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Impact of Encapsulated *Saccharomyces cerevisiae* Yeasts on the Chemical and Sensory Profiles of Sparkling Cider Produced by the *Champenoise* Method

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Abstract: The cider market has been significantly expanding and gaining momentum in Eastern Europe. As such, the aim of this study was to obtain sparkling cider via the *Champenoise* method using two Romanian apple varieties (Topaz and Red Topaz) alongside the employment of two fermentations. Four yeast strains were used in the first fermentation, while encapsulated *Saccharomyces cerevisiae* was used in the second fermentation. The resulting cider was subjected to a comprehensive investigation to quantitatively determine the carbohydrates, organic acids, volatile and phenolic compounds, and amino acids from all the cider samples. A trained panel evaluated the sensory profile of the samples, and a chemometric analysis was used to interpret the data. Secondary fermentation increased the accumulation of malic acid and lactic acid, as well as the volatile profile complexity. The total polyphenol content in the sparkling cider samples increased by almost 20% in the *S. cerevisiae* sample and over 217% in the *P. kluyveri* + *S. cerevisiae* sample compared to the base cider. Additionally, studying the production and consumption trends of sparkling cider offers valuable insights for both producers and consumers. By understanding consumer preferences and refining production techniques, the industry can deliver higher-quality products that better align with market demands.

Keywords: apple sparkling cider; immobilized yeasts; co-fermentation; sensory profile; volatile compounds

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1. Introduction

Cider is an alcoholic beverage made by completely or partially fermenting apple juice. To achieve full alcoholic fermentation, prevent sensory deviation, and provide a consistent and predictable quality, industrial manufacturing frequently uses a selection of *Saccharomyces cerevisiae* yeasts [1]. The importance of non-*Saccharomyces* yeasts has recently been granted attention due to the increasing flavor, body, and complexity of the texture of fermented beverages resulting from their employment. Indigenous yeasts have been involved in the production of distinct aroma compounds, including acids, aldehydes,

esters, higher alcohols, and terpenes, and also a higher glycerol concentration [2]. Sparkling cider, as well as sparkling wine, are high-value products that have gone through two fermentation stages. A high-quality base cider is first produced by fermenting the apple juice, followed by additional fermentation in a closed environment [3]. Sparkling cider can be prepared by different methods: the *Champenoise* method, *Charmat* method, Pét-Nat method, and artificial carbonation. The "Méthode *Champenoise*" requires the secondary fermentation of the base cider in bottles [4], which can lead to the formation of aroma compounds (esters) [5]. However, this method is not economical. In the *Charmat* method, the refermentation is carried out in tanks, and this is becoming popular in industrial cider production [6] because it is much faster and simpler than other methods [7]. The Pét-Nat method involves bottling cider while it is still fermenting and has a certain amount of sugars, and the fermentation is continued in bottles, leading to an accumulation of carbon dioxide in the product [8]. Last but not least, sparkling cider can be obtained by artificial carbonation [4], which has the advantages of being simple, fast, and the cheapest method [9]. The yeast is subjected to stressful conditions such as low temperature, CO_2 pressure, high ethanol, lack of nutrients (such as nitrogen sources), and low pH. During this phase, yeast growth and metabolism [10] are affected. This is due to the fact that the CO_2 formed by yeast respiration is sealed in the bottle to generate an overpressure, which affects the yeast's metabolic behaviors by significantly affecting the tricarboxylic acid cycle proteins and up-regulating the genes for oxidative stress and mitochondrial aerobic respiration [11]. Lipids, amino acids, mannoproteins, and enzymes involved in the formation of aroma compounds are released into the medium outside the cells by the yeast because they constantly come in contact with the cider during alcoholic fermentation. This process significantly affects the sparkling cider's aroma characteristics [12]. Previous studies have been focusing on the yeast strains involved in refermentation over the last few years. *Saccharomyces* yeasts immobilized in alginate beads are suitable for use in cider fermentation [13]. Alginate, a non-toxic biopolymer, is widely favored due to its facilitation of a straightforward encapsulation process feasible at a neutral pH and room temperature, without the need for harsh chemicals. The encapsulation of microbial cells in calcium alginate can be effortlessly accomplished by introducing drops of a cell suspension containing calcium chloride into a sodium alginate solution [14]. Some advantages of the yeast-immobilization systems include: high cell densities, product yield improvement, lowered risk of microbial contamination, better control and reproducibility of the processes, as well as reusage of the immobilization system for batch fermentations and continuous fermentation technologies [15]. High cell densities produced by cell immobilization result in increased volumetric productivities. Additional advantages of using encapsulated yeasts in fermentation include: easier biomass separation and recovery, simplified methodology, decreased risk of microbial contamination of the yeast population, improved efficiency in using carbohydrates, better equipment utilization, and potential cost savings [13].

The aim of this study was to obtain sparkling cider using encapsulated yeasts. Even though, in the production of wine or sparkling cider, the differences between the *Champenoise* method and the *Charmat* method of secondary fermentation are insignificant [16], the *Champenoise* method was chosen for the current study, with the aim of producing a sparkling beverage using the traditional method. Given the growing popularity of cider in Eastern Europe, and particularly among younger consumers in Romania, two apple varieties from the local production were used to prepare the apple juice. Base cider was obtained using different co-inoculations of *Saccharomyces* and non-*Saccharomyces* yeasts and lactic acid bacteria of apple juice. Quantitative determination of the carbohydrates, organic acids, volatile and phenolic compounds, and amino acids from the resulting sparkling cider was performed by means of modern and performant chromatographic methods. A trained panel assessed the sensory profile of each sparkling cider sample, and the results were statistically analyzed.

2. Materials and Methods

2.1. Materials and Experimental Design

Equal amounts of two apple cultivars were selected for this study (Topaz and Red Topaz). They were harvested in September 2022 from an apple orchard located near Cluj-Napoca, Romania (46°48′21.4″ N 23°35′19.6″ E). To avoid microbial contamination, the base cider was made using pasteurized apple juice (11.1 °Brix, pH 3.46, total titratable acidity 6.61 g/L malic acid). As previously described [17], the base cider was prepared using the following yeast strains and the dosage recommended by the manufacturer: *Pichia kluyveri* (Viniflora® FrootZen™, Chr. Hansen, Hoersholm, Denmark), *Saccharomyces cerevisiae* (Viniflora® JAZZ™, Chr. Hansen, Hoersholm, Denmark), and lactic acid bacteria: *Oenococcus oeni* (Viniflora® SPARTA™, Chr. Hansen, Hoersholm, Denmark), and *Lactobacillus plantarum* (Viniflora® NoVa™, Chr. Hansen, Hoersholm, Denmark). This resulted in 4 variants of base cider (Figure 1): *Pichia kluyveri* + *Saccharomyces cerevisiae*, *Lactobacillus plantarum* + *Saccharomyces cerevisiae*, *Oenococcus oeni* + *Saccharomyces cerevisiae*, and *Saccharomyces cerevisiae*, which were further used to obtain the 4 assortments of sparkling cider mentioned below. For the production of the base cider, *Saccharomyces* yeast was co-inoculated with *Pichia* and two lactic acid bacteria to achieve a more diverse aromatic profile, as well as to allow for simultaneous alcoholic and malolactic fermentation, a practice common in both cider and wine production [18,19]. At the end of fermentation, the base cider was clarified (kept at 4 °C for 10 days and decanted).

