Characterization of antioxidant activities and volatile profiles of pineapple beer during the brewing process

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Summary
Pineapple beer brewed with pineapple juice concentrate has a unique taste and is becoming popular. However, little is known about its physico-chemical and aroma characteristics. This study aimed to determine whether pineapple juice concentrate can be used as an adjunct for fruit beer production and elucidate its chemical characteristics. Compared with the control beer without it, addition of pineapple juice concentrate increased total polyphenols concentration (to 506.40 ± 2.50 mg·l^{-1}, expressed as gallic acid equivalents) and flavonoids concentration (to 49.60 ± 0.69 mg·l^{-1}, expressed as catechin equivalents) in final products and markedly enhanced the antioxidant activities. Flavour profiles analysed by using headspace-gas chromatography-ion mobility spectroscopy contained 42 representative compounds (12 esters, 9 alcohols, 11 aldehydes, 2 organic acids, 4 ketones, 2 furans, 1 sulphur compound and 1 olefin). Principal component analysis and orthogonal partial least squares discriminant analysis were used to build the flavour fingerprints and the characteristic flavour compounds were distinguished. Acetic acid, 3-methyl-1-butanol, ethanol, acetone, α-pinene, 3-methylbutanal, 2-propanol, ethyl formate, ethyl 3-methylbutanoate, 2-methylpropanal and dimethyl disulphide could be used to differentiate pineapple beer from control beer. The results are expected to provide a basis for industrial production of pineapple beer.

Keywords
pineapple beer; brewing process; antioxidant activity; volatile organic compound

Fruit beer, a specific beer imparted with fruit flavours, features a vast range of tastes, appearances and other sensory characteristics. As a niche product, it is becoming increasingly popular worldwide. Various types, such as calabura, cornelian cherry, goji, sea buckthorn, banana, raspberry, peach, apricot, grape, plum, orange, and apple beer, are produced [1–6]. Relative to conventional and non-fruit beers, fruit beers are characterized by higher levels of polyphenols and flavonoids from fruits, and these compounds impart antioxidant, antihypotensive, antibiotic, antihaemolytic, anti-inflammatory, and several other bioactive effects to the product. NARDINI and GARAGUSO [5] reported that addition of fruits during the fermentation process significantly improved the phenolics profile of beer and considerably increased the antioxidant activity. Because these effects generally depend on the fruit cultivar and the production process, the appropriate selection of a cultivar and the use of optimal technological processes are essential for preserving high levels of active compounds in the final product.

Pineapple (*Ananas comosus* (L.) Merr.), as an important fruit in the Lingnan area in South China, features high concentrations of minerals and vitamins, polyphenols and flavonoids [7]. Considering the improvement of flavour and the

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preservation of biological activities and aroma provided by alcoholic fermentation, this type of fermentation potentially represents a novel method of pineapple processing. Although aroma profiles of pineapple fruit and products, such as pulp, juice, vinegar or fruit wine, were studied [8], there are no relevant reports on beer brewing with pineapple juice. Thus, it is important to elucidate changes in the volatile compounds during the entire brewing process [9].

Gas chromatography-ion mobility spectroscopy (GC-IMS) is widely used for detecting dangerous chemicals, disease surveillance and in food flavour analysis, owing to the advantages offered by the high separation capacity of GC and the fast response of IMS [10, 11]. Moreover, GC-IMS represents an emerging technique to analyse volatile organic compounds (VOC) in alcoholic products, including Chinese yellow wine, white wine, fruit wine and brandy [12–15]. However, its application in the aroma analysis of beer is very limited [16].

In this study, we aimed to determine whether pineapple juice concentrate can be used as an adjunct for fruit beer and to provide a chemical definition of the unique characteristics of pineapple beer. The bioactive compounds and the antioxidant activities at distinct stages of production were determined, and the flavour characteristics were investigated using HS-GC-IMS coupled to principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The results are expected to provide a scientific basis for enhancing the quality of pineapple beer in industrial production.

MATERIALS AND METHODS

Pineapple juice concentrate with an initial sugar concentration of approximately 60 °Bx was supplied by Guangdong Harvest Canned Foods (Zhanjiang, China). Pilsner malt and rice were from Shun Taimai bud Group (Guangzhou, China) and Sweet Fa Yan Industry (Guangzhou, China), respectively. Cascade and Tsingdao flower hops were obtained from Yakima Chief Hops (Yakima, Washington, USA) and Gansu Tianma Hops (Jiuquan, China), respectively. *Saccharomyces pastorianus* W 34/70 was provided by the Technical University of Munich (Munich, Germany). (+)-Catechin and gallic acid were obtained from Shanghai Yuanye Bio-Technology (Shanghai, China). Folin–Ciocalteu reagent, ketones C4–C9 (98.0 %) and 2,2'-azino-*bis*-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS, 98.0 %) were from Sigma-Aldrich (St. Louis, Missouri, USA). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 98.0 %) and 2,4,6-*tris*-(2-pyridyl)-*s*-triazine (TPTZ, 99.0 %) were obtained from Shanghai Aladdin Biochemical Technology (Shanghai, China).

Brewing process

The wort with an original gravity of 8 °Plato was boiled for 60 min. Tsingdao flower hop (0.14 $g \cdot l^{-1}$) and Cascade hop (0.18 $g \cdot l^{-1}$) were added at 46 min and 59 min, respectively. After boiling, the wort was clarified and cooled to 10–15 °C, and then it was transferred into 200 l of wort in 300 l stainless steel tanks (F&D Process Technology, Guangzhou, China). After that, the propagated *S. pastorianus* W 34/70 (1.0×10^7 $CFU \cdot ml^{-1}$) was inoculated. Fermentation was conducted at 9 °C and the supernatant was transferred to another tank when the apparent extract decreased to 6.0–6.5 %. When the apparent extract decreased to 3.0–4.0 %, 100 $g \cdot l^{-1}$ of pineapple juice concentrate was added for further fermentation. When the real degree of fermentation reached 60.0 % and the total diacetyl concentration did not exceed 0.15 $mg \cdot l^{-1}$, the temperature was decreased to 0 °C and the product was maintained for 3 days for maturation. Samples (described in Tab. 1) were collected and frozen at –20 °C before analysis for no more than one week. The control without pineapple juice concentrate was brewed using identical process.

Total polyphenols and total flavonoids

The total polyphenols concentration was determined according to the method described by

Tab. 1. Characterization of sampling points.

	Wort	Fermentation		Maturation
	Stage 1	Stage 2	Stage 3*	Stage 4
Temperature [°C]	10–15	9	12	0
Pressure [MPa]	0–0.02	0.06–0.08	0.10–0.12	0.10–0.12
Duration [d]	0	1–3	4–9	10–15
Sampling points [d]	0	3	12	15

* – with or without pineapple juice concentrate.

