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Comparative phytochemical profile of the elephant garlic (*Allium ampeloprasum* var. *holmense*) and the common garlic (*Allium sativum*) from the Val di Chiana area (Tuscany, Italy) before and after *in vitro* gastrointestinal digestion

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ABSTRACT

This study is aimed to comparatively investigate the phytochemical profiles, focusing on the nutritional and phytochemical properties of common garlic (*Allium sativum* L.; CG) and elephant garlic (EG) (*Allium ampeloprasum* var. *holmense*) collected from the Val di Chiana area (Tuscany, Italy). The results showed a lower amount of fibers, demonstrating a higher digestibility of the bulb, and sulfur-containing compounds in EG rather than in CG. Untargeted metabolomic profiling followed by supervised and unsupervised statistics allowed understanding the differences in phytochemical composition among the two bulbs, both as raw bulbs, processed following the *in vitro* gastrointestinal digestion process. Typical sulfur-containing compounds, such as alliin and *N*-gamma-glutamyl-S-allyl cysteine, could notably be detected in lower amounts in EG. EG maintains a distinct phytochemical signature during *in vitro* gastrointestinal digestion. Our findings support the distinct sensorial attributes of the bulbs.

1. Introduction

The genus *Allium* includes about 700 bulbous species characterized by high diversity, considering the physiological and morphological aspects. Elephant garlic (EG) (*Allium ampeloprasum* var. *holmense* (Mill.) Asch. et Graebn.) resembles leek (*Allium ampeloprasum* var. *porrum* (L.) J. Gay) and common garlic (CG) (*Allium sativum* L.) in terms of shape and flavor, although it is three times larger than CG (Kim et al., 2018a). Moreover, *A. sativum* is the economically most important species and has been used as a food and a drug. This bulbous species has antifungal, antibacterial, antiviral, antitoxic, and anticancer properties (Kim et al., 2018a; Rattanachaikunsopon & Phumkhachorn, 2009). The bioactive compounds of garlic are reported to have a biological effect on human metabolism (i.e., antithrombotic and fibrinolytic effects on the blood, reducing the effect of LDL cholesterol) (Steiner, Khan, Holbert, & Lin, 1996). These bioactive compounds of CG can be divided into sulfur-containing compounds and sulfur-free polyphenolic compounds. The sulfur-containing compounds (i.e., alliin and its derivatives) are mainly responsible for the antimicrobial activity (Corzo-Martínez, Corzo, & Villamiel, 2007) and the peculiar sensorial attributes of this bulb. By contrast, the sulfur-free polyphenolic compounds play an important role in preventing oxidative damage caused by reactive oxygen species (ROS) (Ma et al., 2011).

Nowadays, EG has been proposed as a substitute for CG in cooking (fresh or processed) because its flavor is very close to that of CG but with a milder impact on human breath and a better digestibility than common garlic (Block, 2011). For this reason, the EG is named "kissingarlic", "garlic for people who do not like garlic" and "garlic-like" (Lu, Ross, Powers, Aston, & Rasco, 2011). In Val di Chiana, an area located in Tuscany (center of Italy), with peculiar weather and soil characteristics, EG was included in the list of Traditional Agri-food Products of the Tuscany Region (Executive Decree Tuscany Region, n. 1569 of April 4th, 2016 https://www.aglionevaldichiana.net/public/Documenti/Decreto_Regione_Toscana.pdf, Aglione della Valdichiana)

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and later in the list of Traditional Agri-food Products of Italy (G.U. n.143 of June 21st, 2016 https://www.aglionevaldichiana.net/public/Documenti/Decreto_MiPAAF.pdf, Aglione della Valdichiana) with the name "Aglione della Valdichiana".

In terms of nutritional and nutraceutical values, the features of EG suggest a reduction in sulfur-compounds among its bioactive substances, although few studies have been performed on its characterization to date. Kim et al. (2018b) evaluated the organo-sulfur compounds in *Allium* species, showing a high content of γ-glutamyl peptides in EG and the highest alliin content in CG. Therefore, they reported higher sulfur-containing volatile compounds in CG than in EG. Although at low concentrations, 13 sulfur compounds were found in CG and six sulfur compounds in EG. In EG and CG bulbs obtained from Poland, EG bulbs showed higher polyphenols and antioxidant activity than CG bulbs (Najda, Błaszczyk, Winiarczyk, Dyduch, & Tchórzewska, 2016). The authors did not find particular differences in terms of the polyphenol profiles between the two bulbs. In EG and CG bulbs obtained from America, EG bulbs showed lower antioxidant activity than CG bulbs, while and the polyphenol content was similar in both the bulbs (Lu et al., 2011). A comparison of the studies by Najda et al. (2016) and Lu et al. (2011) suggests that the origin of both the bulbs may play a pivotal role in determining the polyphenol profile.

Holistic approaches like metabolomics could be very useful to describe and differentiate the bioactive compounds in CG and EG and to ensure geographical traceability (Maietti et al., 2012). This last aspect becomes relevant with the view of a hypothetic award of PDO (Protected Designation of Origin), as proposed for "Aglione della Valdichiana". The qualitative and quantitative differences in secondary metabolites, such as sulfur compounds and polyphenols, can better discriminate the bulbs, identifying potential counterfeits, thus ensuring traceability. It is worthy to note that deep profiling of the phytochemicals in these two bulbs opens the possibility to further investigate the nutraceutical properties rather than desired or unpleasant sensorial attributes.

In this regard, combining *in vitro* gastrointestinal digestion process with untargeted metabolomics may be useful to analyze the bioaccessibility of polyphenols and other health-related compounds. To date, limited information on the changes of bioactive compounds in CG and/or EG during simulated gastrointestinal processes is present in the literature (Bhatt & Patel, 2013; Rosen et al., 2001; Torres-Palazzolo et al., 2018). In recent years, several studies have reported the bioaccessibility of health-promoting compounds, considering it as the percentage of compounds from the food sample released during the simulation of digestion (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002; Rocchetti, Chiodelli, Giuberti, & Lucini, 2018; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). A combination of *in vitro* gastrointestinal digestion with untargeted metabolomics may provide a better understanding of the main changes occurring to bioactive compounds during simulated gastrointestinal processes (Rocchetti et al., 2018).