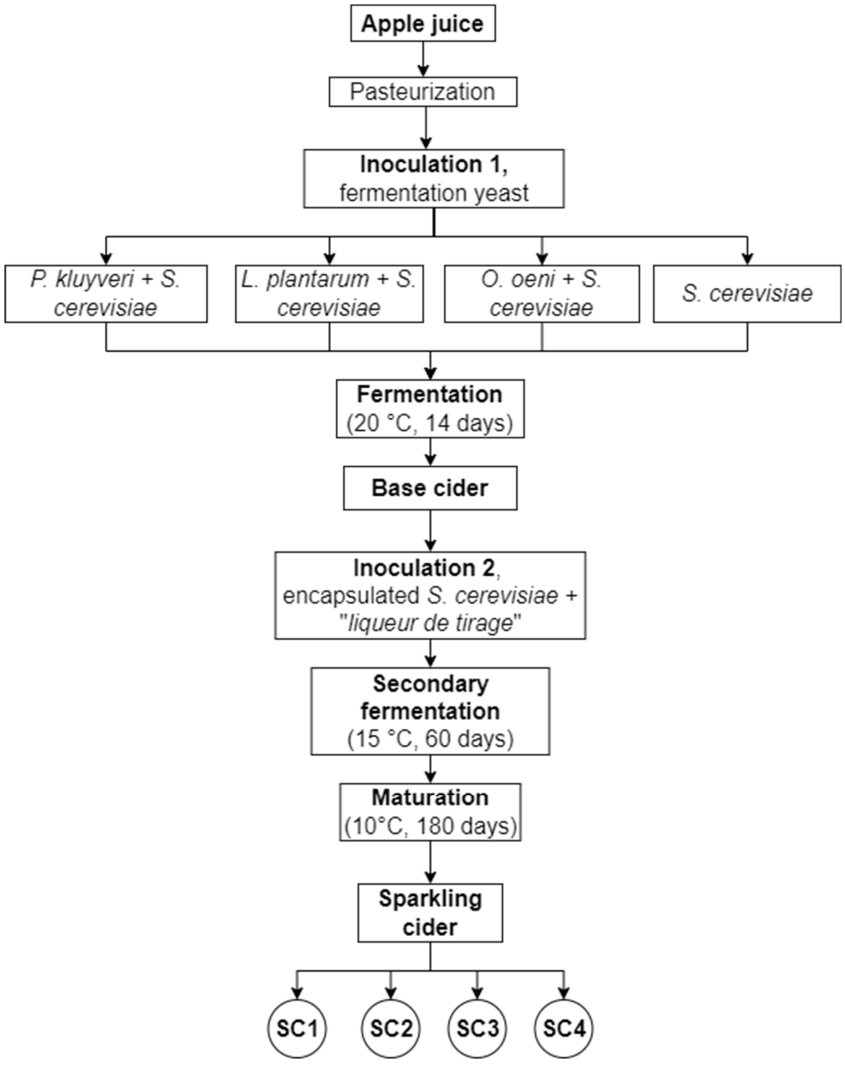

Figure 1. Experimental design and process flow to obtain sparkling cider.

For the secondary bottle fermentation, *Saccharomyces cerevisiae*-encapsulated yeasts (ProElif®, Proenol, Canelas, Portugal) were used. Encapsulated yeasts, base cider, and "*liqueur de tirage*" were added in 750 mL pressure-resistant glass bottles especially designed for sparkling beverages, which were then closed and stored horizontally at 15 °C for 60 days to carry out secondary fermentation. The "*liqueur de tirage*" was made from base cider and sugar (20 g/L) to ensure the conditions needed for secondary fermentation. The amount of encapsulated yeast added was 1.5 g/bottle, ensuring a concentration of 6×10^6 CFU/mL yeast. To enable the beads to gather at the bottle neck, the bottles were gently rotated during the first part of the secondary fermentation. Thus, from each base cider, the sparkling cider variants (SC1—1st fermentation *P. kluyveri* + *S. cerevisiae* and 2nd fermentation *S. cerevisiae*, SC2—*L. plantarum* + *S. cerevisiae* and 2nd fermentation *S. cerevisiae*, SC3—1st fermentation *O. oeni* + *S. cerevisiae* and 2nd fermentation *S. cerevisiae*, SC4—1st and 2nd fermentation *S. cerevisiae*) were obtained. Maturation took place at 10 °C for 180 days. At the end of the maturation stage, the sparkling cider was subjected to sensory analysis. Figure 1 shows the technological process used to obtain the sparkling cider. These experimental variants were carried out in triplicate.

All chemicals and reagents used were of analytical grade or high-performance liquid chromatography (HPLC) grade. Glucose, fructose, maltose, and sulfuric acid 0.5 M were purchased from Chempur, Piekary Śląskie, Poland; malic acid, citric acid and succinic acid, monosodium phosphate (NaH_2PO_4), and acetonitrile were purchased from Merck, Darmstadt, Germany; lactic, acetic, and pyruvic acids were obtained from Sigma-Aldrich, Taufkirchen, Germany; and chlorogenic acid, gallic acid, rutin, quercetin, and catechin were purchased from Sigma, St. Louis, MO, USA. For all solutions used, ultrapure water was generated with the Direct-Q UV (Millipore, Burlington, USA). The reagents and chemicals used for the amino acid analysis were sourced from the EZ: Faast™ kit (Phenomenex, Torrance, CA, USA).

2.2. Glucides and Organic Acids by HPLC

The separation and quantification of glucides and lactic and acetic acids followed the previously reported procedure [20]. Agilent ChemStation software version B.02.01.SR2 (Agilent Technologies, Santa Clara, CA, USA) was employed for data collection and result assessment. Compound identification in the samples was conducted by comparing their retention times with those of standard compounds [21,22]. The compounds isolated from the analyzed apple juice and apple cider samples were identified by comparing their retention times with those of the standards. All experiments were conducted in triplicate.

