



Article

Investigating the Volatiles of Kombucha During Storage Under Refrigerated Conditions

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Abstract

This study investigates the evolution of the chemical components of kombucha aroma during refrigerated storage. Two preparation methods (MT1 and MT2) were used to produce kombucha from a 1:1 mixture of black and green tea. The bottled beverages were stored at 4 °C for three months, and changes in headspace (HS) volatiles were monitored at different time points using solid-phase microextraction (SPME) and GC-MS. A total of 68 volatile substances were identified, with alcohols, acids, and esters dominating the aroma profile. The study revealed significant changes in flavor composition during cold storage, particularly in the first two weeks, with an increase in the number of esters, acids, ketones and terpenoids, as well as the total amount of esters and alkanols. While some changes contribute to the desirable “cider-like” characteristics, others, like certain volatile acids, aliphatic aldehydes and ketones, are associated with off-flavors. These findings suggest that refrigeration alone is not sufficient to completely inhibit microbial activity in freshly prepared kombucha, highlighting the need for further research to correlate chemical changes with sensory properties to establish optimal organoleptic standards and shelf life.

Keywords: kombucha; aroma; volatile substances; solid-phase microextraction; SCOBY; black tea; green tea; fermented beverages



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1. Introduction

Kombucha is a fermented beverage traditionally made from sweetened tea (*Camellia sinensis* L. Kuntze) that is modified by the activity of a symbiotic colony of yeasts, acetic acid bacteria (AAB) and, occasionally, lactic acid bacteria (LAB). Kombucha is usually made from black or green tea, but other types of tea (oolong, white, rooibos and Pu-erh) are also frequently used [1,2]. A variety of flavored kombuchas incorporate fruits and fruit juices, herbs, and spices to enhance flavor and appeal to a broader range of consumers [3,4]. In addition, the rapidly growing market for fermented teas has led to research into the valorization of by-products from various food industries (cocoa mucilage, coffee husks, grape pomace, soybean whey), which offer new opportunities for sustainable development while providing kombucha-style beverages with good sensory acceptability, probiotic potential, and a high content of bioactive substances [5,6].

Kombucha has a history that is shrouded in both fact and legend. Historical documents place the origin of the drink in north-eastern China (Manchuria) around two centuries BC, from which the available evidence suggests a long journey across centuries and continents [7]. Kombucha has recently experienced a surge in popularity and has established itself as a major player in the global beverage market. This is largely due to growing consumer interest in health and wellness, with kombucha often perceived as a healthier alternative to traditional soft drinks [8].

The consumption of kombucha is associated with a number of potential health benefits, including antioxidant activity, antimicrobial and anti-inflammatory effects. Kombucha is traditionally used to support digestive health, boost the immune system and regulate blood sugar levels. Some studies have investigated its potential to lower blood pressure and cholesterol levels, prevent cancer and cardiovascular disease and improve liver function [9,10]. However, many of the reported health benefits of kombucha are based on *in vitro* studies or anecdotal evidence. More well-designed clinical studies are needed to confirm these effects in humans and determine the optimal dosage and consumption habits to maximize health benefits [11,12].

The sensory experience of kombucha is complex and multi-layered: the color typically ranges from pale yellow to amber, depending on the type of tea used as the base ingredient (black, green or herbal) and the duration of fermentation; the taste is described as refreshingly tart, tangy, slightly sweet and effervescent; the aroma profile is described as a blend of fruity, vinegary and sometimes floral or spicy notes [13,14]. Despite the importance of sensory attributes in the development of a mass-market beverage, there is little information on the composition of the volatile fraction, the origin of the aroma components, the dynamic changes in the volatile components during the fermentation process, and the relationship between the key aroma components and sensory perceptions.

Once the optimum fermentation time has been reached, the beverage must be properly preserved to prevent the formation of a biofilm during the storage period. Even though the pH of an optimally fermented product (in terms of taste) is usually below the critical limit for safety (≤ 4.2), kombucha is still a microbiologically unstable system: spoilage due to molds and increases in ethanol and carbon dioxide can occur. The ethanol content can exceed the legal limits for non-alcoholic beverages, especially in sealed containers where the build-up of carbon dioxide stimulates alcoholic fermentation, inhibits the oxidation of ethanol to acetaldehyde/acetic acid and causes an increase in pressure leading to leakage or breakage of the container. Since 2010, there have been reports of unpasteurized kombuchas being recalled from store shelves due to misrepresentation of alcohol content [15,16].

Although heat treatments at low temperatures (60, 65 and 68 °C for 1 min) have been shown to be ineffective in preserving the functional properties of the beverage [17], a properly designed pasteurization (82 °C for 1 min; 75 °C for 15 min) results in a shelf-stable product [16,18]. Other stabilization methods rely on refrigeration (4 °C) alone or together with antifungal preservatives (e.g., sodium benzoate, potassium sorbate), but shelf life should be estimated and validated [16]. Daneluz et al. [19] have recently proposed microfiltration as a mild alternative to thermal pasteurization to improve the microbiological stability and visual appeal of the beverage.

As reported by Nyhan et al. [15], most commercial kombuchas are marketed as raw, unpasteurized products because consumers expect “live” products and probiotic effects. However, there is limited data on the microbial and chemical stability of kombucha during cold storage, and this is especially true for studies combining microbiological data with physico-chemical parameters and descriptive sensory analysis. Grassi et al. [20] monitored microbial dynamics in a flavored green tea-based kombucha and found that the yeast and AAB populations remained viable for up to 90 days during refrigerated storage. In a 90-day

shelf-life study (4 °C), Fabricio et al. [21] measured a consistent presence of viable probiotic *K. marxianus* cells in green tea kombucha fermented by a customized microbial consortium (*Acetobacter aceti*, *Novacetimonas hansenii*, *Komagataeibacter saccharivorans*, *Brettanomyces anomala*, *Kluyveromyces marxianus*). In contrast, Tran et al. [18] observed a decrease in yeast and bacterial populations during an 8-month cold storage of unflavored and flavored kombuchas. Fu et al. [22] also observed that refrigerated storage of green tea fermented with a customized inoculum (*Saccharomyces cerevisiae* Meyen ex Hansen, *Gluconacetobacter* sp. and *Lactobacillus plantarum*) reduced the viability of yeasts, AAB and especially probiotics (LAB), whose survival rate was less than 1% after 8 days of storage. La Torre et al. [23] focused on the effects of long-term (9 months) storage at 4 °C on the phenolic content and antioxidant properties of fermented black tea and suggested a shelf life of four months, beyond which the polyphenolic antioxidant content decreased significantly.

To our knowledge, only the recent study by Tran and co-workers [18] has monitored the changes in volatile profiles of unflavored and flavored kombuchas during a long storage (8 months) at cold temperature to understand how flavoring affects the carbonation and aging of the product. The aim of the present study was therefore to gain new experimental insights into the evolution of the chemical components of kombucha aroma during storage. Changes in the profile of headspace (HS) volatiles obtained by solid-phase microextraction (SPME) and analyzed by GC-MS were monitored in traditional kombucha prepared from a 1:1 mixture of black and green tea, bottled and stored at 4 °C for three months. The study also compared two different methods of producing the finished beverage. This information could help to identify new chemical markers that could be correlated with sensory properties and used to estimate the optimal shelf life of raw and chilled kombucha.

2. Materials and Methods

2.1. Preparation of Kombucha Tea

Two different methods (MT1 and MT2) were used to prepare fermented teas. Using the MT1 method, a batch of 18 L of kombucha was prepared in a stainless-steel container. A mixture of 0.4% *w/v* of organic Gunpowder green tea (Zhejiang, China; <https://storieditecaffè.com/>) and 0.4% of organic CTC (crush, tear, curl) BP (Broken Pekoe) black tea (Assam, India; <https://storieditecaffè.com/>) was infused for 10 min in boiled tap water with a cotton bag. The tea was cooled to 60 °C before adding 10% *w/v* sucrose and further cooled to room temperature before inoculating 10% *w/v* of an actively growing daughter SCOBY (symbiotic culture of bacteria and yeast) (KoRo Handels GmbH, Berlin, Germany) from previous fermentations. The container was immediately covered with clean cloth and the fermentation was carried out in a dedicated room at a controlled temperature (22 ± 2 °C) for 23 days until the pH reached 3.2 ± 0.2. The fermented tea was then bottled in 275 mL glass bottles, which were sealed with a crown cap and stored at 4 °C. The bottles and caps were previously sterilized at 121 °C for 20 min. In the MT2 method, a sweetened tea base is prepared according to the above recipe and cooled to room temperature. A total volume of 18 L of beverage was prepared by mixing 11 L (approx. 60%) of freshly prepared tea and 7 L (approx. 40%) of kombucha fermented for 45 days at 22 ± 2 °C (pH 2.5), so that the pH of the final beverage was 3.2 ± 0.2. The beverage was filled into previously sterilized glass bottles (275 mL) and stored at 4 °C.

In order to analyze the volatile components during storage of the beverages at +4 °C, three bottles were randomly sampled for each method on the day of bottling (time 0) and 1, 2, 3, 4, 6, 8, 10 and 12 weeks after bottling.

2.2. Determination of the Volatile Compounds

Headspace solid-phase microextraction (HS-SPME) was used to characterize the volatile profile of kombucha bottles. Five mL of the filtered beverage was poured into a 20 mL screw cap vial. The vial was equilibrated in a water bath at 40 °C for 15 min and then a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco/Sigma-Aldrich, Milan, Italy) was exposed to the headspace of the vial at 40 °C for 30 min. Volatiles were analyzed using a Trace 1300 gas chromatograph equipped with a Zebron ZB-5 ms capillary column 30 m × 0.25 mm i.d., 0.25 µm film thickness (Phenomenex, Torrance, CA, USA). A Dual Detector Microfluidics kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to split the injected sample 1:1 between an ISQ 7000 single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) (qualitative analysis) and a conventional flame ionization detector (FID) (quantitative analysis). The chromatographic conditions are described in Mozzon et al. [24]. The volatile compounds were identified by comparing mass spectral data and Kovats retention indices (RIs) with the data collected in the NIST/EPA/NIH Mass Spectral Library 2020. A mixture of normal C5–C20 alkanes (Sigma-Aldrich, St. Louis, MO, USA) was used to calculate RIs under the experimental conditions. An automated spreadsheet was used to simplify the calculation of the RIs of the unknown components [25].

