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Metabolites Profiling of Coffee Seeds in Context to their Origin, Processing and Gut Microbiota Interaction

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THE AMERICAN UNIVERSITY IN CAIRO
الجامعة الأمريكية بالقاهرة
Graduate Studies

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A Thesis Submitted by
Amr Abdelwareth Mohamed

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Abstract

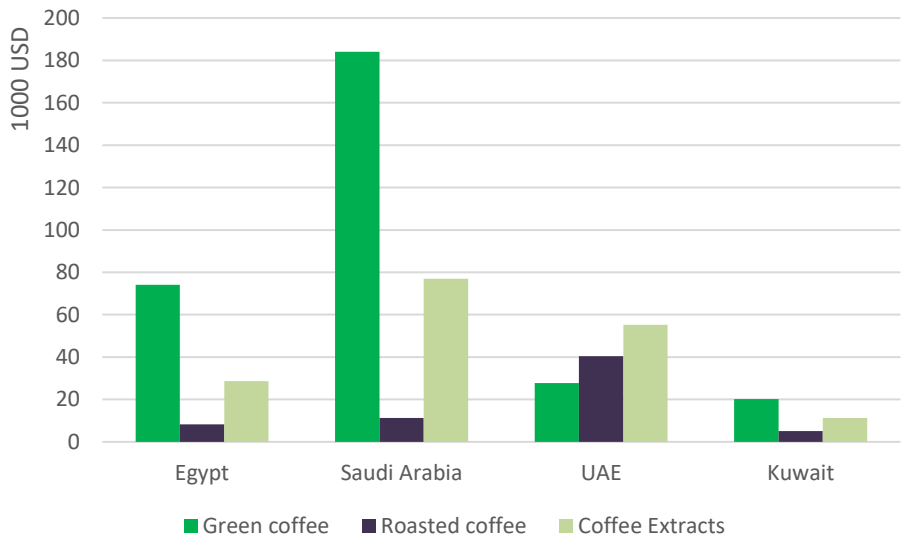
Coffee is among the most consumed beverages worldwide, with the present study to focus on the aroma and non-volatiles composition associated with coffee seeds processing, brewing methods and gut microbiota interaction. The study uncovered a large number of primary and secondary metabolites in diverse coffee products consumed in the Middle East in the context of its origin, roasting, and blends and microbiota mediated biotransformation products. First, aroma of authentic coffee specimens of *Coffea arabica* and *Coffea robusta* alongside with commercial products consumed in the Middle East were analyzed using HS-SPME coupled to GC-MS and modelled using multivariate data analyses (MVA). Results revealed for 102 volatiles with a distinct aroma profile between the different brewing methods. Infusion demonstrated higher esters level, while decoction and maceration were more abundant in sesquiterpenes and terpene alcohols, respectively. Besides, heat-induced products, *i.e*., 4-vinyl guaiacol was identified as potential roasting index in instant coffee and roasted *Coffea robusta* brews. Blending with cardamom further masked the smoky odor of such chemicals by its fragrant terpinyl acetate. Second, non-volatile metabolites in coffee products analysis using GC/MS post-silylation led to the detection of 163 metabolites belonging to 15 different chemical classes. Fructose showed higher abundance in green *Coffea robusta*, while fatty acids *i.e.*, palmitic and stearic acids were more abundant in *Coffea arabica*. Whereas, caffeine was found more abundant in roasted *Coffea robusta* compared to *Coffea arabica*. This study provides the first report on the chemical sensory attributes of Middle Eastern coffee blends and further reveal for the impact of brewing, roasting on its aroma composition. Lastly, insight into the microbiota mediated metabolism of black and green coffee brew is presented using *in vitro* culture and analyzed using GC/MS. Results present the first report regarding how coffee alter gut metabolism through either induction or inhibition of certain metabolic pathways, *i.e.* GABA production likely induced by coffee polyphenols. Likewise, functional food metabolites *i.e.*, purine alkaloids acted themselves as direct substrate in microbiota metabolism.

1 Chapter 1

1.1 Introduction

Coffee is a major worldwide traded commodity that contributes to the economy of several countries [1]. It is recognized as the second most valuable naturally traded product after oil in different world's communities [2]. It is estimated that more than 1.6 billion cups of coffee are consumed on a daily basis [3]. Coffee production reached 10,314,000 tons in 2020 and coffee consumption has reached 9,997,000 tons in 2020/2021. This production is contributed by about 60 tropical and subtropical countries, as reported by the International Coffee Organization [4, 5]. This industry yield is expressed economically to around US $ 200 billion annually [6]. Coffee habitual consumption is attributed mostly for its central nervous system (CNS) stimulant effect asides from its characteristic favorable unique aroma and taste. Recent statistics showed that Saudi Arabia and Egypt have accounted for 75,000 and 77,000 tons of coffee consumption, respectively which accounts for 1.5% of the global coffee consumption [7]. *Coffea arabica* accounts for 60% of the global coffee production, while *Coffea robusta* accounts for the remaining 40% [7]. Evaluation of coffee import in four major countries in the Arab region Egypt, Saudi Arabia, United Arab Emirates and Kuwait shows that most of their imports are in the form of green coffee [8] and reflecting special pattern of consumption based on domestic roastery and wide consumption of mud coffee as indicated in the below figure.

Figure 1-1 Coffee imports per Arab countries in 2014 (FAO Coffee pocket report 2015)



1.2 History of coffee consumption

Current coffee economy relies on remarkable tradition of coffee use and trade from middle ages. *Coffea arabica* shrubs were grown naturally in Ethiopia. They spread to Sudan and the southwest highlands of Arabia. The first documented use of coffee consumption comes back to Yemen in the sixth century AD [1] when it began to be cultivated [9]. Trade and pilgrimage tribes moving in Arab peninsula in middle ages have flourished and contributed to the spread of coffee use to neighboring areas such as Persia where coffee seeds were roasted for the first time [1]. Coffee was identified in the report of Razes Arab physician in the 10th century [9]. Coffee has reached Europe in the 17th century by Turkish conqueror campaigns, through the visit of European travelers, and through Venetian merchants bringing coffee from Mocha port on red sea to Europe [9]. This has triggered several attempts to implant coffee and had led finally to the spread of coffee planting through imperial colonies in west India, Africa and South America where ecological conditions were favoring the growth of this plant. During the 2nd world war, *Coffea robusta* naturally grown in West Africa was used as substituent to *Coffea arabica* which was not reached out in South America. Despite of it inferior flavor, *Coffea robusta* higher caffeine and water extractable content made it good alternative to be used by instant coffee manufacturers during war time [9].

1.3 Coffee species

Among more than 120 species of the genus *Coffea*, arabica coffee (*Coffea arabica*) and robusta coffee (*Coffea canephora* var. *robusta* or *Coffea robusta*) are the most consumed coffee beverages worldwide [10]. Currently, *Coffea arabica* constitutes 60% of the global coffee trade. Along with other coffee species, they belong to family Rubiaceae. *Coffea arabica* have been generated by hybridizing of naturally occurring *C. canephora* and *Coffea eugenioides* in Ethiopia and South Sudan [11]. *Coffea robusta* (*Coffea canephora*) has spread from wild coffee species grown in West Africa to account for *ca*. 40% of coffee trade globally. Despite of its inferior flavor compared with *Coffea arabica*, its low price, higher caffeine content, resistance to coffee diseases such as coffee leaves rust; CLR, and ability to add body to espresso coffee gave it competitive advantage to spread rapidly since world war II. Instant coffee producers favored it due to its higher content of caffeine and water-soluble materials [9, 12, 13]. Currently, it is used in the Arab region in making mixtures with *Coffea arabica* to produce ground coffee (mud coffee) by local roasters [11]. Due to recent challenges of global warming and

environmental changes which have led to extended droughts and emergence of new coffee diseases, special focus has been paid for new wild coffee species that can withstand these conditions. Scientifically noted coffee species other than arabica and robusta coffee are spread in tropical Africa, Madagascar, Comoros islands, Asia and Australia like *Coffea rhamnifolia*, *Coffea fragrans* and *Coffea malabarica* [13], and have yet to be likewise investigated.

1.4 Coffee bioactives

Phytochemically, carbohydrates, lipids, proteins, and chlorogenic acids are the principal components in green coffee seeds amounting for 59-61%, 10-16%, 10%, and 7-10% of its composition, respectively [14]. Sensory attributes, *i.e.*, appearance, odor, flavor, taste, and texture, in coffee seeds are mainly determined by caffeine, trigonelline alongside with sucrose to affect its taste either before or after roasting [15]. Additionally, *ca.*1000 volatile compounds have been recognized in roasted and ground coffee, including acids, aldehydes, alcohols, sulfur compounds, phenolic compounds, pyrazines, pyrroles and furans and to account for its unique aroma [6, 16]. These chemicals constitute to coffee aroma, and therefore, aroma characterization can aid in prioritizing sensory attributes by consumers.

Metabolites of coffee are numerous and could be classified on various basis. Some of these metabolites may be endogenous to coffee seeds like sterols, sugars and amino acids while others may be the outcome of physical and chemical reactions commenced during coffee processing as in roasting and brewing such as furans, pyrazines and melanoidins.

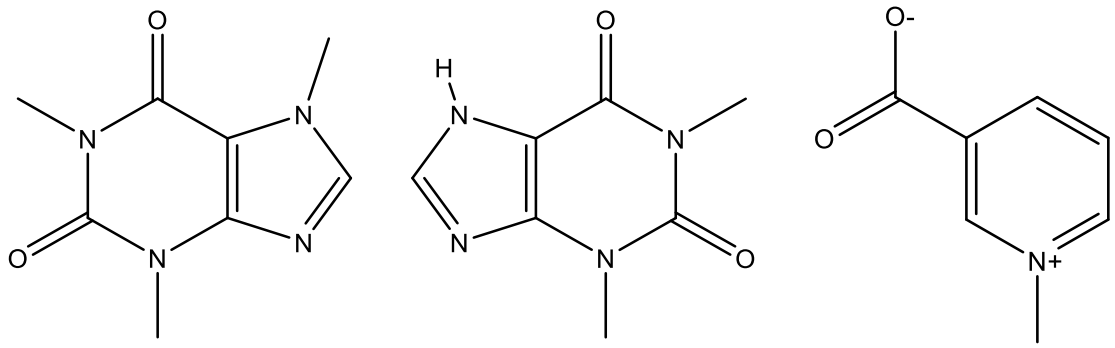
While major coffee metabolites are thermolabile and affected by thermal processes of roasting and brewing such as chlorogenic acid, caffeine one of the major coffee metabolites is heat stable and to show higher levels in roasted compared to green coffee.

Likewise, metabolites could be classified based on their functional role in coffee beverage. Some metabolites are contributing to the flavor with different sensory notes as indicated before, others to impart texture and color to the beverage such as water-soluble solid material and melanoidins. For simplicity we will focus here on the chemical classification based on classes of coffee metabolites in the upcoming sections [1].

1.4.1 Alkaloids

Caffeine is a purine alkaloid that is abundant in tea and coffee. Although caffeine is not the main secondary metabolite of coffee, it is the most recognized for coffee beverage due to its CNS stimulant effect. Along with other purine alkaloids theobromine and theophylline, caffeine is generated from xanthine by methyl transferase. Generally, xanthine alkaloids are naturally present in cocoa, coffee and tea [17]. Content of caffeine varies in different coffee species to range from 4% in robusta coffee seeds to less than 2% in *Coffea arabica*[1]. Some wild species in Madagascar are almost decaffeinated [9]. Other than most of coffee chemicals, xanthine metabolites are characterized by being heat stable thus tending to concentrate upon coffee roasting due to moisture loss and degradation of other components [18].

Figure 1-2 Coffee alkaloids chemical structure



Caffeine Theophylline Trigonelline

Trigonelline is a pyridine alkaloid that is abundant in coffee plant. It constitutes 1-3% of the dry weight of coffee seeds. It is synthesized by methylation of nicotinic acid. Upon coffee roasting, trigonelline tend to degrade into nicotinic acid through demethylation [19].

1.4.2 Hdroxycinnamates

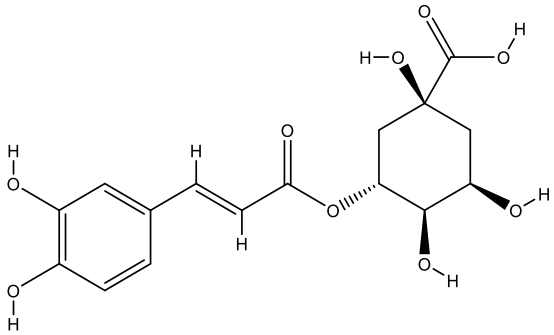
Chlorogenic acid is a general term describing group of coffee phenolic compound that are naturally existing in green coffee to amount for up to 12% of the dry weight of green coffee seed [19]. These group of compounds are formed by esterification of cinnamoyl acid moiety with quinic acid polyalcohol. Cinnamoyl acid moiety may be in the form of caffeic acid, ferulic acid or p-coumaric acid. According to the location of alcohol moiety of quinic acid forming the

esterification bond, different chlorogenic isomer could be generated, with the most abundant chlorogenic acid in green coffee identified as 5-caffeoylquinic acid[20].

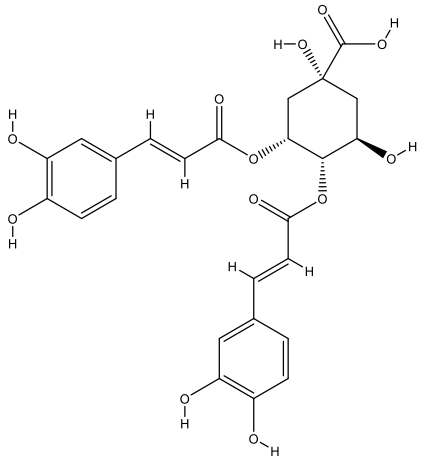
Further, two moieties of caffeic acid quinic acid could bind to single moiety of quinic acid forming dicaffeoyl quinic acid. The most abundant isomer is 4,5-dicaffeoylquinic acid as identified in both *Coffea arabica* and *Coffea robusta* [20].

Chlorogenic acids thermally degrade during roasting by isomerization mainly impacting 5-CQA and lactone formation due to the dehydration of quinic acid moiety which lead to the formation of chlorogenic acid lactones. Seven chlorogenic acid lactones have been reported in roasted coffee, with the most abundant identified as 3-caffeoylquinic acid lactone and 4-caffeoylquinic acid lactone due to lack on cinnamyl conjugation which enabled formation of 1-5 lactone bridge. Lactone content is maximized at medium light roasting (7 min at temp 230 ºC), and to further decline with extended roasting [20].

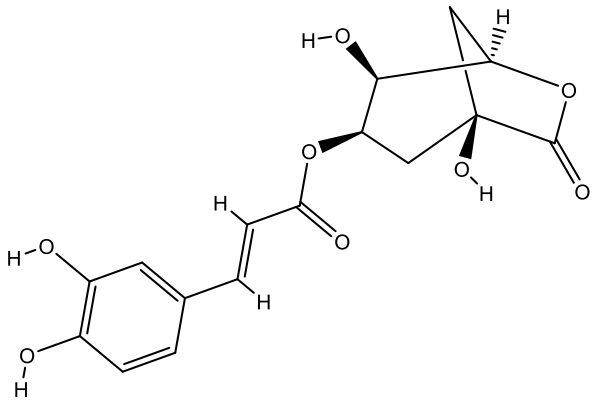
Figure 1-3 Hydroxycinnamates chemical structure



5-Caffeoylquinic acid



4,5-Dicaffeoyl quinic acid



3-Caffeoylquinic acid lactone

1.4.3 Terpenes/lipids

Lipids in coffee are largely constituted by coffee diterpenes, fatty acids, triacyl glycerols along with sterols and tochopherols. These compounds are naturally present in seeds as energy reserve. They also serve as flavor promotors due to capturing of volatile coffee metabolites of hydrophobic nature. Arabica coffee has higher lipid fraction compared to robusta coffee, which explains the superior flavor of the former. They also promote thick texture to coffee brew as with espresso due to their viscosity [19]. Coffee oil constituted by lipid components is concentrated with roasting due to loss of water and degradation of other components such as sugars, phenolics and amino acids [19].

Triacylglycerides account for the major component of coffee lipids, with minor fraction of partial acyl glycerol and free fatty acids. The major fatty acid component of triacyl glycerol is linoleic acid, followed by smaller fractions of palmitic, stearic, lenolenic and oleic acid[19]

Free fatty acids (FFA) and acylglycerols are naturally created in coffee seeds but were also reported to increase significantly in coffee seeds upon storage especially under wet conditions mainly attributed to hydrolysis of triacylgelycerol [19].

Pentacyclic diterpene alcohols derived from ent-kaurenic acid include cafestol, and kahweol present naturally in coffee in either free or conjugated with free fatty acids such as palmitic and linoleic acid. While cafestol is more abundant in *Coffea arabica*, other ditepene alcohol 16-*O*-

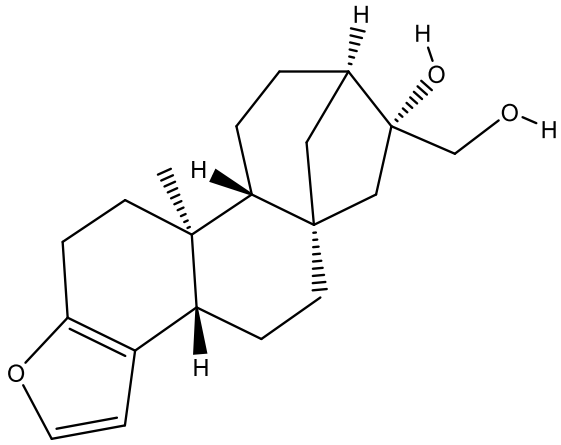
methylcafestol is present only in *Coffea robusta*, and thus can be used as marker for the presence of *Coffea robusta* in coffee mixtures.

Upon roasting, both cafestol and kahweol are converted to dehydrocafestol and dehydrokahweol. The ratio of cafestol to dehydrocafestol decreases with increased roasting temperature and thus could be used as indicator for optimal roasting conditions [19].

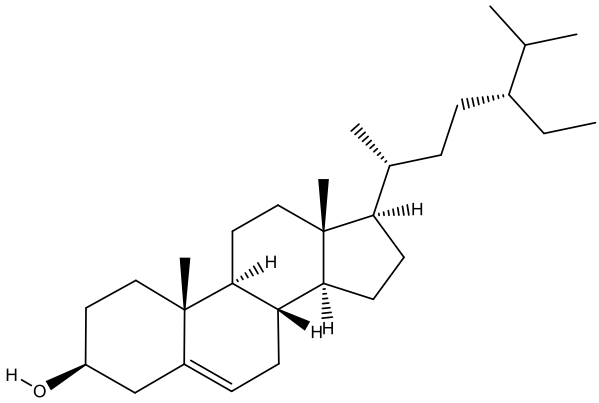
Sterols identified in coffee include cholesterol, 24-methylencholesteriol, campesterol, stigmasterol, beta-sitosterol, delta-5-avenasterol, delta-7-avenasterol, cycloartenol, 24-methylencycloartenol and citrostadienol [19].

Main tocopherols reported in *Coffea arabica*, Coffea robusta are alpha, and beta tocopherols, with total tocopherol found significantly higher in *Coffea arabica*. Tocopherols are minimally affected by roasting process. an exception could be the loss of 25% of β- tocopherol upon *Coffea robusta* roasting [19].

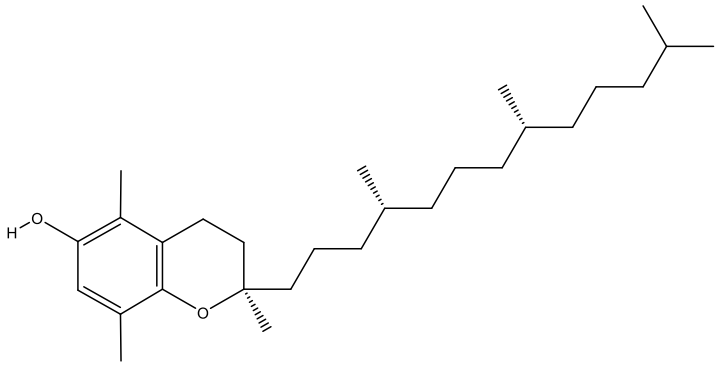
Figure 1-4 Terpenes and sterols chemical structure



Cafestol



β-sitosterol



β-tocopherol

1.4.4 Carbohydrates

Carbohydrates account for 60% of green coffee seeds weight mostly as polysaccharides with less amounts of low molecular weight sugars. Polysaccharides constitute half of the dry weight of green coffee seed in the form of arabinogalactan (type II), galactomannan and cellulose.

Sucrose is the major sugar that is more abundant in *Coffea arabica* rather than *Coffea robusta*, with less amounts of monosaccharides *i.e.,* glucose and fructose. Fructose and glucose levels vary as consequence of postharvest practices. Maltose, isomaltose and melibiose were also reported in immature coffee seeds.

Upon roasting, polysaccharides undergo depolymerization into oligo and monosaccharides, condensation with protein and with several structure modification. Arabinogalactan revealed higher susceptibility to degradation than galactomannan as manifested by reduced molecular weight and branching and increased hot water extractable content.

Roasting degrade sucrose by hydrolysis, dehydration and Milliard reaction, with degradation products to further contribute to the organoleptic properties of coffee drink. Sucrose degradation also imparts acidity to coffee brew *via* degradation to acid such as formic, acetic glycolic or citric acids [21] through intermediate products arabinose, erythrose and 1,6-anhydroglucose.

Polysaccharides constitute most of the coffee beverage fiber intake that are depolymerized by intestinal enzymes and fermented into short chain fatty acids by colon microbiota. Acetylated

galactomannan and type II arabinogalactan from instant coffee modulate cellular immune responses.

1.4.5 Aroma compounds

There are numerous volatile compounds reported in roasted coffee, most of them are in the form of heterocyclic compounds. Major source of volatile compounds in coffee brews is derived from the Milliard reaction which occurs between plant sugars and protein upon roasting. Generally, about 1200 volatile compound have been reported as Milliard reaction products. However, other volatile components may result from degradation of coffee chemicals such as furans derived from the degradation of quinic acid, aromatics to originate from the degradation of cinnamoyl moiety in chlorogenic acid [19].

Coffee aroma is the product of complex mixture of volatile compounds such as thiols, furan, pyrazine, pyridines, thiazole, oxazole and thiophenes. During roasting, oxidation of lipids and carbohydrates occur forming carbonyl compounds which yield furan upon reacting with ammonia from protein degradation [19].

Ammonia and ammonia containing compounds resulting from Milliard reaction play double role in the formation of coffee volatiles. They act as a substrate for nitrogen atom in the heterocyclic compounds and further as catalyst to augment carbohydrate oxidation into carbonyl group compounds/radicals[19].

1.4.6 Miscellaneous metabolites

Flavonoids in coffee exist in the form of isoflavones. Isoflavones reported in coffee include formononetin, daidzein, and genistein. Robusta coffee contains more isoflavones compared to *Coffea arabica*. Isoflavones degrade upon roasting by 30-40%, however, their extractability from coffee brew is enhanced due to the formation of more hydrophilic glycosides[19].

Organic acids are major metabolites that are either inherently present in coffee seed or produced during roasting owing to carbohydrates oxidation. Citric and malic acids are the major acids found more abundant in arabica coffee compared to robusta coffee, and are contributing to its acidity taste [19].

Main minerals in coffee are K, Mg, and Ca to amount for 4% of dry coffee seed weight, found more abundant in *Coffea robusta* compared to *Coffea arabica* [19]

1.5 Preparation of coffee seeds

There are many factors encountered during coffee seed processing and brewing of coffee. These factors affect the metabolite profile of both coffee seed and coffee brew respectively. The tracking of changes in the metabolite profile can help to predict the organoleptic properties and subsequently quality of coffee commodity [22].

Studying the effect of these factors was the subject of researches to define their impact on coffee quality and to identify potential biomarkers dominant in association with these factors. These factors include post-harvest processing of coffee seeds [23], chemical pre-treatment [24], roasting method [25, 26], extraction type [27-30], and or fermentation [31-34].

1.5.1 Roasting of coffee

Roasting process of coffee seeds have shown to be associated with metabolome changes that were identified, and to vary with the degree of roasting. Examples of these changes include reduction of chlorogenic acid and formation of chlorogenic acid lactones with roasting, production of melanoidins out of milliard reaction between sugars and amino acids, pyrazines formation by hydroxyamino acids pyrolysis, pyridine formation from trigonelline and protein pyrolysis [22].

Further processing of ground roasted coffee produces instant coffee products either using spray drying or freeze drying. Such treatment is often associated with a reduction in certain chemicals such as 2-furylmethanol and low molecular weight organic acids, *i.e.*, acetic acid, tartaric, malic, citric formic and 2-oxo-butyric acid. In contrast, sugars *i.e.*, sucrose and ribose, myo-inositol, in addition to acids *i.e.*, fumaric, propionic, glycolic and malonic acids were found to be more enriched in instant coffee compared to ground roasted coffee products [35]. These variations in acid composition are in part attributed to processing method of coffee.

1.5.2 Brewing of coffee

Similar studies applied on different coffee brews showed higher contribution to the roasting process compared with the type of coffee seeds[36]. Furthermore, type of metabolites and their

extent of extraction varied with the different brewing methods of boiled, pour over and cold brew [37].

Decoction, infusion, and pressure methods are the most widely techniques used for coffee brewing [38, 39]. Previous reports have revealed for major influences of coffee processing including roasting, brewing, and blending with condiments associated with product quality, bioactivities, and nutritive values [4, 40, 41]. Examples include the contribution of roasting products, *i.e.*, melanoidins, to the antioxidant and anti-inflammatory effects of coffee extracts [42]. Colzi, *et al.* reported that the composition of volatile organic compounds (VOCs) differed during processing [43].

1.5.3 Coffee additives

Addition of other aromatic herbs such as cardamom improve coffee characteristic flavor and aroma as favored by consumers in several regions, and as typical in the Middle East region [44]. Cardamom is rich in oxygenated monoterpenes, *i.e.*, α-terpinyl acetate, linalool acetate, octyl acetate, and 1,8-cineol, asides from non-oxygenated terpenoids, as limonene and α- and β-pinene [45, 46] to account for its aroma and to be added after coffee seed roasting and prior to its milling.

1.6 Bioavailability and metabolism of coffee metabolites by gut microbiota

Coffee metabolites upon ingestion undergo absorption and metabolism that varies based on different factors such as the metabolite ability to pass the mucosal barrier of the GIT, metabolite nature in terms of hydrophilicity or hydrophobicity, molecular weight, complexation with other compounds either in GIT lumen or in blood, and finally being a substrate for microbiota mediated metabolism for metabolites reaching colon.For example, caffeine is readily absorpable from buccal mucosa and small intestine due to ability to pass the mucosal barrier[47]. In humans, the gastrointestinal (GI) tract harbors approximately 10^{14} Bacterial cells (*i.e.*, 10 times the number of eukaryotic cells in the body), [48]. Bacteria contributing to this intestinal microbiota niche belong to more than 1000 different species harboring more than three million bacterial genes and amount to a biomass of approximately 2 kg, which can be considered as an internal "organ" and provide many functions that are crucial for the hosts well-being. Moreover, the study of intestinal microbiota cannot be separated from its environmental

context. For instance, host genetics, geographical location, nutrition, antibiotics, and other treatments affect the microbiota and its metabolic machinery. The human GI tract hosts a nutrient-rich environment that supports a commensal microbiome providing crucial functions that cannot be carried out alone by the host. These functions are both metabolic (colonic fermentation and production of short chain fatty acids), protective (improving barrier and increasing the resistance to colonization by opportunistic pathogens, secretion of antimicrobial peptides etc.), and structural (maturation of the intestinal epithelium and the immune system), [48]. Microbes present at mucosal sites can also become part of the tumor microenvironment of aerodigestive tract malignancies.[49] In counterpoise, gut microbiota also functions in detoxification of dietary or drug components, can reduce inflammation, and help to maintain a balance in host cell growth and proliferation [50]. Thus, interrogation of gut microbiota metabolism as such and in response to dietary intervention requires a holistic perspective. Assigning microbial communities, their members, and aggregate biomolecular activities into these categories will require a substantial research commitment. Beyond metagenomics, functional approaches, such as meta-transcriptomics, meta-proteomics, and metabolomics (together referred to as meta-omics), are now also rapidly enhancing our knowledge on the gut microbiome. Meta-omic approaches deliver both qualitative and quantitative data on genetic potential, transcripts, proteins, and metabolites present in specific microbial communities under specific conditions. These approaches also have the potential to highlight the systemic influence of microbial communities beyond the gut, deciphering the intricate crosstalk between humans and their microbial ecosystems.

Daily diet has an impact on not only our nutrition but rather health and wellness. This has necessitated an increase in the development and characterization of food products with additional effects than just energy, mineral or vitamin supply the so-called functional foods. Functional foods can be defined as dietary supplements that in addition to their nutritional values, can beneficially modulate body functions towards enhancing physiological responses or reducing a risk of certain disease [51], to the extent that these can be nutraceuticals, *i.e*. foods with clearly established medicinal properties. Upon food ingestion, several mechanical, chemical and enzymatic processes occur within the GIT to mediate for its digestion into nutrients or active ingredients, which are then absorbed to be suitable for use by the body [52].

Gut microbiota mediated metabolic activity can contribute to the metabolism of various dietary compounds as well as transformation of xenobiotics as in functional foods, thus affecting their potential health effects. In contrast, functional food components can themselves also affect the growth and the metabolic activity of gut microbiota and accordingly their composition and or potential functions [53]. For instance, tea phenolics exhibited an inhibitory effect on certain gut microbiota species such as; *Bacteroides spp., Clostridium spp.* (*C. perfringens and C. difficile*), *E. coli and Salmonella typhimurium* with caffeic acid showing the highest inhibitory activity [54]. In parallel, gut microbiota can also affect the pharmacological properties of ingested food products *via* its biotransformation while in the gut pending if not absorbed earlier in the GIT. For example, tannins are solely metabolized by microbial enzymes leading to the formation of conjugated derivatives, which have a different pharmacological profile and being subject to rapid excretion through urine or bile secretions back into the gastro intestinal tract [55]. Whilst most studies have focused on how gut microbes bio transform functional foods, few reports have been made towards how gut microbiota metabolism is affected by food supplements [56]. The main interaction between organisms is of chemical nature. Obtaining a detailed description of how functional foods interact with gut microbiota or do affect its biotransformation can be a key to understand how these organisms regulate our daily food.

Biological transformation of coffee seeds or coffee extracts was largely applied either using *in vitro* microbiota consortium [57] or *in vivo* [58] showed impact of microbiota on the accessibility and changes in the coffee sample metabolome.

1.7 Coffee Health effects

Other than its stimulant effects and characteristic taste promoting its worldwide popularity [59], coffee exhibits health promoting effects attributed to its content of caffeine, chlorogenic acid, diterpene alcohol cafestol and kahweol and other metabolites [59]. Research in coffee effects were based on either epidemiological studies [60, 61] and experimental data or clinical data extracted from studies applied on human beings or laboratory animals [62-64]. All these studies have clearly confirmed the powerful health promoting effects of coffee in terms of its antioxidant [62], antimicrobial [65] activities, reduced the risk of type II diabetes [63], hypo-cholesterolic [66], anti-obesity [67], reduced cardiovascular risk [64], anti-inflammatory [68],

and prophylaxis against neurodegenerative diseases [69]. Coffee consumption was also shown to be associated with reduced cancer risk in urinary tract [70], colorectal cancer and hepatocellular carcinoma [71-73], prostate and bladder cancer [74, 75], pancreatic cancer [76], lung cancer [77].

A major limitation of these studies was that they did not manage to reveal the isolated metabolite-pharmacological action cause-effect relationship in most cases since the demonstrated pharmacological action was either demonstrated to be caused by coffee extract or single metabolite isolate such as caffeine or chlorogenic acid, with the first type of studies not able to derive causality relationship due to different metabolites potential to contribute synergistically or adversely to give certain pharmacological action.

These varying research outcomes may be postulated by many factors such as test environment (in vitro / in vivo), study population, other predisposing factors (such as smoking), and the factor we are most concerned with here which is the metabolite profile of test sample administered.

1.8 Metabolomics analysis of coffee metabolites

Emergence of metabolomics studies in the last decade contributed to identify critical metabolites and revealed the concerted cause-effect relationship of metabolites responsible for pharmacological actions. Metabolomics is one of the omic sciences concerned with the study of mass spectral data related to complex biological extract samples such as plant extracts. It utilizes multivariate statistics to analyze set of spectra related to population of samples then differentiating this population into group of subpopulations and determining the chemical drivers beyond differentiation [78]. Metabolomics comprises a powerful tool to obtain comprehensive analysis of system metabolites without prior knowledge of its content [22]. It has various application such as identification of the species and origin or unknown samples [78] , detections of biomarkers associated with certain types of samples derived from biological matrix such as body fluid or plant extract, in predicting authenticity of food matrix and identify adulteration [58, 79], or in investigation of the impact of different farming or processing conditions on the plant extract metabolite profile [80].

The identification of metabolite is determined by three factors which are its abundance in biological sample (such as coffee brew), farming or processing factor affecting its abundance (like decreased chlorogenic acid with coffee roasting), and the nature of metabolite in relation to the extraction and analysis method (*i.e.*, high molecular weight melanoidins of coffee cannot be determined by GC-MS).

The latter factor represents a constraint of many metabolomics studies due to the use of single hyphenated analytical technique for metabolites detection *i.e.*, liquid chromatography with mass spectroscopy detection; LC-MS for non-volatile polar chemicals versus volatiles profiling using GC-MS. Compared to MS based techniques, NMR is advantageous in terms of versatility and universal detection of all metabolites. Seeds. However, it offer lower sensitivity when compared with hyphenated techniques using mass spectroscopy [22]. Most of coffee volatiles are out of the range of NMR being at low levels [81], more suited to be detected using solid phase micro extraction (SPME) followed by gas chromatography with mass spectroscopy detection; GC-MS [82] for its characterization. Static solid phase micro-extraction (SPME) is a sampling tools that was widely used for sampling of volatile metabolites and aroma compounds in biological samples. Its application has advantages of being rapid and waiving the need of organic solvents like in case of solvent extraction. Fibers based on carboxen , divinyl benzene or liquid PDMS were used with ability to optimize the extraction parameters and sensitivity via either varying the composition of selected fiber or extraction parameters either related to fiber temperature, oven temperature or incubation time [83]. Adsorbed volatile metabolites are desorbed in the gas chromatography injector port upon injection of samples which improves sensitivity of the technique due to sample concentration. SPME along with GC/MS have been widely employed in various applications like determination of coffee sensory flavor profile of coffee brews [84], optimization of coffee brew parameters [85] or trace analysis of acrylamides [86] and polycyclic aromatic hydrocarbons (PAH) [87] in coffee products.

Silylation is another extraction technique that applied the reaction with reagent N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with various nonvolatile components in coffee metabolite matrix to impart them hydrophobicity via forming trimethysiloxane terminals to product compounds [88]. Imparting hydrophobicity to silylated compounds enables gas chromatography separation of wide group of coffee metabolites with higher hydrophilicity such as amino acids, esters, fatty acids and sugars.

Considering the complexity of generated datasets as typical in case of metabolomics, chemo metric data analysis is typically employed for data visualization. Principle component analysis (PCA) is a chemo metric tool that is used to analyze (n) samples of (m) variables. Variables may be represented by identified *m/z* masses in mass spectroscopy or simply wavelengths in spectroscopy spectrum etc…. Out of $(m\ x\ n)$ matrix, PCA generates new set of variables which are called the principle components (PC). Principle components are arranged in descending order in order that PC1 encounters the largest sum of sample variation and so on. Each sample in the matrix is described by its score value on each of the principle components, and to result into what is called score plot which reveals subpopulation variation [78]. High F value and low p value obtained in cross validated ANOVA of multivariate model indicate good separation between groups that is attributed to real differences of metabolic profile of analyzed samples rather than due to data over fitting [22].

While Principle component analysis (PCA-x) represents non targeted (unsupervised) assessment of matrix data, Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) is used to assess supervised data of the matrix. OPLS-DA is characterized by three latent component that explain the model predictive capability (R2X, R2Y and Q2). Predictive capability of the model is confirmed by the random permutation of Y variable. The Score plot obtained by components R2X and R2Y allows reasonably clearer separation when compared with score plot generated by corresponding PCA model due to removal of the structured noise affecting data matrix [80].

Monitoring of metabolite matrix changes and elucidation of biomarkers has been used as a powerful tool to investigate not only the species or geographical origin discrimination of coffee extract but rather extended to different aspect affecting the quality of coffee brew. In that context metabolomics represented an effective substituent to sensory evaluation tests and showing an edge in terms of robustness.

1.9 Study goal

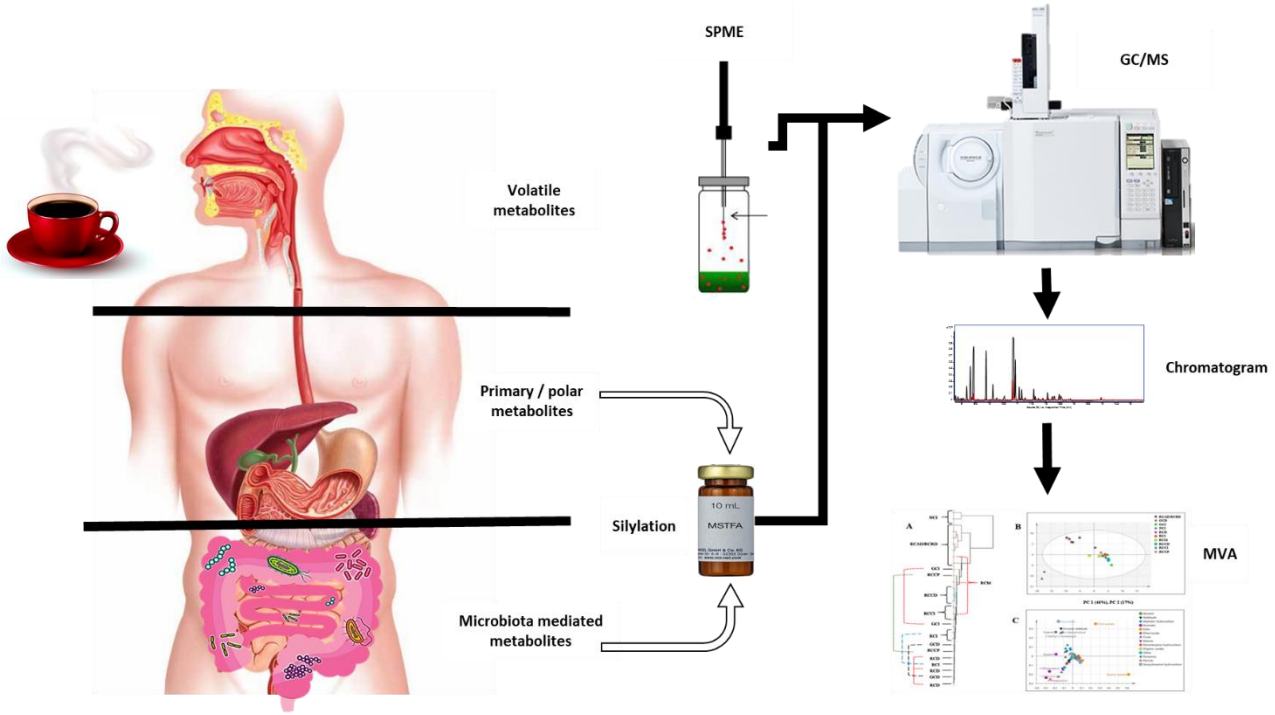
This study employs hyphenated technique of GC-MS. Sample extraction is performed in two ways. The first is through silylation of coffee extract to detect high molecular weight and polar metabolites such as fatty acids and sugars. The second method is through the application of static SPME specific for low molecular weight (volatile compounds) [82].

