacuole. Left, control section without staining; middle, Perls/DAB stained section; right, close up of red rectangle of the middle. B, Fe accumulates in cell walls. Left, control section without staining; right, Perls/DAB stained section. C, Fe heavily accumulated in the back of the squash seed embryo, most probably corresponding to the vasculature as Cucurbitacea seeds, including squash, have a coat usually surrounded by a single vein [53]. Left, squash cross section stained with Perls; middle thin section corresponding to the back part of the seed (such as the pink rectangle shown in the left picture); middle, control section without staining; right, Perls/DAB stained section. Blue coloration in C (left) and black coloration in the rest shows Fe accumulation. Pink arrows point to Fe accumulation. VB: Vascular bundle, V: Vasculature, S: Seed

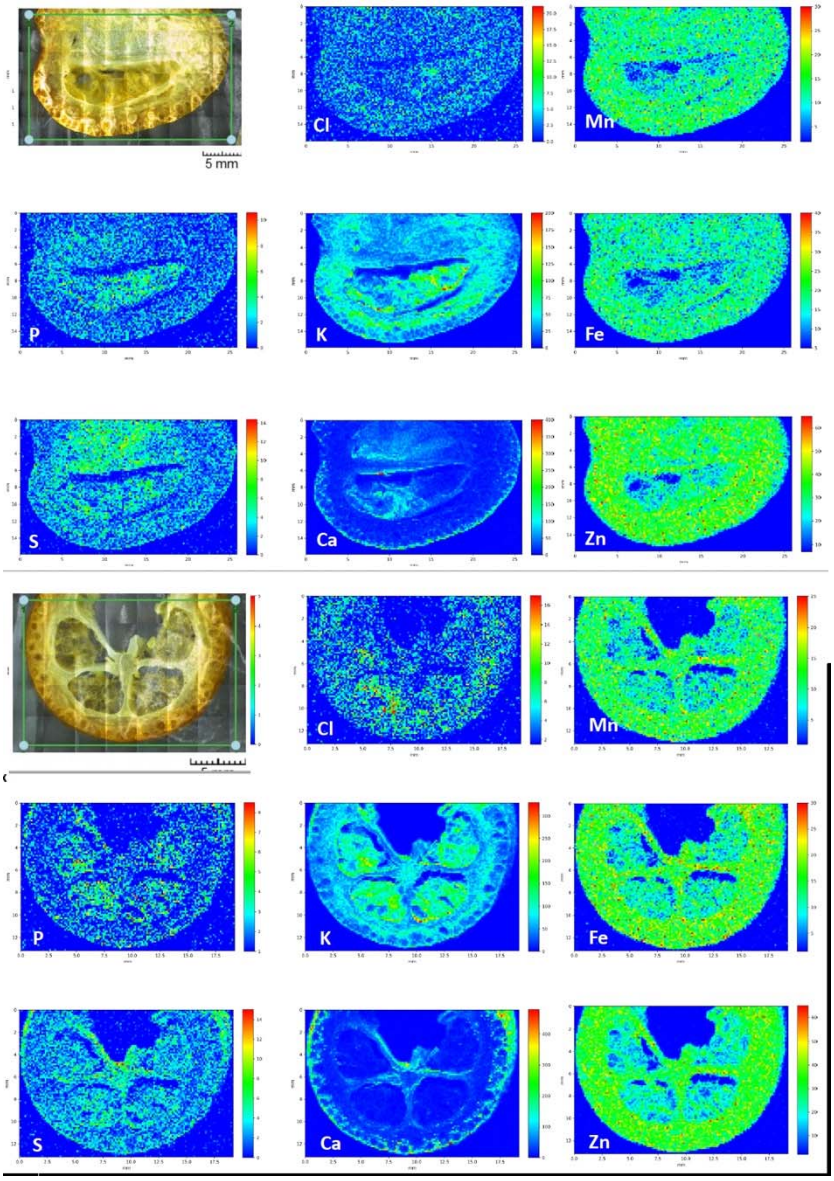


Figure 6: Determining metal localizations on fruit slices by micro XRF mapping. Cross and longitudinal section of Kumquat. The sample was cut, freeze-dried, and analyzed by XRF.

