Dissecting new lipids and their composition in herbal tea using untargeted LC/MS

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Abstract

Herbal teas and beverages have gained global attention because they are rich in natural bioactive compounds, which are known to have diverse biological effects, including antioxidant and anticarcinogenic properties. However, the lipidomic profiles of herbal teas remain unclear. In this study, we applied an untargeted lipidomics approach using high-performance liquid chromatography coupled with linear ion trap-Orbitrap mass spectrometry to comprehensively profile, compare, and identify unknown lipids in four herbal teas: dokudami, kumazasa, sugina, and yomogi. A total of 341 molecular species from five major classes of lipids were identified. Multivariate principal component analysis revealed distinct lipid compositions for each of the herbs. The fatty acid α-linolenic acid (FA 18:3) was found to be abundant in kumazasa, whereas arachidonic acid (FA 20:4) was the most abundant in sugina. Interestingly, novel lipids were discovered for the first time in plants; specifically, short-chain fatty acid esters of hydroxy fatty acids (SFAHFAs) with 4-hydroxy phenyl nonanoic acid as the structural core. This study provides insight into the lipidomic diversity and potential bioactive lipid components of herbal teas, offering a foundation for further research into their health-promoting properties and biological significance.

Keywords: herbal tea, lipidomics, short-chain fatty acid esters of hydroxy fatty acids, multivariate analysis, liquid chromatography, mass spectrometry.

1. Introduction

Tea, a popular nonalcoholic beverage, has been consumed worldwide for over 2000 years since its discovery in China(Pan et al., 2022). As described by the prominent Japanese author, Okakura Kakuzo in The Book of Tea, "Tea started as a medication and developed into a beverage". There are two types of tea: tea made from the leaves, leaf buds, and internodes of the plant *Camellia sinensis*, and herbal tea made from diverse plant species other than *C. sinensis*(Mandiwana et al., 2011). Herbal tea is brewed from a various tisanes, which are mixtures of dried grasses, leaves, seeds, nuts, fruits, bark, flowers, or other botanical elements that give them distinctive flavor and possible health benefits (Liu et al., 2013). Various herbal teas have been reported to have biological significance, including antioxidant, antiglycation, anti-inflammatory, antibacterial, antiviral, anti-allergic, anticarcinogenic, antithrombotic, vasodilatory, antimutagenic, and anti-aging effects (Wargovich et al., 2001).

Houttuynia cordata (heart leaf), also known as dokudami in Japanese, belongs to the family Saururaceae. This traditional herbal tea shows virucidal effects on HSV-1 (Herpes Simplex Virus), HIV (Human Immunodeficiency Virus) and the influenza virus(Hayashi et al., 1995). Moreover, it exhibits antioxidant, antibacterial, antiobesity, and immunomodulatory properties(Lau et al., 2008; Miyata et al., 2010). *Sasa vieitichi* (Japanese bamboo), also known as kumazasa in Japanese, belongs to the family Gramineae. It is used in traditional medicine in Japan and has been shown to possess anticancer, antiulcer, antiviral, antioxidant, anti-inflammatory, and antiobesity properties(Ichimaru et al., 2020). In addition, kumazasa extract was found to reduce obesity-induced insulin resistance (Yoshioka et al., 2017). *Equisetum arvense* (horsetail), also known as sugina in Japanese, belongs to the family Equisetaceae. It has traditionally been used to treat hemorrhage, urethritis, jaundice, and hepatitis (Jinous Asgarpanah, 2012), and has been shown to have antioxidant, anticancer, antimicrobial, antidiabetic, antinociceptive, anti-inflammatory, and antileishmanial properties(Cetojević-Simin et al., 2010; Hassan HF, 2014). *Artemisia princeps* (first wormwood), known as yomogi in Japanese, belongs to the family Asteraceae and has anticancer, anti-inflammatory, and antioxidant effects (Ding et al., 2018).

The bioactive components of these plants can confer all their health-promoting qualities when consumed as tea. Eupafolin, a flavonoid found in *A. princeps*, induces apoptosis of human cervical adenocarcinoma-derived HeLa cells via a caspase-dependent pathway(Chung et al., 2010). A metabolomics study of four South African herbal tea varieties

showed that they contain bioactive compounds such as flavonoids, phenolics, lignans, and megastigmane glycosides, making them suitable for use as health-promoting drinks (Malongane et al., 2018). To date, many studies have focused on the metabolomics of herbal teas. However, investigations of lipids in herbal teas are relatively limited. Lipids are essential components of plants that have a wide variety of functions, both as structural components of the plasma membrane and as bioactive substances with signaling properties (Suh et al., 2022). Bioactive lipids, including PUFAs, carotenoids, phytosterols, and fat-soluble vitamins, exhibit significant biological activities, which can potentially aid in the prevention and management of lifestyle-associated diseases (Rodríguez et al., 2016; Sokoła-Wysoczańska et al., 2018). Hence, the analysis of lipids in food and the identification of novel compounds are of great research interest.

Lipidomics is a technique that is widely used to characterize lipids in foods. In our previous studies, we have applied this technique to identify lipids such as furan fatty acids, *N*-acyl lysophosphatidylethanolamine (LPE), and acyl sterol glycosides, as well as to comprehensively profile the lipids in salmon, shellfish, seaweed, and beans (Gowda et al., 2021a, 2022a; 2022b; Gangadhara et al., 2023). A recent study by Cui et al. demonstrated the application of lipidomics techniques to discriminate between early-spring green tea and late-spring green tea through determining the lipids as characteristic components (Cui et al., 2023). Furthermore, the effects of ultraviolet radiation and nitrogen levels on the lipid composition of tea leaves were assessed using a lipidomics approach (Liu et al., 2017). The importance of lipids and their loss during tea processing are well documented in the literature (Ravichandran & Parthiban, 2000). Li et al. applied an untargeted lipidomics approach and demonstrated that lipidomic changes occur during green tea manufacturing. This study revealed that bioactive lipids such as phosphatidic acids and glycolipids are lost during the fixation process (Li et al., 2021a). Recently, the importance of lipidomics for food quality and authenticity applications has been extensively reviewed (Tietel et al., 2023). Despite the many studies of green tea lipidomics, few have focused on the analysis of the lipid composition of herbal teas. Hence, in this study, we aimed to apply untargeted high-performance liquid chromatography coupled with linear ion trap-Orbitrap mass spectrometry (HPLC/LTQ-Orbitrap-MS) to comprehensively profile the lipid composition of four different varieties of herbal tea. In addition, previously unknown lipids were identified and characterized using MS^n spectral analysis. Therefore, this study aimed to clarify the lipid composition of herbal teas, which may

provide new insights into the bioactive lipids in health-promoting herbal teas.

2. Materials and methods

2.1 Sample collection

Herbal tea samples were sourced locally from Takayama, Japan (coordinates 36.1461° N and 137.2522° E). Dried leaves (500 g) of each species, were collected during the summer season in August. The sample names are as follows: *Houttuynia cordata* (heart leaf), known as dokudami in Japanese, *Sasa vieitichi* (Japanese bamboo), known as kumazasa in Japanese, *Equisetum arvense* (horsetail), known as sugina in Japanese, and *Artemisia princeps* (first wormwood), known as yomogi in Japanese. The dried leaves and twigs were ground into a fine powder and stored in air-tight glass vials in a freezer at -80 °C until further extraction and analysis.

2.2 Materials

All solvents, including isopropanol, chloroform, and methanol, were of liquid chromatography/mass spectrometry (LC/MS) grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1M aqueous ammonium acetate (eluent additive for LC/MS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The EquiSPLASH LIPIDOMIX quantitative standard for mass spectrometry and oleic acid-d9 were purchased for use as internal standards from Avanti Polar Lipids, Inc. (Alabaster, USA).

2.3 Lipid extraction

Lipid extraction from herbal teas was performed using a modified Bligh–Dyer method (Bligh, E.G. and Dyer, 1959; Gowda et al., 2022a). In summary, 20 mg of herbal tea powder (n = 5) was weighed out and transferred to a 2 mL Eppendorf tube. A mixture of 300 µL of methanol and 100 µL of the internal standard (IS) was added. The IS contained pre-mixed EquiSPLASH Lipidomix (Lot: 330731-1EA-013) (1 µg/mL) and oleic acid-d9 (10 µg/mL) in methanol. The mixture was gently vortexed for 10 s, after which 200 µL of chloroform and 40 µL of milli-Q water were added. The solution was vortexed again at 3500 rpm for 5 min and allowed to stand at room temperature for 2 h. After 1 h, the mixture was again vortexed at 3500 rpm for 1 min. The lipid extract was then centrifuged at 15,000 rpm for 10 min and transferred to a new 2 mL

Eppendorf tube. Methanol and chloroform in a 2:1 ratio (*v*/*v*, 1 mL) were added to the residue and the mixture was vortexed for 5 min. It was then centrifuged for 10 min, and the liquid layer from the combined extracts was transferred to the same new 2 mL Eppendorf tube. The extract was filtered through animal charcoal (Lot no. 102K1392). Subsequently, it was placed in a centrifuge evaporator at 4 °C for 5–6 hours. Finally, the extract was redissolved in 100 µL of methanol, centrifuged at maximum speed for 10 minutes, and transferred to LC/MS vials.