2.3. Volatile Compounds by GC/MS

The extraction procedure, liquid–liquid extraction, used by Coelho et al. [23] for the sample was adapted with small modifications. Ultrasonic extraction (made in triplicate) at 0 °C for 25 min was used instead of a magnetic stirrer. The separation and identification of volatile compounds were achieved by gas chromatographic analysis using a GC-MS Shimadzu QP 2010 PLUS Mass Spectrometer (Shimadzu, Kyoto, Japan) coupled with a gas chromatograph (Shimadzu equipped with an AOC-20 i+s injector, Kyoto, Japan) and a wax-type capillary column (ZB-Wax, Phenomenex, Torrance, CA, USA), as described in our previous research work [20]. The results are presented as a percentage of the total peak area (100%).

2.4. Amino Acids by Gas-Chromatography

Samples were subjected to gas chromatography analysis following a protocol established in one of our previous experiments, using a EZ: Faast™ kit (Phenomenex, Torrance, CA, USA) [20]. For the analytical investigation, we employed an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector (FID). Separation of the compounds was performed on a Zebron ZBAAA column. The FID detector temperature was held at 320 °C, and 2.5 µL of the sample was injected at an

injection temperature of 250 °C with a split ratio of 1:15. Helium served as the carrier gas. Each sample underwent duplicate analysis, and data manipulation and processing were carried out using Empower 2.0 software.

2.5. Analysis of Phenolic Compounds by HPLC-DAD ESI+

The analysis of phenolic compound profiles for apple juice and cider was performed following the method described by Coldea et al. [24]. The analysis was carried out using an Agilent 1200 HPLC system with a quaternary pump, solvent degasser, autosampler, UV-Vis detector with photodiode (DAD), and an Agilent model 6110 single-quadrupole mass detector (MS) (Agilent Technologies, Santa Clara, CA, USA). Compound separation was achieved using a Kinetex XB C18 column (Phenomenex, Torrance, CA, USA) [20]. Sample preparation, data acquisition, and data interpretation were performed following the procedure described in Section 2.2. After comparing the retention times, UV-Vis absorption spectra, and mass spectra with those of the standard compounds and the available literature data, the phenolic compounds were identified.

2.6. Sensory Analysis

The flavor profile of each sample was evaluated at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Laboratory for Sensory Analysis of Foods. The panelists were trained (n = 7, 4 men and 3 women aged between 26 and 47 years, including a sommelier and two people working in quality control of alcoholic beverages). The panelists recorded the intensity of perception on a 10 cm linear scale, anchored "Imperceptible" at the left end and "Very intense" at the right end, for 13 sensory attributes: visual appearance (clarity, color), smell (fruity, floral, yeasty), trigeminal sensations (astringency), taste (sour/acid, sweet, bitter), and flavor (fruity, floral, yeasty). Apple cider samples of approximately 50 mL in wine glasses were presented to the panelists at 8–10 °C [17]. Unsalted bread and plain, non-carbonated water were provided as neutralizers between samples to prevent sensory fatigue.

2.7. Statistical Analysis

Triplicate determinations were performed, and the data are presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used to examine the variation of the mean values using SPSS 19.0 software (IBM, New York, NY, USA) and Tukey's honestly significant difference (HSD) test with a confidence interval of 95% or 99%. A p-value below 0.05 was considered as statistically significant. Principal component analysis (PCA) for chemometric analysis was conducted via XLSTAT 2021 software (Addinsoft, New York, NY, USA) [25].

3. Results and Discussion

3.1. Sparkling Cider Analysis

Sparkling cider was obtained by applying the *Champenoise* method and using encapsulated yeasts. As such, Table 1 reports the measured physicochemical properties of the final products. As reported by previous studies [26], at the end of alcoholic fermentation, glucose was completely transformed into cellular energy, ethyl alcohol, carbon dioxide, and other compounds. The main sugars in apple juice, fructose (61.11 g/L) and glucose (42.54 g/L), were almost completely metabolized after the first fermentation. In the base cider, only fructose was still present in a quantity of less than 5.5 g/L. Since there was no residual glucose, and fructose was less than 0.10 g/L in all of the sparkling ciders, it was concluded that yeast fermented all of the available sugars to dryness. It is well known that commercial *Saccharomyces* strains have a high yield in industrial production, being widely used as starter [27], which demonstrates that the encapsulated *S. cerevisiae* yeast used in the secondary fermentation completely fermented the cider sugars. Therefore, considering that the base cider had an alcohol concentration of 6.1–6.2% v/v, in sparkling cider, the ethanol

increase was about 25%, similar to the increase rate reported in previous studies [3] (see Table 1).

Table 1. Physicochemical parameters evaluated in analyzed sparkling cider samples.

	SC1	SC2	SC3	SC4	Significance
Ethanol (% *v*/*v*)	7.80 ± 0.04 ^{a}	7.81 ± 0.03 ^{a}	7.70 ± 0.04 ^{a}	7.80 ± 0.06 ^{a}	ns
Total acidity (g/L malic acid)	2.54 ± 0.03 ^{c}	2.74 ± 0.03 ^{a}	2.41 ± 0.03 ^{d}	2.61 ± 0.03 ^{b}	***
pH	3.92 ± 0.04 ^{ab}	3.87 ± 0.03 ^{b}	3.98 ± 0.04 ^{a}	3.90 ± 0.02 ^{ab}	*
Sugars (g/L)					
Glucose	tr	tr	tr	tr	
Fructose	0.02 ± 0.00 ^{b}	0.03 ± 0.00 ^{b}	0.09 ± 0.01 ^{a}	0.02 ± 0.00 ^{b}	*
Sorbitol	1.58 ± 0.03 ^{a}	1.47 ± 0.02 ^{b}	1.63 ± 0.02 ^{a}	1.61 ± 0.02 ^{a}	*
Erythritol	0.87 ± 0.02 ^{c}	0.95 ± 0.03 ^{b}	0.93 ± 0.03 ^{bc}	1.08 ± 0.03 ^{a}	**
Organic acids (g/L)					
Malic	0.38 ± 0.02 ^{bc}	0.34 ± 0.02 ^{c}	0.42 ± 0.03 ^{ab}	0.46 ± 0.00 ^{a}	**
Lactic	4.51 ± 0.10 ^{a}	4.19 ± 0.07 ^{b}	4.66 ± 0.05 ^{a}	4.29 ± 0.06 ^{b}	**
Pyruvic	0.38 ± 0.02 ^{ab}	0.37 ± 0.02 ^{ab}	0.39 ± 0.02 ^{ab}	0.42 ± 0.02 ^{a}	*
Citric	0.09 ± 0.00 ^{a}	0.09 ± 0.02 ^{a}	0.09 ± 0.02 ^{a}	0.10 ± 0.01 ^{a}	ns
Succinic	0.68 ± 0.03 ^{c}	0.73 ± 0.04 ^{bc}	0.80 ± 0.03 ^{ab}	0.83 ± 0.03 ^{a}	**
Acetic	0.32 ± 0.01 ^{c}	0.54 ± 0.02 ^{c}	0.65 ± 0.03 ^{b}	0.73 ± 0.02 ^{a}	***

Values are expressed as means of three replicates. Values with different lowercase letters in the same row indicate statistically significant differences between samples (Tukey's test). * Significant at $p \leq 0.05$; ** very significant at $p \leq 0.01$; *** extremely significant at $p \leq 0.001$; ns = not significant; tr = traces; SC1 = 1st fermentation, *P. kluyveri* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC2 = 1st fermentation, *L. plantarum* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC3 = 1st fermentation, *O. oeni* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC4 = 1st and 2nd fermentation, *S. cerevisiae*.

