Absorption, Pharmacokinetics, and Urinary Excretion of Pyridines After Consumption of Coffee and Cocoa-Based Products Containing Coffee in a Repeated Dose, Crossover Human Intervention Study

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Scope: The present study assesses the absorption, pharmacokinetics, and urinary excretion of coffee pyridines and their metabolites after daily regular exposure to specific dosages of coffee or cocoa-based products containing coffee (CBPCC), considering different patterns of consumption.

Methods and results: In a three-arm, crossover, randomized trial, 21 volunteers are requested to randomly consume for 1 month: one cup of espresso coffee per day, three cups of espresso coffee per day, or one cup of espresso coffee plus two CBPCC twice per day. The last day of the one-month treatment, blood and urine samples are collected for 24 h. Trigonelline, *N*-methylpyridinium, *N*-methylnicotinamide, and *N*-methyl-4-pyridone-5-carboxamide are quantified. Trigonelline and *N*-methylpyridinium absorption curves and 24-h urinary excretion reflect the daily consumption of different servings of coffee or CBPCC, showing also significant differences in main pharmacokinetic parameters. Moreover, inter-subject variability due to sex and smoking is assessed, showing sex-related differences in the metabolism of trigonelline and smoking-related ones for *N*-methylpyridinium.

Conclusion: The daily exposure to coffee pyridines after consumption of different coffee dosages in a real-life setting is established. This data will be useful for future studies aiming at evaluating the bioactivity of coffee-derived circulating metabolites in cell experiments, mimicking more realistic experimental conditions.

1. Introduction

Coffee is one of the most important non-alcoholic beverages consumed in the world,[1] very popular in Europe, United States and basically in all other countries.[2] Based on large-scale prospective cohorts, systematic reviews and meta-analyses, the regular consumption of coffee has been linked to several protective effects, like the reduction of risk of type 2 diabetes, Alzheimer's disease, some type of cancers, cardiovascular disease development, and overall mortality.[3–8] Indeed, coffee is a complex mixture of dozens of potentially bioactive compounds, namely vitamins, alkaloids, phenolic compounds, diterpenoids, and melanoidins.[2,8,9]

Among the most important and largely investigated coffee alkaloids, besides caffeine, *N*-methyl nicotinic acid, namely trigonelline, is a pyridine commonly recognized as the second most abundant coffee alkaloid,[2,8] synthesized in planta from nicotinic acid and/or nicotinamide.[10] The key reaction of trigonelline biosynthesis pathway is methylation of the pyridine skeleton,

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catalyzed by a trigonelline synthase, namely nicotinic acid *N*-methyltransferase.[10,11] Unlike caffeine, trigonelline is more thermolabile and sensitive to heat treatments, thus during the roasting process it is partially degraded to generate *N*-methylpyridinium and nicotinic acid.[12,13] *N*-methylpyridinium and trigonelline have been recently recognized as good predictors of coffee intake using metabolomic approaches.[14–17] Generally, in espresso coffee, trigonelline ranges between 20 and 80 mg per serving, depending on the coffee cultivars and espresso machine configurations,[18,19] although lower values have been reported for capsule-brewed espresso coffees.[9] The two main trigonelline derivatives generated during coffee roasting are present at lower concentrations, accounting only for a few milligrams per serving.[9,19,20] As it happens for most coffee bioactive compounds, which usually appear in the circulatory system after a relevant set of biological transformations, occurring before and after absorption,[2,21–23] the human metabolism of coffee pyridines needs to be considered when evaluating their implication in coffee health effects. Actually, the human metabolism of coffee pyridines has been previously described.[20,24] Briefly, ingested trigonelline, *N*-methylpyridinium and nicotinic acid are partly excreted in their native form, although some oxidized and methylated derivatives, as nicotinamide-*N*-oxide, *N*-methylnicotinamide, *N*-methyl-pyridone-carboxamide, and *N*-methyl-pyridone-carboxylic acid, have been identified after coffee consumption.[24] Nevertheless, despite the metabolism and pharmacokinetics of coffee pyridines have been well-established in research studies,[20,24] the concentrations in circulation and pharmacokinetic profiles of these compounds under real conditions of coffee consumption are not known so far.[25,26]

To address the metabolism of coffee pyridines under real-life settings, the present study aimed at assessing a daily regular exposure to coffee pyridines on the basis of different patterns of consumption. The absorption, pharmacokinetics, and urinary excretion of the native compounds and their main metabolites after specific dosages of coffee or cocoa-based products containing coffee (CBPCC) along the day were evaluated. Differences among subjects linked to sex and smoking were also taken into account.

2. Results

2.1. Espresso Coffee and CBPCC Pyridine Content and Daily Servings

The quantification of the main pyridines in espresso coffee and CBPCC is reported in Table S2, Supporting Information. 1C arm and PC arm consumed a similar amount of trigonelline (544.5 and 547.3 µmol, respectively), and similar amount of *N*-methylpyridinium (43.9 and 64.3 µmol, respectively), which obviously resulted greater for the 3C group (1633.6 µmol of trigonelline and 131.7 µmol of *N*-methylpyridinium). Last, the three intervention arms were exposed to a limited nicotinic acid amount (3.9, 11.6, and 5.7 µmol for 1C, 3C, and PC group, respectively), since the daily amount provided by the espresso coffee and CBPCC was low.
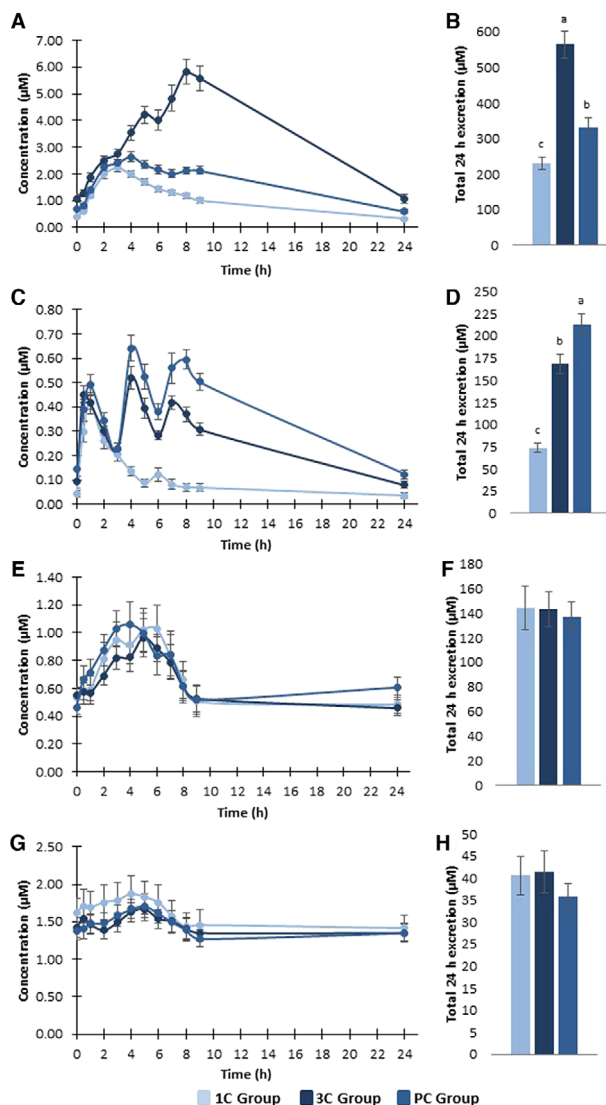