2.4 LC/MS analysis

An Atlantis T3 column (2.1 × 150 mm, 3 µm, Waters, Milford, USA) in a Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan) was used for separation at a 200 µL/min flow rate and 40 °C oven temperature as per our previous report (Gangadhara et al., 2023). Gradient elution was carried out using 10 mM aqueous CH_3COON_4 (solvent A), isopropanol (solvent B), and methanol (solvent C), and the optimal gradient elution program was as follows: 0–1 min, 30% B and 35% C; 1–9 min, 75% B and 15% C; 9–21 min, 82.5% B and 15% C; 21–25 min, 95% B and 5% C; 25–26 min, 30% B and 35% C, with a total elapsed time of 30 min. The injection volume was set at 10 µL for each sample.

Mass spectrometry analysis was performed using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) in both positive and negative ionization modes, and the parameters were identical to those in our previous reports (Gowda et al., 2022b). The MS spectral scan ranges for the negative and positive ionization modes were set to *m/z* 160–1900 and *m/z* 150–1950, respectively. MS/MS spectra were obtained using collision-induced dissociation at a collision energy of 40 V.

The raw data obtained from the Orbitrap MS were processed using MS-DIAL (ver. 4.9) for lipid annotation and integration using the parameters described in our previous study(Gowda et al., 2022a). Both the MS and MS/MS spectra were examined using Xcalibur 2.2 (Thermo Fisher Scientific, Waltham, USA) to guarantee the reliable identification of lipid molecular species. Semi-quantification of the annotated lipids was performed according to the Lipidomics Standards Initiative guidelines (level 2 or level 3) based on the amount of internal standard added and the following formula: (concentration/peak area) of the internal standard = (concentration/peak area) of the analyte. The obtained concentration was normalized to the weight of the sample used for lipid extraction.

2.5 Statistical analysis

The dataset used in this study comprised the molecular lipid species in both positive and

negative ion modes. Data visualization was performed using Microsoft Excel 2021, and the results are presented as mean ± standard error (SE). Subsequently, the data were subjected to one-way analysis of variance (ANOVA) with Tukey's multiple comparison test ($p < 0.05$) to identify statistically significant variables. MetaboAnalyst (version 5.0) was used to conduct cluster correlation analysis. Principal component analysis (PCA) was performed using SIMCA version 17.0.2 (Sartorius Stedim Biotech Umetrics AB, Umeå, Sweden) to obtain an overview of the variation in the data.

3. Results and discussions

3.1 Lipid profile of herbal teas

Using the precise masses and MS/MS behavior established using the software MS-DIAL, an untargeted lipidomic study of four different herbal tea species resulted in the annotation of 341 lipid molecular species. Subsequently, relative quantification of the MS/MS-confirmed lipids was performed using the internal standard method. The results of the untargeted lipidomic analysis of the four herbal tea species are summarized in **Fig. 1A**, which also presents the statistical significance of all the identified lipid molecular species. Of the 341 total species, 286 lipids were found to be statistically significant and are highlighted in blue, while the remaining lipids were not considered to be significant and are depicted in gray. The five major categories of lipids detected in herbal tea using untargeted lipid analysis techniques (Gowda et al., 2022a) include fatty acids (FA), sphingolipids (SP), glycerophospholipids (GP), glycerolipids (GL), and sterol lipids (ST). The plot of the *m/z* value versus the retention time for all the eluted lipid molecular species is shown in **Fig 1B**. FAs eluted first, and STs eluted last. GPs were the dominant category of lipid molecular species. A recent study on the comparative lipid profiles of green tea showed higher levels of GPs and acylglycerolipids in samples collected in early spring (Cui et al., 2023). However, comprehensive lipidomics data for the herbs used in this study are not well documented in the literature. A detailed list of the lipid species identified in the herbal teas and their relative concentrations are provided in Supporting Information Table S1.

3.2 Multivariate analysis of herbal tea lipidome data

Multivariate analysis was applied to a dataset comprising 20 samples ($n = 5$ for each group). PC1 explained 33% of the total variance in the data, whereas PC2 explained 20.6% of the variance. The analysis revealed four distinct clusters, indicating clear differentiation among the

samples (**Fig. 2A**). Importantly, all the samples fell within Hotelling's T2 range, suggesting that they were within the expected limits of variability. In the PCA-X score plot, dokudami and sugina exhibited visible differences, indicating that these two varieties had distinct lipidomic profiles. In contrast, kumazasa and yomogi show high similarity in terms of their lipid molecular species, resulting in their being grouped together. This suggests potential similarity in the lipid compositions of Kumazasa and Yomogi herbal teas. **Fig. 2B** presents the loading plot of the components, which highlights the factors responsible for the variations observed among the herbal tea species in the score plot. The loading plot illustrates the relationships among the 341 lipid molecular species analyzed, revealing that the FA, ST, and SP classes were positively correlated. This suggests that changes or differences in these lipid classes contributed significantly to the separation observed among the herbal tea species in the score plot. The abundance of PUFA-rich GP and GL lipids played a role in distinguishing the herbal tea species, which is consistent with our previous results involving seaweed analysis, which showed the abundance of GPs and GLs in sea mustard and kombu seaweed species. These seaweeds serve as sources of antioxidants and health-promoting foods for human consumption, owing to their high levels of PUFA-rich GPs and GLs (Gowda et al., 2022a). These findings provide insights into the specific lipid classes that contribute to the observed differences among the lipidomic profiles of the studied herbal tea varieties.

3.3 Hierarchical cluster analysis of lipid profile in four herbal teas

The concentrations of lipids in the herbal teas were visually represented using hierarchical clustering heat maps, which showcase the distinct clustering patterns among the sample groups for each major lipid class. In the heat maps, intense red and blue indicate high and low concentrations of the lipid molecular species present in the herbal teas. The hierarchical cluster correlations of the sphingolipids characterized in the herbal teas are shown in **Fig. 3A.** In general, sphingolipids constitute a significant proportion of the lipids present in higher plants (Dunn et al., 2004). Among the sphingolipids, hexosylceramide (HexCer), acylhexosylceramide (AHexCer), and ceramide (Cer) levels were correlated with the four herbal teas. AHexCer lipids appear to be abundant in yomogi, as indicated by the intense red color in the heatmap. In a previous study, 22 sphingolipids, including seven ceramides, were detected and characterized in green tea (*C. sinensis* cv.) by Li et al.(Li et al., 2021a). To the best of our knowledge, the present study is the first to characterize these lipids in these four herbs.

The hierarchical cluster correlations of the GL contents in the four herbs are shown in

Fig. 3B. The concentrations of GLs such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG) were relatively higher in sugina than in the other species. Lipids such as MGDG, SQDG, and DGDG are the most prevalent membrane lipids in plant chloroplasts (Benning & Ohta, 2005). Additionally, they are essential for eukaryotic membrane development, caloric storage, and vital intracellular signaling mechanisms (Voelker, 2013). Moreover, these lipids are most common in plant species, including green tea (Li et al., 2021a), seaweeds (Gowda et al., 2022a) , and purple leaf tea (Chen et al., 2022). Glycerolipids, especially MGDGs, SQDGs and DGDGs are known to be degraded during green tea manufacturing (Li et al., 2021b). However, a comprehensive analysis of GLs in herbal teas at the molecular species level is needed.

A heatmap of the sterol lipid content of the four herbs is depicted in **Fig. 3C**. The concentration of acylated (esterified) sterol glucoside (ASG) is lowest in kumazasa, followed by sugina, yomogi, and dokudami. In general, plant cells contain considerable amounts of sterol esters (SEs), which, in contrast to other sterol lipid classes, are localized in the oil bodies of the cytosol. These phytosterols are known to block cholesterol absorption sites in the human intestine, as observed in some clinical trials, which helps to reduce cholesterol absorption in humans (Ostlund et al., 2003). Similarly, **Fig. 4 A & B** are the heatmap and hierarchical cluster analysis (**Fig. 4 A** only) of the GPs characterized in the herb species. The concentrations of most lipid classes, such as phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidic acid (PA), were higher in yomogi and lower in kumazasa. The hemibismonoacylglycerophosphate (HBMP) and cardiolipin (CL) contents were relatively higher in sugina than in other species. A previous study conducted by Xuejin et al. identified several phospholipids, including HBMP, lysophophatidylcholine (LPC), LPE, PA, PC, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), PI, and phosphatidylserine (PS), in green tea, oolong tea, and black tea made of purple-leaf tea and green-leaf tea. Their findings suggest that PC 34:3 and PC 36:6 are negative contributors to flavor formation in purple-leaf green tea (Chen et al., 2022). However, there are no reports of the characterization of these lipids in the selected herbs. This study represents the first characterization of these lipids in these herbs and expands our knowledge in this field. These findings provide a comprehensive overview of the lipid composition of the studied herbal tea species and highlight the variations in the specific lipid classes. Understanding the lipid profiles of herbal teas contributes to the understanding of their potential health benefits and aids in the exploration of their functional properties. The P:S ratio (ratio of polyunsaturated fatty acids (PUFAs) to saturated fatty acids

(SFAs)) is a widely used index for assessing the nutritional quality of dietary foods. Moreover, it is utilized in the evaluation of dietary impacts on cardiovascular health(Kang et al., 2005). Our previous investigation, which focused on fish and seaweed, provided additional evidence underlining the importance of the P:S ratio in evaluating the health implications of dietary choices (Gowda et al., 2022a; 2022b). The relative amounts of total SFAs, mono-unsaturated fatty acids (MUFAs), and PUFAs found in the herbal teas are summarized in **Table 2.** These results show that the levels of health-beneficial lipids, such as PUFAs, are relatively high in kumazasa, followed by dokudami, sugina, and yomogi. These observations suggest that these herbs have the potential for use as health-promoting beverages.