Erythritol, recognized for its sweet taste, is a by-product of fermentation produced by *Saccharomyces* yeast strains during alcoholic fermentation [28] and by lactic acid bacteria (LAB) during malolactic fermentation [29]. It was found in similar amounts in all sparkling ciders. For instance, it was found in an amount of 0.87 g/L in sample SC1, while the concentration of erythritol in sample SC4 even reached 1.09 g/L. As a result of the secondary fermentation process, the erythritol content increased, with it being found at a proportion of 0.10–0.22 g/L in the base cider. Sorbitol, with its contribution to the sensory qualities of cider, impacts the sweetness, smoothness, and flavor complexity [30], is not commonly found in apple juice [31], but was present in the sparkling cider. The amount of sorbitol varied from 1.47 g/L in SC2 to 1.63 g/L in SC3, similar to that of the base cider, with the differences being statistically significant.

During fermentation, yeasts can metabolize and even produce malic acid, the primary organic acid in apple juice that gives the beverage its distinctively bitter flavor [22]. Even though the amount of malic acid dropped below 0.18 g/L, under the action of yeasts and LAB (*O. oeni* and *L. platarum*) during fermentation, when the base cider was obtained, secondary fermentation caused an increase in the concentration of malic acid. Therefore, all sparkling cider samples were characterized by an increase in malic acid concentration (0.34–0.46 g/L). This phenomenon, in which organic acids are consumed and subsequently produced in secondary fermentation, has also been seen in sparkling wines [32] as a result of yeast's behavior in response to various stress conditions [33].

Malolactic fermentation always leads to the formation of lactic acid [34], which contributes to a decrease in acidity and astringency [21]. Baiano et al. also noted an increase in lactic acid in sparkling cider compared to base cider [35]. In general, secondary fermentation is favorable for the production of lactic acid, which enhances the flavor complexity of wine and cider [36]. Another analysis revealed that, in the late stage of secondary fermentation, lactic acid increased significantly [37]. In the analyzed case, secondary fermentation

resulted in an increase in lactic acid as well (4.19–4.66 g/L), by 28–68% compared to the base cider (1.41–3.32 g/L) [1,35].

Pyruvic and succinic acids are by-products of fermentation and are excreted by yeasts [38]. Factors such as yeast strains and fermentation composition must influence the production of these acids [22]. Pyruvic acid is a necessary precursor for numerous metabolites, while succinic acid is significant because it reacts with other chemicals in order to form esters [38]. The differences in pyruvic acid between our samples were significant, but it was present in relatively low amounts (0.38–0.42 g/L) [3]; the increase was very slight compared to the base cider, where it was present in quantities of 0.24–0.33 g/L.

A minor variation in succinic acid levels between the base and sparkling ciders was previously stated [39]. This was connected to the addition of sugar for secondary fermentation, in which the presence of sugar led yeast to produce succinic acid.

Citric acid is naturally found in fruits, and its concentration varies during fermentation [40]. At the end of secondary fermentation, the citric acid present in the four cider samples decreased to less than 0.10 g/L. This trend is common for citric acid, as its decrease is influenced by the capacity of yeast strains [3] first to synthetize, then to reabsorb and catabolize it [35].

Acetic acid and diacetyl are produced during citric acid metabolism by a wide variety of yeast and LAB species, significantly influencing the fragrance profiles of fermented beverages [41]. One of the primary volatile acids in fermented beverages, acetic acid, plays a significant role in fermentation as the precursor of ethyl acetate and as the substrate for acetyl coenzyme production of fruity acetates [42]. Nevertheless, its concentration in fermented alcoholic beverages is essential, as a concentration above 200 mg/L gives the cider a pungent odor and a vinegar aroma [38]. Recent studies have shown that, during secondary fermentation, acetic acid can form; therefore, sparkling cider will have a higher concentration than base cider (0.07–0.15 g/L) [1,17]. As in previous studies using *S. cerevisiae* yeast for secondary fermentation, acetic acid was formed, but in moderate amounts. Therefore, the sample with the lowest concentration was SC1 (0.32 g/L), followed by SC2 and SC3, the values of which were close (0.54 g/L, and 0.65 g/L, respectively). The highest amount was recorded for the variant in which *S. cerevisiae* (0.73 g/L) was used in both fermentation stages. These values are lower than those reported in other studies [1,43].

3.2. Volatile Compounds in Sparkling Cider

In addition to being present in raw materials, volatile substances can also be produced during the fermentation and maturation of sparkling cider [1]. The apple variety, ripening stage, processing, yeast strain, fermentation conditions, environment, and aging may all have an impact on the synthesis of aroma compounds in cider [44]. Esters, higher alcohols, fatty acids, aldehydes, ketones, terpenes, and lactones are the main volatile elements that contribute to the complex aroma of cider [45]. Table 2 shows the volatile compound concentrations (% of the total peak area) in the sparkling ciders. According to these findings, for sixteen out of the twenty-three volatile compounds identified per sample, very significant and extremely significant statistical differences were recorded between the other compounds, indicating the influence of the microbial strain used in the fermentation process.

Esters are one of the most prevalent volatile by-products of alcoholic fermentation in cider, second only to ethanol [46]. The corresponding higher alcohols are transformed into esters by their reaction with an acid [47]. Most of them are responsible for fruity, floral, and sweet notes and contribute to cider aroma, even in small amounts, by giving the final product typicity [48]. For starters, apple juice contains moderate esters, but most of them are produced by yeast and lactic bacteria in the fermentation process, or by chemical esterification during aging or less contact; therefore, the ester content of cider is constantly changing [49]. Some of the esters in the base cider formed during fermentation were broken down during the secondary fermentation stage.

Table 2. Volatile compounds in sparkling cider (expressed as a percentage of the total peak area).

