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Untargeted Metabolomic Analysis Using High-Resolution Orbitrap Mass Spectrometry for the Comparison of Volatile and Non-Volatile Compounds in Hot and Cold Brew Coffee

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Abstract: Coffee contains several bioactive compounds, such as alkaloids and phenolic compounds, which contribute to its flavor and are influenced by the brewing method. The differences in coffee compounds based on brewing conditions have been studied in previous research, but no studies have yet utilized orbitrap mass spectrometry for this purpose. This study compared non-volatile and aromatic compounds in hot and cold brew coffee using high-resolution orbitrap mass spectrometry, followed by multivariate statistical analysis including principal component analysis and volcano plotting. A total of 163 non-volatile compounds and 93 volatile compounds were identified and annotated, with 18 non-volatile and 13 aroma-active compounds indicating differences between the brewing methods. Notably, certain quinic acids, such as 4,5-dicaffeoylquinic acid, and coumarin derivatives were more abundant in hot brew coffee, indicating that non-volatile compounds are significantly affected by extraction temperature. However, the major non-volatile compounds, including chlorogenic acid and trigonelline, are not affected by brewing conditions. For volatile compounds, phenolic compounds and indole were sensitive to temperature, while pyrazine and furan compounds were more influenced by extraction time. Additionally, in our results, several previously unreported bioactive compounds were detected in coffee, suggesting a need for further research to understand their potential functions and benefits.

Keywords: hot brew coffee; cold brew coffee; volatile; non-volatile; untargeted; high-resolution; orbitrap

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1. Introduction

Coffee, a beverage that is well-known and easily accessible around the world, has experienced steady growth in popularity and production over recent decades. In the 2024/25 period, coffee bean production reached 176.2 million 60 kg bags, consistently remaining within the range of 165 to 175 million bags [1]. Coffee contains several bioactive compounds, such as alkaloids and phenolic compounds, including caffeine, chlorogenic acid, and trigonelline. Some of these compounds are associated with various health benefits, such as antihypertensive, hypoglycemic, and anticancer effects [2–4]. These compounds are also related to the flavor and taste of coffee. The flavor profile of coffee is mainly influenced by its chemical components, where non-volatile compounds affect its taste, while volatile

compounds affect its aroma. The flavor profile of coffee can be affected by various factors, such as geographical characteristics, processing methods, roasting degree, decaffeination, and brewing methods, all of which ultimately influence consumer choice [5]. For example, variations in diterpene composition have been observed among different *Coffea arabica* cultivars, and sensory analyses have indicated that the characteristics of brewed coffee differed based on cultivation altitude [6,7]. The brewing process, recognized as the final step before coffee consumption, has garnered increasing attention from food manufacturers. Coffee can be categorized based on brewing temperature into traditional hot brew and cold brew coffee, with the latter being extracted using water at approximately 25 degrees Celsius. In recent years, the coffee industry has experienced notable shifts. As consumer demand for premium coffee continues to grow, cold brew has gained significant popularity. According to market analysis, the cold brew coffee segment was valued at USD 321 million in 2017 and is projected to reach USD 1.37 billion by 2024 [1,8]. Brewing parameters, such as grind size, extraction time, pressure, and temperature, are crucial factors influencing the sensory quality and flavor perception of coffee [8]. With the expansion of the cold brew coffee market and the growing interest in this beverage, scholarly research comparing the characteristics and differences between cold brew and hot brew coffee has progressively increased. These studies focus on aspects such as flavor, aroma, and chemical compounds like metabolites. According to Gloess et al. [9] and Batali et al. [10], hot brew coffee exhibited a roasted and smoky aroma, whereas cold brew coffee demonstrated fruity, floral, and sweet notes. The brewing method significantly influences the levels of non-volatile bioactive compounds in coffee, such as caffeine and phenolic acids, which are associated with the purported health benefits of coffee beans, including their antioxidant and anti-tumor effects [11–13]. Additionally, a previous study analyzing volatile compounds in medium-roasted Colombian Arabica coffee prepared through both cold brewing and hot brewing methods found that cold brew coffee exhibited a greater concentration of pyrazines and furans compared to hot brew coffee [14].

