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4 **Development of an accurate and direct method for the green food colorants detection**

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Abstract

Color impression represents between 60 to 90% of the final acceptance/rejection choice made by consumers. Consequently, color additives are attribute standards for our daily life in any market and any culture. Currently, authorized natural green food colorants comprise several copper-chelated chlorophyll derivatives. Both the raw materials and the manufacturing processes for the acquisition of these green food colorants are numerous and diverse. Hence, each producer applies its own know-how to obtain ‘signature’ green colorant products. Indeed, the chlorophyll profile of these products is partially known and may substantially differ among batches, while their identification just by HPLC-UV-Vis is not complete. Native chlorophylls do not chelate copper. Therefore, we propose a fast and specific method for copper chlorophyll detection, as indicative (except in a few fermented foods) of probable green food colorant addition or “re-greening” with copper salts. The new method is based on the characteristic isotopic pattern of the copper chlorophyll derivatives and does not require the precise characterization of the corresponding chlorophyll structure. This accurate methodology, based on a specific HPLC-ESI/APCI-HRMS method assisted with powerful post-processing software, is versatile as it can be used for other metallo-chlorophyll complexes also applied to improve the green coloration of food products.

Keywords: copper-chlorophyll, copper-chlorophyllin, food colorants, green colorants, HPLC-MS.

1. Introduction

Chlorophylls, the pigments responsible for green coloration in nature, are cyclic tetrapyrroles carrying a characteristic isocyclic five-membered ring with a function during photosynthesis (Scheer, 2006). Normally, they carry magnesium as the central metal and a $C_{20}H_{40}$ (phytol) alcohol esterifying the propionic acid at C17 (Figure 1A). However, as an outcome of the chlorophyll metabolism (Schelbert et al., 2009), mainly during food processing (Criado, Romero, & Motilva, 2007) and storage, the central magnesium is easily replaced by hydrogens, creating chlorophyll compounds (pheophytins and pheophorbides, Figure 1B) with a brownish coloration. These hues are not attractive to consumers. Consequently, the food industry adds authorized green food colorants to their edible products to make them more appealing, attractive and seemingly healthy. At present, authorized green natural food colorants in most legislations (Viera, Pérez-Gálvez, & Roca, 2019) are comprised of a diverse array of copper-chlorophyll compounds (Figure 1C-D). The chelation of the tetrapyrrole by a divalent cation (copper or zinc) stabilizes the chlorophyll structure, ensuring a permanent and appealing green coloration during the following processes applied in food manufacturing, and during storage. Currently, two green food colorants are mainly utilized by the food industry, the E-141i (or copper chlorophylls/pheophytins) for foods with high fat content, and the E-141ii/21 CFR §73.125 (copper chlorophyllins) applied to foods with a hydrophilic matrix. The use of green food colorants is restricted by the pertinent legislation of each country. For example, in the U.S. only copper chlorophyllins are authorized, to reach a 2% maximum content in citrus-based beverages (FDA regulations), while in the E.U. (Regulation (EC) 1333/2008), the use of both green natural food colorants is banned for certain food products. In addition, some food manufacturing agents apply the so-called “re-greening” process as an alternative method for improving the green coloration. This practice, used with specific vegetable products, consists of the addition of copper salts to form

