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Rapid quantification of isovaleraldehyde in sake by HPLC with post-column fluorescent derivatisation

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Abstract

Why was the work done: Elevated levels of isovaleraldehyde (3-methylbutanal) in sake gives rise to an unfavourable aroma of '*mureka*' or 'stuffy smell'. The concentration of isovaleraldehyde is typically higher in unpasteurised than pasteurised sake. Controlling the concentration of isovaleraldehyde in unpasteurised sake remains a major challenge for quality control. As existing methods for the quantification of isovaleraldehyde in sake require specialised sample preparation, there is a need for a simple and precise method.
How was the work done: High-performance liquid chromatography with fluorescence detection and post-column derivatisation (HPLC-PCD-FLD) for determining the isovaleraldehyde content in sake has been developed with optimisation of the separation of peaks and derivatisation of aldehyde compounds. The new method was compared with the established method of headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS).
What are the main findings: The limit of quantification of the new method was 87 µg/L, and accordingly, the HPLC-PCD-FLD method could determine the concentration of isovaleraldehyde in sake below the reported threshold level. The precision of the HPLC-PCD-FLD method for the analysis of sake containing isovaleraldehyde (> threshold level) either matched or was superior to the HS-SPME-GC-MS method.
Why is the work important: The new approach requires only particle removal for sample preparation, with an rapid analysis time (<1 h per sample), and requires a smaller sample volume (≈ 100 µL) than the alternative method (10 mL). These improvements contribute to a simpler and more efficient workflow for routine analysis of isovaleraldehyde in the quality control of sake.

Keywords

Alcoholic beverage, isovaleraldehyde, post-column derivatisation, sake, sparkling sake, mureka

Introduction

Given the impact on product quality, the avoidance of off-flavours is of paramount importance in the production of sake (Takahashi and Kohno 2016; Makimoto et al. 2020; Endo et al. 2022; Todokoro et al. 2022; Sasaki et al. 2023). An important off-flavour is 3-methylbutanal or isovaleraldehyde which contributes '*mureka*' or 'stuffy smell' to unpasteurised sake. Isovaleraldehyde is formed from the conversion of 3-methyl-1-butanol, via isoamyl alcohol oxidase (IAAOD) from the *kōji* fungus, *Aspergillus oryzae* (Yamashita et al. 1999). Sparkling sake, produced via secondary fermentation with sake yeast to generate carbon dioxide in bottles (natural carbonation) (Akamatsu et al. 2022), carries an increased risk of isovaleraldehyde accumulation due to the lack of filtration and pasteurisation. The lack of these mitigation strategies results in residual IAAOD activity in naturally carbonated sparkling sake. This potentially results in the formation of isovaleraldehyde during storage if appropriate manufacturing and storage protocols are not implemented.

The reported threshold level of isovaleraldehyde in sake is 120 µg/L (Isogai et al. 2005), with commercially unpasteurised sake containing 100–2000 µg/L. The concentration of isovaleraldehyde in unpasteurised sake increases during storage at room temperature due to residual IAAOD activity (Kubodera et al. 2003). Strategies to address this include low temperature storage, high pressure carbonation treatment, and inactivation or removal of IAAOD through heat treatment or filtration. Consequently, unpasteurised sake is typically refrigerated and consumed within a short storage period to minimise the formation of off-flavours associated with isovaleraldehyde (Tanimoto et al. 2008). Therefore, monitoring isovaleraldehyde production is important for maintaining quality standards in both unpasteurised sake and sparkling sake.

Options for a rapid and simple method for quantifying isovaleraldehyde concentrations in sake are currently limited. Although head space gas chromatography with flame ionisation detection (HS-GC-FID) readily identifies and quantifies alcohol and ester compounds such as ethyl hexanoate and isoamyl acetate, HS-GC-FID is generally applied to determine compounds in sake whose concentration is >200 µg/L (Qin et al. 2020; Sabalenka et al. 2023). Therefore, the concentration range of isovaleraldehyde in sake (µg/L to mg/L) poses a challenge for this technique.