Zhao et al. [17]. Gallic acid served as the reference compound and the values were expressed as milligrams of gallic acid equivalents (GAE) per litre of beer. The total flavonoids concentration was determined by the colorimetric method described by Nardini and Garaguso [5], (+)-catechin served as the reference compound and the results were expressed as milligrams of catechin equivalents (CE) per litre of beer.

ABTS assay

The ABTS antioxidant activity (AA_{ABTS}) was determined according to the method of Kawa-Rygielska et al. [4] with some modifications. Briefly, 7 mmol·l^{-1} ABTS solution and 2.45 mmol·l^{-1} potassium persulphate were mixed at a 1 : 1 ratio and allowed to stand in the dark for 16 h to produce ABTS stock solution. This solution was further diluted with distilled water to attain an absorbance of 0.700 ± 0.020 at 734 nm. Next, 60 μl of standard or sample and 3 ml of the ABTS working solution were mixed and the absorbance was measured at 734 nm after 6 min using Varioskan LUX Multimode microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). AA_{ABTS} of samples were determined with reference to a Trolox calibration curve and expressed as millimoles of Trolox equivalent (TE) per litre of beer.

FRAP assay

The ferric reducing antioxidant power (FRAP) assay was conducted according to a previous study [5]. The FRAP reagent was freshly prepared and mixed with 10 mmol·l^{-1} TPTZ solution prepared in 20 mmol·l^{-1} $FeCl_3 \cdot 6H_2O$ solution, 40 mmol·l^{-1} HCl, and 300 mmol·l^{-1} acetate buffer (pH 3.6, in a ratio 1 : 1 : 10), and then 1.0 ml of the diluted sample was mixed with 180 μl of FRAP reagent and incubated at 37 °C for 30 min. The absorbance at 593 nm was determined using Varioskan LUX Multimode microplate reader and the antioxidant activity (AA_{FRAP}) of sample was determined using a calibration curve of ferrous sulphate. The results were reported as millimoles of Fe^{2+} per litre of beer.

Analysis of volatiles

A FlavourSpec instrument (G.A.S., Dortmund, Germany) equipped with an Mxt-WAX capillary column (30 m × 0.53 mm, 1.0 μm) from Restek (Bellefonte, Pennsylvania, USA) was used for analysis of volatiles. An amount of 1 g of the sample was transferred into 20 ml headspace vials (CNW Technologies, Düsseldorf, Germany), and incubated at 60 °C with continuous shaking at 500 Hz for 10 min. A volume of 100 μl of the headspace was automatically injected in splitless mode through a syringe heated to 65 °C and the analytes were separated at a constant temperature of 60 °C. High-purity N2 (99.9 %) was used as the carrier and drift gas, the carrier gas flow rate being programmed as follows: initial flow rate of 2.0 ml·min^{-1} maintained for 2 min, followed by a linear increase to 10.0 ml·min^{-1} within 8 min, and then to 100.0 ml·min^{-1} within 20 min. Analytes were driven to the ionization chamber by a β-ray (3H, 300 MBq) in positive ion mode. The 98 mm drift tube was operated at 45 °C with linear voltage of 500 V·cm^{-1} and the drift gas flow rate of 150 ml·min^{-1}. A series of *n*-ketones (Sinopharm, Beijing, China) were used to calculate the retention index (*RI*). Qualitative analysis was performed by comparing *RI* and the drift time of the standard in the Laboratory Analytical Viewer 2.2.1 (G.A.S.).

Statistical analysis

Sampling was performed by double sampling method and all analyses were performed at least in duplicate. The statistical significance ($p < 0.05$) was analysed using Duncan's test with SPSS 18.0 (SPSS, Chicago, Illinois, USA). PCA and OPLS-DA was performed using SIMCA-P 14.1 (UMetrics, Umea, Sweden).

RESULTS AND DISCUSSION

Antioxidant analysis

The antioxidant level in beer is closely associated with the quality of raw materials and is also affected by the brewing process [18]. Polyphenols and flavonoids are the primary antioxidants in beer and were previously shown to be readily absorbed as well as extensively metabolized in humans [19]. At fermentation stage 2, there was a significant decrease in the total polyphenols concentration, which is mainly attributed to the pH decrease during fermentation, as the precipitation of some colloidal polyphenols occurs when pH is in their isoelectric point range [20]. When pineapple juice concentrate was added, the total polyphenols concentration in the final pineapple beer was significantly higher ($p < 0.05$) than that in the wort and the control sample without pineapple juice concentrate (Tab. 2). A similar trend was noticed for flavonoids, i. e. the flavonoid concentration increased from 39.60 ± 0.70 mg·l^{-1} (expressed as CE) in the wort to 49.60 ± 0.69 mg·ml^{-1} (expressed as CE) in the final pineapple beer, which was also significantly higher than that (46.84 ± 1.08 mg·l^{-1}) in the control sample ($p < 0.05$).

Tab. 2. Total polyphenols and flavonoids concentration and antioxidant activity.

Sample	Total polyphenol [mg·l^{-1}]	Total flavonoid [mg·l^{-1}]	AA_{ABTS} [mmol·l^{-1}]	AA_{FRAP} [mmol·l^{-1}]
Stage 1	300.45 ± 4.06^{c}	39.60 ± 0.70^{d}	1.25 ± 0.10^{b}	6.13 ± 0.11^{b}
Stage 2	278.29 ± 4.72^{e}	44.16 ± 0.50^{c}	1.11 ± 0.05^{c}	6.69 ± 0.28^{b}
Stage 3	498.65 ± 3.91^{b}	49.96 ± 1.24^{a}	2.39 ± 0.05^{a}	12.63 ± 0.65^{a}
Stage 4	506.40 ± 2.50^{ab}	49.60 ± 0.69^{a}	2.45 ± 0.05^{a}	12.66 ± 0.32^{a}
Control	288.20 ± 8.03^{d}	46.84 ± 1.08^{b}	1.04 ± 0.08^{c}	6.61 ± 0.94^{b}

Each value is expressed as mean ± standard deviation (n = 6). Means in the same line with different letters in superscript are significantly different ($p < 0.05$).
Total polyphenols are expressed as milligrams of gallic acid equivalents. Total flavonoids are expressed as milligrams of catechin equivalents.
AA_{ABTS} – antioxidant activity determined by ABTS method (expressed as millimoles of Trolox equivalents), AA_{FRAP} – antioxidant activity determined by ferric reducing antioxidant power method (expressed as millimoles of Fe^{2+}).
Description of samples is given in Tab. 1.

The antioxidant activity determined in beer is closely related to concentrations of polyphenols and flavonoids. In this work, pineapple juice concentrate addition significantly enhanced ($p < 0.05$) the antioxidant activity of the final products, specifically, from AA_{ABTS} (2.45 ± 0.05 mmol·l^{-1}, expressed as TE) to AA_{FRAP} (12.66 ± 0.32 mmol·l^{-1}, expressed as Fe^{2+}), as compared with AA_{ABTS} (1.04 ± 0.08 mmol·l^{-1}) and AA_{FRAP} (6.61 ± 0.94 mmol·l^{-1}) in control. Polyphenols and flavonoids in fruit additives could directly contribute to the characteristics of beer with respect to fermentability, foamability, thermal stability, oxidative stability and fullness [21]. Moreover, beers exhibiting high radical-scavenging activities probably have better flavour stability during storage [22].