the stable chelated version of the chlorophylls. Copper salts are not included in the list of EU-approved food additives (Regulation (EC) 1333/2008), though they have been detected (Aparicio-Ruiz, Riedl, & Schwartz, 2011; Negro et al., 2017) in several commercial products. In consequence, different food colorant legislations, unintentional wrong practices or even conscious adulterations, deliver food products containing unauthorized colorants to the market. Therefore, international food safety agencies and policymakers (EFSA, USDA, FDA) are committed to improving the legal framework to guarantee not only the quality, security, and safety of food additives but also consumer confidence. With this aim, different official calls have been launched for the development of analytical methods to characterize green food colorants (EFSA, 2015a,b,c). The analysis of the chlorophyll composition in these copper colorants started with the development of several chromatographic methods, which were able to separate the different components (Inoue et al., 1988; Scotter, Castle, & Roberts, 2005; Mathiyalagan, Mandal, & Ling, 2019), although Raman spectroscopy has also been proposed (Lian et al. 2015). Hence, the E-141ii/21 CFR §73.125(Figure 1D) contains two main copper chlorins (Cu-chlorin e_6 and Cu-chlorin e_7) and Cu-rhodin g_7 , which were identified by their chromatographic properties and by comparison with authentic standards (Negro et al. 2017; Scotter, Castle, & Roberts, 2005; Delpino-Rius et al., 2018). A further improvement led to the only (to date) published HPLC-MS analysis (Mortensen, & Geppel, 2007) of this colorant, where several new chlorophyll structures were identified, including Cu-purpurin 7, Cu-chlorin p_6 , Cu-isochlorin e_4 , and Cu-rhodochlorin. Additional assignments were tentatively noted but as unknowns. Similarly, initial chromatographic methodologies enabled the characterization of several commercial E-141i yields (Roca, Gallardo-Guerrero, Mínguez-Mosquera, & Gandul-Rojas, 2010), and with the subsequent application of hyphenated techniques (HPLC-MS) a deeper comprehensive knowledge of their composition and spectrometric behavior was achieved (Pérez-Gálvez, Ríos, & Roca, 2015). Nevertheless, both the lack of authentic

standards. and the fact that most of the substituents and ring arrangements in the chlorophyll tetrapyrrole do not modify the UV-Vis spectrum (Figure 1), limit the correct identification of chlorophyll derivatives exclusively by their chromatographic properties, with the result that misidentification of the chlorophyll compounds is likely. In addition, as a result of the diversity of raw materials and the variety of manufacturing processes, each food colorant brand trades green food colorants with differences in quality and quantity. This significantly complicates the correct identification of chlorophyll compounds in the corresponding green food colorants.

Consequently, we have set up a new method that detects the presence of copper-chlorophylls in a food matrix, arising either from green food colorants or from “re-greening”, without the need for proper identification of the chlorophyll structure. This methodology will simplify the work of control laboratories and agencies.

2. Materials and methods

2.1 Samples. Chlorophyll *a* was purchased from Wako (Neuss, Germany), and pheophytin *a* was obtained by the acidification of a solution of chlorophyll *a* in diethyl ether, with two or three drops of 5 M HCl (Sievers, & Hynninen, 1977). Pyrochlorophyll *a* and pyropheophytin *a* were produced from parent chlorophyll *a* and pheophytin *a*, respectively, in pyridine solution blanketed with N₂. For pyrochlorophyll *a*, the solution was heated at 80°C for 4h, while for pyropheophytin *a* it was necessary to increase the temperature to 100°C for 4h (Chen, Ríos, Pérez-Gálvez, & Roca, 2015a). For copper-pyropheophytin *a* synthesis, pyropheophytin *a* standard was dissolved in acetone with an excess of copper (II) ions as chloride and ascorbic acid as antioxidant (Pérez-Gálvez et al., 2015). Five samples of the lipophilic green food colorants (E-141i) and another five samples of hydrophilic green food colorants (E-141ii) were provided by diverse commercial suppliers of natural food colorants. Aliquots of the commercial samples were dissolved in acetone and filtered through 0.22 µm

nylon immediately prior to analysis. Commercial samples of olive oils and canned green vegetables were purchased at a local supermarket. Following a negative analysis for the presence of copper chlorophyll, they were considered to be the control samples. 0.001% w/v of E-141i was added to the control olive oil while 0.001% w/v of E-141ii food colorant was added to the liquid of the control green vegetables.

2.2 Chemicals and Standards. HPLC/MS grade solvents (hexane, methanol, and water) were supplied by Panreac (Barcelona, Spain). Reagent grade solvents (acetone, diethyl ether, N,N-dimethylformamide) and chemicals (ammonium acetate, sodium chloride, anhydrous sodium sulfate) were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.3 Chlorophyll extraction. Based on Minguez-Mosquera & Garrido-Fernández (1989), 15 g of olive oil (control and spiked) and 7 g of canned green vegetables (control and spiked) were homogenized with N,N-dimethylformamide (DMF). DMF phases were washed with hexane and then transferred to 10% NaCl solution with hexane/diethyl ether (1/1 v/v). The upper organic phase was washed with Na₂SO₄ (2%) and later evaporated to dryness under vacuum. The dry residue was dissolved in 1.5 mL of acetone and filtered through 0.22 µm nylon immediately prior to analysis.