To the best of our knowledge, to date, no detailed comparative studies on the nutritional parameters, nutraceutical compounds, and their bioaccessibility after EG and CG digestion have been performed. Therefore, we investigated the nutritional aspects, some mineral elements (including sulfur) as well as phenols and secondary metabolites in EG and CG obtained from Val di Chiana using untargeted metabolomics. Then, the fate of garlic metabolites was investigated for the first time, using an *in vitro* gastrointestinal digestion and an untargeted metabolomics-based approach, to further explore the main differences between the two bulbs from a nutritional standpoint.

2. Materials and methods

2.1. Materials and reagents

All chemicals used in this study were of analytical grade. Perchloric acid, nitric acid, ethanol, methanol, formic acid, acetonitrile, and Na_2CO_3 were purchased from Carlo Erba Reagents (Cornaredo, Milan, Italy). Gallic acid and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Milan, Italy). The commercial kit for sugar assay was purchased from Megazyme (Wicklow, Ireland). For protein determination, the Protein Assay Kit II® (Bio-Rad) was used. The elution solvents, acetonitrile and water (both LCMS grade), were purchased from VWR (Milan, Italy), while formic acid was from LCMS (Sigma Aldrich, Milan, Italy). Finally, for the *in vitro* gastrointestinal digestion step, α-amylase (75 U mL^{-1}; from human saliva Type IX-A, Sigma-Aldrich, Milan, Italy), porcine pepsin (2000 U mL^{-1}; P7000; Sigma-Aldrich Milan, Italy), pancreatin (100 U mL^{-1}; P1750; Sigma-Aldrich Milan, Italy), and bile salts (10 mM; B8631; Sigma-Aldrich Milan, Italy) were used.

2.2. Plant materials

Common garlic and elephant garlic samples were grown in the Val di Chiana area and were offered by local association "Qualità e Sviluppo Rurale S.r.l." to the Department of Agriculture, Food and Environment (DAFE) of the University of Pisa during June 2019. Both the bulbs (50 samples) were derived from the same farm and have been cultivated in the same climatic and edaphic conditions to exclude the contribution of pedo-climatic conditions. Ten bulbs of cloves of EG and CG, uniform in diameter, were randomly selected, manually peeled, and chopped into small pieces. Pieces derived from three randomly bulk groups were selected to represent a sample replicate. Bulb material was lyophilized for the determination of some mineral elements, whereas fiber, sugar content, and the untargeted metabolomics based on high-resolution mass spectrometry; the other part was freeze-dried and stored at –80 °C until analysis. All the analyses were carried out in triplicate.

2.3. Proximate analysis

A part of freeze-dried material was weighed and oven-dried at 65 °C till constant weight, and the percentage of dry matter (% DM) was calculated. Then, the dried samples were used for the determination of phosphorous, potassium, calcium, and magnesium concentration in both the analyzed bulbs. Dry tissues were mineralized for 60 min at 220 °C using a solution of HNO_3: $HClO_4$ (2.5:1 *v/v*). Phosphorus concentration was determined colorimetrically using an Ultrospec 2100 Pro spectrophotometer (GE Healthcare Ltd., Little Chalfont, UK), following the Olsen method, whereas K, Ca, and Mg with an atomic absorption spectrophotometer (Varian AA 24FS, Australia). The results were expressed as the percentage of P, K, Ca, and Mg. Nitrogen, carbon, hydrogen and sulfur determinations were obtained from a Vario MICRO cube (Elementar, USA)

Sugar (sucrose and glucose) quantification was carried out according to Yusof, Rasmusson, and Galindo (2016) and Sotelo, Pérez, Najar-Rodriguez, Walter, and Dorn (2014) with slight modifications. For soluble sugar extraction, 100 mg of dried leaf samples were finely ground in a mortar, suspended in 10 mL of 80% aqueous ethanol (v/v), and placed in an ultrasonic water bath at 60 °C for 30 min. The solution was centrifuged at 10,000 g for 10 min at 10 °C, and the supernatant was filtered using an HPLC filter (pore size: 0.45 μm). Sucrose and glucose were determined using K-SUFRG commercial kit (Megazyme, Wicklow, Ireland), following the manufacturer's protocol. The results were expressed as g·100 g^{-1} dry weight (DW).

Protein determination was performed using the spectrophotometer and the Protein Assay Kit II® (Bio-Rad). Using a bovine serum albumin standard curve, the results were expressed as mg protein per g fresh weight (FW).

The crude fiber and the fiber fractions—neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), hemicellulose, and cellulose—were analyzed according to the method

described by Van Soest, Robertson, and Lewis (1991) using the instrument ANKOM (ANKOM 65 rpm agitation). The results were expressed as a percentage (%).

Briefly, this procedure is based on sequential extraction with neutral and acidic detergents, followed by strong acid extraction. The different fractions were as follows: (i) the soluble fraction extracted using neutral detergent, (ii) hemicelluloses extracted using 2 M hydrochloric acid detergent, (iii) cellulose extracted using 72% sulfuric acid, and (iv) lignin, the difference after the acid extraction.

2.4. Total phenolic content

For total phenolic extraction, the fresh material (1 g) was finely ground in a mortar, suspended in 4 mL 80% aqueous methanol (*v/v*), and placed in an ultrasonic water bath (Digital ultrasonic Cleaner, DU-45, Argo-Lab, Modena, Italy) at 4 °C for 30 min with a power of 180 W. For the determination of total phenolic content, the solution was centrifuged at 10,000 *g* for 7 min, and an amount of the supernatant was added to Folin-Ciocalteu reagent, following Folin-Ciocalteu method described by Dewanto, Wu, Adom, and Liu (2002) with slight modifications. Next, 1.25 mL Na_2CO_3 7% (*w/v*) was added to the solution, and samples were incubated for 90 min in dark conditions. The increase in absorbance was measured spectrophotometrically at 760 nm against a blank. The total phenolic content was expressed as milligrams equivalents of gallic acid per g of fresh weight (mg GAE g^{-1} FW).