- The study aims to identify metabolite changes and biomarkers associated with authentic samples of *Coffea arabica* and *Coffea robusta*. **Sections 3.1 & 3.2**

- Profiling of metabolites in commercial coffee samples collected from different markets in the Middle East in the context of coffee processing prior to brewing (such as seed roasting, cardamom addition to roasted seed) and during brewing (hot infusion, cold maceration, decoction). **Sections 3.3**

- Identify how green and roasted black *Coffea arabica* impact on gut consortium strains, representing the human gut microbiota as analysed using GC/MS at two different time points 0.5 and 24 h post inoculation of coffee extracts in gut culture. **Sections 3.4**

Figure 1-5 Study objective and design



2 Chapter 2: Materials and methods

2.1 Head Space-GC/MS Headspace volatiles analysis of coffee brews

2.1.1 Coffee specimens used in volatiles analysis

Details of the coffee specimens analyzed in this study is provided in **Table 2-1** to include authenticated *C. arabica* and *Coffea robusta* specimens and commercial coffee products of green, roasted and blends produced in the Middle East region. 10 Coffee brew samples represented different brewing methods, roasting levels, and cardamom addition were included, see **Table 2-1**. Roasting was performed in the laboratory at 120 °C for 6 h as previously reported for date pit-based coffee substitute [89] using a Hot Plate Shaker SM 30 AT control (Edmund Buhler GmbH, Germany) set at 120 °C and a shaking speed of 150 rpm.

2.1.2 Fibers and chemicals

The SPME holder and fiber coated with 50/30 µm, 1 cm DVB/CAR/PDMS (Divinyl benzene/carboxen/polydimethylsiloxane) and PDMS (polydimethylsiloxane) were supplied by Supelco® (Oakville, ON, Canada). The fibers were compared for their efficiency and sensitivity toward volatiles of roasted *Coffea arabica* brewed by infusion, *i.e.*, RCI. All chemicals and volatile standards were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

2.1.3 Coffee seeds roasting degree determination using UV spectroscopy

Coffee roasting was determined in relation to relative melanoidins level [90]. About 20 mg of each samples were extracted with 100% methanol (HPLC analytical grade, ≥99.9%) in 1:10 ratio (four replicates) followed by centrifugation to obtain a clear supernatant. An aliquot of 200 µL was used in four replicates in 96-well for the Gen 5 Greener UV Microplate reader (Gen 5 kitted with 96-well quartz cell with 1 nm spectral resolution in the UV/Vis absorption region 200-800 nm) and the absorption spectra were recorded from 200-450 nm. The absorbance at 420 nm (A_{420nm}) was chosen to indicate the melanoidins relative levels and hence the degree of roasting (**Table 2-1**) .

Table 2-1 **A list of coffee specimens, i.e., *Coffea arabica* and *Coffea robusta* seeds, analyzed by HS-SPME including brewing method and sample codes used within chapter 3**

Sample name		**A_{420nm}±SD**	**Degree of roasting (fold ratio compared with green *Coffea arabica*)**	**Producer/Origin**	**Preparation method**	**Sample code**
Authentic coffee specimens	Roasted *Coffea arabica*	0.099±0.008	2.5	Mina Gerais University Arboretum, Brazil	Decoction	RCAD
	Roasted *Coffea robusta*	0.051±0.009	1.3			RCRD
Commercial coffee specimens	Roasted *Coffea arabica* blended with cardamom	0.008±0.003	0.2	Alameed coffee		RCCD
	Green *Coffea arabica*	0.04±0.01	1.0			GCD
	Roasted *Coffea arabica*	0.05±0.02	1.2			RCD
	Green *Coffea arabica*	0.04±0.01	1.0		Infusion	GCI
	Instant coffee	0.25±0.008	6.3	Nescafé Classic		NCI
	Roasted *Coffea arabica*	0.05±0.02	1.2	Alameed coffee		RCI
	Roasted *Coffea arabica* blended with cardamom	0.008±0.003	0.2			RCCI
	Roasted *Coffea arabica*	0.05±0.02	1.2		Maceration	RCM

2.1.4 Coffee volatiles profiling

2.1.4.1 Coffee brewing

With some modifications of Rasheed, *et al.* reported for preparation of different herbal extracts, three methods of coffee brewing were employed [91]. Firstly, the volatiles associated with decoction were released through boiling of 0.5 g of the different coffee powder with 10 mL distilled water inside 20 mL clear glass vials. The aqueous suspension was applied to a direct flame till boiling before removing away from the flame. This step was repeated till obtaining a second froth to mimic a decoction-prepared in a typical coffee beverage. In addition, infusion samples were prepared using same ratio applied in decoction method, except for the addition of boiled water at 100 °C to the powder with stirring at room temperature for 5 min. Moreover, cold maceration was prepared using 1.0 g of coffee powder macerated with 5 mL water for 18 h with occasional swirling.

2.1.4.2 Samples preparation for GC/MS analysis

Each brewed replicate including the decoction-prepared beverage was left to cool while closed, with the supernatant aliquot followed by the addition of 1 µL of aqueous (*Z*)-3-hexenyl acetate as an internal standard (IS) at a concentration of 10 µg/mL. (*Z*)-3-Hexenyl acetate was chosen as an IS as it has not been reported previously among coffee volatiles. The vials were then immediately capped and the SPME fiber manually introduced into the sample vial just above the sample and heated at 50 °C for 30 min. Adsorption was adjusted for 30 min. and a system blank containing no seed was run as a control. Fiber was subsequently retracted inside the needle and sharply introduced inside the injection port of the gas chromatograph [66]. In addition, supernatants of infusion- and maceration-prepared coffee extracts were aliquoted by decantation prior to the addition of IS and volatiles collection.

GC/MS analysis of coffee volatiles was performed as described before by Farag, *et. al.* [92, 93] with typically three replicates analyzed for each specimen using a Shimadzu GC-17A gas chromatograph equipped with DB-5 column (30 ml, 0.25 mm x 0.25 um film thickness; Supelco®) and coupled to Shimadzu QP5050A mass spectrometer. Injector and the interface temperature were set at 220 °C, whereas oven temperature was first set at 40 °C for three min, then increased gradually to 180 °C at a rate of 12 °C.min^{-1}, kept at 180 °C for five min, and finally ramped at a rate of 40 °C.min^{-1} to 240 °C, before being retained at that temperature for

another five min. Helium was used as a carrier gas at a flow rate of 0.9 mL.min^{-1}. Splitless injection was used and the first 5 min of the analysis were considered as solvent delay and omitted from the final chromatograms. For any other subsequent analysis, SPME was reconditioned by placing it in the injection port for two min at 220 °C to ensure a complete elution of any volatile residual. The HP quadrupole mass spectrometer was operated in EI mode at 70 eV and the scan range was set at *m/z* 40-500.

2.1.4.3 GC/MS data processing and multivariate data analysis

Multivariate data analysis (MVA) was employed for samples classification in the context of coffee different genotype (*robusta* vs. *arabica*), coffee processing (green vs. roasted vs. instant coffee), coffee brewing method (decoction vs. infusion vs. maceration) and additives (plain coffee vs. coffee with cardamom) on aroma profile.

VOCs were identified by comparison of peak retention time, Kovat index using a series of alkanes (C_8-C_{20}) analyzed under the same conditions, and with standards whenever available. For peaks identification, peaks were first deconvoluted using AMDIS software (www.amdis.net) prior to spectral matching. In addition, AMDIS was applied for data abundance extraction, where relative content of each metabolite was obtained by area normalization of all responses related to identified hits and average response per injection replicates were calculated per each metabolite. Metabolites showing response in only one replicate were excluded from average calculation. Data was then subjected to PCA, HCA, and OPLS-DA using SIMCA-P version 14.0 software package (Umetrics, Umeå, Sweden). All variables were mean centered and scaled to Pareto.

2.2 Silylated-GC/MS metabolites profiling of coffee nutrients

2.2.1 Coffee specimens and chemicals

Details of investigated coffee specimen's origin and producer included in silylated metabolites analysis are provided in **Table 2-2** to include four authenticated *C. arabcia* and *Coffea robusta* specimens provided by the botanical garden of Mina Gerais University Arboretum in Brazil and 16 commercial coffee products. The commercial products represented different roasting levels and blends consumed mainly in the Middle East region. Besides, some of the green products were roasted in the laboratory at 120 °C for 6 h with shaking. **Table 2-2** showed also the

roasting degree of coffee seeds based on the absorbance of melanoidins indicating the browning level of each sample. The degree of coffee seeds browning was determined following roasting relative to green coffee seeds [90].

Table 2-2 Coffee specimen investigated by GC/MS post silylation (Including origin information, trade names, roasting degree and assigned codes)

Specimen type	Sample information	A_{420nm}±SD	Degree of roasting (fold ratio compared with green *Coffea arabica*)	Country of origin	Provider/Producer	Sample code
Authentic samples	Green *Coffea arabica*	---	---	Brazil	Mina Gerais University Arboretum Brazil	GCA
	Roasted *Coffea arabica*	0.099±0.008	2.5			RCA
	Green *Coffea robusta*	---	---			GCC
	Roasted *Coffea robusta*	0.051±0.009	1.3			RCC
Commercial samples	Green *Coffea arabica*			UAE	Bayara	GCU
	Instant *Coffea arabica* blended with cardamom	0.25±0.008	6.3		Nescafé Arabiana	ICC
	Green *Coffea arabica*	---	---	Saudi Arabia	---	GCS
	Lightly roasted *Coffea arabica*	---	---		---	LRS
	Lightly roasted *Coffea arabica* with Qassim blend	---	---		---*	LRSQ
	Lightly roasted *Coffea arabica* blended with cardamom	---	---		Shahi	LRCS
	Lightly roasted *Coffea arabica*blended with cardamom	---	---		Maatouk	LRCM
	Green *Coffea arabica* Aswan	---	---	Egypt	---	GCE
	Lab-roasted *Coffea arabica* Aswan	---	---		---	BRA
	Green *Coffea arabica*	0.04±0.01	1.0	Kuwait	Alameed coffee	GCK
	Lightly roasted *Coffea arabica*blended with cardamom	0.008±0.003	0.2			LRCK
	Lab-roasted *Coffea arabica*	0.05±0.02	1.2			BRK
	Heavily roasted *Coffea arabica* with cardamom	0.008±0.003	0.2			HRKC
	Instant coffee, *Coffea arabica*	---	---	Ethiopia	Bellarom Gold	ICG
	Lightly roasted *Coffea arabica*blended with cardamom	---	---	Qatar	---	LRCQ
	Instant coffee,*Coffea arabica*	---	---	---	Maxim coffee	ICA

*: Unavailable

2.2.2 Silylated primary and secondary metabolites profiling

2.2.2.1 Samples silylation and GC-MS analysis

Analysis of coffee polar primary metabolites was conducted following the exact protocol detailed in [94]. 100 µL of the coffee extract were evaporated under nitrogen till dryness. Metabolites were silylated *via* reacting with 150 µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) at 60 °C for 45 min. The silylated derivatives were then analyzed using GC/MS on Rtx-5MS (30 m length, 0.25 mm inner diameter, and 0.25 µm film) column. Injections (1 µL) were made in a (1:15) split mode. The temperatures of GC compartments were programmed to be 280 °C at the injection port, 80 °C for 2 min then elevated at a rate of 5 °C/min to 315 °C, then retained at 315 °C for 12 min. Helium was used as a carrier gas at flow rate of 1 mL.min^{-1}. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV and the scan range was set at 50-650 *m*/*z*. To assess variance, three replicates from each sample were analyzed.

2.2.2.2 GC/MS data processing and multivariate data analysis

Data processing and MVA was applied as defined in section 2.1.4.3

2.3 Microbiota mediated biotransformation

2.3.1 Plant material and extraction

Methanol extracts were prepared from finely powdered green GC and black coffee BC seeds derived from *Coffea arabica* by cold maceration over 2 days using 100% methanol until exhaustion. Extracts were then filtered and subjected to evaporation under vacuum at 40 °C until complete dryness. Extracts were placed in tight glass vials and stored at -20 °C until further analysis.

2.3.2 Chemicals and solvents

All solvents and chemicals were of analytical grades and purchased from Sigma-Aldrich, St. Louis, USA.

2.3.3 Gut microbiota culture

The microorganisms consortium used in this study is described as: the extended simplified intestinal human microbiota – SIHUMIx. Microorganisms of the SIHUMIx community, a model for the intestinal microbiota, were selected according to their occurrence in humans, the spectrum of fermentation products formed and the ability to form a stable community" by Becker et al.[95]. Co-cultured bacterial species included: *Anaerostipes caccae* (DSMZ 14662), *Bacteroides thetaiotaomicron* (DSMZ 2079), *Bifidobacterium longum* (NCC 2705), *Blautia producta* (DSMZ 2950), *Clostridium butyricum* (DSMZ 10702), *Clostridium ramosum* (DSMZ 1402), *Escherichia coli K-12* (MG1655) and *Lactobacillus plantarum* (DSMZ 20174), all cultivated as single strains. All bacteria were cultivated in Brain-Heart-Infusion (BHI) medium under anaerobic conditions at 37 °C and 175 rpm shaking for 72 h prior to inoculation. All strains were shown to be able to grow equally in the media. BHI media was prepared by mixing 37 g brain heart infusion, 0.5 g L-cysteine hydrochloride, 0.001 g resazurin, 10 ml Vitamin K hemin solution and 5 g yeast extract in one L of sterile water. Gut bacteria cultured in Brain-Heart-Infusion medium (optical density of 0.1) was left to grow under anaerobic condition at 37 ℃ for 18 h till optical density reached 1.7 prior to functional food extract addition. Details on isolated microbiota consortium strains and its potential metabolic functions is depicted in Table 2-3 and Table 2-4, respectively.

Table 2-3 Microbiota consortium strains isolated from gut and used in growth medium enrichment

Species	Strain	Phylum
Anaerostipes caccae	DSMZ 14662 / CIP 108612 /NCIMB 13811	**Firmicutes**
Bacteroides thetaiotaomicron	DSMZ 2079 / ATCC 29148 / NCTC 10582	**Bacteroidetes**
Bifidobacterium longum	NCC 2705	**Actinobacteria**
Blautia producta	DSMZ 2950	**Firmicutes**
Clostridium butyricum	DSMZ 10702 / ATCC 19398	**Firmicutes**
Clostridium ramosum	DSMZ 1402 / ATCC 25582 / NCIB 10673	**Firmicutes**
Escherichia coli	K-12 MG1655	**Proteobacteria**
Lactobacillus plantarum	DSMZ 20174	**Firmicutes**

Since the microbiota in humans but also in domestic animals is highly diverse and individual, and thus incomprehensible and irreproducible, a simplified but therefore more reliable gut microbiota model system was established cultivating a selection of eight bacterial species that

are representing the core functions of the large intestine microbiota (PMID: 21637015). These consortium is comprised of 8 bacterial species defined in table 2-3 grown together as described by Becker et al. [95]. These species are considered the dominant members of the human gut microbiota representing the extended simplified intestinal human microbiota (SIHUMIx) with functionally important biochemical pathways and interactions that likely occur in the human gut. The established SIHUMIx was used as an *in vitro* model for bioreactor use with the consortium in that composition reproducibly established until it reached the ability to stay essentially unchanged. Functions that are known to be fulfilled by each species are given in Table 2-4. Each culture was performed in triplicate to assess for biological replicates for each treatment, in addition to 3 blank cultures made of SIHUMI and BHI medium without treatment.

Table 2-4 Overview of important functions of SIHUMIx species included in microbial consortium

Species	**Phylum**	**Secondary bile acids conversion**	**Function in the human gut**
Anaerostipes caccae (=Eubacterium entericum)	Firmicutes	yes	short chain fatty acids producer
Bacteroides thetaiotaomicron	Bacteroidetes	yes	mucosal barrier reinforcement immune system modulation nutrients metabolism
Bifidobacterium longum	Actinobacteria	yes	produces acetate catabolism of oligosaccharides
Blautia product	Firmicutes	yes	glucose fermentation among the most abundant members
Clostridium butyricum	Firmicutes	no	short chain fatty acids producer dehydroxylation of bile acids
Clostridium ramosum	Firmicutes	no	conversion of bilirubin to urobilinogen dehydroxylation of bile acids
Escherichia coli	Proteobacteria	no	metabolism of high spectrum of glycoconjugates
Lactobacillus plantarum	Firmicutes	yes	immunomodulation enhancement of the epithelial barrier functions

2.3.4 Gut microbiota coffee extracts incubation *in vitro* assay

A stock solution was prepared of green coffee and black coffee at a concentration of 50 mg/ml in 50:50 methanol: (BHI) growth medium and stored at 4 °C until inoculation. 100 µl and 1 ml of each stock solution was then aliquoted to a final volume of 10 ml BHI media containing the gut microbe culture to achieve a final concentration of 0.5 and 5 mg/ml, respectively. Blank cultures were prepared by adding an equivalent amount of 50 and 500 µl 100% methanol into the culture medium, kept under the same condition, and compared to the culture receiving no solvent treatment.

2.3.5 Biotransformation metabolites profiling

2.3.5.1 Metabolites extraction and GCMS analysis post silylation

200 µl of aliquoted culture harvested at different time points was spiked with xylitol standard solution dissolved in sterile water to reach a final concentration of 10 µg./ml followed by the addition of 800 µl acetonitrile/methanol mixture with incubation at 4 °C for 30 min till complete protein precipitation. Mixture was then centrifuged at 12000 g using Eppendorf centrifuge for 4 min, with 100 µl of the supernatant then aliquoted and subjected to evaporation under nitrogen stream till complete dryness. For metabolites derivatization, 150 µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was then added to the residue and incubated at 60 ◦C for 45 min. Samples were then analyzed using GC-MS (Shiamdzu, Japan). Silylated derivatives were separated on Rtx-5MS (30 m length, 0.25 mm inner diameter, and 0.25 µm film) column. Injections were made in a (1:15) split mode, conditions: injector 280 ˚C, column oven 80 ˚C for 2 min, rate 5˚C/min to 315 ˚C, kept at 315 ˚C for 12 min. He carrier gas at 1 mLmin-1. The transfer line and ion–source temperatures were set at 280 and 180 ˚C, respectively.

2.3.5.2 Data processing and multivariate analysis

Metabolites were identified by comparison of peak retention time, Kovat index and spectrum to reference metabolite in NIST database, and with standards whenever available. For peaks identification, peaks were first deconvoluted using AMDIS software (www.amdis.net) prior to spectral matching. Relative content of each metabolite was obtained by area normalization of all responses related to identified hits. Average response per injection replicates were calculated

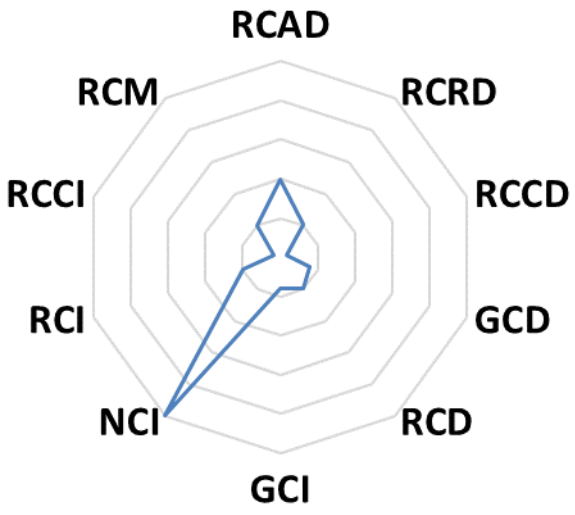
per each metabolite. Metabolites showing response in only one replicate were excluded from average calculation. Data was then subjected to PCA, HCA and OPLS-DA using SIMCA-P version 14.0 software package (Umetrics, Umeå, Sweden). All variables were mean centered and scaled to Pareto Variance.

3 Chapter 3: Results and Discussion

3.1 Coffee seeds roasting degrees

To first determine the roasting levels in coffee specimens prior to aroma analysis, UV spectroscopy was employed by measuring the absorbance of coffee seeds methanol extract at 420 nm specific for melanoidin type compounds. The degree of coffee seeds browning was determined in relation to melanoidins relative levels [90, 96]. **Table 2-1** showed A_{420nm} indicating the browning degree of each coffee sample. In addition, a radar plot for the investigated coffee specimens is presented in **Fig. 3-1**. Results revealed that RCAD and NCI exhibited the highest roasting indexes of 2.5 and 6.3, respectively, relative to green *Coffea arabica* unroasted seed. In contrast, seeds of RCCI demonstrated the least roasting index at 0.2, which may be explained by the interference due to cardamom addition and or dilution.

Figure 3-1 **Radar plot of 420nm absorption in coffee samples (indicating the melanoidin concentration) – Codes are identified in Table 2-1 (RCAD; Authentic *Coffea arabica* roasted decoction, RCRD; Authentic *Coffea canephora* roasted decoction, RCCD; Roasted *Coffea arabica* blended with cardamom decoction, GCD; Green *Coffea arabica* decoction, RCD; Roasted *Coffea arabica* decoction, GCI; Green *Coffea arabica* infusion, RCI; Roasted *Coffea arabica* infusion, NCI; Instant coffee infusion, RCCI; Roasted *Coffea arabica* blended with cardamom infusion, RCM; Roasted *Coffea arabica* maceration)**



3.1.1 GC-MS volatiles identification in brewed coffee specimens

3.2 HS-GC/MS

Two fibers were initially tested for their performance in aroma collection as monitored using GC-MS to include DVB/CAR/PDMS and PDMS fibers. The DVB/CAR/PDMS fiber showed much higher sensitivity in volatiles collection compared to PDMS as shown in Fig. 3-2 for RCI sample. Consequently, DVB/CAR/PDMS was used for aroma collection from all coffee samples followed by GC/MS.

A total of 99 volatile metabolites were identified from all coffee samples analyzed using HS-SPME. Qualitative differences were observed among different seed origins and further with brewing methods as shown in Fig. 3-3, with identified metabolites categorized based on their chemical classes, Table 3-1**.** The increase in volatiles identification, compared with only 37 metabolites using the same platform (HS-SPME) [16], and 77 metabolites using static headspace GC/MS [97], is attributed mostly to data processing step using AMDIS program asides from the improved sensitivity of the SPME collection method. Another reason lies in certain volatiles that were not typical for coffee including, *i.e.*, α-bisabolol, cubenol, menthol, anisaldehyde found almost exclusively in coffee products blended with cardamom as RCCI and RCCD. Volatiles belonged to different classes including alcohols (12), aldehydes (5), aliphatic hydrocarbons (6), aromatic hydrocarbons (3), esters (9), ethers/oxides (6), furans (11), ketones (10), monoterpene hydrocarbons (8), sesquiterpene hydrocarbons (22), pyrazines (4), and pyrroles (4). Heat-induced volatiles, *i.e.*, furans, pyrroles, and pyrazines were more associated with aroma blends in roasted coffee versus esters found the most abundant class in green coffee specimens.

Figure 3-2 **GC/MS chromatograms showing a comparison of SPME fibers extraction of volatiles of roasted *Coffea arabica* brewed by infusion, *i.e.*, RCI**

(DVB/CAR/PDMS (in Black) and PDMS (in Red))

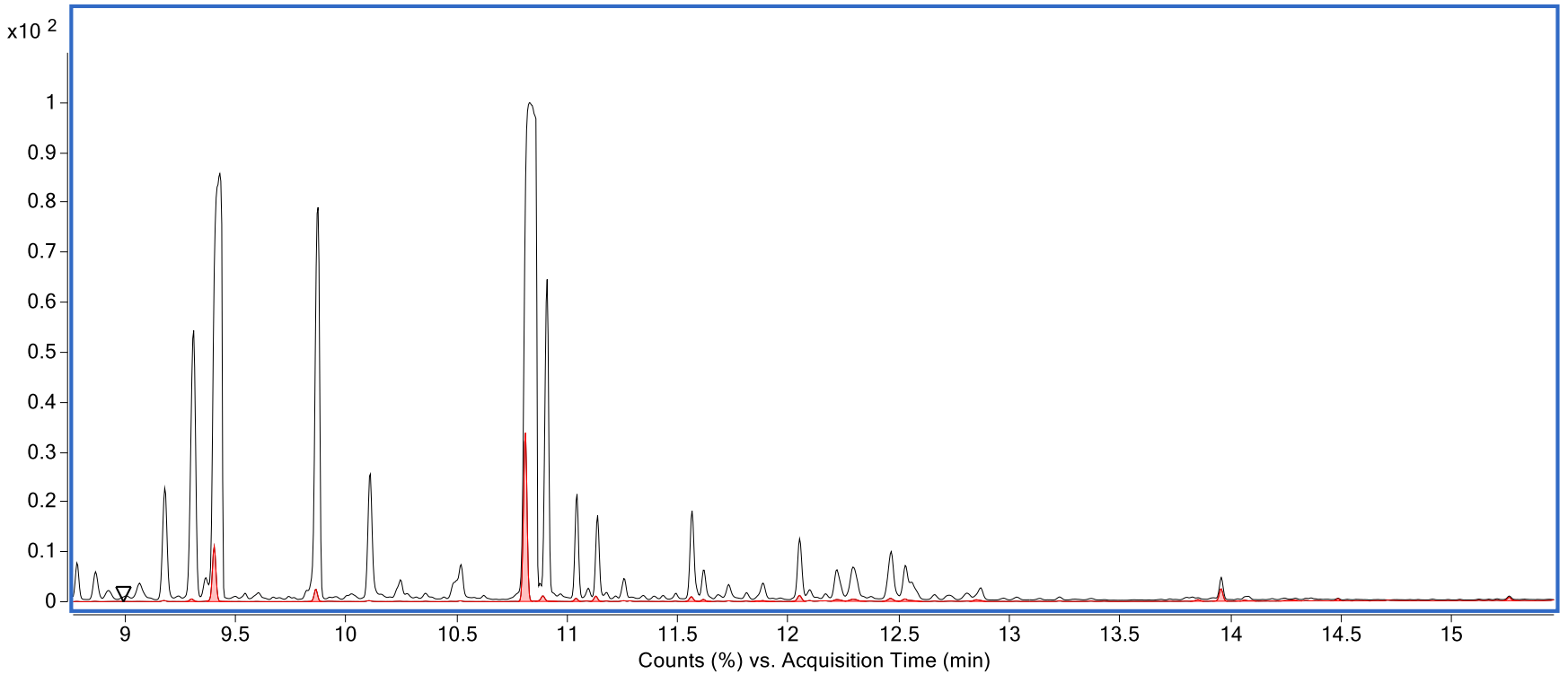


Figure 3-3 **Representative GC chromatograms of coffee specimens extracted by SPME**

(A) Instant coffee (Nescafé Classic) prepared by infusion (NCI), (B) Roasted Coffea arabica prepared by maceration (RCM), (C) Roasted Coffea arabica prepared by decoction (RCD), and (D) Roasted Coffea arabica prepared by infusion (RCI).

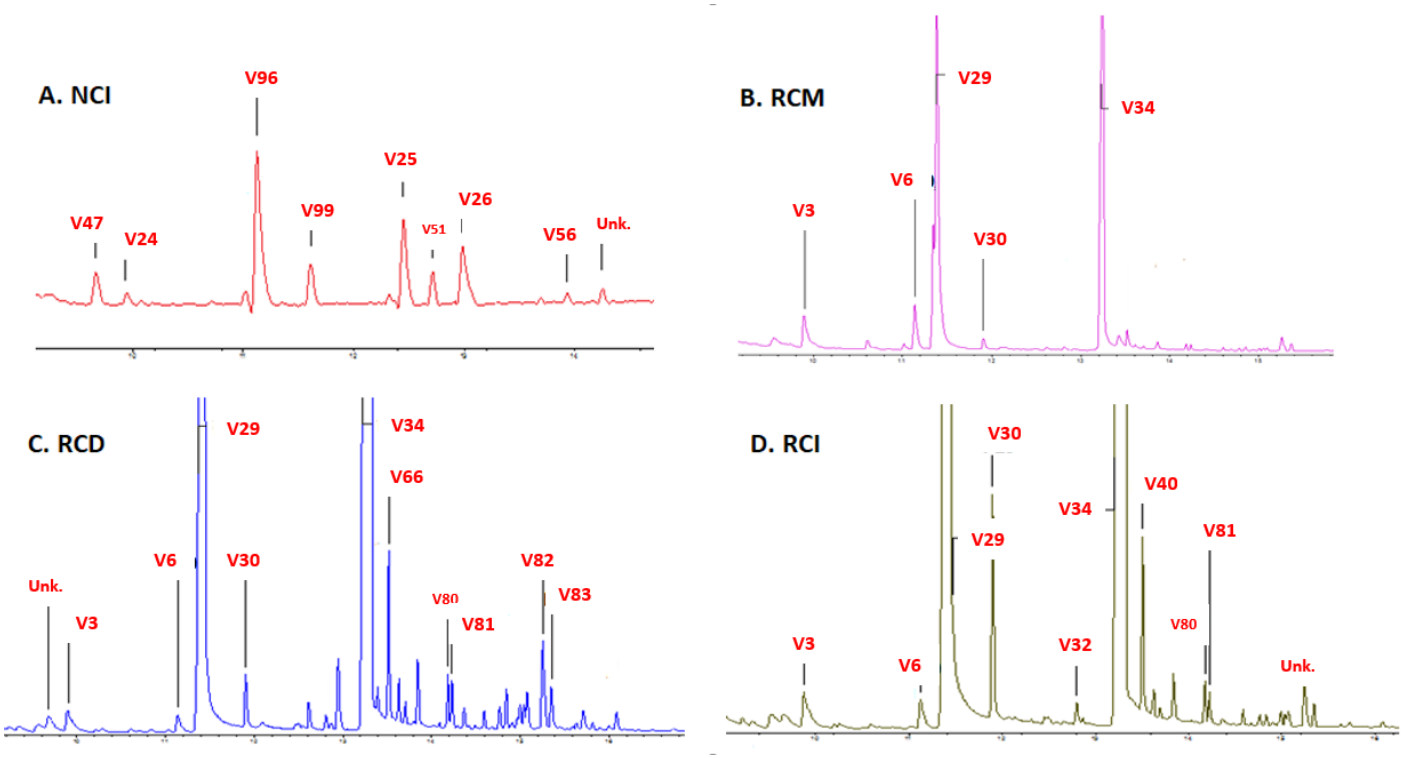


Table 3-1 **Chemical classes of identified volatile metabolites associated with coffee brewing and roasting degrees analyzed using HS-SPME combined with GC/MS analysis.**

Codes are identified in Table 2-1 (RCAD; Authentic *Coffea arabica* roasted decoction, RCRD; Authentic *Coffea canephora* roasted decoction, RCCD; Roasted *Coffea arabica* blended with cardamom decoction, GCD; Green *Coffea arabica* decoction, RCD; Roasted *Coffea arabica* decoction, GCI; Green *Coffea arabica* infusion, RCI; Roasted *Coffea arabica* infusion, NCI; Instant coffee infusion, RCCI; Roasted *Coffea arabica* blended with cardamom infusion, RCM; Roasted *Coffea arabica* maceration)

Peak number	Metabolites	Retention time (min)	Kovat index	RCAD	RCRD	GCD	GCI	NCI	RCD	RCI	RCM	RCCD	RCCI
				n=2	n=4	n=2	n=2	n=2	n=3	n=3	n=3	n=5	n=5
				Total content percentage (Avg. ± SD)									
Alcohols				**4.0**	**-**	**1.67**	**1.95**	**-**	**1.56**	**3.35**	**23.57**	**1.4**	**4.92**
V1	Vinyl hexanol	8.1	962	-	-	-	-	-	-	0.01 ± 0.02	0.11 ± 0.1	-	-
V2	1-Octanol	9.9	1076	-	-	0.56 ± 0.1	0.29 ± 0.35	-	0.5 ± 0.05	1.35 ± 0.23	3.8 ± 2.01	0.25 ± 0.56	1.08 ± 1.25
V3	Linalool	9.9	1075	0.57 ± 0.1	-	0.56 ± 0.27	0.54 ± 0.01		0.49 ± 0.14	1.15 ± 0.13	5.03 ± 2.37	0.28 ± 0.43	1.48 ± 1.3
V4	2,7-dimethyl-4-Octene-2,7-diol	11	1152	-	-	0.05 ± 0.076	0.04 ± 0.06		0.02 ± 0.03	0.04 ± 0.08	0.17 ± 0.3	0.03 ± 0.07	0.14 ± 0.19
V5	Menthol	11.1	1158	-	-	-	-	-	-	-	-	0.02 ± 0.04	0.08 ± 0.19
V6	Terpineol	11.3	1179	3.43 ± 0.8	-	0.49 ± 0.11	1.08 ± 0.26	-	0.39 ± 0.03	0.79 ± 0.1	14.42 ± 9.04	0.2 ± 0.31	1.27 ± 0.92
V7	Geraniol	12.1	1229	-	-	-	-	-	0.11 ± 0.014	-	-	0.08 ± 0.17	0.02 ± 0.04
V8	2,6-Dimethyl-5,7-octadien-2-ol	12.8	1286	-	-	-	-	-	-	-	-	0.02 ± 0.04	0.02 ± 0.05
V9	Nerolidol	15.8	1520	-	-	-	-		0.05 ± 0.1	-	0.04 ± 0.07	0.46 ± 0.76	0.66 ± 1.05
V10	Viridiflorol	16.3	1560	-	-	-	-	-	0.004	-	-	0.03 ± 0.08	0.06 ± 0.06
V11	Cubenol	16.7	1587	-	-	-	-	-	-	-	-	0.03 ± 0.08	0.06 ± 0.06
V12	Bisabolol	16.8	1589	-	-	-	-	-	-	-	-	-	0.05 ± 0.1
Aldehydes				**4.54**	**7.27**	**0.01**	**0.02**	**0.9**	**0.35**	**0.07**	**1.43**	**0.02**	**0.26**
V13	Benzaldehyde	7.9	953	-	-	-	-	0.83 ± 1.17	0.02 ± 0.03	0.01	0.3 ± 0.3	-	0.01 ± 0.01
V14	2-Methoxy-4-methylbenzaldehyde	10.8	1136	-	-	-	-	0.07 ± 0.1	-	-	0.1 ± 0.08	-	-
V15	p-Anisaldehyde	12.4	1255	-	-	-	-	-	-	-	-	0.02 ± 0.05	0.14 ± 0.28

V16	2,4-Decadienal	12.6	1270	-	-	-	-	-	0.18 ± 0.09	-	-	-	-
V17	Cinnamic aldehyde	12.6	1267	4.54 ± 2.45	7.27 ± 1.4	0.01 ± 0.02	0.02 ± 0.03	-	0.15 ± 0.01	0.06 ± 0.08	1.03 ± 0.87	-	0.12 ± 0.15
Aliphatic hydrocarbons				**1.1**	**0.91**	**0.16**	**-**	**-**	**0.32**	**0.15**	**0.13**	**3.06**	**2.08**
V18	Tridecane	13.7	1355	0.29 ± 0.41	0.39 ± 0.16	0.08 ± 0.0	-	-	0.13 ± 0.02	0.07 ± 0.0	0.006	0.02 ± 0.04	0.04 ± 0.04
V19	1-methylene-spiro[4.4]nonane	14.1	1388	-	-	-	-	-	-	-	-	0.01 ± 0.03	0.01 ± 0.01
V20	Pentadecane	14.8	1449	0.81 ± 0.62	0.53 ± 0.49	0.06 ± 0.01	-	-	0.19 ± 0.07	0.08 ± 0.02	0.12 ± 0.01	0.08 ± 0.17	0.05 ± 0.05
V21	Nonadecane	21.4	1836	-	-	-	-	-	-	-	-	0.33 ± 0.69	0.14 ± 0.3
V22	Tetracosane	22	1866	-	-	-	-	-	-	-	-	2.62 ± 5.05	1.81 ± 4.05
V23	Hexadecane	22.5	1893	-	-	0.02 ± 0.03	-	-	-	-	-	0.005	0.04 ± 0.09
Aromatic hydrocarbons				**1.04**	**12.64**	**0.04**	**-**	**38.74**	**0.06**	**0.05**	**0.3**	**0.02**	**0.005**
V24	Guaiacol	9.9	1078	-	4.2 ± 1.16	-	-	5.36 ± 2.54	-	-	-	-	-
V25	4-Ethylguaiacol	12.5	1257	-	4.2 ± 0.56	-	-	13.7 ± 2.85	-	-	-	-	-
V26	4-Vinylguaiacol	13	1299	1.04 ± 0.71	4.24 ± 1.11	0.04 ± 0.0	-	19.69 ± 3.78	0.06 ± 0.01	0.05 ± 0.05	0.3 ± 0.24	0.02 ± 0.03	0.005
Esters				**59.3**	**35.49**	**78.41**	**91.28**	**-**	**75.92**	**84.04**	**58.88**	**78.78**	**71.75**
V27	Vinyl 2-butenoate	8.1	964	-	-	-	0.02 ± 0.03	-	-	-	-	0.01 ± 0.02	0.01 ± 0.02
V28	2-Hydroxyisocaproic acid, acetate	10.6	1123	-	-	0.01 ± 0.01		-	0.01 ± 0.01	0.04 ± 0.01	0.36 ± 0.32	-	0.03 ± 0.04
V29	Octyl acetate	11.4	1176	27.67 ± 2.354	18 ± 2.189	28.17 ± 0.959	15.21 ± 21.506	-	26.57 ± 1.235	29.31 ± 1.583	16.8 ± 8.983	14.77 ± 8.392	17.7 ± 9.962
V30	Linalyl acetate	11.9	1214	0.17 ± 0.25	-	2.27 ± 0.58	1.87 ± 0.06	-	1.46 ± 0.84	2.43 ± 0.44	0.61 ± 0.59	1.97 ± 1.02	3.47 ± 1.14
V31	Isobornyl acetate	12.5	1258	-	-	0.07 ± 0.01	-	-	0.06 ± 0.02	0.07 ± 0.06	-	0.02 ± 0.05	0.08 ± 0.14

V32	Myrcenyl acetate	12.8	1284	-	-	0.11 ± 0.01	0.06 ± 0.0	-	0.08 ± 0.07	0.09 ± 0.08	0.05 ± 0.06	0.03 ± 0.06	0.07 ± 0.07
V33	Methyl geranate	12.9	1289	-	-	0.07 ± 0.0	0.01 ± 0.01	-	0.06 ± 0.02	0.06 ± 0.01	0.01 ± 0.02	0.01 ± 0.03	0.04 ± 0.04
V34	Terpinyl acetate	13	1296	31.17 ± 16.719	17.36 ± 2.665	47.57 ± 0.479	74.1 ± 18.7	-	47.3 ± 0.33	51.76 ± 2.83	40.86 ± 6.7	61.85 ± 5.36	50.22 ± 4.46
V35	Decyl acetate	13.9	1368	0.29 ± 0.07	0.12 ± 0.19	0.14 ± 0.02	0.05 ± 0.03	-	0.39 ± 0.04	0.28 ± 0.07	0.2 ± 0.013	0.13 ± 0.2	0.13 ± 0.12
Ethers/Oxides				**6.96**	**8.53**	**1.11**	**0.8**	**6.59**	**1.4**	**1.57**	**5.68**	**1.62**	**2.4**
V36	Cineol	8.9	1009	-	-	-	0.12 ± 0.17	-	0.2 ± 0.05	1.07 ± 0.29	3.23 ± 0.67	0.92 ± 0.3	1.68 ± 0.30
V37	Linalool oxide	9.5	1049	-	-	-	-	-	-	-	0.08 ± 0.14	-	-
V38	Anethole	12.6	1268	-	-	-	-	0.74 ± 1.05	-	-	-	-	0.07 ± 0.17
V39	Estragole	12.6	1266	-	-	-	-	1.92 ± 2.71	-	-	-	0.06 ± 0.13	-
V40	Eugenol	13.4	1332	6.96 ± 4.89	8.53 ± 1.76	1.11 ± 0.37	0.54 ± 0.14	3.93 ± 5.55	0.94 ± 0.14	0.38 ± 0.18	2.2 ± 2.36	0.19 ± 0.43	0.47 ± 0.4
V41	5-Isopropenyl-2-methylenecyclohexyl hydroperoxide	15.1	1469	-	-	-	0.14 ± 0.1	-	0.26 ± 0.45	0.11 ± 0.2	0.16 ± 0.28	0.45 ± 0.48	0.17 ± 0.24
Furan derivatives				**8.69**	**14.54**	**-**	**-**	**25.42**	**-**	**-**	**0.21**	**-**	**0.03**
V42	Furfural	5.6	834	1.17 ± 1.16	0.89 ± 1.39	-	-	1.22 ± 0.6	-	-	0.02 ± 0.03	-	-
V43	Furfuryl alcohol	6.1	858	1.05 ± 1.0	0.7 ± 0.3	-	-	0.64 ± 0.91	-	-	0.19 ± 0.24		0.006
V44	5-Methylfurfural	7.9	952	3.17 ± 2.92	5.49 ± 3.06	-	-	-	-	-	-	-	-
V45	5-Methyl-2-furaldehyde	7.9	956	3.17 ± 2.92	6.56 ± 5.73	-	-	0.87 ± 1.23	-	-	-	-	-
V46	Furfuryl acetate	8.3	974	0.04 ± 0.06	-	-	-	5.18 ± 2.7	-	-	-	-	-
V47	2-Furfurylfuran	9.7	1060	-	-	-	-	8.56 ± 1.86	-	-	-	-	-
V48	2-Methyl-5-(furan-3-yl)-pent-1-en-3-one	10.7	1130	0.02 ± 0.03	-	-	-	-	-	-	-	-	0.03 ± 0.04
V49	2-Furfuryl-5-methylfuran	11	1151	-	-	-	-	3.7 ± 1.04	-	-	-	-	-
V50	1-Methyloctahydro-2-benzofuran	11.7	1200	-	-	-	-	0.23 ± 0.32	-	-	-	-	-