Table 2 Relative amounts (mean ± SE, in mg/g of herbal tea powder) of the FA classes in four types of herbal tea (SFA-Saturated fatty acids, MUFA-mono-unsaturated fatty acids, PUFA-poly-unsaturated fatty acids)

Fatty acyls (FA)	Dokudami	Kumazasa	Sugina	Yomogi
SFA	0.75 ± 0.05	0.95 ± 0.03	0.51 ± 0.00	0.63 ± 0.01
MUFA	1.13 ± 0.09	1.23 ± 0.00	0.68 ± 0.00	0.23 ± 0.00
PUFA	6.85 ± 1.4	11.06 ± 0.8	5.98 ± 0.28	2.84 ± 0.59

3.4 Identification of short-chain fatty acid esters of hydroxy fatty acids (SFAHFAs) in herbal tea

Fatty acid esters of hydroxy fatty acids (FAHFAs) are endogenous lipids, that act as antidiabetic and anti-inflammatory agents (Yore et al., 2014). Polyunsaturated fatty-acid-esterified FAHFAs exhibit anti-inflammatory properties by stimulating immune cells to limit macrophage activation during inflammatory responses (Kuda et al., 2016). A recent *in vitro* study also revealed the activation of the antioxidant signaling gene Nrf2 (nuclear factor erythroid factor 2-like 2) by an eicosapentaenoic-acid-esterified 12-hydroxy stearic acid (unsaturated-fatty-acid-esterified FAHFA) (Gowda et al., 2020a). FAHFAs show numerous biological activities. Several FAHFA families and regioisomers have been identified in plant-derived foods, including vegetable oils, fruits, grains, cereals, and fermented products (Watanabe et al., 2022).

In one study, teas were found to contain a wide range of structurally diverse FAHFAs with numerous isomers. The investigation focused on post-fermented teas, such as Fu brick, Liubao, Pu-erh, and Zijuan teas, as well as non-post-fermented teas, including green, white, oolong, and black teas, revealing a total of 49 FAHFA families and 138 regioisomers (Zhu et al., 2022). According to recent reports, new C2- and C3-fatty-acid-derived isomeric structures, namely, short-chain fatty acid esters of hydroxy fatty acids (SFAHFA), have been discovered in murine samples (Gowda, Liang, et al., 2020b). Recent studies have revealed that these novel lipids play a significant role in influenza virus infection (Gowda et al., 2021b). These novel lipids are abundant in the rat colon and cecum (Gowda, Hou et al., 2024). Although 2- and 3- hydroxy fatty acids are widely distributed in nature in plants (Zhu et al., 2018), there is no evidence of their derivatives, such as SFAHFAs, in plants.

In this study, we discovered four molecular species of SFAHFAs with 9-phenyl nonanoic acid (PNA), a phenyl-terminated fatty acid, as the hydroxy fatty acid precursor. PNA is mostly found in the seeds of various aroids belonging to the plant family Araceae (subfamily Aroideae) (Meija & Soukup, 2004). Here, we found derivatives of this PNA in herbal teas belonging to four different plant families other than Araceae. The chromatograms of the FAHFAs are shown in **Fig. 5A**. The hierarchical cluster correlation analysis of the major FA classes of lipids is presented in **Fig. 5B**. Specifically, within the PUFA subgroup, α-linolenic acid (FA 18:3) was notably abundant in kumazasa, while arachidonic acid (FA 20:4) was predominant in sugina. Arachidonic acid is an ω-6 fatty acid known for its pro-inflammatory properties, whereas α-linolenic acid is an ω-6 fatty acid with anti-inflammatory properties (Park et al., 2021).

The mass spectra (MS, MS/MS, and MS^3) of the various FAHFAs and their corresponding fragmentation patterns are depicted in **Fig. 6**. All lipid molecular species belonging to the FAHFA family were ionized in negative mode to give $[M-H]^-$ as the precursor ion. The high-resolution MS spectrum of 4-PAHPNA is shown in **Fig. 6 A**. This lipid was ionized in negative mode to produce an $[M-H]^-$ ion at *m/z* 305.1748. This precursor ion was further ionized to give *m/z* 249 in the MS/MS spectra owing to the loss of CH_3CHCO (56 Da), suggesting that the esterified short-chain fatty acid was propanoic acid (Gowda, Liang, et al., 2020b). The fragment ion *m/z* 249 further lost a molecule of H_2O (18 Da) and CO_2 (44 Da) to give ions at *m/z* 231 and *m/z* 205, respectively, indicating that the structural core was hydroxy phenyl nonanoic acid. The ion produced at *m/z* 175 from *m/z* 249 by the neutral loss of CH_3CH_2COOH (74 Da) indicated that hydroxylation occurred at the 4-position of 9-phenyl

nonanoic acid. The fragmentation pattern for determining the hydroxyl position in the SFAHFAs was identical to that proposed in the literature (Yore et al., 2014). These results confirmed that the compound was the propanoic acid ester of 4-hydroxy 9-phenyl nonanoic acid (4-PAHPNA). In **Fig. 6 B**, a similar analysis was performed for the butyric acid esters of 4-hydroxy-9-phenyl nonanoic acid (4-BAHPNA). This lipid was ionized in negative mode to produce an $[M-H]^-$ ion at *m/z* 319.1903. This precursor ion was further ionized to give *m/z* 249 in the MS/MS spectra owing to the loss of CH_3CH_2CHCO (70 Da), suggesting that the esterified short-chain fatty acid was butyric acid. The fragment ion *m/z* 249 further lost a molecule of H_2O (18 Da) and CO_2 (44 Da) to give ions at *m/z* 231 and *m/z* 205, respectively, indicating that the structural core was hydroxy phenyl nonanoic acid. The ion produced at *m/z* 175 from *m/z* 249 by the loss of neutral CH_3CH_2COOH (74 Da) indicated that hydroxylation occurred at the 4-position of 9-phenyl nonanoic acid. These results confirm that the identified compound is 4-BAHPNA. **Fig. 6 C** depicts the analysis of the valeric acid ester of 4-hydroxy-9-phenyl nonanoic acid (4-VAHPNA). This lipid was ionized in the negative mode to produce an $[M-H]^-$ ion at *m/z* 333.2054. This precursor ion was further ionized to give *m/z* 249 in the MS/MS spectra owing to the loss of $CH_3(CH_2)_2CHCO$ (84 Da), suggesting that the esterified short-chain fatty acid was valeric acid. The fragment ion *m/z* 249 further lost a molecule of H_2O (18 Da) and CO_2 (44 Da) to give ions at *m/z* 231 and *m/z* 205, respectively, indicating that the structural core was hydroxy phenyl nonanoic acid. The ion produced at *m/z* 175 from *m/z* 249 by the loss of neutral CH_3CH_2COOH (74 Da) indicated that hydroxylation occurred at the 4-position of 9-phenyl nonanoic acid. These results confirm that the identified compound is 4-VAHPNA. **Table 1** lists all the characterized FAHFAs present in the herbal tea samples.

Table 1. List of detected FAHFAs in four herbal teas as $[M-H]^-$ ions. 4-AAHPNA: Acetic acid esters of 4-hydroxy -9-phenyl nonanoic acid; 4-PAHPNA: Propanoic acid esters of 4-hydroxy 9-phenyl nonanoic acid; 4-BAHPNA: Butyric acid esters of 4-hydroxy 9-phenyl nonanoic acid; 4-VAHPNA: Valeric acid esters of 4-hydroxy 9-phenyl nonanoic acid; FAHFA: Fatty acid esters of hydroxy fatty acids.

Lipid species	Molecular formula	RT (min)	Theoretical mass (*m/z*)	Experimental mass (*m/z*)	Mass error (ppm)
4-AAHPNA	$C_{17}H_{24}O_4$	3.14	291.1602	291.1593	-3.09

4-PAHPNA	$C_{18}H_{26}O_4$	3.63	305.1758	305.1749	-2.94
4-BAHPNA	$C_{19}H_{28}O_4$	4.31	319.1915	319.1903	-3.75
4- VAHPNA	$C_{20}H_{30}O_4$	5.20	333.2071	333.2056	-4.50
FAHFA (16:4/17:4)	$C_{33}H_{48}O_4$	12.35	507.3480	507.3457	-4.53
FAHFA (13:0/17:4)	$C_{30}H_{50}O_4$	13.89	473.3636	473.3619	-3.59

In a similar way, the new FAHFAs characterized in this study and their MS spectra are shown in **Fig. 6 D** and **E**. The fragmentation patterns of the molecular ions were similar. As shown in **Fig. 6 D,** FAHFA (13:0/17:4) gave the molecular ion $[M-H]^-$ at *m/z* 473.3618, which was ionized to give the product ions *m/z* 277 $[HFA17:4-H]^-$ and *m/z* 213 $[FA13:0-H]^-$ in the MS^2 spectra, and the fragment ion *m/z* 277 lost a neutral molecule of H_2O to give *m/z* 259. The MS^3 spectra of *m/z* 277 showed fragment ions at *m/z* 259, *m/z* 233, and *m/z* 231, suggesting loss of the neutral molecules H_2O, CO_2, and HCOOH respectively. The loss of HCOOH suggests that a hydroxyl group may be present at position 2 of FA 17:4, which is similar to our previous results (Gowda et al., 2020a). Similarly, in Fig**. 6 E,** FAHFA (16:4/17:4) gave the molecular ion $[M-H]^-$ at *m/z* 507.3457, which was ionized to give the product ions *m/z* 277 $[HFA17:4-H]^-$ and *m/z* 247 $[FA16:4-H]^-$ in the MS^2 spectra. The fragment ion *m/z* 277 further lost the neutral molecule H_2O to give *m/z* of 259. The MS^3 spectra of *m/z* 277 showed fragment ions at *m/z* 259, *m/z* 233, and *m/z* 217, suggesting the loss of neutral H_2O, CO_2, and CH_3COOH, respectively. This loss of CH_3COOH suggests that a hydroxy group may be present at position 3 of FA 17:4.