2.5. Extraction and untargeted metabolomic profiling by UHPLC-QTOF mass spectrometry

One gram of each lyophilized sample was homogenized in 10 mL of 0.1% formic acid in 80% (*v/v*) methanol solution using a homogenizer-assisted extraction system (Ultra-Turrax, IkaT25, Staufen, Germany) as previously reported by Rocchetti et al. (2019a). Afterward, samples were centrifuged at 6000 *g* for 15 min at 4 °C and then supernatants were filtered with 0.22 nm cellulose syringe filters in vials, which were stored at –18 °C until analysis.

Subsequently, the untargeted metabolomic profile of both bulbs was investigated using UHPLC-ESI-QTOF mass spectrometry, as previously described (Rocchetti et al., 2019a). Briefly, chromatography was carried out in the reverse phase mode using an Agilent Zorbax eclipse plus C18 column and a water-acetonitrile gradient solution (from 6% up to 90% acetonitrile in 33 min) for separation. Formic acid 0.1% (v/v) was added as a phase modifier to both water and acetonitrile. The mobile phase temperature was set to 35 °C, the injection volume was 6 μL, and the flow rate was 220 μL min^{-1}. For mass spectrometry detection, the instrument worked in positive MS-only mode, acquiring accurate masses in the 50–1200 *m/z* range at a rate of 0.8 spectra/s (absolute peak height threshold 3000 counts, relative height threshold 0.0001%). The MS was operated in extended dynamic range mode with a nominal mass resolution of 30,000 FWHM. Nitrogen was used as both sheath gas (10 L min^{-1} at 350 °C) and drying gas (8 L min^{-1} at 330 °C). Besides, the nebulizer pressure was 60 psig, the nozzle voltage was 300 V, and the capillary voltage was 3.5 kV. The annotation of garlic and elephant garlic metabolites was achieved using the software Profinder B.07 from Agilent Technologies, according to the "find-by-formula" algorithm. In particular, the annotations were recursively achieved against two comprehensive databases namely FoodDB (www.fooddb.ca) and Phenol-Explorer (http://phenol-explorer.eu/), fully available in the literature for untargeted studies in food metabolomics and using the entire isotopic profile with a maximum of 5 ppm for mass accuracy. Therefore, in our experimental conditions, Level 2 of compound identification was achieved as set out by the COSMOS Metabolomics Standards Initiative (Rocchetti et al., 2020; Salek et al., 2015; Schrimpe-Rutledge, Codreanu, Sherrod, & McLean, 2016). The obtained dataset was further used for statistics and chemometrics.

2.6. Simulated in vitro gastrointestinal digestion process

The *in vitro* gastrointestinal digestion, simulating the oral, gastric, and pancreatic digestion phases, was applied to lyophilized samples according to the static method described by Minekus et al. (2014). The samples (250 mg) were homogenized with 175 μL simulated salivary fluid (SSF) (Minekus et al., 2014) at pH 7.0, 25 μL salivary α-amylase solution made up in SSF electrolyte stock solution, 1.25 μL $CaCl_2$ 0.3 M and 48.75 μL water. The oral step was run at 37 °C for 2 min. Then, the oral bolus samples were mixed (ratio 1:1) with 375 μL simulated gastric fluid (SGF) at pH 3.0, 80 μL porcine pepsin stock solution made up in SGF electrolyte stock solution (pepsin from porcine gastric mucosa), 0.25 μL $CaCl_2$ 0.3 M, 10 μL HCl 1 M to adjust pH to 3.0, and rest of volume with water. The gastric phase was carried out for 2 h at 37 °C. Then, gastric chyme was mixed (1:1) with 550 μL simulated intestinal fluid (SIF) electrolyte stock solution consisting at pH 7.0, 250 μL of a pancreatin solution (based on pancreatin α-amylase activity) made up in SIF electrolyte stock solution (pancreatin from the porcine pancreas), 125 μL fresh bile (160 mM in fresh bile), 2 μL $CaCl_2$ 0.3 M, 7.5 μL NaOH 1 M to adjust pH to 7.0, and water filling the rest of the volume. The intestinal phase was carried out for 2 h at 37 °C. At selected time points (i.e., gastric and pancreatic phases) corresponding digestion sample tubes for each material were cooled on ice to stop the reaction. The experiment was performed in triplicate. Finally, to depict the fate of bioactive compounds during the *in vitro* gastrointestinal digestion, the digested samples were prepared and analyzed using an untargeted UHPLC-ESI/QTOF mass spectrometry as mentioned above for undigested materials.

2.7. Statistical analysis and chemometrics

The results of the proximate analysis, minerals, and total phenolic content were compared with a two-tailed Student's *t*-test using a significance level of 0.05. Data are expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad (GraphPad, La Jolla, CA, USA).

Metabolomic data were interpreted using Agilent Mass Profiler Professional B.12.06 (from Agilent Technologies). Compounds were filtered by abundance and frequency (only those compounds with an area > 5000 counts and appearing in 100% of samples in at least one condition were considered), normalized at the 75th percentile, and baselined to the median of each compound in all samples. The unsupervised hierarchical cluster analysis (HCA–Euclidean distance) was then used to naively group samples, according to intrinsic similarities in metabolomic profile (Rocchetti et al., 2019a). Besides, a principal component analysis (PCA) was used to obtain information about technical variability by observing the dispersion of replications within treatments. Afterward, the dataset was exported into SIMCA 13 (Umetrics, Malmo, Sweden), Pareto scaled and elaborated for orthogonal partial least squared discriminant analysis (OPLS-DA) supervised modeling, considering the combination of "sample type × digestion phase" as a class membership criterion. Finally, the variable importance in projection (VIP analysis) method was used to evaluate the discrimination potential of the different metabolites (i.e., those compounds possessing a VIP score > 1), and a Fold-Change analysis (FC ≥ 5) was combined with ANOVA ($P < 0.01$, Bonferroni multiple testing correction) in Volcano plot to point out differential metabolites between raw CG and EG samples.

3. Results and discussion

3.1. Nutritional results

The results of the proximate analysis of CG and EG bulbs cultivated in the Val di Chiana area are shown in Table 1. No differences were found in sugar (glucose and sucrose) content of both the analyzed

Table 1
Proximate analysis and minerals of edible garlic and elephant garlic cloves. Data were compared with Student *t*-test (P ≤ 0.05). Significance ns: not significant; *: P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001 for the interaction of factors.