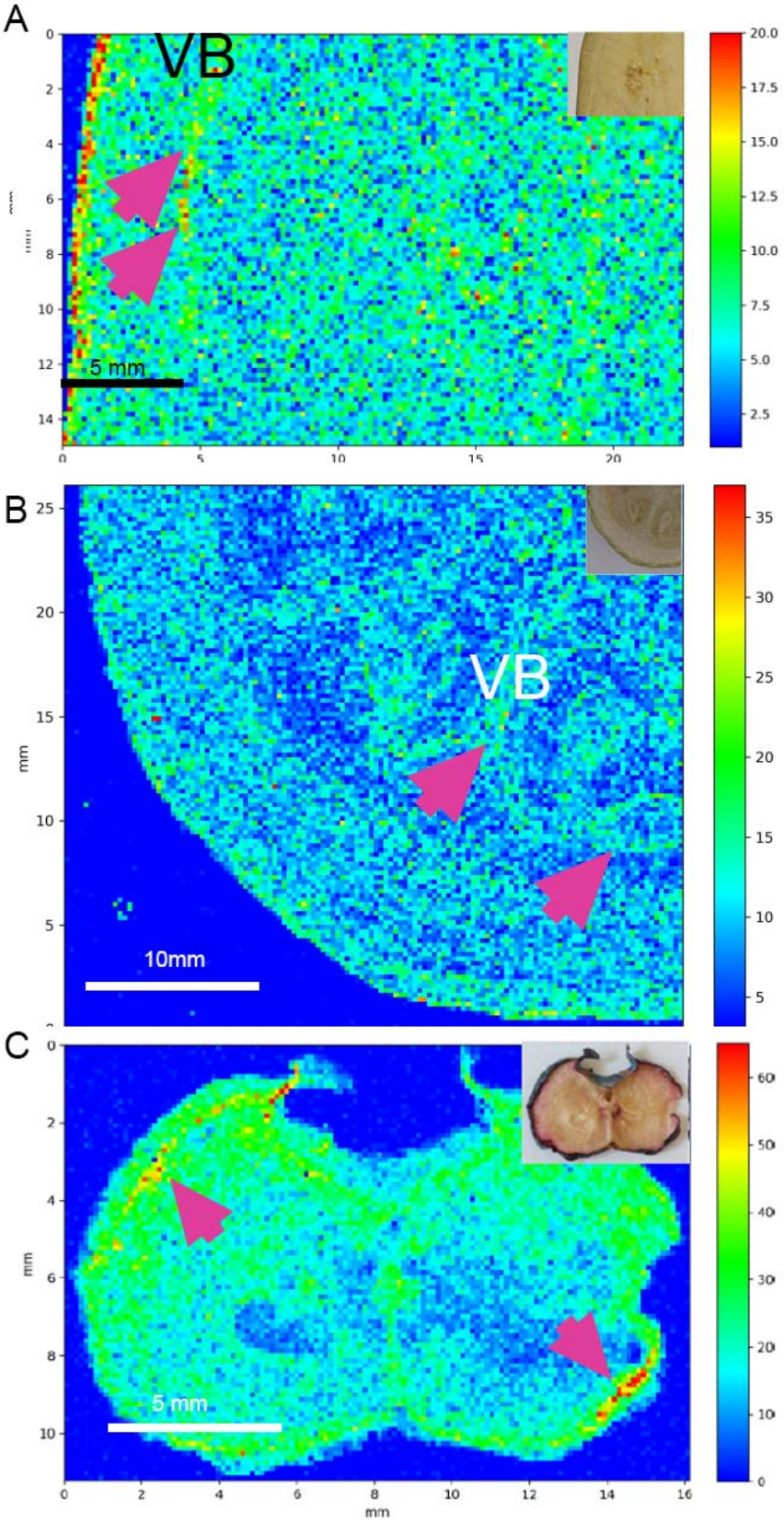
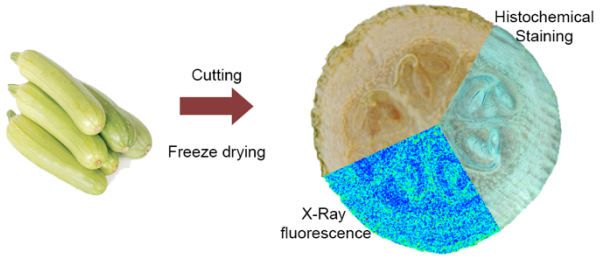