Volatile Compounds	SC1	SC2	SC3	SC4	Significance
Alcohols					
i-BuOH	tr	1.66 ± 0.07 ^{b}	1.74 ± 0.05 ^{b}	1.90 ± 0.06 ^{a}	**
1-BuOH	tr	1.60 ± 0.07 ^{a}	1.33 ± 0.06 ^{b}	1.43 ± 0.04 ^{b}	**
3-Met-BuOH	25.85 ± 0.97 ^{d}	49.08 ± 0.81 ^{a}	39.52 ± 0.74 ^{c}	46.24 ± 0.84 ^{b}	***
1-HexOH	tr	0.94 ± 0.07 ^{b}	0.98 ± 0.04 ^{b}	1.55 ± 0.09 ^{a}	**
R,R)-2,3-ButDiol	tr	2.93 ± 0.16 ^{b}	7.52 ± 0.17 ^{a}	2.73 ± 0.17 ^{b}	**
2,3-ButDiol	2.68 ± 0.10 ^{b}	tr	3.09 ± 0.18 ^{a}	1.77 ± 0.09 ^{c}	***
BnOH	2.23 ± 0.10	tr	tr	tr	
2-PE	25.50 ± 0.94 ^{a}	14.04 ± 0.59 ^{c}	12.20 ± 0.43 ^{d}	17.12 ± 0.59 ^{b}	***
1-Is-2-POH	2.15 ± 0.10	tr	tr	tr	
3-MetTh-1-Prop	1.07 ± 0.06 ^{a}	0.91 ± 0.07 ^{b}	0.66 ± 0.04 ^{c}	0.89 ± 0.05 ^{b}	***
Esters					
1-3-DP	tr	tr	0.43 ± 0.03	tr	
Et-3-MetBut	tr	tr	0.63 ± 0.06	tr	
4-Et-4-OBAc	tr	tr	1.99 ± 0.11	tr	
EtLac	11.61 ± 0.63 ^{c}	16.96 ± 0.89 ^{a}	14.86 ± 0.81 ^{b}	9.93 ± 0.36 ^{c}	***
PheAct	6.73 ± 0.10 ^{b}	1.24 ± 0.05 ^{a}	tr	tr	***
1-MetEt-For	tr	0.73 ± 0.06	tr	tr	
Fatty Acids					
HexAc	3.22 ± 0.09 ^{a}	1.58 ± 0.05 ^{c}	1.63 ± 0.10 ^{c}	2.34 ± 0.16 ^{b}	***
ButAc	1.17 ± 0.04 ^{a}	tr	0.72 ± 0.09 ^{b}	0.66 ± 0.44 ^{b}	**
2-Met-But-Ac	1.93 ± 0.07 ^{a}	tr	0.77 ± 0.05 ^{b}	0.74 ± 0.05 ^{b}	**
OctAc	4.69 ± 0.19 ^{a}	1.67 ± 0.08 ^{c}	1.45 ± 0.07 ^{c}	2.91 ± 0.07 ^{b}	***
4-Met-PenAc	tr	0.61 ± 0.04	tr	tr	
Others					
Met 4-O-metAr	1.49 ± 0.08 ^{a}	0.67 ± 0.08 ^{b}	tr	1.42 ± 0.06 ^{a}	**
GBL	1.77 ± 0.08 ^{a}	tr	0.87 ± 0.07 ^{c}	1.14 ± 0.09 ^{b}	***

Values are expressed as means of three replicates. Values with different lowercase letters in the same row indicate statistically significant differences between samples (Tukey's test). ** very significant at $p \leq 0.01$; *** extremely significant at $p \leq 0.001$; ns = not significant; tr = traces; i-BuOH = Isobutanol; 1-BuOH = 1-butanol; 3-Met-BuOH = 3-Methyl-1-butanol; 1-HexOH = 1-Hexanol; R,R)-2,3-ButDiol = 2,3-Butanediol, [R-(R@,R@)]-; 2,3-ButDiol = 2,3-Butanediol; BnOH = Benzyl Alcohol; 2-PE = 2-phenylethanol; 1-Is-2-POH = 1-Isopropoxy-2-Propanol; 3-MetTh-1-Prop = 3-(Methylthio)-1-Propanol; 1-3-DP = 1,3-Diacetoxypropane; Et-3-MetBut = Ethyl 3-methylbutanoate; 4-Et-4-OBAc = 4-Ethoxy-4-Oxobutanoic Acid; EtLac = Ethyl Lactate; **PheAct** = 2-Phenethyl Acetate; 1-MetEt-For = Formic acid, 1-methylethyl ester; HexAc = Hexanoic acid; ButAc = Butanoic acid; 2-Met-But-Ac = 2-Methylbutanoic acid; OctAc = Octanoic acid; Met-PenAc = 4-methylpentanoic acid; Met 4-O-metAr = Methyl 4-O-methyl-d-arabinopyranoside; GBL = Gamma-Butyrolactone; SC1 = 1st fermentation, *P. kluyveri* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC2 = 1st fermentation, *L. plantarum* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC3 = 1st fermentation, *O. oeni* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC4 = 1st and 2nd fermentation, *S. cerevisiae*.

Recent investigations have shown that the most common volatile compounds in ciders and sparkling ciders are higher alcohols [50,51]. The amount of higher alcohols produced during fermentation through the Ehrlich pathway is significantly influenced by amino acids, which are a source of nitrogen for yeasts [52]. Alcohols may also be produced directly by yeast through the fermentation of sugars [53]. Most of the alcohols were present in both sparkling cider and base cider, but their quantity was variable. Of these, 3-methyl-1-butanol was present in significant quantities in sparkling cider. Isobutanol, 1-butanol, and 1-hexanol were identified in samples SC2–SC4 (0.98–1.90), but missing from SC1. Benzyl alcohol and 1-Isopropoxy-2-Propanol were the alcohols that stood out in sample SC1, although they were lacking in the other variants, and their presence can be attributed to *P. kluyveri*, which is known to have previously been reported for its advantage of increasing the variety of volatile compounds [54].

Alcoholic fermentation can result in the formation of volatile acids. Additionally, fatty acids are present, particularly butanoic and octanoic acids [55]. Fatty acids also contribute

to the fruity, cheesy, fatty, and rancid aromas, and they are produced enzymatically during fermentation [56]. Octanoic and hexanoic acids were found in all samples, with higher values in SC1 (4.69 and 3.22 respectively). Butanoic and 2-methylbutanoic acids were absent from sample SC2, being present in approximately equal amounts in the other samples. Also, 4-methylpentanoic acid was found in only one version of sparkling cider in an insignificant amount (SC2, 0.61).

