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A targeted GC-MS/MS approach for the determination of eight sterols in microgreen and mature plant material

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ABSTRACT

Over the past decades, a remarkable number of scientific studies supported the correlation between an adequate dietary intake of phytosterols (PS) and the reduced risk of cardiovascular diseases. PS are known to inhibit the intestinal absorption of cholesterol, thus promoting the reduction of the low-density lipoproteins (LDL) amount in the bloodstream. Despite the fact that a non-negligible atherogenicity was recognized to PS, thus requiring a careful risk-benefits assessment for plant sterol supplementation, the potential role of PS as cholesterol-lowering agents has been contributing to the spreading awareness of the health benefits associated with the consumption of plant-based foods. In recent years, this has been fueling the market of innovative vegetable products, such as microgreens. Surprisingly, the recent literature concerning microgreens exhibited the lack of studies focusing on the characterization of PS. To fill this gap, a validated analytical method based on the hyphenation of gas chromatography and tandem mass spectrometry is proposed here for the quantitative analysis of the amount of eight phytosterols, namely β-sitosterol, campesterol, stigmasterol, brassicasterol, isofucosterol, and cholesterol, lathosterol and lanosterol. The method was exploited for the characterization of the PS content in 10 microgreen crops, *i.e.*, chia, flax, soybean, sunflower, rapeseed, garden cress, catalogna chicory, endive, kale and broccoli raab. Finally, these results were compared to the PS content of mature forms of kale and broccoli raab. A remarkable amount of PS was detected in chia, flax, rapeseed, garden cress, kale, and broccoli raab microgreens. 100 g (wet weight) of these microgreen crops were found to contain from 20 to 30 mg of the investigated PS. Interestingly, in the case of kale and broccoli raab microgreens, the overall PS content was higher than the one measured in the edible parts of the corresponding mature forms. Additionally, a symmetric change of the PS inner profile was observed between the two growth stages of the latter two crops. Here, the overall decrease of the PS sterol content in the mature forms was associated with the increase of the relative amount of β-sitosterol and campesterol at the expense of minor PS species, such as brassicasterol.

1. Introduction

Phytosterols (PS) are a class of triterpenes that are biosynthetically and structurally related to cholesterol [1,2]. PS account for most plant sterols, while relatively small quantities of cholesterol are found in plant tissues [2].

As all sterols, PS share the 5α-cholestan-3β-ol backbone shown in Fig. S1 [3]. In addition, PS exhibit a distinctive alkylation (mainly ethylation and methylation) of the C24 atom [1,4].

PS can be classified according to the methylation state of the C4 atom of the sterane backbone. Most PS are 4-desmethyl sterols, *i.e.*, they do not exhibit any methyl group linked to C4 [5]. Conversely, 4α-monomethylsterols and 4-dimethylsterols show one and two methyl groups respectively. The latter are both metabolic intermediates in the biosynthetic pathway leading to the 4-desmethyl sterols synthesis, and generally have a low abundance in most plant tissues [6,7].

PS are unsaturated chemical compounds. The saturated equivalents of phytosterols are known as phytostanols and are generally low-

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abundant in plant material [8]. Most 4-desmethyl sterols (*e.g.*, β-sitosterol, campesterol) exhibit a double bond located between C5 and C6 and are commonly named Δ5 phytosterols. Some of these are characterized by the presence of additional C═C bonds at the side chain level (*e.g.*, isofucosterol and brassicasterol) [6].

PS are not synthesized by the human body. Hence, the presence of PS in human blood and tissues is strictly related to the consumption of vegetable oils and plant-based foods [7–9]. PS exhibit poor intestinal absorption and higher biliary elimination rates in respect to cholesterol [9]. Nevertheless, the dietary intake of PS promotes the reduction of LDL cholesterol in blood [10], that is recognized as a major modifiable risk factor for cardiovascular diseases [11]. The health-promoting effects of PS were recognized by authoritative guidelines [7] and, among others, by the European Food Safety Authority (EFSA) [12,13]. This led to their extensive use in functional foods, supplements and pharmaceutical products [14]. On the other hand, since phytosterols were found to be *per se* atherogenic, several other authorities (*e.g.*, the American Heart Association and the National Institute for Health and Care) do not recommend the use of phytosterols and phytostanols as part of the cholesterol-lowering strategy in patients with familial hypercholesterolemia and increased risk of coronary heart disease [7]. Hence, the extended use of phytosterols as supplements, or by means of functional foods, to control the LDL-cholesterol levels should be supported by qualified health professionals after the assessment of patient's clinical characteristics [12].

Among intrinsically functional foods, *i.e.*, foods that naturally include compounds with beneficial effects for human health, microgreens recently gained increasing popularity [15,16]. Here, the term "microgreens" refers to young and tender edible seedlings that are generally harvested a few days after germination when the seed leaves are fully expanded and still turgid [17,18]. Despite the extended research work made so far on nutraceuticals provided by microgreens, poor attention was dedicated to the characterization of PS in microgreen crops [15]. The analysis of the total PS content in food matrices is routinely performed using gas chromatography (GC) coupled to flame ionization detector (FID), after the derivatization of PS as trimethylsilyl (TMS) ethers [19]. However, the analytical strategies based on GC-FID approaches may suffer from the contribution of co-eluting interferents, due to the low specificity of the detection method. Specificity can be increased through the coupling of GC with mass spectrometry (MS). Higher specificity can be reached through the hyphenation of GC with tandem mass spectrometry (MS/MS) strategies [20], as shown by previous studies on the PS characterization in foods and human feces [21,22]. In both cases, electron ionization (EI) was applied. Sample preparation is also a crucial step in total PS analysis. Indeed, plant sterols can be present as free (free sterols, FS) and in their conjugated forms, *i.e.*, bound to both fatty acids (sterol esters, SE) and sugars (sterol glycosides, SG, and acylated sterol glycosides, ASG) [4,23,24]. In a restricted number of plants (mainly cereals), plant sterols can be also found as esters of ferulic acid (steryl ferulates) [24]. FS are mainly present in the plasma membrane, where they play a functional role in the modulation of membrane fluidity and permeability. SE are commonly present in much lower amounts in plant cells, compared to FS [4]. SE are located intracellularly. Here they act as a storage pool of sterols that are required for growth and development stages and are involved in cell membrane homeostasis [24,25]. SG and ASG usually represent a minor portion of the total sterol content in plant tissues. They are believed to act as transport molecules for lipid precursors in cell walls and to play a role in cellulose biosynthesis [5]. Due to the complexity of the forms in which PS are present in plant tissues, the chemical pre-treatment of the sample represents a key step prior to GC-MS analysis. Alkaline hydrolysis (*i.e.*, saponification) is effective in the conversion of SE in FS, but not in the cleavage of the ether bond in SG and ASG (ASG are just converted in SG). Hence, the analytical approaches based on alkaline hydrolysis for the determination of total PS might lead to erroneous results especially in plants (*e.g.*, those belonging to the genus *Solanum*) that are rich in SG and ASG [5,24]. The conversion of SG and ASG in FS can be triggered by acid hydrolysis. Nevertheless, such process is known to promote PS degradation and the formation of artifacts through isomerization processes mainly at the expenses of isofucosterol and Δ7 phytosterols. For these reasons, the enzymatic rather than the chemical hydrolysis is recommended for the transformation of SG and ASG in FS [5]. It is worth noting that soft ionization MS techniques (*i.e.*, ESI, APCI, APPI, MALDI) can play an important role in the characterization of intact SE and SG/ASG [6].

The present study proposes a validated GC-EI-MS/MS method for the quantitative analysis of 8 sterols after alkaline hydrolysis. In particular, β-sitosterol, campesterol, stigmasterol, brassicasterol, isofucosterol (also known as Δ5-avenasterol), cholesterol, lathosterol and lanosterol are included. The corresponding chemical structures are shown in Fig. S2. A sterol profile was established for 10 different microgreen crops, *i.e.*, chia, flax, soybean, sunflower, rapeseed, garden cress, catalogna chicory, endive, kale and broccoli raab. For comparison, the study was also extended to the mature forms of the latter two species.

