Identification of the profile of volatile compounds in commercial kombucha added with hibiscus (*Hibiscus rosa-sinensis*)

Identificação do perfil de compostos voláteis em kombucha comercial adicionado de hibisco (*Hibiscus rosa-sinensis*)

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ABSTRACT

Kombucha is a fermented drink from a symbiotic culture of bacteria and yeasts (SCOBY) and has been consumed due to its sensory and antioxidant characteristics that provide several health benefits, in addition to scientific evidence showing that there are variations in the chemistry of these kombucha. Therefore, this study aimed to investigate the profile of volatile compounds from kombucha commercials using the GC-MS-SPME technique. Five kombucha samples from commercial points on Rio de Janeiro City were analyzed. The profile of volatile compounds was evaluated using the SPME technique using a PDMS/DVB/CAR triple fiber. Volatiles were identified by comparing their mass spectra with a NIST and IRL. The results indicated that the profile of volatile compounds analyzed was constituted by nine classes of compounds, namely, esters, ketones, carboxylic acids, alcohols, aldehydes, amines, hydrocarbons, lactones and terpenes. Among them, the esters were the majority, ranging from 20.0 to 28.0 %, it was also possible to observe that 2-Octenal was the most abundant compound in all samples, ranging from 52.7 to 63.0 %. Thus, it is seen that the profile of volatile compounds in kombucha is found by a complex mixture of compounds belonging to different classes

and which have the esters as the most representative due to the fermentation process that occurs during the production of kombucha.

Keywords: aroma, tea, chromatography, spme.

RESUMO
Kombucha é uma bebida fermentada produzida a partir de uma cultura simbiótica de bactérias e leveduras (SCOBY) e tem sido amplamente consumida devido as suas características sensoriais e antioxidantes que confere diversos benefícios para saúde, além disso as evidências científicas demonstram que existe variações na composição química dessas kombuchas. Portanto, esse estudo teve como objetivo investigar o perfil de compostos voláteis de amostras comercias de kombuchas a partir da técnica de CG-EM-MEFS. Foram analisadas cinco amostras de kombucha em pontos comerciais da cidade do Rio de Janeiro. O perfil de compostos voláteis foi avaliado a partir da técnica de MEFS utilizando um fibra tripla de PDMS/DVB/CAR. Os voláteis foram identificados comparando seus espectros de massa com a NIST e IRL. Os resultados indicaram que o perfil de compostos voláteis das amostras analisadas foi constituído por nove classes de compostos, sendo elas, ésteres, cetonas, ácidos carboxílicos, álcoois, aldeídos, aminas, hidrocarbonetos, lactonas e terpenos. Dentre eles, os ésteres foram os majoritários, variando entre 20.0 a 28.0 %, também foi possível observar que o 2-Octenal, foi o composto mais abundante em todas as amostras, variando entre 52.7 a 63.0 %. Assim, é visto que o perfil de compostos voláteis das kombuchas é constituída por uma mistura complexa de compostos pertencentes a diferentes classes e que possuem os ésteres como mais representativos em função do processo de fermentação que ocorre durante a produção das kombuchas.

Palavras-chaves: aroma, chá, cromatografia, spme.

1 INTRODUCTION

Kombucha is an ancient beverage traditionally prepared with sweetened *Camellia sinensis* tea, through infusions of black, green or *oolong* tea and fermented in a polymeric cellulose film called Symbiotic Culture of Bacteria and Yeasts (SCOBY), for about 7 to 14 days, which gives the characteristics of the drink.[1] After the fermentation process, a refreshing, slightly acidic and carbonated beverage is obtained, containing a diverse content of components, such as sugars (sucrose, glucose and fructose left over from fermentation), ethanol, amino acids.[2] It is a drink that stands out for the presence of many active compounds such as tea polyphenols, hydrolytic enzymes, vitamins (B1, B2, B6, B12 and C), organic acids (acetic acid, gluconic acid, glucuronic acid) and minerals (zinc, copper, iron, manganese and cobalt). These active compounds provide kombucha potential human health benefits, including antioxidant, antimicrobial, anti-inflammatory, antiproliferative, and hepatoprotective effects.[1,3]

Different varieties and amounts of substrates used in the production of kombucha result in drinks with different chemical compositions and sensory aspects, as well as different types of biological activity. In addition, the fermentation time, the composition of the kombucha culture, which can vary according to geographic origin, as well as the concentration and type of sugar used may also affect the sensory characteristics of the final product.[1–3]