Figure 1. Plasma kinetic profile (left) and 24-h cumulative excretion (right) of trigonelline A,B), *N*-methylpyridinium C,D), *N*-methylnicotinamide E,F), and *N*-methyl-4-pyridone-5-carboxamide G,H) after consumption of 1 coffee (1C group), 3 coffees (3C group), and 1 coffee plus four cocoa-based product containing coffee (CBPCC, PC group). Data are expressed as mean ± SEM ($n = 21$). Different letters in the urinary excretion indicate statistical differences of the specific metabolite among the three treatments ($p < 0.05$).

2.2. Plasma Absorption, Pharmacokinetic Parameters, and Urinary Excretion

All the expected metabolites possibly linked to coffee pyridines[24] were monitored. Nicotinic acid, nicotinamide, nicotinamide-*N*-oxide, and *N*-methyl-2-pyridone-5-carboxylic acid were not detected at circulating and urinary excretion level. Instead, four metabolites, namely trigonelline, *N*-methylpyridinium, *N*-methylnicotinamide, and one *N*-methyl-4-pyridone-5-carboxamide were quantified in plasma and urine (**Figure 1**). While *N*-methylnicotinamide and *N*-methyl-4-pyridone-5-carboxamide did not significantly differ among the three

Table 1. Plasma pharmacokinetic parameters of trigonelline, *N*-methylpyridinium, *N*-methylnicotinamide, and *N*-methyl-4-pyridone-5-carboxamide in the three intervention groups.

Compound and treatment	T_{max} [h]	C_{max} [µmol L^{-1}]	C_{min} [µmol L^{-1}]	C_{avg} [µmol L^{-1}]	AUC$_{0-24}$ [µmol L^{-1} × h]
Trigonelline					
1C Group	3.00 ± 0.14[c]	2.33 ± 0.16[b]	0.29 ± 0.04[b]	0.98 ± 0.07[c]	23.53 ± 1.75[c]
3C Group	8.48 ± 0.13[a]	6.13 ± 0.50[a]	0.89 ± 0.13[a]	3.43 ± 0.29[a]	82.44 ± 6.91[a]
PC Group	4.05 ± 0.34[b]	2.80 ± 0.20[b]	0.57 ± 0.10[a]	1.60 ± 0.11[b]	38.49 ± 2.65[b]
p-value	0.000	0.000	0.000	0.000	0.000
N-methylpyridinium					
1C Group	1.52 ± 0.30[c]	0.47 ± 0.04[c]	0.01 ± 0.01[b]	0.09 ± 0.01[c]	2.24 ± 0.30[c]
3C Group	3.24 ± 0.48[b]	0.60 ± 0.04[b]	0.04 ± 0.01[ab]	0.25 ± 0.01[b]	6.10 ± 0.46[b]
PC Group	6.00 ± 0.50[a]	0.84 ± 0.05[a]	0.07 ± 0.01[a]	0.37 ± 0.02[a]	8.84 ± 0.46[a]
p-value	0.000	0.000	0.001	0.000	0.000
N-methylnicotinamide					
1C Group	4.43 ± 0.36	1.28 ± 0.18	0.30 ± 0.05	0.62 ± 0.09	14.76 ± 2.13
3C Group	5.21 ± 1.01	1.18 ± 0.12	0.28 ± 0.04	0.59 ± 0.07	14.06 ± 1.65
PC Group	6.15 ± 1.36	1.34 ± 0.14	0.28 ± 0.05	0.65 ± 0.08	15.67 ± 2.00
p-value	0.480	0.701	0.937	0.653	0.653
N-methyl-4-pyridone-5-carboxamide					
1C Group	5.69 ± 1.42	2.10 ± 0.24	1.19 ± 0.18	1.53 ± 0.19	36.79 ± 4.62
3C Group	8.10 ± 1.78	1.90 ± 0.16	1.06 ± 0.11	1.42 ± 0.21	34.15 ± 4.98
PC Group	6.03 ± 1.39	1.96 ± 0.16	1.14 ± 0.13	1.37 ± 0.10	32.80 ± 2.51
p-value	0.521	0.968	0.802	0.973	0.973

1C group: subjects consumed 1 coffee per day. 3C group: subjects consumed 3 coffees per day. PC group: subjects consumed 1 coffee plus twice cocoa-based products containing coffee (each serving consisted of two CBPCC) per day. Values are expressed as mean ± SEM (n = 21). Different letters indicate statistical differences of the specific metabolite among the three treatments. T_{max}: time to reach the maximum plasma concentration; C_{max}: maximum plasma concentration; C_{min}: minimum plasma concentration; C_{avg}: average plasma concentration calculated as AUC$_{0-24}$/24 h; AUC$_{0-24}$: area under curve.

intervention arms, as demonstrated by their plasma pharmacokinetic curves, pharmacokinetic parameters, and urinary cumulative excretions (Figure 1E–H and **Table 1**), trigonelline and *N*-methylpyridinium absorption curves clearly paralleled the daily consumption of different servings of coffee or CBPCC and were different among treatments (Figure 1A,C and Table 1).