2. Materials and methods

2.1. Chemicals

Methanol (LC-MS grade), hexane (GC-MS grade), orthophosphoric acid (85% w/w), campesterol (24(R)-methylcholest-5-en-3β-ol), stigmasterol (5,22-stigmastadien-3β-ol), cholesterol (cholest-5-en-3β-ol) and lathosterol (5α-cholest-7-en-3β-ol) were purchased from Merck (Darmstadt, Germany). β-sitosterol (24(R)-ethyl-cholest-5-en-3β-ol), isofucosterol ((24*Z*)-Stigmasta-5,24(28)-dien-3β-ol) and lanosterol (lanosta-8,24-dien-3ß-ol) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Brassicasterol was purchased from Tama Chemicals CO. (Kawasaki City, Japan). β-sitosterol-d6 (D6–24(R)-ethylcholest-5-en-3β-ol) and campesterol-d6 (D6–24(R)-methylcholest-5-en-3β-ol) were purchased from Sugaris (Münster, Germany). Isooctane ≥ 99% was purchased from Honeywell Specialty Chemicals GmbH (Seelze, Germany). Chloroform (HPLC grade) and sodium hydroxide were purchased from Carl Roth (Karlsruhe, Germany). Sodium chloride was purchased from VWR GmbH (Darmstadt, Germany). The N-Methyl-N-trimethylsilyl-trifluoracetamide (MSTFA) silylation agent was purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany).

2.2. Microgreen production

Microgreens were grown using seeds of chia (*Salvia hispanica* L.), flax (*Linum usitatissimum* L.), soybean (*Glycine max* (L.) Merr.), sunflower (*Helianthus annuus* L.), rapeseed (*Brassica napus* L., cv. PR44D06), garden cress (*Lepidium sativum* L., cv. Crescione dei Giardini), kale (*Brassica oleracea* var. acephala, cv. Landrace), broccoli raab (*Brassica rapa* L. subsp. sylvestris L. Janch. var. esculenta Hort., cv. Novantina), Catalogna chicory (*Cichorium intybus* L. subsp. intybus var. foliosum, cv. Molfettese) and endive (*Cichorium endivia* L. var. latifolium). Seeds of chia, flax and sunflower were purchased from Selex (Trezzano sul Naviglio, Italy). Soybean seeds were purchased from Dupont-Pioneer (Johnston, IA, USA). Seeds of rapeseed, garden cress and broccoli raab were purchased from Pioneer Hi-Bred Italia Sementi (Sissa, Italy), and from local farms "Tesoro della Terra" (Andria, Italy) and "Riccardo Larosa" (Andria, Italy), respectively. Seeds of Catalogna chicory and endive were purchased from local farm "Sempreverde" (Molfetta, Italy) and from Enza Zaden Italia (Tarquinia, Italy), respectively. The mature forms of kale and broccoli raab were purchased from a local producer. Once harvested, the plants were stored at − 20 °C (24 h) prior to the lyophilization of the edible parts.

Microgreens production occurred in plastic trays by following the guidelines suggested by Di Gioia, Santamaria and Renna [26,27]. The

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seeds were sown on a substrate consisting of a 50/50% mixture of white and black peat (Brill 3 Special, purchased from Brill Substrates, Georgsdorf, Germany) contained in a 10 × 15 cm^2 plastic tray (height 4 cm) having holes on the bottom to enable sub-irrigation after complete germination of seeds. Before sowing, the substrate was swelled with distilled water sprayed through a nebulizer. Different sowing densities, expressed in seeds/cm^2 units, were adopted, according to the average weight of seeds (evaluated from the weight of 1000 seeds): 1 for sunflower, 2 for soybean, 3 for flax, rapeseed and garden cress, and 4 for chia, kale, broccoli raab, Catalogna chicory and endive.

The sowing bed was subsequently irrigated with nebulized distilled water and each tray was covered with a black polyethene film, to keep internal humidity constant, and then transferred into a growth chamber (Bertagnin, Imola, Italy), where it was kept at 20 °C and 70% of relative humidity. Once the total germination was assessed, the black film was re-moved. Each cultivation tray was transferred into a greenhouse, where the seedlings were sub-irrigated daily using a half-strength Hoagland solution [28]. The chemical composition of the latter is shown in Table S1 (see Supplementary Material). Harvesting was carried out once the appearance of the first true leaves was assessed. The seedlings were cut just above the surface of the growing medium, stored for two days at − 20 °C and then freeze-dried for four days in a ScanVac CoolSafe 55–9 Pro-freeze-dryer (LaboGene ApS, Lynge, Denmark).

2.3. Plant tissue homogenization and sample preparation

The lyophilized plant material was weighted and transferred into a tube (2 mL) for bead-beating applications. The tube was prefilled with ceramic beads. An aliquot of plant material, typically ranging from 30 mg to 60 mg was dispersed in a suitable volume of methanol to reach a concentration of 0.05 mg/μL. The mixture was subjected to tissue homogenization using a Precellys 24 tissue homogenizer from Bertin Instruments (Berlin, Germany). The homogenizer was operated at 5000 rpm and two homogenization session were performed. Each session consisted in two cycles of 15 s run time, and a 60 s break interval between both cycles.

The sample preparation for GC-MS/MS analysis, including FS extraction and derivatization steps, followed the work of Kunz and Matysik [22]. In particular, 200 μL of homogenate were transferred into 15 mL polypropylene screw cap tubes. Such amount was spiked with 50 μL of a mixture of β-sitosterol and campesterol deuterated internal standards (β-sitosterol-d6 and campesterol-d6, 10 μg/mL both). Subsequently, the basic hydrolysis, useful for the conversion of SE in FS, was performed. To this purpose, the mixture was incubated at 60 °C for 30 min, after the addition of 500 μL of a methanolic solution of NaOH (1 M). Once cooled down, 700 μL of aqueous NaCl (1 M) were added, and the excess of the base was neutralized through the addition of 40 μL of an aqueous solution of orthophosphoric acid (50% w/w). FS were extracted with 750 μL of isooctane by vortexing. A stable phase separation was achieved by centrifugation for 10 min at 4000 rpm. 500 μL of the supernatant were withdrawn and transferred in amber glass vials (2 mL). This procedure was repeated twice. The two supernatants were pooled to a total volume of 1 mL. Thereafter the sample was vacuum dried and subjected to FS silylation with MSTFA. To the latter purpose, 50 μL of MSTFA were added to the dry residue and the mixture was incubated at 60 °C for 60 min. After cooling, the sample was diluted with 450 μL of hexane and subjected to GC-MS/MS analysis.

2.4. GC-MS/MS instrumentation and operating conditions

Sample analysis was performed on a TQ8050 triple-quadrupole GC-MS/MS instrument equipped with a multifunctional autosampler AOC-6000, an SH Rxi-5Sil MS column (30 m, 0.25 mm, 0.25 μm) (all Shimadzu Deutschland GmbH, München, Germany) and a multi-mode inlet system OPTIC 4 (GL Sciences, Eindhoven, Netherlands).

The separation of trimethylsilyl derivatives was achieved by adopting the method proposed by Kunz and Matysik [22]. Particularly, the following oven temperature program was adopted: 200 °C for 1.0 min, 50 °C/min-290 °C, 5 °C/min-295 °C, 10 °C/min-320 °C, where the temperature was kept for 2.7 min. Helium was selected as carrier gas with a constant flow of 1.2 mL/min and a 1 μL volume of sample was injected in splitless mode.