Although the present study was highly beneficial for the detection and characterization of SFAHFAs in herbs and for comprehensive lipid composition analysis, it had several limitations that must be addressed in future studies. These include the concentrations reported in this study, which are semi-quantitative and not absolute. The identification and confirmation of the novel lipids, specifically SFAHFAs, were primarily based on the analysis of MS spectral information. Commercial standards for these lipid species are unavailable, which limited the ability to conduct comprehensive characterization. Furthermore, while MS/MS analysis provided valuable insights into the position of the hydroxy group within the molecules, detailed information regarding its position of could not be fully elucidated owing to the absence of MS^3 data. FAHFAs were detected, but their unsaturation positions were not confirmed. This study

also provided limited information on the growth conditions and external parameters that affect plant lipid composition.

4. Conclusions

In conclusion, our analysis of the lipid molecular species in four different herbal teas revealed significant variations in their lipid profiles through multivariate data analysis. Our comprehensive study identified over 300 lipid molecular species, shedding light on the complex lipid composition of the selected herbs and emphasizing the presence of bioactive lipids. Notably, we identified and characterized the SFAHFAs in these plant samples for the first time. Moreover, these SFAHFAs are the first phenyl-terminated fatty acid derivatives discovered in plants. This study provides a qualitative overview of the molecular lipid species present in herbal teas and highlights their variations among different varieties. The discovery of these novel SFAHFAs opens new avenues for research, as the FAHFA class has already been extensively studied for its biological significance. The bioactive lipids found in herbal tea have been demonstrated to have numerous activities such as the anti-inflammatory properties of α-linolenic acid, and hence to be beneficial to human health. Further investigations should be conducted to explore the potential biological significance of these SFAHFAs in various biological samples, thereby forming a basis for future research. Our future investigation will focus on identifying other potential sources of lipid molecules in different herbal teas and other food sources. By expanding our understanding of these unique lipids, we aim to contribute to the growing body of knowledge regarding their biological implications and potential applications. This study paves the way for further exploration of the role of lipids in herbal teas and their broad implications for human health and nutrition.

Credit authorship contribution statement: L.R.N: Formal analysis, Methodology, writing-original draft. S.G.B.G: Conceptualisation, Visualization, Supervision, resources, Writing-original draft. D.G.: Data curation, Visualization, resources, Writing- review & editing. F.H.: Formal analysis, Writing- review & editing. H.C.: Supervision, Writing- review & editing. S-P.H.: Supervision, Resources, Writing- review & editing.

Declaration of competing interest: The authors declare that they have no conflict of interest.

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References

Benning, C., & Ohta, H. (2005). Three enzyme systems for galactoglycerolipid biosynthesis are coordinately regulated in plants. *Journal of Biological Chemistry*, *280*(4), 2397–2400. https://doi.org/10.1074/jbc.R400032200

Bligh, E.G. and Dyer, W. J. (1959). A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. *Canadian Journal of Biochemistry and Physiology*, *37*(8),911-917. https://doi.org/10.1139/o59-099

Cetojević-Simin, D. D., Canadanović-Brunet, J. M., Bogdanović, G. M., Djilas, S. M., Cetković, G. S., Tumbas, V. T., & Stojiljković, B. T. (2010). Antioxidative and Antiproliferative Activities of Different Horsetail (Equisetum arvense L.) Extracts. *Https://Home.Liebertpub.Com/Jmf*, *13*(2), 452–459. https://doi.org/10.1089/JMF.2008.0159

Chen, X., Wang, P., Wei, M., Lin, X., Gu, M., Fang, W., Zheng, Y., Zhao, F., Jin, S., & Ye, N. (2022). Lipidomics analysis unravels changes from flavor precursors in different processing treatments of purple-leaf tea. *Journal of the Science of Food and Agriculture*, *102*(9), 3730–3741. https://doi.org/10.1002/JSFA.11721

Chung, K. S., Choi, J. H., Back, N. I., Choi, M. S., Kang, E. K., Chung, H. G., Jeong, T. S., & Lee, K. T. (2010). Eupafolin, a flavonoid isolated from Artemisia princeps, induced apoptosis in human cervical adenocarcinoma HeLa cells. *Molecular Nutrition & Food Research*, *54*(9), 1318–1328. https://doi.org/10.1002/MNFR.200900305

Cui, H. N., Gu, H. W., Li, Z. Q., Sun, W., Ding, B., Li, Z., Chen, Y., Long, W., Yin, X. L., & Fu, H. (2023). Integration of lipidomics and metabolomics approaches for the discrimination of harvest time of green tea in spring season by using UPLC-Triple-TOF/MS coupled with chemometrics. *Frontiers in Sustainable Food Systems*, *7*. https://doi.org/10.3389/fsufs.2023.1119314

Ding, W., Ji, T., Xiong, W., Li, T., Pu, D., & Liu, R. (2018). Realgar, a traditional Chinese medicine, induces apoptosis of HPV16-positive cervical cells through a HPV16 E7-related pathway. *Drug Design, Development and Therapy*, *12*, 3459–3469. https://doi.org/10.2147/DDDT.S172525

Dunn, T. M., Lynch, D. V., Michaelson, L. V., & Napier, J. A. (2004). A post-genomic approach to understanding sphingolipid metabolism in Arabidopsis thaliana. *Annals of*

406 *Botany*, *93*(5), 483–497. https://doi.org/10.1093/aob/mch071
407 Gangadhara, R. M., Gowda, S. G. B., Gowda, D., Inui, K., & Hui, S.-P. (2023). Lipid
408 Composition Analysis and Characterization of Acyl Sterol Glycosides in Adzuki and
409 Soybean Cultivars by Non-Targeted LC-MS. *Foods*, *12*(14), 2784.
410 https://doi.org/10.3390/foods12142784
411 Gowda, S. G. B., Fuda, H., Tsukui, T., Chiba, H., & Hui, S. P. (2020a). Discovery of
412 Eicosapentaenoic Acid Esters of Hydroxy Fatty Acids as Potent Nrf2 Activators.
413 *Antioxidants 2020, Vol. 9, Page 397*, *9*(5), 397.
414 https://doi.org/10.3390/ANTIOX9050397
415 Gowda, S. G. B., Liang, C., Gowda, D., Hou, F., Kawakami, K., Fukiya, S., Yokota, A., Chiba,
416 H., & Hui, S. P. (2020b). Identification of short-chain fatty acid esters of hydroxy fatty
417 acids (SFAHFAs) in a murine model by nontargeted analysis using ultra-high-
418 performance liquid chromatography/linear ion trap quadrupole-Orbitrap mass
419 spectrometry. *Rapid Communications in Mass Spectrometry*.
420 https://doi.org/10.1002/rcm.8831
421 Gowda, S. G. B, Minami, Y., Gowda, D., Furuko, D., Chiba, H., & Hui, S.-P. (2021a).
422 Lipidomic analysis of non-esterified furan fatty acids and fatty acid compositions in
423 dietary shellfish and salmon by UHPLC/LTQ-Orbitrap-MS. *Food Research*
424 *International*, *144*, 110325. https://doi.org/10.1016/j.foodres.2021.110325
425 Gowda, S. G. B., Gowda, D., Ohno, M., Liang, C., Chiba, H., & Hui, S.-P. (2021b). Detection
426 and Structural Characterization of SFAHFA Homologous Series in Mouse Colon
427 Contents by LTQ-Orbitrap-MS and Their Implication in Influenza Virus Infection.
428 *Journal of the American Society for Mass Spectrometry*, *32*(8), 2196–2205.
429 https://doi.org/10.1021/JASMS.1C00138
430 Gowda, S. G. B., Yifan, C., Gowda, D., Tsuboi, Y., Chiba, H., & Hui, S. P. (2022a). Analysis
431 of Antioxidant Lipids in Five Species of Dietary Seaweeds by Liquid
432 Chromatography/Mass Spectrometry. *Antioxidants 2022, Vol. 11, Page 1538*, *11*(8),
433 1538. https://doi.org/10.3390/ANTIOX11081538
434 Gowda, S. G.B., Minami, Y., Gowda, D., Chiba, H., & Hui, S.-P. (2022b). Detection and
435 characterization of lipids in eleven species of fish by non-targeted liquid
436 chromatography/mass spectrometry. *Food Chemistry*, *393*, 133402.
437 https://doi.org/10.1016/j.foodchem.2022.133402
438 Gowda, S. G. B., Hou, F., Gowda, D., Chiba, H., Kawakami, K., Fukiya, S., Yokota, A., & Hui,
439 S. P. (2024). Synthesis and quantification of short-chain fatty acid esters of hydroxy fatty
440 acids in rat intestinal contents and fecal samples by LC-MS/MS. *Analytica chimica*
441 *acta*, *1288*, 342145. https://doi.org/10.1016/j.aca.2023.342145
442 Hassan HF, S. B. (2014). Effect of Some Medical Plant Extracts on Metabolism of Leishmania
443 tropica Promastigotes In vitro. *Journal of Medical Microbiology & Diagnosis*, *03*(05).