Proximate composition		Unit	Garlic (*Allium sativum*)	Elephant garlic (*Allium ampeloprasum* var. *holmense*)	Significance
Protein		g 100 g−1 FW	0.98 ± 0.05	1.22 ± 0,26	*
Carbohydrate	Glucose	g 100 g^{-1} DW	3.55 ± 0.52	3.36 ± 0.14	ns
	Sucrose		0.11 ± 0.04	0.11 ± 0.03	ns
Dietary fibre	Neutral detergent fibre (NDF)	%	9.98 ± 0.16	8.46 ± 0.30	**
	Acid detergent fibre (ADF)		9.09 ± 0.40	8.58 ± 1.09	ns
	Acid detergent lignin (ADL)		3.91 ± 0.34	3.25 ± 0.31	ns
	Hemicellulose		0.48 ± 0.33	0.55 ± 0.12	ns
	Cellulose		2.06 ± 0.23	1.29 ± 0.04	**
Minerals	Nitrogen (N)	%	2.64 ± 0.26	1.88 ± 0.06	**
	Carbon (C)		41.29 ± 0.26	42.38 ± 0.28	**
	Hydrogen (H)		6.56 ± 0.02	6.68 ± 0.04	*
	Sulphur (S)		0.38 ± 0.01	0.65 ± 0.18	*
	Phosphorus (P)		1.17 ± 0.04	0.59 ± 0.01	***
	Potassium (K)		1.04 ± 0.11	0.76 ± 0.02	*
	Calcium (Ca)		0.21 ± 0.01	0.23 ± 0.02	ns
	Magnesium (Mg)		0.05 ± < 0.01	0.06 ± < 0.01	ns
Moisture content		%	62.13 ± 0.57	67.23 ± 0.67	***
Dry matter		%	37.87 ± 0.57	32.77 ± 0.67	***

bulbs, whereas a higher protein content was recorded in EG than in CG. Even the dry matter and the moisture contents were different comparing both the bulbs, with EG reporting a higher moisture content than CG (Table 1). NDF and cellulose resulted significantly lower in the EG as compared to CG, evidencing the superior digestibility of this bulb (Baer, Rumpler, Miles, & Fahey, 1997). These differences probably reflect the differences in variety and species among the two analyzed bulbs as well as the different conditions of growth. Mineral content in the bulb samples differed significantly for P and K exclusively and these minerals were lower in the EG as compared to the CG (Table 1). An interesting result is the higher content of sulfur element found in EG (+41%) as compared with CG. Mineral results are comparable with the findings by Sajid, Butt, Shehzad, and Tanweer (2014) and Odebunmi, Oluwaniyi, and Bashiru (2010).

3.2. Phytochemical discrimination of raw samples

The functional components are represented by the total phenolic content, which was found to be significantly higher in EG than in CG (Fig. 1). This result is in agreement with the findings by Lu et al. (2011), while contrasting observations were made by Najda et al. (2016), who reported very low total phenolic content in both the bulb types. Hence, untargeted metabolomics based on UHPLC-QTOF mass spectrometry was used to comprehensively investigate the differences and similarities in phytochemicals of the two bulb samples, both before and after *in vitro* gastrointestinal digestion. Overall, this approach allowed us to putatively annotate 2745 mass features that were classified according to the FooDB database, together with individual abundances and composite mass spectra (Table S1). Besides, representative total ion current chromatograms for both bulbs and considering the different digestion phases (i.e., raw, gastric, and pancreatic) are reported in the supplementary material (Table S1). Firstly, a Volcano plot was produced to compare raw EG *vs* CG by coupling ANOVA (P ≤ 0.01) and Fold-Change (cut-off ≥ 5) analysis (Table S2). As it can be observed, 161 metabolite species were found to discriminate EG and CG, thus suggesting distinctive chemical fingerprints of the raw matrices before *in vitro* gastrointestinal digestion. Interestingly, only 22% of the discriminant markers were found to be down-accumulated in EG when compared with CG; among these compounds, typical compounds characterizing garlic such as alliin (belonging to α-amino acids), together with two isomeric dipeptides, namely, *N*-gamma-glutamyl-*S*-allyl cysteine and *N*-gamma-glutamyl-*S*-cis-(1-propenyl)cysteine. The down-accumulation of sulfur-containing compounds, such as *N*-gamma-glutamyl-S-allyl cysteine and *N*-gamma-glutamyl-S-cis-(1-propenyl)cysteine, in EG (Table S2), could explain the higher total phenolic content observed in EG compared to CG (Fig. 1). Phan, Netzel, Chhim, Netzel, and Sultanbawa (2019) reported that the total phenolic content can decrease with the increase in organosulfur compounds and terpenoid substances in mature garlic bulbs. In addition, a significant down-accumulation of seven polyphenols [including rosmarinic acid, irigenin, gerberinol, myristicanol B, *ent*-epiafzelechin-(2alpha-7,4alpha-8)-catechin, ramontoside and epiafzelechin-(4alpha-8)-pelargonidin 3′-glucoside] and eight prenol lipids [*trans*-carvyl acetate, 6-acetylfuranofukinol, urodiolenone, sterebin A, dihydrofukinolide, boviquinone 4, 2-(2-methylbutanoyl)-9-(3-methyl-2E-pentenoyl)-2b,9a-dihydroxy-4Z,10(14)-oplopadien-3-one, erinacine D) was observed in EG when compared with CG (Table S2).

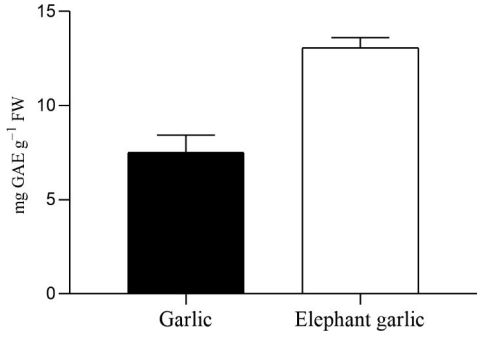

Fig. 1. Total phenolic content of common garlic and elephant garlic. Data were compared with Student *t*-test (P ≤ 0.05). Significance ***: P ≤ 0.001.

