Coffee variety, origin and extraction procedure: implications for coffee beneficial effects on human health

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ABSTRACT

We set up an efficient protocol for the rapid analysis of NMR spectra of green and roasted coffee extracts, enabling the automatic identification and quantification of metabolites in approximately two minutes *per* spectrum. This method allowed for the metabolic profiling and the subsequent evaluation of the content of bioactive compounds and antioxidant activity of coffee samples, depending on their species (Arabica and Robusta), geographical origin and extraction procedure (hydroalcoholic, espresso and moka).

The hydroalcoholic extraction is the most efficient method in terms of yields of low molecular weight compounds (in particular chlorogenic acids), while moka extraction provides the highest amounts of melanoidins. Moreover, that the ratio between health-giving compounds (chlorogenic acids, trigonelline and choline) and caffeine is higher in Arabica coffees.

The data collected provide useful insights for the selection of coffee raw material to be used in the preparation of coffee-based dietary supplements, nutraceuticals and functional beverages.

KEYWORDS

Green coffee beans; roasted coffee beans; Arabica and Robusta coffee; coffee origin, NMR metabolic profiling; antioxidant capacity; chlorogenic acids; melanoidins.

1. INTRODUCTION

Studying the impact that coffee exerts on human health is of great interest, since coffee is one of the most commonly consumed beverages in the world, accounting for 75% of the regular soft drinks consumption (Toci, Farah, Pezza, & Pezza, 2016). Drinking coffee has frequently been discouraged, due to the risks associated with the excessive consumption of this beverage (hypertension, coronary heart disease, anxiety, insomnia, depression, osteoporosis, anaemia, pregnancy-related problems) (Nawrot, Jordan, Eastwood, Rotstein, Hugenholtz, & Feeley, 2003). Nevertheless, the more recent information about coffee phytochemistry and biological properties has progressively led to consider coffee as a potential functional food, as its benefits on human health seem to outdo its negative effects (Messina, Zannella, Monda, Dato, Liccardo, De Blasio, et al., 2015). Specifically, experimental and epidemiological studies have demonstrated that coffee (and some molecules contained in coffee beverages) can provide beneficial effects against several pathologies, among which neurological diseases (Parkinson's and Alzheimer's diseases), cancer (breast and colon cancer), psychoactive responses (alertness, mood change), metabolic disorders (type 2 diabetes), and liver dysfunctions (cirrhosis) (Messina, et al., 2015). Moreover, in recent years, the beneficial properties of green coffee for human health have been pointed out, leading to an increase in the consumption of green–coffee-based beverages (Ludwig, Clifford, Lean, Ashihara, & Crozier, 2014).

The metabolome of green and roasted coffee is rich in compounds with well-known health-giving effects, among which chlorogenic acids, trigonelline and choline.

Chlorogenic acids (quinic esters of hydroxycinnamic acids, CGAs) are the most important class of coffee polyphenols (12-18% dry weight in green coffee), and can be clustered into three main groups: caffeoylquinic acids (CQAs), with 5-*O*-caffeoylquinic acid (5-CQA) being the most abundant, feruloylquinic acids (FQAs), and di-caffeoylquinic acids (di-CQAs) (Clifford & Knight, 2004). Antioxidant and radical scavenging properties of CGAs are well known (Niseteo, Komes, Belščak-Cvitanović, Horžić, & Budeč, 2012), but they present also other interesting biological effects (Upadhyay

& Mohan Rao, 2013), such as a protective effect against cardiovascular diseases, and antihypertensive, anti-inflammatory, anti-viral, anti-fungal, immunoprotective, anti-cancer (Palmioli, Ciaramelli, Tisi, Spinelli, De Sanctis, Sacco, et al., 2017), anti-aging (Amigoni, Stuknytė, Ciaramelli, Magoni, Bruni, De Noni, et al., 2017) and neuroprotective (Ciaramelli, Palmioli, De Luigi, Colombo, Sala, Riva, et al., 2018) actions.

Trigonelline represents approximately 2-3% of total dry weight in green coffee, and is partially degraded during the roasting process (Ludwig, Clifford, Lean, Ashihara, & Crozier, 2014). It is suggested to act as a neuroprotective compound in Alzheimer's and Parkinson's diseases, anti-tumor, hypoglycemic, anti-bacterial, anti-viral and anti-invasive molecule, and it has been shown to reduce diabetic auditory neuropathy and platelet aggregation (Gaur, Bodhankar, Mohan, & Thakurdesai, 2013; Hirakawa, Okauchi, Miura, & Yagasaki, 2005; Jiyin, Shiwen, & Shengya, 2013; Makowska, Szczesny, Lichucka, Giełdoń, Chmurzyński, & Kaliszan, 2014; Yoshinari, Sato, & Igarashi, 2009).

Choline is present in green coffee at a concentration comparable to trigonelline, but it is reduced to 0.1% after roasting. It is an essential nutrient, covering wide-ranging roles in human metabolism, from cell structure to neurotransmitter synthesis. Choline-deficiency is thought to have an impact on pathologies such as liver disease, atherosclerosis and possibly neurological disorders (Zeisel & da Costa, 2009).

A representative coffee metabolite is caffeine, contained both in green and roasted coffee beans and in coffee beverages, with its concentration depending on the preparation methodology and on coffee species and origin. The biological activity of caffeine is controversial. It undoubtedly presents antioxidant properties (Lee, 2000) and some papers report additional biological features: for instance, neuroprotective properties are supported in some studies (Arendash & Cao, 2010; Eskelinen & Kivipelto, 2010), but questioned in others (Shukitt-Hale, Miller, Chu, Lyle, & Joseph, 2013), among which our recent work, where caffeine was completely inactive against Aβ peptide aggregation and toxicity (Ciaramelli, et al., 2018). On the other hand, high consumption of caffeine is associated with several negative effects, such as general toxicity, increased incidence of cancer, cardiovascular effects, among

which cardiac arrhythmias (Chou & Benowitz, 1994) and hypertensive palpitations (Nurminen, Niittynen, Korpela, & Vapaatalo, 1999), gastrointestinal disturbances, effects on bone status and calcium balance, bronchodilatation, male fertility problems and fetus-related issues during pregnancy (Clifford & Knight, 2004; Nawrot, Jordan, Eastwood, Rotstein, Hugenholtz, & Feeley, 2003).

The present work focused on the analysis, *via* an NMR-based protocol, of green and roasted coffee extracts, with the aim of comparing the content of the metabolites characterized by a proven positive biological activity with those having a negative impact on human health. The research looked at coffee beans belonging to two coffee species (*Coffea Arabica* or Arabica *vs Coffea canephora* or Robusta) (Belitz, Grosch, & Schieberle, 2009), coming from different geographies and extracted with three methods (hydroalcoholic, espresso and moka), in order to identify the best coffees to be used as functional foods or for the preparation of nutraceuticals.

