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Discovery of marker peptides of spirulina microalga proteins for allergen detection in processed foodstuffs

Mariachiara Bianco[1], Giovanni Ventura[1], Cosima D. Calvano[1,2*], Ilario Losito[1,2], Tommaso R.I. Cataldi[1,2]

[1]Dipartimento di Chimica, [2]Centro interdipartimentale SMART, Università degli Studi di Bari Aldo Moro, via Orabona 4, 70126, Bari, Italy

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Author for correspondence, email: cosimadamiana.calvano@uniba.it

Abstract

Spirulina (*Arthrospira platensis*) proteins were extracted, digested, and analyzed by LC-ESI-FTMS/MS to find highly conserved peptides as markers of the microalga occurrence in foodstuffs. Putative markers were firstly chosen after *in silico* digestion of allergenic proteins, according to the FAO and WHO criteria, after assuring their presence in food supplements and in (un)processed foodsuffs. Parameters such as sensitivity, sequence size, and uniqueness for spirulina proteins were also evaluated.

Three peptides belonging to C-phycocyanin beta subunit (P72508) were designated as qualifiers (ETYLALGTPGSSVAVGVGK and YVTYAVFAGDASVLEDR) and quantifier (ITSNASTIVSNAAR) marker peptides and used to validate the method for linearity, recovery, reproducibility, matrix effects, processing effects, LOD, and LOQ. The main aim was to determine spirulina in commercial foodstuffs like pasta, cracker, and homemade bread incurred with the microalga. The possible inclusion of the designated peptides in a standardized method, based on multiple reaction monitoring using a linear ion trap MS, was also demonstrated.

1 INTRODUCTION

Currently, novel foods are receiving growing attention for their proven beneficial effects and their contribution to bridging the gap between food availability and nutrition (Alexandratos, Nikos & Bruinsma, 2012; van der Spiegel, Noordam, & van der Fels-Klerx, 2013). Novel foods have been defined as food or food ingredients not yet extensively used for human consumption in the EU before 15 May 1997 (The European Parliament and the Council of the European Union, 2015). According to this definition, the term *novel foods* mainly indicate traditional exotic nutrients newly consumed in the EU, including algae, microorganisms, and insects, which are spreading also in local markets (Hermann, 2009). Among microalgae, spirulina (*Arthrospira platensis*) and chlorella (*Chlorella vulgaris*) are widely employed as healthful ingredients in processed food products (Vaz, Moreira, Morais, & Costa, 2016). Indeed, many studies have been focused on the health benefits related to the high content of proteins, bioactive peptides, vitamins, and polyunsaturated fatty acids, both ω-3 and ω-6 (Aiello et al., 2019; Ibañez & Cifuentes, 2013; Monopoli, Calvano, Nacci, & Palmisano, 2014). However, few studies concerning the negative effects of these novel foods have been reported (Abdelmoteleb et al., 2021; Petrus, Culerrier, Campistron, Barre, & Rougé, 2009; Polikovsky et al., 2019). One health matter of commercial spirulina-based food supplements is related to their contamination with cyanotoxins, specifically microcystins, which are a class of cyanobacteria toxins with several adverse effects on humans, such as hepatotoxicity, vomiting, nausea, and pneumonia. The levels of microcystins recovered in spirulina food supplements do not exceed the attention threshold for adults but they represent a serious concern for children and infants. However, the persistent exposure to microcystins should not be ignored even at low concentrations and regular monitoring during spirulina production is recommended (Papadimitriou, Kormas, & Vardaka, 2021).

Very recently (Bianco, Ventura, Calvano, Losito, & Cataldi, 2021) explored the allergenicity of spirulina and chlorella proteins following the WHO/FAO guidelines (FAO & WHO, 2001) whereby the cross-reactivity takes place when (i) the percentage of identity of amino acid sequences is higher than 35% using a window of 80 amino acids or (ii) there is an identity of at least six contiguous amino acids.

A known allergen of spirulina was identified, *i.e.* C-phycocyanin beta subunit (Uniprot code P72508), along with five putative allergens, namely two thioredoxins (D4ZSU6, K1VP15), a superoxide dismutase (C3V6P3), a glyceraldehyde-3-phosphate dehydrogenase (K1W168), and triosephosphate isomerase (D5A635) related to established allergens of pistachio, shrimp, maize and other food products (Bianco, Ventura, et al., 2022). This microalga is not only commercialized as food supplements in different forms, like tablets, capsules, powder, or sticks, but it is also added as an ingredient in processed food products such as bread, biscuits, pasta, fruit juices, and crackers (Niccolai et al., 2019). For this reason, a study concerning the stability of spirulina allergenic proteins aimed to suggest putative marker peptides useful for allergens discovery and/or quantification can be very useful in the perspective of protecting allergic consumers. Rapid and accurate detection of allergens is crucial since the only way to avoid health problems for these individuals is strict avoidance of food products including allergens (Popping & Diaz-Amigo, 2018; Van Vlierberghe et al., 2020).

Major allergenic food proteins, such as caseins, albumin, and glycinins, obtained from milk, eggs, and soybeans, respectively, have been widely studied to standardize lists of marker peptides for their unequivocal identification in food samples, also taking into account the matrix composition and/or thermal treatments (Julia Heick, Fischer, Kerbach, Tamm, & Popping, 2011; Planque et al., 2017). Despite the potential allergenicity of spirulina was proved (Petrus et al., 2009), no systematic investigation on possible peptide markers of its

allergenic proteins in foodstuffs has been performed so far, to the best of our knowledge. The main objective of this work is thus the definition of marker peptides useful for the unambiguous identification of spirulina allergenic proteins in food supplements and food products.

A workflow including *in silico* approaches and discovery analysis by reversed-phase liquid chromatography (RPLC) coupled to either high-resolution Fourier-transform (FT) tandem mass spectrometry (MS/MS) or linear ion trap (LIT) MS/MS with electrospray ionization (ESI) is described. First, an *in silico* digestion of allergenic proteins was performed to select potential marker peptides for each protein, according to the literature criteria (Downs & Johnson, 2018; Gu et al., 2018; Johnson et al., 2011). These peptides should meet well-defined criteria, such as length between 7-20 amino acids, reproducible digestion pattern (no missed cleavages), and absence of post-translational modifications. Moreover, marker peptides should not contain methionine (M), cysteine (C), and asparagine-glycine motifs (NG), that could undergo chemical changes during thermal processing.

The developed RPLC-ESI(+)-FTMS/MS method was validated for the determination of allergen proteins of spirulina in different food samples, evaluating linearity, limits of detection (LOD) and quantification (LOQ), matrix and processing effects, and recovery of selected marker peptides. The possibility to use the same approach on a low-resolution LIT instrument was also ascertained.

2 MATERIALS AND METHODS

2.1 Chemicals. Water, hexane, acetonitrile, acetone, methanol, and formic acid were purchased from Sigma-Aldrich (Milan, Italy). All solvents used were LC-MS grade except for hexane, acetone, and chloroform (HPLC grade). Tris(hydroxymethyl)aminomethane

hydrochloride (tris-HCl), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, and trypsin from porcine pancreas were obtained from Sigma-Aldrich (Milan, Italy). RapiGest surfactant was obtained from Waters Corporation (Milan, Italy). Standard solutions for mass spectrometer calibration were purchased from Thermo Scientific (Waltham, Massachusetts, United States). *Arthrospira platensis* capsules, powder, sticks, and tablets, used as food supplements (Longlife nutritional supplements, Phoenix srl, Milan, Italy), were purchased from local supermarkets. Cookies and bread incurred or spiked with spirulina, according to the case, were prepared in the laboratory; a fruit juice, subsequently spiked with spirulina, was purchased from a local supermarket. Pasta and crackers including spirulina as an ingredient were purchased from biological supermarkets.

2.2. Standard solutions, and spiked/incurred foodstuffs. *Artrhospira platensis* (spirulina) powder was used to prepare standard solutions for calibration purposes, spike cookies, and fruit juice, and prepared incurred cookies. Specifically, standard solutions were prepared at 2.5-44 $\mu g_{TotProt}$ (total proteins) absolute amounts in 100 µL of solvent. To evaluate the processing effect, incurred cookies were made in the laboratory as follows: 50 g of flour, 22.5 g sugar, 0.125 g salt, 0.25 g ammonium bicarbonate, 11.25 g olive oil, 20 g water, and 1.3 g spirulina powder corresponding to 780 mg $_{TotProt}$. Cookies were cooked at 180 °C for 15/20 minutes; after baking the biscuits' weight descreased to 88.6 g due to water loss. Spiked cookies, used to calculate the matrix effect, were produced by adding a proper amount of spirulina powder directly to blank cookies previously finely ground**.** Calibration curves for incurred and spiked cookies were obtained using concentration levels included in the range 0.5-8.8 $mg_{TotProt}/g_{matrix}$. The processing effect was evaluated as percentage of ratio between

145 slopes of the calibration curve for incurred and spiked cookies, while combined effect was
146 assessed as percentage of ratio between slope of calibration curve for incurred cookies and
147 spirulina powder solutions. Cookies protein extracts fortified with spirulina, used to evaluate
148 the recovery by comparison with protein extracts of spiked cookies, were obtained by adding
149 a spirulina powder solution to protein extracts of blank cookies at two absolute amounts (2.5
150 and 44 $\mu g_{TotProt}$). Incurred small pieces of bread were made with 90 g flour, 0.3 g sugar, 1 g
151 salt, 30 g water, 4 g yeast, and 2 g spirulina powder corresponding to 1.2 $g_{TotProt}$. The bread
152 was cooked at 180 °C for 20 minutes after soaring. Spiked fruit juice was obtained by adding
153 300 mg of powder to 100 mL of mixed fruit juice (apple, pear, banana, and orange). Calibration
154 curves of spirulina juice were obtained in the concentration range 0.5-8.8 $mg_{TotProt}/g_{matrix,}$
155 corresponding to 2.5-44 $\mu g_{TotProt}$ of the absolute amount added. Matrix effect was evaluated
156 for both cookies and fruit juice as percentage of ratio between slopes of calibration lines of
157 spiked samples and spirulina powder solutions. The recovery was estimated on fruit juice and
158 cookies extracts fortified at two absolute amounts (2.5 and 44 $\mu g_{TotProt}$), comparing the peak
159 area of quantifier peptide obtained for spiked samples and for the fortified samples at the
160 same concentration of spirulina. Moreover, for spiked and incurred samples limit of detection
161 (LOD) and limit of quantification (LOQ) were evaluated as three and ten times, respectively,
162 the ratio between the intercept standard deviation and slope of calibration lines.

163 Commercial spirulina-based foodstuffs present different contents of spirulina, based on both
164 the kind of product and the manufacturer. To better evaluate the matrix and processing
165 effects two wholly different matrices, raw liquid fruit juice and solid cooked cookies, were
166 chosen to reduce the analytical variability by exploring the same range of spirulina additions.