The curves obtained for trigonelline followed the same kinetic trend within 3 h in the three groups (Figure 1A). After consumption of the first coffee (t0), the circulating concentration of trigonelline gradually increased in all the intervention arms during the first hours (T_{max} 1C group = 3 h, Table 1). A decrease in the circulating concentration of trigonelline was reported for 1C group after 3 h. However, when the second coffee or the first CBPCC serving were consumed, a second increase within 3–5 h was observed (T_{max} PC group ≈ 4 h, Table 1), in particular for 3C group. As expected, another increase in trigonelline concentration was shown after the consumption of the third coffee or the second CBPCC serving, this increase being particularly relevant again for the 3C group (Figure 1A, T_{max} 3C group ≈ 8.5 h, Table 1). At the end of each treatment (t24), trigonelline plasma concentrations decreased to the basal concentrations (t0) (Figure 1A), in accordance with the behavior expected for these compounds upon daily intake. Minimum plasma concentration (C_{min}) of trigonelline resulted statistically different among treatments, with the 3C group showing the highest levels (Table 1). Other pharmacokinetic parameters also reflected the

higher dose of trigonelline consumed in the 3C arm, as C_{max} (6.1 ± 0.5 µmol L^{-1}) and AUC$_{0-24}$ (82.4 ± 6.9 µmol L^{-1} × h) were significantly higher compared to the values calculated for 1C and PC groups (Table 1). Finally, based on a "real-life" approach addressing the steady state, the C$_{avg}$ was calculated for the three arms to define the mean circulating concentration for trigonelline along the day. Once again, as expected, the highest C$_{avg}$ of trigonelline was reached in the 3C arm (3.4 ± 0.3 µmol L^{-1}), whereas the lowest (1.0 ± 0.1 µmol L^{-1}) was calculated for the 1C group (Table 1). Regarding urinary excretion, the 24-h cumulative excretion of trigonelline reflected the same trend obtained from plasma levels, showing a significantly higher urinary excretion of trigonelline in the 3C group, followed by the PC and 1C groups (Figure 1B).

Like for trigonelline, the pharmacokinetic profile and parameters of *N*-methylpyridinium clearly reflected the different intervention treatments (Figure 1C). The first fast peak slope resulted the same in the three groups and appeared 1 h after the first coffee consumption, indicating a rapid absorption of the native compound (T_{max} 1C group ≈ 1.5 h, Table 1). The plasma concentration of *N*-methylpyridinium in 1C group decreased within 5 h to basal levels, and this concentration range was maintained until 24 h. On the contrary, the consumption of three coffees (3C group) or two servings of CBPCC (PC group) during the test day outlined two additional plasma peaks 1 h after coffee or CBPCC consumption, respectively (Figure 1C). All the plasma
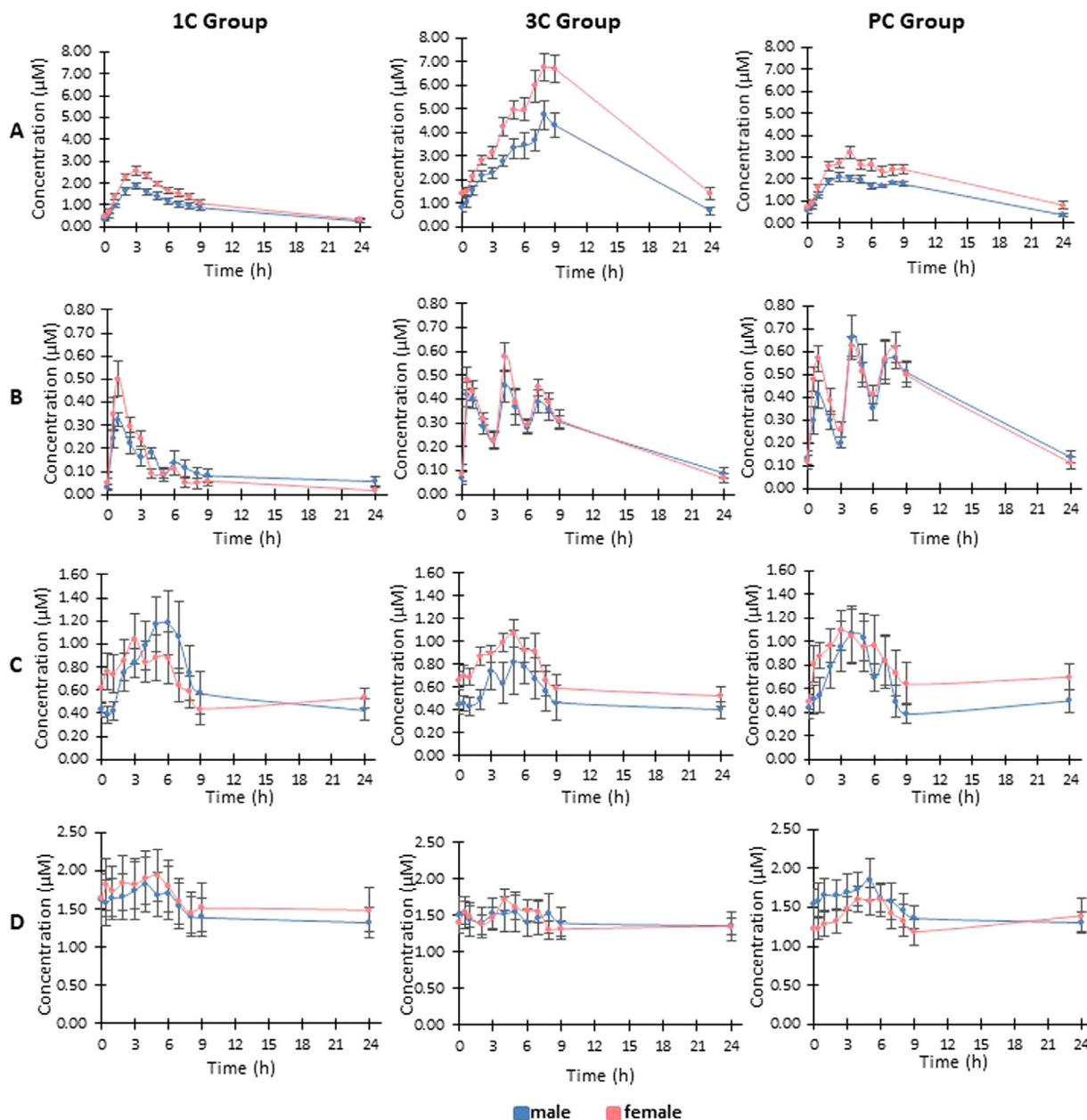
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Figure 2. Plasma kinetic profile of trigonelline A), *N*-methylpyridinium B), *N*-methylnicotinamide C), and *N*-methyl-4-pyridone-5-carboxamide D) in samples collected after consumption of 1 coffee (1C group), 3 coffees (3C group), and 1 coffee plus four cocoa-based product containing coffee (CBPCC, PC group), divided for males and females. Data expressed as mean ± SEM (males = 10 and females = 11).

peaks recorded for *N*-methylpyridinium resulted higher in the PC group compared to the other two gropus. Then, the basal concentrations recorded at t0 for 3C and PC groups were finally reached within 24 h and likely due to its rapid clearance. The C_{min} of *N*-methylpyridinium were pretty similar among the three treatments (0.01–0.07 μmol L^{-1}) despite being statistically different (Table 1). C_{max} and AUC_{0-24}, and, as a consequence, C_{avg} were statistically different among treatments, with PC group showing the highest values, followed by 3C group (Table 1). Similarly, the 24-h cumulative excretion of *N*-methylpyridinium was significantly higher in PC group, followed by 3C group and then 1C arm (Figure 1D).

