Article

Analysis of Phytosterols Content in Italian-Standard Espresso Coffee

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Abstract: This study aims to assess for the first time the content of phytosterols (PS) in espresso coffee (EC) to deepen the knowledge about the phytochemicals and health potentials of coffee brews. PS were extracted by hot saponification from 14 EC samples produced with coffee originating from 13 coffee-producing countries. PS were identified and quantified by high-performance liquid chromatography (HPLC) after derivatization. Among the detected PS, β-sitosterol (4.1–18.2 mg/L) was the most abundant followed by stigmasterol (1.1–4.9 mg/L), campesterol (0.9–4.7 mg/L), and cycloartenol (0.3–2.0 mg/L). Total PS fraction ranged from 6.5 mg/L to 30.0 mg/L with an average level of 15.7 ± 5.8 mg/L. Therefore, a standard cup of EC (25 mL) could provide 0.4 ± 0.1 mg of PS.

Keywords: espresso coffee; phytosterols; β-sitosterol; HPLC; cholesterol lowering

1. Introduction

Espresso coffee (EC) is one of the most common coffee hot brews in the world, obtained from the percolation of hot water under pressure through compacted cakes of roasted ground coffee, where the energy of the water pressure is spent within the cake [1].

EC consumption is continually increasing internationally, being used not only as coffee brew but also in the preparation of other highly consumed coffee beverages such as cappuccino, Frappuccino, or flat white. According to the latest statistics from the International Coffee Organization (ICO), about 10 billion kg of coffee have been sold in 2020, so around 2.7 billion cups of coffee a day are consumed worldwide [2].

Nowadays, coffee brewed from coffee capsule systems is slowly replacing traditional brewing methods. Coffee capsules are small recipients containing previously roasted and ground coffee beans that are used in specially designed systems. These systems greatly increased and facilitated the consumption of homemade EC in the world [3].

EC brewing has been studied in various scientific experiments using different devices (e.g., espresso and capsule machines, etc.) and methodologies to optimize the beverage preparation process [4–9]. Indeed, coffee is a popular beverage with a unique sensory profile and also a complex source of hundreds of bioactive compounds, starting from green beans up to the final cup of coffee. The most studied bioactive compounds in coffee brews include caffeine, trigonelline, diterpenes, and polyphenols such as chlorogenic acids (Figure 1) [10–15].
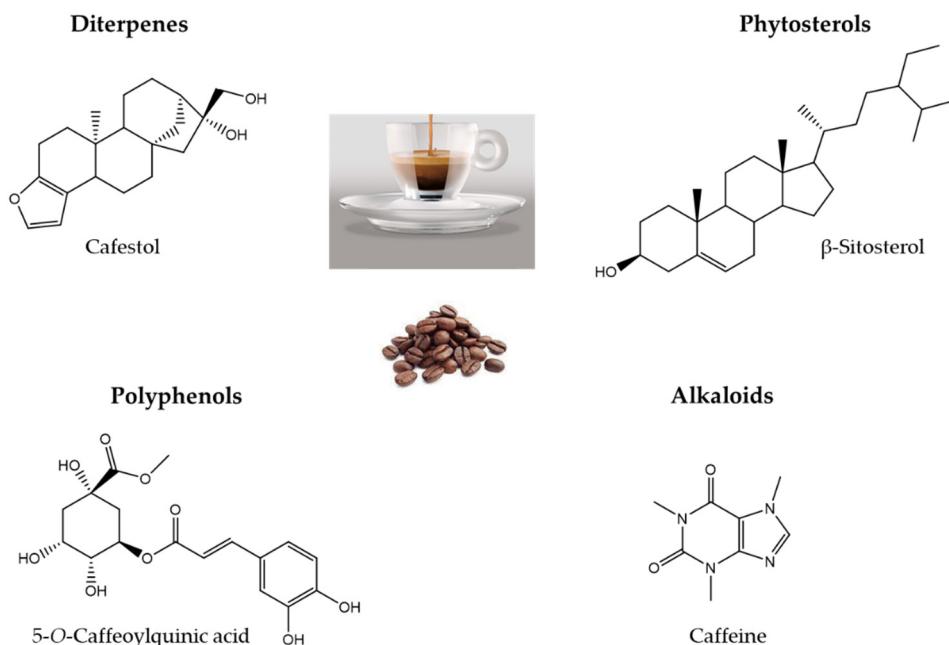
Figure 1. Illustrative summary of the main classes of bioactive compounds present and studied in coffee with an example for each class. Phytosterols represent the only class never studied in coffee brews.

Among all the bioactive compounds investigated in coffee-based products, few studies have explored the presence and levels of phytosterols (PS) in coffee beans and oil [16,17]. However, to our knowledge, PS content has never been assessed in a famous coffee brew such as EC. PS, which include plant sterols/stanols, are common bioactive compounds in plants exhibiting anticancer, hepatoprotective, and blood LDL cholesterol-lowering properties [18,19]. The latter is opposed to the effects of diterpenes, another class of compounds found in coffee, which are responsible for the controversial association between coffee drinking, blood cholesterol increment, and cardiovascular diseases [20]. However, the newest scientific outcomes demonstrate that regular coffee consumption is associated with a reduced risk of type 2 diabetes mellitus, liver disease, CVD, and some types of cancer [21,22]. Thus, considering the health benefits of PS, it is important to assess the human intake of PS through coffee consumption and thus, the potential biological benefits of EC.

PS profiles have been characterized in various foods of the human diet, such as vegetable oil, cereals, fruits, legumes, and vegetables [23–25]. More recently, Nzekoue et al. (2020) studies revealed that coffee spent ground could be a very good and eco-friendly source of PS [26]. Thus, we hypothesized that a notable coffee brew such as EC, very common all over the world, could contain PS. Therefore, this study aims to assess the concentration of phytosterols in our everyday cup of EC. To achieve this objective, 14 coffee samples originated from 13 different countries were collected for EC preparation. PS extraction and analysis from obtained EC were performed through optimized and validated ultrasound-assisted extraction and HPLC methods.