Analysis of volatiles

VOC constitute a group of key substances that contribute to the aroma characteristics of fermented beverages. Beer flavour is widely recognized as affected by not only raw materials, hops and yeasts, but also the fermentation process. However, for pineapple beer, the aroma characteristics remain unelucidated. In this study, the VOC fraction in pineapple beer samples at distinct brewing stages was analysed using HS-GC-IMS. In Fig. 1, the three-dimensional visualization and top-view plots of VOC of the samples at distinct brewing stages are presented. The differences in aroma composition are mainly reflected by the position, quantity, strength and time of the ion peaks, and the peak height indicates the signal intensity of the individual compounds. In relation to the reference values (white), the darkness degree indicates intensity.

In total, 42 typical flavour compounds were identified and 20 unknown compounds were also obtained (Tab. 3). Acetic acid, ethyl formate, ethyl hexanoate, ethyl octanoate and isoamyl acetate at high concentrations were detected in multiple signals that represented the formation of corresponding monomers and/or dimers. The gallery plot (Fig. 2) of the selected signal peak areas provides an intuitive contrast and suggests the characteristic flavour components of each sample and the flavour differences.

During the pineapple beer production process, the 42 VOC identified included 12 esters, 9 alcohols, 11 aldehydes, 2 organic acids, 4 ketones, 2 furans, 1 sulphur compound and 1 olefin (Tab. 3). Among these VOC, acetic acid, propanoic acid, acetaldehyde, 3-methylbutanal, 2-methylbutanal, acetone, ethanol, methanol, 2,5-dimethyl furan and dimethyl disulphide were the most abundant components in wort, while dimethyl disulphide (onion flavour), 2,5-dimethyl furan (meaty) and heptanal (aldehyde, vinous, bitter) were detected only in the wort. These results are consistent with the findings of Alves et al. [23].

The volatile profile becomes more complex concomitantly with yeast fermentation. In this study, two major volatile acids, acetic acid and propanoic acid, were identified. They are known to mostly contribute to pH and have a typical vinegar-like smell accompanied by a sour overripe fruity taste with sharpness [1]. The content of acetic acid substantially increased after pineapple juice concentrate addition and was markedly higher (2 700.13 ± 31.61 μg·kg^{-1}) in the final product than that in the control (2 297.65 ± 119.31 μg·kg^{-1}). Propanoic acid, the degradation product of sugar in wort, indirectly affects ethanol production, although it can also provide precursors for synthesis of esters. Here, we found that the propanoic acid content decreased from 163.33 ± 13.64 μg·kg^{-1} in wort to 134.93 ± 11.46 μg·kg^{-1} in the final beer.

The presence of a large amount of volatile acids is undesirable and will challenge the fermentation of fruit beers.

Alcohols are metabolic by-products in ethanol fermentation that are generated from aromatic and branched-chain amino acids through oxidative decarboxylation of keto acids in the tricarboxylic acid cycle. A higher amount of fermentable sugar from pineapple juice concentrate leads to a higher content of alcohols. Alcohols are generally considered responsible for the malty and fruity character in the final beer, but a strong pungent and fusel-like smell inevitably develops when these alcohols are not well balanced [23]. The highest content of methanol (552.05 ± 9.25 $\mu g \cdot kg^{-1}$) was found in the wort. However, during yeast fermentation, it gradually decreased to 63.47 ± 2.41 $\mu g \cdot kg^{-1}$, far below the limit allowed in beer (50 $mg \cdot kg^{-1}$). Conversely, the levels of 1-propanol (musty and yeasty flavour,

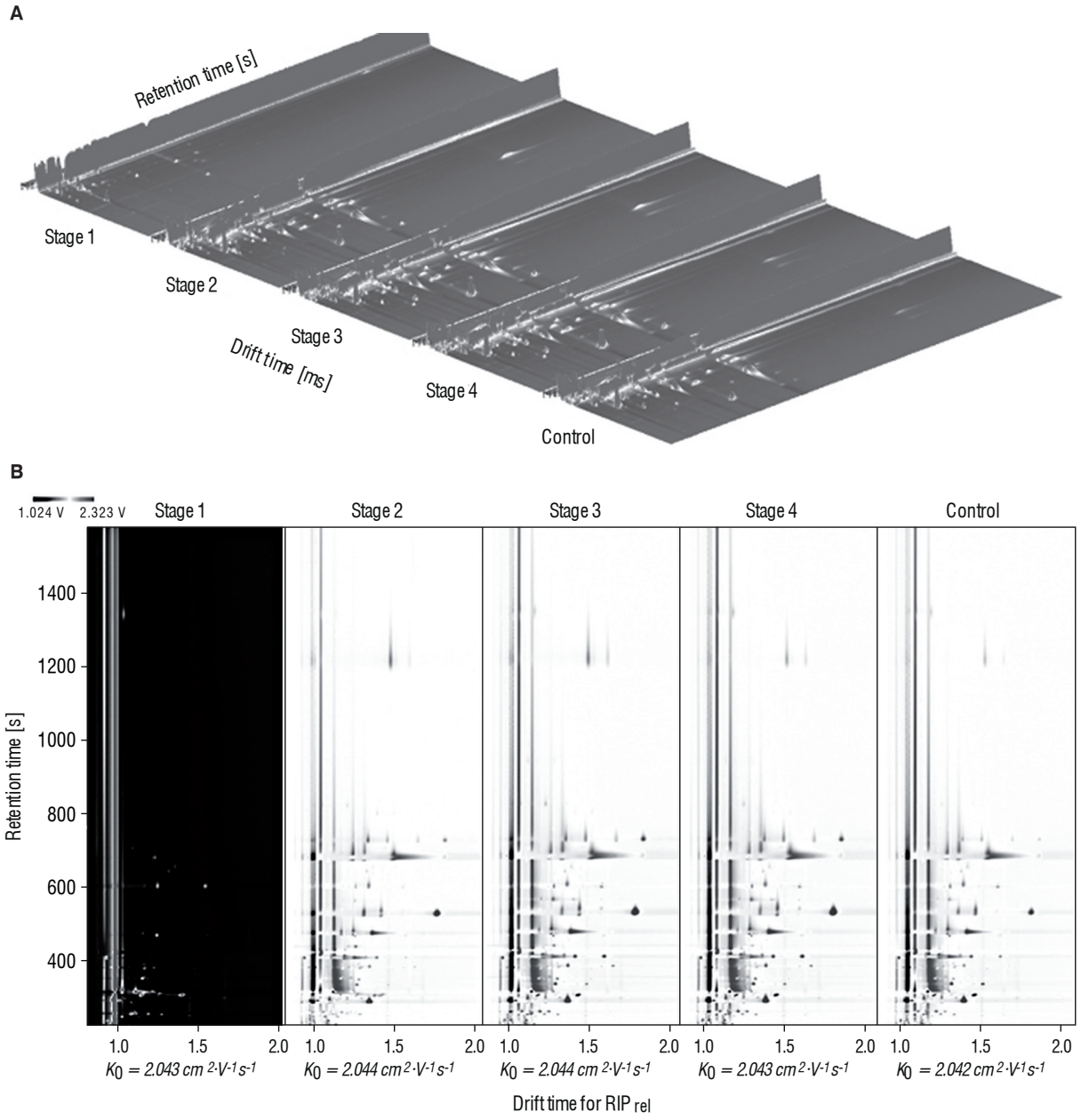

Fig. 1. Volatile fingerprints of pineapple beer samples.

