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Evaluation of the bioavailability and cardiometabolic effects of different dosages of coffee phytochemicals. Insights from an innovative acute/chronic intervention study.

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SUMMARY

CHAPTER 1

COFFEE CONSUMPTION AND OXIDATIVE STRESS: A REVIEW OF HUMAN INTERVENTION STUDIES

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This review summarizes the main literature findings on the effect of coffee consumption on protection against lipid, protein and DNA damage, as well as on the modulation of antioxidant capacity and antioxidant enzymes in humans.

Chapter 1: Coffee Consumption and Oxidative Stress: A Review of Human Intervention Studies

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Abstract: Research on the potential protective effects of coffee and its bioactives (caffeine, chlorogenic acids and diterpenes) against oxidative stress and related chronic disease risk has been increasing in the last years. The present review summarizes the main findings on the effect of coffee consumption on protection against lipid, protein and DNA damage, as well as on the modulation of antioxidant capacity and antioxidant enzymes in human studies. Twenty-six dietary intervention studies (involving acute and chronic coffee intake) have been considered. Overall, the results suggest that coffee consumption can increase glutathione levels and improve protection against DNA damage, especially following regular/repeated intake. On the contrary, the effects of coffee on plasma antioxidant capacity and antioxidant enzymes, as well as on protein and lipid damage, are unclear following both acute and chronic exposure. The high heterogeneity in terms of type of coffee, doses and duration of the studies, the lack of information on coffee and/or brew bioactive composition, as well as the choice of biomarkers and the methods used for their evaluation, may partially explain the variability observed among findings. More robust and well-controlled intervention studies are necessary for a thorough understanding of the effect of coffee on oxidative stress markers in humans.

Keywords: coffee; phenols; DNA damage; lipid damage; protein damage; antioxidant capacity; antioxidant enzymes

1.1. Introduction

Coffee is one of the world's most commonly consumed beverages, just after water and tea, probably thanks to its aromatic bouquet and its stimulating effect on the central nervous system. In 2014, coffee consumption has been estimated to reach over 50 million cups worldwide, with the highest annual consumption registered in Finland, Norway and Denmark (11.4, 8.7 and 8.0 kg per capita, respectively) [1], while per capita consumption for Italy and France was estimated in 5.6 kg and 5.4 kg, respectively [2].

Coffee is made by grinding roasted coffee beans, representing the fruit of the coffee plant, belonging to the Rubiaceae family. The main two species are *Coffea arabica* L. and *Coffea canephora*, originated in Ethiopia and in tropical Africa, respectively. These two species are traditionally used for making Arabica and Robusta coffees, with the former representing the most diffused species worldwide [1]. Even if all coffee brews could be prepared with hot water and ground coffee beans, coffee can assume a number of different forms. In Italian bars, for example, coffee is usually consumed as "espresso", prepared by extracting finely ground powder with high-pressure hot water to produce a 30 mL serving brew. At home, coffee is instead mainly prepared with a three-part coffee-maker (called moka), in which hot water is forced up through the coffee to the top of the machine. Further options include the French press, the American-style (drip) coffee, and many others.

The use of coffee in relation to its effects on health dates back many centuries, although the first studies are linked with the Scientific Revolution. Despite the fact the association between coffee and health has been explored for many decades, the actual role of coffee drinking has been long debated, mainly because some potential negative aspects have been hypothesized. In the nineteenth century, a moderate coffee consumption was considered helpful in reducing fatigue and improving intellectual functions, but overconsumption could move coffee from food to poison. In the 1991, the International Agency for Research on

Cancer (IARC) classified coffee as "possibly carcinogenic to humans" because of a weak positive relationship between coffee consumption and the occurrence of bladder, pancreatic and ovarian cancer [3]. Recently, an international Working Group of scientists from IARC declared inadequate the evidence for the carcinogenicity of coffee drinking overall [4].

Independently from these controversies, coffee has been described as probably the most relevant source of dietary antioxidant compounds [5], which are thought to counteract the action of reactive oxygen species (ROS), the main contributors to the development of oxidative stress. Oxidative stress occurs when the cellular production of oxidant molecules exceeds the availability of antioxidants able to defeat these insults. Antioxidant-rich foods, like coffee, can therefore play an important role against this condition through the scavenging of free radicals.

Coffee contains large amounts of bioactive compounds including caffeine, phenolic compounds, trigonelline, diterpenes and soluble fiber [6]. Caffeine, representing the most widely studied psychoactive molecule in history, is a methylxanthine partially responsible of the bitter characteristics of coffee. The caffeine content in coffee may be affected by genetic and environmental factors, for instance *C. canephora* has double the content of *C. arabica* (1.5–2.5 g and 0.9–1.3 g/100 g dry matter in green seeds, respectively) [6]. The content of caffeine in a serving is also highly variable, depending on the type of roasting (e.g., light, medium or dark), coffee-making method, and extraction (e.g., regular or over extraction), with values ranging from 50 up to over 300 mg per cup [7]. Once ingested, caffeine is rapidly absorbed in the upper gastrointestinal tract, with a peak value within 60 minutes of ingestion [8]. The intake of caffeine has been associated with a high number of biological effects, mainly concerning the stimulation of the central and sympathetic nervous system, typically associated to a feeling of alertness after coffee consumption [9–12]. Recently, the intake of caffeine has been also reported to exert ergogenic effects, as in 2011, the request of a health claim for caffeine in this direction has received a positive opinion from the European Food Safety Authority (EFSA) [13].

Regarding diterpenes, coffee contains cafestol and kahweol, which have been found in higher amount (up to 1.2 g/100 g dry matter) in *C. arabica* seeds. Being poorly soluble in water, these compounds can be trapped by filters so they are present mainly in unfiltered coffee, as well as in espresso. In spite of the anticarcinogenic and hepatoprotective properties that have been reported in in vitro and animal models, a high intake of cafestol and kahweol seems to increase the risk of CHD, mainly through an increase of plasma LDL. A meta-analysis on 11 trials showed an increase of 5.0 mg/dL and 0.9 mg/dL in serum total cholesterol with each 10 mg of cafestol or kahweol consumed per day for four weeks, respectively, with a linear effect up to 100 mg of cafestol/day [14].

Among phenolic compounds, chlorogenic acids (CGAs) are the most abundant in coffee, representing more than 98% of its total phenolic content, as shown in **Figure 1.1**, while the remaining 2% is composed of alkylmethoxyphenols, alkylphenols, methoxyphenols, and other phenolics such as pyrogallol, catechol, and phenol [15].

CGAs are a group of esters formed by hydroxycinnamic acids, such as caffeic acid, ferulic acid and *p*-coumaric acid, bound to quinic acid in a range of conjugated structures known as caffeoylquinic acids, feruloylquinic acids, and *p*-coumaroylquinic acids [16]. The isomers of these compounds depend on the site of esterification that can occur at positions 1, 3, 4 or 5 of the quinic acid moiety [17]. The most abundant and most studied CGA is 5-caffeoylquinic acid (5-CQA), accounting for about 50% of the total content in green coffee beans [15]. As reported for caffeine and diterpenes, the content of CGAs in coffee may vary depending on several factors. For example, genetics has been shown to deeply influence both the occurrence of CGAs, with *C. robusta* generally displaying a higher CGA content than *C. arabica* [18,19], and the proportion of individual CGA subgroups. Environmental factors, like rainfall level and different mean temperatures, have been reported to affect coffee CGA content even in the same cultivar grown in the same growing area over different years [20].

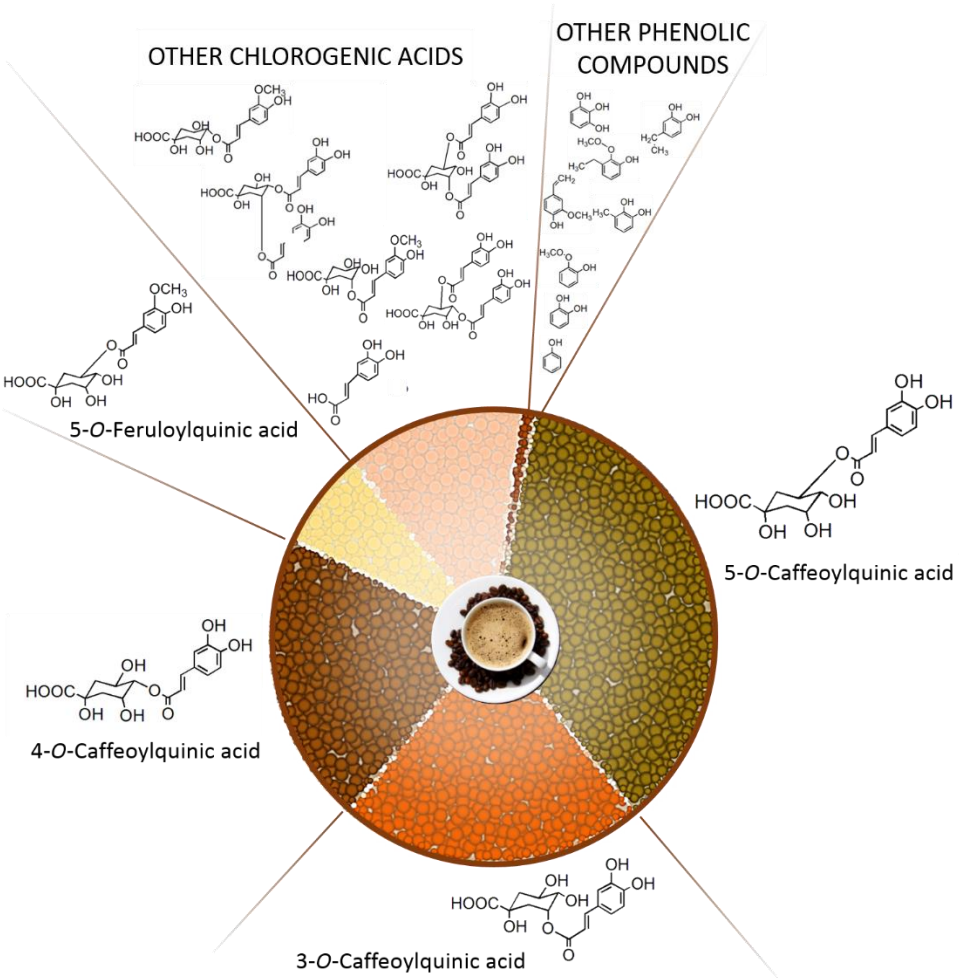

Figure 1.1. Phenolic compounds present in coffee.

As regards the processing of coffee, roasting appears to be a critical process for the evolution of flavor, aroma and color in traditional coffee beverages as well as for their CGA content, with a relevant loss of CQAs (up to 90%) in dark roasted beans compared to unroasted green beans. In addition, CGA levels may be affected by the brewing method so that an over-extraction (~55 mL) has been reported to increase over 85% the total CGAs with respect to the regular extraction (~23 mL) [7]. However, CGAs are generally preserved during coffee brewing, resulting in a final concentration that could be higher than 150 mg per serving [7]. Therefore, in spite of the huge CGA loss during roasting and the differences among preparations, coffee still remains the major dietary source of CGAs, to a level that regular coffee intake by heavy drinkers may lead to a daily intake higher that 1g.

Despite the high amounts of ingested CGAs, only low concentrations of chlorogenic acids in their native forms have been found in blood, and extensive metabolic

transformations were previously described [21]. Actually, if an extremely low amount of these compounds is absorbed in the small intestine, most of CGAs reach the colon, where they can be metabolized by the local microbiota. Metabolites are then absorbed, further conjugated in the liver, and distributed to the tissues [17]. At least ten conjugates, dihydroisoferulic acid 3-O-glucuronide, caffeic acid 3-sulfate, as well as the sulfate and glucuronide derivatives of 3,4-dihydroxyphenylpropionic acid, were identified in human plasma and/or urine after coffee consumption [22].

1.2. Objective and Literature Search Strategy

The present review aims to summarize the main findings of human intervention studies investigating the effects of coffee consumption on oxidative stress. In detail, the effects of coffee consumption on markers of lipid, protein and DNA damage, as well as on markers related to antioxidant capacity and antioxidant enzymes, are reviewed. Most of these markers are considered sensitive and specific biomarkers for antioxidant status and can be useful for a better comprehension of the role of antioxidant-rich foods, including coffee, against oxidative stress and related conditions.

PUBMED, Web of Science and Scopus databases were searched to identify pertinent articles. The searches used the combination of the following terms: ‘’coffee‘’, ‘’antioxidant capacity‘’, ‘’antioxidant activity‘’, ‘’DNA damage‘’, ‘’protein damage‘’, ‘’lipid damage‘’, ‘’oxidation‘’ and ‘’human‘’. Reference lists of the retrieved papers were also searched for additional articles. The search strategy is summarized in **Figure 1.2**.

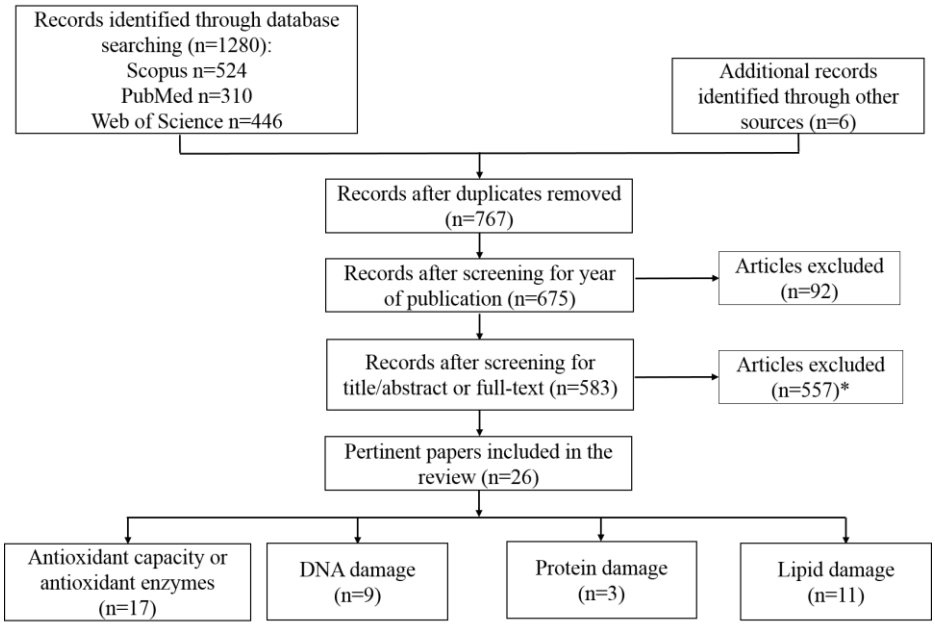

Figure 1.2. Flow diagram of article selection. * Studies were excluded for the following reasons: (1) not in English; (2) did not concern intervention studies; (3) not including in vivo markers of oxidative stress.

A total of 26 pertinent human intervention studies were identified, published in 18 different peer-reviewed journals and conducted in 12 different countries, mainly Austria, Germany and Italy. More than half of the 26 studies were published during the last 5 years, suggesting a growing interest for this topic. The results obtained are reported in Tables 1 and 2 describing the type of coffee used in the investigation, with details about the way of preparation and the content of bioactive compounds in the final brew, when available. Moreover, the duration of the intervention, the number of subjects and their characteristics, the use of a control/placebo food, the selected markers and the main findings are reported.

Three out of the 26 studies investigated the effects of both acute and chronic interventions with coffee, while 8 were only acute studies (i.e., single dose), and 15 were chronic (medium-long term) dietary intervention.

Table 1.1. Role of coffee in the modulation of oxidative stress biomarkers: overview of the acute human intervention studies.

Reference	Subjects	Type of Coffee and Composition	Doses	Study Design	Markers			
					Antioxidant Capacity/Enzymes	Lipid Damage	DNA Damage	Protein Damage
Agudelo-Ochoa et al. [23]	74 healthy subjects (38 males, 37 females), mean age of 38.5 ± 6 9 years, mean BMI 24.1 ± 2.6 kg/m^2 Control group: 13 males, 12 females; 14 subjects aged 20–40 years, 11 aged 41–60 years Group MCCGA: 12 males, 13 females; 14 subjects aged 20–40 years, 11 aged 41–60 years Group HCCGA: 12 males, 12 females; 10 subjects aged 20–40 years, 14 aged 41–60 years	Coffee 1 (MCCGA): Colombian Arabica coffee Composition: total CGAs 105 ± 4.1 mg/100 mL, cafestol 0.19 ± 0.03 mg/100 mL, kahweol 0.22 ± 0.03 mg/100 mL, caffeine 47 ± 1.4 mg/100 mL Coffee 2 (HCCGA): Colombian Arabica coffee Composition: total CGAs 195 ± 6.9 mg/100 mL, cafestol 0.19 ± 0.01 mg/100 mL, kahweol 0.23 ± 0.02 mg/100 mL, caffeine 49 ± 1 mg/100 mL	Coffee group 1: 400 mL of MCCGA coffee Coffee group 2: 400 mL of HCCGA coffee Control group: no coffee	Parallel intervention	↑AC (FRAP)			
Teekachunhatean et al. [24]	11 healthy men, (mean age 21.09 ± 7.97 years, mean BMI 20.80 ± 2.27 kg/m^2)	Coffee 1: Coffee enema, prepared mixing 4 g of ground coffee beans with 100 mL of purified water. Composition: n.d Coffee 2: Coffee for oral procedure: ready-to-drink coffee beverage Composition: n.d	Coffee group 1: Coffee enema (500 mL) Coffee group 2: 180 mL ready-to-drink coffee Control group: n.d	Randomized, two-phase, crossover intervention	=GSH ↓TAC	=MDA		
Bloomer et al. [25]	16 healthy subjects (8 males, 8 females; mean age 29.2 ± 14.4 years, mean BMI 23.3 ± 2.2 kg/m^2)	Coffee: caffeinated and decaffeinate Composition: 175 mg caffeine (caffeinated), 15 mg caffeine (decaffeinated) per 16 ounces	Coffee group 1: 16 ounces of freshly brewed caffeinated coffee following milk shake consumption Coffee group 2: 16 ounces of freshly brewed decaffeinated coffee following milk shake consumption Control group: 16 ounces of bottled water, following milk shake consumption	Parallel intervention	=TAC	=MDA		

Table 1.1. *Cont.*

Reference	Subjects	Type of Coffee and Composition	Doses	Study Design	Markers			
					Antioxidant Capacity/Enzymes	Lipid Damage	DNA Damage	Protein Damage
Leelarungrayub et al. [26]	26 sedentary men Group 1 (Caffeine): 10 males, mean age 20.5 ± 0.53 years, mean BMI 22.84 ± 2.65 kg/m^2 Group 2 (Decaffeinated): 10 males, mean age 20.3 ± 0.48 years, mean BMI 22.27 ± 3.56 kg/m^2 Group 3 (Control): 6 males, mean age 20.17 ± 0.98 years, mean BMI 23.06 ± 3.60 kg/m^2	Coffee 1: Caffeinated coffee Composition: n.d. Coffee 2: Decaffeinated coffee (Instant freeze dried) Composition: n.d.	Coffee group 1: Caffeinated (5 mg caffeine/kg bw) coffee followed by a submaximal exercise test Coffee group 2: decaffeinated coffee followed by a submaximal exercise test Control group: No coffee consumption followed by a submaximal exercise test	Parallel intervention	=TAC	↑MDA		
Moura-Nunes et al. [27]	10 subject (3 males and 7 females), range age 22–57 years, BMI n.d.	Coffee: Instant coffee 100% Arabica prepared dissolving 8 g in 200 mL boiling water. Composition: n.d.	Coffee group: 200 mL instant coffee beverage Control group: 200 mL water	Randomized, controlled, crossover intervention	↑AC (TRAP and FRAP)			
Natella et al. [28]	10 healthy nonsmoker subjects (5 males, 5 females), age and BMI n.d.	Coffee: Coffee Lavazza Qualità Rossa Composition: caffeine 181 ± 10 mg/cup, theobromine 28.9 ± 1.1 mg/cup, total phenols 161 ± 9 mg of GAE/cup, TRAP 10.1 ± 0.6 mM ROO˙eq./cup Tea: Twining Earl Gray Composition: caffeine 130 ± 7 mg/cup, theobromine 5.9 ± 0.4 mg/cup, total phenols 87 ± 9 mg of GAE/cup, TRAP 1.3 ± 0.1 mM ROO·equiv/cup	Coffee group: 200 mL of brewed coffee Control group: 200 mL of Twining Earl Gray tea	Baseline and post-intervention	↑AC (TRAP) ↑SH groups (ns)			
Ochiai et al. [29]	14 healthy men, (mean age 36.2 ± 7.8 years, mean BMI 22.7 ± 1.8 kg/m^2)	Coffee: Coffee polyphenol (CPP) prepared from green coffee beans by hot water extraction Composition: Total CQA content 80.7%	Coffee group: CPP (600 mg CGAs, co-administered with the glucose solution) Control group: 225 mL of a 75-g Glu-equivalent test solution	Single-blind, randomized, controlled, crossover intervention		↑MDA (no differences among treatment) ↑IsoPs (no differences among treatment)		

Table 1.1. *Cont.*

Reference	Subjects	Type of Coffee and Composition	Doses	Study Design	Markers			
					Antioxidant Capacity/Enzymes	Lipid Damage	DNA Damage	Protein Damage
Ochiai et al. [30]	13 healthy men, (mean age 44.9 ± 1.4 years, mean BMI 21.9 ± 0.6 kg/m^2)	Coffee: Coffee bean polyphenol (CBP) beverage Composition: 600 mg CGA/100 mL water	Coffee group: 600 mg CGAs (equivalent to two cups of coffee) in 100 mL of water after a test meal Control group: 100 mL of water after a test meal	Double-blind, randomized, crossover intervention		↑MDA (no differences among treatment) ↓IsoPs		
Sirota et al. [31]	10 healthy subjects Characteristics of the subjects: n.d.	Coffee 1: Turkish roasted ground coffee (A) Composition: 110 mg polyphenols/g·dm Coffee 2: Turkish roasted ground coffee (AG) enriched by 2% freeze-dried powder of green beans Composition: 123 mg polyphenols/g·dm	200 mL coffee A, AG or water together with 250 g red-meat cutlets	Crossover intervention		↓ MDA concentration ↓ MDA absorption after coffee A, less after coffee AG		
Mursu et al. [32]	45 nonsmoking volunteer men (mean age, 26 ± 6 years and BMI < 32 kg/m^2). Only 35 subjects completed the trial	Coffee: Finely ground coffee, repared by filtering through paper (7–8 g of grounds per one 150-mL cup) Composition: 80.9 ± 3.3 mg/100 mL of phenolic acids, with CGA as major compound (~90%)	Coffee group: 1–2 cups (150–300 mL, respectively) Control group: No coffee	Parallel intervention		=LDL-conjugated dienes =Plasma hydroxy fatty acids =F2-IsoPs		
Bakuradze et al. [33]	13 healthy men subjects (mean age 23 ± 2.4 years, mean BMI 23.8 ± 1.6 kg/m^2)	Arabica coffee, freshly prepared in a pad machine Composition: 16.7 ± 0.7 mg/g caffeine, 10.4 ± 0.9 mg/g CGA, 1.1 ± 0.2 NMP and 3.9 ± 0.3 mg/g trigonelline	Coffee group: 200 mL Control group: n.d	Baseline and post intervention			↓SBs	

Legend: AC: antioxidant capacity; BMI: body mass index; CGA: chlorogenic acids; FRAP: ferric-reducing antioxidant power; GAE: gallic acid equivalent; GSH: reduced glutathione; IsoPs: isoprostanes; MDA: malondialdehyde; NMP: *N*-methylpyridinium; SBs: strand breaks; TAC: total antioxidant capacity; TRAP: total reactive antioxidant potential.

Table 1.2. Role of coffee in the modulation of oxidative stress biomarkers: overview of the long-term human intervention studies.

Reference	Subjects	Type of Coffee and Composition	Doses	Study Design	Markers			
					Antioxidant Capacity/Enzymes	Lipid Damage	DNA Damage	Protein Damage
Agudelo-Ochoa et al. [23]	74 healthy subjects (38 males, 37 females), mean age of 38.5 ± 6 9 years, mean BMI 24.1 ± 2.6 kg/m^2 Control group: 13 males, 12 females; 14 subjects aged 20–40 years, 11 aged 41–60 years Group MCCGA: 12 males, 13 females; 14 subjects aged 20–40 years, 11 aged 41–60 years Group HCCGA: 12 males, 12 females; 10 subjects aged 20–40 years, 14 aged 41–60 years	Coffee 1 (MCCGA): Colombian Arabica coffee Composition: total CGAs 105 ± 4.1 mg/100 mL, cafestol 0.19 ± 0.03 mg/100 mL, kahweol 0.22 ± 0.03 mg/100 mL, caffeine 47 ± 1.4 mg/100 Ml Coffee 2 (HCCGA): Colombian Arabica coffee Composition: total CGAs 195 ± 6.9 mg/100 mL, cafestol 0.19 ± 0.01 mg/100 mL, kahweol 0.23 ± 0.02 mg/100 mL, caffeine 49 ± 1 mg/100 mL	Coffee group 1: 400 mL/day of MCCGA for 8 weeks Coffee group 2: 400 mL/day of HCCGA for 8 weeks Control group: no coffee for 8 weeks	Parallel intervention	↓AC (FRAP)			
Teekachunhatean et al. [24]	11 healthy men, mean age 21.09 ± 7.97 years, mean BMI 20.80 ± 2.27 kg/m^2	Coffee 1: (Enema, coffee prepared mixing 4 g of ground coffee beans with 100 mL of purified water. Composition: n.d. Coffee 2: coffee for oral procedure: ready-to-drink coffee beverage Composition: n.d.	Coffee group 1: Coffee enema (500 mL, 3 times/week for 6 visits) Coffee group 2: 180 mL ready-to-drink coffee (2/day for 11 days) Control group: n.d.	Randomized, crossover intervention	=GSH ↓TEAC	=MDA		
Mursu et al. [32]	45 nonsmoking men (mean age, 26 ± 6 years and BMI < 32 kg/m^2). 43 subjects completed the trial	Coffee: finely ground coffee, repared by filtering through paper (7–8 g of grounds per one 150-mL cup) Composition: 80.9 ± 3.3 mg/100 mL of phenolic acids, with CGA as major compound (~90%)	Coffee group 1: 3 cups (450 mL/day) of coffee for 3 weeks Coffee group 2: 6 cups (900 mL/day) of coffee for 3 weeks Control group: No coffee consumption for 3 weeks	Parallel intervention	=GPx	= Serum LDL-conjugated dienes =Plasma hydroxy fatty acids =F2-IsoPs		

Table 1.2. *Cont.*

Corrêa et al. [34]	Twenty healthy subjects (6 males, 14 females), mean age 49 ± 9 years, BMI n.d.	Coffee 1: MLR-Medium Light Roast paper-filtered coffee. 15 g per one 150-mL cup Composition: total phenolic content 11.09 ± 0.29 mg 5-CQAE/mL, total CGAs 1.98 ± 0.02 mg 5-CQAE/mL, caffeine 1.54 ± 0.01 mg/mL Coffee 2: Medium Roast (MR) paper-filtered coffee. 15 g per one 150-mL cup. Composition: total phenolic content 10.53 ± 0.56 mg 5-CQAE/mL, total CGAs 1.24 ± 0.01 mg 5-CQAE/mL, caffeine 1.63 ± 0.02 mg/mL	Coffee group 1: 150 mL MLR for 4 weeks Coffee group 2: 150 mL MR for 4 weeks Control group: n.d.	Randomized, cross-over intervention	↑AC (TAS and ORAC) ↑GPx ↑CAT ↑SOD	=OxLDL =IsoPs		
Hoelzl et al. [35]	29 subjects (13 males: mean age 25.2 ± 5.6 years, mean BMI 23.0 ± 1.7 kg/m^2; 16 females: mean age 29.3 ± 10.9 years, mean BMI 21.8 ± 2.4 kg/m^2)	Coffee: mix of 35% green and 65% roasted coffee water extracts Composition: total CGA 8.91% dm	Coffee group: 800 mL coffee/day over 5 days Control group: 800 mL water/day over 5 days	Randomized, controlled, crossover intervention	=GSH =TAC	↓8-IsoPs =OxLDL =MDA	=EndoIII and FPG sensitive sites =H_2O_2-induced DNA damage	↓3NT
Misik et al. [36]	38 healthy nonsmokers subjects (14 males, 24 females), mean age 27.6 ± 8.0 years, mean BMI 22.3 ± 2.8 kg/m^2	Coffee: coffee brand "Tchibo Beste Bohne" (100% Arabica) prepared by paper filtration. Composition: total CGA 125 mg/100 mL, caffeine 65 mg/100 mL and NMP 3.1 mg/100 mL	Coffee group: 800 mL coffee/day over 5 days Control group: 800 mL water/day over 5 days	Randomized, controlled, crossover intervention	=SOD =GPx =GSH =TAC	=IsoPs =OxLDL =MDA	↓FPG-sensitive sites ↓EndoIII sensitive sites (ns) =H_2O_2-induced DNA damage	=3NT
Revuelta-Iniesta & Al-Dujaili [37]	20 subjects (7 males, 13 females), mean BMI 24.23 ± 4.6 kg/m^2, age n.d.	Coffee 1: BC (black coffee): Sainsbury's Original Blend Cafetière Coffee Composition: polyphenols ranging from 1451 mg GAE/100 mL (Filter method) to 2475 mg GAE/100 mL (French Cafetiere) Coffee 2: GC (green coffee): Ethiopian Harrar 4 (100% Arabica) Composition: polyphenols ranging from 972 mg GAE/100 mL (French Cafetiere) to 2052 mg GAE/100 mL (Italian Cafetiere)	Coffee group 1: 40g/day of GC for 2 weeks Coffee group 2: 40g/day of BC for 2 weeks Control group: n.d.	Randomized, cross-over intervention	=AC (FRAP)			

Table 1.2. *Cont.*

Kotyczka et al. [38]	30 healthy subjects, mean age 26 ± 1 years, mean BMI 23.2 ± 0.5 kg/m^2	Coffee 1: CBs 30 g of roast powder. Dark roast coffee beverage (NMP-CB, 260 °C, 5 min) Composition: rich in NMP (785 µmol/L) and low in CGA (523 µmol/L). Coffee 2: Light roast coffee beverage (CGA-CB, 260 °C, 2 min) Composition: rich in CGA (4538 µmol/L) and low in NMP (56 µmol/L)	Coffee group 1: 500 mL/day of light roast coffee for 4 weeks Coffee group 2: 500 mL/day of dark roast coffee for 4 weeks Control group: n.d.	Randomized, longitudinally, intervention	↑SOD (CGA-CB) ↓SOD (NMP-CB) ↑CAT ↑GPx (CGA-CB) ↓GPx (NMP-CB) ↑tGSH (CGA-CB) ↑tGSH (NMP-CB)	
Steinkellner et al. [39]	First trial: 10 healthy nonsmokers subjects (3 males, 7 females), mean age 26 ± 4 years, mean bw 75 ± 9 kg Second trial: 14 subjects, mean age 25 ± 6 years, mean bw 74 ± 10 kg Third trial: subjects (number n.d.), mean age 26 ± 6 years, mean bw 72 ± 8 kg	Coffee 1: unfiltered coffee: Ground coffee (“Brasil sanft”) boiled in 10.0 L tap water for 5 min and pressed through a metal mesh Composition: n.d. Coffee 2: filtered coffee Composition: n.d.	*First trial:* Coffee group: 7 cups/day (in total 1 L) of unfiltered coffee over 5 days Control group: n.d. *Second trial:* Coffee group 1: 7 cups/day (in total 1 L) of unfiltered coffee for 3 days Coffee group 2: 7 cups/day (in total 1 L) of filtered coffee for 3 days Control group: n.d. *Third trial:* Coffee group: 7 cups/day (in total 1 L) of unfiltered coffee for 5 days Control group: n.d.	First trial: Baseline and post-intervention Second trial: Parallel intervention Third trial: Baseline and post-intervention	=GST in saliva ↑GST in plasma	↓ BPDE-induced DNA damage
Bakuradze et al. [40]	33 healthy males (range age 20–44 years; mean BMI 25.6 ± 3.7 kg/m^2)	Coffee: special roasted and blended Arabica coffee rich in both green and roast bean constituents, especially in CGA and NMP Composition: 72 mg/L NMP, 263.6 mg/L trigonelline, 720 mg/L caffeine	Coffee group: 750 mL/day (in three equal portions) for 4 weeks Control group: 750 mL/day water for 4 weeks	Randomized, controlled, cross-over intervention	↑GSH =GSSG ↑GSR activity	↓ SBs ↓ FPG-sensitive sites

Table 1.2. *Cont.*

Esposito et al. [41]	23 healthy subjects (18 treated and 5 controls), smokers and non smokers Coffee group: 7 males, 11 females; age range 19-25 years, mean BMI M 24.7 ± 2.9 kg/m^2, F 22.8 ± 5.4 kg/m^2 Control group: 2 males, 3 females; age range 20-27 years, mean BMI males 23.0 ± 1.9 kg/m^2, females 22 ± 2.4 kg/m^2;	Coffee: 5.1 ± 0.4 cups/day. 3.6 cups/day moka, 1.5 cups/day espresso; moka 40–50 mL/cup, espresso 25–35 mL/cup. Decaffeinated coffee intake was 24% of the total. Composition: n.d.	Coffee group: 5 cups coffee/day for 1 week Control group: No coffee consumption for 1 week	Parallel intervention	↑ GSH		
Grubben et al. [42]	64 subjects (31 males and 33 females; mean age 43 ± 11 years, mean BMI 24.5 ± 0.5 kg/m^2)	Coffee: blend of arabica and robusta beans. 39 g of ground coffee into a 1 L cafetière coffee-pot (1 L equals six cups) Composition: cafestol mean 34 ± 3 mg/L, kahweol mean 26 ± 1 mg/L	Coffee group: 1 L/day of unfiltered cafetière coffee for 2 weeks Control group: water, milk, tea chocolate drink or broth for 2 weeks	Randomized, controlled, crossover intervention	↑GSH (Colorectal mucosa and plasma)		
Kempf et al. [43]	47 subjects (11 males, 36 females), mean age 54.0 ± 9.0 years, mean BMI 29.2 ± 4.6 kg/m^2	Coffee: Juhla Mokka branded coffee, made with participants' coffee machines at home Composition: n.d.	Coffee group 1: 4 cups/day (150 mL per cup) of coffee for 4 weeks Coffee group 2: 8 cups/day (150 mL per cup) of coffee for 4 weeks Control group: n.d.	Single blind, three stages intervention		↓IsoPs	=3NT
Yukawa et al. [44]	11 healthy men, range age 21–31 years	Coffee: coffee freshly prepared by mixing 8 g of Arabica coffee with 150 mL water Composition: n.d.	Coffee group: 150 mL coffee (3 times per day for 1 week) Control group: mineral water for 1 week (amount not reported)	Baseline and post intervention		↓Susceptibility of LDL to oxidation ↓MDA	

Table 1.2. *Cont.*

Bakuradze et al. [45]	84 healthy subjects, mean age 25.6 ± 5.8 years, mean BMI 22.9 ± 1.9 kg/m^2	Coffee 1: blend (SB) coffee: 100% Arabica Composition: 12.39 ± 0.1 mg/g caffeine, 19.31 ± 0.3 mg/g CGA, 0.39 ± 0.0 mg/g NMP and 6.27 ± 0.1 mg/g trigonelline Coffee 2: market blend (MB) coffee, obtained from equal portions of 4 Arabica and 1 Robusta commercially available regular coffee brands Composition: 12.8 ± 0.2 mg/g caffeine, 10.01 ± 0.3 mg/g CGA, 1.20 ± 0.0 NMP and 3.42 ± 0.2 mg/g trigonelline	Coffee group: 750 mL/day of MB or SB for 4 weeks Control group: n.d.	Randomized, non-controlled, cross-over intervention			↓SBs ↓FPG-sensitive sites
Bakuradze et al.[46]	84 healthy men, range age 19–50 years, mean bw 80.9 ± 12.4 kg Coffee group: 42 men, mean BMI 24.9 ± 3.0 kg/m^2 Control group: 42 men, mean BMI 24.4 ± 3.5 kg/m^2	Coffee: Arabica coffee, freshly prepared in a pad machine Composition: 11.78 ± 0.42 mg/g caffeine, 10.18 ± 0.33 mg/g CGA, 1.10 ± 0.05 NMP and 3.82 ± 0.09 mg/g trigonelline	Coffee group: 750 mL/day of coffee for 4 weeks Control group: 750 mL/day of water for 4 weeks	Randomized, controlled, cross-over intervention			↓SBs
Bichler et al. [47]	8 healthy non-smokers volunteers (age range 20–50 years, BMI n.d.)	Coffee: metal filtered coffee andpaper filtered coffee, both prepared with 50 g of ground coffee per liter Composition: n.d.	Coffee group: 600 mL coffee/day (200 mL metal filtered coffee and 400 mL paper filtered coffee) for 5 days Control group: No coffee consumption		=GPx	↓Endo and FPG-sensitive sites ↓H_2O_2- and Trp-P-2-induced DNA damage
Cardin et al. [48]	37 patients with chronic hepatitis C (29 males, 8 females), mean age 58 ± 11 years, mean BMI 26 ± 5 kg/m^2	Coffee: 100% Coffee Arabica prepared with an Italian-style coffee machine Composition: n.d.	Coffee group: 4 cups of coffee/day for 30 days Control group: no coffee consumption for 4 weeks	Randomized, controlled, cross-over intervention		↓8-OHdG

Legend: 8-OHdG: 8-Hydroxydeoxyguanosine; AC: antioxidant capacity; BMI: body mass index; BPDE: (±)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide; bw: body weight; CAT: catalase; CGA: chlorogenic acids; ENDO III: endonuclease; FPG: formamidopyrimidine DNA glycosylase; FRAP: ferric-reducing antioxidant power; GAE: gallic acid equivalent; GPx: glutathione peroxidase; GSH: reduced glutathione; GSR: glutathione reductase; GSSG: oxidized glutathione; IsoPs: isoprostanes; MDA: malondialdehyde; NMP: *N*-methylpyridinium; NT: nytrotyrosine; ORAC: Oxygen radical absorbance capacity; OxLDL: oxidized LDL; SBs: strand breaks; SOD: superoxide dismutase; TAC: total antioxidant capacity; TAS: total antioxidant status; TEAC: total equivalent antioxidant capacity.

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1.3. Results

1.3.1. Total Plasma Antioxidant Capacity and Antioxidant Enzymes

The effect of coffee consumption on the modulation of plasma antioxidant capacity was evaluated in 10 of the selected studies. Two studies reported both *acute* and *chronic* interventions [23,24], four were acute studies [25–28], while four were chronic intervention studies [34–37]. Three studies were not placebo-controlled [24,34,37] and/or did not provide information about the bioactive composition of coffee [24,26,27]. Total radical trapping antioxidant power (TRAP), Trolox-equivalent antioxidant capacity (TEAC), total antioxidant status (TAS) and oxygen radical absorbance capacity (ORAC) emerged as the most used methods. They differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions and expression of the obtained results.

Only four (three acute and one chronic intervention) out of 10 studies reported a significant increase in total plasma antioxidant capacity following coffee consumption. In particular, Moura-Nunes and colleagues documented that a single serving of 200 mL of coffee increased plasma antioxidant capacity, determined through FRAP and TRAP assays, by 2.6% and 7.6%, respectively, in a group of healthy subjects [27]. Natella and coworkers observed an increase in plasma antioxidant capacity (measured as TRAP) and thiol (SH) groups after consumption of 200 mL of coffee, which was even more pronounced than that observed with the same amount of tea (+6% versus +4%, respectively) [28]. Agudelo-Ochoa et al. [23] reported that a single serving of 400 mL of coffee, providing either 420 mg or 780 mg of chlorogenic acid, significantly increased plasma antioxidant capacity (+6% and +5%, respectively) in a group of healthy volunteers, but these effects were lost following a long term intervention. Finally, Corrêa et al. [34] reported that a 4-week intake of 150 mL/day of medium light roast (MLR) or medium roast (MR) paper-filtered coffee increased the levels of TAS by about 21% and 26% respectively, while ORAC increased only after the consumption of medium light roast paper-filtered coffee. The lack of homogeneous results could be due to differences in the duration of the intervention, the type and the amount of coffee provided, the composition in bioactive compounds, and the method used for the determination of antioxidant capacity.

The effect of coffee in the modulation of endogenous antioxidant enzymes has been assessed in seven chronic intervention trials. The duration of the studies varied from 1 to 4 weeks. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx),

glutathione reductase (GSR) and glutathione S-transferases (GSTs) were the most widely studied enzymes.

Overall, the results obtained are conflicting and do not provide evidence about the role of coffee in the modulation of antioxidant enzymes. For example, Corrêa et al. [34] reported an increase in erythrocyte antioxidant enzyme activity in healthy subjects following a 4-week intervention with 150 mL/day of MLR and MR paper-filtered coffee. In particular, SOD activity increased by 52% and 75% in MLR and MR group, while GPx activity by 62% and 49%, respectively. Moreover, both the interventions significantly increased CAT activity by about 13%. Kotyczka et al. [38] documented that a 4-week intake of light dark roasted coffee (rich in chlorogenic acid) resulted in increased erythrocyte activities of SOD, GPx and CAT by 12%, 25% and 22%, respectively. On the contrary, the intake of dark roasted coffee (poor in chlorogenic acid) decreased erythrocytes SOD and GPx activity by 5.8% and 15%, respectively. Misik and colleagues showed that a 5-day coffee intake (800 mL/day) did not significantly affect SOD and GPx activity in the cytosolic fractions of the lymphocytes of healthy volunteers [36].

The activity of GST and GSR was evaluated in two studies. Steinkellner and colleagues reported that a 5-day intervention with 1 L unfiltered coffee/day increased GST activity in plasma, but not in saliva, in a group of healthy individuals [39]. Bakuradze et al. [40] showed a significant increase in GSR blood level activity following 4-week consumption of 750 mL/day of freshly brewed coffee.

Eight studies (seven chronic interventions and one acute trial) also investigated the role of coffee in the modulation of blood glutathione (GSH) levels as a substrate of GPx and GST enzymes. Four out of seven chronic intervention studies documented an increase in GSH levels [38,40–42], while two long-term studies [35,36] and one study performing both an acute and a chronic intervention [24] did not show any significant effect. Misik and coworkers attributed the lack of effects to the degradation and metabolic conversion of different coffee constituents in the body [36], while for Teekachunhatean and colleagues the short duration of the intervention could be at least partially responsible for the absence of a relevant effect [24].

1.3.2. Protein Damage

The effect exerted by coffee intake on protein damage has been investigated only in three studies [35,36,43]. Those studies differed for the fed coffee type, which was instant coffee [35], coffee homemade with participants' coffee machines [36] or coffee prepared by paper filtration [43]. In two studies [35,43], partially performed by the same research group, a

controlled intervention trial with a cross-over design was scheduled, where subjects were randomized to consume coffee or a control drink (water) for periods of 5 days each, spaced out by a washout phase and dietary restriction. Differently, Kempf and coworkers investigated the effect of two different doses of coffee (4 and 8 cups, corresponding to 600 and 1200 mL respectively) by using a simple experimental design: subjects had to follow 4 weeks of restrictive diet, followed by 4 weeks with 4 cups coffee/day and 4 weeks of 8 cups coffee/day [43]. The studies performed by Hoelzl et al. [35] and Misik et al. [36] were also similar for what concerns the volunteers, being nonsmokers and normal weight young adults in both cases, contrarily to the study by Kempf et al. [43] where subjects with a high risk of type 2 diabetes were recruited. In all the three studies, the marker of protein damage was 3-nytrotirosine (3-NT), a stable post-translational modification in proteins, deriving from the reaction of free or protein-bound tyrosine with reactive nitrogen oxide species including peroxynitrite, nitrogen dioxide and nitrous acid. 3-NT has been suggested to be associated with coronary heart disease (CAD) independently of traditional risk factors [49]. The marker was monitored by LC-MS/MS in two studies [35,36] and by an enzyme immunoassay in the third one [38]. A significant effect of coffee was found only by Hoelzl and colleagues, who observed a significant decrease of 3-NT (16.1%) after 4-week coffee intake [35]. Despite many similarities between the investigations by Hoelzl et al. [35] and Misik et al. [36] (i.e., duration of the intervention, amounts of CGA provided), the latter found no significant effect after coffee intake, in line with the observations of Kempf and colleagues [43]. A possible explanation for such different findings might be linked to the amount of coffee bioactives other than CGA provided with the two different coffee brews. However, Hoelzl et al. [35] provided information only about CGA, making a clear comparison between the two investigations practically impossible.

The small number of investigations about the possible role of coffee consumption on markers of protein damage, together with the heterogeneity of the findings, calls for further studies focusing on this aspect of oxidative damage to biomolecules.

1.3.3. Lipid Damage

The effect of coffee consumption on markers of lipid oxidation has been investigated in 12 studies [24–26,29–32,34–36,43,44]. Five out of 12 studies investigated only the acute effect of coffee consumption [25,26,29–31], five were chronic intervention studies [34–36,43,44], while two studies investigated both acute and chronic effects [24,32].

In these studies, isoprostanes (IsoPs) and malondialdehyde (MDA) were the most frequently considered markers of lipid damage. In detail, isoprostanes are a class of end-

products of peroxidation of arachidonic acid, which are collectively referred as F2-IsoPS due to their similarity to prostaglandin F2α. Among them, 8-isoprostaglandin F2α (8-Iso PGF2α) is commonly used for evaluating oxidative stress, through both chromatographic techniques and immunoassays. MDA is instead a three carbon, low molecular weight aldehyde representing the main product of polyunsaturated fatty acid peroxidation. It is characterized by a high toxicity due to its ability to react with other molecules like DNA and protein [50]. In all the studies evaluating MDA, the reaction with 2-thiobarbituric acid (TBA) was used, so results were reported as TBA reacting substances (TBARS) instead of MDA. In spite of the risk of overestimation of MDA, the TBARS method represents the most common test for evaluating lipid peroxidation.

Besides 8-IsoPGF2 and MDA, further markers of lipid damage and/or protection considered in the present review were oxidized LDL, resistance to LDL oxidation, serum LDL-conjugated dienes and hydroxyl fatty acids. The analysis of the main findings revealed that most of the interventions failed to demonstrate a significant decrease in markers of lipid damage with exception of results found by Ochiai et al. [30] and Sirota et al. [31]. The former reported a significantly reduced urinary 8-epiPGF2α following consumption of a coffee beverage (providing 600 mg of CGAs) when compared with placebo in healthy men. Results showed that consumption of 200 mL Turkish roasted coffee during a meal based on red-meat cutlets resulted in a significant inhibition of postprandial plasma MDA. No effect between treatments and control/placebo were instead found by other authors [25,29,32]. The investigation by Leelarungrayub et al. [26] deserves a special mention, because it reports a significant higher level of MDA in men consuming caffeinated coffee, when compared to decaffeinated coffee or control, followed by a submaximal exercise test. Authors reported that, similarly to what observed in previous investigations, results demonstrated an increased intramuscular fat oxidation following consumption of caffeine-rich foods.

Among medium-long term intervention studies, a significant decrease in isoprostanes was observed only in three studies [30,35,43], while no significant change was reported by Mursu et al. [32] and Misik et al. [36]. Even if the exact composition of the coffee used has not been always provided, the differences in the findings seem to be at least partially attributable to the different composition of the brews.

