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Study of the authentic composition of the novel green foods: Food colorants and coloring foods

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ABSTRACT

The clean label approach is behind the development of the new concept, coloring food, in contrast to regulated food colorants, although few data are available regarding its composition. Consequently, twenty-six commercial green foods (including novel foods) have been analyzed to investigate the authentic composition behind the different labels. It has been identified by HPLC-ESI/APCI-hrTOF-MS^2 the complete array of chlorophylls in the regulated green food colorants, several of them identified for the first time in foods. The coloring food alternative is based on mixing blue (such as spirulina) and yellow (such as safflower) hues. Our data suggest that in the analyzed samples, spirulina is water or solvent extracted before being added to the food. The obtained results showed for the first time, the authentic data on the chemical composition of the new green foods.

1. Introduction

Color is a key constituent of food and beverages, and it plays an important role in the selection of foods by consumers since a proper color may be a synonym for healthy food and improved value. As consumers are currently demanding more natural and healthy foods, the food color industry is finding new ways to naturally retain color in foods and make them attractive and healthy for consumers at the same time. Therefore, besides the regulated food colorants, new strategies are currently being developed with different regulations across countries. For example, plant extracts, fruit and vegetable juices and microalgae are added on foods to improve the color, which are known as "coloring foods" (Schweiggert, 2018). For example, in the United States (US), food colorants (CFR-21, 73–74, 2016) includes those subjected to batch certification (or synthetic pigments), and those exempt from batch certification (natural pigments besides plant extracts and fruit and vegetable juices). In contrast, in Europe, food colors are subject to food additive legislation (EC Regulation 1333/2008), and fruit and vegetable juices (coloring foods or coloring foodstuff) are not considered additives, but food ingredients. Therefore, they are not regulated by the stricter food additive legislation, although they must comply with the EU's general food legislation (EC Regulation 178/2002). The status of food color or coloring food in the EU is confusing, and may lead to an inappropriate use of these ingredients, as there is not a clear regulation to follow (Oplatowska & Elliott, 2017). In fact, the criterion that determines whether an extract is considered a regulated food color or a non-regulated coloring food is an enrichment factor, which decides if there have been or not selective extraction (EC Guidance Notes, 2013). The development of new formulations based on natural extracts are part of the so-called "clean label" strategy, which includes the replacement of food colors with natural ingredients that provide color (coloring foodstuff). In this regard, green is one of the most promising colors, as it is widely present in vegetables and fruits. However, it shows some industrial difficulties, such as stabilization and therefore new alternatives have been developed such as the use of mixtures of blue and yellow extracts to achieve a green color. One of the most-used at present is a combination of spirulina and safflower or spirulina with yellow food colorants such as curcumin (or turmeric) or riboflavin.

But, besides the use of the new coloring foods, the food industry applies the food colorants authorized in each country. For example, in the EU (among other legislations), two natural green food colorants are allowed (EC Regulation 1333/2008): E140 and E141, which are structurally related to chlorophylls. E140 is obtained by direct chlorophyll extraction with organic solvents from natural sources and are marketed in two forms: E140i ("chlorophylls" for lipid foods) and E140ii ("chlorophyllins" for hydrophilic foods), which is obtained after saponifying the former. E140 colorant is rather unstable, and one of the methods to stabilize the color is to form metal-chlorophyll complexes.

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E141 food colorant is obtained by adding copper salts to E140 and therefore may be marketed as E141i ("copper-chorophylls") and as E141ii ("copper chlorophyllins") (Viera et al., 2019). In contrast to the EU, in the US only E141ii is authorized, albeit use is highly restricted (FDA Dietary supplements, 2021). These food colorants have been deeply analyzed by HPLC-MS (Mortensen & Geppel, 2007), and even some bioaccessibility studies have been assessed *in vitro* and *in vivo* (Egner et al., 2000).

The last option to proportionate green color, although less popular, is the application of synthetic food colorants. For example, Green S (E142) is authorized in the EU, but it is banned in the US, Japan and India. The alternative synthetic green food colorant allowed in these countries is Fast green FCF (Tanaka et al., 1995). Also, some synthetic mixtures of blue and yellow are used to achieve a green color, such as brilliant blue (E133) combined with riboflavin (E101), both of which are allowed in the EU and US (FDA Dietary supplements, 2021; EC Regulation 1333/2008), or patent blue (E131), which is only permitted in the EU, in combination with other yellow hues. Although these colorants are permitted and can be found in numerous foods, some studies have shown that they may cause some adverse health effects and allergic reactions (Tanaka et al., 1995) and their safety is constantly re-evaluated.

In summary, the complexity of the question of food colorants leads to confusion among consumers and the industry, as there is a lack of research regarding the composition of these coloring foodstuffs. In addition, new dietary preferences involve the consumption of new foods rich in natural colorant formulations, as detox juices, spirulina and wasabi-containing foods, dietary supplements, etc. The colorant composition of these new foods is poorly investigated and therefore, it should be analyzed. Our hypothesis is that new strategies are being developed, which must be analyzed in depth to decipher the chemical composition behind different methods used to proportionate green color to foods. With such an aim, trendy commercial green foods have been analyzed using a holistic approach to encompass the application of natural food colors, synthetic food colors and the new coloring foodstuff to verify the authentic composition of the green colors. Specifically, due to the absence of knowledge regarding the green coloring foods, the manuscript is focused on investigating the precise composition of such ingredients.

2. Materials and methods.

All the following procedures were carried out under green light to avoid the photooxidation of chlorophylls.

2.1. Raw materials

The study was carried out with 26 different foods bought in different supermarkets and stored following manufacturer instructions until their analysis. They were selected based on the declared composition in the

Table 1
Characteristics of the green food products analysed: apparent color and humidity. Food colorants and coloring foods declared in the labelling and authentic composition.