With the population's interest in health care, there has been significant growth in its industrial scale production and consumption market, which makes it the fastest growing product in the probiotic drinks sector, due to increased global awareness of its products benefits, which leads to improvements in lifestyle and health.[5]

The industrial production of functional foods involves the incorporation of bioactive compounds extracted from plant species that promote color, flavor and texture. *Hibiscus rosa sinensis*, which belongs to the *Malvaceae* family, is considered an ornamental and medicinal plant with a variety of therapeutic properties, and presents studies that demonstrate the presence of bioactive compounds, such as flavonoids, polyphenols, alkaloids, saponins and tannins that have technological potential. To be used in the development of functional foods such as kombucha.[6,7]

Volatile compounds are fundamental to the odor and aroma of tea, the aroma performance is actually made up of the mixture of various volatile flavor compounds (VFCs). In studies, hundreds of volatiles have been described in black tea, with lower numbers in oolong and green tea, due to the lower degree of fermentation in the production of these teas. More than 600 volatile compounds have been identified in fully fermented black tea, and 41 of them have been identified as important contributors to the aroma of black tea.[8,9] Chun Zou et al.[3] identified six groups of volatile compounds in kombucha (esters, alkenes, alcohols, aldehydes, ketones and aromatics). Saturated and unsaturated C_6 and C_9 aldehydes and alcohols are important contributors to the characteristic “fresh green” odor. Terpenes, in semi-fermented and fermented teas, linalool and diastereomers of linalool oxides, with their different flavor characteristics, are present in concentrations of up to 50% of volatiles.[8]

Due to the very process of obtaining the drink, there is great variation in the formulation and concentration of the constituents that end up directly interfering with the composition of the volatile compounds present in kombuchas. In this context, this study aimed to investigate the profile of volatile compounds in commercial kombucha samples using the GC-MS-SPME technique.

2 MATERIAL AND METHODS

2.1 SAMPLES AND MATERIALS

Five samples of the hibiscus-based kombucha (*Hibiscus rosa-sinensis*) drink, from different brands, were collected at commercial points in the Rio de Janeiro Cit. The samples were kept in their original packaging and placed in isothermal boxes with recyclable ice and sent to the UFRJ analysis laboratory. All samples were properly coded as (K1, K2, K3, K4 and K5). Standards of **1-octen-3-one (96 %) and s**aturated alkanes C_7-C_{30} (> 99 %) were purchased from Sigma Aldrich USA.

2.2 EXTRACTION BY SPME

Headspace solid-phase microextraction (SPME) was based on a modified method described by Zou et al.[4] SPME stable flex fiber (50/30 µm, PDMS/DVB/CAR) was used for the headspace experiments. Kombucha samples (2 mL) was placed in a 40 mL sealed glass vial and 1.17 µL of **1-octen-3-one** (internal standard, 1 mg/ mL) was added. After equilibration and stabilization for 15 min, the vial was put into a thermostatic water bath at 70 ºC and the SPME fiber was used for the absorption of volatiles for 30 min. Subsequently, the volatiles were desorbed in mode splitless at the gas chromatography–mass spectrometry (GC–MS) injector (260 ºC) for 5 min.

2.3 GAS CHROMATOGRAPHY

Analyzes were conducted on an Agilent Technologies 7820A system coupled with a mass detector (GC-MS) according to the methodology described by Zou et al.[4] with adaptations. A DB-5 capillary column (30 m x 0.25 mm x 0.25 µm) was used. The oven was conditioned at an initial temperature of 60 °C for 4 min., heating rate of 5 °C min-1 until reaching 180 °C, with a heating rate of 20 °C min-1 to 250 °C for 5 min. The carrier gas was helium at 2 mL min-1. The mass detector operated with 70 e-V ionization energy, the temperatures of the GC transfer line, the ion source and the quadrupole analyzer were set at 250, 230 and 150 °C, respectively.

2.4 IDENTIFICATION OF COMPOUNDS

It was injected 1 µL of an aliphatic hydrocarbon solution (C_7-C_{30}) in the GC-MS under the same conditions described on item 2.2 and used to calculate the linear retention index (LRI) of each compound present in the sample. The LRI of each component was calculated according to conditions described by Zellner et al.[5] The data were analyzed

using the software program Data Analysis (Agilent). The volatile components were identified by comparing their mass spectra with NIST, showing the Match > 800 and calculated LRI for each compound, aided by comparing the LRIs of the compounds in the DB-5 columns with those described in the literature[1,28] for columns of the same polarity and maximum variation of ± 10. The results were expressed as a percentage of volatile compound area.