V51	Difurfuryl ether	12.7	1276		0.05 ± 0.094	-	-	3.72 ± 1.63	-	-	-	-	-
V52	N-Furfurylidene furfurylamine	14.3	1404	0.07 ± 0.1	0.85 ± 0.62	-	-	1.31 ± 1.85	-	-	-	-	-
Ketones				**0.13**	**0.02**	**0.04**	**0.04**	**6.3**	**0.04**	**0.08**	**0.57**	**0.07**	**0.58**
V53	Camphor	10.7	1129	-	-	0.04 ± 0.02	0.04 ± 0.02	-	0.04 ± 0.02	0.08 ± 0.03	0.57 ± 0.31	0.01 ± 0.03	0.08 ± 0.11
V54	Damascenone	13.7	1352	0.02 ± 0.03	0.02 ± 0.05	-	-	-	-	-	-	-	-
V55	Anisyl acetone	13.9	1371	-	-	-	-	-	-	-	-	0.06 ± 0.13	0.5 ± 0.74
V56	Jasmone	13.9	1372	-	-	-	-	6.3 ± 8.91	-	-	-	-	-
V57	Elemenone	16.4	1568	0.1 ± 0.15	-	-	-	-	-	-	-	-	-
Monoterpene hydrocarbons				**0.78**	**-**	**5.64**	**1.86**	**-**	**4.63**	**2.28**	**1.18**	**0.7**	**3.42**
V58	Pinene (isomer)	8	961	-	-	0.32 ± 0.03	0.09 ± 0.12	-	0.16 ± 0.03	0.2 ± 0.09	-	-	0.13 ± 0.18
V59	Sabinene	8	959	-	-	0.29 ± 0.07	-	-	0.04 ± 0.08	0.09 ± 0.15	-	-	0.13 ± 0.18
V60	Unknown monoterpene	8	959	-	-	-	0.02 ± 0.03	-	-	-	-	0.01 ± 0.02	0.01 ± 0.02
V61	Myrcene	8.1	964	-	-	0.29 ± 0.0	0.09 ± 0.12	-	0.16 ± 0.034	0.2 ± 0.09	-	0.04 ± 0.08	0.13 ± 0.17
V62	Limonene	8.8	1005	0.61 ± 0.86	-	4.18 ± 0.98	1.05 ± 0.49	-	3.84 ± 0.79	1.12 ± 1.22	0.88 ± 1.35	0.56 ± 0.78	2.26 ± 1.74
V63	Phellandrene	9.2	1034	-	-	-	-	-	-	-	-	0.01 ± 0.02	-
V64	Terpinene	9.2	1033	-	-	0.04 ± 0.02	0.01 ± 0.02	-	0.02 ± 0.01	0.05 ± 0.0	0.15 ± 0.14	0.009	0.05 ± 0.05
V65	Isoterpinolene	9.6	1055	0.17 ± 0.24	-	0.52 ± 0.07	0.62 ± 0.3	-	0.41 ± 0.11	0.62 ± 0.18	0.15 ± 0.14	0.08 ± 0.12	0.72 ± 0.72
Sesquiterpene hydrocarbons				**11.34**	**13.13**	**8.74**	**2.94**	**-**	**11.81**	**7.26**	**5.88**	**13.76**	**12.27**
V66	Cubebene	13.6	1443	0.19 ± 0.05	0.05 ± 0.1	0.39 ± 0.03	0.11 ± 0.08	-	0.85 ± 0.06	0.56 ± 0.06	0.38 ± 0.25	0.48 ± 0.07	0.48 ± 0.29
V67	8-Isopropenyl-1,5-dimethyl-1,5-cyclodecadiene	13.8	1398	-	-	0.03 ± 0.0	-	-	0.07 ± 0.0	0.05 ± 0.01	0.2 ± 0.057	0.01 ± 0.02	0.05 ± 0.05

V68	Santalen	14.1	1516	-	-	0.04 ± 0.01	-	-	0.07 ± 0.01	0.04 ± 0.0	0.008	0.02 ± 0.06	0.07 ± 0.08
V69	Bergamotene	14.2	1349	0.27 ± 0.38	-	0.69 ± 0.00	0.23 ± 0.08	-	1.17 ± 0.07	0.63 ± 0.08	0.4 ± 0.33	1.22 ± 0.76	1.35 ± 0.65
V70	Caryophyllene	14.2	1359	0.94 ± 0.37	0.25 ± 0.3	1.42 ± 0.06	0.54 ± 0.31	-	1.2 ± 0.17	0.75 ± 0.09	0.47 ± 0.3	0.97 ± 0.36	1.08 ± 0.65
V71	Guaiene	14.2	1468	-	-	1.25 ± 0.05	0.54 ± 0.31	-	0.94 ± 0.08	0.67 ± 0.04	0.43 ± 0.38	0.04 ± 0.1	0.75 ± 0.78
V72	Gurjunene isomer	14.6	1483	-	-	1.51 ± 0.13	0.65 ± 0.46	-	1.65 ± 0.07	1.05 ± 0.09	0.89 ± 0.82	1.06 ± 0.99	1.33 ± 1.41
V73	a-Humulene	14.6	1484	0.07 ± 0.09		0.17 ± 0.02	0.09 ± 0.07	-	0.17 ± 0.01	0.14 ± 0.02	0.08 ± 0.07	0.22 ± 0.06	0.26 ± 0.17
V74	Curcumene	14.8	1443	8.69 ± 1.38	12.11 ± 1.89	0.33 ± 0.05	0.07 ± 0.03	-	-	0.06 ± 0.1	0.31 ± 0.11	-	-
V75	7-epi-α-Cadinene	14.8	1491	-	-	0.25 ± 0.04	-	-	0.46 ± 0.03	0.29 ± 0.06	0.19 ± 0.17	0.07 ± 0.15	0.19 ± 0.18
V76	Muurola-4(14),5-diene	14.9	1491	-	-	-	0.02 ± 0.02	-	-	-	-	0.04 ± 0.09	0.11 ± 0.13
V77	Eudesmene (isomer)	15	1462	0.26 ± 0.37	-	0.56 ± 0.19	0.25 ± 0.25	-	0.7 ± 0.06	0.38 ± 0.06	0.56 ± 0.32	1.07 ± 0.6	0.5 ± 0.42
V78	Farnesene (isomer)	15	1464	0.21 ± 0.13	0.26 ± 0.30	0.07 ± 0	0.14 ± 0.1	-	0.31 ± 0.46	0.11 ± 0.2	0.2 ± 0.35	0.59 ± 0.4	0.43 ± 0.58
V79	Sativen	15	1563	-	0.17 ± 0.24	-	-	-	-	-	-	-	-
V80	Bisabolene	15.1	1400	-	-	0.33 ± 0.08	-	-	0.66 ± 0.12	0.4 ± 0.17	0.21 ± 0.19	0.18 ± 0.4	-
V81	Cadina-1(10),4-diene	15.3	1429	-	-	0.02 ± 0.01	-	-	-	0.05 ± 0.04	0.07 ± 0.06	-	-
V82	Germacrene	15.3	1451	0.62 ± 0.38	0.18 ± 0.21	0.66 ± 0.13	0.3 ± 0.18	-	1.7 ± 0.17	0.88 ± 0.09	0.98 ± 0.28	3.79 ± 1.5	2.45 ± 0.92
V83	Calamenene	15.4	1429	0.09 ± 0.12	0.12 ± 0.14	0.04 ± 0.01	-	-	0.19 ± 0.03	0.13 ± 0.01	0.15 ± 0.03	0.02 ± 0.05	0.08 ± 0.08
V84	Naphthalene, 2,3,4,4a,5,6-hexahydro-1,4a-dimethyl-7-(1-methylethyl)-	15.4	1511	-	-	0.94 ± 0.25	-	-	1.59 ± 0.73	1.04 ± 0.09	0.32 ± 0.56	3.93 ± 1.49	3.0 ± 1.41
V85	Eudesma-3,7(11)-diene	15.6	1462	-	-	0.05 ± 0.01	-	-	0.08 ± 0.02	0.04 ± 0.01	-	0.04 ± 0.1	0.11 ± 0.1
V86	7-epi-eudesmol	15.7	1387	-	-	-	-	-	-	-	-	0.01 ± 0.02	-
V87	7-epi-selinene	16.4	1395	-	-	-	-	-	-	-	-	-	0.05 ± 0.11

Ketones/Oxides				**-**	**-**	**0.001**	**0.02**	**-**	**0.04**	**0.02**	**0.18**	**0.01**	**0.01**
V88	Vinyl acetone	10.6	1124	-	-	0.002	-	-	-	-	-	0.003	0.01 ± 0.01
V89	2-Butylcyclohexanone	12.3	1243	-	-	-	-	-	0.02 ± 0.01	0.02 ± 0.0	0.14 ± 0.13	-	-
V90	Unknown oxide	14.4	1414	-	-	-	-	-	0.003	0.002	-	0.006	-
V91	Ethyl 4-oxo-4-phenylbutanoate	15.1	1466	-	-	-	-	-	0.02 ± 0.03	-	0.05 ± 0.09	-	-
Pyrazines				**1.51**	**4.85**	**-**	**-**	**1.7**	**0.03**	**0.03**	**1.1**	**-**	**0.005**
V92	Methylpyrazine	6.1	858	1.05 ± 1.0	0.7 ± 0.29	-	-	0.64 ± 0.91	-	-	0.19 ± 0.24	-	0.006
V93	2,5-Dimethylpyrazine	7.1	909	0.46 ± 0.5	2.06 ± 2.05	-	-	-	0.03 ± 0.01	0.03 ± 0.02	0.53 ± 0.52	-	0.005
V94	3-ethyl-2,5-dimethyl-Pyrazine	9.7	1062	-	2.04 ± 1.47	-	-	0.78 ± 1.1	-	-	0.35 ± 0.61	-	-
V95	5-Methyl-2,3-diethylpyrazine	10.7	1131	-	0.04 ± 0.08	-	-	0.29 ± 0.40	-	0.003	0.03 ± 0.02	-	-
Pyrroles				**-**	**2.62**	**-**	**-**	**17.46**	**-**	**-**	**-**	**-**	**0.01**
V96	2-Formylpyrrole	9.4	1042	-	0.24 ± 0.28	-	-	0.17 ± 0.23	-	-	-	-	-
V97	1,3-Dimethyl-5-pyrazolone	10.9	1147	-	-	-	-	-	-	-	-	-	0.01 ± 0.01
V98	1-Furfurylpyrrole	11.1	1160	-	2.33 ± 1.26	-	-	15.91 ± 3.27	-	-	-	-	-
V99	Furfuryl methyl disulfide	11.6	1193	-	-	-	-	2.77 ± 0.52	-	-	-	-	-
V100	1-Ethylpyrrole	12.4	1250	-	0.06 ± 0.12	-	-	1.38 ± 0.54	-	-	-	-	-

Analysis of the dried coffee powder blended with cardamom by SPME coupled with GC/MS revealed less detected components (52 compounds) than that detected with extraction of the corresponding specimens, *i.e.*, 68 in RCCI and 58 in RCCD. Among coffee samples, instant coffee showed the least number of aroma compounds with the most different aroma profile being composed mostly of heat-induced by-products such as aromatic hydrocarbons, furans, and pyrroles. Such result is in accordance with previously reported descriptive analysis revealing a distinct sensory variation between instant and fresh filter coffees represented by lack of the typical desirable aromas associated with normal coffee [98]. Previous LC/MS analysis revealed also that instant coffee has a distinctive profile based on non-volatile constituents [99]. Over the next section, major volatiles identified shall be discussed grouped by their class type and/or origin.

3.2.1.1 Esters

Esters amounted for the most abundant volatile class in all coffee specimens, ranging from 35.5-91.3%. They were mostly represented by α-terpinyl acetate (V34) and octyl acetate (V29), **Fig. 3-3** (B-D) and **Table 3**-1. The commercial green coffee seeds brewed by infusion, *i.e.*, GCI, showed the highest levels at 91.3%, while authenticated roasted *Coffea robusta* prepared by decoction (RCRD) was the least at 35.5%. Compared to authenticated roasted *Coffea robusta* (RCRD) esters content (35.5%), higher abundance were detected in authenticated roasted *Coffea arabica* (RCAD) at 59.3% brewed using same method, *i.e.*, decoction. The latter result might indicate that the commercial roasted coffee products, *i.e.*, RCD (75.9%), contained mostly *Coffea arabica*, based on its higher esters levels. Also, green coffee seeds, *i.e.*, GCD and GCI, were found to possess higher levels ranging from 78.4%-91.3%, compared to roasted coffee, *i.e.*, RCD, RCI, and RCM (58.9%-84.0%) and regardless of the brewing method. The higher levels of esters in the commercial product, *i.e.*, RCD at 75.9%, compared to the authenticated counterpart, *i.e.*, RCAD at 59.3%, may be attributed to the method of coffee production invented by the company which is unclear. Production companies typically add some excipients to improve its flavor, where the main differences were attributed to terpinyl acetate and octyl acetate. Also, the degree of roasting varied between the commercial roasted coffee and authenticated coffee samples which might account for such discrepancy in esters level.

The presence of cardamom in certain commercial blends led to an increase in esters level, especially in roasted coffee extracted with decoction as evidenced by an increase in α-terpinyl acetate, compared to products without cardamom (RCCD vs. RCD and RCAD detected at 61.8% vs. 47.3% and 31.2, respectively). α-terpinyl acetate is responsible for the sweet and spicy taste besides an aromatic herbaceous odor characteristic to coffee cardamom blended products. Nevertheless, octyl acetate has been reported previously in coffee seed extracts [100].

Likewise, extraction with infusion resulted in a higher esters level in RCI and GCI at 84.0% and 91.3%, respectively, compared with decoction, *i.e.*, RCD and GCD, at 75.9%-78.4%, and maceration (RCM) at 58.9%. These results suggested that the moderate temperatures employed in infusion were suitable for esters recovery and to decline at higher temperature in case of decoction. Such differences related to coffee brewing methods might be also explained by esters decomposition at the relatively high temperature employed during decoction leading to its lower proportion compared to infusion.

Interestingly, esters were not detected in instant coffee infusion sample (NCI). Similar studies have reported the low level of esters, *i.e.*, diterpene esters, in instant coffee brews [101].

3.2.1.2 Alcohols

Alcohols (V1-V12) constituted the second most abundant class, next to esters, in roasted *Coffea arabica* prepared by maceration (RCM) at 23.5% represented mainly by α-terpineol (V6) at 14.4%, followed by linalool (5.0%)(V3), and 1-octanol (3.8%)(V2). In contrast, alcohols were detected at trace levels (1.7%-1.9%) in most green coffee brewed specimens indicating that alcohols, especially 1-octanol, may be a byproduct for the enhancement of coffee sensory notes [102], *i.e.*, associated with roasting. It was also interestingly to observe that alcohols were detected in authenticated *Coffea arabica* seeds, *i.e.*, RCAD at 4.0%, albeit found completely absent in *Coffea robusta*, *i.e.*, RCRD, and instant coffee samples, *i.e.*, NCI. Therefore, alcohols, especially α-terpineol (V6), ought to be considered among markers for the discrimination between roasted *Coffea arabica* and *Coffea robusta* brew prepared *via* decoction.

3.2.1.3 Furan derivatives

Furans (V42-V52) are produced as sugar by-products during coffee processing, especially roasting [25], and expectedly to be detected at relatively higher levels in roasted robusta coffee

(14.5%) compared to *arabica* coffee (8.7%), and with the highest levels detected in instant coffee at 25.4% of its volatile blend. This information is consistent with previous reports revealing that instant coffee is mainly produced from *Coffea robusta* [99]. Major furans included 2-furfurylfuran (V47) and furfuryl acetate (V46) in instant coffee, while furfuryl alcohol, 5-methyl-2-furfural and 5-methylfurfural were more abundant in authenticated coffee roasted samples, *i.e.*, RCAD and RCRD. Furan derivatives, especially 5-methylfurfural (V44), impart a characteristic sweet, caramel and bready aroma, while furfuryl acetate contributes sweet fruity odor [103]. These results are in accordance with previously characterized furan derivatives associated with coffee roasting [104]. Furans were not detected as expectedly in green coffee seeds, *i.e.*, GCD and GCI, regardless of the employed brewing methods or blend type, *i.e.*, cardamom addition.

3.2.1.4 Sesquiterpene hydrocarbons

Sesquiterpene hydrocarbons (V66-V87) amounted for 5.8%-13.7% in investigated coffee brews aroma, represented mainly by α-curcumene (V74), bergamotene (isomer) (V69), β-caryophyllene (V70), germacrene (V82), and 1,4-cadinadiene (V81). α-Curcumene was found the major volatile in authenticated samples, *i.e.*, RCAD (8.7%) and RCRD (12.1%), compared with other commercial specimens, *i.e.*, germacrene in RCCD (3.79%), RCCI (2.45%), and RCD (1.7%). Sesquiterpene hydrocarbons showed higher levels in decoction compared to infusion and maceration, in case of both green *Coffea arabica* (8.7% for GCD vs. 2.9% for GCI) and likewise roasted, *i.e.*, 11.8% for RCD vs.7.3% for RCI, and 5.9% for RCM.

Cardamom addition in commercial blends led to an increase in sesquiterpene hydrocarbons relative levels in roasted coffee products, *i.e.*, RCCD and RCCI at 13.7% and 12.3%, respectively, compared to roasted coffee without cardamom, *i.e.*, RCD and RCI at 11.8% and 7.3%, respectively. It should be also noted that sesquiterpenes showed lower level in roasted coffee powder blended with cardamom sample at 4.9%, compared to the same brewed samples prepared by either infusion (RCCI) or decoction (RCCD) suggestive for the qualitative differences in aroma blend of brewed coffee from its seeds. Like esters, instant coffee infusion was also devoid of sesquiterpene hydrocarbons.

3.2.1.5 Monoterpene hydrocarbons

Monoterpene hydrocarbons (V58-V65) amounted for 0.7%-5.6% of the total coffee brews' volatiles. They were mainly exemplified by limonene (V62) detected in all specimens, except for RCRD and NCI. Limonene is a chief component of *Coffea arabica* and may impart a fragrant pleasant odor to coffee brews [105, 106]. Monoterpene hydrocarbons showed different abundance in authenticated samples. Monoterpene hydrocarbons showed similar accumulation pattern to sesquiterpenes being enriched in decoction brews (GCD and RCD), compared to infusion or maceration in both green coffee samples, GCD and GCI at 7.8% vs. 2.7%, respectively. Similarly, roasted coffee samples showed similar pattern being detected at 7.0%, 3.7%, 2.1% for decoction RCD, hot RCI, and RCM, respectively. Monoterpenes were also absent in instant coffee infusion as in sesquiterpenes and esters, Fig. 3-3**A** & Table 3-1.

3.2.1.6 Aromatic hydrocarbons

Aromatic hydrocarbons (V24-V26) were mostly represented by 4-vinylguaiacol (V26) followed by 4-ethylguaiacol (V25), and guaiacol (V24). They accounted for the major volatile class in instant coffee sample (NCI) at 38.7%, even higher than furans (25.4%) (V42-V52), Fig. 3-3**A** & Table 3-1. Compared to authenticated *Coffea arabica* decoction levels (RCAD) detected at 1%, much higher levels of aromatic hydrocarbons were detected in *Coffea robusta* (RCRD), *i.e.*, 12.6%. The latter evidence also confirmed the previous relationship between instant coffee and *Coffea robusta* [99] as outlined from furans composition in **section 3.2.1.3.**

Moreover, such volatile class was almost absent in brewed green coffee prepared by either hot infusion and decoction, *i.e.*, GCI at 0.04% and GCD at 0.0%, suggestive that hot brewing method failed to generate these VOCs being mostly produced during the roasting process, *i.e.*, dry heating.

Previous studies have reported that 4-vinylguaiacol and similar products are formed during coffee roasting and to contribute to roasted coffee aroma. These products are derived from thermal degradation of 5-feruloylquinic acid and likewise involved in the formation of coffee melanoidins [107]. Consequently, such class can be considered as potential marker for the identification of instant coffee products, roasting indices, and further discrimination between robusta and arabica coffee. Higher percentage of aromatic hydrocarbons in robusta coffee compared to *arabica* coffee is attributed to its higher phenylpropanoid content, *i.e.*, chlorogenic

acid relative to arabica coffee, where chlorogenic acid serves as the reservoir for aromatics such as 4-vinylguiacol, guaiacol, and phenol *via* successive oxidation process during roasting [107]. Both 4-vinylguiacol and guaiacol contribute to the spicy and smoky sensory notes of coffee [107]. Interestingly, blending with cardamom aided to obscure such odor, with aromatic hydrocarbons detected in RCCD and RCCI at 0.02% and 0.005%, respectively and confirming that cardamom improves the sensory notes of coffee brews.

3.2.1.7 Pyrroles and pyrazines

Likewise, to aromatic hydrocarbons, pyrroles and pyrazines served as indices of the roasting and heat processing of coffee seeds. Although pyrroles were detected at minor levels in all coffee specimens regardless of the brewing method, they amounted as a major class in instant coffee (NCI) at 17.5%, followed by *Coffea robusta* (RCRD) though at much lower levels at 2.6%, confirming the industrial origin of NCI. A major contributor to this class was 1-furfurylpyrrole (V98), as shown in Fig. 3-3**A** for NCI, to exhibit a complex organoleptic properties of vegetable, earthy-green aroma in coffee and other foods [108]. 1-Furfurylpyrrole is a by-product of alanine and glucose during roasting or of proline pyrolysis [109]. Other coffee brews including the authentic *Coffea arabica* (RCAD) and those made from commercial products showed absence of pyrroles regardless of the brewing method. Hence, it may be concluded that *Coffea arabica* is the major constituent of these commercial products.

Moreover, pyrazines were more associated with roasted samples, especially *Coffea robusta* (RCRD) at 4.9%, and absent from green samples either with decoction or infusion, *i.e.*, GCD and GCI.

3.2.1.8 Ethers/oxides and ketones

Ethers/oxides along with ketones were detected in all brewed coffee samples, and in accordance with previous report [16]. Ethers/oxides amounted for 0.8%-8.5% of the brewed coffee aroma, being in general more abundant in roasted coffee (1.4%-8.5%) compared to green coffee (0.8%-1.1%). Interestingly, both authenticated coffee brewed samples RCAD and RCCD showed higher ethers/oxides composition at 7.0% and 8.5%, respectively. Additionally, NCI and RCM contained ethers/oxides at 6.6% and 5.9%, respectively. Major contributors included eugenol (V40) followed by cineol (V36). Eugenol is responsible for the pleasant woody sensory note of coffee flavor[110], while cineol is a major constituent of cardamom and to account for its aroma

[111] expectedly found most enriched in cardamom coffee brewed blends, *i.e.*, RCCD and RCCI. Moreover, ketones were detected only at trace levels in all coffee specimens regardless of the roasting degree and or cardamom blending detected at 0.02%-0.6%, except for NCI which showed much higher levels at 6.3%.

3.2.1.9 Aldehydes, aliphatic hydrocarbons, and others

Aldehydes were detected in all coffee brews and amounted for 0.01% in GCD to 7.3% in RCRD, Table 3-1. Comparison between coffee brews revealed that authentic specimens, i.e., RCAD (4.5%) and RCRD (7.3%) contained the highest aldehydes level represented mainly by (*E*)-cinnamaldehyde (V17). (*E*)-cinnamaldehyde possesses a pungent, cinnamon-like odor. The higher level of cinnamaldehyde in *Coffea robusta* (RCRD) confirmed previous reports indicating the higher abundance of phenylpropanoids in robusta coffee relative to arabica coffee [107]. In contrast to authentic specimens, commercial products showed traces of aldehydes (0.01-1.4%), regardless of the brewing method.

Moreover, aliphatic hydrocarbons were detected in authentic samples of both coffee sp. (0.9%-1.1%) more than commercial products (0.1%-0.3%). However, coffee blends brews, *i.e.*, RCCD and RCCI, were found to be more enriched in aliphatic hydrocarbons owing to the presence of cardamom component, *i.e.*, tetracosane (V22), Table 3-1. Other compounds as sulfur-containing compound, *i.e.*, furfuryl methyl disulfide (P102) appeared to be more associated with instant coffee (NCI), as shown in Fig. 3-3**A**.

3.2.2 Unsupervised multivariate data analyses of coffee brew volatiles dataset

Although differences in aroma profile could be observed by simple visual inspection of chromatograms, as shown in Fig. 3-3, unsupervised MVA, *i.e.*, PCA and HCA were employed for samples classification and markers identification.

3.2.2.1 PCA analysis of the whole coffee volatiles dataset

Hierarchical cluster analysis; HCA was applied at first for all coffee specimens' dataset to determine volatiles heterogeneity among all samples examined without any sort of samples classification. HCA and PCA score plot Fig. 3-4**A** and Fig. 3-4**B**, respectively, showed segregation of commercial instant coffee sample (NCI) as outlier, while authenticated coffee

samples, *i.e.*, RCAD and RCRD, appeared clustered together separately from other commercial coffee products. This is attributed principally to several reasons. Authenticated coffee samples most probably have been subjected to heavier roasting compared to commercial samples. Secondly, while most commercial samples were presumably produced from mixed *Coffea arabica* and *Coffea robusta*, authentic samples are constituted from either coffee species. Lastly, authenticated samples were of geographical origin different from that of commercial products. The principle component PC1/PC2 score plot derived from the whole GC/MS dataset accounted for 62% of the total variance (R2), as identified in Fig. 3-4**B**. While most of the PCA model variability was attributed to the first projection (PC1 = 46%), the second projection (PC2) accounted for 17% of the total variance.

Inspection of the corresponding loading plot Fig. 3-4**C** revealed key aroma compounds responsible for specimens 'segregation observed in score plot (Fig. 3-4**B**). For instance, furans and aromatic hydrocarbons appeared enriched in instant coffee infusion, including 1-furfurylpyrrole (V98), vinyl guaiacol (V26), and ethyl guaiacol (V25). Other furans such as 5-methylfurfural and 5-methyl-2-furaldehyde showed closer correlation with authenticated coffee brew samples rather than instant coffee. In addition, esters such as terpinyl acetate and octyl acetate showed close correlation with green coffee samples than roasted ones.

3.2.2.2 PCA analysis of the roasted coffee samples prepared by decoction, infusion and maceration dataset

To investigate the impact of extraction and or brewing method on similarly processed coffee samples, *i.e.*, roasted coffee samples prepared by either decoction, infusion, and maceration, were modeled together to include RCD, RCI and RCM, as demonstrated in Fig. 3-5. Results showed that the different brewing methods resulted in specimens' separation, as shown in HCA, Fig. 3-5**A**. Maceration (RCM) was observed to be separated from other hot samples (RCI and RCD) along PC1 with positive score values (Fig. 3-5**B**). In contrast, separation of infusion samples (RCI) from decoction (RCD) could only be observed along PC2. The corresponding loading plot shown in Fig. 3-5**C** revealed that maceration samples (RCM) were more enriched in alcohols *i.e.*, terpineol. In contrast, decoction brew was rich in the monoterpene hydrocarbon *i.e.*, limonene versus infusion enrichment in esters, *i.e.*, terpinyl acetate and octyl acetate. Based on these key compounds, aroma of different coffee brews could be expected in relation with brewing method.

Figure 3-4 **(A) Hierarchical cluster analysis (HCA) of all coffee samples along with (B) principal component analysis (PCA) score plot, and (C) loading plot of detected volatile chemical classes.**

(Spatially scattered samples of same origin are noted using parenthesis of varying color) – Codes are identified in Table 2-1 (RCAD; Authentic *Coffea arabica* roasted decoction, RCRD; Authentic *Coffea canephora* roasted decoction, RCCD; Roasted *Coffea arabica* blended with cardamom decoction, GCD; Green *Coffea arabica* decoction, RCD; Roasted *Coffea arabica* decoction, GCI; Green *Coffea arabica* infusion, RCI; Roasted *Coffea arabica* infusion, NCI; Instant coffee infusion, RCCI; Roasted *Coffea arabica* blended with cardamom infusion, RCM; Roasted *Coffea arabica* maceration)

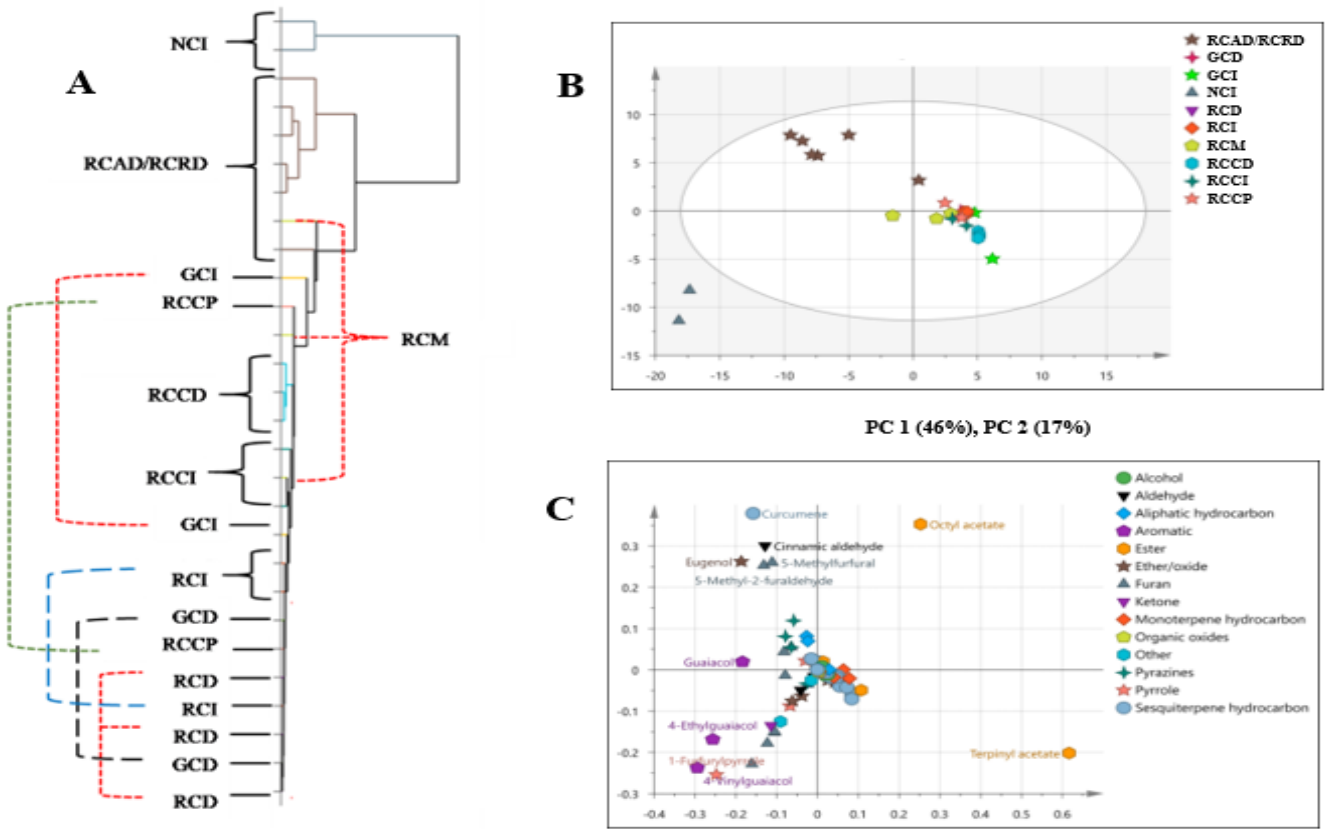
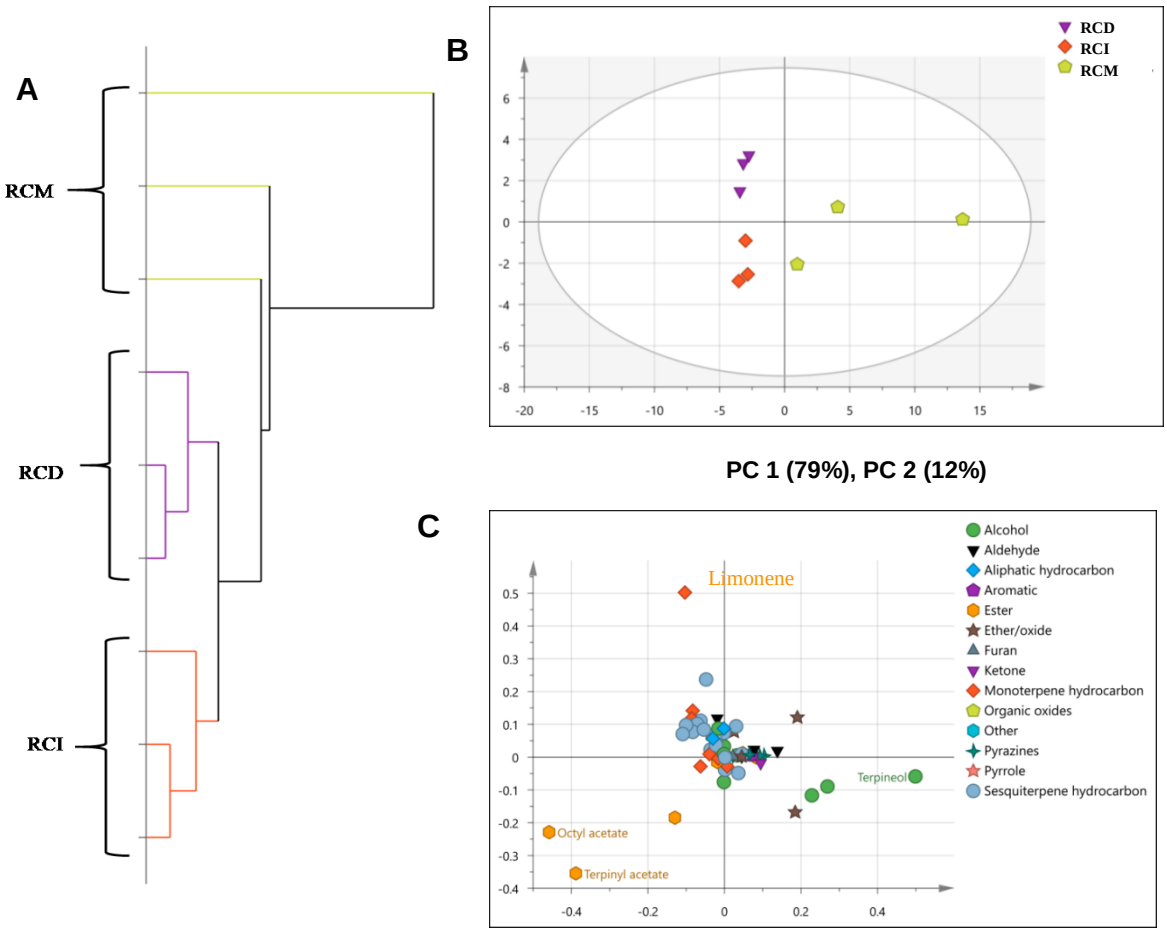


Figure 3-5 **(A) Hierarchical cluster analysis (HCA) of roasted coffee specimens prepared by decoction (RCD), infusion (RCI) and maceration (RCM), (B) principal component analysis (PCA) score plot, and (C) loading plot of the same sample set.**

(RCD: Roasted Coffea arabica decoction; RCI: Roasted Coffea arabica infusion; RCM: Roasted coffee maceration) – Codes are identified in Table 2-1



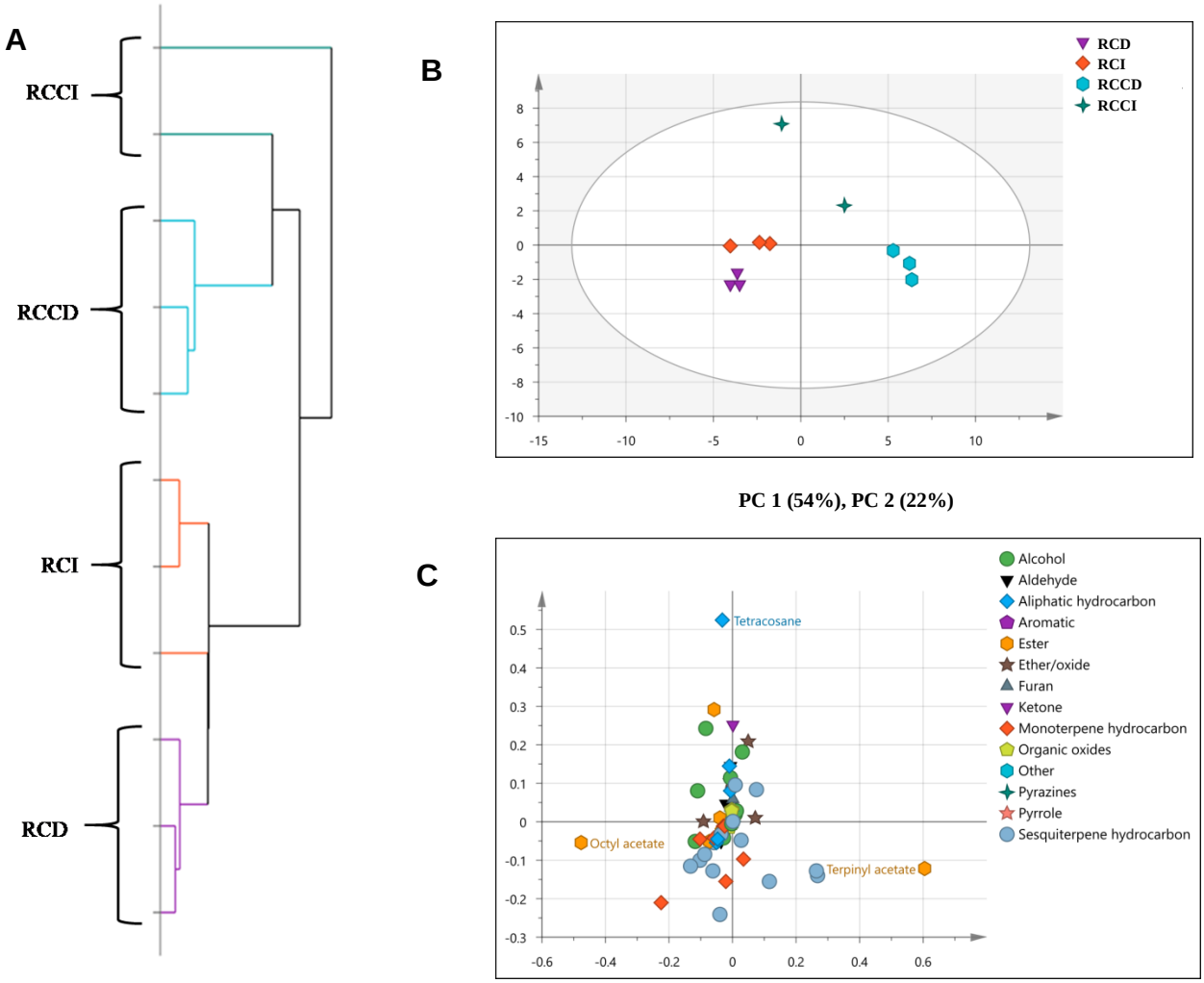
3.2.2.3 PCA analysis of plain coffee versus coffee blended with cardamom

A PCA model was constructed comparing plain roasted coffee versus roasted coffee mixed with cardamom either prepared by decoction or hot infusion; RCI, RCD, RCCI, and RCCD, Fig. 3-6. HCA of coffee brews of roasted coffee samples (Fig. 3-6**A**) demonstrated that RCCI was relatively different and to less extent RCCD than other samples. Score and loading plots of Fig. 3-6 **B** and Fig. 3-6 **C**, respectively revealed that coffee blended with cardamom segregated together in case of infusion and decoction being enriched in terpinyl acetate, while unblended plain roasted coffee (RCI and RCD) prepared by either infusion or decoction were separated on

the other side of the score plot being rich in octyl acetate. These results are in accordance with previous reports revealing for terpinyl acetate as a major constituent of cardamom oil [112].