Food	Food colorant and coloring food declared in the label	Food color	Authentic food colorant and coloring food identified in the food	Humidity (%)
Jellybean	Spirulina extract, curcumin	Light green	Curcumin	0.02
Passionfruit candy	E141, curcumin	Yellowish green	Copper chlorophyllins (E141ii)	1.40
Wasabi peanut	E141	Copper green	Copper chlorophyllins (E141ii)	35.88
Mint chocolate	Chlorophylls and chlorophyllins	Copper green	Copper chlorophyll(E141i) and copper chlorophyllins (E141ii)	1.50
Food coloring	Spirulina and safflower concentrates	Light green	Safflower and c-phycocyanin, natural chlorophylls	8.80
Skin detox juice	Spirulina extract	Brownish green	Copper chlorophylls (E141i) and copper chlorophyllins (E141ii)	92.19
Fruit juice	E141ii	Green	Copper chlorophyllins (E141ii)	96.64
Mint chewing gum	E141	Green	Copper chlorophyllins (E141ii)	0.51
Mint and chocolate ice cream	Chlorophyllins and copper chlorophyllins	Light copper green	Copper chlorophyllins (E141ii)	33.61
Lemon and lime ice cream	Copper complexes of chlorophyllins and curcumin	Copper green	Copper chlorophyllins (E141ii) Safflower and curcumin	69.01
Spirulina and chia cookies	Spirulina powder	Light leafy green	Natural chlorophylls	8.53
Wasabi cheese	Wasabi powder, curcumin	Leafy green	Copper chlorophyllins (E141ii) curcumin	43.22
Juice soft drink	E141 ii	Copper green	Copper chlorophyllins (E141ii)	91.60
Wasabi paste	Copper complexes of chlorophylls and chlorophyllins	Copper green	Copper chlorophyllins (E141ii)	76.75
Canned cherries	E100 and E133	Intense brilliant green	Curcumin, brilliant blue, safflower and riboflavin	34.11
Green pepper mustard	Chlorophyllin /Green pepper/spices	Light green	Copper chlorophylls (E141i) and copper chlorophyllins (E141ii)	40.59
Chilli sauce from Canarian Islands	E100 and E131/coriander/ spices	Green	Natural chlorophylls, curcumin, E131	3.73
Jalapeño chilli pepper	E102/E133/jalapeño chilli/spices/	Dark green	E102/E133	69.85
Antacid pill	Spirulina concentrate, safflower and caramel	Light bluish green	Safflower, c-phycocyanin	1.85
Dietary supplement	Copper chlorophyllin	Dark green	Copper chlorophyllins (E141ii)	4.62
Wasabi coated peanuts	Spirulina concentrate, E141 and wasabi powder	Light copper green	Copper chlorophylls (E141i) and copper chlorophyllins (E141ii), curcumin	3.82
Wasabi coated peanuts	E141i, turmeric, curcumin and caramel	Brilliant green	Copper chlorophyllins (E141ii), curcumin	5.10
Menthol sweets	E141i, E141ii and curcumin	Light green	Copper chlorophyllins (E141ii), curcumin	0.02
Guacamole	E141i and E141ii, avocado, green pepper, green chili, jalapeño pepper	Yellowish green	Copper chlorophyllins (E141ii), natural chlorophylls	79.67
Sweets	Fruit and vegetal concentrate, Spirulina and safflower	Light brownish green	Safflower and curcumin	0.02
Kale juice	Apple and kale puree, Spirulina and safflower concentrate	Dark brownish green	Safflower, natural chlorophylls	72.22

label looking for the maximum variability in the strategies followed to obtain the green color: food colorants (naturals and synthetics) and coloring foodstuff. Table 1 describes the characteristics of the analyzed commercial green foods including color appearance and ingredients declared in the label. The nutritional composition of all of them is depicted in Appendix A.

2.2. Chemicals and reagents

Sodium chloride, tetrabutylammonium acetate, phosphate-buffered saline (PBS), ammonium acetate (98 %) and trifluoroacetic acid were supplied by Sigma-Aldrich Chemical Co. (Madrid, Spain). Acetonitrile and ammonium hydroxide were supplied by Panreac Química S.L.U. (Barcelona, Spain). N, N-dimethylformamide (DMF) was supplied by Scharlab (Barcelona, Spain). Other reagents (acetone, methanol, potassium chloride, calcium chloride (analysis grade)) were supplied by VWR BDH Chemicals (United States of America), and purified water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA.). Standards of chlorophyll pigments: chlorophyll *a* and *b*, pheophorbide *a*, pheophytin *a* and *b*, Cu-pheophorbide *a*, chlorin e_4, rhodin g_7, chlorin e_6, Cu-chlorin e_6, Cu-chlorin e_4, Cu-pyropheophytin *a*, Cu-pheophytin *a*, and purpurin 18*a* were supplied by Cymit S.L. (Barcelona, Spain). The rest of standards were obtained following previous protocol (Chen et al., 2015a,b), except Cu-rhodin g_7, Cu-rhodin g_5, Cu-purpurin 18*a* and purpurin 5b identified following previous reports (Pérez-Gálvez & Roca, 2023). Standards of synthetic colors (Sunset Yellow, Tartrazine, Indigo Carmine, Acid Green S, Brilliant blue, Patent blue, Quinoline yellow and Riboflavin) and coloring foodstuff (curcumin or turmeric, c-phycocyanin and hydroxysafflor yellow A) were supplied by Sigma-Aldrich Chemical Co. (Madrid, Spain). Powdered spirulina was bought at a supermarket.

2.3. Chlorophyll extraction

As previously set-up (Viera et al., 2022), liquid matrices (juices, ice creams and sweets) require freeze-drying before extraction (Telstar LyoQuest-55). Subsequently, the dried residue was extracted several times with 50 mL of DMF saturated with Mg_2CO_3 and mixed with 100 mL of diethyl ether and 400 mL of 10 % (w/v) NaCl solution in a separatory funnel. The mixture was stirred and then left to stand until the complete separation of the organic layer was observed. Next, the solvent phase was washed three times with 400 mL of 10 % (p/v) NaCl solution. The rest of solid foods were freshly extracted several times with DMF until no more color could be extracted (Viera et al., 2022). Next, chlorophylls were transferred to diethyl ether and NaCl and the same protocol explained above was applied. All the filtrates were concentrated on a rotary evaporator under vacuum at 30 °C to dryness and the dried residue was dissolved in acetone for their immediate HPLC analysis.

2.4. Chlorophyll identification by liquid chromatography/electrospray-APCI ionization/high-resolution-time-of-flight mass spectrometry.