https://doi.org/10.4172/2161-0703.1000165

Hayashi, K., Kamiya, M., & Hayashi, T. (1995). Virucidal effects of the steam distillate from Houttuynia cordata and its components on HSV-1, influenza virus, and HIV. *Planta Medica*, *61*(3), 237–241. https://doi.org/10.1055/S-2006-958063/BIB

Ho, C. T., Zheng, X., & Li, S. (2015). Tea aroma formation. *Food Science and Human Wellness*, *4*(1), 9–27. https://doi.org/10.1016/j.fshw.2015.04.001

Ichimaru, Y., Kanaeda, N., Tominaga, S., Suzui, M., Maeda, T., Fujii, H., Nakao, M., & Yoshioka, H. (2020). Sasa veitchii extract induces anticancer effects via inhibition of cyclin D1 expression in MCF-7 cells. *Nagoya Journal of Medical Science*, *82*(3), 509–518. https://doi.org/10.18999/NAGJMS.82.3.509

Jinous Asgarpanah. (2012). Phytochemistry and pharmacological properties of Equisetum arvense L. *Journal of Medicinal Plants Research*, *6*(21), 3689–3693. https://doi.org/10.5897/jmpr12.234

Kang, M. J., Shin, M. S., Park, J. N., & Lee, S. S. (2005). The effects of polyunsaturated:saturated fatty acids ratios and peroxidisability index values of dietary fats on serum lipid profiles and hepatic enzyme activities in rats. *British Journal of Nutrition*, *94*(4), 526–532. https://doi.org/10.1079/BJN20051523

Kuda, O., Brezinova, M., Rombaldova, M., Slavikova, B., Posta, M., Beier, P., Janovska, P., Veleba, J., Kopecky, J., Kudova, E., & Pelikanova, T. (2016). Docosahexaenoic Acid–Derived Fatty Acid Esters of Hydroxy Fatty Acids (FAHFAs) With Anti-inflammatory Properties. *Diabetes*, *65*(9), 2580–2590. https://doi.org/10.2337/DB16-0385

Lau, K. M., Lee, K. M., Koon, C. M., Cheung, C. S. F., Lau, C. P., Ho, H. M., Lee, M. Y. H., Au, S. W. N., Cheng, C. H. K., Lau, C. B. S., Tsui, S. K. W., Wan, D. C. C., Waye, M. M. Y., Wong, K. B., Wong, C. K., Lam, C. W. K., Leung, P. C., & Fung, K. P. (2008). Immunomodulatory and anti-SARS activities of Houttuynia cordata. *Journal of Ethnopharmacology*, *118*(1), 79–85. https://doi.org/10.1016/J.JEP.2008.03.018

Li, J., Hua, J., Yuan, H., Deng, Y., Zhou, Q., Yang, Y., Dong, C., Zeng, J., & Jiang, Y. (2021a). Investigation on green tea lipids and their metabolic variations during manufacturing by nontargeted lipidomics. *Food Chemistry*, *339*(July 2020), 128114. https://doi.org/10.1016/j.foodchem.2020.128114

Li, J., Hua, J., Yuan, H., Deng, Y., Zhou, Q., Yang, Y., Dong, C., Zeng, J., & Jiang, Y. (2021b). Investigation on green tea lipids and their metabolic variations during manufacturing by nontargeted lipidomics. *Food Chemistry*, *339*, 128114. https://doi.org/10.1016/j.foodchem.2020.128114

Liu, Y., Ahmed, S., & Long, C. (2013). Ethnobotanical survey of cooling herbal drinks from southern China. *Journal of Ethnobiology and Ethnomedicine*, *9*(1), 1–8. https://doi.org/10.1186/1746-4269-9-82/FIGURES/4

Malongane, F., McGaw, L. J., Nyoni, H., & Mudau, F. N. (2018). Metabolic profiling of four

South African herbal teas using high resolution liquid chromatography-mass spectrometry and nuclear magnetic resonance. *Food Chemistry*, *257*, 90–100. https://doi.org/10.1016/J.FOODCHEM.2018.02.121

Mandiwana, K. L., Panichev, N., & Panicheva, S. (2011). Determination of chromium(VI) in black, green and herbal teas. *Food Chemistry*, *129*(4), 1839–1843. https://doi.org/10.1016/J.FOODCHEM.2011.05.124

Meija, J., & Soukup, V. G. (2004). Phenyl-terminated fatty acids in seeds of various aroids. *Phytochemistry*, *65*(15), 2229–2237. https://doi.org/10.1016/J.PHYTOCHEM.2004.06.033

Miyata, M., Koyama, T., & Yazawa, K. (2010). Water Extract of Houttuynia cordata Thunb. Leaves Exerts Anti-Obesity Effects by Inhibiting Fatty Acid and Glycerol Absorption. *Journal of Nutritional Science and Vitaminology*, *56*(2), 150–156. https://doi.org/10.3177/JNSV.56.150

Ostlund, R. E., Racette, S. B., & Stenson, W. F. (2003). Inhibition of cholesterol absorption by phytosterol-replete wheat germ compared with phytosterol-depleted wheat germ. *The American Journal of Clinical Nutrition*, *77*(6), 1385–1389. https://doi.org/10.1093/AJCN/77.6.1385

Pan, S. Y., Nie, Q., Tai, H. C., Song, X. L., Tong, Y. F., Zhang, L. J. F., Wu, X. W., Lin, Z. H., Zhang, Y. Y., Ye, D. Y., Zhang, Y., Wang, X. Y., Zhu, P. L., Chu, Z. S., Yu, Z. L., & Liang, C. (2022). Tea and tea drinking: China's outstanding contributions to the mankind. *Chinese Medicine (United Kingdom)*, *17*(1), 1–40. https://doi.org/10.1186/S13020-022-00571-1/FIGURES/17

Park, J., Choi, J., Kim, D.-D., Lee, S., Lee, B., Lee, Y., Kim, S., Kwon, S., Noh, M., Lee, M.-O., Le, Q.-V., & Oh, Y.-K. (2021). Bioactive Lipids and Their Derivatives in Biomedical Applications. *Biomolecules & Therapeutics*, *29*(5), 465–482. https://doi.org/10.4062/biomolther.2021.107

Ravichandran, R., & Parthiban, R. (2000). Lipid occurrence, distribution and degradation to flavour volatiles during tea processing. *Food Chemistry*, *68*(1), 7–13. https://doi.org/10.1016/S0308-8146(99)00143-0

Rodríguez, J., Martín, M. J., Ruiz, M. A., & Clares, B. (2016). Current encapsulation strategies for bioactive oils: From alimentary to pharmaceutical perspectives. *Food Research International*, *83*, 41–59. https://doi.org/10.1016/j.foodres.2016.01.032

Sokoła-Wysoczańska, E., Wysoczański, T., Wagner, J., Czyż, K., Bodkowski, R., Lochyński, S., & Patkowska-Sokoła, B. (2018). Polyunsaturated Fatty Acids and Their Potential Therapeutic Role in Cardiovascular System Disorders—A Review. *Nutrients*, *10*(10), 1561. https://doi.org/10.3390/nu10101561

Suh, M. C., Uk Kim, H., & Nakamura, Y. (2022). Plant lipids: trends and beyond. *Journal of Experimental Botany*, *73*(9), 2715–2720. https://doi.org/10.1093/JXB/ERAC125

Voelker, D. R. (2013). Glycerolipid Structure, Function, and Synthesis in Eukaryotes. *Encyclopedia of Biological Chemistry: Second Edition*, 412–418. https://doi.org/10.1016/B978-0-12-378630-2.00510-7

Wargovich, M. J., Woods, C., Hollis, D. M., & Zander, M. E. (2001). Herbals, Cancer Prevention and Health. *The Journal of Nutrition*, *131*(11), 3034S-3036S. https://doi.org/10.1093/JN/131.11.3034S

Watanabe, A., Balas, L., Saigusa, D., Ogura, J., Durand, T., Mano, N., & Yamaguchi, H. (2022). Analytical evaluation of fatty acid esters of hydroxy fatty acid quantity in fermented brown rice and rice bran (FRBA). *Food Chemistry Advances*, *1*, 100040. https://doi.org/10.1016/J.FOCHA.2022.100040

Yore, M. M., Syed, I., Moraes-Vieira, P. M., Zhang, T., Herman, M. A., Homan, E. A., Patel, R. T., Lee, J., Chen, S., Peroni, O. D., Dhaneshwar, A. S., Hammarstedt, A., Smith, U., McGraw, T. E., Saghatelian, A., & Kahn, B. B. (2014). Discovery of a Class of Endogenous Mammalian Lipids with Anti-Diabetic and Anti-inflammatory Effects. *Cell*, *159*(2), 318–332. https://doi.org/10.1016/J.CELL.2014.09.035

Yoshioka, H., Mori, M., Fujii, H., & Nonogaki, T. (2017). Sasa veitchii extract reduces obesity-induced insulin resistance and hepatic steatosis in obese mice fed a high-fat diet. *Nagoya Journal of Medical Science*, *79*(3), 279–290. https://doi.org/10.18999/NAGJMS.79.3.279

Yu, X., Hu, S., He, C., Zhou, J., Qu, F., Ai, Z., Chen, Y., & Ni, D. (2019). Chlorophyll Metabolism in Postharvest Tea (Camellia sinensis L.) Leaves: Variations in Color Values, Chlorophyll Derivatives, and Gene Expression Levels under Different Withering Treatments. *Journal of Agricultural and Food Chemistry*, *67*(38), 10624–10636. https://doi.org/10.1021/ACS.JAFC.9B03477/ASSET/IMAGES/LARGE/JF9B03477_0007.JPEG

