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Article

Untargeted HR-MS Profiling and Discrimination of *Coffea arabica* L. Leaves under Different Postharvest Production Processes

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ABSTRACT: Coffee byproducts, such as coffee leaves, are components of the coffee plant that are still underexplored. Considering their potential application in the food chain, defining their phytochemical profile and its susceptibility to processing is essential. A comprehensive HR-MS profiling performed using ultrahigh-performance liquid chromatography coupled with traveling wave ion mobility spectrometry/quadrupole time-of-flight mass spectrometry was applied to dive into the phytochemicals occurring in *Coffea arabica* L. leaves (cv. red Caturra), following three postharvest approaches used to obtain an oxidized, a slightly oxidized, and a nonoxidized, herbal infusion-like botanical product. Coffee signature compounds, such as caffeine and 5-caffeoylquinic acid, were also quantified, evidencing that each processing step had a peculiar impact on the extracts. Slightly oxidized and oxidized samples showed comparable levels of caffeine and 5-caffeoylquinic acid, while the content of both compounds was significantly different in nonoxidized leaves. The untargeted HR-MS approach followed by chemometrics allowed a clear clustering of the three different treatments, and 472 identifications were putatively assigned. The large majority (95.4%) of the identified metabolites fall within 8 chemical superclasses, the most represented being phenylpropanoids (38.0%), sterol lipids (18.2%), and glycerophospholipids (17.7%). Among all of the identified metabolites, 45% are significantly overaccumulated in nonoxidized samples, 37% in the oxidized samples, and, finally, 18% in the slightly oxidized samples. Besides providing exhaustive profiling, such an approach confirms the suitability of untargeted HR-MS for discrimination of commercial batches of coffee leaves and highlights how its exploitation could support future investigations.

KEYWORDS: *coffee byproducts, Coffea arabica, novel food, innovative foods, botanical products, postharvest*

INTRODUCTION

Genus *Coffea* (Rubiaceae) includes 124 species, two of which are commercially used to produce coffee beverages: *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehner (*C. robusta*).[1] The global dimension of this market requires extensive cultivation, from which the main consolidated product is currently represented by coffee seeds, both green and roasted. This implies the availability of a variety of byproducts created in primary and secondary coffee manufacturing, such as flowers, pulp, husk, parchment, silver skin, and, in particular, leaves, whose commercial exploitation could be considered if proper knowledge on their composition, properties, and management is collected.[2] As the commercial exploitation of byproducts is advisable to create further economic benefits, the scientific community started evaluating such materials, highlighting the presence of substances deemed beneficial for health and appreciated from an organoleptic standpoint.[3−6] The marketing of some of these byproducts is already allowed under the novel food regulatory framework in force within the European Union.[7] Since 2020, the publication of such regulation has allowed coffee leaf infusions to be placed on the EU market as a traditional food from a third country, assigning at the same time critical values for compounds such as caffeine (<80 mg/L) and chlorogenic acid (<100 mg/L).[8]

Coffee leaves have been traditionally used to produce infusions in Indonesia, Jamaica, India, Java, Sumatra, Ethiopia, and South Sudan since the XIX century, and in multiple tropical countries coffee leaves are consumed by local communities for cultural and medical reasons.[4] For instance, Novita (2018) described the traditional production of "Kahwa daun", a herbal tea made from coffee leaves produced by people in West Sumatra, Indonesia, by drying and toasting leaves above a cooking fire.[9] Most of these preparations rely on the direct use of fresh leaves or make recourse to very simple drying methods, whose scale-up to large batches necessary for commercial production requires careful standardization.

Coffee leaves harbor a wide array of compounds, including phenols, alkaloids, flavonoids, phytosterols, tannins, xanthones, amino acids, terpenes, and anthocyanins.[3,10−12] In particular, coffee leaves are discriminated from beans by the presence of xanthones and, in particular, mangiferin.[13] Their phytochemical profile is influenced by multiple environmental, genetic, and climatic factors, but further variability is determined by postharvest handling, which can reduce, enhance, or modulate the relative abundance of compounds relevant for bioactive or

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organoleptic purposes.[3] Recently, DePaula et al.[14] highlighted the possibility for coffee farmers and consumers worldwide to support sustainability in coffee production by means of coffee leaves' tea products. Considering the similar signature of phytochemicals and the overlap in cultivation occurring in some areas of the world, previous reports have suggested that mimicking the different tea styles and postharvest managements available for *Camellia sinensis* leaves may lead to obtain distinct phytochemical profiles also from coffee leaves. For instance, treating young coffee leaves with a green tea process and mature leaves with a black tea process provided the highest and the lowest phenolic content, respectively. The differences were considered a consequence of the reduced polyphenol oxidase and peroxidase activities occurring in coffee leaves treated with milder postharvest conditions.[15]

However, the potential of coffee leaves as a source of phytochemicals and their potential exploitation in the food and beverages industry is still underexplored. Processing is often based on local procedures, with little to no knowledge of their effects on the phytochemical contents. There is a general lack of standardized protocols for processing allowing for comparison and quality assessment, thus creating a scenario in which the lack of adequate information hampers product development.

Current investigations suggest that understanding how the postharvest processing handling of coffee leaves affects their content is essential for producing innovative botanicals based on this plant material.[16] At the same time, the availability of further postharvest management protocols, especially in areas of the world where the overlap between coffee and tea cultivation is absent, could lead to botanicals based on Coffea leaves with different profiles.

In this regard, untargeted HR-MS may provide an efficient and sensitive approach for fingerprinting the phytochemical composition of coffee leaves. It may allow, also in the absence of analytical standards, us to annotate plant metabolites by taking advantage of the large online repositories and database available for plant metabolomics, such as PlantCyc.[17] The application of HR-MS-based techniques has recently allowed a more thorough description of the wide variety of secondary metabolites occurring in coffee leaves, along with the simultaneous detection of entire molecular families.[18−25] However, few papers have investigated the potential effect of such techniques on the postharvest processing of coffee leaves and the phytochemical modifications involved.[21] This approach allows the untargeted comparison of large analytical data in several herbal plants and spices, leading to not only the discrimination of plants with distinct geographical origin but also the classification of different styles of postharvest management.[26−28]

In the present study, a quantitative determination of signature metabolites was combined with an untargeted HR-MS analysis to dive into the phytochemical profile of coffee leaves following three different processing treatments. Such protocols have been developed in Ecuador according to local knowledge with increasing intensity. In particular, the milder process was based on conventional herbal drying (NOL), while in the other protocols, coffee leaves underwent partial (SOL) and complete (HOL) oxidation.

