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Influence of Fermentation with *Hanseniaspora* sp. Yeast on the Volatile Profile of Fermented Apple

Giovana de Arruda Moura Pietrowski,*,[†] Caroline Mongruel Eleutério dos Santos,[§] Elenise Sauer,[†] Gilvan Wosiacki,[§] and Alessandro Nogueira[§]

[†]Department of Food, Federal Technological University of Paraná, Ponta Grossa Campus, Av. Monteiro Lobato s/n, Km 04, CEP 84016-210 Ponta Grossa, PR, Brazil

[§]Department of Food Engineering, State University of Ponta Grossa, Av. Gal. Carlos Cavalcanti, 4748 − Uvaranas, CEP 84030-900 Ponta Grossa, PR, Brazil

ABSTRACT: This study aims to evaluate the aromatic profile of fermented apple, obtained by the action of strains of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* using two methods of analysis: static headspace and solid phase microextraction (SPME). The results obtained confirm that the strains of the *Hanseniaspora* genus contributed positively to the aroma profile of fermented apple, producing considerable amounts of esters and higher alcohols. In comparing the methods of analysis of aromatic compounds using headspace and SPME, it was verified that by using the headspace method it was possible to capture amounts that were up to 16 times greater than the value of the volatile compounds obtained by SPME. However, when using SPME, 5 times more compounds were obtained than when using headspace. Even so, in the conditions of this study it was noted that headspace was more efficient in the extraction of the aromatics of fermented apple when taking into consideration the cost effectiveness of both methods.

KEYWORDS: *volatile compounds, non-Saccharomyces, headspace, SPME*

INTRODUCTION

In Brazil the production of fermented apple is carried out by large companies that specialize in extraction and alcoholic fermentation. After the complete exhaustion of sugars and with the presence or absence of natural malolactic fermentation, the beverage is then sold to companies that use it as raw material in the production of ciders and vinegars.[1,2] Among these products, those that are most produced are fermented sparkling apple juice or cider.

Various factors that affect the composition and concentration of volatile compounds must be considered in the preparation of fermented apple, such as variety, ripeness, and quality of the raw material, processing steps (pressing, enzymatic treatment, clarification, and heat treatment), and storage conditions. Therefore, it becomes essential to monitor the various biochemical transformations that occur in apple must, mainly due to the action of yeasts that play an important role in the manufacture of fermented fruit.[3,4]

Aroma plays a significant role in the quality of fermented apple and cider. The alcoholic fermentation of apple must, made only with *Saccharomyces* sp. type yeasts, provides sensory results such as a neutral aroma; however, with the presence of the *Hanseniaspora* sp. strain, sensory notes appear as "fruity", or flavoured with fruit, due to the presence of esters such as ethyl acetate and phenyl ethyl acetate.[1]

The primary aromatic compounds of apples and apple juice, obtained in various ways, have been studied extensively over the past 30 years. However, with respect to secondary aromas, derived from the fermentation process, the amount of information is much smaller. The major volatile compounds in fermented apples and ciders are alcohols, esters, fatty acids, and ketones. Of these, ethanol, 1-butanol, 1-hexanol, 3-methylbutyl acetate, 2-phenylethyl acetate, butyl acetate, and hexanoic acid are typically dominant. Terpenes and phenolic derivatives have also been identified, but to a lesser extent.[5]

According to Simões et al., the aroma of fermented apple and cider is made up of alcohols such as 2-phenylethanol, butanol, 2.3-butanediol, and isobutanol and esters such as ethyl acetate. They also note that some of the compounds that are characteristic of apples such as butanol and others from fermentation such as amyl alcohols and ethyl acetate contribute to the aromatic profile of the fermented apple.[3]

The chromatographic analysis of organic compounds that are present in different products, whether foods or not, always requires a pretreatment of the sample. This is due to the existence of macromolecules such as proteins or sugars that are incompatible with the chromatographic columns or the concentration of substances at trace level. There are various extraction techniques for these organic compounds. However, choosing an appropriate analytical extraction technique is still a challenge because volatile compounds are very unstable and subject to changes and losses during extraction. In some cases it becomes necessary to combine extraction techniques to obtain a representative extract of volatiles from the sample.[6]