A – three-dimensional topographical plots, B – top-view plot.
RIP_{rel} – relative reactant ion peak, K_0 – ion mobility. Description of samples is given in Tab. 1.

with a slightly sweet fruity nuance of apple), 2-methyl-1-propanol (whiskey) and 3-methyl-1-butanol (banana, nail polish remover) increased markedly during fermentation and peaked at maturation. 2-Propanol (alcoholic flavour), which was undetected in wort, was determined at approximately 80 $\mu g \cdot kg^{-1}$ during fermentation, and low levels of 1-pentanol (alcohol, medicinal, solvent-like) and 1-hexanol (coconut) were maintained during the entire beer production, being not strongly correlated with yeast metabolism. 3-Methyl-1-butanol and 2-methyl-1-propanol are closely associated with beer drinkability [23]. With the exception of ethanol, 3-methyl-1-butanol was the most abundant alcohol in beer, followed by 2-methyl-1-propanol.

Volatile esters most notably affect beer flavour and were investigated in various beers [1, 23, 24]. The composition, odour thresholds and interactions of volatile esters can readily interfere with the overall flavour perception. In this study, the esters were produced by fermentation and pineapple juice concentrate addition as an adjunct strongly promoted the accumulation of esters. The key esters identified were ethyl formate (fruity, solvent), ethyl acetate (pineapple, sweet, flowery), ethyl butanoate (papaya, butter, sweet, apple, fruity), isoamyl acetate (fruity, banana, pear, solvent, apple, sweet), ethyl hexanoate (sour apple, aniseed, fruity) and ethyl octanoate (apple, sweet, fruity, sour apple).

Aldehydes are intermediate compounds in alcohol formation. They are associated with beer staling as the major source of off-flavour substances produced by Strecker degradation or yeast metabolism [24]. Thus, when certain odour threshold values for the aldehydes are exceeded, beer typically presents a strong immature, cardboardy and rancid aroma. Acetaldehyde (fruity, solvent), 2-methylbutanal (potato, almond) and 3-methylbutanal (potato almond) were the key aldehydes present at high levels in wort, followed by nonanal. These compounds are mainly formed by Maillard reactions and Strecker degradation initiated during wort production. Butanal (green malt, green leaves) was enriched through the production process, whereas the contents of other aldehydes, such as Strecker aldehydes (methional, heptanal, octanal, nonanal), were decreased. 2-Methylpropanal (malty, fruity) and 3-methylbutanal

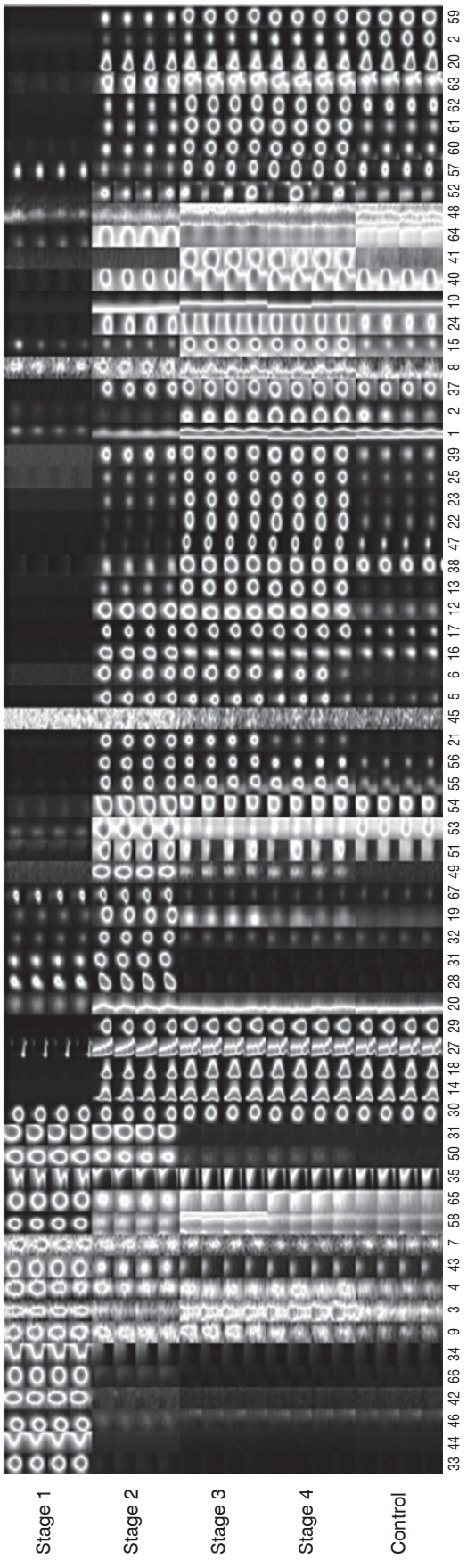

Fig. 2. Gallery plot of selected signal peak areas.
Description of samples is given in Tab. 1. Peak codes are identified in Tab. 3.

Tab. 3. Content of volatile organic compounds in pineapple beer samples.