For what concerns the other markers of lipid damage, only Yukawa et al. [44] found a modest reduction of LDL oxidation susceptibility and a decrease of MDA levels following consumption of 3 coffees/day for 1 week. No significant effect was instead found by Mursu et al. [32] on serum LDL-conjugated dienes and plasma hydroxyl fatty acids, or by

Teekachunhatean et al. [24] on MDA levels [24] and by Hoelzl et al. [35] on both MDA and oxidized LDL.

1.3.4. DNA Damage

The role of coffee on markers of DNA damage has been investigated in nine studies (eight chronic interventions and one acute study), four of which performed by the same research group [33,40,46,47]. Eight out of nine studies measured the levels of DNA damage through the comet assay, a single cell gel electrophoresis technique widely used also in human biomonitoring and dietary intervention studies [51,52]. Three of these studies [33,46,47] investigated the effect of coffee on spontaneous DNA strand breaks (SBs), which directly reflect the background DNA oxidative damage within cells. Background SBs may derive from endogenous and/or exogenous exposure to DNA damaging agents and/or may reflect incomplete DNA repair. The consumption of coffee was associated to reduce DNA SBs in healthy volunteers. Bakuradze et al. [33] reported that the ingestion of 800 mL of coffee (200 mL every 2 h) significantly reduced (up to 30%) DNA SBs in a short-term human intervention study. The same research group documented that coffee consumption (3 × 250 mL per day) was associated with DNA-protective effects ($p < 0.001$) in a 4-week, double-blind, randomized, crossover intervention [46]. Finally, the same authors showed that a daily consumption of 750 mL of fresh dark roast coffee decreased by 27% spontaneous DNA SBs in a 4-week, randomized, controlled trial [47].

Five out of eight studies investigated the effects of coffee consumption on oxidized DNA bases through the exploitation of specific enzymes such as formamidopyrimidine-DNA glycosylase (FPG) and/or endonuclease III (Endo III), able to detect oxidized purines and pyrimidine bases, respectively [53,54]. Some studies also evaluated the resistance to oxidatively induced DNA damage, using H_2O_2 [35,36,48], BPDE [39] and Trp-P-2 [47] as oxidative agents.

Bichler and coworkers showed that the consumption of 600 mL coffee (400 mL paper filtered and 200 mL metal filtered/day) for 5 days reduced both endogenous (FPG and Endo III-sensitive sites by 64% and 48%, respectively) and oxidatively induced DNA damage (measured as DNA resistance to H_2O_2 and Trp-P-2 by 17% and 35%, respectively) in a group of healthy volunteers [47]. Steinkellner and colleagues documented that a 5-day intervention with 1 L unfiltered coffee/day increased cell protection from (±)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide oxidative insult in a group of healthy subjects [39]. Misik et al. [36] reported a significant reduction in the levels of FPG (by 12.3%) and Endo III-sensitive sites (by 10%),

but not DNA resistance to H_2O_2-oxidative treatment, following the administration for 5 days of 800 mL/day of paper filtered coffee. Hoelzl and colleagues showed that the intake of a comparable amount of instant coffee co-extracted from green and roasted beans did not significantly affect the levels of FPG and Endo III-sensitive sites, and H_2O_2-induced DNA damage, in a group of healthy individuals [35].

Another marker widely used to measure oxidized base lesion is 8-oxo-2′-deoxyguanosine (8-OHdG), as, among all purine and pyridine bases, guanine is the most prone to oxidation and a common biomarker reflecting the balance between oxidative damage and repair rate [55]. The role of coffee in the modulation of 8-OH-dG was evaluated only in one study, with positive results [48]. The study was performed in a group of patients affected by chronic hepatitis C and the authors documented that 8-OHdG levels were significantly lower during coffee intake (30-day consumption of 4 cups of coffee/day), with almost a three-fold decrease.

1.4. Conclusions

During the last five years, coffee has been the objective of several studies for its potential role in human health, with a specific focus on the prevention of several chronic degenerative diseases. The current review summarized the main findings of 26 studies performed in humans, with the aim of comparing results on the effect of coffee consumption on the main markers of oxidative damage to lipid, DNA and protein, as well as on the modulation of antioxidant capacity and antioxidant enzymes in humans. Studies were performed on healthy subjects with the exception of one study in which patients with chronic hepatitis C were recruited.

Overall, a high heterogeneity among studies was observed, mainly in terms of doses and duration of the interventions, and, in several studies, information concerning the polyphenol content of the coffee used was lacking. Only a few studies provided the content of CGAs, caffeine and other bioactive compounds in the fed coffee brew, and, in general, they did not describe in great detail the way coffee was prepared (i.e., grams of coffee used for each dose). This lack of information about the composition of the brews makes the comparison among studies extremely difficult, with an objective evaluation of a dose-response effect almost impossible. Therefore, the need for more detailed information about the chemical composition of coffees in future studies appears crucial for a more accurate analysis of results.

Despite these complications, the main findings of the reviewed works seem to suggest that consumption of coffee may increase glutathione levels and reduce the levels of DNA

damage. These effects are more evident in chronic interventions than in acute studies, letting hypothesize that a long-term exposure to coffee and/or its bioactive compounds is needed to obtain such putative health effects. On the contrary, an extreme heterogeneity of the results has been observed for total plasma and serum antioxidant status, as well as for protein and lipid damage. This could be attributed to the different biomarkers and methods used for their evaluation. Based on the difficulties described above, a comprehensive understanding of the beneficial effects of coffee on oxidative stress markers, through the development of robust and well-controlled intervention studies, is required.

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1.5. References

1. International Coffee Organization. Infographics on the Global Coffee Trade. Available online: http://www.ico.org/coffee-trade-statistics-infographics.asp?section=Statistics (accessed on 1 March 2016).
2. International Coffee Council. Trends in Coffee Consumption in Selected Importing Countries. Available online: http://www.ico.org/documents/icc-109-8e-trends-consumption.pdf (accessed on 4 June 2016).
3. WHO. *Coffee, Tea, Mate, Methylxanthines and Methylglyoxal*; International Agency for Research on Cancer: Lyon, France, 1991.
4. Loomis, D.; Guyton, K.Z.; Grosse, Y.; Lauby-Secretan, B.; El Ghissassi, F.; Bouvard, V.; Benbrahim-Tallaaa, L.; Guhaa, N.; Mattocka, H.; Straifa, K. Carcinogenicity of drinking coffee, mate, and very hot beverages. *Lancet Oncol*. **2016**, *17*, 877–878.
5. Vinson, J.A. Polyphenols: Total amounts in foods and beverages and U.S. per capital consumption. Abstract number AGFD 10. In Proceedings of the American Chemical Society 230th National Meeting, Washington, DC, USA, 28 August 2005.
6. Farah, A. Coffee Constituents. In *Coffee: Emerging Health Effects and Disease Prevention*; Chu, Y.F., Ed.; Wiley-Blackwell: Oxford, UK, 2012; pp. 21–58.
7. Ludwig, I.A.; Mena, P.; Calani, L.; Cid, C.; Del Rio, D.; Lean, M.E.; Crozier, A. Variations in caffeine and chlorogenic acid contents of coffees: What are we drinking? *Food Funct.* **2014**, *5*, 1718–1726.
8. Lang, R.; Dieminger, N.; Beusch, A.; Lee, Y.M.; Dunkel, A.; Suess, B.; Skurk, T.; Wahl, A.; Hauner, H.; Hofmann, T. Bioappearance and pharmacokinetics of bioactives upon coffee consumption. *Anal. Bioanal. Chem.* **2013**, *405*, 8487–8503.
9. Lean, M.E.J.; Ashihara, H.; Clifford, M.N.; Crozier, A. Purine Alkaloids: A Focus on Caffeine and Related Compounds in Beverages. In *Teas, Cocoa and Coffee: Plant Secondary Metabolites and Health*; Crozier, A., Ashihara, H., Tomás-Barbéran, F., Eds.; Wiley-Blackwell: Oxford, UK, 2011; pp. 25–44.
10. Godos, J.; Pluchinotta, F.R.; Marventano, S.; Buscemi, S.; Li Volti, G.; Galvano, F.; Grosso G. Coffee components and cardiovascular risk: Beneficial and detrimental effects. *Int. J. Food Sci. Nutr.* **2014**, *65*, 925–936.
11. Cappelletti, S.; Piacentino, D.; Sani, G.; Aromatario M. Caffeine: Cognitive and physical performance enhancer or psychoactive drug? *Curr. Neuropharmacol.* **2015**, *13*, 71–88.
12. Owens, J.A.; Mindell, J.; Baylor, A. Effect of energy drink and caffeinated beverage consumption on sleep, mood, and performance in children and adolescents. *Nutr. Rev.* **2014**, *72* (Suppl. S1), 65–71.
13. EFSA Panel on Dietetic Products Nutrition and Allergies (EFSA NDA Panel). Scientific Opinion on the substantiation of health claims related to caffeine and increase in physical performance during short-term high-intensity exercise (ID 737, 1486, 1489), increase in endurance performance (ID 737, 1486), increase in endurance capacity (ID 1488) and

reduction in the rated perceived exertion/effort during exercise (ID 1488, 1490) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J.* **2011**, *9*, 2053.
14. Urgert, R.; Katan, M.B. The cholesterol-raising factor from coffee beans. *Ann. Rev. Nutr.* **1997**, *17*, 305–324.
15. Tresserra-Rimbau, A.; Medina-Remón, A.; Estruch, R.; Lamuela-Raventós, R.M. Coffee Polyphenols and High Cardiovascular Risk Parameters. In *Coffee and Health and Disease Prevention*; Preedy, V.R., Ed.; Academic Press: San Diego, CA, USA, 2014; pp. 387–394.
16. Arai, K.; Terashima, H.; Aizawa, S.I.; Taga, A.; Yamamoto, A.; Tsutsumiuchi, K.; Kodama, S. Simultaneous determination of trigonelline, caffeine, chlorogenic acid and their related compounds in instant coffee samples by HPLC using an acidic mobile phase containing octanesulfonate. *Anal. Sci.* **2015**, *31*, 831–835.
17. Stalmach, A.; Mullen, W.; Barron, D.; Uchida, K.; Yokota, T.; Cavin, C.; Steiling, H.; Williamson, G.; Crozier, A. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. *Drug Metab. Dispos.* **2009**, *37*, 1749–1758.
18. Farah, A.; Donangelo, C.M. Phenolic compounds in coffee. *Braz. J. Plant Physiol.* **2006**, *18*, 23–36.
19. Ky, C.L.; Louarn, J.; Dussert, S.; Guyot, B.; Hamon, S.; Noirot, M. Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild *Coffea arabica* L. and *C. canephora P. accessions*. *Food Chem.* **2001**, *75*, 223–230.
20. Komes, D.; Aleksandra, V. Effects of varieties and growing conditions on antioxidant capacity of coffee. In *Processing and Impact on Antioxidants in Beverages*; Preedy, V., Ed.; Academic Press: San Diego, CA, USA, 2014; pp. 77–85.
21. Rodriguez-Mateos, A.; Vauzour, D.; Krueger, C.G.; Shanmuganayagam, D.; Reed, J.; Calani, L.; Mena, P.; Del Rio, D.; Crozier, A. Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: An update. *Arch. Toxicol.* **2014**, *88*, 1803–1853.
22. Fumeaux, R.; Menozzi-Smarrito, C.; Stalmach, A.; Munari, C.; Kraehenbuehl, K.; Steiling, H.; Crozier, A.; Williamson, G.; Barron, D. First synthesis, characterization, and evidence for the presence of hydroxycinnamic acid sulfate and glucuronide conjugates in human biological fluids as a result of coffee consumption. *Org. Biomol. Chem.* **2010**, *8*, 5199–5211.
23. Agudelo-Ochoa, G.M.; Pulgarín-Zapata, I.C.; Velásquez-Rodriguez, C.M.; Duque-Ramírez, M.; Naranjo-Cano, M.; Quintero-Ortiz, M.M.; Lara-Guzmán, O.J.; Muñoz-Durango, K. Coffee consumption increases the antioxidant capacity of plasma and has no effect on the lipid profile or vascular function in healthy adults in a randomized controlled trial. *J. Nutr.* **2016**, *146*, 524–531.
24. Teekachunhatean, S.; Tosri, N.; Sangdee, C.; Wongpoomchai, R.; Ruangyuttikarn, W.; Puaninta, C.; Srichairatanakool, S. Antioxidant effects after coffee enema or oral coffee consumption in healthy Thai male volunteers. *Hum. Exp. Toxicol.* **2012**, *31*, 643–651.
25. Bloomer, R.J.; Trepanowski, J.F.; Farney, T.M. Influence of acute coffee consumption on postprandial oxidative stress. *Nutr. Metab. Insights* **2013**, *6*, 35–42.
26. Leelarungrayub, D.; Sallepan, M.; Charoenwattana, S. Effects of acute caffeinated coffee consumption on energy utilization related to glucose and lipid oxidation from short submaximal Treadmill exercise in sedentary Men. *Nutr. Metab. Insights* **2011**, *4*, 65–72.
27. Moura-Nunes, N.; Perrone, D.; Farah, A.; Donangelo, C.M. The increase in human plasma antioxidant capacity after acute coffee intake is not associated with endogenous non-enzymatic antioxidant components. *Int. J. Food Sci. Nutr.* **2009**, *60* (Suppl. S6), 173–181.
28. Natella, F.; Nardini, M.; Giannetti, I.; Dattilo, C.; Scaccini, C. Coffee drinking influences plasma antioxidant capacity in humans. *J. Agric. Food Chem.* **2002**, *50*, 6211–6216.
29. Ochiai, R.; Sugiura, Y.; Shioya, Y.; Otsuka, K.; Katsuragi, Y.; Hashiguchi, T. Coffee polyphenols improve peripheral endothelial function after glucose loading in healthy male adults. *Nutr. Res.* **2014**, *34*, 155–159.
30. Ochiai, R.; Sugiura, Y.; Otsuka, K.; Katsuragi, Y.; Hashiguchi, T. Coffee bean polyphenols ameliorate postprandial endothelial dysfunction in healthy male adults. *Int. J. Food Sci. Nutr.* **2015**, *66*, 350–354.
31. Sirota, R.; Gorelik, S.; Harris, R.; Kohen, R.; Kanner, J. Coffee polyphenols protect human plasma from postprandial carbonyl modifications. *Mol. Nutr. Food Res.* **2013**, *57*, 916–919.
32. Mursu, J.; Voutilainen, S.; Nurmi, T.; Alfthan, G.; Virtanen, J.K.; Rissanen, T.H.; Happonen, P.; Nyyssönen, K.; Kaikkonen, J.; Salonen, R.; et al. The effects of coffee consumption on lipid peroxidation and plasma total homocysteine concentrations: A clinical trial. *Free Radic. Biol. Med.* **2005**, *38*, 527–534.
33. Bakuradze, T.; Lang, R.; Hofmann, T.; Schipp, D.; Galan, J.; Eisenbrand, G.; Richling, E. Coffee consumption rapidly reduces background DNA strand breaks in healthy humans: Results of a short-term repeated uptake intervention study. Mol. Nutr. Food Res. 2016, 60, 682–686.
34. Corrêa, T.A.F.; Monteiro, M.P.; Mendes, T.M.N.; de Oliveira, D.M.; Rogero, M.M.; Benites, C.I.; Vinagre, C.G.; Mioto, B.M.; Tarasoutchi, D.; Tuda, V.L.; et al. Medium light and medium roast paper-filtered coffee increased antioxidant capacity in healthy volunteers: Results of a randomized trial. *Plant Foods Hum. Nutr.* **2012**, *67*, 277–282.
35. Hoelzl, C.; Knasmüller, S.; Wagner, K.H.; Elbling, L.; Huber, W.; Kager, N.; Ferk F, Ehrlich, V.; Nersesyan, A.; Neubauer, O.; et al. Instant coffee with high chlorogenic acid levels protects humans against oxidative damage of macromolecules. *Mol. Nutr. Food Res.* **2010**, *54*, 1722–1733.
36. Mišík, M.; Hoelzl, C.; Wagner, K.H.; Cavin, C.; Moser, B.; Kundi, M.; Simic, T.; Elbling, L.; Kager, N.; Ferk F.; et al. Impact of paper filtered coffee on oxidative DNA-damage: Results of a clinical trial. *Mutat. Res.* **2010**, *692*, 42–48.
37. Revuelta-Iniesta, R.; Al-Dujaili, E.A. Consumption of green coffee reduces blood pressure and body composition by influencing 11β-HSD1 enzyme activity in healthy individuals: A pilot crossover study using green and black coffee. *BioMed Res. Int.* **2014**, *2014*, 482704, doi:10.1155/2014/482704.

38. Kotyczka, C.; Boettler, U.; Lang, R.; Stiebitz, H.; Bytof, G.; Lantz, I.; Hofmann, T.; Marko, D.; Somoza, V. Dark roast coffee is more effective than light roast coffee in reducing body weight, and in restoring red blood cell vitamin E and glutathione concentrations in healthy volunteers. *Mol. Nutr. Food Res.* **2011**, *55*, 1582–1586.
39. Steinkellner, H.; Hoelzl, C.; Uhl, M.; Cavin, C.; Haidinger, G.; Gsur, A.; Schmid, R.; Kundi, M.; Bichler, J.; Knasmüller, S. Coffee consumption induces GSTP in plasma and protects lymphocytes against (±)-anti-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide induced DNA-damage: Results of controlled human intervention trials. *Mutat. Res.* **2005**, *591*, 264–275.
40. Bakuradze, T.; Boehm, N.; Janzowski, C.; Lang, R.; Hofmann, T.; Stockis, J.P.; Albert, F.W.; Stiebitz, H.; Bytof, G.; Lantz, I.; et al. Antioxidant-rich coffee reduces DNA damage, elevates glutathione status and contributes to weight control: Results from an intervention study. *Mol. Nutr. Food Res.* **2011**, *55*, 793–797.
41. Esposito, F.; Morisco, F.; Verde, V.; Ritieni, A.; Alezio, A.; Caporaso, N.; Fogliano, V. Moderate coffee consumption increases plasma glutathione but not homocysteine in healthy subjects. *Aliment. Pharmacol. Ther.* **2003**, *17*, 595–601.
42. Grubben, M.J.A.L.; Den Braak, V.; Jong, D.; Rijt, V.; Ruijter, D. The effect of unfiltered coffee on potential biomarkers for colonic cancer risk in healthy volunteers: A randomized trial. *Aliment. Pharmacol. Ther.* **2000**, *14*, 1181–1190.
43. Kempf, K.; Herder, C.; Erlund, I.; Kolb, H.; Martin, S.; Carstensen, M.; Koenig, W.; Sundvall, J.; Bidel, S.; Kuha, S.; et al. Effects of coffee consumption on subclinical inflammation and other risk factors for type 2 diabetes: A clinical trial. *Am. J. Clin. Nutr.* **2010**, *91*, 950–957.
44. Yukawa, G.S.; Mune, M.; Otani, H.; Tone, Y.; Liang, X.M.; Iwahashi, H.; Sakamoto, W. Effects of coffee consumption on oxidative susceptibility of low-density lipoproteins and serum lipid levels in humans. *Biochemistry* **2004**, *69*, 70–74.
45. Bakuradze, T.; Parra, G.A.M.; Riedel, A.; Somoza, V.; Lang, R.; Dieminger, N.; Hofmann, T.; Winkler, S.; Hassmannd, U.; Marko, D.; et al. Four-week coffee consumption affects energy intake, satiety regulation, body fat, and protects DNA integrity. *Food Res. Int.* **2014**, *63*, 420–427.
46. Bakuradze, T.; Lang, R.; Hofmann, T.; Eisenbrand, G.; Schipp, D.; Galan, J.; Richling, E. Consumption of a dark roast coffee decreases the level of spontaneous DNA strand breaks: A randomized controlled trial. *Eur. J. Nutr.* **2015**, *54*, 149–156.
47. Bichler, J.; Cavin, C.; Simic, T.; Chakraborty, A.; Ferk, F.; Hoelzl, C.; Schulte-Hermann, R.; Kundi, M.; Haidinger, G.; Angelis, K.; et al. Coffee consumption protects human lymphocytes against oxidative and 3-amino-1-methyl-5*H*-pyrido [4,3-*b*] indole acetate (Trp-P-2) induced DNA-damage: Results of an experimental study with human volunteers. *Food Chem. Toxicol.* **2007**, *45*, 1428–1436.
48. Cardin, R.; Piciocchi, M.; Martines, D.; Scribano, L.; Petracco, M.; Farinati, F. Effects of coffee consumption in chronic hepatitis C: A randomized controlled trial. *Dig. Liver Dis.* **2013**, *45*, 499–504.
49. Ahsan, H. 3-Nitrotyrosine: A biomarker of nitrogen free radical species modified proteins in systemic autoimmunogenic conditions. *Hum. Immunol.* **2013**, *74*, 1392–1399.
50. Del Rio, D.; Stewart, A.J.; Pellegrini, N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* **2005**, *15*, 316–328.
51. Collins, A.; Koppen, G.; Valdiglesias, V.; Dusinská, M.; Kruszewski, M.; Møller, P.; Rojas, E.; Dhawan, A.; Benzie, I.; Coskun, E.; et al. The comet assay as a tool for human biomonitoring studies: The ComNet project. *Mutat. Res. Rev. Mutat. Res.* **2014**, *759*, 27–39.
52. Hoelzl, C.; Knasmüller, S.; Misík, M.; Collins, A.; Dusinská, M.; Nersesyan, A. Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: Recent results and trends. *Mutat. Res.* **2009**, *681*, 68–79.
53. Dušinská, M.; Collins, A. Detection of oxidized purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay. *Altern. Lab. Anim.* **1996**, *24*, 405–411.
54. Collins, A.R.; Duthie, S.J.; Dobson, V.L. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* **1993**, *14*, 1733–1735.
55. Cooke, M.S.; Evans, M.D.; Burd, R.M.; Patel, K.; Barnard, A.; Lunec, J.; Hutchinson, P.E. Induction and excretion of ultraviolet-induced 8-oxo-2′-deoxyguanosine and thymine dimers in vivo: Implications for PUVA. *J. Investig. Dermatol.* **2001**, *116*, 281–285.

CHAPTER 2

NIACIN, ALKALOIDS AND (POLY)PHENOLIC COMPOUNDS IN THE MOST WIDESPREAD ITALIAN CAPSULE-BREWED COFFEES

This chapter has been submitted and is presently in its second revision on Scientific Report (manuscript number SREP-18-29398).

In this study, we compared the content of caffeine, trigonelline, *N*-methylpyridinium (NMP), niacin, and chlorogenic acids of 65 different capsule-brewed coffees, commercialised by 5 of the most representative brands in Italy.

Chapter 2: Niacin, alkaloids and (poly)phenolic compounds in the most widespread Italian capsule-brewed coffees.

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Abstract: Coffee is one of the most popular beverages worldwide and, nowadays, one of the most practical way for its preparation is by prepacked capsules. The aim of this study was comparing the content in caffeine, trigonelline, *N*-methylpyridinium (NMP), niacin, and chlorogenic acids of 65 different capsule-brewed coffees, commercialised by 5 of the most representative brands in Italy. Coffees were prepared from capsules following manufacturer's instructions and analysed with an optimized UHPLC-MS/MS method able to assess all these phytochemicals in one single run. Inter-lot and capsule variability were also studied for a subset of coffee capsules. Except for decaffeinated coffees, caffeine amount accounted between 54 and 208 mg/serving. Regular espresso coffees showed higher trigonelline, NMP, and niacin concentrations than large (*lungo*) and decaffeinated samples, with average serving amounts of 17.96, 1.78, and 0.66 mg, respectively. Regarding chlorogenic acids, caffeoylquinic acids were the most relevant ones (20-117 mg/serving). Feruloylquinic acids were quantified between 8 and 50 mg/serving. Coumaroylquinic acids, hydroxycinnamate dimers, caffeoylshikimic acids, and caffeoylquinic lactones were also present at lower concentrations. Multivariate analysis provided comprehensive information on the phytochemical profile of the different types of coffee, showing a great variability among coffees with some brand-related insights. This study supports the need for accurately characterizing espresso coffees while investigating the beneficial effects of coffee on human health.

List of Abbreviations: CGAs, Chlorogenic acids; CID, Collision Induced Dissociation; CSA*n*, Caffeoylshikimic acid isomer; CQL*n*, Caffeoylquinic lactone isomer; EC, Espresso Coffee; LOD, Limit of Detection; LOQ, Lower Limit of Quantification (LLOQ); *n*-CQA, *n*-*O*-caffeoylquinic acid; *n*-CouQA, *n*-*O*-coumaroylquinic acid; *n*-FQA, *n*-*O*-feruloylquinic acid, NMP, *N*-methylpyridinium; *n*-FQL, *n*-O-feruloylquinic lactone, S/N, Signal to Noise; SRM, Selected Reaction Monitoring; ULOQ, Upper Limit of Quantification.

2.1. Introduction

Coffee is one of the most consumed beverages in the world, prepared from the roasted seeds of *Coffea* plant cultivars[1]. The two main botanical species used for the production of coffee are *Coffea arabica* and *Coffea canephora* var. Robusta, which differ between them for the higher sucrose, lipid, and trigonelline contents of the former with respect to the latter, which in turn shows higher levels caffeine and chlorogenic acids (CGAs)[2]. However, the roasting process, besides modifying the volatile profile, deeply alters the chemical composition of the beans, increasing the production of melanoidins, main contributors of colour and flavour, at the expense of sucrose, aminoacids and CGAs[2].

Several epidemiological and intervention studies confirmed that the consumption of three to six cups of coffee may have beneficial effects on cardiovascular diseases and diabetes mellitus, decreasing blood pressure, inflammatory markers and blood cholesterol[3–6]. These effects are mainly attributed to the wide spectrum of bioactive compounds contained in coffee beverages, like niacin, the pyridine alkaloids trigonelline and *N*-methylpyridinium (NMP), the purine alkaloid caffeine and to phenolic compounds[7,8]. Caffeine (1,3,7-trimethylxanthine) has been claimed to have no adverse effects in healthy adults when the daily intake is lower than ~400 mg, equivalent to three to six coffee cups[9]. Besides its psychoactive effects, caffeine has been described to be able to increase metabolic rate, perhaps through increased lipid oxidation and thermogenic mechanisms[10]. Trigonelline contributes to the flavour and taste of coffee, and the roasting process leads to the formation of two main trigonelline derivatives, namely NMP and nicotinic acid, a water-soluble B vitamin, also known as niacin. This last compound is highly bioavailable in coffee, more than in other food sources[11]. Concerning their biological effects, trigonelline and its derivatives have been related to anti-diabetic, neuroprotective, and anti-proliferative activities [12]. Coffee also represents one of the major dietary sources of CGAs[13], which are quinate esters of hydroxycinnamic acids such as caffeic, ferulic, and *p*-coumaric acids,

mainly substituted in the 3′, 4′ and 5′ position[14]. Among them, caffeoylquinic acids (CQAs) are the most relevant compounds, followed by feruloylquinic acids, CGA dimers, and other derivatives such as caffeoylquinic lactones and cinnamoylshikimate esters[8]. Generally, CGAs have been endorsed with several biological activities, such as inhibiting reactive oxygen species production, improving endothelial function by modulating nitric oxide production and/or thromboxane activation, and reducing blood LDL-cholesterol levels[15].

According to the most recent statistics of the International Coffee Organization, about 1.5 billion coffee cups are consumed every day in the world, prepared in several different ways according to the geography and culture of the country (www.ico.org). The most common form in southern Europe, mainly in Italy, is espresso coffee (EC), prepared from roasted and grounded coffee beans. The beverage is prepared by using a coffee machine which, under high pressure, allows the percolation of a limited amount of hot water through a ground coffee compacted powder in a short time, producing a small volume (15-30 mL) of creamy coffee[14]. Among the different coffee machines used for EC preparation, domestic and bar machines have been flanked by portable electric machines which use small sealed plastic capsules or filters, pre-packed with given amounts of coffee powders[16]. Today, a 25% of the total coffee market share in Italy (https://www.statista.com/statistics/693074/market-share-of-coffee-pods-and-capsule-for-offices-in-italy/) has been taken over by several different brands of coffee capsules, with different coffee powder mixtures, characterized by mixes of coffee cultivars, roasting degrees, and production countries, as well as by the occasional presence of aromas or additives. A recent work of our research group investigated the content of caffeine and CGAs of several EC differing for mixture, country, preparation mode and volume of the beverage[14]. A wide difference in caffeine and CGAs, mostly on the basis of different powder mixtures as well as the volume of consumption, was observed, highlighting that "one cup of coffee" might not be a reproducible serving in order to provide information about any bioactive compound[14].

To date, no studies have investigated comprehensively the main bioactive compounds in coffee capsules. Thus, the present work aimed to characterize the (poly)phenolic and alkaloid profile as well as the niacin content of up to 65 different coffees prepared with coffee capsules belonging to the five most common brands in Italy, in accordance with specific manufacturers' instructions and specific machines. Capsule variability was also

assayed in order to show the variability existing between different lots of the same coffee powder as well as for the coffee machine along the day.

2.2. Material and methods

2.2.1. Materials

Capsules from sixty-five different types of coffee capsules (named 1 to 65) belonging to five different brands (named A to E), together with their relative coffee machine, were purchased on local markets in Parma or through online stores during 2016 (23 capsules, named A1-A23, from brand A; 15 capsules, B24-B38, brand B; 10 capsules, C39-C48, brand C; 10 capsules, D49-D58, brand D; and 7 capsules, E59-E65, brand E). Two lots for each type of capsule were purchased. The type of coffee – regular, large (*lungo*) or decaffeinated EC – and the amount of coffee powder for each capsule is provided in **Supplementary Table 2.1**.

3-*O*-Caffeoylquinic acid (3-CQA), 4-*O*-caffeoylquinic acid (4-CQA), 5-*O*-caffeoylquinic acid (5-CQA), caffeine, trigonelline, NMP, and niacin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and formic acid were also purchased from Sigma-Aldrich. Water for UHPLC analysis was purchased from VWR Chemicals (Fontenay-sous-bois, France).

2.2.2. Preparation of coffee brews

Two different lots for each type of coffee were considered and, for each lot, two capsules were extracted. Extractions were performed by using the coffee capsule with its relative brand machine, and according to the manufacturer's instructions in terms of extraction time and coffee volume. Concerning the amount of coffee, brand A and D machines stop automatically for a pre-determined amount of coffee, while machines of the brand B, C and E leave the consumer free to stop the extraction at the desired time. For this reason, the extraction volume for the capsules belonging to these last brands was standardized by calculating the extraction time useful to reach the volume of coffee suggested by the manufacturer. Pressure of extraction was the one set for each brand machine: A, 19 bar; B, 15 bar; C, 15 bar; D, 19 bar; and E, 20 bar (data refers to the manufacturers' information booklets). Bidistilled water was used for capsule extraction.

Before each coffee extraction, a washing cycle with bidistilled water was carried out to avoid carry-over effects (this was assessed by analysing the caffeine content in the washing water). Two preliminary coffee extractions were performed prior to sample collection, in order to allow the machine to reach a steady extraction temperature. Each brewed coffee was collected in a graduated glass cylinder for volume and temperature measurements.

In order to study the inter-capsule variability, two cycles of ten consecutive extractions (including a washing step between sequential uses) were carried out for a subset of coffee capsules, with at least a type of coffee for each brand, including decaffeinated coffees: 17 (regular), 20 (*lungo*), and 23 (decaffeinated) for brand A; 26 (regular), brand B; 43 (regular), brand C; 54 (regular), brand D; and 62 (regular), brand E. These cycles were performed in two different days.

Aliquots of the brewed coffees were sampled and stored at -80 °C until analysis.

2.2.3. Liquid chromatography-mass spectrometry (UHPLC-ESI-MS/MS) analysis and method validation

An aliquot of coffee brew was centrifuged at 17,968 *g* for 10 min at 4 °C and generally diluted 1:50 in 0.1% formic acid in water (v:v) prior to UHPLC-ESI-MS/MS analysis. Ten out of the sixty-five samples (A-13, C-43, C-48, C-49, C-50, D-55, D-56, E-59, E-62, E-63) felt outside the calibration curve ranges of caffeine, and for this reason they were diluted 100 folds only for caffeine quantification. A method for the analysis of all the considered coffee phytochemicals in a single chromatographic run was developed and validated. Samples were analysed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS, LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization (ESI) probe (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were carried out using a XSelect HSS T3 2.5 µm particle size (50x2.1 mm, Waters, Milford, MA, USA). Volume injected was 5 µL, column oven was set to 30°C, and elution flow rate was 0.3 mL/min. The initial gradient was 97% of 0.1% aqueous formic acid and 3% of acetonitrile 0.1% formic acid, reaching 32% acidified acetonitrile at 6.5 min. From 6.5 to 7 min the acidified acetonitrile was increased to 80%, followed by 1.5 min of 80% acetonitrile and then 1.5 min at the start conditions to re-equilibrate the column.

A selective full scan MS^2 mode analysis was developed to identify and quantify the coffee phytochemicals. The MS worked with capillary temperature at 275 °C, while the source was set at 300 °C. The sheath gas flow was 60 units, while auxiliary gas pressure was

set to 5 units. The source voltage was 4 kV. Positive ionization mode was used for the analysis of caffeine, trigonelline, NMP, and niacin, while negative ionization was used for the phenolic compounds. The capillary and tube lens voltages were 3 and 45 V (positive ionization) and -33 and -98 V (negative ionization), respectively. Collision induced dissociation (CID) equal to 30 (arbitrary units) for all the compounds except for trigonelline, NMP, and niacin, where a CID equal to 38 was applied. Pure helium gas was used for CID.

Data processing was performed using Xcalibur software from Thermo Scientific. All compounds were identified by comparison with exact standards, when available, and published mass spectral and chromatographic data. Quantification was carried out in selected reaction monitoring mode by selecting the relative base peak at the corresponding mass to charge ratio (*m/z*), and through external calibration with commercial standards, when available, or with a reference compound selected on the basis of structural similarity. Details on the identification and quantification of the coffee bioactives are presented in **Table 2.1**. IUPAC nomenclature has been used for the CGAs.

Table 2.1: Mass spectral characteristics of the main alkaloids, niacin and phenolic compounds in the coffee samples.

N.	Compound	Ion.Mode	Rt[1] (min)	$[M-H]^+$ or $[M-H]^-$ (*m/z*)	MS[2] (*m/z*)	MSI MI level[3]	Quantified as
1	NMP	+	0.48	95	**79**[3]; 83	1	NMP
2	Trigonelline	+	0.45	138	**92**; 94	1	Trigonelline
3	Niacin	+	0.64	124	**106**; 80	1	Niacin
4	Caffeine	+	3.61	195	**138**	1	Caffeine
5	3-CQA	-	3.18	353	**191**;179;135	1	3-CQA
6	4-CQA	-	3.86	353	**173**; 191; 179	1	4-CQA
7	5-CQA	-	3.76	353	**191**;179	1	5-CQA
8	3-FQA	-	3.72	367	**193**: 191	2	3-CQA
9	4-FQA	-	4.33	367	**173**; 193	2	4-CQA
10	5-FQA	-	4.70	367	**191**;173	2	5-CQA
11	3-CouQA	-	4.20	337	**191**;179	2	3-CQA
12	4-CouQA	-	4.60	337	**173**; 191	2	4-CQA
13	CSA1	-	3.94	335	**179**; 161	2	5-CQA
14	CSA2	-	4.43	335	**173**; 179	2	5-CQA
15	CQL1	-	4.64	335	**161**; 135	2	5-CQA
16	CQL2	-	4.80	335	**161**; 135	2	5-CQA
17	4-FQL	-	5.72	349	**175**; 193	2	4-CQA

Legend: *n*-CQA: *n*-*O*-caffeoylquinic acid; CSA: *n*-*O*-caffeoylshikimic acid; *n*-FQA: *n*-*O*-feruloylquinic acid; *n*-FQL: *n*-*O*-feruloylquinic lactone; *n*-CouQA: *n*-*O*-coumaroylquinic acid; CQL: *n*-*O*-caffeoylquinic lactone; NMP: *N*-methylpyridinium. [1]Rt, retention time; [3]the fragment ions used for quantification are highlighted in bold; [3]Metabolite standards initiative (MSI) metabolite identification (MI) levels[31].

The method was validated for selectivity, calibration curve, range, limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), intra-

day and inter-day precision, and accuracy. Method validation was carried out according to Food and Drug Administration (FDA) guidelines[17]. Selectivity was assessed by analysing acidified water (0.1% formic acid) spiked or not with the selected standard compounds at the LLOQ (**Supplementary Table 2.2**), and the method was revealed to be highly sensitive. The evaluation of the range of calibration curves was based on data fitting to linear or quadratic regressions, prioritizing linear fitting. Acceptable fitting was estimated by using the coefficient of determination (R^2). The LOD and LLOQ for each compound were determined as the concentration in which the fragment ion used for quantification showed a signal-to-noise (S/N) ratio ≥3 and ≥10, respectively. The intra-day precision (repeatability) and inter-day precision (semi-reproducibility) of the method, reported as the relative standard deviation (% RSD), was evaluated at the LLOQ of each compound (L1) and at two higher concentration levels (L2 and L3). Each solution was injected randomly three times per day in three different days. The acceptance criteria were RSD <20% for L1 and <15% for both L2 and L3. Accuracy was calculated in terms of recovery rate for the L2 concentration level of each compound, as the ratio between the mean recorded concentration and the spiked concentration, multiplied by 100.

2.2.4. Statistical Analysis

The SPSS statistical package (SPSS Inc., Chicago, IL, USA, version 25) was used. All data were expressed as mean ± SD (n=4). Principal component analysis (PCA) with varimax was performed to explore the variability among coffee capsules using amount of bioactives per coffee serving as well as the concentration of bioactives in the coffee (mg/mL).

2.3. Results and Discussion

In a recent historical review, it was claimed that "coffee is never 'just coffee'"[18], as beans can grow in different parts of the world and be roasted in different ways, coffee can be prepared following several brewing methods and be served in a wide range of sizes. However, in Italy, the espresso coffee consumed in a "bar" is still the most common choice, and the crave to have something similar available at home recently boosted the market of coffee machines that are easy to use, clean and that allow the preparation of coffees that are close to what people can buy in a bar.

To date, to the best of our knowledge, this is the first work fully focused on the characterization of the main alkaloids and phenolic acids in ECs prepared from capsules of

the most representative brands in Italy. Most of these brands also commercialize their products worldwide.

It is worth mentioning that the alkaloid and phenolic profiles of ECs prepared by capsules may result different from the ones prepared with moka pots or bar machines. Pressure and temperature of the machines, as well as physico-chemical characteristics of the coffee powders may be different, which makes comparisons with the literature difficult.

2.3.1. Physical properties of coffees brewed from capsules

For each of the five considered brands, regular size, *lungo* and decaffeinated versions of capsule ECs were present. Concerning the volume of a serving, regular ECs ranged from 19 mL to 53 mL, while *lungo* coffees fell in the range of 90 mL to 200 mL. Decaffeinated coffees had an average volume of 44 mL, with a spike of 90 mL for sample #A-21, which was a *lungo* decaffeinated coffee (**Table 2.2**). It should be noted that serving volumes for regular ECs were slightly higher than those found for ECs served at Italians bars (ranging from 13 to 31 mL)[14].

The temperatures registered along all the brewed coffees ranged between 60.0 °C to 78.5 °C, without relevant differences among the different ECs and brands (**Supplementary Table 2.3**).

Table 2.2. Characterization of the espresso coffees from capsules for volume, main alkaloids, niacin and chlorogenic acid contents. Values are expressed as mean ± SD (n=4).

ID Capsule	Volume (mL)	Caffeine (mg/serving)	Trigonelline (mg/serving)	NMP (mg/serving)	Niacin (mg/serving)	Total CQAs (mg/serving)	Other phenolic acids (mg/serving)	Ratio caffeine/ total CQAs
A-1	28.0±1.4	72.42±14.55	8.43±0.63	1.44±0.26	0.77±0.10	23.68±0.27	55.47±0.14	2.99±0.07
A-2	29.5±0.7	1.82±0.20	8.38±1.60	1.19±0.05	0.43±0.07	25.99±1.30	44.77±2.24	0.07±0.00
A-3	82.7±5.9	75.09±11.70	29.38±4.72	1.61±0.12	0.55±0.05	63.11±8.81	114.49±0.12	1.20±0.17
A-4	31.5±4.9	63.55±3.94	16.68±2.64	1.18±0.03	0.39±0.01	39.68±1.98	69.27±3.46	1.60±0.08
A-5	30.5±0.7	60.41±0.59	13.17±0.48	1.54±0.25	0.47±0.02	36.26±1.81	58.20±2.91	1.67±0.08
A-6	31.2±2.5	73.92±5.69	10.70±1.27	1.91±0.34	0.51±0.03	29.62±2.44	59.97±0.02	2.50±0.21
A-7	34.0±2.4	58.35±5.95	12.80±1.84	1.03±0.22	0.34±0.01	32.72±2.89	63.59±0.13	1.79±0.16
A-8	31.5±0.7	2.39±0.05	10.18±1.85	1.41±0.09	0.58±0.07	24.05±1.20	50.14±2.51	0.10±0.00
A-9	27.7±0.9	87.24±20.90	7.30±1.35	1.71±0.29	1.01±0.09	19.97±1.40	50.25±0.11	4.38±0.31
A-10	30.2±1.2	59.77±4.81	10.37±0.36	1.79±0.06	0.53±0.03	26.08±4.78	51.35±0.67	2.33±0.43
A-11	92.5±5.2	92.52±2.04	15.32±4.48	2.51±0.13	0.47±0.03	36.40±0.85	103.32±0.20	2.54±0.06
A-12	30.0±1.4	91.74±5.35	13.78±2.32	1.45±0.05	0.57±0.01	40.01±2.00	77.71±3.89	2.29±0.11
A-13	29.0±2.4	135.63±17.08	6.78±1.25	2.08±0.15	1.01±0.11	22.39±3.36	66.09±0.11	6.13±0.92
A-14	94.5±4.2	83.43±0.49	15.71±3.82	2.41±0.01	0.42±0.04	39.34±4.53	97.84±1.22	2.13±0.25
A-15	34.7±0.5	54.91±12.57	12.69±1.74	1.57±0.16	0.56±0.11	33.58±0.79	75.40±0.76	1.68±0.02
A-16	28.0±0.8	96.43±11.64	12.12±0.33	2.16±0.22	0.91±0.01	37.67±1.78	67.12±0.02	2.56±0.12

A-17	28.0±1.4	63.13±8.36	12.55±0.42	1.53±0.28	0.42±0.01	38.78±1.94	57.93±2.90	1.63±0.08
A-18	35.0±2.1	66.05±4.57	16.43±2.24	1.21±0.10	0.35±0.01	46.90±5.47	87.13±0.18	1.42±0.17
A-19	35.0±2.8	63.20±3.76	11.57±0.74	1.58±0.27	0.47±0.08	31.29±1.56	56.13±2.81	2.02±0.10
A-20	95.0±3.2	89.51±1.36	17.53±2.00	2.92±0.31	0.65±0.13	44.45±3.51	102.14±0.57	2.02±0.16
A-21	89.7±0.5	3.63±0.01	20.01±4.84	1.62±0.18	0.57±0.05	54.28±5.02	105.47±0.15	0.07±0.01
A-22	32.7±1.2	58.02±12.73	13.29±1.24	1.47±0.25	0.44±0.01	32.87±1.55	70.74±0.03	1.77±0.08
A-23	31.2±0.9	1.86±0.09	15.68±0.74	0.98±0.07	0.33±0.07	33.74±1.69	51.92±0.42	0.06±0.00
B-24	33.5±1.7	125.35±19.71	17.31±2.94	1.96±0.15	0.78±0.01	51.89±7.23	89.22±0.11	2.49±0.41
B-25	38.7±2.5	142.10±24.62	16.56±3.24	1.24±0.06	0.55±0.08	57.00±2.70	97.11±0.08	2.50±0.12
B-26	48.5±3.8	89.47±7.76	20.55±1.57	1.52±0.24	0.45±0.09	55.34±0.17	84.36±0.05	1.62±0.00
B-27	50.0±3.7	74.81±5.46	12.49±1.94	2.00±0.11	0.55±0.10	30.87±5.40	66.60±0.02	2.46±0.43
B-28	46.7±4.1	2.16±0.21	14.17±3.08	1.77±0.14	0.78±0.09	35.91±6.53	73.01±3.65	0.06±0.01
B-29	51.0±3.5	130.87±9.78	20.60±3.42	2.17±0.15	0.84±0.14	52.94±4.10	93.25±0.16	2.48±0.19
B-30	54.2±7.1	2.92±0.59	13.47±1.70	2.24±0.36	0.90±0.15	36.56±0.62	81.91±0.42	0.08±0.00
B-31	206.3±13.7	130.77±6.99	20.26±1.71	5.88±1.19	1.16±0.17	46.98±2.70	141.33±1.58	2.79±0.16
B-32	183.8±7.5	178.29±11.99	47.32±7.95	2.97±0.58	0.56±0.08	116.68±5.31	223.42±1.89	1.53±0.07
B-33	126.3±13.2	116.07±5.46	20.54±2.12	3.29±0.32	0.73±0.13	47.34±5.72	110.16±1.20	2.47±0.30
B-34	106.3±7.5	185.92±20.67	18.26±2.25	2.92±0.01	0.92±0.05	58.78±3.66	121.40±0.10	3.17±0.20
B-35	31.5±1.2	99.33±19.89	20.83±3.46	1.41±0.03	0.59±0.05	54.66±3.42	81.25±0.03	1.78±0.05
B-36	30.7±2.2	114.63±19.89	12.43±2.96	2.04±0.28	0.93±0.08	33.88±4.04	72.22±0.04	3.41±0.41
B-37	31.2±3.8	107.66±11.02	18.60±2.73	1.46±0.03	0.56±0.07	52.83±6.12	86.82±0.32	2.05±0.24
B-38	47.5±2.3	87.13±2.60	22.33±3.95	1.95±0.22	0.57±0.14	62.88±8.41	98.79±0.03	1.40±0.19
C-39	48.5±0.7	123.17±9.18	26.69±2.54	2.17±0.24	0.73±0.12	86.61±4.33	114.71±5.74	1.42±0.07
C-40	47.7±4.9	1.88±0.16	30.50±0.19	2.06±0.25	0.75±0.16	73.71±0.49	93.59±0.14	0.03±0.00
C-41	46.2±2.6	134.77±12.06	26.49±1.72	2.26±0.13	0.87±0.09	74.26±13.93	109.27±0.14	1.85±0.35
C-42	48.0±2.5	152.59±11.12	14.32±0.36	2.48±0.09	0.93±0.13	50.41±7.66	113.17±0.03	3.06±0.47
C-43	47.5±2.3	200.05±9.64	19.55±3.80	3.02±0.29	0.97±0.06	65.60±1.43	122.89±0.75	3.05±0.07
C-44	100.0±7.1	190.32±5.40	33.47±1.03	3.36±0.20	1.05±0.09	98.97±4.95	156.46±7.82	1.92±0.10
C-45	51.0±1.4	150.20±3.24	23.10±4.04	2.32±0.00	0.62±0.09	68.19±3.41	114.40±5.72	2.20±0.11
C-46	48.0±4.5	157.31±2.10	19.22±1.05	3.04±0.43	1.17±0.15	51.94±1.81	112.05±0.16	3.03±0.11
C-47	49.5±2.6	122.10±9.31	22.00±0.82	2.00±0.35	0.60±0.05	57.63±2.19	101.41±0.02	2.12±0.08
C-48	47.7±3.5	207.99±4.20	16.04±2.90	1.73±0.21	0.75±0.01	69.42±12.62	141.61±0.02	3.05±0.55
D-49	25.0±6.3	117.11±15.60	24.52±1.44	0.81±0.11	0.31±0.03	67.85±10.85	91.17±0.19	1.75±0.28
D-50	22.0±3.4	105.87±2.97	24.48±3.19	1.16±0.01	0.50±0.07	58.02±4.53	81.93±0.03	1.83±0.14
D-51	27.0±2.8	53.87±1.66	28.72±1.11	1.43±0.28	0.66±0.05	53.19±2.66	77.10±3.85	1.01±0.05
D-52	28.5±0.7	4.02±0.36	19.87±0.71	1.65±0.02	0.71±0.00	41.03±2.05	69.32±3.47	0.10±0.00
D-53	132.5±3.5	121.86±10.70	21.10±0.37	3.15±0.27	0.73±0.10	54.88±2.74	108.97±5.45	2.22±0.11
D-54	29.0±0.0	108.95±9.26	15.10±0.10	1.98±0.30	0.59±0.06	50.70±2.54	80.74±4.04	2.15±0.11
D-55	29.5±0.7	134.10±3.89	11.16±0.26	3.43±0.12	1.07±0.01	31.35±1.57	68.40±3.42	4.28±0.21
D-56	27.0±2.8	125.67±0.40	18.72±1.11	1.68±0.25	0.73±0.04	46.41±2.32	78.22±3.91	2.71±0.14
D-57	32.5±2.1	108.73±5.62	33.57±2.25	1.63±0.21	0.47±0.03	75.67±3.78	99.64±4.98	1.44±0.07
D-58	32.0±1.4	103.28±2.16	24.31±1.42	1.19±0.02	0.48±0.02	59.89±2.99	81.66±4.08	1.72±0.09
E-59	33.7±3.9	148.57±27.55	17.21±3.94	1.78±0.06	0.76±0.04	56.40±3.74	103.39±0.07	2.59±0.25
E-60	36.0±3.3	4.57±0.87	32.33±5.99	1.45±0.29	0.68±0.03	62.98±14.90	101.08±0.61	0.07±0.02
E-61	35.0±0.8	107.52±10.62	29.44±7.35	2.19±0.03	0.74±0.05	63.25±11.31	87.19±0.02	1.73±0.31
E-62	35.7±4.0	172.28±40.02	18.99±0.52	2.00±0.10	0.83±0.00	58.00±4.59	109.27±0.05	2.98±0.24
E-63	36.0±5.3	141.98±16.38	19.91±3.96	1.94±0.23	0.82±0.06	61.60±9.53	100.25±0.07	2.33±0.36

E-64	37.5±2.0	127.10±13.97	24.35±4.13	1.89±0.21	0.72±0.01	67.94±9.68	100.93±0.57	1.89±0.27
E-65	35.7±1.2	123.99±16.43	25.06±4.53	1.82±0.38	0.79±0.02	68.50±10.07	105.24±0.75	1.83±0.27

Legend: CQAs: caffeoylquinic acids; NMP: *N*-methylpyridinium.