3.3. Amino Acid Profile of Sparkling Cider

Amino acids (AA) are crucial components for yeasts, and their amount in ciders is closely related to the variety of cider apples used. They are also important during every step of the production process [43]. In apple juice, the primary source of naturally assimilable nitrogen for yeast consists mainly of amino acids. These amino acids serve as the essential nutrient supply necessary for yeast growth and development during fermentation [57]. Numerous studies have examined the free amino acid content of sparkling wines and the changes that take place during aging [43,58]. As mentioned previously, amino acids can be precursors of volatile compounds [55], but as they are also a source of nutrients for yeast, they are consumed during fermentation. In order for the yeast to carry out secondary fermentation, in addition to sugars, they also need nutrients. Therefore, the amino acids found in the base cider have been almost completely consumed by the yeast. Similar research has shown that, at the end of secondary fermentation (*Champenoise* method), amino acids are found in the lowest amounts [43]. Table 3 summarizes the concentration of amino acids in sparkling cider, which are found in very low quantities. Alanine, sarcosine, and isoleucine were identified only in trace amounts. In sample SC1, the amino acid concentration was 3.85 mg/L, followed by SC2 and SC3, with 5.4 mg/L and 8.78 mg/L, respectively. In sample SC4, no amino acids were identified.

Table 3. Amino acids (mg/L) in sparkling cider samples.

Amino Acid Compounds	SC1	SC2	SC3	SC4	Significance
ALA	tr	tr	tr	tr	
SAR	tr	tr	tr	tr	
βAIB	tr	0.23 ± 0.03 [a]	0.25 ± 0.04 [a]	tr	ns
ILE	tr	tr	tr	tr	
ASN	tr	tr	1.23 ± 0.03	tr	
GLU	1.04 ± 0.04	tr	tr	tr	
GLN	tr	0.83 ± 0.03 [a]	0.82 ± 0.00 [a]	tr	ns
ORN	tr	tr	2.14 ± 0.04	tr	
LYS	tr	1.44 ± 0.03 [a]	1.44 ± 0.03 [a]	tr	ns
HIS	0.80 ± 0.03 [b]	2.90 ± 0.05 [a]	2.90 ± 0.02 [a]	tr	*
TRP	2.01 ± 0.06	tr	tr	tr	
Total	3.85	5.40	8.78	-	

Values are expressed as means of three replicates. Values with different lowercase letters in the same row indicate statistically significant differences between samples (Tukey's test). * significant at $p \leq 0.05$; ns = not significant; tr = traces; ALA = alanine; βAIB = β-aminoisobutyric acid; GLN = glutamic acid; HIS = histidine; ILE = isoleucine; LYS = lysine; ORN = ornithine; SAR = sarcosine; ASN = asparagine; GLN = glutamine; TRP = tryptophan; SC1 = 1st fermentation, *P. kluyveri* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC2 = 1st fermentation, *L. plantarum* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC3 = 1st fermentation, *O. oeni* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC4 = 1st and 2nd fermentation, *S. cerevisiae*.

3.4. Phenolic Profile of Sparkling Cider

The composition of apple cider determines its qualities, while polyphenols play a significant role in the production process. Polyphenols represent a quality parameter primarily because they significantly influence organoleptic quality, color, bitterness, taste, and astringency [59]. Also, polyphenols facilitate the pressing process and higher juice

production by inhibiting the pectolytic disintegration of apple tissue [60]. They can also influence the fermentation process by preventing microbial spoilage and some cider defects [27]. Additionally, proteins and phenolic compounds work synergistically to maintain the colloidal stability of cider [59]. Moreover, they help with the natural clarifying of the must throughout the fermentative stage and play a beneficial role in the appearance of any defects that can appear during cider storage [60]. The phenolic content of cider is known to be significantly influenced by the apple variety and climatic conditions [61]. The concentration of polyphenolic compounds is reported in Table 4.

Table 4. Phenolic compounds (mg/L) in sparkling apple ciders.

Phenolic Compounds	SC1	SC2	SC3	SC4	Significance
ASA	1.91 ± 0.03 ^{c}	1.82 ± 0.04 ^{c}	2.06 ± 0.03 ^{b}	5.42 ± 0.08 ^{a}	***
GAG	7.71 ± 0.24 ^{d}	9.97 ± 0.34 ^{c}	13.54 ± 0.27 ^{a}	12.17 ± 0.14 ^{b}	***
DBA	2.65 ± 0.12 ^{b}	3.37 ± 0.09 ^{a}	3.67 ± 0.07 ^{a}	3.11 ± 0.06 ^{ab}	**
GAL	0.46 ± 0.04 ^{c}	0.87 ± 0.07 ^{b}	0.72 ± 0.04 ^{b}	1.59 ± 0.08 ^{a}	**
GEN	4.04 ± 0.15 ^{b}	3.23 ± 0.09 ^{c}	1.87 ± 0.11 ^{d}	8.14 ± 0.18 ^{a}	***
PRT	0.90 ± 0.08 ^{c}	1.35 ± 0.06 ^{b}	1.65 ± 0.07 ^{a}	1.17 ± 0.08 ^{b}	**
NCL	12.58 ± 0.32 ^{b}	14.12 ± 0.20 ^{a}	11.95 ± 0.13 ^{c}	12.31 ± 0.14 ^{bc}	**
PD B1	35.29 ± 1.21 ^{a}	34.44 ± 0.83 ^{a}	34.51 ± 0.63 ^{a}	35.42 ± 0.96 ^{a}	ns
CAT	16.90 ± 0.64 ^{a}	17.50 ± 0.58 ^{a}	15.25 ± 0.42 ^{b}	17.16 ± 0.29 ^{a}	*
CHL	316.38 ± 11.29 ^{a}	246.26 ± 5.91 ^{b}	224.85 ± 4.47 ^{c}	254.47 ± 4.49 ^{b}	**
PD B2	137.96 ± 3.35 ^{a}	121.86 ± 3.86 ^{b}	99.88 ± 2.33 ^{c}	119.21 ± 1.84 ^{b}	***
EPC	50.21 ± 1.13 ^{ab}	51.59 ± 1.26 ^{a}	47.77 ± 1.22 ^{b}	52.37 ± 1.22 ^{a}	*
CQA	11.94 ± 0.66 ^{c}	21.11 ± 0.97 ^{a}	9.96 ± 0.27 ^{c}	17.48 ± 0.94 ^{b}	**
QRS	6.61 ± 0.34 ^{c}	16.52 ± 0.81 ^{a}	10.26 ± 1.05 ^{b}	15.32 ± 0.85 ^{a}	**
QGS	7.61 ± 0.38 ^{ab}	8.26 ± 0.40 ^{a}	6.77 ± 0.55 ^{b}	8.86 ± 0.81 ^{a}	*
QAS	8.27 ± 0.37 ^{c}	11.68 ± 0.88 ^{b}	12.38 ± 0.54 ^{b}	15.09 ± 0.76 ^{a}	**
PXG	12.11 ± 0.49 ^{a}	8.55 ± 0.48 ^{c}	6.41 ± 0.43 ^{d}	10.32 ± 0.34 ^{b}	***
QMG	13.76 ± 0.91 ^{a}	10.76 ± 1.03 ^{b}	11.32 ± 1.01 ^{ab}	13.77 ± 0.82 ^{a}	*
PHZ	7.54 ± 0.54 ^{a}	8.51 ± 0.26 ^{a}	2.24 ± 0.35 ^{c}	4.04 ± 0.27 ^{b}	***
PT C1	19.97 ± 0.91 ^{a}	19.09 ± 0.57 ^{a}	19.13 ± 0.51 ^{a}	19.52 ± 1.29 ^{a}	ns