2. MATERIAL AND METHODS

2.1 Green and roasted coffee extraction.

Ground coffee beans were received from Beyers Koffie, Belgium. We analysed green and medium-roasted coffees from 10 different countries (3 coffee samples for each origin), 5 of them belonging to the species Arabica (Colombia, Brazil A, Burundi, Kenya, Guatemala) and 5 to the species Robusta (Tanzania, Uganda, Vietnam, Brazil R, India). Each sample was analysed in triplicate.

Coffee extracts were obtained according to three different procedures. Espresso and moka extractions were selected as they are the two most common procedures for the preparation of roasted-coffee-based beverages, in order to shed a light on the chemical composition of the coffee extracts which are part of consumers' diet. Being extensively exploited for natural extracts' preparation, hydroalcoholic extraction was also investigated, in order to evaluate its possible employment for the preparation of coffee-based nutraceuticals.

Hydroalcoholic extraction. 200 mg of ground green or roasted coffee were extracted with 20 mL of a mixture of acidified (with 0.1 M HCl) water (pH 4.5; 70%) and methanol (30%) by sonication at 37 kHz

for 15 min at 30 °C in an ultrasound bath (Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany). After 1 h, solutions were filtered through cotton wool and 0.45 µm PTFE filters (Pall Corporation, Port Washington, NY, USA), concentrated under reduced pressure at 40 °C and freeze-dried. The extraction yield was calculated for each sample. The lyophilized samples were stored at -20 °C (Amigoni, et al., 2017).

Espresso extraction. The espresso coffee extracts were prepared from 14 g of ground green or roasted coffee, with a two-cups coffee machine (Gaggia Baby Espresso Machine, Gaggia Milano, Italy) for a volume of coffee brew of 50 mL, corresponding to 2 cups of espresso coffee. Fixed espresso coffee-brewing conditions were followed using a pressure in the espresso machine pump equal to 9 bar and extraction times of 30 s at 90 °C. A volume of 5 mL (in triplicate) was freeze-dried and weighted to calculate the extraction yield and stored at -20 °C.

Moka extraction. The moka coffee brews were prepared with an Italian moka machine (Bialetti traditional moka, 3 cups model, Bialetti Industrie Spa, Italy), from approximately 12 g of ground roasted coffee for a starting volume of 100 mL of drinking water and a recovered volume of coffee brew of approximately 75 mL, corresponding to 3 moka coffee cups. The extraction time and heating temperature were approximately 10 min at 93 °C. A volume of 5 mL (in triplicate) was freeze-dried and weighted to calculate the extraction yield and stored at -20 °C.

2.2 Nuclear Magnetic Resonance (NMR) spectroscopy.

NMR spectroscopy has already been used to analyse both green (GCEs) and roasted coffee extracts (RCEs) and proved to be suitable for metabolomics studies on this food matrix, aimed at identifying the coffee species (Arabica *vs* Robusta) and geographical origin, as well as monitoring the authenticity and quality control of the product (Arana, Medina, Alarcon, Moreno, Heintz, Schäfer, et al., 2015; Bosco, Toffanin, de Palo, Zatti, & Segre, 1999; Cagliani, Pellegrino, Giugno, & Consonni, 2013; Consonni,

Cagliani, & Cogliati, 2012; Toci, de Moura Ribeiro, de Toledo, Boralle, Pezza, & Pezza, 2018; Wei, Furihata, Hu, Miyakawa, & Tanokura, 2011).

The procedure for NMR sample preparation and spectrum acquisition was adapted from (Amigoni, et al., 2017). Freeze-dried samples of coffee extracts were suspended in D_2O at a final concentration of 5 to 20 mg/mL. All samples were sonicated (37 kHz, 20 min, Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany) and centrifuged (9425*xg*, 15 min, 20 °C, ScanSpeed 1730R Labogene, Lynge, Sweden) before NMR analyses. The 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS, final concentration 1 mM) was added to the supernatant as an internal reference for concentrations and chemical shift. The pH of each sample was verified with a microelectrode (Mettler Toledo, Columbus, OH, USA) for 5 mm NMR tubes and adjusted to 7.4 with NaOD or DCl. The pH values of samples in D_2O were corrected for the isotope effect. The acquisition temperature was 25 °C. All spectra were acquired on an AVANCE III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA), equipped with a QCI (^{1}H, ^{13}C, $^{15}N/^{31}P$ and 2H lock) cryogenic probe. ^{1}H-NMR spectra were recorded with water suppression (cpmgpr1d pulse sequences in Bruker library) and 256 scans, spectral width 20 ppm, relaxation delay 4 s. They were processed with 0.3 Hz line broadening, automatically phased and baseline corrected. Chemical shifts were internally calibrated to the DSS peak at 0.0 ppm. The identification and assignment of compounds were done with the support of 2D NMR experiments and compared with reported assignments (Wei, Furihata, Hu, Miyakawa, & Tanokura, 2011). The $^{1}H,^{1}H$-TOCSY (Total Correlation SpectroscopY) spectra were acquired with 40 scans and 512 increments, a mixing time of 80 ms and a relaxation delay of 2 s. $^{1}H,^{13}C$-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 64 scans and 256 increments, and a relaxation delay of 2 s. NMR spectra processing and peak piking were performed by MestReNova software package of Mestrelab (MestReNova v 12.0.0-20080, 2017, Mestrelab Research, Santiago de Compostela, Spain). For metabolite quantification, the Simple Mixture Analysis (SMA) tool integrated in MestreNova software package of Mestrelab (MestReNova v 12.0.0-20080, 2017, Mestrelab Research, Santiago de Compostela, Spain) was exploited

(Cobas, Seoane, Domínguez, Sykora, & Davies, 2011) to set a semi-automatic protocol for the identification and quantification of metabolites, building specific libraries for the analyzed matrices. In this protocol, the GSD (global spectrum deconvolution) algorithm was employed to deconvolute the overlapping regions and to perform the absolute quantification for metabolites with resonances in crowded spectral areas, too. When possible, for the different compounds, the concentration was calculated looking at the mean value of the different assigned signals (Airoldi, Ciaramelli, Fumagalli, Bussei, Mazzoni, Viglio, et al., 2016). Metabolite concentrations were reported as means (M) ± standard deviations (SD) of triplicate experiments.

2.3 Statistical analysis.

Statistical analysis was performed exploiting the real-time interactive web-based application Metaboanalyst 4.0 (Chong, Soufan, Li, Caraus, Li, Bourque, et al., 2018). To discriminate the different coffee samples through NMR spectra, multivariate statistical analysis was carried out. Data were uploaded as a table, reporting the concentrations of metabolites for each sample, as determined from the use of the quantification protocol applied through SMA tool of MestReNova Software. Samples were classified as belonging to the species Arabica (A) and Robusta (R), and according to the extraction method. Data underwent unsupervised multivariate analyses, through Principal Component Analysis, PCA. As the presence of citrate and myoinositol in RCEs samples is extraction-method-dependent (see Table 2), these metabolites were not considerate for PCA analysis.