2. Materials and Methods

2.1. Reagents and Standards

Analytical standards of Campesterol ($C_{28}H_{48}O$, CAS No. 474–62-4) were provided by Carbosynth (Berkshire, United Kingdom), while β-sitosterol ($C_{29}H_{50}O$, CAS No. 83–46-5), stigmasterol ($C_{29}H_{48}O$, CAS No. 83–48-7), cycloartenol ($C_{30}H_{50}O$, CAS No. 469–38-5), and hexacosanol (1-hexacosanol) as internal standard (I.S, $C_{26}H_{54}O$, CAS No. 79983–71-4) were supplied by Sigma-Aldrich (Milano, Italy). Dansyl chloride ($C_{12}H_{12}ClNO_2S$, CAS No. 506–52-5) and 4-dimethylaminopyridine (DMAP) were provided by Sigma-Aldrich (Milano, Italy). All solvents used were HPLC or analytical grade. The deionized water

(58 M cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA), ethanol was supplied by Fisher Scientific (Loughborough, UK), hexane was supplied by Carlo Erba (Milan, Italy), while HPLC-grade acetonitrile and dichloromethane were supplied by Merck (Darmstadt, Germany). All the solvents and solutions were filtered through a 0.45 μm filter from Supelco (Bellefonte, PA, USA) before use. Stock standard solutions (1000 μg/mL) were prepared by dissolving 10 mg of standard in 10 mL of chloroform (Merck, Darmstadt, Germany) and were used to prepare standard working solutions at various concentrations by proper dilution in acetonitrile.

2.2. Coffee Samples and Espresso Machines

Coffee samples for EC preparation were all 100% Arabica originating from 13 different countries (Table 1) and provided in single dose capsules or roasted coffee beans (Figure 2).

Table 1. Espresso coffee origins and labels. C: capsules; R: roasted coffee beans.

Sample N°.		Sample Origin
1	C	Brazil
2	C	Ethiopia (1)
3	C	Colombia
4	C	Costa Rica
5	R	Guatemala
6	R	EI Salvador
7	R	Yemen
8	R	Dominican Rep.
9	R	Ethiopia (2)
10	R	Rwanda
11	R	Burundi
12	R	Uganda
13	R	Kenya
14	R	Timor-Leste

Figure 2. Coffee samples forms for EC preparation.

Coffee samples in capsules (C) were roasted by Illycaffe and were from single-origin countries (Brazil, Ethiopia, Colombia, Costa Rica). For EC preparation, capsules were applied into automatic capsule coffee machine "X7.1—Macchina da Caffè Iperespresso bianca", which was manufactured by Illycaffe S.p.A. (Via Flavia 110, 34, 147 Trieste, Italy).

EC from roasted coffee bean samples (R) was also from single-origin countries (Guatemala, EI Salvador, Yemen, Dominican Rep., Ethiopia (2), Rwanda, Burundi, Uganda, Kenya, and Timor-Leste) and was prepared using the grinding machine (Mythos 1) and the semiautomatic espresso machine (Vittoria Arduino, VA388 Black Eagle) provided by the espresso machine manufacturing company Simonelli Group S.p.A. (Belforte del Chienti, Italy).

2.3. EC Preparation

Two different preparations of espresso coffee were conducted. The first was performed using a single-dose capsule containing 7 g of coffee, while the second was completed with

roasted and ground coffee beans (7 g of coffee cake). The extraction conditions of EC with capsule coffee machine (19 bars; 80 ± 5 °C) and semi-automatic espresso machine (9 bars; 90 ± 2 °C) were maintained constant following standard procedures for Certified Italian EC preparation: coffee temperature in the cup of 67 ± 3 °C, percolation time of 25 s, viscosity at 45 °C > 1.5 mPas, total fat $\geq$ 2 mg/mL, caffeine < 100 mg/cup, and volume in the cup (inclusive of foam) 25 ± 2.5 mL [27,28]. Moreover, considering the influence of water on EC quality [20], minimally mineralized water was used and was bought always from the same commercial brand (Blues, Italy). This water is commercially available and its parameters are: total solid at 180 °C 22.0 mg/L: HCO_3^-—9.5 mg/L; Ca^{2+}—2.8 mg/L; Mg^{2+}—0.45 mg/L; SiO_2—7.3 mg/L; NO_3^-—1.0 mg/L; Na^+—1.8 mg/L; SO_4^{2-}—3.6 mg/L; Cl^-—0.21 mg/L; K^+—0.20 mg/L; F^-—<0.10 mg/L.

2.4. Phytsterols Extraction from EC

A measure of 10 mL of EC in a round flask was saponified with 25 mL of KOH (50% *w/w*) and 100 mL of ethanol. Saponification was performed in a water bath at 80 °C for 40 min. After cooling, the saponified samples were extracted three times with hexane (50 + 25 + 25 mL). The extracts were collected and dried using a rotary evaporator and then, reconstituted with 1 mL of hexane. The obtained extract was spiked with the internal standard (hexacosanol 10 μg/mL) and once again dried under nitrogen. The reconstitution was performed with 2 mL of dansyl chloride and DMAP solution (8 mg/mL in dichloromethane) the obtained solution was heated at 40 °C for 30 min following the method proposed by Nzekoue et al. (2020) [29]. After derivatization, samples were dried under nitrogen, dissolved in 1 mL of acetonitrile, and filtered on a 0.45 μm PTFE filter (Supelco Bellefonte, PA, USA) for HPLC analysis.

2.5. HPLC-DAD Analyses

PS were analyzed by an HPLC-DAD system (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) following a previously validated method [18]. PS separation was performed with a Gemini C18 analytical column (250 × 3.0 mm, 5 μm, Phenomenex, Torrance, CA, USA) as stationary phase. The mobile phase consisted of H_2O (A) and methanol (B) at a flow rate of 0.5 mL/min following a gradient elution mode: 0 min, 30% B; 0–10 min, 100% B; 10–75 min, isocratic condition, 100% B; 75–80 min, 30% B; 80–85 min, 30% B. The injection volume was 20 μL, the column temperature was 40 °C, and 254 nm was used as wavelength of detection.

2.6. Statistical Analyses

All the results are expressed as mean $\pm$ standard deviation (SD) with data measurements and analyses repeated in triplicate (n = 3). Relative standard deviation (%RSD) was determined to assess the precision of the obtained levels. Statistically significant differences between the samples were determined by one-way analysis of variance (ANOVA) with a significant level α of 0.05.