2.4 Liquid chromatography/APCI-ESI/Time-of-Flight Mass Spectrometry. The liquid chromatograph in the HPLC-ESI/APCI-HRMS system was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed by injection of 30 µL of the sample in a stainless-steel column (20 × 0.46 cm i.d.), packed with 3 µm-size C18 Spherisorb ODS-2 (Teknokroma, Barcelona, Spain). The gradient was described previously (Pérez-Gálvez et al., 2015) using 1.0 mL/min as a flow rate. Briefly, the mobile phases were (A) water/1 M ammonium acetate/methanol (1/1/8, v/v/v) and (B) methanol/acetone (1/1, v/v). The gradient program starts with 75% A, then changes to 25% A in 8 min, isocratic 2 min, changes to 10% A in 8 min, then to 100% B in 5 min, isocratic 15

min, and returns to initial conditions in 5 min. Sequential detection was performed with a photodiode array detector at 430 nm, although the complete spectrum from 350 nm to 800 nm was recorded. The LOD for Cu-pyropheophytin *a* was determined following the ISO 11843, being for the present conditions of 44 ppb (Wilrich, 1995). A split post-column of 0.25 mL/min was introduced directly on the mass spectrometer APCI source or ESI interface. Mass spectrometry was performed using a micrOTOF-QII High Resolution Time-of-Flight mass spectrometer (UHR-TOF) with Qq-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an APCI source (for analysis of phytylated chlorophyll derivatives, E141-i colorants) or ESI (for analysis of dephytylated chlorophyll derivatives, E141-ii/21 CFR §73.125 colorants) interface (Chen et al., 2015a; Chen, Ríos, Roca, & Pérez-Gálvez, 2015b). The scan range applied was m/z 50-1500 Da and mass resolving power was always over 18000 ($m/\Delta m$). The instrument was operated in positive ion mode. Mass spectra and data were acquired through bbCID mode, automatically providing MS and then MS/MS spectra, which were used for structural confirmation of the compounds detected. The collision energy was estimated dynamically based on appropriate values for the mass and stepped across a +/- 10% magnitude range to ensure good quality fragmentation spectra. The instrument control was performed using Compass 1.3 for micrOTOF-Q II+Focus Option Version 3.0.

2.5 Data analysis. The first workflow for data analysis follows the data evaluation described in our previous publications (Chen et al., 2015a, b). Briefly, the *in-house* mass database created *ex professo* comprises monoisotopic masses, elemental composition and, optionally, retention time and characteristic product ions if known, for chlorophyll derivatives from *a* and *b* series, including pheophytins, pheophorbides, chlorophyllides, their allomerized and pyro-derivatives compounds, and all the chlorins, rhodins, purpurins and glyoxylic acid derivatives described in the literature. Additionally, this database includes the corresponding metallo-chlorophyll complexes with copper. Data evaluation was performed with Bruker Daltonics

DataAnalysis 4.1. Theoretical isotopic distributions were calculated with the Compass isotope pattern calculator (Bruker, Bremen, Germany). The filtering rules applied in the new workflow for data analysis are assisted by Bruker Daltonics Data Analysis 4.1 and Bruker Daltonics Target AnalysisTM.

3. Results and Discussion

3.1 Theoretical principle

As stated above, the natural chlorophylls present in foods are magnesium chlorophylls, and hydrogen-chlorophylls (pheophytins and pheophorbides). Except for an alteration in table olives (Aparicio-Ruiz et al., 2011) and other fermented foods, the presence of copper chlorophylls probably means the addition of green food colorants or the application of copper salts. Consequently, it is only necessary to develop a method to detect the presence of copper chlorophyll derivatives, regardless of whether these are known or unknown and of their structural features. In this sense, the application of the isotopic pattern could provide a fast and reliable procedure to detect their presence. In fact, this approach has been applied as an authenticity marker of virgin olive oil (Faber et al., 2014). The point is that metal elements naturally present more natural isotopes than organic elements, producing a characteristic wide distribution of the isotopic peaks of the organometallic compound. For example, copper presents two stable isotopes, ⁶³Cu, and ⁶⁵Cu, with a relative intensity abundance of 100 and 44.61, respectively. To the contrary, magnesium shows three stable isotopes, ²⁴Mg, ²⁵Mg and ²⁶Mg with abundances of 100, 12.66 and 13.93, respectively. As a consequence of the isotope profile of copper, the isotopic distribution of copper chlorophyll derivatives (Figure 2) shows an isotopic pattern that resembles the significant abundance of $\delta\text{Cu}^{64.9272}$ (44.6%) to the isotope pattern of copper. As an example, Figure 2A displays the isotopic pattern of copper pyropheophytin *a* (C₅₃H₇₀CuN₄O₃), the main chlorophyll derivative in lipophilic green food colorants (E-141i) (Roca et al., 2010; Fang et al., 2015; Arrizabalaga-Larrañaga, Rodríguez,