Chromatographic separation was performed in a Dionex Ultimate3000RS UHPLC (Thermo Fisher Scientific, Waltham, MA, USA). The column used was a Mediterranea Sea18 column (200 × 4.6 mm, 3 μm particle size) (Teknokroma, Barcelona, Spain), protected by a guard column (10 × 4.6 mm) filled with the same material. Chromatographic separation was performed using the elution gradient described by Chen et al. (2015a,b) with the mobile phases: A, water/1 M ammonium acetate in water/methanol (1/1/8, v/v/v) and B, methanol/acetone (1/1, v/v). Online UV–visible spectra were recorded from 350 to 800 nm with the photodiode array detector, and sequential detection was performed at 410, 430, 450, and 666 nm. The HPLC/ESI-HRQqTOF operated for mass analysis using a micrOTOF-QII High-Resolution Time-of-Flight mass spectrometer (HR-TOF) with Qq-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with electrospray ionization (ESI) interface for polar chlorophylls and with an APCI interface for non-polar chlorophylls. The instrument control was performed using Bruker Daltonics HyStar 3.2. Mass spectra were acquired in bbCID mode and data were used to perform multitarget-screening using TargetAnalysis 1.2 software (Bruker Daltonics, Bremen, Germany). Data evaluation was performed with Bruker Daltonics DataAnalysis 4.1. The experimental values of mass to charge ratio and isotopic pattern corresponding to those elemental compositions were compared with the theoretical data, obtaining the mass error and mSigma values, which should be below the tolerance limits (mass error < 5 ppm and mSigma value < 50). The consistency of the elemental composition and formulas of the experimental product ions was checked by applying the same procedure to the product ions and with the assistance of the SmartFormula3D algorithm (Chen et al., 2015a).

2.5. Chlorophyll quantification by HPLC-UV–Visible

The identified chlorophyll derivatives were quantified by reverse-phase HPLC using a Hewlett-Packard HP 1100 liquid chromatograph following the chromatographic separation above described. Next, the data was collected and processed with an LC HP ChemStation (Rev. A.05.04; Santa Clara, CA, USA). Pigment quantification was performed with the corresponding calibration curves (amount vs integrated peak area). The calibration equations were obtained by least squares linear regression analysis over a concentration range according to the observed levels of these pigments in the samples. Triplicate injections were made for five different volumes of each standard solution.

2.6. Extraction, identification and quantification of C-phycocyanin

Standard of C-phycocyanin was used for identification. Samples were extracted based on previous methods (Chandralekha et al., 2021) with slight adaptations. Briefly, 2 g of each food were mixed with 2 mL of PBS. Samples were stirred and centrifuged at 6000 rpm and 4 °C for 10 min. Supernatants were collected and extraction was repeated until no blue color remained in the sample (at least three times). Distilled water was added to level up the volume. Collected supernatants were centrifuged at 13000 rpm for 10 min to eliminate residues, and samples were transferred to an HPLC vial until analysis. The pigments were separated by reversed phase HPLC using the same chromatograph and HPLC column as before to perform the identification based on the C-phycocyanin standard. The gradient elution was performed following Kissoudi et al., (2018), with slight modifications. The column was pre-equilibrated with 20 % (v/v) aqueous acetonitrile solution containing 0.1 % (v/v) trifluoroacetic acid and samples were eluted using a linear gradient from 20 to 100 % (v/v) ACN in 40 min, followed by a 10 min of elution with 100 % ACN. The flow rate was set at 1 mL min^{-1}. The on-line UV–visible spectra were recorded from 220 to 800 nm with the photodiode–array detector and the detection was performed at 620 nm. Data were collected and processed with an LC HP ChemStation (Rev.A.05.04; Santa Clara, CA, USA). For c-phycocyanin quantification, extracts were scanned in the range from 400 nm to 750 nm, using a spectrophotometer (Shimadzu UV-1900i) and the concentration of c-phycocyanin as mg/ml was determined using the following formula (Yacobi et al., 2015), and converted to mg/Kg dw:

$$PC = (A_{617} - 0{,}474A_{652})/5{,}34$$

where A_{617} and A_{652} are the absorbance at 617 nm and 652 nm, respectively.

2.7. Extraction, identification and quantification of coloring foodstuff and synthetic yellow, blue and green food colorants

Extraction of synthetic colors, were carried out following previous procedures (De Araújo Siqueira-Bento et al., 2015), with slight

modifications. Briefly, 2 g of each food were mixed with 2 mL of methanol:ammonium hydroxide 2 M (80:20) and samples were stirred and centrifuged at 6000 rpm and 4 °C for 10 min. Supernatants were collected and extraction step was repeated twice more. All the supernatants were collected and topped up to 8 mL with purified water. Safflower and curcumin were obtained with successive methanol extractions, following He et al., 2018 and Ahmed Nasef et al., 2019, respectively. A 1 mL aliquot was filtrated through 0.45 nylon filter and transferred to an HPLC vial until analysis. Identification and quantification were performed after separation of pigments by reversed phase HPLC using the same liquid chromatograph and column as previously. The elution gradient for separation was performed following Wu et al., (2021). The mobile phase composition was 20 mM of ammonium acetate (A) paired with LC–MS grade acetonitrile (B). Gradient conditions initiated with 5 % B before incorporating a linear increase to 98 % B over 15 min and held for 5 min. Finally, the gradient was returned to the initial conditions at 20 min and re-equilibrated for 5 min to complete the whole run. The on-line UV–visible spectra were recorded from 350 to 800 nm with the photodiode–array detector and sequential detection was performed at the maximum absorption wavelength for each standard. Data were collected and processed with an LC HP ChemStation (Rev.A.05.04; Santa Clara, CA, USA). Quantification of pigments was developed using the corresponding calibration curves (amount vs integrated peak area). The calibration equations were obtained by least-squares linear regression analysis over a concentration range according to the observed levels of these pigments in the samples.

2.8. Determination of humidity.

The percentage of moisture in the foods was determined in duplicate using the moisture analyzer OHAUS MB25.

2.9. Statistical analysis

Data were expressed as means ± standard deviation (SD).

3. Results and discussion

As previously stated, at present three different approaches co-exist in the food market to obtain green hues. Therefore, we have applied three different tactics to analyze the commercial green foods depending on the food label declaration: green food colorants, coloring foodstuff, and synthetic colorants.