2.3. Data Analysis

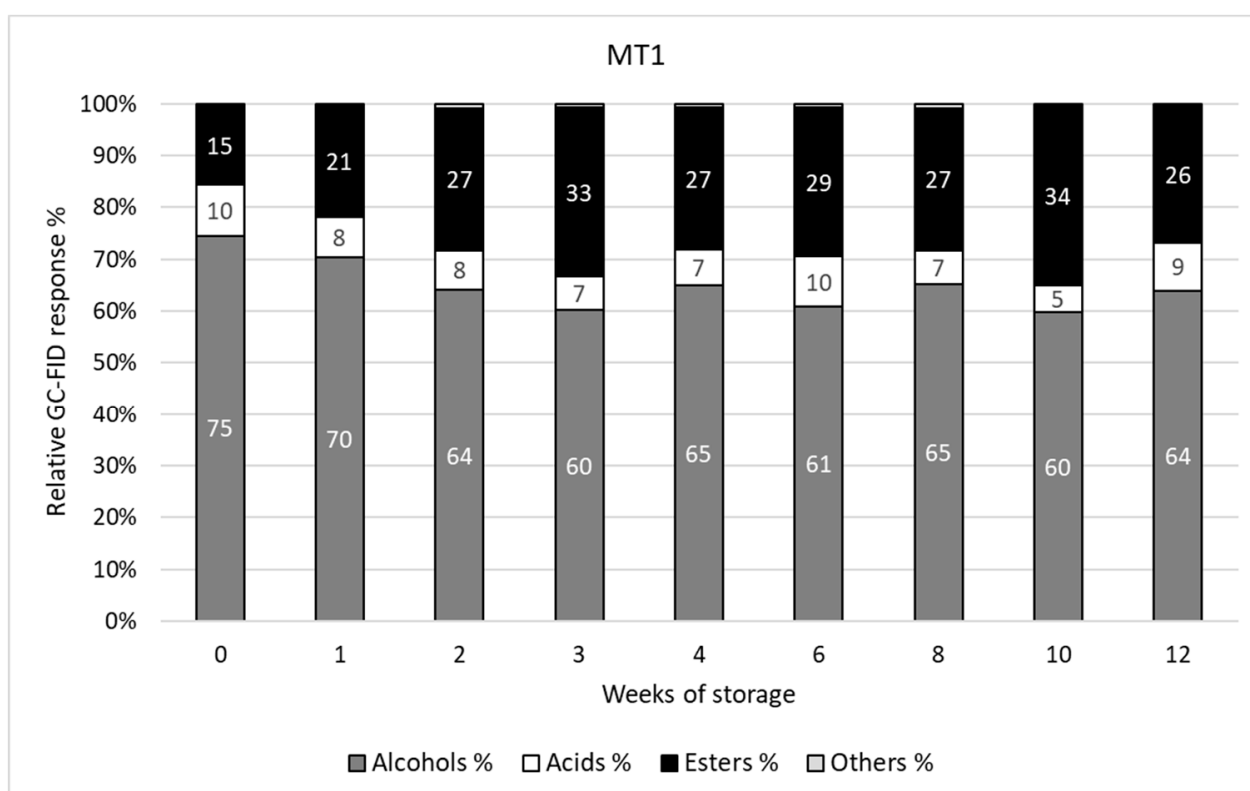
All experimental data were expressed as mean ± standard deviation of absolute GC-FID response. The effect of storage time on the dependent variables (aroma components) was analyzed using a one-way ANOVA, separately for each preparation method. Tukey–Kramer’s Honest Significant Difference (HSD) test was used to compare multiple means. Two-way (factorial) ANOVA was applied to the analytical data to test and estimate the effect of the independent factors (preparation method, refrigerated storage time) and their interaction on the measured variables. Principal component analysis (PCA) was performed on the auto-scaled data matrix to explore the structure of the experimental data and the relationships between the aroma composition and the independent factors. The significance level was always set at $p < 0.05$. All statistical analyzes were performed using JMP® Version 10 software (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

The studies on the aroma of traditional kombucha are very recent and cover the last 7 years (2018–2025) (Table A1). SPME has been widely used to adsorb or absorb the volatiles on fibers coated with DVB/carboxen/PDMS [21,26–41] or Carboxen/PDMS [42–45], but other sampling techniques have also been used: Suffys et al. [46] and Tran et al. [18] captured volatile organic compounds using the stir bar sorptive extraction (SBSE) method, Ferremi Leali et al. [47] quantified alcohols, esters, fatty acids and benzenoids with a solid-phase extraction (SPE) cartridge (poly(styrene-divinylbenzene)) and Savary et al. [48] used an automatic sampler for HS analysis integrated with the gas chromatograph. The analytes were usually separated on polar GC capillary columns (DB-WAX type) [1,18,21,27,31–34,36–38,41,43,46,47,49,50] and less frequently on apolar DB-5 equivalents [28,30,35,40,42,44,45,50–53] or DB-1 equivalents [26,54]. Out of the chorus, Xu et al. [55] separated 53 volatiles in the aroma of refermented kombucha beverages using a polyethylene glycol capillary column modified with nitroterephthalic acid.

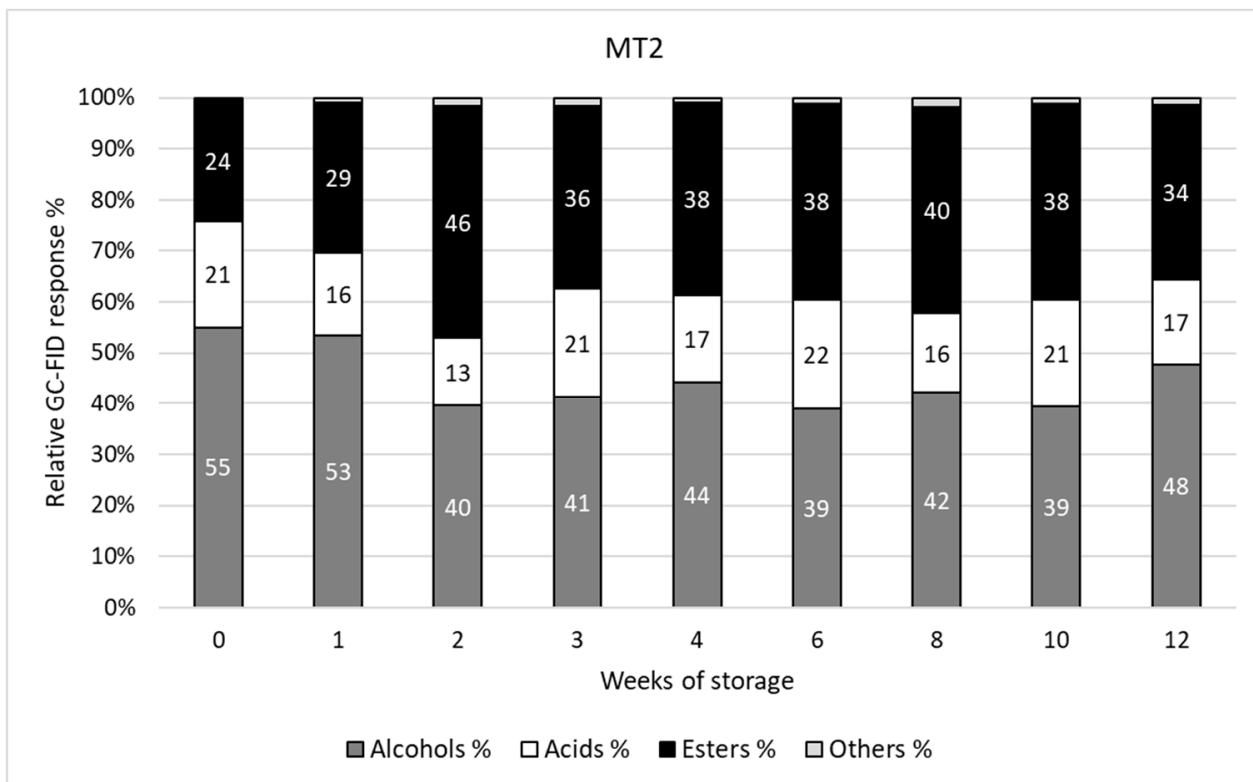
A total of 68 volatile substances were identified in the static HS of the kombucha samples (Table A1). Based on their structural similarity, the volatiles were categorized into aldehydes (2), aliphatic alcohols (5), carboxylic acids (9), esters (24), ketones (8), terpenes (14), phenols (3) and others (3) (alkenylbenzenes, benzofurans and benzopyrans). Most of

the volatiles identified in the HS of the analyzed samples have been previously detected in the aroma of kombuchas from traditional Chinese teas (black tea, oolong tea, dark tea, green tea, yellow tea and white tea) with the exception of isoamyl propionate and nonanoate, ethyl 3-nonenoate, α -isomethylionone, D-camphor and menthone. The aroma profiles of the analyzed samples were dominated by alcohols, acids and esters during the entire storage period, which together accounted for 98.0–99.7% of the total volatiles. According to Njieukam et al. [43], it is possible to produce kombucha without the addition of SCOBY but only with the fermented liquid from the previous batch, as we did in the MT2 method. This resulted in beverages characterized by lower relative amounts of alcohols (35.9–60.3% vs. 56.1–76.3%) and higher proportions of acids (10.3–26.3% vs. 4.6–12.8%) and esters (23.3–51.8% vs. 13.6–38.8%) than MT1 kombuchas (Figure 1). Alcohols (ethanol, phenylethanol, 3-methyl-1-butanol), acids (acetic, isobutyric, valeric, 2-methylhexanoic, octanoic, decanoic, dodecanoic) and esters (ethyl acetate, phenethyl acetate, ethyl laurate) were also found to be the predominant volatiles in refermented green tea kombucha [55], in traditional black and green tea kombuchas [26,28,34,38,49] and in sweetened black tea inoculated with *Gluconacetobacter intermedius* and *Starmerella davenportii* [53]. During the first two weeks of storage, a significant increase in the number of volatiles was observed, especially in esters, which increased from 8 to 22 in MT1 samples and from 6 to 22 in MT2 samples. The number of acids, ketones and terpenoids also increased from 5–7 to 9, from 1–2 to 5–7 and from 7–9 to 11–12, respectively. Only in the MT2 beverage was the increase in the number of esters accompanied by a significant increase in their total amount. The total amount of alcohol almost doubled during storage of the MT2 kombucha, while only a slight increase was observed in the MT1 samples (Figure 2).