The EI ion source was held at 280 °C and operated at 70 eV electron energy. The solvent delay was set for the first 6 min. All compounds of interest were monitored in the multiple reaction monitoring (MRM) mode. The responses of each compound were normalized to the closest eluting deuterated internal standard. Specifically, the responses of cholesterol, brassicasterol, lathosterol, campesterol and stigmasterol were normalized to campesterol-d6, while the responses of β-sitosterol, lanosterol and isofucosterol were normalized in respect to β-sitosterol-d6 internal standard.

2.5. Calibration solutions and calibration lines

The calibration lines were built by monitoring the instrumental response of seven calibration levels prepared from a methanol/chloroform (1:1 v/v) standard solution mix containing cholesterol (20 μg/mL), brassicasterol (40 μg/mL), lathosterol (2 μg/mL), campesterol (100 μg/mL), stigmasterol (60 μg/mL), β-sitosterol (300 μg/mL), lanosterol (4 μg/mL) and isofucosterol (60 μg/mL). The level 0, *i.e.*, the "solvent blank calibration level", was obtained by spiking 200 μL of pure methanol with 50 μL of a mixture containing the two deuterated internal standard, namely β-sitosterol-d6 and campesterol-d6, both at a concentration of 10 μg/mL. Calibration levels from 1 to 5 were prepared by diluting the standard mix using the following dilution factors, respectively: 1:200, 1:40, 1:20, 1:4 and 1:2. Level 6 corresponded to the standard mix itself. 200 μL of each calibration solution were spiked with the mixture of the two deuterated internal standards (as stated for level 0) and subjected to the sample preparation procedure (including the basic hydrolysis and the extraction and derivatization steps) described in Section 2.3, prior to the GC-MS/MS analysis. For each sterol, the calibration lines were built using a simple linear regression model by evaluating the normalized instrumental response (see Section 2.4) as a function of the sterol amount (μg/mL). For all sterols, the calibration line exhibited a coefficient of determination (R^2) always higher than 0.996.

3. Results and discussion

3.1. Method development

As can be inferred from Fig. 1, the GC method offers a rapid and efficient separation of sterol TMS derivatives. Notably, the retention time of lanosterol was close to the one observed for β-sitosterol and isofucosterol and a substantial overlap was observed between the peaks of lanosterol and isofucosterol. However, the lack of chromatographic resolution was easily tackled through the selection of MS/MS transitions that were highly specific for each of the co-eluting species. For all sterols, high specificity was confirmed by the agreement between quantifier/qualifier ratio observed for calibrator and sample.

Table 1 shows the MS/MS transitions selected for all the sterol TMS derivatives. Those for cholesterol, lathosterol and lanosterol were already proposed by Kunz and Matysik [22]. On the other hand, slight modifications of the method were introduced for stigmasterol, campesterol, sitosterol and the deuterated standards of the latter two species.

Quantifier and qualifier ion transitions for brassicasterol and isofucosterol were identified using the Shimadzu MRM optimization tool. To such purpose, several product ion spectra were acquired at different collision energies. For both isofucosterol and brassicasterol, the precur-

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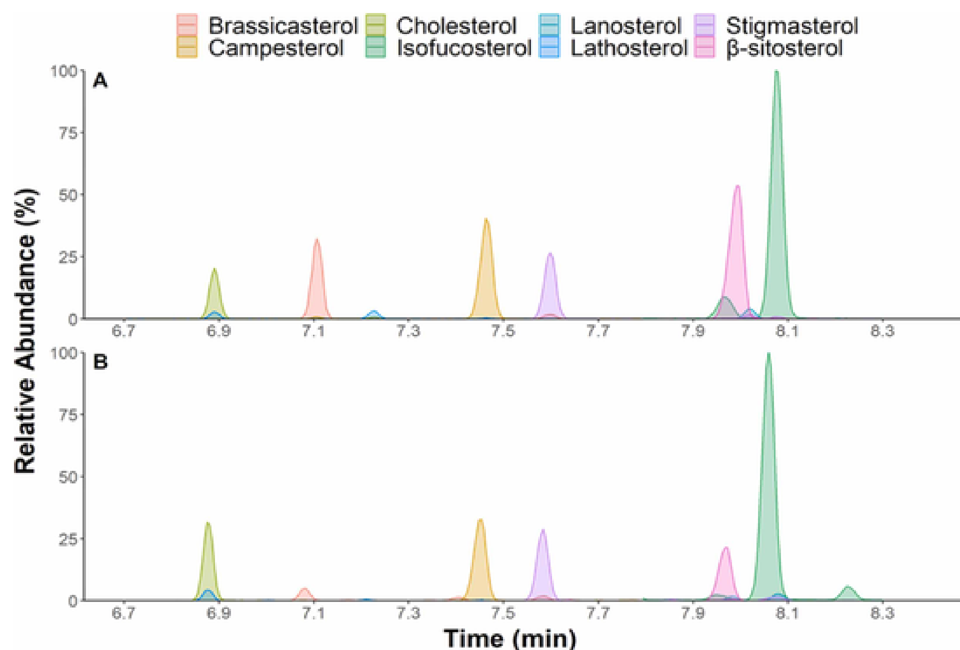

Fig. 1. GC-EI-MS/MS total ion current chromatograms referring to the sterol standard mix (A) represented by the calibration level 4 (see Section 2.5), and the flax microgreen sample (B). Each colored signal emerges from the sum of peak areas related to the quantifier and qualifier ion transitions that were selected for each sterol species. Signals of deuterated internal standards are not shown.

Table 1
MS/MS transitions and collision energies (CE) selected for sterol trimethylsilyl derivatives. For each species, the retention time (t_R) is shown in the form of mean ± standard deviation. These t_R data refer to gas chromatography separations performed in four different days on the standard mix corresponding to the fourth calibration level (see Section 2.5). The nominal *m/z* ratios of the $M^{+\bullet}$ molecular ions are also reported.

Species (molecular ion *m/z*)	t_R (min)	Quantifier ion transition (*m/z*)	CE (V)	1st Qualifier ion transition (*m/z*)	CE (V)	2nd Qualifier ion transition (*m/z*)	CE (V)
Cholesterol (m/z 458)	6.8898 ± 0368.00 > 14530			458.00 > 14520		368.00 > 353.4010	
Brassicasterol (m/z 470)	7.105 ± 0.0380.00 > 25510			380.00 > 69.25		365.00 > 69.10	25
Lathosterol (m/z 458)	7.2255 ± 0458.00 > 21310			458.00 > 2299.00		443.00 > 353.06	
Campesterol-d6 (m/z 478)	7.436 ± 0.0388.00 > 16612			478.00 > 388.20		478.00 > 373.2015	
Campesterol (m/z 472)	7.464 ± 0.0472.00 > 38240			472.00 > 367.40		382.00 > 367.0010	
Stigmasterol (m/z 484)	7.598 ± 0.0394.00 > 25500			484.00 > 394.00		484.00 > 379.4010	
β-Sitosterol-d6 (m/z 492)	7.951 ± 0.0402.00 > 16450			492.00 > 387.00		477.00 > 387.45	
β-Sitosterol (m/z 486)	7.987 ± 0.0486.00 > 38140			471.00 > 381.40		396.00 > 255.0010	
Lanosterol (m/z 498)	8.017 ± 0.0483.00 > 39300			498.00 > 187.00		498.00 > 393.09	
Isofucosterol (m/z 484)	8.076 ± 0.0386.00 > 29630			386.00 > 281.30		296.00 > 281.3010	

sor ions were accurately selected after the analysis of the corresponding EI-MS spectra. The latter are shown in Fig. 2. $[M - CH_3]^+$, $[M - (H_3C)_3SiOH]^{+\bullet}$, $[M - CH_3 - (H_3C)_3SiOH]^+$, along with the ion generated from the 129 Da neutral loss are typical fragments that are commonly encountered in the EI-MS spectra of Δ5 sterols in the high *m/z* spectral region [29]. These signals (detected at *m/z* 455, 380, 365 and 341, respectively), along with that of the molecular ion (*m/z* 470), are clearly distinguishable in the EI-MS spectrum of brassicasterol (see Fig. 2A).