Figure 3-6 **(A) Hierarchical cluster analysis (HCA)of coffee brews of roasted coffee samples (plain and mixed with cardamom) prepared either by decoction or infusion, (B) principal component analysis (PCA) score plot, and (C) loading plot of the same sample data set.**

(RCD: Roasted *Coffea arabica* decoction; RCI: Roasted *Coffea arabica* infusion; RCCD: Roasted *Coffea arabica* with cardamom decoction; RCCI; Roasted *Coffea arabica* with cardamom infusion) – Codes are identified in Table 2-1



3.2.3 Supervised multivariate data analyses of coffee brew volatiles dataset

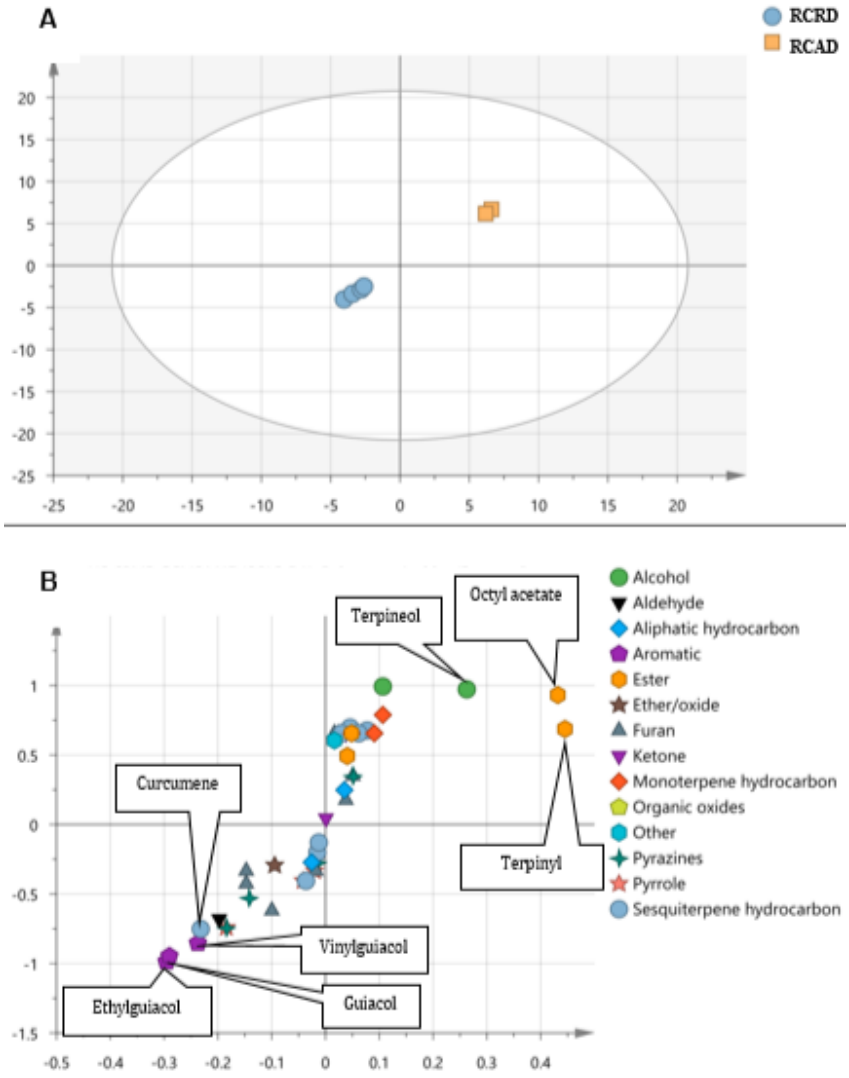
To confirm the hypothesis generated using the unsupervised models, supervised models, *i.e.*, OPLS-DA have been used to further enhance group discrimination and reduce model noise that may be associated with PCA [113]. Supervised models were further applied to verify the

findings regarding the effect of coffee species, roasting and brewing method as earlier discussed in Fig. 3-5.

3.2.3.1 OPLS Analysis of *Coffea arabica* versus *Coffea robusta*

As demonstrated in Fig. 3-7**A**, OPLS-DA score plot showed a clear separation of *Coffea robusta* preparations RCRD from *Coffea arabica* RCAD when modeled together. RCAD samples were found enriched in esters, *i.e.*, octyl and terpinyl acetate, whereas RCRD were found abundant in aromatic hydrocarbons, furans and pyrazines, Fig. 3-7**B**.

Figure 3-7 **(A) Orthogonal projections to latent structures (OPLS) model score plot, and (B) S-plot of decoction brewing of *Coffea arabica* (RCAD) against *Coffea robusta* samples (RCRD)**
(Codes are identified in Table 2-1)



3.2.3.2 OPLS Analysis of instant coffee versus granular coffee brewed dataset

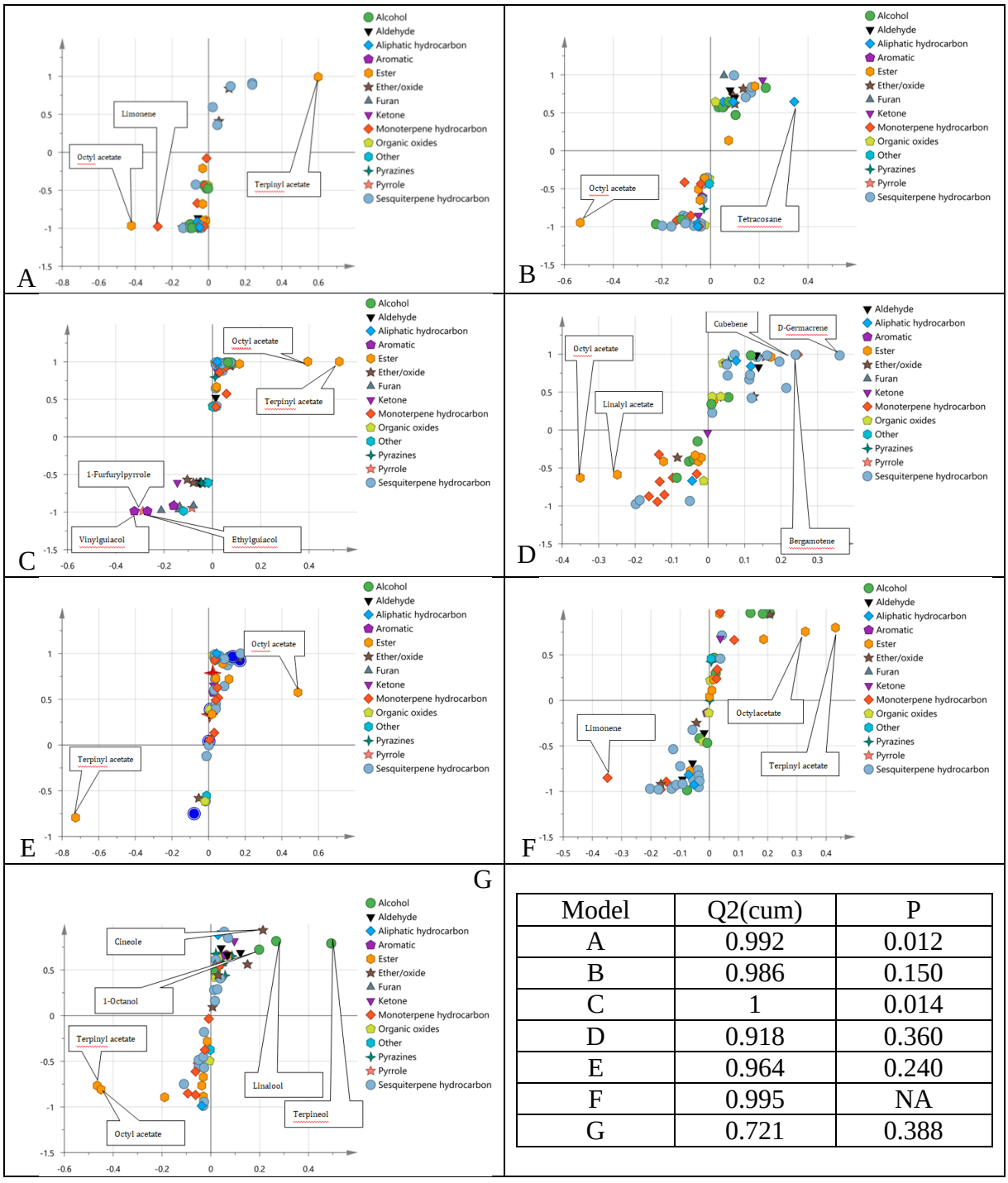
Instant coffee samples (NCI) constituted another type of coffee processing and was found most distant in PCA analysis (Fig. 3-4 **B)**. They were modelled against roasted coffee brewed by infusion using OPLS model. Instant coffee brew showed higher abundance of aromatics, *i.e.*, 4-vinylguaiacol and ethyl guaiacol, in addition to furan compounds, *i.e.*, 1-methylfurfural. In contrast, roasted granular coffee infusion showed higher esters level, *i.e.*, octyl acetate and terpinyl acetate as revealed from S-plot, Fig. 3-8 **C** which imparts better more flowery pleasant flavor of roasted coffee brew compared to smoky flavor of instant coffee.

3.2.3.3 OPLS analysis of samples prepared by decoction versus infusion dataset

Differences between brewing methods accounted for the clear segregation among coffee samples prepared by using decoction, infusion, and maceration. To confirm such differences, decoction, maceration, and infusion methods for roasted coffee samples were modeled one at a time, *i.e.*, RCD vs. RCI, and RCI vs. RCM as depicted in Fig. 3-8 **D** and Fig. 3-8 **G**, respectively. Roasted coffee samples showed relative higher abundance of esters, *i.e.*, terpinyl acetate and octyl acetate in infusion versus limonene enrichment in decoction (Fig. 3-8 **D**). In contrast, OPLS model of maceration brewed roasted coffee showed higher abundance of alcohols, *i.e.*, terpineol and linalool, while infusion samples were richer in esters, *i.e.*, terpinyl and octyl acetate, (Fig. 3-8 **E**). This demonstrates higher fragrance potential of maceration brew compared to infusion counterpart.

Figure 3-8 **OPLS analysis S-Plot of SPME extracted coffee samples**

OPLS model S-Plot of SPME extraction of roasted coffee with cardamom samples (RCCD) to the left against plain roasted coffee extracted by decoction (RCD) to the right(A), Roasted coffee with cardamom (RCCI) to the right samples against plain roasted coffee extracted by infusion (RCI) to the left (B), Roasted coffee (RCI) to the left samples against instant coffee samples (NCI) to the right both extracted by infusion (C), Roasted coffee samples (RCD) to the left against green coffee samples (GCD) to the right both extracted by decoction (D), Roasted coffee (RCI) to the left samples against green coffee samples both (GCI) to the right extracted by infusion (E), Roasted coffee samples extracted by infusion (RCI) to the left and decoction (RCD) to the right (F), Roasted coffee samples extracted by infusion (RCI) to the right and maceration (RCM) to the left (G). Q2(cum) and p value are defined per each model– Codes are identified in Table 2-1

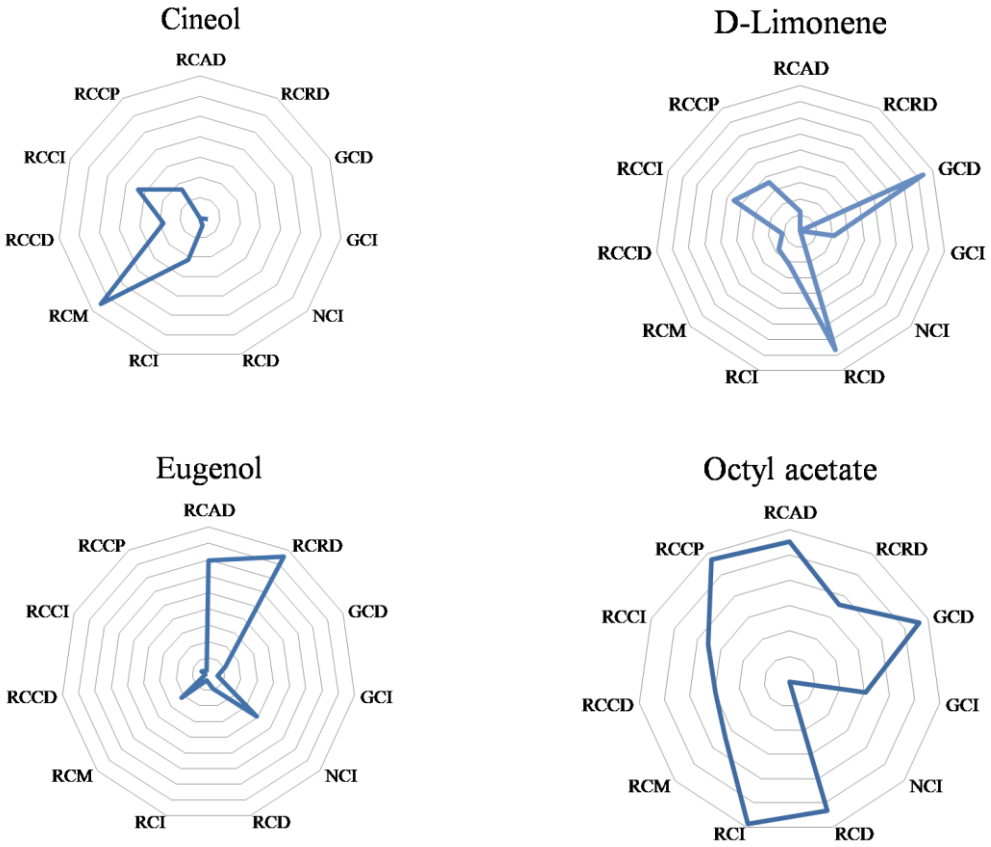


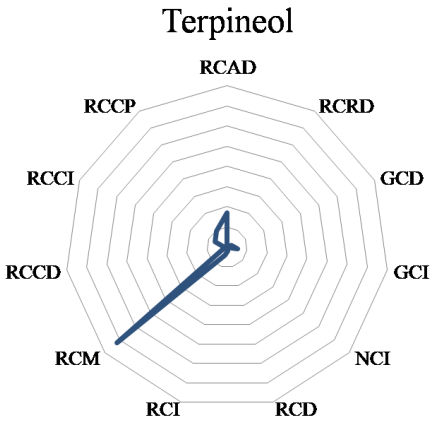
Model	Q2(cum)	P
A	0.992	0.012
B	0.986	0.150
C	1	0.014
D	0.918	0.360
E	0.964	0.240
F	0.995	NA
G	0.721	0.388

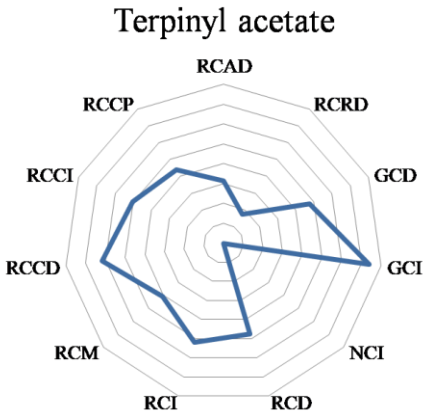
To sum up the potential markers that can be used for the discrimination between investigated coffee brews, a radar plot of coffee volatile chemical markers is depicted in Fig. 3-9, the radar plot traced key VOCs to include cineol, D-limonene, eugenol, octyl acetate, terpineol, and terpinyl acetate. The figure demonstrated that terpineol and cineol were potential markers for RCM, limonene for GCD and RCD, eugenol for RCRD and RCAD, while terpinyl acetate and octyl acetate for several coffee brews.

Figure 3-9 **Radar plot of chief coffee volatile chemical markers, i.e., cineol, D-limonene, eugenol, octyl acetate, terpineol, and terpinyl acetate, in investigated coffee brews.**

Codes are identified in Table 2-1 (RCAD; Authentic *Coffea arabica* roasted decoction, RCRD; Authentic *Coffea canephora* roasted decoction, RCCD; Roasted *Coffea arabica* blended with cardamom decoction, GCD; Green *Coffea arabica* decoction, RCD; Roasted *Coffea arabica* decoction, GCI; Green *Coffea arabica* infusion, RCI; Roasted *Coffea arabica* infusion, NCI; Instant coffee infusion, RCCI; Roasted *Coffea arabica* blended with cardamom infusion, RCM; Roasted *Coffea arabica* maceration)







3.3 Silylated metabolites profiling via GC/MS

Silylation of coffee samples analyzed by GC/MS was performed to monitor higher molecular weight and compound of higher polarity primary and secondary metabolites such as simple sugars, amino acids and fatty acids in coffee samples.

3.3.1 Analysis of non volatile primary and & secondary metabolites by GC/MS post silylation

A total of 144 non-volatile primary and secondary metabolites belonging to 15 different chemical classes were identified in the different extracts of coffee products post-silylation by GC/MS, Table 3-2. Metabolites identified were belonging to volatile metabolites identified in previous section which went silylation or higher molecular weight and polar metabolites not highlighted before in this manuscript. Metabolites were categorized to include sugars (19 compounds), fatty acids (20 compounds), organic acids (25 compounds), alkaloids (2 compounds), sugar alcohols (7 compounds), nitrogenous compounds (17 compounds), alcohols (2 compounds), phenol/phenolic acids (7 compounds), amino acids/peptides (11 compounds), steroids (9 compounds) and terpenoids (8 compounds). These classes represented the major constituents compared to less abundant classes i.e., aromatics (5 compounds), Millard reaction products (7 compounds) and nucleotides (4 compounds) along with phosphoric acid as detailed over the coming subsections. Codes in the coming table and sections are derived from table 2-2 in chapter 2.

Table 3-2 **Relative percentages of silylated primary metabolites in investigated coffee specimens post silylation using GC/MS. Each value represents mean ± S.D**

(Codes identified in this table are following table 2-2)

Peak number	Identified metabolite	RT (min)	GCE	BRA	GCU	GCA	GCC	ICC	ICG	GCK	BRK	HRKC	LRCK	LRCQ	RCA	RCC	GCS	LRCS	LRSQ	LRS
			Total content percentage (Avg.±SD)																	
Alcohols			6.9	4.1	7.6	3.6	1.5	2.4	5.3	4.0	3.4	3.4	4.5	3.9	2.9	4.8	4.9	4.3	4.8	4.4
S1	Glycerol	11.6	5.8 ± 0.3	2.95 ± 0.18	6.24 ± 0.58	0.97 ± 0.07	0.94 ± 1.10	2.09 ± 0.02	4.11 ± 0.32	2.23 ± 0.21	1.31 ± 0.21	1.41 ± 0.04	3 ± 0.10	1.83 ± 0.26	0.69 ± 0.07	2.08 ± 0.01	2.8 ± 0.01	2.94 ± 0.17	3.29 ± 0.03	2.14 ± 0.21
S2	2-Methyl propanetriol	18.6	-	0.01 ± 0.01	-	0.64 ± 0.907	-	-	-	-	-	-	0.04 ± 0.0	0.04 ± 0.02	-	-	-	-	-	-
Alkaloids			14.0	15.0	8.1	11.4	20.8	10.4	6.4	13.3	9.6	10.5	6.7	9.2	10.1	16.0	8.2	19.2	10.2	10.3
S3	Trigonelline	17.5	0.99 ± 0.11	0.19 ± 0.03	1.46 ± 0.03	1.42 ± 0.08	0.51 ± 0.72	0.37 ± 0.14	0.87 ± 0.86	1.97 ± 0.19	0.53 ± 0.18	0.13 ± 0.02	0.55 ± 0.08	0.67 ± 0.44	0.59 ± 0.19	0.3 ± 0.06	1.51 ± 0.18	0.1 ± 0.05	2.17 ± 0.64	2.05 ± 0.36
S4	Caffeine	25.3	12.97 ± 0.2	14.8 ± 0.87	6.66 ± 1.40	10.0± 0.49	20.26 ± 11.5	9.99 ± 0.35	5.54 ± 5.22	11.32 ± 0.86	9.03 ± 1.04	10.38 ± 1.3	6.17 ± 0.67	8.52 ± 1.2	9.48 ± 0.9	15.67 ± 1.13	6.67 ± 0.21	19.06 ± 0.47	8.03 ± 1.74	8.21 ± 0.72
Amino acids/peptides			1.9	1.1	0.9	4.2	1.0	3.9	8.3	2.9	0.9	4.0	1.5	1.2	2.2	2.0	1.6	1.9	1.1	1.4

S5	Alanine	7.4	0.39 ± 0.03	0.12 ± 0.03	0.23 ± 0.06	0.4 ± 0.02	0.18 ± 0.25	0.26 ± 0.01	0.16 ± 0.21	0.45 ± 0.03	-	-	-	-	0.02 ± 0.02	0.02 ± 0.03	0.34 ± 0.08	-	0.18 ± 0.12	0.11 ± 0.16
S6	Leucine	8.7	0.04 ± 0.04	-	-	0.01 ± 0.02	0.03 ± 0.04	0.03 ± 0.03	-	0.02 ± 0.03	-	-	-	-	-	-	-	-	-	-
S7	Valine	10.1	0.11 ± 0	0.01 ± 0.02	0.02 ± 0.02	0.09 ± 0.00	0.03 ± 0.05	0.09 ± 0.00	0.06 ± 0.01	0.08 ± 0.01	-	-	-	-	-	-	0.05 ± 0.00	-	0.02 ± 0.03	0.09 ± 0.06
S8	Serine	11.2	0.21 ± 0.02	-	0.05 ± 0.01	0.49 ± 0.03	0.15 ± 0.21	0.19 ± 0.02	-	0.39 ± 0.05	-	-	-	-	-	-	0.16 ± 0.01	-	0.04 ± 0.05	0.22 ± 0.03
S9	Proline	14.1	0.02 ± 0.02	-	-	0.01 ± 0.02	-	0.02 ± 0.00	0.27 ± 0.00	-	-	0.08 ± 0.02	0.01± 0.00	-	0.05 ± 0.01	0.01 ± 0.01	-	0.04 ± 0.01	-	-0.01
S10	Aspartic acid	15.5	0.18 ± 0.03	-	0.07 ± 0	0.39 ± 0.00	0.12 ± 0.17	0.33 ± 0.04	-	0.32 ± 0.05	-	-	0.01 ± 0.02	0.01 ± 0.02	-	-	0.09 ± 0.13	-	-	0.1 ± 0.15
S11	5-oxo-proline	17.8	0.47 ± 0.03	0.98 ± 0.02	0.4 ± 0.01	0.63 ± 0.04	0.24 ± 0.33	2.69 ± 0.08	6.8 ± 2.19	0.51 ± 0.02	0.93 ± 0.17	3.66 ± 0.44	1.47 ± 0.38	1.18 ± 0.21	1.94 ± 0.02	1.65 ± 0.09	0.74 ± 0.02	1.72 ± 0.01	0.81 ± 0.1	0.84 ± 0.21
S12	Glycyl-glutamic acid	18.2	0.32 ± 0.00	-	0.12 ± 0.023	1.38 ± 0.00	0.17 ± 0.24	0.13 ± 0.04	-	1.04 ± 0.14	-	-	-	-	-	-	0.16 ± 0.04	-	-	0.06 ± 0.08
S13	Phenylalanine	18.4	0.11 ± 0.031	-	-	0.05 ± 0.01	0.03 ± 0.04	0.1 ± 0.05	-	0.05 ± 0.00	-	-	-	-	-	-	-	-	-	-

S14	Glutamic acid	20.2	-	-	-	0.78 ± 0.11	-	0.08 ± 0.00	-	-	-	-	-	-	-	-	-	-	-
S15	Ser-Leu	21.8	-	-	-	-	-	0.03 ± 0.04	1.04 ± 0.03	-	-	0.3 ± 0.01	-	-	0.21 ± 0.04	0.32 ± 0.10	-	0.11 ± 0.02	-	-
Aromatics			0.1	0.2	0.2	0.2	0.1	0.1	0.5	0.2	0.4	0.5	0.2	0.3	0.5	1.4	0.2	0.2	0.1	0.2
S16	4-tert-Butylcatechol	8.6	0.02 ± 0.00	-	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	-	0.02 ± 0.01	0.01 ± 0.01	-	0.01 ± 0.01	0.01 ± 0.00	-	0.01 ± 0.01	0.01 ± 0.01	0.01± 0.00	0.02 ± 0.01	-	0.01 ± 0.01
S17	Hydroquinone	14.9	0.06 ± 0.00	0.08 ± 0.00	0.1 ± 0.00	0.08 ± 0.01	0.03 ± 0.02	-	0.17 ± 0.01	0.08 ± 0.01	0.2 ± 0.01	0.17 ± 0.02	0.06 ± 0.0	0.1 ± 0.03	0.14 ± 0.02	0.7 ± 0.28	0.08 ± 0.01	0.08 ± 0.02	0.03 ± 0.00	0.11 ± 0.04
S18	4-Hydroxyphenylethanol	16.3	0.02 ± 0.01	-	-	-	0.01± 0.00	-	0.25 ± 0.04	0.01 ± 0.01	-	0.15 ± 0.01	-	-	0.06 ± 0.00	0.1 ± 0.01	-	0.04 ± 0.03	-	0.01 ± 0.01
S19	Unknown	17.4	0.05 ± 0.00	0.09 ± 0.02	0.07 ± 0	0.11 ± 0.01	0.06 ± 0.01	0.13 ± 0.02	0.07 ± 0.02	0.08 ± 0.01	0.22 ± 0.02	0.14 ± 0.02	0.1 ± 0.03	0.15 ± 0.0	0.27 ± 0.02	0.56 ± 0.11	0.1 ± 0.01	0.11 ± 0.02	0.04 ± 0.01	0.11 ± 0.02
S20	3-Hydroxyanthranillic acid	20.5	-	-	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.01	-	-	-	0.03 ± 0.00	-	-	0.02 ± 0.00	-	0.08 ± 0.01	0.01 ± 0.01	-	-	0.02 ± 0.01
Fatty acids/glycerides			26.9	26.6	41.1	25.1	12.2	18.8	1.2	33.9	37.1	27.4	20.2	23.4	22.8	20.9	17.4	21.1	15.2	22.2

S21	Nonanoic acid	13.9	0.03 ± 0.01	0.03 ± 0.04	0.03 ± 0.03	-	0.01 ± 0.01	-	-	0.04 ± 0.00	0.01 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.01 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.05 ± 0.01	0.02 ± 0.03	0.01 ± 0.01
S22	Decanoic acid	16.3	-	-	-	-	-	0.05 ± 0.00	-	-	-	-	-	-	-	-	-	-	0.01 ± 0.01	-
S23	Dodecanoic acid	21	0.02 ± 0.00	0.03 ± 0.02	0.02 ± 0.0	0.01 ± 0.01	0.01 ± 0.01	0.12 ± 0.02	-	0.03 ± 0.01	0.06 ± 0.02	0.02 ± 0.0	0.01 ± 0.01	-	-	0.02 ± 0.01	-	0.03 ± 0.01	0.17 ± 0.02	-
S24	Azelaic acid	24.1	-	-	-	-	-	-	-	-	-	-	0.01 ± 0.01	-	0.01 ± 0.01	0.01 ± 0.02	-	-	-	-
S25	cis-9-Hexadecenoic acid	28.6	-	0.05 ± 0.07	0.02 ± 0.03	0.06 ± 0.04	-	-	-	0.02 ± 0.02	0.26 ± 0.03	-	0.06 ± 0.01	0.06 ± 0.09	-	-	0.02 ± 0.00	0.01 ± 0.02	0.15 ± 0.21	-
S26	Methyl-linoleic acid	30	0.21 ± 0.16	0.04 ± 0.06	2.76 ± 0.01	0.48 ± 0.00	0.08 ± 0.03	-	-	0.73 ± 0.11	-	0.12 ± 0.00	0.32 ± 0.18	0.14 ± 0.04	-	-	0.15 ± 0.05	0.65 ± 0.78	0.68 ± 0.22	0.09 ± 0.13
S27	Methyl-9-Octadecenoic acid	30.1	0.03 ± 0.05	-	-	0.03 ± 0.02	-	-	-	0.07 ± 0.02	-	-	0.03 ± 0.03	-	-	-	-	-	0.13 ± 0.05	-
S28	Methyl-stearic acid	30.6	0.04 ± 0.02	0.02 ± 0.03	0.45 ± 0.01	0.04 ± 0.04	-	0.04 ± 0.01	-	0.07 ± 0.03	-	-	0.06 ± 0.02	-	-	-	0.03 ± 0.04	0.12 ± 0.16	0.1 ± 0.01	0.02 ± 0.02

S29	Heptadecanoic acid	30.9	-	-	-	-	-	-	-	-	-	-	0.02 ± 0.03	0.04 ± 0.05	-	0.01 ± 0.01	-	-	-	-
S30	9 - Octadecenoic acid	32.2	5.37 ± 0.56	4.52 ± 0.24	5.07 ± 0.1	3.56 ± 1.0	1.62 ± 0.32	0.07 ± 0.01	0.22 ± 0.12	3.74 ± 0.56	3.87 ± 0.62	3.7 ± 0.46	3.33 ± 0.22	3.7 ± 1.2	3.76 ± 1.28	3.08 ± 0.04	2.18 ± 0.06	3.99 ± 0.15	2.39 ± 0.05	3.87 ± 0.24
S31	Unknown	35.2	0.12 ± 0.00	0.33 ± 0.03	0.06 ± 0.08	0.13 ± 0.01	0.26 ± 0.22	0.02 ± 0.03	-	0.21 ± 0.04	0.29 ± 0.06	0.16 ± 0.04	0.19 ± 0.02	0.15 ± 0.02	0.11 ± 0.16	0.1 ± 0.14	0.18 ± 0.04	0.25 ± 0.01	0.07 ± 0.1	0.12 ± 0.06
S32	cis-11-Eicosenoic acid	35.6	0.12 ± 0.00	0.08 ± 0.02	0.12 ± 0.04	0.13 ± 0.05	0.02 ± 0.03	-	-	0.18 ± 0.04	0.2 ± 0.05	0.14 ± 0.05	0.06 ± 0.01	0.08 ± 0.05	0.12 ± 0.02	0.13 ± 0.01	0.08 ± 0.01	0.06 ± 0.1	-	0.13 ± 0.06
S33	Eicosanoic acid	36	2.26 ± 0.00	2.62 ± 0.07	2.66 ± 0.03	2.27 ± 0.28	1.56 ± 0.57	-	0.15 ± 0.03	3.84 ± 0.48	3.28 ± 0.33	2.2 ± 0.21	1.55 ± 0.03	1.8 ± 0.27	1.99 ± 0.17	2.83 ± 0.07	1.94 ± 0.22	1.75 ± 0.031	0.87 ± 0.18	1.81 ± 0.02
S34	Hexadecanoic acid	38.3	16.33 ± 0.61	16.93 ± 0.88	25.59 ± 0.76	16.58 ± 0.32	7.66 ± 2.86	3.78 ± 0.21	0.85 ± 0.30	22.17 ± 2.62	25.47 ± 0.70	18.45 ± 1.24	12.97 ± 0.08	16.01 ± 2.06	15.99 ± 1.23	12.51 ± 0.56	11.62 ± 0.05	12.2 ± 0.04	10.13 ± 0.9	15.05 ± 2.78
S35	Docosanoic acid	39.1	0.46 ± 0.18	0.44 ± 0.02	0.55 ± 0.23	-	0.41 ± 0.05	-	-	0.19 ± 0.26	1.04 ± 0.08	0.41 ± 0.11	0.21 ± 0.1	0.13 ± 0.18	0.42 ± 0.04	0.88 ± 0.04	0.28 ± 0.01	0.32 ± 0.02	-	0.11 ± 0.16
S36	Nonadecanoic acid	40.4	-	-	0.18 ± 0.12	0.26 ± 0.07	-	-	-	0.71 ± 0.48	0.54 ± 0.0	0.07 ± 0.0	-	-	0.01 ± 0.02	-	0.51 ± 0.15	-	-	0.56 ± 0.04

S37	9-Octadecenoic acid	40.7	1.49 ± 0.13	1.19 ± 0.09	3.01 ± 0.64	0.95 ± 0.16	0.29 ± 0.21	-	-	1.34 ± 0.18	1.34 ± 0.13	1.78 ± 0.08	0.99 ± 0.03	0.92 ± 0.11	-	0.58 ± 0.03	0.04 ± 0.0	0.81 ± 0.005	-	0.05 ± 0.07
S38	Unknown ester	41.1	0.29 ± 0.00	0.26 ± 0.01	0.35 ± 0.02	0.24 ± 0.08	0.06 ± 0.08	2.16 ± 0.18	-	0.25 ± 0.03	0.25 ± 0.05	0.23 ± 0.09	0.21 ± 0.00	0.19 ± 0.03	0.23 ± 0.0	0.2 ± 0.01	0.15 ± 0.00	0.23 ± 0.03	0.49 ± 0.22	0.16 ± 0.08
S39	Tetracosanoic acid	41.9	0.12 ± 0.05	0.08 ± 0.03	0.18 ± 0.0	0.33 ± 0.25	0.18 ± 0.12	-	-	0.31 ± 0.1	0.35 ± 0.01	0.1 ± 0.14	0.12 ± 0.02	0.16 ± 0.01	0.14 ± 0.00	0.27 ± 0.04	0.18 ± 0.07	0.13 ± 0.00	-	0.19 ± 0.04
S40	Unknown	45.9	-	-	-	-	-	12.54 ± 3.26	-	-	-	-	-	-	-	-	-	-	-	-
Inorganic			0.5	0.2	0.2	0.5	0.5	6.1	2.6	0.5	0.4	0.6	0.5	0.8	0.5	0.3	0.7	0.2	0.6	0.8
S41	Phosphoric acid	11.6	0.53 ± 0.08	0.19 ± 0.03	0.2 ± 0.04	0.51 ± 0.07	0.48 ± 0.04	6.09 ± 0.45	2.57 ± 0.07	0.55 ± 0.12	0.39 ± 0.15	0.63 ± 0.07	0.51 ± 0.07	0.8 ± 0.12	0.5 ± 0.0	0.26 ± 0.02	0.69 ± 0.12	0.19 ± 0.08	0.64 ± 0.11	0.76 ± 0.03
Nitrogenous compounds			4.3	11.8	5.1	5.2	1.3	3.8	6.7	7.6	10.7	4.7	5.6	4.0	5.1	6.4	6.6	6.3	5.2	5.7
S42	Unknown	7.1	0.86 ± 0.09	2.28 ± 0.61	1.33 ± 0.52	0.89 ± 0.02	0.22 ± 0.31	0.48 ± 0.01	0.3 ± 0.09	1.63 ± 0.08	1.95 ± 0.52	1.09 ± 0.31	1.29 ± 0.3	1.01 ± 0.61	1.27 ± 0.04	1.62 ± 0.31	1.84 ± 0.95	1.66 ± 0.25	0.93 ± 0.31	0.93 ± 0.33
S43	Sarcosine	7.5	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.02	0.03 ± 0.00	0.01 ± 0.02	0.02 ± 0.00	0.01 ± 0.01	0.04 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.03	0.02 ± 0.01	0.04 ± 0.00	0.03 ± 0.03	0.05 ± 0.00	0.04 ± 0.01	0.03 ± 0.02

S44	Hydroxylamine	7.6	2.69 ± 0.31	8.56 ± 1.2	2.94 ± 0.08	3.57 ± 0.25	0.74 ± 0.86	1.75 ± 0.12	2.87 ± 0.09	5.1 ± 2.39	8.03 ± 3.29	2.42 ± 0.39	3.12 ± 0.28	2.3 ± 0.27	2.64 ± 0.11	4.02 ± 0.04	3.39 ± 0.33	3.48 ± 0.18	2.6 ± 0.91	3.02 ± 1.12
S45	6-methylpyridine	9.8	-	-	-	-	-	0.03 ± 0.0	0.31 ± 0.03	-	-	0.06 ± 0.0	0.01± 0.00	-	0.03 ± 0.01	0.03 ± 0.01	-	0.03 ± 0.01	-	-
S46	Urea	11	-	-	-	0.02 ± 0.02	-	0.05 ± 0.04	0.02 ± 0.00	-	-	-	0.05 ± 0.00	-	-	-	-	0.03 ± 0.05	0.02 ± 0.01	-
S47	Anthranilic acid, linalyl ester	11.1	-	-	-	-	-	0.25 ± 0.06	-	-	-	-	0.12 ± 0.02	-	-	-	-	0.01 ± 0.02	0.15 ± 0.04	-
S48	Nicotinic acid	12.2	0.16 ± 0.11	-	0.44 ± 0.24	0.36 ± 0.28	0.17 ± 0.25	0.26 ± 0.01	0.94 ± 0.1	0.41 ± 0.14	0.15 ± 0.0	0.46 ± 0.01	0.35 ± 0.02	0.31 ± 0.33	0.58 ± 0.113	0.15 ± 0.07	0.53 ± 0.03	0.33 ± 0.02	0.7 ± 0.14	1.17 ± 0.69
S49	Glycine	12.4	0.24 ± 0.0	0.13 ± 0.0	0.02 ± 0.03	0.15 ± 0.06	0.05 ± 0.08	0.16 ± 0.04	-	0.11 ± 0.02	0.12 ± 0.03	-	0.06 ± 0	0.07 ± 0.05	-	0.05 ± 0.03	0.15 ± 0.04	0.04 ± 0.01	0.03 ± 0.02	0.24 ± 0.16
S50	Cadaverine	13.4	0.18 ± 0.0	0.42 ± 0.1	0.24 ± 0.12	0.15 ± 0.0	0.05 ± 0.07	0.1 ± 0.01	0.05 ± 0.02	0.31 ± 0.01	0.29 ± 0.09	0.18 ± 0.01	0.22 ± 0.04	0.14 ± 0.07	0.17 ± 0.0	0.22 ± 0.04	0.38 ± 0.22	0.28 ± 0.03	0.14 ± 0.08	0.15 ± 0.11
S51	Epistephamiersine	15.8	-	-	-	0.01 ± 0.00	0.01 ± 0.00	0.01± 0.00	0.01 ± 0.01	-	-	0.02 ± 0.02	-	0.01 ± 0.01	-	0.03 ± 0.05	-	0.01± 0.00	-	0.02 ± 0.01