Overall, it has been suggested that the biological and health-promoting properties of garlic are primarily derived from its polyphenols and organosulfur compounds (Phan et al., 2019). These trends were confirmed by Kim et al. (2018b), reporting a high level of bioactive γ-glutamyl peptides in both EG and CG. However, considering that organosulfur compounds are extremely unstable and susceptible to further transformation into volatile compounds (such as allicin and diallyl-sulfides), recent attention has been placed on polyphenols due to their potential role in health-related benefits to humans. On the other hand, 125 additional compounds (i.e., the remaining 78% of discriminant metabolites outlined by Volcano plot analysis) were proposed in this work as chemical markers of EG. The most represented classes among the discriminant markers were those of steroids and derivatives (27 compounds), glycerophospholipids (24 compounds), prenol lipids (13 compounds), polyphenols (12 compounds), amino acids and derivatives (8 compounds), organooxygen compounds (8 annotations) and fatty

acyls (7 compounds). Therefore, our findings revealed a high abundance of compounds belonging to lipids and steroids (mainly saponins) in EG. These results are not surprising as plants belonging to the genus *Allium* have been previously reported as a good source of bioactive saponin compounds, responsible for many of their reported pharmacological activities (e.g. antiproliferative, antifungal, and antispasmodic activities). In this regard, previous studies (Lanzotti, 2005; Petropoulos et al., 2018) showed that saponins, such as spirostane, cholestane, and oleane-type structures, are widely represented in *Allium*, thus confirming our findings.

Additionally, an abundance of *N*-gamma-L-glutamyl-L-methionine and eruboside B was noticed in the EG sample (Table S1). *N*-Gamma-L-glutamyl-L-methionine belongs to the organosulfur compounds with important biological effects (lipid-lowering, antidiabetic, anticancer, anti-asthmatic, antiplatelet, and anti-atherosclerotic activities) previously described by Kim et al. (2018b), while eruboside B is a typical garlic compound that improves the antimicrobic properties of *Allium* vegetables (Nakamoto, Kunimura, Suzuki, & Kodera, 2020). However, it is important to take into account that genotype has a greater impact on the metabolomic profile of CG and EG bulbs (Najda et al., 2016); therefore, both genotype and pedoclimatic conditions represent two critical parameters that need to be taken into account to reach the quality improvement of the final products.

3.3. *In vitro* gastrointestinal digestion and discrimination of both bulb samples

Moreover, once the differences between EG and CG were represented in the raw matrices, multivariate statistics (based on both unsupervised and supervised methods) was used to depict the changes occurring during the *in vitro* gastrointestinal digestion process of both bulbs. The unsupervised hierarchical cluster analysis (i.e., HCA) and principal component analysis (PCA) carried out on the UHPLC-QTOF mass spectrometry data allowed to identify a clear separation trend (Fig. 2 and Table S1), outlining a stronger impact of each digestion phase (i.e., both gastric and pancreatic phases) on the phytochemical composition of both bulb samples. Besides, the PCA score plot revealed that the two principal components were able to explain > 65% of the total variance (Table S1). Overall, the initial differences in phytochemical profiles between EG and CG may be notably conserved, even during the digestion process. Therefore, the results from unsupervised statistics suggest that a further application of the OPLS-DA score plot would help to point out the most discriminant compounds, driving the trends observed. The OPLS-DA score plot illustrates a modification of the metabolite profiles shifting from raw samples to digested samples (Fig. 3). This supervised model allowed us to confirm the unsupervised findings (Fig. 2). First, confirmation of the differences existing on the raw matrices was noticed on the left part of the graph, confirming the results of Volcano plots previously discussed (Table S2). Besides, the second latent vector t [2] showed a clear impact of both gastric and pancreatic phases of digestion, driven also by the different matrix incubated (i.e., EG *vs* CG) (Fig. 3).

Furthermore, a variable selection method (VIP; variable importance in projection) was used to reduce the number of variables and better explain the differences observed in the *in vitro* digestion process (Table 2). Markers that are assigned a VIP score of > 1 are organized in chemical class and reported together with the Log Fold-Change (FC) values for each main comparison (i.e., EG *vs* CG on raw, gastric, and pancreatic samples) (Table 2). Overall, 85 compounds matched this criterion (including some isomeric compounds), mainly belonging to the classes of polyphenols, amino acids, benzenoids, sulfur-containing compounds, fatty acyls, glycerophospholipids, heteroaromatic compounds, indoles, prenol lipids, pyrrolizines, quinolines, steroids, and derivatives, tetrahydrofurans, and other compounds. Overall, when considering the comparison EG *vs* CG during the gastric phase of digestion, the most affected compounds were found to be hovenidulcigenin A (a prenol lipid; LogFC = –18.55), LysoPC(18:1(11Z)) (a glycerophospholipid; LogFC = –18.36) and 4-[(2-hydroxy-1-naphthalenyl)azo]benzenesulfonic acid (a benzenoid; LogFC = –9.28). To date, the bioactive and pharmacological role of the hovenidulcigenin A and 4-[(2-hydroxy-1-naphthalenyl)azo]benzenesulfonic acid has not been established in the literature, while the LysoPC species play