Besides providing an exhaustive profiling of major coffee leaf phytochemicals, such an approach was applied to evaluate the suitability of untargeted HR-MS in the discrimination of commercial batches of this innovative botanical.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. For quantitative purposes, reference standards were used where possible: 3-caffeoylquinic acid (3CQA), 4-caffeoylquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA), and caffeine (Ph. Eur. Grade), from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). LC-MS-grade methanol and acetonitrile were purchased from Scharlab Italia srl (Milan, Italy); bidistilled

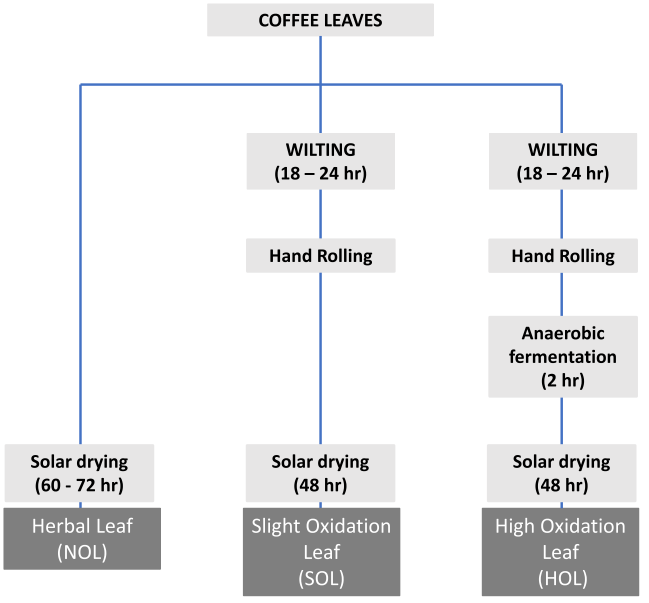

Figure 1. Process applied to the coffee leaves considered in this study.

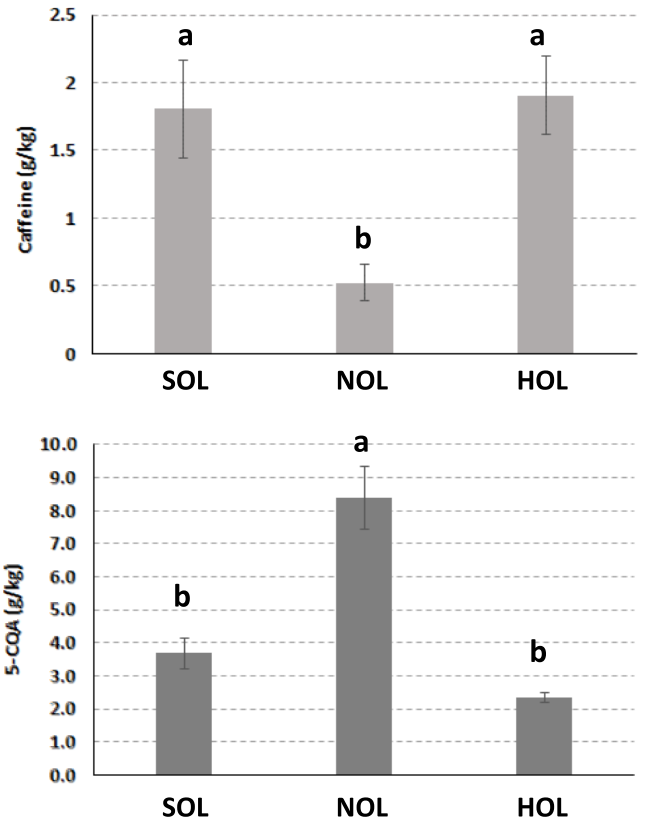

Figure 2. Caffeine (A) and 5-CQA (B) contents in coffee leaf samples. Values with different letters differ significantly by Bonferroni's post hoc test ($p < 0.05$).

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water was obtained using the Milli-Q System (Millipore, Bedford, Massachusetts). MS-grade ammonium acetate, acetic acid, and formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, California) were also used.

2.2. Plant Material. *C. arabica* leaves (red Caturra cv.) were collected in 2020 from Terrazas del Pisque, Pichincha, Ecuador (altitude: 2100 m), from 3-year-old plants. Leaves were collected mimicking the "two leaves and a bud" approach used to produce the highest tea quality.[26] The apical bud and the first three leaves of the young stems were picked from 12 different and healthy coffee plants to avoid differences caused by light-shadow and growing land. The processing step (Figure 1) was performed following the local approach aimed at standardizing coffee leaves' production. Three different leaf-processing procedures were followed: (i) herbal leaf (NOL) processing: fresh leaves are directly dried by direct solar exposure. The drying step was performed for about 60−72 h, until cracking. (ii) Slight oxidation leaf (SOL) processing: fresh leaves are wilted for 18−24 h after harvest under shade on African beds, allowing spontaneous enzymatic activity. After oxidation, leaves are rolled by hand and exposed to direct solar radiation drying. The drying step was performed for about 48 h, until cracking. (iii) High oxidation leaf (HOL) processing: fresh leaves are wilted for 18−24 h after harvest under shade on African beds, allowing spontaneous enzymatic activity. After oxidation, leaves are rolled by hand to facilitate cell disruption and then further oxidated under anaerobic conditions by putting the material in plastic bags. After 2 h, the plant material was exposed to direct solar radiation drying. The drying step was performed for about 48 h, until cracking.

2.3. Sample Preparation. Batches were obtained from bulk mixes of coffee leaves exposed to the selected treatment. For SOL and HOL treatments, one bulk batch of about 5 kg of dried leaves was considered, while for NOL two separate bulk batches (5 kg each) were considered due to the slightly higher variability of the applied conditions. Technical replicate aliquots (50 g each, $n = 3$) were obtained from bulk batches by thorough mixing. For each aliquot, 250 mg of leaves milled using a mortar and pestle were subjected to hydroalcoholic extraction with 5 mL of 80% methanol. The extract was treated with ultrasonication at room temperature for 30 min and centrifuged for 10 min at 10 000 rpm at 4 °C, and then the supernatants were transferred in a sealed vial prior to analysis.

Quality control samples (QC; $n = 5$) were prepared by thoroughly mixing 10 μL of each extract in a vial. QC samples were randomly injected across the batch analysis and used for monitoring instrumental errors.