2.3. Differences among Subjects due to Sex and Smoking

Splitting data by males (*n* = 10) and females (*n* = 11), the general kinetic trends previously described for the three intervention arms were maintained. Different absorption curves were observed between men and women in the three treatments for some metabolites (**Figure 2**a–d). In particular, trigonelline reached higher circulating concentrations for females compared to males (Figure 2a). This was confirmed by the pharmacokinetic parameters (Table S3, Supporting Information), that were generally higher, although not always significantly, in the female group. In general, it should be noted that a significant main effect of the sex
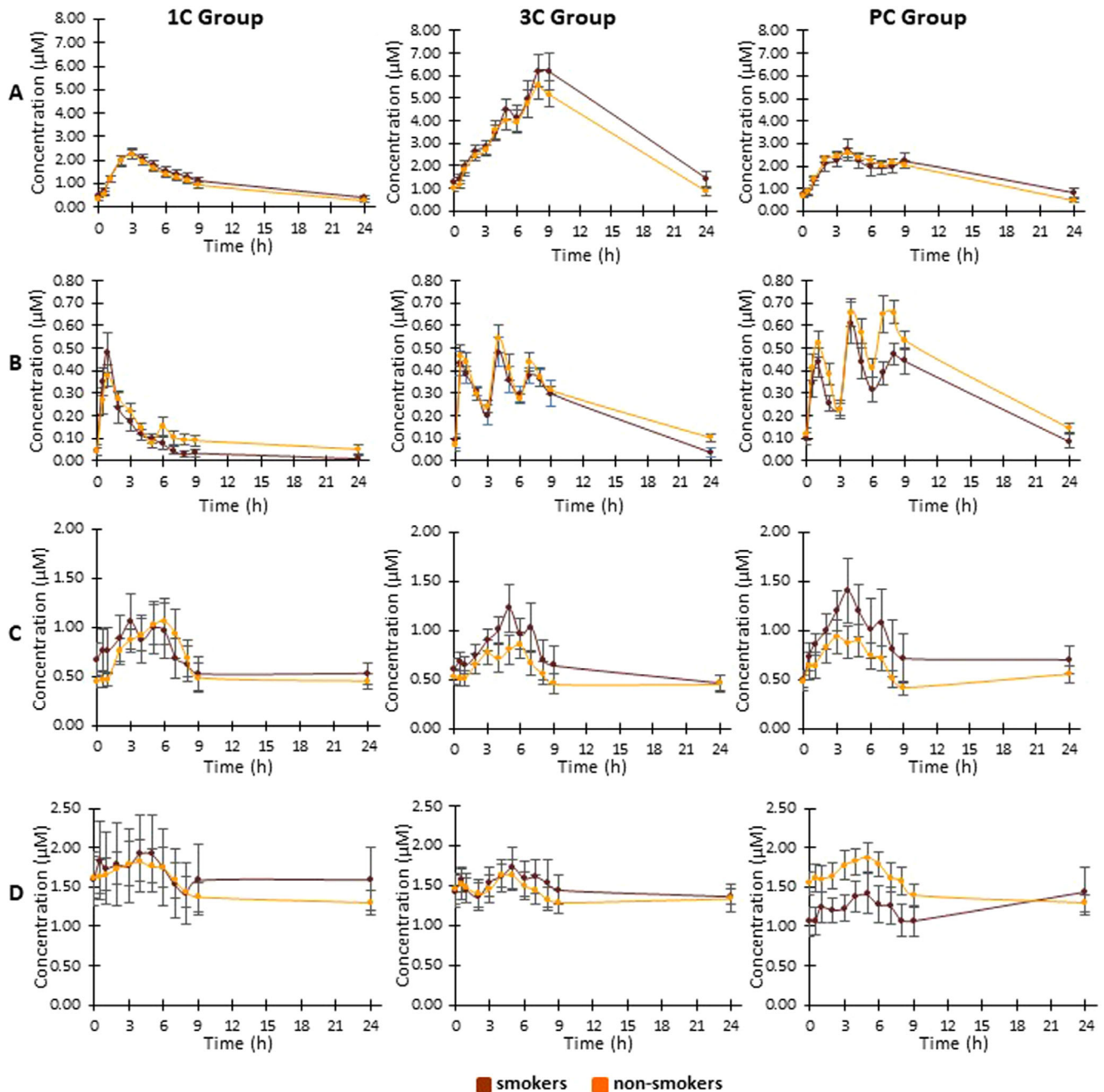
Figure 3. Plasma kinetic profile of trigonelline A), *N*-methylpyridinium B), *N*-methylnicotinamide C), and *N*-methyl-4-pyridone-5-carboxamide D) in samples collected after consumption of 1 coffee (1C group), 3 coffees (3C group), and 1 coffee plus four cocoa-based product containing coffee (CBPCC, PC group), divided for smokers and non-smokers. Data are expressed as mean ± SEM (smokers = 8 and non-smokers = 13).

was showed for the C_{min}, C_{max}, C_{avg}, and AUC_{0-24} of trigonelline, as well as the C_{min} and T_{max} of *N*-methylnicotinamide (Table S3, Supporting Information). This was not observed, however, when pyridine urinary excretion was considered, since no significant main effects of the sex were observed (all the *p*-values for a main effect of sex were >0.05 (Figure S1, Supporting Information).

When subjects were divided into smokers ($n = 8$) and non-smokers ($n = 13$), general trends were confirmed, but small differences among participants were observed for some metabolites

(**Figure 3**a–d). In this case, trigonelline did not show any statistically significant differences between smokers and non-smokers (*p*-value for a main effect of smoking >0.05, Figure 3a). On the contrary, a significant main effect of smoking was observed for the C_{avg} and AUC_{0-24} of *N*-methylpyridinium, highlighting higher concentrations in smokers than in non-smokers (Table S4, Supporting Information). This trend was not observed by the excreted amount of any metabolite in urine (*p*-values for main effects of smoking >0.05, Figure S2, Supporting Information).