3. Results and Discussion

3.1. Extraction of PS in EC

EC can be considered as an extraction method for ground coffee using hot and high-pressured water. Indeed, during EC preparation, various bioactive compounds including alkaloids, polyphenols, and PS are removed from coffee to water due to the penetration of water inside vegetable cells. PS are naturally present in plant cells in 2 forms: free form and conjugated forms, the latter including phytosteryl esters (PSE), phytosteryl glycosides (PSG), and acylated phytosteryl glycosides (APSG) [30]. However, through EC preparation, just PS in the free form are extracted. Indeed, various studies reported that the extraction of conjugated PS in food matrix requires a prior hydrolysis step in acidic conditions to break down ether and ester bonds linking PS in plant cell membranes [31,32].

To separate unsaponifiable compounds such as PS from emulsions such as EC, a saponification step followed by extraction with apolar solvent is recommended [26]. Saponification is necessary to hydrolyze ester bonds in triacylglycerides and free glycerol and fatty acid salts with higher polarities. Consequently, PS can be extracted with the unsaponifiable fraction using an apolar solvent such as hexane. This process thus allows to obtain a more selective extract with low impurities by separating PS from esters and other fats components.

3.2. HPLC Analysis

The applied HPLC method was performed through a pre-column derivatization step using a developed dansylation technique. Moreover, the derivatization provides chromophores on PS structures giving a specific UV-spectrum allowing to further confirm PS identification (Figure 3). The gradient elution allowed the separation of analytes. However, due to their high structural similarity, campesterol and stigmasterol were not fully separated. A similar issue is reported in published HPLC methods for PS analysis [33]. The quantification was performed by comparing the area of each analyte normalized by the area of I.S. in the standard solution and in EC sample and calculated through the calibration curves plot.
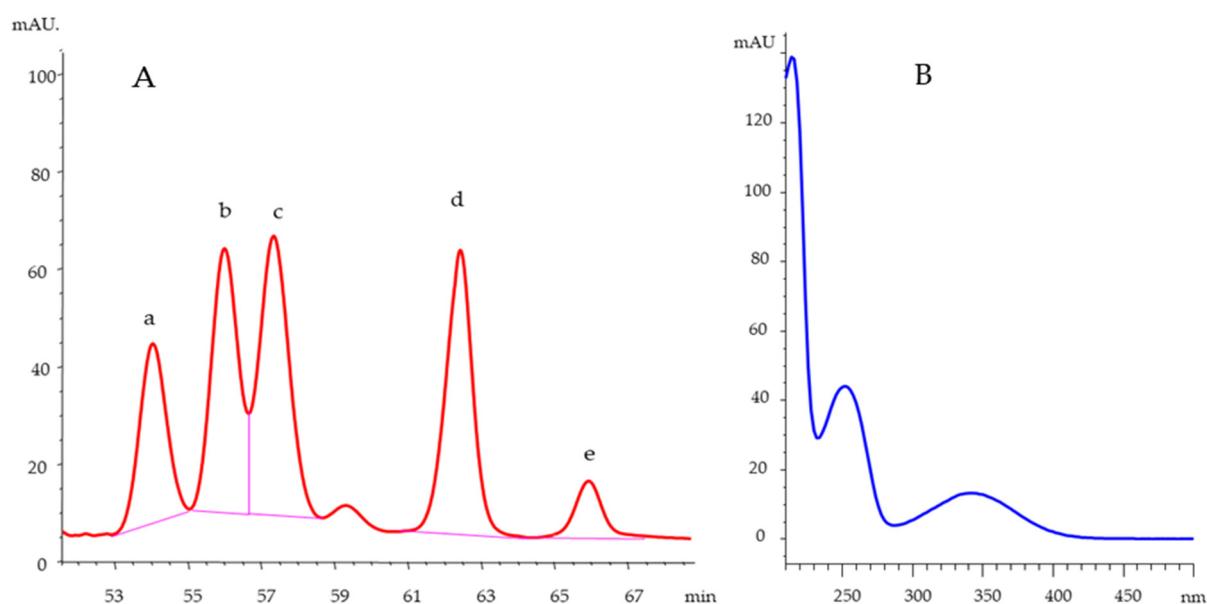

Figure 3. (**A**) Chromatogram of phytosterols standard solution at 100 mg/L: a for cycloartenol, b for campesterol, c for stigmasterol, d for β-sitosterol, and e for hexacosanol (internal standard); (**B**) UV spectrum of derivatized phytosterols.

Figure 4 shows a chromatogram of the EC sample from roasted Costa Rica coffee. Compared to other HPLC methods where PS are detected at 210 nm, which is non-selective, the developed method allowed the identification of PS at a more selective detection wavelength (254 nm), thus limiting the observation of interfering compounds. This is due to the pre-column derivatization step, which added chromophoric groups into PS structures.
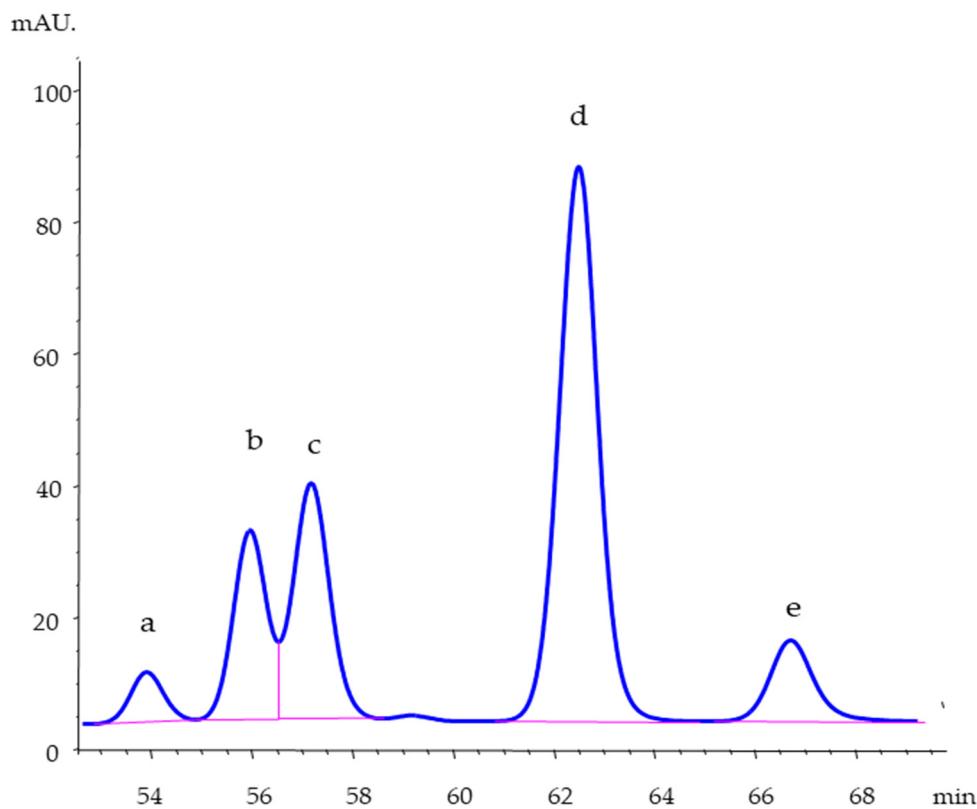
Figure 4. Chromatogram of phytosterols in espresso coffee from Costa Rica coffee-origin (sample No. 4), a for cycloartenol, b for campesterol, c for stigmasterol, d for β-sitosterol, and e for hexacosanol (internal standard).