2.3 Protein extraction from spirulina-based food supplements and foodstuffs. Two protocols for protein extraction from spirulina powder were tested, based on cold acetone or Tris-HCl buffer solutions (pH=8), respectively. For the first one, 9.0 mL of cold acetone were added to 0.100 g of spirulina powder. After vigorously vortexing, the sample was kept at 4 °C for 1 h and shacked every 10 minutes. The sample was centrifuged at 5000 *g* for 1 h and the solvent was discharged. Upon washing the pellet twice with 1.0 mL of cold acetone, it was dried under a nitrogen flow (De Ceglie, Calvano, & Zambonin, 2014). The extraction based on the Tris-HCl buffer solution, following a recently described protocol (Bianco, Calvano, Ventura, Losito, & Cataldi, 2022), was applied both to spirulina powder and to all the other investigated samples. Among the latter, spirulina capsules, sticks and tablets were ground and the proteins were extracted by adding 9.5 mL of 50 mM Tris-HCl to ca. 0.1 g of ground product. After vortexing, solutions were incubated for 1 h at 55 °C in an ultrasound bath and stirred every 20 minutes. Then, 0.50 mL of methanol was added, and the samples were incubated for 10 minutes in an ultrasound bath to promote protein extraction. Afterward, 3.5 mL of hexane were added, and the mixture was vigorously vortexed to allow fat partitioning in the organic solvent. Samples were centrifuged for 15 minutes at 5000 *g*. Finally, the organic phase was discharged, and 100 µL of the aqueous phase was collected and dried under a nitrogen flow. The protein extraction from foodstuffs including spiked and incurred cookies, incurred bread, spiked fruit juice, pasta, and crackers was performed by applying the protocol based on 50 mM Tris-HCl but starting from 0.500 g of each sample.

2.4 Protein digestion. 50 µL of RapiGest (0.1 % w/v in 50 mM NH_4HCO_3) and 50 µL of 50 mM NH_4HCO_3 were added to dried samples and to 600 µg of pellet. Samples were vigorously vortexed to dissolve them into the surfactant solution. Thereafter, 10 µL of 50 mM DTT were added and the samples were incubated for 30 minutes at 60°C followed by the addition at

room temperature of 10 µL of 150 mM IAA; after standing in the dark for 30 minutes, 5.0 µL of trypsin (0.1 µg/ µL in 25mM NH_4HCO_3) were added and the samples were incubated overnight at 37 °C (Bianco, Calvano, et al., 2022). The enzymatic reaction was stopped with formic acid (pH ca. 2). The samples were dried using a gentle nitrogen flow and resuspended in 100 µL of the mobile phase, *i.e.* H_2O/ACN (95/5 v/v with 0.1% of formic acid) (see section 2.5). When using strong denaturing agents such as sodium dodecyl sulphate, urea, or thiourea, for protein solubilization before their digestion, a preliminary purification is applied to remove detergents and, to this aim, micro solid-phase extraction (µSPE) can be used. However, as previously demonstrated (Bianco, Ventura, et al., 2022; van der Werf, Calvano, Palmisano, & Sabbatini, 2012), the use of RapiGest™ as an alternative denaturing anionic detergent improved the efficiency of digestion, simplifying, at the same time, the purification protocol since complete removal of the detergent can be easily accomplished by acidification and centrifugation. The absence of a desalting step allowed us to speed up the whole process also lowering the loss of proteins.

2.5 RPLC-ESI-FTMS instrumentation and operating conditions. Liquid chromatography coupled to mass spectrometry analyses were performed using an Ultimate 3000 UHPLC chromatographic station coupled to a quadrupole-Orbitrap spectrometer (Q-Exactive, Thermo Scientific, Waltham, MA, USA) equipped with a higher collisional-energy dissociation (HCD) cell by a HESI (heated electrospray ionization) source (Thermo Scientific). The chromatographic separations were accomplished at 40°C using a Phenomenex Aeris WIDEPORE 200 Å C18 column (250 x 2.1 mm, 3.6µm) equipped with Phenomenex AJO 8783 WIDEPORE C18 (2 x 2.1 mm ID) security guard cartridge and a mobile phase based on H_2O (solvent A) and ACN (solvent B) both containing 0.1% of formic acid. Specifically, the gradient used during each chromatographic run, at a flow rate of 0.200 mL/min, was the following: 0 −

2 min at 5% solvent B; 2 − 20 min linear from 5% to 60% (v/v) of B; 20 – 22 min linear from 60% to 100% B; 22−26 min isocratic at 100% of B; 26−30 min back to the initial composition, followed by 5 min equilibration time. Mass spectrometry analyses were carried out in full scan and data-dependent mode in positive polarity. The ESI and ion optic parameters adopted were the following: sheath gas flow rate, 10 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); spray voltage, 3.5 kV in positive polarity; capillary temperature, 200 °C; S-lens radio frequency level, 100 arbitrary units. Positive MS full-scan spectra were acquired in the *m/z* range 150-2500 with 70k of resolution using an automatic gain control (AGC) target of $1x10^6$ and an injection time of 200 ms. For data-dependent analyses, MS full-scan spectra were acquired in the *m/z* range 500–2000 with 70k of resolution using an automatic gain control (AGC) target of 3 $x10^6$. The Full-MS/$ddMS^2$ analyses on top 5 ions experiments were performed using NCE fixed at 30 with a 17.5k resolution, AGC of 1 $x10^5$, IT fill time of 50 ms, isolation window of 4 m/z, minimum AGC of 8.00 $x10^3$, and dynamic exclusion of 10 s. Tandem mass spectra of selected peptides were also acquired, after RPLC separation, by a Velos Pro (Thermo Scientific) double-stage linear ion trap mass spectrometer, operating collisional induced dissociation (CID) in a low energy regime. Collisional energy equal to 35% and an isolation window width for precursor ions of 4 *m/z* were adopted. The control of the LC-MS instrumentation and the first processing of data was performed by the Xcalibur software 2.2 SP1.48 (Thermo Scientific).

2.6 Identification of microalgae proteins using Full-MS/$ddMS^2$ analysis and putative peptide marker. The identification of microalgae proteins in all spirulina samples (powder, capsules, sticks, and tablets) was carried out by using Full-MS/$ddMS^2$ raw files as input data for the ProteomeDiscoverer™ software (version 2.4, Thermo Fisher Scientific) and a *A. platensis* database downloaded from Uniprot (https://www.uniprot.org/uniprot/

?query=Arthrospira+platensis#) on August 20, 2021. The Processing and Consensus workflows for ProteomeDiscoverer™ investigation were PWF_QE_Basic_SequestHT.pdProcessingWF and CWF_Basic.pdConsensusWF, respectively. The research criteria used are reported in our previously work (Bianco, Ventura, et al., 2022). The identification of putative peptide markers of allergen proteins was performed *in silico* using the PeptideMass (Exapsy) (https://web.expasy.org/peptide_mass/) software.

3 RESULTS AND DISCUSSION

3.1 Protein extraction and allergens identification in spirulina powder and food supplements

To assess the best performance in terms of the number of extracted proteins and allergens recognition, two methods for protein extraction from spirulina powder were explored: (i) aqueous Tris-HCl buffer solution (Bianco, Calvano, et al., 2022) and (ii) cold acetone solvent (De Ceglie et al., 2014). Digests of protein extracts obtained using each protocol were analyzed by reversed-phase liquid chromatography coupled to high resolution/accuracy mass spectrometry with electrospray ionization, operated in positive ion mode, and using a data-dependent method for tandem MS analysis (RPLC-ESI(+)-FT-ddMS/MS). Collected raw files were processed using Proteome Discoverer™, applying the specific spirulina *species* database (*i.e. A. platensis*) obtained from Uniprot and reduced to remove redundancy by an in-house written MATLAB® pipeline. Here, identical proteins having the same amino acid sequences but registered with different accession numbers were removed, thus leaving only a single one in each case. The original database of *A. platensis* contained 25,226 protein sequences while after redundancy removal 23,354 sequences were worked out. A comparison of the results obtained for both extraction methods revealed that the use of Tris-HCl buffer allowed the

identification of a greater number of algal proteins (127) compared to cold acetone precipitation (26). The lists of identified proteins are reported in **Table S1** and **Table S2** (Supplementary Material). Accordingly, the Tris-HCl buffer was applied throughout this work to all the examined spirulina-containing samples.

Spirulina digests of protein extracts obtained from the investigated food supplements were also analyzed *via* RPLC-ESI(+)-FT-ddMS/MS and the raw files were processed for protein search exploiting the reduced database of *A. platensis*. The complete lists of identified proteins in spirulina powder, tablets, capsules, and sticks with coverage up to 10% are respectively reported in **Tables S1**, **S3**, **S4**, and **S5** (Supplementary Material). Although 127 proteins were identified in the sample of spirulina powder, the number was relatively lower in the case of tablets, capsules, and sticks, namely 50, 103, and 104, respectively. **Figure S1** shows the Eulero-Venn diagram reporting the identified proteins in all the analyzed food supplements containing spirulina. As expected, common proteins were the most abundant ones, namely, those involved in the photosynthesis process, such as phycocyanins, allophycocyanins, phycobilisomes, and photosystems (Glazer, 1989; Singh, Sonani, Prasad Rastogi, & Madamwar, 2015).

According to earlier results obtained from spirulina samples (Bianco, Ventura, et al., 2022), the inspection of recognized proteins in **Tables S1-S5** (Supplementary Material) highlights the occurrence of allergenic proteins (listed in bold). Among them, the well-known food allergen C-phycocyanin beta subunit (P72508) (Petrus et al., 2009) was identified in all four food supplements, together with proteins sharing high similarity with it according to FAO/WHO guidelines (FAO & WHO, 2001), *i.e.* proteins with the following UniProt codes: A9UKJ0, Q6XAW9, Q6XAW5, Q208D1, corresponding to Phycocyanin beta subunit. Among proteins exhibiting high similarity with known food allergens, two thioredoxins, *i.e.*, D4ZSU6 and

K1VP15, which are both analogous to a Maize allergen (Allergen.org code: Zea m 25), were identified in all the examined samples. Strikingly, the superoxide dismutase protein (C3V6P3), exhibiting a percentage of sequence identity (PID) of 40.7 % with a pistachio allergen (Pis v 4), was found only in the spirulina powder sample. Except for tablets containing spirulina, a triosephosphate isomerase (D5A635) and a glyceraldehyde-3-phosphate dehydrogenase (K1W168), matching with known fish food allergens Pan h 13 and Cra c 8 (Bianco, Ventura, et al., 2022), were both retrieved in all food supplements. While all mentioned allergens were identified in protein extracts obtained using the buffered Tris-HCl solution, only the C-phycocyanin beta subunit (P72508) and the Phycocyanin beta subunit (A9UKJ0) were recognized in cold acetone extracts (**Table S2,** Supplementary Material).