A
VB
5 mm
B
VB
10mm
mm
C
5 mm
mm

Figure 7: Micro XRF mapping confirmed the histochemical methods on tissue-level Fe hotspots. A, aubergine; B, squash; C, blueberry. Pink arrows point to Fe accumulations. VB: vascular bundle



Graphical abstract, a low-cost, high-throughput protocol to map metal localizations in everyday fruits.

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Figures and captions

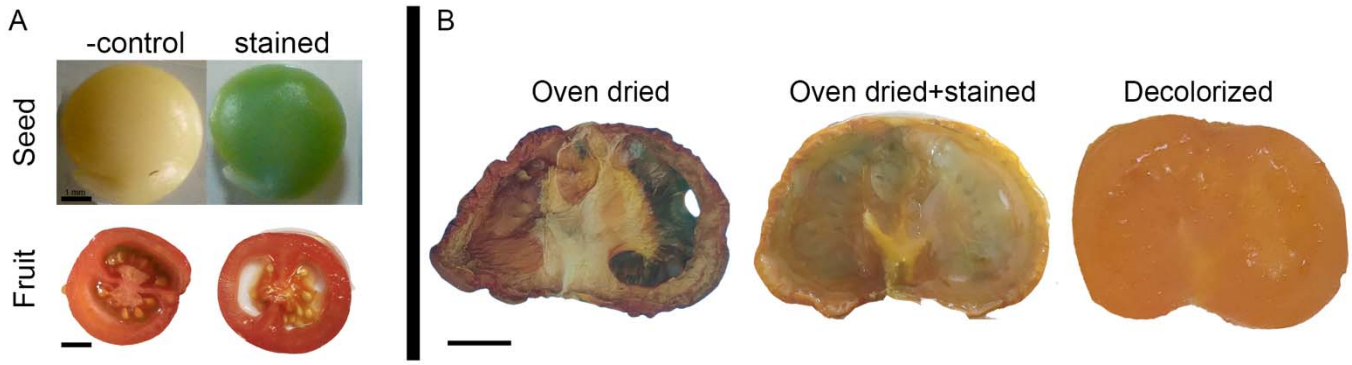

Figure 1: The histochemical approach to fruits requires a new protocol for sample preparation. A, Effectiveness of direct staining of seeds compared with fleshy fruits. Lentil seed coat was removed, and lentil embryos were submerged in Perls staining solution for two hours. Cut tomato pieces were immersed directly in Perls solution overnight. B, Common obstacles for histochemical approach on fruits include high water content and bright colors. Left, a tomato slice was dried in an oven; simple drying led to structure loss. Middle, dried tomato ineffectively stained by Perls. The oven-dried tomato slice was incubated in Perls solution for two hours. Right, fixatives decolorized the tomato but led to structure loss. A freshly cut tomato slice was treated with fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1) overnight with continuous shaking. Blue coloration shows Fe. Bar 1cm unless otherwise indicated.