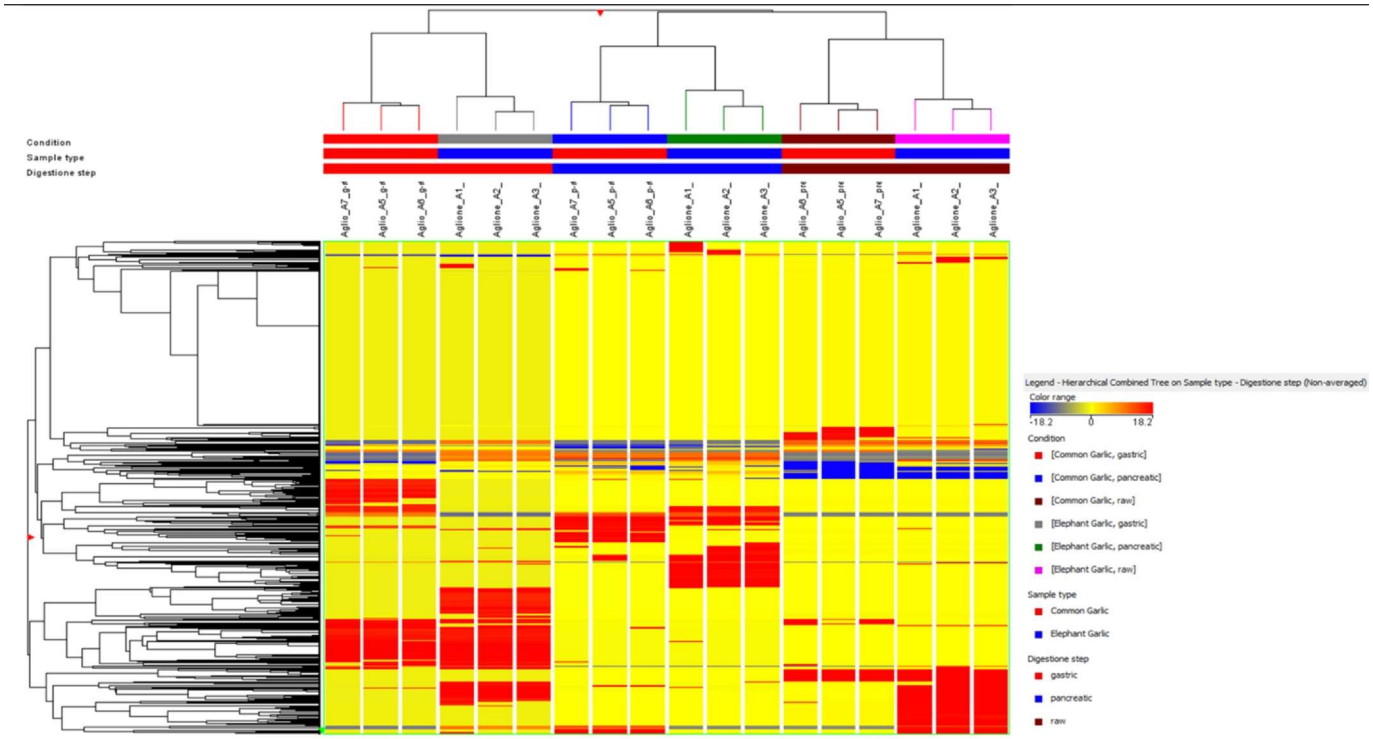

Fig. 2. Unsupervised hierarchical cluster analysis (HCA) based on Fold-Change heat map (similarity: Euclidean; linkage rule: ward) for raw and *in vitro* digested elephant garlic (EG) and common garlic (CG) samples.

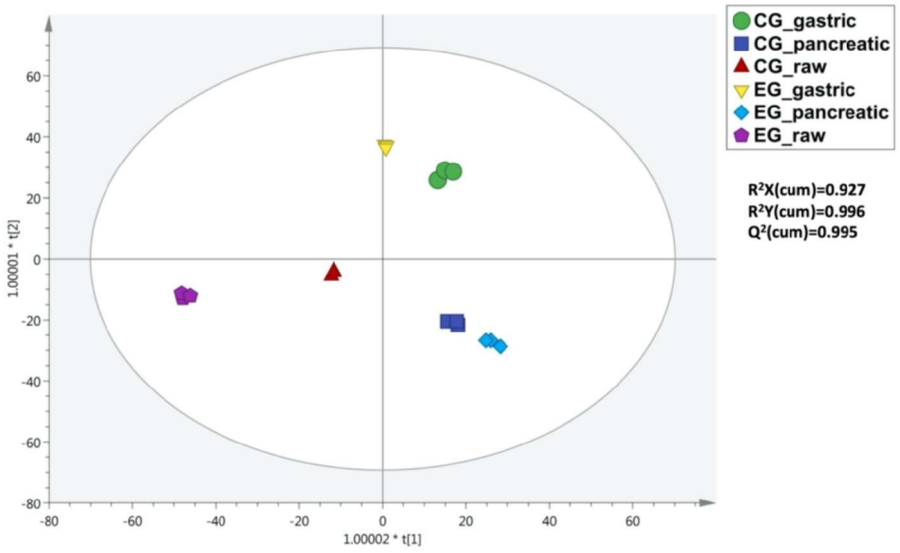

Fig. 3. Supervised OPLS-DA prediction model for raw and *in vitro* digested elephant garlic (EG) and common garlic (CG) samples.

an important role as lipid mediators in cellular responses and pathophysiology. They are involved in the activation of inflammatory responses, and their potential role as the vaccine has been discussed (Wi, 2014).

Moreover, the gastric phase of digestion mainly affected CG polyphenols composition. The discriminant compounds, namely, 4-hydroxybenzoic acid 4-O-glucoside, phloretin, and butein/naringenin were characterized in EG during the gastric phase by LogFC values > 2. The 4-hydroxybenzoic acid is a phenolic acid that can be converted into more useful compounds such as resveratrol, muconic acid, gastrodin, ubiquinone with a wide variety of biological and pharmaceutical activities as antibacterial, antioxidant, anticancer, hypolipidemic, and prevention of heart diseases activities (Wang, Bilal, Hu, Wang, & Zhang, 2018). Phloretin is one of the best-known dihydrochalcone with antifungal, antiviral, anti-inflammatory, anticancer, and estrogenic activity, and can improve the fluidity of biological membranes, increasing the penetration of drugs (Behzad et al., 2017). Butein and naringenin are flavonoids with important health roles such as antioxidant, antitumor, cardioprotective, antiviral, and antibacterial activity (Bordoloi et al., 2019; Salehi et al., 2019).

Finally, analyzing the results of the pancreatic phase, EG was characterized by an overall down-accumulation of several lipid-derived compounds, mainly belonging to fatty acyls (6 annotated compounds) and steroids (4 annotated compounds). Afterward, the polyphenol 4-hydroxybenzoic acid 4-O-glucoside showed significantly higher LogFC values (i.e., 9.95) in EG when compared to CG also during the pancreatic digestion phase. Other metabolites characterizing EG during the pancreatic phase of digestion were pantoyllactone glucoside (fatty acyls), (2S,4S)-monatin (alpha-amino acid), followed by several isomeric compounds classified as "other compounds" (Table 2). The OPLS-DA model also allowed to detect discriminant compounds characterizing the raw bulbs; for example, AS 1–5 (belonging to the class of organic compounds known as glycosyl-*n*-acylsphingosines and typical in garlic) was found to characterize the raw EG (LogFC = 19.91) sample, but it was highly affected by the *in vitro* digestion process (Table 2).