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3. Discussion

This randomized crossover intervention study represented an innovative way to assess the kinetic profile and urinary excretion of pyridines under real-life conditions of coffee consumption. Moreover, the consumption of confectionary products containing coffee was also taken into account, as an alternative source for the intake of coffee phytochemicals. Among the potential metabolites derived from coffee, pyridines, trigonelline, *N*-methylpyridinium, *N*-methylnicotinamide, and *N*-methyl-4-pyridone-5-carboxamide were detected and quantified in plasma and urine samples. Looking at the curves obtained when coffee or CBPCC were consumed, it could be asserted that trigonelline was mainly absorbed in the small intestine,[20,29] as demonstrated by its C_{max} at around 3 h in 1C group (Table 1). Trigonelline did not decrease during the first half of the sampling day in the 3C and PC groups, and, actually, a sort of plasma accumulation could be hypothesized upon repeated intake, as previously reported for both caffeine and trigonelline,[20] probably due to their long elimination half-life (about 5 h).[20,24] On the contrary, *N*-methylpyridinium showed a different behavior, with a faster plasma appearance, compared to trigonelline, after coffee or CBPCC consumption, indicating a rapid intestinal absorption ($T_{max} = 1.5$ h in 1C group). Its circulating concentration rapidly decreased within 4 h, indicating also a fast plasma clearance, as previously demonstrated by its short half-life time (about 2 h).[24]

Although not explicative of the different study treatments, both *N*-methylnicotinamide and *N*-methyl-4-pyridone-5-carboxamide curves changed after coffee consumption. These metabolites, derived from nicotinic acid, increased after the consumption of the first coffee and slowly disappeared within the 24-h period to reach basal concentrations (Figure 1E,G). At hepatic level, nicotinic acid is quickly converted into nicotinamide, which represents the functional part of the coenzyme nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), playing a key role in numerous dehydrogenase-catalyzed reactions in living organisms.[24,30] The amide form is then *N*-methylated via *S*-adenosylmethionine giving rise to *N*-methylnicotinamide, which is finally oxidized to yield *N*-methyl-pyridone-carboxyxamide isomers, both *N*-methyl-2-pyridone-5-carboxamide and *N*-methyl-4-pyridone-5-carboxamide.[11,24,31,32] Nevertheless, though these compounds are major nicotinic acid metabolites, they were unaffected by the different treatments and showed quite constant concentrations in plasma, in line with previous reports.[20,24] Jacob and colleagues suggested that differences in plasma concentration of nicotinic acid metabolites appeared only with large changes in niacin intake.[33] The low amount of nicotinic acid in coffee and CBPCC, ranging from 0.5 to 1.4 mg per serving in the best case (Table S2, Supporting Information), can justify the absence of differences in the kinetic curves of these metabolites among the three treatments. Differently from previous studies reporting nicotinamide and two *N*-methyl-pyridone-carboxamides at plasma level,[24] as well as nicotinamide, two *N*-methyl-pyridone-carboxamides, nicotinic acid, nicotinamide-*N*-oxide, and *N*-methyl-2-pyridone-5-carboxylic acid at urinary level,[24,34] no traces of these metabolites were detectable in any plasma and urine samples in the present work. Because of the same reason previously mentioned, the lack of detection of these pyridine

metabolites could be due to the low amount of nicotinic acid in the products used in this study and to the low urinary excretion reported for some minor pyridine metabolites.[34]

Differences among subjects were observed in the appearance and kinetic parameters of some metabolites linked to sex and smoking habits. Inter-person variability is a key topic to properly address the bioavailability and potential health effects of food phytochemicals, but further information is still needed.[35] Trigonelline concentrations were higher in females probably due to their lower body weight and blood volume, causing a higher dilution in the blood of male volunteers, as previously suggested.[24] Trigonelline C_{max} and C_{avg} were, for instance, 33% and 36% higher in female compared to male in the 3C group (Table 3, Supporting Information). These differences might condition the individual physiological response to the sustained consumption of trigonelline, so care should be taken about this sex-dependent difference in the case of coffee pyridines. Regarding smoking, although it has been proposed that smokers may present higher trigonelline levels than non-smokers,[36] trigonelline concentrations did not change due to smoking habits. On the contrary, to date no information is available for possible differences in *N*-methylpyridinium metabolism linked to sex or smoking habits.

Coffee consumption has been associated with risk reduction for various health outcomes at three to four cups a day,[7,37] and trigonelline appeared to be implicated in both hypoglycemic and hypolipidemic effects,[38–40] indicating its possible contribution to the preventive role of coffee in the framework of type 2 diabetes. However, the mechanisms behind the potential health benefits of coffee and the compounds potentially responsible for these benefits are still poorly understood. Moreover, the doses tested in in vitro mechanistic studies might be considered a rough estimation due to the lack of more comprehensive and realistic approaches.[26,41] The biological effects associated with trigonelline or *N*-methylpyridinium have been generally obtained by exposing cell lines to concentrations not in line with the physiological concentrations reachable in normal coffee consumption settings. Some positive results on antioxidant activity, on functional neurite outgrowth or on dopamine release have been obtained using trigonelline concentrations comparable to those recorded for the 3C group,[42] whereas chemopreventive properties[43] and estrogenic activity[44,45] were observed for trigonelline using nano- to picomolar concentrations. Generally, concentrations higher than 10 µmol L^{-1} for trigonelline and 1 µmol L^{-1} or *N*-methylpyridinium[8,43,46–49] should be considered too high in common daily coffee intake scenarios. This work presented an innovative approach that allowed to estimate the daily average plasma concentration (C_{avg}) of coffee pyridines in a real-life setting. Concentrations of 1.0, 3.4, and 1.6 µmol L^{-1} for trigonelline, and 0.1, 0.3, and 0.4 µmol L^{-1} for *N*-methylpyridinium in the 1C, 3C, and PC groups, respectively, can be considered physiological and able to mimic sustained exposure to coffee pyridines in low and medium to high coffee consumers. In this context, the C_{avg} calculated in the present work for the main coffee pyridine metabolites will be useful for future in vitro studies applying more adequate concentrations. In addition, the C_{avg} reported for trigonelline was perfectly in line with the values reported in a cohort of 3503 participants, where the median plasma concentration of trigonelline, calculated based on coffee cup consumption, was 1.63 µmol L^{-1} when

only one cup of coffee was consumed and 3.56 µmol L^{-1} when 2–4 cups were consumed.[36] Moreover, trigonelline has been reported as a suitable biomarker of coffee intake, considering its high correlation with intake and its scarce presence in other components of the diet.[15,17]

In conclusion, this study established, for the first time, the daily exposure to trigonelline, *N*-methylpyridinium, *N*-methylnicotinamide, and *N*-methyl-4-pyridone-5-carboxamide after the consumption of different coffee dosages in a real-life setting of coffee consumption. Contrary to the majority of scientific works, which investigated the metabolism of coffee bioactive compounds using a "one shot uptake," this study represents a valid approach to simulate realistic situations of coffee consumption. This work also served to confirm that repeated coffee servings along the day may lead to more persistent pyridine metabolite levels compared to a single serving. This data will be useful for future studies aiming at evaluating the bioactivity of coffee-derived circulating metabolites in cell experiments, mimicking more realistic experimental conditions. In addition, it may be useful to better understand the role that some drivers of subject variability, like sex and smoking, may have in the bioavailability of coffee pyridines.