To address this limitation, highly sensitive and reliable methods have been developed (Kishikawa et al. 2019). Existing techniques for determining the isovaleraldehyde concentration in sake include high performance liquid chromatography with ultraviolet detection (HPLC-UV), employing dinitrophenyl hydrazide (DNPH) derivatisation and stir bar sorptive extraction (SBSE) combined with GC-MS (Isogai et al. 2005; Mitsui and Kondo 2018). Headspace solid-phase microextraction (HS-SPME) combined with GC-MS is a well established technique for analysing volatile organic compounds in alcoholic beverages (Kang et al. 2016) and has been adapted for isovaleraldehyde analysis in sake, shochu (Osafune et al. 2020), wine (Bueno et al. 2014), and Chinese rice wine (Yu et al. 2019). While these methods are effective for measuring isovaleraldehyde at sub-mg/L levels, they require time consuming pre-treatments, such as adjusting the ethanol concentration to 10% (v/v) in samples for SBSE-GC-MS and HS-SPME-GC-MS. In addition, the global shortage of helium used aa a carrier gas can cause further complexity (Siddhantakar et al. 2023). Pre-column derivatisation and the subsequent extraction of derivatised compounds are also required for HPLC-UV using the DNPH derivatisation method. HPLC with fluorescent detection, employing cyclohexane-1, 3-dione for carbonyl compounds, offers potential for the analysis of sub-mg/L aldehyde levels (Stahovec and Mopper 1984; Suzuki 1985; Tanaka et al. 2013), but its application to sake analysis has not been previously reported.

This study asought to develop a straightforward, cost effective, and high throughput method for determining the concentration of isovaleraldehyde in both sake and sparkling sake. HPLC with post-column derivatisation using cyclohexane-1,3-dione derivatisation (PCD) and fluorescent detection (FLD) offers potential for a simple, rapid, and sensitive method for isovaleraldehyde. Accordingly, we investigated whether the HPLC-PCD-FLD method was applicable to quantify isovaleraldehyde in sake. Further, the HPLC-PCD-FLD method was

optimised to achieve separation and derivatisation of isovaleraldehyde with a single column oven, leading to advantages in versatility and reduced equipment costs. Finally, the performance of the method was compared to the established HS-SPME-GC-MS method.

Materials and methods

Reagents and materials

Seven commercial sake samples and 19 commercial sparkling sake samples were used in this work. Isovaleraldehyde (3-methylbutanal, CAS no. 590-86-3), ethanol (CAS no. 64-17-5), ammonium acetate (CAS no. 631-61-8), acetic acid (CAS no. 64-19-7), cyclohexane-1, 3-dione (CAS no. 504-02-9), and HPLC-grade methanol (CAS no. 67-56-1) were purchased from FujiFilm Wako Pure Chemical Corporation (Tokyo, Japan). Pentanal (CAS no. 110-62-3) was purchased from Merck (Darmstadt, Germany). All aqueous solutions were produced with water (Purelite water purification system, Organo Corporation, Tokyo, Japan).

High-performance liquid chromatography

The HPLC-PCD-FLD method employed a Vanquish™ Duo UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) comprising of VF-P32-A-01 pumps, a VF-A10-A auto-injector, a VH-C10-A-03 column oven, and a VF-D51-A fluorescent detector, equipped with a BEH C18 column (2.1×100 mm, 1.7 µm, Waters Corp., Milford, MA, USA) and PEEK coil for the post-column reaction (0.50 mm×10190 mm, Shonan Maruhachi Estec, Kanagawa, Japan) (Figure 1A). The mobile phase comprised of 0.3% (w/v) acetic acid solution and methanol (95:5, v/v), and the post-column derivatisation reagent consisted of 2.5 mol/L ammonium acetate, 1.75 mol/L acetic acid, and 40 mmol/L cyclohexane-1,3-dione in ultrapure water. Analysis was performed using the isocratic mode at a mobile phase flow rate of 0.3 mL/min at 85°C for 20 min with the derivatisation reagent flow rate of 0.3 mL/min. The injection volume of sake was 20 µL. Sake samples were centrifuged at 15,000 *g* at 4°C for 10 min, and the supernatants filtered using an Ekicrodisc 13 (0.2 µm pore size, Nihon Pall, Tokyo, Japan) before injection. As the sample volume was decreased by centrifugation and filtration, the sake sample was at least 100 µL.