Particularly, signals corresponding to $[M - (H_3C)_3SiOH]^{+\bullet}$ (*m/z* 380) and $[M - CH_3 - (H_3C)_3SiOH]^+$ (*m/z* 365) product ions were selected for the identification of the quantifier and qualifier ion transitions. However, a different fragmentation pattern, along with the absence of the molecular ion signal (expected at *m/z* 484) characterized the EI-MS spectrum of the isofucosterol TMS derivative (see Fig. 2B). Indeed, the methyl and trimethylsilanol neutral losses, along with their combination, and the loss of 129 Da, occurred from the *m/z* 386 ion. The latter was tentatively identified as the result of a pericyclic rearrangement (retro-ene reaction) as shown in Fig. 3. This fragmentation mechanism has been previously proposed by Münger et al. [30] for sterols having an additional C-C double bond involving the C(24) and C(24¹) atoms. For the isofucosterol TMS derivative, the *m/z* 386 ion and the corresponding trimethyl silanol neutral loss (*m/z* 296) were selected as precursor ions for the identification of both the quantifier and qualifier ion transitions.

For both the brassicasterol and isofucosterol, the precursor ion spectra were acquired at 7 different collision energies (V), namely 5, 10, 15, 20, 25, 10 and 35. To such purpose, appropriate amounts of brassicasterol and isofucosterol were dissolved in methanol/chloroform 1:1 (v/v) to a final concentration of 100 µg/mL for each sterol. Thence, the mixture was vacuum dried. The dry residue was subjected to silylation using MSTFA following the same reaction conditions described in Section 2.3.

3.2. Method validation

All microgreens showed a highly heterogeneous distribution in terms of absolute amount of each sterol (see Section 3.3). Hence, a mixture of homogenates of five different microgreen species was assumed to be more representative for all plant materials. Specifically, the following microgreen species were selected: flax (high amount of cholesterol and stigmasterol), soybean (high amount of lathosterol), sunflower (high content of lanosterol), rapeseed (high content of sitosterol,

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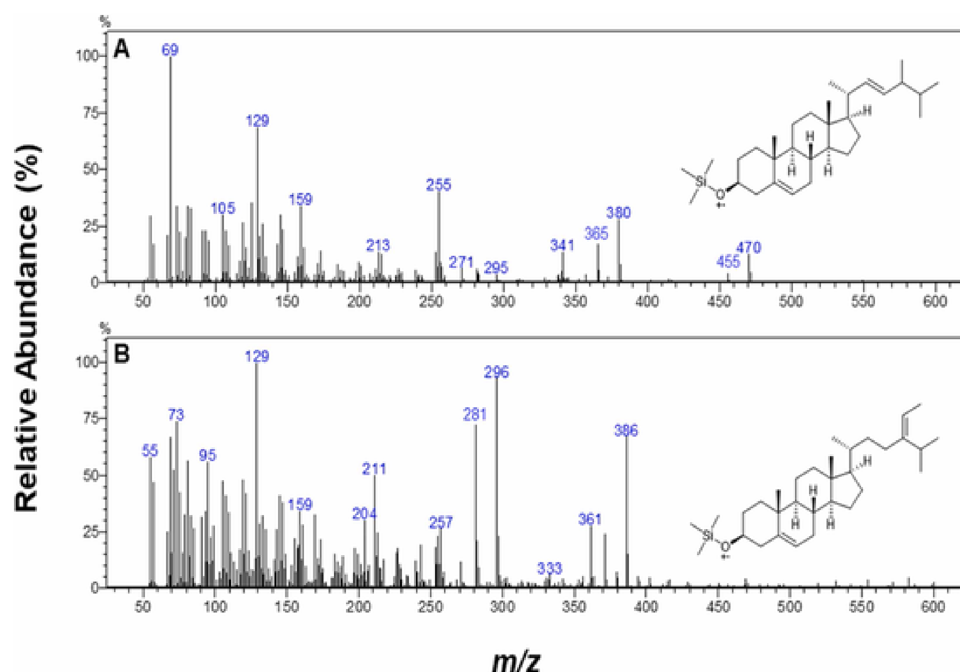

Fig. 2. EI-MS spectra of the trimethylsilyl derivatives of (A) brassicasterol (m/z 470) and (B) isofucosterol (m/z 484).

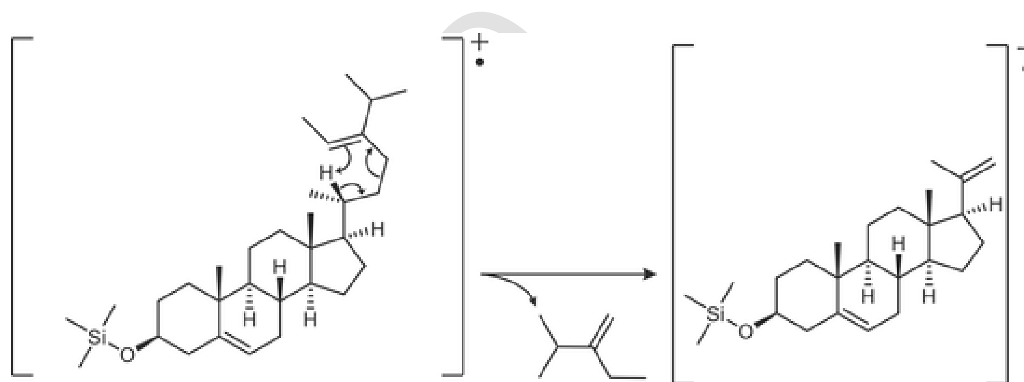

Fig. 3. Putative mechanism proposed for the formation of the m/z 386 ion from the molecular ion of the isofucosterol trimethylsilyl derivative.

campesterol and brassicasterol), and garden cress (high amount of isofucosterol). This model matrix (M1) was employed for the validation of the GC-MS/MS method. Particularly, M1 was obtained by mixing 40 μL of flax, soybean, sunflower, rapeseed and garden cress methanol homogenates. The model matrix was diluted to the same overall volume (200 μL) using 1:2 (M2) and 1:10 (M3) dilution factors. M1, M2 and M3 samples were subjected to the sample preparation procedure described in Section 2.3. Specifically, 5 homogenization and mix preparation/extraction/derivatization replicates were produced for each dilution level for the intra-day precision evaluation. Inter-day precision was estimated over 4 days using one homogenization and mix preparation/extraction/ derivatization replicate for each dilution level. The results are shown in Table 2.

In conventional GC-EI-MS approaches, no matrix effect is typically observed for analyte ionization process. Indeed, the energy of the ionizing electron beam (70 eV) is sufficiently high to assume the competition for ionization among co-eluting analytes to be negligible [31]. However, the presence of the matrix can still affect the GC-MS signal. This can be caused by the different interactions between the analytes and the active surfaces (free silanol groups) of the glass liner and the stationary phase, when the analyte molecules are dissolved in pure solvent rather than in the matrix [32]. Nonetheless, the use of appropriate analytical internal standards can help to compensate the matrix effect [31].

In the case of microgreen samples, a "blank matrix" (*i.e.*, a matrix containing all the phytochemicals except for the PS of interest) was not available for the proper evaluation of the matrix effect for all the investigated PS. Therefore, the influence of the matrix on signal intensity and retention time was investigated only for the deuterated campesterol and β-sitosterol. On one hand, the retention time (t_R) and the GC-MS/MS peak area observed for campesterol-d6 and β-sitosterol-d6 in the calibration levels (see Section 2.5) were considered representative for the pure solvent. On the other hand, the t_R and peak area values measured for the same spiked amount of the two deuterated standards in 200 μL of pooled model matrix (M1) were assumed to be representative for the possible presence of matrix effects. Five replicates of the spiked M1 were prepared and subjected to the sample preparation described in Section 2.3, along with the calibration levels. All the samples were processed in the same day. Fig. S4 (see Supplementary Material) displays the 95% confidence intervals for the difference of means for both t_R and the analytical response (peak area). In all cases, the 0 value was included in the calculated confidence intervals, thus indicating that the matrix effect on the two investigated parameters was not statistically significant.