2.4 Determination of antioxidant activity.

The extract antioxidant activity was determined by Folin-Ciocalteu's method assay as previously reported (Amigoni, et al., 2017; Ciaramelli, et al., 2018). Briefly, 80 µL of diluted samples (or standards/blank) and 40 µL of Folin's reagent were dispensed in a cuvette containing 400 µL of H_2O. Then, 480 µL of Na_2CO_3 10.75% (w/v) solution was added and, after 30 min of incubation at room temperature, absorbance was read at 760 nm with Varian Cary® 50 Scan UV–Visible Spectrophotometer

using plastic 10 mm-cuvettes relative to a blank solution. Samples were diluted to 0.2 mg/mL and standard solutions of 5-CQA (0–200 μg/mL) were used for calibration (linear fitting $R^2 > 0.98$, n = 7). Results were expressed as mg of 5-CQA equivalent (5-CQA eq)/g of freeze-dried coffee extract. Data were reported as means (±SD) of triplicate measurements.

2.5 Determination of high molecular weight (HMW) melanoidin content.

Melanoidins were determined spectrophotometrically at the selected wavelength of 405 nm as already reported (Bekedam, Schols, van Boekel, & Smit, 2006; Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002). Since a pure melanoidin standard was not available, the standard calibration curve was built using a HMW (high molecular weight) coffee fraction (MW>10 KDa), obtained by ultrafiltration from a Tanzania roasted coffee sample as described in a previous work (Ciaramelli, et al., 2018). A stock solution of HMW fraction (2 mg/mL in H_2O) was prepared from freeze-dried sample and diluted standard solutions (0-500 μg/mL in H_2O) were used for calibration (linear fitting $R^2 > 0.99$, n = 5). Roasted coffee samples were diluted to 0.2 mg/mL. Results were expressed as mg of HMW melanoidins/g of freeze-dried coffee extract. Data were reported as means (±SD) of triplicate measurements.

3. RESULTS

Coffee samples prepared with hydroalcoholic, espresso and moka extractions were characterized by NMR spectroscopy. Representative NMR spectra acquired on GCEs (1) and RCEs (2) from Burundi samples after hydroalcoholic (A) and espresso (B) extractions are depicted in Figure 1.

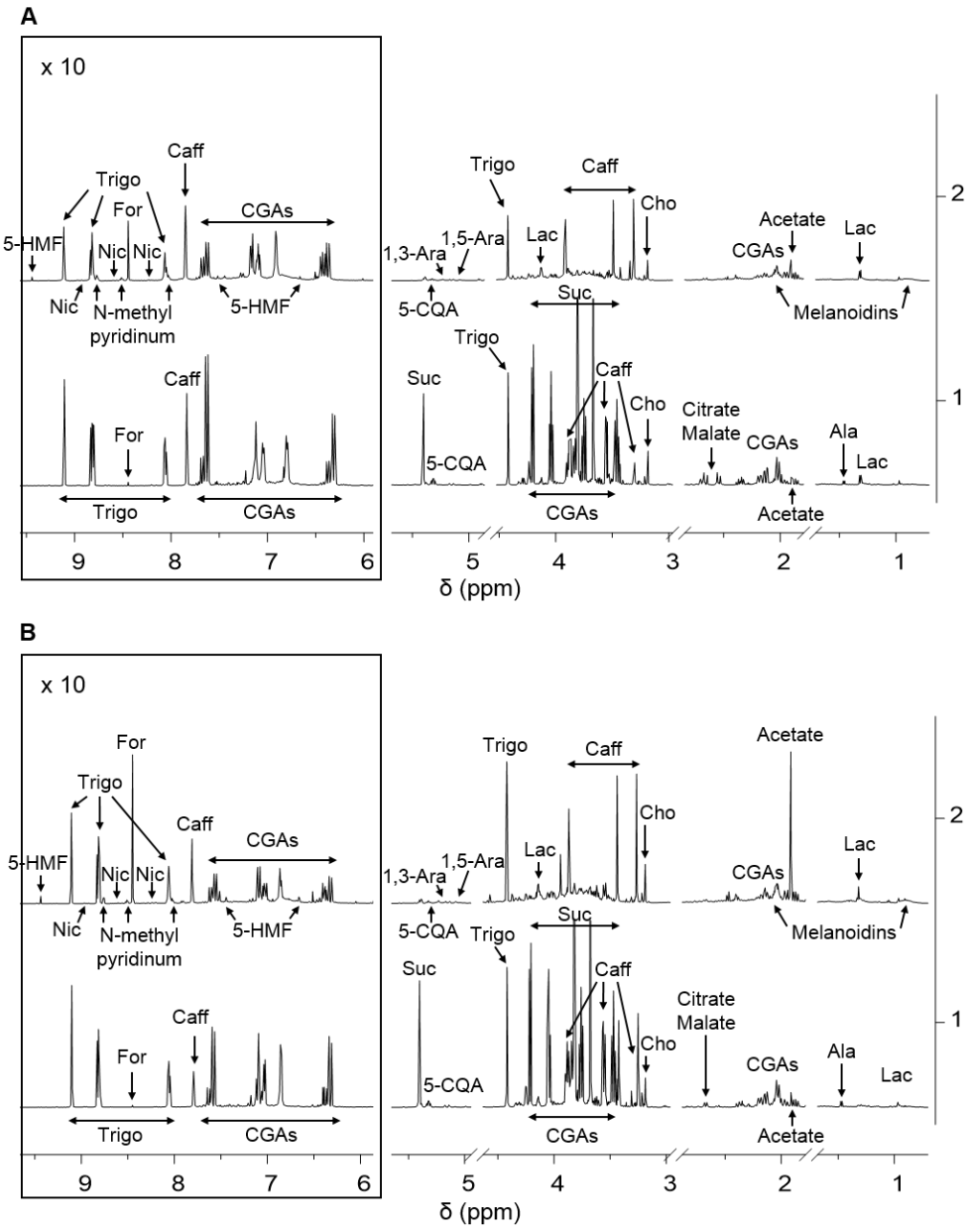

Figure 1. ^{1}H-NMR spectra of green (**1**) and roasted (**2**) coffees from Burundi extracted with hydroalcoholic (**A**) and espresso (**B**) procedures. Each sample, containing 5 mg/mL of extract, was dissolved in D_2O, pH 7.4, DSS 1 mM. Spectra were acquired at 25 °C and 600 MHz. Assignments of the resonances of the most important metabolites are shown (5-HMF, 5-hydroxymethylfurfural; Trigo, trigonelline; Nic, nicotinic acid; For, formate; Caff, caffeine; CGAs, chlorogenic acids; Suc, sucrose; 5-CQA, 5-*O*-caffeoylquinic acid; 1,3-Ara, 1,3-arabinofuranose unit; 1,5-Ara, 1,5-arabinofuranose unit; Cho, choline; Ala, alanine; Lac, lactate).