2.4. Targeted LC/MS Analysis. For caffeine and chlorogenic acids, the method developed by Angelino et al.[29] was applied with slight modification. Briefly, samples were analyzed using an UHPLC Ultimate 3000 (Dionex, Sunnyvale, California) equipped with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher, San Jose, California) fitted with a heated-electrospray ionization (ESI) probe (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were carried out using an ACE UltraCore SuperC18 2,5 μm particle size (2, 10 mm × 100 mm, Advanced Chromatography Technologies Limited, Aberdeen, Scozia). The volume injected was 2 μL, column oven was set to 40 °C, and elution flow rate was 0.35 mL/min. Eluent A was 0.1% aqueous formic acid and eluent B was 0.1% formic acid in acetonitrile. Initial conditions were set at 95% eluent A and kept for 11 min; then, eluent B was increased to 80% in 4 min and decreased to the initial conditions in 6 min. The total run was 21 min. The MS worked with capillary temperature at 275 °C, while the source was set at 300 °C. The sheath gas flow was 50 units, while auxiliary gas pressure was set to 10 units. The source voltage was 3.5 kV. Caffeine was monitored in ESI+ considering the following MRM transitions: 195 ≥ 138, 195 ≥ 110 m/z; on the contrary, chlorogenic acid isomers were monitored in ESI- using the following transitions: 353 → 191, 353 → 179 m/z for 3- and 5-CQA (retention time: 2.35 min, 4.65 min, respectively); 353 → 173, 353 → 179 for 4-CQA (retention time: 5.09 min). Data processing was performed using Xcalibur software from Thermo Scientific. For quantification, a calibration was performed over 5 concentration levels (0.5−1 to 2−5 to 10 g/kg), with three replicates at each concentration. The working standard solution were prepared in 80% methanol. A good linearity was achieved with $r^2 > 0.999$ for caffeine and chlorogenic acids.

2.5. Untargeted UHPLC-TWIMS-QTOF Analysis. An ACQUITY I-Class UPLC separation system coupled to a VION IMS QTOF mass spectrometer (Waters, Wilmslow, UK) equipped with an electrospray ionization (ESI) interface was employed for untargeted analysis. Samples were injected (2 μL) and chromatographically separated using a reversed-phase C18 BEH ACQUITY column of 2.1 mm × 100 mm, 1.7 μm particle size (Waters, Milford, Massachusetts). A gradient profile was applied using water (eluent A) and acetonitrile (eluent B) both acidified with 0.1% formic acid as mobile phases. The initial conditions were set at 1%; after 1.5 min of isocratic step, a linear change to 100% B in 13.5 min. 100% B was achieved in 15 min and held for 5 min to allow for column washing before returning to initial conditions. Column reconditioning was achieved over 5 min, providing a total run time of 25 min. The column was maintained at 45 °C and a flow rate of 0.35 mL/min was used.

Mass spectrometry data were collected in positive and negative electrospray mode over the mass range of m/z 50−1000. Source settings were maintained using a capillary voltage, 1.5 kV (positive) and 2.0 kV (negative); source temperature, 120 °C; desolvation temperature, 650 °C; and desolvation gas flow, 950 L/h. The TOF analyzer was operated in sensitivity mode and data were acquired using HDMSE, which is a data-independent approach (DIA) coupled with ion mobility. The optimized ion mobility settings included the following: nitrogen flow rate, 90 mL/min (3.2 mbar); wave velocity, 650 m/s, and wave height, 40 V. The device within the Vion was calibrated using the Major Mix IMS calibration kit (Waters, Wilmslow, UK) to allow for CCS values to be determined in nitrogen. The calibration covered the CCS range from 130 to 306 Å^2. The TOF was also calibrated prior to data acquisition and covered the mass range from 151 to 1013 Da. TOF and CCS calibrations were performed for both positive and negative ion mode. Data acquisition was conducted using UNIFI 1.8 (Waters, Wilmslow, UK).

2.6. Data Processing and Multivariate Modeling. Data processing and compound identification were conducted using Progenesis QI Informatics (Nonlinear Dynamics, Newcastle, UK). Each UHPLC-MS run was imported as an ion-intensity map, including m/z (m/z range 50−1000) and retention time, which were then aligned in the retention-time direction (0−20 min). From the aligned runs, an aggregate run representing the compounds in all samples was used for peak picking. This aggregate was then compared with all runs, so that the same ions were detected in every run. Isotope and adduct deconvolution were applied to reduce the number of features detected. Unsupervised principal components analysis (PCA) with Pareto scaling was performed to check the quality of the raw data. Afterward, the variables were filtered, retaining entities with coefficients of variation lower than 30% across the QCs. From the analysis of the variance (ANOVA) significant features were selected, retaining those presenting, simultaneously, a fold change > 2 and a Benjamini−Hochberg false discovery rate (FDR) adjusted p-value (q-value) < 0.01. In parallel, multivariate supervised models, including partial least-squares discriminant analysis (PLS-DA), were built and validated using SIMCA software (v. 16.0.2, Sartorius Stedim Data Analytics, Sweden). Cross-validation of the PLS-DA model using the one-third leaving out approach and permutation testing were applied to validate and to exclude overfitting by inspecting model parameters (goodness-of-fit R2Y and goodness-of-prediction Q2Y). The variable influence in projection analysis (VIP) was further used to identify the compounds that had the highest discrimination potential (VIP value threshold > 1.2). The resulting significant features of both ANOVA p-values < 0.01 and VIP > 1.2 were subjected to the identification. Metabolites were identified by publicly available database searches including Lipid Metabolites and Pathways Strategy (LIPID MAPS)[30] and the Plant Metabolic Pathways Database,[17] as well as by fragmentation patterns, retention times, and CCS. Metabolites were annotated based on the International Standard Initiative for Metabolomics.[31,32]