Since the matrix effect was found to be negligible, we decided to calculate the limit of quantification (LOQ) for each of the 8 sterols of interest without performing any matrix-spiking experiments. To such pur-

Table 2
Results of the intra and inter-day precision evaluation for three dilution degrees (M1, M2 and M3) of the model matrix, corresponding to a mix of microgreen homogenates. Coefficient of variation (CV) values higher than 15% for intra-day and 20% for the inter-day experiments are highlighted in red. The limit of quantification (LOQ) is also shown for each sterol.

		Intra-day precision		**Inter-day precision**		
		Mean amount (n=5) [µg/mL]	CV (%)	Mean amount (n=4) [µg/mL]	CV (%)	**LOQ** [µg/mL]
Cholesterol	M1	2.517	8	2.882	6	0.5
	M2	1.316	10	1.381	7	
	M3	0.267	21	0.311	17	
Brassicasterol	M1	3.193	10	3.578	11	1.9
	M2	1.662	8	1.736	22	
	M3	0.146	31	0.395	80	
Lathosterol	M1	0.087	5	0.099	14	0.04
	M2	0.052	12	0.056	13	
	M3	0.018	8	0.019	37	
Campesterol	M1	18.842	7	20.125	7	4
	M2	10.386	9	9.835	11	
	M3	1.404	18	2.023	31	
Stigmasterol	M1	11.003	4	11.265	9	4
	M2	5.984	9	5.697	16	
	M3	0.949	12	1.253	39	
β-Sitosterol	M1	57.112	3	63.167	10	17
	M2	34.440	10	34.073	12	
	M3	4.330	15	5.026	45	
Lanosterol	M1	0.393	5	0.410	9	0.19
	M2	0.218	16	0.218	22	
	M3	0.045	10	0.047	60	
Isofucosterol	M1	8.035	4	9.564	9	1.9
	M2	5.031	16	4.876	6	
	M3	1.174	10	1.370	30	

pose, we referred to an adaptation of the LOQ quantification method that has already been exploited in previous literature studies [33,34]. In particular, the LOQ (µg/mL) values were calculated from the following regression model, by setting a 20% value of the coefficient of variation (CV). The mathematical expression used for the calculation of the LOQ is also shown hereafter.

$$CV(\%) = \boldsymbol{a} \bullet x^{\boldsymbol{b}} \rightarrow LOQ = \left(\frac{20}{\boldsymbol{a}}\right)^{-b}$$

The values of the $\boldsymbol{a}$ and $\boldsymbol{b}$ parameters were estimated after the curve fitting of the experimental data referring to the inter-day essay data. Specifically, the CV(%) and the average µg/mL (x) concentration were calculated, for each sterol, from the four replicates (day 1, day 2, day 3 and day 4) produced for each of the three dilution steps of the model matrix, *i.e.*, M1, M2 and M3. The data points and the corresponding fitting curves are shown in Fig. S3 (see Supplementary Material), while the calculated LOQ values are shown in Table 2.

3.3. Quantitative characterization of sterols in microgreen and mature plant material

Table 3 shows the results of the GC-MS/MS analysis of the total (free and esterified) sterols in flax, chia, soybean, sunflower, rapeseed, garden cress, kale, broccoli raab, catalogna chicory and endive microgreens and in the mature forms of kale and broccoli raab. The same data are displayed as cumulative stacked bar charts in Fig. 4, both in terms of absolute (mg/kg dry weight, Fig. 4A) and percent amounts (Fig. 4B). The latter reflects the inner sterol profile of each plant material. Fig. 5 offers an alternative and more intuitive graphical representation of the data in Table 3. Although more than 250 plant sterol species have been proposed in literature [4,5,20], the overall amount (free and esterified forms) of the eight sterol species that were selected for the present study, is consistent with the total sterol content that is averagely expected in plants, *i.e.*, 1–3 mg/g dry weight [23] (see Fig. 4A). β-sitosterol, campesterol and stigmasterol are recognized as the most abundant sterols in plants [2,4,10]. Their free forms are known to contribute to membrane fluidity and perme-

Table 3
Total amount of 8 sterols determined in 10 microgreen and 2 mature ("macro") plant material. The values are expressed as mg per kg of plant material dry weight (DW). Two significant digits are adopted. All the entries labeled with (*) were quantified below the limit of quantification (LOQ). Only one significant digit is reported for the latter values. The < LOD symbol was introduced when the sterol amount was below the limit of detection.

Species	Total Sterols [mg/kg DW]											
	Chia	Flax	Soybean	Sunflower	Rapeseed	Garden cress	Kale	Macro Kale	Broccoli Raab	Macro Broccoli Raab	Catalogna Chicory	Endive
Cholesterol	< LOD	1.9×10^2	1.4×10	< LOD	2.9×10	3.8×10	3.8×10	2 *	4.3×10	3.3×10	< LOD	< LOD
Brassicasterol	< LOD	3×10 *	< LOD	< LOD	3.3×10^2	< LOD	9.2×10	< LOD	2.3×10^2	< LOD	< LOD	< LOD
Lathosterol	< LOD	1.9	3.4	0.1 *	2.0	1.8	0.7 *	< LOD	3.1	0.7 *	< LOD	< LOD
Campesterol	2.0×10^2	5.5×10^2	5×10 *	1.0×10^2	8.2×10^2	2.7×10^2	6.2×10^2	2.7×10^2	6.0×10^2	3.7×10^2	1.7×10^2	1.7×10^2
Stigmasterol	1.6×10^2	4.2×10^2	2.6×10^2	3.3×10^2	6 *	5×10 *	2×10 *	< LOD	4 *	< LOD	2.0×10^2	4.7×10^2
β-sitosterol	2.0×10^3	6.5×10^2	5.7×10^2	1.2×10^3	1.8×10^3	1.2×10^3	1.4×10^3	1.1×10^3	1.6×10^3	1.3×10^3	9.0×10^2	7.3×10^2
Lanosterol	4.4	1.1×10	2 *	2.2×10	4 *	4.5	2 *	0.5 *	4.8	1 *	2 *	4.3
Isofucosterol	1.2×10^2	3.0×10^2	5.0×10	7.0×10	1.4×10^2	4.0×10^2	6.1×10	1×10 *	1.1×10^2	3×10 *	3.2×10^2	2.8×10^2

ability. Particularly, the major effect on membrane ordering was attributed to campesterol, followed by β-sitosterol [25]. In both cases, however, the membrane-packing efficiency was found to be lower than cholesterol [4]. This seems to be related to the size of the alkyl branching at C24 (see Figs. S1 and S2). In the case of stigmasterol, the ordering effect in the plasma membrane is further reduced by the presence of an additional carbon-carbon double bond (see Fig. S2); the latter lowers the flexibility of the side chain [25]. Among the investigated plant materials, the amount of β-sitosterol and campesterol covered more than the 50% of the total sterol amount. Particularly, both these PS accounted for almost the totality of the sterol content in the mature forms of kale and broccoli raab (see Fig. 4). As can be intuitively deduced from Fig. 5, β-sitosterol was the most abundant sterol in many of the plant materials that we investigated. This is consistent with the sterol content that was observed for many vegetable and edible oils, as reviewed by Piironen et al. [25] and Poudel *et* al. [35]. β-sitosterol was also reported to be the most abundant sterol in the model plant *Arabidopsis thaliana* [4]. However, as recently surveyed by Poudel et al. [35], the relative amount of the major PS (*e.g.* β-sitosterol, campesterol and stigmasterol) in fresh vegetables is heavily depending on crop type. For example, campesterol was observed to be the most abundant phytosterol in sweet potatoes and bamboo shoots, *i.e.*, those vegetables that showed the highest PS content among those reviewed by the authors.