4. Experimental Section

Chemicals: Nicotinic acid, nicotinamide, nicotinamide-*N*-oxide, *N*-methylpyridinium iodate, *N*-methylnicotinamide, and trigonelline hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade solvents were purchased from VWR International (Radnor, PA, USA). Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

Participants: The subject characteristics and the criteria for the recruitment have been already published.[25] Briefly, 21 volunteers, 10 males (2 smokers) and 11 females (6 smokers), aged 25.9 ± 0.5, weight 67.0 ± 2.7 kg, and BMI 22.3 ± 0.6 kg m^{-2}, were recruited in Parma (Italy). Those volunteers who agreed to participate were asked to sign the written informed consent to participate in the study. Information to the volunteers was provided before and separately from the consent form. All the volunteers completed the intervention study.

Dosage Information and Study Design: The study was approved by the Ethics Committee for Human Research of the University of Parma (AZOSPR/0015693/6.2.2.), registered on ClinicalTrials.gov on May 21, 2017 (NCT03166540), and the study protocol has been previously published.[25] The human intervention study was of a short term, randomized crossover trial and followed a repeat-dose, 3-arm design. Briefly, subjects were requested to follow their habitual diet and to randomly consume the following treatments for 1 month: one cup of espresso coffee per day (at 9.00 a.m., namely 1C group), three cups of espresso coffee per day (at 9.00 a.m., 12.00 noon, and 3.00 p.m., namely 3C group), or one cup of espresso coffee plus two CBPCC twice per day (coffee at 9.00 a.m. and two CBPCC at 12.00 p.m., and 3.00 p.m., namely PC group). Randomization list was generated using Random Number Generator Pro (Segobit Software).[25] To standardize the products consumed and cup volume (≈35 mL), a coffee machine (Essenza EN 97.W, De'Longhi Appliances S.r.l, Treviso, Italy), coffee capsules (Capriccio, Nespresso Italia S.p.a., Assago, Italy), and CBPCC (Pocket Coffee, Ferrero Commerciale Italia S.r.l., Alba, Italy) were supplied to all volunteers. The coffee capsules were selected based on their bioactive content after profiling several espresso coffee capsules available in the Italian market.[9] The number of CBPCC was chosen considering that the amount of caffeine provided by a cup of espresso coffee was ≈60 mg per serving, while two CBPCC provided, together, ≈30 mg of caffeine and thus were approximately the same as half coffee cup in terms of caffeine content.

Minimal recommendations to avoid other sources of coffee- and cocoa-like phytochemicals, besides what introduced through the assigned coffee/CBPCC serving, were provided for the 2 days prior to each sampling day (the last day of each intervention period) and on the sampling day. Volunteers were asked to standardize the time of coffee and CBPCC consumption during the intervention period and, in particular, during the last 3 days of each treatment. The day before the sampling day, time of dinner, and its composition were standardized and only water was allowed as a drink overnight. Volunteers' food intake and compliance with the study requirements were assessed by means of three-day dietary records, administered during each intervention period at two time points, that is, in the middle of each intervention arm, including 2 weekdays and a weekend day, and at the end of each intervention arm, including 2 days prior to the sampling day and the sampling day.

During the sampling day of each study arm, subjects attended the ambulatory of the Endocrinology Unit of the Department of Medicine and Surgery at the University of Parma, where fasting blood was collected prior to coffee consumption (t0). After baseline blood drawing, all subjects consumed (at about 9.00 a.m.) a cup of coffee, together with a low-phenolic breakfast (a sponge milky cake). This was the only dose of coffee for treatment 1C, whereas treatment 3C consumed two more cups of coffee (at 12.00 noon and 3.00 p.m.), while treatment PC consumed 2 CBPCC twice during the day (at 12.00 noon and 3.00 p.m.). Starting just after the consumption of the first coffee, blood samples were collected through a venous catheter at selected time points (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 24 -t24- h). 5 h after the consumption of the first coffee, participants received a standardized lunch (ham and cheese sandwich) free of coffee/cocoa-related phytochemicals and water was available ad libitum. 24 h after receiving the first coffee of the sampling day, blood samples were taken to check for return to baseline of the circulating phytochemicals, and then subjects were switched to another arm, based on the randomization protocol. For each blood drawing, 3 mL of venous EDTA-blood was collected. Blood was immediately centrifuged at 2400 × *g* for 15 min to separate the plasma, which was frozen at −80 °C until analyses. Additionally, urine from each volunteer was collected at baseline (t0) and at different collection periods within 0–3 h, 3–6 h, 6–9 h, and 9–24 h. The volume of urine collected during each period was measured and two 2 mL samples were stored at −80 °C until analysis.

Coffee and CBPCC Pyridine Extraction: Pyridines in espresso coffee were extracted according to Lang and colleagues,[13] with minor modifications. Coffee, prepared with the same coffee machine used by the volunteers, was diluted 1:50 or 1:200 v/v with pure acetonitrile. The solution was centrifuged at 13 765 × *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, diameter 13 mm) in vial prior to UHPLC-ESI-MS^2 analysis. The CBPCC, having an important lipid fraction, was extracted adapting the methodology reported by Pedan and colleagues.[27] CBPCC was first heated up to 40 °C for 10 min and dissolved in 25 mL of *n*-hexane to eliminate the lipid fraction. Samples were vortexed for 5 min, centrifuged at 2647 × *g* for 10 min at room temperature and the supernatant was eliminated. This purification step was repeated twice. Pyridines from defatted CBPCC were then extracted with 25 mL of 70% aqueous methanol acidified with formic acid (1% v/v). The solution was sonicated for 10 min, centrifuged at 2647 × *g* for 10 min at room temperature and 1 mL of the supernatant was collected and diluted 1:2 or 1:100 with pure acetonitrile. The extract was centrifuged at 13 765 × *g* for 5 min at room temperature and filtered (0.22 µm nylon filter, diameter 13 mm) in vial prior to analysis. Nine samples from three different lots were considered for each product (espresso coffee and CBPCC) and each sample was extracted in triplicate.

Plasma and Urine Extraction After Coffee and CBPCC Consumption: An aliquot of 100 µL of human plasma or 10 µL of urine was precipitated with pure acetonitrile (1:10 or 1:100 v/v, respectively). Biological samples were vortexed for 1 min, centrifuged at 13 765 × *g* for 10 min at room temperature and supernatants were finally analyzed by UHPLC-ESI-MS^2.