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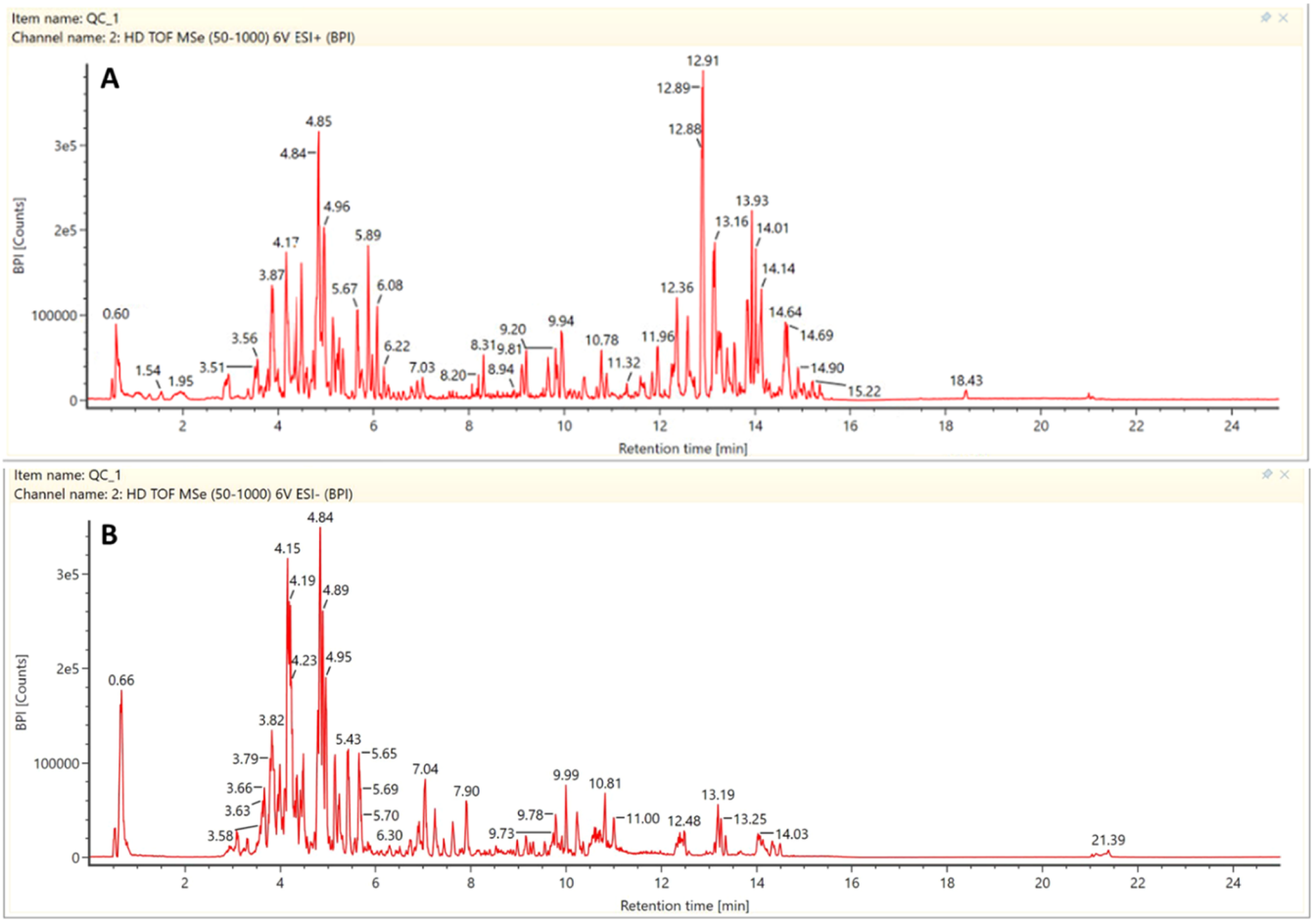

Figure 3. Ultrahigh-performance liquid chromatography coupled to high-resolution tandem mass spectrometry base peak chromatograms of the QC pool of coffee leaves extract obtained using positive (A) and negative (B) ionization modes.

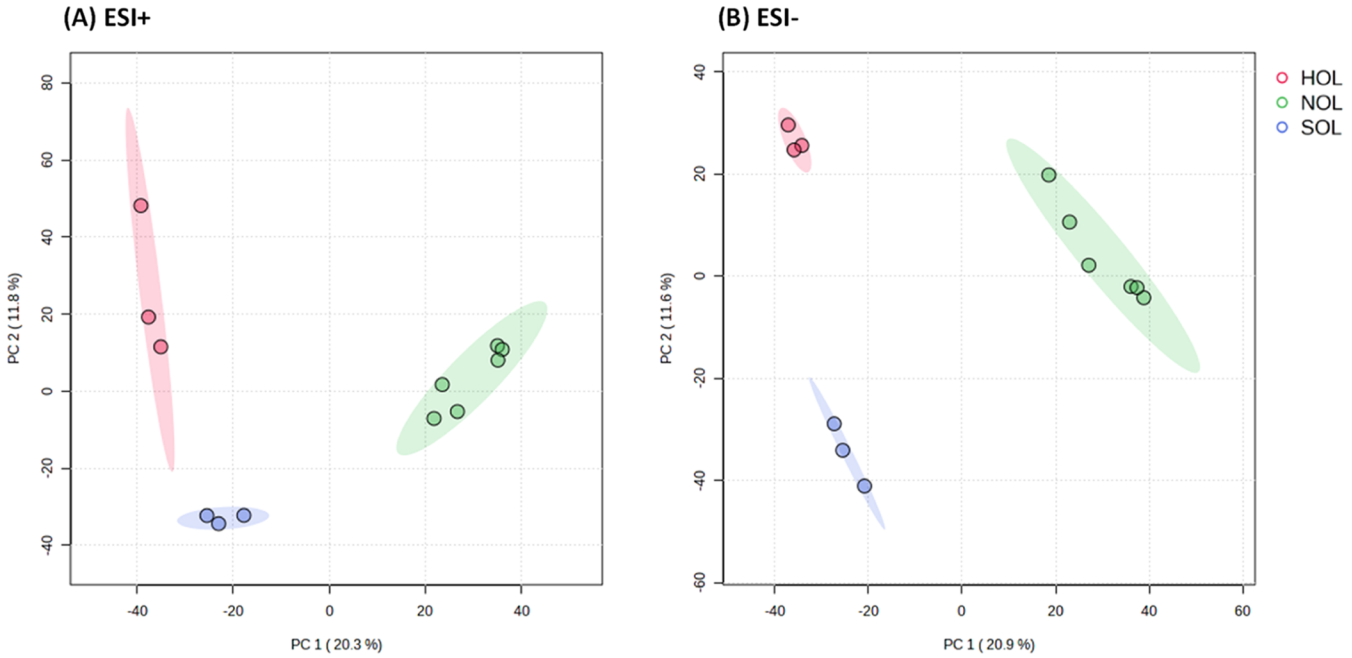

Figure 4. Unsupervised principal components analysis (PCA) models built from filtered positive (A) and negative (B) ionization data sets. Green dots: NOL; blue dots: SOL; red dots: HOL.