Values are expressed as mean of three replicates. Values with different lowercase letters in the same row indicate statistically significant differences between samples (Tukey's test). * significant at $p \leq 0.05$; ** very significant at $p \leq 0.01$; *** extremely significant at $p \leq 0.001$; ns = not significant; ASA = p-Anisaldehida; GAG = Gallic acid-glucoside; DBA = 2,3-Dihydroxybenzoic acid; GAL = Gallic acid; GEN = Gentisic acid; PRT = Protocatechuic acid; NCL = Neochlorogenic acid; PD B1 = Procyanidin dimer B1; CAT = Catechin; CHL = Chlorogenic acid; PD B2 = Procyanidin dimer B2; EPC = Epicatechin; CQA = p-Coumaroylquinic acid; QRS = Quercetin-rutinoside; QGS = Quercetin-glucoside; QAS = Quercetin-arabinoside; PXG = Phloretin-xylosyl-glucoside; QMG = Quercetin-(malonyl-glucoside); PHZ = Phloridzin; PT C1 = Procyanidin trimer C1; SC1 = 1st fermentation, *P. kluyveri* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC2 = 1st fermentation, *L. plantarum* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC3 = 1st fermentation, *O. oeni* + *S. cerevisiae*, and 2nd fermentation, *S. cerevisiae*; SC4 = 1st and 2nd fermentation, *S. cerevisiae*.

As shown in other reported studies, the concentration of phenolic compounds increased during secondary fermentation [60,62]. At the end of the fermentation, the amount of polyphenols was higher in all samples (536.09–674.51 mg/L) than in the base cider (310.36–522.63 mg/L), and moreover, in one sample, it doubled. Of the 20 phenolic compounds analyzed, four of them showed small variations compared to the base cider (p-anisaldehide, protocatechuic acid, p-coumaroylquinic acid, phloridzin). Following secondary fermentation, five of them slightly decreased (2,3-Dihydroxybenzoic acid, gentisic acid, catechin, phloretin-xylosyl-glucoside and procyanidin trimer C1), while the concentration of the remaining ones increased. In terms of the total polyphenol content in sparkling cider samples, compared to base cider, it increased by 19.86% in sample SC4 and by more than 217% in sample SC1, the latter being also the highest (from 310.36 mg/L to 674.51 mg/L). In contrast, other studies have shown that secondary fermentation can lead to a decrease in polyphenol content. This result can be caused by a variety of mech-

anisms, such as spontaneous clarity due to interaction and subsequent precipitation of polyphenol–protein complexes and polyphenol polysaccharide aggregation, enzymatic browning, polymerization of procyanidins, and yeast cell absorption [35].

Polyphenols (flavonols, flavan 3-ols, dihydrochalcones, procyanidins, and hydroxycinnamic acids and derivatives) are one of the important quality indicators of cider [59] because they significantly contribute to organoleptic quality, particularly color, bitterness, flavor, and astringency. Chlorogenic acid, ranging between 224.83 and 316.13 mg/L, predominated among the polyphenols in the analyzed sparkling cider samples, followed by procyanidin dimer B2, epicatechin, and procyanidin dimer B1. Phenolic acids play a minor role in the fruity aroma. However, during maturation or processing, numerous simple aromatic phenols may be released from glycosylated precursors via enzymatic or chemical processes [63]. Usually, in terms of polyphenols' influence on sensory attributes, they are associated with bitterness and astringency [64].

3.5. Sensory Analysis

Apple cider is a fermented product; therefore, the sour/acidic perception is very intense. The panelists evaluated a total of 13 sensory attributes in the cider samples on a 10 cm non-structured intensity scale. PCA (Figure 2) revealed the first component (F1, 46.23%) to be highly correlated with SC3, while the second component (F2, 32.72%) was highly correlated with SC1 and SC2. Therefore, samples SC3 and SC4 were perceived with higher sourness and an intense yeast smell, attributes specific to fermented products, while samples SC1 and SC2 had completely different profiles, with a floral and fruity smell and flavor. These results are probably due to co-fermentations between *Pichia* and *Lactobacillus* with *Saccharomyces*, for which other studies have also shown the production of numerous aroma compounds [17,65]. Sample SC4, obtained through fermentation with *Saccharomyces* yeast, was noted for its pleasant and clear visual aspect upon sensory analysis, but exhibited a less diverse range of aromatic characteristics.

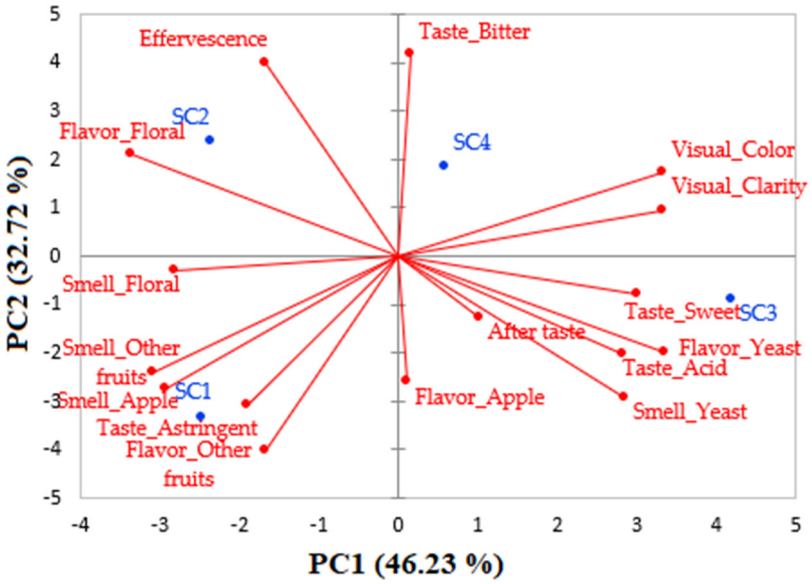

Figure 2. Principal component analysis of the sensory profile of the analyzed sparkling cider.