NMR profiles confirmed the complete disappearance of sucrose in roasted extracts, together with a significant reduction of the amount of CGAs, due to melanoidin formation during the roasting process. The same process is responsible for the appearance of *N*-methyl pyridinium, nicotinic acid, 5-hydroxy-methyl furfural and 2-furylmethanol, and the decrease in trigonelline and choline quantities. The identification of metabolites was based on the analysis of mono (^{1}H) and bi-dimensional (^{1}H,^{1}H-TOCSY, ^{1}H,^{13}C-HSQC) NMR spectra and was in agreement with data from previous literature (Arana, et al., 2015; Consonni, Cagliani, & Cogliati, 2012; Wei, Furihata, Hu, Miyakawa, & Tanokura, 2011).

After the manual identification of the compounds, specific libraries were built for each type of coffee (GCE or RCE) and for each extraction procedure, using the Simple Mixture Analysis (SMA) tool implemented in the MestReNova 12.0 software (Mestrelab, 2018). All the libraries developed with this approach are available as .exp files (Airoldi, Ciaramelli, & Palmioli, 2018). SMA allows for the simultaneous quantification of all the metabolites contained in a complex mixture also for the simultaneous analyses of multiple samples (up to 30 spectra at the same time in this study). For each group of spectra, the plug-in output is represented by a table containing the list of the identified metabolites and their relative concentrations. In addition, the metabolite assignments can be rapidly checked trough the visual inspection of the spectra, where metabolite labels are automatically reported (Supplementary Material – Figure S1). This procedure allows for a considerable reduction in the time required for spectra analysis and for the determination of the concentration of metabolites. In particular, the manual procedure takes between 30 and 45 minutes *per* spectrum, against the two minutes of the SMA-based approach.

Table 1, showing concentrations of metabolites determined in GCEs, and Table 2, reporting concentrations of metabolites for RCEs, allow to directly compare the nature and the concentrations of metabolites obtained under different extraction conditions.

Green coffees		Metabolites (µg/mg of extract)																			
		5-CQA		Acetate		Alanine		Caffeine		CGAs		Choline		Formate		Lactate		Sucrose		Trigonelline	
Origin		M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
Colombia	H	87,29	9,47	0,47	0,09	1,51	0,12	36,81	5,86	181,08	15,49	26,71	3,73	0,17	0,19	10,78	2,16	323,58	47,52	21,47	3,04
	E	48,60	2,21	1,28	0,05	1,87	0,08	29,00	1,77	125,01	5,02	26,46	0,84	0,39	0,03	13,51	0,39	304,87	12,05	27,86	0,99
Brazil A	H	83,33	17,90	0,55	0,07	2,16	0,30	35,44	5,72	173,32	25,59	23,82	4,70	0,29	0,20	12,05	2,68	284,85	58,52	28,87	6,55
	E	41,28	1,88	1,46	0,03	2,45	0,03	28,90	1,72	112,73	4,50	23,44	0,70	0,21	0,10	14,47	0,32	264,48	9,88	33,87	0,79
Burundi	H	93,55	6,01	0,78	0,11	1,82	0,13	36,52	2,19	191,26	5,37	33,13	2,50	0,24	0,15	15,31	0,92	389,43	29,64	31,07	3,17
	E	52,32	3,44	1,31	0,02	1,81	0,09	28,38	2,50	132,10	9,33	26,16	1,58	0,32	0,04	13,54	0,88	293,23	16,92	31,11	2,12
Guatemala	H	121,88	11,57	0,55	0,08	2,25	0,18	45,05	4,50	195,82	12,20	31,67	1,73	0,31	0,00	14,15	1,53	378,12	24,66	32,09	0,98
	E	46,36	1,30	1,27	0,01	2,10	0,04	32,56	0,99	120,47	2,76	24,91	0,47	0,43	0,02	13,24	0,32	289,81	4,96	31,38	0,86
Kenya	H	124,95	17,01	0,55	0,13	1,85	0,24	49,20	5,06	217,78	26,07	41,11	5,50	0,31	0,09	14,02	1,36	496,33	68,67	30,21	5,09
	E	46,89	3,62	1,00	0,05	1,48	0,12	32,33	1,47	121,41	7,03	27,85	0,71	0,28	0,17	11,56	0,33	325,18	8,54	26,65	1,03
Tanzania	H	96,08	16,75	0,57	0,11	1,62	0,12	69,41	13,81	256,92	45,35	18,46	2,30	0,25	0,09	11,63	1,83	216,65	30,86	20,42	2,37
	E	57,75	3,77	1,59	0,14	2,26	0,17	51,07	3,10	176,92	13,27	19,45	1,69	0,32	0,04	15,97	1,41	211,48	19,06	26,06	2,04
Uganda	H	109,85	3,61	1,04	0,08	2,26	0,13	67,94	3,16	274,82	9,87	21,82	0,35	0,30	0,04	13,93	0,57	258,71	10,23	30,75	1,24
	E	54,21	3,94	1,67	0,08	2,30	0,09	41,33	2,09	159,91	9,94	17,10	0,95	0,33	0,03	14,16	0,63	200,13	14,69	27,20	1,48
Vietnam	H	110,25	11,87	1,12	0,18	2,30	0,31	71,83	7,79	286,36	22,82	20,38	1,51	0,22	0,07	13,32	0,51	239,71	19,01	24,25	2,76
	E	49,84	0,68	1,75	0,15	2,21	0,06	34,89	0,88	152,00	1,81	16,04	0,41	0,30	0,03	13,42	0,25	194,82	2,81	20,14	0,46
Brazil R	H	128,50	2,61	0,67	0,06	1,92	0,13	83,89	3,81	349,59	6,68	16,31	0,15	0,25	0,08	12,62	0,48	204,01	2,79	22,90	0,85
	E	67,91	2,68	1,12	0,05	2,06	0,10	51,33	1,19	214,83	5,23	13,63	0,36	0,31	0,17	13,75	0,34	158,60	5,21	22,65	0,71
India	H	129,44	7,35	0,98	0,06	3,05	0,16	91,92	9,90	329,75	29,82	24,45	0,80	0,26	0,11	14,00	1,05	288,44	8,41	25,53	0,75
	E	56,04	0,74	1,90	0,05	2,67	0,05	48,81	1,66	161,03	3,46	17,80	0,34	0,57	0,01	13,98	0,20	197,73	4,68	22,24	0,54

Table 1. Mean concentrations (M) and corresponding standard deviations (SD) of metabolites contained in GCEs (µg/mg coffee extract), obtained with different extraction procedures (H: hydroalcoholic; E: espresso), determined from ^{1}H-NMR spectra.