3.2 Potential allergen marker peptides of spirulina in food supplements

In **Table 1** are listed all the potential marker peptides of allergenic spirulina proteins recognized by *in silico* digestion using the Expasy PeptideMass tool (https://www.expasy.org/). Following the literature guidelines (Gu et al., 2018; Johnson et al., 2011), including reproducibility both in terms of extraction and ionization, some marker peptides were selected. Five potential marker peptides of the C-phycocyanin beta subunit were chosen (see **Table 1**). Among putative allergenic proteins, two marker peptides were retrieved for thioredoxins (D4ZU6/K1VP15), and six for superoxide dismutase (C3V6P3), five for triosephosphate isomerase (D5A635), and nine for glyceraldehyde-3-phosphate dehydrogenase (K1W168). The uniqueness and specificity of each peptide were delineated by querying BLASTp (*i.e.*, Basic Local Alignment Search Tool protein to protein) using the reviewed UniprotKB database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_

LOC=blasthome) and the ProteinProspector MS-Homology tool against SwissProt.2021.06.18 (https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology) (Gu et al., 2018; Nitride et al., 2019; Pilolli et al., 2020). As a result, a list of proteins containing the examined peptides was generated. Notably, putative allergenic proteins D4ZU6, K1VP15, C3V6P3, D5A635, and K1W168 are not yet listed in the SwissProt or reviewed UniProt databases. Therefore, several designated marker peptides were neither associated with any other proteins nor with putative allergenic proteins. This is the case of two marker peptides of thioredoxin proteins (D4ZU6/K1VP15), five superoxide dismutases (C3V6P3), four triosephosphate isomerases (D5A635), and six glyceraldehyde-3-phosphate dehydrogenases (K1W168). When more studies on this topic will be available, marker peptides reported in **Table 1** will be suitable for the identification and quantification of putative allergenic proteins. Since these proteins will be no longer deemed in this study, our attention was focused on C-phycocyanin beta subunit (P72508), which is listed in the SwissProt and reviewed UniProt DBs. The following three marker peptides were outlined by both BLASTp and MS-Homology, *i.e.,* sequences E_{115}TYLALGTPGSSVAVGVGK$_{133}$ at *m/z* 903.484^{2+} I_{44}TSNASTIVSNAAR$_{57}$ at *m/z* 702.875^{2+} and Y_{92}VTYAVFAGDASVLEDR$_{108}$ at *m/z* 938.459^{2+}. The potential occurrence of interferences due to peptides having isomeric amino acid mutations, such as isoleucine/leucine (I/L), glutamic acid/glutamine (E/Q), glutamic acid/lysine (E/K), glutamine/lysine (Q/K), isoleucine/asparagine (I/N), leucine/asparagine (L/N), and asparagine/aspartic acid (N/D) was assessed (Korte, Lepski, & Brockmeyer, 2016; Pilolli et al., 2020). To this purpose, *in silico* generated mutated amino acid sequences of P72508 marker peptides were processed by BLASTp and MS-Homology, but no proteins were rescued. Thus, E_{115}TYLALGTPGSSVAVGVGK$_{133}$, I_{44}TSNASTIVSNAAR$_{57}$, and Y_{92}VTYAVFAGDASVLEDR$_{108}$ are emblematic peptides of P72508 protein. The same was not true for peptides

SLFAEQPQLIAPGGNAYTSR and VVSQADTR, since they were generated also by other proteins. Indeed, three other proteins, with Uniprot code P07119, P14877, P20777, share the SLFAEQPQLIAPGGNAYTSR sequence with P72508, while proteins P84341 and P08039 share the VVSQADTR sequence. All these proteins are related to the Phycocyanin C beta subunit of other cyanobacteria species. Extracted ion current (XIC) chromatograms, referred to as ions of the selected marker peptides and obtained for the spirulina powder sample, are reported in **Figure 1A**. In the present chromatographic conditions, marker peptide ITSNASTIVSNAAR (*m/z* 702.875^{2+}) was eluted at 12.0 minutes, exhibiting the most intense peak generated from the C-phycocyanin beta subunit protein. The other two less intense marker peptides ETYLALGTPGSSVAVGVGK (*m/z* 903.484^{2+}) and YVTYAVFAGDASVLEDR (*m/z* 938.459^{2+}) exhibited, respectively, a retention time of 14.5 min and 15.7 min. To confirm the amino acid sequences of these marker peptides, higher-energy collisional dissociation (HCD) tandem mass spectra were collected in all the analysed samples. The analysis showed that HCD MS/MS spectra of the chosen marker peptides, I_{44}TSNASTIVSNAAR$_{57}$ at *m/z* 702.875^{2+}, E_{115}TYLALGTPGSSVAVGVGK$_{133}$ at *m/z* 903.484^{2+} and Y_{92}VTYAVFAGDASVLEDR$_{108}$ at *m/z* 938.459^{2+}, shown, respectively, in plots A-C of **Figure 2**, exhibited *y* and *b*-types main product ions, together with the a_2-b_2 couple, commonly observed in the HCD regime (Michalski, Neuhauser, Cox, & Mann, 2012).

3.3 Marker peptides of spirulina allergens in processed foodstuffs

To evaluate the matrix effect, cookies spiked with spirulina powder were prepared and the protein extraction protocol was carried out. Besides, to examine the influence of thermal stability on the marker peptides of spirulina, thus mimicking the production processes, homemade incurred cookies were prepared (Pilolli, De Angelis, & Monaci, 2018). Food

processing may affect allergenic proteins, inducing structural changes that include unfolding, aggregation, chemical modification, and cross-linking to matrix components (Korte, Oberleitner, & Brockmeyer, 2019). As demonstrated in the XIC chromatograms of **Figures 1B** and **1C**, the marker peptide ITSNASTIVSNAAR (*m/z* 702.875^{2+}) was observed in both spiked and incurred samples. Different absolute intensities were most likely due to matrix and/or processing effects. For the same reasons, ETYLALGTPGSSVAVGVGK (*m/z* 903.484^{2+}) and YVTYAVFAGDASVLEDR (*m/z* 938.459^{2+}), were not or barely detected in incurred cookies (see **Figure 1C**). Moreover, tandem mass spectra were acquired on peptides occurring in the tryptic digests related to protein extracts of spiked and incurred cookies using a low-resolution LIT MS analyzer harboring a conventional collisional induced dissociation (CID) cell. Typically, low collisional energy and a multiple reaction monitoring (MRM) acquisition mode is employed in proteomic studies to enhance sensitivity (J. Heick, Fischer, & Pöpping, 2011; Korte & Brockmeyer, 2016) and were thus applied also in the present case. Starting from the major *y*-type product ions detected in FT tandem mass spectra of ETYLALGTPGSSVAVGVGK (*m/z* 903.484^{2+}) and YVTYAVFAGDASVLEDR (*m/z* 938.459^{2+}) peptides, it was possible to confirm their occurrence in both processed samples using CID-MS/MS and MRM. **Figure 3** shows the extracted ion currents of y_{11} (PGSSVAVGVGK), y_{13} (GTPGSSVAVGVGK), and y_{14} (LGTPGSSVAVGVGK) product ions related to the precursor ion at *m/z* 903.5^{2+} (plot A) and y_9 (GDASVLEDR), y_{10} (AGDASVLEDR) and y_{11} (FAGDASVLEDR) related to the ion at *m/z* 938.5^{2+} (plot B) in spiked cookies. Likewise, the same product ions were observed in incurred samples and are displayed in **Figure S2**. Compared to the more stable ITSNASTIVSNAAR at *m/z* 702.875^{2+}, the detection of both P72508 marker peptides is possible by using the proposed approach, despite they are extremely susceptible to matrix effects and processing, including heating. Conceivably, these three C-phycocyanin beta subunit marker peptides were

considered throughout this work. Specifically, peptides ETYLALGTPGSSVAVGVGK (*m/z* 903.484^{2+}) and YVTYAVFAGDASVLEDR (*m/z* 938.459^{2+}) were chosen as "qualifier" markers and detected by MRM acquisition mode according to other allergens detection methods (Hands, Sayers, Nitride, Gethings, & Mills, 2020; Korte, Monneuse, et al., 2016). The peptide ITSNASTIVSNAAR (*m/z* 702.875^{2+}) was designated as a "quantifier" marker, being much less affected by matrix and processing treatments; it can be thus used for method validation of allergenic protein P72508 in commercial foodstuffs.

The stability of the chosen marker peptides was ascertained on other foodstuffs, including homemade incurred bread, and commercial pasta and crackers containing spirulina as an ingredient. **Figure S3** reports XIC chromatograms of the quantifier peptide at *m/z* 702.875^{2+} in crackers, bread, and pasta samples, plots A, B, and C, respectively. The existence of other signals at the same *m/z* ratio can be inferred in both plots A and C of **Figure S3**, most likely related to isobaric/isomeric peptides arising from other proteins included in commercial crackers and pasta samples, as confirmed by MS/MS analysis. As expected, the quantifier marker peptide at *m/z* 702.875^{2+} is distinguishable without interfering signals.

3.4 Method validation to identify and quantify allergenic proteins of spirulina

Typically, primary food processing including heating, pressurization, and sterilization is necessary to make most foods edible. This food processing may lead to the modification of proteins. The proposed methodology was tested on a variety of samples to authenticate the presence/absence of marker peptides of spirulina allergenic proteins in both native and processed foods. Spirulina powder, incurred and spiked cookies, as well as spiked fruit juice samples, were analyzed in the same experimental conditions for method validation. Linearity, precision, matrix and processing effects, recovery, the limits of detection (LOD) and quantitation (LOQ), both expressed as $\mu g_{TotProt}/g_{matrix}$ were established for spiked and incurred

samples (Bianco, Calvano, et al., 2022; Pilolli et al., 2018). All parameters were evaluated using the peptide ITSNASTIVSNAAR, chosen as a quantifier marker of the phycocyanin allergenic protein. Spiked samples of cookies and fruit juice were prepared by adding spirulina powder before protein extraction, while incurred cookies were obtained by mixing spirulina powder with other ingredients during biscuits dough preparation. All samples were prepared in the concentration range 0.5-8.8 $mg_{TotProt}/g_{matrix}$. Calibration curves for spirulina powder, prepared in the same concentration range, spiked and incurred cookies, and spiked fruit juice were obtained by interpolating peak areas of the quantifier marker peptide (ITSNASTIVSNAAR) at *m/z* 702.875^{2+} versus concentration. LOD and LOQ were defined for all samples, except for powder solutions, considering the composition and features of the different foodstuffs. LOD and LOQ were calculated respectively as three and ten times the ratio between the intercept standard deviation and slope (Miller & Miller, 2010) of calibration curves of spiked and incurred samples acquired over a defined concentration range (Pilolli et al., 2018). These data are reported in **Table 2**. Good linearity was observed in spiked and incurred samples, with correlation coefficients better than 0.990 in all cases. The proposed analytical method allowed us to obtain LOD and LOQ values very suitable to identify and quantify allergenic proteins of spirulina according to its common content as ingredient in several processed food products, that generally ranges between 3-6% (w/w) in pasta products, 2-3% in cookies, and 0.8-2% in crackers. The marketed products such as crackers may contain spirulina flour at a content of 0.8% w/w which corresponds to 8 $mg_{spirulina}/g_{matrix}$; if an averaged amount of total protein equal to 60% is considered, this addiction leads to 4.8 $mg_{TotProt}/g_{matrix}$, i.e. 4800 ppm. The lowest concentration experimented with the present protocol is approximately 550 ppm, being about ten times lower than that added to foodstuffs. Therefore, the method might be able to identify spirulina allergens due to cross-contamination. Furthermore, recovery was

evaluated for solid/liquid sample matrices by rationing the peak area of the quantifier marker peptide at *m/z* 702.875^{2+} obtained from spiked cookies/fruit juices and the one retrieved for the corresponding protein extracts fortified with spirulina. Two absolute amounts (2.5 $\mu g_{TotProt}$ and 44 $\mu g_{TotProt}$ i.e. 25 µg/mL and 440 µg/mL) were evaluated and data obtained from both samples are summarized in **Table 3**. Recoveries ranged from 30 to 87% for cookies and from 49 to 107% for fruit juice. The recovery of proteins from spirulina samples was investigated by Parimi et al. (Parimi et al., 2015). It was found that it depends on pH that affects the solubility, being the result of protein denaturation and clustering with an irregular trend and on the incomplete cell lysis, which does not release the intracellular proteins. This last factor can be critical at lower protein concentrations since the membrane disruption can be hindered by the elevated occurrence of non-proteic components. The used protocol is still efficient for spirulina allergen extraction also at lower concentrations, especially for liquid samples. The analytical repeatability and reproducibility, including extraction and digestion steps of spirulina proteins along with the stability of selected marker peptides were assessed by analyzing three independent extracts of cookies and fruit juice, preliminarily spiked at a concentration level of 4.4 $mg_{Tot.Prot.}/g_{matrix}$ by injecting each sample three times for three working days. The intra-day and inter-day variabilities were evaluated respectively within and between spiked samples for chosen quantifier marker peptide, calculating the RSD values on peak area obtained from XIC chromatograms. The RSD values for cookies and fruit juice samples were 1.4-5.2 and 1.1-3.4 for repeatability (intra-day within-sample) respectively, suggesting a negligible instrumental variability while were 13.2-18.4 and 11.5-15.7 for reproducibility, respectively. Moreover, the matrix effect was assessed for both cookies and fruit juice samples. The matrix effect was evaluated in terms of a ratio between the calibration curves' slope of the spiked sample and the spirulina powder solution, following the reported