2.3.2. Identification and quantification of niacin, alkaloids and phenolic compounds in capsule coffees

The UHPLC-ESI-MS/MS analysis developed and validated for this study allowed the identification and quantification of up to 17 different compounds in ECs in a single chromatographic run (**Figure 2.1**). Their mass spectral characteristics are showed in **Table 2.1**. Interestingly, niacin and very polar pyridine alkaloids trigonelline and NMP, present a good peak resolution under the chromatographic conditions used (**Figure 2.1**). Only the most relevant phenolic compounds, contributing significantly to the total amount of CGAs in coffee, were taken into account in this analysis[19,20]. Besides 3-, 4-, and 5-*O*-caffeoylquinic acids, other phenolics quantified in this study were 3-, 4-, 5-feruloylquinic acids; 3- and 4-*O*-coumaroylquinic acids; caffeoylshikimic acids, caffeoylquinic lactones, and 4-*O*-feruloylquinic lactone. Concerning caffeoylshikimic isomers (CSA1 and CSA2, **Table 2.1**), these were tentatively identified as 4-*O*-caffeoylshikimic acid and 3-*O*-caffeoylshikimic acid, by comparing their elution profile and fragmentation patterns with those found by Jaiswal *et al.*[21]. Nevertheless, 3-*O*-caffeoylshikimic acid was not fully confirmed because of the presence of a fragment ion at *m/z* 173, that was not reported by Jaiswal *et al.*[21]. Similarly, caffeoylquinic lactone isomers CQL1 and CQL2 were tentatively identified as 3-*O*-caffeoylquinic lactone and 4-*O*-caffeoylquinic lactone according to Jaiswal *et al.*[21].

Overall, this validated analytical method allowed a high throughput and accurate fingerprinting of main coffee bioactives, in a unique chromatographic run lasting 10 min. It could be used for further studies assessing objectively the intake of coffee bioactive compounds in epidemiological studies.

Since volumes of ECs changed in relation to the manufacturer's instruction, results on phytochemical composition have been expressed both as mg per serving and mg/mL (**Table 2.2** and **Supplementary Tables 2.3 to 2.5**).

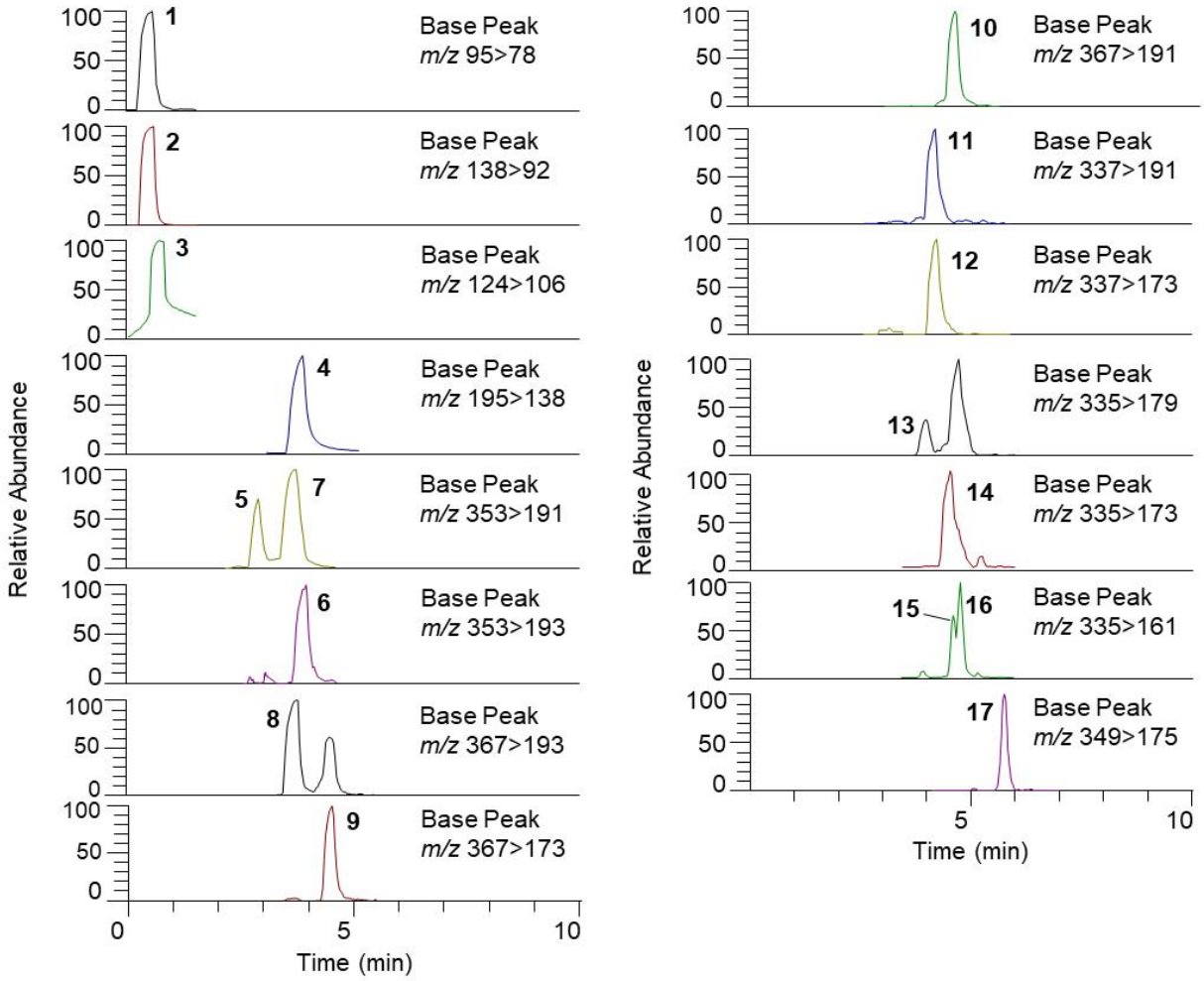

Figure 2.1. UHPLC-MS/MS profile of capsule-brewed coffees with detection of the considered compounds by SRM.
Legend: *m/z* 95 for *N*-methylpyridinium (**1**); *m/z* 138 for trigonelline (**2**); *m/z* 124 for niacin (**3**); *m/z* 195 for caffeine (**4**); *m/z* 353 for caffeoylquinic acids (**5-7**); *m/z* 367 for feruloylquinic acids (**8-10**); *m/z* 337 for coumaroylquinic acids (**11-12**); *m/z* 335 for caffeoylshikimic acids and caffeoylquinic lactones (**13-16**); *m/z* 349 for feruloylquinic lactones (**17**). See **Table 2.1** for further details.

2.3.3. Alkaloid and niacin contents of the capsule espresso coffees

The alkaloids considered in the present work were caffeine, trigonelline and NMP. The caffeine content was highly variable (**Table 2.2**). The average amount of caffeine per cup was 108.6 mg/serving for regular ECs and 137.4 mg/serving for *lungo* ECs. Considering individual brands (excluding decaffeinated coffees), ECs belonging to brand C had the highest content of caffeine, (156.0 ± 32.4 mg/serving), while coffees from brand A reached an average content of 73.6 ± 21.3 mg/serving. All the other brands showed an average content of caffeine over 100 mg/serving. Regarding caffeine concentrations in caffeinated coffees, **Supplementary Table 2.3** reports a caffeine range between 0.64 mg/mL and 4.89 mg/mL. The results of the present work are in agreement with those of Albanese *et al.*[16],

who evaluated the caffeine content of coffees brewed from pods by using machines working at pressures comparable to the ones used for this experiment. Authors found amounts of caffeine in pod-brewed coffees (pure Arabica, pure Robusta, and blends) ranging from 2.59 mg/mL to 4.65 mg/mL. Furthermore, our data fell within the range of values found by Andueza *et al.*[22] under similar conditions of pressure and temperature, reporting an average of caffeine around 2 mg/mL. On the contrary, a recent paper of Jeon *et al.*[23] showed lower values (< 1 mg/mL) in home-made coffees prepared with grounded coffee beans packed in paper filters and extracted with hot water (no information on pressure available). The caffeine content of coffees prepared with bar espresso machines was studied by Caprioli *et al.*[24]. Different temperatures (88 °C, 92 °C and 98 °C), pressures (values between 7 and 11 bar), and types of coffees (pure Arabica, 95% Robusta, and blends) were assessed, and the caffeine content ranged from 111.2 mg to 255.1 mg per serving. These values were slightly higher when compared to the average amount of caffeine in the EC described in the present study (**Table 2.2**) which are, however, in agreement with those reported by Ludwig *et al.*[14], who evaluated several coffee samples brewed with bar espresso machines in Spain, Italy, and Scotland.

Concerning trigonelline, NMP, and niacin, servings of these compounds resulted higher in *lungo* ECs, compared to regular and decaffeinated ECs (**Table 2.2**). Trigonelline amounts ranged between 15.32 and 47.32 mg/serving for *lungo* ECs, 8.38 and 33.74 mg/serving for decaffeinated ECs and 6.78 and 17.88 mg/serving for regular ECs. NMP amounts ranged between 1.61 and 6.16 mg/serving for *lungo* ECs, 0.98 and 1.60 mg/serving for decaffeinated ECs and between 0.81 and 3.43 mg/serving for regular ECs. Concerning niacin, *lungo* ECs showed a concentration range of 0.42-1.16 mg/serving, decaffeinated coffee 0.31-0.93 mg/serving and regular ECs 0.31-1.21 mg/serving. However, when concentrations (mg/mL) were considered (**Supplementary Table 2.3**), regular ECs resulted in higher trigonelline, NMP, and niacin concentrations, with average values of 0.52 mg/mL, 0.05 mg/mL, and 0.02 mg/mL, respectively. These values almost doubled the respective average values recorded for decaffeinated and *lungo* ECs (**Supplementary Table 2.3**). Caprioli *et al.*[24] also considered the amounts of trigonelline and niacin in coffees brewed with bar espresso machines. Trigonelline values ranged from 22.0 to 49.4 mg/serving for 95% Robusta blends and between 52.5 and 72.7 mg/serving for Arabica, relevantly higher than what reported in **Table 2.2** for most of the ECs. Andueza *et al*. reported trigonelline concentrations close to 1 mg/mL[22], slightly higher than those found in the present study, although some individual samples (#D-50, #D-51, and #E-61) were over 1 mg/mL (**Supplementary Table 2.3**).

Regarding niacin amounts, data are in line with those reported by Caprioli *et al.*[24] and with the USDA Food Composition Databases[25] for espresso coffee items. Last, in the case of NMP, Lang's group[26,27] found average NMP concentrations of 491 nmol/L (~0.046 mg/mL) in brewed coffee, in agreement with our findings (**Supplementary Table 2.3**).

Table 2.3: Inter-lot variation in the content of main capsule espresso coffee phytochemicals.

ID Capsule	NMP (% CV)	Trigonelline (% CV)	Niacin (% CV)	Caffeine (% CV)	3-CQA (% CV)	4-CQA (% CV)	5-CQA (% CV)
A-1	17.88	7.42	13.21	20.10	2.65	5.45	4.18
A-2	3.78	19.05	15.28	10.96	8.22	15.50	11.04
A-3	7.55	16.06	9.09	15.58	18.37	17.78	7.66
A-4	2.31	15.81	1.66	6.20	9.37	2.97	10.16
A-5	16.33	3.62	4.85	0.97	4.15	8.76	7.04
A-6	17.98	11.92	5.41	7.69	10.22	11.82	2.95
A-7	21.70	14.38	3.40	10.20	19.38	8.60	3.63
A-8	6.71	18.20	11.68	2.17	0.15	0.22	4.82
A-9	16.91	18.55	8.88	23.96	10.69	13.04	3.17
A-10	3.18	3.43	4.73	8.05	21.90	21.34	11.73
A-11	5.15	29.22	5.38	2.20	4.37	2.52	2.04
A-12	3.59	16.84	2.35	5.83	12.80	2.16	0.45
A-13	7.20	18.49	10.79	12.60	21.06	3.56	16.96
A-14	0.54	24.34	8.75	0.58	18.77	16.25	0.03
A-15	10.01	13.72	18.96	22.89	20.79	18.22	18.26
A-16	10.43	2.70	0.99	12.07	4.89	8.60	1.63
A-17	18.50	3.32	2.39	13.24	5.80	11.63	15.93
A-18	8.40	13.64	3.89	6.92	21.10	12.98	0.74
A-19	17.20	6.41	16.64	5.95	2.33	5.99	1.48
A-20	10.72	11.43	20.36	1.52	17.99	11.64	6.16
A-21	10.98	24.19	8.96	0.20	13.26	10.29	4.16
A-22	16.72	9.36	2.98	21.94	15.47	3.68	6.97
A-23	7.52	4.70	22.00	4.94	14.93	4.61	8.01
B-24	7.89	16.97	1.19	15.72	18.94	8.30	11.99
B-25	5.04	19.56	14.29	17.33	16.11	0.36	8.41
B-26	15.66	7.64	20.98	8.68	2.42	12.46	3.81
B-27	5.62	15.53	17.62	7.29	21.87	19.97	8.63
B-28	8.15	21.72	10.99	9.69	21.21	17.08	15.33
B-29	6.91	16.60	16.73	7.47	10.57	5.04	6.07
B-30	16.21	12.59	16.97	20.04	9.58	19.51	5.02
B-31	20.23	8.43	14.27	5.35	13.83	9.89	7.42
B-32	19.39	16.81	14.40	6.72	4.09	13.24	0.84
B-33	9.86	10.31	17.56	4.70	20.85	12.35	3.12
B-34	0.38	12.30	5.27	11.12	4.80	1.94	16.49
B-35	2.15	16.62	9.11	20.03	12.10	16.04	8.41
B-36	13.63	23.83	8.17	17.35	12.65	14.46	8.66
B-37	2.19	14.65	11.99	10.23	5.15	17.88	15.71
B-38	11.44	17.68	24.95	2.98	22.78	13.94	0.77
C-39	11.25	9.53	16.86	7.45	5.83	0.28	3.50
C-40	12.32	0.62	21.83	8.56	3.06	0.83	3.01
C-41	5.62	6.50	9.97	8.94	21.55	22.45	12.38
C-42	3.81	2.49	14.39	7.29	17.61	14.32	12.61
C-43	9.48	19.43	6.06	4.82	2.09	2.81	1.73
C-44	6.03	3.09	8.36	2.84	3.27	14.12	13.46
C-45	0.12	17.47	14.84	2.15	12.60	8.23	7.59
C-46	14.30	5.47	13.11	1.34	1.38	2.27	6.92
C-47	17.33	3.71	7.88	7.63	0.45	9.11	4.84
C-48	12.05	18.07	1.49	2.02	14.09	19.77	21.27
D-49	13.16	5.86	11.41	13.32	21.10	10.66	13.19
D-50	1.03	13.02	13.99	2.81	14.50	6.66	10.29
D-51	19.28	3.85	8.27	3.08	8.17	3.66	7.45

D-52	1.19	3.58	0.61	8.86	14.85	0.68	11.88
D-53	8.73	1.74	14.26	8.78	10.44	2.55	7.83
D-54	15.15	0.66	9.53	8.50	15.71	3.71	1.42
D-55	3.40	2.31	0.80	2.90	4.62	1.58	0.10
D-56	14.88	5.94	6.09	0.32	12.21	16.67	10.32
D-57	13.20	6.69	6.07	5.17	6.93	14.55	6.01
D-58	2.03	5.83	5.13	2.10	0.58	15.76	14.27
E-59	3.30	22.90	5.49	18.55	15.14	1.93	2.10
E-60	20.18	18.52	5.15	19.13	24.72	19.01	25.36
E-61	1.19	24.95	6.93	9.88	21.93	15.85	13.05
E-62	4.95	2.71	0.52	23.23	1.52	8.93	15.96
E-63	11.65	19.89	7.54	11.53	21.78	15.73	4.59
E-64	11.15	16.95	2.08	10.99	15.61	16.52	10.32
E-65	20.80	18.08	3.06	13.25	18.22	20.98	3.50

Legend: *n*-CQA: *n*-*O*-caffeoylquinic acid; NMP: *N*-methylpyridinium.

2.3.4. Chlorogenic acids in capsule espresso coffee

The most relevant CGAs in coffee beverages are CQAs, mainly 3-, 4- and 5-CQA. The total amount per serving of these three compounds is shown in **Table 2.2**. *Lungo* coffees have the highest average amount of CQAs (60.4 ± 26.6 mg/serving), followed by regular (49.6 ± 16.0 mg/serving) and decaffeinated (43.0 ± 17.4 mg/serving) ECs. Concerning the various brands, brand C coffees revealed to have the highest average content of CQAs (69.5 ± 15.1 mg/serving), followed by E (63.1 ± 4.5 mg/serving), D (53.9 ± 12.7 mg/serving), B (52.5 ± 20.2 mg/serving), and brand A (35.2 ± 10.1 mg/serving). The coffee presenting the highest CQA content was sample #B-32 (116.7 ± 5.3 mg/serving), 3-CQA, 4-CQA and 5-CQA representing 36.1%, 25.7%, and 38.2% of the total CQAs, respectively (**Supplementary Table 2.4**). This pattern, registered for most of the tested coffees, was in accordance with literature data for capsule- and filter-brewed coffees[28], whereas Crozier *et al.*[29] indicated that 5-CQA was the main CQA in EC, representing about 50% of total CQAs. The mg/serving of total CQAs was also considered by Ludwig *et al.*[14] for ECs brewed in different countries and from different blends. Data in **Table 2.2** is in line with the CQA amounts per serving reported by these authors for Italian and Scottish ECs, while these were lower with respect to the quantity of CQAs contained in Spanish ECs. Concerning CGA concentrations, our data confirmed the ranges reported by Moeenfard *et al.*[28], with particular high amounts of 3-CQA and 5-CQA (about 1 mg/mL each) for ECs #D-49 and #D-50 (**Supplementary Table 2.4**). These 5-CQA concentrations close to 1 mg/mL, two- to five-fold higher than those recorded for most of the capsule ECs (**Supplementary Table 2.4**), were also registered by Andueza *et al.*[22,30] for a set of blended ECs brewed under different extraction conditions.

Table 2.2 also shows that ECs belonging to brand C had the highest content in the sum of these phenolics, hitting an average 118.0 ± 18.5 mg/serving, while brand D coffees had the lowest values, with an average of 83.7 ± 12.8 mg/serving. EC #B-32 reached 223.4 ± 1.9 mg/serving, almost double with respect to most of the other coffee samples. Concentration data (mg/mL, **Supplementary Tables 2.4 and 2.5**) were in line with previous results for individual phenolics. These observations back the need for accurately investigating the content in CGAs, and not only in CQAs, of coffee samples. This should be key to comprehensively establish the amount of phenolic compounds provided by a cup of coffee, a point required to further support observational studies with an objective assessment of the intake of these major dietary bioactives[13,14].

2.3.5. Degree of roasting

Caffeine/CQA ratio was used as marker of the degree of roasting of the ECs[14]. The caffeine/total CQA ratio, not taken into account for the decaffeinated ECs, was similar among the different brand capsules, ranging from an average of 2.12 for brand D ECs to 2.29 for brand B capsules (**Table 2.2**). For instance, considering each type of capsule, EC #D-51 showed the lowest caffeine/total CQA ratio (1.01 ± 0.05), while EC #A-9 showed the highest value (4.38 ± 0.31). Data are in line with those reported by Ludwig *et al.*[14] for coffees prepared in different European countries and, particularly, the range of caffeine/total CQA ratio was close to the Italian ECs, from 1.3 to 5.3.

2.3.6. Inter-lot and capsule variability

The inter-lot and capsule variation in the content of main coffee phytochemicals was assessed to better understand how some factors may alter the content in bioactives of capsule ECs. The overall variability for the main compounds between the two lots analysed for each of the 65 coffees fell in the range 10-20% (**Table 2.3**).

The capsule variability along one day was studied only in a subset of samples for caffeine, trigonelline, NMP, and total CQAs (**Figure 2.2A-2.2D**). Trigonelline and NMP showed the greatest variability, ranging from 8% to 23% and 10% to 21%, respectively. A slightly smaller inter-capsule variability was observed for caffeine, varying between 3% and 20% for the considered coffees. Finally, all the considered brands and capsules showed an average inter-capsule variability along the day lower than 14% for total CQAs.

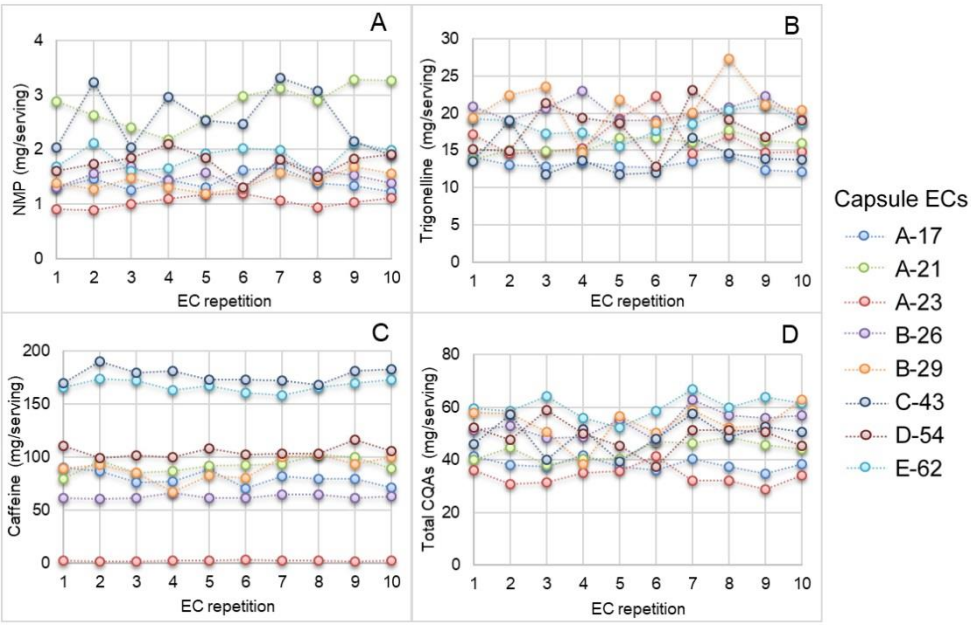

Figure 2.2. Capsule variability of caffeine (A), trigonelline (B), *N*-methylpyridinium (C) and total caffeoylquinic acids (D) of capsule espresso coffee samples. Capsules were representative of regular, *lungo*, and decaffeinated coffees belonging to the five considered brands. CQAs, caffeolylquinic acids; EC, espresso coffee; NMP, *N*-methylpyridinium.

2.3.7. Global assessment of the phytochemical profile of the capsule espresso coffees

Multivariate unsupervised PCA, considering the mg/serving of the studied compounds, was carried out in order to better understand the variability in the phytochemical profile of the main coffee capsules commercialised in the Italian market (**Figure 2.3A**). Two principal components (PCs) explained up to 73.8% of the total variability. PC1 accounted for 40.4% of the observed variability and was positively load by trigonelline, the three CQA isomers (3-CQA, 4-CQA, and 5-CQA), the two caffeoylquinic lactones (CQL1 and CQL2), and the two coumaroylquinic acid isomers (3-CouQA and 4-CouQA). PC2 explained a 33.4% of the total variability and had positive loadings from caffeine, niacin, NMP and the phenolic compounds not loading PC1.

Individual scores for each EC revealed interesting insights with regard to the composition of ECs prepared with coffee capsules (**Figure 2.3B**). A pattern where the brand seemed to be a factor conditioning the phytochemical composition of the ECs was observed. Briefly, according to EC scores, most of the brand A coffees had negative values for both PCs, which pointed out that most of the ECs prepared using these capsules are poor in coffee bioactives. Most of the brand B ECs showed neutral scores for PC1, while they displayed a great variability with regard to their scores for PC2. This variability was mainly influenced

by the serving size, since ECs #B-31 to B-34 were large – *lungo* – (or very large) ECs. Regarding brand D ECs, these showed positive PC1 scores and negative PC2 ones, which may account for a high content in trigonelline and caffeoylquinic acids and lactones, paralleled to a low content in other alkaloids and phenolic acids. In the case of brands C and E, most of their ECs presented positive scores for both PCs, with a higher variability for PC2. This indicated that they were, comprehensively, the capsule ECs providing the highest amount of coffee bioactives. Contrary to brand B, variability within these ECs was not related to the serving size. This may indicate that the differences in the amount of phytochemicals provided by coffees belonging to brands C and E may be linked to the different types of coffee used for the preparation of the capsules. Finally, while brands C and E offered a great versatility in their product portfolio in terms of coffee bioactives, brands A and B exhibited a homogenous pattern of phytochemicals, despite the putative differences existing in the coffees used for the preparation of each capsule.

An additional PCA, considering the mg/mL of the compounds in coffee samples, was also carried out in order to limit the effect of serving size in the phytochemical profile of the capsules (**Supplementary Figure 2.1A and 2.1B**). Similar to results found in **Figure 2.3A** and **3B**, two PCs explained up to 73.8% of the total variability and the main aspects associated with the aforementioned brand-related pattern were observed, with minor variations. Positive PC1 scores and negative PC2 ones have been found for brand D ECs (mainly D-49, D-50, D-51 and D-57), influenced by the high concentrations in trigonelline and caffeoylquinic acids and lactones. On the contrary, brand E ECs were mainly influenced by their concentrations in feruloylquinic acids.

This brand-related pattern was fully unexpected since a high variability in terms of coffee variety, origin, and roasting degree is claimed by each brand when commercialising their different capsules. Consequently, the configuration of a general phytochemical pattern for every brand was not initially hypothesised. Nevertheless, this pattern could be currently attributed to i) the impact that the brewing machine used might have on the extraction of coffee bioactives or ii) the main processing steps used within each company for the preparation of all their capsules. In this regard, Andueza *et al.*[22] demonstrated by using multivariate analysis the effect of the brewing pressure used in the final profile of coffee components. Further information would be required to confirm the reasons underlying this brand-based pattern in the phytochemical content of ECs produced from capsules.

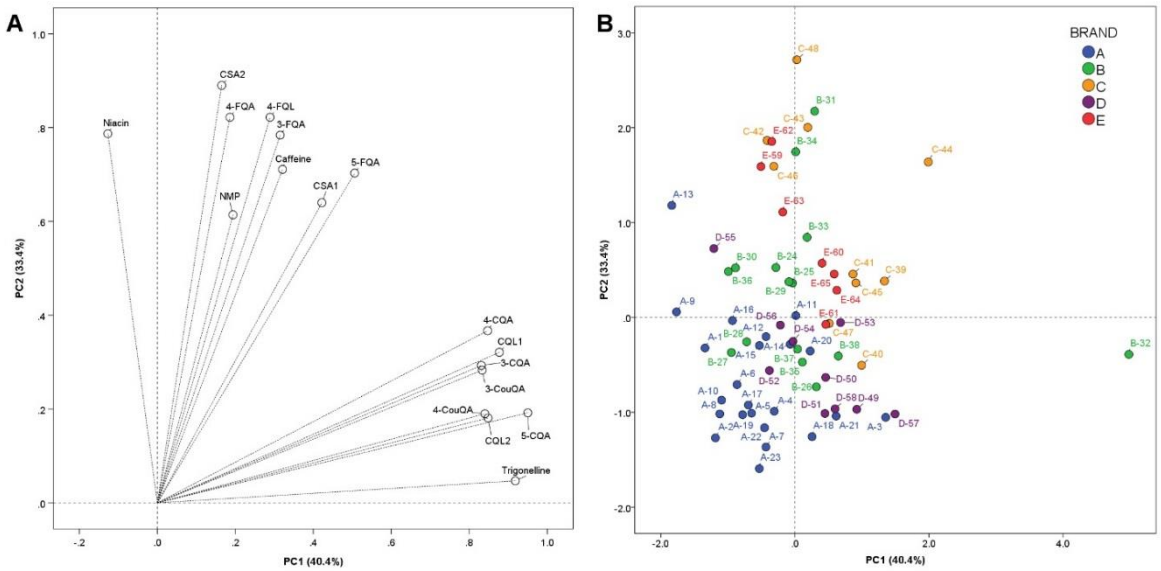

Figure 2.3. Loading plot (A) and score plot (B) obtained from the PCA with varimax of the considered bioactive compounds and capsule espresso coffees. Legend: *n*-CQA: *n*-*O*-caffeoylquinic acid; CSA*n*: caffeoylshikimic acid isomer; CQL*n*: caffeoylquinic lactone isomer; *n*-CouQA: *n*-*O*-coumaroylquinic acid; *n*-FQA: *n*-*O*-feruloylquinic acid; *n*-FQL: *n*-*O*-feruloylquinic lactone; NMP: *N*-methylpyridinium.

In conclusion, the alkaloid and phenolic profiles as well as the niacin content of the most representative capsule ECs in the Italian market showed a wide variability both among capsules of the same brand and among different brands. These differences in the composition of ECs prepared by using coffee capsules may be of interest for further studies when a specific set of bioactives is specifically investigated. Moreover, this variability is of note for future studies, since it demonstrates that "coffee is never 'just coffee'" and that the content of bioactives in a cup of coffee may vary significantly.[31]

2.4. References

1. Dechamp, E.; Breitler, J.-C.; Leroy, T.; Etienne, H. Coffee (Coffea arabica L.). *Methods Mol. Biol.* **2015**, *1224*, 275–291, doi:10.1007/978-1-4939-1658-0_22.
2. Wang, X.; Lim, L.-T. Physicochemical Characteristics of Roasted Coffee. In *Coffee in Health and Disease Prevention*; Preedy, V. R. B. T.-C. in H. and D. P., Ed.; Academic Press: San Diego, 2015; pp. 247–254 ISBN 978-0-12-409517-5.
3. Chrysant, S. G. The impact of coffee consumption on blood pressure, cardiovascular disease and diabetes mellitus. *Expert Rev. Cardiovasc. Ther.* **2017**, *15*, 151–156, doi:10.1080/14779072.2017.1287563.
4. Kempf, K.; Herder, C.; Erlund, I.; Kolb, H.; Martin, S.; Carstensen, M.; Koenig, W.; Sundvall, J.; Bidel, S.; Kuha, S.; Jaakko, T. Effects of coffee consumption on subclinical inflammation and other risk factors for type 2 diabetes: a clinical trial. *Am. J. Clin. Nutr.* **2010**, *91*, 950–957.
5. Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E. L. Coffee, Caffeine, and Health Outcomes: An Umbrella Review. *Annu. Rev. Nutr.* **2017**, *37*, 131–156, doi:10.1146/annurev-nutr-071816-064941.
6. Loftfield, E.; MC, C.; Caporaso, N.; Yu, K.; Sinha, R.; Freedman, N. Association of coffee drinking with mortality by genetic variation in caffeine metabolism: Findings from the UK biobank. *JAMA Intern. Med.* **2018**, In press: 10.1001/jamainternmed.2018.2425.
7. Martini, D.; Del Bo', C.; Tassotti, M.; Riso, P.; Del Rio, D.; Brighenti, F.; Porrini, M. Coffee Consumption and Oxidative Stress: A Review of Human Intervention Studies. *Molecules* **2016**, *21*, 979;

10.3390/molecules21080979, doi:10.3390/molecules21080979.
8. Ludwig, I. A.; Clifford, M. N.; Lean, M. E. J.; Ashihara, Hi.; Crozier, A. Coffee: biochemistry and potential impact on health. *Food Funct.* **2014**, *5*, 1695–1710, doi:10.1039/C4JA00138A.
9. Nawrot, P.; Jordan, S.; Eastwood, J.; Rotstein, J.; Hugenholtz, A.; Feeley, M. Effects of caffeine on human health. *Food Addit. Contam.* **2003**, *20*, 1–30, doi:10.1080/0265203021000007840.
10. Heckman, M. A.; Weil, J.; de Mejia, E. G. Caffeine (1, 3, 7-trimethylxanthine) in foods: A comprehensive review on consumption, functionality, safety, and regulatory matters. *J. Food Sci.* **2010**, *75*, 77–87, doi:10.1111/j.1750-3841.2010.01561.x.
11. Stadler, R. H.; Varga, N.; Hau, J.; Vera, F. A.; Welti, D. H. Alkylpyridiniums. 1. Formation in model systems via thermal degradation of trigonelline. *J. Agric. Food Chem.* **2002**, *50*, 1192–1199.
12. Perrone, D.; Donangelo, C. M.; Farah, A. Fast simultaneous analysis of caffeine, trigonelline, nicotinic acid and sucrose in coffee by liquid chromatography-mass spectrometry. *Food Chem.* **2008**, *110*, 1030–1035, doi:10.1016/j.foodchem.2008.03.012.
13. Zamora-Ros, R.; Knaze, V.; Rothwell, J. A.; Hémon, B.; Moskal, A.; Overvad, K.; Tjønneland, A.; Kyrø, C.; Fagherazzi, G.; Boutron-Ruault, M. C.; Touillaud, M.; Katzke, V.; Kühn, T.; Boeing, H.; Förster, J.; Trichopoulou, A.; Valanou, E.; Peppa, E.; Palli, D.; Agnoli, C.; Ricceri, F.; Tumino, R.; de Magistris, M. S.; Peeters, P. H. M.; Bueno-de-Mesquita, H. B.; Engeset, D.; Skeie, G.; Hjartåker, A.; Menéndez, V.; Agudo, A.; Molina-Montes, E.; Huerta, J. M.; Barricarte, A.; Amiano, P.; Sonestedt, E.; Nilsson, L. M.; Landberg, R.; Key, T. J.; Khaw, K. T.; Wareham, N. J.; Lu, Y.; Slimani, N.; Romieu, I.; Riboli, E.; Scalbert, A. Dietary polyphenol intake in Europe: the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Eur. J. Nutr.* **2015**, *55*, 1359–1375, doi:10.1007/s00394-015-0950-x.
14. Ludwig, I. A.; Mena, P.; Calani, L.; Cid, C.; Del Rio, D.; Lean, M. E. J.; Crozier, A. Variations in caffeine and chlorogenic acid contents of coffees: what are we drinking? *Food Funct.* **2014**, *5*, 1718–1726, doi:10.1039/c4fo00290c.
15. Tajik, N.; Tajik, M.; Mack, I.; Enck, P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. *Eur. J. Nutr.* **2017**, *56*, 2215–2244, doi:10.1007/s00394-017-1379-1.
16. Albanese, D.; Di Matteo, M.; Poiana, M.; Spagnamusso, S. Espresso coffee (EC) by POD: Study of thermal profile during extraction process and influence of water temperature on chemical-physical and sensorial properties. *Food Res. Int.* **2009**, *42*, 727–732, doi:10.1016/j.foodres.2009.02.027.
17. US Food and Drug Administration *Guidance for Industry: Bioanalytical Method Validation*; Rockville, MD, USA, 2001;
18. Morris, J. Why espresso? Explaining changes in European coffee preferences from a production of culture perspective. *Eur. Rev. Hist.* **2013**, *20*, 881–901, doi:10.1080/13507486.2013.833717.
19. Baeza, G.; Sarriá, B.; Bravo, L.; Mateos, R. Exhaustive Qualitative LC-DAD-MSnAnalysis of Arabica Green Coffee Beans: Cinnamoyl-glycosides and Cinnamoylshikimic Acids as New Polyphenols in Green Coffee. *J. Agric. Food Chem.* **2016**, *64*, 9663–9674, doi:10.1021/acs.jafc.6b04022.
20. Jaiswal, R.; Matei, M. F.; Subedi, P.; Kuhnert, N. Does roasted coffee contain chlorogenic acid lactones or/and cinnamoylshikimate esters? *Food Res. Int.* **2014**, *61*, 214–227, doi:10.1016/j.foodres.2013.09.040.
21. Jaiswal, R.; Sovdat, T.; Vivan, F.; Kuhnert, N. Profiling and characterization by LC-MSnof the chlorogenic acids and hydroxycinnamoylshikimate esters in maté (Ilex paraguariensis). *J. Agric. Food Chem.* **2010**, *58*, 5471–5484, doi:10.1021/jf904537z.
22. Andueza, S.; Maeztu, L.; Dean, B.; De Peña, M. P.; Bello, J.; Cid, C. Influence of water pressure on the final quality of Arabica espresso coffee. Application of multivariate analysis. *J. Agric. Food Chem.* **2002**, *50*, 7426–7431, doi:10.1021/jf0206623.
23. Jeon, J.-S.; Kim, H.-T.; Jeong, I.-H.; Hong, S.-R.; Oh, M.-S.; Park, K.-H.; Shim, J.-H.; El-Aty, A. M. A. Determination of chlorogenic acids and caffeine in homemade brewed coffee prepared under various conditions. *J. Chromatogr. B* **2017**, *1064*, 115–123, doi:10.1016/j.jchromb.2017.08.041.
24. Caprioli, G.; Cortese, M.; Maggi, F.; Minnetti, C.; Odello, L.; Sagratini, G.; Vittori, S. Quantification of caffeine, trigonelline and nicotinic acid in espresso coffee: the influence of espresso machines and coffee cultivars. *Int. J. Food Sci. Nutr.* **2014**, *65*, 465–469, doi:10.3109/09637486.2013.873890.
25. USDA USDA Food Composition Database. https://ndb.nal. usda.gov/.
26. Lang, R.; Wahl, A.; Skurk, T.; Yagar, E. F.; Schmiech, L.; Eggers, R.; Hauner, H.; Hofmann, T. Development of a Hydrophilic Liquid Interaction Chromatography−High-Performance Liquid Chromatography−Tandem Mass Spectrometry Based Stable Isotope Dilution Analysis and Pharmacokinetic Studies on Bioactive Pyridines in Human Plasma and Urine after Coffee. *Anal. Chem.* **2010**, *82*, 1486–1497, doi:10.1021/ac902616k.
27. Lang, R.; Wahl, A.; Stark, T.; Hofmann, T. Urinary N-methylpyridinium and trigonelline as candidate dietary biomarkers of coffee consumption. *Mol. Nutr. Food Res.* **2011**, *55*, 1613–1623, doi:10.1002/mnfr.201000656.
28. Moeenfard, M.; Rocha, L.; Alves, A. Quantification of caffeoylquinic acids in coffee brews by HPLC-DAD. *J. Anal. Methods Chem.* **2014**, *2014*, 965353; 10.1155/2014/965353, doi:10.1155/2014/965353.
29. Crozier, T. W. M.; Stalmach, A.; Lean, M. E. J.; Crozier, A. Espresso coffees, caffeine and chlorogenic acid intake: Potential health implications. *Food Funct.* **2012**, *3*, 30–33, doi:10.1039/c1fo10240k.
30. Andueza, S.; Maeztu, L.; Pascual, L.; Ibáñez, C.; de Peña, M. P.; Cid, C. Influence of extraction temperature on the final quality of espresso coffee. *J. Sci. Food Agric.* **2003**, *83*, 240–248, doi:10.1002/jsfa.1304.
31. Sumner, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W.-M.; Fiehn, O.; Goodacre, R.; Griffin, J. L.; Hankemeier, T.; Hardy, N.; Harnly, J.; Higashi, R.; Kopka, J.; Lane, A. N.; Lindon, J. C.; Marriott, P.; Nicholls, A. W.; Reily, M. D.; Thaden, J. J.; Viant, M. R. Proposed minimum reporting

standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* **2007**, *3*, 211–221, doi:10.1007/s11306-007-0082-2.

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Author Contributions

P.M. and D.D.R. conceived the study; D.A., M.T. and P.M. designed and performed the experiments; P.M. performed the statistical analysis; D.A. and P.M. wrote the manuscript; F.B. and D.D.R. critically revised and improved the meaning of the manuscript.

All authors read and approved the final version of the manuscript.

Competing Interests: The authors declare that they have no competing interests.

2.5. Supplemental material

Supplementary Table 2.1. Powder amount of the coffee capsules used for the coffee preparations.

ID Capsule	Coffee characteristic	Powder amount (g)
A-1	Caffeinated	5
A-2	Decaffeinated *lungo*	5
A-3	Caffeinated *lungo*	5.5
A-4	Caffeinated	5
A-5	Caffeinated	5
A-6	Caffeinated	5
A-7	Caffeinated	5
A-8	Decaffeinated	5
A-9	Caffeinated	5
A-10	Caffeinated	5
A-11	Caffeinated *lungo*	6
A-12	Caffeinated	5
A-13	Caffeinated	5
A-14	Caffeinated *lungo*	5.5
A-15	Caffeinated	5
A-16	Caffeinated	5
A-17	Caffeinated	5
A-18	Caffeinated	5
A-19	Caffeinated	5
A-20	Caffeinated *lungo*	6
A-21	Decaffeinated *lungo*	5.5
A-22	Caffeinated	5
A-23	Decaffeinated	5
B-24	Caffeinated	7.5
B-25	Caffeinated	7
B-26	Caffeinated	6
B-27	Caffeinated	5.2
B-28	Decaffeinated	6
B-29	Caffeinated	8
B-30	Decaffeinated	7
B-31	Caffeinated *lungo*	8
B-32	Caffeinated *lungo*	10
B-33	Caffeinated *lungo*	7
B-34	Caffeinated *lungo*	9
B-35	Caffeinated	6.5
B-36	Caffeinated	7
B-37	Caffeinated	7
B-38	Caffeinated	7
C-39	Caffeinated	7.5
C-40	Decaffeinated	7.5
C-41	Caffeinated	7.5
C-42	Caffeinated	7.5
C-43	Caffeinated	7.5
C-44	Caffeinated *lungo*	8
C-45	Caffeinated	7.5
C-46	Caffeinated	7.5
C-47	Caffeinated	7
C-48	Caffeinated	7.5
D-49	Caffeinated	6.7
D-50	Caffeinated	6.7
D-51	Caffeinated	6.7

D-52	Decaffeinated	6.7
D-53	Caffeinated *lungo*	6.2
D-54	Caffeinated	6.7
D-55	Caffeinated	6.7
D-56	Caffeinated	6.7
D-57	Caffeinated	6.7
D-58	Caffeinated	6.7
E-59	Caffeinated	7
E-60	Decaffeinated	7
E-61	Caffeinated	7
E-62	Caffeinated	7
E-63	Caffeinated	7
E-64	Caffeinated	7
E-65	Caffeinated	7

Supplementary Table 2.2. Parameters for quantification of niacin and the main alkaloids and caffeoylquinic acids in coffee capsule samples by UHPLC-MS^2 in SRM mode (MS/MS).

No.	Compound	Calibration curve	R^2	LOD (µg/mL)	LLOQ (µg/mL)	ULOQ (µg/mL)	Precision intra-day (% RSD)			Precision inter-day (% RSD)			Accuracy (%)
							L1	L2	L3	L1	L2	L3	
1	NMP	$y = -1.4x^2 + 159.6x$	0.987	0.09	0.2	4.7	12.0	9.5	7.7	15.4	13.8	7.9	110.5
2	Trigonelline	$y = -56.1x^2 + 21745x$	0.995	<0.01	<0.01	27.4	7.6	4.3	6.5	14.1	13.5	13.4	105.8
3	Niacin	$y = 11755x$	0.991	0.01	0.6	12.3	13.3	11.5	6.0	12.9	11.7	9.5	87.4
4	Caffeine	$y = 167464x$	0.995	<0.02	0.02	38.8	8.4	6.1	3.2	10.5	6.4	2.3	88.7
5	3-CQA	$y = -20.8x^2 + 15381x$	0.998	0.03	0.2	70.9	5.9	7.9	2.1	16.9	12.7	9.7	92.6
6	4-CQA	$y = -34.9x^2 + 16234x$	0.999	0.03	0.2	70.9	2.8	10.9	8.9	11.3	10.9	4.5	101.8
7	5-CQA	$y = -136.1x^2 + 58230x$	0.991	<0.03	<0.03	70.9	8.5	3.2	2.6	11.1	7.7	6.9	91.5

Legend: LLOQ, Lower Limit of Quantification; LOD, Limit of Detection; n-CQA: *n*-*O*-caffeoylquinic acid; NMP, *N*-methylpyridinium; RSD, Relative Standard Deviation; ULOQ, Upper Limit of Quantification.

LODs and LLOQs presented as <0.1 indicate a signal-to-noise over 10 (S/N>10), at the indicated concentration. Lower concentrations were not considered for these compounds since all the coffee samples analyze presented values much higher than the LLOQ for these major coffee compounds.

Supplementary Table 2.3. Temperature and 3-, 4- and 5-caffeoylquinic acid contents in the considered coffee capsules expressed as mg/serving.