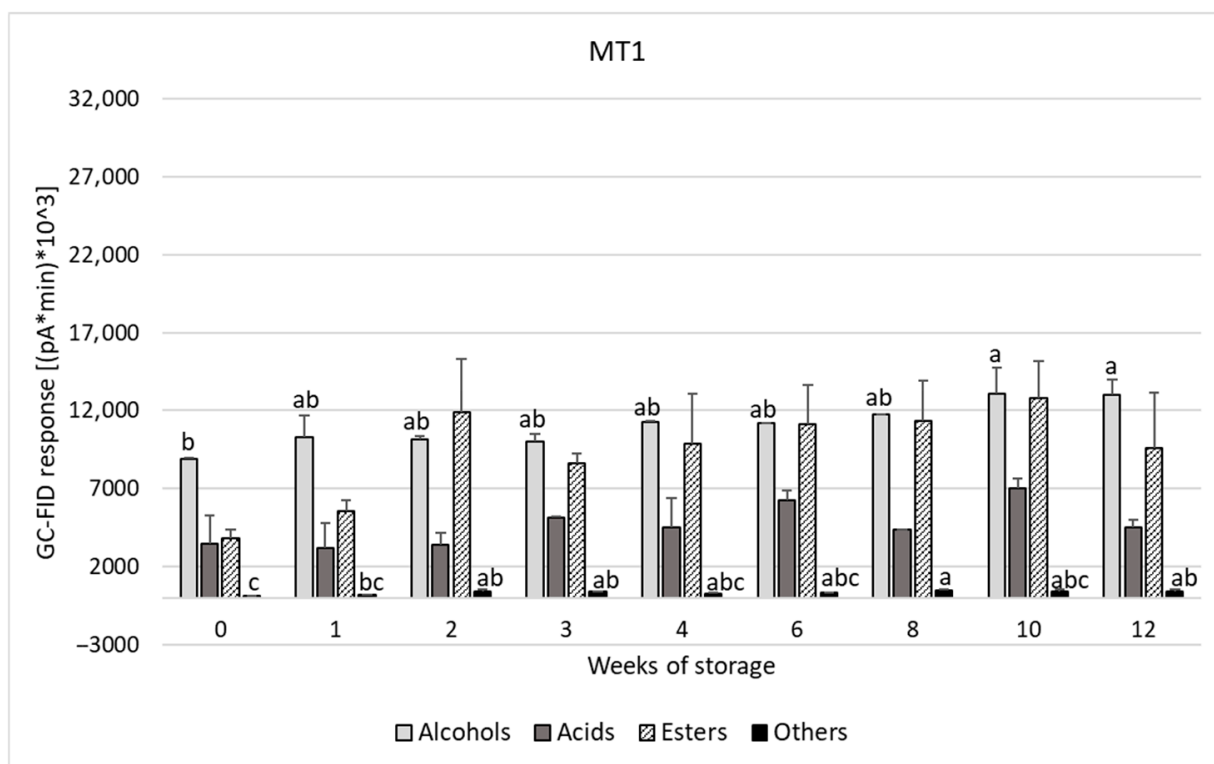
(a)

Figure 1. Cont.



(b)

Figure 1. Average contents (relative peak areas) of the different classes of volatiles during cold storage of: (a) kombucha produced according to the MT1 method; (b) kombucha produced according to the MT2 method.



(a)

Figure 2. Cont.

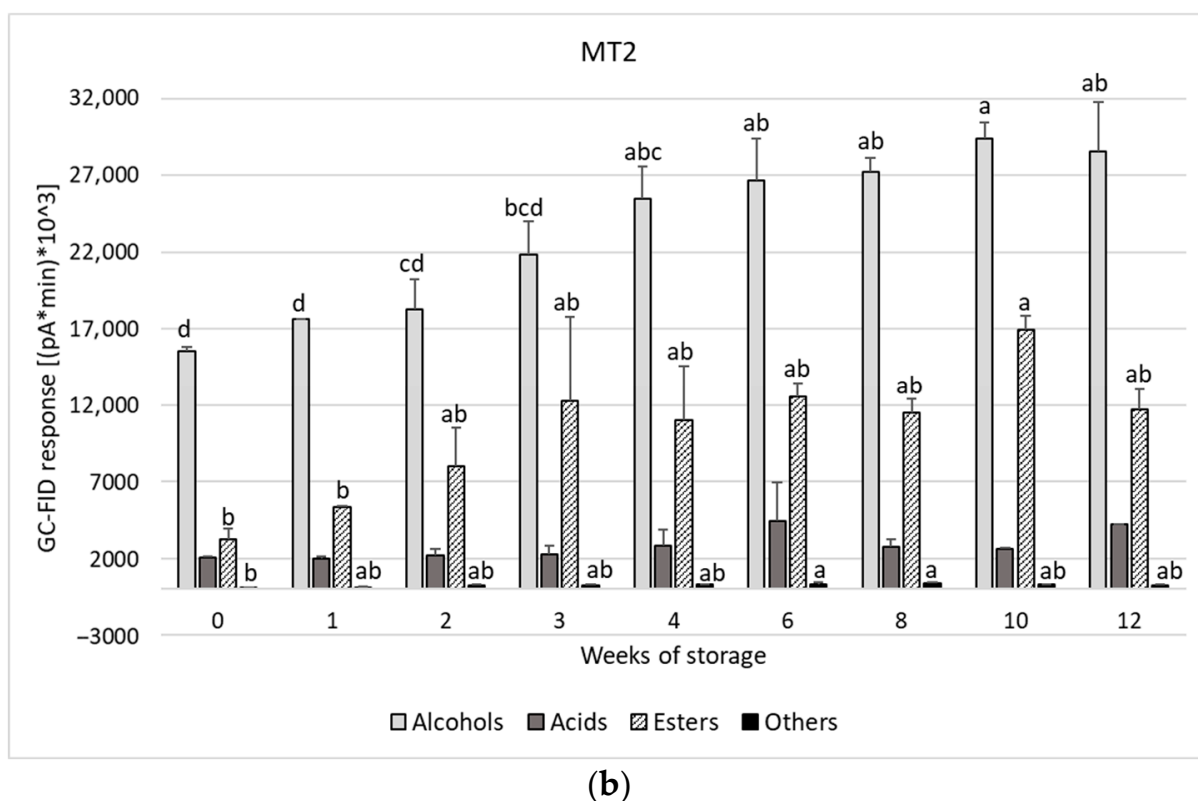


Figure 2. Total amount (absolute GC-FID response; mean \pm SD) of the different classes of volatiles during cold storage of: (a) kombucha produced according to the MT1 method; (b) kombucha produced according to the MT2 method. Lower case letters indicate significant differences between storage times at an alpha level of 0.05 according to an HSD test.

Ethanol is the most abundant aliphatic alcohol detected in all kombucha products, followed by 3-methyl-1-butanol (isoamyl alcohol) and phenylethyl alcohol (Tables 1 and 2). These results are in agreement with the data reported by Wu et al. [50] for Fu-brick fermented tea. All alkanols showed an increasing trend during cold storage of MT1 and MT2 fermented teas, indicating significant yeast activity. Indeed, yeasts can synthesize ethanol (alcoholic, slightly sweet) from residual sugars by glycolysis and higher alcohols by the Ehrlich pathway, which is based on the transamination of an amino acid (valine, phenylalanine), decarboxylation of the keto acid (α -keto-isovalerate, phenylpyruvate) and subsequent reduction in the acid to volatile alcohol (isoamyl alcohol, phenylethanol) [1,27,37,43,44]. Our results are in agreement with those of Talebi et al. [56], who monitored the ethanol concentration of two kombucha batches over a period of 60 days at 4 and 22 °C. The authors found that the ethanol content peaked after 14 days of storage at both temperatures and then leveled off. Ethanol, isoamyl alcohol and phenylethyl alcohol were not detected in unfermented black and green teas [28,43], further supporting their microbiological origin. The “cider-like” character of the beverage is attributed to the metabolites of yeast activity, especially higher alcohols and esters. Isoamyl alcohol can contribute to “fusel” notes at higher concentrations, but can add complexity at lower concentrations [21].

Table 1. Levels of volatile substances (peak areas [(pA × min) × 10³]; mean ± SD, n = 3) ¹ sampled by SPME in the headspace of kombucha beverages prepared by method MT1.