Conversely, the impact of stigmasterol on the PS profile was highly variable among the investigated crops. Particularly, a very little amount of stigmasterol was observed in Brassica microgreen species (*i.e.*, rapeseed, garden cress, kale and broccoli raab), while no stigmasterol was detected in mature kale and broccoli raab. Results for rapeseed microgreens are consistent with literature data referring to rapeseed oil, where only traces of stigmasterol were detected [36]. It is also worth noting that the percentage of β-sitosterol (57%), campesterol (26%) and brassicasterol (10%) is in accordance with reports about rapeseed oil by Phillips et al. [14,36]. The reliability of data comparison is supported by the fact that the authors estimated the amount of free and esterified PS focusing on the same major sterols (*i.e.*, β-sitosterol, campesterol, stigmasterol, isofucosterol, brassicasterol and cholesterol) with the addition of minor plant stanol species, namely campestanol and sitostanol. The latter produced a very little impact on the sterol profile of rapeseed oil,.

Brassica microgreens, except for garden cress, exhibited the highest amount of brassicasterol. Indeed, such plant sterol is known to be characteristic of Brassica crops [37]. On the other hand, no brassicasterol was found in mature kale and broccoli raab, while it was detected in flax microgreen. Particularly, flax exhibited the most uniformly distributed sterol profile, along with the highest cholesterol levels in respect to the other investigated plant materials.

As previously mentioned, stigmasterol is known to be one of the most common PS in many plant tissues. In the present case, stigmasterol levels were found to be higher than campesterol in soybean, sunflower and endive microgreens. This result is consistent with other literature data referring to soybean sprouts [38] and mature endive varieties [39]. Isofucosterol was also dominant in endive and catalogna chicory microgreen. Significant amount of isofucosterol were also detected in flax and garden cress crops. As for lathosterol and lanosterol, both were detected in very low amount in all the investigated microgreen and mature plant materials. Along with cholesterol, lathosterol was found to play a role in the biosynthesis of phytoecdysteroids (*i.e.*, phytohormones involved in plant defense) [6]. Lanosterol represents the universal precursor for cholesterol in both animals and fungi. Conversely, limited information is available about the role of lanosterol in plant sterol biosynthesis. Indeed, cycloartenol is recognized as the main PS precursor. Notably, the sunflower microgreen showed the highest amount of lanosterol.

4. Conclusions

The total amount (including free and esterified forms) of eight sterols (β-sitosterol, campesterol, stigmasterol, brassicasterol, isofucosterol, cholesterol, lanosterol and lathosterol) was characterized in 10 microgreen species, *i.e.*, chia, flax, soybean, sunflower, rapeseed, garden cress, kale, broccoli raab, catalogna chicory and endive. The study was also extended to the mature forms of kale and broccoli raab. The quantitative analysis of sterols was performed using a validated GC-EI-MS/MS method.

Among the investigated plant materials, chia, rapeseed and broccoli raab microgreens showed the highest sterol content, corresponding to 250–300 mg per 100 g of dry weight. According to literature a daily intake of 300–400 mg of phytosterols is capable of producing a 5% reduction of the intestinal cholesterol absorption [8,12]. Assuming that water is averagely responsible for the 90% of plant wet weight, however, a 100 g portion of these microgreens would only provide one tenth of the aforementioned amount. As far as we know, there are no literature studies focusing on which plant sterol is more effective in determining the reduction of the intestinal absorption of cholesterol. β-sitosterol was found to be the most abundant sterol in all microgreen crops under study, followed by campesterol and stigmasterol. Stigmasterol levels were higher than campesterol in soybean, sunflower, catalogna chicory

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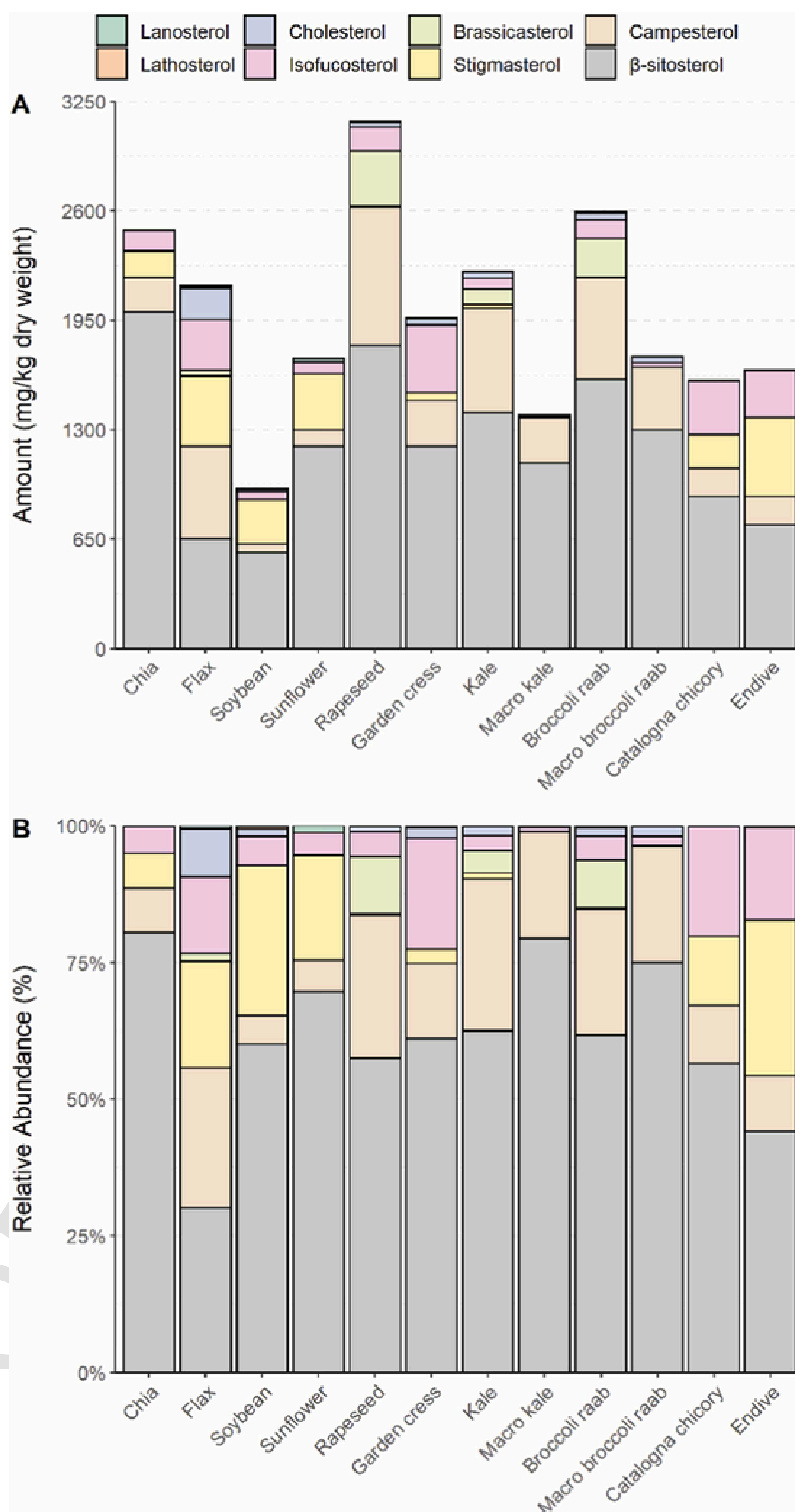

Fig. 4. Stacked bar charts showing the absolute (A) and percent (B) amount of 8 sterols in 10 microgreen and 2 mature ("macro") plant samples.

and endive microgreens. Conversely, very low amounts of stigmasterol characterized the overall sterol content of Brassica microgreens. The latter showed the highest amount of brassicasterol, except for garden cress. On the other hand, the content of isofucosterol was higher than campesterol in both the Asteraceae (*i.e.*, catalogna chicory and endive) and garden cress microgreens. Among all the investigated plant materials, flax exhibited the highest amount in cholesterol and the most equally distributed sterol profile.