ID Capsule	Temperature (°C)	Caffeine (mg/mL)	Trigonelline (mg/mL)	NMP (mg/mL)	Niacin (mg/mL)
A-1	70.0±1.4	2.68±0.54	0.30±0.04	0.06±0.00	0.03±0.00
A-2	71.5±0.7	0.06±0.01	0.28±0.06	0.04±0.00	0.01±0.00
A-3	73.3±1.7	0.91±0.07	0.36±0.03	0.02±0.00	0.01±0.00
A-4	71.5±0.7	2.25±0.17	0.46±0.05	0.04±0.01	0.01±0.00
A-5	70.5±2.1	1.98±0.07	0.43±0.01	0.05±0.01	0.02±0.00
A-6	70.0±1.4	2.11±0.08	0.38±0.07	0.06±0.01	0.02±0.00
A-7	70.5±2.1	1.72±0.18	0.36±0.07	0.03±0.01	0.01±0.00
A-8	70.5±0.7	0.08±0.00	0.32±0.07	0.04±0.00	0.02±0.00
A-9	68.0±2.2	3.17±0.64	0.26±0.05	0.06±0.01	0.04±0.00
A-10	70.5±2.4	1.97±0.09	0.34±0.02	0.06±0.00	0.02±0.00
A-11	75.3±0.5	1.00±0.01	0.16±0.03	0.03±0.00	0.01±0.00
A-12	70.5±2.1	3.06±0.03	0.44±0.07	0.05±0.00	0.02±0.00
A-13	68.0±1.8	4.72±0.93	0.25±0.01	0.07±0.01	0.04±0.00
A-14	71.8±1.0	0.88±0.00	0.17±0.03	0.03±0.00	0.00±0.00
A-15	70.5±1.0	1.53±0.30	0.37±0.05	0.05±0.00	0.02±0.00
A-16	69.3±1.5	3.45±0.50	0.43±0.02	0.08±0.01	0.03±0.00
A-17	71.0±0.0	2.27±0.41	0.45±0.01	0.05±0.01	0.01±0.00
A-18	70.3±2.2	1.89±0.02	0.47±0.09	0.03±0.00	0.01±0.00
A-19	70.0±1.4	1.81±0.04	0.33±0.01	0.04±0.00	0.01±0.00
A-20	72.8±2.4	0.94±0.01	0.18±0.02	0.03±0.00	0.01±0.00
A-21	74.5±0.6	0.04±0.00	0.22±0.04	0.02±0.00	0.01±0.00
A-22	71.0±1.2	1.76±0.33	0.41±0.03	0.04±0.01	0.01±0.00
A-23	69.8±1.0	0.06±0.00	0.51±0.00	0.03±0.00	0.01±0.00
B-24	67.0±2.9	3.74±0.51	0.52±0.10	0.06±0.00	0.02±0.00
B-25	70.3±1.7	3.65±0.48	0.38±0.04	0.03±0.00	0.01±0.00
B-26	71.8±2.6	1.85±0.01	0.42±0.08	0.03±0.00	0.01±0.00
B-27	73.3±2.5	1.50±0.00	0.26±0.02	0.04±0.01	0.01±0.00
B-28	71.3±1.7	0.05±0.01	0.31±0.05	0.04±0.01	0.02±0.00
B-29	72.5±1.0	2.59±0.30	0.39±0.06	0.04±0.00	0.02±0.00
B-30	70.3±1.5	0.05±0.00	0.25±0.06	0.05±0.00	0.02±0.00
B-31	75.0±4.7	0.64±0.02	0.10±0.02	0.03±0.00	0.01±0.00
B-32	74.4±6.6	0.97±0.09	0.26±0.05	0.02±0.00	0.00±0.00
B-33	71.3±7.4	0.93±0.16	0.15±0.02	0.03±0.00	0.01±0.00
B-34	78.5±1.3	1.75±0.15	0.17±0.02	0.03±0.00	0.01±0.00
B-35	68.8±1.3	3.25±0.74	0.69±0.13	0.04±0.00	0.02±0.00
B-36	66.0±3.6	3.75±0.87	0.41±0.07	0.07±0.01	0.03±0.00
B-37	68.0±2.5	3.46±0.62	0.58±0.11	0.05±0.00	0.02±0.00
B-38	70.8±2.6	1.84±0.11	0.47±0.07	0.04±0.00	0.01±0.00
C-39	70.0±0.0	2.54±0.23	0.55±0.06	0.04±0.00	0.02±0.00
C-40	65.8±3.0	0.04±0.00	0.65±0.09	0.05±0.00	0.02±0.00
C-41	65.3±3.2	2.93±0.46	0.57±0.00	0.06±0.00	0.02±0.00
C-42	68.8±1.5	3.19±0.42	0.30±0.01	0.05±0.00	0.02±0.00
C-43	67.0±1.2	4.21±0.07	0.41±0.09	0.06±0.01	0.02±0.00
C-44	71.0±0.0	1.91±0.08	0.34±0.01	0.03±0.00	0.01±0.00
C-45	69.0±0.0	2.95±0.15	0.45±0.07	0.05±0.00	0.01±0.00
C-46	67.5±2.5	3.30±0.28	0.40±0.02	0.06±0.00	0.03±0.00
C-47	70.5±0.6	2.48±0.33	0.44±0.01	0.04±0.00	0.01±0.00
C-48	68.5±3.7	4.37±0.31	0.35±0.07	0.04±0.00	0.02±0.00
D-49	59.8±3.5	4.80±0.69	1.02±0.22	0.03±0.01	0.01±0.00
D-50	57.5±1.3	4.89±0.81	1.12±0.07	0.05±0.01	0.02±0.00
D-51	62.5±0.7	2.00±0.15	1.07±0.07	0.05±0.00	0.02±0.00
D-52	61.0±0.0	0.14±0.01	0.70±0.01	0.06±0.00	0.02±0.00
D-53	75.0±1.4	0.92±0.11	0.16±0.01	0.02±0.00	0.01±0.00
D-54	65.5±0.7	3.76±0.32	0.52±0.00	0.07±0.01	0.02±0.00
D-55	64.5±0.7	4.55±0.24	0.38±0.02	0.12±0.01	0.04±0.00
D-56	60.5±2.1	4.68±0.51	0.69±0.03	0.07±0.01	0.03±0.00
D-57	64.0±4.2	3.35±0.05	1.04±0.14	0.05±0.01	0.01±0.00
D-58	64.0±2.8	3.23±0.21	0.76±0.08	0.04±0.00	0.02±0.00
E-59	67.3±2.4	4.47±0.47	0.64±0.08	0.05±0.00	0.02±0.00
E-60	69.5±1.7	0.13±0.03	0.84±0.11	0.04±0.01	0.02±0.00
E-61	64.5±8.3	3.07±0.24	1.00±0.08	0.06±0.00	0.02±0.00
E-62	68.3±1.7	4.81±0.58	0.54±0.08	0.06±0.00	0.02±0.00
E-63	68.5±2.4	4.02±0.90	0.55±0.05	0.05±0.00	0.02±0.00
E-64	67.8±3.2	3.41±0.56	0.66±0.09	0.05±0.01	0.02±0.00
E-65	66.5±5.3	3.47±0.49	0.70±0.12	0.05±0.01	0.02±0.00

Values are expressed as mean ± SD (n=4). Legend: NMP, *N*-methylpyridinium.

Supplementary Table 2.4: 3-, 4- and 5-caffeoylquinic and feruloylquinic acid and 3-, 4-coumaroylquinic acid contents in the considered coffee capsules expressed as mg/serving and mg/mL.

ID Capsule	3-CQA (mg/serving) (mg/mL)	4-CQA (mg/serving) (mg/mL)	5-CQA (mg/serving) (mg/mL)	3-FQA (mg/serving) (mg/mL)	4-FQA (mg/serving) (mg/mL)	5-FQA (mg/serving) (mg/mL)	3-CouQA (mg/serving) (mg/mL)	4-CouQA (mg/serving) (mg/mL)
A-1	**9.69±0.26**	**6.17±0.34**	**7.82±0.33**	**2.50±0.46**	**1.15±0.18**	**4.81±0.71**	**1.90±0.31**	**2.27±0.25**
	0.35±0.01	0.22±0.00	0.28±0.03	0.09±0.02	0.04±0.01	0.17±0.03	0.06±0.01	0.08±0.01
A-2	**12.51±1.03**	**6.15±0.95**	**7.33±0.81**	**2.42±0.24**	**0.99±0.03**	**4.57±0.71**	**1.44±0.21**	**1.68±0.12**
	0.42±0.05	0.21±0.04	0.25±0.03	0.08±0.01	0.03±0.00	0.16±0.03	0.05±0.01	0.06±0.01
A-3	**23.40±4.30**	**14.56±2.59**	**25.15±1.93**	**4.50±0.45**	**1.04±0.03**	**9.28±0.61**	**2.78±0.09**	**6.30±0.56**
	0.28±0.04	0.17±0.01	0.30±0.00	0.05±0.00	0.01±0.00	0.11±0.00	0.03±0.00	0.08±0.01
A-4	**17.41±1.63**	**10.67±0.32**	**11.60±1.18**	**3.25±0.47**	**1.30±0.26**	**7.91±0.17**	**1.95±0.17**	**3.05±0.31**
	0.56±0.14	0.34±0.04	0.41±0.05	0.10±0.00	0.05±0.01	0.25±0.05	0.07±0.01	0.10±0.01
A-5	**15.30±0.64**	**9.16±0.80**	**11.80±0.83**	**2.46±0.26**	**1.26±0.16**	**6.93±0.07**	**2.23±0.37**	**2.30±0.33**
	0.50±0.01	0.30±0.02	0.39±0.02	0.08±0.01	0.04±0.01	0.23±0.00	0.07±0.01	0.08±0.01
A-6	**13.05±1.33**	**7.00±0.83**	**9.57±0.28**	**2.63±0.54**	**1.10±0.07**	**4.61±0.38**	**2.15±0.02**	**2.50±0.28**
	0.44±0.04	0.22±0.01	0.31±0.01	0.08±0.01	0.04±0.00	0.15±0.01	0.07±0.00	0.08±0.01
A-7	**13.58±2.63**	**7.78±0.67**	**11.36±0.41**	**2.36±0.05**	**1.31±0.22**	**5.16±0.34**	**2.11±0.51**	**3.11±0.35**
	0.40±0.08	0.23±0.02	0.34±0.02	0.07±0.00	0.04±0.01	0.15±0.01	0.06±0.01	0.09±0.01
A-8	**10.90±0.02**	**5.44±0.01**	**7.71±0.37**	**2.21±0.05**	**1.30±0.05**	**4.47±0.06**	**1.71±0.16**	**3.17±0.16**
	0.35±0.01	0.17±0.00	0.25±0.02	0.07±0.00	0.04±0.00	0.14±0.01	0.05±0.00	0.10±0.01
A-9	**8.59±0.92**	**5.20±0.68**	**6.18±0.20**	**2.30±0.29**	**1.35±0.01**	**4.10±0.47**	**1.53±0.03**	**1.97±0.03**
	0.31±0.03	0.19±0.02	0.22±0.01	0.08±0.01	0.05±0.00	0.15±0.02	0.05±0.00	0.07±0.00
A-10	**10.65±2.33**	**6.63±1.41**	**8.80±1.03**	**2.04±0.07**	**1.24±0.17**	**5.11±0.21**	**1.81±0.05**	**2.19±0.38**
	0.36±0.07	0.21±0.05	0.29±0.04	0.07±0.00	0.04±0.01	0.17±0.01	0.06±0.00	0.07±0.01
A-11	**12.26±0.54**	**10.75±0.27**	**12.59±0.26**	**3.01±0.26**	**2.14±0.16**	**6.89±0.40**	**3.39±0.36**	**4.58±0.26**
	0.14±0.01	0.11±0.01	0.14±0.01	0.03±0.00	0.02±0.00	0.07±0.00	0.04±0.00	0.05±0.00
A-12	**18.23±2.33**	**10.35±0.22**	**11.43±0.05**	**4.51±0.21**	**1.23±0.03**	**7.77±0.37**	**2.79±0.19**	**3.67±0.10**
	0.61±0.11	0.35±0.01	0.38±0.02	0.15±0.00	0.04±0.00	0.26±0.00	0.09±0.01	0.12±0.00
A-13	**9.57±2.01**	**6.21±0.22**	**6.61±1.12**	**6.23±1.01**	**2.86±0.41**	**9.86±0.57**	**2.07±0.35**	**1.76±0.15**
	0.34±0.03	0.21±0.01	0.23±0.02	0.22±0.03	0.11±0.01	0.34±0.01	0.07±0.01	0.06±0.01
A-14	**16.25±3.05**	**9.14±1.49**	**13.95±0.00**	**3.35±0.38**	**1.82±0.22**	**7.36±0.11**	**2.09±0.04**	**4.26±0.85**
	0.17±0.03	0.10±0.01	0.15±0.00	0.04±0.00	0.02±0.00	0.08±0.00	0.02±0.00	0.04±0.01
A-15	**13.67±2.84**	**9.08±1.65**	**10.83±1.98**	**2.56±0.23**	**1.94±0.12**	**5.88±0.63**	**2.53±0.03**	**3.41±0.80**
	0.39±0.08	0.26±0.05	0.31±0.06	0.07±0.01	0.06±0.00	0.17±0.02	0.07±0.00	0.12±0.00
A-16	**16.18±0.79**	**9.14±0.79**	**12.35±0.20**	**2.87±0.53**	**1.23±0.10**	**7.26±0.41**	**1.73±0.22**	**2.69±0.06**
	0.58±0.01	0.33±0.02	0.44±0.00	0.10±0.01	0.04±0.01	0.26±0.02	0.06±0.01	0.10±0.00
A-17	**16.38±0.95**	**9.95±1.16**	**12.45±1.98**	**3.23±0.29**	**1.18±0.04**	**8.01±0.13**	**1.74±0.07**	**2.09±0.36**
	0.59±0.06	0.36±0.06	0.45±0.09	0.12±0.02	0.04±0.00	0.29±0.02	0.06±0.00	0.08±0.01
A-18	**19.38±4.09**	**11.53±1.50**	**15.99±0.12**	**2.65±0.33**	**0.98±0.07**	**8.04±0.04**	**2.63±0.09**	**3.51±0.49**
	0.45±0.03	0.33±0.06	0.46±0.02	0.07±0.01	0.03±0.00	0.23±0.01	0.08±0.01	0.10±0.01
A-19	**13.84±0.32**	**8.34±0.50**	**9.11±0.14**	**2.33±0.13**	**1.10±0.03**	**6.52±0.41**	**2.11±0.04**	**2.63±0.05**
	0.40±0.02	0.24±0.01	0.26±0.02	0.07±0.00	0.03±0.00	0.19±0.00	0.06±0.00	0.08±0.00
A-20	**17.68±3.18**	**11.09±1.29**	**15.68±0.97**	**2.73±0.40**	**1.13±0.11**	**7.53±0.61**	**2.52±0.10**	**5.34±1.21**
	0.19±0.03	0.12±0.01	0.16±0.01	0.03±0.00	0.01±0.00	0.08±0.01	0.03±0.00	0.05±0.01
A-21	**23.61±3.13**	**10.01±1.03**	**20.65±0.86**	**2.92±0.50**	**1.42±0.15**	**8.03±0.22**	**2.20±0.18**	**5.19±0.15**
	0.26±0.03	0.11±0.01	0.23±0.01	0.03±0.01	0.02±0.00	0.09±0.00	0.02±0.00	0.06±0.00
A-22	**12.93±2.00**	**8.80±0.32**	**11.15±0.78**	**2.40±0.16**	**0.97±0.13**	**6.35±0.75**	**2.09±0.41**	**2.79±0.46**
	0.40±0.07	0.27±0.02	0.34±0.01	0.07±0.00	0.03±0.00	0.19±0.02	0.06±0.01	0.08±0.01
A-23	**14.79±2.21**	**7.94±0.37**	**11.01±0.88**	**2.19±0.17**	**1.00±0.07**	**5.34±0.26**	**1.79±0.04**	**2.77±0.51**
	0.47±0.07	0.25±0.01	0.35±0.02	0.07±0.00	0.03±0.00	0.17±0.01	0.06±0.00	0.09±0.02
B-24	**22.24±4.21**	**14.54±1.21**	**15.10±1.81**	**6.63±0.82**	**1.98±0.37**	**11.75±0.88**	**2.85±0.51**	**3.36±0.67**
	0.67±0.14	0.43±0.05	0.45±0.06	0.19±0.01	0.06±0.01	0.35±0.03	0.09±0.02	0.10±0.02
B-25	**25.66±4.13**	**13.71±0.05**	**17.62±1.48**	**8.24±0.04**	**2.40±0.53**	**16.72±1.26**	**2.42±0.02**	**3.76±0.09**
	0.66±0.13	0.35±0.02	0.45±0.02	0.21±0.01	0.06±0.01	0.43±0.01	0.06±0.00	0.10±0.01
B-26	**24.37±0.59**	**11.92±1.49**	**19.05±0.73**	**4.94±0.83**	**1.73±0.20**	**9.76±1.39**	**2.67±0.28**	**3.90±0.19**
	0.50±0.04	0.25±0.06	0.39±0.02	0.10±0.01	0.03±0.00	0.24±0.00	0.05±0.00	0.08±0.00
B-27	**14.30±3.13**	**7.40±1.48**	**9.17±0.79**	**4.23±0.27**	**1.41±0.03**	**8.84±1.04**	**1.91±0.23**	**2.37±0.35**
	0.28±0.06	0.15±0.03	0.19±0.03	0.09±0.01	0.03±0.00	0.18±0.03	0.04±0.00	0.05±0.00
B-28	**14.69±3.12**	**9.38±1.60**	**11.84±1.81**	**3.97±0.72**	**1.67±0.05**	**7.23±0.11**	**2.49±0.34**	**3.06±0.20**
	0.32±0.05	0.20±0.01	0.25±0.01	0.08±0.01	0.04±0.00	0.16±0.01	0.05±0.00	0.07±0.00
B-29	**22.78±2.41**	**13.34±0.67**	**16.82±1.02**	**4.94±1.06**	**2.15±0.31**	**11.07±1.82**	**2.74±0.60**	**3.63±0.06**
	0.45±0.06	0.26±0.02	0.33±0.03	0.09±0.02	0.04±0.01	0.22±0.04	0.05±0.01	0.07±0.00
B-30	**17.45±1.67**	**7.74±1.51**	**9.52±0.48**	**4.33±0.69**	**2.54±0.29**	**8.96±0.23**	**2.10±0.18**	**4.23±0.62**
	0.36±0.04	0.15±0.03	0.18±0.03	0.08±0.02	0.05±0.00	0.17±0.02	0.04±0.01	0.08±0.00
B-31	**21.01±2.90**	**9.93±0.98**	**16.04±1.19**	**3.91±0.75**	**2.84±0.34**	**9.61±0.98**	**5.09±0.32**	**7.45±0.83**
	0.10±0.02	0.05±0.01	0.08±0.00	0.02±0.00	0.01±0.00	0.05±0.00	0.02±0.00	0.04±0.00
B-32	**42.34±1.73**	**29.86±3.95**	**44.47±0.37**	**8.20±0.03**	**3.47±0.20**	**22.72±1.21**	**6.99±0.25**	**13.35±0.54**
	0.23±0.02	0.16±0.03	0.24±0.00	0.05±0.01	0.02±0.00	0.12±0.01	0.04±0.00	0.07±0.00

B-33	**17.67±3.69**	**11.97±1.48**	**17.70±0.55**	**7.88±1.45**	**2.63±0.03**	**10.74±2.19**	**3.51±0.67**	**6.17±1.24**
	0.14±0.03	0.10±0.02	0.14±0.02	0.07±0.01	0.02±0.00	0.08±0.01	0.02±0.00	0.05±0.00
B-34	**27.75±1.33**	**15.14±0.29**	**15.88±2.62**	**11.78±2.04**	**4.07±0.23**	**20.67±1.07**	**3.63±0.48**	**4.63±0.80**
	0.24±0.04	0.13±0.01	0.15±0.02	0.11±0.02	0.04±0.00	0.20±0.01	0.03±0.01	0.04±0.01
B-35	**23.95±2.90**	**12.68±2.03**	**18.02±1.52**	**5.22±0.35**	**1.78±0.18**	**11.64±0.12**	**2.94±0.24**	**3.35±0.28**
	0.76±0.06	0.40±0.05	0.57±0.07	0.17±0.00	0.06±0.01	0.37±0.01	0.09±0.00	0.11±0.00
B-36	**14.94±1.89**	**8.77±1.27**	**10.16±0.88**	**4.29±0.36**	**2.12±0.04**	**8.17±1.32**	**2.79±0.18**	**3.02±0.29**
	0.48±0.03	0.29±0.02	0.33±0.01	0.14±0.00	0.07±0.00	0.27±0.03	0.09±0.01	0.10±0.00
B-37	**23.55±1.21**	**14.27±2.55**	**15.02±2.36**	**5.73±0.97**	**1.93±0.30**	**11.93±0.69**	**2.75±0.52**	**3.09±0.67**
	0.74±0.07	0.43±0.08	0.49±0.06	0.19±0.03	0.06±0.00	0.39±0.01	0.09±0.01	0.10±0.02
B-38	**27.05±6.16**	**14.96±2.09**	**20.86±0.16**	**4.35±0.86**	**1.24±0.16**	**11.06±0.45**	**3.38±0.19**	**4.32±0.25**
	0.57±0.12	0.31±0.03	0.44±0.01	0.09±0.02	0.03±0.00	0.23±0.00	0.07±0.00	0.09±0.00
C-39	**37.61±2.19**	**21.14±0.06**	**27.86±0.98**	**8.30±1.49**	**2.01±0.12**	**14.08±1.68**	**5.28±0.92**	**3.96±0.01**
	0.78±0.03	0.44±0.01	0.57±0.01	0.17±0.03	0.04±0.00	0.29±0.03	0.11±0.02	0.08±0.00
C-40	**33.98±1.04**	**16.72±0.14**	**23.00±0.69**	**5.99±0.16**	**1.88±0.20**	**13.50±0.28**	**4.62±0.22**	**4.20±0.12**
	0.72±0.07	0.35±0.04	0.49±0.08	0.13±0.02	0.04±0.01	0.28±0.03	0.10±0.02	0.09±0.01
C-41	**31.32±6.75**	**18.47±4.15**	**24.48±3.03**	**6.06±1.01**	**1.95±0.16**	**13.67±3.04**	**3.92±0.66**	**4.54±0.28**
	0.67±0.10	0.40±0.06	0.53±0.03	0.13±0.01	0.04±0.01	0.29±0.05	0.08±0.01	0.10±0.01
C-42	**21.55±3.80**	**13.28±1.90**	**15.57±1.96**	**7.85±0.18**	**3.44±0.17**	**16.05±0.81**	**3.14±0.34**	**4.42±0.36**
	0.45±0.05	0.28±0.02	0.32±0.02	0.16±0.01	0.07±0.01	0.33±0.00	0.07±0.00	0.09±0.00
C-43	**27.66±0.58**	**18.49±0.52**	**19.45±0.34**	**9.92±0.42**	**3.00±0.07**	**21.13±3.19**	**3.71±0.02**	**4.81±0.38**
	0.58±0.00	0.39±0.00	0.41±0.00	0.21±0.00	0.06±0.00	0.45±0.05	0.08±0.00	0.10±0.01
C-44	**40.60±1.33**	**23.61±3.33**	**34.75±4.68**	**11.05±1.17**	**2.75±0.00**	**18.83±1.96**	**5.54±0.19**	**5.49±0.52**
	0.41±0.04	0.24±0.02	0.35±0.02	0.11±0.00	0.03±0.00	0.19±0.03	0.06±0.01	0.06±0.01
C-45	**27.31±3.44**	**18.29±1.51**	**22.60±1.72**	**6.11±0.70**	**1.39±0.03**	**14.49±1.27**	**4.43±0.71**	**4.52±0.55**
	0.53±0.05	0.36±0.02	0.44±0.02	0.12±0.02	0.03±0.00	0.28±0.02	0.09±0.01	0.09±0.01
C-46	**21.69±0.30**	**12.61±0.29**	**17.64±1.22**	**7.48±0.15**	**2.26±0.15**	**14.82±1.61**	**3.28±0.39**	**3.52±0.67**
	0.47±0.03	0.27±0.01	0.38±0.00	0.16±0.01	0.05±0.00	0.31±0.07	0.07±0.00	0.08±0.01
C-47	**23.00±0.10**	**14.48±1.32**	**20.15±0.97**	**5.98±1.00**	**1.89±0.04**	**14.33±1.06**	**3.61±0.58**	**4.30±0.13**
	0.47±0.03	0.29±0.01	0.41±0.00	0.12±0.01	0.04±0.00	0.29±0.00	0.06±0.01	0.09±0.01
C-48	**25.82±3.64**	**19.60±3.87**	**24.00±5.11**	**15.86±1.45**	**4.59±0.24**	**29.39±5.68**	**2.70±0.45**	**4.40±0.56**
	0.56±0.08	0.41±0.06	0.50±0.08	0.33±0.01	0.10±0.00	0.61±0.09	0.06±0.01	0.09±0.01
D-49	**28.95±6.11**	**15.44±1.65**	**23.45±3.09**	**4.87±1.14**	**1.18±0.01**	**12.22±2.65**	**3.11±0.09**	**3.87±0.90**
	1.18±0.08	0.54±0.05	0.96±0.14	0.20±0.01	0.05±0.01	0.49±0.03	0.14±0.02	0.16±0.01
D-50	**24.69±3.58**	**14.64±0.98**	**18.69±1.92**	**4.05±0.08**	**1.52±0.07**	**9.36±0.46**	**3.42±0.17**	**3.52±0.40**
	1.13±0.05	0.66±0.15	0.86±0.08	0.19±0.03	0.06±0.01	0.43±0.06	0.16±0.02	0.16±0.01
D-51	**23.10±1.89**	**13.34±0.49**	**16.74±1.25**	**2.22±0.22**	**0.91±0.03**	**6.66±0.04**	**3.29±0.56**	**3.45±0.19**
	0.86±0.16	0.50±0.03	0.63±0.11	0.08±0.00	0.03±0.00	0.25±0.03	0.14±0.01	0.13±0.01
D-52	**17.03±2.53**	**10.61±0.07**	**13.39±1.59**	**3.88±0.47**	**1.31±0.25**	**8.46±0.46**	**2.90±0.32**	**2.88±0.07**
	0.60±0.07	0.37±0.01	0.47±0.04	0.14±0.02	0.05±0.00	0.30±0.01	0.10±0.01	0.10±0.00
D-53	**24.42±2.55**	**11.65±0.30**	**18.82±1.47**	**3.86±0.04**	**1.19±0.08**	**10.68±1.31**	**3.97±0.46**	**5.98±0.24**
	0.18±0.01	0.09±0.00	0.14±0.01	0.03±0.00	0.01±0.00	0.08±0.01	0.03±0.00	0.05±0.00
D-54	**20.42±3.21**	**13.72±0.51**	**16.56±0.23**	**3.06±0.30**	**1.66±0.08**	**8.66±1.45**	**3.21±0.34**	**3.63±0.05**
	0.70±0.11	0.47±0.02	0.57±0.01	0.11±0.01	0.06±0.00	0.30±0.05	0.11±0.01	0.13±0.00
D-55	**13.14±0.61**	**8.13±0.13**	**10.08±0.01**	**3.21±0.32**	**1.73±0.04**	**5.98±0.27**	**2.53±0.42**	**2.91±0.37**
	0.45±0.03	0.28±0.00	0.34±0.01	0.11±0.01	0.06±0.00	0.20±0.00	0.09±0.02	0.10±0.01
D-56	**19.51±2.38**	**12.72±2.12**	**14.19±1.46**	**4.39±0.47**	**1.51±0.24**	**8.81±0.22**	**3.51±0.55**	**2.76±0.48**
	0.72±0.01	0.47±0.03	0.53±0.00	0.16±0.00	0.06±0.00	0.33±0.03	0.13±0.01	0.10±0.01
D-57	**33.21±2.30**	**16.44±2.39**	**26.01±1.56**	**3.53±0.15**	**1.42±0.16**	**10.57±0.30**	**3.68±0.51**	**5.05±0.03**
	1.02±0.00	0.50±0.04	0.80±0.00	0.11±0.00	0.04±0.01	0.33±0.03	0.11±0.01	0.16±0.01
D-58	**26.14±0.15**	**13.76±2.17**	**19.99±2.85**	**4.63±0.51**	**1.64±0.06**	**9.63±0.34**	**3.44±0.49**	**3.68±0.05**
	0.82±0.04	0.43±0.09	0.63±0.12	0.15±0.02	0.05±0.00	0.30±0.02	0.11±0.02	0.12±0.00
E-59	**25.05±3.79**	**15.08±0.29**	**16.27±0.34**	**11.79±0.70**	**3.74±0.42**	**19.64±2.69**	**1.92±0.21**	**2.98±0.36**
	0.80±0.13	0.45±0.05	0.49±0.04	0.35±0.02	0.11±0.02	0.58±0.02	0.06±0.01	0.09±0.00
E-60	**31.95±7.90**	**13.67±2.60**	**17.36±4.40**	**9.36±2.17**	**4.13±0.18**	**17.45±4.15**	**3.16±0.69**	**3.52±0.69**
	0.82±0.10	0.39±0.07	0.46±0.08	0.26±0.05	0.11±0.00	0.45±0.07	0.08±0.01	0.10±0.02
E-61	**30.06±6.59**	**13.96±2.21**	**19.23±2.51**	**3.94±0.43**	**1.52±0.06**	**10.37±2.01**	**3.72±0.64**	**3.43±0.31**
	0.72±0.01	0.40±0.07	0.55±0.08	0.11±0.02	0.04±0.00	0.30±0.06	0.11±0.01	0.10±0.01
E-62	**24.61±0.37**	**15.86±1.42**	**17.53±2.80**	**10.76±0.57**	**3.78±0.65**	**17.99±1.86**	**2.28±0.26**	**3.32±0.27**
	0.70±0.07	0.47±0.04	0.51±0.01	0.30±0.02	0.11±0.00	0.51±0.01	0.06±0.00	0.10±0.01
E-63	**28.51±6.21**	**16.22±2.55**	**16.87±0.77**	**8.87±0.66**	**2.34±0.17**	**17.61±0.61**	**1.94±0.12**	**3.17±0.30**
	0.79±0.09	0.45±0.02	0.47±0.03	0.25±0.01	0.07±0.00	0.50±0.03	0.06±0.00	0.09±0.00
E-64	**29.40±4.59**	**18.01±2.97**	**20.53±2.12**	**5.92±1.40**	**1.66±0.17**	**14.96±1.76**	**3.94±0.07**	**4.06±0.56**
	0.79±0.09	0.47±0.06	0.55±0.03	0.16±0.02	0.04±0.00	0.40±0.02	0.11±0.01	0.11±0.01
E-65	**32.96±6.01**	**16.13±3.38**	**19.41±0.68**	**6.52±0.93**	**1.75±0.40**	**15.08±1.32**	**3.89±0.52**	**3.73±0.16**
	0.92±0.16	0.45±0.09	0.54±0.01	0.18±0.02	0.05±0.01	0.42±0.03	0.11±0.02	0.10±0.00

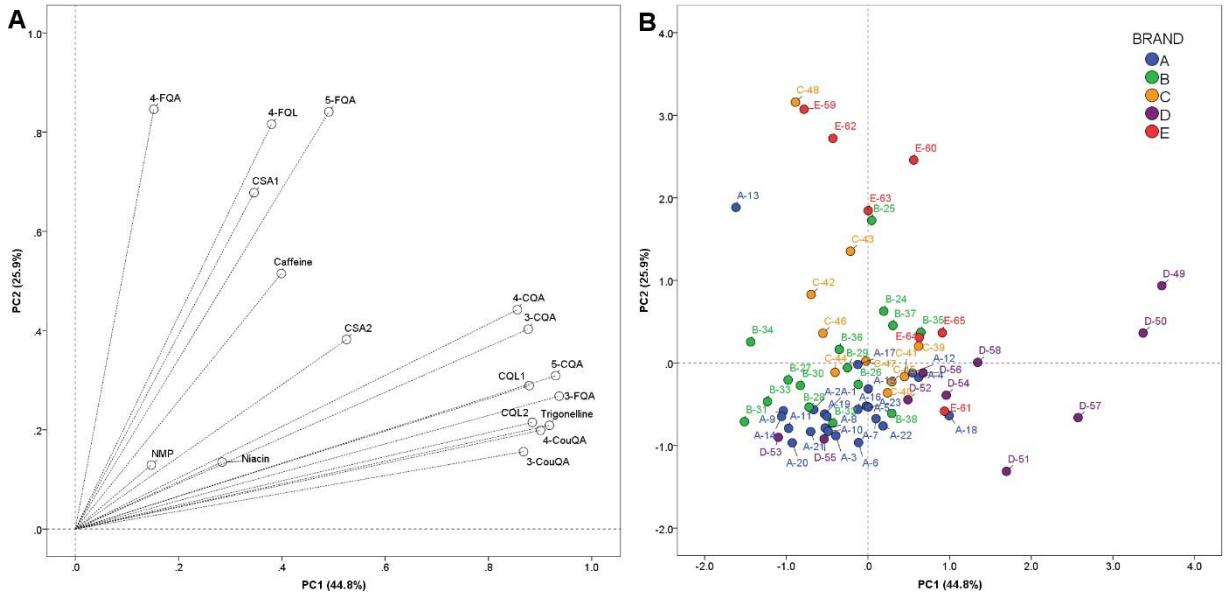
Values are expressed as mean ± SD (n=4). Legend: *n*-CQA: *n*-*O*-caffeoylquinic acid; *n*-CouQA: *n*-*O*-coumaroylquinic acid; *n*-FQA: *n*-*O*-feruloylquinic acid.

Supplementary Table 2.5. Main caffeoylshikimic and caffeoylquinic acids and feruloyl quinic lactone contents in the considered coffee capsules expressed as mg/serving and mg/mL.

ID Capsule	CSA1 (mg/serving) (mg/mL)	CSA2 (mg/serving) (mg/mL)	CQL1 (mg/serving) (mg/mL)	CQL2 (mg/serving) (mg/mL)	4-FQL (mg/serving) (mg/mL)
A-1	**3.31±0.77**	**3.92±0.37**	**18.72±0.87**	**14.44±2.26**	**2.33±0.23**
	0.14±0.01	0.14±0.01	0.67±0.07	0.55±0.06	0.08±0.01
A-2	**2.51±0.22**	**2.52±0.41**	**15.91±0.70**	**11.22±0.86**	**1.52±0.27**
	0.09±0.01	0.09±0.02	0.54±0.04	0.38±0.04	0.06±0.00
A-3	**4.46±0.40**	**3.51±0.44**	**41.88±6.74**	**37.69±5.54**	**2.96±0.29**
	0.05±0.01	0.04±0.00	0.50±0.05	0.39±0.05	0.04±0.01
A-4	**3.42±0.09**	**3.17±0.07**	**25.01±0.72**	**17.88±0.71**	**2.33±0.03**
	0.11±0.01	0.10±0.02	0.81±0.15	0.57±0.07	0.08±0.01
A-5	**2.40±0.27**	**3.09±0.16**	**20.73±1.70**	**15.00±0.85**	**1.80±0.07**
	0.08±0.01	0.10±0.00	0.68±0.04	0.49±0.02	0.06±0.00
A-6	**3.88±0.29**	**3.30±0.52**	**19.60±0.28**	**18.37±4.92**	**1.82±0.18**
	0.12±0.02	0.11±0.02	0.63±0.04	0.51±0.07	0.06±0.01
A-7	**2.92±0.10**	**2.27±0.33**	**23.23±0.73**	**19.22±3.85**	**1.99±0.24**
	0.09±0.00	0.07±0.01	0.69±0.03	0.57±0.11	0.06±0.01
A-8	**2.40±0.43**	**3.20±0.05**	**17.23±2.98**	**12.71±1.99**	**1.74±0.23**
	0.08±0.01	0.10±0.00	0.55±0.08	0.40±0.05	0.06±0.01
A-9	**3.04±0.66**	**4.75±0.39**	**17.08±0.26**	**12.24±1.50**	**1.97±0.37**
	0.09±0.01	0.17±0.01	0.62±0.02	0.44±0.06	0.07±0.01
A-10	**3.04±0.53**	**2.91±0.32**	**16.63±2.17**	**15.25±2.64**	**1.59±0.30**
	0.10±0.02	0.10±0.01	0.55±0.05	0.51±0.08	0.05±0.01
A-11	**5.75±0.14**	**5.21±0.58**	**37.34±2.35**	**31.24±2.59**	**3.92±0.84**
	0.06±0.00	0.06±0.00	0.41±0.01	0.34±0.04	0.04±0.01
A-12	**4.04±0.34**	**5.46±0.07**	**25.40±0.13**	**19.80±0.97**	**3.04±0.04**
	0.13±0.00	0.18±0.01	0.85±0.04	0.66±0.00	0.10±0.01
A-13	**3.49±0.36**	**4.95±0.43**	**17.95±2.65**	**12.51±1.46**	**4.49±0.51**
	0.12±0.00	0.17±0.00	0.62±0.04	0.43±0.02	0.16±0.03
A-14	**4.32±0.98**	**4.35±0.88**	**34.74±4.20**	**30.50±6.19**	**4.19±0.78**
	0.05±0.00	0.04±0.01	0.37±0.05	0.32±0.08	0.05±0.00
A-15	**5.25±0.57**	**3.32±0.42**	**24.09±1.80**	**21.56±2.99**	**4.32±0.15**
	0.15±0.02	0.10±0.01	0.69±0.06	0.62±0.09	0.13±0.00
A-16	**3.39±0.71**	**4.25±0.40**	**23.26±0.02**	**17.68±0.87**	**2.75±0.32**
	0.12±0.03	0.15±0.01	0.83±0.02	0.63±0.02	0.10±0.01
A-17	**2.50±0.49**	**3.02±0.50**	**19.96±1.68**	**13.79±2.60**	**2.42±0.30**
	0.09±0.02	0.11±0.02	0.72±0.10	0.50±0.12	0.09±0.02
A-18	**3.23±0.48**	**2.92±0.02**	**30.31±0.29**	**29.43±2.75**	**3.29±0.66**
	0.09±0.01	0.08±0.01	0.87±0.04	0.83±0.04	0.09±0.01
A-19	**2.51±0.09**	**2.69±0.06**	**20.65±3.48**	**13.65±0.38**	**1.95±0.02**
	0.07±0.01	0.08±0.01	0.65±0.08	0.39±0.02	0.06±0.00
A-20	**4.46±0.18**	**4.18±0.82**	**37.77±0.62**	**32.87±5.73**	**4.02±0.86**
	0.05±0.00	0.04±0.01	0.40±0.01	0.35±0.06	0.04±0.01
A-21	**3.92±0.71**	**3.55±0.12**	**38.30±2.28**	**36.50±1.56**	**3.54±0.30**
	0.04±0.01	0.04±0.00	0.43±0.02	0.41±0.02	0.04±0.00
A-22	**2.29±0.38**	**3.08±0.34**	**21.93±3.18**	**26.26±1.15**	**2.60±0.48**
	0.07±0.01	0.09±0.01	0.67±0.08	0.78±0.02	0.08±0.01
A-23	**2.58±0.46**	**2.00±0.19**	**18.27±0.08**	**13.68±2.72**	**2.59±0.42**
	0.08±0.01	0.06±0.01	0.58±0.01	0.44±0.09	0.08±0.01
B-24	**4.26±0.39**	**5.61±0.08**	**28.58±1.47**	**20.60±0.79**	**3.68±0.30**
	0.13±0.01	0.17±0.01	0.85±0.06	0.62±0.01	0.11±0.01
B-25	**3.67±0.06**	**4.46±0.48**	**29.04±0.69**	**21.56±0.82**	**4.76±0.31**
	0.09±0.00	0.12±0.00	0.75±0.01	0.56±0.00	0.12±0.00
B-26	**3.54±0.66**	**3.73±0.34**	**29.46±0.59**	**21.99±1.73**	**2.61±0.49**
	0.07±0.02	0.08±0.00	0.61±0.04	0.46±0.07	0.06±0.01
B-27	**2.90±0.20**	**3.74±0.13**	**22.06±1.39**	**16.28±0.38**	**2.84±0.23**
	0.06±0.01	0.07±0.01	0.44±0.06	0.33±0.02	0.06±0.00
B-28	**3.88±0.43**	**4.59±0.74**	**25.92±4.48**	**17.52±2.94**	**2.68±0.18**
	0.08±0.00	0.10±0.01	0.55±0.04	0.37±0.03	0.06±0.01
B-29	**4.00±0.37**	**5.28±0.20**	**33.63±1.29**	**22.32±3.22**	**3.60±0.60**
	0.08±0.00	0.10±0.01	0.66±0.00	0.44±0.05	0.07±0.01
B-30	**4.82±1.03**	**5.58±0.14**	**28.57±2.27**	**17.41±2.95**	**3.66±0.75**
	0.09±0.01	0.10±0.02	0.53±0.03	0.33±0.03	0.06±0.00
B-31	**4.93±0.44**	**7.00±0.38**	**43.54±3.96**	**43.49±10.05**	**12.36±0.93**
	0.02±0.00	0.03±0.00	0.21±0.00	0.21±0.03	0.06±0.01
B-32	**4.44±0.30**	**4.76±0.05**	**77.50±12.32**	**75.46±11.01**	**5.20±0.41**
	0.03±0.01	0.03±0.00	0.42±0.05	0.41±0.05	0.03±0.00
B-33	**6.31±0.67**	**4.62±0.87**	**40.38±7.66**	**23.79±4.69**	**4.98±0.88**

	0.05±0.00	0.03±0.00	0.32±0.02	0.19±0.02	0.04±0.00
B-34	**3.68±0.48**	**4.75±0.24**	**36.28±1.25**	**25.73±1.49**	**6.10±0.11**
	0.03±0.00	0.04±0.00	0.34±0.02	0.24±0.02	0.06±0.00
B-35	**3.40±0.21**	**3.49±0.42**	**26.95±2.55**	**19.56±0.41**	**2.91±0.21**
	0.11±0.00	0.11±0.01	0.85±0.04	0.62±0.01	0.09±0.01
B-36	**3.62±0.05**	**5.21±0.18**	**22.83±3.07**	**16.86±1.52**	**3.35±0.25**
	0.12±0.00	0.17±0.02	0.74±0.05	0.55±0.02	0.11±0.01
B-37	**3.22±0.24**	**4.07±0.82**	**29.17±5.16**	**21.65±0.31**	**3.05±0.20**
	0.11±0.00	0.13±0.02	0.88±0.16	0.65±0.01	0.10±0.00
B-38	**4.39±0.15**	**4.87±0.72**	**36.23±0.01**	**25.89±0.83**	**3.10±0.10**
	0.09±0.00	0.10±0.01	0.76±0.02	0.54±0.00	0.07±0.00
C-39	**5.00±0.22**	**5.58±0.76**	**34.51±2.12**	**30.95±4.99**	**5.04±0.32**
	0.10±0.00	0.11±0.01	0.71±0.05	0.64±0.09	0.10±0.01
C-40	**3.46±0.65**	**4.24±0.83**	**29.63±4.59**	**22.83±5.01**	**3.35±0.29**
	0.06±0.01	0.07±0.00	0.63±0.11	0.51±0.11	0.07±0.01
C-41	**4.92±0.39**	**5.91±0.72**	**36.65±1.69**	**27.76±0.75**	**3.99±0.10**
	0.11±0.02	0.13±0.01	0.79±0.02	0.60±0.03	0.09±0.01
C-42	**5.67±0.48**	**7.77±0.41**	**32.80±0.64**	**26.38±2.09**	**5.63±0.62**
	0.12±0.02	0.16±0.02	0.68±0.05	0.55±0.08	0.12±0.02
C-43	**5.31±1.11**	**6.88±0.49**	**33.86±3.28**	**27.48±3.05**	**6.27±1.21**
	0.11±0.03	0.15±0.01	0.72±0.09	0.58±0.08	0.13±0.02
C-44	**6.92±0.86**	**6.63±0.01**	**54.19±2.29**	**38.47±3.35**	**6.58±0.39**
	0.07±0.00	0.07±0.00	0.54±0.02	0.38±0.01	0.07±0.00
C-45	**5.14±0.99**	**6.40±0.62**	**39.98±7.45**	**27.54±3.47**	**4.42±0.07**
	0.10±0.02	0.13±0.01	0.78±0.12	0.54±0.05	0.09±0.00
C-46	**5.22±0.50**	**5.89±0.89**	**37.50±1.96**	**26.04±3.56**	**6.15±0.62**
	0.11±0.00	0.13±0.03	0.79±0.05	0.54±0.02	0.13±0.00
C-47	**4.35±0.14**	**3.88±0.12**	**32.09±0.38**	**26.73±1.70**	**4.25±0.32**
	0.09±0.01	0.08±0.00	0.65±0.03	0.54±0.00	0.09±0.00
C-48	**5.03±0.80**	**7.09±1.28**	**36.92±6.49**	**26.09±1.10**	**9.52±0.85**
	0.11±0.01	0.15±0.02	0.77±0.10	0.55±0.00	0.20±0.01
D-49	**3.91±0.93**	**3.12±0.70**	**29.98±4.42**	**25.38±5.17**	**3.66±0.75**
	0.16±0.01	0.13±0.01	1.28±0.08	1.04±0.09	0.15±0.02
D-50	**4.38±0.83**	**4.02±0.42**	**27.66±3.46**	**20.91±3.49**	**3.11±0.60**
	0.20±0.00	0.18±0.02	1.27±0.09	0.95±0.03	0.14±0.00
D-51	**4.15±0.56**	**3.77±0.20**	**30.10±1.93**	**20.30±0.23**	**2.25±0.24**
	0.18±0.00	0.14±0.01	1.12±0.05	0.76±0.09	0.08±0.02
D-52	**3.66±0.37**	**4.30±0.18**	**22.28±1.92**	**17.04±0.79**	**2.63±0.11**
	0.13±0.01	0.15±0.00	0.78±0.05	0.60±0.01	0.09±0.00
D-53	**4.64±0.40**	**4.85±0.39**	**38.47±2.56**	**31.88±3.71**	**3.46±0.16**
	0.03±0.00	0.04±0.00	0.29±0.01	0.24±0.03	0.03±0.00
D-54	**4.29±0.19**	**4.32±0.34**	**29.12±2.28**	**19.66±0.34**	**3.12±0.27**
	0.15±0.01	0.15±0.01	1.00±0.08	0.68±0.01	0.11±0.01
D-55	**4.15±0.47**	**4.92±0.22**	**24.18±2.81**	**15.94±0.86**	**2.84±0.00**
	0.14±0.01	0.17±0.00	0.82±0.08	0.54±0.02	0.10±0.00
D-56	**3.94±0.67**	**4.71±0.55**	**26.04±1.70**	**19.70±2.09**	**2.85±0.19**
	0.15±0.01	0.17±0.00	0.97±0.04	0.73±0.00	0.11±0.00
D-57	**4.12±0.32**	**3.50±0.55**	**37.34±2.34**	**27.17±0.69**	**3.26±0.34**
	0.13±0.02	0.11±0.01	1.15±0.00	0.84±0.08	0.10±0.00
D-58	**3.41±0.12**	**2.49±0.07**	**28.67±0.41**	**21.16±0.61**	**2.90±0.03**
	0.11±0.01	0.08±0.01	0.90±0.03	0.66±0.05	0.09±0.00
E-59	**4.25±0.01**	**5.74±0.64**	**26.72±1.82**	**20.27±0.03**	**6.28±1.24**
	0.13±0.01	0.17±0.04	0.80±0.13	0.61±0.06	0.19±0.02
E-60	**4.43±0.35**	**5.42±0.78**	**28.30±2.94**	**20.33±2.66**	**4.55±0.18**
	0.12±0.01	0.15±0.02	0.79±0.08	0.57±0.08	0.13±0.00
E-61	**4.46±0.25**	**5.73±0.87**	**28.60±4.24**	**22.64±0.44**	**2.79±0.12**
	0.13±0.01	0.16±0.03	0.82±0.14	0.65±0.03	0.08±0.00
E-62	**5.16±0.09**	**6.79±0.18**	**30.70±1.87**	**22.63±4.35**	**5.89±0.72**
	0.15±0.02	0.20±0.04	0.76±0.04	0.63±0.05	0.17±0.01
E-63	**4.34±0.48**	**6.70±1.17**	**29.20±2.00**	**21.49±2.05**	**4.54±0.15**
	0.12±0.00	0.19±0.01	0.82±0.03	0.60±0.01	0.13±0.01
E-64	**4.91±0.04**	**5.20±0.15**	**32.38±4.21**	**23.72±3.52**	**4.60±0.01**
	0.13±0.01	0.14±0.00	0.86±0.06	0.64±0.07	0.12±0.01
E-65	**5.05±0.21**	**5.98±0.52**	**35.05±3.56**	**23.92±0.31**	**3.74±0.27**
	0.14±0.00	0.17±0.01	1.00±0.06	0.55±0.06	0.10±0.01

Values are expressed as mean ± SD (n=4). Legend: CSA*n*: caffeoylshikimic acid isomer; CQL*n*: caffeoylquinic lactone isomer; *n*-FQL: *n*-*O*-feruloylquinic lactone.