3.6. Multivariate Statistical Analysis

Unsupervised multivariate methods, such as PCA and heat map analysis (HMA), were employed to distinguish between various types of cider samples based on their compositional profiles (including sugars, organic acids, amino acids, phenolic compounds, volatile compounds) and sensory attributes. Initially, PCA was conducted to explore the quantitative data related to these components, aiming to identify distinctive biomarkers for each cider sample type.

Figure 3a–e present the distribution of cider samples in the PC1–PC2 scores plot. The first two components of the PCA model explained 85.48% of the variance, primarily based on sugar composition (Figure 3a). Notably, a distinct separation between SC3 and SC4 samples was observed, particularly regarding organic acid composition (Figure 3b), with all organic acids except lactic acid corresponding to these samples.

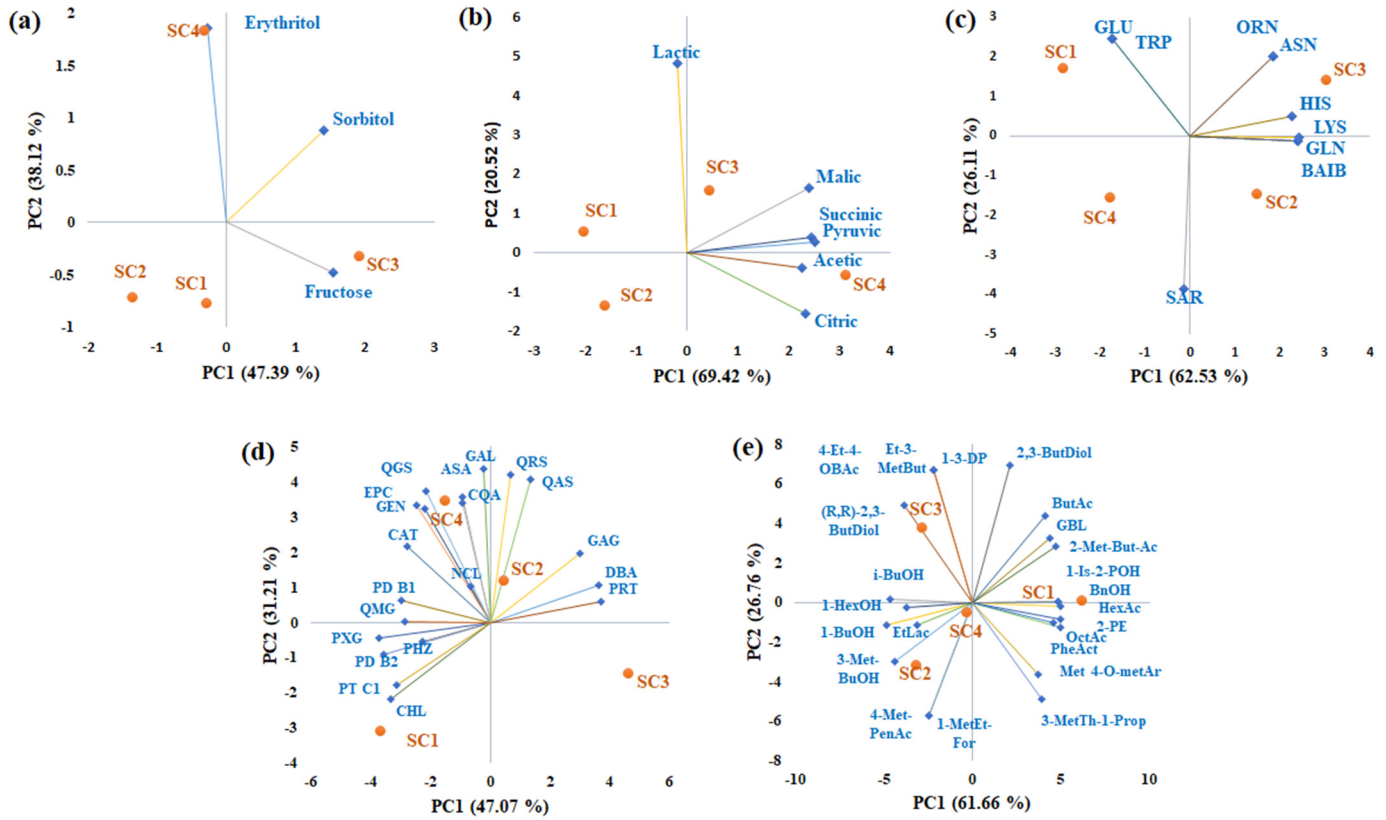

Figure 3. PCA results (scores and loading biplots) of different cider samples based on: (**a**) sugars; (**b**) organic acids; (**c**) amino acids; (**d**) volatile compounds; and (**e**) phenolic compounds; Abbreviations are as described in Table 2 for volatile compounds, Table 3 for amino acids, and Table 4 for phenolic compounds.

According to the PCA results, which were based on amino acid composition (explaining 88.64% of the variability, with PC1 accounting for 62.53% and PC2 for 26.11%, respectively) (Figure 3c), the amino acids effectively differentiated the samples from each other. For instance, it was the amino acids glutamic acid and tryptophan which differentiated sample SC1, while ornithine and asparagine distinguished sample SC3. Moreover, the amino acids glutamine, lysine, and β-aminoisobutyric acid corresponded to samples SC2 and SC3.

PCA based on phenolic compounds (Figure 3d) explained 78.28% of the variance (with PC1 accounting for 47.07% and PC2 for 31.21%, respectively). The majority of quantified phenolic compounds were distributed across quadrants I, II, and III, while sample SC3, positioned in quadrant IV, stood out from the others due to it exhibiting the lowest composition of total phenolic compounds. PCA based on volatile composition (Figure 3e) explained 88.42% of the variance (with PC1 accounting for 61.66% and PC2 for 26.76%, respectively) and revealed that the samples SC1, SC2, and SC3, obtained from co-fermentations, were differentiated from each other, being positioned in different quadrants. Each of them corresponded to certain volatile compounds, which differentiated them from each other.

4. Conclusions

Four types of sparkling cider, obtained via fermentation of base cider with encapsulated yeasts using the *Champenoise* method, were analyzed in this study. In terms of phenol content, secondary fermentation resulted in a significant increase in their content. As for aromatic compounds, the samples obtained by co-fermentation were noted for their higher content of compounds influencing the sensory characteristics of the finished product. Following sensory analysis, co-inoculation of *S. cerevisiae*, *P. kluyveri*, and *L. plantarum* followed by secondary fermentation resulted in samples with organoleptic characteristics different from the other samples. These findings hold practical significance for advancing our understanding of the extensive compositional and aromatic diversity present in traditional apple varieties. This is particularly crucial given that cider production typically involves blending varieties to achieve a desirable balance of aroma and taste. This, coupled with the optimization of applied technology, is essential for producing high-quality natural beverages.

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