3.3. Analysis of Phytosterols in EC Samples

The determination of bioactive compounds in coffee and coffee brew is an important topic in food science research. However, among the different classes of compounds investigated in coffee, phytosterols have received little attention. In this study, 14 samples of EC of different origins were analyzed to assess their PS content. The analysis revealed the presence of 4 PS: β-sitosterol, campesterol, stigmasterol, and cycloartenol (Figure 5).

The analytical method was validated showing good linearity ($R^2 \geq 0.998$) and reproducibility (intraday $\leq 0.5\%$ and interday $\leq 3.9\%$) for the four detected PS. Moreover, this method showed a high sensitivity with limit of detection (LOD) ranging between 9–15 ng/mL, and limit of quantification (LOQ) ranging from 29 to 50 ng/mL (Table S1).

Table 2 reports the levels of PS in the 14 EC analyzed. In all the EC samples studied, β-sitosterol was the most concentrated PS, with levels ranging from 4.8 mg/L (sample No. 6 from Ethiopia 2) to 18.0 mg/L (sample No. 4 from Costa Rica). Important levels of β-sitosterol were also found in sample No. 2 from Ethiopia 1 (13.3 ± 0.4 mg/L), sample No. 13 from Uganda (11.0 ± 0.5 mg/L), and sample No. 12 from Timor-Leste (10.4 ± 2.2 mg/L). Campesterol (0.9–4.7 mg/L) and stigmasterol (1.1–4.9 mg/L) were also abundant PS in EC. The highest levels of campesterol were found in sample No. 4 (4.6 ± 0.1 mg/L) followed by sample No. 12 (3.3 ± 0.2 mg/L), while the highest levels of stigmasterol were found in sample No. 4 (4.9 ± 0.1 mg/L) followed by sample No. 2 (4.3 ± 0.3 mg/L).
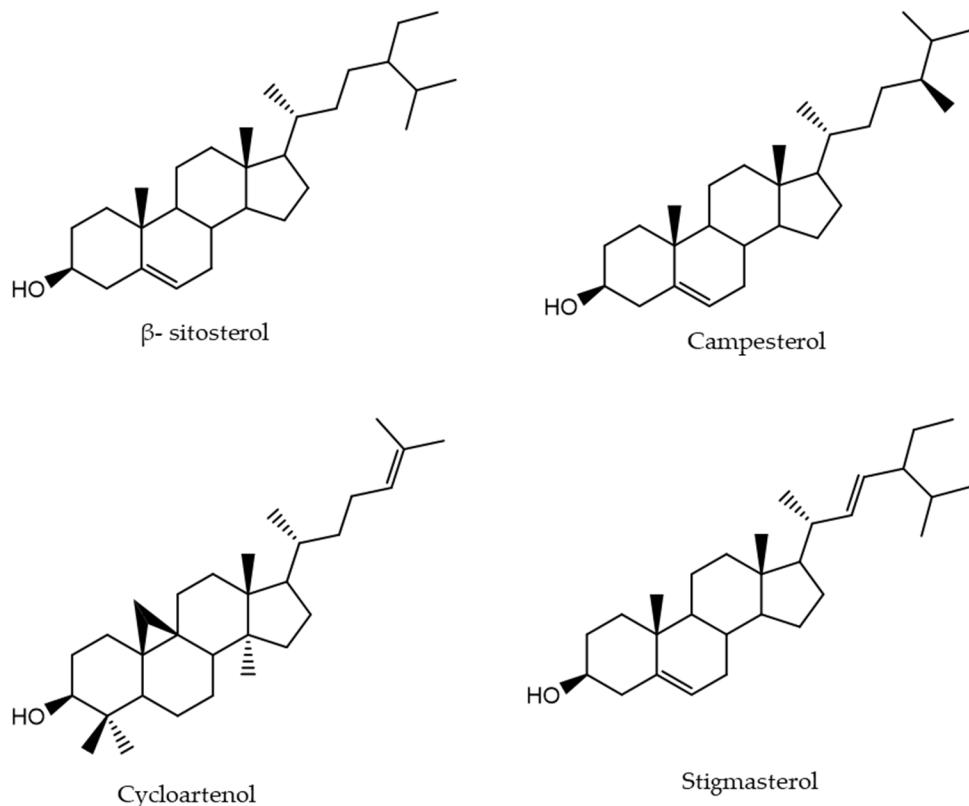
Figure 5. Chemical structures of detected phytosterols in espresso coffee.

Table 2. Concentration (mg/L) of phytosterols in different espresso coffee samples. Data are expressed as mean ± standard deviation, (*n* = 3 technical replicates). Total levels with different upper-case letters are statistically different with α of 0.05.