Codes	Compound	*RI*	*Rt* [s]	*Dt* (RIP_{rel})	Content [$\mu g \cdot kg^{-1}$]				
					Stage 1	Stage 2	Stage 3	Stage 4	Control
Acids									
1	Acetic acid (monomer)	1 512.8	1 338.625	1.05327	711.68 ± 77.19^{e}	1 301.02 ± 54.42^{d}	2 535.12 ± 27.91^{b}	2 700.13 ± 31.61^{a}	2 297.65 ± 119.31^{c}
2	Acetic acid (dimer)	1 515.0	1 343.823	1.15115	45.25 ± 8.06^{e}	61.93 ± 2.90^{d}	204.44 ± 6.49^{b}	268.84 ± 16.69^{a}	162.87 ± 31.65^{c}
4	Propanoic acid	1 555.3	1 438.014	1.09275	163.33 ± 13.64^{a}	140.80 ± 12.09^{b}	127.69 ± 9.37^{d}	134.93 ± 11.46^{c}	115.41 ± 7.49^{e}
Esters									
38	Ethyl formate (monomer)	856.7	269.239	1.06448	16.43 ± 0.91^{e}	97.75 ± 6.36^{d}	223.02 ± 2.45^{a}	198.27 ± 3.39^{b}	159.69 ± 5.78^{c}
47	Ethyl formate (dimer)	856.7	269.215	1.23076	11.76 ± 0.75^{e}	37.77 ± 0.97^{d}	239.33 ± 4.91^{a}	224.56 ± 5.33^{b}	115.17 ± 3.34^{c}
29	Ethyl acetate	895.2	295.616	1.34037	34.16 ± 1.75^{e}	3 457.95 ± 22.78^{d}	4 527.84 ± 32.14^{b}	4 662.83 ± 21.82^{a}	4 070.87 ± 13.32^{c}
25	Propyl acetate	983.1	355.918	1.48257	11.51 ± 0.79^{e}	23.84 ± 1.17^{c}	45.90 ± 2.21^{b}	49.61 ± 1.72^{a}	18.72 ± 1.48^{d}
26	Ethyl propanoate	961.7	341.236	1.46261	23.98 ± 3.07^{d}	78.09 ± 0.79^{c}	187.94 ± 5.42^{b}	211.32 ± 8.06^{b}	372.84 ± 5.07^{a}
22	Ethyl butanoate	1 041.1	414.123	1.56489	15.00 ± 2.08^{e}	66.65 ± 4.22^{d}	582.47 ± 17.65^{b}	684.32 ± 18.36^{a}	201.62 ± 4.09^{c}
23	2-Methylpropyl acetate	1 019.5	390.526	1.61852	12.15 ± 2.82^{e}	59.74 ± 1.10^{c}	173.12 ± 8.03^{b}	187.88 ± 6.98^{a}	39.32 ± 0.45^{d}
39	Methyl 2-methylbutanoate	1 017.9	388.703	1.55047	8.40 ± 0.70^{e}	37.35 ± 1.94^{c}	55.77 ± 1.22^{b}	58.23 ± 0.88^{a}	29.05 ± 1.01^{d}
21	Ethyl 3-methylbutanoate	1 068.4	444.012	1.26304	21.88 ± 1.91^{e}	257.84 ± 4.33^{a}	226.84 ± 5.24^{b}	109.37 ± 4.74^{c}	34.28 ± 2.45^{d}
16	Isoamyl acetate (monomer)	1 128.4	532.545	1.30974	51.19 ± 10.55^{e}	1 187.39 ± 18.42^{a}	943.70 ± 19.69^{c}	973.66 ± 26.36^{b}	729.33 ± 12.00^{d}
17	Isoamyl acetate (dimer)	1 129.8	535.080	1.75358	71.04 ± 8.99^{e}	3 253.04 ± 56.40^{c}	4 935.10 ± 74.27^{b}	5 351.10 ± 112.21^{a}	1 767.08 ± 24.10^{d}
12	Ethyl hexanoate (monomer)	1 239.5	732.005	1.33899	21.55 ± 1.42^{e}	570.57 ± 24.95^{a}	507.60 ± 12.01^{c}	512.11 ± 23.38^{b}	228.74 ± 3.51^{d}
13	Ethyl hexanoate (dimer)	1 238.9	730.958	1.80823	27.16 ± 5.7^{d}	364.45 ± 15.47^{b}	934.12 ± 32.69^{a}	938.24 ± 74.11^{a}	113.82 ± 6.94^{c}
5	Ethyl octanoate (monomer)	1 459.1	1213.300	1.47317	124.55 ± 8.17^{e}	2 089.86 ± 107.64^{a}	1 836.24 ± 56.07^{b}	1 503.23 ± 261.88^{c}	710.76 ± 81.46^{d}
6	Ethyl octanoate (dimer)	1 460.3	1215.991	2.02878	73.39 ± 2.30^{e}	304.41 ± 23.26^{b}	342.42 ± 19.65^{a}	254.56 ± 54.54^{c}	87.86 ± 5.95^{d}
45	Ethyl heptanoate	1 335.1	923.654	1.40674	40.75 ± 5.03^{b}	47.05 ± 7.05^{a}	38.04 ± 5.91^{d}	40.31 ± 4.38^{c}	40.88 ± 4.96^{b}
Alcohols									
34	Methanol	902.8	300.860	0.97991	552.05 ± 9.25^{a}	210.4 ± 12.78^{b}	71.76 ± 3.87^{d}	63.47 ± 2.41^{e}	76.87 ± 0.57^{c}
27	Ethanol	937.2	324.456	1.12834	3 156.04 ± 59.14^{e}	19 580.24 ± 27.61^{d}	20 944.41 ± 131.72^{b}	21 077.45 ± 27.52^{a}	20 373.13 ± 83.77^{c}
20	1-Propanol	1 041.6	414.647	1.25182	52.19 ± 7.20^{e}	1 081.76 ± 4.48^{d}	1 198.45 ± 14.03^{b}	1 165.99 ± 20.38^{c}	1 311.71 ± 9.26^{a}
24	2-Propanol	990.0	360.637	1.23061	8.06 ± 0.69^{e}	89.84 ± 1.65^{a}	88.53 ± 3.36^{b}	78.15 ± 3.80^{c}	56.44 ± 1.21^{d}
15	1-Butanol	1 148.0	568.035	1.18754	68.04 ± 18.31^{d}	89.40 ± 3.38^{c}	204.25 ± 2.58^{a}	204.92 ± 2.29^{a}	143.93 ± 4.05^{b}
18	2-Methyl-1-propanol	1 101.0	482.690	1.35609	81.39 ± 6.43^{e}	2 117.07 ± 16.13^{d}	3 165.82 ± 12.06^{b}	3 250.17 ± 4.50^{a}	2 720.52 ± 18.55^{c}
11	1-Pentanol	1 257.9	764.455	1.25492	21.93 ± 1.99^{d}	47.41 ± 3.90^{b}	34.29 ± 2.35^{c}	36.65 ± 4.91^{c}	40.74 ± 1.86^{a}
14	3-Methyl-1-butanol	1 214.0	686.993	1.47502	289.41 ± 13.47^{e}	6 672.75 ± 25.29^{d}	7 961.58 ± 45.76^{b}	8 287.88 ± 90.67^{a}	7 445.24 ± 26.90^{c}
8	1-Hexanol	1 364.1	991.276	1.32634	57.80 ± 5.36^{b}	51.84 ± 2.71^{c}	71.83 ± 5.60^{a}	72.14 ± 1.30^{a}	57.96 ± 3.70^{b}
Aldehydes									
35	Acetaldehyde	807.2	235.314	0.98115	435.48 ± 4.10^{a}	341.55 ± 18.56^{b}	311.82 ± 11.84^{b}	317.51 ± 8.72^{b}	349.06 ± 5.74^{b}