Zhu, Q. F., Ge, Y. H., An, N., Li, N., Xiao, Y., Huang, G. X., Zhang, L. L., Feng, Y. Q., & Wu, J. L. (2022). Profiling of Branched Fatty Acid Esters of Hydroxy Fatty Acids in Teas and Their Potential Sources in Fermented Tea. *Journal of Agricultural and Food Chemistry*, *70*(17), 5369–5376. https://doi.org/10.1021/acs.jafc.2c01210

Zhu, Q. F., Yan, J. W., Zhang, T. Y., Xiao, H. M., & Feng, Y. Q. (2018). Comprehensive Screening and Identification of Fatty Acid Esters of Hydroxy Fatty Acids in Plant Tissues by Chemical Isotope Labeling-Assisted Liquid Chromatography-Mass Spectrometry. *Analytical Chemistry*, *90*(16), 10056–10063. https://doi.org/10.1021/acs.analchem.8b02839

Figure 1

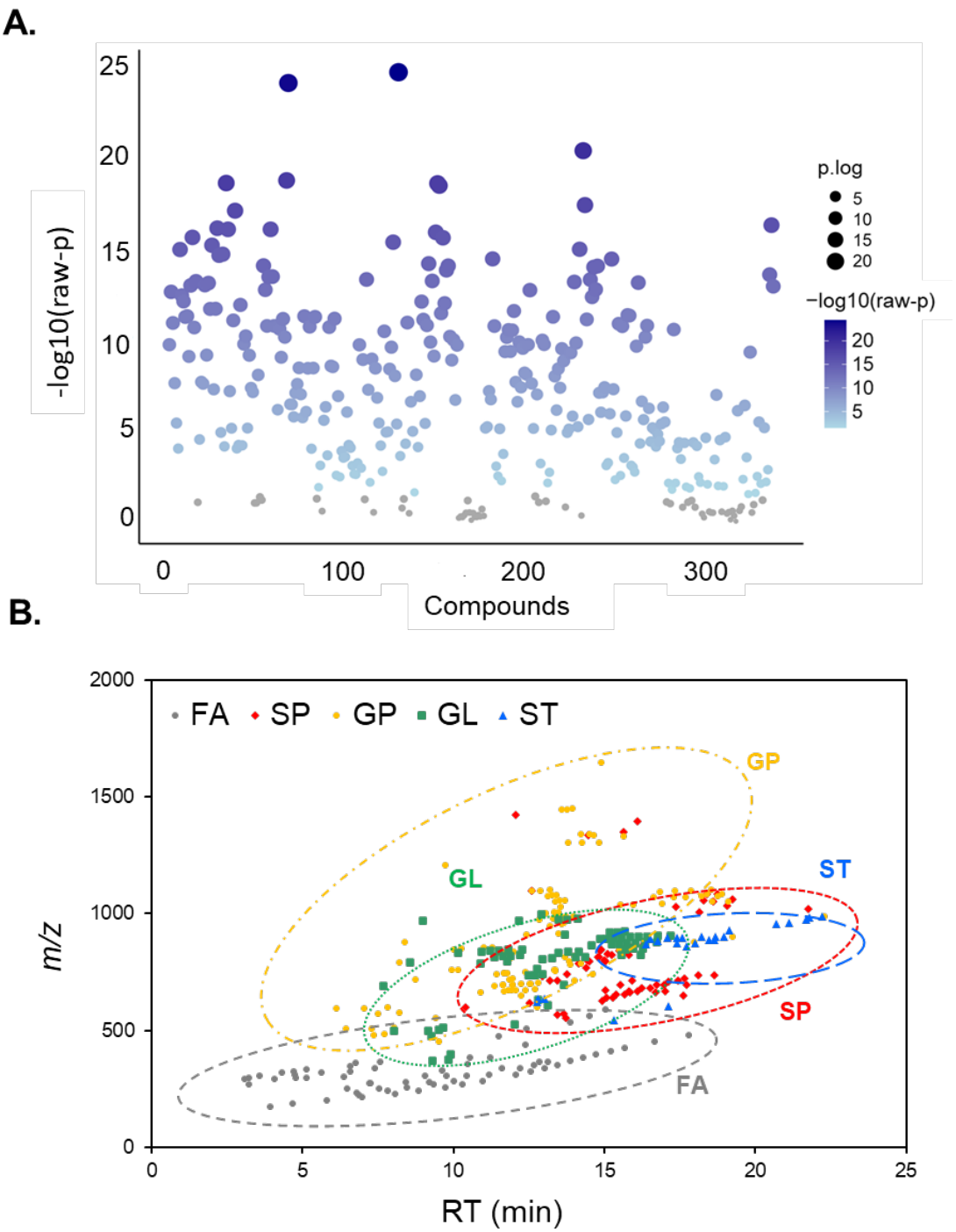


Fig. 1. Lipid profiles of four different herbal teas. **A**. One-way ANOVA analysis of the 341 identified lipid molecular species (Tukey's HSD with $p < 0.05$). Here, 286 lipids are significant (dark and light blue points), while 55 lipids are not (grey points). **B**. Plot of *m/z* vs RT (min) showing an overview of the elution profile of the lipids that were identified in the herbal teas. Fatty acyls (FA), sphingolipids (SP), glycerophospholipids (GP), glycerolipids (GL), and sterol lipids (ST).

Figure 2

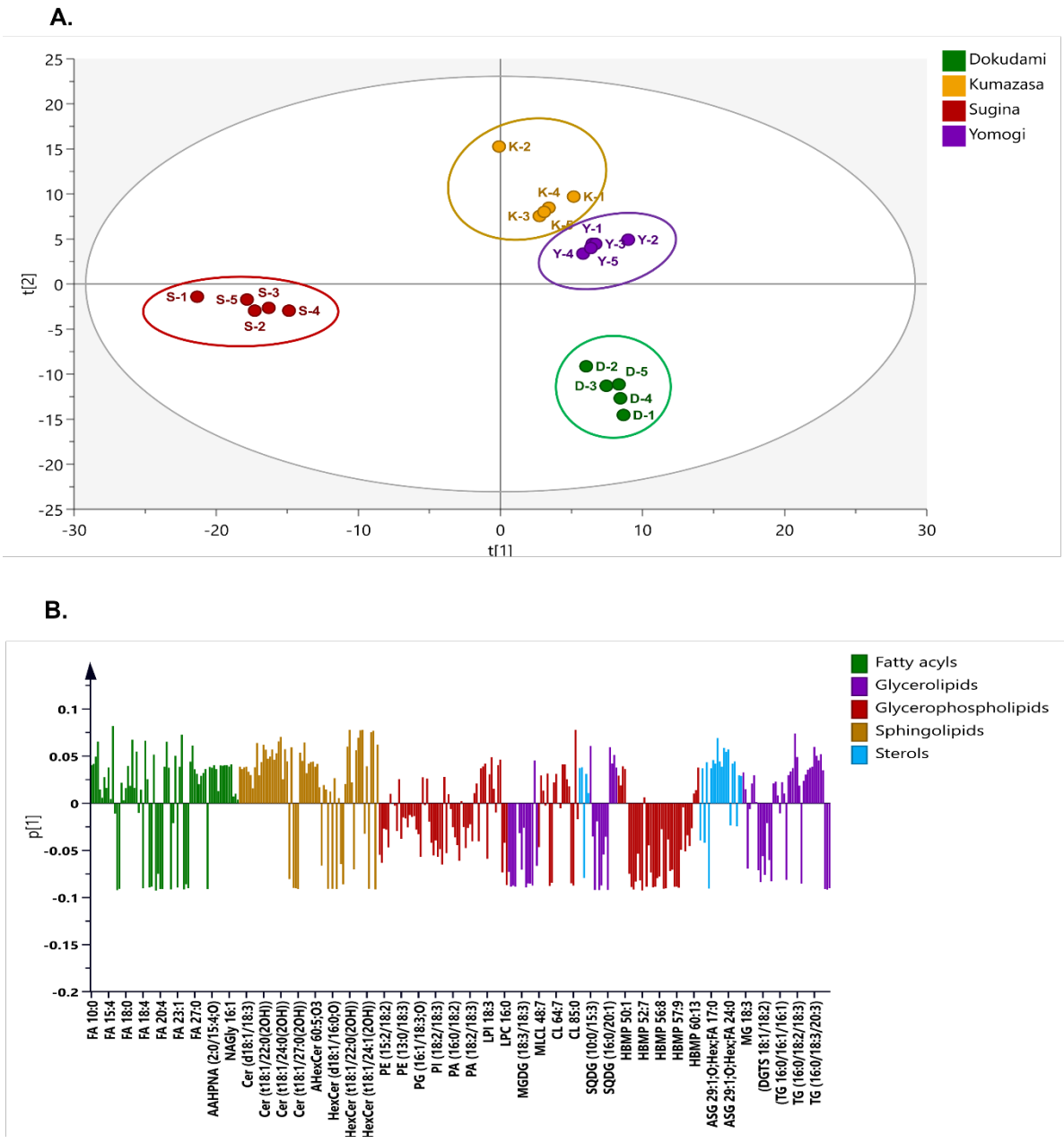


Fig. 2. Multivariate analysis of lipids detected in herbal teas. **A.** PCA score plot of the lipidomics data with samples color-coded according to their respective varieties. *X*-axis: t[1] first score or PC1, and *Y*-axis: t[2] second score or PC2 (n = 20). **B.** PCA analysis loading plot of the PC1 component.