Various forms of extraction of aromatic compounds have been used for studies of cider, such as solid phase extraction (SPE), purge and trap (P&T) or dynamic headspace (DHS), direct injection, liquid−liquid extraction (LLE), solvent-assisted

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evaporation (SAFE), static headspace (SHS), and solid phase microextraction (SPME).[4,5,7−9]

When using the headspace or static headspace techniques, the vapor phase (where all of the volatile compounds are concentrated) must be collected with a syringe after the balance of the gaseous phase and liquid phase has occurred. The vapor phase is then injected directly into the chromatograph. In this way, there is little sample handling and the loss of volatiles is minimized. However, those compounds with high boiling points, and which are found only in trace amounts, are not easily captured by this technique.[10]

Considering the current trend for low-alcohol drinks, and modifications in flavors with an emphasis on the aromatic profile, this study aims to evaluate the aromatic profile of fermented apple, obtained by the action of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* strains using two methods of analysis: static headspace and SPME.

■ MATERIALS AND METHODS

Materials. Samples of Gala apples (90 kg) from the 2009−2010 harvest were obtained in the city of Ponta Grossa, Paraná, Brazil.

The microorganisms used were *H. uvarum* and *H. guilliermondii*, which had been previously identified by analysis of molecular profiles (PCR-fingerprinting) using the PCR mini/microsatellite (MSP-PCR) technique with synthetic oligonucleotide (GTG) 5,[11] with DNA extraction using the method of Brandão et al.[12] For sequencing, the D1/D2 domains of the larger subunit of the rRNA gene were amplified,[13] and the sequences obtained were compared with those deposited in the GenBank database using the Basic Local Alignment Search Tool.[14]

The reagents and standards used were of chromatographic grade (≥99.7%, Fluka; ≥98.5%, Merck; ≥97−99%, Aldrich; analytical grade, Interchim). The SPME fiber utilized was coated with Carbowax/divinylbenzene (CW/DVB) of 70 μm thickness (Supelco).

The standards of aromas were ethyl ethanoate (Fluka), ethanol (Merck), ethyl propanoate (Interchim), ethyl 3-methylbutanoate (Interchim), propyl ethanoate (Interchim) 2-methylpropyl ethanoate (Interchim), ethyl butanoate (Interchim), butyl ethanoate (Fluka), hexanone (Interchim), 3-methylbutyl ethanoate (Aldrich), 2-heptanone (Interchim), 3-methyl-1-butanol (Merck), 2-methyl-1-butanol (Interchim), 2-hexanol (Interchim), hexyl ethanoate (Fluka), 2-octanone (Interchim), ethyl hexanoate (Interchim), 2-hydroxyethyl propanoate, (Aldrich), 1-hexanol (Fluka), ethyl octanoate (Aldrich), ethyl decanoate (Aldrich), butanoic acid (Fluka), diethyl butanedioate (Fluka), 2-phenylethanol (Aldrich), ethyl dodecanoate (Aldrich), and octanoic acid (Fluka).

Processes. *Processing of Apple Juice.* The apples were selected, washed, and crushed in a microprocessor (Metvisa CPU, type MPA). The grated mass was wrapped in packages of porous plastic, which were superimposed and subjected to a pressure of 3.0 kgf/cm^2 in a hydraulic press (Eureka, Hoppe Ind. Ltd., Brazil) for 5 min. The apple must was clarified by hydrolysis of pectin (Pectinex Ultra SP, Novozymes, Brazil), at a rate of 3.0 mL/hL (120 min at 20−25 °C), and after sedimentation, the supernatant was racked, filtered on qualitative filter paper, bottled in 600 mL glass bottles, sealed with a metal cover, and pasteurized at 80−85 °C for 20 min.[15]