Tab. 3. *continued*

Codes	Compound	*RI*	*Rt* [s]	*Dt* (RIP_{rel})	Content [$\mu g \cdot kg^{-1}$]				
					Stage 1	Stage 2	Stage 3	Stage 4	Control
32	Propanal	837.0	255.764	1.14704	27.39 ± 0.85^{e}	99.02 ± 4.96^{a}	34.34 ± 0.39^{d}	42.17 ± 3.24^{b}	36.22 ± 3.42^{c}
31	2-Methylpropanal	845.4	261.532	1.28425	68.58 ± 1.48^{b}	121.95 ± 1.30^{a}	4.71 ± 0.53^{c}	3.88 ± 0.55^{d}	3.65 ± 0.38^{d}
37	Butanal	909.8	305.642	1.29714	5.95 ± 1.23^{e}	59.00 ± 1.60^{d}	82.48 ± 2.10^{b}	84.39 ± 1.93^{a}	78.41 ± 1.06^{c}
3	Methional	1 506.4	1 323.639	1.08941	70.67 ± 5.12^{a}	41.79 ± 5.44^{d}	53.33 ± 4.92^{b}	54.88 ± 3.07^{b}	46.84 ± 4.85^{c}
36	2-Methylbutanal	919.5	312.308	1.15496	186.16 ± 0.82^{a}	165.02 ± 6.63^{b}	8.34 ± 0.36^{c}	6.16 ± 0.64^{d}	8.28 ± 0.53^{c}
28	3-Methylbutanal	920.4	312.920	1.40773	782.23 ± 15.05^{b}	1 235.47 ± 11.90^{a}	64.11 ± 1.75^{c}	49.26 ± 2.48^{e}	54.52 ± 1.88^{d}
42	Hexanal	1 097.1	475.567	1.56512	23.88 ± 2.76^{a}	9.39 ± 1.00^{b}	6.64 ± 0.86^{d}	7.70 ± 0.86^{c}	7.78 ± 1.25^{c}
46	Heptanal	1 193.2	650.211	1.33640	69.29 ± 1.19^{a}	23.78 ± 1.10^{d}	25.09 ± 1.11^{c}	27.11 ± 0.49^{b}	24.21 ± 1.02^{d}
9	Octanal	1 297.8	836.533	1.40309	37.88 ± 2.16^{a}	35.11 ± 3.01^{b}	35.30 ± 3.99^{b}	29.38 ± 4.68^{c}	25.91 ± 1.70^{d}
7	Nonanal	1 403.2	1 082.777	1.47484	98.18 ± 8.48^{a}	88.42 ± 5.92^{c}	86.75 ± 2.63^{c}	83.63 ± 7.99^{d}	92.57 ± 10.29^{b}
Ketones									
30	Acetone	850.8	265.203	1.12210	860.33 ± 13.99^{b}	742.00 ± 3.97^{e}	819.44 ± 3.49^{d}	865.11 ± 9.11^{a}	832.52 ± 10.10^{c}
10	Acetoin	1 292.9	826.216	1.05622	71.30 ± 2.50^{e}	501.4 ± 26.32^{c}	801.25 ± 23.67^{a}	669.96 ± 76.69^{b}	708.30 ± 4.05^{d}
19	2,3-Pentanedione	1 070.3	446.109	1.21565	33.39 ± 2.47^{e}	113.44 ± 2.78^{a}	97.55 ± 4.46^{b}	76.26 ± 6.78^{c}	47.05 ± 0.98^{d}
43	4-Methyl-2-pentanone	1 016.5	387.202	1.48607	18.11 ± 1.04^{a}	17.07 ± 0.85^{b}	11.64 ± 0.62^{c}	11.90 ± 0.66^{c}	10.53 ± 0.52^{d}
Others									
44	Dimethyl disulfide	1 017.9	388.722	0.98017	132.99 ± 3.08^{a}	17.87 ± 0.57^{b}	10.73 ± 0.42^{c}	8.38 ± 0.51^{d}	10.39 ± 0.35^{c}
33	2,5-Dimethyl furan	899.8	298.762	1.02855	265.95 ± 7.60^{a}	22.93 ± 1.28^{b}	5.58 ± 0.21^{bc}	5.07 ± 0.48^{c}	6.20 ± 0.25^{c}
41	2-Methyl-3-(methylthio) furan	1 344.5	945.604	1.10424	22.03 ± 1.78^{c}	18.24 ± 2.15^{e}	50.22 ± 1.22^{a}	40.89 ± 7.51^{b}	19.38 ± 3.09^{d}
40	α-Pinene	1 018.8	389.729	1.21571	8.76 ± 1.43^{e}	16.04 ± 1.32^{d}	73.60 ± 2.39^{a}	72.35 ± 0.86^{c}	72.91 ± 1.40^{b}
Unknown compounds									
48	Peak 48	1 323.4	896.351	1.08984	48.09 ± 7.94^{a}	39.89 ± 0.68^{b}	46.62 ± 3.99^{ab}	43.91 ± 3.64^{ab}	43.53 ± 2.41^{ab}
49	Peak 49	1281	805.280	1.39096	17.78 ± 1.39^{c}	56.04 ± 6.53^{a}	31.06 ± 1.97^{b}	29.75 ± 1.65^{b}	17.78 ± 0.31^{c}
50	Peak 50	1 199.2	660.823	1.36497	75.16 ± 1.09^{a}	54.52 ± 4.02^{b}	20.05 ± 2.72^{d}	24.67 ± 2.49^{c}	12.60 ± 1.24^{e}
51	Peak 51	1 060.7	435.622	1.08468	32.43 ± 3.23^{c}	145.44 ± 9.95^{a}	89.19 ± 14.15^{b}	91.87 ± 24.36^{b}	97.08 ± 2.95^{b}
52	Peak 52	1 060.7	435.622	1.19569	25.29 ± 3.89^{b}	89.88 ± 7.96^{a}	100.49 ± 22.01^{a}	100.00 ± 38.06^{a}	81.55 ± 8.31^{a}
53	Peak 53	1 018.1	388.953	1.08343	34.43 ± 0.88^{d}	79.17 ± 2.17^{a}	56.08 ± 0.72^{c}	61.80 ± 1.00^{b}	63.45 ± 1.68^{b}
54	Peak 54	1 015.7	386.331	1.28425	9.89 ± 1.07^{d}	89.34 ± 0.90^{a}	78.06 ± 3.09^{c}	81.31 ± 0.79^{b}	80.68 ± 2.06^{bc}
55	Peak 55	820.2	244.228	1.09092	16.32 ± 1.01^{d}	281.72 ± 15.38^{b}	319.29 ± 5.88^{a}	329.16 ± 3.06^{a}	187.39 ± 6.09^{c}
56	Peak 56	803.4	232.692	1.13831	8.88 ± 1.99^{e}	257.14 ± 4.31^{a}	186.44 ± 4.07^{b}	154.30 ± 1.35^{c}	81.60 ± 2.07^{d}
57	Peak 57	822.5	245.801	0.95621	201.98 ± 3.19^{e}	225.57 ± 3.68^{d}	361.54 ± 7.13^{b}	422.33 ± 5.01^{a}	285.75 ± 4.15^{c}
58	Peak 58	1 017.4	388.191	1.23639	55.81 ± 2.15^{a}	38.21 ± 2.14^{b}	27.90 ± 1.28^{c}	28.79 ± 4.26^{c}	22.76 ± 0.62^{d}
59	Peak 59	962.9	342.046	1.27000	12.34 ± 0.76^{d}	259.65 ± 4.22^{c}	502.45 ± 8.07^{b}	505.48 ± 5.55^{b}	586.00 ± 6.06^{a}