Supplementary Figure 2.1. Loading plot (A) and score plot (B) obtained from the PCA with varimax of the considered bioactive compounds and capsule espresso coffees, by using bioactive concentrations (mg/mL). Legend: *n*-CQA: *n*-*O*-caffeoylquinic acid; CSA*n*: caffeoylshikimic acid isomer; CQL*n*: caffeoylquinic lactone isomer; *n*-CouQA: *n*-*O*-coumaroylquinic acid; *n*-FQA: *n*-*O*-feruloylquinic acid; *n*-FQL: *n*-*O*-feruloylquinic lactone; NMP: *N*-methylpyridinium.

CHAPTER 3

THE POCKET-4-LIFE PROJECT, BIOAVAILABILITY AND BENEFICIAL PROPERTIES OF THE BIOACTIVE COMPOUNDS OF ESPRESSO COFFEE AND COCOA-BASED CONFECTIONERY CONTAINING COFFEE: STUDY PROTOCOL FOR A RANDOMIZED CROSS-OVER TRIAL.

This chapter has been published on “TRIALS” (Mena, Pedro, et al. "The Pocket-4-Life project, bioavailability and beneficial properties of the bioactive compounds of espresso coffee and cocoa-based confectionery containing coffee: study protocol for a randomized cross-over trial." *Trials* 18.1 (2017): 527.)

A 3 arm crossover design was described with the aim to define the bioavailability and beneficial properties of coffee bioactive compounds on the basis of different levels of coffee consumption. Moreover, the contribution of cocoa-based products containing coffee to the pool of circulating metabolites and their putative bioactivity was taken into account.

The results of this study will be described in chapters 4 - 7.

Chapter 3: The Pocket-4-Life project, bioavailability and beneficial properties of the bioactive compounds of espresso coffee and cocoa-based confectionery containing coffee: study protocol for a randomized cross-over trial

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Abstract

Background: Coffee is an important source of bioactive compounds, including caffeine, phenolic compounds (mainly chlorogenic acids), trigonelline, and diterpenes. Several studies have highlighted the preventive effects of coffee consumption on major cardiometabolic diseases, but the impact of coffee dosage on markers of cardiometabolic risk is not well understood. Moreover, the pool of coffee-derived circulating metabolites and the contribution of each metabolite to disease prevention still need to be evaluated in real life settings. The aim of this study will be to define the bioavailability and beneficial properties of coffee bioactive compounds on the basis of different levels of consumption, by using an innovative experimental design. The contribution of cocoa-based products containing coffee to the pool of circulating metabolites and their putative bioactivity will also be investigated.

Method/Design: A 3-arm, crossover, randomized trial will be conducted. Twenty-one volunteers will be randomly assigned to consume three treatments in a random order for 1 month: 1 cup of espresso coffee/day, 3 cups of espresso coffee/day, and 1 cup of espresso coffee plus 2 cocoa-based products containing coffee twice per day. The last day of each treatment, blood and urine samples will be collected at specific time points, up to 24 hours following the consumption of the first product. At the end of each treatment the same protocol will be repeated, switching the allocation group. Besides the bioavailability of the coffee/cocoa bioactive compounds, the effect of the coffee/cocoa consumption on several

cardiometabolic risk factors (anthropometric measures, blood pressure, inflammatory markers, trimethylamine N-oxide, nitric oxide, blood lipids, fasting indices of glucose/insulin metabolism, DNA damage, eicosanoids, and nutri-metabolomics) will be investigated.

Discussion: Results will provide information on the bioavailability of the main groups of phytochemicals in coffee and on their modulation by the level of consumption. Findings will also show the circulating metabolites and their bioactivity when coffee consumption is substituted with the intake of cocoa-based products containing coffee. Finally, the effect of different levels of 1 month-coffee consumption on cardiometabolic risk factors will be elucidated, likely providing additional insights on the role of coffee in the protection against chronic diseases.

Trial registration: ClinicalTrials.gov, NCT03166540. Registered on 21 May 2017.

Keywords (3-10): coffee, cocoa, bioavailability, pharmacokinetic, cardiometabolic risk factors, caffeoylquinic acid, flavan-3-ol, caffeine, trigonelline, diterpene.

3.1. Background

Coffee is one of the most popular beverages worldwide, but, far from statistics, coffee is much more than a popular beverage. It is a moment of pleasure in the daily life of millions of people. This wide consumption ranks coffee as the main food source of four recognized bioactive constituents within the Mediterranean diet: the purine alkaloid caffeine (1,3,7-trimethylxanthine), the pyridine alkaloid trigonelline, (poly)phenolic substances (mainly chlorogenic acids), and the pentacyclic diterpenes cafestol and kahweol [1]. This unique combination of phytochemicals, with proven biological properties, turns coffee into a dietary agent able to impact on human health [2].

The beneficial properties associated to regular coffee consumption have been clearly described by an important number of systematic reviews and meta-analyses [3-6]. Roasted coffee is associated to a reduced risk of several chronic pathologies related to inflammation processes, such as atherosclerotic heart disease, stroke, and type 2 diabetes, as well as neurodegenerative conditions [4-10]. Most of these observations have also emphasized the dose-response inverse relationship of long-term coffee consumption with disease risk. Similarly, the non-linear U-shaped curve linking coffee consumption and cardiovascular disease (CVD) might be due to a combination of beneficial and detrimental effects [4, 11]. However, although observational studies provide the first line of evidence on a causal relation between coffee intake and risk of cardiometabolic diseases, randomized trials are required to definitively address this point [6, 12, 13]. This need for intervention studies has been scarcely tackled and, despite some progresses have been made [14-16], most of research carried out so far has failed to elucidate the rationale behind the potential preventive effects of coffee consumption. One of the main causes behind these scarce evidence-based explanations can be attributed to the lack of association of the physiological responses with the coffee bioactives in circulation. In this sense, coffee is a complex mixture, with dozens of chemicals appearing in the circulatory system after consumption, absorption and metabolism in humans, and where each single circulating metabolite may exert different effects within the human body [17, 18]. The complete pool of coffee-derived circulating metabolites and the contribution of each metabolite to disease prevention are still unknown.

The co-presence in circulation of different plant-derived metabolites with proven biological activities is a key factor on the prevention of cardiometabolic diseases through adequate dietary habits [1, 19-21]. Among the vegetal matrixes with high content in bioactive phytochemicals, cocoa is gaining increasing attraction. Actually, many initiatives

(EU FP7 project FLAVIOLA and the COSMOS trial, for instance) have been carried out assessing the efficacy of flavan-3-ols (main phenolics in cocoa/chocolate) towards surrogate markers of cardiovascular function, with some of these obtaining positive health claims [22-26]. In addition, cocoa and chocolate present high amounts of the alkaloid theobromine, an important caffeine metabolite [27]. Cocoa-based products represent, therefore, an interesting target that could synergize the preventive cardiometabolic effects of regular coffee consumption. In this sense, chocolate confectionary containing coffee, combining the phytochemical content of coffee and cocoa, could be regarded as a potential candidate to enhance the circulating levels of putatively protective metabolites in the context of a balanced diet.

This work will help to define the bioavailability and beneficial properties of coffee bioactive compounds on the basis of different levels of coffee consumption. Moreover, the contribution of cocoa-based products containing coffee to the pool of circulating metabolites and their putative bioactivity will be taken into account. This innovative study design guarantees adherence to real life settings and patterns of consumption, which will serve to solve critical gaps within the framework of nutritional intervention studies with coffee.

3.2. Methods/Design

3.2.1. Objectives

With final aim of studying the bioavailability of coffee/cocoa bioactive compounds and their effects in cardiometabolic health, the objectives of this intervention will be:

i) Assessing the bioavailability of the four main groups of phytochemicals in roasted coffee (methylxanthines, phenolic compounds, trigonelline, and diterpenes), its modulation by the level of consumption, and establishing the daily average concentration of coffee-derived plasma circulating metabolites;

ii) investigating the effect of different levels of coffee consumption on cardiometabolic risk factors;

iii) evaluating circulating metabolites and their putative bioactivity when substituting coffee consumption with the intake of cocoa-based products containing coffee.

3.2.2. Protocol and study design

A human study will be carried out to achieve the above-described goals. The human intervention study will consist of a short-term, randomized cross-over trial, addressed at measuring the daily mean concentrations of each coffee/cocoa-derived circulating metabolite (CCDCM) for the four main groups of coffee/cocoa phytochemicals (methylxanthines, trigonelline, phenolics, diterpenes). On the basis of different patterns of consumption, this free-living study (although some minimal dietary restrictions will be provided two days before sampling times) will also take into consideration the effects of repeated doses on the bioavailability of coffee/cocoa bioactives.

The study will follow a repeat-dose, 3-arm, cross-over design (a detailed study design is shown in **Figure. 3.1**, and **Figure. 3.2** shows the SPIRIT chart). This design has been chosen according to ILSI's guidelines for intervention trials with dietary products [28]. This protocol was also developed in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) Statement (SPIRIT checklist presented as Additional file 1).

Subjects will be assigned to consume the following treatments in a random order for 1 month (including the sampling day -the last day of each intervention period-):

1) 1 cup of espresso coffee/day ("low consumers") at 9.00 A.M.

2) 3 cups of espresso coffee/day ("high consumers") at 9.00 A.M., 12.00 P.M. and 3.00 P.M.

3) 1 cup of espresso coffee at 9.00 A.M. and 2 cocoa-based products containing coffee two-times per day, at 12.00 P.M. and 3.00 P.M. This group will be named "medium consumers", considering the caffeine content of the cocoa-based product containing coffee.

Minimal recommendations to avoid other sources of coffee/cocoa-related phytochemicals besides what introduced through the assigned treatment, and to standardize the time of coffee consumption, will be provided for the two days prior to each sampling day and on the sampling day. Dinner timing and composition will also be standardized the day before the sampling day. Only water could be drunk during the night. At the sampling day, the subjects will refer in the morning at the ambulatory where fasting baseline blood and urine samples will be collected. Then, low and high consumers will drink one or three cups of espresso coffee, respectively (without sugar, sweeteners, and milk for the first coffee; with 5 g of sugar for the last two coffees), while medium consumers will drink a cup of espresso coffee and 2 cocoa-based products containing coffee twice during the day, following the

aforementioned timing. After ingestion of the first coffee together with a phytochemical-free breakfast (a sponge milky cake), blood and urine samples will be collected at selected time points along the following 24-h (**Figure. 3.3**). Five hours after the consumption of the first coffee, participants will receive a standardized mixed meal (ham and cheese sandwich) free of coffee/cocoa-related phytochemicals. Water will be available ad libitum. Twenty-four hours after receiving the treatment, blood and urine samples will also be taken in order to assess return to baseline. In addition, anthropometric characteristics and blood pressure (BP) will be measured.

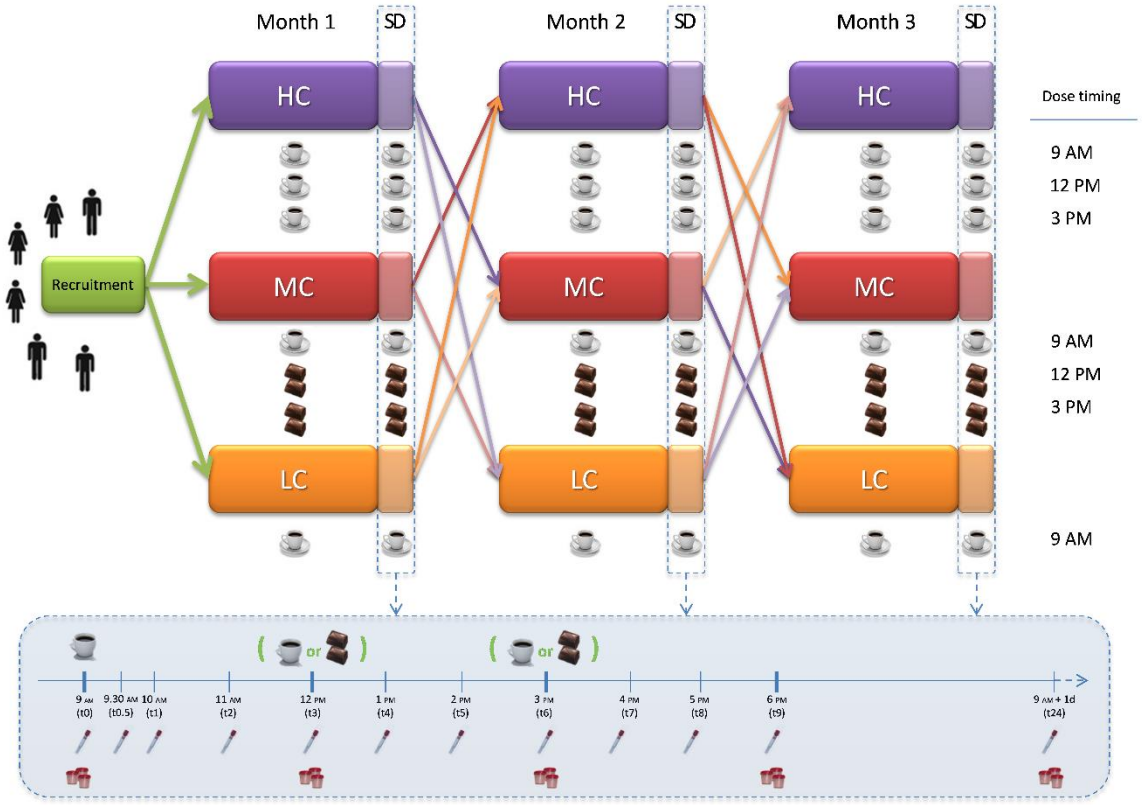

Figure 3.1. Study design and sampling day (in the blue box) scheme. HC high consumers, LC low consumers, MC medium consumers, SD sampling day

	STUDY PERIOD					
	Enrolment	Allocation	Post-allocation			
TIMEPOINT	*Pre-intervention*	0	*Baseline*	*Visit 1*	*Visit 2*	*Visit 3*
ENROLMENT:						
Eligibility screen	X					
Informed consent	X					
Allocation		X				
INTERVENTIONS:						
Low consumers (1 coffee/day)				X*	X*	X*
High consumers (3 coffees/day)				X*	X*	X*
Medium consumers (1 coffee + 4 Pocket coffee/day)				X*	X*	X*
ASSESSMENTS:						
Sociodemographic variables	X					
Lifestyle-related variables	X		◆—	—	—	—◆
Dietary intakes	X		◆—	—	—	—◆
CCDCMs			X	X	X	X
TMAO			X	X	X	X
Inflammatory markers			X	X	X	X
Anthropometric measures			X	X	X	X
Blood pressure			X	X	X	X
Nitric Oxide			X	X	X	X
Blood lipids			X	X	X	X
Glucose/insulin metabolism markers			X	X	X	X
DNA damage (Comet assay)			X	X	X	X
DNA damage (oxidation catabolites)			X	X	X	X
Eicosanoids			X	X	X	X
Nutri-metabolomics			X	X	X	X

* Randomly assigned treatments

Figure 3.2. SPIRIT figure: summarizes the allocation, interventions, and outcomes of the study

3.2.3. Testing materials

Volunteers will be supplied with a single-serve coffee machine (Essenza EN 97.W, De'Longhi Appliances S.r.l, Treviso, Italy) and coffee capsules (Capriccio, Nespresso Italia S.p.a., Assago, Italy) to standardize brewing method, raw material, and cup volume (approximately 45 mL). Volunteers will also be supplied with the cocoa-based product containing coffee (Pocket Coffee, Ferrero Commerciale Italia S.r.l., Alba, Italy).

3.2.4. Participant selection

Twenty-one healthy volunteers will be recruited in Parma (North Italy) for the study, using announcements placed in university, hospital, and public places. All the subjects involved in the nutritional intervention will be previously informed on the details of the protocol and about the risks associated to participation, and they will be asked by the study staff for their written informed consent to participate in the study (information to the volunteers will be provided before and separately from the consent form). Personal data collection will include name, surname, e-mail, phone number, age, height, weight, and dietary habits of the volunteers.

3.2.5. Inclusion and exclusion criteria

Inclusion criteria will include being adult, healthy, of normal weight (BMI 18–25), and regular coffee consumers of 1–5 cups per day. Exclusion criteria will include clinically diagnosis for metabolic, renal or digestive disorders, regular consumption of medication, antibiotic therapy taken within the last 3 months, intense physical activity, pregnancy or lactation, and regular intake of coffee exceeding 5 coffees/day. These criteria are set in order to avoid likely confounding factors and according to similar works in the field [29].

3.2.6. Data and sample collection

Socio-demographic variables will be assessed through a generic questionnaire filled at recruitment. The questionnaire will also contain questions useful to identify possible exclusion criteria (e.g. diagnosis for diseases, regular consumption of medication, food allergy). Dietary habits of volunteers will also be evaluated during the enrolment, through a semi-quantitative food frequency questionnaire (FFQ) for the assessment of dietary total antioxidant capacity [30]. In addition, participants' food intake and compliance with the study requirements will be assessed by means of 3-day dietary records, administered

throughout each intervention period at two time points: i) in the middle of each intervention period during two weekdays and a weekend day, and ii) at the end of each intervention period, 2 days prior to the sampling day and the sampling day. The habitual physical activity level of each participant will be measured through a validated International Physical Activity Questionnaires [31].

Blood sample collection will be carried out in the ambulatory unit of the Department of Medicine and Surgery. Blood collection will be carried out by a physician. A venous catheter will be inserted into the antecubital vein and blood samples from each subject will be collected in specific tubes at the time-points indicated at **Figure 1.3**. Blood at 24 h after first coffee consumption will be taken by venipuncture. Urine samples will be collected during different periods of time (**Figure 1.3**) using urine collectors.

Blood samples will be centrifuged and plasma, serum, and peripheral blood mononuclear cells (PBMCs) will be collected, aliquoted, and stored at -80 °C for further processing. Urine samples will be aliquoted and stored at -80°C for further processing.

3.2.7. Measurements

The primary selected endpoint of the study is the quantification of the daily mean concentration of coffee-derived plasma circulating phenolic metabolites, whereas the study of the bioavailability of other coffee-derived circulating bioactives, the bioavailability of cocoa-derived circulating phytochemicals, and the assessment of cardiometabolic markers will be considered secondary endpoints.

Anthropometric measures

At the beginning and end of each intervention period, body weight, height, and waist circumference will be assessed, and BMI will be calculated.

Blood pressure (BP)

Systolic and diastolic BP of each volunteer will be obtained after a 5-min rest in a seated position in the morning of the beginning and end of each intervention period.

Daily mean concentration of coffee/cocoa-derived plasma circulating phenolic metabolites

The determination of CCDCMs will be performed in all the samples. Samples will be subjected to UHPLC-MS^n analysis (linear ion trap MS for identification and triple quadrupole MS for quantification purposes). Plasma samples will be extracted according to Zhang et al. [32], while urine and coffee samples will be centrifuged, diluted, and filtered. Methylxanthines, trigonelline, other pyridine metabolites, diterpenes, and phenolic

metabolites will be determined as previously described [33-36]. Accurate calibration and absolute quantification will be achieved by comparison with authentic standards when commercially available or with standards previously synthesized [37, 38].

Pharmacokinetic studies

Metabolite data will be analyzed using the WinNonlin software. Pharmacokinetic parameters will include maximum, minimum, and average plasma concentration (C_{max}, C_{min}, and C_{avg}), degree of fluctuation, area under the curve from 0–24 hours ($AUC_{0–24}$), times of maximum and minimum plasma concentrations (T_{max} and T_{min}), and they will be calculated for each volunteer and metabolite (**Figure 3.3**). Urinary excretion kinetics will also be estimated.

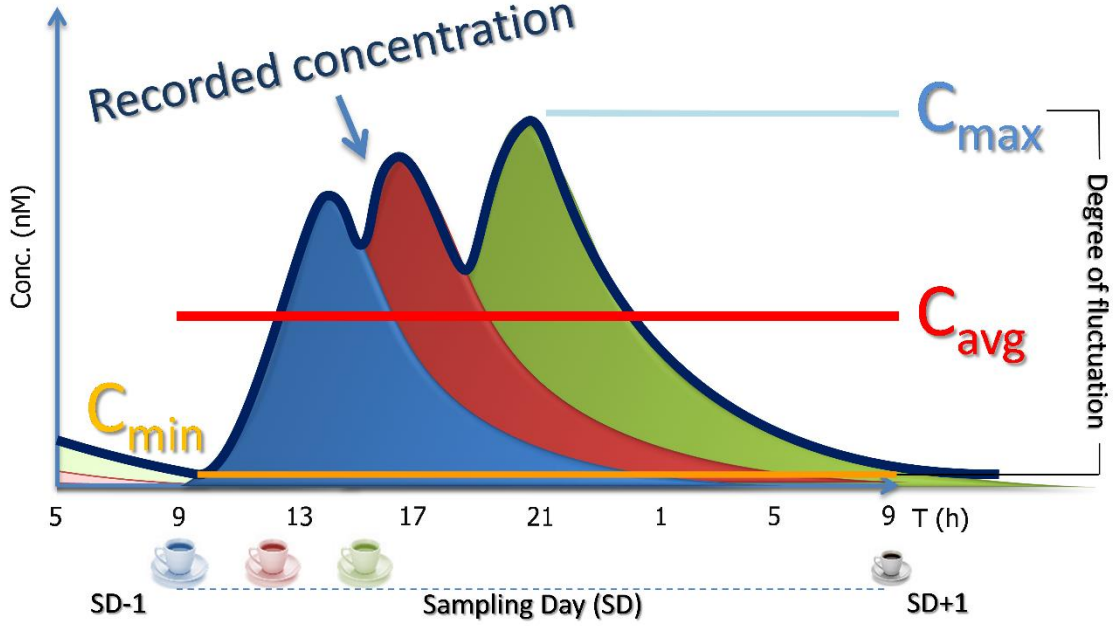

Figure 3.3. Modelled pharmacokinetic profile recorded after repeated consumption of coffee along day. C_{max} maximum concentration, C_{avg} average concentration, C_{min} minimum concentration, SD sampling day

Trimethylamine N-oxide (TMAO)

TMAO is a novel biomarker of cardiovascular risk produced from L-carnitine with involvement of the gut microbiota and, thus, influenced by the diet [39, 40]. It will be quantified in baseline (0 h) plasma samples before and after each treatment by UHPLC-MS^n. Before the analysis, each sample will be added with TMAO-d9 as internal standard and then extracted with acidified acetonitrile as previously described [41]. Samples will be centrifuged and the supernatants collected for the UHPLC-MS^n analysis.

Inflammatory markers

Some markers associated with inflammatory processes linked to atherosclerosis onset will be studied with a Bio-Plex Pro™ Human Cytokine Assays (Bio-Rad Laboratories S.r.l., Segrate, Italy). The concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, MCP-1 (MCAF), and TNF-α will be measured by using a Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories). These analyses will be performed in the baseline (0 h) plasma before and after each treatment.

Nitric oxide (NO)

Increased NO bioavailability is inversely associated with endothelial dysfunction. NO acts as an endogenous vasodilatory factor involved in the regulation of the endothelial function, maintaining an anti-inflammatory environment in the vessel [42]. Plasmatic NO, assessed as nitrate/nitrite, will be determined at the beginning and end of each intervention period using Nitrate/Nitrite Fluorometric Assay Kit (Vinci-Biochem S.r.l., Vinci, Italy).

Blood lipids

Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides will be analyzed by standardized hospital routine analysis at the beginning and end of each intervention period.

Fasting indices of glucose/insulin metabolism

In order to investigate the effect of the chronic coffee consumption on diabetes and insulin resistance prevention, plasma glucose and insulin concentrations will be monitored at fasting state before and after each intervention period. The analysis of glycaemia will be performed by means of an automatic analyzer with a combined enzymatic-electrochemical system (YSI 2900 STAT PLUS, Yellow Spring Instruments). Plasmatic insulin concentration will be monitored by using routine blood analysis. Fasting insulin sensitivity will be determined using the quantitative insulin sensitivity check index (QUICKI) [43]. Fasting insulin secretion capacity will be evaluated as the Homeostatic Model Assessment (HOMA) for beta cell function (HOMA-B) [44] and for insulin resistance (HOMA-IR) [45].

DNA damage (Comet assay)

H_2O_2-induced DNA damage (i.e. oxidatively induced DNA damage) and FPG-sensitive sites of DNA (i.e. endogenous oxidative base damage) will be evaluated by the Comet assay, a simple technique for the evaluation of DNA damage in all types of eukaryotic cells and tissues. DNA damage will be evaluated in PBMCs before and after each treatment, but also in parallel with the pharmacokinetic study in order to determine the effect of both acute and long-term consumption [46].

DNA damage (oxidation catabolites)

DNA oxidation catabolites (8-hydroxy-2´-deoxyguanosine, 8-hydroxyguanosine, 8-nitroguanosine, guanosine-3′-5′-cyclic monophosphate, and 8-nitroguanosine-3′-5′-cyclic monophosphate) will be evaluated in plasma (collected using heparinized blood tubes) before and after each treatment by UHPLC-MS/MS, but also in parallel with the pharmacokinetic study in order to determine the effect of both acute and long-term consumption [47].

Eicosanoids (oxidative stress, vascular, and inflammatory markers)

Eicosanoids are biomarkers that have been used for tracking changes in lipid peroxidation and vascular events. Eicosanoid family comprises prostaglandins, thromboxanes, and isoprostanes. These markers will be evaluated in urine before and after each treatment by UHPLC-MS/MS [48].

Nutri-metabolomics

Urine and plasma samples at the beginning and end of each intervention period will be subjected to untargeted HR-LC-MS/MS metabolomics approach, in order to assess potential differences between control (before) and treatment (after), as well as among the three treatments described. This explorative investigation will be applied as a complementary assay to generate a comprehensive picture of the impact of coffee and the cocoa-based product containing coffee on human health.

3.2.8. Sample size calculation, randomization and statistical analysis

The sample size has been calculated considering the primary outcome, the daily mean concentration of coffee-derived plasma circulating phenolic metabolites, and according to Lenth [49]. Considering the lack of literature dealing with this outcome, the AUC of dihydrocaffeic acid-3′-*O*-sulfate, one of the most representative coffee-derived phenolic metabolites [50], has been used for sample size calculations. Keeping a 80% power and an α of 5%, and considering data from Stalmach et al. [50], 15 subjects will have to complete an acute intervention to detect a change of 600 nmol/h/L^{-1} in dihydrocaffeic acid-3′-*O*-sulfate plasma concentration with a SD of 870 nmol/h/L^{-1}. A total of 21 subjects will be recruited to allow for dropouts and for nonparametric statistical analysis (15% additional subjects required). Once selected the volunteers, a randomization list will be generated using a randomized block design by means of Random Number Generator Pro (Segobit Software). This list will be blind for the PI and volunteers, as it will be made by a colleague not involved in subject enrollment, and using a numbered sequence in sealed, opaque envelopes.

Before any comparison is performed, the normality of each variable will be evaluated to choose the most appropriate statistical test. If normally distributed, data will be expressed as mean ± standard deviation and analyzed using general linear models for repeated measures with post-hoc comparisons. If data are not normally distributed, they will be reported as median and interquartile range and the Friedman test with post hoc pairwise comparisons will be performed. Multivariate analyses will be carried out to understand individual responses to coffee consumption on the basis of metabolite production. All the analyses will be carried out using SPSS 23.0. P-value <0.05 will be regarded as statistically significant.

3.2.9. Confidentiality of data

Each participant will be assigned to a unique code provided by the PI, so that all personal information, including questionnaire information and samples, will be confidential. Information will be collected exclusively by the PI (or the specialized staff officially involved in the study) and will be stored in a dedicated non-web connected computer. Sample codification will be hidden to the researchers analyzing the samples (single-blind study). Recruited volunteers will sign an authorization to the use of personal information and data. The identity of the participants will not be revealed in any published data or in presentation of the information obtained as a result of this study. All the data collected for this study will be treated as confidential.

3.3. Discussion

There is growing evidence that regular coffee consumption is associated to several beneficial properties. However, research has emphasised how differences in the number of cups consumed on a daily basis affect the prevalence of cardiometabolic disorders [12, 51-57] and biomarkers of cardiometabolic risk [12, 55, 58]. There is a linear inverse dose-response relationship between coffee consumption and diabetes, and every additional cup of coffee in a day is associated with a 5% to 10% lower risk of new-onset disease [6, 54, 59]. In the case of CVD, a non-linear U-shaped curve links coffee consumption and CVD risk, with the largest risk reduction observed at a level of about 3 cups/day, although high rates of variability have been observed [53, 60, 61]. These difficulties in the definition of the adequate coffee intake to promote cardiometabolic health make dietary recommendations

for coffee consumption almost impossible [9]. Thus, randomized trials addressing the impact of coffee dose on markers of cardiometabolic risk are needed [6, 12, 13].

The need for intervention studies considering coffee dosage has been scarcely tackled, and the biological actions behind the preventive effects of coffee consumption has not been elucidated yet. The main reasons behind this missing information might be 1) the lack studies considering the association of the physiological responses with coffee bioactives in circulation, and 2) the high inter-individual variation observed for the selected cardiometabolic endpoints. Regarding point 1, it should be noted that ascertaining the exact metabolites appearing in circulation after consuming a cup of coffee is a key point to fully unravel the bioactive(s) responsible for its preventive effects. Nevertheless, despite substantial research on caffeine, trigonelline, and phenolic compounds [34], there is a lack of fundamental knowledge on this critical topic, even more when it comes to different patterns of consumption (number of daily servings and repeated daily doses, which represents a common scenario among coffee consumers). In addition, the circulating metabolites derived from coffee diterpenes [36, 62, 63], potent cholesterol-raising compounds, are still unknown. This information is paramount to draw a more realistic physiological picture and, hence, to better understand the biological properties of long-term coffee consumption [34, 55]. On the other hand, heterogeneity in individual responsiveness to food components (point 2) can obscure associations between diet and health, and limit the understanding of the exact role of the different coffee bioactives. Individual responses to coffee consumption, likely driven by variations in the bioavailability of key metabolites [64], may be affected by gender [15, 54], age [65], weight or BMI [15, 54], health status [12], genetic polymorphisms [66, 67], smoking [54], physical activity [68], dietary habits, and gut microbiota composition [69-72], among other factors. The gut microbiota has recently emerged as one of the key drivers for diet: cardiometabolic health interactions, due to its ability to produce several metabolites that modulate host physiology at many levels [71, 73, 74]. Likewise, coffee phenolics are highly metabolized by the gut microbiota and are able, in turn, to modulate the microbiota composition [72, 75-77]. This bidirectional relation may deeply condition both the cardiometabolic response and the types and levels of circulating bioactives after coffee consumption.

In summary, despite an overwhelming number of published studies, there is a paradoxical lack of knowledge on the bioactives responsible for the observed beneficial effects of coffee, and on their ability to regulate physiological processes involved in its preventive efficacy. This missing information becomes even more apparent when the effect

of dosage and repeated doses during the day are taken into account. Finally, although some factors influencing inter-individual variability have been tackled, most remain still unexplored. This study will try to bridge these major gaps by linking coffee consumption to individual CCDCM profiles and metabolic responses. To do that, in a real life setting, the design of this study is extremely innovative, joining under the same protocol both acute and short-term observations, maximizing its prospects and reducing all the operational constraints associated with separate intervention studies.

Abbreviations

AUC: Area under the curve; BMI: Body mass index; BP: Blood Pressure; CCDCMs: Coffee/cocoa-derived circulating metabolites; CVD: cardiovascular disease; FFQ: food frequency questionnaire; FPG: Formamidopyrimidine DNA glycosylase; HDL: High Density Lipoprotein; HOMA: Homeostatic Model Assessment; HR-LC-MS/MS: high resolution-liquid chromatography mass spectrometry; IL: Interleukin; LDL: Low Density Lipoprotein; MCP-1(MCAF): Monocyte Chemotactic Protein 1; NO: Nitric oxide; PBMCs: peripheral blood mononuclear cells; PI: Principal Investigator; QUICKI: quantitative insulin sensitivity check index; TMAO: Trimethylamine N-oxide; TNF-α: Tumor Necrosis Factor alpha; UHPLC-MS^n: Ultra-High Pressure Liquid Chromatography-Mass Spectrometry.

Declarations

Ethics approval and consent to participate

The Ethics Committee of the University of Parma approved the study on 12 October 2016 (AZOSPR/0036174/6.2.2.). The protocol was modified to include further analysis and the ethical approval for these amendments was granted on 19 April 2017 (AZOSPR/0015693/6.2.2.). All trial participants will provide full written informed consent.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have read and approved the final version of this manuscript. PM was responsible for trial conception and design, and drafted the manuscript. MT contributed to the development of the study protocol. DM provided methodological assistance and assisted with manuscript writing and proof reading. AR provided methodological assistance, and assisted with protocol development and proof reading. FB provided critical revision of the manuscript. DDR was responsible for trial conception, design, and critical revision and final approval of this manuscript.

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3.4. References

1. Tresserra-Rimbau A, Rimm EB, Medina-Remón A, Martínez-González MA, de la Torre R, Corella D, et al. Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study. Nutr Metab Cardiovasc Dis. **2014**;24:639-47.
2. Ludwig IA, Clifford MN, Lean MEJ, Ashihara H, Crozier A. Coffee: biochemistry and potential impact on health. Food Funct. **2014**;5:1695-717.
3. Van Dam RM, Hu FB. Coffee consumption and risk of type 2 diabetes: A systematic review. J Am Med Assoc. **2005**;294:97-104.
4. Ding M, Bhupathiraju SN, Satija A, van Dam RM, Hu FB. Long-term coffee consumption and risk of cardiovascular disease: A systematic review and a dose-response meta-analysis of prospective cohort studies. Circulation. **2013**;129:643-59
5. Malerba S, Turati F, Galeone C, Pelucchi C, Verga F, La Vecchia C, Tavani A. A meta-analysis of prospective studies of coffee consumption and mortality for all causes, cancers and cardiovascular diseases. Eur J Epidemiol. **2013**;28:527-39.
6. Huxley R, Lee CMY, Barzi F, Timmermeister L, Czernichow S, Perkovic V, et al. Coffee, decaffeinated coffee, and tea consumption in relation to incident type 2 diabetes mellitus: A systematic review with meta-analysis. Arch Intern Med. **2009**;169:2053-63.
7. de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. Lancet Neurol. **2006**;5:525-35.
8. Lindsay J, Laurin D, Verreault R, Hébert R, Helliwell B, Hill GB, McDowell I. Risk factors for Alzheimer's disease: A prospective analysis from the Canadian Study of Health and Aging. Am J Epidemiol. **2002**;156:445-53.
9. Bidel S, Tuomilehto J. The emerging health benefits of coffee with an emphasis on type 2 diabetes and cardiovascular disease. Eur Endocrinol. **2013**;9:99-106.
10. Larsson SC. Coffee, tea, and cocoa and risk of stroke. Stroke. **2014**;45:309-14.
11. Mostofsky E, Rice MS, Levitan EB, Mittleman MA. Habitual coffee consumption and risk of heart failure a dose-response meta-analysis. Circ Heart Fail. **2012**;5:401-5.

12. Nordestgaard AT, Thomsen M, Nordestgaard BG. Coffee intake and risk of obesity, metabolic syndrome and type 2 diabetes: a Mendelian randomization study. Int J Epidemiol. **2015**;44:551-65.
13. Shang F, Li X, Jiang X. Coffee consumption and risk of the metabolic syndrome: A meta-analysis. Diabetes Metab. **2016**;42:80-7.
14. Kempf K, Kolb H, Gärtner B, Bytof G, Stiebitz H, Lantz I, et al. Cardiometabolic effects of two coffee blends differing in content for major constituents in overweight adults: a randomized controlled trial. Eur J Nutr. **2015**;54:845-54.
15. Gavrieli A, Fragopoulou E, Mantzoros CS, Yannakoulia M. Gender and body mass index modify the effect of increasing amounts of caffeinated coffee on postprandial glucose and insulin concentrations; a randomized, controlled, clinical trial. Metabolism. **2013**;62:1099-106.
16. Rakvaag E, Dragsted LO. Acute effects of light and dark roasted coffee on glucose tolerance: a randomized, controlled crossover trial in healthy volunteers. Eur J Nutr. **2016**;55:2221-30.
17. Rodriguez-Mateos A, Vauzour D, Krueger CG, Shanmuganayagam D, Reed J, Calani L, et al. Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. Arch Toxicol. **2014**;88:1803-53.
18. Del Rio D, Rodriguez-Mateos A, Spencer JPE, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal. **2013**;18:1818-92.
19. Landberg R, Sun Q, Rimm EB, Cassidy A, Scalbert A, Mantzoros CS, et al. Selected dietary flavonoids are associated with markers of inflammation and endothelial dysfunction in U.S. women. J Nutr. **2011**;141:618-25.
20. Chiva-Blanch G, Badimon L, Estruch R. Latest evidence of the effects of the Mediterranean diet in prevention of cardiovascular disease. Curr Atheroscler Rep. **2014**;16:446.
21. Zamora-Ros R, Forouhi NG, Sharp SJ, González CA, Buijsse B, Guevara M, et al. Dietary intakes of individual flavanols and flavonols are inversely associated with incident type 2 diabetes in European populations. J Nutr. **2014**;144:335-43.
22. Rodriguez-Mateos A, Cifuentes-Gomez T, Gonzalez-Salvador I, Ottaviani JI, Schroeter H, Kelm M, et al. Influence of age on the absorption, metabolism, and excretion of cocoa flavanols in healthy subjects. Mol Nutr Food Res. **2015**;59:1504-12.
23. Vogiatzoglou A, Mulligan AA, Bhaniani A, Lentjes MAH, McTaggart A, Luben RN, et al. Associations between flavan-3-ol intake and CVD risk in the Norfolk cohort of the European Prospective Investigation into Cancer (EPIC-Norfolk). Free Radic Biol Med. **2015**;84:1-10.
24. Sansone R, Rodriguez-Mateos A, Heuel J, Falk D, Schuler D, Wagstaff R, et al. Cocoa flavanol intake improves endothelial function and Framingham Risk Score in healthy men and women: a randomised, controlled, double-masked trial: the Flaviola Health Study. Br J Nutr. 2015;114:1246-55.
25. EFSA. Scientific Opinion on the substantiation of a health claim related to cocoa flavanols and maintenance of normal endothelium-dependent vasodilation pursuant to Article 13(5) of Regulation (EC) No 1924/2006. EFSA J. **2012**;10.
26. Kwok CS, Boekholdt SM, Lentjes MAH, Loke YK, Luben RN, Yeong JK, et al. Habitual chocolate consumption and risk of cardiovascular disease among healthy men and women. Heart. **2015**; 101:1279-87.
27. Kim J, Shim J, Lee CY, Lee KW, Lee HJ. Cocoa Phytochemicals: Recent Advances in Molecular Mechanisms on Health. Crit Rev Food Sci Nutr. **2014**;54:1458-72.
28. Welch RW, Antoine JM, Berta JL, Bub A, de Vries J, Guarner F, et al. Guidelines for the design, conduct and reporting of human intervention studies to evaluate the health benefits of foods. Br J Nutr. **2011**;106 Suppl 2:S3-15.
29. Renouf M, Marmet C, Giuffrida F, Lepage M, Barron D, Beaumont M, et al. Dose-response plasma appearance of coffee chlorogenic and phenolic acids in adults. Mol Nutr Food Res. **2014**;58:301-9.
30. Pellegrini N, Salvatore S, Valtueña S, Bedogni G, Porrini M, Pala V, et al. Development and validation of a food frequency questionnaire for the assessment of dietary total antioxidant capacity. J Nutr. **2007**;137:93-8.
31. Craig CL, Marshall AL, Sjöström M, Bauman AE, Booth ML, Ainsworth BE, et al. International physical activity questionnaire: 12-Country reliability and validity. Med Sci Sports Exerc. **2003**;35:1381-95.
32. Zhang Y, Mehrotra N, Budha NR, Christensen ML, Meibohm B. A tandem mass spectrometry assay for the simultaneous determination of acetaminophen, caffeine, phenytoin, ranitidine, and theophylline in small volume pediatric plasma specimens. Clin Chim Acta. **2008**;398:105-12.
33. Bianco G, Abate S, Labella C, Cataldi TR. Identification and fragmentation pathways of caffeine metabolites in urine samples via liquid chromatography with positive electrospray ionization coupled to a hybrid quadrupole linear ion trap (LTQ) and Fourier transform ion cyclotron resonance mass spectrometry and tandem mass spectrometry. Rapid Commun Mass Spectrom. **2009**;23:1065-74.
34. Lang R, Dieminger N, Beusch A, Lee YM, Dunkel A, Suess B, et al. Bioappearance and pharmacokinetics of bioactives upon coffee consumption. Anal Bioanal Chem. **2013**;405:8487-503.
35. Marmet C, Actis-Goretta L, Renouf M, Giuffrida F. Quantification of phenolic acids and their methylates, glucuronides, sulfates and lactones metabolites in human plasma by LC-MS/MS after oral ingestion of soluble coffee. J Pharm Biomed Anal. **2014**;88:617-25.
36. Van Cruchten STJ, De Waart DR, Kunne C, Hooiveld GJEJ, Boekschoten MV, Katan MB, et al. Absorption, distribution, and biliary excretion of cafestol, a potent cholesterol-elevating compound in unfiltered coffees, in mice. Drug Metab Dispos. **2010**;38:635-40.

37. Curti C, Brindani N, Battistini L, Sartori A, Pelosi G, Mena P, et al. Catalytic, Enantioselective Vinylogous Mukaiyama Aldol Reaction of Furan-Based Dienoxy Silanes: A Chemodivergent Approach to γ-Valerolactone Flavan-3-ol Metabolites and δ-Lactone Analogues. Adv Synth Catal. **2015**;357:4082-92.
38. Brindani N, Mena P, Calani L, Benzie I, Choi SW, Brighenti F, et al. Synthetic and analytical strategies for the quantification of phenyl-gamma-valerolactone conjugated metabolites in human urine. Mol Nutr Food Res. **2017**; doi: 10.1002/mnfr.201700077.
39. Klick DE, Hines RN. Mechanisms regulating human FMO3 transcription. Drug Metab Rev. 2007;39:419-42.
40. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. **2011**;472:57-63.
41. Miller CA, Corbin KD, da Costa KA, Zhang S, Zhao X, Galanko JA, et al. Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose-response study. Am J Clin Nutr. **2014**;100:778-86.
42. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation. **2006**;113:1708-14.
43. Pacini G, Mari A. Methods for clinical assessment of insulin sensitivity and beta-cell function. Best Pract Res Clin Endocrinol Metab. **2003**;17:305-22.
44. Stadler M, Pacini G, Petrie J, Luger A, Anderwald C. Beta cell (dys)function in non-diabetic offspring of diabetic patients. Diabetologia. **2009**;52:2435-44.
45. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. **1985**;28:412-9.
46. Del Bo C, Fracassetti D, Lanti C, Porrini M, Riso P. Comparison of DNA damage by the comet assay in fresh versus cryopreserved peripheral blood mononuclear cells obtained following dietary intervention. Mutagenesis. **2015**;30:29-35.
47. Garcia-Flores LA, Medina S, Cejuela-Anta R, Martinez-Sanz JM, Abellan A, Genieser HG, et al. DNA catabolites in triathletes: effects of supplementation with an aronia-citrus juice (polyphenols-rich juice). Food Funct. **2016**;7:2084-93.
48. Medina S, Domínguez-Perles R, Moreno DA, García-Viguera C, Ferreres F, Gil JI, Gil-Izquierdo Á. The intake of broccoli sprouts modulates the inflammatory and vascular prostanoids but not the oxidative stress-related isoprostanes in healthy humans. Food Chem. **2015**;173:1187-94.
49. Lenth RV. Some Practical Guidelines for Effective Sample Size Determination. Am Stat. **2001**;55:187-93.
50. Stalmach A, Mullen W, Barron D, Uchida K, Yokota T, Cavin C, et al. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. Drug Metab Dispos. **2009**;37:1749-58.
51. Freedman ND, Park Y, Abnet CC, Hollenbeck AR, Sinha R. Association of coffee drinking with total and cause-specific mortality. New Eng J Med. **2012**;366:1891-904.
52. Saito E, Inoue M, Sawada N, Shimazu T, Yamaji T, Iwasaki M, et al. Association of coffee intake with total and cause-specific mortality in a Japanese population: the Japan Public Health Center-based Prospective Study. Am J Clin Nutr. **2015**;101:1029-37.
53. Andersen LF, Jacobs DR, Jr., Carlsen MH, Blomhoff R. Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. Am J Clin Nutr. **2006**;83:1039-46.
54. Jiang X, Zhang D, Jiang W. Coffee and caffeine intake and incidence of type 2 diabetes mellitus: A meta-analysis of prospective studies. Eur J Nutr. **2014**;53:25-38.
55. Sartorelli DS, Fagherazzi G, Balkau B, Touillaud MS, Boutron-Ruault MC, De Lauzon-Guillain B, Clavel-Chapelon F. Differential effects of coffee on the risk of type 2 diabetes according to meal consumption in a French cohort of women: The E3N/EPIC cohort study. Am J Clin Nutr. **2010**;91:1002-12.
56. Pereira MA, Parker ED, Folsom AR. Coffee consumption and risk of type 2 diabetes mellitus: An 11-year prospective study of 28 812 postmenopausal women. Arch Intern Med. **2006**;166:1311-6.
57. Salazar-Martinez E, Willett WC, Ascherio A, Manson JE, Leitzmann MF, Stampfer MJ, Hu FB. Coffee Consumption and Risk for Type 2 Diabetes Mellitus. Ann Intern Med. **2004**;140:1-8.
58. Grosso G, Stepaniak U, Micek A, Topor-Madry R, Pikhart H, Szafraniec K, Pajak A. Association of daily coffee and tea consumption and metabolic syndrome: results from the Polish arm of the HAPIEE study. Eur J Nutr. **2015**;54:1129-37.
59. Koloverou E, Panagiotakos DB, Pitsavos C, Chrysohoou C, Georgousopoulou EN, Laskaris A, Stefanadis C. The evaluation of inflammatory and oxidative stress biomarkers on coffee-diabetes association: results from the 10-year follow-up of the ATTICA Study (2002-2012). Eur J Clin Nutr. **2015**;69:1220-5.
60. Crippa A, Discacciati A, Larsson SC, Wolk A, Orsini N. Coffee consumption and mortality from all causes, cardiovascular disease, and cancer: a dose-response meta-analysis. Am J Epidemiol. **2014**;180:763-75.
61. Zhao Y, Wu K, Zheng J, Zuo R, Li D. Association of coffee drinking with all-cause mortality: A systematic review and meta-analysis. Public Health Nutr. **2015**;18:1282-91.
62. Boekschoten MV, Engberink MF, Katan MB, Schouten EG. Reproducibility of the serum lipid response to coffee oil in healthy volunteers. Nutr J. **2003**;2:1-8.
63. De Roos B, Meyboom S, Kosmeijer-Schuil TG, Katan MB. Absorption and urinary excretion of the coffee diterpenes cafestol and kahweol in healthy ileostomy volunteers. J Intern Med. **1998**;244:451-60.