3.1. Composition of green food colorants in commercial foods

Commercial foods were assayed to identify the chlorophyll compounds present in the four green food colorants on the market (Viera et al., 2019). When available, chlorophyll standards were used for the purposes of identification. On the contrary, chromatographic and mass spectroscopic data (including main product ions) were compared with data from previous studies. All the details of the 27 chlorophylls identified are set out in Table 2, while the scheme of the structural configurations is depicted in Fig. 1 (Appendix B details in detail all the structures). All the chlorophyll compounds have been previously identified in green food colorants by mass spectrometry (Mortensen & Geppel, 2007) and more recently by high resolution mass spectrometry (Chen et al., 2015a,b; Pérez-Gálvez & Roca, 2023). Polar chlorophylls or non-phytylated chlorophylls (up to 12 min. in Table 2), include rhodins, chlorins, pheophorbides and their copper-containing derivatives, and they are characteristics of water soluble green food colorants: E140ii ("chlorophyllins") and E141ii ("copper chlorophyllins") (Viera et al., 2019). Meanwhile, non-polar chlorophylls or phytylated chlorophylls (approx. from 15 min. in Table 2), such as pheophytins, derivatives and copper-containing pheophytins are the main components in oil soluble green food colorants: E140i ("chlorophylls") and E141i ("copper chlorophylls"), as expected (Pérez-Gálvez et al., 2020).

The quantification of chlorophylls in commercial foods (Table 3) showed a wide range of concentrations of green food colorants. High concentrations were found in guacamole and wasabi paste with more than 10 mg chlorophylls/100 gr dw and even somewhat higher in fruit juice and dietary supplements with around 40 mg/100 gr dw. On the contrary, low chlorophyll foods contained<2 mg/kg dw (wasabi peanuts, mint chocolate, mint chewing gum or menthol sweets) and even<0.25 mg/kg dw as detox juice or in ice cream. Previous reports have quantified copper chlorophylls (including all types) in quantities around 3–20 mg/kg mainly in jams, ice creams, juices and candies (Delpino-Rius et al., 2018; Mathiyalagan et al., 2019). The higher quantities quantified in this paper can be explained as new foods have been considered in this paper as dietary supplements, detox juices, spirulina and wasabi foods, considering the evolution towards more natural products.

The constant use of green food colorants can be verified in Table 3, which shows that 85 % of the analyzed food products contained copper chlorophyll complexes, while only three of them contained natural chlorophylls without copper. These foods (spirulina cookie, chilli sauce and kale juice) consist of pheophorbides and pheophytins coming not from the addition of chlorophyll food colorant (E140) but from natural chlorophylls present in the raw material. The original chlorophylls present in the ingredients of these foods (spirulina, pepper and fruits) are transformed during the food processing into pheophorbides and pheophytins, including to pyropheophytins, which is indicative of heat treatments (Viera et al., 2022). But, in the rest of the analyzed foods, the green color stabilization is mainly achieved by the addition of copper chlorophylls (E141i) and copper chlorophyllins (E141ii) (Table 3). The chlorophyll profile of foods with Cu-chlorophyllin colorant is dominated by Cu-chlorins, mainly by Cu-chlorin e_4 and Cu-chlorin e_6 since they are the main components of the E141ii food colorants, as previously identified (Mortensen & Geppel, 2007). In minor quantities, chlorins, rhodins and Cu-pheophorbide are also present in these foods. Of interest is the presence of purpurin 18*a* and copper-purpurin 18*a* in a few foods whose presence in polar colorants has only been recently described along with a description of the chemical pathway that takes place during the manufacturing of the food colorant (Pérez-Gálvez & Roca, 2023). Regarding the foods with non-polar copper-chlorophyll complexes (or E141i), as in chocolate, mustard and wasabi coated peanuts, the main chlorophyll component was copper-pyropheophytin as assumed, since it has been shown that this compound represents more than 75 % of the chlorophyll profile in different brands of E141i (Pérez-Gálvez et al., 2020). Regardless of the type of colorant use, as can be seen in Table 3, the great majority (75 %) of the foods were more than 80 % copper chlorophylls complexes. However, almost 20 % of the food products with green colorants did not declare them on the label (Table 1) and 33 % of the foods claimed an incorrect green colorant on their labels. For example, chlorophyll and chlorophyllins (E140) was declared when in fact copper complexes of chlorophyll and chlorophyllins (E141) were added, or copper chlorophyll (E141i) was declared when copper chlorophyllin (E141ii) was added. In summary, only 58 % of the foods containing green food colorants had correctly indicated the composition on the label.

When it comes to spirulina as food, it refers to *Arthrospira platensis* and, as with all cyanobacteria species, it contains as characteristic pigments chlorophylls exclusively from the *a* series (but not the *b* series), and c-phycocyanins. Interestingly, from the eight foods analyzed that were declared to contain spirulina (Table 1), only one (spirulina cookies) contained chlorophylls from the *a* series (Table 3) and two of them (skin detox juice and one of the wasabi coated peanuts) consisted of green food colorant (copper chlorophyllin and copper chlorophyll).

Table 2
Chromatographic and mass spectrometry characteristics of chlorophylls identified in commercial green foods.