S52	2-Methyl-5-phenyl-pyrrole	16.3	-	-	-	-	-	0.01 ± 0.0	-	-	-	-	0.02 ± 0.0	-	-	-	-	-	0	-
S53	Aminobutyric acid	18	1.33 ± 0.09	0.22 ± 0.04	0.39 ± 0.01	0.49 ± 0.04	0.27 ± 0.38	0.35 ± 0.04	-	1.03 ± 0.48	-	-	0.05 ± 0.00	0.03 ± 0.00	-	-	1.11 ± 0.25	-	0.41 ± 0.51	0.88 ± 0.09
S54	2-Piperidinecarboxylic acid	21.4	0.03 ± 0.0	-	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.03	0.03 ± 0.0	-	0.03 ± 0.0	-	-	-	-	-	-	0.25 ± 0.2	-	0.17 ± 0.01	0.06 ± 0.08
S55	1,3,5-Triazine	23.2	0.01 ± 0.02	0.02 ± 0.03	0.05 ± 0.07	0.02 ± 0.03	-	-	-	0.01 ± 0.02	0.08 ± 0.12	0.04 ± 0.06	0.01 ± 0.01	0.03 ± 0.05	0.03 ± 0.037	0.01 ± 0.021	0.01 ± 0.015	0.01 ± 0.019	0.01 ± 0.02	0.04 ± 0.057
S56	Glucosamine	25.7	-	-	-	-	0.04 ± 0.06	0.14 ± 0.00	-	-	-	-	-	-	-	-	-	-	-	-
S57	9-Octadecenamide	34.8	-	-	-	-	-	-	-	-	-	-	-	-	0.12 ± 0.17	-	-	-	-	-
S58	Oleanitrile	36.9	-	0.05 ± 0.07	0.03 ± 0.04	-	-	-	-	-	0.01± 0.00	-	-	-	-	-	-	0.01 ± 0.02	-	-
Nucleotides			0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0

S59	Uracil	13.3	0.01 ± 0.00	-	-	-	0.01 ± 0.01	0.01 ± 0.01	0.06 ± 0.00	-	-	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	-	0.01 ± 0.01	-	-
S60	2'-Deoxyuridine	17.9	-	-	-	-	-	-	-	-	-	-	-	-	0.07 ± 0	-	-	-	-	-
S61	Uridine	32.5	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02 ± 0.03	-	-	-	-
S62	Guanosine	41.0	-	-	-	-	0.02 ± 0.03	-	-	-	-	-	-	-	-	-	-	-	-	-
Organic acids			7.1	8.3	13.4	11.9	4.0	9.1	23.2	11.1	15.4	20.9	8.5	9.0	19.3	12.8	10.5	11.5	8.7	8.6
S63	Lactic acid	6.5	0.43 ± 0.06	0.64 ± 0.03	0.42 ± 0.21	0.84 ± 0.43	0.28 ± 0.31	2.95 ± 0.0	4.2 ± 0.02	0.73 ± 0.02	0.54 ± 0.03	2.62 ± 0.19	0.93 ± 0.05	0.49 ± 0.03	2.3 ± 0.12	1.9 ± 0.05	0.59 ± 0.03	1.75 ± 0.08	0.51 ± 0.05	0.42 ± 0.06
S64	Acetic acid	6.8	0.02 ± 0.01	0.1 ± 0.08	0.07 ± 0.03	0.13 ± 0.07	0.07 ± 0.1	1.31 ± 0.07	6.95 ± 0.02	0.02 ± 0.01	0.26 ± 0.04	4.24 ± 0.38	0.44 ± 0.14	0.34 ± 0.03	4.25 ± 0.06	2.2 ± 0.06	0.19 ± 0.06	1.87 ± 0.08	0.18 ± 0.02	0.21 ± 0.01
S65	Hexanoic acid	6.8	0.01 ± 0.01	-	0.01 ± 0.02	0.01 ± 0.01	-	-	-	-	0.02 ± 0.00	-	0.02 ± 0.01	-	-	-	0.01 ± 0.00	-	0.01 ± 0.01	-
S66	2-Propenoic acid	7.1	-	-	0.01 ± 0.01	0.02 ± 0.01	-	-	0.05 ± 0.02	0.01± 0.00	0.01 ± 0.0	0.02 ± 0.01	-	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	-	0.01 ± 0.00

S67	3-Hydroxybutyric acid	8.8	-	-	-	-	-	-	0.01 ± 0.01	-	-	-	0.03 ± 0.01	-	-	-	-	-	0.01 ± 0.0	0.01 ± 0.01
S68	2,4-Hexadienoic acid	10.2	-	-	-	0.01 ± 0.00	-	-	-	-	0.01 ± 0.00	-	-	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.01	-	-	-	0.01 ± 0.00
S69	4-Hydroxybutyric acid	10.7	-	-	0.01 ± 0.02	-	0.02 ± 0.03	0.04 ± 0.01	0.29 ± 0.01	-	-	0.09 ± 0.02	0.02 ± 0.01	-	0.05 ± 0.02	0.03 ± 0.01	0.01 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.01 ± 0.01
S70	2-Butenedioic acid	12.4	-	-	-	0.1 ± 0.06	-	-	0.26 ± 0.05	-	-	0.12 ± 0.04	0.01 ± 0.02	0.05 ± 0.07	0.14 ± 0.02	0.05 ± 0.07	0.04 ± 0.06	-	-	0.09 ± 0.12
S71	Succinic acid	12.7	0.32 ± 0.05	0.26 ± 0.02	0.37 ± 0.17	0.15 ± 0.02	0.36 ± 0.5	1.19 ± 0.04	1.53 ± 0.12	0.22 ± 0.07	0.13 ± 0.07	0.55 ± 0.03	1.34 ± 0.08	1.05 ± 0.25	0.37 ± 0.05	0.54 ± 0.04	0.46 ± 0.01	0.51 ± 0.01	0.72 ± 0.08	0.47 ± 0.12
S72	Methyl-butanedioic acid	13.0	-	-	-	-	-	0.01 ± 0.01	0.62 ± 0.05	-	-	0.09 ± 0.00	0.01 ± 0.00	-	0.07 ± 0.01	0.03 ± 0.01	-	0.02 ± 0.00	0.01± 0.00	-
S73	Methylmaleic acid	13.6	0.01 ± 0.01	0.02 ± 0.01	-	0.03 ± 0.01	-	0.04 ± 0.01	1.82 ± 0.19	0.01 ± 0.01	0.02 ± 0.00	0.66 ± 0.02	0.04 ± 0.04	0.02 ± 0.00	0.44 ± 0.00	0.36 ± 0.02	0.02 ± 0.00	0.16 ± 0.01	-	0.01 ± 0.01
S74	Malic acid	14.8	1.11 ± 0.12	1.04 ± 0.03	3.29 ± 3.6	0.85 ± 0.08	1.24 ± 1.76	0.96 ± 0.02	0.94 ± 0.08	0.89 ± 0.09	6.47 ± 1.12	5.53 ± 0.29	0.88 ± 0.06	0.96 ± 0.11	4.56 ± 5.73	1.94 ± 0.08	1.53 ± 0.14	3.35 ± 0.71	1.5 ± 0.2	1.31 ± 0.49

S75	Pentanedioic acid	15.0	-	-	-	-	-	0.01± 0.00	0.19 ± 0.03	-	-	0.04 ± 0.00	0.01± 0.00	-	0.03 ± 0.01	0.01 ± 0.00	0.01± 0.00	-	0.01 ± 0.00	0.01± 0.00
S76	Butanoic acid	15.2	-	-	-	-	-	0.13 ± 0.01	1.3 ± 0.09	-	0.02 ± 0.03	0.73 ± 0.03	0.08 ± 0.04	0.04 ± 0.0	0.82 ± 0.03	0.44 ± 0.02	0.02 ± 0.02	0.47 ± 0.0	-	0.01 ± 0.02
S77	Dihydroxybutanoic acid	15.7	0.04 ± 0.01	0.04 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.04	0.79 ± 0.11	1.08 ± 0.07	0.01 ± 0.02	0.01 ± 0.0	0.08 ± 0.03	0.24 ± 0.2	0.08 ± 0.01	0.65 ± 0.1	0.42 ± 0.03	0.08 ± 0.00	0.16 ± 0.03	0.12 ± 0.03	0.08 ± 0.02
S78	3-Methyl-Pentanoic acid	16.5	-	-	-	-	-	-	0.16 ± 0.01	-	-	0.13 ± 0.0	-	-	0.06 ± 0.03	-	-	0.09 ± 0.02	-	-
S79	2-deoxy-Pentonic acid-lactone	17.5	-	-	-	-	-	0.02 ± 0.03	-	-	-	-	0.11 ± 0.02	0.04 ± 0.05	0.18 ± 0.02	-	-	0.01 ± 0.02	-	-
S80	Valeric acid	17.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03 ± 0.04	-	-
S81	2-Hydroxyglutaric acid	18.1	0.05 ± 0.01	-	0.03 ± 0.04	0.03 ± 0.03	-	0.1 ± 0.00	0.31 ± 0.03	0.04 ± 0.03	-	0.09 ± 0.00	0.06 ± 0.01	0.04 ± 0.01	0.09 ± 0.00	0.08 ± 0.00	0.04 ± 0.01	0.07 ± 0.03	0.04 ± 0.03	0.01 ± 0.00
S82	Trihydroxybutyric acid	18.8	-	-	-	-	-	0.12 ± 0.01	-	-	-	0.03 ± 0.01	0.02 ± 0.03	-	-	0.04 ± 0.03	-	0.02 ± 0.01	0.04 ± 0.06	-

S83	3-Methylvaleric acid	19.3	-	-	-	-	-	-	0.35 ± 0.05	-	-	0.09 ± 0.0	0.01 ± 0.01	-	0.07 ± 0.01	0.06 ± 0.02	-	0.04 ± 0.01	-	-
S84	3-deoxy-Pentonic acid	20.9	0.03 ± 0.01	-	0.02 ± 0.01	0.04 ± 0.03	0.02 ± 0.04	0.37 ± 0.04	1.33 ± 0.0	0.02 ± 0.01	0.05 ± 0.02	2.15 ± 0.09	0.13 ± 0.08	0.06 ± 0.01	0.9 ± 0.11	1.37 ± 0.19	0.06 ± 0.01	0.91 ± 0.18	0.03 ± 0.00	0.06 ± 0.03
S85	Trihydroxypentanoic acid	21.1	0.04 ± 0.06	-	-	-	0.06 ± 0.08	0.12 ± 0.02	-	-	0.17 ± 0.05	0.13 ± 0.0	0.56 ± 0.03	0.39 ± 0.01	0.24 ± 0.02	-	0.11 ± 0.02	0.2 ± 0.02	0.44 ± 0.00	0.18 ± 0.04
S86	Citric acid	24.6	3.06 ± 1.89	4.13 ± 1.79	7.99 ± 2.3	8.25 ± 2.05	1.33 ± 1.87	0.38 ± 0.03	1.83 ± 0.21	7.07 ± 0.85	6.02 ± 0.58	3.13 ± 1.21	3.09 ± 0.15	4.98 ± 1.37	3.79 ± 0.10	2.53 ± 0.15	5.69 ± 1.58	1.22 ± 0.59	4.35 ± 0.77	4.08 ± 0.2
S87	β-D-Glucopyranuronic acid	34.9	0.19 ± 0.01	0.18 ± 0.01	0.21 ± 0.02	0.05 ± 0.01	0.25 ± 0.36	0.15 ± 0.01	-	0.19 ± 0.04	0.12 ± 0.0	0.11 ± 0.01	0.42 ± 0.02	0.11 ± 0.0	0.16 ± 0.08	0.32 ± 0.01	0.12 ± 0.05	0.24 ± 0.01	0.27 ± 0.10	0.15 ± 0.1
Phenolic			3.7	3.7	2.7	2.4	1.4	2.7	2.0	2.8	3.9	1.8	1.8	2.3	1.9	3.2	2.5	2.4	2.3	2.5
S88	Phenol	6.4	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.01	-	-	0.03 ± 0.00	0.08 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.23 ± 0.05	0.03 ± 0.00	0.04 ± 0.00	-	0.04 ± 0.02
S89	Hydrocaffeic acid	27.1	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	-	0.06 ± 0.00	0.27 ± 0.03	0.03 ± 0.0	0.05 ± 0.01	0.11 ± 0.01	0.02 ± 0.0	0.03 ± 0.0	0.06 ± 0.01	0.09 ± 0.01	0.01 ± 0.02	0.05 ± 0.02	0.01± 0.00	0.02 ± 0.0
S90	Methyl-cinnamic acid	28.3	0.01 ± 0.0	0.06 ± 0.01	0.77 ± 0.24	0.04 ± 0.01	0.03 ± 0.01	-	-	0.13 ± 0.11	0.03 ± 0.01	0.04 ± 0.03	0.05 ± 0.05	0.03 ± 0.01	0.04 ± 0.0	0.15 ± 0.03	0.02 ± 0.02	0.2 ± 0.11	0.4 ± 0.33	0.02 ± 0.02

S91	3,4-Dimethoxy-cinnamic acid	28.7	-	-	-	-	-	-	0.04 ± 0.05	-	-	0.04 ± 0.03	-	-	0.01 ± 0.00	0.02 ± 0.00	-	0.01 ± 0.01	-	-
S92	Ferulic acid	29.9	0.57 ± 0.02	0.5 ± 0.07	0.19 ± 0.01	0.2 ± 0.02	0.09 ± 0.05	0.31 ± 0.02	0.2 ± 0.03	0.28 ± 0.01	0.37 ± 0.01	0.17 ± 0.04	0.2 ± 0.01	0.22 ± 0.03	0.2 ± 0.01	0.87 ± 0.06	0.3 ± 0.08	0.31 ± 0.01	0.15 ± 0.02	0.23 ± 0.06
S93	Caffeic acid	30.8	3.03 ± 0.1	3.12 ± 0.28	1.73 ± 0.077	1.98 ± 0.11	1.13 ± 1.22	1.73 ± 0.13	1.34 ± 0.15	2.25 ± 0.25	3.37 ± 0.02	1.38 ± 0.2	1.53 ± 0.08	1.94 ± 0.28	1.51 ± 0.1	1.88 ± 0.04	2.1 ± 0.25	1.78 ± 0.28	1.68 ± 0.1	2.1 ± 0.61
S94	Catechin	43.2	0.08 ± 0.00	-	0.01 ± 0.01	0.12 ± 0.02	0.1 ± 0.14	0.6 ± 0.06	0.12 ± 0.02	0.05 ± 0.03	-	-	0.01 ± 0.02	0.06 ± 0.01	-	-	0.07 ± 0.00	-	0.03 ± 0.00	0.08 ± 0.00
Heat-induced roasting by-products			0.1	0.3	0.2	0.1	0.0	0.4	1.7	0.3	0.2	0.7	0.2	0.2	1.0	0.5	0.1	0.6	0.3	0.1
S95	4-Hydroxypyridine	6	0.13± 0.00	0.24 ± 0.02	0.23 ± 0.04	0.15 ± 0.01	0.03 ± 0.05	0.08 ± 0.00	0.3 ± 0.02	0.31 ± 0.13	0.21 ± 0.02	0.24 ± 0.01	0.19 ± 0.02	0.15 ± 0.03	0.22 ± 0.01	0.24 ± 0.06	0.13 ± 0.01	0.28 ± 0.02	0.25 ± 0.13	0.11 ± 0.00
S96	2-Methylpyran-4-one	12	-	-	-	-	-	0.02 ± 0.0	0.16 ± 0.02	-	-	0.07 ± 0	0.01	0.01 ± 0.01	0.04 ± 0.01	-	-	0.05 ± 0.01	-	-
S97	2-Furanacetic acid	15.2	-	-	-	-	-	0.08 ± 0.0	-	-	-	-	-	-	0.05 ± 0.08	-	-	-	-	-

S98	Dihydro-Furanone	15.5	-	-	-	-	-	-	-	-	-	-	-	-	0.01 ± 0.02	-	-	-	-	-
S99	2,5-dimethyl-3,6-bis(3-methylbutyl)-pyrazine	15.8	-	-	-	-	-	-	-	-	-	-	0.003	-	0.03 ± 0.01	-	-	-	-	0.01 ± 0.01
S100	4H-Pyran-4-one	16.8	-	0.01 ± 0.00	-	-	-	0.02 ± 0.01	0.03 ± 0.01	-	0.01± 0.00	0.01 ± 0.00	-	0.02 ± 0.01	0.1 ± 0.01	0.01± 0.00	0.01 ± 0.00	-	-	0.02 ± 0.00
S101	2-Furancarboxylic acid	18.6	-	-	-	-	-	0.23 ± 0.01	1.22 ± 0.13	-	-	0.42 ± 0.04	0.03 ± 0.02	-	0.53 ± 0.04	0.28 ± 0.05	-	0.28 ± 0.0	0.01± 0.00	0.01± 0.00
Steroids			1.2	0.9	2.1	3.0	1.7	0.0	0.1	3.8	2.8	2.9	2.1	2.1	3.0	1.7	2.7	2.3	1.4	2.7
S102	Unknown steroid	41.0	0.01 ± 0.01	-	0.21 ± 0.1	0.28 ± 0.03	-	-	-	0.68 ± 0.25	0.51 ± 0.12	0.1 ± 0.08	-	-	0.03 ± 0.04	-	0.39 ± 0.13	0.06 ± 0.04	0.1 ± 0.07	0.66 ± 0.03
S103	Pregnane-diol-11-one	40.6	-	-	-	-	-	-	0.05 ± 0.01	-	-	0.14 ± 0.01	-	-	0.07 ± 0.04	-	0.07 ± 0.02	-	-	0.03 ± 0.04
S104	Androst-4-en-3-one	36.5	-	-	-	-	-	-	-	-	-	-	0.12 ± 0.01	-	-	-	-	-	-	-

S105	Campesterol	47.3	0.13 ± 0.1	0.05 ± 0.07	0.16 ± 0.11	0.37 ± 0.19	0.18 ± 0.02	-	-	0.41 ± 0.23	0.39 ± 0.12	0.26 ± 0.04	0.24 ± 0.03	0.1 ± 0.02	0.4 ± 0.07	0.09 ± 0.04	0.28 ± 0.11	0.26 ± 0.0	-	0.26 ± 0.15
S106	Pregn-en-20-one	43.4	-	-	-	-	-	-	-	-	-	0.17 ± 0.03	-	-	-	0.16 ± 0.07	-	0.03 ± 0.04	-	-
S107	Pregnan-11-one	41.9	-	-	0.14 ± 0.01	0.13 ± 0.03	-	-	-	-	-	0.12 ± 0.02	0.03 ± 0.01	0.05 ± 0.08	0.26 ± 0.15	-	-	-	-	-
S108	β-Sitosterol	48.6	0.48 ± 0.02	0.87 ± 0.16	0.99 ± 0.08	1.2 ± 0.10	0.63 ± 0.22	-	-	1.42 ± 0.35	1.17 ± 0.21	1.35 ± 0.19	1.14 ± 0.05	1.23 ± 0.36	1.37 ± 0.1	0.47 ± 0.14	1.21 ± 0.1	1.05 ± 0.07	1.31 ± 0.16	1.18 ± 0.1
S109	Squalene	41.6	0.01 ± 0.01	0.01± 0.00	0.02 ± 0.04	0.01 ± 0.02	0.14 ± 0.09	-	-	0.01 ± 0.01	0.05 ± 0.02	-	0.02 ± 0.00	-	0.01 ± 0.02	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	-	-
S110	Stigmasterol	47.7	0.54 ± 0.1	-	0.56 ± 0.09	1.01 ± 0.14	0.72 ± 0.28	-	-	1.23 ± 0.1	0.63 ± 0.22	0.81 ± 0.07	0.56 ± 0.00	0.76 ± 0.00	0.88 ± 0.19	0.92 ± 0.03	0.76 ± 0.06	0.87 ± 0.05	-	0.59 ± 0.21
Sugars			20.2	11.7	10.9	17.6	42.2	24.4	26.5	11.3	9.5	14.8	40.1	34.3	24.1	20.9	31.9	18.5	34.3	30.3
S111	L-Threonic acid	18.4	0.2 ± 0.01	0.21 ± 0.05	0.16 ± 0.07	0.27 ± 0.15	0.16 ± 0.23	1.25 ± 0.1	5.02 ± 0.24	0.09 ± 0.04	0.14 ± 0.06	0.11 ± 0.01	0.51 ± 0.19	0.29 ± 0.12	0.53 ± 0.01	0.17 ± 0.02	0.47 ± 0.01	0.31 ± 0.02	0.58 ± 0.25	0.45 ± 0.04
S112	Stephasunoline	19.9	-	-	-	-	-	-	-	-	-	0.04 ± 0.00	-	-	0.04 ± 0.01	-	-	0.01 ± 0.00	-	-

S113	Arabinonic acid-lactone	20.5	-	-	-	-	-	0.09 ± 0.01	0.16 ± 0.04	-	-	-	0.06 ± 0.01	0.07 ± 0.03	0.18 ± 0.0	-	0.04 ± 0.02	-	0.06 ± 0.08	0.03 ± 0.05
S114	Arabinose	20.8	-	-	-	-	-	0.03 ± 0.01	0.04 ± 0.01	-	-	0.02 ± 0.02	0.02 ± 0.03	-	0.1 ± 0.01	0.02 ± 0.04	-	0.01 ± 0.01	-	-
S115	Xylulose	21.8	0.03 ± 0.01	0.14 ± 0.04	0.09 ± 0.05	0.15 ± 0.02	0.01 ± 0.01	-	-	0.11 ± 0.03	0.13 ± 0.03	-	0.02 ± 0.03	0.04 ± 0.02	-	-	0.05 ± 0.01	-	0.11 ± 0.01	0.07 ± 0.02
S116	Levoglucosan	22	0.08 ± 0.01	0.11 ± 0.01	0.16 ± 0.07	0.11 ± 0.01	0.2 ± 0.18	1.38 ± 0.13	0.17 ± 0.23	0.17 ± 0.00	0.16 ± 0.01	0.36 ± 0.02	0.12 ± 0.01	0.12 ± 0.04	0.37 ± 0.02	0.16 ± 0.01	0.1 ± 0.03	0.22 ± 0.02	-	0.04 ± 0.06
S117	2-Keto-L-gluconic acid	23.7	0.06 ± 0.03	-	0.33 ± 0.13	0.06 ± 0.01	0.06 ± 0.08	-	-	0.04 ± 0.00	-	-	0.33 ± 0.24	0.12 ± 0.01	-	-	0.12 ± 0.01	-	0.97 ± 0.55	0.08 ± 0.01
S118	3-deoxy-Ribo-dexonic acid-lactone	24	-	0.11 ± 0.01	-	-	-	0.38 ± 0.36	6.9 ± 2.76	-	0.63 ± 0.02	2.24 ± 0.0	0.22 ± 0.01	0.28 ± 0.08	3.38 ± 0.16	1.47 ± 0.06	0.13 ± 0.08	1.31 ± 0.5	-	0.09 ± 0.12
S119	Fructofuranose	24.4	0.76 ± 0.23	0.02 ± 0.00	0.05 ± 0.00	0.77 ± 0.17	3.2 ± 4.43	7.14 ± 0.07	0.86 ± 0.11	0.01 ± 0.01	0.17 ± 0.02	-	2.9 ± 1.29	6.41 ± 1.04	0.66 ± 0.22	0.15 ± 0.21	1.84 ± 0.37	0.29 ± 0.02	2.63 ± 2.18	3.27 ± 1.17
S120	Xylopyranose	25	-	-	-	-	-	-	-	-	-	-	-	-	0.08 ± 0.04	0.08 ± 0.12	-	0.11 ± 0.03	-	-

s121	3-deoxy-Arabino-Hexonic acid	25.5	-	-	-	-	-	0.39 ± 0.55	2.1 ± 2.97	-	0.75 ± 0.53	7.77 ± 1.3	0.89 ± 0.26	0.73 ± 0.25	8.86 ± 2.48	4.37 ± 0.35	0.14 ± 0.2	4.54 ± 0.28	0.23 ± 0.06	0.11 ± 0.16
S122	Psicopyranose	25.8	-	-	-	-	0.89 ± 1.27	0.09 ± 0.13	0.88 ± 0.4	-	-	-	0.06 ± 0.00	0.09 ± 0.08	-	-	0.04 ± 0.03	-	0.01 ± 0.01	0.01 ± 0.02
S123	Psicose	26	0.07 ± 0.02	-	-	0.03 ± 0.01	-	-	-	-	-	-	-	-	-	-	0.17 ± 0.02	-	0.05 ± 0.07	0.16 ± 0.07
S124	Fructose	27	10.71 ± 0.03	0.82 ± 0.24	3.15 ± 0.17	4.66 ± 0.92	16.78 ± 18.2	6.03 ± 0.96	-	2.86 ± 0.25	3.61 ± 0.97	-	29.56 ± 1.1	20.59 ± 2.56	2.27 ± 0.6	0.69 ± 0.67	15.14 ± 0.88	5.98 ± 0.75	17.44 ± 3.41	12.27 ± 1.91
S125	Gluconic acid	27.5	-	0.04 ± 0.0	0.05 ± 0.02	-	-	-	-	-	0.02 ± 0.03	-	0.04 ± 0.04	0.17 ± 0.05	-	-	0.03 ± 0.02	0.01 ± 0.01	-	0.05 ± 0.03
S126	Methyl 6-deoxy-galactopyranoside	27.7	-	-	-	-	-	0.12 ± 0.01	0.16 ± 0.02	-	-	0.07 ± 0.03	-	-	0.05 ± 0.06	0.16 ± 0.03	-	0.33 ± 0.01	-	-
S127	Inositol	28.1	8.25 ± 0.7	10.07 ± 0.11	6.93 ± 0.73	11.53 ± 0.40	20.79 ± 12.71	6.53 ± 0.1	10.18 ± 0.08	8.02 ± 0.77	3.83 ± 0.48	4.11 ± 0.04	5.35 ± 0.03	5.38 ± 0.00	7.24 ± 0.17	13.52 ± 1.31	13.67 ± 0.23	5.3 ± 0.04	12.1 ± 0.1	13.72 ± 0.86
S128	Galactonic acid-lactone	35.2	-	0.09 ± 0.06	-	-	-	-	-	-	-	0.04 ± 0.06	-	-	0.11 ± 0.00	0.06 ± 0.04	-	0.04 ± 0.02	-	-

S129	L-Threonic acid	18.4	0.2 ± 0.01	0.21 ± 0.05	0.16 ± 0.07	0.27 ± 0.15	0.16 ± 0.23	1.25 ± 0.1	5.02 ± 0.24	0.09 ± 0.04	0.14 ± 0.06	0.11 ± 0.01	0.51 ± 0.19	0.29 ± 0.12	0.53 ± 0.01	0.17 ± 0.02	0.47 ± 0.01	0.31 ± 0.02	0.58 ± 0.25	0.45 ± 0.04
Sugar alcohols			13.0	15.9	6.8	13.8	13.2	12.5	15.5	7.6	4.8	6.5	6.8	9.0	6.1	8.8	11.9	11.2	11.4	10.1
S130	L-Threitol	17.6	0.21 ± 0.13	0.06 ± 0.02	0.04 ± 0.01	0.18 ± 0.06	0.07 ± 0.09	0.4 ± 0.02	0.14 ± 0.02	0.06 ± 0.02	0.02 ± 0.03	0.02 ± 0.00	0.11 ± 0.01	0.06 ± 0.01	0.05 ± 0.03	0.03 ± 0.01	0.1 ± 0.00	0.02 ± 0.00	0.35 ± 0.1	0.11 ± 0.03
S131	Ribitol	22.4	0.28 ± 0.01	0.19 ± 0.03	0.36 ± 0.29	0.12 ± 0.05	0.15 ± 0.21	0.62 ± 0.07	0.43 ± 0.06	0.04 ± 0.01	0.01 ± 0.02	-	0.16 ± 0.0	0.1 ± 0.01	-	0.15 ± 0.04	0.25 ± 0.01	0.12 ± 0.06	0.17 ± 0.01	0.26 ± 0.04
S132	Xylitol	22.4	0.28 ± 0.01	0.19 ± 0.03	0.36 ± 0.29	0.12 ± 0.05	0.15 ± 0.21	0.62 ± 0.07	0.43 ± 0.06	0.03 ± 0.01	0.01 ± 0.02	0.07 ± 0.01	0.16 ± 0.0	0.1 ± 0.01	0.06 ± 0.02	0.15 ± 0.04	0.25 ± 0.01	0.12 ± 0.06	0.17 ± 0.01	0.26 ± 0.04
S133	Sorbitol	26.8	0.36 ± 0.03	0.31 ± 0.05	0.14 ± 0.0	7.77 ± 2.87	1.35 ± 0.86	1.22 ± 0.24	0.9 ± 1.27	-	-	0.19 ± 0.08	-	0.68 ± 0.12	1.27 ± 1.8	-	1.84 ± 2.3	0.22 ± 0.17	1.04 ± 1.1	0.28 ± 0.1
S134	Myoinositol	29.8	11.77 ± 0.02	15.05 ± 0.1	5.72 ± 0.3	5.37 ± 0.04	11.26 ± 7.44	9.6 ± 0.11	13.62 ± 0.11	7.12 ± 0.17	4.51 ± 0.93	6.08 ± 0.16	6.02 ± 0.15	7.76 ± 0.49	4.55 ± 0.32	8.41 ± 0.84	9.03 ± 0.10	10.66 ± 0.47	8.93 ± 0.26	8.8 ± 0.62
S135	Myo-Inositol phosphate	35.4	0.04 ± 0.04	0.1 ± 0.02	0.11 ± 0.09	0.17 ± 0.03	0.07 ± 0.1	-	-	0.33 ± 0.06	0.27 ± 0.03	0.11 ± 0.04	0.25 ± 0.05	0.21 ± 0.01	0.14 ± 0.07	0.08 ± 0.06	0.24 ± 0.03	0.01 ± 0.01	0.53 ± 0.26	0.14 ± 0.04
S136	Maltitol	42.2	0.1 ± 0.02	0.02 ± 0.01	0.06 ± 0.03	0.1 ± 0.05	0.16 ± 0.1	-	-	0.07 ± 0.02	-	-	0.09 ± 0.03	0.07 ± 0.01	-	-	0.17 ± 0.08	-	0.2 ± 0.07	0.2 ± 0.02
Terpenes			0.1	0.1	0.7	0.7	0.2	5.4	0.0	0.7	1.0	1.2	1.2	0.3	0.5	0.2	0.7	0.5	4.6	0.7

S137	Cineol	6.2	-	-	0.01 ± 0.00	-	-	2.93 ± 0.81	-	-	-	-	0.04 ± 0.01	0.01 ± 0.01	-	-	-	0.01± 0.00	0.07 ± 0.02	-
S138	Linalool	10.6	-	-	0.01 ± 0.01	-	-	1.07 ± 0.11	-	-	-	-	0.21 ± 0.0	0.03 ± 0.04	-	-	-	0.02 ± 0.03	0.2 ± 0.05	-
S139	1-methyl-1-(4-methyl-3-cyclohexen-1-yl)ethoxy]-	12.9	-	-	0.03 ± 0.01	-	-	0.55 ± 0.03	-	0.03 ± 0.01	-	0.02 ± 0.02	0.29 ± 0.03	0.04 ± 0.05	-	-	0.01 ± 0.02	0.04 ± 0.01	0.19 ± 0.00	-
S140	Geraniol	13.9	-	-	-	-	-	0.37 ± 0.01	-	-	-	-	0.06 ± 0.02	-	-	-	-	-	0.07 ± 0.09	-
S141	4-Allyl-2-methoxyphenol	16.6	-	-	-	-	-	-	-	-	-	0.12 ± 0.01	0.07 ± 0.02	-	-	-	0.07 ± 0.02	0.15 ± 0.05	3.3 ± 0.35	-
S142	Isoeugenol	19.6	-	-	-	-	0.01± 0.00	0.01 ± 0.01	-	-	-	-	-	-	-	-	-	0.01 ± 0.01	-	-
S143	3,7,11-Trimethyl-2,6,10-dodecatriene	21.5	-	-	-	-	-	0.37 ± 0.00	-	-	-	-	0.23 ± 0.00	-	-	-	-	0.03 ± 0.02	0.14 ± 0.01	-
S144	Tocopherol	43.9	0.05 ± 0.00	0.11 ± 0.00	0.62 ± 0.08	0.7 ± 0.22	0.15 ± 0.02	-	-	0.64 ± 0.19	0.95 ± 0.18	1.02 ± 0.12	0.25 ± 0.12	0.19 ± 0.02	0.46 ± 0.03	0.15 ± 0.02	0.65 ± 0.07	0.23 ± 0.12	0.61 ± 0.08	0.74 ± 0.08

3.3.1.1 Sugars

Sugars constituted the most abundant class in coffee samples at *ca*. 23.9% (9.5-42.2%) of the total metabolite abundances and represented by 19 compounds, with highest levels in authentic green *Coffea robusta* (GCC) followed by lightly roasted *Coffea arabica* blended with cardamom (LRCK) at 42.2% and 40.1%, respectively. The lowest levels were in lab-roasted *Coffea arabica* from Kuwait (BRK) and green *Coffea arabica* from United Arab of Emirates (GCU) at *ca*. 10%, respectively. These observations indicated that in general sugar level in green *Coffea robusta* was more abundant than *Coffea arabica* in authentic samples, though with roasting under same conditions opposite pattern was observed. Generally, roasting decreases the sugar content in most of the samples consistent with previous reports likely due to their cleavage to other furan derivatives contributing to the coffee seeds browning and characteristic odor [4]. Coffee specimens blended with cardamom represented by Kuwait- and Qatar-specimens, *i.e.*, LRCK and LRCQ showed increase in sugar levels likely derived from cardamom seeds.

Major sugar forms included inositol (S127) and L-threonic acid (S129), in addition to fructose (S124), 3-deoxy-arabino-hexonic acid (S121) and 3-deoxy-ribo-hexanoic acid-lactone (S118).

Fructose (S124) and inositol the principal sugar components in most samples showed different patterns upon roasting. While fructose dropped with roasting especially in instant coffee product, *i.e.*, ICG almost absent as well as heavily roasted coffee product, *i.e.*, HRKC. Inositol a sugar alcohol was found to be relatively heat-stable and not affected by roasting process.

3.3.1.2 Lipids

Plant seeds are considered typically rich reservoir of lipids, with fatty acids detected in coffee samples at 23.0% (1.2-41.1%) close to sugars (23.5%) and represented by 20 compound. Contrary to sugars, roasting in the Aswan green coffee sample, *i.e.*, GCE at 26.9 % and Kuwait green coffee sample, *i.e.*, GCK at 33.9 showed no obvious decrease in their lab heated counterpart samples (BRA 26.6%, BRK 37.1%). Except in case of instant coffee product (ICG), that showed the least fatty contents at 1.2% being derived from an aqueous coffee extract post drying.

The major abundant fatty acids included hexadecanoic (S34) or palmitic acid (16:0) followed by octadecenoic (S37) or oleic acid (18:1), found in all coffee samples. However, eicosanoic acid (20:0) or arachidic acid (S33) were found in all coffee samples except in instant Arabian coffee blended with cardamom, *i.e.*, ICC.

3.3.1.3 Organic acids

Organic acids were represented by 25 compounds, and to demonstrate large variation in levels among specimens. Organic acids amounted for 12.1% (4.0-23.2%) of the total detected metabolites represented by acetic acid (P 64), malic acid (P 76), and succinic acid (P 73). These acids are known to be byproducts of sugar decomposition [4] and are associated with the acrid and sour flavor of foods and beverages [114].

Generally, organic acids showed higher abundance in authentic *Coffea arabica* samples, i.e., GCA and RCA at 11.9 and 19.3%, compared to authentic *Coffea robusta, i.e.*, GCC and RCC at 4.0 and 12.8%. Such pattern was also observed in commercial products that organic acids content showed increase with roasting ranging from green coffee samples (4.0%-13.4%), through lightly roasted (8.5%-11.5%), heavily roasted (20.9%), and instant coffee (23.2%). Instant coffee composed of *Coffea arabica* (ICG) showed the highest levels at 23.2% owing to the contributions of acetic acid in addition to lactic acid. Major organic acid in green coffee specimens included citric acid (S86) at i.e., GCA (8.3%), GCU (8.0%), and GCK (7.1%). Citric acid has a pleasant acidic taste to be a well-known food additive [115], and found at lowest levels in instant coffee products *i.e.*, 0.4%- 1.8%.

3.3.1.4 Alkaloids

Caffeine and trigonelline contributed to 11.8% (6.4-20.8%) of all coffee metabolites abundance. Highest alkaloids abundance was detected in authentic green *Coffea robusta*, *i.e.*, GCC, and Saudi Arabian lightly roasted *Coffea arabica* blended with cardamom, *i.e.*, LRCS at *ca*. 20%. In contrast, instant *Coffea arabica* (ICG) and lightly roasted blended with cardamom, *i.e.*, LRCK showed the lowest alkaloids content with 6.4% and 6.7%, respectively.

Caffeine (S4) was the major alkaloid in all investigated samples detected at highest levels in GCC and LRCS at *ca*. 20% and 19.1%, respectively. In contrast, instant coffee, *i.e.*, ICG and LRCK showed the lowest levels at 6%. Roasting did not appear to much influence caffeine

levels likely due to its thermal stability being detected in authentic green and roasted *Coffea arabica*, *i.e.*, GCA and RCA at comparable levels *ca*. 10.0%, respectively. In contrast to caffeine, roasting affected trigonelline (S3) level exemplified in roasted and unroasted seeds GCE (1.0%) vs. BRA (0.2%), and GCA (1.4%) vs. RCA (0.6%), Table 3-2. The pattern of trigonelline reduced content was directly correlated with the degree of roasting in the order that trigonelline abundance scored 0.5-2.0% in green coffee seeds, down to 0.1-0.6% in heavily roasted coffee seeds. These results were consistent with literature confirming that caffeine is a heat-stable alkaloid, while trigonelline is a heat-labile, and that caffeine contribution the bitter taste of coffee seeds either in green or roasted products [15], and could account for the less perceived bitterness in roasted coffee due to trigonelline degradation.

3.3.1.5 Sugar alcohols

Sugar alcohols represented major forms in coffee seeds at *ca*.10.3% (4.8-15.9%) of the total metabolites in coffee samples, with the highest level in ICG at 15.5% versus lowest in BRK at 4.8%. Interestingly, instant coffee products showed higher abundance of sugar alcohols, *i.e.*, ICC (12.5%) and ICG (15.5%) among coffee samples and suggestive that they mediate for its sweet perception. Myo-inositol (S134) was the major sugar alcohol and its abundance in instant coffee is likely due to its relatively higher stability compared to free sugars [116].

The effect of roasting on sugar alcohols content appeared clearly in RCA (6.1%), RCC (8.8%), and BRK (4.8%) compared to green analogues, *i.e.*, GCA (13.8%), GCC (13.2%), and GCK (7.6%). Other blended roasted samples showed higher abundances of sugar alcohols i.e., LRCS (11.2%) and LRSQ (11.4%) attributed to other constituents like cardamom rich in carbohydrates.

3.3.1.6 Miscellanious

Other lower abundance secondary metabolites were detected including phenols/phenolic acids 2.6% (1.4-3.9%) represented mainly by caffeic acid (S93) in Egyptian coffee green (GCE) and lab. roasted (BRA) seeds, amino acids/peptides especially in instant coffee products, nitrogenous compounds 5.9% (1.3-11.8%) in BRA. Steroids exemplified by campesterol (S105), stigmasterol (S110) and β-sitosterol (S108) were detected in all samples except instant products likely due to the polar nature of coffee decoction prepared for instant coffee prior to its evaporation. Terpenes detected at highest levels (0.9%) in cardamom containing blends as

ICC and LRSQ and likely to add to these blends aroma exemplified by cineol (S137) as major volatile.

3.3.2 Multivariate data analyses of coffee silylated extracts dataset

Unsupervised MVA was employed for assessment of heterogeneity in coffee metabolome (primary nutrients) post silylation in context of coffee species (*robusta* vs. *arabica*), coffee processing (green, roasted vs. instant coffee), and additives (plain coffee vs. coffee blended with cardamom and Qassim blend) in an untargeted manner.