64. Guertin KA, Loftfield E, Boca SM, Sampson JN, Moore SC, Xiao Q, et al. Serum biomarkers of habitual coffee consumption may provide insight into the mechanism underlying the association between coffee consumption and colorectal cancer. Am J Clin Nutr. **2015**; 101:1000-11.
65. Lof M, Sandin S, Yin L, Adami HO, Weiderpass E. Prospective study of coffee consumption and all-cause, cancer, and cardiovascular mortality in Swedish women. Eur J Epidemiol. **2015**;30:1027-34.
66. Cornelis MC, Byrne EM, Esko T, Nalls MA, Ganna A, Paynter N, et al. Genome-wide meta-analysis identifies six novel loci associated with habitual coffee consumption. Mol Psychiatry. **2015**;20:647-56.
67. Cornelis MC, El-Sohemy A, Kabagambe EK, Campos H. Coffee, CYP1A2 genotype, and risk of myocardial infarction. J Am Med Assoc. **2006**;295:1135-41.
68. Salonen JT, Happonen P, Salonen R, Korhonen H, Nissinen A, Puska P, et al. Interdependence of associations of physical activity, smoking, and alcohol and coffee consumption with serum high-density lipoprotein and non-high-density lipoprotein cholesterol--a population study in eastern Finland. Prev Med. **1987**;16:647-58.
69. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. Nature. **2013**;500:585-8.
70. Anhe FF, Varin TV, Le Barz M, Desjardins Y, Levy E, Roy D, Marette A. Gut Microbiota Dysbiosis in Obesity-Linked Metabolic Diseases and Prebiotic Potential of Polyphenol-Rich Extracts. Curr Obes Rep. **2015**;4:389-400.
71. Wong JM. Gut microbiota and cardiometabolic outcomes: influence of dietary patterns and their associated components. Am J Clin Nutr. **2014**;100 Suppl 1:369S-77S.
72. Mills CE, Tzounis X, Oruna-Concha MJ, Mottram DS, Gibson GR, Spencer JP. In vitro colonic metabolism of coffee and chlorogenic acid results in selective changes in human faecal microbiota growth. Br J Nutr. **2015**;113:1220-7.
73. Neves AL, Chilloux J, Sarafian MH, Rahim MB, Boulange CL, Dumas ME. The microbiome and its pharmacological targets: therapeutic avenues in cardiometabolic diseases. Curr Opin Pharmacol. **2015**;25:36-44.
74. Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, et al. Low-grade inflammation, diet composition and health: Current research evidence and its translation. Br J Nutr. **2015**;114:999-1012.
75. Tomas-Barberan F, García-Villalba R, Quartieri A, Raimondi S, Amaretti A, Leonardi A, Rossi M. In vitro transformation of chlorogenic acid by human gut microbiota. Mol Nutr Food Res. **2013**;58:1122-31.
76. Ludwig IA, Paz de Peña M, Cid C, Crozier A. Catabolism of coffee chlorogenic acids by human colonic microbiota. BioFactors. **2013**;39:623-32.
77. Cowan TE, Palmnas MS, Yang J, Bomhof MR, Ardell KL, Reimer RA, et al. Chronic coffee consumption in the diet-induced obese rat: impact on gut microbiota and serum metabolomics. J Nutr Biochem. **2014**;25:489-95.

CHAPTER 4

ABSORPTION AND PHARMACOKINETICS OF PYRIDINES AFTER COFFEE AND COCOA-BASED CONFECTIONERY CONTAINING COFFEE CONSUMPTION IN A REPEAT DOSE 3-ARM CROSS-OVER HUMAN INTERVENTION STUDY.

This chapter reports the pharmacokinetics of trigonelline and N-methylpyridinium, and their principal metabolites, after specific dosages of coffee or cocoa-based product containing coffee. Moreover, the influence of gender and smoke are described.

Chapter 4: Absorption and pharmacokinetics of pyridines after coffee and cocoa-based confectionery containing coffee consumption in a repeat dose 3-arm cross-over human intervention study

4.1. Introduction

Coffee is one of the most important beverages consumed in the world, very popular in Europe, United States and basically all other countries [1]. An important number of systematic reviews and meta-analyses linked the regular consumption of coffee with several beneficial properties, as the reduction of type 2 diabetes, Alzheimer's disease [2] and cardiovascular disease development [3].

Coffee is a complex mixture containing dozens of potentially bioactive compounds, namely alkaloids, phenolic compounds, vitamins (especially B3), diterpenoid and melanoidin [4];[5];[1]. Among the most important and studied coffee alkaloids, besides caffeine, the betaine N-methyl nicotinic acid, namely trigonelline, is commonly recognized as the second most abundant [6]. Unlike caffeine, trigonelline is sensitive to heat treatments and it has been reported that part of the native trigonelline degrades during the roasting treatment, forming niacin and N-methylpyridinium [7]. Generally, in coffee brews, trigonelline amount ranges between 40 and 110 mg depending on the applied production process [8].

In general, coffee bioactive compounds appear in the human circulatory system after a relevant set of biological transformation occurring before and after absorption, and each single metabolite may exert different biological effects within the human body [9]; [4]; [5].

Trigonelline has been described in a great number of *in vitro* an *in vivo* (human and animal models) studies for its potential health benefits. In particular, it seems to be involved in the modulation of cell signaling and in the inhibition of cytokine release [10] and in the reduction of serum total cholesterol and triglycerides in rats [11]. In humans, plasmatic levels of this compound have been linked to an insulin level reduction in overweight men after the consumption of the compound [12].

The process of coffee roasting has been described as able to degrade trigonelline into N-methylpyridinium and niacin [13] in a time dependent way [7], with a content of N-

methylpyridinium in coffee estimated to be around 0.86 mg/g [14]. Additionally, in recent years, metabolomic studies identified N-methylpyridinium and trigonelline as novel markers of coffee intake, as in observational studies using untargeted approaches, plasma N-methylpyridinium and trigonelline resulted very effective in separating coffee drinkers from non-coffee drinkers [15]. The purpose of the present study was to assess the daily exposure to trigonelline and N-methylpyridinium, and to evaluate the pharmacokinetics of their principal metabolites, after specific dosages of coffee or cocoa-based product containing coffee (CBPCC).

4.2. Materials and Methods

4.2.1. Chemicals

Trigonelline hydrochloride, N-methylpyridinium iodate, nicotinamide, nicotinic acid, nicotinamide-N-oxide and methylnicotinamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trimethylamine N-oxide-d9 (TMAO-d9), used as internal standard (IS), was purchased by Cambridge Isotope Laboratories (Tewksbury, MA, USA). HPLC-grade solvents were purchased from VWR International (Radnor, Pennsylvania, USA). Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

4.2.2. Coffee and CBPCC extraction

Compounds present in coffee and CBPCC were extracted according to Sánchez-Salcedo and colleagues [16], making small changes depending on the matrix. The coffee samples, prepared with the coffee machine, were diluted in 3 fractions (1:5, 1:50 and 1:200) with H_2O acidified with formic acid (0.1%, v/v). The solution was centrifuged at 17,968 *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, dimeter 13 mm) in vial prior to analysis. Each sample was extracted in triplicate.

The CBPCC was firstly heated up to 40 °C for 10 minutes and dissolved in 25 mL of n-exane to eliminate the lipid fraction. Samples were vortexed for 5 minutes, centrifuged at 2647 *g* for 10 min at room temperature and the supernatant was eliminated. The purification step was repeated twice. The compounds were finally extracted from the purified pellet with 25 ml of 70% aqueous methanol acidified with formic acid (1% v/v). The solution was sonicated for 10 minutes, centrifuged at 2647 *g* for 10 min at room temperature and 1 mL of

the supernatant was collected and diluted 1:2 and 1:100 with H_2O acidified with formic acid (0.1%). The extract was centrifuged at 17,968 *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, dimeter 13 mm) in vial prior analysis. Each sample was extracted in triplicate.

4.2.3. Coffee and CBPCC quantification by liquid chromatography-mass spectrometry (UHPLC/MS)

Coffee and CBPCC extracts were analyzed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Separations were performed using a XSELECT HSS T3 (50x2.1 mm), 2.5 µm particle size (Waters, Ireland). Volume injection was 5 µL and column oven was set to 40°C. Compound elution was performed at a flow rate of 0.4 mL/min. The gradient started with 95% of 0.1% aqueous formic acid (solvent A) and 5% of acidified acetonitrile (0.1% formic acid, v/v) (solvent B). Isocratic conditions were maintained for 0.5 min, followed by a 5.5-min linear gradient to increase solvent B from 5% to 60%. Solvent B was then increased to 80% by a 1-min linear gradient and maintained for 1 min, prior re-establishing the start conditions to re-equilibrate the column in 3 min. For coffee and CBPCC samples, H-ESI-II worked with a capillary temperature of 275 °C, while the source heater temperature was set to 300 °C. The sheath gas flow was 60 units and auxiliary gas was 5 units. The source voltage was 5 kV, whereas the capillary and tube lens voltage were +3 and +45 V, respectively. Analyses were carried out using a full MS^2 method, monitoring specific parental ion, and fragmentation was obtained using a collision induced dissociation (CID) equal to 35 (arbitrary units). Pure helium gas was used for CID. Chromatograms and spectral data were acquired using XCalibur software 2.1 (Thermo Fisher Scientific Inc.) and quantification was performed with calibration curves built with the standard compounds.

4.2.4. Subjects

Twenty-one volunteers were recruited in Parma (PR) using announcements placed in university, hospital and public place. Inclusion criteria included being adult, healthy, of average weight (BMI 18-25), and regular coffee consumers of 1-5 cups per day. Exclusion criteria included clinically diagnosis for metabolic, renal or digestive disorder, regular

consumption of medication, antibiotic therapy taken within the last 3 months, intense physical activity (LAF ≥ 2,10 – LARN 2014), pregnancy or lactation, and regular intake of coffee exceeding 5 coffee/day. Volunteers were supplied with a single-serve machine and coffee capsule to standardize brewing method, raw material and cup volume.

4.2.5. Study Design

The study was approved by the Ethics Committee of the University of Parma on April 2017 (AZOSPR/0015693/6.2.2.) and registered on Clinical Trial (NCT03166540). All participants provided full written informed consent. The study followed a repeat-dose, 3-arm, crossover design. Randomization list was generated using Random Number Generator Pro (Segobit Software).

During a run-in period of 1 month, a total of 14 participants, paired by gender, were requested to follow their habitual diet and a regular intake of 1 (1-coffe group, i.e. 7 low coffee consumers) or 3 coffee capsule/day (3-coffee group, i.e. 7 high coffee consumers), whereas 7 volunteers were requested to consume 1 coffee capsule at breakfast and 2 cocoa-based product containing coffee two times per day (CBPCC group, i.e. medium coffee consumers). Minimal recommendation to avoid other sources of coffee and cocoa phytochemicals, besides what introduced through the assigned coffee/cocoa dose, were provided for the two days prior each sampling day and on the sampling day. Moreover, volunteers were asked to standardize the time of coffee and CBPCC consumption during the test period. During the sampling day, fasting blood was collected prior coffee consumption. Then, low and high coffee consumers drunk 1 or 3 coffees, respectively, while medium consumers consumed 1 coffee and 2 CBPSS twice during the day. After the consumption of the first coffee, together with a phytochemical-free breakfast, blood was collected at specific time points within 24 h. Blood sampling after the consumption of the first coffee was performed 0.5-1-2-3-4-5-6-7-8-9-24 h using EDTA tubes. Blood was immediately centrifuged at 1600 *g* for 15 min at 20 °C; plasma was collected and stored at -80°C until extraction.

4.2.6. Plasma extraction after coffee and CBPCC consumption

An aliquot of 100 µL of human plasma was added with 5 µL of TMAO-d9 as IS and protein fraction was precipitated with acetonitrile (1:10, v/v). Plasma was vortexed for 1

min, centrifuged at 13765 *g* for 10 min (room temperature) and supernatant was finally analyzed by UHPLC-ESI-MS.

4.2.7. Plasma analyses by UHPLC/MS

Analysis of plasma extract was performed using an Accela UHPLC 1250 equipped with linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc., San Jose, CA, USA). Separation was carried out by means of an XBridge® BEH HILIC (2.1 x 100mm, 2.5 µM particle size) (Waters, Milford, MA, USA). The mobile phase consisted of acetonitrile containing 0.1% formic acid as solvent A acidified water (0.1% formic acid, v/v) as solvent B and acidified ammonium formate (20 mmol/L) containing 1% of formic acid (v/v) as solvent C.

The gradient started with 10% C, which was maintained constant during the whole analysis, 89% A and 1% B. The mobile phases comprised a program of 0-1 min of 89% A and 1% B; 1-4 min linear gradient to reach 80% A and 10% B; 4-4.5 min to decrease solvent A from 80% to 27% and to increase solvent B from 10% to 63%. The gradient was maintained for 1 min and the starting conditions were re-established in 1.5 min and maintained for 3 min until the end of the analysis (10 min) to re-equilibrate the column. The flow rate was 0.5 mL/min, the injection volume was 5 µL, and the column temperature was set at 35 °C. The analytical mass spectrometric conditions were optimized by infusion of pure standard of trigonelline, working in positive ionization mode. The compounds analyzed were reported in the **Table 4.1**.

During the analysis, the H-ESI interface worked with a capillary temperature of 275°C. The source heater was set at 250 °C, the auxiliary gas flow (N_2) was set at 10 (arbitrary units) and the sheath gas flow (N_2) at 50. The source voltage was 3.8 kV, whereas the capillary voltage and tube lens voltage were +36 V and +75 V, respectively. Chromatograms and spectral data were acquired using XCalibur software 2.1 (Thermo Fisher Scientific Inc.) and quantification was performed with calibration curves built with the standard compounds.

Table 4.1. Overview on mass transition, chromatographic proprieties of the HILIC-HPLC-MS/MS method developed for the analysis of the pyridines.
*detected with the standard. [1]: retention time.

Compound	Rt[1] (min)	$[M]^+$ (m/z)	MS^2 (m/z)	References
Nicotinic acid*	0.77	124	**78**; 106	-
Nicotinamide*	0.80	123	**80**; 96	-
Nicotinamide-N-oxide*	1.17	139	**122**; 106	-
N-methylpyridinium*	2.37	94	**79**; 68	-
Methylnicotinamide*	2.67	137	**94**; 106	-
Trigonelline*	3.73	138	**92**; 94	-
N-methyl-4-pyridone-5-carboxamide	-	153	136	[17]
N-methyl-2-pyridone-5-carboxamide	-	153	136	[17]
N-methyl-2-pyridone-3-carboxamide	-	153	136	[17]
N-methyl-2-pyridone-5-carboxylic	-	154	126	[17]

4.2.8. Statistical analyses

Data were expressed as mean ± standard error of the mean (SEM). PKsolver add-on program was used to perform pharmacokinetic data analysis in Microsoft Excel [18]. A difference was considered significant at $p<0.05$. The statistical analysis was performed with the Statistical Package for Social Sciences software (IBM SPSSVR Statistics, Version 22.0. IBM Corp., Chicago, IL). Comparison among treatments was analyzed by repeated measurement General Linear Model using Greenhouse–Geisser correction if epsilon was lesser than 0.75 or Huynh–Feldt correction if epsilon was greater than 0.75 when the assumption of sphericity was violated, and Bonferroni post hoc tests for multiple comparisons.

A paired-sample t-test between t0 and t24 was performed for comparison within the same group before and after product consumption.

T-test were used for comparisons between groups (males/females and smokers/non-smokers).

4.3. Results

4.3.1. Coffee and CBPCC composition

The content of the main pyridines of each products used for the intervention study are reported in **Table 4.2**.

Table 4.2. Quantification (mg/serving) of pyridines in the products used for the 3 treatments. CBPCC (chocolate based product containing coffee). Coffee serving is considered 1 coffee (one espresso coffee corresponds to 40 mL in volume), CBPCC serving is considered 2 cocoa based products containing coffee.

Compound	Coffee (mg/serving)	CBPCC (mg/serving)
Nicotinic acid	0.45 ± 0.06	0.24 ± 0.02
N-methylpyridinium	4.02 ± 0.50	7.44 ± 0.43
Trigonelline	70.91 ± 3.23	2.03 ± 0.04

4.3.2. Subjects characteristics

All the volunteers recruited, completed the intervention study. In the **Table 4.3** were reported the characteristics of partecipants.

Table 4.3. Characteristics of participants in the study. Data are expressed as means ± SD.

Volunteers	21
Male	10
Female	11
Age (years)	25.9 ± 0.5
Height (m)	1.72 ± 0.02
Weight (kg)	66.5 ± 2.6
BMI (kg/m^2)	22.3 ± 0.5

4.3.3. Plasma absorption and pharmacokinetic parameters

Among the expected monitored metabolites possibly deriving from coffee consumption (**Table 4.1**)[19], only 3 compounds, namely trigonelline, N-methylpyridinium and methylnicotinamide, have been quantified in plasma samples. Other metabolites did not reach relevant concentrations during the test period (data not shown). Area under the curve

(AUC_{0-24}), average plasma concentration (C_{avg}), maximum plasma concentration (C_{max}), minimum plasma concentration (C_{min}), time to reach C_{max} (t_{max}) and time to reach C_{min} (t_{min}) are reported in the **Table 4.4** for trigonelline, N-methylpyridinium and methylnicotinamide.

Figure 4.1A compares the plasmatic appearance of trigonelline after the consumption of the 3 treatments. The basal plasmatic levels of trigonelline are statistically different among the treatments ($p<0.05$), depending on the different daily coffee intake during the run-in period of one month (3 coffee group: 1.10 ± 0.13 µmol/L, CBPCC group: 0.67 ± 0.08 µmol/L and 1 coffee group: 0.41 ± 0.06 µmol/L). C_{max} after 1 coffee-treatment (2.33 ± 0.02 µmol/L) was recorded 3 hours after coffee ingestion, with a C_{avg} of 0.98 ± 0.02 µmol/L. When the volunteers consumed 3 coffees, three consecutive increments at t3, t5 and t8 were highlighted. C_{max} for high consumers was calculated at 6.06 ± 0.11 µmol/L, whereas C_{avg} resulted 3.41 ± 0.06 µmol/L. When the subjects followed the CBPCC treatment, C_{max} for trigonelline was 2.75 ± 0.04 µmol/L, reached 4.91 ± 0.21 h after the first intake, and the C_{avg} was at 1.58 ± 0.02 µmol/L. After 24 hours, the plasmatic concentration of trigonelline returned to the basal level, with the same statistical differences among the treatments as observed at t0.

N-methylpyridinium (**Figure 4.1B**) also showed three different pharmacokinetic profiles among the treatments. At t0, no significant differences ($p>0.05$) in N-methylpyridinium levels among the treated groups. After the 1 coffee treatment Cmax (0.46 ± 0.01 µmol/L) was reported 1 hour after coffee consumption and a quick decrease was observed within 4 hours. When subjects followed 3 coffee and CBPCC treatments C_{max} (0.61 ± 0.01 and 0.82 ± 0.01 µmol/L, respectively) was at t4, i.e. one hour after the consumption of the second coffee or of the first praline. At t6 the subjects consumed the last coffee or the second daily chocolate product, and at t7 a third peak could be observed. At t0 and t24, there were no significant differences among the 3 treatments.

In **Figure 4.1C** the plasmatic concentration of methylnicotinamide after the 3 different treatments are reported. There were no significant statistical differences among the treatments. For this metabolite, the pharmacokinetic parameters were not influenced by the coffee dosage.

In **Figure 4.2** the plasmatic concentration of the 3 quantified metabolites in each treatment were represented, by dividing subjects between male and female (11 women and 10 men). N-methylpyridinium and methylnicotinamide did not show any statistical differences among the groups. On the contrary, trigonelline levels showed significant differences between male and female in each treatment (1 coffee fig. 2.A1, 3 coffees fig.

2.A2 and CBPCC fig. 2.A3). Finally, when subjects were divided into smokers and non smokers (**Figure 4.3**), no statistical differences appeared among the treatment for any of the metabolites.

Table 4.4. Plasma pharmacokinetic parameters of trigonelline, N-methylpyridinium and methylnicotinamide after ingestion of 1 coffee, 3 coffees and 1 coffee plus 4 CBPCC (chocolate based product containing coffee). Different letters in the same row indicate differences among the three treatment groups ($p < 0.05$).

	Pharmacokinetic parameters	1 coffee	3 coffees	CBPCC
Trigonelline	AUC_{0-24} (µmol/L*h)	23.64 ± 0.37^{c}	81.84 ± 1.48^{a}	37.80 ± 0.57^{b}
	C_{avg} (µmol/L)	0.98 ± 0.02^{c}	3.41 ± 0.06^{a}	1.58 ± 0.02^{b}
	C_{max} (µmol/L)	2.33 ± 0.03^{b}	6.06 ± 0.11^{a}	2.75 ± 0.04^{b}
	C_{min} (µmol/L)	0.30 ± 0.01^{c}	0.95 ± 0.03^{a}	0.56 ± 0.02^{b}
	t_{max} (h)	3.00 ± 0.03	8.41 ± 0.03	4.91 ± 0.21
	t_{min} (h)	0 ± 0	0 ± 0	0 ± 0
N-methylpyridinium	AUC_{0-24} (µmol/L*h)	2.28 ± 0.06^{b}	6.13 ± 0.07^{a}	8.72 ± 0.14^{a}
	C_{avg} (µmol/L)	0.09 ± 0.00^{b}	0.26 ± 0.00^{a}	0.36 ± 0.01^{a}
	C_{max} (µmol/L)	0.46 ± 0.01^{b}	0.61 ± 0.01^{a}	0.82 ± 0.01^{a}
	C_{min} (µmol/L)	0.01 ± 0.00	0.04 ± 0.00	0.07 ± 0.00
	t_{max} (h)	1.50 ± 0.06	3.41 ± 0.11	6.95 ± 0.21
	t_{min} (h)	0 ± 0	0 ± 0	0 ± 0
Methylnicotinamide	AUC_{0-24} (µmol/L*h)	15.06 ± 0.46	13.77 ± 0.36	16.39 ± 0.45
	C_{avg} (µmol/L)	0.63 ± 0.02	0.57 ± 0.01	0.68 ± 0.02
	C_{max} (µmol/L)	1.32 ± 0.04	1.15 ± 0.03	1.42 ± 0.04
	C_{min} (µmol/L)	0.30 ± 0.01	0.25 ± 0.01	0.28 ± 0.01
	t_{max} (h)	4.45 ± 0.08	4.98 ± 0.22	7.00 ± 0.34
	t_{min} (h)	0 ± 0	0 ± 0	0 ± 0

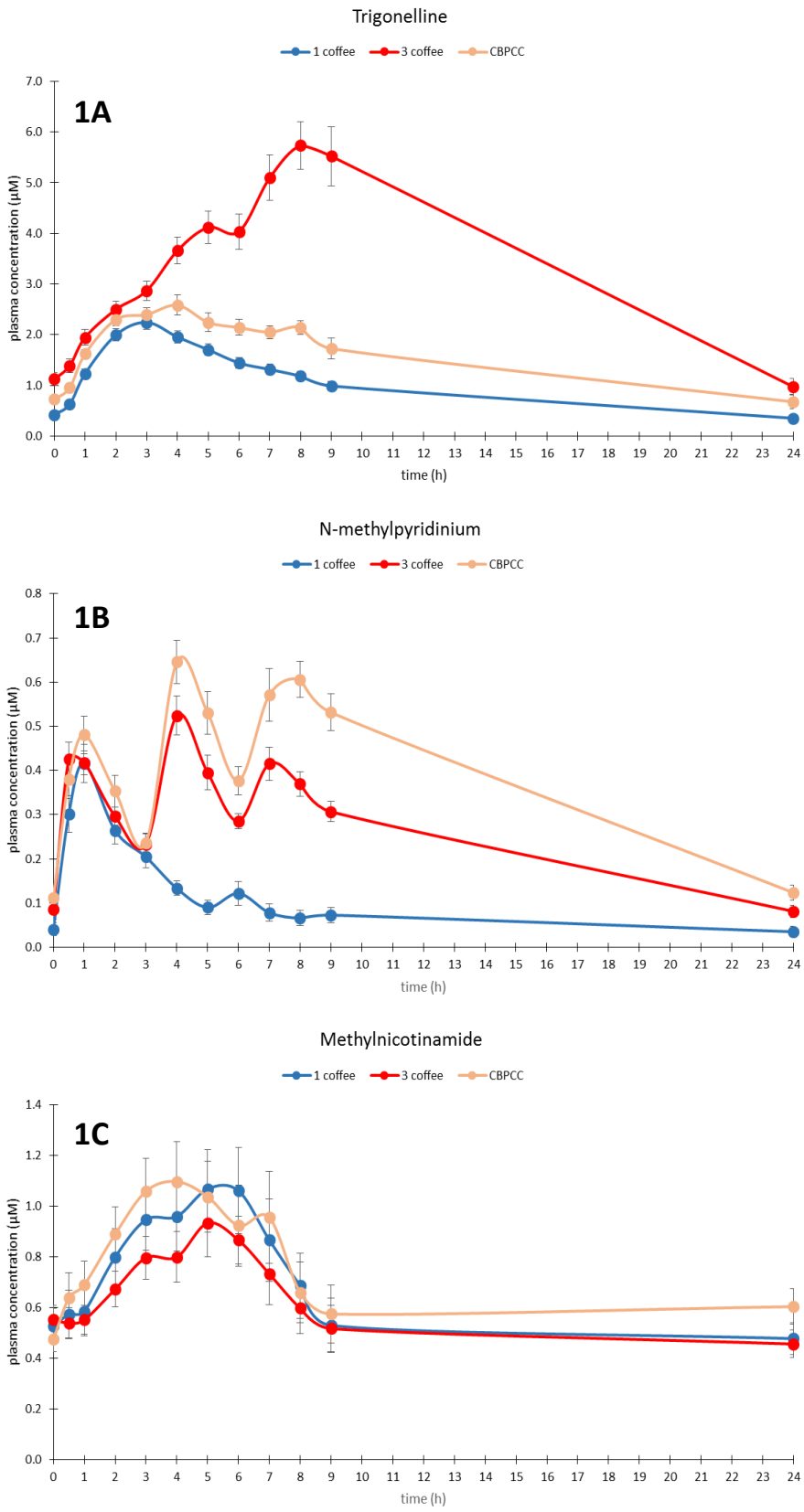

Figure 4.1. Plasma concentrations over time of trigonelline (1A), N-methylpyridinium (1B) and methylnicotinamide (1C) in all plasma samples collected after 1 coffee consumption (grey line), 3 coffee consumption (black line) and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption (dove grey). Data are expressed as mean ± SEM (n=21).

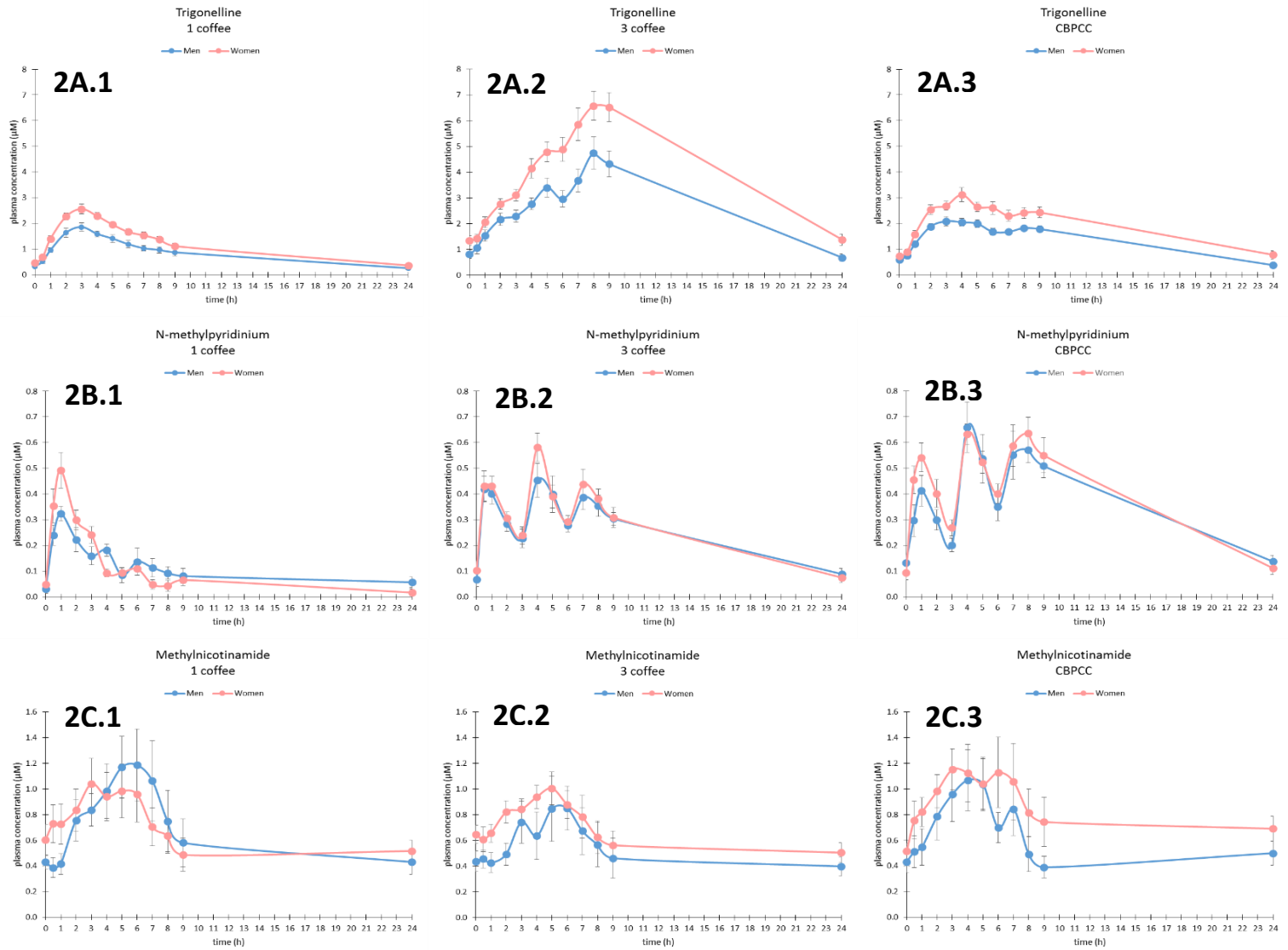

Figure 4.2. Time point of plasmatic concentration of trigonelline (2.A1, 2.A2 and 2.A3), N-methylpyridinium (2.B1, 2.B2 and 2.B3) and methylnicotinamide (2.C1, 2.C2 and 2.C3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and four cocoa-based product containing coffee (CBPCC) consumption and separated in women (black line) and men (dove grey line). Data expressed as mean ± SEM (men=10 and women=11).

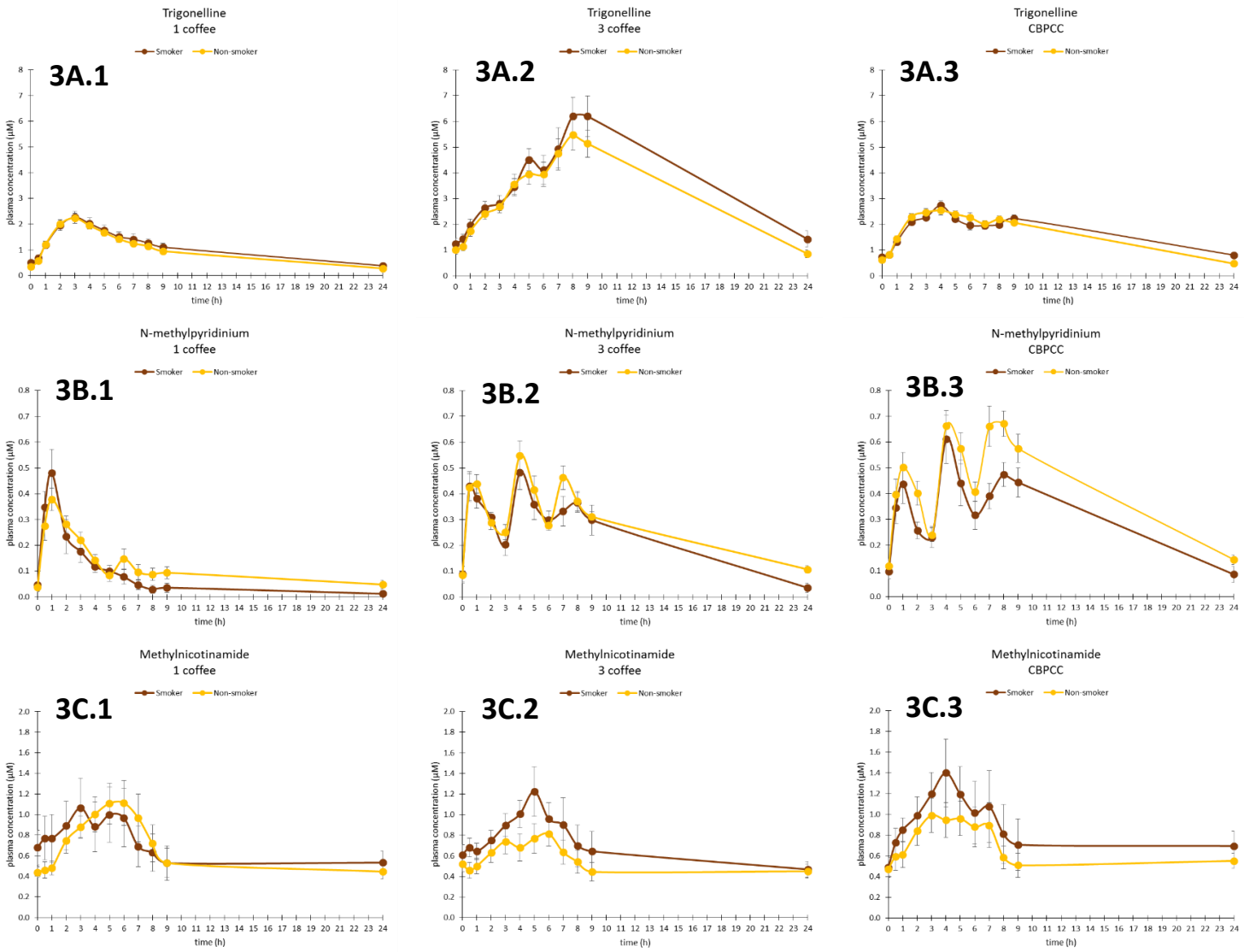

Figure 4.3. Time point of plasmatic concentration of trigonelline (3.A1, 3.A2 and 3.A3), N-methylpyridinium (3.B1, 3.B2 and 3.B3) and methylnicotinamide (3.C1, 3.C2 and 3.C3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and four cocoa-based product containing coffee (CBPCC) consumption and separated in smokers (black line) and non-smokers (dove grey line) within the 3 coffee treatments. Data are expressed as mean ± SEM (smokers=8 and non-smokers=13).

4.4. Discussion

This randomized cross-over intervention study evaluated the plasmatic concentration of the main pyridines of coffee, namely trigonelline, N-methylpyridinium and nicotinic acid, after specific coffee dosage consumption, in a real life setting.

Trigonelline rapidly appeared in plasma, 30 min after the consumption of the first coffee. This time of appearance is consistent with previous observations reporting the absorption of trigonelline in the stomach and in the small intestine, without phase II modification [19]. The results of an intervention study conducted by Lang, including six female and seven male volunteers who consumed 350 mL of a standard coffee beverage, showed a similar trend. The C_{max} in Lang' study was detected at 3.14 h [17], very close to what reported in the present study after the consumption of one coffee (T_{max} = 3.00 h). The plasmatic concentration of trigonelline reflected the coffee consumption, as there was a clear dose dependency in the definition of the average plasmatic concentration of trigonelline. Several in vitro studies endorsed trigonelline with specific biological effects, but the concentration used was by far exceeding what has been observed as average exposure in this work, making most of the conclusions drawn quite doubtful [20-23].

N-methylpyridinium was reported to be rapidly absorbed after coffee ingestion, reaching its C_{max} within 1 hour after coffee consumption [17], and the present study confirmed this observation. N-methylpyridinium has been reported to activate cellular means of detoxification [24] and to promote phase II-enzyme activity[25, 26]. As reported in **Table 4.4**, the CBPCC contained a higher amount of N-methylpyridinium with respect to coffee, this difference being reflected on the pharmacokinetic parameters of the CBPCC treatment, which led to a higher C_{max}, AUC_{0-24} and, consequently, C_{avg} compared to the 3 coffee treatment.

The plasmatic absorption profile reported for methylnicotinamide could be misleading. Although the increase in plasma concentration was concomitant with the consumption of the first coffee, the same effect was not evident after the consumption of the subsequent coffee or CBPCC. In this framework, Lang and colleagues reported that methylnicotinamide was almost unaffected by coffee consumption [17]. In contrast with previous studies which reported other pyridine metabolites after coffee consumption, namely N-methyl-4-pyridone-5-carboxamide and N-methyl-4-pyridone-5-carboxamide, in the present intervention study

these metabolites, together with nicotinic acid, nicotinamide and nicotinamide-N-oxide were not detected [17].

Finally, when volunteers were divided in male/female and smokers/non-smokers, the plasmatic trend of trigonelline showed significant differences depending on gender, with women showing higher circulating concentration for trigonelline and N-methylpyridinium compared to men, probably due to their different BMI, as explained by Lang et al.[17]. Smoking habits did not significantly influence the pharmacokinetics of any of the quantified metabolites.

4.5. Conclusion

This study established, for the first time, the daily exposure to trigonelline and N-methylpyridinium, after the consumption of different coffee dosages. The different coffee dosages were consumed at different hours to simulate a normal daily coffee consumption, considering low consumers (1 coffee per day) or high consumers (3 coffees per day). These results will be useful for future studies aiming at evaluating the bioactivity of coffee-derived circulating metabolites in cell experiments mimicking more realistic experimental conditions.

4.6. References

1. Ludwig IA, Clifford MN, Lean MEJ, Ashihara H, Crozier A: Coffee: biochemistry and potential impact on health. *Food Funct* **2014**, 5:1695-1717.
2. Ding M, Bhupathiraju SN, Satija A, van Dam RM, Hu FB: Long-Term Coffee Consumption and Risk of Cardiovascular Disease: A Systematic Review and a Dose-Response Meta-Analysis of Prospective Cohort Studies. *Circulation* **2013**, 129:643-659.
3. Grosso G, Godos J, Galvano F, Giovannucci EL: Coffee, Caffeine, and Health Outcomes: An Umbrella Review. *Annu Rev Nutr* **2017**, 37:131-156.
4. Rodriguez-Mateos A, Vauzour D, Krueger CG, Shanmuganayagam D, Reed J, Calani L, Mena P, Del Rio D, Crozier A: Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. *Archives of Toxicology* **2014**, 88:1803-1853.
5. Del Rio D, Rodriguez-Mateos A, Spencer JPE, Tognolini M, Borges G, Crozier A: Dietary (Poly)phenolics in Human Health: Structures, Bioavailability, and Evidence of Protective Effects Against Chronic Diseases. *Antioxidants & Redox Signaling* **2013**, 18:1818-1892.
6. Viani R, Horman I: THERMAL BEHAVIOR OF TRIGONELLINE. *Journal of Food Science* **1974**, 39:1216-1217.
7. Lang R, Yagar EF, Wahl A, Beusch A, Dunkel A, Dieminger N, Eggers R, Bytof G, Stiebitz H, Lantz I, Hofmann T: Quantitative Studies on Roast Kinetics for Bioactives in Coffee. *Journal of Agricultural and Food Chemistry* **2013**, 61:12123-12128.
8. Maeztu L, Andueza S, Ibanez C, Paz de Pena M, Bello J, Cid C: Multivariate methods for characterization and classification of espresso coffees from different botanical varieties and types of roast by foam, taste, and mouthfeel. *J Agric Food Chem* **2001**, 49:4743-4747.
9. Ludwig IA, Mena P, Calani L, Cid C, Del Rio D, Lean MEJ, Crozier A: Variations in caffeine and chlorogenic acid contents of coffees: what are we drinking? *Food Funct* **2014**, 5:1718-1726.
10. Morani AS, Bodhankar SL, Mohan V, Thakurdesai PA: Ameliorative effects of standardized extract from Trigonella foenum-graecum L. seeds on painful peripheral neuropathy in rats. *Asian Pacific Journal of Tropical Medicine* 2012, 5:385-390.

11. Zhou J, Zhou S, Zeng S: Experimental diabetes treated with trigonelline: Effect on β cell and pancreatic oxidative parameters. *Fundamental and Clinical Pharmacology* **2013**, 27:279-287.
12. van Dijk AE, Olthof MR, Meeuse JC, Seebus E, Heine RJ, van Dam RM: Acute Effects of Decaffeinated Coffee and the Major Coffee Components Chlorogenic Acid and Trigonelline on Glucose Tolerance. *Diabetes Care* **2009**, 32:1023-1025.
13. Lang R, Yagar EF, Eggers R, Hofmann T: Quantitative investigation of trigonelline, nicotinic acid, and nicotinamide in foods, urine, and plasma by means of LC-MS/MS and stable isotope dilution analysis. *J Agric Food Chem* **2008**, 56:11114-11121.
14. Lang R, Dieminger N, Beusch A, Lee Y-M, Dunkel A, Suess B, Skurk T, Wahl A, Hauner H, Hofmann T: Bioappearance and pharmacokinetics of bioactives upon coffee consumption. *Analytical and Bioanalytical Chemistry* **2013**, 405:8487-8503.
15. Rothwell JA, Madrid-Gambin F, Garcia-Aloy M, Andres-Lacueva C, Logue C, Gallagher AM, Mack C, Kulling SE, Gao Q, Praticò G, et al: Biomarkers of intake for coffee, tea, and sweetened beverages. *Genes and Nutrition* **2018**, 13.
16. Sánchez-Salcedo EM, Mena P, García-Viguera C, Hernández F, Martínez JJ: (Poly)phenolic compounds and antioxidant activity of white (Morus alba) and black (Morus nigra) mulberry leaves: Their potential for new products rich in phytochemicals. *Journal of Functional Foods* **2015**, 18:1039-1046.
17. Lang R, Wahl A, Skurk T, FiratYagar E, Schmiech L, Eggers R, Hauner H, Hofmann T: Development of a hydrophilic liquid interaction chromatography-high- performance liquid chromatography-tandem mass spectrometry based stable isotope dilution analysis and pharmacokinetic studies on bioactive pyridines in human plasma and urine after coffee consumption. *Analytical Chemistry* **2010**, 82:1486-1497.
18. Zhang Y, Huo M, Zhou J, Xie S: PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Computer Methods and Programs in Biomedicine* **2010**, 99:306-314.
19. Lang R, Fromme T, Beusch A, Lang T, Klingenspor M, Hofmann T: Raw coffee based dietary supplements contain carboxyatractyligenin derivatives inhibiting mitochondrial adenine-nucleotide-translocase. *Food and Chemical Toxicology* **2014**, 70:198-204.
20. Tohda C, Kuboyama T, Komatsu K: Search for Natural Products Related to Regeneration of the Neuronal Network. *Neurosignals* **2005**, 14:34-45.
21. Hossain SJ, Aoshima H, Koda H, Kiso Y: Effects of coffee components on the response of GABA(A) receptors expressed in Xenopus oocytes. *J Agric Food Chem* 2003, 51:7568-7575.
22. Temraz TA, Houssen WE, Jaspars M, Woolley DR, Wease KN, Davies SN, Scott RH: A pyridinium derivative from Red Sea soft corals inhibited voltage-activated potassium conductances and increased excitability of rat cultured sensory neurones. *BMC Pharmacol* **2006**, 6:10.
23. Satheeshkumar N, Mukherjee PK, Bhadra S, Saha BP: Acetylcholinesterase enzyme inhibitory potential of standardized extract of Trigonella foenum graecum L and its constituents. *Phytomedicine* **2010**, 17:292-295.
24. Boettler U, Sommerfeld K, Volz N, Pahlke G, Teller N, Somoza V, Lang R, Hofmann T, Marko D: Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression. *J Nutr Biochem* **2011**, 22:426-440.
25. Somoza V, Lindenmeier M, Wenzel E, Frank O, Erbersdobler HF, Hofmann T: Activity-guided identification of a chemopreventive compound in coffee beverage using in vitro and in vivo techniques. *J Agric Food Chem* **2003**, 51:6861-6869.
26. Boettler U, Volz N, Pahlke G, Teller N, Kotyczka C, Somoza V, Stiebitz H, Bytof G, Lantz I, Lang R, et al: Coffees rich in chlorogenic acid or N-methylpyridinium induce chemopreventive phase II-enzymes via the Nrf2/ARE pathway in vitro and in vivo. *Mol Nutr Food Res* **2011**, 55:798-802.

CHAPTER 5

PHARMACOKINETICS OF PHENOLIC METABOLITES AFTER COFFEE AND COCOA-BASED CONFECTIONERY CONTAINING COFFEE CONSUMPTION IN A REPEAT DOSE 3-ARM CROSS-OVER HUMAN INTERVENTION STUDY.

This chapter reports the pharmacokinetics of 7 metabolites derived from chlorogenic acids introduced after specific dosages of coffee or a cocoa-based product containing coffee. Moreover, the influence of gender and smoke are described.

Chapter 5: Pharmacokinetics of phenolic metabolites after coffee and cocoa-based confectionery containing coffee consumption in a repeat dose 3-arm cross-over human intervention study.

5.1. Materials and Methods

5.1.1. Chemicals

All chemicals and solvents were of analytical grade. 3-O-Caffeoylquinic acid (3-CQA), 4-O-caffeoylquinic acid (4-CQA) and 5-O-caffeoylquinic acid (5-CQA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ferulic acid-4'-*O*-sulphate, ferulic acid-4'-*O*-glucuronide, isoferulic acid-3'-*O*-sulphate, isoferulic acid-3'-*O*-glucuronide, dihydroisoferulic acid-3'-*O*-glucuronide, caffeic acid-4'-*O*-glucuronide and dihydrocaffeic acid-3'-*O*-sulphate were purchased from Toronto Research Chemicals (Toronto, ON, Canada). HPLC-grade solvents were purchased from VWR International (Radnor, Pennsylvania, USA). Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

5.1.2. Coffee and CBPCC extraction

Compounds present in coffee and CBPCC were extracted according to Sánchez-Salcedo and colleagues [1], making small changes depending on the matrix. The coffee samples, prepared with the coffee machine, were diluted in 3 fraction (1:5, 1:50 and 1:200) with H_2O acidified with formic acid (0.1%, v/v). The solution was centrifuged at 17,968 *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, dimeter 13 mm) in vial prior analysis. Each sample was extracted in triplicate.