455 method by Pilolli et al. (Pilolli et al., 2018). Reported data in **Table 3** suggest limited matrix
456 effects for both cookies (72.6±1.9%) and fruit juice (78.3±1.5%). In contrast with other
457 allergenic proteins found in milk, egg, peanut, hazelnut, and soybean (Pilolli et al., 2018;
458 Planque et al., 2017), the signal intensity of the P72508 marker peptide, at least in the samples
459 examined in this study, was minimally affected. The processing effect, basically due to thermal
460 heating (baking), was assessed for cookies in terms of the percentage ratio between the slopes
461 of the calibration lines obtained for incurred and spiked cookies. The combined effect (*i.e.* due
462 to both matrix and processing) was evaluated for cookies as the percentage ratio of the slopes
463 of calibration lines obtained for incurred cookies and spirulina powder solutions (see **Table 3**)
464 (Bianco, Calvano, et al., 2022). A significant signal intensity decrease of the chosen marker
465 peptide, due to the baking process, was observed, which means a processing effect much
466 greater than the matrix one. Since matrix and processing effects can contribute to the
467 variation of signal intensity of marker peptides, these effects should be considered very
468 carefully for each type of investigated matrix, to obtain a reliable allergen quantification. Both
469 the effects should be calculated for each foodstuff under investigation considering the
470 different food compositions and treatments that impact the final analytical signal of the
471 allergenic peptides. To quantify an allergenic protein, calibration curves should be built for
472 spiked and/or incurred samples and properly applied to calculate the signal variation due to
473 matrix and/or processing effects.
474 To date, no relevant clinical limits have been defined for spirulina as an allergenic food
475 ingredient capable of causing adverse reactions, due to the low number of people sensitized
476 to this microalga. It is likely that in the future, with increasing consumption of spirulina
477 products, the number of allergic subjects will increase, and clinical limits might be defined as
478 happened with milk, egg, soy, and others (Allen et al., 2014).

4 CONCLUSIONS

A new analytical protocol of unprocessed spirulina-containing food supplements was devised that includes extraction, enzymatic digestion of proteins, and LC-ESIMS/MS analysis, which allowed us to establish the occurrence of reliable marker peptides of allergenic proteins. These marker peptides were adopted to quantify the same allergen in a variety of food products. *In silico* evaluation and careful selection criteria, ensured that each marker peptide sequence is conserved and unique for spirulina proteins and three marker peptides of the allergenic C-phycocyanin beta subunit (P72508) were chosen. The proposed method was validated with commercial samples such as pasta and crackers and with spirulina-incurred homemade bread, leading to important implications for manufacturing certification and food labeling purposes. To the best of our knowledge, this is the first study proposing reliable marker peptides for allergenic protein detection in novel food matrices like microalgae.

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Conflict of interest statement

The authors have declared that no competing interest exists.

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660

Table 1. Peptides identified of putatively allergenic proteins in the tryptic digests of the spirulina (*A. platensis*) protein extracts: (A) known allergenic protein C-phycocyanin beta subunit (P72508), and (B) proteins having high similarity with known food allergens.[a] Possible marker peptides are reported in bold.

	Protein	Peptide	Assigned to protein by MS-homology & BLASTp	Uniqueness
A	**P72508**	**ETYLALGTPGSSVAVGVGK**	✓	✓
	C-phycocyanin	**ITSNASTIVSNAAR**	✓	✓
	beta subunit	**YVTYAVFAGDASVLEDR**	✓	✓
		SLFAEQPQLIAPGGNAYTSR	✓	X
		VVSQADTR	✓	X
B[b]	**D4ZSU6/K1VP15**	**LNTDENPQVASQYGIR**	X	✓
	thioredoxins	**STLANTLEK**	X	✓
	C3V6P3	**TANAVNPMVEGK**	X	✓
	superoxide	**SGIFNNAAQVWNHSFFWK**	X	✓
	dismutase	**TPLLTLDVWEHPLLSELP**	X	✓
		IQADFGSFDAFIQEFK	X	✓
		ATYNDPSK	X	✓
		HHAAYVK	X	X
	D5A635	**QYFGETNETVNLR**	X	✓
	triosephosphate	**TQAEAQEFLK**	X	✓
	isomerase	**AAQNHGLTPILCVGETK**	X	✓
		FVVIGHSER	X	✓
		IIIAGNWK	X	X
	K1W168	**GILAYSDLPLVSSDYR**	X	✓
	glyceraldehyde-	**VPTPNVSVVDFVAQVEK**	X	✓
	3-phosphate	VVDLAEIVAK	X	X
	dehydrogenase	VVAWYDNEWGYSQR	X	X
		VLQDNFGIIK	X	✓
		TTFTEEVNK	X	✓
		VLITAPGK	X	X
		GENTNLEVTAINDTSDPR	X	✓
		VPTPNVSVVDFVAQVEK	X	✓

[a] Proteins with high similarity with known food allergens: thioredoxins D4ZSU6 and K1VP15, superoxide dismutase (C3V6P3), triosephosphate isomerase (D5A635), and glyceraldehyde-3-phosphate dehydrogenase (K1W168). [b] Proteins not yet contained in the SwissProt or the reviewed UniProt database; for this reason, MS-Homology & BLASTp do not assign these peptides to proteins from which they are generated.

669

670 **Table2.** Calibration curve parameters, limits of detection (LOD) and quantification (LOQ),
671 expressed as $\mu g_{TotProt}/g_{matrix}$, of incurred and spiked samples considering **ITSNASTIVSNAAR**
672 as marker peptide of the C-phycocyanin beta subunit allergen.

Sample	Slope	R^2	LOD $\mu g_{Tot.Prot.}/g_{matrix}$	LOQ $\mu g_{Tot.Prot.}/g_{matrix}$
Incurred cookies	$(207\pm6)*10^3$	0.990	450	1499
Spiked cookies	$(307\pm7)*10^4$	0.994	336	1121
Spiked fruit juice	$(331\pm4)*10^4$	0.998	196	653

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678 **Table3.** Recovery, matrix, processing, and combined effects in spiked cookies and fruit juice.

Sample	Recovery % (2.5 $\mu g_{TotProt}$)	Recovery % (44 $\mu g_{TotProt}$)	Matrix effect	Processing effect	Combined effect
Cookies	30±2*	87±2	72.6±1.9	6.7±0.3	4.9±0.2
Fruit juice	49±2	107±15	78.3±1.5	-	-

679 *RSD values were calculated on three replicate analyses.

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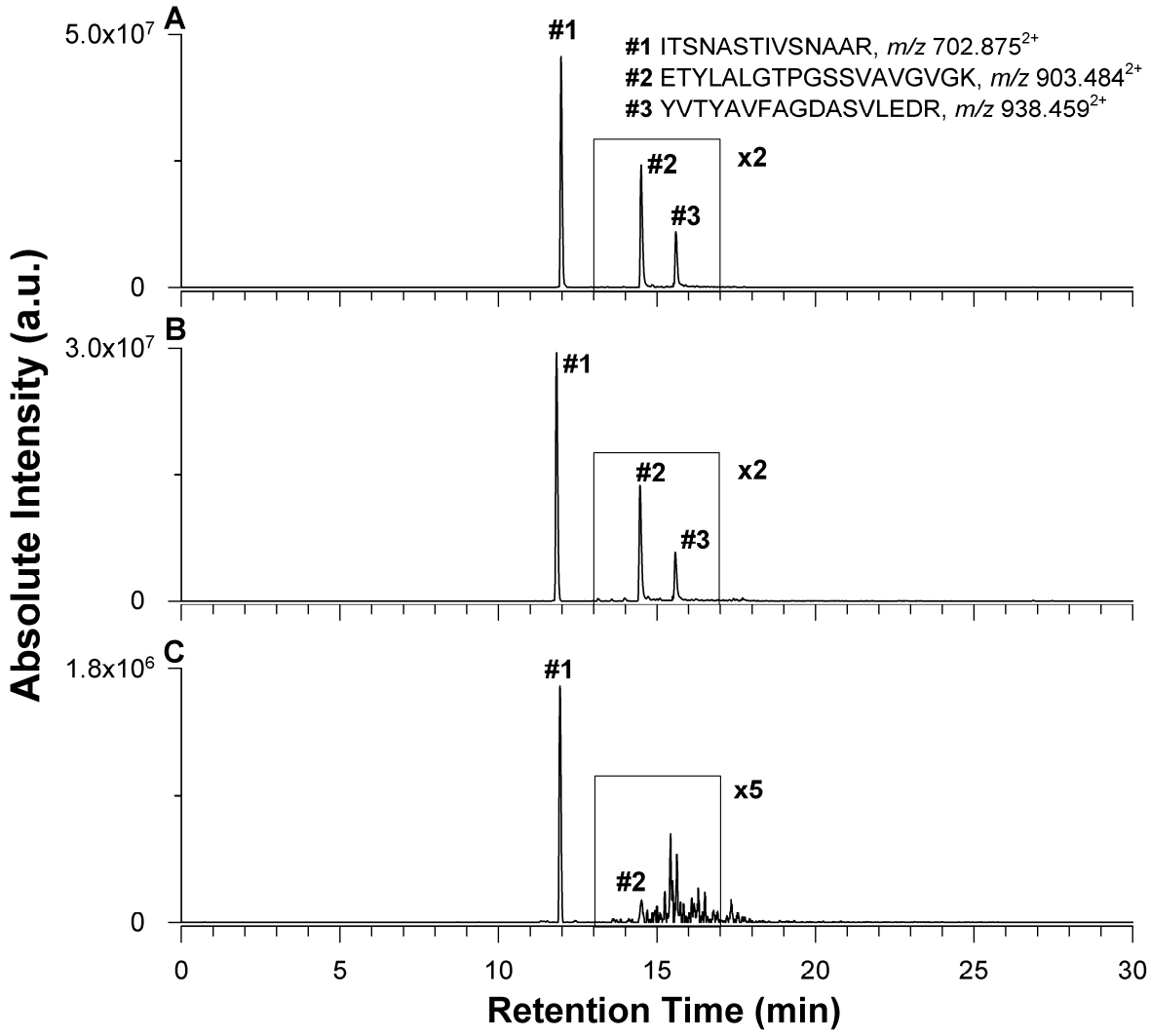

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684 **Figure 1**. XIC chromatograms by RPLC-ESI-FTMS in positive ion mode of the marker peptides
685 of C-phycocyanin beta subunit (P72508) ITSNASTIVSNAAR at *m/z* 702.875^{2+} (#1),
686 ETYLALGTPGSSVAVGVGK at *m/z* 903.484^{2+} (#2), and YVTYAVFAGDASVLEDR at *m/z* 938.459^{2+}
687 (#3) occurring in spirulina powder samples (**A**), spiked cookies (**B**), and incurred cookies (**C**).
688 Traces were obtained using an extraction window of 10 ppm centered on the monoisotopic
689 *m/z* ratio of each protonated peptide.