Roasted coffees		Metabolites (µg/mg of extract)																													
		1,3-arabinofuranose unit		1,5-arabinofuranose unit		2-furylmethanol		5-CQA		5-hydroxymethyl-furfural		Acetate		Caffeine		CGAs		Choline		Citrate		Formate		Lactate		Myoinositol		Nicotinic acid		N-methylpyridinium		Trigonelline	
Origin		M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
Colombia	H	8,53	1,91	6,22	1,06	0,64	0,32	35,24	5,19	1,64	0,20	1,39	1,97	39,50	8,91	126,12	30,36	1,96	0,49	16,33	3,92	4,29	0,36	6,10	0,90	30,46	7,54	0,07	0,10	0,91	0,29	18,22	4,12
	E	7,74	0,31	7,20	0,48	1,72	0,16	28,23	2,46	3,02	0,25	8,68	0,70	35,21	2,03	115,56	5,31	1,48	0,01	ND	ND	10,37	0,40	5,22	0,23	ND	ND	0,67	0,22	1,73	0,24	22,10	0,14
	M	11,96	0,54	8,61	0,41	1,67	0,27	33,95	1,97	2,58	0,08	7,14	1,08	37,16	1,55	126,08	5,56	1,55	0,04	ND	ND	8,12	0,29	5,46	0,34	29,64	0,70	0,94	0,02	1,86	0,05	22,08	0,44
Brazil A	H	8,47	2,51	6,66	1,99	0,74	0,54	31,40	8,01	1,18	0,29	1,92	1,78	38,44	9,10	111,53	27,92	2,38	0,48	16,53	3,12	5,44	0,72	6,36	1,12	31,67	8,20	0,32	0,45	2,48	1,07	20,24	5,10
	E	5,93	0,05	5,44	0,07	1,69	0,09	19,78	1,30	1,71	0,06	10,46	0,50	28,68	0,32	86,51	0,36	1,77	0,01	ND	ND	8,39	0,08	5,16	0,40	ND	ND	0,37	0,06	2,38	0,05	22,06	0,09
	M	10,61	0,40	7,83	0,49	1,41	0,11	30,12	0,25	1,73	0,33	7,88	0,33	33,34	1,12	100,15	6,04	1,85	0,05	ND	ND	7,79	0,19	6,14	0,63	29,37	0,77	0,99	0,34	2,84	0,32	22,51	0,53
Burundi	H	10,32	1,23	7,73	1,23	1,04	0,39	47,33	5,14	1,77	0,23	3,86	0,63	42,53	3,00	166,24	15,56	2,73	0,04	21,62	1,82	4,34	0,44	7,50	0,43	40,03	1,53	0,56	0,10	1,42	0,49	25,95	1,06
	E	6,65	0,11	6,28	0,19	1,72	0,13	23,27	0,84	2,36	0,17	9,20	0,18	30,13	0,05	108,36	2,39	1,73	0,01	ND	ND	10,81	0,12	5,07	0,12	ND	ND	0,19	0,03	1,26	0,06	25,49	0,16
	M	11,84	0,42	8,63	0,32	1,39	0,12	36,02	2,21	2,48	0,14	5,98	0,68	31,94	0,41	126,55	2,19	1,75	0,01	ND	ND	7,92	0,26	5,81	0,48	30,66	0,31	0,93	0,11	1,82	0,08	24,59	0,29
Guatemala	H	9,75	0,18	7,13	0,25	0,62	0,03	43,25	1,34	1,54	0,03	2,89	0,18	49,45	3,91	155,19	3,79	2,31	0,18	18,61	1,67	3,50	0,10	8,19	0,44	33,86	2,38	0,16	0,01	1,03	0,28	24,60	0,30
	E	7,94	0,40	7,70	0,23	1,53	0,15	26,93	0,95	3,04	0,12	8,63	0,53	38,39	0,37	120,58	4,85	1,59	0,02	ND	ND	12,16	0,32	5,09	0,10	ND	ND	0,74	0,04	1,79	0,15	28,43	1,04
	M	12,59	0,30	9,66	0,28	1,68	0,05	36,43	0,30	2,68	0,10	7,11	0,34	44,76	0,78	144,68	3,64	1,76	0,04	ND	ND	9,00	0,34	5,75	0,46	29,94	0,55	1,05	0,03	2,06	0,11	28,41	0,12
Kenya	H	11,94	1,16	7,81	0,99	0,73	0,05	59,29	2,16	2,32	0,22	2,56	0,13	52,17	4,98	197,71	8,10	1,93	0,16	18,37	1,85	3,97	0,27	7,43	0,50	26,18	18,80	0,00	0,00	0,36	0,16	24,67	2,21
	E	9,33	0,25	7,41	0,18	1,56	0,21	31,30	0,67	4,04	0,11	7,25	1,42	38,71	1,23	133,22	4,13	1,27	0,07	ND	ND	10,95	0,70	4,51	0,22	ND	ND	0,58	0,02	1,09	0,04	25,94	0,71
	M	14,74	0,41	9,41	0,53	1,69	0,04	47,95	8,72	3,70	0,03	6,73	0,92	43,89	1,18	169,48	5,51	1,43	0,04	ND	ND	8,43	0,27	5,21	0,04	32,34	0,64	0,74	0,09	1,15	0,08	27,77	0,40
Tanzania	H	8,87	2,10	6,52	1,83	0,62	0,30	33,79	8,80	1,54	0,34	1,82	1,36	66,31	8,39	137,66	25,48	2,34	0,13	14,92	0,73	2,77	0,28	5,13	0,60	33,93	6,33	0,65	0,76	1,80	0,91	13,83	3,14
	E	7,18	0,27	6,92	0,38	1,65	0,19	26,04	1,26	3,06	0,09	9,16	0,72	51,69	1,23	115,86	3,11	1,74	0,04	ND	ND	6,19	0,31	4,38	0,17	ND	ND	1,00	0,06	2,16	0,09	15,75	0,43
	M	10,66	0,92	7,68	0,53	1,38	0,08	31,71	6,36	2,22	0,12	6,70	0,51	49,07	3,73	119,23	11,10	1,73	0,13	ND	ND	5,03	0,28	4,38	0,27	28,26	1,78	1,05	0,11	1,94	0,09	15,31	1,03
Uganda	H	8,62	1,46	6,25	1,13	0,58	0,18	35,87	6,17	1,45	0,21	4,34	0,51	69,49	8,84	151,65	23,00	2,49	0,29	15,82	1,72	1,77	0,16	5,86	0,74	34,22	4,72	0,23	0,04	2,04	0,37	16,92	1,97
	E	6,97	0,09	7,44	0,18	1,48	0,05	26,28	0,22	3,09	0,01	9,10	0,27	51,04	0,24	121,00	0,58	1,66	0,00	ND	ND	6,71	0,04	4,46	0,03	ND	ND	1,08	0,02	2,47	0,01	19,63	0,09
	M	11,23	0,35	8,78	0,32	1,29	0,07	34,66	0,80	2,55	0,06	6,75	0,71	53,50	4,25	134,11	3,56	1,77	0,03	ND	ND	5,38	0,19	5,06	0,12	30,72	0,46	1,27	0,10	2,49	0,10	19,41	0,34
Vietnam	H	9,06	0,82	6,05	0,58	0,61	0,04	39,18	3,30	1,55	0,17	4,94	0,39	81,31	6,53	166,06	14,29	2,86	0,20	16,36	1,49	2,51	0,32	6,49	0,58	37,23	3,05	0,31	0,08	1,22	0,29	15,43	1,22
	E	6,62	0,11	6,33	0,11	1,40	0,03	23,92	0,38	2,69	0,02	8,14	0,21	48,35	1,22	113,62	1,97	1,63	0,04	ND	ND	7,30	0,16	4,04	0,11	ND	ND	0,93	0,07	1,86	0,05	15,59	0,23
	M	11,16	0,15	8,13	0,13	1,12	0,14	32,89	0,33	2,28	0,14	6,03	0,28	46,57	5,91	132,63	4,57	1,82	0,03	ND	ND	5,99	0,03	4,61	0,02	30,21	0,52	1,09	0,12	1,72	0,20	16,18	0,46
Brazil R	H	10,18	0,31	7,31	0,37	0,59	0,08	42,54	1,64	1,02	0,72	4,12	0,18	70,30	3,49	148,57	13,96	2,20	0,04	15,74	0,16	1,81	0,17	6,71	0,68	36,63	0,34	0,27	0,06	1,47	0,15	12,16	1,14
	E	7,81	0,34	8,18	0,34	1,50	0,22	27,46	2,14	3,11	0,17	9,21	0,54	53,76	3,63	124,19	10,77	1,44	0,06	ND	ND	8,17	0,29	4,66	0,21	ND	ND	1,10	0,04	2,24	0,02	16,05	1,04
	M	12,66	0,44	9,93	0,09	1,44	0,21	37,50	5,97	2,65	0,05	6,65	0,31	61,92	1,36	147,39	7,06	1,55	0,02	ND	ND	6,13	0,10	4,71	0,04	31,68	1,38	1,24	0,05	2,34	0,08	15,66	0,50
India	H	8,52	0,70	5,87	0,47	0,53	0,04	40,65	2,70	1,59	0,10	4,02	0,15	75,74	1,77	161,80	5,91	2,96	0,09	17,61	0,91	1,58	0,24	7,02	0,56	36,37	2,09	0,51	0,08	0,89	0,11	13,90	0,51
	E	7,58	0,46	7,82	0,45	1,49	0,18	29,76	1,30	3,33	0,12	10,11	0,78	63,79	1,56	139,89	5,86	2,21	0,16	ND	ND	9,06	0,69	5,43	0,45	ND	ND	0,98	0,09	2,07	0,04	19,52	1,10
	M	10,71	0,53	8,18	0,46	1,38	0,15	34,66	1,52	2,42	0,20	7,25	0,21	65,64	1,93	148,22	0,84	2,18	0,05	ND	ND	6,38	0,20	5,37	0,12	32,49	0,63	0,90	0,32	1,77	0,32	17,83	0,11