The CBPCC was firstly heated up to 40 °C for 10 minutes and dissolved in 25 mL of n-exane to eliminate the lipid fraction. Samples were vortexed for 5 minutes, centrifuged at 2647 *g* for 10 min at room temperature and the supernatant was eliminated. The purification step was repeated twice. The compounds were finally extracted from the purified pellet with 25 ml of 70% aqueous methanol acidified with formic acid (1% v/v). The solution was sonicated for 10 minutes, centrifuged at 2647 *g* for 10 min at room temperature and 1 mL of

the supernatant was collected and diluted 1:2 and 1:100 with H_2O acidified with formic acid (0.1%). The extract was centrifuged at 17,968 *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, dimeter 13 mm) in vial prior analysis. Each sample was extracted in triplicate.

5.1.3. Coffee and CBPCC quantification by liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

Coffee and CBPCC extracts were analyzed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Separations were performed using a XSELECT HSS T3 (50x2.1 mm), 2.5 µm particle size (Waters, Ireland). Volume injection was 5 µL and column oven was set to 40°C. Compound elution was performed at a flow rate of 0.4 mL/min. The gradient started with 95% of 0.1% aqueous formic acid (solvent A) and 5% of acidified acetonitrile (0.1% formic acid, v/v) (solvent B). Isocratic conditions were maintained for 0.5 min, followed by a 5.5-min linear gradient to increase solvent B from 5% to 60%. Solvent B was then increased to 80% by a 1-min linear gradient and maintained for 1 min, prior re-establishing the start conditions to re-equilibrate the column in 3 min. For coffee and CBPCC samples H-ESI-II worked with a capillary temperature of 275 °C, while the source heater temperature was set to 300 °C. The sheath gas flow was 60 units and auxiliary gas was 5 units. The source voltage was 5 kV, whereas the capillary and tube lens voltage were -50 and -142.75 V, respectively. Analyses were carried out using a full MS^2 method, monitoring specific parental ion, and fragmentation was obtained using a collision induced dissociation (CID) equal to 35 (arbitrary units). Pure helium gas was used for CID. Chromatograms and spectral data were acquired using XCalibur software 2.1 (Thermo Fisher Scientific Inc.) and quantification was performed with calibration curves built with the standard compounds.

Table 5.1: Mass spectral characteristics of the main phenolic compounds in the coffee samples.

Compound	Ion.Mode	Rt[1] (min)	[M-H]$^-$ (*m/z*)	MS[2] (*m/z*)	MSI MI level[3]	Quantified as
3-CQA	-	3.18	353	**191**;179;135	1	3-CQA
4-CQA	-	3.86	353	**173**; 191; 179	1	4-CQA
5-CQA	-	3.76	353	**191**;179	1	5-CQA
3-FQA	-	3.72	367	**193**: 191	2	3-CQA
4-FQA	-	4.33	367	**173**; 193	2	4-CQA
5-FQA	-	4.70	367	**191**;173	2	5-CQA

Legend: *n*-CQA: *n*-*O*-caffeoylquinic acid; *n*-FQA: *n*-*O*-feruloylquinic acid. [1]Rt, retention time; [3]the fragment ions used for quantification are highlighted in bold; [3]Metabolite standards initiative (MSI) metabolite identification (MI) levels.

5.1.4. Subjects recruitment

Twenty-one volunteers were recruited in Parma (PR) using announcements placed in university, hospital and public place. Inclusion criteria included being adult, healthy, of average weight (BMI 18-25), and regular coffee consumers of 1-5 cups per day. Exclusion criteria included clinically diagnosis for metabolic, renal or digestive disorder, regular consumption of medication, antibiotic therapy taken within the last 3 months, intense physical activity (LAF $\geq$ 2,10 – LARN 2014), pregnancy or lactation, and regular intake of coffee exceeding 5 coffee/day. Volunteers were supplied with a single-serve machine and coffee capsule to standardize brewing method, raw material and cup volume.

5.1.5. Study Design

The study was approved by the Ethics Committee of the University of Parma on April 2017 (AZOSPR/0015693/6.2.2.) and registered on Clinical Trial (NCT03166540). All participants provided full written informed consent. The study followed a repeat-dose, 3-arm, crossover design. Randomization list was generated using Random Number Generator Pro (Segobit Software).

During a run-in period of 1 month, a total of 14 participants, paired by gender, were requested to follow their habitual diet and a regular intake of 1 (1-coffe group, i.e. 7 low coffee consumers) or 3 coffee capsule/day (3-coffee group, i.e. 7 high coffee consumers), whereas 7 volunteers were requested to consume 1 coffee capsule at breakfast and 2 cocoa-based product containing coffee two times per day (CBPCC group, i.e. medium coffee consumers). Minimal recommendation to avoid other sources of coffee and cocoa

phytochemicals, besides what introduced through the assigned coffee/cocoa dose, were provided for the two days prior each sampling day and on the sampling day. Moreover, volunteers were asked to standardize the time of coffee and CBPCC consumption during the test period. During the sampling day, fasting blood was collected prior coffee consumption. Then, low and high coffee consumers drunk 1 or 3 coffees, respectively, while medium consumers consumed 1 coffee and 2 CBPSS twice during the day. After the consumption of the first coffee, together with a phytochemical-free breakfast, blood was collected at specific time points within 24 h. Blood sampling after the consumption of the first coffee was performed 0.5-1-2-3-4-5-6-7-8-9-24 h using EDTA tubes. Blood was immediately centrifuged at 1600 *g* for 15 min at 20 °C; plasma was collected and stored at -80°C until extraction.

5.1.6. Plasma extraction after coffee and CBPCC consumption

Plasma samples of all volunteers were extracted using a solid phase extraction (SPE) method previously reported by Feliciano and colleagues [2]. Briefly, 350 µL of plasma samples were diluted with 350 µL of *o*-phosphoric acid 4% (v/v). After plate activation, 600 µL of the diluted plasma samples were loaded on a 96 well µ-SPE HLB plate (Oasis® HLB µElution Plate 30 µm, Waters, Milford, Massachusetts, MA, USA). Samples were then washed with 200 µL of 0.2% (v/v) acetic acid. Finally, samples were eluted with 60 µL o methanol for UHPLC-ESI-MS/MS analysis.

5.1.7. Plasma analyses by UHPLC-MS/MS

Analysis of plasma extract was performed using an UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (QqQ-MS/MS, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc., San Jose, CA, USA). Separation was carried out by means of a Kinetex EVO C18 (100 x 2.1 mm) column, 2.6 µm particle size (Phenomenex, Torrace, CA, USA). For UHPLC, mobile phase A was water containing 0.2% formic and mobile phase B was acid acetonitrile containing 0.2% formic acid in water. The gradient started with 5% A, isocratic conditions were maintained for 0.5 min, and reached 95% A after 6.5 min, followed by 1 min at 95%. The starting gradient was then immediately reestablished and maintained for 4 min to re-equilibrate the column. The flow rate was 0.4

mL/min, the injection volume was 5 µL, and the column temperature was set at 40 °C. The applied mass spectrometry (MS) method consisted in the selective determination of each target precursor ion by the acquisition of characteristic product ions in selective reaction monitoring (SRM) mode (**Table 5.2**), applying a negative ionization. To optimize the method, all the available standard compounds were infused into the MS to set the best mass parameters and to check the actual fragmentation patterns. Finally, for all the analyses, the spray voltage was set at 3 kV, the vaporizer temperature at 300 °C, and the capillary temperature operated at 270°C. The sheath gas flow was 60 units, and auxiliary gas pressure was set to 10 units. Ultrahigh purity argon gas was used for collision-induced dissociation (CID). The S-lens values were defined for each compound based on infusion parameter optimization (**Table 5.2**). Quantification was performed with calibration curves of standards (**Table 5.2**). Data processing was performed using Xcalibur software (Thermo Scientific Inc., Waltham, MA, USA). All data were expressed as mean values ± SEM Metabolite identification was carried out by comparing the retention time with authentic standards and MS/MS fragmentation patterns. Quantification were performed with calibration curve

During the analysis, the H-ESI interface worked with a capillary temperature of 275°C. The source heater was set at 250 °C, the auxiliary gas flow (N_2) was set at 10 (arbitrary units) and the sheath gas flow (N_2) at 50. The source voltage was 3.8 kV, whereas the capillary voltage and tube lens voltage were +36 V and +75 V, respectively. Chromatograms and spectral data were acquired using XCalibur software 2.1 (Thermo Fisher Scientific Inc.) and quantification was performed with calibration curves built with the standard compounds.

Table 5.2. Overview on mass transition, chromatographic proprieties of the HILIC-HPLC-MS/MS method developed for the analysis of the pyridines.

Compound	Rt[1] (min)	$[M-H]^-$	SRM transition	S-Lens
Ferulic acid-4'-*O*-sulphate	4.46	273	**193**; 134; 178	92
Ferulic acid-4'-*O*-glucuronide	3.75	369	**193**; 178; 175	92
Isoferulic acid-3'-*O*-sulphate	4.90	273	**193**; 134; 178	92
Isoferulic acid-3'-*O*-glucuronide	4.32	369	**193**; 178; 175	92
Dihydroisoferulic acid-3'-*O*-glucuronide	4.23	371	**195**; 113	90
Caffeic acid-4'-*O*-glucuronide	2.61	355	**179**; 135	87
Dihydrocaffeic acid-3'-*O*-sulphate	4.31	261	**181**; 137	96

Legend: [3]the fragment ions used for quantification are highlighted in bold

5.1.8. Statistical analyses

Data were expressed as mean ± standard error of the mean (SEM). PKsolver add-on program was used to perform pharmacokinetic data analysis in Microsoft Excel [3]. A difference was considered significant at $p<0.05$. The statistical analysis was performed with the Statistical Package for Social Sciences software (IBM SPSSVR Statistics, Version 22.0. IBM Corp., Chicago, IL). Comparison among treatments were analyzed by repeated measurement General Linear Model using Greenhouse–Geisser correction if epsilon was lesser than 0.75 or Huynh–Feldt correction if epsilon was greater than 0.75 when the assumption of sphericity was violated, and Bonferroni post hoc tests for multiple comparisons.

A paired-sample t-test between t0 and t24 was performed for comparison within the same group before and after product consumption.

T-test were used for comparisons between groups (males/females and smokers/non-smokers).

5.2. Results

5.2.1. Coffee and CBPCC composition

The content of the main phenolic compounds of each products used for the intervention study are reported in **Table 5.3**.

Table 5.3. Quantification (mg/serving) of the main phenolic compounds in the products used for the 3 treatments. CBPCC (chocolate based product containing coffee). Coffee serving is considered 1 coffee (one espresso coffee corresponds to 40 mL in volume), CBPCC serving is considered 2 cocoa based products containing coffee.

Compound	Coffee (mg/serving)	CBPCC (mg/serving)
Phenolic compounds	55.90 ± 0.76	11.66 ± 0.25

5.2.2. Subjects characteristics

All the volunteers recruited, completed the intervention study. In the **Table 5.4** were reported the characteristics of partecipants.

Table 5.4. Characteristics of participants in the study. Data are expressed as means ± SD.

Volunteers	21
Male	10
Female	11
Age (years)	25.9 ± 0.5
Height (m)	1.72 ± 0.02
Weight (kg)	66.5 ± 2.6
BMI (kg/m^2)	22.3 ± 0.5

5.2.3. Plasma absorption and pharmacokinetic parameters

The phenolic compounds selectively analysed in plasma samples were ferulic acid 4'-*O*-sulphate (F4S), ferulic acid4'-*O*-glucuronide (F4G), isoferulic acid 3'-*O*-sulphate (isF3S), isoferulic acid 3'-*O*-glucuronide (isF3G), dihydroisoferulic acid 3'-*O*-glucuronide (DisF3G), caffeic acid 4'-*O*-glucuronide (C4G) and dihydrocaffeic acid-*O*-sulphate (DCS). The pharmacokinetic profiles of quantified compounds are illustrated in **Figures 5.1, 5.2** and **5.3**. The pharmacokinetics characteristics for each quantified metabolite, including area under the curve from t_0 to t_{24} (AUC_{0-24}), average concentration (C_{avg}), peak plasma concentration (C_{max}), minimum concentration (C_{min}), and the time when the maximum concentration was reached (T_{max}) are reported in the **Table 5.5**.

No statistically significant differences were found among the t0 of each treatment for each metabolite analysed and, at the same time, no statistically significant differences were found among treatments at t24. The highest post-ingestion C_{max} values were detected after consumption of three coffees, except for DCS, that showed the highest C_{max} after CBPCC intake.

Table 5.5. Pharmacokinetics parameters of ferulic acid-4'-*O*-sulphate (F4S), ferulic acid-4'-O-glucuronide (F4G), isoferulic acid-3'-*O*-sulphate (isF3S), isoferulic acid-3'-*O*-glucuronide (isF3G), dihydroisoferulic acid-3'-O-glucuronide (DisFG), caffeic acid-4'-*O*-glucuronide (C4G) and dihydrocaffeic acid-*O*-sulphate after ingestion of 1 coffee, 3 coffee and 1 coffee plus 4 CBPCC (chocolate based product containing coffee). Data expressed as mean ± SEM (n=21). Different letters in the same row indicate differences among the three treatment groups.

		1 coffee	**3 coffee**	**CBPCC**	***p.value***
F4S	AUC_{0-24} (nmol/L*h)	79.80 ± 13.70	137.35 ± 24.11	111.51 ± 27.19	>0.05
	C_{avg} (nM)	3.33 ± 0.57	5.72 ± 1.00	4.63 ± 1.13	>0.05
	C_{max} (nM)	14.67 ± 2.29	18.53 ± 2.46	14.41 ± 2.61	>0.05
	C_{min} (nM)	0.42 ± 0.12	0.66 ± 0.16	0.34 ± 0.09	>0.05
	t_{max} (h)	4.14 ± 1.16	4.88 ± 0.66	4.83 ± 1.16	
F4G	AUC_{0-24} (nmol/L*h)	1966.98 ± 263.75^{b}	2957.05 ± 351.48^{a}	1657.49 ± 260.32^{b}	**<0.05**
	C_{avg} (nM)	81.96 ± 10.99^{b}	123.21 ± 14.64^{a}	69.06 ± 10.85^{b}	**<0.05**
	C_{max} (nM)	179.19 ± 17.54^{b}	312.22 ± 36.10^{a}	214.49 ± 39.96^{ab}	**<0.05**
	C_{min} (nM)	30.63 ± 7.96	19.16 ± 5.78	15.55 ± 6.11	>0.05
	t_{max} (h)	4.64 ± 0.68	6.50 ± 0.41	5.13 ± 0.63	
isF3S	AUC_{0-24} (nmol/L*h)	5.69 ± 0.76^{b}	9.41 ± 1.04^{a}	7.00 ± 1.32^{ab}	**<0.05**
	C_{avg} (nM)	0.24 ± 0.03^{b}	0.39 ± 0.04^{a}	0.29 ± 0.06^{ab}	**<0.05**
	C_{max} (nM)	1.14 ± 0.17	1.39 ± 0.12	1.33 ± 0.21	>0.05
	C_{min} (nM)	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	>0.05
	t_{max} (h)	4.38 ± 1.16	5.50 ± 1.11	3.33 ± 0.55	
isF3G	AUC_{0-24} (nmol/L*h)	517.17 ± 73.28^{b}	1253.81 ± 116.02^{a}	607.26 ± 83.51^{b}	**<0.001**
	C_{avg} (nM)	21.55 ± 3.05^{b}	52.24 ± 4.83^{a}	25.28 ± 3.48^{b}	**<0.001**
	C_{max} (nM)	69.75 ± 7.43^{b}	121.57 ± 9.16^{a}	67.37 ± 6.31^{b}	**<0.001**
	C_{min} (nM)	3.45 ± 1.72	5.19 ± 1.27	2.66 ± 1.35	>0.05
	t_{max} (h)	1.33 ± 0.20	6.14 ± 0.37	3.33 ± 0.56	
DisFG	AUC_{0-24} (nmol/L*h)	418.54 ± 78.12	492.58 ± 63.47	357.40 ± 76.06	>0.05
	C_{avg} (nM)	17.44 ± 3.25	20.52 ± 2.64	14.89 ± 3.17	>0.05
	C_{max} (nM)	42.16 ± 7.19	39.11 ± 5.38	33.61 ± 5.90	>0.05
	C_{min} (nM)	2.15 ± 0.76	5.85 ± 1.23	2.13 ± 0.83	>0.05
	t_{max} (h)	8.02 ± 1.32	9.07 ± 1.45	7.48 ± 0.983	
C4G	AUC_{0-24} (nmol/L*h)	12.77 ± 1.79	23.38 ± 3.96	11.99 ± 3.69	>0.05
	C_{avg} (nM)	0.53 ± 0.07	0.97 ± 0.17	0.50 ± 0.15	>0.05
	C_{max} (nM)	1.97 ± 0.27	2.69 ± 0.35	1.88 ± 0.38	>0.05
	C_{min} (nM)	0.10 ± 0.04	0.09 ± 0.04	0.02 ± 0.02	>0.05
	t_{max} (h)	3.26 ± 1.18	5.26 ± 0.47	3.43 ± 0.65	
DCS	AUC_{0-24} (nmol/L*h)	202.98 ± 69.32	173.16 ± 47.66	297.31 ± 109.34	>0.05
	C_{avg} (nM)	8.46 ± 2.89	7.22 ± 1.99	12.39 ± 4.56	>0.05
	C_{max} (nM)	31.88 ± 8.19	25.75 ± 6.62	43.46 ± 14.36	>0.05
	C_{min} (nM)	1.18 ± 1.03	0.27 ± 0.17	0.06 ± 0.04	>0.05
	t_{max} (h)	7.55 ± 1.34	5.93 ± 0.72	6.50 ± 1.10	

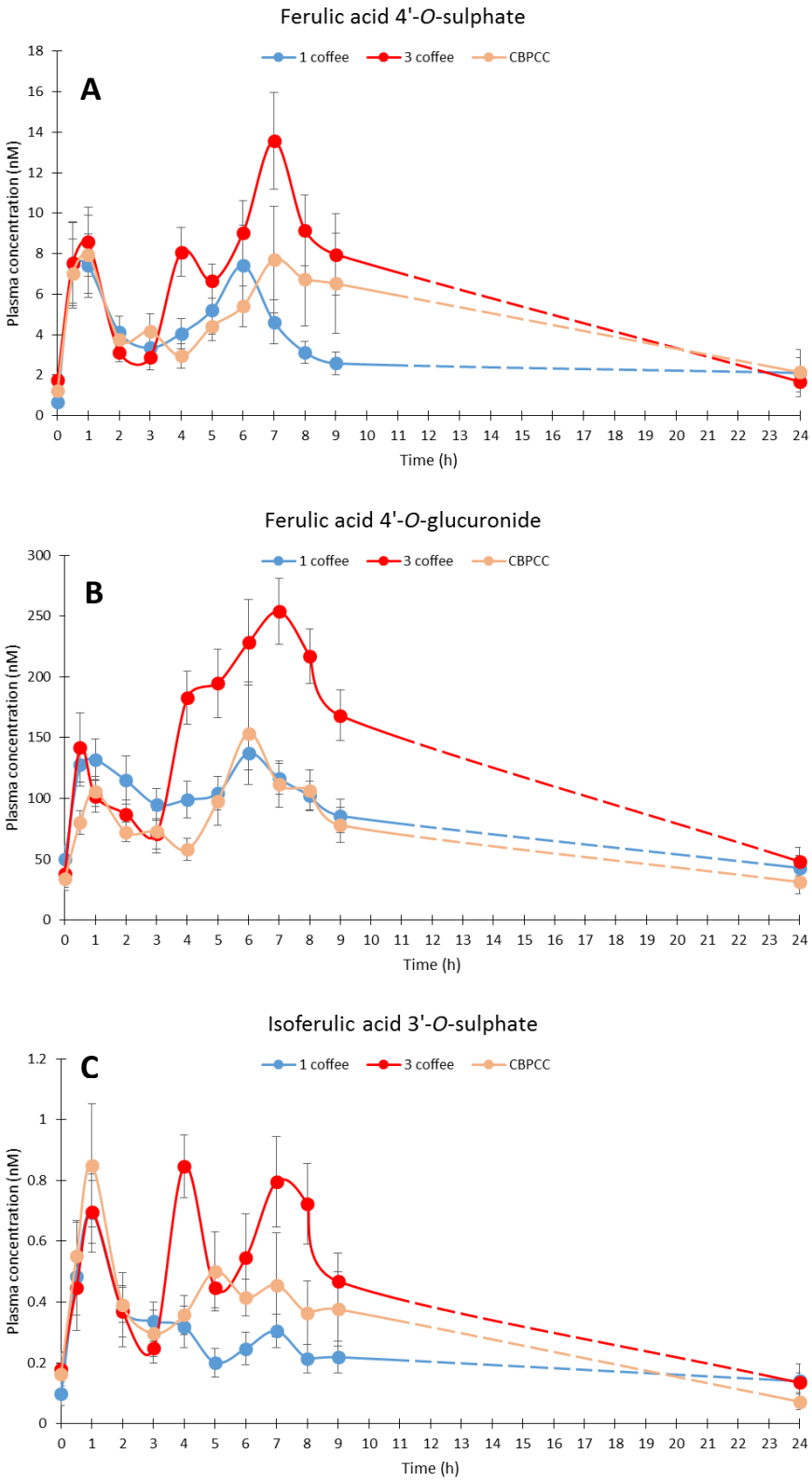

Figure 5.1. Plasma concentrations over time of ferulic acid 4'-*O*-sulphate (A), ferulic acid 4'-*O*-glucuronide (B) and isoferulic acid 3'-*O*-sulphate (C), in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption. Data expressed as mean ± SEM (n=21).

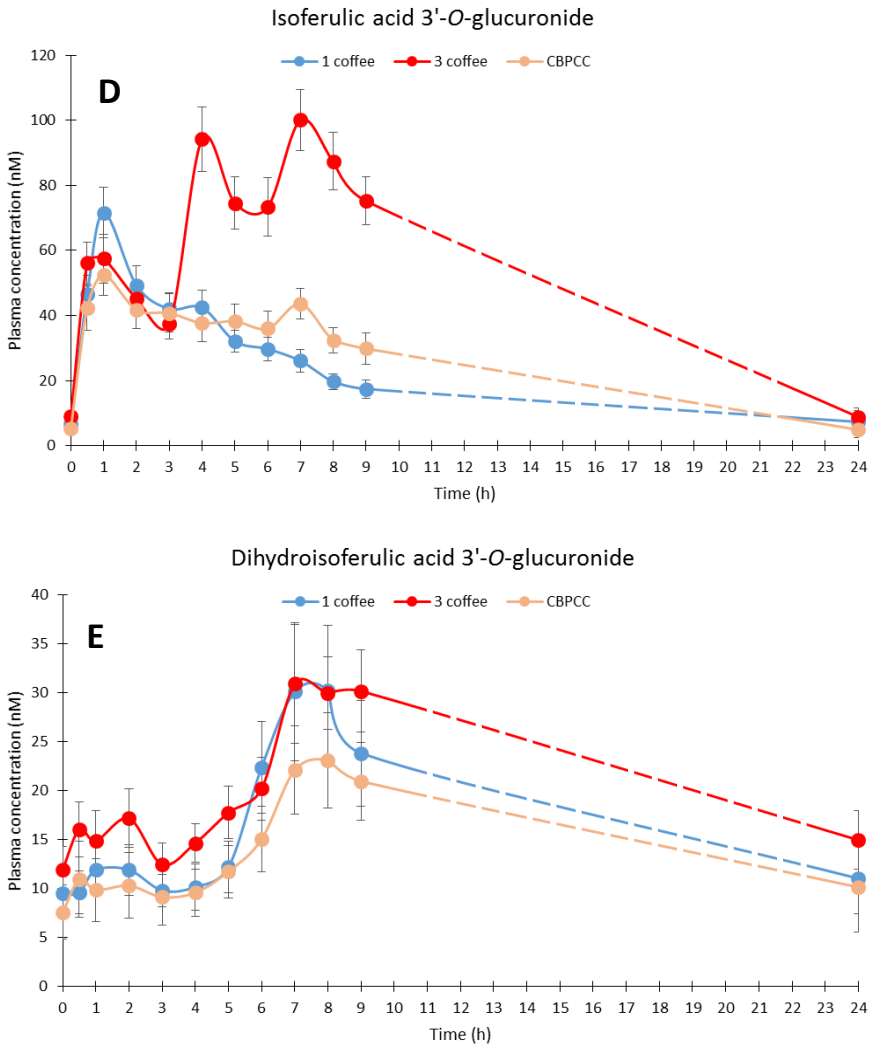

Figure 5.2. Plasma concentrations over time of isoferulic acid 3'-*O*-glucuronide (D) and dihydroferulic acid 3'-*O*-glucuronide (E) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption. Data expressed as mean ± SEM (n=21).

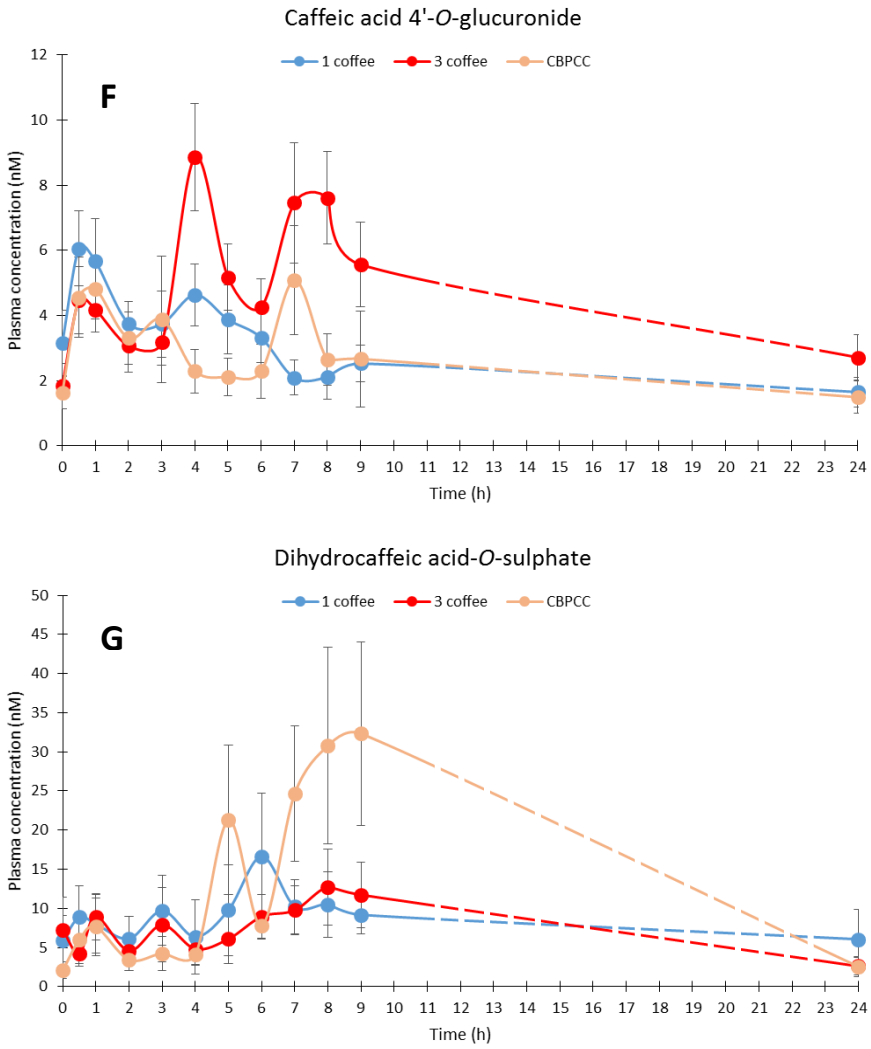

Figure 5.3. Plasma concentrations over time of caffeic acid 4'-*O*-glucuronide (F) and dihydrocaffeic acid-3'-*O*-sulphate (G) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption. Data expressed as mean ± SEM (n=21).

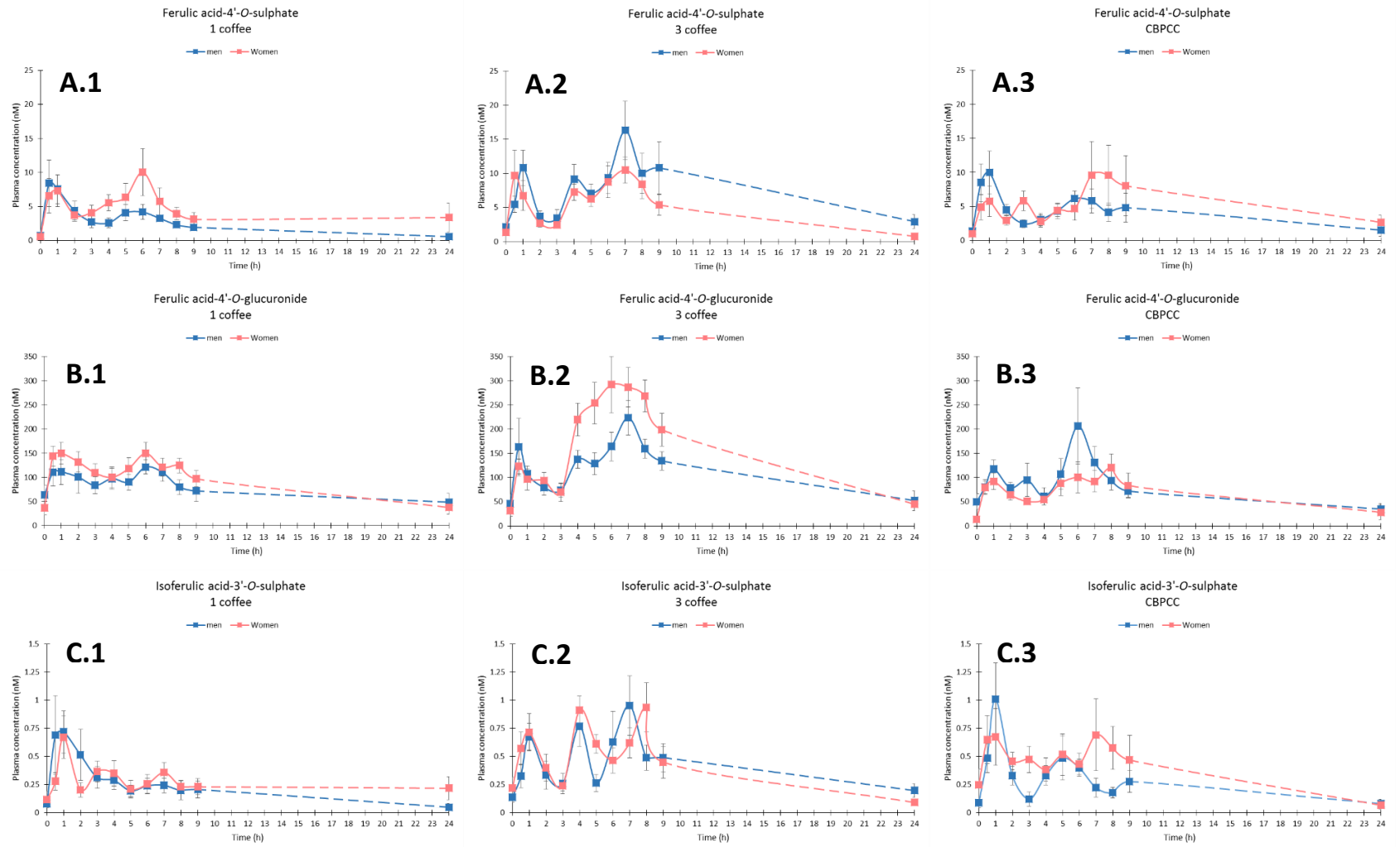

Figure 5.4. Time-points of plasmatic concentration of ferulic acid 4'-*O*-sulphate (A.1, A.2 and A.3), ferulic acid 4'-*O*-glucuronide (B.1, B.2 and B.3) and isoferulic acid 3'-*O*-sulphate (C.1, C.2 and C.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in women and men. Data expressed as mean ± SEM (men=10 and women=11).

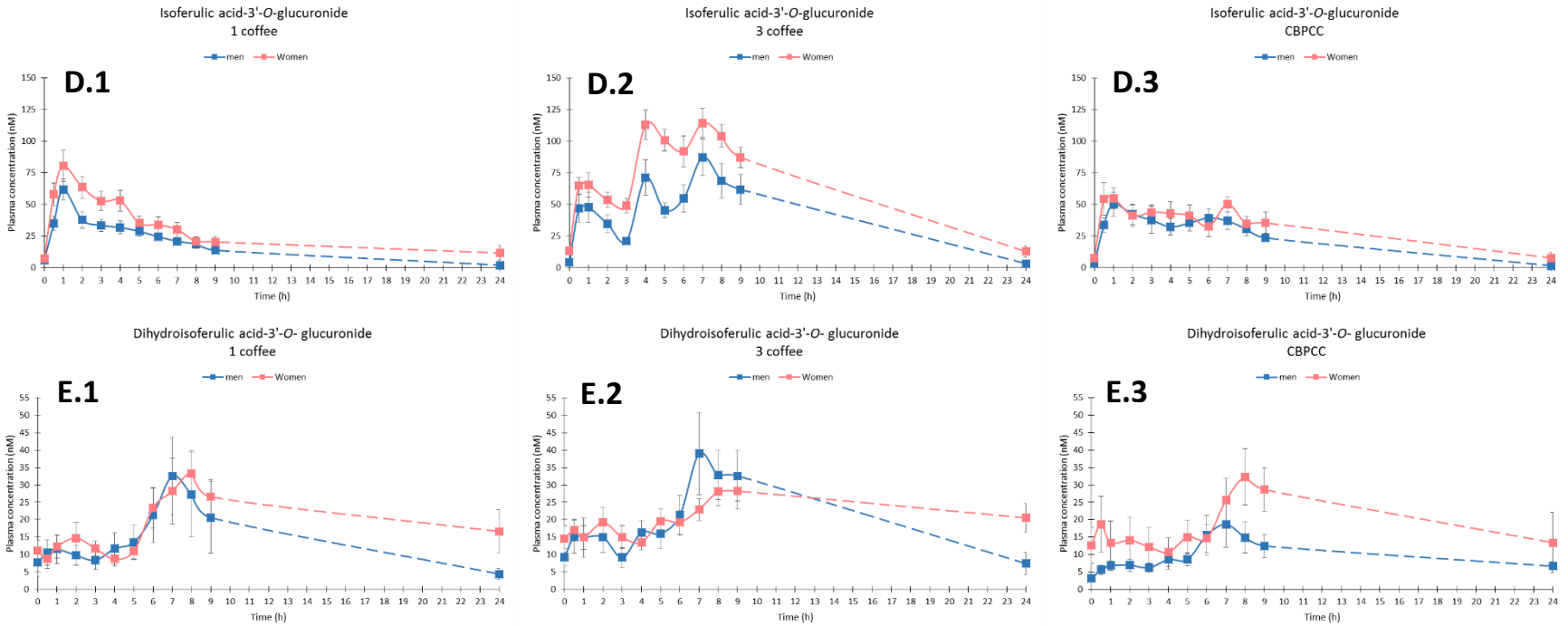

Figure 5.5. Time-points of plasmatic concentration of isoferulic acid 3'-*O*-glucuronide (D.1, D.2 and D.3) and dihydroferulic acid 3'-*O*-glucuronide (E.1, E.2 and E.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in women and men. Data expressed as mean ± SEM (men=10 and women=11).

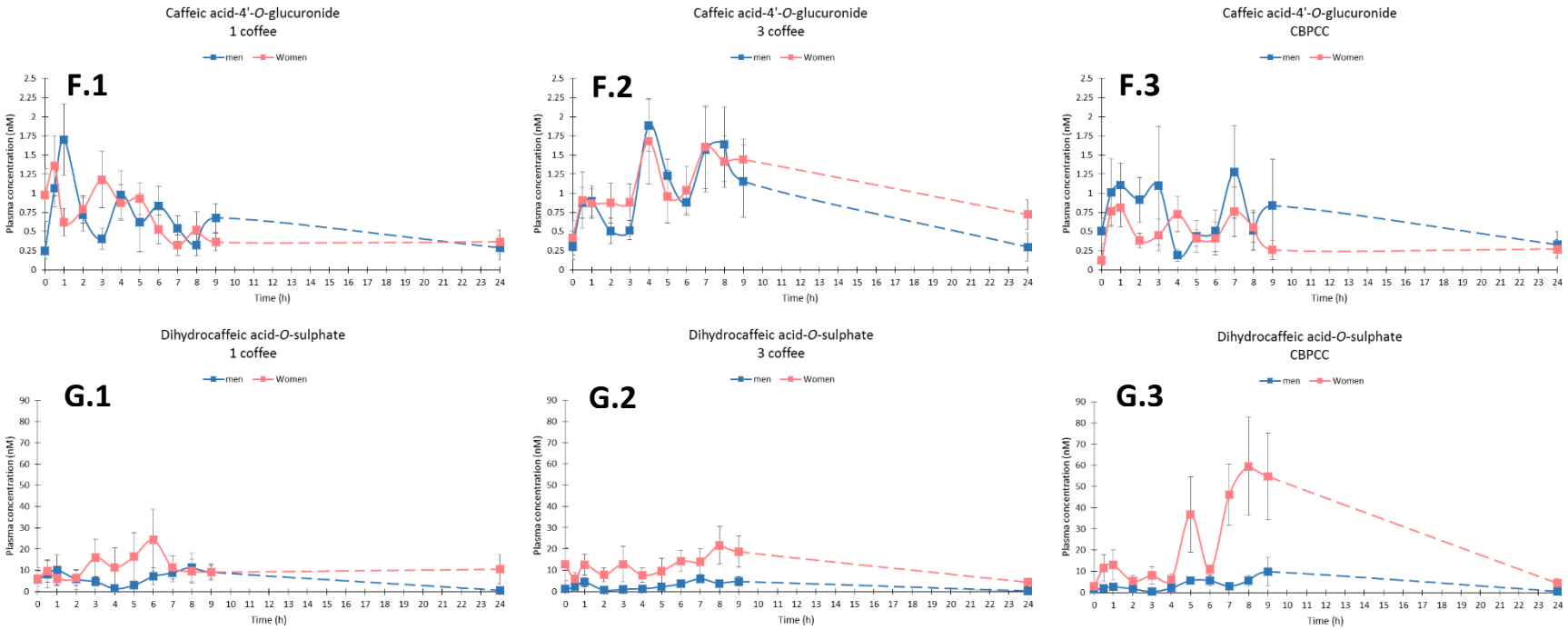

Figure 5.6. Time-points of plasmatic concentration of caffeic acid 4'-*O*-glucuronide (F.1, F.2 and F.3) and dihydrocaffeic acid-*3'*-*O*-sulphate (G.1, G.2 and G.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in women and men. Data expressed as mean ± SEM (men=10 and women=11).

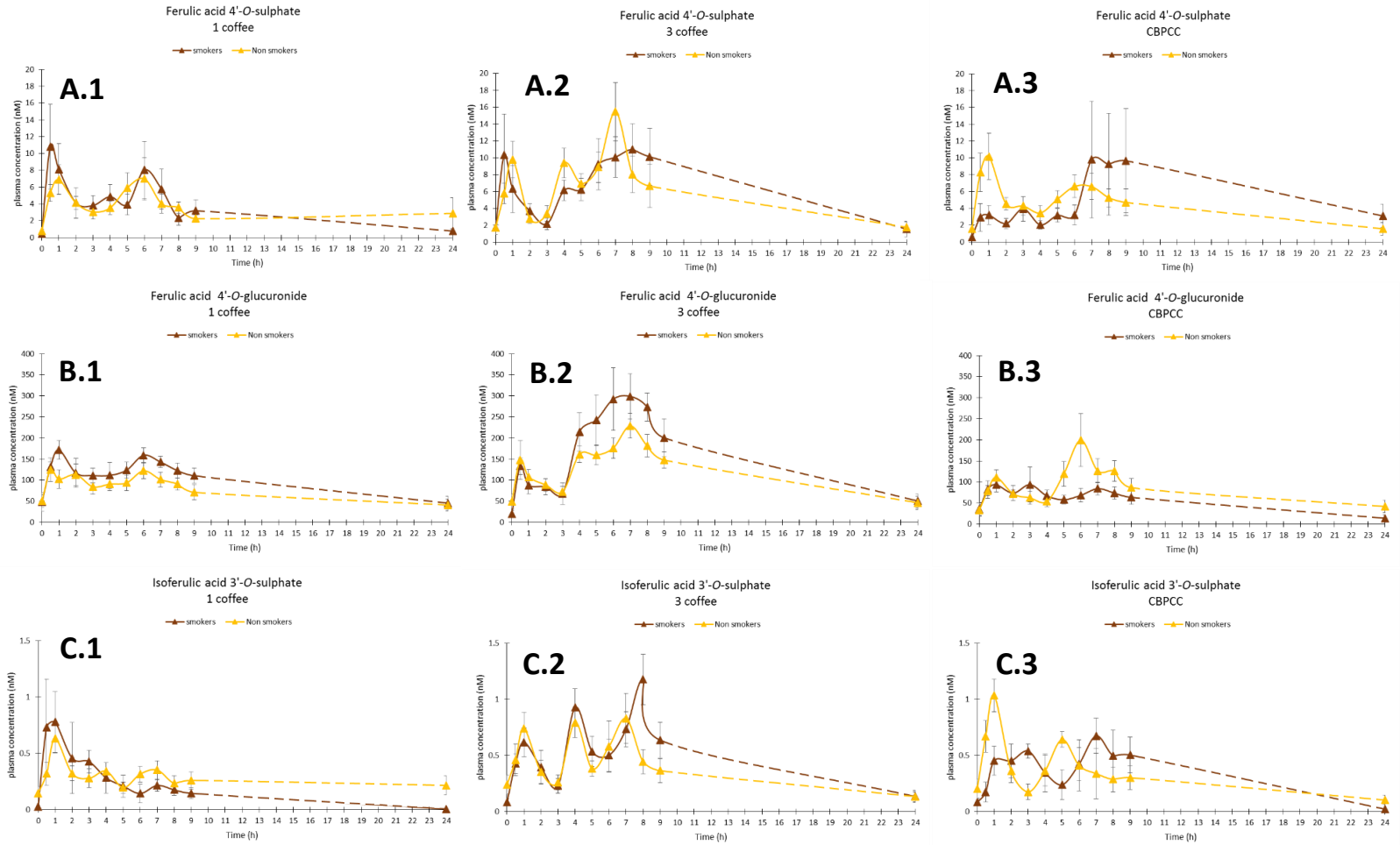

Figure. 5.7. Time-points of plasma concentration of ferulic acid 4'-O-sulphate (A.1, A.2 and A.3), ferulic acid 4'-O-glucuronide (B.1, B.2 and B.3) and isoferulic acid 3'-O-sulphate (C.1, C.2 and C.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in smokers and non-smokers. Data expressed as mean ± SEM (smokers=8 and non-smokers=13).

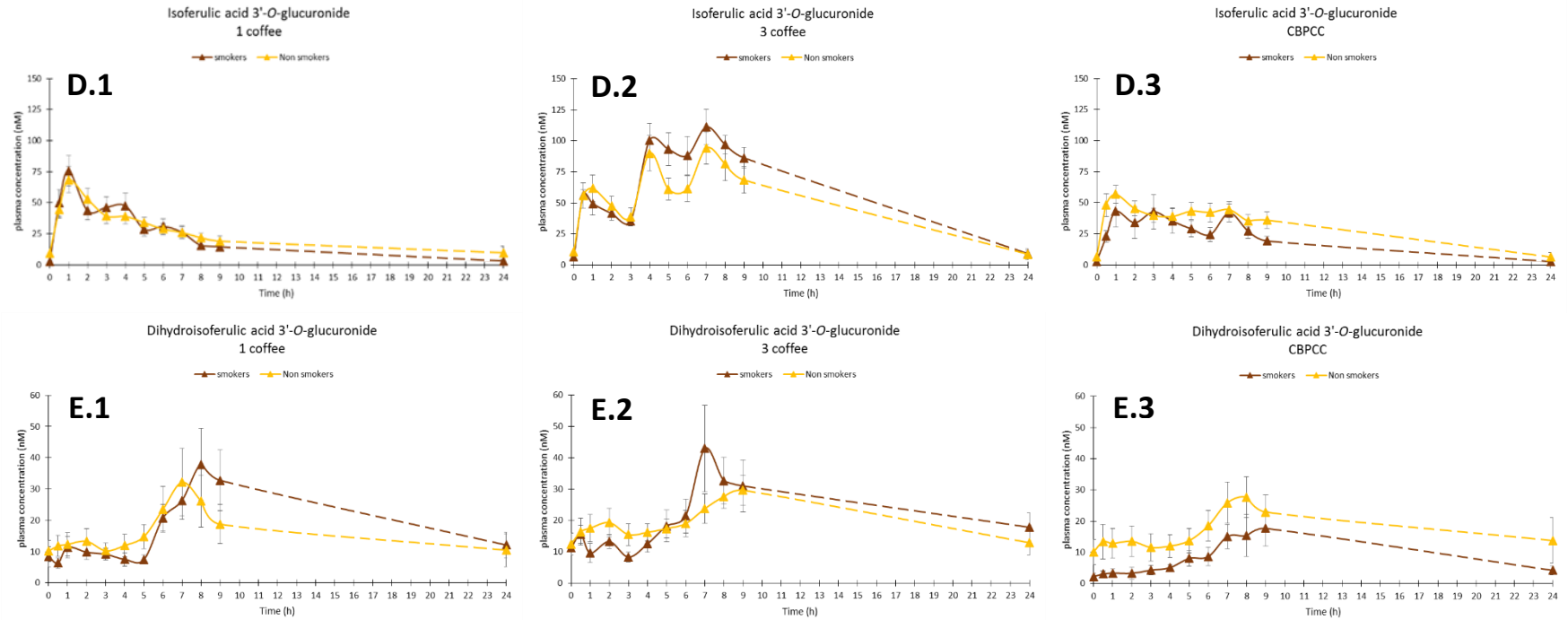

Figure 5.8. Time-points of plasma concentration of isoferulic acid 3'-*O*-glucuronide (D.1, D.2 and D.3), dihydroisoferulic acid 3'-*O*-glucuronide (E.1, E.2 and E.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in smokers and non-smokers. Data expressed as mean ± SEM (smokers=8 and non-smokers=13).