Samples	Stigmasterol	Campesterol	β-Sitosterol	Cycloartenol	Total
1	1.5 ± 0.1	1.0 ± 0.0	5.8 ± 0.2	0.3 ± 0.1	8.6 ± 0.3 [a]
2	4.3 ± 0.3	3.2 ± 0.1	13.3 ± 0.4	1.2 ± 0.1	22.0 ± 1.0 [bc]
3	1.9 ± 0.3	1.6 ± 0.3	8.4 ± 0.1	0.5 ± 0.0	12.4 ± 3.8 [ad]
4	4.9 ± 0.1	4.6 ± 0.1	18.0 ± 0.3	2.0 ± 0.1	29.5 ± 0.6 [c]
5	1.8 ± 0.1	1.5 ± 0.0	6.3 ± 0.3	0.7 ± 0.1	10.3 ± 0.2 [ad]
6	1.1 ± 0.1	1.0 ± 0.1	4.8 ± 0.1	0.5 ± 0.0	7.5 ± 1.4 [a]
7	2.0 ± 0.4	2.0 ± 0.6	8.8 ± 0.2	1.0 ± 0.3	13.7 ± 3.6 [abd]
8	2.1 ± 0.0	1.8 ± 0.2	8.1 ± 0.1	0.8 ± 0.1	12.8 ± 0.4 [ad]
9	3.0 ± 0.1	2.6 ± 0.0	9.4 ± 0.2	1.0 ± 0.0	16.0 ± 0.2 [abd]
10	3.1 ± 0.2	2.8 ± 0.4	10.9 ± 0.9	1.4 ± 0.3	18.2 ± 0.2 [bd]
11	2.8 ± 0.1	2.7 ± 0.1	10.5 ± 0.5	1.1 ± 0.3	16.4 ± 1.0 [abd]
12	3.4 ± 1.0	3.3 ± 0.2	10.4 ± 2.2	1.5 ± 0.4	18.6 ± 2.8 [bd]
13	2.9 ± 0.1	2.6 ± 0.2	11.0 ± 0.5	1.1 ± 0.0	17.5 ± 1.0 [bd]
14	3.0 ± 0.8	2.5 ± 1.0	9.7 ± 2.0	1.2 ± 0.4	16.4 ± 2.2 [abd]

Looking at the proportions of each PS in EC, we observed that β-sitosterol, stigmasterol, campesterol, and cycloartenol were, respectively, 61.3 ± 1.0%, 17.1 ± 0.5%, 15.2 ± 0.6%, and 6.4 ± 0.9% of total PS in EC. These percentages are in the range of the proportions of PS in Arabica coffee oil reported in the literature: 46.7–53.8%, 20.5–23.8%, 14.7–17.0%, respectively, for β-sitosterol, campesterol, and stigmasterol [16,17,34].

The total concentrations of PS varied between the samples, with total levels ranging between 7.5 ± 1.4 mg/L (sample No. 6) and 29.5 ± 0.6 mg/L (sample No. 4). One-way ANOVA test was performed to compare the results of the total PS content in the 14 EC

samples. Statistically significant differences were observed between some EC samples showing thus that the coffee origin impacts the PS levels of coffee beverages. Indeed, sample No. 4 showed a significantly higher content of PS than all the other samples except sample No. 2. Other statistically significant differences can be noted for example the lower concentration of PS in sample No. 6 compared to samples No. 2, 4, 10, 12, and 14.

3.4. Discussion

PS are steroidal compounds present in the membrane lipid bilayer of plant cells [31]. Although more than 200 PS structures have been reported, β-sitosterol, campesterol, and stigmasterol are the most occurring PS in foods representing, respectively, 65%, 30%, and 5% of total PS dietary intake [35]. The most studied sources of PS in common dietary patterns include vegetable oils, nuts, cereals, vegetables, and fruits [36,37]. The idea to study the PS content of EC comes from the high intake of this beverage all around the world, and the proportion of lipids inside EC (40–200 mg lipids/100 mL) [38]. Indeed, the lipids fraction of coffee beans has been analyzed in various studies reporting high levels of triacylglycerols (75% of total lipids) and a large proportion of the unsaponifiable fraction made mainly of diterpenes (19% of total lipids), sterols (5% of total lipids), and tocopherols (0.05% of total lipids) [17].

Few studies assessed the content of secondary metabolites from the unsaponifiable fraction of coffee brews focusing on diterpenes and tocopherols [39]. Diterpenes in coffee brew mainly refer to diterpene alcohols of the kaurene family with cafestol, kahweol, and 16-*O*-methylcafestol representing the principal compounds [40]. They principally occur in esterified forms with fatty acids and according to preparation conditions, their total content in EC can vary from 0.2–1.4 mg/25 mL for EC prepared from capsules [41] and 0.8–1.5 mg/25 mL for EC prepared from roasted coffee beans [42]. Despite their health benefits including anti-inflammatory, antioxidant, anti-angiogenic, and anti-carcinogenic properties, the interest of diterpenes in coffee brew comes from their LDL-cholesterol-elevating effects [43]. The effects of these single compounds suggested controversial impacts of coffee brew consumption on blood cholesterol levels, and consequently, the increasing of the risk of coronary heart disease (CHD). Indeed, some studies revealed that the occurrence of diterpenes varies according to coffee brews and preparation techniques. For example, diterpenes in boiled coffee Turkish coffee, and French-pressed coffee show the highest levels of diterpenes (1–2 mg/25 mL) while filtered coffee brew shows the lowest diterpenes content ($\leq$0.2 mg/25 mL) [44,45]. Although some case–control studies have reported a positive association between coffee drinking and CHD, the majority of cohort studies conducted in different countries with high consumption of EC such as Italy [46], Sweden [47], Finland [48], or the USA [49] did not observe any association between coffee brew intake and CHD for women and men. More recently, Groni et al. (2015) conducted a prospective cohort study on 30,449 women and 12,800 men in Italy, after which, they concluded that the intake of more than two cups/day of Italian-style EC is not associated with plasma cholesterol changes (LDL, HDL, or total cholesterol) [50].