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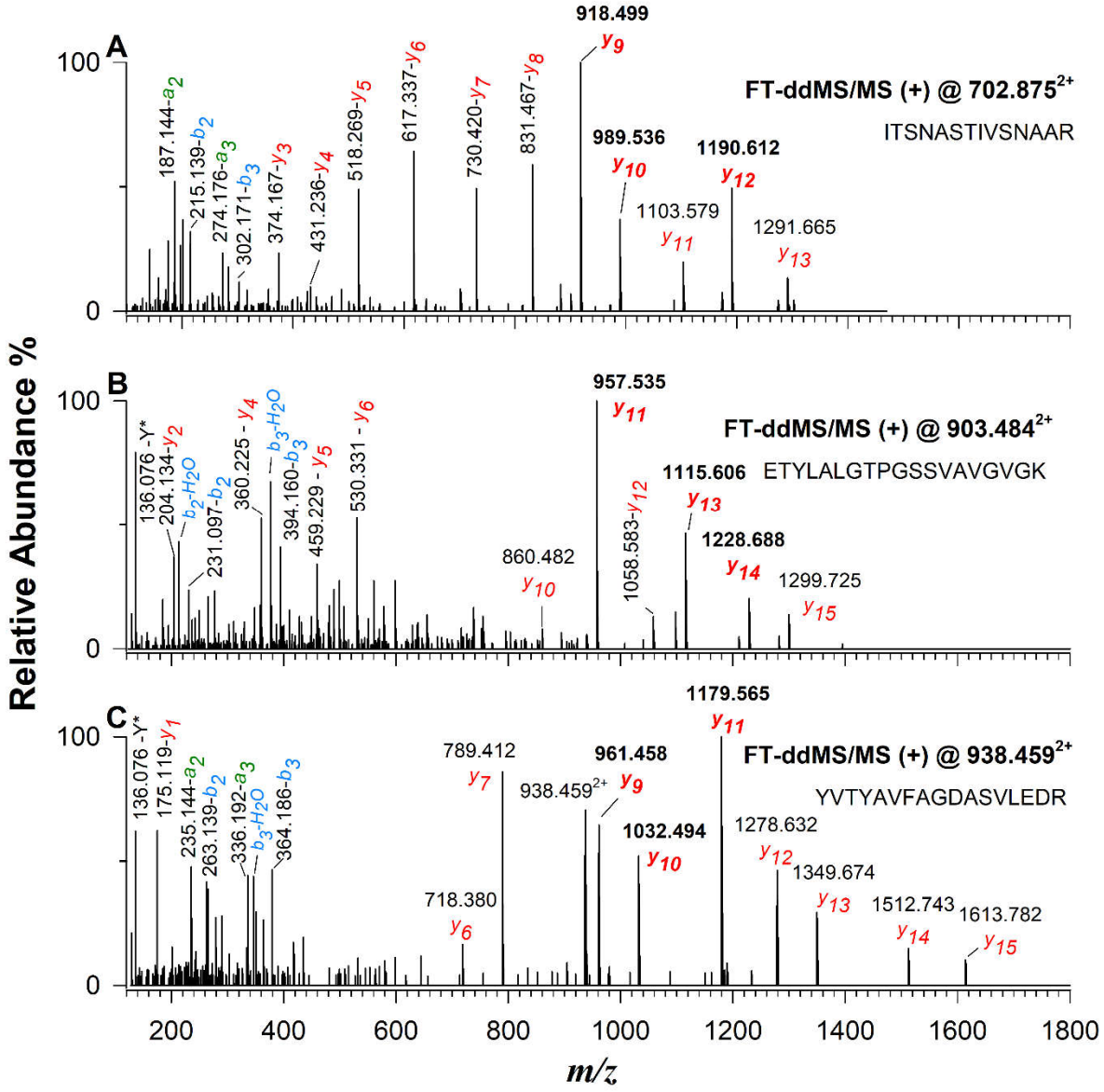


692

693 **Figure 2**. High-resolution HCD-MS/MS spectra of marker peptides of the spirulina allergenic

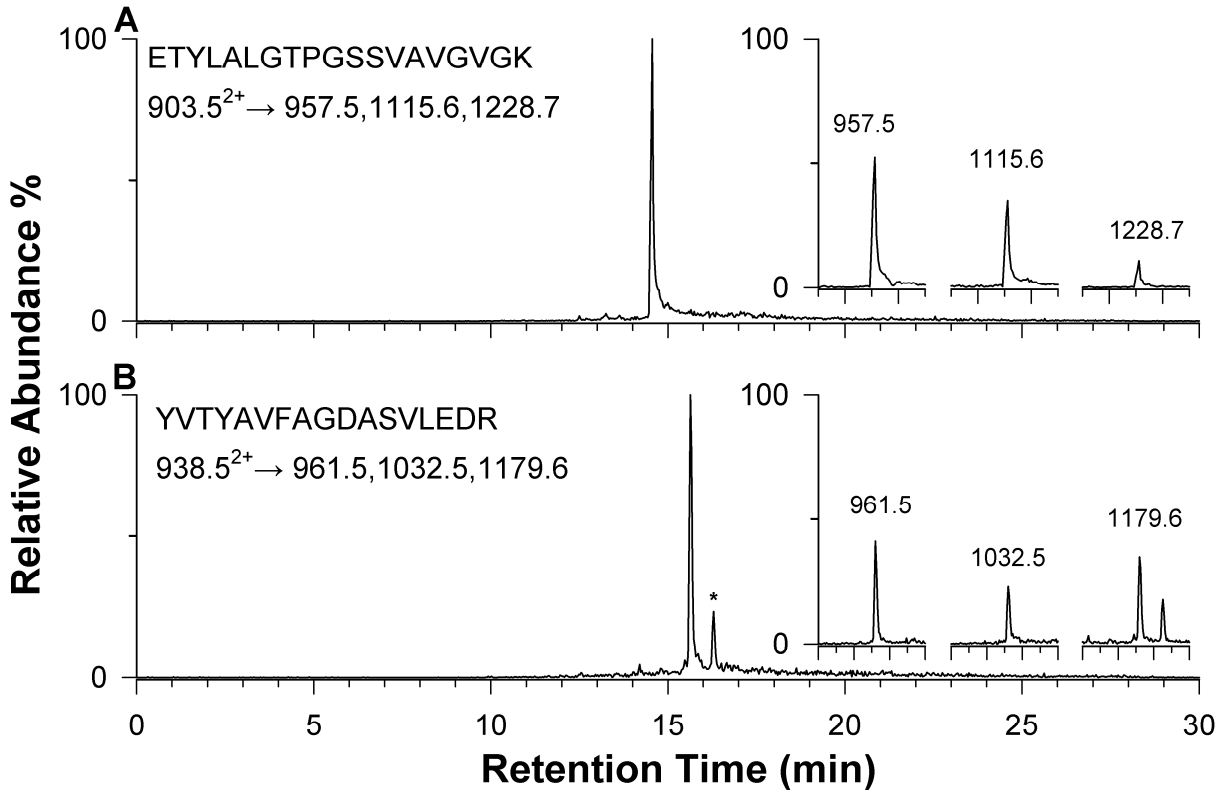
694 protein P72508 (C-phycocyanin beta subunit): (A) ITSNASTIVSNAAR at *m/z* 702.875^{2+}, (B)

695 ETYLALGTPGSSVAVGVGK at *m/z* 903.484^{2+}, and (C) YVTYAVFAGDASVLEDR at *m/z* 938.459^{2+}.

696 Major y-type product ions, useful for multiple ion monitoring (MRM) acquisitions, are labeled

697 in bold.

698



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701 **Figure 3**. Chromatograms by RPLC-ESI-LIT-MS/MS in multiple reaction monitoring (MRM) of
702 marker peptides of the spirulina allergenic protein C-phycocyanin beta subunit in spiked
703 cookies at 2.5 $mg_{TotProt/gmatrix}$: (A) ETYLALGTPGSSVAVGVGK at *m/z* 903.5^{2+}, and (B)
704 YVTYAVFAGDASVLEDR at *m/z* 938.5^{2+}. Major *y-type* product ions detected in CID-MS/MS
705 spectra were used to extract the ion currents (see bold product ions in **Figure 2**). Insets show
706 the individual measured transitions.

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Supplementary information

Discovery of marker peptides of spirulina microalgae proteins for allergen detection in processed foodstuffs

Mariachiara Bianco[1], Giovanni Ventura[1], Cosima D. Calvano[1,2*], Ilario Losito[1,2], Tommaso R.I. Cataldi[1,2]

[1]Dipartimento di Chimica, [2]Centro interdipartimentale SMART, Università degli Studi di Bari Aldo Moro, via Orabona 4, 70126, Bari, Italy

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Author for correspondence, email: cosimadamiana.calvano@uniba.it

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742 **Table S1.** Identified proteins in a sample of spirulina powder (*A. platensis*)
743 extracted using an aqueous buffer solution of Tris-HCl. Possible allergenic
744 proteins are reported in bold.

ACCESSION	PROTEIN	COVERAGE %
B1NJ40	Phycocyanin beta subunit (Fragment)	100
A0A0C4VYY9	ApcA protein	84
A0A4Y5T2P3	Phycocyanin alpha subunit (Fragment)	83
T1YX51	Allophycocyanin beta-subunit	74
A0A0C4W3I9	CpcA protein	71
G9C3E8	Phycocyanin beta chain	58
P72508	**C-phycocyanin beta subunit**	**58**
A9UKJ0	**Phycocyanin beta subunit**	**58**
Q6XAW9	**Phycocyanin beta subunit (Fragment)**	**56**
Q6XAW5	**Phycocyanin beta subunit (Fragment)**	**56**
K1W174	Nucleoid-associated protein	52
D4ZSU6	**Thioredoxin**	**50**
K1VP15	**Thioredoxin**	**50**
Q5SCD5	**Phycocyanin beta subunit (Fragment)**	**48**
G9BN03	GvpC	46
Q208D1	**Phycocyanin beta chain**	**45**
K1WBU5	10 kDa chaperonin	45
K1W7U2	Cytochrome c-550	44
K1X5W6	Elongation factor Tu	42
D4ZR56	RNA-binding protein	40
K1VXV4	Histone-like DNA-binding protein	37
K1X7B8	50S ribosomal protein L7/L12	37
K1WZD2	Photosystem I reaction center subunit II	36
D5A424	Phycobilisome 7.8 kDa linker polypeptide,	36
A0A4Y7LLS6	Ribosome hibernation promoting factor	33
K1X048	Glutathione-dependent peroxiredoxin	32
D4ZZN4	Uncharacterized protein	32
A0A4Y5T334	Phycocyanin beta subunit (Fragment)	32
K1W1T8	Elongation factor Ts	31
D4ZZD6	Uncharacterized protein	30
D4ZZX7	30S ribosomal protein S21	28
K1X8N9	ATP synthase subunit alpha	27
K1VVX1	Nucleoside diphosphate kinase	27
K1W1R1	Beta-18 Phycobilisome core subunit	27
K1VYQ4	Phosphoglycerate kinase	26
D5A193	Photosystem II 12 kDa extrinsic protein	26
S4UUT6	Nitrogen regulatory protein P-II	25
K1VTM7	Uncharacterized protein	24
K1WCN6	Uncharacterized protein	24
A0A4Y7LKB7	ATP synthase subunit beta	24
K1VPJ3	50S ribosomal protein L9	24