Processing of Fermented Apple. The clarified must was packaged in 1450 mL glass fermenters (previously sterilized in an autoclave at 121 °C for 15 min) with a working volume of 1200 mL, as shown in Figure 1, and kept in a climate room at a temperature of 20 °C.[16] Antibiotic chloramphenicol was added to the must at 0.01% (99%, Henrifarma Supplier) to control bacterial growth. The yeasts were inoculated with an initial population of approximately 4.0×10^6 cfu/mL. To reach this population, one colony of each strain of *Hanseniaspora* sp., isolated in YMA (Merck) in 5 mL of GPBY broth−glucose peptone broth yeast (Merck), was inoculated, and after 24 h of incubation at 25 °C, this culture was transferred to 100 mL of the same broth, time, and temperature, reaching a population of

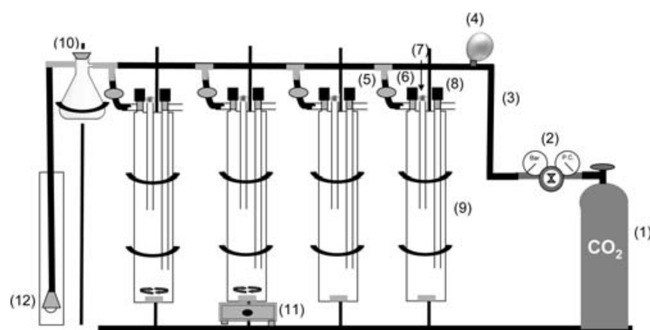

Figure 1. Anaerobic fermenting system: (1) carbon dioxide cylinder to pressurize the system; (2) gauge; (3) pipe passing carbon dioxide through the system; (4) safety valve to prevent gas leak; (5) 0.20 μm filter; (6) screw cap for input and output of gas in the fermenter; (7) rubber stopper for inoculation of yeast in the must; (8) screw cap to remove the sample; (9) 1450 mL fermenter; (10) security container to prevent return of water to the fermentation system; (11) magnetic agitator (Quimis); (12) butt to maintain the anaerobic fermentation system.

approximately 10^{12} cfu/mL. From this population, a sufficient volume was collected to achieve the desired population in the fermenters (about 2.0×10^6 cfu/mL). On the 10th day, fermentation was stopped and the fermented apple was centrifuged at 10200*g* at 5 °C (Himac Centrifuge CR21GII) for 20 min, then racked, bottled, and stored at low temperature (−18 °C). The ethyl alcohol content was determined by ebuliometry.

Instrumental Analysis. *Collection of Volatile Compounds: Headspace.* The capture of compounds using headspace was performed according the method of Saerens et al., with modifications.[17] Samples of fermented apple were placed in glass vials with a capacity of 20 mL, in amounts of 6 mL. Then, 50 μL of internal standard (heptanoic acid, Merck) was added. The compounds were identified by the retention time of standard compounds. Prior to the collection of the volatile compounds the samples remained at 60 °C under agitation in the oven of the automatic injector (Young Lin Instrument gas chromatograph) for 10 min.

Collection of Volatile Compounds: SPME. The SPME was carried out according to the method of Reid et al., with modifications.[18] The samples of fermented apple were placed in glass vials with a capacity of 20 mL, in amounts of 6 mL. Then 2.2 g of NaCl was added (as per the recommendations of the manufacturer, Supelco) and also 40 μL of the same internal standard (heptanoic acid, 16.3 mg/mL, Merck). For the SPME, the vials with samples were maintained at 60 °C for 5 min and were then kept at the same temperature and stirred for 10 min, during which the microfiber remained exposed in the headspace of the bottle for the adsorption of compounds. After extraction, the SPME was directly exposed in the injection port of the gas chromatograph (GC) for the thermal desorption (44 min at 220 °C) of the compounds.