Medina, Santos, & Moyano, 2019), which was calculated with the Compass Isotope Pattern software (section 2.3). For this copper chlorophyll compound, the abundance of the third isotope is higher (0.62747) than the second one (0.59716), as can be confirmed in Figure 3A with the authentic standard of copper pyropheophytin *a*. On the contrary, the substitution of copper by hydrogen (C₅₃H₇₂N₄O₃, Figure 2B), or pyropheophytin *a*, causes the third isotope (0.59739) to always be lower than the second one (0.18147). Experimentally, Figure 3B verified the isotopic pattern of the pyropheophytin *a* standard. The same behavior can be observed for the corresponding magnesium derivative (Figure 2C, pyrochlorophyll *a* and tested with the authentic standard in Figure 3D), where the relative abundance of the isotopes decreases, being for the second and third isotope 0.72375 and 0.39632, respectively.

The significant different isotope cluster analysis between copper and non-copper chlorophylls was the founding principle of our methodology, constituting the first filtering rule for the screening. But additional filtering rules can be applied to the samples for an even more refined and rigorous result. Figure 4 outlines the complete workflow that allowed a high-throughput screening in complex food matrices. After HPLC injection, the mass spectrum was acquired in a full scan mode. ESI interface is recommended for the polar food matrix, while APCI source provides better results for non- polar foods (Chen et al., 2015a,b). The massive positive hits list obtained should first be screened, as stated previously, according to the restriction imposed by the characteristic isotopic pattern of copper compounds, which would considerably reduce the list of candidates. However, this result can contain false positives, that is, compounds with copper in their structure which are not chlorophyll derivatives. To avoid this confounding possibility, additional constraints should be applied. Thus, the elemental composition of chlorophylls contains four atoms of nitrogen, such that this chemical feature should be fulfilled with the maximum limit for the mass error (below 5 ppm), and the second list of candidates obtained. Finally, the third filtering rule is

based on the typical UV-Vis spectrum featured by chlorophyll pigments. Chlorophylls compounds should depict two maxima wavelengths, one in the blue spectral region (Soret band) *ca.* 430 nm, and a second in the red zone (Scheer, 2006), *ca.* 660 nm. The application of these three filtering rules ensured the recognition of all the copper chlorophyll compounds present in a food matrix without the need for a complete structural identification.

This pipeline methodology could also be applied for the detection of zinc chlorophylls in a food matrix. The aforementioned “re-greening” procedure is sometimes developed with zinc salts (Ngo, & Zhao, 2007), yielding zinc complexes of chlorophylls. In fact, the isotopic pattern of zinc is even more peculiar, with five stable isotopes ^{64}Zn , ^{66}Zn , ^{67}Zn , ^{68}Zn and ^{70}Zn with relative abundances 100, 57.958, 8.498, 39.413 and 1.307, respectively. Consequently, its isotopic behavior generates zinc chlorophylls with a distinct isotopic pattern. As an example, the isotope profile of zinc pyropheophytin *a* ($\text{C}_{53}\text{H}_{70}\text{ZnN}_4\text{O}_3$) is shown in Figure 2D. Therefore, the substitution of the restriction of the copper isotopic pattern by the zinc pattern in the workflow implies that the proposed methodology could also be applied for detecting the presence of zinc chlorophylls. In this sense, the method is not restricted to the detection of copper chlorophylls. On the contrary, it is a versatile procedure.