D4ZR96	D-fructose 1,6-bisphosphatase class	23
A0A3G2BBA0	Gas vesicle protein C	23
D4ZMN8	DNA-binding protein	22
K1VSS0	Phycobilisome linker polypeptide	22
K1W8A9	Cytochrome b559 subunit alpha	22
K1W460	Alkyl hydroperoxide reductase/ Thiol specific	22
A0A0C4W3G7	CpcI protein	22
K1WLU1	60 kDa chaperonin	21
K1X2B2	Uncharacterized protein	21
D4ZPG9	50S ribosomal protein L29	21
D5A649	Cytochrome c6	21
K1WP33	30S ribosomal protein S6	21
D4ZNG4	Photosystem II manganese-stabilizing	21
K1X5E2	Fructose-1,6-bisphosphate aldolase	21
K1WH83	Putative RNA-binding region protein	20
K1WET8	Phosphoribulokinase	20
D4ZXK1	50S ribosomal protein L21	20
K1WBP7	Phycobiliprotein ApcE	19
C7DQP7	CpcD (Fragment)	19
K1WAM8	ATP synthase subunit b	19
A0A4Y7LIW3	Ketol-acid reductoisomerase (NADP(+))	18
D4ZR50	Putative bacterioferritin comigratory protein	18
C3V6P3	**Superoxide dismutase (Fragment)**	**18**
K1W168	**Glyceraldehyde-3-phosphate dehydrogenase**	**18**
K1X3I4	Uncharacterized protein	18
D4ZWF8	SpoVT-AbrB domain-containing protein	17
K1W2D0	30S ribosomal protein S16	17
K1W9C7	50S ribosomal protein L1	17
K1WD80	Transketolase	17
K1WDU3	Acyl carrier protein	17
K1W3Y1	Uncharacterized protein	17
D4ZYS5	Ferredoxin--NADP reductase	17
K1X441	Uncharacterized protein	17
K1W362	Peptidyl-prolyl cis-trans isomerase	17
K1W1I3	50S ribosomal protein L23	17
K1WT22	Flavin reductase-like, FMN-binding protein	16
K1WD48	50S ribosomal protein L24	16
K1VYN4	PpiC-type peptidyl-prolyl cis-trans isomerase	15
K1VQH4	RNP-1 like RNA-binding protein	15
D4ZP30	2Fe-2S ferredoxin	15
K1X603	Putative DNA-binding protein HU-beta	15
D4ZNY4	CP12 domain-containing protein	15
K1WPY7	Adenylate kinase	15
K1WC13	60 kDa chaperonin	15
K1VV20	Ribulose-bisphosphate carboxylase	14
A0A6H9GWQ9	Photosystem I reaction center subunit IX	14
K1VXA7	ATP synthase subunit delta	14
D4ZNP7	Ribulose-phosphate 3-epimerase	14
A0A6H9GGU0	Uncharacterized protein	14

K1WT08	Peptidyl-prolyl cis-trans isomerase	14
D4ZUX6	30S ribosomal protein S10	13
A0A6H9GKP9	Uncharacterized protein	13
K1WF39	Glycine cleavage system H protein	13
K1VVC8	Trigger factor	13
D4ZSV0	Uncharacterized protein	13
K1VYM2	Uncharacterized protein	13
K1VPI8	Uncharacterized protein	13
A0A6H9H3H4	RHH_1 domain-containing protein	13
K1WD08	Uncharacterized protein	12
K1VVN1	Carbon dioxide concentrating mechanism	12
K1VYM5	Glutamine synthetase	12
D4ZVX9	Uncharacterized protein	12
P13550	Elongation factor G	12
K1WD53	30S ribosomal protein S3	12
D4ZPH7	30S ribosomal protein S5	12
D4ZTM3	Usp domain-containing protein	12
K1WPC6	Ribose-5-phosphate isomerase A	11
K1WDR4	Enolase	11
K1W8D0	ATP synthase subunit b	11
Q6WJF7	Superoxide dismutase (Fragment)	11
D4ZMS9	Uncharacterized protein	11
D4ZQA8	Photosystem I iron-sulfur center	11
K1WG10	Uncharacterized protein	11
A0A2U3HXL5	Adenosylhomocysteinase	11
A0A6H9GCD2	Uncharacterized protein	11
K1WJN7	50S ribosomal protein L11	11
D4ZPH4	30S ribosomal protein S8	11
K1VQN1	PSI-F	10
K1W3H8	Single-strand binding protein	10
D4ZS76	Probable 30S ribosomal protein PSRP-3	10
K1X4P0	Serine hydroxymethyltransferase	10
D5A0F4	Uncharacterized protein	10
K1XDI1	30S ribosomal protein S11	10
D4ZPP8	Photosystem II reaction center Psb28 protein	10
D4ZVQ6	Molybdopterin biosynthesis protein	10
D5A635	**Triosephosphate isomerase**	**10**

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747 **Table S2.** Identified proteins in a sample of spirulina powder (*A. platensis*)
748 extracted by cold acetone precipitation. Possible allergenic proteins are
749 reported in bold.

ACCESSION	PROTEIN	COVERAGE %
A0A0C4VYY9	ApcA protein	54
A0A5B8AFT4	C-phycocyanin alpha subunit	43
G9BN03	GvpC	35
A0A4Y5T334	Phycocyanin beta subunit (Fragment)	32
P72508	**C-phycocyanin beta subunit**	**27**
K1W8A9	Cytochrome b559 subunit alpha	27
G9C3E8	Phycocyanin beta chain	27
A9UKJ0	**Phycocyanin beta subunit**	**27**
T1YX51	Allophycocyanin beta-subunit	22
K1VSS0	Phycobilisome linker polypeptide	22
K1X5W6	Elongation factor Tu	21
K1WZD2	Photosystem I reaction center subunit II	18
S4UUT6	Nitrogen regulatory protein P-II	17
K1W1Z4	Photosystem II lipoprotein Psb27	15
B2BN96	CpcD	15
D5A649	Cytochrome c6	15
A0A4Y7LLS6	Ribosome hibernation promoting factor	15
K1WIE0	Ribulose bisphosphate carboxylase large chain	14
A0A0C4W3G7	CpcI protein	13
K1W1T8	Elongation factor Ts	12
D4ZPH8	50S ribosomal protein L15	12
Q6WJF7	Superoxide dismutase (Fragment)	11
D4ZXK1	50S ribosomal protein L21	11
K1X5E2	Fructose-1,6-bisphosphate aldolase	10
D4ZWF8	SpoVT-AbrB domain-containing protein	10
K1VYQ4	Phosphoglycerate kinase	10

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753 **Table S3**. Identified proteins in tablet extracts containing spirulina (*A.*
754 *platensis).* Possible allergenic proteins are reported in bold.

ACCESSION	PROTEIN	COVERAGE %
A0A0C4VYY9	ApcA protein	84
Q6XAW9	**Phycocyanin beta subunit (Fragment)**	**56**
Q6XAW5	**Phycocyanin beta subunit (Fragment)**	**56**
P72508	**C-phycocyanin beta subunit**	**53**
A9UKJ0	**Phycocyanin beta subunit**	**53**
T1YX51	Allophycocyanin beta-subunit	53
P72509	C-phycocyanin alpha subunit	53
Q5SCD5	**Phycocyanin beta subunit (Fragment)**	**48**
K1WBU5	10 kDa chaperonin	45
D4ZSU6	**Thioredoxin**	**41**
D4ZZN4	Uncharacterized protein	32
A0A4Y5T334	Phycocyanin beta subunit (Fragment)	32
D4ZS76	Probable 30S ribosomal protein PSRP-3	31
D4ZR56	RNA-binding protein	30
K1VV20	Ribulose-bisphosphate carboxylase	30
E0Z0C7	CcmK1	29
K1W1R1	Beta-18 Phycobilisome core subunit	28
K1X048	Glutathione-dependent peroxiredoxin	24
D4ZMN8	DNA-binding protein	22
K1W475	Photosystem I reaction center subunit IV	22
D4ZXX3	50S ribosomal protein L7/L12	22
D4ZV18	Cytochrome c-550	21
D4ZUX7	Elongation factor Tu	20
D5A649	Cytochrome c6	19
D4ZQB0	Acyl carrier protein	17
K1WDU3	Acyl carrier protein	17
I3RMQ3	Phycocyanin beta subunit (Fragment)	17
C7DT12	CpcI	15
K1X5E2	Fructose-1,6-bisphosphate aldolase	15
D5A2Q1	CaMKII_AD domain-containing protein	15
D4ZTI9	Uncharacterized protein	14
D5A193	Photosystem II 12 kDa extrinsic protein	14
A0A6H9GGU0	Uncharacterized protein	14
K1WZD2	Photosystem I reaction center subunit II	13
K1WF39	Glycine cleavage system H protein	13
D5A0Q3	ATP synthase subunit alpha	13
K1X8N9	ATP synthase subunit alpha	13
D4ZR93	Thioredoxin	13
D4ZR96	D-fructose 1,6-bisphosphatase class 2/sedoheptulose 1,7-bisphosphatase	13
D4ZR75	30S ribosomal protein S6	12
D4ZNP7	Ribulose-phosphate 3-epimerase	12
D4ZTM3	Usp domain-containing protein	12
D4ZMS9	Uncharacterized protein	11
D4ZQA8	Photosystem I iron-sulfur center	11

D4ZR50	Putative bacterioferritin comigratory protein	11
K1W8A9	Cytochrome b559 subunit alpha	11
A0A4Y7LKB7	ATP synthase subunit beta	11
A0A3G2BBC4	Gas vesicle protein C	10
D4ZRX7	Uncharacterized protein	10
K1VU04	Uncharacterized protein	10

758 **Table S4**. Identified proteins in capsules extracts containing spirulina (*A.*
759 *platensis).* Possible allergenic proteins are reported in bold.