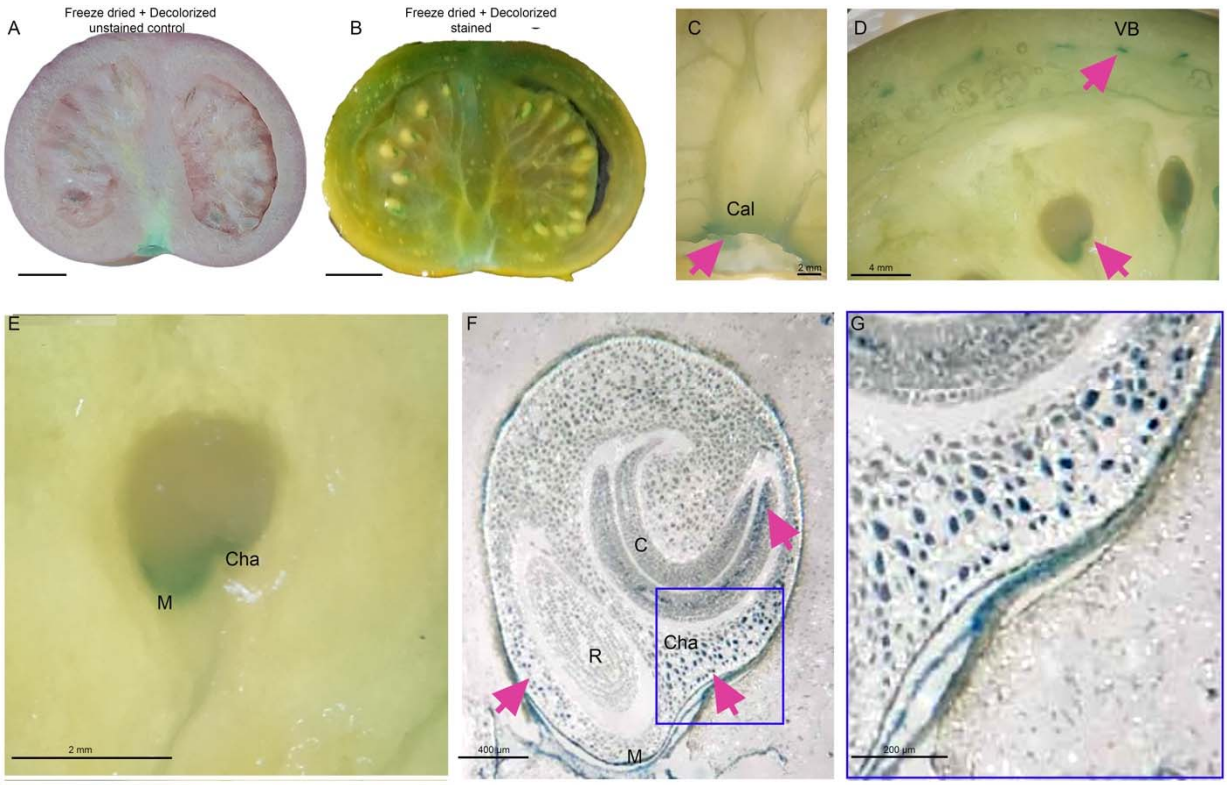

Figure 2: A newly developed protocol allowed histochemical methods to apply to tomatoes and revealed preferential Fe accumulation patterns. A, freeze-dried, decolorized tomato slice. B, freeze-dried, decolorized, and stained tomato slices. C-E, close-up pictures of B. F, G; stained thin cross sections of the tomato slice. For A, freshly cut tomato pieces were first freeze-dried and then decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). For B, after fixation, pieces were submerged in Perls solution for 2-4 hours. To see the staining inside the seeds, freshly cut tomato pieces were first freeze-dried and then decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). For F, G; after fixation, samples were embedded in wax, and 10 µm cuts were prepared on slides, following Perls staining. Blue coloration shows iron. Pink arrows point to blue coloration. Bar 1cm unless otherwise indicated. Cal: calyx detachment site, VB: vascular bundle, M: micropylar region, Cha: chalazal region, C: cotyledon, R: radicle