Table 2. Mean concentrations (M) and corresponding standard deviations (SD) of metabolites contained in RCEs (μg/mg coffee extract), obtained with different extraction procedures (H: hydroalcoholic; E: espresso; M: moka), determined from ^{1}H-NMR spectra. ND: not detectable because under the detection limit.

Due to their different chemical composition, the number of compounds detected and quantified in GCEs and RCEs is different. Among the three tested procedures, the hydroalcoholic extraction is the most performant in terms of low molecular weight (LMW) compounds' quantity *per* dry weight of extract (Supplementary material – Figure S2), especially for GCEs. The different yield in LMW molecules extracted with different protocols is less significant for RCEs, where the total amount of these metabolites is decreased by the reactions occurring during the roasting process. Notably, citrate concentration in RCEs could be determined only in spectra obtained with the hydroalcoholic procedure, as the amount of this metabolite extracted with the other two methods is very low. Similarly, espresso extraction prevents from quantifying myoinositol.

Multivariate statistical analysis performed on the concentrations of metabolites contained in the extracts showed that PCA (Principal Component Analysis) provides a clear discrimination between Arabica and Robusta coffees, independently of the extraction method. The separation between the two classes along PC1 is evident in the score plots reported in Figure 2, regardless of the type of coffee (green, Figure 2A1, or roasted, Figure 2A2) and the extraction method employed (hydroalcoholic, espresso or moka). The discrimination of Arabica and Robusta species on the basis of the sample metabolic profiling is not surprising and is in agreement with previous data (Cagliani, Pellegrino, Giugno, & Consonni, 2013). However, this analysis provided an additional and very interesting result. In fact, PCA loading plots (Figure 2B1 and 2B2) revealed that coffee molecular components presenting significant biological activities, in particular CGAs, caffeine, trigonelline and choline, are among the metabolites discriminating between the two species (including also sucrose for GCEs).