3.2 Practical application of the methodology in complex food matrices

The common protocol for identifying chlorophylls by HPLC-ESI/APCI-HRMS started with the acquisition of the extracted ion chromatograms (EICs) in full scan mode (bbCID). As an example, Figure 4 shows the EICs acquired in two samples of E-141i (Figure 5A) and E-141ii/21 CFR §73.125 (Figure 5B), although the method was applied to five samples of each food colorant. With the *in-house* library created *ex professo* for the detection of chlorophyll derivatives, if available, it was possible to annotate those EICs through the automated search of the chlorophyll derivatives. Alternatively, the analysis could be performed manually, increasing the time, effort and complexity of the data analysis. Whatever the method applied,

the list of potential positive hits included 139 and 40 annotated EICs for the E-141i and E-141ii/21 CFR §73.125 colorants, respectively. This should then be filtered according to the threshold values for mass accuracy and isotopic pattern (mass error below 5 ppm and mSigma value below 50), to obtain the list of filtered hits. Although this filtered list would already indicate the presence or absence of copper chlorophyll derivatives, its subsequent curation with the tandem MS characteristics of the filtered compounds was necessary to yield a match-list of identified chlorophyll derivatives in the samples. This second filtering process was performed by applying the same filters, mass accuracy, and isotopic pattern, to the product ions which were derived from the protonated molecular ions included in the list of filtered compounds. Nevertheless, this workflow is operative only for the known compounds, which were included in the *in-house* library, while the unknown chlorophyll derivatives, both non-metallo and metallo-derivatives, would not be included in the match list.

4. Instead of applying this laborious and not always easy workflow, we could take advantage of the characteristic isotopic pattern of the copper chlorophyll derivatives and use it as a filtering rule to the annotated EICs, following the methodology described in Figure 4. Then, the next step is to select those protonated molecular ions for which isotopic distribution fulfills a specified m/z difference and intensity ratio between isotopic species. Hence, the m/z difference between the first and the third isotopic peak of the copper-chlorophyll derivatives is 2.00057 Da and the intensity ratio of those peaks is 0.62747 (Figure 2). These theoretical values were screened in the experimental bbCID spectrum with the application of threshold values to the m/z difference (± 1 ppm) and to the intensity ratio ($\pm 5\%$), and the corresponding isotopic chromatogram was acquired. The new chromatograms obtained (Figure 5C-D) show the position in the analysis where copper chlorophyll derivatives were located, independently of whether they are known or unknown. Thus, some copper chlorophyll derivatives that were not annotated with the

application of the first workflow (not-known chlorophyll compounds), would emerge on the scene. Specifically, the application of this first filtering rule over the two green food colorants reduced the hits in the non-polar colorant (E-141i) sample from 139 to 14 hits (Figure 5C). The same screening in the polar green food colorant (E-141ii/21 CFR §73.125) decreased the possible positives from 40 to 11 (Figure 5D). Next, the second and third filtering rules were applied to the samples to guarantee the identification of copper chlorophylls and excluding other copper compounds. After both screenings, only copper chlorophylls were detected, specifically obtaining 11 copper chlorophyll compounds in the green food colorant E-141i (Table 1) and 9 copper chlorophyll compounds in the polar green food colorant E-141ii/21 CFR §73.125 (Table 1). Among these, only a few copper chlorophylls could be identified following an intricate and expert-curated characterization based on retention time, UV-Vis spectrum, elemental composition, exact mass, isotopic pattern and MSⁿ profile. However, in spite of this complex analysis, sometimes pigments are only tentatively described due to the lack of authentic standards or insufficient previous references. In addition, for half of the peaks, it was challenging to identify the chlorophyll structure. Nevertheless, the only assumption we can assert is that all the compounds listed in Table 1 are copper chlorophyll compounds. In this sense, with the proposed protocol, it is possible to confirm the presence of copper chlorophylls in the samples analyzed with the exactitude of a high-resolution technique but without the need for a precise and accurate chlorophyll identification. In this regard, previous methodologies base the detection of green food colorants on the identification of copper chlorophylls compounds. Whatever technique is developed (HPLC-UV-VIS, hyphenated MS, high resolution MS), it means that precise and accurate identification of each chlorophyll compound is required to guarantee the determination. Such a degree of certainty frequently implies laborious calibrations and quantifications, exact *m/z* determinations, MSⁿ analysis, etc. However, the