	t_R (min)	Molecular formula	$[M + H]^+$ (*m/z*) Calcd./Meas.	Mass error (ppm)	mSigma	Main product ions (*m/z*)
Rhodin g_7	2.1	$C_{34}H_{35}N_4O_7$	611.2501/611.2477	3.8	32.4	551.2244[a] $[M + H\text{-acetic acid}]^+$
Cu-rhodin g_7	2.6	$C_{34}H_{32}CuN_4O_7$	672.1640/672.1648	−1.2	29.6	626.1604[b] $[M + H\text{-}H_2O\text{-}CO]^+$
Chlorin e_6	3.1	$C_{34}H_{37}N_4O_6$	597.2508/597.2690	3.0	29.2	551.2526 [a] $[M + H\text{-}H_2O\text{-}CO]^+$
Chlorin e_4	3.8	$C_{33}H_{36}N_4O_4$	553.2810/553.2827	−3.2	45.5	479.2464 [a] $[M + H\text{-propionic acid}]^+$
Chlorophyllide *a*	4.2	$C_{35}H_{35}MgN_4O_5$	615.2452/615.2424	4.6	8.5	555.2218[c] $[M + H\text{-}CH_2COOH]^+$
Cu-chlorin e_6	4.4	$C_{34}H_{35}CuN_4O_6$	658.1847/658.1827	3.0	43.9	614.1901[b] $[M + H\text{-}CO_2]^+$
Cu-isorhodin g_5	4.8	$C_{33}H_{32}CuN_4O_5$	628.1742/628.1717	3.9	29.3	610.1679 $[M + H\text{-}H_2O]^+$
Pheophorbide *b*	7.0	$C_{35}H_{35}N_4O_6$	607.2551/607.2597	−7.6	47.1	547.2296[c] $[M + H\text{-acetic acid}]^+$
Cu-chlorin e_4	7.2	$C_{33}H_{35}CuN_4O_4$	614.1949/614.1947	0.3	22.5	568.1816[b] $[M + H\text{-}H_2O\text{-}CO]^+$
Purpurin 5*b*	7.5	$C_{33}H_{32}N_4O_8$	581.2395/581.2403	−1.5	41.6	563.2301[a] $[M + H\text{-}H_2O]^+$
Pheophorbide *a*	8.1	$C_{35}H_{36}N_4O_5$	593.2758/593.2782	−4.0	44.7	533.2508[c] $[M + H\text{-acetic acid}]^+$
Pyropheophorbide *a*	8.3	$C_{33}H_{34}N_4O_3$	535.2704/535.2725	−3.9	44.4	447.2202[c] $[M + H\text{-}CH\text{-}CH_3\text{-}CH_2\text{-}COOH]^+$
Purpurin 18*a*	8.4	$C_{33}H_{32}N_4O_5$	565.2445/565.2467	−3.7	11.1	503.2400 [a] $[M + H\text{-}H_2O\text{-}CO_2]^+$
13^2-OH pheophorbide *a*	8.7	$C_{35}H_{36}N_4O_6$	609.2708/609.2713	−0.9	30.5	503.2436[c] $[M + H\text{-COO-}CH_3\text{-}H_2O]^+$
Cu-purpurin 18*a*	8.6	$C_{33}H_{30}CuN_4O_5$	626.1585/626.1599	−2.3	18.3	564.1588 $[M + H\text{-}H_2O\text{-}CO_2]^+$
Cu-pheophorbide *a*	9.7	$C_{35}H_{35}CuN_4O_5$	654.1898/654.1898	−1.9	45.6	636.1720 $[M + H\text{-}H_2O]^+$
Cu-pyropheophorbide *a*	11.3	$C_{35}H_{35}CuN_4O_5$	596.1843/596.1815	4.7	17.2	568.1817 $[M + H\text{-}CO]^+$
Chlorophyll *b*	15.5	$C_{55}H_{71}MgN_4O_6$	907.5219/907.5214	0.5	15.3	569.1995[d] $[M + H\text{-phytyl}]^+$
Chlorophyll *a*	18.0	$C_{55}H_{73}MgN_4O_5$	893.5426/893.5389	4.2	40.3	555.2196[d] $[M + H\text{-phytyl}]^+$
15^1-OH-lactone-pheophytin *a*	22.5	$C_{55}H_{75}N_4O_7$	903.5630/903.5664	−3.7	27.8	549.2575[d] $[M + H\text{-COOH-OH}]^+$
13^2-OH-pheophytin *b*	22.9	$C_{55}H_{73}N_4O_7$	901.5474/901.5518	−5.0	43.3	607.2540[d] $[M + H\text{-phytyl –OH}]^+$
13^2-OH-pheophytin *a*	25.2	$C_{55}H_{74}N_4O_6$	886.5681/886.5570	3.7	21.0	593.2715[d] $[M + H\text{-phytyl-OH}]^+$
Pheophytin *b*	25.3	$C_{55}H_{73}N_4O_6$	885.5525/885.5531	−0.7	27.9	547.2332[d] $[M + H\text{-COO-phytyl}]^+$
Pheophytin *a*	27.4	$C_{55}H_{75}N_4O_5$	871.5732/871.5738	−0.7	22.6	593.2735[d] $[M + H\text{-phytyl}]^+$
Pheophytin *á*	28.1	$C_{55}H_{75}N_4O_5$	871.5732/871.5747	−1.7	25.4	593.2751[d] $[M + H\text{-phytyl}]^+$
Pyropheophytin *a*	31.1	$C_{53}H_{73}N_4O_3$	813.5677/813.5672	0.6	25.4	535.2692[d] $[M + H\text{-phytyl}]^+$
Cu-pyropheophytin *a*	35.5	$C_{53}H_{71}CuN_4O_3$	874.4817/874.4855	−4.4	44.4	596.1825[e] $[M + H\text{-phytadiene}]^+$

[a] In agreement with Pérez-Gálvez and Roca (2023); [b]In agreement with Mortensen and Geppel (2007); [c]In agreement with Chen et al. (2015a); [d]In agreement with Chen et al (2015b); [e]in agreement with Pérez-Gálvez et al (2020).

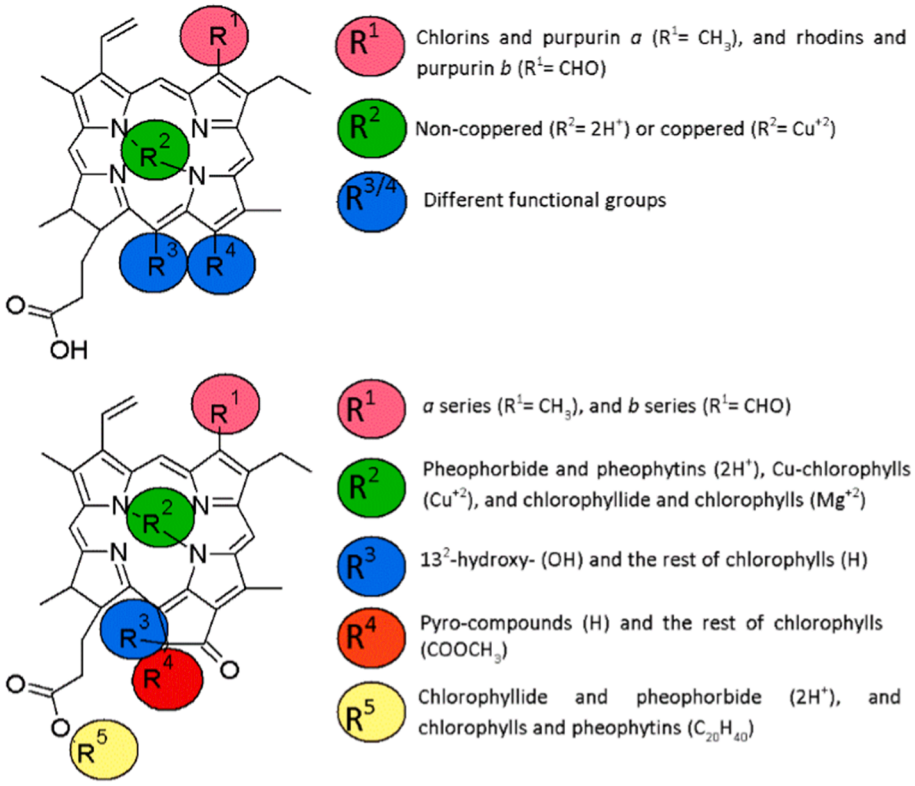

Fig. 1. Scheme of the structural configuration names for all the chlorophylls compounds identified by HPLC-ESI/APCI-*hr*TOF-MS/MS in the foods analysed. A detailed description is given in Appendix A.