Derivatisation of the aldehyde was performed in the reaction coil in the column oven at 85°C. Before every injection, the column was washed with a high methanol mobile phase (0.3%, w/v acetic acid solution/methanol = 10:90, v/v) for 5 min to remove residues, and the column was equilibrated with the mobile phase for 20 min. The wavelengths of excitation and emission in the fluorescence detector were 366 nm and 440 nm, respectively. The limit of quantification (LOQ) and the limit of detection (LOD) were calculated by multiplying the standard deviation (SD) obtained from the analysis of 100 µg/L of standard samples (n = 10) by 10 or 3.3, respectively.

A spike recovery test was performed by analysing a mixture of 180 µL of sake with 20 µL of a isovaleraldehyde standard (5000 µg/L). The recovery was calculated based on the determined value from the spike test. The isovaleraldehyde concentration was determined by calculating the peak area of the corresponding peak (Figures 1B and C).

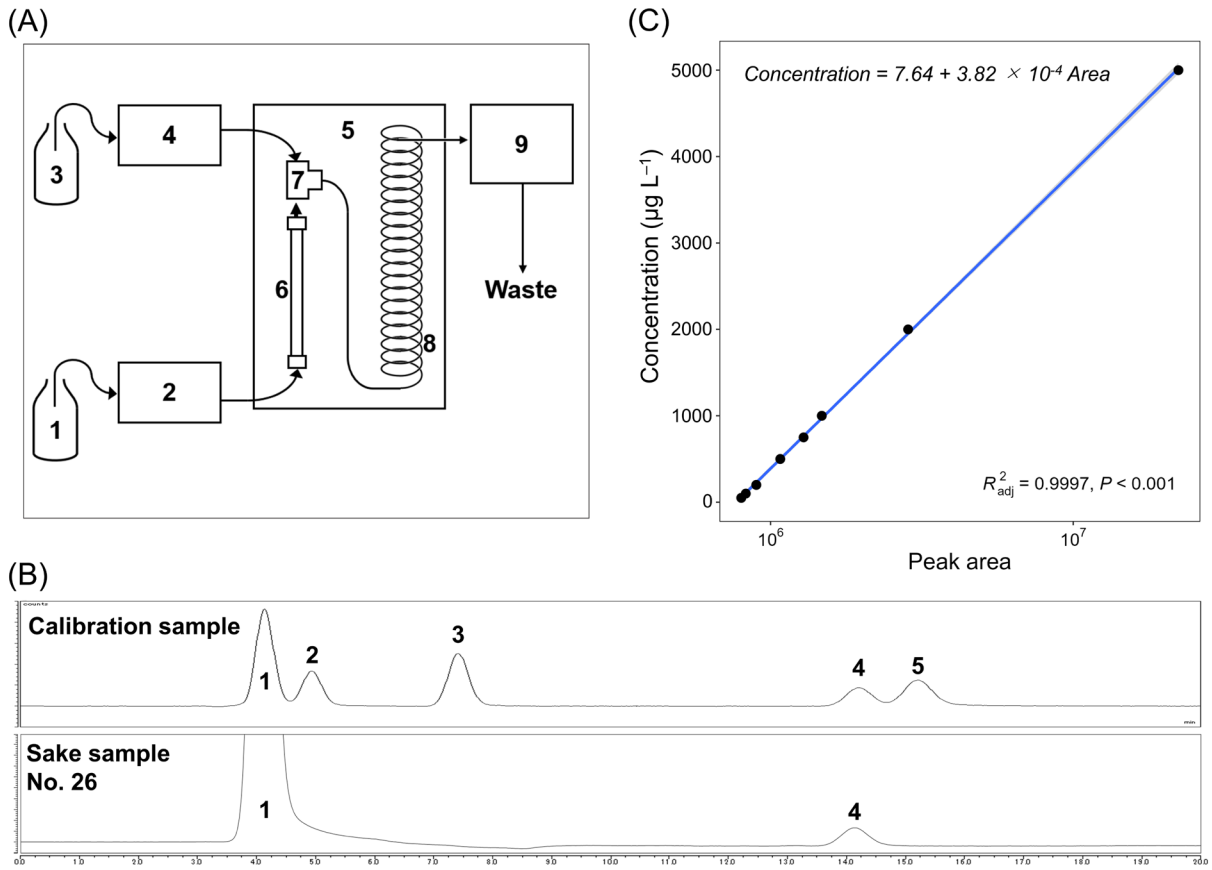
Headspace solid-phase microextraction-gas chromatography-mass spectrometry