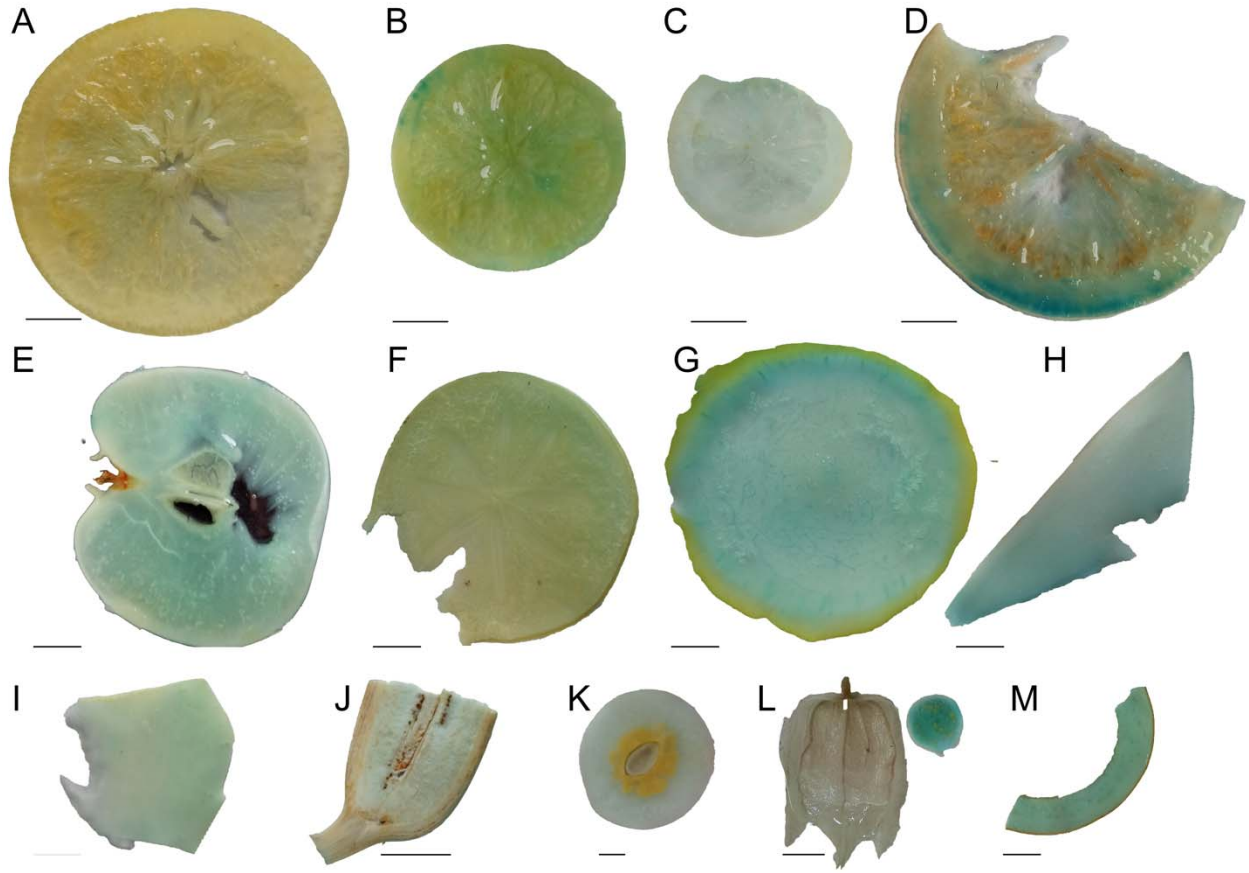

Figure 3: **The newly developed protocol revealed Fe accumulation in various fruits.** Fruits were either cut cross-sectional or longitudinal with a V- blade (For the V-blade, refer to supplementary figure 1). These pieces were then freeze-dried and decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). After fixation, pieces were submerged in Perls' staining solution between 2-16 hours. From A to M; orange, mandarin, lemon, grapefruit, apple, persimmon, honey melon, mango, pumpkin, plantain, nectarin, cape gooseberry, avakado. Note that despite the differences in fruit types, the optimized protocol preserved the structure and decolorized the tissues efficiently. For the complete list of fruits successfully stained, refer to supplementary table 1. Blue coloration shows Fe. Bar 1cm unless otherwise indicated.