Tab. 3. *continued*

Codes	Compound	*RI*	*Rt* [s]	*Dt* (RIP_{rel})	Content [$\mu g \cdot kg^{-1}$]				
					Stage 1	Stage 2	Stage 3	Stage 4	Control
60	Peak 60	982.3	355.376	1.27904	18.03 ± 0.54^{d}	126.47 ± 0.57^{c}	232.73 ± 4.61^{a}	235.57 ± 4.96^{a}	140.42 ± 2.06^{b}
61	Peak 61	1 021.6	392.805	1.34496	11.72 ± 0.88^{c}	186.35 ± 3.52^{b}	409.26 ± 12.70^{a}	417.56 ± 10.98^{a}	187.15 ± 1.67^{b}
62	Peak 62	1 042.2	415.365	1.38632	16.36 ± 1.85^{d}	207.50 ± 1.57^{c}	528.71 ± 4.18^{a}	524.82 ± 4.47^{a}	358.77 ± 5.23^{b}
63	Peak 63	1 026.3	397.932	1.28034	10.02 ± 1.04^{e}	54.10 ± 2.02^{d}	62.55 ± 1.82^{b}	71.74 ± 3.16^{a}	58.38 ± 2.11^{c}
64	Peak 64	989.8	360.504	1.20666	30.35 ± 1.66^{a}	89.21 ± 4.86^{a}	85.52 ± 3.40^{ab}	83.57 ± 1.83^{bc}	78.67 ± 2.19^{c}
65	Peak 65	1 228.6	712.774	1.11090	38.70 ± 1.51^{a}	16.36 ± 1.40^{b}	14.08 ± 0.82^{c}	16.38 ± 1.16^{b}	10.41 ± 0.19^{d}
66	Peak 66	1 041.5	414.576	0.94624	218.73 ± 5.77^{a}	47.95 ± 4.40^{b}	9.59 ± 0.55^{cd}	9.29 ± 1.00^{d}	15.31 ± 0.76^{c}
67	Peak 67	850.8	265.193	1.20894	131.71 ± 1.11^{b}	188.97 ± 4.22^{a}	45.33 ± 1.60^{c}	44.82 ± 0.76^{c}	47.52 ± 2.02^{c}

Each value is calculated using 10 μl of 4-methyl-2-pentanol (20 $mg \cdot l^{-1}$) as an internal standard and the result is expressed as mean ± standard deviation ($n = 4$). Means in the same line with different letters in superscript are significantly different ($p < 0.05$).
RI – retention index calculated using *n*-ketones C4–C9 as external standard on Mxt-WAX column, *Rt* – retention time in the capillary GC column, *Dt* – migration time in the drift tube, RIP_{rel} – relative reactant ion peak. Description of samples is given in Tab. 1.

increased substantially during fermentation by the third day but decreased after addition of pineapple juice concentrate. Similar to the control beer, the final beer product contained high levels of acetoin (669.96 ± 76.69 $\mu g \cdot kg^{-1}$) and acetaldehyde (317.51 ± 8.72 $\mu g \cdot kg^{-1}$), followed by nonanal, butanal, methional (boiled potato, metallic), 3-methylbutanal, heptanal and octanal, which might potently affect the final flavour of beer at their relatively lower levels (27–84 $\mu g \cdot kg^{-1}$.

Acetone (aromatic, pungent, fruity) is the oxidation product of iso-t-acids, the main bitter component from hops [25]. Acetone did not appear to be related to the fermentation process and its levels were not markedly different between the finished beer and the control. Acetoin (fruity, mouldy, and woody note) was enriched through the beer production. 2,5-Dimethyl furan (meaty, gravy) and dimethyl disulphide (cooked cabbage) are mainly provided by wort or pineapple juice concentrate, being metabolized as the fermentation progresses. α-Pinene (citrus and lemon odour) is mainly derived from pineapple as a characteristic aroma [26]. It was detected at higher values after the addition of pineapple juice concentrate and remained relatively stable during the later brewing process.

Principal component analysis

The significant variations of VOC during the pineapple beer brewing process cannot be readily distinguished through visual comparison alone, so it is necessary to use statistical analysis to comprehensively investigate these changes. PCA is an unsupervised multivariate data analysis approach that uses the signal strengths of metabolomics data to highlight differences between samples. It is widely used and facilitates elucidation of the overall aroma characteristics of the food fermentation process [27]. In Fig. 3, the corresponding points from each group clustered together, whereas the samples from distinct brewing stages were well separated on the score plot, which demonstrated that the volatile compounds changed markedly during the brewing process. The cumulative variance contribution of the first two principal components accounted for 91.0 % of the accumulative variability and could represent all flavour components well for the flavour analysis. According to Song et al. [28], all VOC could be classified into six categories based on the original peak area. The eigenvalues and contribution rates for the first two PC and their eigenvectors and load matrices were calculated (data not shown). The contribution rate for PC1 (70.1 %) reflected acids, esters and alcohols, while the contribution rate

for PC2 (28.4 %) reflected aldehydes. Moreover, the aroma profiles of green beer (stage 3) and final product (stage 4) were very similar, although they were separated in the score map. Anyway, the results indicated that the aroma fingerprints of the samples from the distinct brewing stages of pineapple beer were successfully constructed using the HS-GC-IMS. It is also worth noting that the addition of pineapple juice concentrate enriched, to a certain extent, the aroma compared to the control beer.

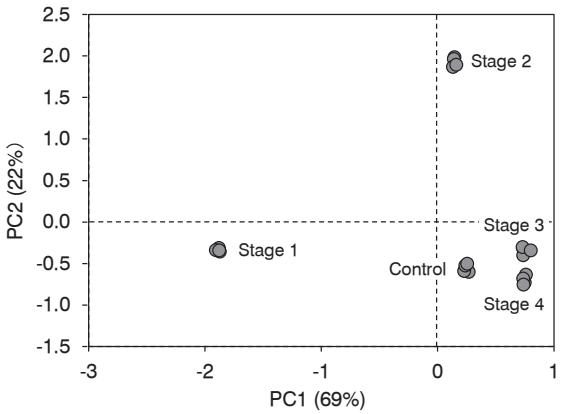

Fig. 3. Score plot of principal component analysis. Description of samples is given in Tab. 1.