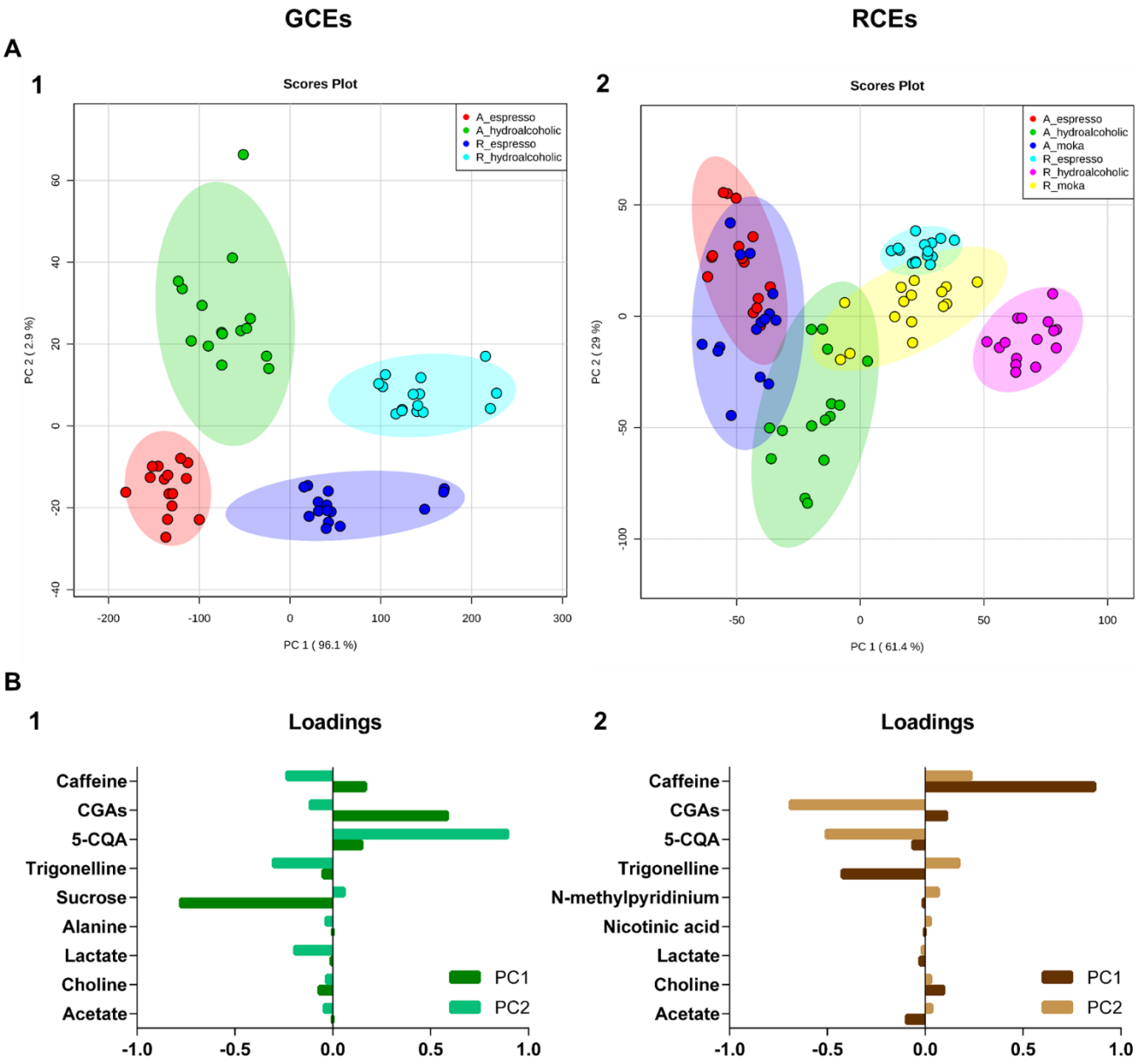

Figure 2. (**A**) PCA score plots obtained for GCEs (**1**) and RCEs (**2**), prepared with different extraction procedures. The explained variances are shown in brackets. Ellipses display 95% confidence regions. (**B**) PCA loadings relative to the PCA score plots (Figure 2A) obtained for GCEs (**1**) and RCEs (**2**).

As already shown by the data reported on Table 1, the content of CGAs and caffeine within GCEs is always higher in Robusta extracts, with the hydroalcoholic procedure being the most efficient for the extraction of both the metabolites. The higher CGAs extraction efficiency of the hydroalcoholic procedure was also confirmed by PCA analysis. Indeed, the score plot reported on Figure 2A1 shows a

separation along PC2 among the samples prepared with hydroalcoholic and espresso procedures, with chlorogenic acids representing the variable contributing the most to PC2 (Figure 2B). The same separation can be observed for RCEs, albeit with less significance (Figure 2A2). Conversely, the average quantity of choline and trigonelline in Arabica is higher than in Robusta coffees (Table 1 and 2).

The evidence just discussed prompted us to calculate the ratio between the content of compounds with beneficial effects on human health (CGAs, trigonelline and choline) and that of caffeine, which presents negative implications for health. The results obtained for GCEs are summarized in Figure 3A, highlighting that Arabica coffees have the highest ratios, independently on the extraction method, with Burundi origin showing the best values.

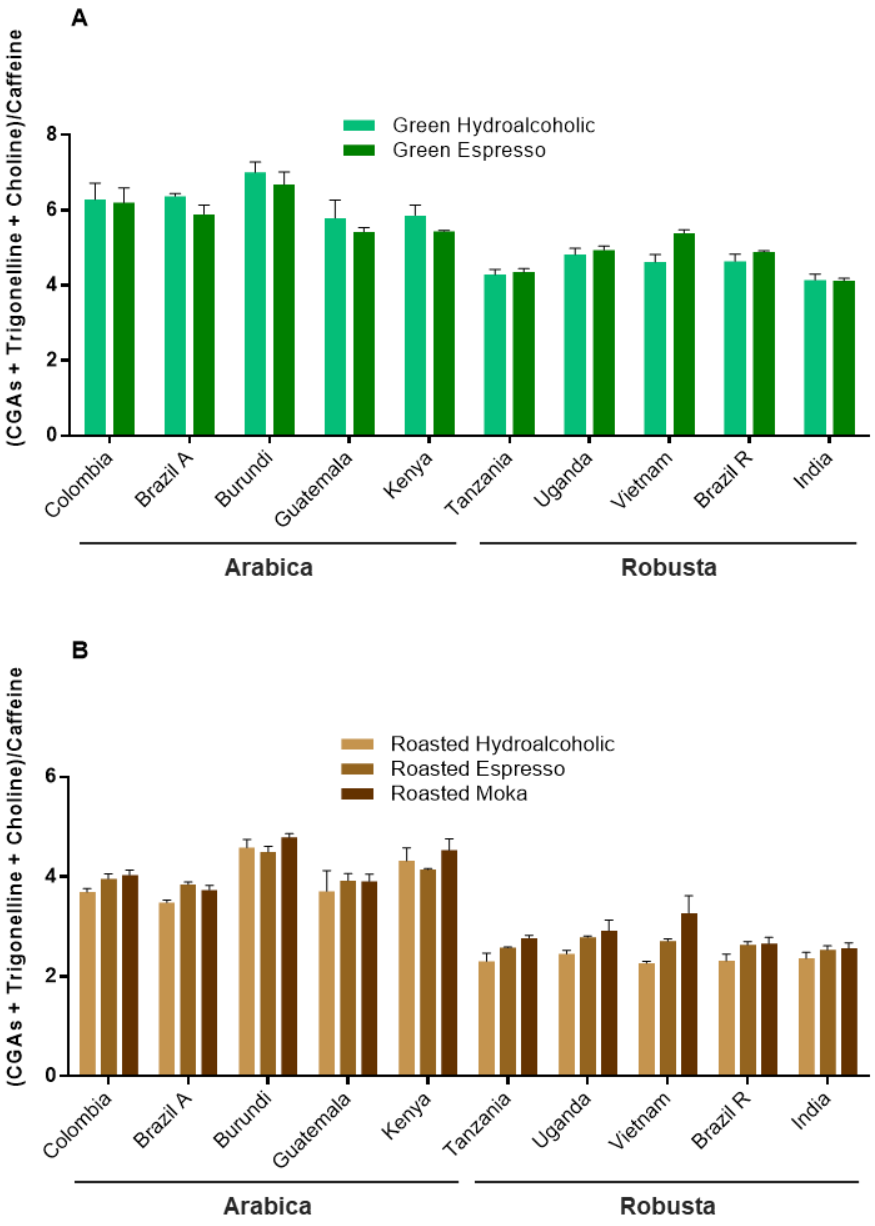

Figure 3. Ratios between the concentrations of beneficial metabolites (CGAs + trigonelline + choline) and caffeine, expressed in µg/mg of coffee extract, in GCEs (**A**) and RCEs (**B**) obtained with different extraction procedures. Results are reported as the mean ± SD of three independent experiments.

For RCEs (Figure 3B) the difference in the ratio beneficial metabolites/caffeine between Arabica and Robusta coffees is even more significant, with Burundi and Kenya origins presenting the highest values.