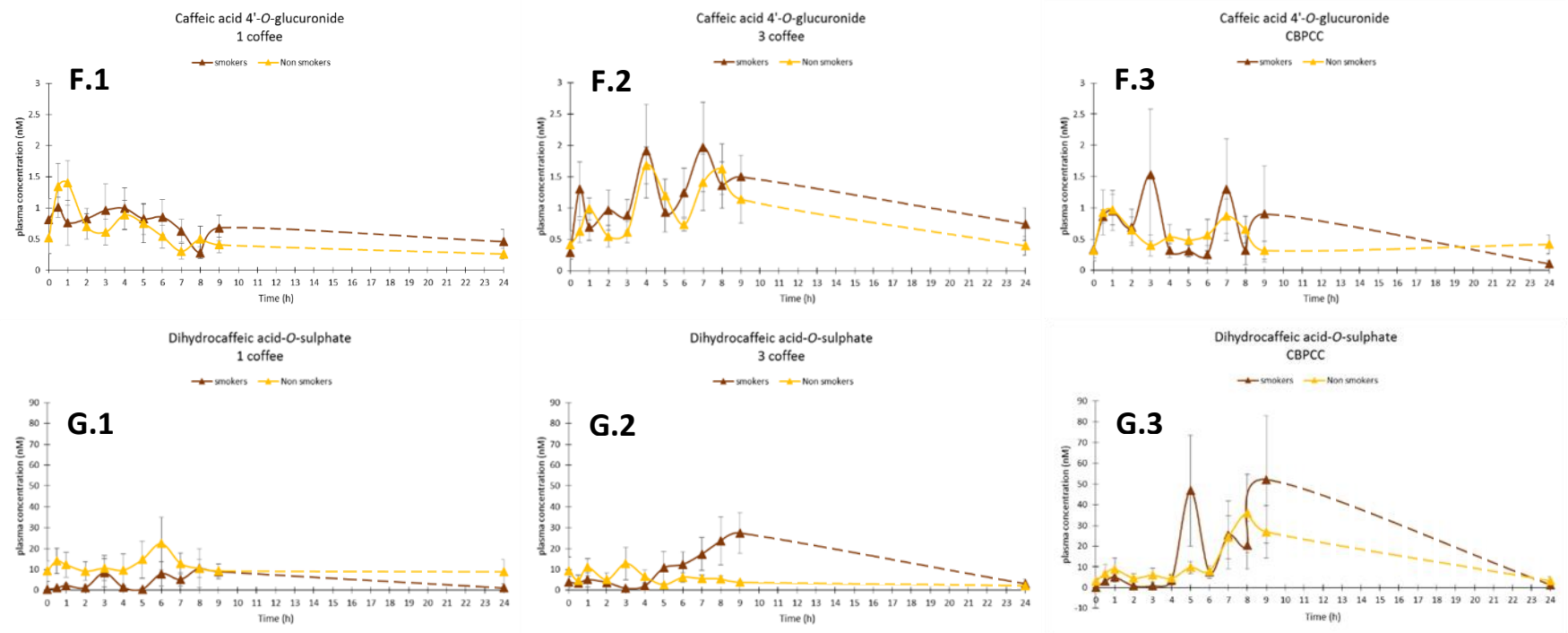

Figure 5.9. Time-points of plasma concentration of caffeic acid 4'-*O*-glucuronide (F.1, F.2 and F.3), dihydrocaffeic acid-*O*-sulphate (G.1, G.2 and G.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in smokers and non-smokers. Data expressed as mean ± SEM (smokers=8 and non-smokers=13).

5.3. Discussion

The most relevant observations that could be made after this intervention study with regards to phenolic compounds are:

- The maximum concentrations found in plasma after the intake of the highest dose of coffee phenolics were reached on average at 6.18 hours after the first dose. Summing the concentrations of all phenolic metabolites at the tmax a maximum concentration equal to 411.82 nM was reached, and this should be taken into account when in vitro stuydies are carried out in order to evaluate the effects of coffee phenolic metabolites on health or disease-related mechanisms in cells. The average daily concentrations of the main metabolites range from 133.81 nM to 210.27 nM and this should also be kept into account when working on cell models with long exposure to bioactive metabolites.
- The effects of multiple consumption of coffee are paramount for many of the quantified metabolites. Actually, in the three-coffee branch, three peaks clearly appear. The first one is similar to the peak obtained after 1 coffee treatment, the second peak, around t4, is due the consumption of the second coffee (at t3), but the third and last peak, occurring around t7, corresponds to the consumption of the third cup of coffee (at t6), but with the contribution of the fraction of metabolites originating from the colonic transformation of caffeoylquinic acids that were introduced with the first cup of coffee. This is a very important, and unprecedented observation, that highlights the real consequences of the consumption of multiple (and physiological) doses of coffee on the presence and levels of circulating phenolic metabolites in humans.
- When the volunteers were divided in males/females and smokers/non-smokers, significant differences were observed in the plasmatic trends for F4S, F4G, isF3G and DisF3G between genders. To the best of our knowledge, this difference has been reported for first time. The highest plasmatic concentrations observed in female volunteers could be partly explained by the lower BMI, but a specific sex-related metabolism could not be excluded and warrants further investigation. To maintain the intervention study as close as possible to real life conditions, smoke wasn't among the exclusion criteria, and, actually, several smokers were enrolled. Significant differences were observed between smokers

and non-smokers in the plasma profile of several metabolites, even after correction for sex and BMI, raising another important issue about the different effects coffee might exert in different categories of consumers.

5.4. Conclusion

Several studies have demonstrated that chlorogenic acids are bioavailable and potentially beneficial to humans. However, considering that: 1) their concentration in coffee depends on a wide range of factors; 2) that a considerable inter-individual variability occurs in the metabolism of these compounds in humans; and 3) that the ingested amount necessary to promote each of their potential health benefits is still unknown, these results have the merit of highlighting the daily exposure to 7 metabolites derived from chlorogenic acids after the consumption of different coffee dosages. The different coffee dosages were served at different hours, to mimic a normal day. These results will be useful for future studies aiming at understanding, for instance, the bioactivity of coffee-derived circulating metabolites in cell experiments mimicking more realistic experimental conditions.

5.5. References

1. Sánchez-Salcedo EM, Mena P, García-Viguera C, Hernández F, Martínez JJ: (Poly)phenolic compounds and antioxidant activity of white (Morus alba) and black (Morus nigra) mulberry leaves: Their potential for new products rich in phytochemicals. *Journal of Functional Foods* **2015**, 18:1039-1046.
2. Feliciano RP, Mecha E, Bronze MR, Rodriguez-Mateos A: Development and validation of a high-throughput micro solid-phase extraction method coupled with ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry for rapid identification and quantification of phenolic metabolites in human plasma and urine. *Journal of Chromatography A* **2016**, 1464:21-31.
3. Zhang Y, Huo M, Zhou J, Xie S: PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Computer Methods and Programs in Biomedicine* **2010**, 99:306-314.

CHAPTER 6

PHARMACOKINETICS OF XANTHINE AND THE MAIN METABOLITES AFTER COFFEE AND COCOA-BASED CONFECTIONERY CONTAINING COFFEE CONSUMPTION IN A REPEAT DOSE 3-ARM CROSS-OVER HUMAN INTERVENTION STUDY.

This chapter reports the pharmacokinetics of caffeine, theobromine, paraxanthine and theophylline after specific dosages of coffee or cocoa-based product containing coffee. Moreover, the influence of gender and smoke are described.

Chapter 6: Pharmacokinetics of xanthine and the main metabolites after coffee and cocoa-based confectionery containing coffee consumption in a repeat dose 3-arm cross-over human intervention study.

6.1. Materials and Methods

6.1.1. Chemicals

All chemicals and solvents were of analytical grade. Caffeine, theobromine, paraxanthine and theophylline were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade solvents were purchased from VWR International (Radnor, Pennsylvania, USA). Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

6.1.2. Coffee and CBPCC extraction

Compounds present in coffee and CBPCC were extracted according to Sánchez-Salcedo and colleagues [1], making small changes depending on the matrix. The coffee samples, prepared with the coffee machine, were diluted in 3 fraction (1:5, 1:50 and 1:200) with H_2O acidified with formic acid (0.1%, v/v). The solution was centrifuged at 17,968 *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, dimeter 13 mm) in vial prior analysis. Each sample was extracted in triplicate.

The CBPCC was firstly heated up to 40 °C for 10 minutes and dissolved in 25 mL of n-exane to eliminate the lipid fraction. Samples were vortexed for 5 minutes, centrifuged at 2647 *g* for 10 min at room temperature and the supernatant was eliminated. The purification step was repeated twice. The compounds were finally extracted from the purified pellet with 25 ml of 70% aqueous methanol acidified with formic acid (1% v/v). The solution was sonicated for 10 minutes, centrifuged at 2647 *g* for 10 min at room temperature and 1 mL of the supernatant was collected and diluted 1:2 and 1:100 with H_2O acidified with formic acid (0.1%). The extract was centrifuged at 17,968 *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, dimeter 13 mm) in vial prior analysis. Each sample was extracted in triplicate.

6.1.3. Coffee and CBPCC quantification by liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

Coffee and CBPCC extracts were analyzed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Separations were performed using a XSELECT HSS T3 (50x2.1 mm), 2.5 µm particle size (Waters, Ireland). Volume injection was 5 µL and column oven was set to 40°C. Compound elution was performed at a flow rate of 0.4 mL/min. The gradient started with 95% of 0.1% aqueous formic acid (solvent A) and 5% of acidified acetonitrile (0.1% formic acid, v/v) (solvent B). Isocratic conditions were maintained for 0.5 min, followed by a 5.5-min linear gradient to increase solvent B from 5% to 60%. Solvent B was then increased to 80% by a 1-min linear gradient and maintained for 1 min, prior re-establishing the start conditions to re-equilibrate the column in 3 min. For coffee and CBPCC samples H-ESI-II worked with a capillary temperature of 275 °C, while the source heater temperature was set to 300 °C. The sheath gas flow was 60 units and auxiliary gas was 5 units. The source voltage was 5 kV, whereas the capillary and tube lens voltage were +3 and +45 V, respectively. Analyses were carried out using a full MS^2 method, monitoring specific parental ion, and fragmentation was obtained using a collision induced dissociation (CID) equal to 35 (arbitrary units). Pure helium gas was used for CID. Chromatograms and spectral data were acquired using XCalibur software 2.1 (Thermo Fisher Scientific Inc.) and quantification was performed with calibration curves built with the standard compounds.

Table 6.1. Mass spectral characteristics of the main xanthine compounds in the products used for the treatments.

Compound	Ion.Mode	Rt[1] (min)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Quantified as
Caffeine	+	3.61	195	**138**	Caffeine
Theobromine	+	3.86	181	**162**	Theobromine
Paraxanthine	+	3.76	181	**124**; 96	Paraxanthine
Theophylline	+	3.72	181	**96;** 124	Theophylline

6.1.4. Subject recruitment

Twenty-one volunteers were recruited in Parma (PR) using announcements placed in university, hospital and public place. Inclusion criteria included being adult, healthy, of average weight (BMI 18-25), and regular coffee consumers of 1-5 cups per day. Exclusion criteria included clinically diagnosis for metabolic, renal or digestive disorder, regular consumption of medication, antibiotic therapy taken within the last 3 months, intense physical activity (LAF ≥ 2,10 – LARN 2014), pregnancy or lactation, and regular intake of coffee exceeding 5 coffee/day. Volunteers were supplied with a single-serve machine and coffee capsule to standardize brewing method, raw material and cup volume.

6.1.5. Study Design

The study was approved by the Ethics Committee of the University of Parma on April 2017 (AZOSPR/0015693/6.2.2.) and registered on Clinical Trial (NCT03166540). All participants provided full written informed consent. The study followed a repeat-dose, 3-arm, crossover design. Randomization list was generated using Random Number Generator Pro (Segobit Software).

During a run-in period of 1 month, a total of 14 participants, paired by gender, were requested to follow their habitual diet and a regular intake of 1 (1-coffe group, i.e. 7 low coffee consumers) or 3 coffee capsule/day (3-coffee group, i.e. 7 high coffee consumers), whereas 7 volunteers were requested to consume 1 coffee capsule at breakfast and 2 cocoa-based product containing coffee two times per day (CBPCC group, i.e. medium coffee consumers). Minimal recommendation to avoid other sources of coffee and cocoa phytochemicals, besides what introduced through the assigned coffee/cocoa dose, were provided for the two days prior each sampling day and on the sampling day. Moreover, volunteers were asked to standardize the time of coffee and CBPCC consumption during the test period. During the sampling day, fasting blood was collected prior coffee consumption. Then, low and high coffee consumers drunk 1 or 3 coffees, respectively, while medium consumers consumed 1 coffee and 2 CBPSS twice during the day. After the consumption of the first coffee, together with a phytochemical-free breakfast, blood was collected at specific time points within 24 h. Blood sampling after the consumption of the first coffee was performed 0.5-1-2-3-4-5-6-7-8-9-24 h using EDTA tubes. Blood was immediately centrifuged at 1600 *g* for 15 min at 20 °C; plasma was collected and stored at -80°C until extraction.

6.1.6. Plasma extraction after coffee and CBPCC consumption

Plasma samples of all volunteers were extracted using a solid phase extraction (SPE) method previously reported by Feliciano and colleagues [2]. Briefly, 350 µL of plasma samples were diluted with 350 µL of *o*-phosphoric acid 4% (v/v). After plate activation, 600 µL of the diluted plasma samples were loaded on a 96 well µ-SPE HLB plate (Oasis® HLB µElution Plate 30 µm, Waters, Milford, Massachusetts, MA, USA). Samples were then washed with 200 µL of 0.2% (v/v) acetic acid. Finally, samples were eluted with 60 µL o methanol for UHPLC-ESI-MS/MS analysis.

6.1.7. Plasma analyses by UHPLC-MS/MS

Analysis of plasma extract was performed using an UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (QqQ-MS/MS, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc., San Jose, CA, USA). Separation was carried out by means of a Kinetex EVO C18 (100 x 2.1 mm) column, 2.6 µm particle size (Phenomenex, Torrace, CA, USA). For UHPLC, mobile phase A was acetonitrile containing 0.2% formic acid and mobile phase B was 0.2% formic acid in water. The gradient started with 5% A, isocratic conditions were maintained for 0.5 min, and reached 95% A after 6.5 min, followed by 1 min at 95%. The starting gradient was then immediately reestablished and maintained for 4 min to re-equilibrate the column. The flow rate was 0.4 mL/min, the injection volume was 5 µL, and the column temperature was set at 40 °C. The applied mass spectrometry (MS) method consisted in the selective determination of each target precursor ion by the acquisition of characteristic product ions in selective reaction monitoring (SRM) mode (Table 1), applying a negative ionization. To optimize the method, all the available standard compounds were infused into the MS to set the best mass parameters and to check the actual fragmentation patterns. Finally, for all the analyses, the spray voltage was set at 3 kV, the vaporizer temperature at 300 °C, and the capillary temperature operated at 270°C. The sheath gas flow was 60 units, and auxiliary gas pressure was set to 10 units. Ultrahigh purity argon gas was used for collision-induced dissociation (CID). The S-lens values were defined for each compound based on infusion parameter optimization (**Table 6.2**). Quantification was performed with calibration curves of standards (**Table 6.2**). Data processing was performed using Xcalibur software (Thermo Scientific Inc., Waltham, MA, USA). All data were expressed as mean values ± SEM Metabolite identification was carried

out by comparing the retention time with authentic standards and MS/MS fragmentation patterns. Quantification were performed with calibration curve

During the analysis, the H-ESI interface worked with a capillary temperature of 275°C. The source heater was set at 250 °C, the auxiliary gas flow (N_2) was set at 10 (arbitrary units) and the sheath gas flow (N_2) at 50. The source voltage was 3.8 kV, whereas the capillary voltage and tube lens voltage were +36 V and +75 V, respectively. Chromatograms and spectral data were acquired using XCalibur software 2.1 (Thermo Fisher Scientific Inc.) and quantification was performed with calibration curves built with the standard compounds.

Table 6.2. Overview on mass transition, chromatographic proprieties of the HILIC-HPLC-MS/MS method developed for the analysis of the pyridines. The fragment ions used for quantification are highlighted in bold

Compound	Rt[1] (min)	$[M+H]^+$	SRM transition	S-Lens
Caffeine	4.46	195	**138**; 110	103
Theobromine	1.18	181	**163**; 85	74
Paraxanthine	0.75	181	**124**; 96	74
Theophylline	0.74	181	**124**; 96	74

6.1.8. Statistical analyses

Data were expressed as mean ± standard error of the mean (SEM). PKsolver add-on program was used to perform pharmacokinetic data analysis in Microsoft Excel [3]. A difference was considered significant at $p<0.05$. The statistical analysis was performed with the Statistical Package for Social Sciences software (IBM SPSSVR Statistics, Version 22.0. IBM Corp., Chicago, IL). Comparison among treatments were analyzed by repeated measurement General Linear Model using Greenhouse–Geisser correction if epsilon was lesser than 0.75 or Huynh–Feldt correction if epsilon was greater than 0.75 when the assumption of sphericity was violated, and Bonferroni post hoc tests for multiple comparisons.

A paired-sample t-test between t0 and t24 was performed for comparison within the same group before and after product consumption.

T-test were used for comparisons between groups (males/females and smokers/non-smokers).

6.2. Results

6.2.1. Coffee and CBPCC composition

The content of the main phenolic compounds of each products used for the intervention study are reported in **Table 6.3**.

Table 6.3. Quantification (mg/serving) of the main phenolic compounds in the products used for the 3 treatments. CBPCC (chocolate based product containing coffee). Coffee serving is considered 1 coffee (one espresso coffee corresponds to 40 mL in volume), CBPCC serving is considered 2 cocoa based products containing coffee.

Compound	Coffee (mg/serving)	CBPCC (mg/serving)
Caffeine	63.90 ± 0.76	46.66 ± 0.25
Theobromine	11.05 ± 0.12	45.91 ± 0.15

6.2.2. Subjects characteristics

All the volunteers recruited, completed the intervention study. In the **Table 6.4** were reported the characteristics of partecipants.

Table 6.4. Characteristics of participants in the study. Data are expressed as means ± SD.

Volunteers	21
Male	10
Female	11
Age (years)	25.9 ± 0.5
Height (m)	1.72 ± 0.02
Weight (kg)	66.5 ± 2.6
BMI (kg/m^2)	22.3 ± 0.5

6.2.3. Plasma absorption and pharmacokinetic parameters

The pharmacokinetics of caffeine (**Figure 6.1A**), theobromine (**Figure 6.1B**) and Paraxanthine + theophylline (**Figure 6.1C**), were reported in the **Figure 6.1**. **Table 6.5** reports the pharmacokinetics parameter for the same three components.

The concentrations of caffeine in plasma are reported in **Figure 6.1A**. The t0 levels of caffeine were significantly different among the three treatments, indicating that the consumption of different doses of coffee results in modification in the basal level of this compound. The concentration of caffeine over time follows a predictable pattern, with a visible increase post consumption after every dose ingested.

The pharmacokinetic of theobromine (3,7-dimethylxanthine), described in **Figure 6.1B**, showed a similar trend in the treatments with 1 coffee and 3 coffees. The same conclusions drawn for caffeine with respect to basal levels after 1 month treatment also stand for theobromine, with three coffees a day associated with a significantly higher basal level. Differences could be spotted, instead, for the CBPCC treatment, with two high increments appearing after the consumption of the cocoa products at t_3 and t_6, with a sustained higher concentration over time. This is due to the presence, in chocolate, of a relevantly higher amount of theobromine.

Paraxanthine (1,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) were quantified together because of chromatographical resolution problems. A similar baseline trend and pharmacokinetics behavior were observed for these compounds as for caffeine and theobromine.

Table 6.5: Pharmacokinetic parameters of caffeine, theobromine and paraxanthine + theopylline after ingestion of 1 coffee, 3 coffees and 1 coffee plus 4 CBPCC (chocolate based product containing coffee). Data expressed as mean ± SEM (n=21). Different letters in the same row indicate differences among the treatment groups (*pvalue* <0.001).

		1 coffee	3 coffees	CBPCC
Caffeine	AUC_{0-24} (µmol/L*h)	114.72 ± 13.13[b]	337.75 ± 37.26[a]	254.52 ± 23.26[a]
	C_{avg} (µM)	4.78 ± 0.55[b]	14.07 ± 1.55[a]	10.59 ± 0.97[a]
	C_{max} (µM)	12.85 ± 0.99[b]	22.89 ± 1.92[a]	20.19 ± 1.38[a]
	C_{min} (µM)	0.89 ± 0.26[b]	5.97 ± 1.39[a]	3.11 ± 0.72[a]
	t_{max} (h)	1.33 ± 0.19	6.17 ± 0.35	6.40 ± 0.42
Theobromine	AUC_{0-24} (µmol/L*h)	3.22 ± 1.36[c]	7.50 ± 1.74[b]	55.50 ± 5.18[a]
	C_{avg} (µM)	0.13 ± 0.06[c]	0.31 ± 0.07[b]	2.31 ± 0.22[a]
	C_{max} (µM)	0.53 ± 0.19[b]	0.83 ± 0.14[b]	4.35 ± 0.45[a]
	C_{min} (µM)	0.02 ± 0.02[c]	0.04 ± 0.03[b]	0.83 ± 0.14[a]
	t_{max} (min)	1.26 ± 0.52	2.76 ± 0.66	7.20 ± 0.40
Paraxanthine + Theophylline	AUC_{0-24} (µmol/L*h)	58.29 ± 4.96	165.80 ± 15.69	122.37 ± 11.37
	C_{avg} (µM)	2.43 ± 0.21	6.91 ± 0.65	5.10 ± 0.47
	C_{max} (µM)	4.26 ± 0.32	10.67 ± 0.92	8.52 ± 0.66
	C_{min} (µM)	0.84 ± 0.17	3.53 ± 0.51	2.76 ± 0.42
	t_{max} (min)	5.05 ± 0.50	6.81 ± 0.56	7.43 ± 0.95

To check for potential differences based on sex and smoking behaviours, data have been expressed and analysed separately for males and females (**Figure 6.2**), and smokers and non-smokers (**Figure 6.3**).

Statistically significant differences were observed for both caffeine and theobromine. In detail, the pharmacokinetic parameters of caffeine were lower after consumption of 1 coffee compared to 3 coffee and CBPCC consumption. Conversely, the pharmacokinetic parameter values of theobromine were the highest after CBPCC consumption, followed by 3 coffee and 1 coffee consumption.

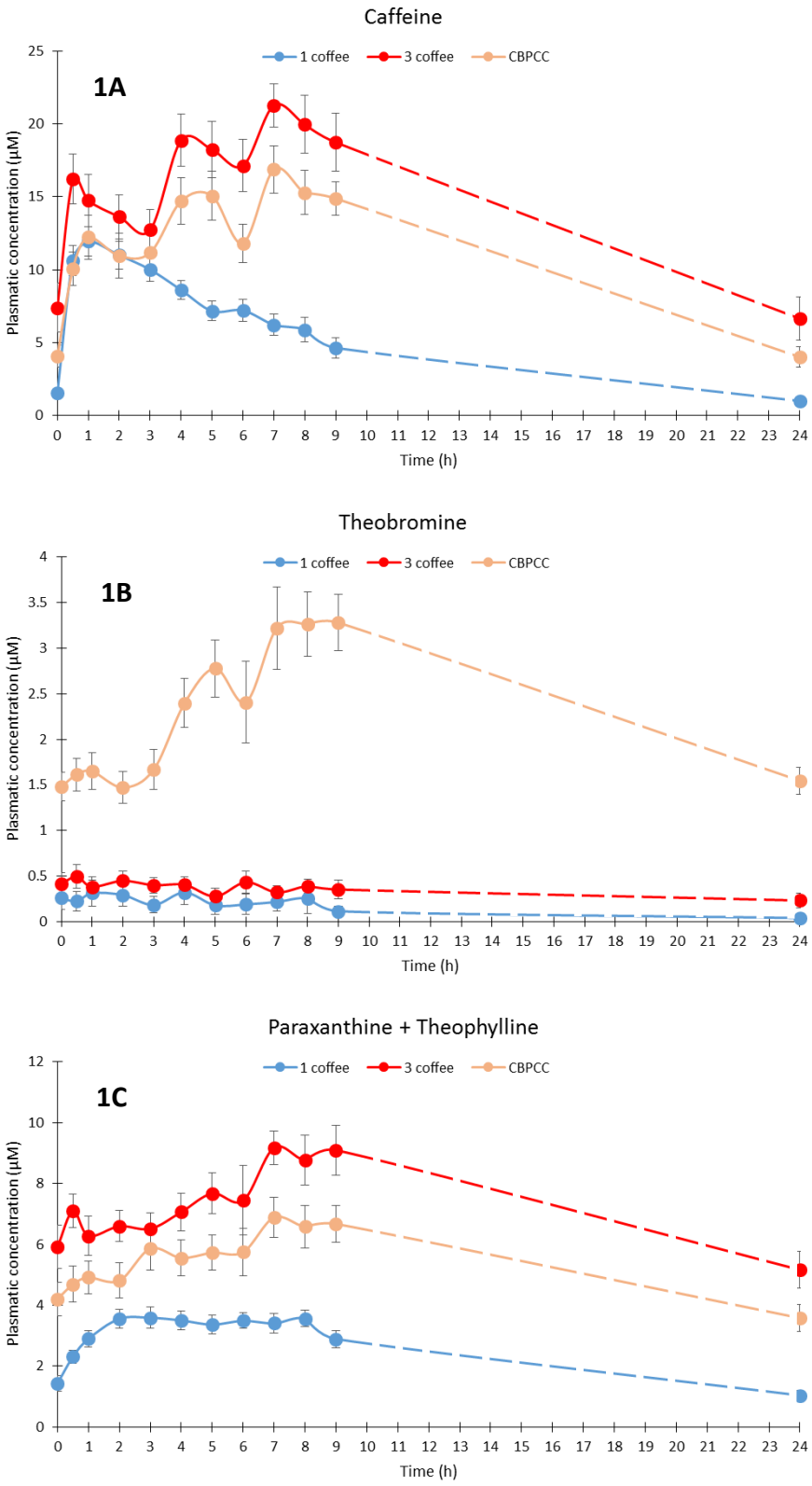

Figure 6.1. Time-points concentration of caffeine (1A), theobromine (1B) and paraxanthine + theophylline in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four CBPCC (chocolate based product containing coffee) consumption. Data expressed as mean ± SEM (n=21).

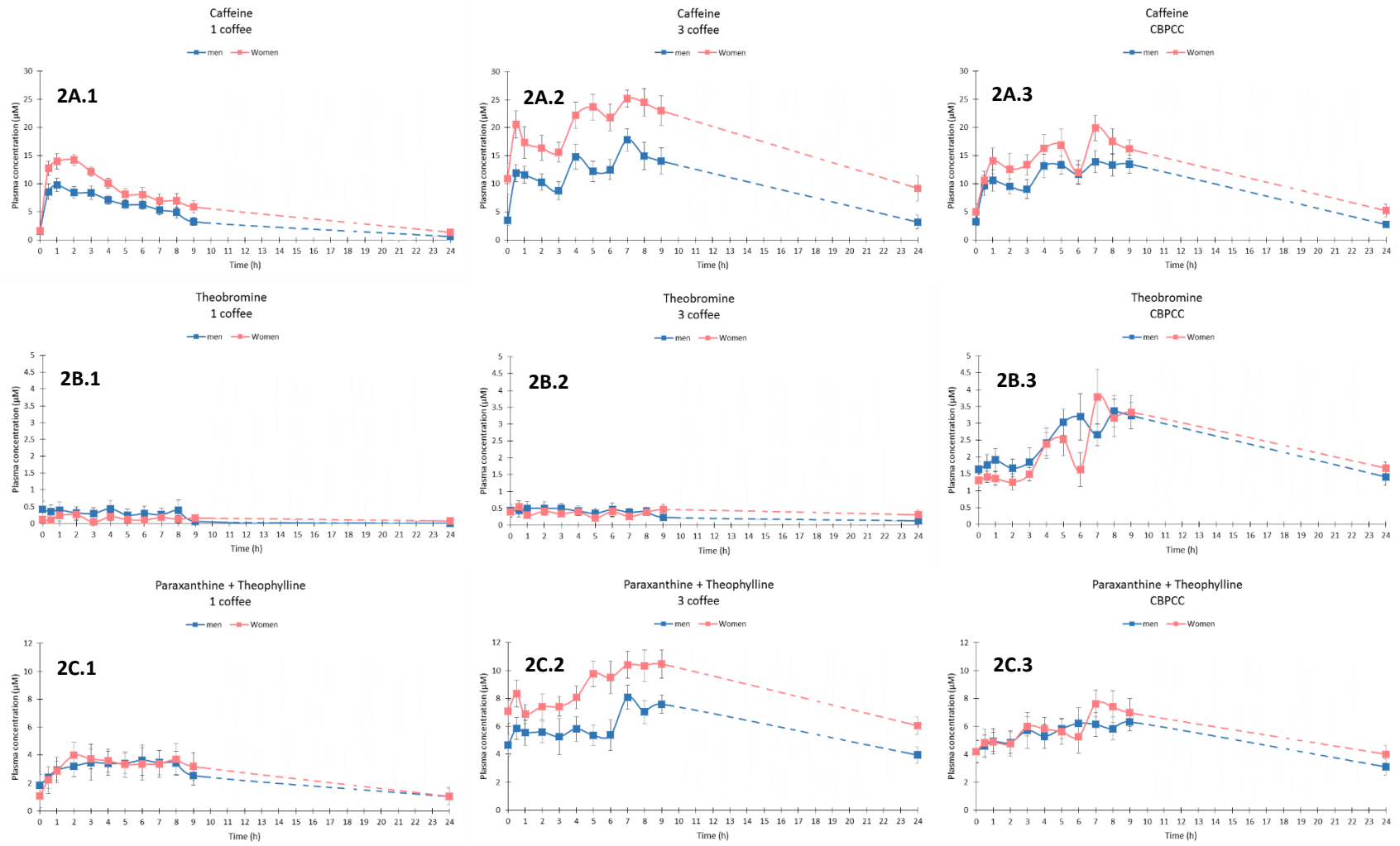

Figure 6.2. Time-points of plasmatic concentration of caffeine (2A.1, 2A.2 and 2A.3), theobromine (2B.1, 2B.2 and 2B.3) and paraxanthine + theophylline (2C.1, 2C.2 and 2C.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in women and men. Data expressed as mean ± SEM (men=10 and women=11).

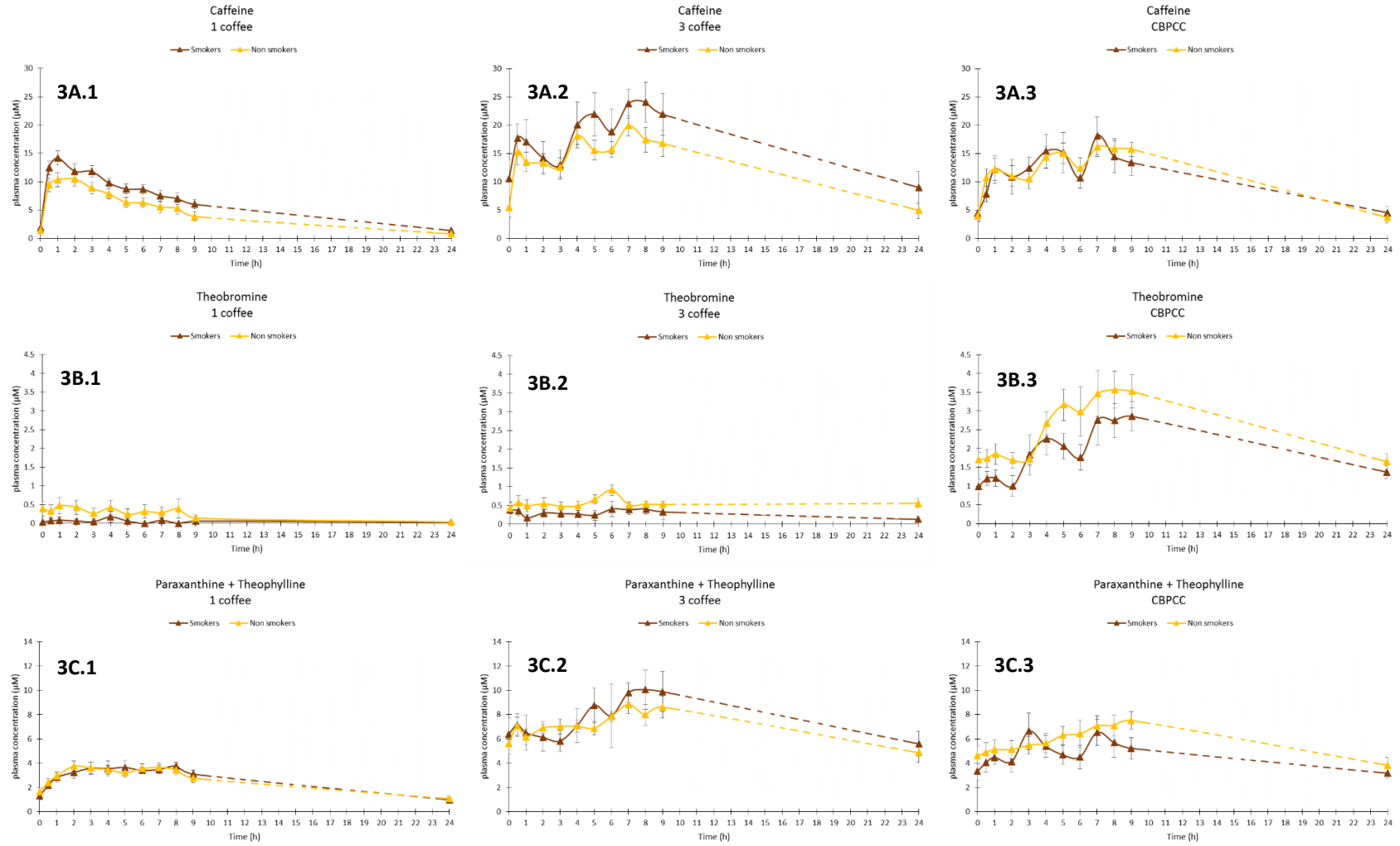

Figure 6.3. Time-points of plasma concentration of caffeine (3A.1, 3A.2 and 3A.3), theobromine (3B.1, 3B.2 and 3B.3) and paraxanthine + theophylline (3C.1, 3C.2 and 3C.3) in all plasma samples collected after 1 coffee consumption, 3 coffee consumption and 1 coffee and four chocolate-based product containing coffee (CBPCC) consumption and separated in smokers and non-smokers. Data expressed as mean ± SEM (smokers=8 and non-smokers=13).

6.3. Discussion

The most relevant observations that could be made after this intervention study with regards to circulating xanthines are:

- The maximum concentrations found in plasma after the intake of the highest dose of coffee were reached on average at 7.27 hours after the first dose. Summing the concentrations of all xanthine metabolites at the tmax a maximum concentration equal to 34.39 µM was reached, and this should be taken into account when in vitro studies are carried out in order to evaluate the effects of coffee xanthines on health or disease-related mechanisms in cells. The average daily concentrations of the main metabolites range from 7.34 µM to 21.29 µM and this should also be kept into account when working on cell models with long exposure to bioactives.
- The effects of multiple consumption of coffee are paramount for many of the quantified metabolites. Actually, in the three-coffee branch, three peaks clearly appear. It is although interesting to observe some sort of cumulative effect, where the second peak always builds up over the first, letting hypothesise the need of a multiple dose approach to really appreciate the concentrations reached after intake of coffee. This is particularly true for caffeine. This is a very important, and unprecedented observation, that highlights the real consequences of the consumption of multiple (and physiological) doses of coffee on the presence and levels of circulating xanthines in humans.
- When the volunteers were divided in males/females and smokers/non-smokers, significant differences were observed in the plasmatic trends for caffeine between genders. To the best of our knowledge, these differences have been reported for first time. The highest plasmatic concentrations observed in female volunteers could be partly explained by the lower BMI, but a specific sex-related metabolism could not be excluded and warrants further investigation. To maintain the intervention study as close as possible to real life conditions, smoke wasn't among the exclusion criteria, and, actually, several smokers were enrolled. No significant differences were observed between smokers and non-smokers in the plasma profile.

6.4. Conclusions

The results of this study highlight the pharmacokinetics trends of xanthines after coffee consumptions in a real life setting. This innovative design, combining a chronic study with an acute study allowed a better understanding of both the acute and the medium term effects of coffee intake on plasma xanthine concentrations.

6.5. References

1. Sánchez-Salcedo EM, Mena P, García-Viguera C, Hernández F, Martínez JJ: (Poly)phenolic compounds and antioxidant activity of white (Morus alba) and black (Morus nigra) mulberry leaves: Their potential for new products rich in phytochemicals. *Journal of Functional Foods* **2015**, 18:1039-1046.
2. Feliciano RP, Mecha E, Bronze MR, Rodriguez-Mateos A: Development and validation of a high-throughput micro solid-phase extraction method coupled with ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry for rapid identification and quantification of phenolic metabolites in human plasma and urine. *Journal of Chromatography A* **2016**, 1464:21-31.
3. Zhang Y, Huo M, Zhou J, Xie S: PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Computer Methods and Programs in Biomedicine* **2010**, 99:306-314.

CHAPTER 7

EFFECT OF COFFEE AND COCOA-BASED CONFECTIONERY CONTAINING COFFEE CONSUMPTION ON CARDIOMETABOLIC RISK FACTORS IN A REPEAT DOSE 3-ARM CROSS-OVER HUMAN INTERVENTION STUDY.

This chapter reports the effect of prolonged dosages of coffee or cocoa-based product containing coffee consumption on specific cardiometabolic risk factors.

Chapter 7: Effect of coffee and cocoa-based confectionery containing coffee consumption on cardiometabolic risk factors in a repeat dose 3-arm cross-over human intervention study.

7.1. Material and Methods

7.1.1. Subjects recruitment

Twenty-one volunteers were recruited in Parma (PR) using announcements placed in university, hospital and public place. Inclusion criteria included being adult, healthy, of average weight (BMI 18-25), and regular coffee consumers of 1-5 cups per day. Exclusion criteria included clinically diagnosis for metabolic, renal or digestive disorder, regular consumption of medication, antibiotic therapy taken within the last 3 months, intense physical activity (LAF $\geq$ 2,10 – LARN 2014), pregnancy or lactation, and regular intake of coffee exceeding 5 coffee/day. Volunteers were supplied with a single-serve machine and coffee capsule to standardize brewing method, raw material and cup volume.

7.1.2. Study design

The study was approved by the Ethics Committee of the University of Parma on April 2017 (AZOSPR/0015693/6.2.2.) and registered on Clinical Trial (NCT03166540). All participants provided full written informed consent. The study followed a repeat-dose, 3-arm, crossover design. Randomization list was generated using Random Number Generator Pro (Segobit Software).

During a run-in period of 1 month, a total of 14 participants, paired by gender, were requested to follow their habitual diet and a regular intake of 1 (1-coffe group, i.e. 7 low coffee consumers) or 3 coffee capsule/day (3-coffee group, i.e. 7 high coffee consumers), whereas 7 volunteers were requested to consume 1 coffee capsule at breakfast and 2 cocoa-based product containing coffee two times per day (CBPCC group, i.e. medium coffee consumers). Minimal recommendation to avoid other sources of coffee and cocoa phytochemicals, besides what introduced through the assigned coffee/cocoa dose, were provided for the two days prior each sampling day and on the sampling day. Moreover, volunteers were asked to standardize the time of coffee and CBPCC consumption during the

test period. At the end of 1-month treatments, blood samples were collected using EDTA tubes for the evaluation of cardiometabolic markers. Blood was immediately centrifuged at 1600 *g* for 15 min at 20 °C; plasma was collected and stored at -80°C until extraction.

7.1.3. Data collection

Height, weight, waist circumference, systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the subjects were measured and the body mass index (BMI) was calculated at baseline and at the end of the intervention. Height was measured at baseline barefoot to the nearest 0.5 cm. Weight was measured with a precision of 100 g, with volunteers wearing light clothes, using Seca digital scale. In addition, waist circumference was measured to the nearest 0.5 cm approximately between the lower margin of the last rib and top of the iliac crest at the level of navel. SBP and DBP were measured twice after a 10-min rest using a Citizen digital blood pressure monitor with 1mmHg precision.

Blood was obtained from an intravenous catheter into tubes containing EDTA, heparin or nothing depending on the analysis. Analyses were performed in a single-blind manner by the personnel of the Laboratory of University Hospital "Virgen de la Arrixaca", Murcia, Spagna. Fasting plasma glucose, total cholesterol, HDL cholesterol, triglycerides, insulin, total folates, homocysteine and hs-CRP were assessed by a central laboratory using standard methods. LDL cholesterol was calculated using the Friedewald formula.

Nitric oxide was quantified using a nitrate/nitrite fluorimetric kit (Vinci-Biochem S.r.l., Vinci, Italia).

Trimethylamine-*N*-oxide (TMAO) was determined in plasma samples by means of a UHPLC-MS/MS method. Samples were defrosted at room temperature, shaken, diluted 1:10 with acetonitrile and centrifuged at 13765 *g* for 10 minutes before being transferred in vials for the analysis. Analysis of extracted plasma was performed using an Accela UHPLC 1250 equipped with linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc., San Jose, CA, USA). Separation were carried out by means of an Xbridge BEH HILIC XP (100x2.1 mm) with a porosity of 2.5 µm (Waters, USA).

The effect of daily coffee consumption on the inflammatory status was monitored by analysing interleukin 8 (IL-8), tumor necrosis factor (TNFα) and vascular endothelial growth factor (VEGF) before and after each treatment arm of the study using Bio-Plex Pro™ Human Cytokine 3-Plex customized Immunoassay (Bio-Rad Laboratories, Inc.). Determination of biomarkers was performed according to the instruction of the manufacturer.

7.2. Results

Twenty-one volunteers (10 male and 11 female) were recruited for the study, and everyone completed the trial. The parameters detected after one month of specific coffee dosage are reported in **Table 7.1**.

Table 7.1: Characteristics of study participants n=21 (average ± SEM) at baseline (t0) and after consumption of 4 cocoa-based products containing coffee and 1 espresso (CBPCC) per day or 1 espresso or 3 espresso coffees per day for 1 month. BMI: Body Mass Index; WC: Waist circumference; TAG: Triglycerides; TMAO: Trimethylamine-N-oxide; Il-8: Interleukin 8; TNFα: Tumor necrosis factor; VEGF: Vascular endothelial growth factor. (n=21; mean ± SEM).

	Base line	**CBPCC**	**1 Coffee**	**3 Coffee**
Weight (kg)	66.6 ± 2.6	66.7 ± 2.6	66.6 ± 2.7	66.6 ± 2.7
Body Mass Index (kg/m^2)	22.3 ± 0.5	22.3 ± 0.5	22.3 ± 0.5	22.3 ± 0.5
Waist circumference (cm)	71.7 ± 1.6	73.4 ± 1.6	72.9 ± 1.7	73.0 ± 1.8
BPS (mmHg)	116.6 ± 2.7	113.7 ± 2.6	115.5 ± 2.1	113.0 ± 2.2
BPD (mmHg)	73.7 ± 1.7	73.1 ± 1.6	72.3 ± 1.2	71.1 ± 1.9
Cholesterol TOT (mg/dL)	176.4 ± 6.9	170.6 ± 6.2	169.5 ± 7.2	172.6 ± 6.5
Cholesterol LDL (mg/dL)	93.9 ± 5.7	88.0 ± 5.3	90.7 ± 6.0	89.7 ± 5.8
Cholesterol HDL (mg/dL)	66.3 ± 3.6	66.8 ± 3.6	66.0 ± 3.1	65.8 ± 3.5
TAG (mg/dL)	81.2 ± 4.3	79.6 ± 5.4	82.0 ± 5.8	84.1 ± 5.0
Glucose (mg/dL)	86.3 ± 1.6	86.6 ± 1.4	87.6 ± 1.8	86.3 ± 1.5
Insulin (microU/mL)	10.6 ± 3.7	8.5 ± 0.6	10.4 ± 1.2	8.4 ± 0.9
Nitric oxide (µmol/L)	12.9 ± 1.4	12.7 ± 1.5	12.0 ± 1.7	11.7 ± 1.7
TMAO (µmol/L)	3.1 ± 0.3	2.5 ± 0.2	2.5 ± 0.3	3.2 ± 0.4
IL-8 (pg/mL)	9.4 ± 1.1	10.7 ± 1.3	9.6 ± 1.1	10.1 ± 1.2
TNFα (pg/mL)	7.4 ± 0.7	8.1 ± 0.8	8.1 ± 0.7	8.1 ± 0.7
VEGF (pg/mL)	61.6 ± 12.3	60.1 ± 12.8	59.7 ± 11.2	69.5 ± 14.8

None of the anthropometric, blood pressure, metabolic (lipidemic and glycaemic) or inflammatory parameters was influenced by coffee or CBPCC coffee consumption.

GENERAL CONCLUSIONS

Coffee has been the *leitmotiv* of this Doctoral Thesis, which was aimed to investigate the content of several bioactive compounds in coffee, their bioavailability as well as to explore the potential role of coffee consumption on human health in a framework of physiological consumption. To this aim, the main activities described in this work included:

- **Coffee Consumption and Oxidative Stress: A Review of Human Intervention Studies.** This review summarized the main findings of 26 studies performed in humans, with the aim of comparing results on the effect of coffee consumption on the main markers oxidative damage to lipid, DNA, and protein, as well as the modulation of antioxidant capacity and antioxidant enzymes in humans. The main findings of the reviewed works seem to suggest that consumption of coffee may increase glutathione levels and reduce the DNA damage, although high heterogeneity among studies was observed.
- **Niacin, alkaloids and (poly)phenolic compounds in the most widespread Italian capsule-brewed coffees.** The alkaloid and phenolic profiles as well as the niacin content of the most representative capsule of espresso coffee in the Italian market showed a wide variability among both capsules of the same brand and of different brands. These differences in the composition of espresso coffee prepared by using coffee capsules may be of interest for further studies when a specific set of bioactives is specifically investigated.
- **The Pocket-4-Life project, bioavailability and beneficial properties of the bioactive compounds of espresso coffee and cocoa-based confectionary containing coffee: study protocol for a randomized cross-over trial.** In this part of research, an innovative experimental design was developed with the aim to allow the simultaneous evaluation of the effect of acute and chronic consumption of different dosages of coffee and cocoa-based confectionary containing coffee on the bioavailability of the main phytochemical groups and on specific markers of cardio-metabolic health.
- **Absorption and pharmacokinetics of pyridines after coffee and cocoa-based confectionery containing coffee consumption in a repeat dose 3-arm cross-over human intervention study.** The results of this part of research showed, for the first time, the daily exposure to trigonelline and N-methylpyridinium, after the consumption of different coffee dosages. These results will be useful for

future studies aiming at evaluating the bioactivity of coffee-derived circulating metabolites in cell experiments mimicking more realistic experimental conditions.

- **Pharmacokinetics of phenolic metabolites after coffee and cocoa-based confectionery containing coffee consumption in a repeat dose 3-arm cross-over human intervention study.** The results of this part of research demonstrated a dose-response relationship between the coffee intake and the concentration of 3 out of the 7 metabolites studied. A considerable inter-individual variability occurred in the metabolism of these compounds in humans. The ingested amount necessary to promote each of their potential health benefits is still unknown. These results have the merit of highlighting the daily exposure to 7 metabolites derived from chlorogenic acids after the consumption of different coffee dosages. They will be useful for future studies aiming at understanding, for instance, the bioactivity of coffee-derived circulating metabolites in cell experiments mimicking more realistic experimental conditions.
- **Pharmacokinetics of xanthine and the main metabolites after coffee and cocoa-based confectionery containing coffee consumption in a repeat dose 3-arm cross-over human intervention study.** The results from this part of research reported the pharmacokinetics trend of xanthines after coffee consumption in a real life setting. Statistically significant differences in Cmax values, Tmax, Cmin and AUC_{0-24} were observed between treatments, depending on the coffee dosage. Similarly to what highlighted for phenolics, these results will be useful for future studies aiming at understanding, for instance, the exposure of coffee-derived circulating metabolites in cell experiments mimicking more realistic experimental conditions.
- **Effect of coffee and cocoa-based confectionery containing coffee consumption on cardiometabolic risk factors in a repeat dose 3-arm cross-over human intervention study.** This part of the work was aimed at evaluating the effect of different coffee dosages, chronically consumed for one month, on several cardiometabolic risk factors. None of the parameters detected was influenced by coffee consumption.

In conclusion, the findings of this Doctoral Thesis contribute to provide advance of knowledge about the content of bioactive compounds in coffee, the metabolic response of

these bioactives and the effects on health following coffee consumption in a real life settings with an innovative approach. The results already obtained and the findings of the ongoing analyses, will be a starting point for future studies aimed to better elucidate the role of coffee on human health