Orthogonal partial least squares discriminant analysis

The disadvantage of PCA is that it presents a collinearity problem when the number of samples is lower than the number of variables in the model-building process. Conversely, OPLS-DA fully considers the Y-variable information and can be applied to evaluate the correlations between volatile compounds and food samples [29]. So, OPLS-DA was used for sample data separation to determine the characteristic VOC contributions to each brewing stage and build a classification model. In Fig. 4, the black boxes represent the different samples and the remaining compounds are characteristic flavour substances. The results explained 49.5 % of the total difference and most of the characteristic compounds were located between R^2 50 % and 100 % ellipses, with R^2X = 34.2 % and R^2Y = 15.3 %. The flavours differed most significantly between the wort and beer samples across fermentation. Specific compounds were detected at distinct stages, for example, aldehydes and furans dominated in the wort, while esters and alcohols dominated in the subsequent stages. Such variations can be assigned to specific VOC. The wort group in the fourth quadrant, for example, was positively correlated with heptanal, 2,5-dimethyl furan, propanoic acid, methanol, 4-methyl-2-pentanone, acetaldehyde, nonanal and dimethyl disulphide. In the first quadrant (stage 1), the characteristic compounds were 2,3-pentanedione, ethyl hexanoate and propanal. Most of the characteristic compounds in the third quadrant positively correlated with their flavour types in stages 3 and 4, including acetic acid, ethyl butanoate, acetone, 3-methyl-1-butanol, ethyl octanoate, ethanol, 1-propanol, isoamyl acetate, ethyl hexanoate, ethyl formate, butanal, 2-methylpropyl acetate, methyl 2-methylbutanoate, isoamyl acetate, furan, 2-methyl-3-(methylthio)-1-pentanol, 2-propanol and

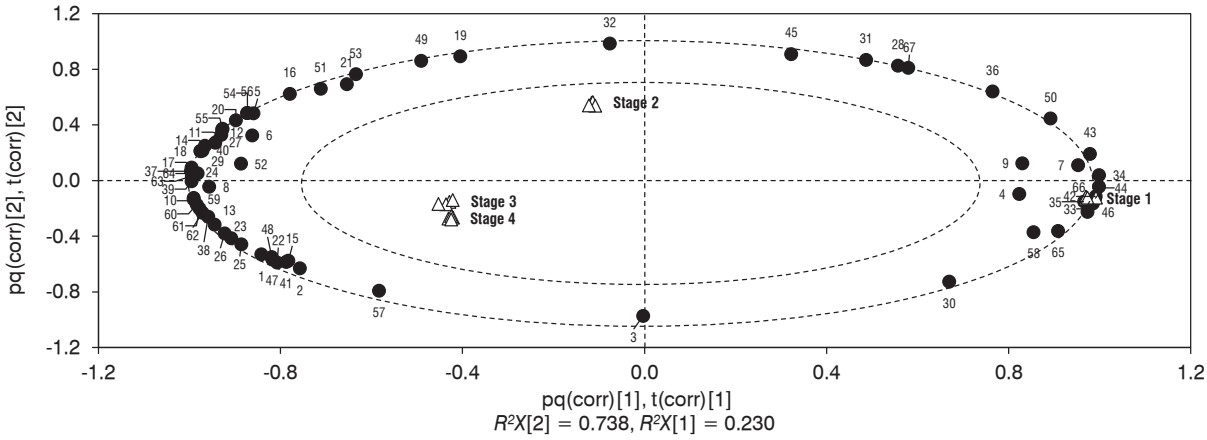

Fig. 4. Orthogonal partial least squares discriminant analysis model.

Description of brewing stages is given in Tab. 1. Coumpond codes were given in Tab. 3.
R^2X[1], R^2X[2] – represent the variabilities of the first and second principal component, respectively.

1-pentanol. Moreover, the density of the characteristic flavour compounds clearly increased as the brewing process progressed, which reflected the increasing complexity of the flavour. Wort was markedly poorer in terms of flavour compounds than samples from the subsequent brewing stages. The fermentable sugar and nitrogen sources from pineapple juice concentrate increased the levels of nutrients for yeast growth and metabolism, and thus a relatively richer volatile profile was found at stage 3. Esters dominated in late fermentation as expected, the precursors of the esters being generally produced by primary fermentation.

To circumvent the problems caused by small sample sizes, a permutation test with 200 random arrangements of samples was conducted and then statistical inference was performed to validate the robustness of the model [30]. The intercepts of Q^2 and R^2 featuring values of –0.716 and 0.321, respectively, were lower than the original points, which supported the validity of the original model. Variable influence on projection (*VIP*) is typically used to evaluate the contribution of *X* variables to the OPLS-DA model, and *VIP* values > 1 are generally considered indicative of key variables. Here, 24 such compounds were obtained, including octanal, ethyl 3-methylbutanoate, methional, 1-hexanol, ethyl octanoate, acetone, propanal, 2,3-pentanedione, acetic acid, furan, 2-methyl-3-(methylthio) propanoic acid, propyl acetate, ethyl propanoate, 2-methylpropanal, ethyl hexanoate, 2-methylpropyl acetate, 1-butanol, 3-methylbutanal, ethyl heptanoate and ethanol, together with six unknown compounds (peaks 49, 50, 57, 58, 65 and 67). These results showed that OPLS-DA facilitated identification of characteristic flavour substances and discrimination of aroma models. Moreover, acetic acid, 3-methyl-1-butanol, ethanol, acetone, α-pinene, 3-methylbutanal, 2-propanol, ethyl formate, ethyl 3-methylbutanoate, 2-methylpropanal and dimethyl disulphide could be used to differentiate pineapple beer from the control.

CONCLUSION

Demand for beers featuring fruity aromas has grown steadily in recent years, and the inclusion of fruit juices or fruit extracts in the beer brewing is recognised as adding new organoleptic and functional characteristics to the products. However, successfully developing a fruit beer that, besides being rich in bioactive compounds, is attractive in appearance, possesses a unique aroma and can be readily produced, is challenging. Although pineapple juice concentrate represents a promising adjunct for beer brewing because the juice offers new styles, unique flavours and nutritious properties, it has not been used in the industrial production of pineapple beer by brewing companies. In this study, our results showed that 100 $g \cdot l^{-1}$ of pineapple juice concentrate as an adjunct for beer brewing considerably increased the levels of bioactive compounds and enhanced the antioxidant activities. OPLS-DA was successfully used to construct the flavour fingerprint of beer during the brewing process. The results indicated that this approach provides useful and comprehensive insights that can help understand the impact of each brewing stage on volatile organic compoounds. In this work, acetic acid, 3-methyl-1-butanol, ethanol, acetone, α-pinene, 3-methylbutanal, 2-propanol, ethyl formate, ethyl 3-methylbutanoate, 2-methylpropane, and dimethyl disulphide could be used effectively to differentiate pineapple beer from the control, and we believe that this method will help to produce high-quality beer. However, certain compounds that could potentially affect beer flavour (e.g., terpenes and phenolic acids) were not detected, and 20 potential metabolite markers were not identified, which was limited by the GC-IMS technology. Anyway, this study could not only provide a basis for research aimed at enhancing the functionality and sensory characteristics of pineapple beer, but also facilitate the discrimination of novel fruit beers appearing in the growing market.

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