To our knowledge, among all the studies on the unsaponifiable fraction of coffee, the present research is the first to assess the PS levels in a coffee beverage. The assessment of PS in EC is important considering the potent blood LDL cholesterol-lowering properties of PS [18] and the high consumption of coffee beverage in the world. It is important to highlight that the aim of this study was not to compare the different coffee origins but to have a wider spectrum of the PS content in EC by analyzing coffee brews obtained from 14 different sources. The mean levels of β-sitosterol (0.1–0.5 mg/25 mL), campesterol (0.02–0.12 mg/25 mL), stigmasterol (0.03–0.12 mg/25 mL), and cycloartenol (0.01–0.05 mg/25 mL) in one cup of standard EC are reported in Figure 6.
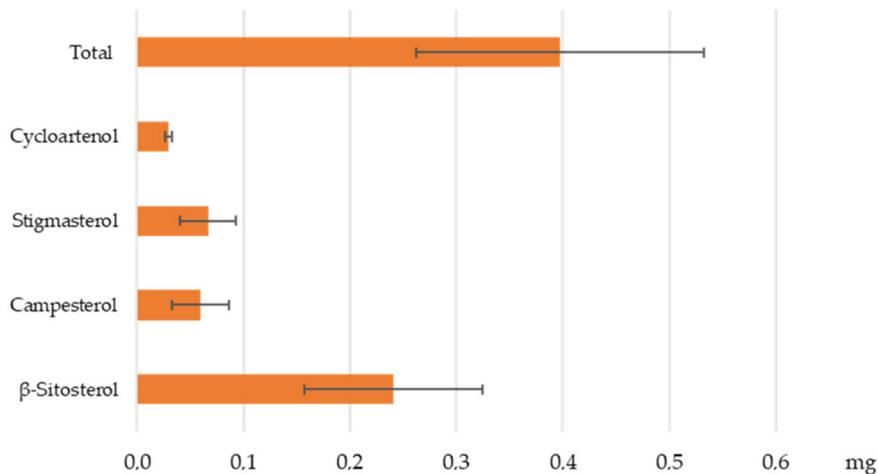
Figure 6. Mean levels of phytosterols (mg) in one cup of espresso coffee (25 mL). Results are expressed as mean $\pm$ standard deviation, with 14 biological replicates from 14 different samples (*n* = 14).

PS in coffee mostly belong to the family of 4-desmethylsterols, which are known to reduce LDL-blood cholesterol [51]. As reported in various analyses on coffee oil, β-sitosterol, campesterol, and stigmasterol are the most abundant PS in Arabica and Robusta coffee types [51,52]. Pacetti et al. [53] analyzed the unsaponifiable fraction of green coffee oil and observed various of PS including β-sitosterol, campesterol, citrostadienol, cycloartenol, Δ5-avenasterol, Δ7-avenasterol, 24-methylencycloartanol, and stigmasterol.

In this study, the four main PS in coffee were identified and quantified (β-sitosterol, campesterol, stigmasterol, and cycloartenol). Analyses performed in this study showed that the intake of one cup of EC (25 mL) according to coffee origin can provide between 0.2 mg and 0.7 mg of PS. However, the findings obtained do not suggest that the consumption of coffee brew can lower blood cholesterol. Indeed, according to Regulation (EU) No 432/2012 [54], the beneficial effect of PS is obtained from an intake $\geq$800 mg of PS/day, a quantity that cannot be obtained from normal coffee consumption.

4. Conclusions

This study reports for the first time the levels of PS in Italian-standard EC. HPLC analyses allowed the identification and quantification of four cholesterol-lowering compounds: β-sitosterol, campesterol, stigmasterol, and cycloartenol for a total level ranging between 0.2 and 0.7 mg/25 mL of brew. PS in EC could contrast the hypercholesterolemic effects of diterpenes report in the literature. However, further studies are still necessary to confirm this hypothesis. The methodology used can find applications in future experiments to assess more accurately the PS content of other coffee brews and determine how coffee preparation could affect PS levels in coffee.