ACCESSION	PROTEIN	COVERAGE %
A0A4Y5T2P3	Phycocyanin alpha subunit (Fragment)	83
A0A0C4VYY9	ApcA protein	67
P72508	**C-phycocyanin beta subunit**	**66**
A9UKJ0	**Phycocyanin beta subunit**	**66**
Q6XAW9	**Phycocyanin beta subunit (Fragment)**	**64**
Q6XAW5	**Phycocyanin beta subunit (Fragment)**	**64**
P72509	C-phycocyanin alpha subunit	56
T1YX51	Allophycocyanin beta-subunit	53
Q208D1	**Phycocyanin beta chain**	**53**
K1WBU5	10 kDa chaperonin	51
D4ZSU6	**Thioredoxin**	**50**
Q5SCD5	**Phycocyanin beta subunit (Fragment)**	**48**
K1X603	Putative DNA-binding protein HU-beta	47
D4ZV18	Cytochrome c-550	44
D4ZS76	Probable 30S ribosomal protein PSRP-3	37
K1WZD2	Photosystem I reaction center subunit II	37
D4ZUX6	30S ribosomal protein S10	34
D4ZR56	RNA-binding protein	33
D4ZUF5	Phycobilisome rod-core linker polypeptide	32
K1W2D0	30S ribosomal protein S16	32
Q8GLJ5	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	28
K1W7U2	Cytochrome c-550	28
A0A0C4W3G7	CpcI protein	28
K1WLU1	60 kDa chaperonin	26
D4ZZX7	30S ribosomal protein S21	26
K1W1R1	Beta-18 Phycobilisome core subunit	26
K1W168	**Glyceraldehyde-3-phosphate dehydrogenase**	**25**
K1X5E2	Fructose-1,6-bisphosphate aldolase	25
K1VV20	Ribulose-bisphosphate carboxylase	25
D5A243	Glyceraldehyde-3-phosphate dehydrogenase	25
D4ZXX3	50S ribosomal protein L7/L12	25
C7DT12	CpcI	23
D4ZR50	Putative bacterioferritin comigratory protein	23
D4ZPG9	50S ribosomal protein L29	23
D4ZMN8	DNA-binding protein	22
K1W1I3	50S ribosomal protein L23	22
K1W475	Photosystem I reaction center subunit IV	22
A0A6H9GII7	Thioredoxin	22
K1W8A9	Cytochrome b559 subunit alpha	22
K1X2B2	Uncharacterized protein	21
K1X5W6	Elongation factor Tu	20
D4ZUX7	Elongation factor Tu	20
K1VVX1	Nucleoside diphosphate kinase	20

K1X048	Glutathione-dependent peroxiredoxin	20
K1X8N9	ATP synthase subunit alpha	20
S4UUT6	Nitrogen regulatory protein P-II	20
D5A649	Cytochrome c6	19
D5A0Q6	ATP synthase subunit b	19
K1W1H7	50S ribosomal protein L5	19
A0A3G2BBC4	Gas vesicle protein C	19
A0A3G2BBA0	Gas vesicle protein C	19
K1VX49	Uncharacterized protein	19
K1W460	Alkyl hydroperoxide reductase/ Thiol specific antioxidant/	19
B2BN94	CpcH	18
D5A0Q3	ATP synthase subunit alpha	18
K1VYQ4	Phosphoglycerate kinase	18
A0A3G2BBA3	Gas vesicle structural protein	18
P13577	30S ribosomal protein S7	18
K1W174	Nucleoid-associated protein SPLC1_S532150	18
D5A0K0	Uncharacterized protein	17
D4ZQB0	Acyl carrier protein	17
K1WDU3	Acyl carrier protein	17
K1WAM8	ATP synthase subunit b	17
K1WET8	Phosphoribulokinase	16
A0A2U3HXL5	Adenosylhomocysteinase	15
D4ZPG2	50S ribosomal protein L4	15
I3RMQ8	Phycocyanin beta subunit (Fragment)	15
D4ZYS5	Ferredoxin--NADP reductase	15
K1W9C7	50S ribosomal protein L1	15
A0A4Y7LLS6	Ribosome hibernation promoting factor	15
K1VXA7	ATP synthase subunit delta	14
K1WP33	30S ribosomal protein S6	14
K1WBP7	Phycobiliprotein ApcE	14
K1W1G7	50S ribosomal protein L13	14
A0A4Y7LKB7	ATP synthase subunit beta	14
D4ZWE3	Phycobiliprotein ApcE	14
D5A424	Phycobilisome 7.8 kDa linker polypeptide, allophycocyanin-	13
K1WRJ6	Transketolase domain protein	13
K1WF39	Glycine cleavage system H protein	13
D4ZRE6	Starvation-inducible DNA-binding protein	13
K1VYM5	Glutamine synthetase	12
D5A327	60 kDa chaperonin	12
D4ZR75	30S ribosomal protein S6	12
D4ZNG4	Photosystem II manganese-stabilizing polypeptide	12
K1W8D0	ATP synthase subunit b	12
D4ZPG1	50S ribosomal protein L3	12
D4ZW50	Pentapeptide repeat-containing protein	12
K1VTF0	Cytochrome b6-f complex iron-sulfur subunit	11
D4ZQA8	Photosystem I iron-sulfur center	11

K1WDQ6	Ferredoxin	11
K1W1H1	30S ribosomal protein S13	11
D4ZXK1	50S ribosomal protein L21	11
D4ZPH7	30S ribosomal protein S5	11
D4ZPD7	Transketolase	11
K1WJN7	50S ribosomal protein L11	11
D4ZPH4	30S ribosomal protein S8	11
D5A0F4	Uncharacterized protein	10
K1XDI1	30S ribosomal protein S11	10
D5A6F5	Elongation factor Ts	10
D4ZPP8	Photosystem II reaction center Psb28 protein	10
K1WD48	50S ribosomal protein L24	10
K1WD08	Uncharacterized protein	10
D5A635	**Triosephosphate isomerase**	**10**

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762 **Table S5**. Identified proteins in stick extracts containing spirulina (*A. platensis).*
763 Possible allergenic proteins are reported in bold.

ACCESSION	PROTEIN	COVERAGE %
A0A0C4W3I9	CpcA protein	71
A0A0C4VYY9	ApcA protein	68
T1YX51	Allophycocyanin beta-subunit	61
Q6XAW9	**Phycocyanin beta subunit (Fragment)**	**60**
Q6XAW5	**Phycocyanin beta subunit (Fragment)**	**60**
P72508	**C-phycocyanin beta subunit**	**57**
A9UKJ0	**Phycocyanin beta subunit**	**57**
K1WBU5	10 kDa chaperonin	51
G9BN03	GvpC	50
K1VP15	**Thioredoxin**	**49**
Q5SCD5	**Phycocyanin beta subunit (Fragment)**	**48**
K1W1R1	Beta-18 Phycobilisome core subunit	47
K1W7U2	Cytochrome c-550	44
K1WGI4	Photosystem II 12 kDa extrinsic protein	43
Q208D1	**Phycocyanin beta chain**	**42**
K1VSS0	Phycobilisome linker polypeptide	36
D5A243	Glyceraldehyde-3-phosphate dehydrogenase	36
K1WZD2	Photosystem I reaction center subunit II	36
K1W168	**Glyceraldehyde-3-phosphate dehydrogenase**	**36**
B2BN61	CpcH	34
K1W460	Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Malallergen	31
D4ZR50	Putative bacterioferritin comigratory protein	31
K1WF39	Glycine cleavage system H protein	31
K1VYN4	PpiC-type peptidyl-prolyl cis-trans isomerase	30
K1VV20	Ribulose-bisphosphate carboxylase	30
K1WD80	Transketolase	29
K1X048	Glutathione-dependent peroxiredoxin	29
K1VTM7	Uncharacterized protein	28
K1VX49	Uncharacterized protein	28
K1W9Y7	Uncharacterized protein	28
A0A0C4W3G7	CpcI protein	27
K1WGQ8	Uncharacterized protein	27
K1X8N9	ATP synthase subunit alpha	26
S4UUT6	Nitrogen regulatory protein P-II	25
A0A3G2BBA0	Gas vesicle protein C	25
D4ZNG4	Photosystem II manganese-stabilizing polypeptide	24
K1VXV4	Histone-like DNA-binding protein	24
K1WLU1	60 kDa chaperonin	24
K1X5W6	Elongation factor Tu	23
K1WBP7	Phycobiliprotein ApcE	23
D5A424	Phycobilisome 7.8 kDa linker polypeptide, allophycocyanin-associated, core	22
D4ZQA8	Photosystem I iron-sulfur center	22
K1W8A9	Cytochrome b559 subunit alpha	22

D4ZXX3	50S ribosomal protein L7/L12	22
K1W8D0	ATP synthase subunit b	21
K1X2B2	Uncharacterized protein	21
D5A649	Cytochrome c6	21
K1VTF0	Cytochrome b6-f complex iron-sulfur subunit	21
D4ZTX4	NAD(P)H-quinone oxidoreductase subunit M	21
K1WAM8	ATP synthase subunit b	20
K1VYM5	Glutamine synthetase	20
K1VU04	Uncharacterized protein	20
A0A4Y7LKB7	ATP synthase subunit beta	20
K1X5E2	Fructose-1,6-bisphosphate aldolase	20
C7DQP7	CpcD (Fragment)	19
K1VZR0	Uncharacterized protein	18
D5A240	Nucleoid-associated protein NIES39_C00240	17
K1WDU3	Acyl carrier protein	17
K1VVN1	Carbon dioxide concentrating mechanism protein CcmM	17
K1WH93	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C	16
D4ZZD6	Uncharacterized protein	16
D4ZYS5	Ferredoxin--NADP reductase	16
K1WLC1	Uncharacterized protein	15
K1VYQ4	Phosphoglycerate kinase	15
K1XAL8	Uncharacterized protein	15
A0A6H9GWQ9	Photosystem I reaction center subunit IX	14
K1VXA7	ATP synthase subunit delta	14
K1X1W3	Uncharacterized protein	14
K1WP33	30S ribosomal protein S6	14
K1VPJ1	Uncharacterized protein	14
A0A6H9GKP9	Uncharacterized protein	13
K1X1F1	NAD(P)H-quinone oxidoreductase subunit O	13
K1XDJ4	50S ribosomal protein L29	13
K1WKJ5	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	13
K1WDC7	Uncharacterized protein	13
A0A6H9H3H4	RHH_1 domain-containing protein	13
K1WD08	Uncharacterized protein	12
K1X441	Uncharacterized protein	12
K1VPI8	Uncharacterized protein	12
D4ZVX9	Uncharacterized protein	12
K1WAG4	Uncharacterized protein	12
D4ZNP7	Ribulose-phosphate 3-epimerase	12
K1WC53	Orange carotenoid protein	12
D5A5G1	Uncharacterized protein	12
A0A6H9H1T3	Uncharacterized protein	12
K1WFH8	Peptidyl-prolyl cis-trans isomerase cyclophilin type	12
D4ZTM3	Usp domain-containing protein	12
K1W8T1	Phycobilisome protein	11
Q6WJF7	Superoxide dismutase (Fragment)	11

K1WIE0	Ribulose bisphosphate carboxylase large chain	11
D4ZXK1	50S ribosomal protein L21	11
K1WG10	Uncharacterized protein	11
D4ZU92	Uncharacterized protein	10
K1WAL6	Uncharacterized protein	10
D4ZXK3	50S ribosomal protein L27	10
D4ZMN8	DNA-binding protein	10
K1WET8	Phosphoribulokinase	10
A0A6H9GL65	Uncharacterized protein	10
D4ZPP8	Photosystem II reaction center Psb28 protein	10
D5A278	PSI-F	10
K1WC13	60 kDa chaperonin	10
K1WD48	50S ribosomal protein L24	10
K1WRJ6	Transketolase domain protein	10
D5A635	**Triosephosphate isomerase**	**10**

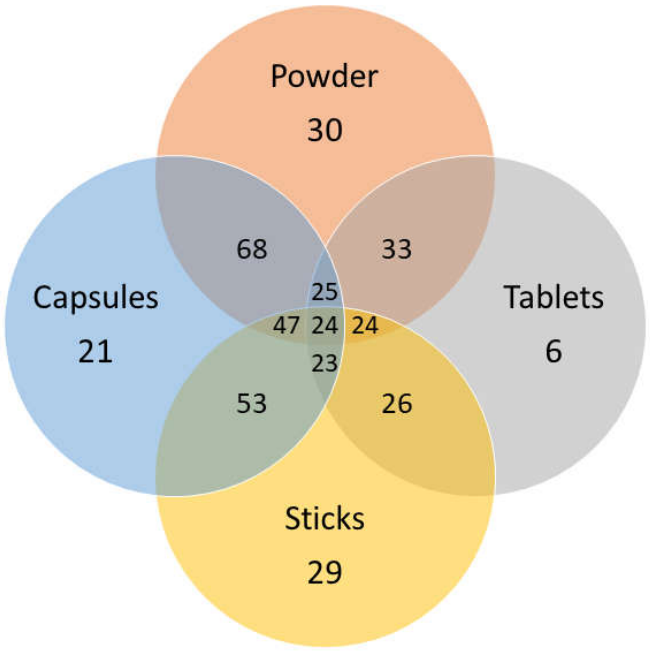


Figure S1. Identified proteins in four *spirulina* food supplements by RPLC-ESI(+)-Full-MS/ddMS^2 analysis.