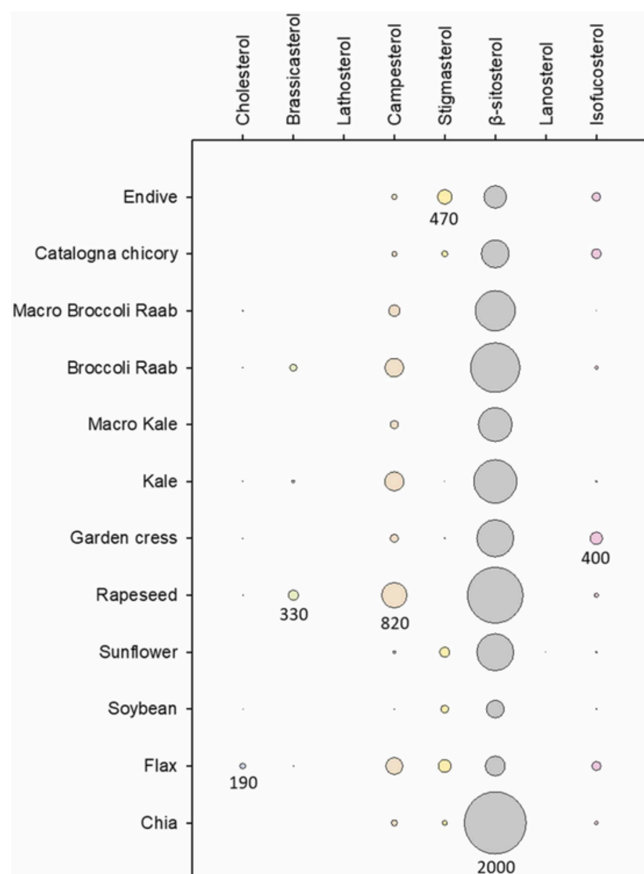

Fig. 5. Bubbleplot providing an intuitive graphical representation of the data enclosed in Table 3 (see main text). Here, the dimension of each bubble is proportional to the sterol amount detected in the corresponding plant material. For a more reliable quantitative interpretation of the graph, the figure shows the maximum value observed for each sterol concentration (mg/kg, dry weight) among the investigated plant material. These values are shown under the corresponding circle in the plot.

The discrepancies observed between the micro and macro forms of kale and broccoli raab represent another relevant outcome of the study described in this paper. Indeed, both mature forms exhibited a lower total sterol amount, compared to the respective microgreens. Interestingly, this was mainly due to the reduction of the campesterol content, along with the remarkable decrease of the levels of both brassicasterol and isofucosterol in mature kale and broccoli raab.

Generally, our results suggest that phytosterols might represent important compounds with potential nutraceutical properties provided in a non-negligible amount by microgreens. The described analytical approach could be advantageously applied in the future to evaluate if specific conditions for microgreen cultivation would be able to further increase the vegetal sterols content.

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CRediT authorship contribution statement

A. Castellaneta : Conceptualization, Investigation. **I. Losito :** Conceptualization, Investigation. **B. Leoni :** Investigation, Resources. **M. Renna :** Investigation, Resources. **C. Mininni :** Investigation, Resources. **P. Santamaria :** Investigation, Resources. **C.D. Calvano :** Investigation, Resources. **T.R.I. Cataldi :** Investigation, Resources. **G. Liebisch :** Investigation, Resources. **S. Matysik :** Conceptualization, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106361.

References

[1] W.D. Nes, Biosynthesis of cholesterol and other sterols, Chem. Rev. 111 (2011) 6423–6451, https://doi.org/10.1021/cr200021m.
[2] P.D. Sonawane, J. Pollier, S. Panda, J. Szymanski, H. Massalha, M. Yona, T. Unger, S. Malitsky, P. Arendt, L. Pauwels, E. Almekias-Siegl, I. Rogachev, S. Meir, P.D. Cárdenas, A. Masri, M. Petrikov, H. Schaller, A.A. Schaffer, A. Kamble, A.P. Giri, A. Goossens, A. Aharoni, Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism, Nat. Plants 3 (2017) 16205, https://doi.org/10.1038/nplants.2016.205.
[3] G.P. Moss, Nomenclature of steroids (Recommendations 1989), Pure Appl. Chem. 61 (1989) 1783–1822, https://doi.org/10.1351/pac198961101783.
[4] J.N. Valitova, A.G. Sulkarnayeva, F.V. Minibayeva, Plant sterols: diversity, biosynthesis, and physiological functions, Biochemistry 81 (2016) 819–834, https://doi.org/10.1134/S0006297916080046.
[5] R.A. Moreau, L. Nyström, B.D. Whitaker, J.K. Winkler-Moser, D.J. Baer, S.K. Gebauer, K.B. Hicks, Phytosterols and their derivatives: structural diversity, distribution, metabolism, analysis, and health-promoting uses, Prog. Lipid Res. 70 (2018) 35–61, https://doi.org/10.1016/j.plipres.2018.04.001.
[6] R.A. Moreau, B.D. Whitaker, K.B. Hicks, Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses, Prog. Lipid Res. 41 (2002) 457–500, https://doi.org/10.1016/S0163-7827(02)00006-1.
[7] U. Makhmudova, P.C. Schulze, D. Lütjohann, O. Weingärtner, Phytosterols and cardiovascular disease, Curr. Atheroscler. Rep. 23 (2021) 68, https://doi.org/10.1007/s11883-021-00964-x.
[8] H. Gylling, P. Simonen, Phytosterols, phytostanols, and lipoprotein metabolism, Nutrients 7 (2015) 7965–7977, https://doi.org/10.3390/nu7095374.
[9] T. Sudhop, D. Lütjohann, K. von Bergmann, Sterol transporters: targets of natural sterols and new lipid lowering drugs, Pharmacol. Ther. 105 (2005) 333–341, https://doi.org/10.1016/j.pharmthera.2004.10.011.
[10] J. Ying, Y. Zhang, K. Yu, Phytosterol compositions of enriched products influence their cholesterol-lowering efficacy: a meta-analysis of randomized controlled trials, Eur. J. Clin. Nutr. 73 (2019) 1579–1593, https://doi.org/10.1038/s41430-019-0504-z.
[11] B.A. Ference, H.N. Ginsberg, I. Graham, K.K. Ray, C.J. Packard, E. Bruckert, R.A. Hegele, R.M. Krauss, F.J. Raal, H. Schunkert, G.F. Watts, J. Borén, S. Fazio, J.D. Horton, L. Masana, S.J. Nicholls, B.G. Nordestgaard, B. van de Sluis, M.-R. Taskinen, L. Tokgözoğlu, U. Landmesser, U. Laufs, O. Wiklund, J.K. Stock, M.J. Chapman, A.L. Catapano, Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel, Eur. Heart J. 38 (2017) 2459–2472, https://doi.org/10.1093/eurheartj/ehx144.
[12] A. Poli, F. Marangoni, A. Corsini, E. Manzato, W. Marrocco, D. Martini, G. Medea, F. Visioli, Phytosterols, cholesterol control, and cardiovascular disease, Nutrients 13 (2021) 2810, https://doi.org/10.3390/nu13082810.
[13] Scientific opinion on the substantiation of a health claim related to 3 g/day plant sterols/stanols and lowering blood LDL-cholesterol and reduced risk of (coronary) heart disease pursuant to Article 19 of Regulation (EC) No 1924/2006, EFSA J., 10, 2012, p. 2693. ⟨https://doi.org/10.2903/j.efsa.2012.2693⟩.
[14] R. Zhang, Y. Han, D.J. McClements, D. Xu, S. Chen, Production, characterization, delivery, and cholesterol-lowering mechanism of phytosterols: a review, J. Agric.