Pyridine Quantification by Liquid Chromatography-Mass Spectrometry (UHPLC-ESI-MS2) in Products and Biological Samples: Both food and biological 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 XBridge BEH HILIC (2.1 × 100 mm, 2.5 μm particle size) (Waters, Milford, MA, USA). Volume injection was 5 μL and column oven was set to 35 °C. Compound elution was performed at a flow rate of 0.5 mL min^{-1}. For separation, mobile phase A was ammonium formate (20 mmol L^{-1}) containing 1% of formic acid, phase B was acetonitrile containing 0.1% formic acid, and phase C was acidified water (0.1% formic acid). The gradient started with 10% A, which was maintained constant during the whole analysis, 89% B and 1% C. The mobile phases comprised a program of 0–1 min, 1% B; 1–4 min, to reach 10% B; 4–4.5 min, 10–63% B, maintained for 1 min; 5.5–7 min, 1% B to return to the starting conditions and maintained until the end of the analysis (10 min). The analytical mass spectrometric conditions were optimized by infusion of a pure standard of trigonelline, working in positive ionization mode. Analyses were carried out using a full MS^2 method, monitoring specific mass to charge (*m/z*) transitions (Table S1, Supporting Information), and fragmentation was obtained using a collision induced dissociation (CID) equal to 35 (arbitrary units). Pure helium gas was used for CID. 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 units. The source voltage was 3.8 kV, whereas the capillary voltage and tube lens voltage were +36 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 available standard compounds in a concentration range of 0.01–100 μmol L^{-1}.

Statistical Analyses: The sample size was calculated considering as primary outcome the daily mean concentration of a coffee derived plasma circulating phenolic metabolite, as previously reported.[25] Sample size was not calculated for secondary outcomes but 21 subjects can be considered appropriate also to observe significant differences in pyridine pharmacokinetic parameters, based on previously published data in which 13 volunteers (6 female and 7 male) were assessed.[24] Subgroup analysis by sex should be also powered (with 10 males and 11 females) attending to the data provided by Lang et al.[24]

Product pyridine content were expressed as mean ± standard deviation (SD). Plasma and urinary 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,[28] including area under curve (AUC$_{0-24}$), maximum (C_{max}), minimum (C_{min}) and average (C_{avg}) plasma concentration (the latter calculated as AUC$_{0-24}$/24 h), and time to reach the maximum (T_{max}) plasma concentration.

The Kolmogorov–Smirnov test was used to check for data normality. Comparison among treatments were performed by repeated measurement ANOVA, testing the assumption of sphericity through the Mauchly's test and, when the assumption of sphericity was violated, using Greenhouse–Geisser correction if epsilon was less than 0.75 or Huynh–Feldt correction if epsilon was above 0.75; Bonferroni post hoc tests were used for multiple comparisons. In addition, a mixed between-within subjects analysis of variance was conducted to assess the impact of sexes (male and female) or of smoking habits (smoker and non-smoker) among the three treatments. A paired sample *t*-test was performed for comparison between t0 and t24 data within the same group before and after product consumption. A difference was considered significant at $p < 0.05$. The statistical analysis was performed with the Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0. IBM Corp., Chicago, IL).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Conflict of Interest

This study was funded by Soremartec Italia S.r.l. (Ferrero Group, Alba, Italy). The funder approved the final trial protocol prior to its implementation, but it was not involved in the design of the study, data analysis, and interpretation nor the drafting of this manuscript. The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

L.B. and M.T. contributed equally to this work. P.M., D.D.R., and F.B. designed the study. M.T., A.R., D.M., M.A., A.D.C., and R.B. conducted the study. M.T. and L.B. performed the analysis. M.T., A.R., and P.M. analyzed and interpreted data. L.B. and M.T. drafted the manuscript. P.M. and D.D.R. edited the manuscript. All authors critically read and approved the final version of the manuscript.

Keywords

[1] N. D. Freedman, Y. Park, C. C. Abnet, A. R. Hollenbeck, R. Sinha, *N. Engl. J. Med.* **2012**, *366*, 1891.

[2] I. A. Ludwig, M. N. Clifford, M. E. Lean, H. Ashihara, A. Crozier, *Food Funct.* **2014**, *5*, 1695.

[3] J. V. Higdon, B. Frei, *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 101.

[4] M. Ding, S. N. Bhupathiraju, A. Satija, R. M. van Dam, F. B. Hu, *Circulation* **2014**, *129*, 643.

[5] M. Ding, A. Satija, S. N. Bhupathiraju, Y. Hu, Q. Sun, J. Han, E. Lopez-Garcia, W. Willett, R. M. van Dam, F. B. Hu, *Circulation* **2015**, *132*, 2305.

[6] M. J. Gunter, N. Murphy, A. J. Cross, L. Dossus, L. Dartois, G. Fagherazzi, R. Kaaks, T. Kühn, H. Boeing, K. Aleksandrova, A. Tjønneland, A. Olsen, K. Overvad, S. C. Larsen, M. L. R. Cornejo, A. Agudo, M. J. S. Pérez, J. M. Altzibar, C. Navarro, E. Ardanaz, K.-T. Khaw, A. Butterworth, K. E. Bradbury, A. Trichopoulou, P. Lagiou, D. Trichopoulos, D. Palli, S. Grioni, P. Vineis, S. Panico, et al., *Ann. Intern. Med.* **2017**, *167*, 236.

[7] G. Grosso, J. Godos, F. Galvano, E. L. Giovannucci, *Annu. Rev. Nutr.* **2017**, *37*, 131.

[8] G. R. Romualdo, A. B. Rocha, M. Vinken, B. Cogliati, F. S. Moreno, M. A. G. Chaves, L. F. Barbisan, *Food Res. Int.* **2019**, *123*, 567.

[9] D. Angelino, M. Tassotti, F. Brighenti, D. Del Rio, P. Mena, *Sci. Rep.* **2018**, *8*, 17874.

[10] H. Ashihara, *Nat. Prod. Commun.* **2016**, *11*, 1047.

[11] EFSA Panel on Dietetic Products, Nutrition and Allergies, *EFSA J.* **2014**, *12*, 3759.

[12] R. H. Stadler, N. Varga, J. Hau, F. A. Vera, D. H. Welti, *J. Agric. Food Chem.* **2002**, *50*, 1192.

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[13] R. Lang, E. F. Yagar, R. Eggers, T. Hofmann, *J. Agric. Food Chem.* **2008**, *56*, 11114.

[14] R. Lang, A. Wahl, T. Stark, T. Hofmann, *Mol. Nutr. Food Res.* **2011**, *55*, 1613.