3.2. Composition of coloring foods and mixtures of food colors in commercial foods

All the coloring foods or coloring foodstuff, curcumin/turmeric, riboflavin and synthetic colors used to obtain green hues were identified using authentic standards (as described in Materials and Methods) and analyzed using different HPLC methods, according to their nature. The majority of them were determined by adapting the method of Wu et al.,

Table 3
Quantification of chlorophylls and copper chlorophylls in green commercial foods (mg/kg dw).

Pigment	Passion fruit candy	Wasabi peanut	Mint chocolate	Skin detox juice	Fruit juice	Mint chewing gum	Mint and chocolate ice cream	Lemon and lime ice cream	Spirulina and chia cookies	Wasabi cheese	Juice soft drink	Wasabi paste	Green pepper mustard	Chilli sauce	Dietary supplement	Wasabi coated peanuts	Wasabi coated peanuts	Menthol sweets	Guacamole	kale juice
Chlorin					8.33 (0.06)		0.38 (0.03)													
Rhodin g_7					27.67 (0.01)						0.39 (0.04)				56.01 (4.92)					
Cu-chlorin	3.55 (0.23)	1.26 (0.08)	0.06 (<0.01)		330.26 (0.08)	1.37 (0.01)	11.88 (0.05)	0.1 (0.01)		9.98 (0.18)	2.74 (0.14)	171.64 (3.61)			315.30 (23.95)	0.72 (0.56)	7.01 (0.26)	1.17 (<0.01)	73.45 (4.23)	
Cu-rhodin		0.03 (0.00)			6.84 (0.11)		1.6 (0.00)	0.04 (0.01)		traces		3.60 (0.26)			15.28 (0.51)			0.01 (<0.01)		
Chld *a*									0.06 (<0.01)											
Pheo *a*				0.02 (0.00)	2.49 (0.02)	0.19 (<0.01)	0.10 (<0.01)	traces	0.19 (<0.01)			1.37 (0.17)	0.94 (0.02)	4.10 (0.45)	4.76 (0.47)				0.65 (0.00)	0.18 (0.01)
Pheo *b*												3.20 (0.14)								
Cu-Pheo *a*	0.17 (0.01)	0.11 (0.00)	0.07 (0.01)	0.01 (0.00)	1.86 (0.00)		0.13 (0.00)	traces		0.74 (0.00)	0.13 (<0.01)	18.94 (1.30)	1.66 (0.09)		15.28 (0.51)	0.82 (<0.01)		0.04 (0.01)	0.52 (0.03)	
Purpurin		0.01 (0.00)					15.32 (1.23)		0.07 (0.00)		0.04 (<0.01)									
Cu-purpurin	0.33 (0.01)	0.12 (0.00)				0.27 (0.00)	1.15 (0.00)	0.06 (<0.01)		3.33 (0.22)		45.57 (0.03)			35.97 (2.80)		3.63 (0.08)		6.56 (0.01)	
Chl *a*									29.76 (1.93)								0.24 (0.05)			
Chl *b*																	0.25 (0.03)			
Phy *a*			0.22 (0.02)	0.02 (<0.01)			0.13 (0.00)	traces	21.94 (1.21)		0.01 (0.00)		0.50 (0.03)	5.96 (0.04)			1.96 (0.18)		26.08 (0.20)	7.43 (0.06)
Phy *b*																	0.23 (0.04)		2.25 (0.27)	0.83 (<0.01)
Cu-pyrophy *a*			0.52 (0.04)	traces									5.41 (0.52)			0.71 (0.00)				
Pyrophy *a*			0.06 (0.00)	0.01 (0.00)										0.45 (0.01)			0.5 (0.20)		8.26 (<0.01)	0.27 (0.00)
Cu-chls	4.05 (0.31)	1.51 (0.18)	0.67 (0.05)	0.01 (0.00)	33.95 (0.19)	1.64 (0.01)	14.82 (1.05)	0.21 (0.02)		14.05 (1.03)	2.91 (0.15)	239.70 (7.57)	7.07 (0.81)		366.5 (21.21)	2.25 (0.21)	10.64 (0.37)	1.22 (0.07)	80.52 (4.33)	
Non cu-chls	0.00	0.01 (0.00)	0.28 (0.23)	0.04 (<0.01)	38.49 (0.10)	0.19 (0.01)	15.93 (1.15)	0.01 (<0.01)	51.32 (3.36)	0.00	0.04 (<0.01)	4.57 (0.27)	1.47 (0.05)	10.50 (0.46)	60.78 (4.45)	0.00	3.18 (0.02)	0.00	34.51 (0.84)	8.71 (0.07)
a series	4.05 (0.31)	1.51 (0.15)	0.94 (0.02)	0.04 (<0.01)	342.93 (0.17)	1.83 (0.22)	29.09 (1.38)	0.17 (0.01)	51.32 (3.36)	14.05 (1.03)	2.92 (0.11)	237.5 (5.14)	8.34 (0.83)	9.61 (0.42)	371.32 (18.74)	2.25 (0.01)	13.34 (<0.01)	1.20 (0.01)	112.7 (4.90)	7.88 (0.06)
b series	0.00	0.03 (<0.01)	Traces	0.01 (0.00)	34.51 (0.12)	0.00	1.66 (0.12)	0.04 (0.01)	0.00	Traces	0.39 (<0.01)	6.79 (0.40)	0.19 (<0.01)	0.89 (0.03)	56.01 (4.02)	0.00	0.48 (0.01)	0.01 (<0.01)	2.25 (0.18)	0.83 (0.01)
Total chls	**4.05 (0.31)**	**1.53 (0.10)**	**0.94 (0.02)**	**0.05 (<0.01)**	**377.44 (0.29)**	**1.83 (0.12)**	**30.75 (2.20)**	**0.214 (0.02)**	**51.32 (3.36)**	**14.05 (1.03)**	**3.31 (0.26)**	**244.3 (8.54)**	**8.53 (0.86)**	**10.50 (0.46)**	**427.3 (27.66)**	**2.25 (0.11)**	**13.82 (1.07)**	**1.22 (0.01)**	**115.0 (5.17)**	**8.71 (0.05)**