Chromatographic Analysis of Volatile Compounds. The analysis of aromatic compounds was performed by gas chromatography according to the methods of Xu et al. and Saerens et al., with modifications: Young Lin Instrument (YL 6100 GC) equipped with FID, capillary column (Phenomenex), flame ionization detector, 30 m in length with an internal diameter of 25 mm, and 0.25 μm thick ZB-WAX film. The injector temperature was 220 °C and the detector temperature, 230 °C. The carrier gas was nitrogen, with a flow of 2.5 mL/min, and the injection technique was split 1:1.2. The analysis conditions were programmed with an initial temperature of 40 °C for 5 min with an increase of 10 °C/min to 150 °C, maintaining this temperature for 10 min. The temperature was then increased at 10 °C/min to 200 °C for 5 min and again increased at 10 °C/min to 220 °C, at which it remained for 16 min.[5,17]

For the tests using headspace, the GC automatic injection method was used with a volume of 1500 μL, whereas for the tests using SPME the injection was manual, with the fiber exposed in the interior of the injector port to allow the desorption of volatile compounds.[17,18]

The composition of the reference stock solution used for the identification and quantitation of volatile compounds in the fermented apple is presented in Table 1. For the headspace analysis 1:25 diluted stock standard solution was used, and for the extraction using SPME, 1:30 diluted stock standard solution was used. The dilutions of stock solution were subjected to the same analysis conditions as described for the samples of fermented apple, including the addition of internal standard.

Table 1. Composition of Reference Stock Solution for Analysis of Volatile Compounds in Fermented Apple

standard component	concentration (mg/100 mL)	standard component	concentration (mg/100 mL)
ethanal	260;[a] 325[b]		
ethyl ethanoate	133.8	2-hexanol	18.5
ethanol	15860	hexyl ethanoate	9.0
ethyl propanoate	41.5	2-octanone	30.6
3-methylethyl butanoate	34.2	ethyl hexanoate	42.7
propyl ethanoate	45.6	2-hydroxyethyl propanoate	130.9
2-methylpropyl ethanoate	35.1	1-hexanol	7.6
ethyl butanoate	8.1	ethyl octanoate	16.5
butyl ethanoate	5.3	ethyl decanoate	8.7
hexanone	30.5	butanoic acid	18.1
3-methylbutyl ethanoate	31.8	diethyl butanedioate	10.2
2-heptanone	30.1	2-phenylethanol	390.5
3-methyl-1-butanol	241.6	ethyl dodecanoate	8.1
2-methyl-1-butanol	32.3	octanoic acid	75.2

[a]Concentration used in headspace. [b]Concentration used in SPME.

The compounds were identified by comparing retention times with those obtained in the reference solution. For headspace, three repetitions of each sample were carried out, and for SPME two repetitions were carried out. To quantitate the concentrations of identified volatile compounds, eq 1 was used, where C is the concentration of component (mg/L), A the concentration of the substance in the reference solution (mg/L), h the peak area of the substance in the sample, H the peak area of substance in the reference, I the peak area of internal standard in the reference, and i the peak area of internal standard in the sample.[19]

$$C = A \times (h/H) \times (I/i) \quad (1)$$

Statistical Analysis. Analysis of variance (ANOVA) and Tukey's test ($p < 0.05$) were carried out for differentiation of averages using Excel 2007 software.

RESULTS AND DISCUSSION

Aromatic Profile of the Fermented Apple. The aromatic composition obtained in the fermentation of apple must by strains of *H. guilliermondii* and *H. uvarum* demonstrated the presence of esters, aldehydes, acids, ketones, and alcohols in the headspace analysis method and also in the SPME method, as shown in Table 2.

The contribution of the species that were investigated for aromatic compounds is evident in Table 2, noting that both *H. guilliermondii* and *H. uvarum* were able to synthesize all of the quantified compounds, because the values detected in the fermented apple were always higher than those found in the apple must used for the fermentations. This failed to occur with butyl ethanoate, 1-hexanol, and 2-heptanone, which appear to have been metabolized by the investigated strains, as shown by the SPME results. It also failed to occur with hexyl ethanoate, which showed the same concentration in the must and in the fermented apple using *H. uvarum* by the SPME method.