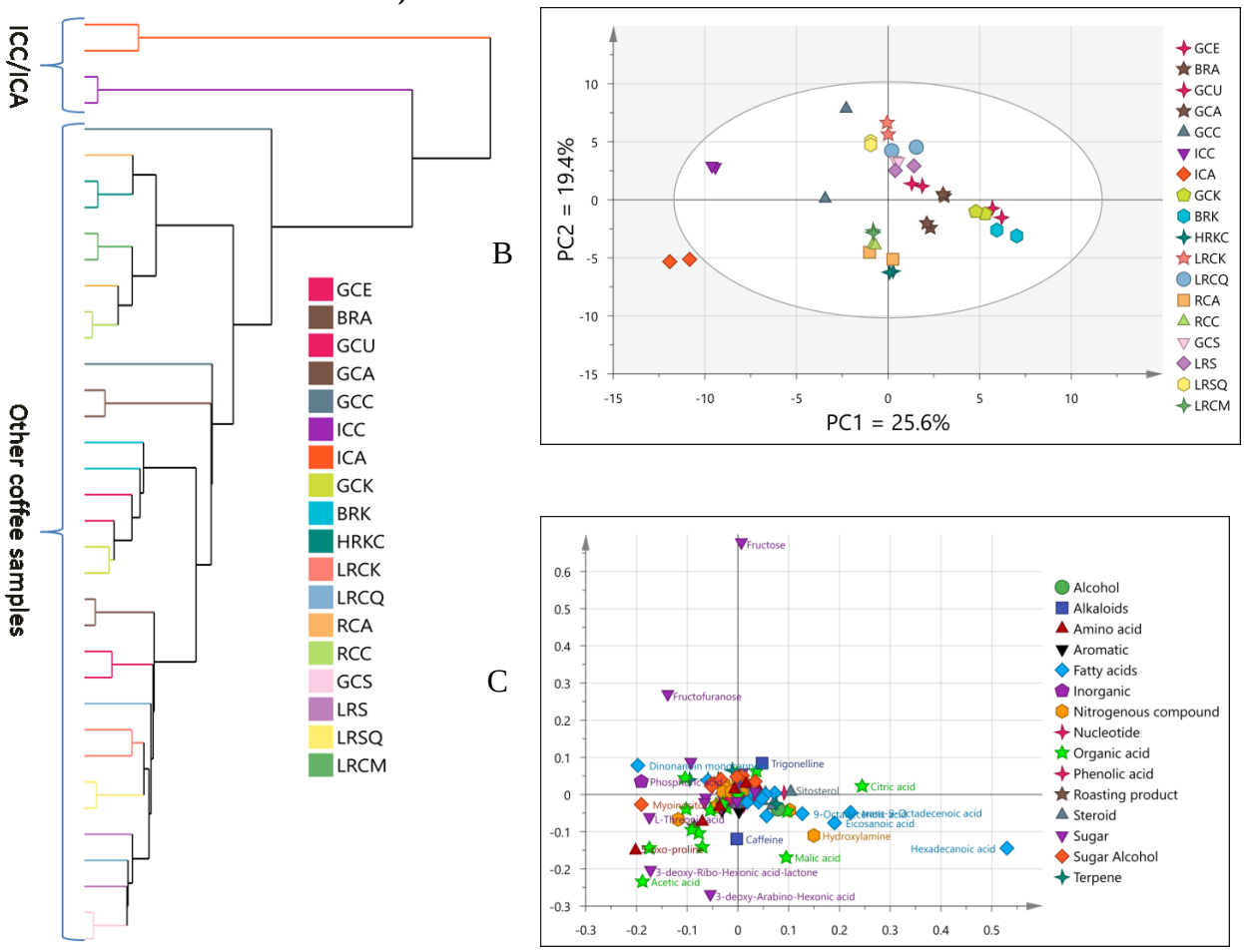
3.3.2.1 Unsupervised analysis of the whole samples' dataset

HCA of the whole GC/MS silylated metabolites dataset, **Fig. 3-10 A** demonstrated a clear segregation of instant coffee samples, *i.e.*, ICC and ICA, from all other coffee specimens. Likewise, PCA score plot (**Fig. 3-10 B**) revealed a remarkable similar segregation along PC1 accounting for 25.6% of the total variance versus 19.4% for PC2. Less clear classification was apparent along PC1 of robusta coffee samples, *i.e.*, GCC and RCC with negative score values opposite to *Coffea arabica* along PC1, while green and lightly roasted commercial samples were on the opposite side along PC2 from roasted specimens. Inspection of the corresponding loading plot (**Fig. 3-10 C**) for variables mediating for such segregation revealed that *Coffea arabica* were enriched in fatty acids i.e., palmitic acid, stearic acid, and arachidic acid, while *Coffea robusta* was more rich in sugars i.e., fructose. Instant coffee samples segregation was attributed to higher relative levels of organic acids *i.e.*, lactic and acetic, and phosphoric acid. Higher content of phosphoric acid in instant coffee may be related to the degree of roasting [117]. While phosphoric, acetic, lactic and L-threonic acids increased with roasting, malic acid and citric acid showed slight decrease or remained constant and all contribute to the perceived acidity of coffee.

Figure 3-10 **PCA of silylated GCMS coffee samples dataset HCA plot (A), scatter plot (B) and loading plot (C) .**

Samples were categorized per origin and preparation method (BRA: Lab-roasted Aswan-green; BRK: Lab-roasted Kuwait-green; GCA: Green *Coffea arabica*; GCC: Green C. canephora var. robusta or *Coffea robusta*; GCE: Aswan-green, Egypt; GCK: Kuwait-green ; GCS: Saudi Arabia-green; GCU: Emirates-green; HRKC: Kuwait-heavily roasted with cardamom; ICA: Instant coffee *Coffea arabica*; ICC: Instant Arabian coffee blended with cardamom; ICG: Instant coffee 100% *Coffea arabica*; LRCK: Kuwait-lightly roasted blended with cardamom; LRCM: Saudi Arabia-lightly roasted blended with cardamom(Maatouk); LRCQ: Qatar-lightly roasted blended with cardamom; LRCS: Saudi Arabia-lightly roasted blended with cardamom(Shahi); LRS:

Saudi Arabia-lightly roasted ; LRSQ: Saudi Arabia-lightly roasted with Qassim blend; RCA: Roasted *Coffea arabica*; RCC: Roasted C. *canephora* var. robusta or *Coffea robusta*)-Codes are identified in table 2-2



3.3.2.2 Unsupervised analysis of the commercial samples' dataset

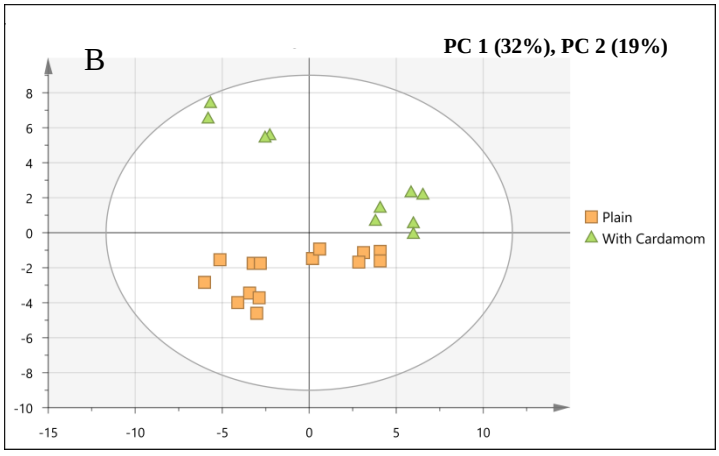
To reveal for metabolites heterogeneity among commercial samples from Middle East of various origin, authenticated and instant coffee samples were excluded from the model. Included samples represented different origin, roasting levels, and blends, *i.e.*, cardamom. Among these different categories, only cardamom blends that appeared clearly separated from other commercial coffee specimens as revealed from HCA, Fig. 3-11 **A** and PCA aligned alongside PC2, Fig. 3-11 **B** that showed differentiation of coffee samples fortified with cardamom, *i.e.*, HRCK and LRCM. Loading plot revealed predominance of inositol sugar in plain coffee, whereas samples blended with cardamom showed higher abundance of fructose, 3-deoxyarabinohexenoic acid, and 5-deoxy-ribohexonic acid lactone.

3.3.2.3 Unsupervised analysis of authentic samples dataset

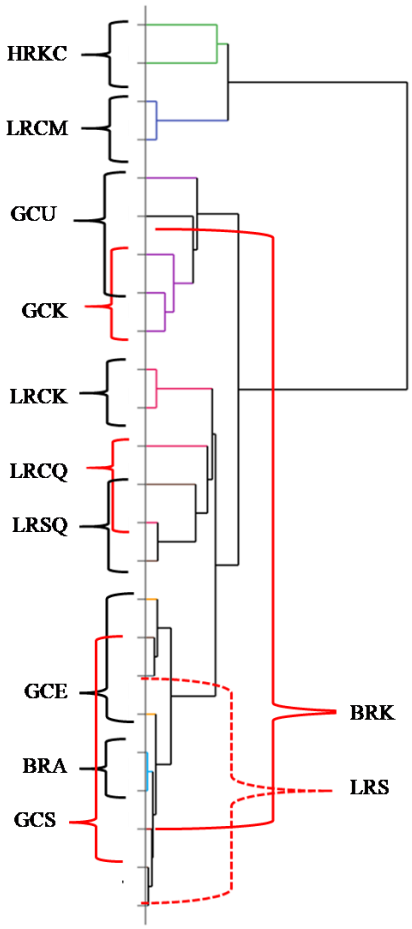
PCA was utilized to determine metabolites heterogeneity among *Coffea arabica* and *Coffea robusta* in either roasted or green form. PCA score plot **(Fig. 3-12 B)** prescribed by PC1 and PC2 accounting for 39.8% and 27.6%, respectively revealed segregation of roasting specimen along PC2, while gentoype along PC1 as expectedly being a larger variance. PCA loading plot **(Fig. 3-12 C)** identified caffeine and cyclitol sugars, *i.e.*, inositol and myoinositol as being more abundant in robusta coffee. In contrast, *Coffea arabica* showed enrichment in organic acids i.e., malic acid, acetic and citric acid, sugar acids i.e., 3-deoxy arabino-hexonic acid, and 3-deoxy ribo-hexonic acid and fatty acids i.e., hexadecanoic acid or palmitic acid. The abundance of organic acids in *Coffea arabica* than *Coffea robusta*, especially citric acid was consistent with previous investigations [117]. This finding might explain the higher acidity assigned to roasted *Coffea arabica* compared to robusta coffee. Likewise, abundance of myoinositol in *Coffea robusta* compared to *Coffea arabica* falls in alignment with previous reports [117].

Figure 3-11 **Principal Component Analysis (PCA) score plot (B) and loading plot (C) of commercial silylated of roasted coffee samples along with hierarchical cluster analysis (HCA) (A).**

Sample codes(BRA: Lab-roasted Aswan-green; BRK: Lab-roasted Kuwait-green; GCE: Aswan-green, Egypt; GCK: Kuwait-green ; GCS:Saudi Arabia-green; GCU: Emirates-green; HRKC: Kuwait-heavily roasted with cardamom; LRCK: Kuwait-lightly roasted blended with cardamom; LRCM:Saudi Arabia-lightly roasted blended with cardamom; LRCQ: Qatar-lightly roasted blended with cardamom; LRCS: Saudi Arabia-lightly roasted blended with cardamom; LRS: Saudi Arabia-lightly roasted ; LRSQ: Saudi Arabia-lightly roasted with Qassim blend) Codes are identified in table 2-2



A



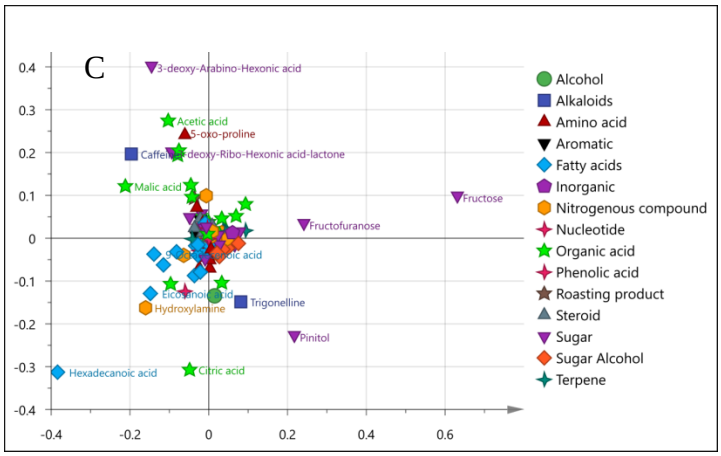
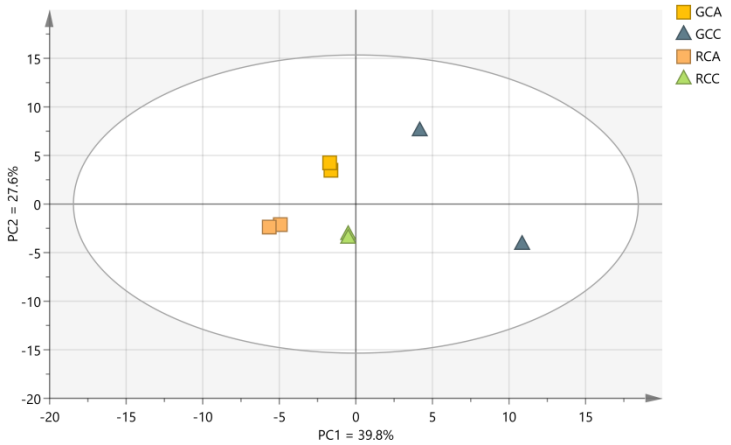
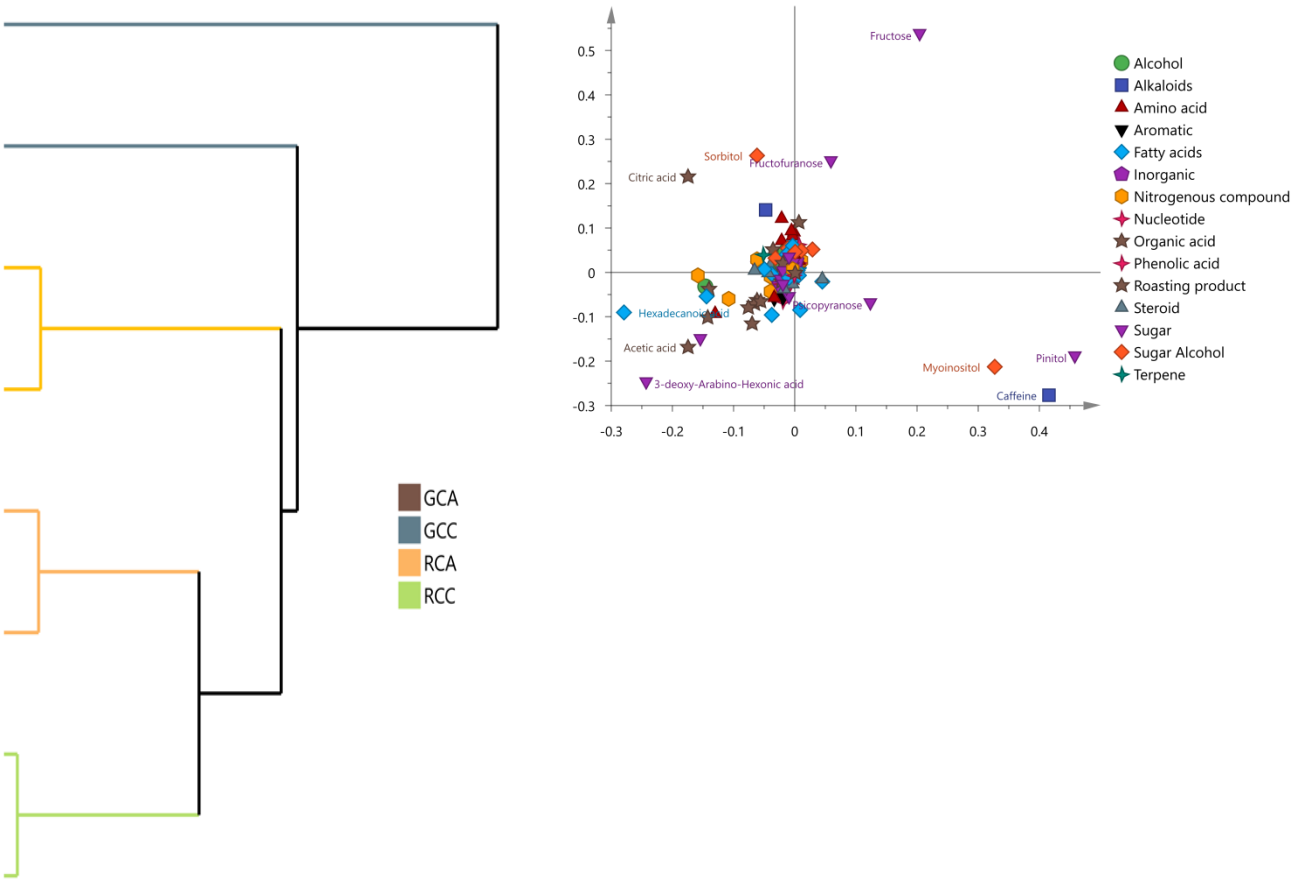


Figure 3-12 **PCA score plot of authentic coffee samples analyzed by silylated GC-MS (GCA: Green *Coffea arabica*; GCC: Green C. canephora var. robusta or *Coffea robusta*; RCA: Roasted *Coffea arabica*; RCC: Roasted C. canephora var. robusta or *Coffea robusta*) Codes are identified in table 2-2**





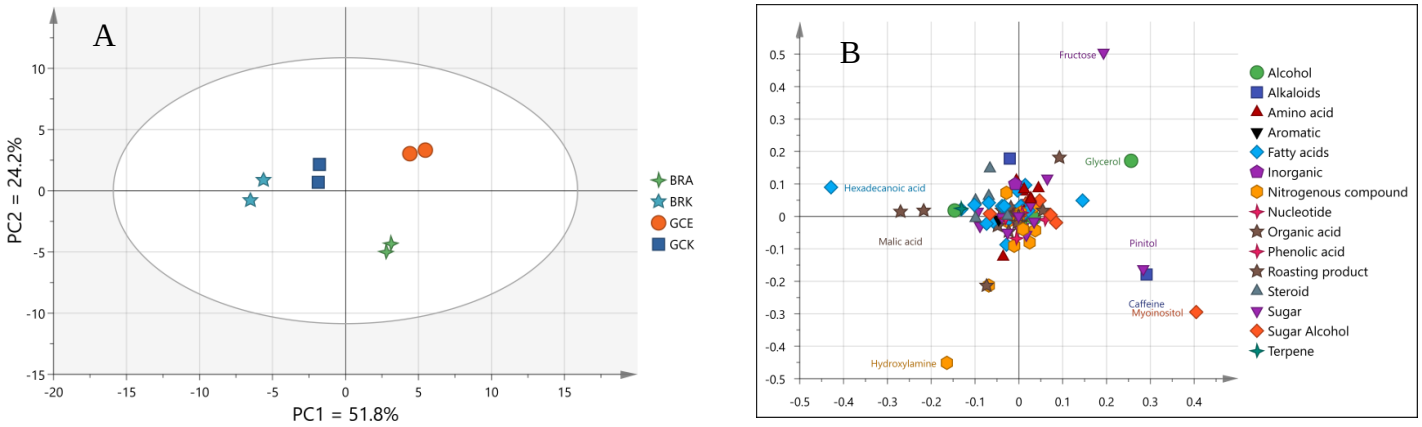
3.3.2.4 Unsupervised multivariate data analysis modelling the effect of roasting

To investigate the effect of roasting on coffee seeds, the commercial green coffee from different geographical origin, *i.e.*, GCE and GCK, were compared with their roasted counterparts, i.e., BRA and BRK, respectively. The PCA scatter plots based on silylated products **(Fig. 3-13A)** could differentiate between the four investigated specimens. The roasted samples were positioned left to their green coffee counterparts. Besides, each pair of samples of the same geographical origin were apart indicating a higher contribution of the geographical origin to the variability compared to the coffee processing.

The corresponding loading plots **(Fig. 4-13 B)** and OPLS-DA S-plot of GCE vs BRA indicated that caffeine, sugar alcohols i.e., inositol and myoinositol were increased by roasting in consistent with previous reports [118]. On the other hand, green coffee showed higher abundance of fructose along with glycerol, trigonelline, aminobutyric acid, and octadecenoic acid. Octadecanoic acid or stearic acid is a fatty acid that is formed during somatic

embryogenesis of coffee along with fructose sugar [119]. Trigonelline and γ-aminobutyric acid higher abundance in green coffee specimens are in accordance with previous report on the effect of roasting on coffee metabolome using NMR [118] .

Figure 3-13 **PCA score plot of green coffee samples detected by GCMS post silylation (A) along with loading plots (B)**
(BRA: Lab-roasted Aswan-green; BRK: Lab-roasted Kuwait-green; GCE: Aswan-green, Egypt; GCK: Kuwait-green) - Codes are identified in table 2-2



3.3.3 Conclusion

As derived from the pattern of relative abundance of metabolites in different coffee brews it was noted that thermal processing of coffee in terms of roasting temperature, roasting time, brewing conditions affected the metabolite profile and changed the relative abundance of different metabolites. Previous research has focused on the caffeine chlorogenic acid. However, in this research we have revealed special pattern associated with maceration for example showing higher abundance of alcohols as terpineole compared with hot extraction method more characterized by abundance of esters or terpenes. The difference in metabolite abundance with certain coffee processing was attributed to various factors as its stability or degradation at processing method parameters, degradation of other metabolites in the sample matrix which may lead to increase in the relative abundance of such metabolite (like increased caffeine with roasting due to degradation of other components). And, by the processing conditions themselves like, temperature, time and pressure. Utilization of these insights in future will bring more understanding of processing conditions of coffee to realize pharmacological action related to either of these metabolites.

3.4 Microbiota mediated biotransformation

This part of our reserch was dedicated to investigate how green coffee and black coffee as functional food impact the metabolism of microbiota mimicking those species in human colon. Two different time aliquots were obtained from functional foods (including green and black coffee arabica) amended cultures, the first one was at 0.5 h a time at which no significant biotransformation is expected to occur, and at 24 h at which most of the biochemical and enzymatic changes would have occurred. Results revealed that while addition of coffee alter microbiota metabolism through either stimulation or inhibition of its metabolic pathways, not surprisingly functional food metabolites themselves can also act as substrate for microbiota metabolism.

3.4.1 GC/MS metabolite profiling of gut microbiota treated functional foods

GC/MS was employed to characterize microbiota culture metabolism and monitor changes occurring 0.5 and 24 h post exposure to the different functional food extracts, including black and green *Coffea arabica* authentic samples. Metabolites detected (as such or as volatile per-trimethylsilylated derivatives) comprised mostly microbial low molecular weight primary metabolites *viz.* organic acids, alcohols, amino acids, fatty acids, inorganic compounds, nitrogenous compounds, nucleic acids, phenolics, steroids and sugars in addition to few secondary metabolites representative of certain functional foods [120]. A total of 114 metabolite peaks (Table 3-3) were detected from all untreated blank and coffee treated cultures. The relative percentile levels of all metabolite classes detected for cultures harvested at 0.5 and 24 h from different functional foods (including coffee) and blank cultures is presented in (Fig. 3-14).

Figure 3-14 **Relative percentile levels of metabolite classes detected using GC/MS for cultures harvested at 0.5 and 24 h from functional foods amended culture: BC, GC versus blank**

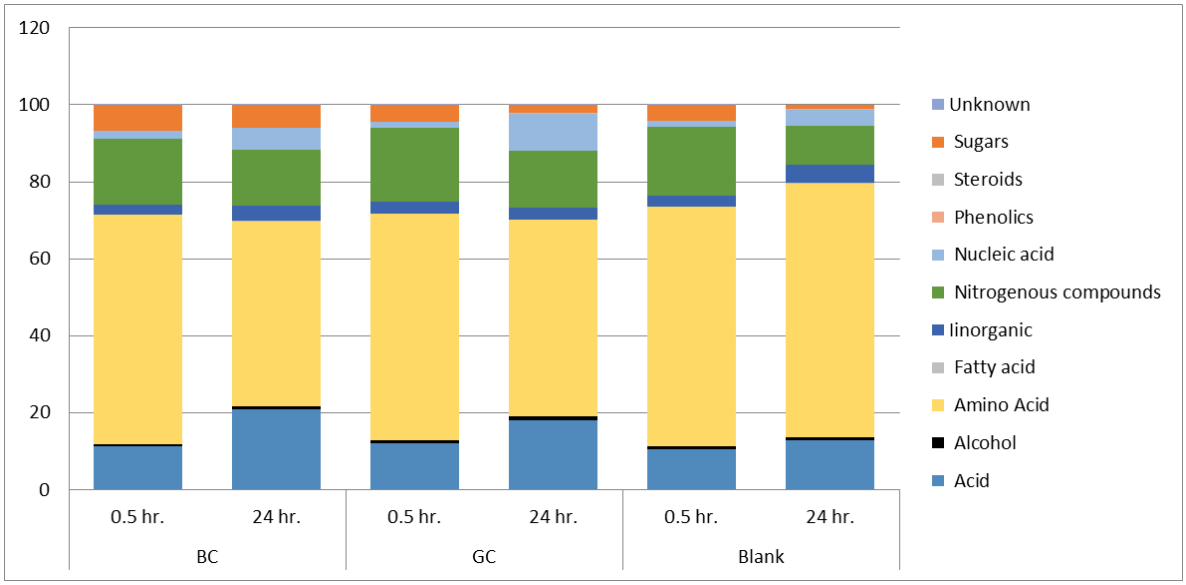


Table 3-3 **Primary metabolites analysis using GC-MS from functional foods amended gut culture: BC and GC versus methanol blank harvested at 0.5 and 24 h post inoculation. Results are expressed as relative percentile (average ± std deviation, n=3) of the total peak areas. * Green (GC) and black (BC) coffee (*Coffea arabica*).**

Category	Metabolite	BC		GC		Blank	
Acid	2-Deoxytetronic acid	0.03±0.01	0.08±0.01	0.02±0.01	0.10±0.03	0.02±0.01	0.09±0.00
	2-Hydroxy-3-methylvaleric acid	3.93±1.47	4.63±2.12	4.07±0.51	4.36±2.04	4.01±0.32	2.14±2.64
	2-Hydroxyglutaric acid	0.05±0.01	0.12±0.04	0.04±0.00	0.10±0.01	0.03±0.00	0.12±0.00
	2-Hydroxyisocaproic acid	0.22±0.03	0.31±0.12	0.22±0.05	0.48±0.09	0.23±0.02	0.27±0.03
	3-Deoxytetronic acid	0.18±0.32	0.68±0.60	0.07±0.12	0.23±0.39	-	-
	3-Hydroxypropionic acid	0.06±0.03	-	0.05±0.01	-	0.04±0.00	-
	4-Hydroxybutyric acid	0.03±0.04	0.06±0.01	0.03±0.02	0.03±0.03	0.01±0.00	-
	Amino isobutyric acid	0.01±0.00	0.40±0.05	0.01±0.00	0.38±0.09	0.01±0.00	0.55±0.14
	Fumaric acid	0.31±0.08	0.46±0.03	0.26±0.08	0.39±0.06	0.21±0.01	0.49±0.09
	Glyceric acid	0.01±0.01	0.07±0.01	0.01±0.01	0.06±0.01	0.01±0.00	0.02±0.01
	Glycolic acid	0.31±0.05	-	0.03±0.03	-	0.02±0.00	-
	Tartaric acid	0.25±0.12	0.62±0.30	0.17±0.11	0.36±0.17	0.20±0.01	0.21±0.30
	Lactic acid	0.83±0.05	1.17±0.51	0.98±0.31	2.62±1.36	1.54±1.63	-
	Aspartic acid	0.01±0.01	0.54±0.47	0.04±0.07	0.72±0.55	0.02±0.03	0.42±0.03
	Threonic acid	0.05±0.01	0.09±0.02	0.02±0.00	0.10±0.03	0.04±0.02	0.06±0.00
	Malic acid	0.14±0.03	0.38±0.03	0.12±0.02	0.40±0.02	0.10±0.01	0.37±0.07
	Methyl succinic acid	0.01±0.00	0.06±0.01	-	-	-	-
	Oxalic acid	0.04±0.00	-	0.42±0.05	-	0.17±0.21	-
	Phosphoric acid	0.10±0.01	0.47±0.03	0.10±0.03	0.07±0.02	0.05±0.01	0.03±0.00
	2-desoxy-ribonic acid	0.02±0.04	0.14±0.13	0.06±0.02	0.35±0.10	0.07±0.04	0.17±0.00

Category	Metabolite	BC		GC		Blank	
	Succinic acid	4.08±0.54	9.91±2.93	5.09±0.10	6.48±1.01	3.46±0.84	7.28±2.03
	Succinyl acetone	0.13±0.07	0.17±0.05	0.05±0.03	0.14±0.05	0.06±0.00	0.11±0.03
	Tartaric acid(another silylated derivative)	0.33±0.18	0.62±0.30	0.21±0.17	0.64±0.13	0.27±0.02	0.46±0.30
	Total	**11.15**	**20.98**	**12.09**	**18**	**10.57**	**12.79**
Alcohol	Butane	0.02±0.00	0.07±0.10	0.06±0.02	0.05±0.05	0.03±0.00	-
	Ethylene glycol	0.16±0.03	0.07±0.06	0.08±0.07	0.11±0.03	0.17±0.08	0.20±0.17
	Nonanol	0.06±0.02	0.08±0.02	0.05±0.01	0.12±0.11	0.06±0.02	-
	Propyleneglycol	0.51±0.14	0.45±0.26	0.42±0.05	0.70±0.36	0.38±0.07	0.55±0.05
	Total	**0.75**	**0.68**	**0.6**	**0.99**	**0.64**	**0.75**
Amino acid	Alanine	7.42±1.94	1.31±0.30	10.31±2.60	1.26±0.84	9.59±1.28	1.55±0.71
	Aspartate	0.03±0.01	0.01±0.02	0.02±0.01	0.02±0.02	0.03±0.00	-
	Phenylalanine	4.63±2.10	6.76±3.53	4.48±0.95	10.93±1.55	4.00±0.82	9.93±0.76
	Citrulline	0.36±0.21	-	0.32±0.17	-	0.28±0.05	-
	Cysteine	0.20±0.02	0.09±0.04	0.19±0.06	0.08±0.02	0.17±0.04	0.03±0.05
	DL-Phenylalanine	1.02±0.10	2.93±0.97	0.88±0.26	2.36±0.36	1.03±0.20	3.92±1.28
	Glutamic acid	0.54±0.93	1.13±1.96	1.70±1.63	1.12±1.94	1.10±1.56	4.95±0.43
	Glycine	0.84±0.09	0.84±0.20	0.68±0.07	0.99±0.94	0.66±0.08	1.06±0.60
	Homocysteine	0.07±0.02	0.12±0.13	0.05±0.02	-	0.03±0.02	-
	Homoserine	0.06±0.02	0.06±0.01	0.05±0.01	0.04±0.02	0.06±0.01	0.03±0.00
	Isoleucine	6.12±2.04	2.01±1.53	3.94±2.51	1.72±1.39	6.62±0.26	3.23±0.07
	N-acetyl-glutamic acid	0.13±0.04	-	0.14±0.02	-	0.06±0.09	0.11±0.15
	Isoleucine	2.12±0.32	2.46±1.17	2.28±0.37	1.95±0.67	1.76±0.56	1.77±0.08
	Leucine	-	-	0.02±0.02	-	0.01±0.01	-
	Norvaline	1.32±0.30	0.29±0.45	0.23±0.40	-	0.77±1.08	-
	Serine	0.03±0.00	-	-	-	0.01±0.00	-
	Threonine	2.61±0.62	3.33±0.73	2.74±0.20	3.20±0.69	2.07±0.02	2.27±0.18
	Threonine	1.48±0.29	4.12±1.99	1.11±0.15	3.60±0.36	1.04±0.61	2.60±1.14
	Methionine	0.19±0.34	1.11±1.00	0.43±0.19	2.88±1.16	0.96±0.95	4.48±0.38
	N,N-Dimethylglycine	0.51±0.05	0.18±0.32	0.37±0.03	-	0.25±0.05	-
	4-hydroxyproline	-	-	0.02±0.02	-	-	-
	Leucine	0.73±0.67	3.34±1.16	0.23±0.39	3.92±1.25	3.97±0.30	5.89±0.17
	Norleucine	0.01±0.00	0.02±0.00	0.01±0.01	-	0.01±0.00	-
	Ornithine	13.90±1.17	2.50±0.22	14.13±3.21	0.83±1.44	13.68±2.39	3.79±0.06
	Ornithine	0.60±0.16	-	0.72±0.23	0.02±0.03	0.44±0.10	-
	Proline	0.59±0.24	0.60±0.05	0.49±0.24	0.61±0.10	0.85±0.14	0.79±0.00
	Pyroglutamic acid	3.91±0.87	6.51±3.29	3.21±0.74	6.18±1.52	2.38±0.75	9.80±0.72
	Serine	0.01±0.01	-	-	-	0.05±0.03	-
	Lysine	0.48±0.34	-	0.35±0.11	-	0.56±0.02	-
	Tryptophan	0.46±0.79	-	-	-	1.21±0.16	-
	Tyr,(N,O,O-TMS)	2.14±0.42	1.88±0.49	1.62±0.61	3.64±0.66	1.63±0.17	3.16±0.14
	Tyrosine	0.16±0.07	0.96±0.14	0.04±0.08	0.92±0.67	0.11±0.05	2.58±0.18
	Valine	5.55±0.78	5.53±1.15	6.93±0.41	4.89±0.95	5.63±0.59	4.09±1.44
	Valine	1.25±0.39	-	1.28±0.44	-	1.36±0.25	-
	Total	**59.48**	**48.09**	**58.98**	**51.16**	**62.36**	**66.05**
Fatty acid	Palmitic acid	0.01±0.02	0.03±0.06	0.07±0.06	0.05±0.09	-	0.05±0.07
	Total	**0.01**	**0.03**	**0.07**	**0.05**	**0**	**0.05**

Category	Metabolite	BC		GC		Blank	
Inorganic	Phosphoric acid	2.68±0.43	4.04±1.86	3.07±0.18	3.00±0.12	2.93±0.77	4.90±2.03
	Total	**2.68**	**4.04**	**3.07**	**3**	**2.93**	**4.9**
Nitrogenous compound	dihydro-2,4-pyrimidine-dione	0.01±0.02	-	0.02±0.01	0.02±0.03	0.04±0.00	-
	3-Methyl-piperazine-2,5-dione	1.75±0.12	1.59±0.19	1.52±0.31	2.04±1.23	1.36±0.17	1.47±0.04
	4-Hydroxypyridine,trimethylsilylether	0.04±0.00	0.05±0.01	0.03±0.00	0.03±0.02	0.01±0.00	-
	Aminoethanol	0.03±0.05	-	-	0.06±0.10	0.06±0.09	0.07±0.10
	Aminomalonic acid	0.14±0.03	0.02±0.03	0.09±0.03	0.14±0.20	0.07±0.02	-
	Amphetamine	0.36±0.05	1.98±0.34	0.24±0.03	1.74±0.21	0.03±0.00	0.80±0.18
	Cadaverine	1.20±0.37	0.39±0.51	1.11±0.59	0.52±0.31	1.35±0.14	0.04±0.06
	Ethanolamine	0.03±0.02	0.03±0.05	0.01±0.02	0.06±0.06	0.04±0.01	0.08±0.11
	Ethanolamine	0.08±0.07	0.23±0.08	0.14±0.05	0.15±0.13	0.03±0.04	-
	GABA	2.16±0.23	3.62±0.26	2.15±0.41	3.62±0.12	2.15±0.08	1.04±0.13
	HIS	0.82±0.14	-	0.78±0.20	-	0.57±0.33	-
	4-aminomethylcyclohexane carboxylic acid	0.04±0.01	0.06±0.00	0.04±0.01	0.05±0.00	0.03±0.00	0.04±0.00
	N,N-bis[Trimethylsiloxyethyl],trimethylsilanamine	0.02±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.00	0.01±0.02
	Nicotinic acid	0.15±0.02	0.31±0.09	0.10±0.02	0.24±0.08	0.08±0.01	0.18±0.01
	Phenyl ethanolamine	0.09±0.07	-	-	-	-	-
	2,5-diketo- Piperazine	0.05±0.01	-	0.07±0.02	-	0.08±0.00	-
	Putrescine	0.90±0.08	1.16±0.34	1.14±0.43	1.28±0.08	0.97±0.37	1.51±0.21
	3,6-dihydro pyrazine	0.03±0.01	0.01±0.01	0.02±0.01	0.03±0.03	0.03±0.00	0.01±0.01
	Piperidine carboxylate	0.01±0.01	0.02±0.01	0.01±0.00	0.02±0.00	0.02±0.00	0.02±0.01
	Amino propanoate	7.73±1.55	1.31±0.30	10.31±2.60	1.26±0.84	9.59±1.28	1.55±0.71
	Tryptamine	0.11±0.19	-	0.14±0.12	-	0.37±0.08	-
	Unknown nitrogenous compound	0.97±0.10	3.13±0.24	0.96±0.27	2.99±0.71	0.71±0.16	2.34±0.21
	Urea	0.26±0.09	0.56±0.48	0.28±0.13	0.56±0.51	0.20±0.02	0.74±0.73
	Total	**16.98**	**14.51**	**19.2**	**14.83**	**17.85**	**9.91**
Nucleic acid	purine	-	-	-	4.10±0.51	-	-
	Purin-6-yl-aminoacetic acid	-	-	0.02±0.04	-	0.05±0.00	-
	Adenine	0.13±0.04	0.69±0.15	0.08±0.01	0.47±0.17	0.11±0.01	0.09±0.01
	Adenosine	-	0.33±0.02	-	0.02±0.03	-	-
	Hypoxanthine	0.94±0.10	1.43±0.31	0.61±0.14	2.07±0.39	0.61±0.08	2.23±0.06
	Inosine	0.09±0.03	0.77±0.15	-	0.05±0.02	-	-
	Thymine	0.19±0.06	0.20±0.02	0.22±0.03	0.25±0.09	0.17±0.05	0.15±0.01
	Uracil	0.77±0.14	2.26±0.14	0.69±0.17	2.61±0.57	0.60±0.05	1.69±0.43
	Total	**2.12**	**5.69**	**1.63**	**9.56**	**1.55**	**4.16**
Phenolic	4-Hydroxyphenylacetic acid	0.01±0.00	0.04±0.00	0.01±0.00	0.06±0.02	0.01±0.00	0.15±0.01
	Gallic acid	-	0.01±0.00	-	0.02±0.01	-	-
	Hydrocinnamic acid	-	-	-	0.09±0.16	-	0.23±0.03
	Resorcinol	0.01±0.01	0.01±0.02	0.01±0.01	-	-	-
	Total	**0.02**	**0.06**	**0.02**	**0.17**	**0.01**	**0.38**
Steroid	2-Methoxyoestradiol	0.04±0.01	0.05±0.01	0.03±0.01	0.07±0.01	0.02±0.00	0.02±0.01
	Total	**0.04**	**0.05**	**0.03**	**0.07**	**0.02**	**0.02**
Sugar	Hexopyranose	0.06±0.05	0.26±0.08	0.16±0.02	0.22±0.04	0.19±0.02	0.21±0.01
	Trehalose	0.52±0.89	-	-	-	-	-

Category	Metabolite	BC		GC		Blank	
	3-Deoxy-arabino-Hexonicacid	0.56±0.16	2.49±0.28	-	0.95±1.00	-	-
	Glucose	0.01±0.01	-	0.01±0.01	-	0.00±0.01	-
	Mannopyranose	-	-	0.33±0.29	-	-	-
	Erythritol	0.01±0.01	0.02±0.02	0.00±0.01	0.08±0.04	0.01±0.00	0.04±0.02
	Fructose methyloxime	-	-	0.01±0.01	-	0.06±0.01	-
	Galactinol	0.66±0.25	1.08±0.04	0.34±0.07	0.72±0.10	0.36±0.06	0.61±0.03
	Threose	-	-	-	0.01±0.01	0.01±0.02	-
	Lactitol	0.04±0.03	0.15±0.04	0.01±0.01	0.17±0.03	-	0.12±0.02
	Ribitol,5TMS	-	0.37±0.64	-	-	-	-
	Trehalose	4.14±0.49	-	3.41±0.43	-	3.38±0.44	-
	Unknown sugar	0.72±0.14	1.50±0.45	-	-	-	-
	Xylulose-O-methyloxime	-	-	0.01±0.01	-	-	-
	Total	**6.72**	**5.86**	**4.28**	**2.15**	**4.01**	**0.99**
Unknown	Unknown	0.03±0.00	0.01±0.03	0.03±0.02	0.03±0.03	0.06±0.01	-
	Total	**0.03**	**0.01**	**0.03**	**0.03**	**0.06**	**0**

GC/MS Metabolite profiling revealed that amino acids form the major class in cultures harvested at 0.5 h ranging from 59.0 % to 59.5% in those amended with BC and GC food extracts compared to 62.4% in blank culture at the same time point (Table 3-3), suggesting that amino acids are mostly derived from the microbial culture itself. Following amino acids, nitrogenous compounds represented the second most abundant class, ranging from 17.0% to 19.2% in BC and GC extract fortified media against 17.9% in blank gut microbial culture at 0.5 h. Compared to amino acids and nitrogenous compounds that showed comparable levels at 0.5 h incubation, sugars (4.3-6.7%) showed a larger variation among cultures fortified with BC and GC extracts compared to blank culture with 4.0%, supporting the conclusion that such difference is attributed to coffee seeds individual compositions. Sugars are a major primary metabolite class in most plant foods as analyzed using GC/MS [121]. Other minor classes identified in microbial cultures included inorganic metabolites (2.7-3.1%), phenolic compounds (0.02%) and nucleic acids (1.6-2.1 %).