Figure 3

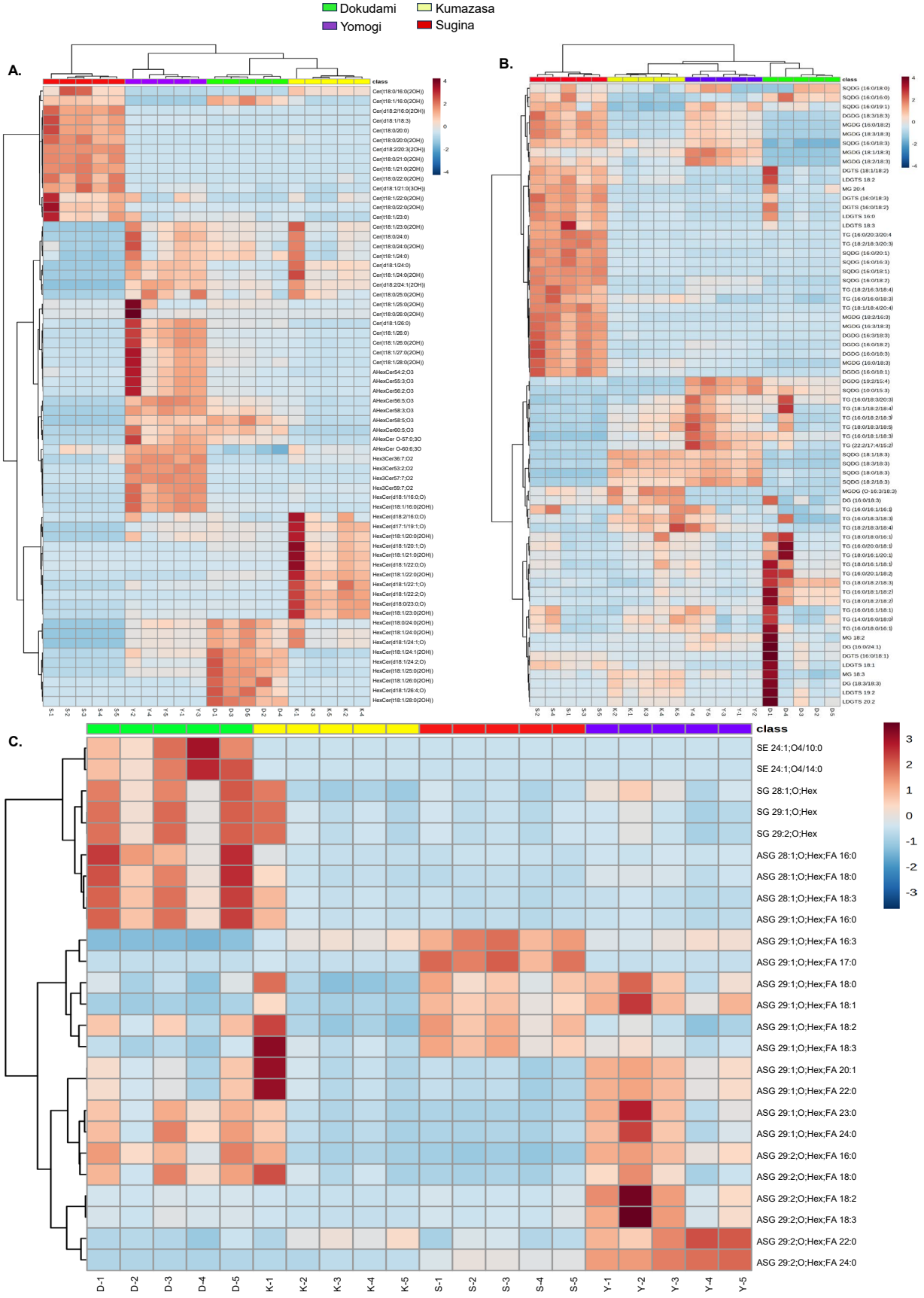

Dokudami
Kumazasa
Yomogi
Sugina
A.
B.
C.
class
SE 24:1;O4/10:0
SE 24:1;O4/14:0
SG 28:1;O;Hex
SG 29:1;O;Hex
SG 29:2;O;Hex
ASG 28:1;O;Hex;FA 16:0
ASG 28:1;O;Hex;FA 18:0
ASG 28:1;O;Hex;FA 18:3
ASG 29:1;O;Hex;FA 16:0
ASG 29:1;O;Hex;FA 16:3
ASG 29:1;O;Hex;FA 17:0
ASG 29:1;O;Hex;FA 18:0
ASG 29:1;O;Hex;FA 18:1
ASG 29:1;O;Hex;FA 18:2
ASG 29:1;O;Hex;FA 18:3
ASG 29:1;O;Hex;FA 20:1
ASG 29:1;O;Hex;FA 22:0
ASG 29:1;O;Hex;FA 23:0
ASG 29:1;O;Hex;FA 24:0
ASG 29:2;O;Hex;FA 16:0
ASG 29:2;O;Hex;FA 18:0
ASG 29:2;O;Hex;FA 18:2
ASG 29:2;O;Hex;FA 18:3
ASG 29:2;O;Hex;FA 22:0
ASG 29:2;O;Hex;FA 24:0
D-1
D-2
D-3
D-4
D-5
K-1
K-2
K-3
K-4
K-5
S-1
S-2
S-3
S-4
S-5
Y-1
Y-2
Y-3
Y-4
Y-5

Fig. 3. Hierarchical cluster analysis of the major classes of lipids observed in the herbal teas. A. sphingolipids (SP), B. glycerolipids (GL), and C. sterol lipids, (clustering method: ward, distance measure: Euclidean).

Figure 4

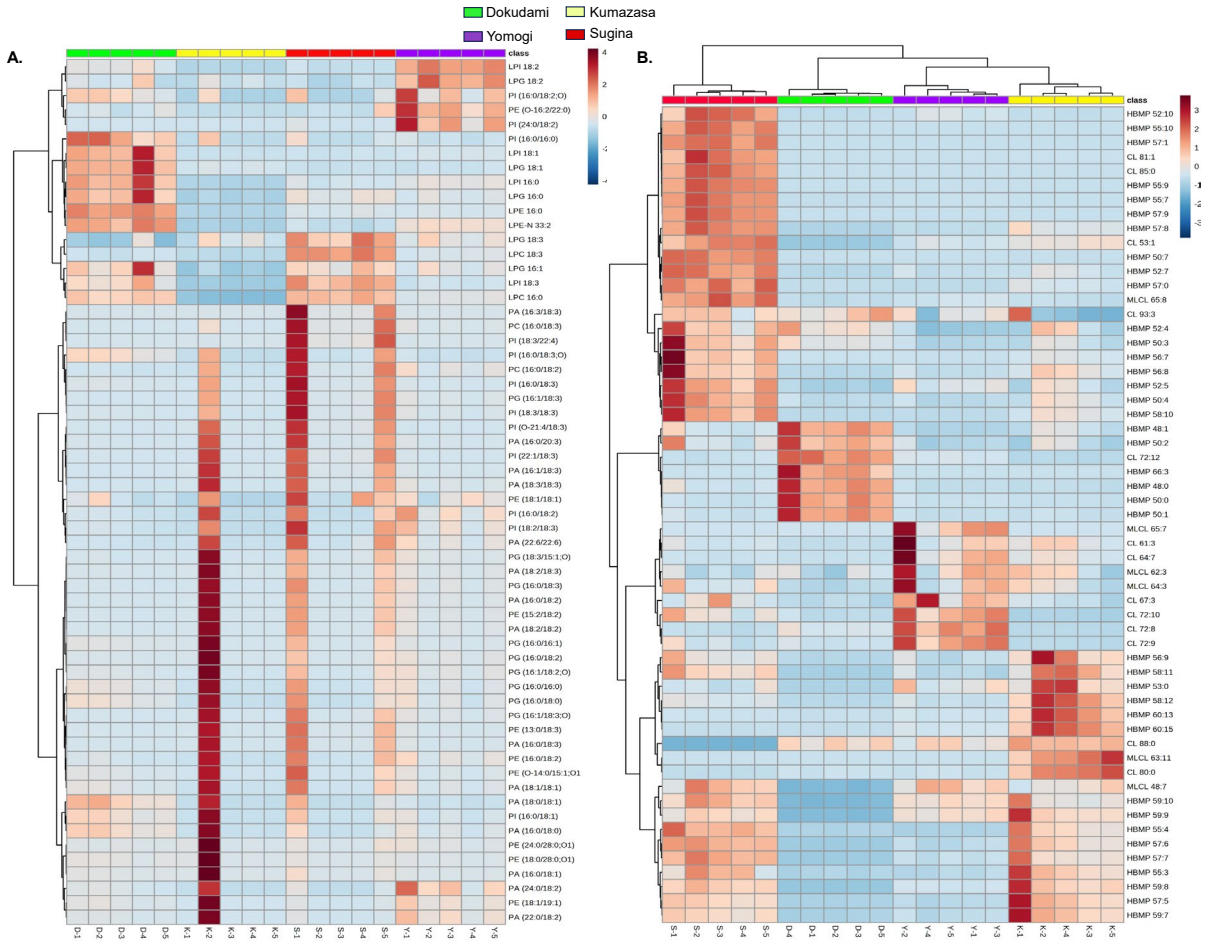


Fig. 4. Heatmap showing the concentration distributions of glycerophospholipids (GP) observed in the herbal teas. **A**. GPs including lysophospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylglycerol (PG). **B.** GPs including hemibismonoacylglycerophosphate (HBMP), cardiolipin (CL), and monolysocardiolipins (MLCL) (clustering method: ward; distance measure: Euclidean).