Table 2. Concentration of Volatile Compounds of Headspace and Extracted by SPME from Fermented Apple Treated with Strains of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii*[a]

volatile compound	apple must		*H. uvarum*		*H. guilliermondii*	
	headspace	SPME	headspace	SPME	headspace	SPME
ethyl ethanoate	1.14 ± 0.11	0.58 ± 0.05	146.99a ± 0.75	7.63A ± 0.70	9.90b ± 0.60	0.98B ± 0.09
2-methylpropyl ethanoate	nd	nd	nd	nd	nd	0.31 ± 0.02
ethyl butanoate	0.05 ± 0.00	0.03 ± 0.00	nd	nd	0.05 ± 0.00	0.72 ± 0.07
butyl ethanoate	nd	0.15 ± 0.02	nd	0.11A ± 0.02	nd	0.11A ± 0.02
3-methylbutyl ethanoate	0.06 ± 0.01	0.01 ± 0.00	0.63 ± 0.03	0.09 ± 0.01	nd	nd
hexyl ethanoate	0.07 ± 0.01	0.01 ± 0.00	0.18 ± 0.01	0.01 ± 0.00	nd	nd
2-hydroxyethyl propanoate	16.07 ± 0.64	2.01 ± 0.11	47.99a ± 3.96	4.30A ± 0.24	45.17b ± 3.73	3.79B ± 0.21
ethyl octanoate	nd	nd	0.10a ± 0.01	nd	0.02b ± 0.00	nd
ethyl decanoate	nd	nd	0.29a ± 0.03	0.01 ± 0.00	0.09a ± 0.00	nd
diethyl butanedioate	2.90 ± 0.03	0.08 ± 0.02	6.24a ± 0.37	0.36A ± 0.09	4.33a ± 0.21	0.36A ± 0.09
ethyl dodecanoate	1.34 ± 0.00	0.08 ± 0.00	4.26a ± 0.36	0.16B ± 0.00	2.94a ± 0.22	0.33A ± 0.00
ethanal	3.50 ± 0.30	0.63 ± 0.01	38.04a ± 0.56	5.25A ± 0.04	21.47b ± 0.56	2.79B ± 0.02
butanoic acid	nd	0.04 ± 0.00	4.85a ± 0.25	0.08A ± 0.01	3.30a ± 0.27	0.06B ± 0.01
octanoic acid	nd	0.07 ± 0.00	nd	0.45A ± 0.01	nd	0.27B ± 0.00
3-methyl-1-butanol	0.88 ± 0.08	0.01 ± 0.00	21.99a ± 0.15	1.96A ± 0.22	12.41b ± 0.37	1.13B ± 0.13
2-hexanol	nd	0.06 ± 0.01	0.28a ± 0.02	nd	0.13a ± 0.01	nd
2-phenylethanol	nd	0.02 ± 0.00	nd	0.20A ± 0.01	nd	0.19A ± 0.01
2-heptanone	nd	0.30 ± 0.03	nd	nd	0.04 ± 0.00	nd
2-octanone	nd	nd	0.06 ± 0.00	nd	nd	0.01 ± 0.00

[a]Concentration of volatile compounds identified in mg/L. nd, not detected. Data are the average of three replicates for headspace and two replicates for SPME ± DP. Different lower case letters in the same row indicate significant difference existing between the quantities in headspace samples ($p < 0.05$). Different capital letters in the same row indicate significant difference between the quantities available in SPME samples ($p < 0.05$).

In the results for both headspace and SPME it was noted that the particular strain of yeast used influenced the volatile profile because the results differed significantly from one strain to another. In general, the amount of compounds produced by *H. uvarum* was always larger than that of *H. guilliermondii*, except for ethyl butanoate and 2-heptanone, which were not detected in *H. uvarum* by the headspace method. Ethyl butanoate, 2-methylpropyl ethanoate, and 2-octanone were not detected in *H. uvarum* by SPME. Butyl ethanoate and diethyl butanedioate had the same concentration in both strains by this method. Rojas and co-workers worked with SPME and the same yeast species and found the opposite result; that is, the *H. guilliermondii* strain produced larger amounts of compounds such as 3-methylbutyl ethanoate, ethyl ethanoate, ethyl octanoate, ethyl decanoate, and isobutyl ethanoate in comparison to *H. uvarum*.[20] However, it should be noted that these authors worked with fermentations in synthetic media, which have a chemically defined composition and which meet all of the nutritional requirements of the microrganisms under study.