proposed methodology does not require any of the previous studies, as it takes advantage of the peculiar isotopic pattern of copper to accurately detect the presence of copper chlorophyll compounds. An additional advantage of the new methodology described is the feasibility of application in complex matrices. As it can be seen in Figure 4, the detailed workflow could be applied independently of the chlorophyll extraction methodology used. At present, specific chlorophyll extraction methods have been validated for complicated food matrices, i.e., high lipid content, complex extracellular polymers, etc. However, regardless of the solvent mixture, disruption method, or technique employed for chlorophyll extraction, the pipeline methodology presented can be applied satisfactorily. To exemplify the suitability of the proposed procedure we have checked the presence or absence of copper chlorophyll derivatives in complex food sample matrices. Figure 6 showed the application of the workflow to olive oil (Figure 6A-D) and processed vegetable (Figure 6 E-H) samples: both control (Figure 6 A, C, E, and G) and green food colorant spiked (Figure 6 B, D, F, and H). After chlorophyll extraction, following the protocol described in materials and methods, samples were analyzed by HPLC-HRMS, olive oil samples through APCI, and the vegetable samples using ESI, as advised previously. The acquisition of the EICs in full scan mode annotated between 60 and 150 potential positive hits (Figure 6 A-B, E-F) depending on the food sample. But, the application of the isotopic pattern filtering rule substantially reduces these lists. In control samples, the demand for an m/z difference of 2.00057 (indicative copper-chlorophyll derivatives presence) showed no positive results (Figure 6 C and G). On the contrary, samples with E-141 added showed the presence of three copper compounds in olive oil samples (Figure 6 D) and only one positive hit in the processed vegetable sample (Figure 6 H). The application of the second and third filtering rules confirmed their nature as copper chlorophylls. To confirm the obtained results, the list of positive results were analyzed to verify their identity with the *in*

house library following parameters including mass accuracy and isotopic pattern (mass error below 5 ppm and mSigma value below 50), retention time, elemental composition and UV-Vis spectrum. In the E141-spiked olive oil samples, three copper chlorophyll compounds were identified (Figure 6 D): copper pyropheophytin *a* and *b* and copper pheophytin *a* (peaks 9, 10 and 11, Table 1) as expected were the main components of the E-141i food colorant (Figure 5C, Roca *et al.* 2010; Pérez-Gálvez *et al.* 2015). In the E141-ii spiked vegetable sample, copper chlorin *e₄* was identified (Figure H, peak 8, Table 1). This compound has been described as the main component of E-141ii (Mortensen, & Geppel, 2007) in addition to being identified in samples with added copper chlorophylls (Delpino-Rius *et al.* 2018; Harp, Scholl, Gray, & Delmonte, 2020).

Conclusion

We propose a simple, fast and reliable method for detecting the presence of green food colorants as well as the application of the “re-greening” practice, that is, the addition of copper salts to food products with the aim of ‘artificially’ preserving/increasing their green color. The basis of the methodology relies on the application of the characteristic isotopic pattern of the copper chlorophyll derivatives as a filtering rule to detect the presence or absence of these coloring products in foods. The most practical advantage is the accurate determination of copper chlorophylls in complex matrices without the indispensable characterization of the chlorophyll structure. Finally, the versatility of the method is suitable for use with the feasibility of the other metallo-chlorophyll detection.

Author contributions

Antonio Pérez-Gálvez: data curation, investigation, software, and original writing; Isabel Viera: formal analysis and methodology and María Roca: conceptualization, investigation, funding acquisition, project administrator, supervision and review/editing writing.

Declaration of interest

Declarations of interest: none.

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442 **Figure Legends**

443 **Figure 1:** Outline of chlorophyll structures. A, structure of chlorophyll; B, main structure of
444 H-chlorophyll (pheophytin/pheophorbide) derivatives; C, main structure of Cu-complexes of
445 chlorophylls; D, main structure of commercial Cu-chlorophyllins. R, R₁, R₂ and R₃ indicate

the locations of different functional groups. R could be CH₃ or CHO, R₁ could be H or phytol, R₂ could be H, HCOOCH₃, OHCOOCH₃, COCOOH, COH, CH₂COOH, CH₂COOCH₃, CH₂CH₃, CH₃, or COOH, and R₃ could be H or COOH. Structures B and C could be present in colorant E-141i while structure D is limited to colorant E-141ii.

Figure 2: Theoretical isotopic pattern and natural abundance of: A, Cu-pyropheophytin *a*; B, pyropheophytin *a*; C, pyrochlorophyll *a* (“Mg-pyropheophytin *a*”); D, Zn-pyropheophytin *a*.