As the antioxidant property (AOC) of coffee extracts, in particular of GCEs, is one of the main features prompting their use as potential nutraceutical, we measured the AOC of our samples with the Folin-Ciocalteu's method (Figure 4A) (Amigoni, et al., 2017; Ciaramelli, et al., 2018).

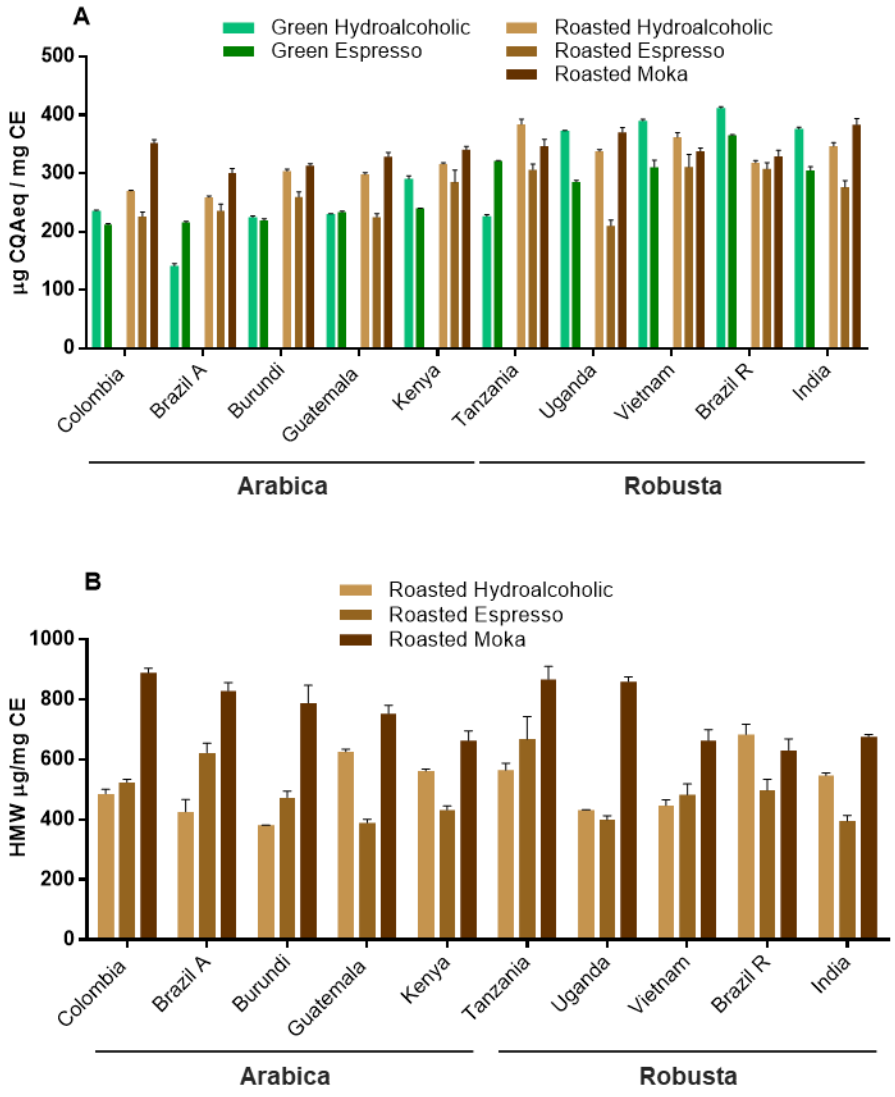

Figure 4. **A**) Antioxidant capacity (AOC) of coffee extracts assessed by Folin-Ciocalteu's assay. Results are expressed as µg of CQA equivalents *per* mg of freeze-dried coffee extracts and reported as mean ± SD of triplicate measurements in three independent experiments. **B**) Melanoidin content in roasted coffee extracts as determined spectrophotometrically at 405 nm, expressed as µg of high molecular weight (HMW) melanoidins *per* mg of extract. Results are reported as the mean ± SD of three independent experiments.

For GCEs, a significant difference was found between coffees belonging to Arabica and Robusta species, with the latter presenting higher AOCs. This finding fits with previous data (Babova, Occhipinti, & Maffei, 2016) and extracts chemical compositions, given that CGAs, the main class of compounds responsible for the antioxidant activity in green coffee, is most abundant in Robusta coffees. According to our findings, after the roasting process, the differences in AOCs disappeared due to the significant reduction in CGAs concentration and to the formation of melanoidins. Thus, all RCEs display very similar AOCs, comparable with the values obtained for the best GCEs. Given that melanoidins also contribute to the beneficial effects of coffee, presenting some important biological properties, among which antioxidant, antimicrobial, anticancer, and detoxifying action (Langner & Rzeski, 2014), their content was measured through a colorimetric assay (Figure 4B) (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002). Indeed, being a class of heterogeneous brown-coloured macromolecules, whose chemical structure is complex and largely unknown, an accurate detection and quantification by liquid state NMR spectroscopy was not feasible. Notably, our data show that the extraction procedure that afforded the best yields in melanoidins is moka, while no correlation between melanoidin content and coffee species or geographical origin was found.

4. CONCLUSIONS

The NMR-based methodology applied to the analysis of green and roasted coffee extracts revealed the influence of species, geographical origin and extraction method on the potential beneficial effects of coffee on human health.

In particular, we identified CGAs, trigonelline and choline as the coffee components of interest for their positive bioactivity, and, for each extract, we measured the ratio between their amount and that of caffeine, which is instead associated with negative effects for human health. The ratio between healthy metabolites and caffeine was higher for coffee samples belonging to Arabica species, regardless of the

extraction procedure, with the roasting process slightly widening the difference between Arabica and Robusta, and the geographical origin exerting little influence.

However, green coffee samples belonging to Arabica species showed lower AOC values than Robusta ones, because of their reduced content of antioxidant molecules such as CGAs and caffeine. Melanoidin formation during roasting largely fills this gap, as Arabica and Robusta roasted coffees present considerable and very similar AOC values, comparable to those of Robusta green coffees.

Regarding the different extraction methods, our study highlights that moka coffee beverages retain the highest content of high molecular weight melanoidins, while the hydroalcoholic procedure performs better in extracting low molecular weight compounds, in particular chlorogenic acids.

All together, these results suggest that not only green coffees, but also roasted coffees, in particular those belonging to Arabica species, can be an important source of bioactive compounds and are characterised by beneficial properties, presenting an average caffeine content lower than Robusta ones. This finding is of interest, as Arabica coffee is the most commonly used for the preparation of beverages and the most valuable from a commercial point of view, due to its aromatic superiority.

The analytical approach presented here provides useful insights for the selection of coffee raw material to be used in the preparation of coffee-based dietary supplements, nutraceuticals and functional beverages. At the same time, this work represents a versatile method for the rapid and reliable analysis of food matrixes aimed at evaluating the content of molecular components associated with health-giving effects.

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Authors declare no conflicts of interest.

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