In the present study, the headspace method was found to produce considerable amounts of ethyl ethanoate, 2-hydroxyethyl propanoate, diethyl butanedioate, and ethyl dodecanoate, lesser amounts of 3-methylbutyl ethanoate, hexyl ethanoate, ethyl octanoate, and ethyl decanoate for both yeast strains, and ethyl butanoate only in the case of *H. guilliermondii* (Table 2). Rojas et al. found similar results in relation to ethyl ethanoate, for both *H. guilliermondii* and *H. uvarum*.[20] Although the difference in amount between the two strains was large, this was the most abundant compound produced by both strains.

Xu et al., using SPME, found that ethyl ethanoate, 2-hydroxyethyl propanoate, and 3-ethyl hexanoate were also the esters produced in greater concentrations in ciders produced by strains of *Hanseniaspora valbyensis*, suggesting that this is a characteristic of this strain of yeast.[21] However, Mingorance-Cazorla et al., using strains of *H. uvarum* to ferment grape must and orange juice, after distillation and direct injection in the GC, obtained ethyl ethanoate only in the fermentation of the orange juice.[22]

The fermentations of wine and cider share many similarities in microbiological terms, and the volatile compounds formed in these fermentations will have the same effect on the perception of aroma, whatever its origin.[20] Cortes and Blanco claim that ethyl octanoate, ethyl hexanoate, and 3-methylbutyl ethanoate are the most influential compounds in determining secondary aromas with fruity notes in Treixadura wine.[23] According to Rojas et al., the mixture of 3-methylbutyl ethanoate, ethyl octanoate, ethyl decanoate, hexyl ethanoate, and 2-phenylethyl acetate determines fruity and floral aromas in wines.[20]

With regard to the presence of these compounds in fermented apple, it is clear that only 3-methylbutyl ethanoate was detected by both methods when using the *H. uvarum* strain (Table 2). Ethyl hexanoate was not produced by any of the studied strains, and ethyl octanoate was produced in small quantities by the two strains; however, it was identified only when using headspace.

Peng et al. found nine key components in the aroma of Shaanxi cider (China) obtained from Fuji apples: ethyl ethanoate, acetic acid isobutyl ester, isopentyl alcohol acetate, ethyl octanoate, ethyl 4-hydroxybutanoate, isopentyl alcohol, 3,4,5-trimethyl-4-heptanol, nonyl alcohol, and 3-methylthio-1-propanol, which could account for 85.61% of total variation in the components of the aroma of the product.[8] Of these compounds, four (ethyl ethanoate, 3-methylbutyl ethanoate, ethyl octanoate, and 3-methyl-1-butanol) were found in the fermented apple (Table 2).

The *H. uvarum* strain showed a higher content of ethanol in the final product (1.9° GL) than the *H. guilliermondii* strain (1.3 °GL), confirming the results of ethanol production by non-*Saccharomyces* yeasts found by Ciani et al.[24] However, in fermentations of pure *H. valbyensis*, Xu et al. obtained an ethanol content of 6% (v/v) in cider.[21]

Increasing the concentration of ethanol leads these molecules to form agglomerates, which increase the solubility of the esters in the liquid phase, thereby decreasing the concentration of esters in the headspace.[25] This factor explains the lower perception of some aromatic compounds in higher concentrations of ethanol, a fact that was not observed in this study, which found low concentrations of ethanol.

According to Garden-Cerdan and Ancin-Azpilicueta, wild strains or non-*Saccharomyces* contribute little to the formation of 3-methyl-1-butanol.[26] However, Xu et al. confirmed that the *H. valbyensis* strain was capable of producing 45.35 mg/L of 3-methyl-1-butanol in the fermentation of pure cultures and, when combined with *Saccharomyces* sp. production, peaked at 232.05 mg/L, depending on the time of inoculum.[21] Although they are different species, in the fermentation of apple must, the *H. uvarum* and *H. guilliermondii* strains have demonstrated that they can produce concentrations of 21.99 and 12.41 mg/L, respectively, of 3-methyl-1-butanol when using headspace.