Figure 5

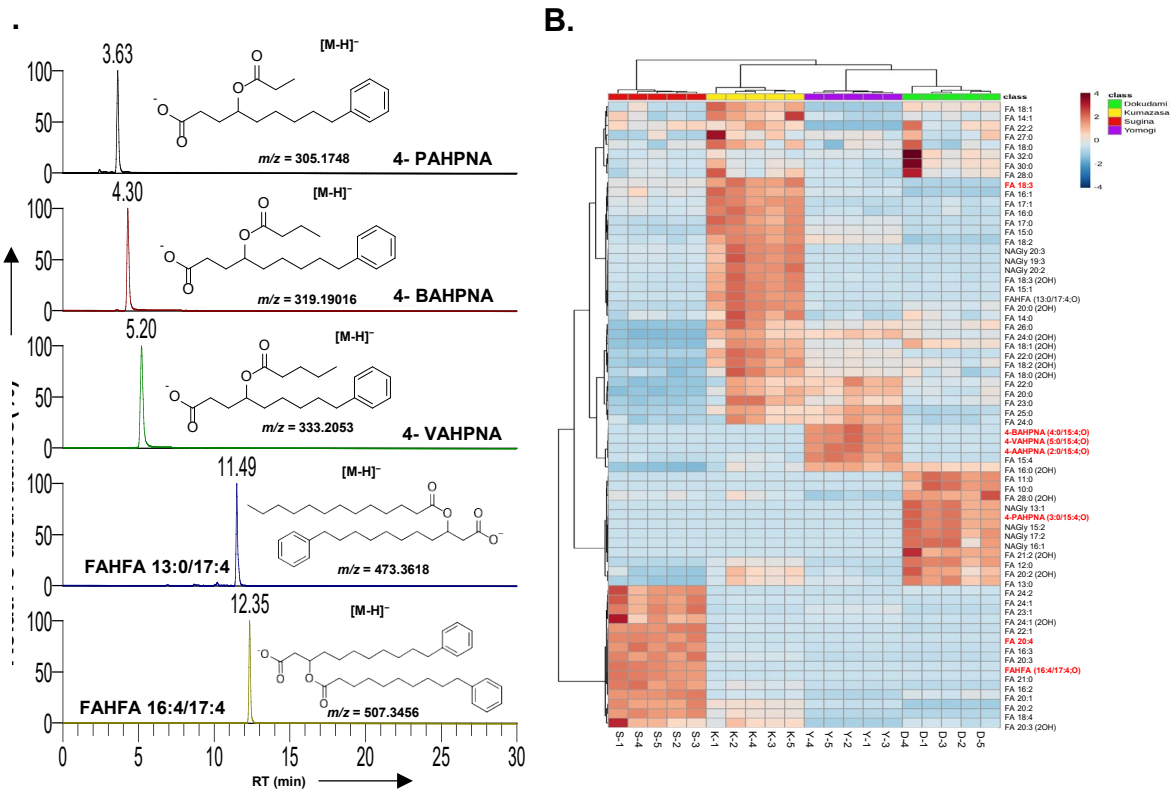


Fig. 5. **A.** Extracted ion chromatograms of FAHFAs detected in the study. **B.** Hierarchical cluster correlation analysis of concentrations of fatty acyls distributed between the different herbal teas. (Clustering method: ward; distance measure: Euclidean).

Figure 6

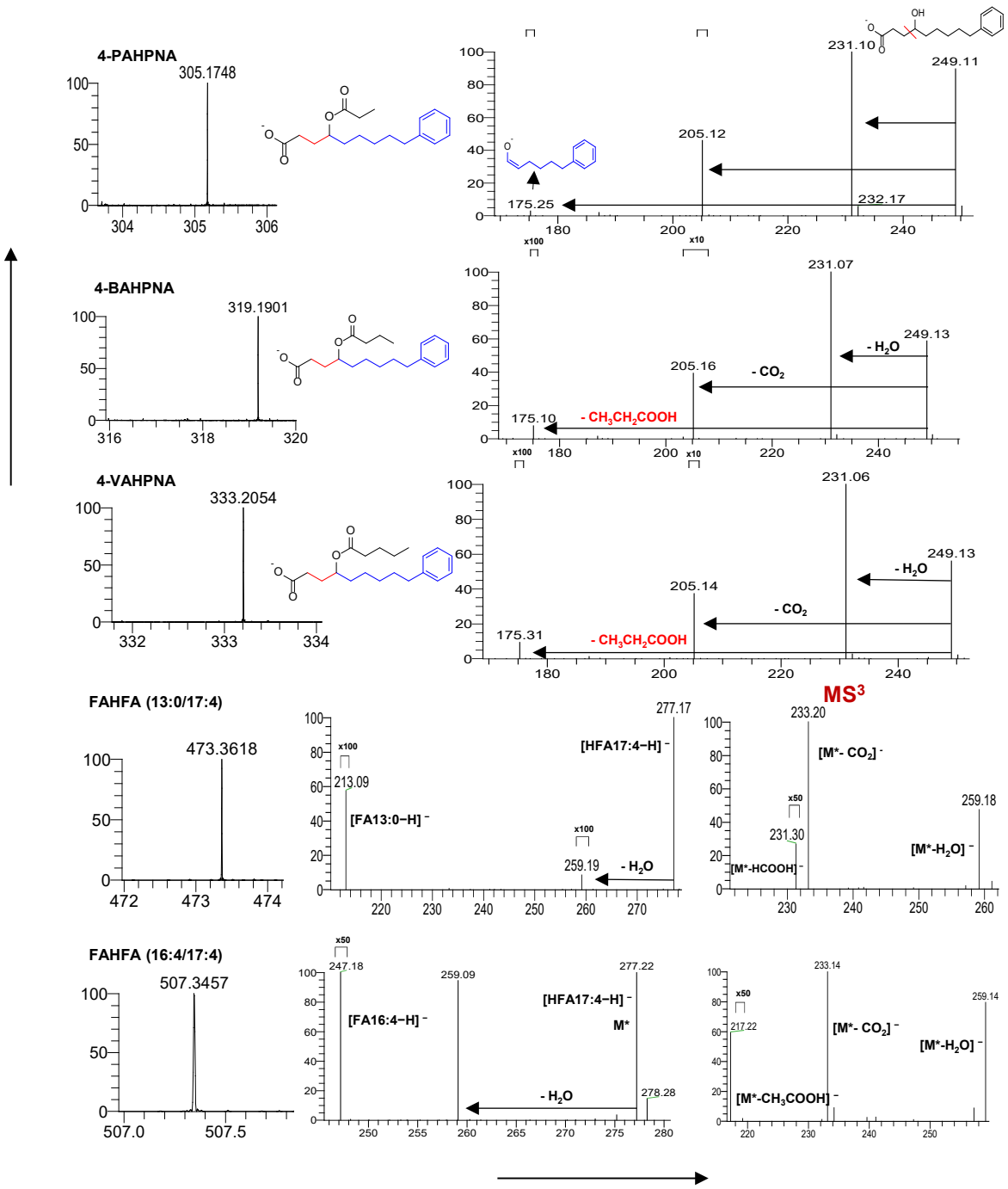
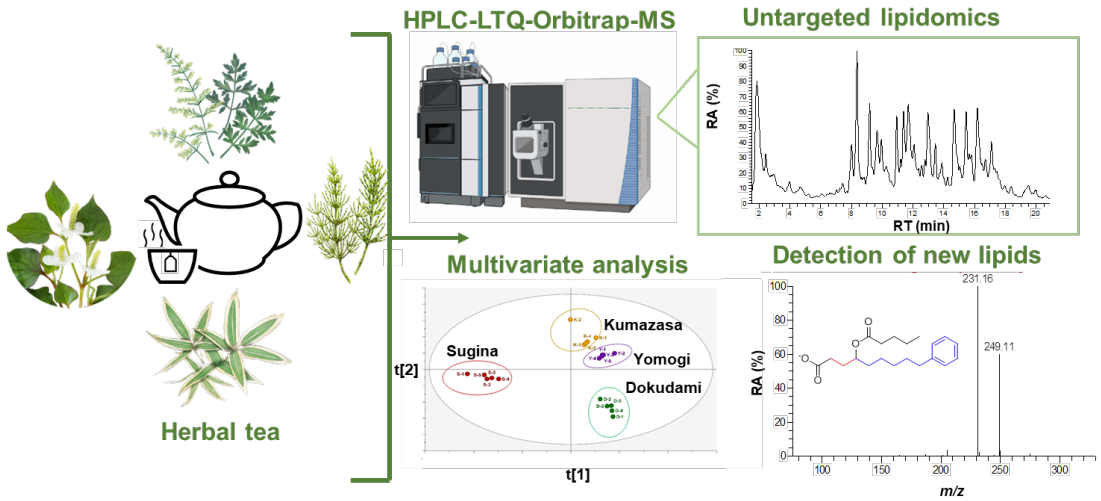


Fig. 6. High-resolution mass spectra of representative FAHFAs detected and characterized in the study. Mass spectra (MS) and MS/MS of **A.** propanoic acid esters of 4-hydroxy-9-phenyl nonanoic acid (4-PAHNA), **B.** butyric acid esters of 4-hydroxy-9-phenyl nonanoic acid (4-BAHPNA), **C.** valeric acid esters of 4-hydroxy-9-phenyl nonanoic acid (4-VAHPNA), **D.** FAHFA (13:0/17:4) and **E.** FAHFA (16:4/17:4). M* is the precursor ion of hydroxy fatty acid.

596 **Graphical abstract**

597



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