Traces: <0.01 mg/kg dw. Jellybeans, food coloring and antacid pills (Table 1) do not contain chlorophylls. Abbreviations: chld: chlorophyllide; pheo: pheophorbide; chl: chlorophyll; phy: pheophytin; pyrophy: pyropheophytin, chlorin stands for chlorin e_4 and chlorin e_6, cu-chlorin stands for cu-chlorin e_6 and cu-chlorin e_4, cu-rhodin stands for cu-rhodin g_7 and cu-rhodin g_5, pheophorbide *a* stands for pheophorbide *a*, 13^2-OH-pheophorbide *a* and pyropheophorbide *a*, Cu-pheophorbide a stands for cu-pheophorbide *a* and cu-pyropheophorbide *a*, purpurin stands for purpurin 5*b* and purpurin 18*a*, cu-purpurin stands for cu-purpurin 18*a*, pheophytin a stands for 15^1-OH-lactone pheophytin *a*, 13^2-OH-pheophytin *a*, pheophytin *a* and pheophytin *á*, pheophytin b stands for 13^2-OH-pheophytin *b* and pheophytin *b*. See Table 2 for identification.

(2021). But, for spirulina, a more specific method was needed, due to its chromatographic characteristics, and an adaptation of Kissoudís et al. method (2018) was selected. The chromatographic and spectroscopic characteristics of all of the analyzed standards are shown in Table 4 along with the different nomenclatures followed in the EU and US regulations). The quantification of all of them is shown in Fig. 2, and is, to the best of our knowledge, the first time that the quantity of coloring foodstuffs present in foods has been analyzed.

It can be observed that the most widely used color in green foods was curcumin (turmeric), which was present in almost all the samples studied as it is considered a regulated natural food colorant (FDA Color Additive Status List, 2021; EC Regulation 1333/2008). Curcumin (or turmeric) is an oil-soluble yellow-colored curcuminoid which is a lipophilic polyphenol extracted from *Curcuma longa* L. (turmeric) (Bresson et al., 2017) and recently, the hydrogenation of curcuminoids has been approved as a Novel Food (Turck et al., 2021). It can be seen in the table that curcumin exhibit a widespread use, being applied to foods with completely different textures and solubility from aqueous lemon ice to fatty cheese, including sweets, sauces and snacks. The observed quantities varied also among foods being below 3 mg/kg dw in sweets but between 30 up to 90 mg/kg dw in the rest of foods. To the best of our knowledge, different percentages have been published for turmeric powder, tincture or extract (Tayyem et al., 2006) but not authentic quantities in foods have been described. Due to its biological properties curcumin is receiving a lot of attention, especially among food supplements. But, curcumin represents only the 2–5 % of turmeric powder, and around 80 % of total curcuminoids in curcumin. The rest of curcuminoids consist of demethoxycurcumin (17 %), and bidemethoxycurcumin (3 %), whose effect on human organism have not been studied yet. In consequence, several national Food Safety Agencies (BfR 2021) point to a precautionary principle until further research elucidate possible non-desired effects of curcumin and derivatives in human health.

Another yellow pigment found in the analyzed foods was safflower, one of the main coloring components of the aqueous extraction of *Carthamus tinctorius* L. (Bratinova, 2015). Its quantification showed important differences among foods: in ice cream and cherries its presence was rather low (below 3 mg/kg dw, Fig. 2a), but in food coloring and antacid the content was very high (between 200 up to 400 mg/kg dw, Fig. 2b). The comparison with other foods is complicate as to the best of our knowledge no previous report has been published regarding safflower content in commercial foods. Anyhow, the quantities quantified in this paper are significant, having into account that, as mentioned before, in the US its use is no longer permitted (FDA Color Additive Status List, 2021), while in the EU is only considered a food coloring and not a food colorant and therefore, no acceptable daily ingestion (ADI) has been established. Surprisingly, 33 % of the foods analyzed with safflower in their composition have not declared it on the label (Table 1).

Table 4
Chromatographic and spectroscopic characteristics of coloring foods and synthetic colorants analyzed.

Common name	EU code	FDA code	λ_{max} (nm)	t_R (min)
Safflower	–	banned	405	6.1
Curcumin	E100	Turmeric and turmeric oleorresin	430	14.0
Riboflavin	E101	Riboflavin	370, 450	7.0
Tartrazine	E102	FD&C Yellow No. 5	430	5.2
Patent blue	E131	banned	640	9.5
Brilliant blue	E133	FD&C Blue No. 1	630	7.8
Sunset Yellow	E110	FD&C Yellow 6	480	6.5
Indigo Carmine	E132	FD&C Blue No. 2	615	5.6
Acid S	E142	banned	640	6.2
Spirulina	Arthrospira platensis	Aqueous spirulina extract	620	25.0

All of them were analysed through Wu et al. (2021)́s method except Spirulina which was determined with Kissoudís method (2018). The authors declare that they have no conflict of interest that could have influenced the research shown in the paper.

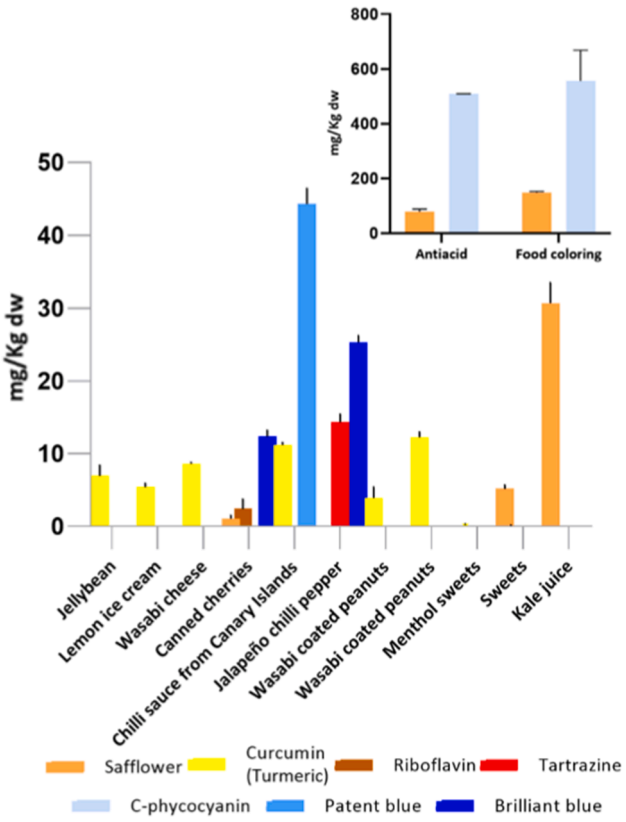

Fig. 2. Quantification (mg/kg dw +/- SD) of coloring foods and synthetic colorants in the commercial foods described in Table 1. The inserted figure refers specifically to the content of antiacid and food coloring.