Metabolomics is a scientific approach used to quantitatively and qualitatively describe the overall levels of endogenous biological metabolites and their responses to external and internal factors. This method is renowned for its high efficiency, precision, and comprehensiveness, making it widely applicable in fields such as plant studies [15], pharmaceuticals [16], and food science [17–19]. This approach is frequently employed across various disciplines for both qualitative and quantitative analysis of metabolites from whole organisms, tissues, and cells, with the aim of identifying key differential metabolites. Untargeted metabolomics, coupled with high-resolution mass spectrometry (HR-MS), is a powerful tool for comparative analyses, providing comprehensive insights into the metabolic profiles of different samples, such as cold and hot brew coffee.

Orbitrap mass spectrometry (OMS), a type of HR-MS, is especially valuable for food authentication due to its improved dynamic range, simplified mass calibration, and tandem mass spectrometry capabilities [20]. Also, OMS exhibits high mass accuracy, with performance levels of 5 ppm under standard calibration and 3 ppm when using an internal standard. It allows for streamlined sample pretreatment, facilitating quicker and more efficient identification and quantification in complex matrices [21]. Thus, OMS has proven to be a highly sensitive and selective method, significantly boosting analytical efficiency, and to be especially valuable for analyzing substances, such as metabolites, newly emerging contaminants, and transformation products [22].

Several studies have analyzed untargeted chemical compounds in coffee using HR-MS, including time-of-flight mass spectrometry (TOF) and other mass spectrometry techniques, including triple quadrupole (QQQ) with liquid (LC) and gas chromatography (GC). For instance, Vezzulli et al. [23] reported on the metabolite differences between coffee species

such as *Coffea arabica* and *Coffea canephora* var. *Robusta* using LC-QTOF analysis. Pérez Míguez et al. [24] examined the variation in metabolic compounds in coffee depending on the roasting process, also using LC-QTOF analysis. Additionally, Cai et al. [25] presented comparative profiling data of both volatile and non-volatile compounds in hot and cold brew coffee using LC-Q-ion trap mass spectrometry and GC-MS, respectively. However, there is a lack of comprehensive metabolomic studies utilizing HR-OMS technology to investigate the effects of different brewing methods on coffee.

Therefore, the objective of this study was to compare non-volatile compounds, such as alkaloids and phenolic compounds, in hot and cold brew coffee through untargeted metabolomics analysis using LC-HR-OMS. Additionally, aromatic compounds, such as furans and pyrazines, were analyzed using GC-HR-OMS, followed by multivariate statistical analysis. Also, unlike other studies, this research standardized the extraction time across all conditions to accurately assess the impact of extraction temperatures. The results of this study are expected to provide a clearer understanding of the differences between hot brew and cold brew coffee and facilitate a comparison of analytical data obtained from orbitrap and other HR-MS techniques.

2. Materials and Methods

2.1. Materials

Green coffee beans produced in Ethiopia were provided for our research by Lotte Chilsung Beverage Co., Ltd. (Seoul, Republic of Korea). To analyze untargeted metabolites in hot and cold brew coffee using HR-OMS, high-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Other reagents, including ammonium formate and formic acid, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Preparing Coffee

The green coffee beans were roasted at 190–210 °C for 30 min using a roasting machine (Model Probat BRZ 2, Probat, Emmerich am Rhein, Germany) and then ground with a semi-automatic grinder (Model K32S20, Mahlkonig GmbH & Co. KG, Hamburg, Germany) with the dial set to 4. The ground coffee was then sieved to obtain particles sized between 50 and 100 mesh, which were used for both hot and cold brew coffee preparation. For hot brew coffee, a total of 24 g of ground coffee was placed in a coffee filter paper. Approximately 300 g of purified water was then poured directly over the filter, and heated to brew the coffee. The coffee extract was released at a temperature of 95 °C, and the brewing process took approximately 3 min until the extraction volume reached 240 mL. For cold brew coffee, a total of 24 g of ground coffee was placed in a coffee filter. About 300 g of purified water was then poured directly over the filter. The coffee extract was released at a temperature of 25 °C, and the brewing process took approximately 3 min, until the extraction volume reached 240 mL.