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Food Chem. 70 (2022) 2483–2494, https://doi.org/10.1021/acs.jafc.1c07390.
[15] J. Teng, P. Liao, M. Wang, The role of emerging micro-scale vegetables in human diet and health benefits—an updated review based on microgreens, Food Funct. 12 (2021) 1914–1932, https://doi.org/10.1039/D0FO03299A.
[16] I. Marchioni, M. Martinelli, R. Ascrizzi, C. Gabbrielli, G. Flamini, L. Pistelli, L. Pistelli, Small functional foods: comparative phytochemical and nutritional analyses of five microgreens of the brassicaceae family, Foods 10 (2021) 427, https://doi.org/10.3390/foods10020427.
[17] Z. Xiao, G.E. Lester, Y. Luo, Q. Wang, Assessment of vitamin and carotenoid concentrations of emerging food products: edible microgreens, J. Agric. Food Chem. 60 (2012) 7644–7651, https://doi.org/10.1021/jf300459b.
[18] M.C. Kyriacou, Y. Rouphael, F. Di Gioia, A. Kyratzis, F. Serio, M. Renna, S. De Pascale, P. Santamaria, Micro-scale vegetable production and the rise of microgreens, Trends Food Sci. Technol. 57 (2016) 103–115, https://doi.org/10.1016/j.tifs.2016.09.005.
[19] C.T. Srigley, S.L. Hansen, S.A. Smith, A. Abraham, E. Bailey, X. Chen, S.H. Chooi, L.M. Clement, M. Dao, A.R. Fardin Kia, B. Mitchell, M. Mogla, J.A. Ruano Ortiz, K. Persons, E. von Kries, G. Ware, J. Wubben, R. Cantrill, Sterols and stanols in foods and dietary supplements containing added phytosterols: a collaborative study, J. Am. Oil Chem. Soc. 95 (2018) 247–257, https://doi.org/10.1002/aocs.12011.
[20] G. Gachumi, A. El-Aneed, Mass spectrometric approaches for the analysis of phytosterols in biological samples, J. Agric. Food Chem. 65 (2017) 10141–10156, https://doi.org/10.1021/acs.jafc.7b03785.
[21] Y.-Z. Chen, S.-Y. Kao, H.-C. Jian, Y.-M. Yu, J.-Y. Li, W.-H. Wang, C.-W. Tsai, Determination of cholesterol and four phytosterols in foods without derivatization by gas chromatography-tandem mass spectrometry, J. Food Drug Anal. 23 (2015) 636–644, https://doi.org/10.1016/j.jfda.2015.01.010.
[22] S. Kunz, S. Matysik, A comprehensive method to determine sterol species in human faeces by GC-triple quadrupole MS, J. Steroid Biochem. Mol. Biol. 190 (2019) 99–103, https://doi.org/10.1016/j.jsbmb.2019.03.014.
[23] A. Mamode Cassim, P. Gouguet, J. Gronnier, N. Laurent, V. Germain, M. Grison, Y. Boutté, P. Gerbeau-Pissot, F. Simon-Plas, S. Mongrand, Plant lipids: key players of plasma membrane organization and function, Prog. Lipid Res. 73 (2019) 1–27, https://doi.org/10.1016/j.plipres.2018.11.002.
[24] A. Ferrer, T. Altabella, M. Arró, A. Boronat, Emerging roles for conjugated sterols in plants, Prog. Lipid Res. 67 (2017) 27–37, https://doi.org/10.1016/j.plipres.2017.06.002.
[25] V. Piironen, D.G. Lindsay, T.A. Miettinen, J. Toivo, A.-M. Lampi, Plant sterols: biosynthesis, biological function and their importance to human nutrition, J. Sci. Food Agric. 80 (2000) 939–966, https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<939::AID-JSFA644>3.0.CO;2-C.
[26] F. Di Gioia, M. Renna, P. Santamaria, Sprouts, microgreens and "baby leaf" vegetables. Minimally Processed Refrigerated Fruits & Vegetables, 2017, pp. 403–432, https://doi.org/10.1007/978-1-4939-7018-6_11.
[27] P. Santamaria, F.Di Gioia, Microgreens: Novel, Fresh and Functional Food to Explore All the Value of Biodiversity, 2015.
[28] D.R. Hoagland, D.I. Arnon, The water-culture method for growing plants without soil, Circ. Calif. Agric. Exp. Stn. 347 (1950).
[29] A. Rahier, P. Benveniste, Mass spectral identification of phytosterols, in: W.D. Nes, E.J. Parish (Eds.), Analysis of Sterols and Other Biologically Significant Steroids, first ed., Academic Press, Inc., San Diego, CA, 1989, pp. 223–251.
[30] L.H. Münger, S. Boulos, L. Nyström, UPLC-MS/MS based identification of dietary steryl glucosides by investigation of corresponding free sterols, Front. Chem. 6 (2018), https://doi.org/10.3389/fchem.2018.00342.
[31] L. Silvestro, I. Tarcomnicu, S. Rizea, Matrix effects in mass spectrometry combined with separation methods — comparison HPLC, GC and discussion on methods to control these effects, in: Tandem Mass Spectrometry - Molecular Characterization, InTech, 2013, https://doi.org/10.5772/55982.
[32] T. Fujiyoshi, T. Ikami, T. Sato, K. Kikukawa, M. Kobayashi, H. Ito, A. Yamamoto, Evaluation of the matrix effect on gas chromatography – mass spectrometry with carrier gas containing ethylene glycol as an analyte protectant, J. Chromatogr. A 1434 (2016) 136–141, https://doi.org/10.1016/j.chroma.2015.12.085.
[33] D.A. Armbruster, M.D. Tillman, L.M. Hubbs, Limit of detection (LQD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs, Clin. Chem. 40 (1994) 1233–1238, https://doi.org/10.1093/clinchem/40.7.1233.
[34] H.-F. Schött, S. Krautbauer, M. Höring, G. Liebisch, S. Matysik, A validated, fast method for quantification of sterols and gut microbiome derived 5α/β-stanols in human feces by isotope dilution LC–high-resolution MS, Anal. Chem. 90 (2018) 8487–8494, https://doi.org/10.1021/acs.analchem.8b01278.
[35] P. Poudel, S.A. Petropoulos, F. Di Gioia, Plant tocopherols and phytosterols and their bioactive properties, in: Natural Secondary Metabolites, Springer International Publishing, Cham, 2023, pp. 285–319, https://doi.org/10.1007/978-3-031-18587-8_8.
[36] K.M. Phillips, D.M. Ruggio, J.I. Toivo, M.A. Swank, A.H. Simpkins, Free and esterified sterol composition of edible oils and fats, J. Food Compos. Anal. 15 (2002) 123–142, https://doi.org/10.1006/jfca.2001.1044.
[37] L. Teh, C. Möllers, Genetic variation and inheritance of phytosterol and oil content in a doubled haploid population derived from the winter oilseed rape Sansibar × Oase cross, Theor. Appl. Genet. 129 (2016) 181–199, https://doi.org/10.1007/s00122-015-2621-y.
[38] J.-H. Han, Y.-X. Yang, M.-Y. Feng, Contents of phytosterols in vegetables and fruits commonly consumed in China, Biomed. Environ. Sci. 21 (2008) 449–453, https://doi.org/10.1016/S0895-3988(09)60001-5.
[39] F. D'Acunzo, D. Giannino, V. Longo, M. Ciardi, G. Testone, G. Mele, C. Nicolodi, M. Gonnella, M. Renna, G. Arnesi, A. Schiappa, O. Ursini, Influence of cultivation sites on sterol, nitrate, total phenolic contents and antioxidant activity in endive and stem chicory edible products, Int. J. Food Sci. Nutr. 68 (2017) 52–64, https://doi.org/10.1080/09637486.2016.1221386.

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