Regarding the potential biomarkers proposed in this study, 4-hydroxybenzoic acid 4-O-glucoside, 5-nonadecylresorcinol, and tryptophan were found to be among the most representative compounds in the undigested EG (Table 2). According to the literature (Bento-Silva et al., 2020), phenolic acids such as 4-hydroxybenzoic acid 4-O-glucoside can be released from the food matrix in the stomach, further enhancing their release and absorption. The absorption of phenolic acids from beverages occurs at a greater extent than from solid food matrices. Most of the phenolic acids exist as conjugated or bound to dietary fiber, thus reaching the colon and becoming available for further metabolism by the gut microbiota (Mosele, Maciá, & Motilva, 2015; Rocchetti et al., 2019b). Besides, hydrolysis by intestinal or microbial esterases can promote the release of phenolic acids in the intestine, supporting their absorption across the gastrointestinal barrier and enter the peripheral blood circulation. Therefore, besides a clear species effect, our findings suggested that consuming EG could be a valid strategy to promote the bioaccessibility of bioactive phenolic acids, such as 4-hydroxybenzoic acid 4-O-glucoside. Another phenolic compound namely 5-nonadecylresorcinol is an alkylresorcinol with cytotoxic activity, and it can significantly inhibit the growth of various cell lines, such as lung cancer cells, human epithelial cells, breast cancer cells, epithelioid cervix carcinoma cells, and human central nervous system tumor cell line (Liu, Winter, Stevenson, Morris, & Leach, 2012). The tryptophan is an essential amino acid for human health because the human body is not able to synthesize it. Recently, Li et al. (2019) reported a high antioxidant activity of this amino acid and the addition of tryptophan to the walnut protein-derived peptides can inhibit of xanthine oxidase, a critical enzyme in human health, because of its ability to catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Therefore, the inhibition of the xanthine oxidase may be able to alleviate the development of hyperuricemia (Li et al., 2019). The different accumulation of health-related compounds in both analyzed bulbs, during the *in vitro* gastrointestinal digestion process, supports the potential exploitation of EG as a source of bioactive compounds with important biological and pharmacological roles in addition to CG.

4. Conclusions

In this study, we compared the phytochemical compounds of CG and EG obtained from the Val di Chiana area (Tuscany, Italy), focusing on bioactive compounds. Although these two bulbs are similar in shape and aspect, they belong to different *Allium* species. The differences are reflected in the proximate results and in two distinctive metabolomic profiles. First, the EG samples showed a lower fiber content, which is supportive of a higher digestibility of this bulb, confirming the lower sulfur-containing compounds found in EG than in CG. The total phenolic content was 2-fold higher in EG than in CG. Finally, the untargeted metabolomics approach using UHPLC-QTOF mass spectrometry allowed us to identify a higher number of organosulfur compounds in CG than in EG. These sulfur-containing metabolites are

Table 2
Bioactive compounds identified by VIP (Variable Importance in Projection) selection method following OPLS-DA model, in the raw matrix of elephant garlic and common garlic and during the *in vitro* gastrointestinal digestion of the two analysed bulbs. Compounds are provided together with VIP scores (measure of variable's importance in the OPLS-DA model) and Log Fold-Change in raw material and during different phases (gastric and pancreatic phases) of the *in vitro* digestion. [1]ns: not significative.