Romano et al. reported that the *Hanseniaspora* strain produced a smaller amount of 3-methyl-1-butanol (40 mg/L) and a higher concentration of ethyl ethanoate (100 mg/L) than *Saccharomyces* (250 mg/L, <10 mg/L, respectively), indicating that *S. cerevisiae* produced a much greater concentration of higher alcohols. In the present research a concentration below 40 mg/L of 3-methyl-1-butanol was obtained for both strains, but in relation to ethyl ethanoate, *H. uvarum* produced almost 50% more (using headspace) (Table 2) than the data reported by these authors.[27]

Ethanal was quantified in the two yeast strains by both headspace and SPME (Table 2). According to Gil and co-workers, ethanal is the most important aldehyde in wine production and is commonly known as a byproduct of alcoholic fermentation, the production of which can be influenced by the type of yeast strain. At lower concentrations, ethanal contributes fruity notes; however, at high concentrations (>200 mg/L) this compound negatively influences the aroma of wine.[28] Thus, under the conditions in which it was measured in the present study (38.04 and 21.47 mg/L for *H. uvarum* and *H. guilliermondii*, respectively) it contributed to a fruity aroma in the fermented product.

According to Ciani et al. and Rojas et al., esters formed in fermentations have the greatest impact on the aroma of products.[20,24] Andorrá et al. comment that *Hanseniaspora* species are considered to be major producers of esters, the majority of which contribute to the floral and fruity aroma of wines.[29] However, Zohre and Erten warn that the primary ester, ethyl ethanoate, at concentrations above 200 mg/mL, produces an unpleasant aroma of glue or solvent, a factor that was not found in the present study.[30] Suárez Valles et al. emphasize that the aromatic profile of cider is marked by the relationship between the low concentration of ethanol and ethyl ethanoate. In our opinion, the results of our study show the positive influence of the investigated strains on the aromatic quality of fermented apple.[31]

The higher alcohols also contribute to the aromatic complexity of fermented beverages at concentrations below 300 mg/L; however, when >400 mg/L, they may have a negative effect in terms of the aroma of the product.[32]

Comparison of Headspace and SPME Methodologies. It is worth highlighting the significant difference in the levels of the compounds acquired using the headspace and SPME methods. The sum of the esters in the headspace for *H. uvarum* was 206.67 and 62.50 mg/L for *H. guilliermondii*. When using SPME, the same compounds added values of 12.67 and 6.59 mg/L, respectively, for the same strains. The alcohols when using headspace amounted to 22.27 and 12.54 mg/L, and when using SPME they were 2.16 and 1.32 mg/L for *H. uvarum* and *H. guilliermondii*, respectively (Table 2). This variation was repeated for all other compounds, probably due to several factors that will now be discussed.

It is considered that in the analysis of volatile organic compounds, in most cases the analytes are first transferred through a gas phase, to be subsequently analyzed, usually by gas chromatography.[33] In the headspace method, compounds were collected directly from this phase, whereas when using SPME, they had to pass through an adsorption phase and a later desorption phase to be identified and quantified, which might justify the lower amount found when using this method. It should be noted that the extraction time used in this research was 10 min at 60 °C, whereas Xu et al. used 30 min at 50 °C;[5] Boylston et al., 30 min at 40 °C;[34] Peng et al., 30 min at 30 and 45 °C, respectively,[4,8] and Azhu-Valappil et al., 45 min at 36 °C,[35] each of the latter extracting volatiles from cider.

According to Dean, the most important phase of the SPME method is the adsorption of analytes in the fiber, so the choice of sorbent is essential as it must have a strong affinity for the organic compounds to be evaluated.[36] The preconcentration may occur from any aqueous sample or the gas phase. Thus, for the analysis of apples, apple juice, cider, and synthetic media, Azhu Valappil et al., Xu et al., Zierler et al., and Buzzini et al. used DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) as a coating for the fiber.[5,35,37,38] A coating of polydimethylsiloxane (PDMS) was used in the studies of Peng et al., Xiaobo and Jiewen, Reid et al., Boylston et al., and Young et al.,[4,8,18,34,39,40] and Zierler et al. used CAR/PDMS (carboxen/polydimethylsiloxane).[37]

Having been aware of the use of Carbowax/divinylbenzene coating as reported in the literature, in this study we decided to opt for the use of this fiber to assess its performance in fermented apple and also because it comprises two components that have this adsorbency: Carbowax (moderately polar adsorbent) and divinylbenzene (suspended in Carbowax phase, which has a high affinity for small amines). Carbowax/divinylbenzene is suitable for SPME of alcohols and polar compounds and is, therefore, appropriate to the target volatile compounds.