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Table 1. Summary of the Significant Identified Metabolites with Their Statistical Values

compound name	*m/z*	retention time (min)	CCS (Å^2)	*q* value	highest mean	fragmentation score	Δmass (ppm)	superclass	subclass	annotation level[31,32]
trigonelline	**138.0545**	0.61		8.9×10^{-03}		**96.8**	**−3.37**	alkaloids	pyridine alkaloids	I
adenosine	268.1035	1.10	156.91	6.5×10^{-06}	NOL	89.9	−2.15	nucleotides/nucleosides	purines	II
mangiferoxanthone A	841.1461	3.35		4.3×10^{-04}		91.2	−1.00	benzopyrans	xanthones	II
dihydroxy dimethoxyflavone hexoside	499.1216	3.42	217.49	3.9×10^{-07}	NOL	69.9	1.14	phenylpropanoids	flavonoids	III
rhamnazin hexoside	515.1154	3.43	213.21	4.5×10^{-05}	NOL	69.5	−1.20	phenylpropanoids	flavonoids	II
quercetin acetylhexosyl hexoside	707.1223	3.72	232.93	7.9×10^{-03}	SOL	65.6	0.44	phenylpropanoids	flavonoids	II
isoflavonoid diglycoside I	567.1333	3.78	233.71	1.7×10^{-04}	HOL	79.7	−2.09	phenylpropanoids	flavonoids	III
pentahydroxyflavanone glucoside	489.1014	3.78	213.85	2.0×10^{-03}	HOL	78.8	2.21	phenylpropanoids	flavonoids	III
laricitrin acetylglycoside	519.1123	3.78	224.87	1.3×10^{-03}	HOL	70.8	−1.90	phenylpropanoids	flavonoids	II
coniferyl alcohol	163.0747	3.80	134.44	2.2×10^{-07}	NOL	79.3	−3.55	benzenoids	phenols	II
luteolin rhamnoside dimethyl ether	483.1280	3.97	212.06	1.3×10^{-05}	HOL	72.4	3.96	phenylpropanoids	flavonoids	II
luteolin 5,3′-dimethyl ether hexoside	499.1216	4.64	231.34	2.0×10^{-04}	NOL	66.2	1.01	phenylpropanoids	flavonoids	II
isomangiferin	421.0764	5.07	189.7	1.2×10^{-06}	NOL	89.9	−2.90	benzopyrans	xanthones	II
epigallocatechin-epicatechin-gallate ester	711.1337	5.19	267.92	9.1×10^{-04}	HOL	68.8	−1.08	phenylpropanoids	flavonoids	II
tetrahydroxy dimethoxyflavone	369.0589	5.67	181.52	7.2×10^{-05}	NOL	67.2	2.22	phenylpropanoids	flavonoids	III
scutellarein dimethyl ether acetylhexoside	483.1297	5.72	227.77	6.8×10^{-03}	NOL	66.5	2.20	phenylpropanoids	flavonoids	II
hydroxyflavan I	447.0694	6.32	205.33	2.6×10^{-06}	HOL	66.6	1.67	phenylpropanoids	flavonoids	III
N-linolenoyl glutamic acid	430.2570	7.15	203.89	4.1×10^{-05}	NOL	89.5	1.43	fatty acyls	*N*-acyl amines	II
LysoPA (18:1)	452.2788	7.28	212.88	4.1×10^{-05}	NOL	70.9	3.79	glycerophospholipids	glycerophosphates	II
mangiferin	421.0787	7.39	188.82	5.4×10^{-06}	NOL	98.2	2.68	benzopyrans	xanthones	I
sorbifolin	301.0699	7.45	166.01	8.0×10^{-03}	NOL	68.5	−2.71	phenylpropanoids	flavonoids	II
7-dehydrodesmosterol	405.3133	8.24	204.63	9.9×10^{-04}	HOL	81.7	1.37	sterol lipids	phytosterols	II
pelargonidin	272.0662	8.30	158.47	7.0×10^{-04}	NOL	66.3	−4.44	phenylpropanoids	flavonoids	II
caffeoylshikimate	300.0619	8.30	164.29	6.3×10^{-04}	NOL	74.2	−2.66	phenylpropanoids	cinnamates	II
ladanein	315.0857	8.31	169.00	4.7×10^{-04}	NOL	67.0	−2.10	phenylpropanoids	flavonoids	II
breverin	345.0961	8.40	178.68	2.0×10^{-04}	NOL	68.3	−2.18	phenylpropanoids	flavonoids	II
LysoPC(18:3)	518.3235	9.19	228.86	3.2×10^{-06}	HOL	74.1	−1.21	glycerophospholipids	glycerophosphocholines	II
apigenin dimethyl ether	299.0907	9.62	164.33	8.4×10^{-03}	NOL	64.4	−2.31	phenylpropanoids	flavonoids	II
hexadecasphinganine derivative I	527.3699	9.70	228.64	1.4×10^{-05}	HOL	64.8	−1.47	sphingolipids	ceramides	III
PS O-34:4	706.4786	9.80	294.00	2.5×10^{-06}	NOL	71.3	−2.75	glycerophospholipids	glycerophosphoserines	II
PA(22:0)	527.3702	10.20	232.64	2.4×10^{-04}	HOL	73.7	−1.18	glycerophospholipids	glycerophosphates	II
PG(21:0)	537.3546	10.21	246.62	3.8×10^{-05}	NOL	74.5	−0.99	glycerophospholipids	glycerophosphoglycerols	II
ST 29:1;O2	423.3609	10.27	211.77	1.2×10^{-05}	SOL	64.7	2.91	sterol lipids	phytosterols	II
octadecatetraenoic acid	277.2154	10.44	172.33	2.6×10^{-04}	HOL	69.6	−3.05	fatty Acyls	unsaturated fatty acids	II
oxocholestadienol I	421.3068	10.74	207.98	1.1×10^{-03}	SOL	66.9	−2.36	sterol lipids	phytosterols	II
cholecalciferol derivative	527.3707	11.00	226.65	2.7×10^{-04}	HOL	69.3	−0.07	sterol lipids	secosteroids	III
ST 30:4;O	423.3605	11.12	209.84	1.7×10^{-05}	HOL	94.0	−3.76	sterol lipids	phytosterols	II
acetoxy ergostentriol I	515.3685	11.22	232.94	2.4×10^{-03}	NOL	65.3	−4.52	sterol lipids	phytosterols	III
ST 27:4;O5	409.2716	11.29	206.42	7.4×10^{-03}	HOL	81.1	−4.86	sterol lipids	phytosterols	II
oleanolic acid glucuronide	633.3967	11.45	246.50	2.0×10^{-05}	NOL	84.9	−4.81	prenol lipids	C30 isoprenoids	II
PC 25:1;O	650.4379	11.46	264.90	3.0×10^{-05}	HOL	72	−1.94	glycerophospholipids	oxidized glycerophosphocholines	II