Figure 3: *hr*-MS spectra of authentic chlorophyll standards. A, Cu-pyropheophytin *a*; B, pyropheophytin *a*; C, pyrochlorophyll *a* (“Mg-pyropheophytin *a*”)

Figure 4: Proposed workflow to identify copper-complexes of chlorophylls, based on the application of the isotopic filtering rule.

Figure 5: Practical application of the proposed methodology on lipophilic (A and C) and hydrophilic (B and D) green food colorants. Full scan MS spectra (A and B); extracted ion chromatogram resulting from the application of the isotopic filtering rule (first screening, C and D).

Figure 6: Practical application of the proposed methodology on food samples: olive oil (A to D) and processed green vegetable (E to H) samples. Control samples (A, C, E and G) were spiked with the E-141i (B and D) and E-141ii (F and H) green food colorants. Full scan MS spectra (A, B, E and F); extracted ion chromatogram resulting from the application of the isotopic filtering rule (first screening, C, D, G and H).

465 **Table 1:** List of positive hits of copper-complexes of chlorophylls from colorant E141-i and E141-ii/21 CFR §73.125, starting from isotopic
 466 cluster analysis (Figure 5 C and D) after the second and third screening (N₄, mass error and UV-Vis spectrum).

Compound	Peak number	R _t (min)	Elemental composition	mSigma	error	<i>m/z</i> experimental [M+H] ⁺
Food colorant E141-i						
unknown ^a	1 ^b	9.8				612.1794
Cu-pyropheophorbide <i>a</i>	2 ^b	10.2	C ₃₃ H ₃₂ CuN ₄ O ₃	20.2	-1.6	596.1842
unknown ^a	3 ^b	11.8				570.2053
Cu-pheophytin <i>b</i>	4 ^b	21.5	C ₅₅ H ₇₀ CuN ₄ O ₆	22.3	1.1	946.4665
Cu-13 ² OH-pheophytin <i>a</i>	5 ^b	25.1	C ₅₅ H ₇₂ CuN ₄ O ₆	25.6	3.2	948.4823
Cu-15 ¹ -OH-lactone-pheophytin <i>a</i>	6 ^b	26.4	C ₅₅ H ₇₂ CuN ₄ O ₇	19.4	3.1	964.4773
unknown ^a	7 ^b	27.5				864.4611
unknown ^a	8 ^b	27.8				972.4819
Cu-pyropheophytin <i>b</i>	9 ^b	28.0	C ₅₃ H ₆₈ CuN ₄ O ₄	31.2	-1.1	888.4608
Cu-pheophytin <i>a</i>	10 ^b	29.5	C ₅₅ H ₇₂ CuN ₄ O ₅	18.7	-1.1	932.4871
Cu-pyropheophytin <i>a</i>	11 ^b	33.0	C ₅₃ H ₇₀ CuN ₄ O ₃	15.9	1.2	874.4818
Food colorant E141-ii						
unknown ^a	1 ^c	2.5				570.2053
unknown ^a	2 ^c	3.0				568.1895
Cu-rhodin <i>g</i> ₇	3 ^c	3.3	C ₃₄ H ₃₂ CuN ₄ O ₇	26.8	3.0	672.1642
unknown ^a	4 ^c	4.7				612.1793
Cu-chlorin <i>e</i> ₆	5 ^c	4.9	C ₃₄ H ₃₄ CuN ₄ O ₆	20.3	3.0	658.1849
Cu-chlorin <i>p</i> ₆	6 ^c	5.9	C ₃₃ H ₃₂ CuN ₄ O ₆	31.1	1.4	644.1700
unknown ^a	7 ^c	7.0				594.1688
Cu-chlorin <i>e</i> ₄	8 ^c	7.8	C ₃₃ H ₃₄ CuN ₄ O ₄	13.5	1.9	614.1947
Cu-pyropheophorbide <i>a</i>	9 ^c	9.0	C ₃₃ H ₃₂ CuN ₄ O ₃	17.2	1.7	596.1844

467 ^aThis compound fulfills the isotopic filtering analysis and presents a featured chlorophyll-type UV-vis, but the structural arrangement is still elusive. ^bFigure 5 panel C.

468 ^cFigure 5 panel D.

A

B

C

D

472

473

474

475

First screening

Isotope cluster analysis

D

476

477