2.5 STATISTICAL ANALYSIS

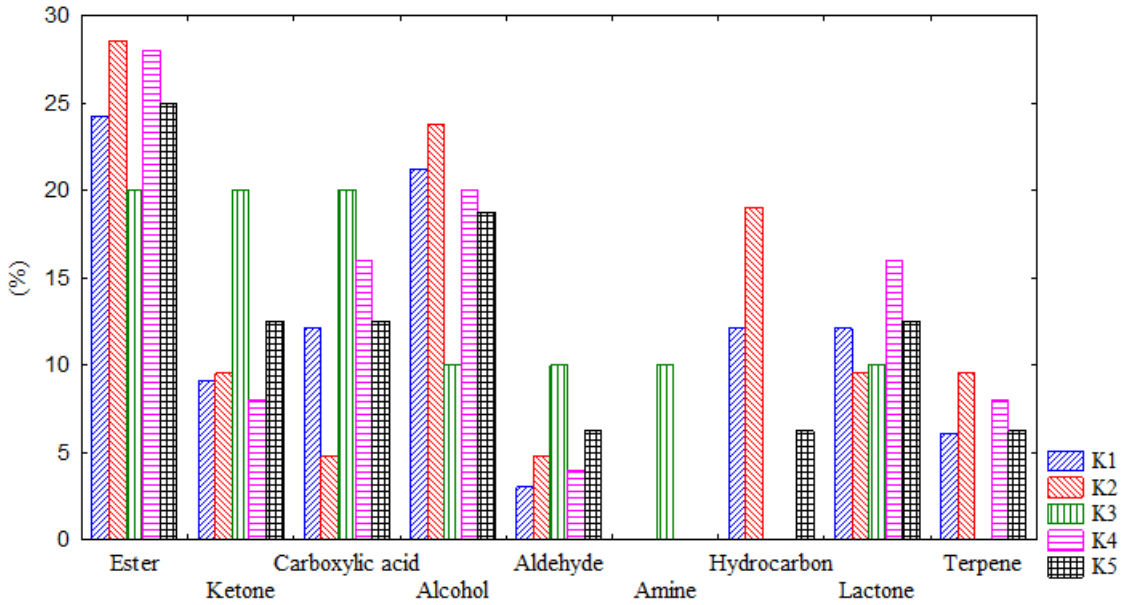
The graphics were plotted in the Statistica 7 software. The Principal Component Analysis (PCA) was used to summarize the information in some key components and was calculated using the correlation matrix. Statistical analyzes were performed using Minitab software v. 17.

3 RESULTS AND DISCUSSION

3.1 IDENTIFICATION OF COMPOUNDS

Overall, a total of 46 volatile compounds were tentatively identified in the kombucha samples. Nine groups of volatiles (esters, ketone, carboxylic acid, alcohols, aldehydes, amines, hydrocarbons lactones and terpenes) were identified, as illustrated in Figure 1.

Figure 1. Percentage of volatile compounds tentatively identified among different kombucha samples (K1, K2, K3, K4 e K5).



Source: Author himself (2021)

Among these different classes, the esters were the main compounds detected in all beverages, ranging from 20.0 to 28.0 % and probably play an important role in the sensorial profile of kombuchas. Zou et al.[6] identified six groups of volatiles (esters, alkenes, alcohols, aldehydes, ketones, and aromatics) and observed that the concentrations of esters, increased significantly after the fermentation process, during the production of kombucha. But the types of esters have a certain difference among them, according to the type of kombucha from which they originate.

There is a great variation in the concentration of these constituents due to the process of obtaining the beverage itself. The use of different vegetable matrices in the formulation of kombucha also contributed to the production of diversified beverages in terms of chemical and sensorial aspects.[4,6] According to Tran et al.[9] does not exist a common pattern in the changes of other volatiles formed for each type of kombucha during the fermentation process.