2.3. Analysis of Non-Volatile Compounds

For analyzing non-volatile compounds in hot and cold brew coffee, a modified version of the method by Cai et al. and Li et al. [25,26] was followed. Briefly, the hot and cold brew coffee was vigorously shaken for 1 min before sampling. A 500 µL aliquot of coffee was mixed with 500 µL of 80% methanol solution, and then the mixture was vortexed for 10 min. Following centrifugation at 4000 rpm for 5 min using a centrifuge (Model Combi R515, Hanil Scientific, Gimpo, Republic of Korea), the supernatant was filtered through a 0.2 µm syringe filter (Sartorius, Göttingen, Germany). Finally, 1 µL of the sample was injected into the LC-HR-OMS system (Model Q-Exactive, Thermo Scientific, Waltham, MA,

USA) featuring an electrospray ionization (ESI) source. Chromatographic separation was performed using a C18 column (Zorbax Eclipse Plus, 2.1 × 100 mm, particle size 100 µm, Agilent, Santa Clara, CA, USA) maintained at 30 °C. The separation was conducted using mobile phases comprising water with 0.1% formic acid and 5 mM ammonium formate (solvent A) and 90% acetonitrile with 0.1% formic acid and 5 mM ammonium formate (solvent B), which were eluted following a specified gradient profile: 5–60% solvent B over 10 min, 60–100% solvent B over 5 min, 100–5% solvent B over 2 min, and then maintaining 5% solvent B for 3 min, resulting in a total analysis time of 20 min. The ESI probe temperature was set to 250 °C, the spray voltage to 3 kV, the ion transfer line temperature to 300 °C, the sheath gas pressure to 35 psi, and the auxiliary gas flow to 10 (arbitrary units). Full MS and data-dependent MS^2 ($ddMS^2$)/top N (N = 4) scan modes were conducted in both positive and negative ion monitoring modes, respectively, with the following parameters: a resolution of 80,000 for full MS in the m/z range of 100 to 1000, a maximum trap filling time of 80 milliseconds, a resolution of 35,000 for fragment scans, and collision energies of 20, 40, and 60 eV with nitrogen as the collision gas.

2.4. Analysis of Volatile Compounds

For analyzing volatile compounds in hot and cold brew coffee using solid-phase microextraction (SPME), a modified version of the method by Pan et al. [27]. was followed. A 4 mL coffee sample was placed into a 20 mL headspace vial containing 2.5 g of NaCl. The vial was closed with a PTFE-silicone septum and equilibrated at 60 °C for 10 min with continuous agitation. Subsequently, a 50/30 µm divinylbenzene/carboxen™/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber was exposed to the coffee headspace for 45 min at the same temperature. The fiber was then removed and inserted into the gas chromatography injector at 250 °C for 5 min.

The GC-HR-OMS (Model Q-Exactive GC, Thermo Scientific, Waltham, MA, USA) analysis was conducted following the procedure outlined by Heo et al. [28]. Volatile compounds were separated using DB-WAX fused silica capillary columns (30 m × 0.25 mm I.D., 0.25 µm film thickness, Agilent, Santa Clara, CA, USA). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature was initially maintained at 40 °C for 10 min, then increased at a rate of 8 °C/min to 260 °C, and then maintained at that temperature for 10 min. Mass spectrometry was performed in the electron impact mode at 70 eV, scanning a range of m/z 35–500 at a resolution of 60,000.

2.5. Data Processing and Multivariate Statistical Analysis

The raw data signals (n = 5) from HR-OMS were processed using Compound Discoverer, software version 3.3 (Thermo Scientific, Waltham, MA, USA). All statistical analyses were conducted based on the intensity area under the peaks for each qualitatively identified molecular mass. To conduct qualitative analysis, LC-HR-OMS raw data were identified by matching the mass spectrum and tandem mass spectrum with the mzCloud and mzVault databases in Compound Discoverer. Additionally, GC-HR-OMS raw data were identified by matching the mass spectrum with the NIST 17 standard database in Compound Discoverer after deconvolution. To visualize the differences between hot and cold brew coffee, principal component analysis (PCA) and volcano plotting were conducted using Compound Discoverer software. The PCA reduces the dimension of data by transforming it into a smaller set of uncorrelated variables to highlight key patterns. Volcano plotting is a graphical representation that shows the relationship between the fold change in log scale and statistical significance calculated by Student's t-test with the data.