	Weeks of Storage at 4 °C								
	0	1	2	3	4	6	8	10	12
Aldehydes									
Nonanal	0 ± 0 b	0 ± 0 b	3 ± 1 ab	3 ± 2 ab	4 ± 1 ab	6 ± 1 ab	18 ± 5 a	17 ± 9 a	8 ± 7 ab
Alkanols									
Ethanol	11,936 ± 476 c	14,014 ± 257 bc	14,270 ± 1524 bc	17,256 ± 2195 abc	20,545 ± 1523 ab	21,327 ± 1948 a	22,174 ± 759 a	23,106 ± 1291 a	23,300 ± 3094 a
3-Methyl-1-butanol	3576 ± 214 b	3612 ± 226 b	3984 ± 384 b	4303 ± 153 b	4591 ± 527 ab	4856 ± 720 ab	4656 ± 141 ab	5808 ± 250 a	4660 ± 149 ab
2-Ethyl-1-hexanol	30 ± 2 abc	0 ± 0 c	8 ± 2 c	25 ± 0 bc	10 ± 0 c	45 ± 2 abc	64 ± 6 abc	109 ± 4 ab	116 ± 68 a
2-Phenylethyl alcohol	39 ± 11 bc	0 ± 0 c	34 ± 14 c	297 ± 117 abc	350 ± 25 ab	424 ± 117 a	364 ± 11 a	413 ± 10 a	526 ± 199 a
Carboxylic acids									
Acetic acid	1738 ± 11	1513 ± 79	1155 ± 214	1283 ± 44	2097 ± 1004	3517 ± 2281	2066 ± 434	1635 ± 49	3080 ± 258
Propanoic acid, 2 methyl-	0 ± 0 c	0 ± 0 c	0 ± 0 c	30 ± 10 a	15 ± 3 abc	19 ± 1 ab	11 ± 1 abc	22 ± 11 ab	9 ± 3 bc
Butanoic acid, 3-methyl-	209 ± 51	301 ± 24	404 ± 76	354 ± 209	222 ± 24	231 ± 66	222 ± 14	272 ± 31	218 ± 28
Butanoic acid, 2-methyl-	48 ± 7	61 ± 4	87 ± 19	90 ± 34	76 ± 2	82 ± 11	78 ± 2	76 ± 5	83 ± 20
n-Hexanoic acid	13 ± 4	25 ± 2	27 ± 2	21 ± 5	21 ± 0	35 ± 29	26 ± 5	44 ± 7	33 ± 13
Hexanoic acid, 2-ethyl-	0 ± 0	0 ± 0	2 ± 0	2 ± 0	3 ± 2	4 ± 3	1 ± 0	1 ± 0	0 ± 1
n-Octanoic acid	34 ± 11 e	63 ± 17 de	274 ± 29 bc	259 ± 111 bc	213 ± 10 cde	289 ± 51 bc	239 ± 33 bcd	489 ± 53 a	410 ± 32 ab
n-Nonanoic acid	0 ± 0	0 ± 0	124 ± 32	75 ± 76	37 ± 3	37 ± 24	25 ± 15	45 ± 15	71 ± 58
n-Decanoic acid	0 ± 0	0 ± 0	102 ± 39	175 ± 56	105 ± 0	188 ± 66	68 ± 1	48 ± 35	280 ± 212
Esters									
Ethyl acetate	2882 ± 557 d	4571 ± 10 cd	5329 ± 1115 bcd	7518 ± 1168 abc	7738 ± 1827 abc	8059 ± 113 abc	7980 ± 70 abc	8732 ± 852 ab	8969 ± 489 a
Isobutyl acetate	6 ± 5	19 ± 1	7 ± 4	14 ± 7	16 ± 9	17 ± 8	15 ± 0	27 ± 6	25 ± 0
Butanoic acid, ethyl ester	0 ± 0 c	0 ± 0 c	2 ± 1 bc	5 ± 3 abc	9 ± 2 ab	11 ± 1 a	12 ± 2 a	11 ± 2 a	8 ± 2 ab
Butanoic acid, 2-methyl-, ethyl ester	0 ± 0	2 ± 0	12 ± 9	70 ± 84	102 ± 9	109 ± 20	87 ± 3	79 ± 9	63 ± 17
Butanoic acid, 3-methyl-, ethyl ester	0 ± 0	0 ± 0	3 ± 0	6 ± 4	7 ± 0	7 ± 3	6 ± 1	6 ± 0	6 ± 3
1-Butanol, 3-methyl-, acetate	316 ± 115	677 ± 45	559 ± 312	786 ± 299	669 ± 245	735 ± 190	599 ± 31	750 ± 79	520 ± 216
Hexanoic acid, methyl ester	0 ± 0	0 ± 0	4 ± 0	1 ± 1	9 ± 13	5 ± 7	10 ± 8	0 ± 0	3 ± 4
Hexanoic acid, ethyl ester	3 ± 1	11 ± 4	47 ± 30	168 ± 169	158 ± 42	189 ± 50	142 ± 4	146 ± 7	108 ± 67
1-Butanol, 3-methyl-, propanoate	0 ± 0	0 ± 0	1 ± 1	4 ± 5	5 ± 1	4 ± 1	3 ± 1	3 ± 0	2 ± 2
Heptanoic acid, ethyl ester	0 ± 0	0 ± 0	12 ± 7	30 ± 25	26 ± 11	40 ± 18	36 ± 2	37 ± 4	24 ± 15
Octanoic acid, ethyl ester	18 ± 9 b	33 ± 4 b	1295 ± 778 ab	2290 ± 2388 ab	1451 ± 997 ab	2254 ± 600 ab	1816 ± 725 ab	4265 ± 288 a	1395 ± 334 ab
Acetic acid, 2-phenylethyl ester	2 ± 1 b	6 ± 2 ab	23 ± 8 ab	32 ± 9 a	29 ± 6 ab	30 ± 7 a	22 ± 2 ab	24 ± 0 ab	24 ± 14 ab
3-Nonenoic acid, ethyl ester	0 ± 0	0 ± 0	1 ± 1	3 ± 3	4 ± 2	5 ± 1	4 ± 1	4 ± 1	4 ± 4
Nonanoic acid, ethyl ester	0 ± 0 b	0 ± 0 b	279 ± 162 ab	237 ± 186 ab	170 ± 132 ab	222 ± 110 ab	120 ± 9 ab	939 ± 623 a	130 ± 58 ab
4-Decenoic acid, ethyl ester	0 ± 0 b	0 ± 0 b	0 ± 0 b	2 ± 2 ab	1 ± 0 ab	2 ± 0 ab	2 ± 0 ab	4 ± 1 a	1 ± 0 ab
9-Decenoic acid, ethyl ester	0 ± 0	0 ± 0	13 ± 7	53 ± 68	19 ± 10	41 ± 2	24 ± 10	67 ± 21	12 ± 3
Decanoic acid, ethyl ester	14 ± 7	23 ± 3	312 ± 97	873 ± 970	483 ± 228	714 ± 47	562 ± 89	1676 ± 880	376 ± 91
Octanoic acid, 3-methylbutyl ester	0 ± 0	0 ± 0	23 ± 4	41 ± 47	27 ± 21	31 ± 11	23 ± 4	70 ± 24	14 ± 1
Octanoic acid, 2-methylbutyl ester	0 ± 0 c	0 ± 0 c	3 ± 0 a	1 ± 1 bc	1 ± 0 bc	1 ± 0 bc	1 ± 0 bc	2 ± 0 ab	1 ± 1 bc
Nonanoic acid, 3-methylbutyl ester	0 ± 0 b	0 ± 0 b	11 ± 2 ab	9 ± 6 ab	9 ± 5 ab	7 ± 2 ab	4 ± 1 ab	20 ± 11 a	4 ± 1 ab
Dodecanoid acid, ethyl ester	2 ± 0	2 ± 0	19 ± 5	75 ± 82	51 ± 13	42 ± 6	26 ± 1	43 ± 12	33 ± 17
Decanoic acid, 3-methylbutyl ester	0 ± 0	0 ± 0	7 ± 0	27 ± 33	23 ± 9	20 ± 0	9 ± 1	22 ± 6	7 ± 1

Table 1. Cont.

	Weeks of Storage at 4 °C									
	0	1	2	3	4	6	8	10	12	
Ketones										
5-Hepten-2-one, 6-methyl-Damascenone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 1	2 ± 1	0 ± 0	0 ± 0	
α-Ionone	0 ± 0	1 ± 0	2 ± 0	2 ± 2	1 ± 0	0 ± 0	1 ± 0	1 ± 0	1 ± 0	
α-Isomethylionone	0 ± 0	5 ± 5	2 ± 0	2 ± 0	1 ± 0	1 ± 0	2 ± 0	1 ± 0	1 ± 0	
β-Ionone	1 ± 1 ab	3 ± 0 a	3 ± 1 a	2 ± 0 a	2 ± 0 ab	0 ± 0 b	1 ± 0 ab	2 ± 0 ab	0 ± 0 b	
	0 ± 0	0 ± 0	4 ± 4	1 ± 0	1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	
Phenols										
3-Ethylphenol	0 ± 0	0 ± 0	14 ± 6	21 ± 9	8 ± 6	12 ± 5	6 ± 2	17 ± 9	8 ± 11	
4-Ethyl-2-methoxy-phenol	0 ± 0 b	0 ± 0 b	5 ± 1 ab	7 ± 3 ab	3 ± 0 ab	7 ± 2 ab	7 ± 0 ab	7 ± 1 ab	10 ± 5 a	
2,4-Di-tert-butylphenol	11 ± 3 c	19 ± 1 bc	27 ± 7 abc	45 ± 3 ab	48 ± 12 ab	41 ± 8 ab	30 ± 4 abc	52 ± 12 a	37 ± 10 abc	
Terpenes										
β-Myrcene	0 ± 0	0 ± 0	51 ± 11	11 ± 14	63 ± 89	119 ± 84	70 ± 58	1 ± 0	12 ± 17	
o-Cymene	0 ± 0	0 ± 0	3 ± 0	2 ± 3	3 ± 4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	
D-Limonene	2 ± 2 b	4 ± 1 b	11 ± 2 b	4 ± 2 b	15 ± 11 b	7 ± 7 b	47 ± 11 a	4 ± 1 b	5 ± 5 b	
Eucalyptol	7 ± 1	13 ± 0	12 ± 7	15 ± 0	9 ± 2	12 ± 4	18 ± 2	17 ± 1	12 ± 4	
Linalool oxide, (Z)-	5 ± 6 b	17 ± 0 ab	23 ± 12 ab	32 ± 3 ab	24 ± 9 ab	27 ± 1 ab	35 ± 1 a	37 ± 1 a	34 ± 14 a	
Linalool	23 ± 5 b	39 ± 3 ab	51 ± 21 ab	67 ± 3 ab	54 ± 14 ab	63 ± 10 ab	82 ± 6 a	76 ± 4 a	64 ± 22 ab	
D-Camphor	1 ± 1 b	3 ± 1 ab	4 ± 1 ab	5 ± 1 a	4 ± 2 ab	5 ± 1 ab	4 ± 0 ab	5 ± 0 a	4 ± 0 ab	
Menthone	0 ± 1	2 ± 0	2 ± 1	3 ± 0	2 ± 1	2 ± 0	2 ± 0	1 ± 1	0 ± 1	
α-Terpineol	0 ± 0	2 ± 0	2 ± 0	3 ± 3	2 ± 1	2 ± 0	3 ± 1	1 ± 0	2 ± 2	
cis-β-Farnesene	0 ± 0	0 ± 0	2 ± 2	2 ± 1	2 ± 0	3 ± 0	2 ± 0	4 ± 1	5 ± 3	
Others										
trans-Nerolidol	1 ± 0 b	3 ± 0 ab	9 ± 3 ab	12 ± 1 a	10 ± 2 ab	10 ± 1 ab	9 ± 0 ab	11 ± 3 ab	12 ± 6 a	
Styrene	2 ± 2	6 ± 0	15 ± 9	15 ± 3	10 ± 7	9 ± 2	14 ± 5	20 ± 9	29 ± 25	
Edulan 1	0 ± 0 b	2 ± 0 b	2 ± 1 ab	2 ± 0 ab	1 ± 1 ab	1 ± 0 ab	2 ± 0 a	2 ± 0 a	2 ± 1	

¹ Lower case letters indicate significant differences between storage times at an alpha level of 0.05 according to an HSD test.

Table 2. Levels of volatile substances (peak areas [(pA × min) × 10³]; mean ± SD, n = 3) ¹ sampled by SPME in the headspace of kombucha beverages prepared by method MT2.