Alcohols were also common in the volatile profile of beverages, ranging from 12.0 to 24.0 %. In the kombucha K2 sample, hydrocarbons were the most characteristics class of compounds. Amines were identified only in K3 samples. Low percentages of compounds belonging to the class of aldehydes (4.0 to 10.0 %) and terpenes (7.0 to 10.0 %) were determined in the different samples evaluated. While Silva Junior et al.[10] determined the terpenes were the main volatile compounds described in kombucha beverages, which was found to have favored the sensorial profile of kombucha pitanga-flavored and kombucha umbu-cajá-flavored. Zou et al.[6] also noted that the concentrations of alcohols raised significantly, levomenthol, for example, was produced in all kinds of kombucha samples after fermentation. In the samples of the present study, the alcohol levomenthol was not identified. During the kombucha fermentation process yeasts firstly metabolized sugar to produce alcohols, and then acetic acid bacteria used alcohols to produce acids. Alcohols and acids form esters through esterification.[9]

The sample K1 exhibited 33 compounds, esters and alcohols were the most representative 24.2 and 21.2 %. In the K4 sample, 25 compounds were identified and esters and carboxylic acids also were the major ones with 28.0 and 20.0 % respectively. In the K5 sample, 16 compounds were identified, among these, 20.0 % of esters, 20.0 % ketones and 20.0 % of carboxylic acids, from the total of alcohols were found 10.0%, aldehydes 10.0 %, amines 10.0 % and 10.0 % of lactones, respectively. This sample had lower percentages of volatile compounds compared to the others, however lactic acid was only identified in the K3 sample, although lactic acid is characteristic of the kombucha

fermentation process, it lowers the pH of the kombucha, which contributes to the antimicrobial abilities[11]. Silva Junior et al.[10] when evaluating the impact of the addition of pitanga (*Eugenia uniflora* L.) and umbu-cajá (*Spondia tuberosa*) fruits on the parameters volatiles of beverage kombucha identified the acids acetic, butyric, citric, succinic, and malic acids in all kombuchas, except for the malic acid in sample pitanga-flavored kombucha.

Table 1 shows the volatile compounds that were tentatively identified in the different samples of hibiscus-based kombucha (*Hibiscus rosa-sinensis*).

Table 1 - Volatile compounds identified in the headspace of samples of hibiscus-based kombucha (*Hibiscus rosa-sinensis*) by mass spectrometry and linear retention index

Compound	R_T	K1 (%)	K2 (%)	K3 (%)	K4 (%)	K5 (%)	Match	CAS	LRI_{cal}	LRI_{lit}	Ref.
Ester											
Ethyl acetate	1.22	--	1.13	0.89	--	--	875	141-78-6	788	781	[12]
Ethyl Butanoate	2.03	1.49	--	--	2.61	2.01	829	105-54-4	840	850	[13]
1-Octenyl acetate	6.37	--	1.61	0.10	--	--	847	2442-10-6	1113	NI	-
3-Octenyl acetate	6.88	1.38	--	--	0.35	6.33	959	2442-12-06	1123	1126	[14]
Vinyl Hexanoate	7.20	1.81	1.74	--	4.81	--	828	3050-69-9	1162	1164	[15]
Isopentyl 3-hydroxy-2-methylenebutanoate	7.50	0.36	1.46	--	--	1.80	807	80758-72-1	1181	NI	-
Ethyl Hexanoate	7.88	2.26	1.74	--	3.26	--	859	123-66-0	1204	1212	[16]
Isopentyl Hexanoate	9.19	0.48	--	--	0.99	--	878	2198-61-0	1291	1292	[17]
Isoamyl Hexanoate	9.26	0.36	--	--	1.03	--	934	540-07-8	1295	1295	[17]
Pentyl Hexanoate	9.33	--	0.33	--	--	2.78	936	540-07-8	1300	1290	[18]
Ketone											
2-Butanone	1.43	--		0.58	--	--	803	590-90-9	802	804	[19]
4-Methyl-2-hexanone	2.83	0.62	3.47	--	1.08	2.45	846	105-42-0	895	896	[20]
2-Nonanone	6.69	0.65	--	--	0.05	0.97	888	821-55-6	1131	1133	[21]
2-Butyl-1,3-cyclohexandione	10.60	0.44	--	--	--	--	887	18456-90-1	1390	NI	-
Cyclohexanone	15.56	--	0.69	--	--	--	823	98361-31-0	1304	1304	[22]
Carboxylic Acid											
2,3-Dihydroxypropionic acid	3.39	--	--	3.78	--	--	852	473-81-4	930	NI	-
2-Ethylhexanoic acid	7.07	1.94	--	--	2.94	--	884	149-57-5	1155	1158	[23]