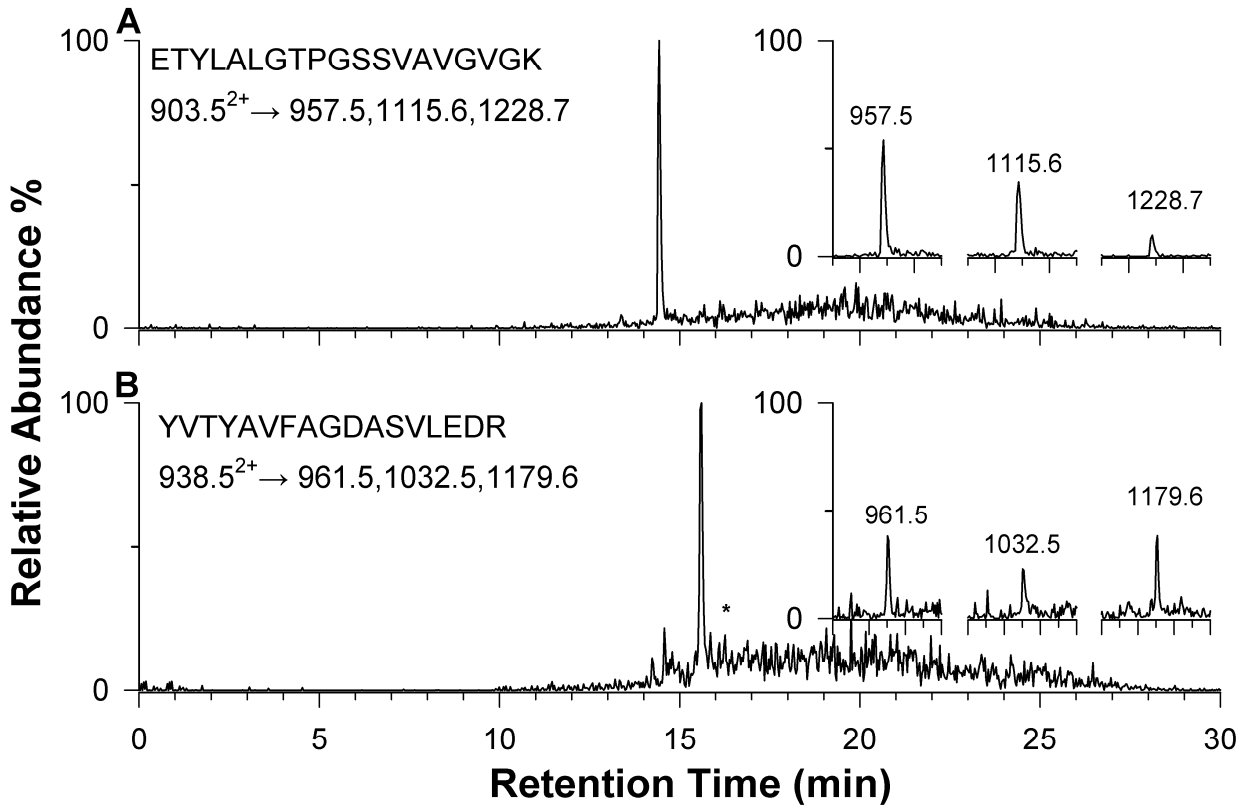


Figure S2. Chromatograms by RPLC-ESI-LIT-MS/MS in multiple ion monitoring (MRM) of spirulina marker peptides in incurred cookies at 2.5 $mg_{TotProt/gmatrix}$: (A) ETYLALGTPGSSVAVGVGK at *m/z* 903.5^{2+}, and (B) YVTYAVFAGDASVLEDR at *m/z* 938.5^{2+}. Major *y-type* product ions in CID-MS/MS were used to extract the ion currents. Insets report the individual measured transitions.

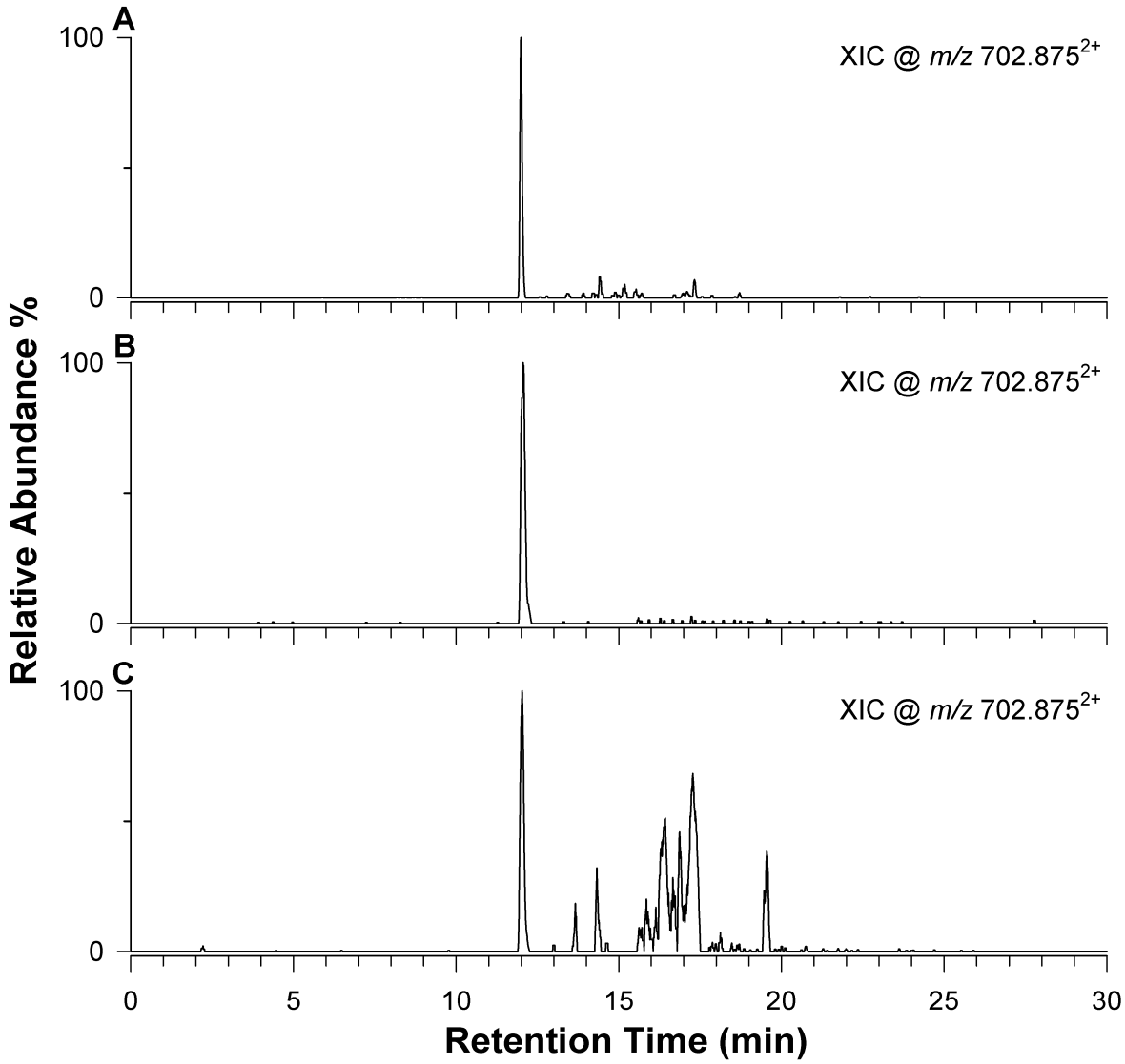
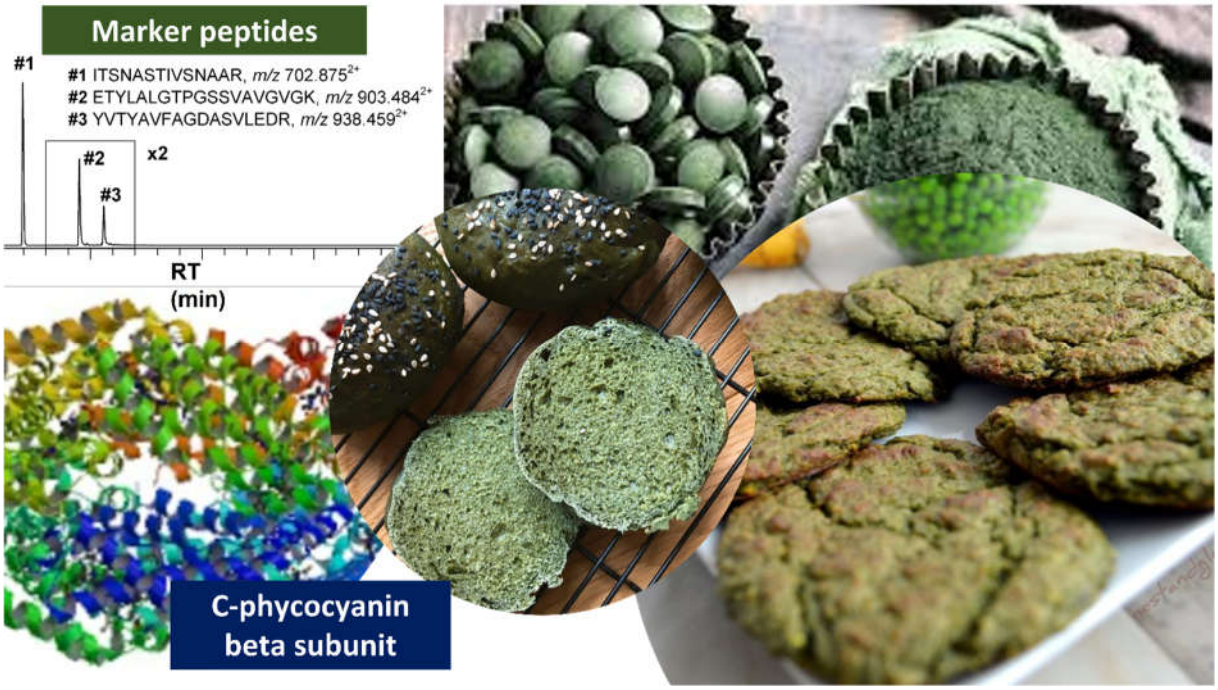


Figure S3. XIC chromatograms by RPLC-ESI-FTMS of C-phycocyanin beta subunit *quantifier* marker peptide ITSNASTIVSNAAR (*m/z* 702.875^{2+}) in crackers (A), bread (B), and pasta (C). Traces were obtained using an extraction window of 10 ppm centered on the selected ion.



Graphical abstract

Spirulina proteins were extracted from unprocessed food supplements, such as capsules and powder, purified, and digested. The LC-HRMS/MS analysis allowed us to identify one selected quantifier and two qualifier peptides of C-phycocyanin beta subunit, a known allergenic protein. The method was validated, and the marker peptides were detected also in processed samples such as bread, biscuits, and pasta.