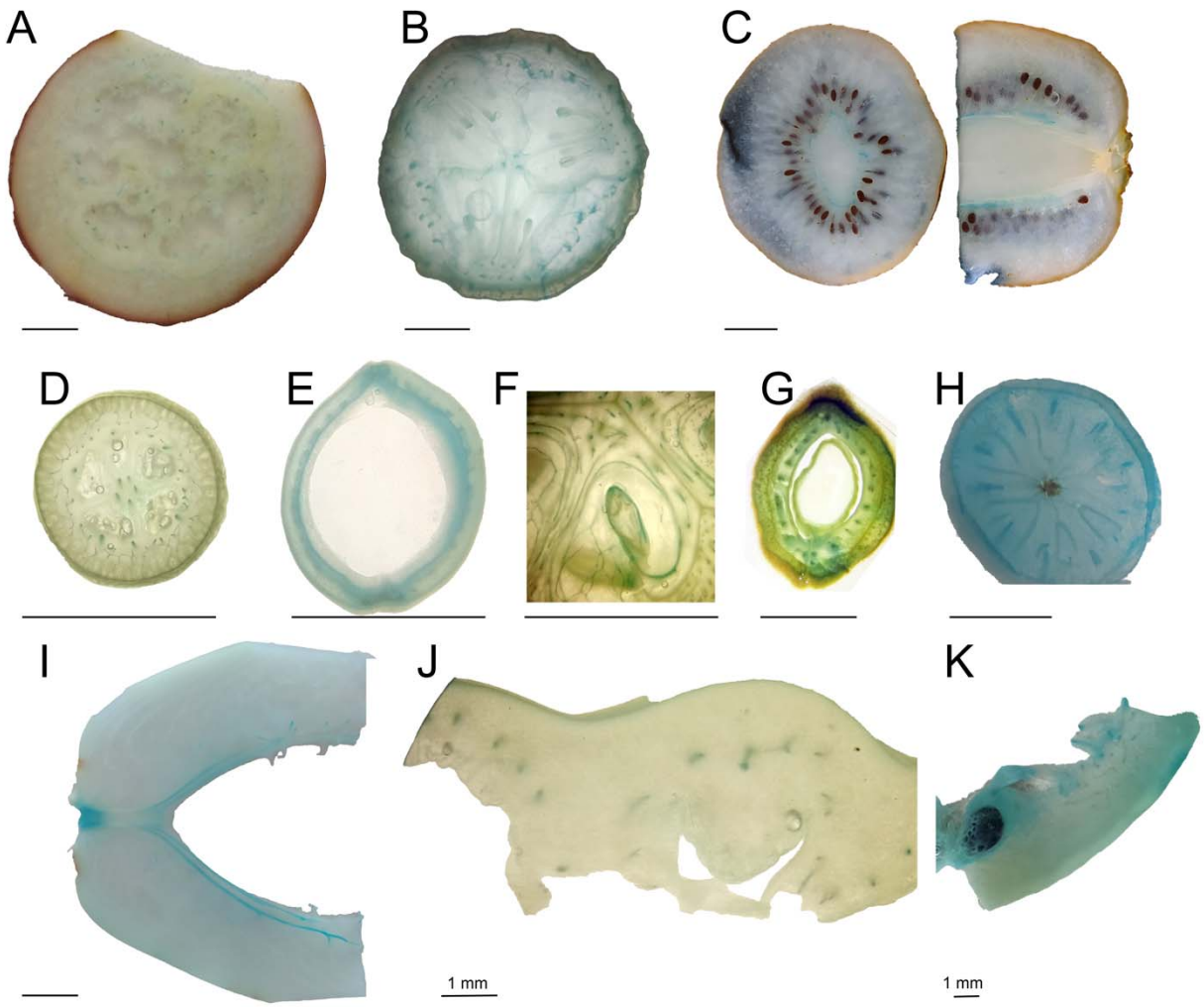

Figure 4: Fe accumulation was detected in the main vasculatures of fruits. Fruits were either cut cross-sectional or longitudinal with a V- blade(For the V-blade, refer to supplementary figure 1). These pieces were then freeze-dried and decolorized using fixatives (Methanol: Chloroform: Glacial acetic acid; 6:3:1). After fixation, pieces were submerged in Perls' staining solution between 2-16 hours. For this figure, fruit pictures with apparent Fe accumulation in the vascular bundles were chosen. From A to K; eggplant, cucumber, kiwi, kumquat, haricot, squash, green almond, blueberry, papaya, pepper, passion fruit. Bar 1cm, unless otherwise indicated.

Figure 5: Fe accumulation in fruits could be detected at the subcellular level. A, Fe accumulated inside the epidermal cells of blueberry, most probably bound to polyphenols in the vacuole. Left, control section without staining; middle, Perls/DAB stained section; right, close up of red rectangle of the middle. B, Fe accumulates in cell walls. Left, control section without staining; right, Perls/DAB stained section. C, Fe heavily accumulated in the back of the squash seed embryo, most probably corresponding to the vasculature as Cucurbitacea seeds, including squash, have a coat usually surrounded by a single vein [53]. Left, squash cross section stained with Perls; middle thin section corresponding to the back part of the seed (such as the pink rectangle shown in the left picture); middle, control section without staining; right, Perls/DAB stained section. Blue coloration in C (left) and black coloration in the rest shows Fe accumulation. Pink arrows point to Fe accumulation. VB: Vascular bundle, V: Vasculature, S: Seed