Class	Metabolites	VIP score (OPLS-DA)	LogFC [EG *vs* CG] raw	LogFC [EG *vs* CG] gastric	LogFC [EG *vs* CG] pancreatic
Polyphenols	4-Hydroxybenzoic acid 4-O-glucoside	1.02 ± 0.56	11.42	2.16	9.95
	Rosmarinic acid	1.06 ± 0.25	–18.70	–0.83	ns
	Sinapoylspermine	1.22 ± 0.23	[1]ns	–0.37	–17.73
	Bisdemethoxycurcumin	1.22 ± 0.27	0.35	0.64	2.31
	5-Nonadecylresorcinol	1.00 ± 0.53	21.89	ns	ns
	Phloretin	1.06 ± 0.14	0.49	9.80	ns
	Butein/Naringenin	1.24 ± 0.32	ns	11.23	0.01
Aminoacids and derivatives	L-Proline	1.02 ± 0.58	–2.56	–1.01	–6.90
	N-acetyl lysine methyl ester	1.23 ± 0.33	9.24	9.28	–0.33
	Tryptophan	1.23 ± 0.35	19.61	–0.46	–0.29
	Cinnamoylglycine	1.22 ± 0.36	0.57	1.08	–0.30
	(2S,4S)-Monatin	1.22 ± 0.33	0.10	0.64	19.61
Benzenoids	*N*-Acetylarylamine/ *N*-benzylformamide/2-Phenylacetamide	1.22 ± 0.32	17.95	1.23	–0.31
	2-(2-Methylpropoxy)naphthalene	1.23 ± 0.33	ns	9.28	–0.33
	4-[(2-Hydroxy-1-naphthalenyl)azo]benzenesulfonic acid	1.23 ± 0.11	0.56	–9.28	ns
	1,1′-[1,12-Dodecanediylbis(oxy)]bisbenzene	1.24 ± 0.35	ns	16.89	0.03
Sulphur containing compounds	*N*-gamma-glutamyl-S-allylcysteine/ *N*-gamma-glutamyl-S-trans-(1-propenyl) cysteine	1.01 ± 0.38	–7.01	0.64	2.31
Fatty acyls	Methyl (R)-3-methyl-2-oxopentanoate	1.22 ± 0.19	ns	0.17	20.27
	Pantoyllactone glucoside	1.23 ± 0.27	0.23	0.57	18.85
	Sativic acid/ Pinellic acid/9,12,13-TriHOME/(9S,10E,12S,13S)-9,12,13-Trihydroxy-10-octadecenoic acid/5,8,12-Trihydroxy-9-octadecenoic acid/ 9,10,13-TriHOME	1.23 ± 0.13	ns	–0.01	–19.08
	Cervonoyl ethanolamide	1.24 ± 0.38	–7.76	7.80	0.16
Glycerophospholipids	LysoPC(18:1(11Z))	1.24 ± 0.36	–0.65	–18.36	–0.15
	LysoPC(20:3(8Z,11Z,14Z))	1.24 ± 0.39	ns	–1.32	0.15
	LysoPC(20:4(8Z,11Z,14Z,17Z))	1.24 ± 0.37	ns	–1.32	–0.01
	1–16:0–2–18:1-phosphatidylcholine	1.25 ± 0.16	0.41	17.52	ns
	1–18:3–2–18:1-phosphatidylcholine	1.25 ± 0.18	0.34	18.55	0.16
	PE-NMe2(16:0/16:0)	1.25 ± 0.32	0.15	–1.01	ns
Heteroaromatic compounds	5-(2-Furanyl)-3,4-dihydro-2H-pyrrole	1.22 ± 0.32	17.96	1.23	–0.31
	3,4-Dihydro-4-[(5-methyl-2-furanyl)methylene]-2H-pyrrole	1.25 ± 0.19	20.92	0.18	0.09
Indoles and derivatives	3-(1H-Indol-3-yl)-2-propenoic acid	1.22 ± 0.36	0.55	1.10	–0.30
	5-Methoxyindoleacetate/ Indolelactic acid/ Methyl 1-methoxy-1H-indole-3-carboxylate/ Methyl oxindole-3-acetate	1.22 ± 0.36	0.57	1.09	–0.30
	Indole-3-ethanol/ Tryptophol	1.25 ± 0.19	20.91	0.18	0.09
Prenol lipids	Hovenidulcigenin A	1.24 ± 0.38	ns	–18.55	0.06
	Hydroxysintaxanthin 5,6-epoxide	1.25 ± 0.34	ns	–1.32	–0.006
Pyrrolizines	2,3-Dihydro-1H-pyrrolizine-5-carboxaldehyde	1.22 ± 0.32	17.96	1.23	–0.31
	1-(2,3-Dihydro-1H-pyrrolizin-5-yl)-2-propen-1-one	1.25 ± 0.19	20.92	0.18	0.09
Quinolines and derivatives	Edulitine	1.22 ± 0.36	0.57	1.08	–0.30
	Graveolinine/ Graveoline	1.24 ± 0.40	ns	1.32	0.19
	6-Methylquinoline	1.25 ± 0.19	20.91	0.18	0.09
Steroids and derivatives	Taurochenodesoxycholic acid	1.22 ± 0.45	ns	18.64	0.26
	Withaphysacarpin/14alpha-Hydroxyixocarpanolide/2,3-Dihydrowithanolide E/ Perulactone B	1.22 ± 0.23	ns	–0.28	–17.4
	Lithocholic acid glycine conjugate	1.24 ± 0.37	21.31	ns	0.19
	Cucurbitacide E	1.24 ± 0.38	ns	–1.32	–0.07
	2-Hydroxyestrone sulfate	1.24 ± 0.32	–18.01	–0.36	0.04
	3b-Hydroxy-5-cholenoic acid	1.24 ± 0.35	ns	–1.32	–0.04
Tetrahydrofurans	Tetrahydrofurfuryl acetate/ Botryodiplodin	1.23 ± 0.19	ns	0.17	20.27
Other compounds	2-Aminoacetophenone	1.22 ± 0.32	17.96	1.23	–0.31
	L-Menthone 1,2-glycerol ketal	1.24 ± 0.39	–16.87	ns	0.19
	Avenalumin II	1.24 ± 0.40	ns	1.32	0.19
	Canavaninosuccinate	1.23 ± 0.28	0.21	0.65	19.61
	4-hydroxysphinganine	1.24 ± 0.39	ns	1.32	0.13
	4-Hydroxycyclohexylcarboxylic acid	1.22 ± 0.19	ns	0.17	20.27
	Dihydro-2,4-dimethyl-6-(2-methylpropyl)-4H-1,3,5-dithiazine	1.23 ± 0.33	9.27	9.30	–0.33
	Ethyl levulinate	1.22 ± 0.19	ns	0.17	20.27
	AS 1–5	1.01 ± 0.53	19.91	ns	ns
	N-(2,5-Dihydroxyphenyl)pyridinium(1 +)	1.22 ± 0.35	0.52	1.11	–0.31
	(S)-Pterosin K	1.22 ± 0.29	0.20	0.63	–10.32
	6-Chloro-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine	1.22 ± 0.36	0.56	1.08	–0.30
	Dictyoquinazol C	1.24 ± 0.32	ns	–1.32	0.03
	3-[(5-Methyl-2-furanyl)methyl]-1H-pyrrole	1.25 ± 0.19	20.91	0.18	0.09
	(R)-Boschniakine	1.25 ± 0.19	20.91	0.18	0.09
	Acetyl-methylpyridine derivatives	1.22 ± 0.32	17.96	1.23	–0.32

responsible for several biological effects of *Allium* vegetables, in addition to the unpleasant smell that garlic leaves in the breath of humans. This opens the possibility of using EG rather than CG for food tasting purposes. The untargeted metabolomics approach also identified 125 key metabolites in raw EG, including lipid-derived molecules, polyphenols, and amino acid-derived compounds. Clear differences were outlined between EG and CG during *in vitro* gastrointestinal digestion process, evidencing a higher impact of the gastric phases on the phytochemical modifications, with 4-hydroxybenzoic acid 4-O-glucoside, 5-nonadecylresorcinol, and tryptophan proposed as biomarkers of the consumption of EG. Taken together, the present findings indicate distinct phytochemical profiles among EG and CG, with distinct bioactive and functional properties and sensorial attributes. Our dataset also contributes to identifying some putative biomarkers that could be exploited for the traceability of *Allium ampeloprasum* var. *holmense*.

CRediT authorship contribution statement

Costanza Ceccanti: Conceptualization, Data curation, Formal analysis, Writing - original draft. **Gabriele Rocchetti:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing. **Luigi Lucini:** Validation, Writing - review & editing, Supervision. **Gianluca Giuberti:** Methodology, Investigation, Formal analysis, Writing - review & editing. **Marco Landi:** Conceptualization, Writing - review & editing, Supervision. **Stefano Biagiotti:** Resources, Supervision. **Lucia Guidi:** Validation, Writing - review & editing, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128011.

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