Riboflavin is the water-soluble vitamin B_2, that industrially can be obtained by chemical synthesis or from microbiological sources. Riboflavin was found only in cherries, in at around 17 mg/Kg dw. In the US, it is included in the list of Specific Substances Affirmed as GRAS (Generally Recognized As Safe), while in the EU as food colorant (E101) has been recently re-evaluated and included in a group of ADI "not specified" (EC, 2016). Moreover, the EFSA concluded that riboflavin produced by fermentation using genetically modified *Bacillus subtilis* can be used as a coloring matter. However, responsible use of this compound is recommended by the FAO/WHO (FAO & WHO, 2020).

Regarding blue tones, c-phycocyanin is the blue pigment present in spirulina found in food coloring and antiacid (Fig. 2b) at similar quantities, around 500 mg/Kg dw. Despite the widespread use of spirulina and although there are studies related to fortification with spirulina, comparison with quantities in commercial foods have been unproductive. At all events, the surprising fact detected was that in 75 % of the foods that declared on the label the presence of spirulina, that pigment was not found when the foods were analyzed. Moreover, for some foods the specific term used on the label was "spirulina extract", while for others was "spirulina concentrated". None of the foods with spirulina declared on their label contained, at the same time, the characteristics pigments of *Arthrospira* as it has been specified before, c-phycocyanin and chlorophyll *a*. In the best situation, the analyzed foods incorporate one or the other. With the aim of clarifying what had been added to foods, lyophilized spirulina was bought and extracted with acetone, obtaining an intense green extract with chlorophyll *a,* followed by a

buffer extraction that generates a deep-blue extract riched in c-phycocyanin (Appendix C). We found out that with a simple extraction step both fractions of spirulina could be easily separated. This means that several foods do not include the whole organism to the food. The term "spirulina" in food refers to the dried biomass of the cyanobacteria *Arthrospira platensis*, in contrast to the genus "Spirulina". In the EU, use of *Arthrospira platensis* as a novel food is recognized (Araujo & Peteiro, 2021), without an E-number label requirement, and in the US, water soluble extract from the dried biomass of cyanobacteria *Arthrospira platensis* is included in the list of additives exempt from batch certification (CFR-21, 73–74, 2016). The use of spirulina as a coloring food has quickly expanded, as it shows health-promoting properties (Campos Assumpção de Amarante et al., 2020). Moreover, due to its natural origin, it is perceived as a healthy ingredient by consumers, and can be marketed as clean-label, which makes it interesting for both consumers and food industry at the same time. Perhaps, a clearer label could be expected.

3.3. Composition on synthetic food colorants in commercial foods

From all the synthetic colors analyzed (Table 4), only three of them were found in the foods - tartrazine (E102), patent blue (E131) and brilliant blue (E133) - and all of them were clearly declared on their labels. Surprisingly, no synthetic green color (E1 142) was found, but rather a combination of synthetic yellow and blue hues to achieve the green color. The yellow tartrazine was found only in Jalapeño chilli pepper at 50 mg/kg dw, which is slightly lower than in previous studies, around 160 mg/kg in soft drinks (Feng et al., 2011) and around 300 mg/kg in yogurt (Soponar et al., 2008). In the EU, tartrazine is a synthetic food color included in group III with a maximum limit and recently revised the established ADI at 7.5 mg/kg bw/day (European Food Safety Authority, Food additive re-evaluations, 2012). In the US, the FDA consider that tartrazine may be safely used for coloring foods generally in quantities consistent with GMP (US-FDA). Thus, the quantity found in the chilli pepper was according to the current legislation.

Regarding blue color, two synthetic colorants were found in foods: brilliant blue and patent blue. Brilliant blue was found in canned cherries and Jalapeño chilli pepper in combination with other yellow hues, and the quantities varied from 19 to 40 mg/Kg dw. This concentration accords with other published data (Feng et al., 2011), where similar concentrations were found. After some revisions, the current ADI for this color by EFSA is 6 mg/kg bw/day (EFSA, Food additive re-evaluations, 2012) while in the US in amounts consistent with GMP (CFR-21, 73–74, 2016), which it is impossible to exceed with the quantities found. Finally, patent blue was found in a quantity of around 33 mg/Kg dw in only one sample (chilli sauce from Canary Islands), perhaps due to its restrictions. In 2013, the EFSA re-evaluated the ADI for this colorant and established a new one at 5 mg/kg bw/day (EFSA, Food additive re-evaluations, 2012). As in the case of Brilliant blue, with a normal ingestion it is quite unlikely to exceed the limit. Although currently synthetic colorants are not popular, it should be stressed that all the food analyzed complied with the synthetic food colorants legislation and were found in low quantities.

4. Conclusions

At present, consumers are well-informed and therefore, the nutritive and healthy capacity are one of the most valuable parameters to select a specific food. This knowledge is behind the clean-label strategy, which ideally seeks to provide clearer and greater information to the consumer. The results obtained in this paper demonstrate that an important regulation is needed in this field. The use of natural extracts and coloring foodstuff is rapidly increasing, since they can be categorized as "natural" and "healthy" (Echegaray et al., 2023). Those new extracts may include a large variety of ingredients and different uses and processing methods have not been studied in depth. In this research, several of the 27 chlorophylls identified by HPLC-ESI/APCI-*hr*TOF-MS^2 in the analysed foods, have been described for the first time in foods. Therefore, in view of the outcome of the present research, it is necessary to develop a complete database with the authentic composition in coloring foods and food colors of foods to guarantee the quality criteria.

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CRediT authorship contribution statement

Marta Herrera: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Isabel Viera:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **María Roca:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Software, Visualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112974.

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