3. Results

3.1. Non-Volatile Compounds in Hot and Cold Brew Coffee

Metabolic profiling using LC-HR-OMS was performed on hot and cold brew coffee. Compound Discoverer conducted peak alignment and baseline correction, resulting in the identification of approximately 1400 peaks in the positive ion mode and 1300 peaks in the negative ion mode in brewed coffee samples. The process of annotation followed the methods of Souza et al. [29] and Sukor et al. [30] with some modifications The analysis commenced with the exclusion of peaks that lacked MS/MS fragmentation data, exhibited a ΔMass (ppm) exceeding 2.0, had a peak rating of 8.0 or below, or presented a CV (%) value of less than or equal to 10%. The remaining data were subjected to further analysis using MS/MS fragmentation patterns, which were compared against several spectral databases to enhance the accuracy of compound identification. Key databases referenced in this process included the mzCloud spectral library, mzVault, ChemSpider™, and a mass list of natural and flavonoid compounds. Subsequently, the peaks exhibiting a matching rate of 80% or higher with these databases were annotated (Figure 1), ensuring that only highly confident matches were retained. As a result of this annotation process, a total of 163 compounds were identified as metabolites present in both hot and cold brew coffee. Specifically, 93 compounds were annotated as being in the positive ion mode, while 70 compounds were identified as being in a negative ion mode, offering a comprehensive metabolic profile of the coffee samples. A detailed list of these annotated compounds, along with their respective data, is provided in Table S1.

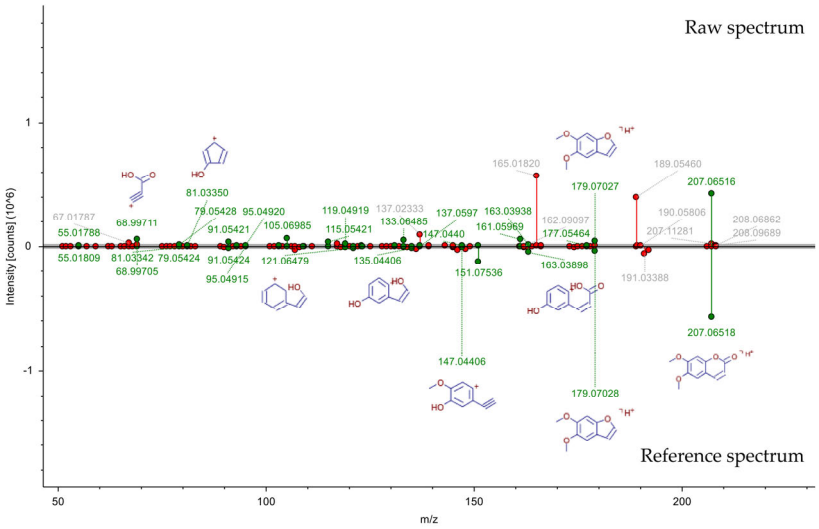

Figure 1. Mirror match of fragmentation of scoparone by mzCloud spectral library: best matching rate, 86.2%. The green color indicates matching, while the red color indicates non-matching.

Among these compounds, to evaluate the variation and distribution of hot and cold brew coffee based on its non-volatile chemical compound profile, a PCA score plot was examined. As illustrated in Figure 2, the first two principal components (PC1 and PC2) accounted for a combined total of 91.5% (positive ion mode) and 94.4% (negative ion mode) of the variance across the groups. This high percentage of explained variance highlights the model's strong capability to differentiate between the samples, effectively distinguishing the brewing conditions used in our study. The clear separation observed in the PCA plot demonstrates the robustness of the statistical model in capturing the chemical differences associated with each method of extraction. Also, volcano plot analysis was employed, integrating Student's t-test (p-value < 0.05) with Fold Change analysis (Log_2 Fold Change > 1.0), to examine the chemical variations among the hot and cold brew coffee samples in positive

and negative ion modes, respectively (Figure 3). As shown in the results, the volcano plot comparing hot and cold brew coffee revealed 18 significant compounds (in both positive and negative ion modes), with 17 compounds exhibiting significant up-regulation in hot brew coffee, having the exception of 3-(3-pyridinyl)propanoic acid (detected in the positive ion mode). Detailed up-regulated compound information, including regarding molecule weight, formula, and CAS number, is provided in Table 1.

Table 1. Differential non-volatile compounds in hot and cold brew coffee using high-resolution orbitrap mass spectrometry with volcano plotting up-regulation (p-value < 0.05, Log_2 Fold Change > 1.0).