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Table 1. continued

compound name	m/z	retention time (min)	CCS (Å^2)	q value	highest mean	fragmentation score	Δmass (ppm)	superclass	subclass	annotation level[31,32]
6,8-nonadien-2-ol	179.0834	11.50	133.25	8.1×10^{-05}	NOL	66.7	1.17	fatty Acyls	fatty alcohols	II
PI 38:7	845.4936	11.60	295.81	1.4×10^{-07}	HOL	88.9	−3.08	glycerophospholipids	glycerophosphoinositols	II
ceriferol	379.2961	12.02	195.98	9.3×10^{-04}	SOL	76.4	−2.88	prenol lipids	C25 isoprenoids	II
ST 27:2;O	407.3285	12.23	206.48	8.6×10^{-06}	SOL	77.0	0.15	sterol lipids	phytosterols	II
ST 28:2;O	421.3448	12.32	209.90	1.3×10^{-04}	HOL	96.1	1.67	sterol lipids	phytosterols	II
ST 27:1;O2	425.3391	12.84	211.71	1.2×10^{-03}	SOL	93.2	0.26	sterol lipids	phytosterols	II
MGDG 34:8;O	783.4646	13.22	292.50	2.1×10^{-03}	HOL	78.8	−0.98	glycerolipids	glycosyldiacylglycerols	II
MGDG 36:8	719.4845	13.24	269.88	1.6×10^{-03}	NOL	65.1	−4.84	glycerolipids	glycosyldiacylglycerols	II
PI O-38:7	831.5182	13.27	302.72	4.0×10^{-03}	NOL	93.5	1.32	glycerophospholipids	glycerophosphoinositols	II
PI(30:0)	811.4964	13.41	296.41	2.2×10^{-05}	NOL	97.3	−0.42	glycerophospholipids	glycerophosphoinositols	II
nonenone	179.0830	13.57	131.62	3.1×10^{-03}	NOL	76.7	−2.06	organooxygen compounds	ketones	II
miraxanthin V	309.0854	14.16	167.46	8.6×10^{-05}	HOL	85.5	−4.65	benzenoids	phenols	III
ST 28:1;O2	417.3711	14.79	223.71	2.6×10^{-04}	HOL	96.7	−3.94	sterol lipids	phytosterols	II
hydroxymethyl stigmastadienol	425.3771	14.81	215.60	2.9×10^{-03}	HOL	95.6	−1.48	sterol lipids	phytosterols	III
Hex2Cer 36:2;O2	852.6180	14.89	320.41	4.1×10^{-06}	SOL	96.3	−1.65	sphingolipids	ceramides	III
MGDG 34:2	773.5171	14.92	288.30	1.5×10^{-06}	HOL	74.8	−0.44	glycerolipids	glycosyldiacylglycerols	III
ergostenetetrol derivative I	485.3587	14.95	227.71	1.3×10^{-05}	NOL	97.9	−3.07	sterol lipids	phytosterols	III
MGDG 36:5	799.5327	15.04	294.41	6.2×10^{-05}	HOL	65.2	−0.52	glycerolipids	glycosyldiacylglycerols	II
PS (42:7)	884.5440	17.48	315.30	4.3×10^{-04}	SOL	78.3	3.20	glycerophospholipids	glycerophosphoserines	II

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RESULTS AND DISCUSSION

Targeted Analysis. Coffee signature compounds such as caffeine and 5-CQA were quantified in the three different groups of processed leaves. Results are reported as g/kg in Figure 2. The extract obtained from leaves treated with the herbal protocol had a content of caffeine of 0.5 and 8.3 g/kg of 5-CQA, while the former was more than tripled and the latter was more than halved in oxidized samples. It should be noted that each type of postharvest management has different consequences on the chemical compositions of the extracts, and this should be carefully considered wherever coffee leaves are used for both research and commercial purposes. In particular, NOL leaves underwent direct sun-drying according to a protocol identical to the one usually in place for the production of herbal infusions, in which the enzymatic oxidation of polyphenols and chlorophyll is prevented in order to preserve the original color and taste.[33] On the contrary, by causing cell breaking, the hand-rolling process used to produce SOL and HOL leaves facilitates browning and enzymatic degradation, albeit with different intensities.[34] The oxidative process mediated by enzymatic activity and oxygen exposure leads to the formation of quinones and to the cleavage of glycosidic bonds. It is noteworthy to highlight that the higher caffeine content of extracts obtained from SOL and HOL leaves (slightly below 2 g/kg) may pose a higher risk to produce beverages not compliant with the critical value assigned under EU regulations (<80 mg/L). At the same time, it is clear that the overall exposure to caffeine would be quite lower if the production follows the milder herbal infusion style.

In fact, SOL and HOL samples showed comparable levels of caffeine and 5-CQA, while the contents of both compounds were significantly different compared with that of NOL leaves, although with a different trend. Regarding caffeine, its stability during the fermentation/oxidation process is well known, while the prolonged wilting may increase its extractability from leaf tissues, as observed in SOL and HOL-like compared to NOL samples.[35] This is also consistent with previous reports suggesting a decrease in caffeine extractability after the kill-green process, and a strong, increased extractability following cell breaking due to the hand-rolling process.[36] On the contrary, the higher content of 5-CQA found in NOL leaves compared to SOL and HOL samples can be due to the milder process and mainly due to the limited oxidation and lack of fermentation step.[37] In this regard, it is noteworthy to mention that while the processes used to produce HOL and SOL samples bear some similarities to the usual protocols used for oolong and black tea, the one applied to obtain NOL samples is different from the usual green tea process, as no steam/thermal treatment was applied to deactivate the enzymatic activity.

Multivariate Modeling. An untargeted analysis approach based on UHPLC-TWINS-QTOF was used to explore the phytochemical signature of different *C. arabica* leaves. Ultrahigh-performance liquid chromatography-traveling wave ion mobility spectrometry quadrupole time-of-flight data sets, obtained in positive and negative ionization modes (see Figure 3), were separately submitted for data analysis. A total of 12 828 and 8089 features were initially peak picked for positive and negative modes, respectively.

After a preliminary data quality assessment check and data filtration, the principal components analysis (PCA) models were built to investigate the metabolome and, therefore, differences between the samples. PCA models based on filtered data (Figure 4A,B) clearly indicate a scenario where leaves were successfully separated in both positive and negative ionization modes.

Subsequently, significant features were selected, retaining those presenting, simultaneously, fold change > 2, ANOVA FDR adjusted p-value (q-value) < 0.01, and CV% in QC < 30%. This filtering step returned a data set with 4292 significant features for positive and 2934 for negative ion mode, which were subjected to the identification. With mass fragmentation and isotope matching, 472 identifications were putatively assigned following the Metabolomics Standards Initiative annotation guidelines.[31,32] Significant metabolites with their statistical values and annotation level are listed in Supporting Information, Table 1.

DIFFERENTIAL METABOLITES INTERPRETATION

The large majority (95.4%) of the identified metabolites fall within 8 chemical superclasses. Among them the most represented are phenylpropanoids (38.0%), sterol lipids (18.2%), and glycerophospholipids (17.7%), as reported in Figure 5.