References

1. Illy, A.; Viani, R.; Suggi Liverani, F. *Espresso Coffee: The Science of Quality*, 2nd ed.; Elsevier: Amsterdam, The Netherlands, 2005.
2. International Coffee Organization World Coffee Consumption, Data as of November 2020. Available online: https://ico.org/ (accessed on 1 August 2021).
3. Global Coffee Pods and Capsules Market–Growth, Trends and Forecasts (2020–2025), 2020, Mordor Intelligence. Available online: https://www.prnewswire.com/ (accessed on 1 August 2021).
4. Severini, C.; Caporizzi, R.; Fiore, A.G.; Ricci, I.; Onur, O.M.; Derossi, A. Reuse of spent espresso coffee as sustainable source of fibre and antioxidants. A map on functional, microstructure and sensory effects of novel enriched muffins. *LWT* **2020**, *119*, 108877. [CrossRef]
5. Caprioli, G.; Cortese, M.; Cristalli, G.; Maggi, F.; Odello, L.; Ricciutelli, M.; Sagratini, G.; Sirocchi, V.; Tomassoni, G.; Vittori, S. Optimization of espresso machine parameters through the analysis of coffee odorants by HS-SPME–GC/MS. *Food Chem.* **2012**, *135*, 1127–1133. [CrossRef] [PubMed]
6. Parenti, A.; Guerrini, L.; Masella, P.; Spinelli, S.; Calamai, L.; Spugnoli, P. Comparison of espresso coffee brewing techniques. *J. Food Eng.* **2014**, *121*, 112–117. [CrossRef]
7. Labbe, D.; Sudre, J.; Dugas, V.; Folmer, B. Impact of crema on expected and actual espresso coffee experience. *Food Res. Int.* **2016**, *82*, 53–58. [CrossRef]
8. Navarini, L.; Rivetti, D. Water quality for espresso coffee. *Food Chem.* **2010**, *122*, 424–428. [CrossRef]
9. Caprioli, G.; Cortese, M.; Sagratini, G.; Vittori, S. The influence of different types of preparation (espresso and brew) on coffee aroma and main bioactive constituents. *J. Food Compos. Anal.* **2015**, *66*, 505–513. [CrossRef] [PubMed]
10. Andueza, S.; Maeztu, L.; Pascual, L.; Ibanez, C.; de Pena, M.P.; Cid, C. Influence of extraction temperature on the final quality of espresso coffee. *J. Sci. Food Agric.* **2003**, *322*, 190–193. [CrossRef]
11. Khamitova, G.; Angeloni, S.; Fioretti, L.; Ricciutelli, M.; Sagratini, G.; Torregiani, E.; Vittori, S.; Caprioli, G. The impact of different filter baskets, heights of perforated disc and amount of ground coffee on the extraction of organics acids and the main bioactive compounds in espresso coffee. *Int. Food Res. J.* **2020**, *133*, 199–220. [CrossRef]
12. Pimpley, V.; Patil, S.; Srinivasan, K.; Desai, N.; Murthy, P.S. The chemistry of chlorogenic acid from green coffee and its role in attenuation of obesity and diabetes. *Prep. Biochem. Biotechnol.* **2020**, *50*, 969–978.
13. Sandi, D.; Araújo, J.M.A. Extraction of coffee diterpenes and coffee oil using supercritical carbon dioxide. *Food Chem.* **2007**, *101*, 1087–1094.
14. Jeszka-Skowron, M.; Zgoła-Grześkowiak, A.; Grześkowiak, T. Analytical methods applied for the characterization and the determination of bioactive compounds in coffee. *Eur. Food Res. Technol.* **2015**, *240*, 19–31. [CrossRef]
15. Angeloni, S.; Mustafa, A.; Abouelenein, D.; Alessandroni, L.; Acquaticci, L.; Nzekoue, F.; Petrelli, R.; Sagratini, G.; Vittori, S.; Torregiani, E.; et al. Characterization of the Aroma Profile and Main Key Odorants of Espresso Coffee. *Molecules* **2021**, *26*, 3856. [CrossRef]
16. Voldřich, H.Č.V.S.M.; Ševčík, R. Differentiation of coffee varieties according to their sterolic profile. *J. Food Nutr. Res.* **2007**, *46*, 28–34.
17. Speer, K.; Kölling-Speer, I. The lipid fraction of the coffee bean. *Braz. J. Plant Physiol.* **2006**, *18*, 201–216. [CrossRef]
18. Nzekoue, F.K.; Alesi, A.; Vittori, S.; Sagratini, G.; Caprioli, G. Development of a functional whey cheese (ricotta) enriched in phytosterols: Evaluation of the suitability of whey cheese matrix and processing for phytosterols supplementation. *LWT* **2021**, *139*, 110479. [CrossRef]
19. Feng, S.; Wang, L.; Belwal, T.; Li, L.; Luo, Z. Phytosterols extraction from hickory (*Carya cathayensis Sarg.*) husk with a green direct citric acid hydrolysis extraction method. *Food Chem.* **2020**, *315*, 126217. [CrossRef] [PubMed]
20. Cornelis, M.C.; El-Sohemy, A. Coffee, caffeine, and coronary heart disease. *Curr. Opin. Lipidol.* **2007**, *18*, 13–19. [CrossRef]
21. Gökcen, B.B.; Şanlier, N. Coffee consumption and disease correlations. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 336–348. [CrossRef] [PubMed]
22. O'Keefe, J.H.; Di Nicolantonio, J.J.; Lavie, C.J. Coffee for cardioprotection and longevity. *Prog. Cardiovasc. Dis.* **2018**, *61*, 38–42. [CrossRef] [PubMed]
23. Wang, M.; Huang, W.; Hu, Y.; Zhang, L.; Shao, Y.; Wang, M.; Zhang, F.; Zhao, Z.; Mei, X.; Li, T.; et al. Phytosterol profiles of common foods and estimated natural intake of different structures and forms in China. *J. Sci. Food Agr.* **2018**, *66*, 2669–2676. [CrossRef]

24. Asl, P.J.; Niazmand, R.; Jahani, M. Theoretical and experimental assessment of supercritical CO$_2$ in the extraction of phytosterols from rapeseed oil deodorizer distillates. *J. Food Eng.* **2020**, *269*, 109748.

25. Vu, D.C.; Lei, Z.; Sumner, L.W.; Coggeshall, M.V.; Lin, C.H. Identification and quantification of phytosterols in black walnut kernels. *J. Food Compos. Anal.* **2019**, *75*, 61–69. [CrossRef]

26. Nzekoue, F.K.; Khamitova, G.; Angeloni, S.; Sempere, A.N.; Tao, J.; Maggi, F.; Xiao, J.; Sagratini, G.; Vittori, S.; Caprioli, G. Spent coffee grounds: A potential commercial source of phytosterols. *Food Chem.* **2020**, *325*, 126836. [CrossRef]

27. Khamitova, G.; Angeloni, S.; Borsetta, G.; Xiao, J.; Maggi, F.; Sagratini, G.; Vittori, S.; Caprioli, G. Optimization of espresso coffee extraction through variation of particle sizes, perforated disk height and filter basket aimed at lowering the amount of ground coffee used. *Food Chem.* **2020**, *314*, 126220. [CrossRef]

28. Caprioli, G.; Cortese, M.; Odello, L.; Ricciutelli, M.; Sagratini, G.; Tomassoni, G.; Torregiani, E.; Vittori, S. Importance of espresso coffee machine parameters on the extraction of chlorogenic acids in a certified Italian espresso by using SPE-HPLC-DAD. *J. Food Res.* **2013**, *2*, 55. [CrossRef]

29. Nzekoue, F.K.; Caprioli, G.; Ricciutelli, M.; Cortese, M.; Alesi, A.; Vittori, S.; Sagratini, G. Development of an innovative phytosterol derivatization method to improve the HPLC-DAD analysis and the ESI-MS detection of plant sterols/stanols. *Int. Food Res. J.* **2020**, *131*, 108998. [CrossRef] [PubMed]

30. Zhu, D.; Nyström, L. Phytosterols. In *Whole Grains and Their Bioactives: Composition and Health*; Johnson, J., Wallace, T., Eds.; Wiley: Hoboken, NJ, USA, 2019; pp. 427–466.

31. Duong, S.; Strobel, N.; Buddhadasa, S.; Stockham, K.; Auldist, M.J.; Wales, W.J.; Moate, P.J.; Orbell, J.D.; Cran, M.J. Influence of acid hydrolysis, saponification and sample clean-up on the measurement of phytosterols in dairy cattle feed using GC–MS and GC with flame ionization detection. *J. Sep. Sci.* **2018**, *41*, 3467–3476. [CrossRef] [PubMed]

32. Moreau, R.A.; Nyström, L.; Whitaker, B.D.; Winkler-Moser, J.K.; Baer, D.J.; Gebauer, S.K.; Hicks, K.B. Phytosterols and their derivatives: Structural diversity, distribution, metabolism, analysis, and health-promoting uses. *Prog. Lipid Res.* **2018**, *70*, 35–61. [CrossRef] [PubMed]

33. Ito, M.; Ishimaru, M.; Shibata, T.; Hatate, H.; Tanaka, R. High-performance liquid chromatography with fluorescence detection for simultaneous analysis of phytosterols (stigmasterol, β-sitosterol, campesterol, ergosterol, and fucosterol) and cholesterol in plant foods. *Food Anal. Methods* **2017**, *10*, 2692–2699. [CrossRef]

34. Speer, K.; Kölling-Speer, I. Lipids: Production, quality and chemistry. In *Coffee*; Farah, A., Ed.; Royal Society of Chemistry: London, UK, 2019; pp. 458–504.