Compounds	CAS [1]	Formula	Molecule Weight	Reference Ion	Peak Rating
6-Methyl-2-pyridinemethanol	1122-71-0	C7H9NO	123.0683	[M + H] + 1	9.2
2-Methylbenzoic acid	118-90-1	C8H8O2	136.0524	[M − H] − 1	9.2
Tyrosol	501-94-0	C8H10O2	138.0681	[M − H] − 1	9
3-(3-pyridinyl)propanoic acid [2]	3724-19-4	C8H9NO2	151.0632	[M + H] + 1	8.3
6-Methoxyquinoline	5263-87-6	C10H9NO	159.0683	[M + H] + 1	8.8
4-Coumaric acid	7400-08-0	C9H8O3	164.0473	[M − H] − 1	8.7
Norharman	244-63-3	C11H8N2	168.0687	[M + H] + 1	9.6
3-Dehydroshikimate	2922-42-1	C7H8O5	172.0371	[M − H] − 1	9.2
Indole-3-acetic acid	87-51-4	C10H9NO2	175.0633	[M − H] − 1	8.8
1-(4-Methylphenyl)pyrrolidine-2,5-dione	–	C11H11NO2	189.0790	[M + H] + 1	8.7
Scoparone	120-08-1	C11H10O4	206.0579	[M + H] + 1	8.5
Euparin	532-48-9	C13H12O3	216.0787	[M − H] − 1	9
Resveratrol	510-36-0	C14H12O3	228.0786	[M − H] − 1	10
Naringenin	480-41-1	C15H12O5	272.0686	[M − H] − 1	8.6
N-Caffeoyltryptophan	109163-69-1	C20H18N2O5	366.1215	[M − H] − 1	9.2
Mascaroside II	–	C26H34O9	490.2206	[M + H] + 1	10
Mascaroside I	–	C26H36O10	508.2311	[M + H] + 1	9.4
4,5-Dicaffeoylquinic acid	57378-72-0	C25H24O12	516.1268	[M − H] − 1	9.6
		C25H24O12	516.1272	[M + H] + 1	8.8

[1] Minus sign '–' indicates the CAS was not available in the literature. [2] This compound was only up-regulated in cold brew coffee.

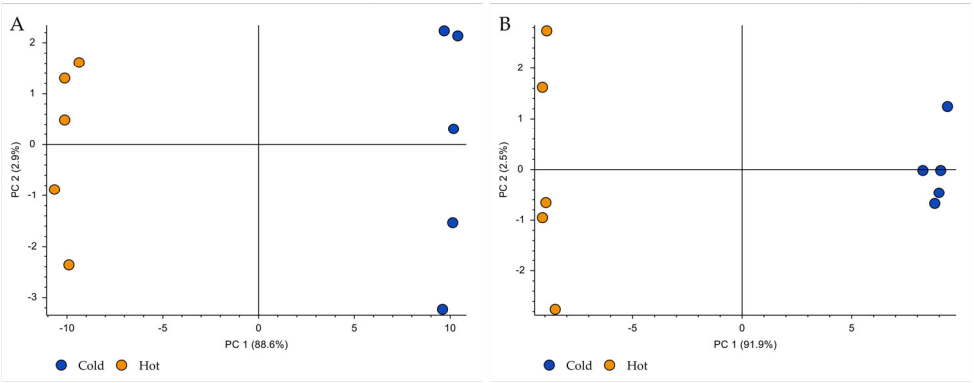

Figure 2. PCA score plot for non-volatile compounds in hot and cold brew coffee: (**A**) positive ion mode, (**B**) negative ion mode.

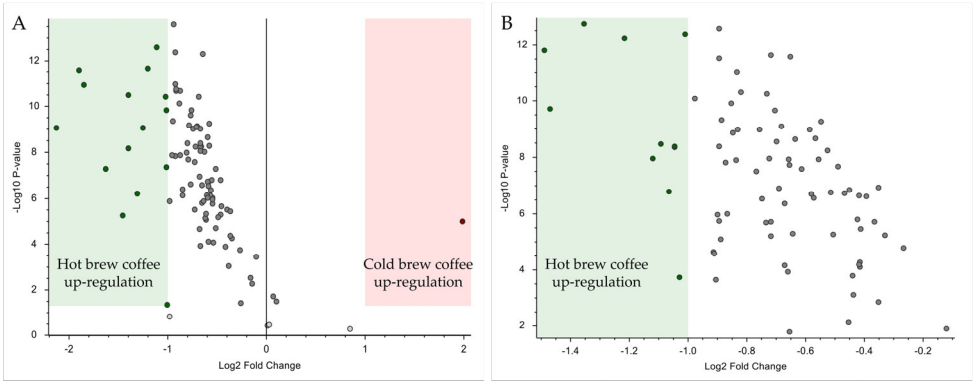

Figure 3. Volcano plotting for non-volatile compounds in hot and cold brew coffee: (**A**) positive ion mode; (**B**) negative ion mode.