After incubation for a period of 24 h, samples were aliquoted and analyzed using the same protocol to reveal for metabolite changes occurring in culture. A general decrease in amino acid levels by 16% (0.8 fold) in BC & GC treated samples, indicate that amino acids may serve as nutrient substrate for gut microbial growth. Amino acids are utilized by bacteria as building blocks for microbial protein assembly essential for bacterial growth, or to be fermented as an

energy source [122]. This amino acid decrease trend in BC & GC treated samples 24 h post incubation is contrasting the untreated blank samples, showing even a slight 1.1-fold increase in amino acid content. For nitrogenous compounds, a decrease was revealed in all cultures treated and blank. Decreases was observed with green coffee and black coffee to be 0.8 fold reductions. Bacteria can utilize nitrogenous compounds such as amino propanoate as a single carbon source or energy source [123]. Interestingly, a significant decrease in sugar levels is observed in green coffee by 0.5 fold and black coffee by 0.87 fold compared to 0.25 fold decrease in blank samples. Why sugars are consumed faster in treated samples, and in parallel free amino acid contents are reduced (used for energy production too, or for protein production?) is a matter of speculation, but since plants often contain bioactive, partially antibiotic compounds such as polyphenols, extra energy might be required for microbiota survival. The most significant decrease of sugars and amino acids in coffee treated samples was observed in GC (0.5 fold), i.e. polyphenol rich plant products. Metabolite classes that showed an opposite pattern, i.e. an increase over time (0.5 versus 24 h) included organic acids and low molecular weight phenolics in coffee amended cultures compared to blank. Increase is likely attributed for microbiota degradation of high molecular weight plant sugars and phenolics to its simpler organic and phenolic acids. Detection of high molecular weight phenolics cannot be achieved using GC/MS and has yet to be performed using liquid chromatography mass spectrometry LC/MS [124]. Several bacterial species are reported to possess hydrolytic enzymes necessary for plant polyphenols degradation, e.g. through dehydroxylation, decarboxylation, ring cleavage or oxidation, ultimately generating simpler phenolic and organic compounds [125]. Likewise, nucleic acids increase upon incubation, especially in case of a GC amended culture at 6-fold increase after 24 h compared to 0.5 h treatment versus a 3-fold increase in case of the blank culture. A pie chart showing relative percentile levels of the different metabolite classes analyzed in microbiota culture fortified with the different coffee treatments at 0.5 and 24 h is given in Fig.3-15. Provided below is an overview of the major changes observed for metabolite classes in blank culture compared to those amended with coffee extracts.

Figure 3-15 **Pie chart showing relative percentile levels of the different metabolite classes analyzed using GC/MS post-silylation in microbiota culture fortified with black coffee BC and green coffee GC harvested at 0.5 and 24 h**

3.4.1.1 Amino acids

Ornithine, alanine and isoleucine were the most abundant amongst the detected amino acids, and showed a decline of 0.8, 0.14 and 0.32 fold, respectively, upon incubation in all cultures. An exception to this pattern was observed in case of phenylalanine, glutamic and pyroglutamic acid in both coffee treated and blank cultures. Phenylalanine showed ca. 1.8-fold increase in all coffee fortified samples compared to 2.5-fold increase in blank culture, likewise pyroglutamic acid showed an average of 1.7-fold increase compared to 4-fold increase in blank at 24 h treatment, i.e. the increase in treated cultures also was reduced vs. blank. Despite, its reduction or even complete depletion upon GC treatment, glutamic acid showed 4.5-fold increase. This might be attributed to specific producers of these amino acids *i.e. E. coli* induced production of phenylalanine [126] and *L. plantarum* mediated production of glutamic and pyroglutamic acid [127], both present in the consortium culture. Amino acids play multiple roles in gut microbiota either via protein fermentation or internally to synthesize essential amino acids to be further utilized as building blocks for cellular composition [128], to serve as signaling molecules between microbial cells [129] or within the host [130], or to be used as an energy source [131]. Nevertheless, such increase failed to lead to an increase in the overall amino acid percentile levels as the aforementioned reduction of other amino acids was much more significant with larger negative fold changes.

3.4.1.2 Other nitrogenous compounds

The most pronounced decrease in nitrogenous compounds was detected for 3-amino propanoate at comparable levels in coffee extracts amended and blank cultures (ca. 0.16-fold). Human gut microbiota produces various short chain fatty acids (SCFAs) with propanoate as major metabolic product of anaerobic fermentation Fig. 3-16.

Along with butanoate, propanoate is a major component in microbiota metabolic pathways for the synthesis of other SCFAs [132]. It does play a significant role during irritable bowel syndrome if reduced by medication [133]. Bacterial genera such as *Bacteroides*, *Blautia*, *Eubacterium*, *Escherichia* and *Clostridium* can ferment non-digestible dietary carbohydrates and amino acids to propionate through either 1,2-propanediol or succinate pathways (Fig. 3-17) [134, 135].

Figure 3-16 **Aminopropanoate accumulation pattern in Black coffee (A), Green coffee (B), and Blank (H) at 0.5 h and 24 h harvest time (both at 0.5mg/mL and 5.0 mg/mL dosing level)**

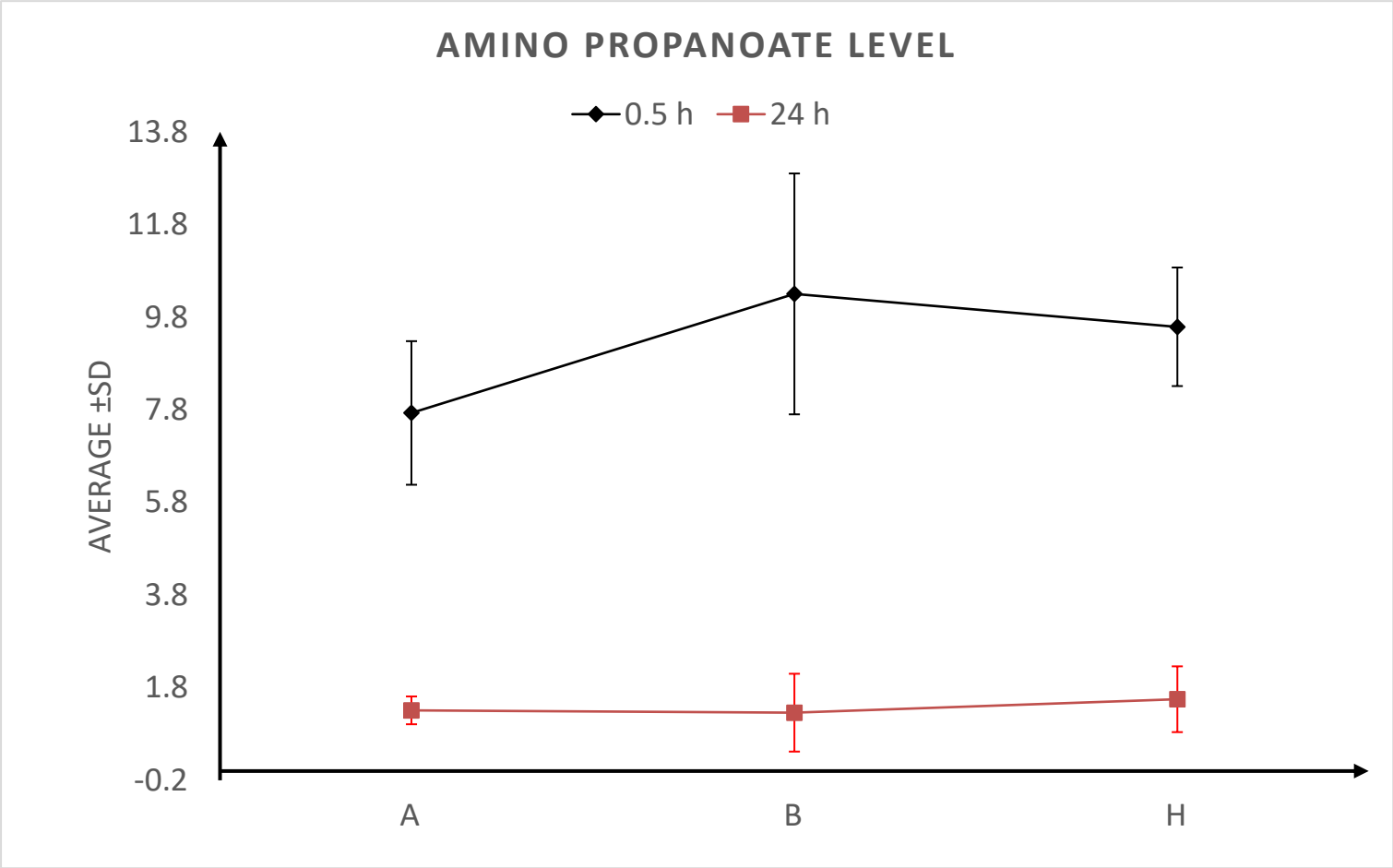
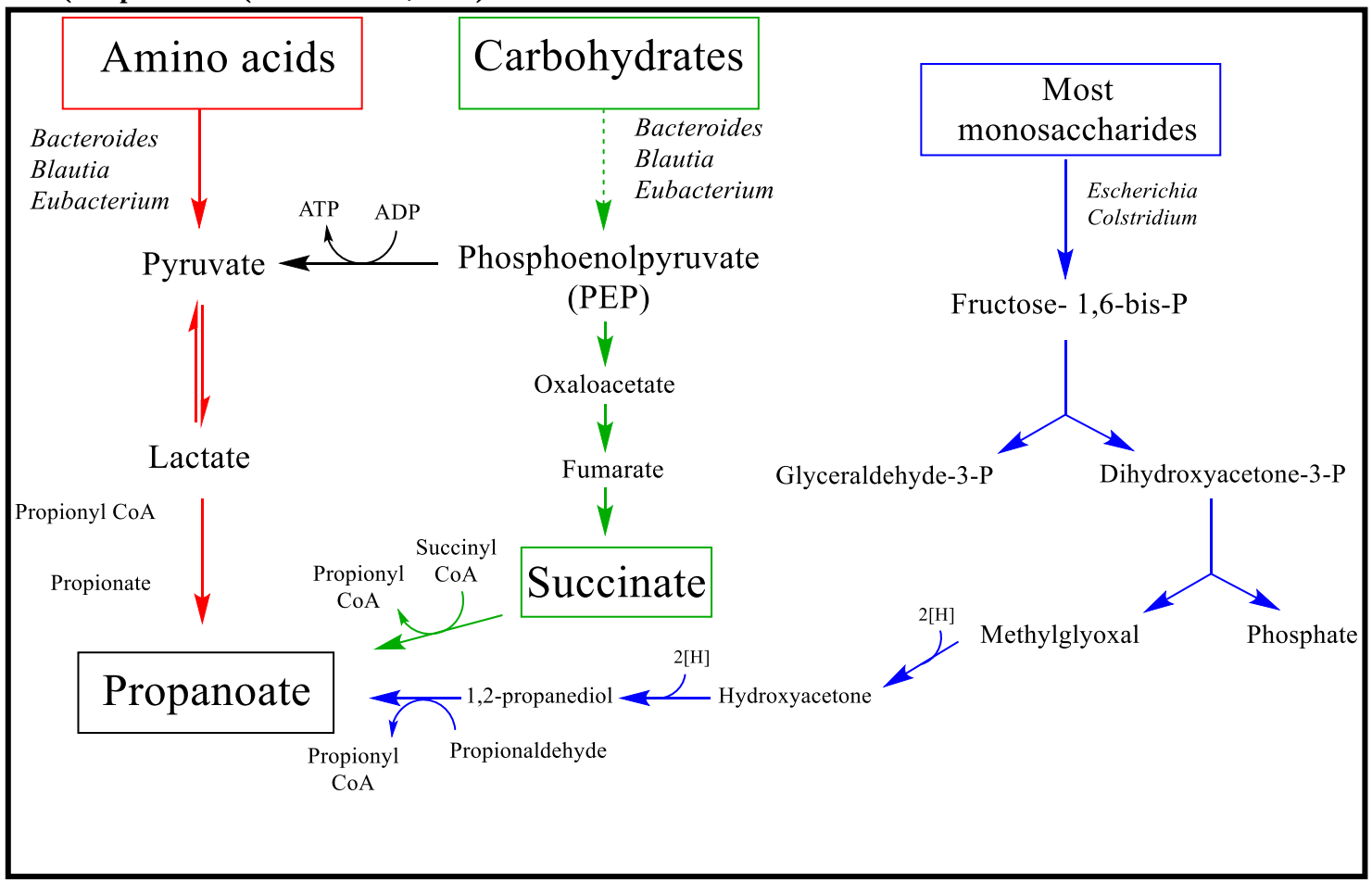


Figure 3-17 **Microbial pathways for formation of propanoate ion from carbohydrates and amino acids through succinate (green), lactate utilization (red) and 1,2-propandiol (blue) pathways**
(Adapted from (Louis & Flint, 2017).



In contrast, other nitrogenous compounds *viz*. gamma amino butyric acid (GABA) increased significantly in coffee extract amended cultures over time compared to a reduction in case of blank (Fig. 3-18), likely attributed to bacterial fermentation of plant derived amino acids i.e. L-glutamic acid to GABA [136]. Whether an increase in GABA levels occurs similarly in the gut upon fermentation of coffee is unclear, but if so it will contribute to a biological effect yet to be determined in its extent. GABA is an inhibitory neurotransmitter that has many reported pharmacological effects. It also is involved in various neurological disorders including epilepsy, seizures, convulsions, Huntington's disease, and Parkinsonism [137]. The gut microbiota related brain axis effect (Brain-Gut-Microbiome Axis) is a hot topic regarding all CNS disorders, and alterations in brain-gut-microbiome communication is found to be involved in the pathogenesis of several disorders [138].

Other detected nitrogenous compounds include cadaverine derived from lysine via its decarboxylation [139]. Gut microbiota is reported to synthesize cadaverine under high protein diet [140]. This is in general related to biogenic amino compounds including putrescine and spermidine produced *via* decarboxylation of other amino acids (Fig. 3-19).

Polyamines have been reported to stimulate cell division of gut microbiota, e.g., of *E.coli* [141] and also to impart health benefits to the host such as regulation of growth and aging, and prevention of metabolic and neurodegenerative disorders [142]. Microbiota cell uptake of polyamines may explain the lower cadaverine levels at 24 h. However, the relatively high levels of putrescine may indicate biosynthesis overweighing microbiota cell uptake and can be explained by the fact that putrescine is synthesized from ornithine [142, 143] and showing an increase with incubation time (Fig.3-20**)**. Polyamine generation reaction is found to favor of SCFA synthesis, driven by certain bacterial strains such as *E.coli* [144] also present in this microbial consortium (Table 2-3). In this study, a reduction in putrescine was observed to be concurrent with higher lactic acid and succinic acid levels in certain cultures, which support the competition between polyamine and SCFA synthesis pathways on amino acid substrates.

Figure 3-18 **GABA accumulation pattern in Black coffee (A), Green coffee (B), and Blank (H) at 0.5 h and 24 h harvest time (both at 0.5mg/mL and 5.0 mg/mL dosing level)**

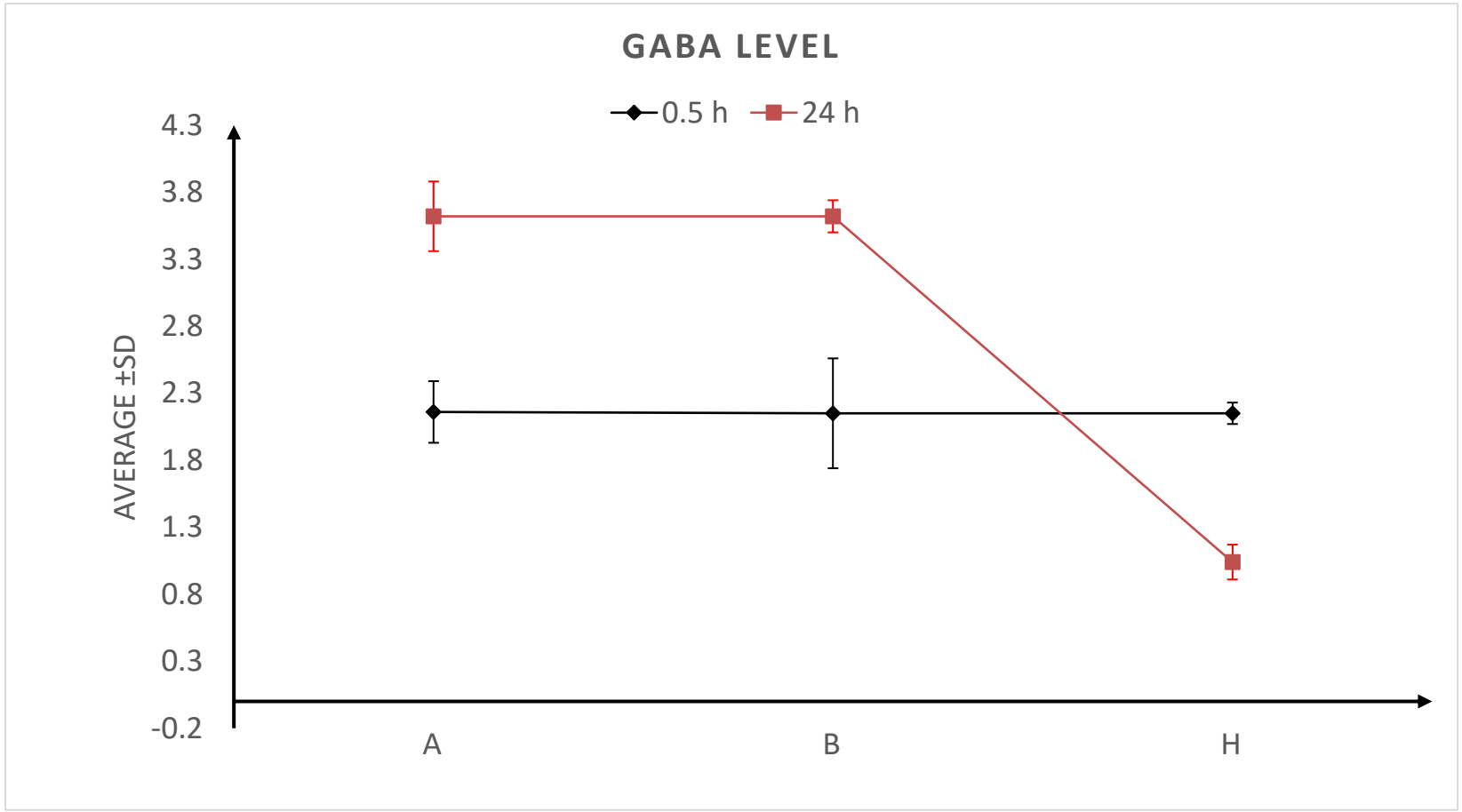


Figure 3-19 **Cadaverine accumulation pattern in Black coffee (A), Green coffee (B), and Blank (H) at 0.5 h and 24 h harvest time (both at 0.5mg/mL and 5.0 mg/mL dosing level)**

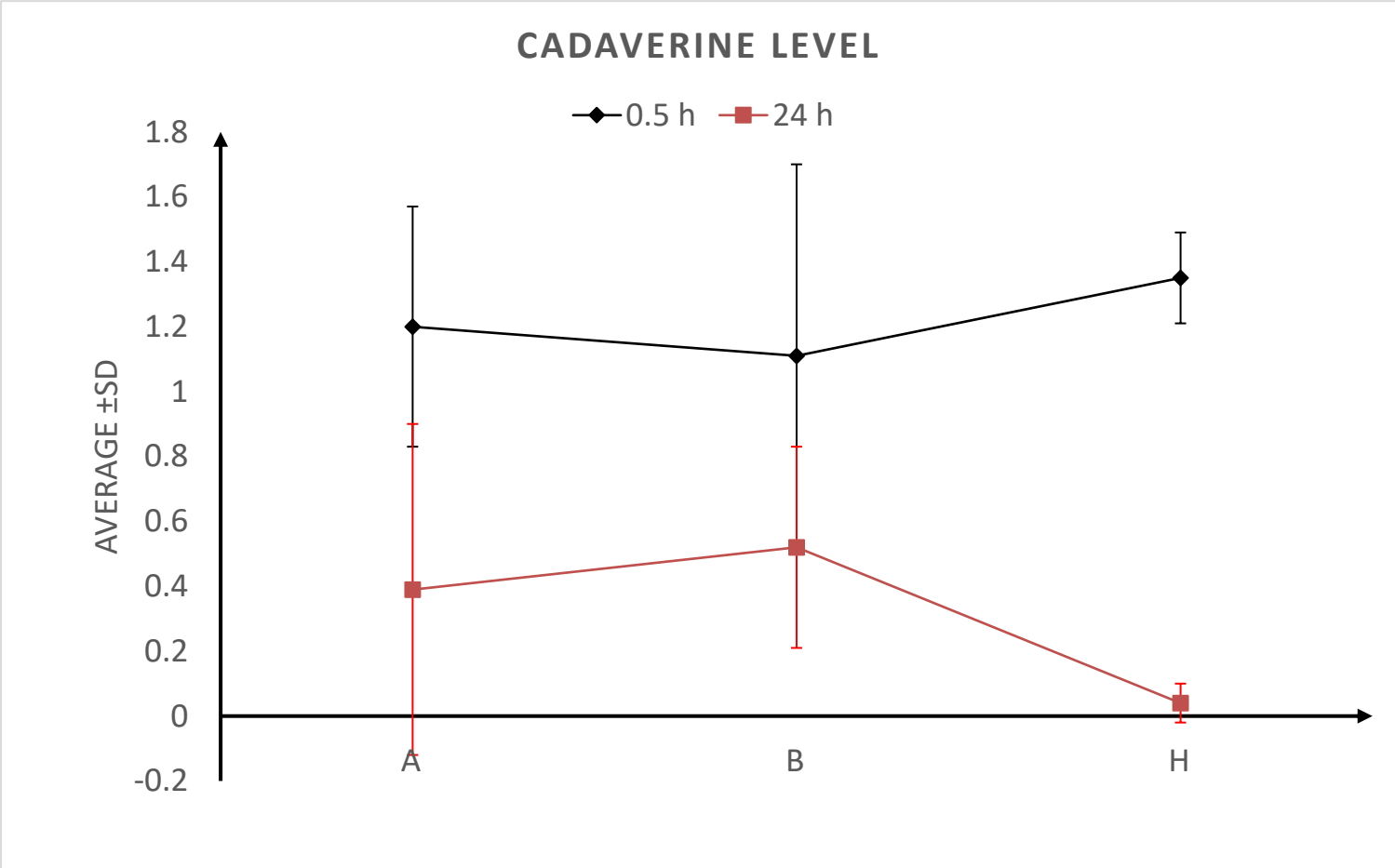
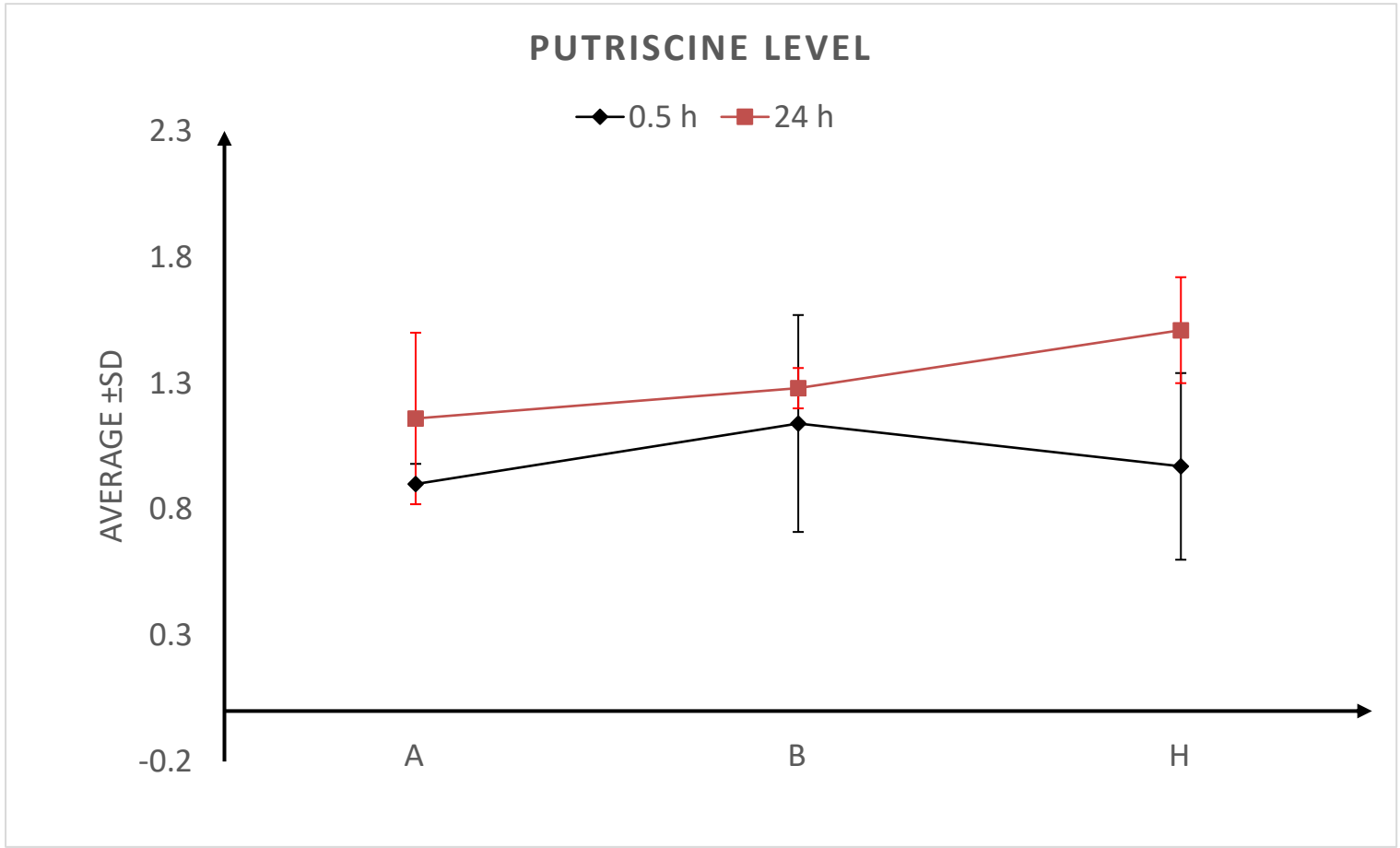


Figure 3-20 **Putriscine accumulation pattern in Black coffee (A), Green coffee (B), and Blank (H) at 0.5 h and 24 h harvest time (both at 0.5mg/mL and 5.0 mg/mL dosing level)**



3.4.1.3 Sugars

Sugars identified included monosaccharides, disaccharides and sugar alcohols. Generally, sugars showed lower abundance 24 h post incubation as expected due to their utilization as energy source and as carbon source for the generation of SCFA [145] as evidenced in strains of Bifidobacterium [146] (Table 3-3**; Fig. 3-21)**.

A neuroprotection sugar that could exert its effect at the gut level is trehalose which showed one of the most pronounced decreases, being completely depleted upon incubation likely degraded by trehalase enzyme secreted by the microbiota [147]. Only oral intake of trehalose subsequently influenced by gut microbiota but not i.v. injection was found to exert the reported neuro-protection. An increasing body of evidence for gut microbiota effects on CNS suggests that trehalose exerts its neuroprotective role through microbiota-gut-brain signaling [148]. BC cultures showed the highest levels of trehalose at 4-5% followed by GC (3-4%). Sugar abundance appeared to be dependent on the functional food type *i.e.*, 3-deoxy-D-arabino-hexonic acid found exclusively in GC & BC Table 3-3. The type of carbohydrates in functional foods was reported to influence gut microbiota composition [56].

3.4.1.4 Organic Acids

Microbiota is known to metabolize carbohydrate into short chain fatty acids (SCFA) i.e. lactic acid to be used as an energy source [149]. Also proteins and free fatty acids act also as precursors for SCFA production by microbiota [129]. For example, glycine, glutamate, ornithine and threonine act as precursor of acetate, whereas threonine, lysine and glutamate yield butyrate, and threonine can be used as substrate for propionate production [129]. A *ca*. 2-fold increase in SCFA levels was observed upon incubation in all coffee amended cultures. It was not observed in blank untreated culture (Table 3-3**, Fig. 3-22**).

Figure 3-21 **Sugar compounds accumulation pattern in Black coffee (A), Green coffee (B), and Blank (H) at 0.5 h and 24 h harvest time (both at 0.5mg/mL and 5.0 mg/mL dosing level)**

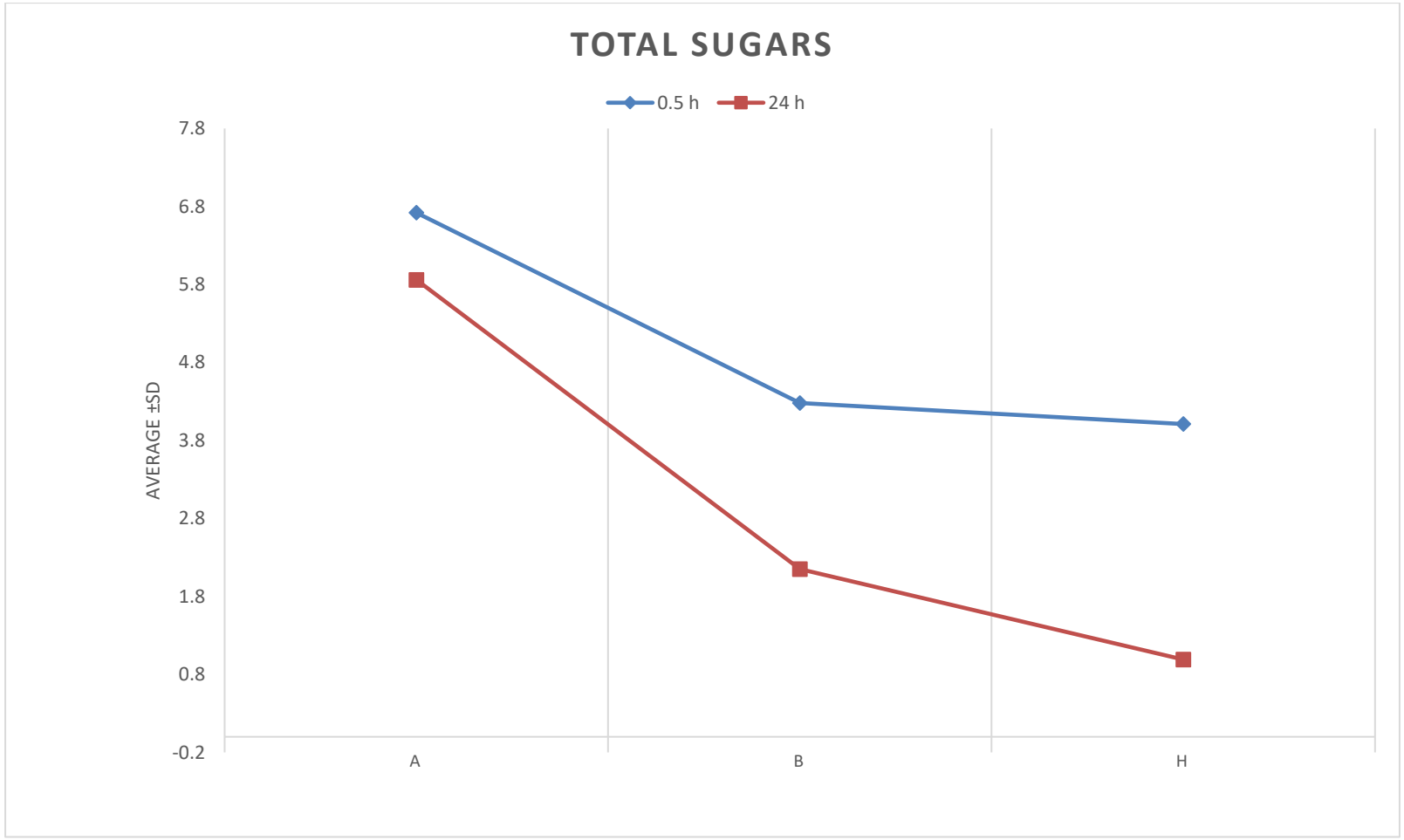
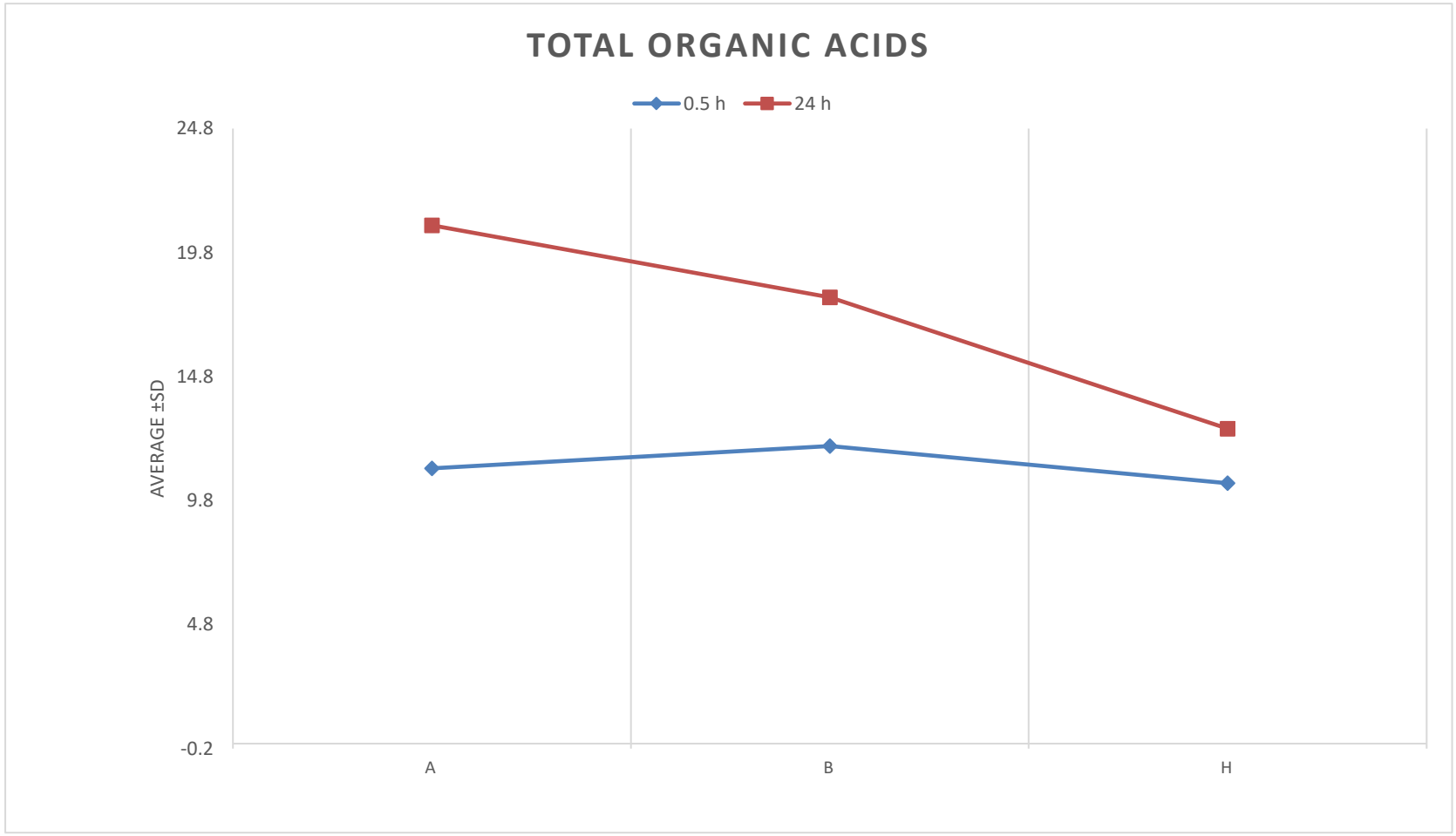


Figure 3-22 **Organic acid compounds accumulation pattern in Black coffee (A), Green coffee (B), and Blank (H) at 0.5 h and 24 h harvest time (both at 0.5mg/mL and 5.0 mg/mL dosing level)**

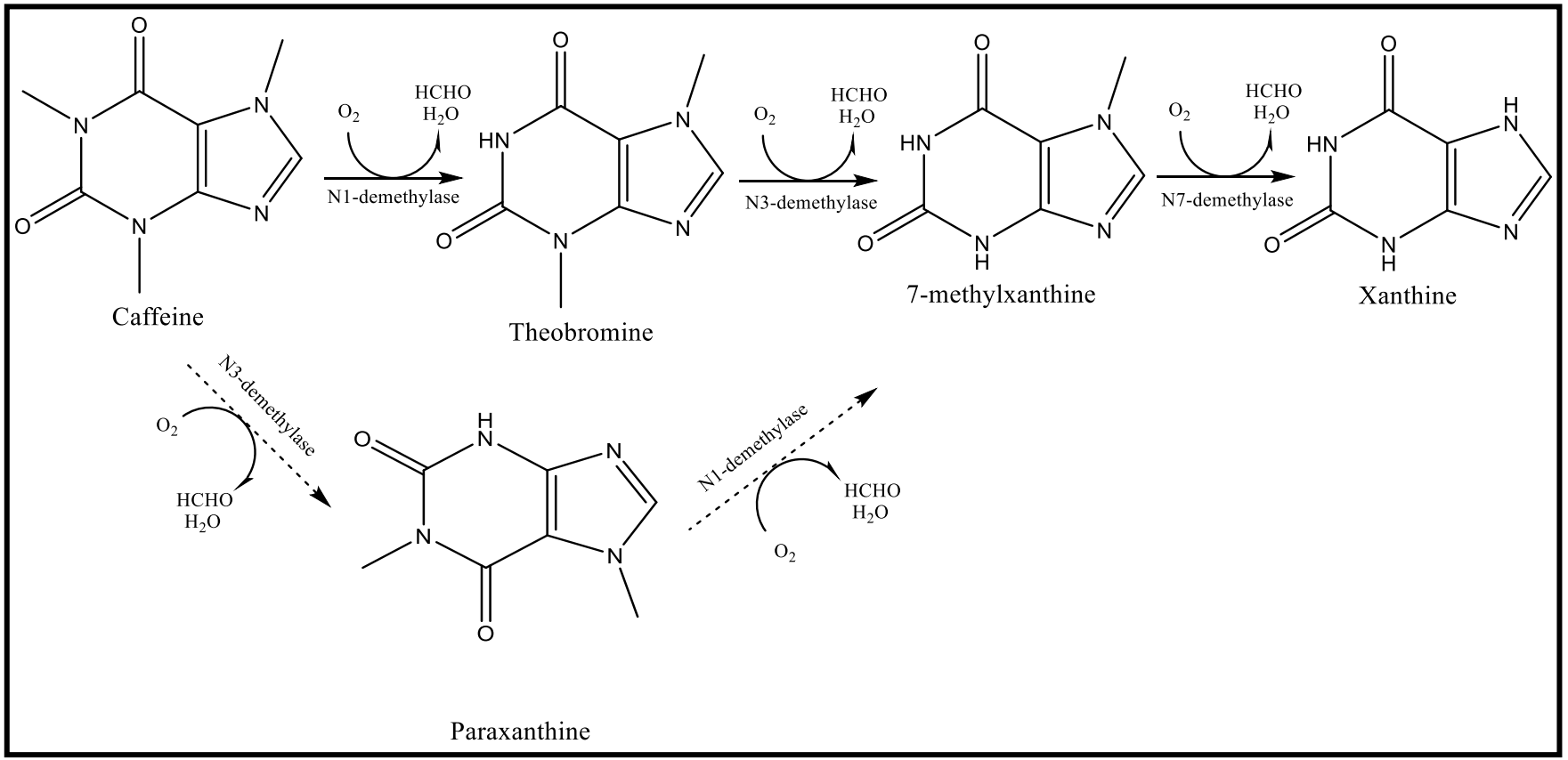


A major acid that showed such a pattern is succinic acid, likely produced as a sugar fermentation product [150-152]. Elevated succinate levels within the gut lumen have been associated with microbiome disturbances (dysbiosis), as well as in patients with inflammatory bowel disease (IBD) [153]. Other sources of succinic acid in the body involve the tricarboxylic acid (TCA) cycle within host cells, though unlikely to function here under hypoxic conditions. Lactate, another carbohydrate fermentation product [154] mediated *via* lactic acid bacteria (LAB) *i.e. Lactobacillus plantarum*, [155] also increased upon incubation at 24 h compared to trace levels in blank.