Isovaleraldehyde was extracted from samples using a headspace solid-phase microextraction (HS-SPME) method. Samples were adjusted to 10% (v/v) ethanol concentration with pure water or ethanol. Aliquots (10 mL) of sample and 0.05 mL of internal standard (pentanal at 200 mg/L) were transferred to a 20 mL headspace vial. Volatile compounds were extracted using a polydimethylsiloxane/divinylbenzene SPME fibre (65 µm thickness, 1 cm length; Merck). Prior to extraction, the samples were equilibrated at 40°C for 5 min. The SPME fibre was exposed to the headspace of the sample vial for 10 min at 40°C, removed, and inserted into the GC injection port.

Analysis employed an Agilent 7890B/5975 GC-MS system equipped with a DB-5MS UI column (30 m length, 0.25 mm i.d, 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA).

Figure 1.

(A) Configuration of the HPLC system. 1: mobile phase. 2: pump for the mobile phase. 3: derivatisation reagent. 4: pump for the derivatisation reagent. 5: column oven. 6: column. 7: tee fitting. 8: reaction coil for fluorescent derivatisation. 9: fluorescence detector. **(B) chromatogram of aldehyde mixtures (top) and sake sample (bottom)**. 1: acetaldehyde and an unknown peak. 2: propionaldehyde. 3: butyraldehyde and isobutyraldehyde. 4: isovaleraldehyde. 5: valeraldehyde. The mixture contained 1000 µg/L of each compound. **(C) Calibration curve of isovaleraldehyde using the HPLC-PCD-FLD method**. The concentration of the standard samples used for the calibration curve were 50, 100, 200, 500, 750, 1000, 2000, and 5000 µg/L.



Splitless injection was performed for 2 min at 250°C. The GC oven temperature programme was 40°C for 2 min, ramp to 80°C at 5°C/min, then a further ramp to 250°C at 10°C/min, with a final hold at 250°C for 2 min.

The carrier gas was helium at a constant flow rate of 1 mL/min. The transfer line, quadrupole, and ionisation source temperatures in the GC-MS system were set at 240, 150, and 230°C, respectively. Electron impact mass spectra were operated in the simultaneous selected ion monitoring (SIM) mode with ionisation voltage at 70 eV. Each sample was analysed in triplicate. The ions monitored in SIM mode were m/z 86 for both isovaleraldehyde and pentanal. The concentration of isovaleraldehyde was determined by calculating the ratio of the peak area of isovaleraldehyde to the peak area of the internal standard of pentanal.