3.2. Volatile Compounds in Hot and Cold Brew Coffee

Metabolic profiling using GC-HR-OMS was conducted on hot and cold brew coffee. Compound Discoverer performed peak alignment, baseline correction, and deconvolution, resulting in the identification of approximately 1400 peaks in hot brew coffee and 1300 peaks in cold brew coffee. This process followed the method of Sapozhnikova [31] with some modifications. Firstly, peak detection is carried out using high-resolution deconvolution with a mass tolerance of less than 5 ppm. The deconvolution of raw data are particularly advantageous for reducing and eliminating interferences. After deconvolution, peaks with a CV (%) below 10% were identified, with an S/N threshold greater than 3 and a total ion chromatogram (TIC) threshold of 5,000,000, where TIC threshold refers to the total intensity area of all ions at a specific retention time. And then, a library search was conducted using the NIST 17 standard database in Compound Discoverer. Among these 1300–1400 peaks, 93 compounds were annotated as metabolic compounds in hot and cold brew coffee (Table S2). The annotated metabolic compounds in hot and cold brew coffee were classified into alcohol, aldehyde, ester, fatty acid, furan, hydroquinone, indole, ketone, pyrazine, phenol, and others.

In order to assess the differences and distribution patterns between hot and cold brew coffee based on their respective profiles of volatile chemical compounds, a PCA score plot was employed. As shown in Figure 4A, the first two principal components (PC1 and PC2) together explained a significant portion of the overall variance, accounting for 93.0% of the total variation observed among the different groups. These two components were instrumental in distinguishing the coffee samples, providing insight into how the volatile profiles differ between hot and cold brew coffees. By capturing the majority of the variance within the first two components, the PCA score plot effectively demonstrates the underlying chemical distinctions that contribute to the sensory and flavor characteristics of the respective coffee types. In addition, a volcano plot analysis was conducted to investigate the volatile compound differences between the hot and cold brew coffee samples. This analysis combined Student's t-test (p-value < 0.05) with Fold Change analysis (Log_2 Fold Change > 0.9) to identify significant variations (Figure 4B). As shown in the results, the volcano plot comparing hot and cold brew revealed 13 significant compounds, with 10 compounds exhibiting significant up-regulation in hot brew coffee. Detailed information on the up-regulated compounds, including molecular weight, odor, and CAS number, is provided in Table 2.

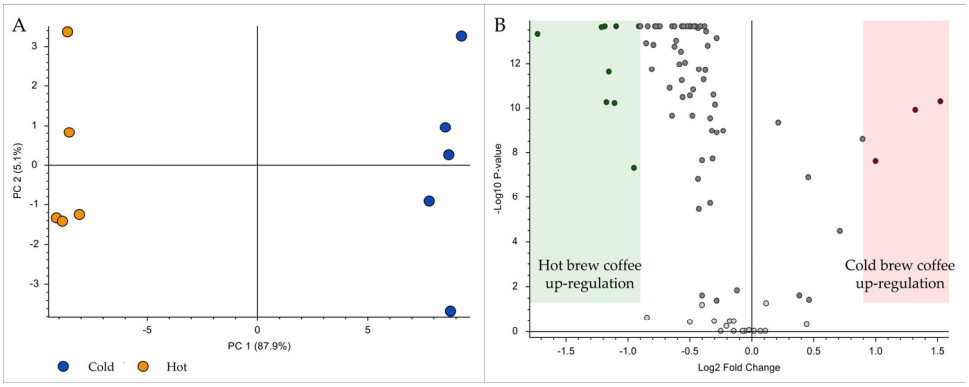

Figure 4. PCA score plot and volcano plotting for volatile compounds in hot and cold brew coffee: (**A**) PCA score plot; (**B**) volcano plotting.

Table 2. Differential volatile compounds in hot and cold brew using high-resolution orbitrap mass spectrometry with volcano plotting of up-regulation (p-value < 0.05, Log_2 Fold Change > 0.9).