3.4.1.5 Nucleic acids

Nucleic acids were enhanced *i.e*. purines were detected in GC 24 h post incubation. Increase in its levels is attributed to gut microbiota metabolism effected by or derived from caffeine catabolism, with caffeine found to be enriched in coffee. Nucleic acids showed a general pattern of an increase upon incubation in GC, BC fortified cultures and blank as observed in case of uracil and to a lesser extent in adenine (Table 3-3). Hypo was also detected in all coffee amended cultures as well as in blank as a byproduct of microbial oxidation of nucleic acids through a salvage pathway to act as a nitrogen source as well as energy sources and promoting protective functions to the colonic epithelium [151, 156]. Positive correlations of hypoxanthine and uracil with *Bacteroidetes species Alloprevotella* [157] was reported. In contrast, xanthine was detected exclusively in GC and BC amended cultures as a metabolic microbiota product of methylxanthines *i.e*. caffeine and theobromine enriched in green coffee [158, 159] (Fig. 3-23).

Figure 3-23 Microbial metabolism of methyl xanthines



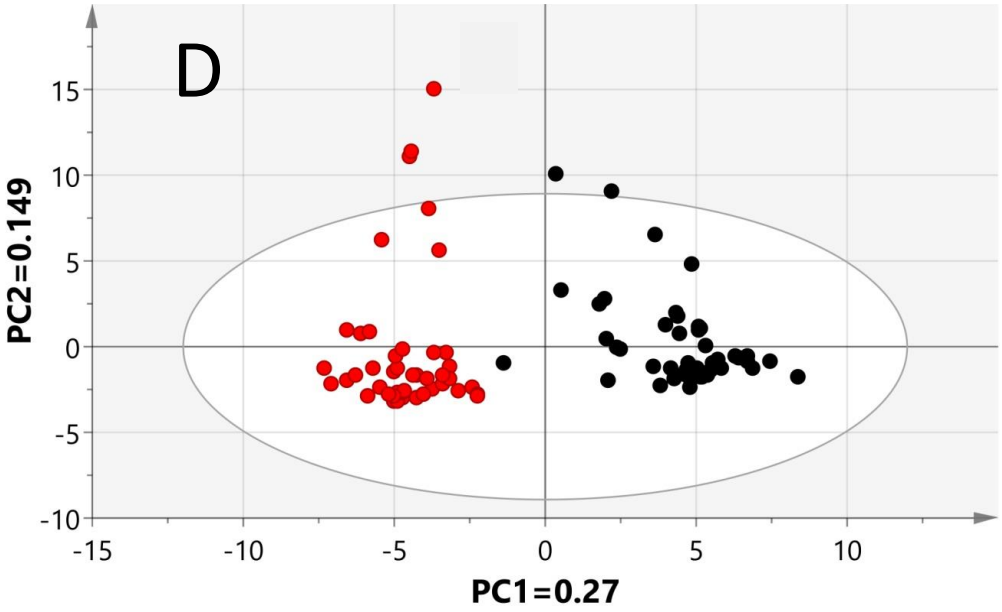
3.4.2 Unsupervised and supervised multivariate data analyses of GC/MS dataset

Multivariate data analyses were employed to further determine the impact of treatment on microbiota metabolism in an untargeted manner. Untargeted multivariate analysis tools such as principle component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) can help reveal for differences between samples and postulate hypothesis related to the effect of applied functional food on microbiota metabolism. Principle component analysis (PCA) is an untargeted multivariate analysis tool that is used to bring multidimensional datasets into a two-dimensional plane that can be graphically represented as scatter plots. In the two-dimensional plane, data point coordinates on principle component 1 (PC1) and PC2 vectors are assigned to account for the maximum variability of multidimensional data [160, 161].

3.4.2.1 PCA analysis of the whole dataset

Investigation of the impact of different functional foods (including green and black coffee) on microbiota metabolism was investigated in our previous work [162]. PCA was applied to the whole sample dataset to determine metabolome heterogeneity among all samples examined with no sort of samples classification. The principle component PC1/PC2 score plot derived from the whole GC-MS data accounted for 42% of the total variance (R2). This is not a high value and shows that variance between samples is high and needs more dimensions to cover all aspects. The whole pool of samples in the score plot, however, could be segregated into two big clusters distributed along PC 1 (Fig. 3-24). These two major clusters appeared to be based upon incubation time that is (0.5 h versus 24 h incubation time as revealed in (Fig. 3-24**)**, suggesting this to be the most-variable parameter overcoming others. This has pinpointed that monitored metabolites are mostly derived from microbiota culture and not much represented by metabolites from amended functional foods since either of the identified big clusters (Fig. 3-24) contained functional food extract treated samples along with their corresponding blank samples.

Figure 3-24 **PCA analysis of GC/MS metabolites dataset at 0.5 and 24 h for different functional food treatments (including BC & GC) and blank classified based on incubation time (0.5 h [Black], and 24 h [Red])**



HCA analysis confirmed PCA results (Fig. 3-25) by showing clear samples segregation aliquoted at 0.5 h (highlighted in a green box) compared to samples harvested at 24 h (blue box) disregarding treatment type (either blank or functional food treated samples) or dose level (0.5 mg/mL & 5 mg/mL). Results of whole sample data set modelling either from PCA or HCA fall in agreement with another report showing the impact of transient time on gut microbiota metabolites composition [131].

To pinpoint for metabolites mediating for such segregation and contributing to samples segregation with time course, the corresponding loading plot was inspected. Among detected metabolites, amino acids *i.e.* phenylalanine, ornithine and to a lesser extent organic acids, especially lactic acid, accounted for most of the variability observed along PC1 (**Fig. 3-26**).

3.4.2.2 PCA classification of GC and BC versus blank

Another PCA attempt was adopted to model each of green coffee and black coffee extract amended culture vs. the blank at the two time points at 0.5 and 24 h (Fig. 3-28).

Both functional food and blank samples aliquoted at 0.5 h were positioned on the right side of PC1 with positive p values. Whereas its counterpart aliquots harvested at 24 h were aligned on

the left side of PC1, with an overall covered variance from PC1 and PC2 from each dataset (Fig. 3-28) showing in general higher variance coverage than of the whole sample dataset (Fig. 3-24). The score plots of BC (**A**) and GC (**B**) treated cultures showed consistent patterns of samples segregation being mostly based upon harvest time. Both blank and treated samples aliquoted at 0.5 h showed overlapping masses clustering at the positive side of PC1 versus samples aliquoted at 24 h showing two well-separated clusters distributed along PC2, which indicated no metabolome difference detected at the 0.5 h harvest point between blank samples and functional food. The separation of BC and GC treated samples at the positive side of PC2 versus its blank samples being located at the opposite side suggested for contributions of functional food metabolites or its biotransformed products in such a discrimination.

Figure 3-25 **HCA plot of whole various functional food treated sample dataset showing classification of samples based on incubation time. Samples harvested at 0.5 h are shaded in green and lay on the left side of the HCA plot. Samples harvested after 24 h are shaded in blue and lay on the left side of HCA plot irrespective of their origin or dosing concentration**

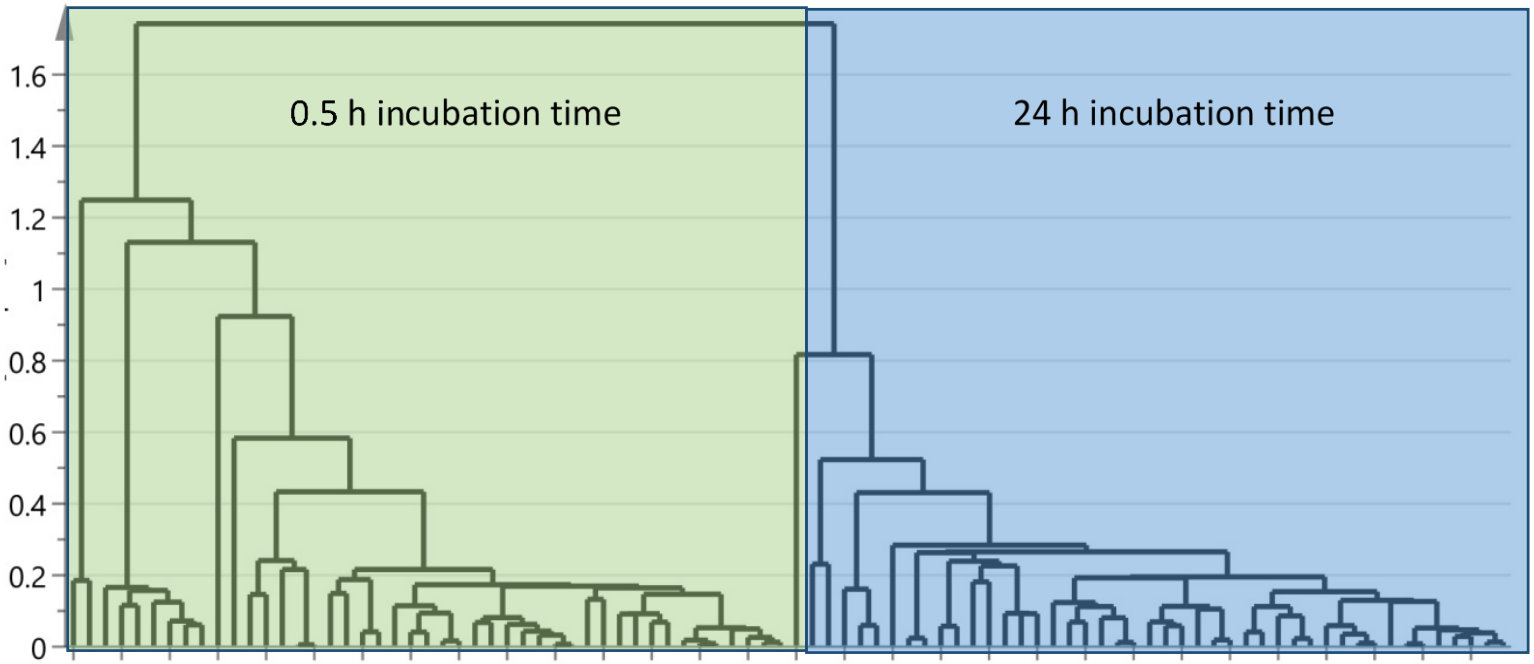


Figure 3-26 **PCA Loading plot of whole various functional food treated sample dataset presented in Fig. 3-23**

Figure is showing metabolites prevalent at the 0.5 h incubation with positive p value (to the right) metabolites prevalent at the 24 h with negative p value (to the left).

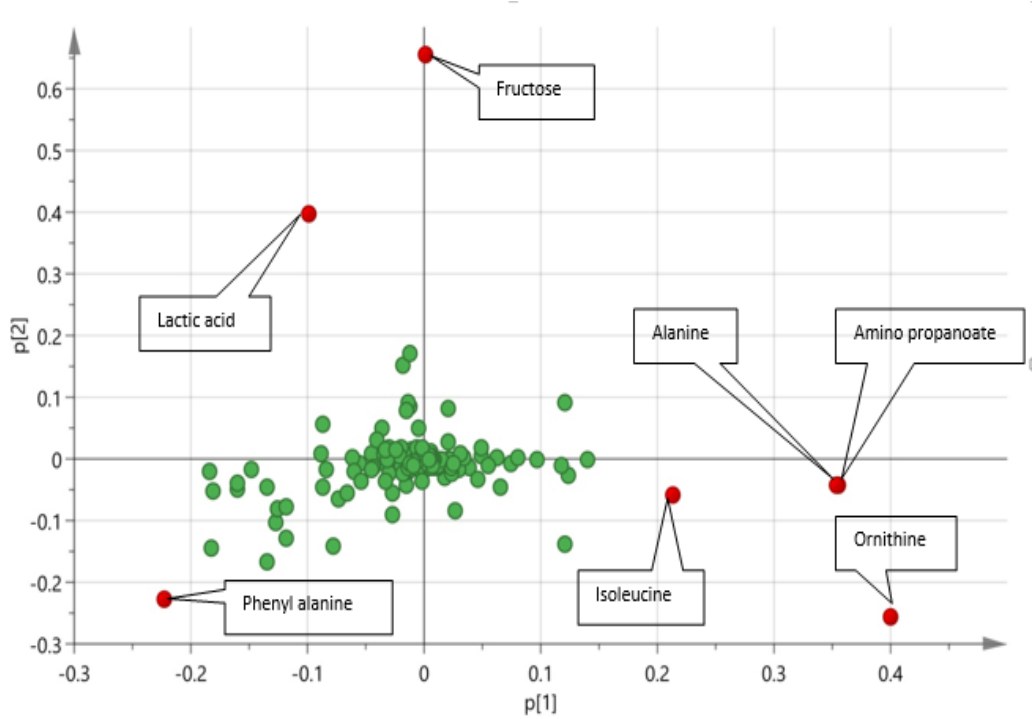
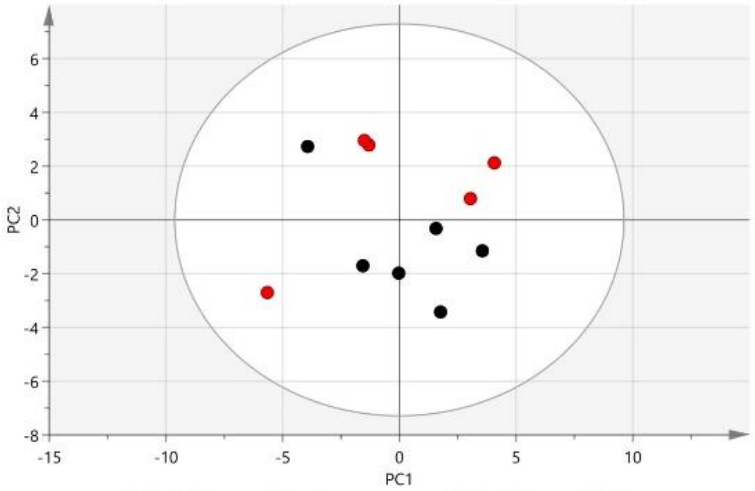
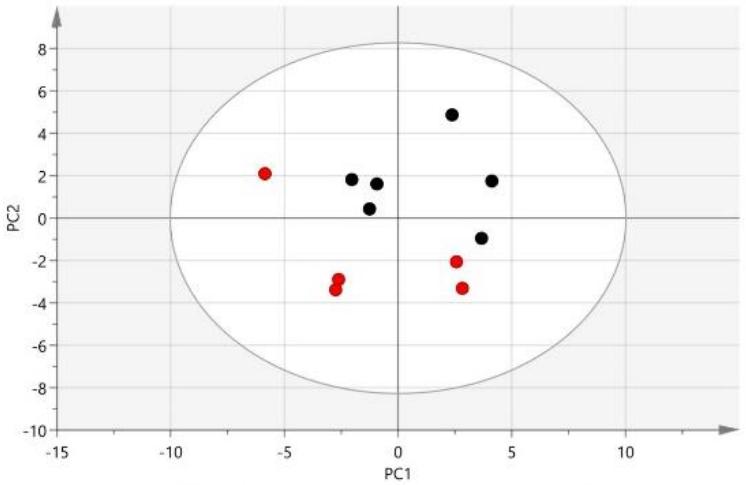


Figure 3-27 PCA score plot of black coffee (A), green coffee (B) treated samples (black) and its blank (red) at 0.5 h



PC1=0.27 PC2=0.16

A



PC1=0.31 PC2=0.21

B

3.4.2.3 PCA classification of BC and GC versus blank at 0.5 h

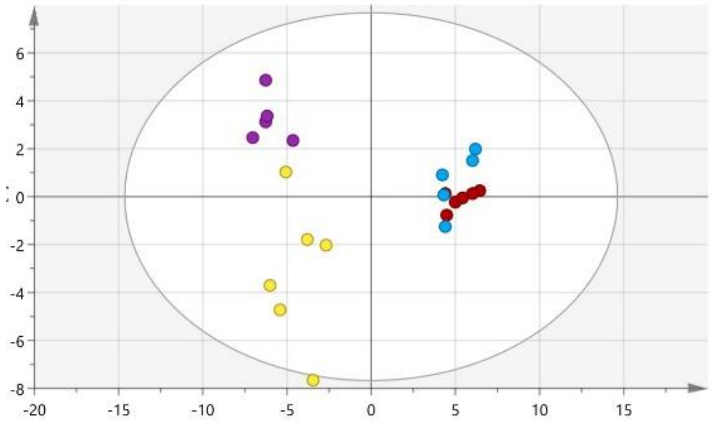
To better reveal for contributions from untransformed BC and GC metabolites in sample segregations as observed in Fig. 3-28, we attempted to model each of BC and GC culture amended along with its corresponding blank sample aliquoted at 0.5 h, a time at which no significant biotransformation by microbiota is expected to occur (Fig. 3-27). For BC (A), GC (B), lower variance coverage was revealed compared to that in (Fig. 3-28), and overlap between treatment and blank samples at both dose levels indicate that the functional food metabolite interference at 0.5 h is negligible.

3.4.2.4 OPLS-DA classification based upon incubation time at 0.5 h versus 24 h

Considering that unsupervised data analysis failed to provide clear segregation in response to treatment for each respective time point, supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was further employed to model each treatment separately viz. BC, GC. Samples were classified as such by pooling samples for each treatment for the 2 dose levels 0.5 and 5 mg/ml as one class group at 0.5 h (a) versus 24 h (b) as another class group (Fig. 3-29).

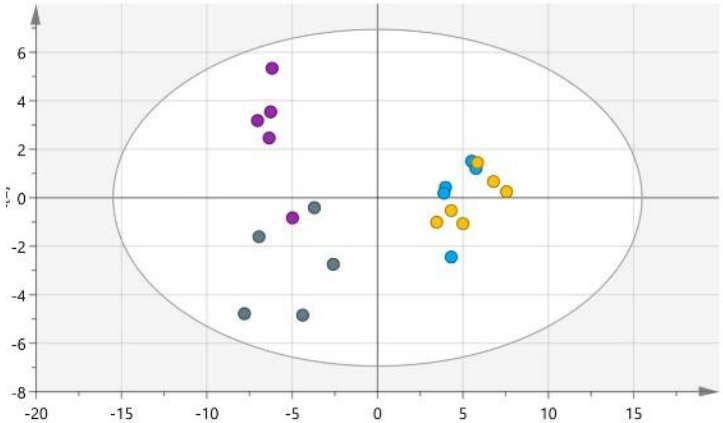
Figure 3-28 **PCA score plot of black coffee (A) and green coffee (B) treated samples against blank at 0.5 and 24 h (24 h to the right and 0.5 h to the left)**

A



PC1=0.46 PC2=0.13

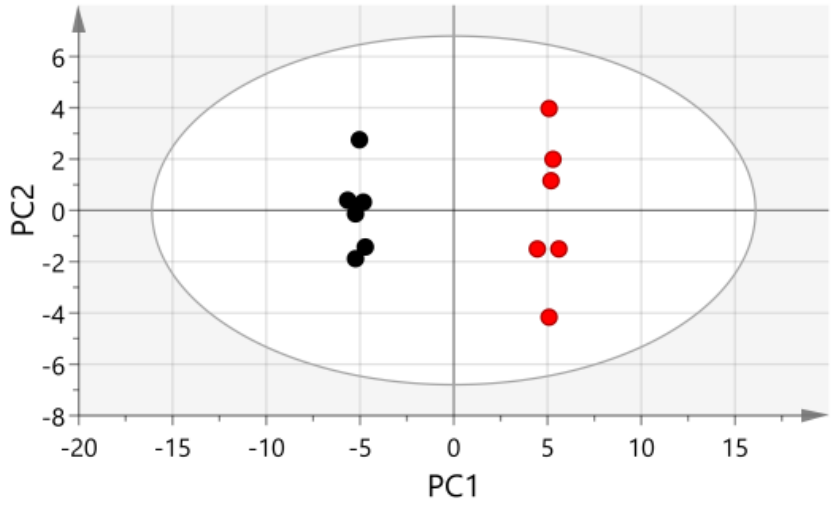
B

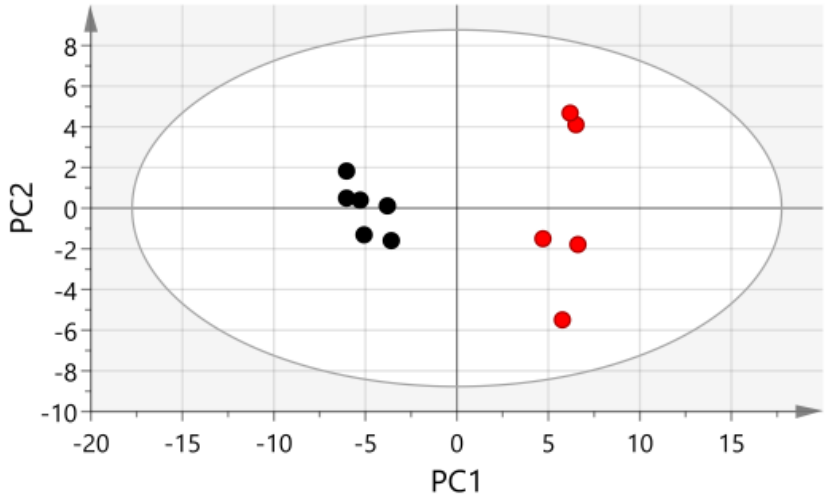


PC1=0.51 PC2=0.10

Figure 3-29 **OPLS model of black coffee (A) and Green coffee (B) classified based upon incubation time 24 h. Samples (Red color) modeled against 0.5 h (Black color) for both dosing levels of 0.5 mg/mL and 5.0 mg/mL**

A B





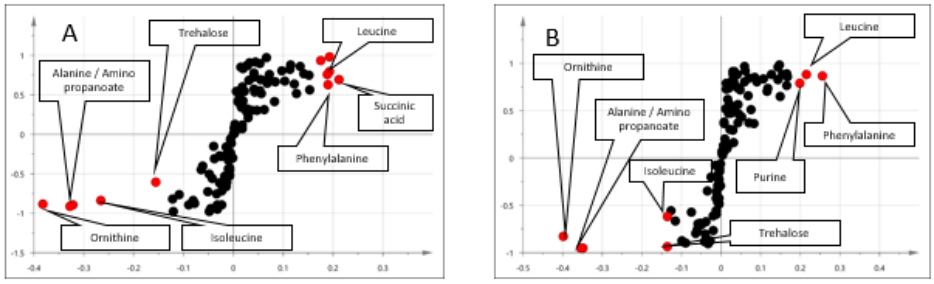
Compared to PCA (Fig. 3-28), each OPLS model showed clearer sample segregations (Fig. 3-29). Model validation was based on estimating the total variance (R2), prediction goodness parameter (Q2) and p-value as detailed in (Table 3-4). All of the models showed high repeatability, prediction and significantly low regression p-values suggestive of no overfitting of these models. Investigation of the S-loading plot allows to visualize both the covariance and the correlation structure between the X-variables and the predictive score t[1] [163]. S-Plot for each respective OPLS-DA model (**Fig. 3-30**) revealed for multiple findings. Metabolites with a positive p [1] value indicate an increase upon incubation, whereas metabolites with a negative p[1] value indicate higher abundance at 0.5 h and a decrease upon incubation (**Fig. 3-30 A-B)**.

Table 3-4 OPLS Model total variance and prediction goodness parameters of coffee treated samples and blank classified based upon incubation time

Treatment or model	Total variance (R2)	Prediction goodness parameter (Q2)	p-Value
Black coffee (BC)	0.996	0.963	0.000
Green coffee (GC)	0.976	0.913	0.000
Blank	99.9	98.1	0.000

Metabolites belonging to amino acids/nitrogenous compounds i.e., alanine, amino propanoate, isoleucine, valine and ornithine exhibited significant model influence at 0.5 h (negative p[1] on S-plot). In contrast, other metabolites related to the same classes such as leucine, methionine, glutamic acid and phenyl alanine showed significant model influence at 24 h (positive p[1] on S-plot). These findings propose mixed microbial metabolic activities that are either to utilize some of these amino acids as a substrate to form other compounds (catabolism) [129] or to be synthesized from other metabolites (anabolism). To provide stronger evidence for metabolites reprograming and to understand metabolic pathways.

Figure 3-30 **S-Plot of OPLS model of black coffee; BC (A) and green coffee; GC (B) at 0.5 (to the left) and 24 h (to the right).**



Other than primary metabolites converting into SCFA, succinic acid showed an increase with time (+ve p value in S-plot) acting as fermentation product of secondary metabolites. e.g., chlorogenic acid (CA) was reported to be fermented into succinic acid by microbiota [164], and could account for its 2.4-fold increase in BC at 24 h (Table 3-3). CA is the main active constituent in coffee recognized as its slimming factor.

3.4.2.5 OPLS-DA classification based upon functional food type versus blank at 24 h

Considering that all of the above metabolic changes (**Fig. 3-30**) were related to the time course metabolism of microbiota and to help identify other metabolic changes specifically influenced by certain functional food treatment, OPLS-DA was performed by modelling each treatment at 24 h versus its blank sample at the same point for the 2 different dose levels. Derived S-plots for each case are presented in (Fig. 3-31) and with metabolites that showed the strongest variation influence as reflect by their p-values listed in Table 3-5.

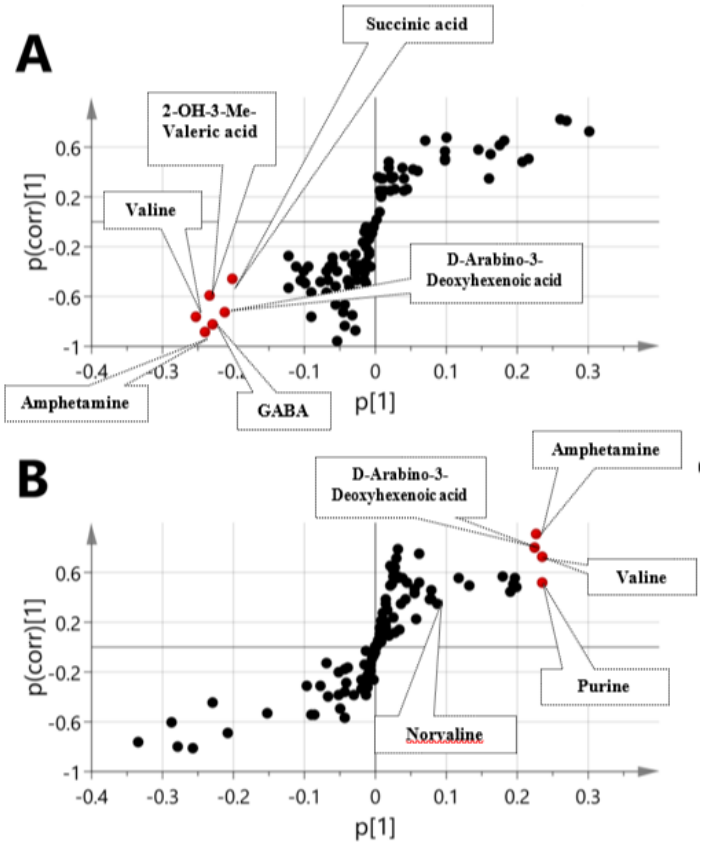
Table 3-5 **Major metabolites differentiating coffee treated samples against blank at 24 h post incubation as revealed from OPLS analysis.**
Symbols (++++) indicate very high influence of S-plot, (+++) high influence, (++) intermediate, and (+) low influence, Black coffee (BC), Green coffee (GC).

Metabolite	Category	BC	GC
Lactic acid	Acid	(+)	(++)
Succinic acid		(++)	(++)
3-Deoxytetronic acid			(+)
Isoleucine	Amino acid	(+)	(++)
Threonine		(+)	
Ornithine		(+)	

Valine		(++)	(++)
GABA	Nitrogenous compound	(++)	(+)
Amphetamine		(++)	(++)
Norvaline ester derivative		(+)	
Purine	Nucleic acid		(++)
Uracil			(+)
2-Hydroxy-3-methylvaleric acid	Phenolic	(++)	(++)
D-arabino-3-deoxy Hexonic acid	Sugar	(++)	(++)
Unknown sugar		(+)	

Metabolites such as sugars, organic acids, i.e. lactic and succinic acid, and GABA were detected as significant contributors to variation in almost coffee treated cultures compared with blank samples at 24 h (Fig. 3-30**A-B**). Gut microbiota such as *Lactobacillus* species and *E.coli* have been reported to secrete GABA as an acid resistance mechanism at low pH which may explain co-appearance of GABA with organic acid increase and other low acidic metabolites such as polyphenols in OPLS –derived S plot of several functional food treatments (Fig. 3-30**A**). GABA can also be utilized by bacteria as carbon source through conversion to succinate which enters the tricarboxylic acid cycle [165] and this may explain the coexistence of GABA and succinic acid (r2=0.40) as strong influencers in S-Plots of functional food treated samples.

Figure 3-31 **S-Plot of OPLS model of black coffee; BC (A) and green coffee; GC (B) versus blank at 24 h.**
Metabolites with higher abundance in functional food treated samples have positive p value in BC and negative value in GC



4 Chapter 4: Conclusion

This study presents on volatiles profile in coffee and its blends, and further its different brews analyzed using HS-SPME coupled with GC/MS. Such setup identified several volatiles in coffee brews and revealed for differences associated with coffee seeds origin, roasting degree, brewing method, and effect of other condiments. In addition, data processing with MVA revealed for several volatile markers strongly associated with coffee seeds genotype exemplified by pyrazines, furans, and aromatic hydrocarbons compounds more abundant in *Coffea robusta* than *Coffea arabica*. Markers to reveal for coffee roasting indices included aromatic hydrocarbons and furans. Instant coffee showed the most distinct aroma profile represented by guaiacol derivatives along with furans as 1-methylfurfural due to its different processing nature. Brewing method as a less influencing factor on coffee aroma revealed that coffee decoction marked by the abundance of monoterpenes *i.e.*, limonene, and sesquiterpenes. germacrene, cubebene and bergamotene. However, higher esters and alcohols were markedly

correlated with the mild preparation methods including infusion and maceration, respectively. Fortification of coffee with condiments, *i.e.*, cardamom, prepared by decoction showed higher terpinyl acetate than plain coffee samples. Hence, the presence of cardamom has proven its function improving coffee aroma by overcoming the smoky odor of heat-induced products, especially in case of aromatic hydrocarbons. All these markers associated with coffee samples provided a valuable tool for determining coffee brews quality more likely to be associated with consumers' preference towards certain type of coffee exemplified by analyzing commercial samples in the Middle East region for the first time. As a future direction, LC/MS represents a potential tool for targeting non-volatile metabolites in brews, including primary and secondary metabolites. This work should complement the current study in revealing coffee organoleptic characters, including the taste, nutritive values, beside its health benefits.

Silylated-GC/MS results pointed out that sugars played a major role in differentiation of coffee samples, where fructose showed higher abundance in green *Coffea robusta*, other sugars such as 3-deoxy-ribohexenoic acid lactone and 3-deoxyarabinohexenoic acid were more abundant in either plain roasted coffee or roasted coffee with cardamom. Moreover, cyclitol sugars as inositol and myoinositol were associated with coffee roasting. Caffeine showed an increase in roasted coffee due to its thermal stability contrary to trigonelline. Also, it showed higher abundance in *Coffea robusta* compared with *Coffea arabica*. Fatty acids as arachidic acid, palmitic acid and stearic acid were more abundant in *Coffea arabica* than *Coffea robusta*. Yet, stearic acid showed higher abundance in green coffee compared to roasted coffee, but tricaprelyl glycerine was characteristic of instant coffee. Characteristic acids of instant coffee products included phosphoric acid, lactic acid and threonic acid. Other acids such as malic acid, citric acid and acetic acid showed higher association with *Coffea arabica* compared to *Coffea robusta*.

Microbiota mediated metabolism results provide insights into gut microbiota altered metabolisms in response to green and black coffee extracts at the metabolite level and define general and specific biomarkers for each type. In general, green and black coffee amendment to gut microbiota appeared to alter its metabolism in two different scenarios. First, functional food components can serve as a substrate to microbiota metabolism as in case of purine alkaloids such as caffeine acting as precursors of purine by microbiota demethylation. An alternative mechanism is that functional food components modify the extent, existing metabolic

pathways are activated within microbiota, showing e.g. GABA production in presence of higher acidity induced by metabolites such as organic acids.

This study demonstrated the metabolic pathways adopted by microbiota in the generation of SCFA through either nitrogen metabolism of amino acids or carbon metabolism of sugars lead to an ultimate increase of SCFA under consumption of (most members) of these metabolic precursors (Fig. 4-1). However, most of these changes are related to the change the microbiota environment encounters with time and is not specific for certain functional foods with a few exceptions.

Figure 4-1 **Schematic diagram outlining the metabolic pathways adopted by microbiota to convert amino acids and sugars into SCFA through either Carbon metabolism or Nitrogen metabolism**

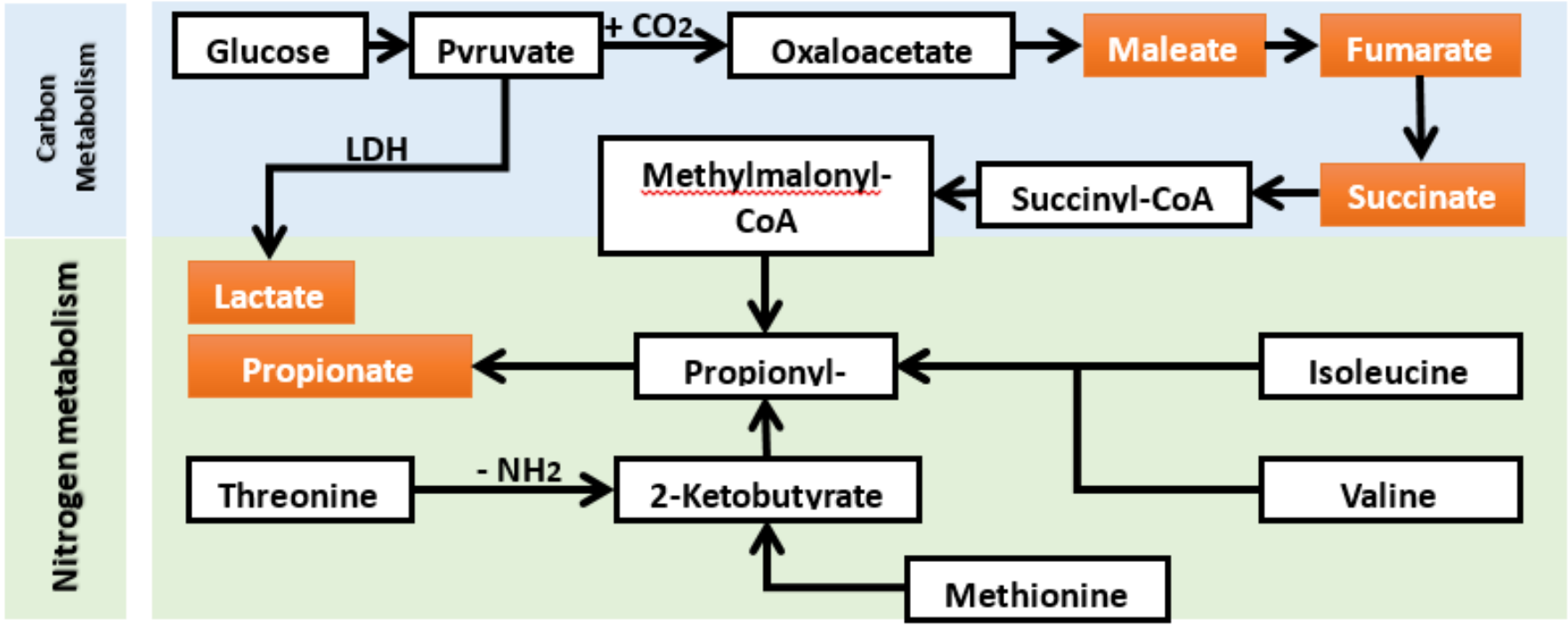


Fig. 4-1 outlines the mechanism of major changes in microbiota primary metabolite classes *viz*. sugars, nitrogenous compounds, acids and amino acids in response to coffee treatments.

Further studies that apply high resolution visualization of metabolite pools, such as nanoscale secondary ion mass spectrometry, coupled with stable isotope tracers will serve to provide further insight into the potential roles of free metabolites and their exact metabolic origin considering the complexity of microbial metabolisms. It also remains to clarify, if additional information on non-volatile metabolites, which might be captured by LC/MS, and a correlation to the strain composition changes with time, will be useful or not. These data, coupled with the ongoing outputs from rapidly developing additional "omics" studies (i.e., genomics, transcriptomics, and proteomics) will aid in further elucidating the metabolic cross-talk both within and between partners, which is essential for maintaining a functional healthy gut environment.

The above mentioned limitation of this study to metabolites amenable to GC-MS detection, i.e. those of primary origin except for a few simple phenolics, may underpin the impact of secondary metabolites not detected by this analytical platform such as plant polyphenols or not represented in this study at all as with plant terpenoids in leading to a variation in microbiota composition and metabolism. Still, clear evidence for functional food comparable metabolites composition impact on microbiota culture metabolism was observed, exemplified in xanthine rich extracts i.e. GC and BC. Further, clinical studies ought to be considered in the future by analyzing stool metabolism in humans exposed to selected functional foods for defined periods, which would provide a true translation of this in vitro yet fundamental assay results to future research in the field of functional food - gut microbiota interaction.

Building on the current research future directions shall be summarized in the below points"

- Utilization of the identified biomarkers either in SPME extracted or silylated metabolites for monitoring coffee processing parameters and differentiating coffee species in quantitative mode for QC analysis.
- Replicating the study of effect of GC and BC on microbiota metabolism using in vivo setup rather than relying on in vitro microbiota consortium
- Utilization of other analysis techniques like NMR and LC-MS for characterization of metabolite profile to widen the pool of identified metabolites and reveal much more of the metabolite gut microbiota interaction

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