	Weeks of Storage at 4 °C								
	0	1	2	3	4	6	8	10	12
Aldehydes									
Octanal	0 ± 0 b	0 ± 0 b	2 ± 2 b	2 ± 0 b	1 ± 1 b	1 ± 1 b	15 ± 5 ab	3 ± 2 b	7 ± 3 ab
Nonanal	7 ± 4 b	3 ± 1 b	10 ± 3 b	15 ± 4 b	3 ± 1 b	4 ± 2 b	51 ± 5 a	12 ± 7 b	30 ± 20 ab
Alkanols									
Ethanol	7668 ± 7 b	9005 ± 1384 ab	8758 ± 130 ab	8594 ± 382 ab	9818 ± 175 ab	9869 ± 69 ab	10,284 ± 57 ab	11,263 ± 1502 a	11,142 ± 931 a
3-Methyl-1-butanol	1157 ± 141 b	1275 ± 22 ab	1373 ± 84 ab	1392 ± 139 ab	1397 ± 121 ab	1245 ± 45 ab	1360 ± 77 ab	1695 ± 232 a	1708 ± 112 a
1-Hexanol	3 ± 0 bc	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	1 ± 2 c	6 ± 0 ab	7 ± 1 a	8 ± 1 a
2-Ethyl-1-hexanol	13 ± 5 a	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	5 ± 4 ab	10 ± 0 ab	10 ± 7 ab
2-Phenylethyl alcohol	1 ± 0 d	0 ± 0 d	0 ± 0 d	0 ± 0 d	22 ± 10 cd	37 ± 11 bc	56 ± 3 ab	75 ± 16 a	85 ± 11 a
Carboxylic acids									
Acetic acid	3086 ± 1736	2667 ± 1503	2124 ± 1030	3716 ± 31	3247 ± 2050	5058 ± 517	3201 ± 65	5690 ± 661	2923 ± 339
Propanoic acid, 2 methyl-	31 ± 10 abc	35 ± 10 abc	82 ± 13 abc	92 ± 5 a	45 ± 7 abc	43 ± 3 abc	13 ± 1 c	30 ± 42 bc	0 ± 0 c
Butanoic acid, 3-methyl-	249 ± 31 c	348 ± 15 bc	580 ± 100 ab	608 ± 4 ab	718 ± 190 a	554 ± 52 abc	589 ± 2 ab	650 ± 76 ab	719 ± 72 a
Butanoic acid, 2-methyl-	56 ± 5 c	75 ± 10 bc	126 ± 30 ab	129 ± 3 ab	145 ± 33 a	110 ± 9 abc	122 ± 3 ab	133 ± 9 ab	145 ± 14 a
n-Hexanoic acid	7 ± 2 c	15 ± 4 bc	16 ± 0 bc	24 ± 2 bc	41 ± 19 ab	30 ± 2 bc	42 ± 2 ab	43 ± 11 ab	61 ± 6 a
Hexanoic acid, 2-ethyl-	2 ± 0 c	6 ± 1 bc	11 ± 3 a	13 ± 1 a	8 ± 1 ab	10 ± 1 ab	9 ± 0 ab	6 ± 1 bc	9 ± 1 ab
n-Octanoic acid	34 ± 8 c	53 ± 4 c	249 ± 23 b	261 ± 22 b	144 ± 36 bc	219 ± 44 b	206 ± 60 b	240 ± 3 b	401 ± 56 a
n-Nonanoic acid	0 ± 0 b	0 ± 0 b	62 ± 30 ab	104 ± 38 a	44 ± 18 ab	45 ± 21 ab	44 ± 7 ab	75 ± 22 ab	68 ± 45 ab
n-Decanoic acid	0 ± 0 b	0 ± 0 b	143 ± 51 a	203 ± 20 a	102 ± 30 ab	154 ± 10 a	135 ± 18 a	108 ± 31 ab	158 ± 41 a
Esters									
Ethyl acetate	3104 ± 141 b	4326 ± 615 ab	5678 ± 1553 ab	5381 ± 135 ab	5253 ± 812 ab	5926 ± 137 ab	5273 ± 795 ab	6320 ± 1143 a	4886 ± 412 ab
Isobutyl acetate	42 ± 20 ab	73 ± 24 a	49 ± 8 ab	40 ± 8 ab	67 ± 2 ab	26 ± 7 ab	34 ± 13 ab	27 ± 2 ab	19 ± 6 b
Butanoic acid, ethyl ester	0 ± 0 c	0 ± 0 c	19 ± 2 abc	15 ± 6 abc	7 ± 6 bc	42 ± 6 ab	51 ± 28 a	9 ± 7 bc	22 ± 1 abc
Butanoic acid, 2-methyl-, ethyl ester	0 ± 0	2 ± 0	10 ± 3	8 ± 1	16 ± 7	9 ± 10	14 ± 4	10 ± 3	8 ± 5
Butanoic acid, 3-methyl-, ethyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 1	4 ± 0	4 ± 1	4 ± 2	3 ± 2
1-Butanol, 3-methyl-, acetate	653 ± 340	1110 ± 69	1153 ± 182	1028 ± 249	691 ± 320	608 ± 178	792 ± 297	931 ± 384	572 ± 333
Hexanoic acid, methyl ester	0 ± 0 c	0 ± 0 c	2 ± 0 bc	2 ± 0 bc	4 ± 1 bc	8 ± 2 a	4 ± 1 ab	4 ± 1 bc	4 ± 1 bc
Hexanoic acid, ethyl ester	3 ± 2	5 ± 1	142 ± 156	20 ± 3	48 ± 17	49 ± 17	44 ± 20	29 ± 11	20 ± 14
Heptanoic acid, ethyl ester	0 ± 0 b	0 ± 0 b	13 ± 1 ab	9 ± 1 ab	22 ± 7 a	21 ± 9 a	23 ± 9 a	15 ± 5 ab	8 ± 5 ab
Butanedioic acid, diethyl ester	0 ± 0	0 ± 0	9 ± 4	9 ± 0	6 ± 3	15 ± 16	0 ± 0	0 ± 0	0 ± 0
Octanoic acid, ethyl ester	9 ± 5	14 ± 1	2561 ± 600	1433 ± 253	2037 ± 943	2270 ± 1056	2765 ± 766	2850 ± 716	1892 ± 1212
Benzeneacetic acid, ethyl ester	0 ± 0 b	0 ± 0 b	3 ± 1 a	3 ± 0 a	2 ± 1 ab	2 ± 1 ab	3 ± 0 a	2 ± 0 ab	2 ± 1 ab
Acetic acid, 2-phenylethyl ester	0 ± 0 c	2 ± 0 bc	13 ± 3 a	14 ± 0 a	11 ± 3 ab	11 ± 4 ab	11 ± 1 ab	10 ± 3 ab	10 ± 3 ab
Nonanoic acid, ethyl ester	0 ± 0	4 ± 4	711 ± 460	322 ± 163	434 ± 10	524 ± 441	513 ± 285	1011 ± 155	734 ± 764
4-Decenoic acid, ethyl ester	0 ± 0 c	0 ± 0 c	2 ± 1 abc	1 ± 1 bc	4 ± 2 abc	4 ± 2 ab	5 ± 1 a	4 ± 0 bc	3 ± 1 bc
9-Decenoic acid, ethyl ester	0 ± 0	0 ± 0	23 ± 8	7 ± 1	22 ± 12	29 ± 17	32 ± 7	26 ± 1	22 ± 15
Decanoic acid, ethyl ester	8 ± 4	9 ± 1	1356 ± 751	283 ± 51	1088 ± 921	1456 ± 852	1633 ± 404	1370 ± 15	1249 ± 773
Octanoic acid, 3-methylbutyl ester	0 ± 0	0 ± 0	22 ± 16	6 ± 0	21 ± 19	19 ± 12	23 ± 9	21 ± 3	16 ± 9
Octanoic acid, 2-methylbutyl ester	0 ± 0	0 ± 0	6 ± 5	4 ± 0	6 ± 5	6 ± 3	7 ± 2	7 ± 2	5 ± 2
Nonanoic acid, 3-methylbutyl ester	0 ± 0	0 ± 0	5 ± 3	3 ± 1	6 ± 3	5 ± 3	5 ± 3	8 ± 1	5 ± 4
Dodecanoid acid, ethyl ester	0 ± 0	0 ± 0	59 ± 27	28 ± 1	102 ± 76	91 ± 27	79 ± 22	80 ± 10	62 ± 12
Decanoic acid, 3-methylbutyl ester	0 ± 0	0 ± 0	5 ± 2	3 ± 0	13 ± 12	9 ± 3	9 ± 3	8 ± 1	5 ± 1

Table 2. Cont.

	Weeks of Storage at 4 °C								
	0	1	2	3	4	6	8	10	12
Ketones									
5-Hepten-2-one, 6-methyl-	2 ± 1 c	4 ± 1 bc	9 ± 0 a	8 ± 3 ab	3 ± 1 bc	4 ± 1 abc	5 ± 1 abc	3 ± 1 c	3 ± 1 c
2,2,6-Trimethylcyclohexanone	0 ± 0 b	2 ± 0 ab	3 ± 0 a	3 ± 0 a	1 ± 1 ab	1 ± 0 ab	1 ± 0 ab	2 ± 1 ab	1 ± 1 ab
(E,E)-3,5-Octadien-2-one	0 ± 0 b	0 ± 0 b	6 ± 3 a	5 ± 0 a	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b
Damascenone	1 ± 0 b	4 ± 0 ab	6 ± 1 a	7 ± 0 a	4 ± 1 ab	4 ± 1 ab	5 ± 1 ab	6 ± 1 ab	5 ± 2 ab
α-Ionone	0 ± 0 c	2 ± 0 bc	4 ± 1 ab	4 ± 1 a	2 ± 1 bc	2 ± 1 ab	3 ± 0 ab	2 ± 0 ab	2 ± 1 ab
Dehydro-β-ionone	0 ± 0 c	2 ± 0 bc	5 ± 1 a	6 ± 0 a	4 ± 1 ab	4 ± 1 ab	5 ± 0 ab	4 ± 1 ab	4 ± 1 ab
β-Ionone	0 ± 0 d	3 ± 0 c	5 ± 0 ab	6 ± 0 a	4 ± 0 bc	3 ± 1 bc	5 ± 0 ab	5 ± 0 ab	4 ± 1 bc
Phenols									
3-Ethylphenol	1 ± 0 c	3 ± 1 c	22 ± 9 ab	33 ± 1 a	12 ± 7 bc	16 ± 3 abc	16 ± 3 abc	20 ± 4 ab	23 ± 1 ab
4-Ethyl-2-methoxy-phenol	0 ± 0 b	5 ± 0 ab	15 ± 4 a	17 ± 1 a	10 ± 0 ab	9 ± 5 ab	15 ± 0 a	14 ± 7 a	16 ± 0 a
2,4-Di-tert-butylphenol	8 ± 3 b	24 ± 2 ab	45 ± 14 a	36 ± 9 ab	33 ± 10 ab	33 ± 6 ab	28 ± 14 ab	13 ± 1 ab	39 ± 3 ab
Terpenes									
β-Myrcene	0 ± 0 c	0 ± 0 c	24 ± 2 bc	23 ± 6 bc	4 ± 3 c	65 ± 28 a	47 ± 10 ab	6 ± 3 bc	40 ± 1 bc
o-Cymene	5 ± 3	8 ± 0	14 ± 4	7 ± 2	4 ± 1	7 ± 2	5 ± 2	7 ± 7	3 ± 1
D-Limonene	18 ± 5	29 ± 6	45 ± 21	27 ± 4	29 ± 2	27 ± 1	90 ± 29	75 ± 79	50 ± 20
γ-Terpinene	5 ± 0	7 ± 0	11 ± 4	8 ± 1	6 ± 3	8 ± 0	9 ± 1	15 ± 12	10 ± 4
Bornylene	2 ± 0	3 ± 0	5 ± 1	5 ± 1	4 ± 2	3 ± 2	5 ± 1	5 ± 1	4 ± 2
Eucalyptol	0 ± 0 b	2 ± 2 b	2 ± 0 b	3 ± 0 b	2 ± 0 b	1 ± 0 b	8 ± 2 a	1 ± 0 b	1 ± 1 b
Linalool oxide, (Z)-	3 ± 0 bc	3 ± 1 c	8 ± 2 bc	8 ± 0 b	5 ± 3 bc	5 ± 1 bc	7 ± 0 bc	8 ± 0 a	10 ± 1 a
Linalool	33 ± 5 b	62 ± 4 ab	101 ± 14 a	107 ± 0 a	70 ± 14 ab	72 ± 19 ab	113 ± 5 a	102 ± 18 a	86 ± 34 ab
α-Terpineol	5 ± 0 b	8 ± 0 b	22 ± 4 a	21 ± 1 a	20 ± 1 a	20 ± 1 a	19 ± 0 a	22 ± 2 a	24 ± 4 a
β-Cyclocitral	2 ± 1	4 ± 0	6 ± 1	6 ± 1	4 ± 1	4 ± 3	5 ± 1	5 ± 1	3 ± 1
cis-β-Farnesene	0 ± 0 b	0 ± 0 b	4 ± 1 a	6 ± 0 a	3 ± 1 ab	3 ± 1 a	4 ± 0 a	4 ± 1 a	5 ± 1 a
trans-Nerolidol	5 ± 1 b	6 ± 1 b	31 ± 12 a	35 ± 2 a	30 ± 8 a	31 ± 4 a	30 ± 2 a	35 ± 6 a	34 ± 6 a
Others									
Dihydroactinidiolide	0 ± 0 b	0 ± 0 b	3 ± 1 a	2 ± 0 a	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b
Edulan 1	0 ± 0 b	2 ± 0 ab	3 ± 0 a	3 ± 0 a	2 ± 0 ab	2 ± 1 ab	2 ± 1 ab	2 ± 0 ab	2 ± 1 ab

¹ Lower case letters indicate significant differences between storage times at an alpha level of 0.05 according to an HSD test.