[15] K. A. Guertin, S. C. Moore, J. N. Sampson, W. Y. Huang, Q. Xiao, R. Z. Stolzenberg-Solomon, R. Sinha, A. J. Cross, *Am. J. Clin. Nutr.* **2014**, *100*, 208.

[16] J. A. Rothwell, Y. Fillatre, J. F. Martin, B. Lyan, E. Pujos-Guillot, L. Fezeu, S. Hercberg, B. Comte, P. Galan, M. Touvier, C. Manach, *PLoS One* **2014**, *9*, e93474.

[17] J. A. Rothwell, P. Keski-Rahkonen, N. Robinot, N. Assi, J. A. Rothwell, P. Keski-Rahkonen, N. Robinot, N. Assi, C. Casagrande, M. Jenab, P. Ferrari, M.-C. Boutron-Ruault, Y. Mahamat-Saleh, F. R. Mancini, H. Boeing, V. Katzke, T. Kühn, K. Niforou, A. Trichopoulou, E. Valanou, V. Krogh, A. Mattiello, D. Palli, C. Sacerdote, R. Tumino, A. Scalbert, *Mol. Nutr. Food Res.* **2019**, *63*, 1900659.

[18] L. Maeztu, S. Andueza, C. Ibanez, M. Paz de Pena, J. Bello, C. Cid, *J. Agric. Food Chem.* **2001**, *49*, 4743.

[19] G. Caprioli, M. Cortese, F. Maggi, C. Minnetti, L. Odello, G. Sagratini, S. Vittori, *Int. J. Food Sci. Nutr.* **2014**, *65*, 465.

[20] R. Lang, N. Dieminger, A. Beusch, Y. M. Lee, A. Dunkel, B. Suess, T. Skurk, A. Wahl, H. Hauner, T. Hofmann, *Anal. Bioanal. Chem.* **2013**, *405*, 8503.

[21] A. Rodriguez-Mateos, D. Vauzour, C. G. Krueger, D. Shanmuganayagam, J. Reed, L. Calani, P. Mena, D. Del Rio, A. Crozier, *Arch. Toxicol.* **2014**, *88*, 1803.

[22] D. Del Rio, A. Rodriguez-Mateos, J. P. Spencer, M. Tognolini, G. Borges, A. Crozier, *Antioxid. Redox Signaling* **2013**, *18*, 1818.

[23] S. Martínez-López, B. Sarriá, M. Gómez-Juaristi, L. Goya, R. Mateos, L. Bravo-Clemente, *Food Res. Int.* **2014**, *63*, 446.

[24] R. Lang, A. Wahl, T. Skurk, E. F. Yagar, L. Schmiech, R. Eggers, H. Hauner, T. Hofmann, *Anal. Chem.* **2010**, *82*, 1486.

[25] P. Mena, M. Tassotti, D. Martini, A. Rosi, F. Brighenti, D. Del Rio, *Trials* **2017**, *18*, 527.

[26] P. Mena, D. Del Rio, *J. Agric. Food Chem.* **2018**, *66*, 8221.

[27] V. Pedan, N. Fischer, S. Rohn, *Anal. Bioanal. Chem.* **2016**, *408*, 5905.

[28] Y. Zhang, M. Huo, J. Zhou, S. Xie, *Comput. Methods Programs Biomed.* **2010**, *99*, 306.

[29] S. Yuyama, in *Tryptophan, Serotonin, and Melatonin: Basic Aspects and Applications* (Eds: G. Huether, W. Kochen, T. J. Simat, H. Steinhart), Springer, Boston, MA **1999**, p. 723.

[30] A. Garten, S. Petzold, A. Korner, S. Imai, W. Kiess, *Trends Endocrinol. Metab.* **2009**, *20*, 130.

[31] M. L. W. Chang, B. C. Johnson, *J. Biol. Chem.* **1961**, *236*, 2096.

[32] P. Wong, A. Bachki, K. Banerjee, B. Leyland-Jones, *J. Pharm. Biomed. Anal.* **2002**, *30*, 773.

[33] R. A. Jacob, M. E. Swendseid, R. W. McKee, C. S. Fu, R. A. Clemens, *J. Nutr.* **1989**, *119*, 591.

[34] J. I. Kremer, K. Gompel, T. Bakuradze, G. Eisenbrand, E. Richling, *Mol. Nutr. Food Res.* **2018**, *62*, 1700735.

[35] C. Morand, F. A. Tomas-Barberan, *Eur. J. Nutr.* **2019**, *58*, 13.

[36] O. Midttun, A. Ulvik, O. Nygard, P. M. Ueland, *Am. J. Clin. Nutr.* **2018**, *107*, 941.

[37] R. Poole, O. J. Kennedy, P. Roderick, J. A. Fallowfield, P. C. Hayes, J. Parkes, *Br. Med. J.* **2017**, *359*, j5024.

[38] O. Yoshinari, H. Sato, K. Igarashi, *Biosci., Biotechnol., Biochem.* **2009**, *73*, 1033.

[39] R. Farias-Pereira, C. S. Park, Y. Park, *Food Sci. Biotechnol.* **2019**, *28*, 1287.

[40] A. E. van Dijk, M. R. Olthof, J. C. Meeuse, E. Seebus, R. J. Heine, R. M. van Dam, *Diabetes Care* **2009**, *32*, 1023.

[41] A. Fardet, E. Rock, *Adv. Nutr.* **2014**, *5*, 430.

[42] J. Zhou, L. Chan, S. Zhou, *Curr. Med. Chem.* **2012**, *19*, 3523.

[43] U. Boettler, K. Sommerfeld, N. Volz, G. Pahlke, N. Teller, V. Somoza, R. Lang, T. Hofmann, D. Marko, *J. Nutr. Biochem.* **2011**, *22*, 426.

[44] G. Yoo, C. D. Allred, *Food Chem. Toxicol.* **2016**, *87*, 23.

[45] K. F. Allred, K. M. Yackley, J. Vanamala, C. D. Allred, *J. Nutr.* **2009**, *139*, 1833.

[46] J. C. Liao, K. T. Lee, B. J. You, C. L. Lee, W. T. Chang, Y. C. Wu, H. Z. Lee, *Food Nutr. Res.* **2015**, *59*, 29884.

[47] L. Sharma, N. A. Lone, R. M. Knott, A. Hassan, T. Abdullah, *Food Chem. Toxicol.* **2018**, *121*, 283.

[48] S. Anwar, U. Bhandari, B. P. Panda, K. Dubey, W. Khan, S. Ahmad, *J. Pharm. Biomed. Anal.* **2018**, *159*, 100.

[49] S. V. Somoza, M. Lindenmeier, E. Wenzel, O. Frank, H. F. Erbersdobler, T. Hofmann, *J. Agric. Food Chem.* **2003**, *51*, 6861.