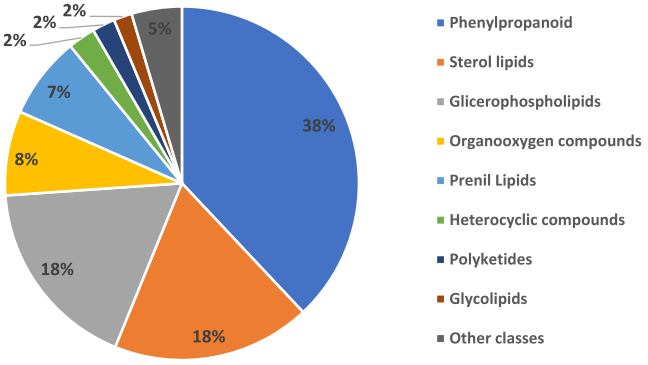

Figure 5. Distribution of classes of differential metabolites identified in this study.

Superclasses, identified based on the Classyfire classification system,[38] can be then divided into subclasses. Flavonoids, the main group among phenylpropanoid compounds, are the most represented metabolites in coffee leaves (164 compounds), followed by phytosterols (85 compounds). Among all of the identified metabolites, 45% are significantly overaccumulated in NOL samples, 37% in HOL samples, and, finally, 18% in SOL samples.

The overall higher amount of secondary plant metabolites in NOL samples compared to SOL and HOL can be ascribed to the milder process adopted in the former. In addition, a prolonged oxidation step in HOL manufacturing may lead to the partial degradation of metabolites compared with less intensive treatments. Finally, the polymerization of phenolic compounds commonly described in black tea leaves gives rise to higher-molecular-weight compounds, which can show a lower extractability under the conditions herein applied.

The secondary metabolite classes differentially accumulated across the coffee leaf samples were used for the calculation of the Heat Map reported in Figure 6. A clear clusterization of NOL, SOL, and HOL samples was obtained.

It should be underlined that differences in coffee leaf batches were expected based on the process applied, which was

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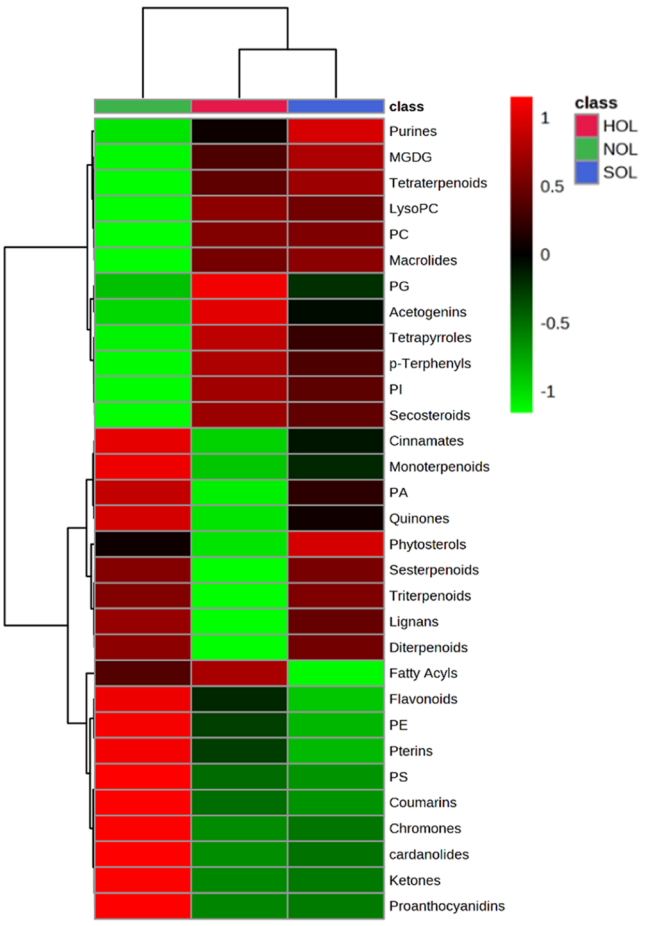

Figure 6. Heat map calculated over the average group values for the annotated secondary metabolite classes.

expected to cause oxidative and/or thermal degradation. Since such phenomena are not biochemically mediated but a consequence of thermal stress, an extensive degradation of phytochemicals was likely expected, giving rise to oxygenated analogues. Such an accumulation of oxidated compounds was indeed observed in our samples, following the NOL < SOL < HOL trend.

NOL samples were richer in flavonoids compared to SOL and HOL groups, which undergo mild and full oxidative fermentation, respectively. This is consistent with the literature describing a lower content of flavonoids in black tea compared to green tea samples.[3]

More in detail, flavonoids, lignans, mono-, di-, and triterpenoids are more abundant in SOL leaves, while their presence is significantly lower in SOL samples, probably on account of the oxidative process (Figure 7). The lowest content of sterol lipids was also observed in HOL samples.

Mangiferin, a xanthone previously found to occur in coffee leaves but not in coffee beans,[35,39] has been identified in all of the samples, together with its analogues isomangiferin and mangiferoxanthone A, being higher in NOL compared to oxidized leaves (Figure 8). The presence of mangiferine and isomangiferine in coffee leaves is of particular interest due to the health-related properties often associated with such compounds.[38−40] Therefore, coffee leaf extracts are valuable also for functional beverages, and these compounds may offer an additional layer of benefit to the already rich phenolic profile. It is thus important that the process does not affect significantly their content and, in this regard, nonoxidative treatment seems to represent the most interesting choice. Furthermore, the aa xanthone dimer known as mangiferoxanthone A was putatively annotated in coffee leaf samples. This compound has been described previously as a symmetric homodimer of mangiferin.[41] Although little is known about the biological activity of this compound, several studies have