Five carboxylic acids (acetic, isovaleric, 2-methyl-butanoic, caproic and caprylic) were detected in all samples, while nonanoic and decanoic (capric) acids appeared after 1–2 weeks of storage, 2-methyl-hexanoic acid became detectable in the second week of storage of MT1 beverage and isobutyric acid appeared after 3 weeks of storage of MT1 kombucha and disappeared after 12 weeks of storage of MT2 product (Tables 1 and 2). Acetic acid was the predominant volatile carboxylic acid, followed by isovaleric acid and octanoic (caprylic) acid. Acetic acid imparts the “vinegary” taste to products and its production is mainly associated with the oxidizing activity of AAB on ethanol produced by yeast during kombucha fermentation, although other bacteria and some yeasts may also contribute to acetic acid production [1,57,58]. Other volatile acids, such as isobutyric, isovaleric, octanoic and decanoic, are often associated with negative effects (rancid, sweaty and cheesy notes) on the sensory properties of alcoholic beverages [1,27]. Saturated short- and medium-chain carboxylic acids (acetic, isobutyric, isovaleric, 2-methylbutanoic, hexanoic, octanoic, nonanoic and decanoic) were not detected in black and green tea [28,43]. On the contrary, they were produced in significant amounts in mono- and co-cultures of yeasts (*Brettanomyces bruxellensis* and *Hanseniaspora valbyensis*) and AAB (*Acetobacter indonesiensis*) and for this reason were classified as “fermentative” flavors [37]. Several other yeast (*Saccharomyces*, *Dekkera*, *Brettanomyces*) and bacterial genera (*Clostridium*, *Faecalibacterium*, *Lactobacillus*, *Acinetobacter*) have been recognized as capable of producing carboxylic acids [1,47,54,59–61]. Although no significant changes were observed in the HS levels of acetic acid during cold storage of the beverages, the increase in the number of acids detected (recipes MT1 and MT2) and their amounts (recipe MT2) showed significant microbial activity.

Esters were the most populated class of volatile substances. Twenty-four different substances were identified in the HS of the experimental kombuchas (Table A1), mainly ethyl esters (15) and isoamyl esters (5). This was consistent with the semi-quantitative aroma composition data, which showed that ethanol and isoamyl alcohol were largely the most abundant alkanols in all samples (Tables 1 and 2). Ethyl acetate was the most represented ester detected in all samples, which is consistent with the literature [43,45], followed by ethyl octanoate, isoamyl acetate, ethyl nonanoate and ethyl decanoate, which together accounted for 86–95% and 77–93% of the total esters in samples MT1 and MT2, respectively. Three ethyl esters of unsaturated fatty acids (UFAs) (3-nonenoic acid, 4-decenoic acid and 9-decenoic acid) were identified in the aroma of the samples after 1–2 weeks of refrigerated storage. A plethora of UFAs (2-hexenoic acid, 2-heptenoic acid, 2-octenoic acid, 3-nonenoic acid, 9-decenoic acid, 3-decenoic acid, 2-decenoic acid, 9-tetradecenoic acid, 7-hexadecenoic acid, 9-hexadecenoic acid, 10-heptadecenoic acid, 9-octadecenoic acid, 11-octadecenoic acid, 10-nonadecenoic acid) and ethyl esters of UFAs (ethyl-3-methyl-2-butenate, ethyl-4-decenoate, ethyl-3-hexenoate) have already been detected in fermented teas [21,26,29,34,36,43,46,53,55,62]. However, the presence of the ethyl ester of 3-nonenoic acid was reported for the first time.

Esters and acids are generally considered to be the most important factors for the organoleptic properties of fermented teas, as they are associated with the common descriptors of these products (acidic, vinegary refreshing and fruity). The esters in particular are crucial for the fruity, floral and sweet notes in the aroma of kombucha. Ethyl acetate is often described as a solvent-like aroma at high concentrations, but as fruity (pineapple, pear) at lower concentrations. Other esters such as isoamyl acetate (banana), ethyl hexanoate (apple, pineapple), ethyl octanoate (fruity, waxy), ethyl decanoate (sweet, fruity), ethyl 2-phenylacetate (fruity, sweet, honey) and 2-phenylethyl acetate (floral, rose, sweet) contribute to the overall fruity character. The specific ester profile is highly dependent on SCOBY and fermentation conditions and contributes significantly to the nuanced dif-

ferences between kombucha varieties [1]. The semi-quantitative data showed a general increase in the number and amount of esters during cold storage, especially during the first two weeks, again indicating a movement in the microbial population. Indeed, the formation of ethyl and acetate esters has been shown to be closely linked to yeast metabolism of fatty acids and amino acids. AAB are also able to esterify acyl-CoA with ethanol to form ethyl acetate, and some other bacterial genera (e.g., *Butyricoccus*) have been found to correlate with ester content in fermented teas [1,27,39,44,47,54,63]. In addition, ethyl esters of acetic, phenylacetic, 2-methylbutyric, isobutyric, hexanoic, octanoic and decanoic acids were not detected in black and green tea, suggesting their fermentative origin [28,43].

The less represented volatiles (aliphatic aldehydes, ketones, terpenes, phenols, alkenyl-benzenes, benzofurans and benzopyrans) made up a really small proportion of the total volatiles (0.2–1.0% in MT1 samples; 0.6–2.0% in MT2 samples) but still play an important role in defining the kombucha flavor. Similar values (0.02% to 1.15%) were found by Meng et al. [34] in black tea kombucha samples from different regions of China.

A group of closely related chemical substances known as “rose ketones” (the norisoprenoids damascenone, α -ionone, dehydro- β -ionone, α -isomethylionone and β -ionone) were identified in the HS of the samples. They originate from the oxidative degradation of carotenoids, were first identified in kombucha by Ferremi Leali et al. [47] and were also detected in unfermented teas [21]. Aliphatic aldehydes (octanal, nonanal) and ketones can contribute to a range of aromas, from fruity and green to cheesy and pungent, but are generally associated with off-flavors in fermented beverages, such as rancid, greasy, buttery, rubbery, peppery and mushroomy odor descriptors [21,27]. Norisoprenoids are known for their low odor threshold and are highlighted as key aromas (floral, sweet, honey) in green tea and pu-erh tea [29,64], which can enhance floral, sweet and honey notes in synergy with other volatile compounds [26]. Carbonyl compounds can be formed in various ways, including the microbial oxidation of alcohols, the degradation of amino acids and the oxidation of lipids. Their production is influenced by the specific microbial strains and fermentation conditions. These pathways are often complex and less well understood than the primary fermentation pathways [27,45,49]. However, Tran et al. [37] classified the aldehydes and ketones in kombucha flavor as “varietal” because they were detected at higher concentrations in sweetened black tea than in fermented products. In the analyzed samples, most of the carbonyl compounds were detectable from the first week of storage, with the exception of 6-methyl-5-hepten-2-one, damascenone, α -isomethylionone and nonanal. Therefore, microbial activity played a key role in their accumulation during cold storage of the beverages.

Five monoterpene hydrocarbons (β -myrcene, o-cymene, D-limonene, γ -terpinene, bornylene), seven oxygenated monoterpenes (eucalyptol, linalool oxide, linalool, D-camphor, menthone, α -terpineol, β -cyclocitral), two sesquiterpenes (cis- β -farnesene, trans-nerolidol) and three volatile phenols (4-ethylphenol, 4-ethyl-2-methoxy-phenol, 2,4-di-tert-butylphenol) were identified in the HS of the analyzed kombucha samples (Table A1). Linalool and 2,4-di-tert-butylphenol were the most abundant terpenes and phenols, respectively. Some qualitative and quantitative differences were found between the MT1 and MT2 methods: D-camphor and menthone were only detected in the MT1 samples, while γ -terpinene, bornylene and β -cyclocitral were only observed in the MT2 samples; nerolidol was more abundant in MT2 beverages than in MT1 beverages (Tables 1 and 2). Terpenoids and volatile phenolic substances were mainly extracted from tea materials and persist in kombucha [64], but some authors [38] could not detect terpenes in kombucha made from black tea and black tea with dried pineapple by-products (peels and cores). Terpenes and volatile phenols have been reported to be present in brewed tea in their glycosylated form, emphasizing the key role of yeast-derived glycosidase enzymes in the release

of these compounds [14,47]. In addition, yeasts are also able to synthesize mono- and sesquiterpenes via the methylerythritol 4-phosphate pathway and the mevalonate pathway, respectively [29,47] and convert non-volatile tea polyphenols into 2,4-di-tert-butylphenol, 4-ethyl-2-methoxy-phenol (4-ethylguaiacol) and 4-ethylphenol [1,28,29], which may explain the general increase in terpenoids during cold storage of beverages (Tables 1 and 2). In addition, 4-ethylguaiacol and 4-ethylphenol were not detected in black and green tea [28,43], suggesting a contribution of their fermentative origin. Some of these compounds can also be biotransformed by the microbial consortium, resulting in new flavor compounds. The type of tea used (black, green, oolong, white or herbal) significantly influences the aromatic profile. Especially in flavored teas and herbal teas, terpenes can make an important contribution to the aroma [34]. However, the role of typical tea aroma seems to be of less importance, as the flavor profile of kombucha is dominated by the vinegary and cidery traits related to the activity of AAB and yeast, respectively [14]. Phenolic compounds can add spicy, smoky or medicinal notes, while terpenes contribute a wide range of aromas, including citrus, floral, herbal and woody notes. Examples include linalool (floral, lavender), limonene (citrus), β -myrcene (woody, floral) and β -farnesene (fragrant, floral and fruity) [29,43].

Styrene was detected in the MT1 samples but not in the MT2 samples, with levels apparently increasing during storage, but not significantly due to the large standard deviations (Tables 1 and 2). Aromatic volatile organic compounds such as styrene and naphthalene were also detected at relatively low levels in the kombucha beverages by other authors [31,33,46,55]. Styrene, in particular, has been reported to be naturally present in a variety of products such as fruits, vegetables, nuts, beverages and meats and contributes to a sweet or floral aroma [33]. Dihydroactinidiolide has already been detected in tea infusions and kombuchas [28,31,36,41,46,49]. It can result from the Maillard reaction in the production of sweetened tea infusions [28].