The choice between the direct immersion of SPME in the sample and the headspace of the sample is also an important factor in the use of SPME. Thus, the SPME of headspace should be considered for the extraction of volatile compounds in solid or liquid samples when the normal boiling point of the analytes is <200 °C; otherwise, the direct immersion of SPME is likely to be required.[33] In this way, the choice to expose the fiber in headspace is justified because most of the desired compounds (esters and higher alcohols) have a boiling point below this temperature. However, six compounds have been found that have a boiling point above 200 °C (octanoic acid, 2-phenylethanol, ethyl octanoate, ethyl decanoate, diethyl butanedioate, and ethyl dodecanoate).

On the basis of results already discussed, it was observed that a larger number of compounds was captured by headspace compared to SPME. However, by studying the unidentified compounds, it is clear that when using SPME, 57 compounds were captured, 5 times more than for headspace, by which only 11 compounds were found.

As the method of identification used in this study was the comparison of retention time standards, it was found that SPME was less effective than headspace because, despite having picked up a much larger number of compounds, their concentration was much lower. Thus, even though SPME captured 46 more unidentified compounds than headspace, these compounds could not be identified because this study did not use mass spectrometry coupled with gas chromatography, as used by other authors.[4,5,8,18,34,35,39,41]

Therefore, under the conditions when these tests were performed, that is, in the absence of mass spectrometry, the cost of using SPME was not justified.

Slack et al. consider that SPME has several advantages in the analysis of volatile organic compounds, among which they refer to the low cost of the fibers compared to the cost of other methods of extraction of volatile analytes, a fact that was not observed in this research in the conditions studied, where headspace was more efficient at keeping costs low.[33]

It is important to note that it was not our aim to optimize the conditions of adsorption of the compounds in SPME, which would certainly improve the acquisition of compounds in terms of both quantity and quality. The objective was to compare the results using the same form of acquisition of compounds for SPME and headspace using equivalent sample conditioning time (10 min), temperature (60 °C), and agitation for both methods.

To perform the desorption of the fiber compounds, the same injector temperature as headspace was used, that is, 220 °C, which lies within the 200−240 °C range recommended in the SPME manual (Supelco, Bellefonte, PA, USA; Sigma-Aldrich Co.).

Even with the existence of advanced methods for the identification and quantitation of volatile compounds, extraction techniques remain the most complex step of the analysis. According to Queiroz et al., the chosen extraction technique should take into consideration the following characteristics: simplicity, extraction time, cost, provision of extracts free of possible interferences, and high recoveries with good accuracy and precision. In this study, it was possible to analyze the results regarding each of the aforementioned aspects in both methods and therefore choose the headspace technique as the most viable, given the same analysis conditions.[42]

The strains of *H. uvarum* and *H. guilliermondii* showed the ability to produce esters, aldehydes, acids, ketones, and alcohols. Among these compounds the presence of ethyl ethanoate, ethyl octanoate, 3-methylbutyl ethanoate (only in *H. uvarum*), 3-methyl-1-butanol, and ethanal was observed, which, according to the literature, contribute to the fruity aroma of fermented apples and wine. Thus, both strains have potential for the production of aromatic compounds and can be applied in the preparation of fermented apples to improve the aromatic quality of the final product.

The capture of volatile compounds from fermented apples using static headspace demonstrated satisfactory results, showing the quantitation of the compounds of interest and at

a low cost in the applied conditions. However, SPME requires optimal extraction conditions, as well as gas chromatography coupled to mass spectrometry, to obtain results that justify the cost/benefit ratio.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 55 42 32204824. Fax: 55 42 32204810. E-mail: gampietrowski@brturbo.com.br.

Notes

The authors declare no competing financial interest.

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