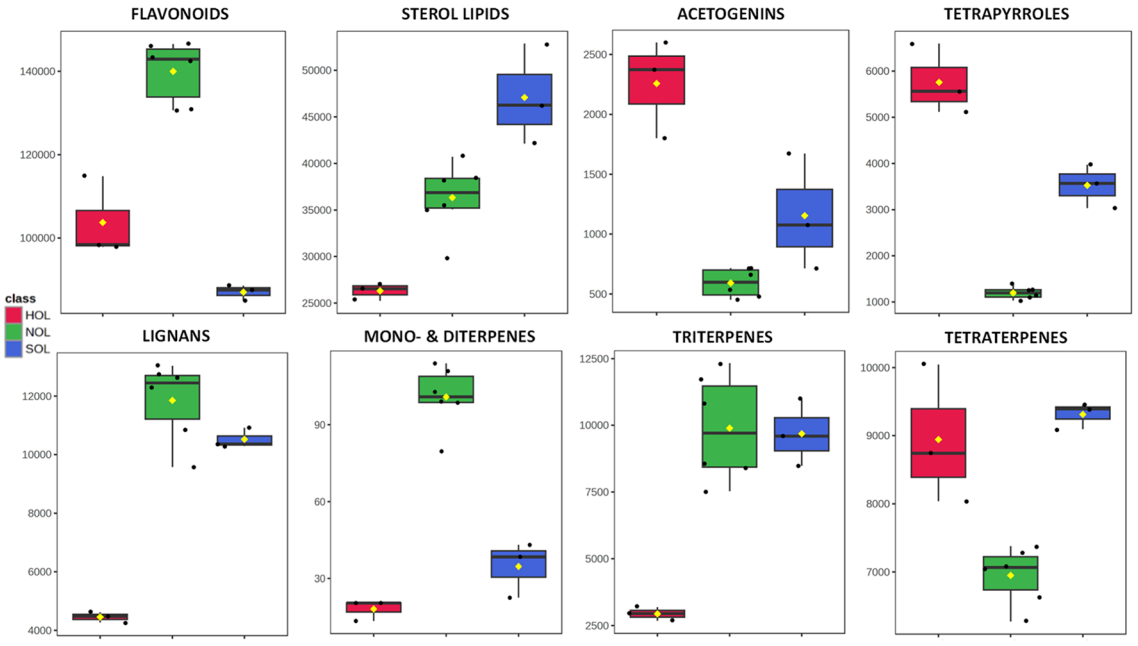

Figure 7. Boxplot analysis of selected metabolite classes. Black dots represent the normalized abundances of selected features in all samples. The notch indicates a 95% CI around the median of each group, defined as ±1.58*IQR/sqrt(n). The mean concentration is indicated with a yellow diamond.

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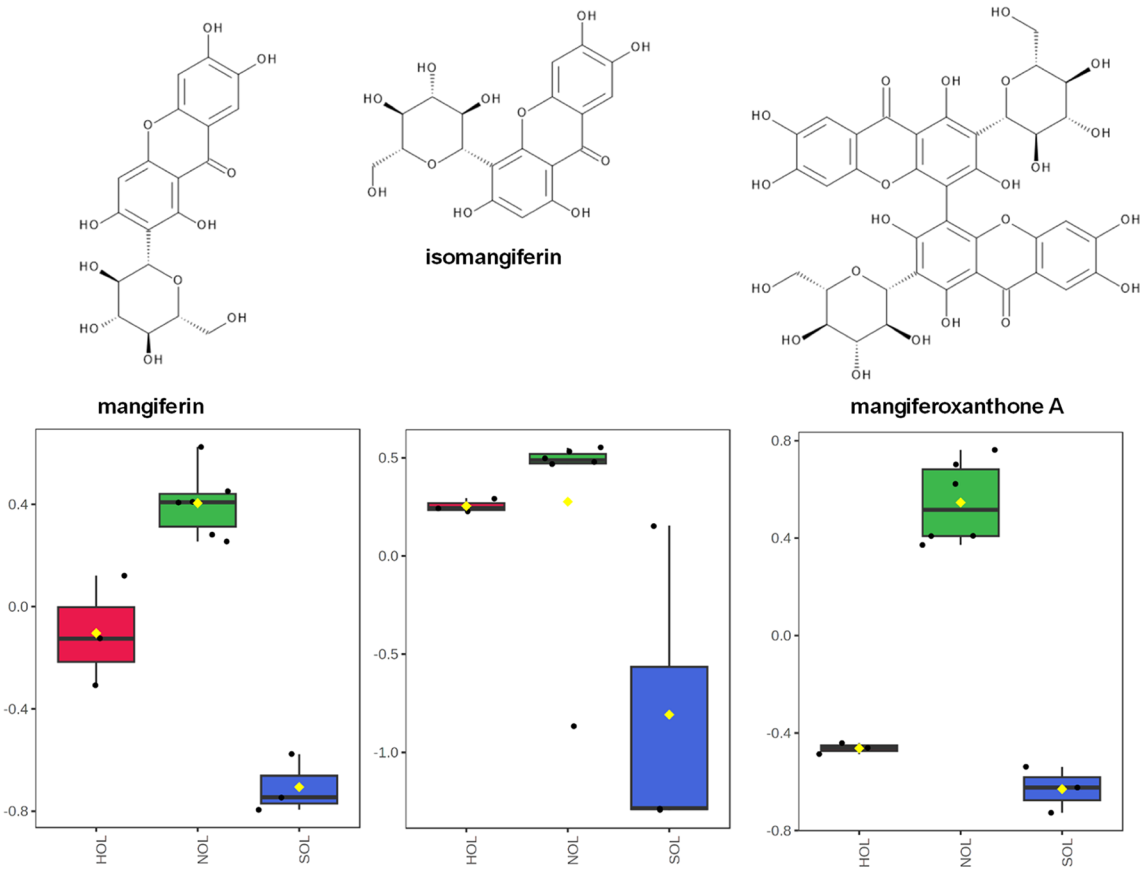

Figure 8. Boxplot analysis of mangiferin and its analogues. Black dots represent the normalized abundance of selected features in all samples. The notch indicates 95% CI around the median of each group, defined as ±1.58*IQR/sqrt(n). The mean concentration is indicated with a yellow diamond.

reported that dimerization may increase the bioactivity compared to its monomer.[42]

In conclusion, postharvest handling induces remarkable effects on coffee leaves' composition, and, although starting from the same plant material, it may lead to the creation of distinct botanical products whose bioactivity and organoleptic properties should be evaluated separately. While this phenomenon may have some positive relapses from a commercial and even product identity perspective, it could also lead to potential misunderstandings if the production process and overall composition are not properly described or taken into account in future investigations. Furthermore, the different content of key secondary metabolites in our extracts suggests that a very careful standardization approach should be enforced, also when the regulations propose critical values for specific compounds, such as in the case of coffee leaves in the European Community.[7] In this regard, the intake of compounds such as caffeine from final products such as food supplements and infusions could be extremely variable even if the starting botanical source is uniform. In other words, both from a research perspective and under commercial purposes, a simple "coffee leaf extract" definition without a clear explanation of postharvest management may be limited and misleading. At the same time, untargeted analysis offers a powerful tool that is useful to discriminate and clusterize the different botanical products obtainable from coffee leaves. Its recourse may be valuable for the description of extracts and plant materials used to evaluate the bioactivities of these products, but also for standardization and identity control in future coffee leaves trade.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.4c00054.

Significant metabolites detected in *Coffea arabica* L. leaves by means of UHPLC-TWIMS-QTOF metabolomic profiling with their statistical values (PDF)

Table supp Mat (XLSX)

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Notes

The authors declare the following competing financial interest(s): B.S., A.N., and M.P. are employees of Lavazza S.p.A.

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