35. Shahzad, N.; Khan, W.; Shadab, M.D.; Ali, A.; Saluja, S.S.; Sharma, S.; Al-Allaf, F.A.; Abduljaleel, Z.; Ibrahim, I.A.A.; Abdel-Wahab, A.F.; et al. Phytosterols as a natural anticancer agent: Current status and future perspective. *Biomed. Pharm.* **2017**, *88*, 786–794. [CrossRef]

36. Yang, R.; Xue, L.; Zhang, L.; Wang, X.; Qi, X.; Jiang, J.; Yu, L.; Wang, X.; Zhang, W.; Zhang, Q.; et al. Phytosterol contents of edible oils and their contributions to estimated phytosterol intake in the Chinese diet. *Foods* **2019**, *8*, 334. [CrossRef]

37. Martins, C.M.; Fonseca, F.A.; Ballus, C.A.; Figueiredo-Neto, A.M.; Meinhart, A.D.; de Godoy, H.T.; Izar, M.C. Common sources and composition of phytosterols and their estimated intake by the population in the city of São Paulo, Brazil. *Nutrition* **2013**, *29*, 865–871. [CrossRef] [PubMed]

38. Ratnayake, W.M.N.; Hollywood, R.; O'grady, E.; Stavric, B. Lipid content and composition of coffee brews prepared by different methods. *Food Chem. Toxicol.* **1993**, *31*, 263–269. [CrossRef]

39. Alves, R.C.; Casal, S.; Oliveira, M.B.P. Tocopherols in coffee brews: Influence of coffee species, roast degree and brewing procedure. *J. Food Compos. Anal.* **2010**, *23*, 802–808. [CrossRef]

40. Rendón, M.Y.; dos Santos Scholz, M.B.; Bragagnolo, N. Physical characteristics of the paper filter and low cafestol content filter coffee brews. *Int. Food Res. J.* **2018**, *108*, 280–285. [CrossRef]

41. Pacetti, D.; Boselli, E.; Balzano, M.; Frega, N.G. Authentication of Italian Espresso coffee blends through the GC peak ratio between kahweol and 16-O-methylcafestol. *Food Chem.* **2012**, *135*, 1569–1574. [CrossRef] [PubMed]

42. Moeenfard, M.; Silva, J.A.; Borges, N.; Santos, A.; Alves, A. Diterpenes in espresso coffee: Impact of preparation parameters. *Eur. Food Res. Technol.* **2015**, *240*, 763–773. [CrossRef]

43. Urgert, R.; Katan, M.B. The cholesterol-raising factor from coffee beans. *Ann. Rev. Nutr.* **1997**, *17*, 305–324. [CrossRef] [PubMed]

44. Novaes, F.J.M.; Bayan, F.C.; Neto, F.R.A.; Resende, C.M. The occurrence of cafestol and kahweol diterpenes in different coffee brews. *Coffee Sci.* **2019**, *14*, 265–280. [CrossRef]

45. Naidoo, N.; Chen, C.; Rebello, S.A.; Speer, K.; Tai, E.S.; Lee, J.; Buchmann, S.; Koelling-Speer, I.; van Dam, R.M. Cholesterol-raising diterpenes in types of coffee commonly consumed in Singapore, Indonesia and India and associations with blood lipids: A survey and cross sectional study. *Nutr. J.* **2011**, *10*, 1–10. [CrossRef]

46. Tavani, A.; Bertuzzi, M.; Negri, E.; Sorbara, L.; La Vecchia, C. Alcohol, smoking, coffee and risk of non-fatal acute myocardial infarction in Italy. *Eur. J. Epidemiol.* **2001**, *17*, 1131–1137. [CrossRef]

47. Rosner, S.A.; Åkesson, A.; Stampfer, M.J.; Wolk, A. Coffee consumption and risk of myocardial infarction among older Swedish women. *Am. J. Epidemiol.* **2007**, *165*, 288–293. [CrossRef] [PubMed]

48. Kleemola, P.; Jousilahti, P.; Pietinen, P.; Vartiainen, E.; Tuomilehto, J. Coffee consumption and the risk of coronary heart disease and death. *Arch. Intern. Med.* **2000**, *160*, 3393–3400. [CrossRef] [PubMed]

49. Lopez-Garcia, E.; Willett, W.C.; Rimm, E.B.; van Dam, R.; Manson, J.E.; Stampfer, M.J.; Hu, F.B. Coffee consumption and coronary heart disease in men and women: A prospective cohort study. *Circulation* **2006**, *111*, E210. [CrossRef] [PubMed]
50. Grioni, S.; Agnoli, C.; Sieri, S.; Pala, V.; Ricceri, F.; Masala, G.; Saieva, C.; Panico, S.; Mattiello, A.; Chiodini, P.; et al. Espresso coffee consumption and risk of coronary heart disease in a large Italian cohort. *PLoS ONE* **2015**, *10*, e0126550. [CrossRef] [PubMed]
51. Williamson, K.; Hatzakis, E. NMR analysis of roasted coffee lipids and development of a spent ground coffee application for the production of bioplastic precursors. *Food Res. Int.* **2019**, *119*, 683–692. [CrossRef]
52. Guercia, E.; Berti, F.; Navarini, L.; Demitri, N.; Forzato, C. Isolation and characterization of major diterpenes from C. canephora roasted coffee oil. *Tetrahedron Asymmetry* **2016**, *27*, 649–656. [CrossRef]
53. Wuerges, K.L.; Santos, A.C.F.D.; Mori, A.L.B.; Benassi, M.D.T. Contents of diterpenes in espresso coffee brews prepared from commercial capsules. *Coffee Sci.* **2016**, *11*, 276–284.
54. European Commission. Commission Regulation (EU) No 432/2012. *Off. J. Eur. Union* **2012**, *55*, 136/1–136/40.