PCA was performed on the average levels of volatiles of the kombucha samples to investigate the relationships between the variables and storage time. The analysis revealed nine principal components (PCs) with eigenvalues greater than 1, which in total explained 94.5% of the total variance, while the first two PCs explained 67.8% of the sample variance, indicating a good fit of the multivariate technique. The distribution of samples on the score plot (Figure 3a) showed the ability of PC1 to discriminate between recipes (MT1 samples in the left quadrants and MT2 samples in the right quadrants), while cold storage caused a shift in samples along PC2. The loading plot (Figure 3b) showed the specific contribution of the variables to the model fit. PC1 was mainly influenced by a group of monoterpenes (bornylene, β -cyclocitral, α -terpineol) and norisoprenoids (dehydro- β -ionone, damascenone) with positive loadings while a miscellaneous of volatiles (3-methyl-1-butanol, D-camphor, ethanol, eucalyptol, ethyl 3-nonenoate, styrene) had the highest negative loadings. The presence of bornylene, β -cyclocitral and dehydro- β -ionone as well as higher average amounts of α -terpineol and damascenone were the key variables that drove MT2 samples on the positive loadings along PC1. Higher levels of aliphatic and terpenic alcohols (ethanol, 3-methyl-1-butanol, eucalyptol) as well as the presence of D-camphor, styrene and ethyl 3-nonenoate lifted the MT1 samples to the left quadrants. Samples taken after 1 week of storage were located together the initial samples in the lower quadrants of the score plot, indicating a similar flavor composition. However, samples taken after 2–12 weeks of storage were far away in the upper left and right quadrants for recipes MT1 and MT2, respectively, indicating that the main chemical changes in flavor composition occurred early during storage. The increase in octanoic acid and ethyl esters (ethyl octanoate, ethyl heptanoate, ethyl 9-decenoate and ethyl acetate), which were

characterized by high loadings on PC2, shifted the samples along PC2 with increasing storage time.

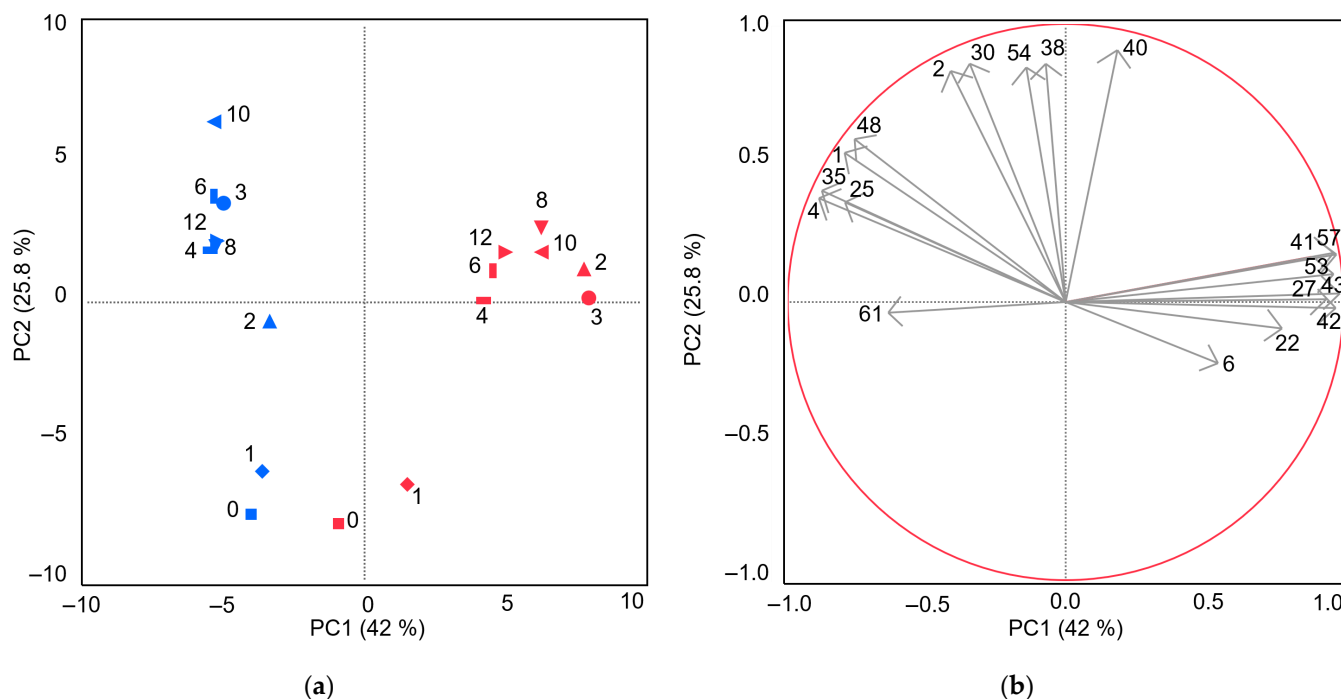


Figure 3. PCA (a) score plot and (b) loading plot of the most significant variables. The samples are colored blue (MT1) or red (MT2). Different shapes and numbers on the score plot refer to the storage time (number of weeks). The numbers on vectors of the loading plot are the volatile substances as indicated in Table A1.

Two-way (factorial) ANOVA showed a significant effect of preparation method and/or refrigerated storage time on most of the measured variables with only two exceptions (methyl hexanoate and α -ionone), while the interaction between the two factors was significant for 30 variables out of 68 (Table A2).

4. Conclusions

Knowledge about the chemical and microbiological behavior of traditional and flavored kombuchas during storage of the final product is limited and inconsistent. This currently represents a significant limitation for the development of a microbiologically unstable product that is often marketed as raw and unpasteurized [15–23]. Despite the low pH of the beverages (3.2 ± 0.2), refrigeration ($4\text{ }^{\circ}\text{C}$) proved to be ineffective in inhibiting the activity of the microbial consortium in freshly prepared kombuchas as significant qualitative and quantitative changes in the composition of the volatile fraction occurred, mainly between the first and second week of cold storage. This emphasizes the need for a deeper knowledge of the kinetics of chemical changes during storage in order to estimate the optimal shelf life of raw and chilled kombucha. In particular, octanoic acid and ethyl esters (ethyl octanoate, ethyl heptanoate, ethyl 9-decenoate and ethyl acetate) were found to be potential markers for flavor changes during storage.

Even if the same ingredients were used (teas, sugar, SCOBY), the preparation method had a strong influence on the aroma of the beverages. Kombucha prepared with the addition of fermented liquid from the previous batch (MT2) was characterized by the presence of bornylene, β -cyclocitral and dehydro- β -ionone, as well as higher average amounts of α -terpineol and damascenone, while higher levels of aliphatic and terpenic

alcohols (ethanol, 3-methyl-1-butanol, eucalyptol) and the presence of D-camphor, styrene and ethyl 3-nonenoate distinguished the kombucha produced by the addition of SCOBY (MT1) from MT2 beverages.

Further studies are needed to correlate the chemical changes in the flavor composition with the sensory properties to help manufacturers control the organoleptic standards and their stability.

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Abbreviations

The following abbreviations are used in this manuscript:

AAB	Acetic acid bacteria
ANOVA	Analysis of variance
CAR	Carboxen
DVB	Divinylbenzene
FID	Flame ionization detector
GC	Gas chromatography
HS	Headspace
LAB	Lactic acid bacteria
PCA	Principal component analysis
PDMS	Polydimethylsiloxane
RI	Retention index (Kovats)
RT	Retention time
SBSE	Stir-bar sorptive extraction
SD	Standard deviation
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
UFA	Unsaturated fatty acid

Appendix A

Table A1. Identified volatile substances in the headspace of kombucha samples prepared from a 1:1 mixture of black and green tea and stored at 4 °C for 12 weeks.

Peak # ¹	RT [min]	RI _{cal}	NIST Mass Spectral Library			Name	CAS Number	Chemical Family	Previously Identified by ² :
			MF	RMF	RI _{lit}				
20	9.52	1004	830	919	1003	Octanal	124-13-0	Aldehyde	[18,21,26–30,42,49–51]
32	12.23	1105	839	848	1104	Nonanal	124-19-6	Aldehyde	[18,21,26–32,42–44,46,48–52,54,55]
1	1.89		954	954	427	Ethanol	64-17-5	Alkanol	[1,18,26–28,31,33,35–37,40,41,44–50]

Table A1. Cont.