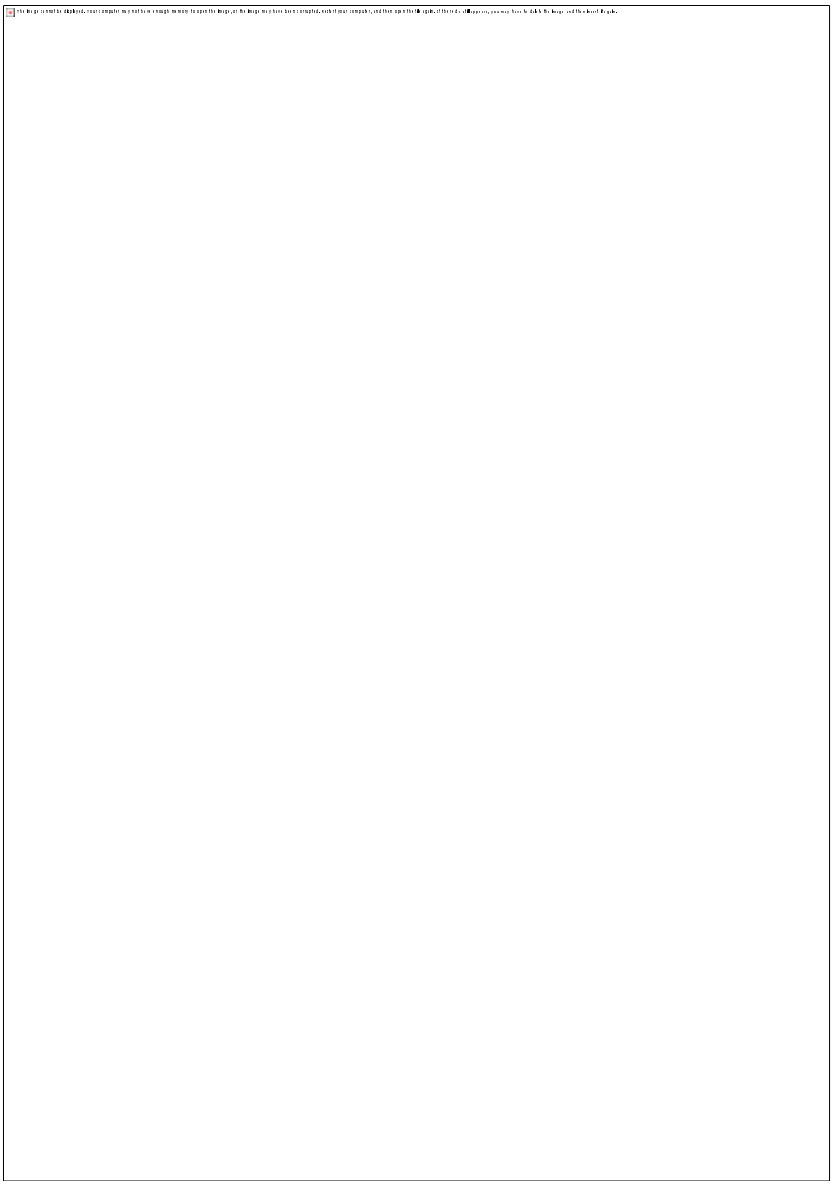

Figure 6: Determining metal localizations on fruit slices by micro XRF mapping. Cross and longitudinal section of Kumquat. The sample was cut, freeze-dried, and analyzed by XRF.

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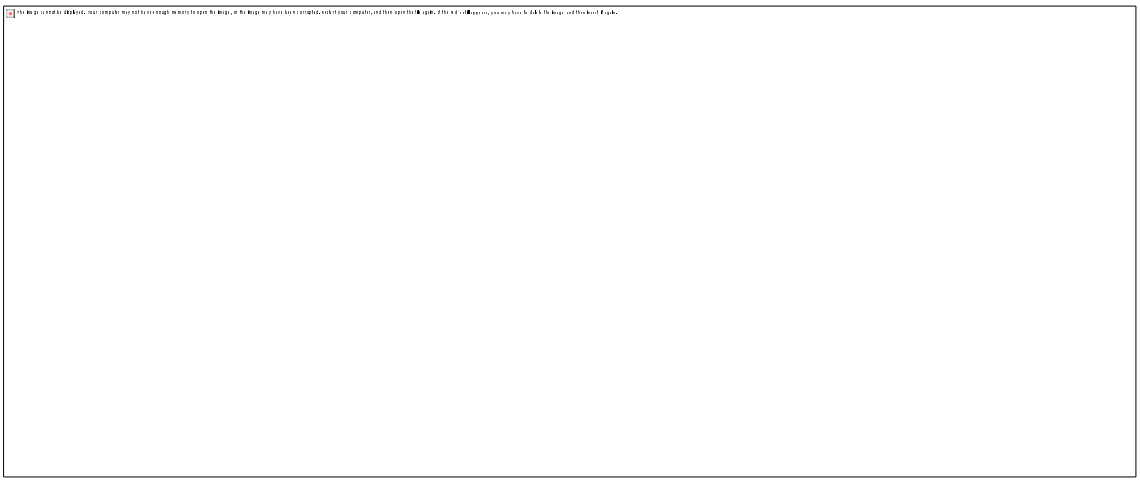
Figure 7: Micro XRF mapping confirmed the histochemical methods on tissue-level Fe hotspots. A, aubergine; B, squash; C, blueberry. Pink arrows point to Fe accumulations. VB: vascular bundle



Graphical abstract, a low-cost, high-throughput protocol to map metal localizations in everyday fruits.