Hexanoic acid	7.70	0.61	0.31	--	0.77	0.89	890	2051-49-2	1373	1370	[24]
lactic acid	9.25	--	--	9.50	--	--	886	50-21-5	1294	1298	[25]
Octanoic acid	10.36	0.58	--	--	1.58	--	809	124-07-2	1193	1187	[26]
Hexadecanoic acid	17.13	0.48	--	--	1.48	--	820	57-10-3	1964	1968	[27]
Octadecenoic acid	19.01	0.11	--	--	0.37	1.30	853	57-11-4	2164	NI	-
Alcohol											
2-Octen-1-ol	6.27	17.97	20.33	1.30	--	--	817	18409-17-1	1106	1100	[28]
2,6-Dimethyl-4-heptanol	6.59	1.81	1.66	--	--	--	843	108-82-7	1125	NI	-
Phenylethyl alcohol	6.99	1.99	3.45	--	0.99	6.50	879	60-12-8	1150	1144	[29]
4-Dodecanol	7.41	1.28	--	--	3.28	--	815	10203-32-4	1175	1182	[30]
3,7-Dimethyl-1-octanol	8.35	0.63	--	--	1.02	--	888	106-21-8	1235	1230	[31]
3-Octanol	8.65	1.40	--	--	1.33	--	829	589-98-0	1255	1254	[32]
3,7,11-Trimethyl-1-dodecanol	9.03	--	0.27	--	--	5.36	828	6750-34-1	1180	NI	-
2-Hexadecanol	18.30	0.15	0.12	--	0.10	1.10	820	14852-31-4	2087	2085	[33]
Aldehyde											
2-Octenal	6.50	53.78	52.58	2.81	63.00	60.80	915	2548-87-0	1120	NI	-
Amine											
Isobutylhydrazine	6.66	--	--	1.40	--	--	857	42504-87-0	1124	NI	-
Hydrocarbon											
Tetradecane	7.54	--	0.79	--	--	--	803	10374-74-0	1183	NI	-
1-Ethyl-2,4,5-trimethylbenzene	8.11	0.44	0.56	--	--	--	883	17851-27-3	1219	1220	[34]
Pentamethylbenzene	8.27	0.52	--	--	--	--	903	700-12-9	1230	1235	[35]
2,3-dimethyl-undecane	8.41	1.17	--	--	--	--	895	17312-77-5	1239	NI	-
1,1,2-Tridecadiene	8.48	0.48	0.51	--	--	2.30	833	21964-48-7	1243	1249	[36]

4-Methyl-docosane	13.61	--	0.41	--	--	--	836	25117-30-0	1631	NI	-
Lactone											
γ-butyrolactone	6.78	--	--	0.09	--	--	810	13861-97-7	1132	1126	[37]
δ-Nonalactone	9.04	0.24	--	--	1.24	--	864	3301-94-8	1281	1284	[38]
δ-Tetradecalactone	11.31	--	0.70	--	--	0.57	874	2721-22-4	1444	1447	[39]
δ-Dodecalactone	11.65	0.53	--	--	0.53	--	830	713-95-1	1471	1470	[39]
δ-Tridecalactone	17.03	0.35	--	--	1.35	--	829	7370-92-5	1954	1966	[39]
δ-Octadecalactone	18.23	0.07	0.11	--	0.15	3.10	895	1227-51-6	2079	2081	[39]
Terpene											
Linalool	6.80	1.74	3.84	--	3.44	1.74	940	78-70-6	1138	1140	[40]
α-Terpineol	8.03	1.21	1.57	--	2.01	--	912	98-55-5	1214	NI	-

*Relative peak areas, obtained by GC-MS, using DB-5 column. NI = Not identified.

According to the data in Table 1, it was possible to observe that several compounds were identified in the kombucha samples and these substances are mostly related to fruity and floral odor notes.

Among the esters, ethyl butanoate was detected in samples K1 = 1.49 %; K4 = 2.61 % and K5 = 2.05 %, contributing with fruity and pineapple scent notes,[41] 3-octenyl acetate was identified in samples K1 = 1.38 %, K4 = 0.35 % and K5 = 6.33 %, being related to pomegranate odor notes,[42] and ethyl hexanoate (K1 = 2.26%; K2 = 1.74% and K4 = 3.26%) as reported by Selli et al.[41] is described with ripe banana odor notes. According to Savary et al.[43] esters are compounds that have sweet and fruity odorous notes.