Peak # ¹	RT [min]	RI_cal	NIST Mass Spectral Library			Name	CAS Number	Chemical Family	Previously Identified by ² :
			MF	RMF	RI_lit				
4	3.69	737	942	947	736	3-Methyl-1-butanol	123-51-3	Alkanol	[18,21,26,27,29–31,33,35–38,40–42,45,46,48,49,51,52]
12	6.20	874	790	870	868	1-Hexanol	111-27-3	Alkanol	[21,26,27,34,35,37,39,43,46,50,51]
23	10.20	1032	788	942	1030	2-Ethyl-1-hexanol	104-76-7	Alkanol	[18,26–31,33–35,37–40,45,47,51,53,54]
34	12.49	1116	882	922	1119	2-Phenylethyl alcohol	60-12-8	Alkanol	[1,18,26,28–34,36–41,43–53,55]
14	6.70	894	818	929	893	Styrene	100-42-5	Alkenylbenzene	[31,33,46,55]
64	22.74	1539	822	857	1532	Dihydroactinidiolide	17092-92-1	Benzofuran	[28,31,36,41,46,49]
50	17.60	1315	820	842	1314	Edulan 1	41678-29-9	Benzopyran	[29,30]
3	2.67	660	910	916	610	Acetic acid	64-19-7	Carboxylic acid	[1,18,26,28,31,33–38,40–46,48–52,54,55]
5	4.13	767	911	913	765	Propanoic acid, 2 methyl-	79-31-2	Carboxylic acid	[1,21,26,27,31–34,36,38,41,43,46,48–50,54,55]
10	5.93	863	956	956	850	Butanoic acid, 3-methyl-	503-74-2	Carboxylic acid	[18,21,26–29,31,32,34,36,37,39,41,43,44,47–51,54,55]
11	6.08	869	942	947	861	Butanoic acid, 2-methyl-	116-53-0	Carboxylic acid	[18,26,28,29,51,54]
16	8.94	984	736	906	990	n-Hexanoic acid	142-62-1	Carboxylic acid	[21,26–29,32,41,47,48]
33	12.44	1114	738	825	1123	Hexanoic acid, 2-ethyl-	149-57-5	Carboxylic acid	[21]
38	14.08	1177	908	921	1180	n-Octanoic acid	124-07-2	Carboxylic acid	[1,21,26–29,31–39,41,43,44,46–50,54]
46	16.45	1269	916	935	1273	n-Nonanoic acid	112-05-0	Carboxylic acid	[21,26,28,29,31,34,36,37,41,43,46,48,49]
51	18.84	1367	930	941	1372	n-Decanoic acid	334-48-5	Carboxylic acid	[1,21,26–28,31–38,40,41,43–46,48–50]
2	2.46	640	743	786	612	Ethyl acetate	141-78-6	Ester	[1,18,21,26–28,31,33,34,36–38,42–45,47–51,54,55]
6	4.23	773	894	899	772	Isobutyl acetate	110-19-0	Ester	[18,21,53,54]
7	4.72	801	771	868	801	Butanoic acid, ethyl ester	66-25-1	Ester	[21]
8	5.71	850	832	915	849	Butanoic acid, 2-methyl-, ethyl ester	7452-79-1	Ester	[1,28,38,48]
9	5.79	853	733	800	853	Butanoic acid, 3-methyl-, ethyl ester	108-64-5	Ester	[21,26,47,54]
13	6.31	879	969	969	876	1-Butanol, 3-methyl-, acetate	123-92-2	Ester	[18,26,27,34,37,39,44,47,48,50,51,54,55]
15	7.44	926	856	897	925	Hexanoic acid, methyl ester	106-70-7	Ester	[46]
19	9.36	998	916	926	999	Hexanoic acid, ethyl ester	123-66-0	Ester	[1,18,21,33,34,38,39,43,45–47,50,55]
21	9.73	1013	874	906	1014	1-Butanol, 3-methyl-, propanoate	105-68-0	Ester	
30	12.00	1097	909	917	1098	Heptanoic acid, ethyl ester	106-30-9	Ester	[48]
39	14.12	1178	763	807	1181	Butanedioic acid, diethyl ester	123-25-1	Ester	[21,55]
40	14.63	1196	936	954	1196	Octanoic acid, ethyl ester	106-32-1	Ester	[26–28,30–32,34,36,39,40,43–45,47,48,55]
44	15.84	1246	880	918	1247	Benzeneacetic acid, ethyl ester	101-97-3	Ester	[1,21,26,28,38,44–46,50,53–55]
45	16.14	1257	925	952	1258	Acetic acid, 2-phenylethyl ester	103-45-7	Ester	[1,18,21,26,29–31,33,34,37–39,43,46,47,50,53,54]
48	16.97	1289	883	915	1283	3-Nonenoic acid, ethyl ester	91213-30-8	Ester	
49	17.11	1294	943	945	1295	Nonanoic acid, ethyl ester	123-29-5	Ester	[31,32,36,48]
52	19.17	1380	798	845	1375	4-Decenoic acid, ethyl ester	76649-16-6	Ester	[21]
54	19.30	1385	824	843	1388	9-Decenoic acid, ethyl ester	67233-91-4	Ester	[31,34,55]
55	19.50	1393	946	951	1396	Decanoic acid, ethyl ester	110-38-3	Ester	[1,26,28,31–34,36,40,43–45,47–50,55]
58	20.69	1447	884	943	1446	Octanoic acid, 3-methylbutyl ester	2035-99-6	Ester	[31]
59	20.75	1449	763	785	1453	Octanoic acid, 2-methylbutyl ester	67121-39-5	Ester	

Table A1. Cont.

Peak # ¹	RT [min]	RI_cal	NIST Mass Spectral Library			Name	CAS Number	Chemical Family	Previously Identified by ² :
			MF	RMF	RI_lit				
65	22.88	1546	671	753	1559	3-Methylbutyl nonanoate	7779-70-6	Ester	
67	23.89	1592	922	935	1594	Dodecanoic acid, ethyl ester	106-33-2	Ester	[1,21,28,30–33,38,40,44,45,48,55]
68	24.96	1645	852	870	1645	Decanoic acid, 3-methylbutyl ester	2306-91-4	Ester	[21,27,31,34,37,49]
17	9.01	986	886	900	986	5-Hepten-2-one, 6-methyl-	110-93-0	Ketone	
26	10.42	1041	765	837	1036	2,2,6-Trimethylcyclohexanone	2408-37-9	Ketone	[26]
28	11.29	1073	717	901	1072	(E,E)-3,5-Octadien-2-one	38284-27-4	Ketone	[21,43,46,50]
53	19.24	1383	879	917	1386	Damascenone	23726-93-4	Ketone	[21,26–28,39,44,45,47,51,53,54]
56	20.24	1426	831	837	1426	α -Ionone	127-41-3	Ketone	[31,34,54]
57	20.37	1432	763	784	1485	Dehydro- β -ionone	1203-08-3	Ketone	[27,29,46]
61	21.41	1478	809	836	1480	α -Isomethylionone	127-51-5	Ketone	
62	21.53	1483	911	917	1486	β -Ionone	79-77-6	Ketone	[21,26,28,29,31,34,36,46,50]
18	9.16	991	871	924	991	β -Myrcene	123-35-3	Monoterpene (hydrocarbon)	[29,31,34–36,40,47,51,55]
22	10.14	1030	938	953	1022	<i>o</i> -Cymene	527-84-4	Monoterpene (hydrocarbon)	[29,35]
24	10.27	1035	923	925	1031	D-Limonene	5989-27-5	Monoterpene (hydrocarbon)	[18,26,29,30,34–36,40,46,47,51,53–55]
27	11.05	1064	790	870	1060	γ -Terpinene	99-85-4	Monoterpene (hydrocarbon)	[26,28,29,31,36,46,47]
43	15.60	1236	840	903	908	Bornylene	464-17-5	Monoterpene (hydrocarbon)	[26,49,53,54]
25	10.37	1039	808	827	1032	Eucalyptol	470-82-6	Monoterpene (oxygenated)	[18,21,54]
29	11.37	1075	806	811	1074	Linalool oxide, (Z)-	5989-33-3	Monoterpene (oxygenated)	[28,46,47]
31	12.10	1100	948	949	1099	Linalool	78-70-6	Monoterpene (oxygenated)	[18,26–34,36,40,46,47,49–54]
35	13.49	1155	794	889	1144	D-Camphor	464-49-3	Monoterpene (oxygenated)	
36	13.68	1162	792	907	1157	Menthone	14073-97-3	Monoterpene (oxygenated)	
41	14.71	1199	923	935	1198	α -Terpineol	98-55-5	Monoterpene (oxygenated)	[27,29,31–33,35,37–40,42,43,45–47,51,52,54]
42	15.33	1225	891	916	1220	β -Cyclocitral	432-25-7	Monoterpene (oxygenated)	[21,46,51]
37	13.89	1170	712	813	1169	4-Ethylphenol	123-07-9	Phenol	[1,28,33,34,38,40,43,46,47,50,51,55]
47	16.65	1277	910	926	1282	4-Ethyl-2-methoxyphenol	2785-89-9	Phenol	[1,27,28,31,33,34,36,38–40,43,46,50,51,54,55]
63	22.06	1507	949	951	1514	2,4-Di-tert-butylphenol	96-76-4	Phenol	[21,26,29–31,34,36,39,43,50,51,53]
60	20.87	1455	875	899	1457	cis- β -Farnesene	28973-97-9	Sesquiterpene (hydrocarbon)	[31,53]
66	23.28	1564	951	957	1564	trans-Nerolidol	40716-66-3	Sesquiterpene (oxygenated)	[21,55]

¹ Elution sequence on a DB-5 equivalent GC column under the experimental conditions. ² In the aroma of kombuchas from traditional Chinese teas (black tea, oolong tea, dark tea, green tea, yellow tea and white tea). #, elution number; RT, retention time; RI_cal, Kovats retention index calculated from the experimental RT; RI_lit, Kovats retention index from literature (DB-5 equivalent column); MF (Match Factor), direct match of peak *m/z* values and relative intensities; RMF (Reverse Match Factor), match factor obtained by ignoring all peaks that are in the sample spectrum but not in the library spectrum. MF, RMF > 900 excellent match, 800–900 good match, 700–800 fair match.

Table A2. Analysis of variance ¹ applied to a full factorial linear model in which the preparation method (MT) and the storage time (ST) are the factors potentially influencing the level of volatiles.

Analyte	MT	ST	MT × ST
Octanal	***	***	***
Nonanal	***	***	**
Ethanol	***	***	***
3-Methyl-1-butanol	***	***	**
1-Hexanol	***	***	***
2-Ethyl-1-hexanol	***	***	***
2-Phenylethyl alcohol	***	***	***
Styrene	***		
Dihydroactinidiolide	***	***	***
Edulan 1		***	
Acetic acid	***	*	
Propanoic acid, 2 methyl-	***	***	***
Butanoic acid, 3-methyl-	***	**	**
Butanoic acid, 2-methyl-	***	***	
n-Hexanoic acid		***	
Hexanoic acid, 2-ethyl-	***	***	**
n-Octanoic acid	**	***	*
n-Nonanoic acid		**	
n-Decanoic acid		***	
Ethyl acetate	***	***	*
Isobutyl acetate	***	*	**
Butanoic acid, ethyl ester	***	***	**
Butanoic acid, 2-methyl-, ethyl ester	***	**	*
Butanoic acid, 3-methyl-, ethyl ester	***	***	
1-Butanol, 3-methyl-, acetate	*		
Hexanoic acid, methyl ester			
Hexanoic acid, ethyl ester	**		
1-Butanol, 3-methyl-, propanoate	***		
Heptanoic acid, ethyl ester	**	***	
Butanedioic acid, diethyl ester	**		
Octanoic acid, ethyl ester		***	
Benzeneacetic acid, ethyl ester	***	***	***
Acetic acid, 2-phenylethyl ester	***	***	
3-Nonenoic acid, ethyl ester	***	*	*
Nonanoic acid, ethyl ester	*	**	
4-Decenoic acid, ethyl ester	**	***	
9-Decenoic acid, ethyl ester		*	
Decanoic acid, ethyl ester	*	**	
Octanoic acid, 3-methylbutyl ester	*	*	
Octanoic acid, 2-methylbutyl ester	***	*	
Nonanoic acid, 3-methylbutyl ester	*	**	
Dodecanoic acid, ethyl ester	*	*	
Decanoic acid, 3-methylbutyl ester	*		
5-Hepten-2-one, 6-methyl-	***	***	***
2,2,6-Trimethylcyclohexanone	***	**	**
(E,E)-3,5-Octadien-2-one	***	***	***
Damascenone	***	*	
α-Ionone			
Dehydro-β-ionone	***	***	***
α-Isomethylionone	***	***	***
β-Ionone	***	***	*
β-Myrcene		*	
o-Cymene	***	*	
D-Limonene	***	*	

Table A2. Cont.

Analyte	MT	ST	MT × ST
γ-Terpinene	***		
Bornylene	***		
Eucalyptol	***	**	
Linalool oxide, (Z)-	***	***	*
Linalool	***	***	
D-Camphor	***	*	*
Menthone	***	*	*
α-Terpineol	***	***	***
β-Cyclocitral	***		
3-Ethylphenol	**	***	
4-Ethyl-2-methoxy-phenol	***	***	
2,4-Di-tert-butylphenol	*	***	**
cis-β-Farnesene	**	***	
trans-Nerolidol	***	***	*

¹ Number of * represents different level of significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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