Statistical analyses

One way analysis of variance (ANOVA) was used to evaluate the HPLC-PCD-FLD and HS-SPME-GC-MS methods for the measurement of isovaleraldehyde in sake and sparkling sake. The analytical method (HPLC-PCD-FLD or HS-SPME-GC-MS) was included in the model as a fixed effect and the sample type (sake and sparkling sake) was included as a random effect to account for variations in the substrate of the samples. Linear regression analyses were applied to test (i) the relationship between the peak area and concentration of isovaleraldehyde from the HPLC-PCD-FLD method and (ii) the relationship between the isovaleraldehyde concentration in the samples using the different analytical methods. All the statistical analyses were performed using R version 4.1.2 (R Core Team 2021), and a *P*-value < 0.05 was considered significant for all results.

Results and discussion

The HPLC-PCD-FLD method achieved good separation of the isovaleraldehyde peak from the other aldehydes (Figure 1B), with a calculated limit of quantification of 87 µg/L (Table 1). The new method is capable of quantifying the concentration of isovaleraldehyde below the best estimated threshold (120 µg/L) (Isogai et al. 2005). Additionally, the results from the spike recovery results 88.7 ± 10.3% (mean ± SD) (Table 2). This indicates only minimal matrix effects on the measurement of sake or sparkling sake using the HPLC-PCD-FLD method. Collectively, these results suggest that the HPLC-PCD-FLD method offers adequate accuracy for the determination of isovaleraldehyde in sake samples.

There was no significant effect of the HPLC-PCD-FLD or HS-SPME-GC-MS methods on the determination of isovaleraldehyde in sake and sparkling sake (P = 0.518, F = 0.42, one-way ANOVA), suggesting that the new method is a viable alternative to the conventional method, particularly for isovaleraldehyde concentrations greater than the best estimated threshold from the sensory analysis. The calibration curve of the HPLC-PCD-FLD method showed excellent linearity, accomplished without an internal standard (adjusted R^2 = 0.9997, Figure 1C). Notably, the isovaleraldehyde values determined using the HPLC-PCD-FLD method exhibited a robust correlation with those from the HS-SPME-GC-MS method (adjusted R^2 = 0.9931, Figure 2A and Table 3). Although no significant effect of the two methods for isovaleraldehyde levels was observed, the HPLC-PCD-FLD yielded consistently higher results in sake (average of 21%). This may have been reflect sample preparation using the HS-SPME-GC-MS method, but this will require further study.

The most significant finding in this study is the lower relative standard deviation of the HPLC-PCD-FLD method compared to the HS-SPME-GC-MS method, particularly for samples with an isovaleraldehyde concentration exceeding the LOQ (Figure 2B). This finding suggests that (i) the accuracy for determining the isovaleraldehyde concentration in sake – using the HPLC-PCD-FLD method - is comparable to the HS-SPME-GC/MS method where the isovaleraldehyde concentration exceeds the best estimated threshold, and (ii) the HPLC-PCD-FLD method is a suitable tool for quantifying isovaleraldehyde in both sake and sparkling sake.

The conditions for post-column derivatisation were investigated in detail, including the column oven temperature, reaction coil length, and flow rate of the mobile phase and derivatisation reagent. An increase in column oven temperature, a decrease of the flow rate of the mobile phase and derivatisation reagent, an increase in the concentration of the derivatisation reagent, and an increase in the length of the reaction coil resulted in an improvement in derivatisation efficiency. Conversely, decreasing the flow rate of mobile phase caused peak broadening, and an increase in the concentration of the derivatisation reagent resulted in disturnabce of the baseline. Additionally, the mobile phase also influenced derivatisation efficiency. The peak resolution and derivatisation efficiency were improved by using a solution of methanol–acetic acid as the mobile phase rather than methanol-ammonium acetate or methanol-water. The gradient elution contributed to improvement of peak shapes and peak resolution but caused base line drift.

The HPLC-PCD-FLD method requires no specialised expensive equipment (e.g. post-column reactors), additional chemical reagents, large sample volumes,

Table 1.

The limit of quantification (LOQ) and the limit of detection (LOD) in the isovaleraldehyde analysis.