Up-Regulation	Compounds	Molecule Weight	CAS [1]	Odor
Cold brew	Cedrenol	220.1827	28231-03-0	Woody, sweet [32]
	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	276.1725	82304-66-3	
	Dicyclohexyl adipate	310.2144	849-99-0	
Hot brew	Catechol	110.0368	120-80-9	
	Indole	117.0578	120-72-9	Animal, floral, moth ball [25]
	3-Methylindole	131.0735	83-34-1	Animal [32]
	1,3-Benzenediol, 4-ethyl-	138.0681	2896-60-8	
	3-Methoxy-1,2-benzenediol	140.0473	934-00-9	
	2-Naphthalenol	144.0575	135-19-3	Slight phenolic odor [32]
	2-Methoxy-4-vinylphenol	150.0681	7786-61-0	Smoky, woody, powdery [32]
	2-Amino-5-methoxybenzaldehyde	151.0633	26831-52-7	
	1-(2,3-Dihydroxyphenyl)ethanone	152.0473	—	
	4-Vinylphenol	162.0681	2628-16-2	Sweet, vanilla [32]

[1] Minus sign '—' indicates the CAS was not available in the literature.

4. Discussion

In our results, 163 non-volatile compounds were identified and annotated in hot and cold brew coffee (Table S1). These compounds were classified into alkaloids, including caffeine, theobromine, and trigonelline; phenolic compounds such as chlorogenic acid, caffeic acid, and quinic acid; and organic acids, amino acids, coumarins, furans, vitamins, pyrazines, and others. These compounds play a crucial role in determining the sensory profile of coffee, contributing significantly to its flavor characteristics [33]. Additionally, they provide various bioactive functions, including antioxidant properties and stimulatory effects. Bitterness was a major sensory attribute that impacted consumer acceptability. Both caffeine and chlorogenic acid played a role in contributing to this bitterness, while chlorogenic acid additionally contributed to astringency [34]. In our results, the major non-volatile compounds in coffee, including caffeine, chlorogenic acid, and trigonelline, showed no significant differences between hot and cold brew coffee, suggesting these compounds

are less influenced by the coffee brewing condition. According to Fuller and Rao [35], the caffeine and 3-chlorogenic acid levels in coffee vary with different roast degrees and particle sizes. Their findings indicated that roasting temperature influenced the 3-chlorogenic acid content. However, no significant difference was observed between cold and hot brewed coffee, which is in agreement with our results. Intragranular pore diffusion limits the extraction of chlorogenic acid, despite its high solubility in water. This characteristic allows for efficient extraction of chlorogenic acid at both high and low temperatures, enhancing its extraction under various brewing conditions [36]. Trigonelline plays a role in the overall aroma profile of brewed coffee. While it typically has a mild, bitter taste, it breaks down quickly during the roasting process, forming potent volatile compounds like pyridines and pyrroles. Córdoba et al. and Stankek et al. [14,37] investigated the variation in trigonelline content in coffee as influenced by different brewing methods. In their results, most coffee varieties showed no significant variation, though some exceptions were observed. Specifically, Brazilian coffee beans exhibited higher trigonelline levels in cold brew extraction, whereas Guatemalan beans demonstrated higher trigonelline concentrations in hot brew extraction.

Our findings indicate that 17 non-volatile compounds exhibited significant differences between hot brew and cold brew coffee (Table 1 and Figure 3). Among these compounds, one of the most prominent in coffee is 4,5-dicaffeoylquinic acid, which is a derivative of quinic acid. Silveira et al. [38] reported that the extraction efficiency of quinic acids tends to increase proportionally with rising temperatures. Notably, a sharp enhancement in extraction was observed when the temperature exceeded 95 °C, suggesting that higher thermal conditions significantly promote the solubility and release of quinic acids. Additionally, coumarin derivatives, including 4-coumaric acid and scoparone, showed relatively higher extraction efficiencies in hot brew coffee compared to cold brew coffee. Similarly, Doctor et al. [39] reported findings that coumarin derivatives exhibit increased extraction efficiency as temperature rises. Other compounds, such as alkaloids, including norharman and polyphenol, tyrosol, and shikimic acid, exhibited relatively higher extraction efficiencies in hot brew coffee. This finding underscores the critical role of temperature in optimizing the extraction of bioactive compounds during the brewing process, particularly at elevated temperatures where extraction kinetics are more favorable [40]. 3-(3-Pyridinyl)propanoic acid, as a derivative of pyridine, was shown to be present in relatively high amounts in cold brew coffee. However, no other literature demonstrating similar results could be found. Therefore, future research should aim to verify the reproducibility of this substance in cold brew coffee and investigate the underlying causes.