Among the ketones, 2-butanone, 2-nonanone, 4-methyl-2-hexanone and cyclohexanone were detected in the different kombucha samples and have been associated with fruity, sweet, vanilla, caramel, nutty, hazelnut and roasted chocolate notes.[44]

Carboxylic acids were also found, such as 2,3-dihydroxypropionic acid only in sample K3 (3.78 %), hexanoic acid (K1 = 0.61 %, K2 = 0.31%, K4 = 0.77 and K5 = 0.89), in addition to octanoic acid in the samples K1 and K5. As reported by Sampaio et al.[45] these compounds impart

negative aroma notes, related to a repulsive, vinegary, stinky and bitter odor. It is worth noting that these compounds are the result of the beverage fermentation process and that their composition may vary depending on the conditions used at this stage.

Regarding alcohols, 2-octen-1-ol was identified only in samples K1, K2 and K3 and is described with typical mushroom notes and is considered an off-flavor in fermented beverages.[46] In addition, 2-octen-1-ol was quite representative in samples K1 = 17.97 % and K2 = 20.33 %, thus contributing negatively to the aroma of K1 and K2 beverages. While phenylethyl alcohol detected in all samples (K1 = 1.99 %, K2 = 3.45 %, K4 = 0.99 % and K5 = 6.50 %), except for K3. According to Savchuk et al.[47] phenylethyl alcohol is a common macro component in beers and wines, in addition to exhibiting floral, rose and honey scent notes.[48] The 3-octanol, detected only in samples K1 and K4, contributes with refreshing and mint notes.[49]

In the case of aldehydes, only 2-octenal was found, in a very representative way, in all samples studied (K1 = 53.78 %, K2 = 52.58 %, K3 = 2.81 %, K4 = 63.00 % and K5 = 60.80 %). and is often associated with pungent, green and greasy odorous notes.[50] As stated by Melo et al.[44] aldehydes are often related to the degradation of lipids and, depending on their concentration, they introduce pleasant (floral and fruity) or unpleasant notes to the matrix.

Among the hydrocarbons, tetradecane, 1-ethyl-2,4,5-trimethylbenzene, pentamethylbenzene, 2,3-dimethyl-undecane, 4-methyl-docosane and 1,12-tridecadiene were identified, being the last one the most representative in sample K5 = 2.30%. According to Jati and Sari[51] hydrocarbons stand out for being related to floral, sweet and balm scent notes.

Regarding lactones, γ-butyrolactone were identified only in the K3 sample, δ-nonalactone (K1 = 0.24 % and K4 = 1.24 %), δ-tetradecalactone (K2 = 0.70% and K5 = 0.57 %), δ-dodecalactone (K1 = 0.53% and K4 = 0.53 %), δ-tridecalactone (K1 = 0.35 % and K4 = 1.35 %) and δ-octadecalactone detected in all samples, except in sample K3. These substances along with ketones contribute fruity and sweet odor notes. Lactones are cyclic esters formed from the condensation of an alcohol functional group and a carboxylic acid group in the same molecule.[44]

From the terpene class, linalool and α-terpineol were detected in the samples (K1 = 1.74 %, K2 = 3.84 %, K4 = 3.44 % and K5 = 1.74 %) and (K1 = 1.21 %, K2 = 1.27 % and K4 = 2.04 %), respectively. These compounds are classified as monoterpenes and are present in the sweet, citrusy odor of orange and lemon.[52] According to Melo et al.[44] linalool contributes floral, citrus, fennel and woody scent notes. Since, in fermented beverages like kombucha terpenes are considerably important for the sensory profile.[10]