In our results, 93 volatile compounds were identified and annotated in hot and cold brew coffee (Table S2). These compounds were classified into pyrazines which are associated with nutty and roasted aromas, such as 2-methylpyrazine, 2-ethylpyrazine, and 2-ethyl-5-methylpyrazine; phenolic compounds which are associated with smoky aromas, such as cresol; furans which are associated with sweet and nutty aromas, such as 2-methylfuran and furfural; and pyridines; alcohols; and others [32].

Pyrazines and furans are among the most abundant volatile compounds in coffee, primarily generated through the Maillard reaction during the roasting process of coffee beans. In previous studies, pyrazines, including 2-methylpyrazine and 2-ethylpyrazine, were typically found to exhibit higher extraction efficiencies in cold brew coffee compared to hot brew coffee [25,36]. However, in our results, most pyrazines showed no significant difference between cold brew and hot brew extractions (Table 2). This discrepancy suggests that extraction time, rather than temperature, may play a more critical role in the extraction of pyrazines. While previous studies utilized relatively longer extraction times for cold brew, our study maintained consistent extraction times across both methods, focusing

specifically on temperature variations. Additionally, Maksimowski et al. [41] reported that the content of pyrazines increases as the extraction time is extended under conditions of 25 °C. A similar trend was observed with furans such as furfural and 2-methylfuran.

On the other hand, phenolic compounds, including catechol, 4-ehtly-1,3-benzenediol, 2-methoxy-4-vinylphenol (smoky and woody aroma), and 4-vinylphenol (sweet and vanilla aroma), as well as indole and 3-methylindole (animalic aroma), were present in relatively higher amounts in hot brew coffee compared to cold brew coffee in our results [32]. According to Cai et al. [25], 4-vinylguaiacol (2-methoxy-4-vinylphenol) was present in relatively higher amounts in hot brew coffee. Additionally, Pan et al. [36] reported that 4-ethylguaiacol and indole were present in higher concentrations in hot brew coffee, which is in agreement with our results. This finding suggests that extraction temperature, rather than time, may play a more critical role in the extraction of phenolic compounds and indole. In particular, indole was not detected in some other studies, suggesting that its presence may be influenced by factors such as coffee variety and roasting conditions [36].

Cedrenol, a compound classified as a terpenoid, is known for its woody and sweet flavor. In our study, it was found to be present in relatively higher concentrations in cold brew coffee compared to hot brew coffee. In general, elevated temperatures increase the saturated vapor pressure, leading to greater losses of some volatile compounds [42].

In this study, we utilized HR-OMS and advanced software, such as Compound Discoverer, to identify and annotate a total of 163 non-volatile compounds and 93 volatile compounds in both hot brew and cold brew coffee, significantly more than in previous research. Among the annotated compounds, in addition to those previously mentioned, we identified several chemicals, such as esculetin, scoparone, scopoletin, and resveratrol, which have not yet been reported in relation to coffee. Given that many of these compounds exhibit potent antioxidant properties and hold potential health benefits, further in-depth studies are warranted to explore their biological relevance and possible applications.

5. Conclusions

In conclusion, we identified and annotated a total of 163 non-volatile compounds and 93 volatile compounds in both hot brew and cold brew coffee using HR-OMS. The major classes of non-volatile compounds were alkaloids, including caffeine and trigonelline, and phenolic compounds, including chlorogenic acid. The main classes of volatile compounds were pyrazines, phenolic compounds, and furans. The influence of hot and cold brewing on the coffee's non-volatile and volatile compound profiles showed notable differences, with 18 distinct non-volatile compounds and 13 aroma-active compounds identified. Our analysis revealed that certain quinic acids, such as 4,5-dicaffeoylquinic acid, and coumarin derivatives were present in higher amounts in hot brew coffee. Based on this, we can conclude that some non-volatile compounds are significantly influenced by temperature during the extraction process. Regarding volatile compounds, phenolic compounds and indole are significantly influenced by temperature during the extraction process. On the other hand, a comparison with other studies revealed that pyrazine and furan compounds are more affected by extraction time than by temperature. Finally, among the annotated compounds, several previously unreported bioactive compounds in coffee were detected. Although our study qualitatively identified compounds not previously reported in previous research, the quantification of each compound was not assessed. Therefore, further research should focus on the quantification of these compounds, while concurrently evaluating the functionality and toxicity of the major compounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages11010010/s1: Table S1: Non-Volatile compounds identi-

fied in hot and cold brew coffee using liquid chromatography high-resolution orbitrap mass spectrometry; Table S2: Volatile compounds identified in hot and cold brew coffee using gas chromatography high-resolution orbitrap mass spectrometry.

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