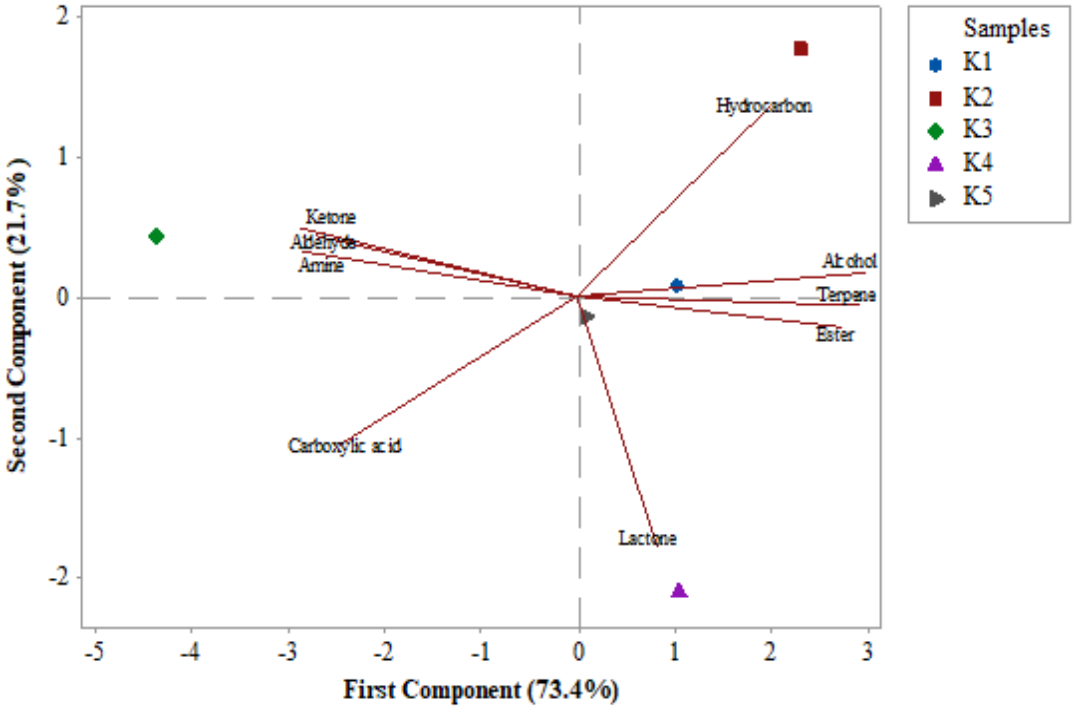
3.2 MULTIVARIATE ANALYSIS

To better understand the relationship between the samples and the identified variables, the principal component analysis method (PCA) was performed, which, by the direction and size of the vectors, indicates their contributions to the discrimination of samples of kombucha

commercial. The figure 2 illustrates the scores of the major components, PC1 and PC2, which explained respectively 73.4 % and 21.7 % and demonstrated the variance stored in each main component expressed by the eigenvalues of the standardized matrix. It was verified in the present study that the two main components explained 95.1 % of the total variance observed among the samples.

Each sample was located near the vectors that describe with greater intensity its characteristics. Vectors close to each other, suggest attributes with statistically significant correlations. The PC1 (X-axis) is mainly related to the samples K4, K1 and K2 and were characterized by the vectors of hydrocarbons, alcohols, terpenes, esters and lactones, suggesting that these sources are possibly more correlated with each other and have a very similar profile of volatile compounds belonging to this class. While PC2 (Y-axis) was discriminated by the K3 sample and was characterized by volatiles belonging to the classes of aldehydes, amines, ketones and carboxylic acids. Sample K5 was located in the center of the graph, indicating that it was not influenced by any vector.

Figura 2. Score and factorial load of Principal Component Analysis of volatile compounds in commercial kombucha



Source: Author himself (2021)

4 CONCLUSION

In the present study, it was possible to identify that volatile compounds belonging to the classes of esters and alcohols were the most representative. Furthermore, it is seen that these

compounds are mainly formed during the fermentation process, which explains the greater prevalence of esters, since they are compounds resulting from the esterification reactions that occur as a function of the concentration of alcohols and carboxylic acids, which are the precursors for the formation of volatile compounds characteristic of the aroma of kombucha.

It was also verified that the volatile profile of commercial kombuchas added with Hibiscus is constituted by a complex mixture of volatile compounds belonging to different classes, among which the most important are ethyl butanoate, 2,6-dimethyl-4-heptanol, linalool, 3-octenyl acetate, ethyl caproate, 3-octanol, hexadecanoic acid and 2-octen-1-ol, with emphasis still on the 2-octenal that was more abundant in the evaluated samples representing more than 50.0% of the volatile fraction. It was also seen that the substances identified in the volatile profile of the different kombucha samples studied are mainly related to fruity and floral odor notes.

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