Detection method	LOQ (µg/L)	LOD (µg/L)	Determination range (µg/L)
HPLC-PCD-FLD	87	29	87-5000
HS-SPME-GC-MS	11	4	11-2100

Table 2.

Repeatability (n = 10) and recovery (n = 6) for isovaleraldehyde by HPLC-PCD-FLD

Repeatability (n = 10)			Recovery (n = 6)		
Spiked level in blank (µg/L)	Mean value (µg/L)	Coefficient of variation (%)	Spiked level in sake (µg/L)	Recovery rate (%)	Standard deviation (%)
100	115	8.7	500	88.7	10.3

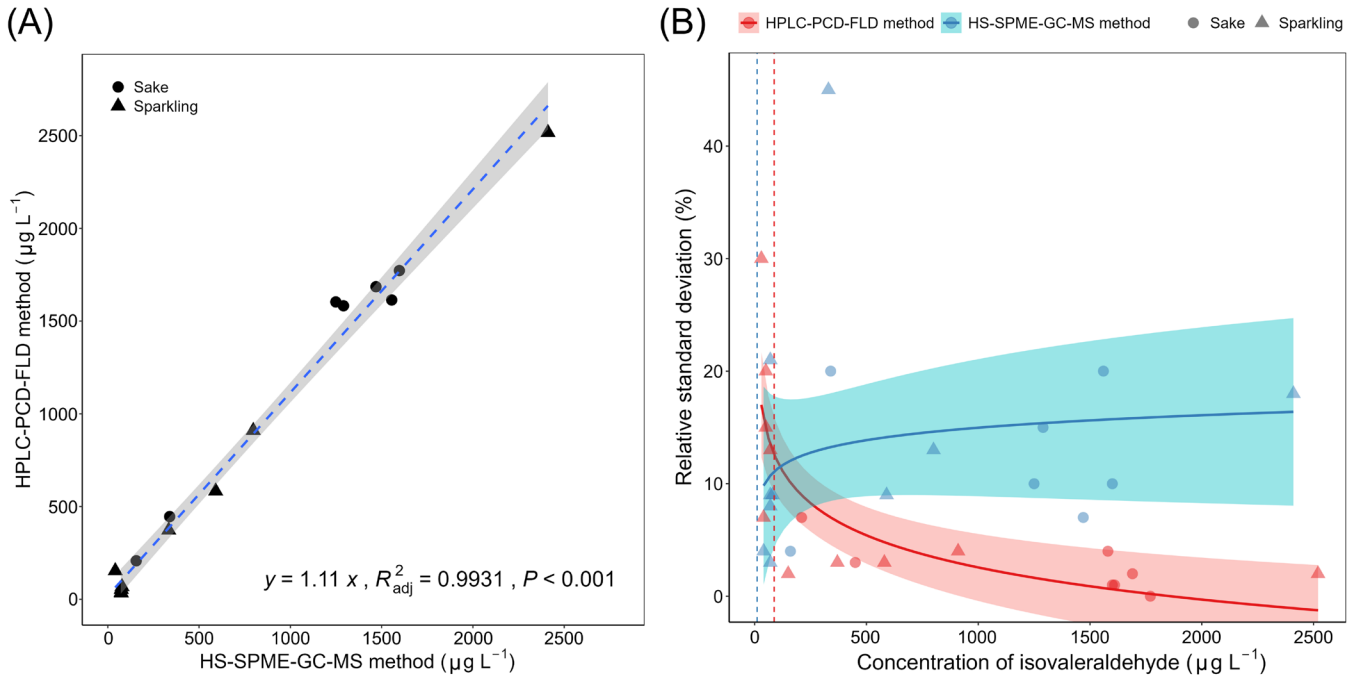
Table 3.

Isovaleraldehyde in commercial samples of sake and sparkling sake (n = 26) determined by the HPLC-PCD-FLD and HS-SPME-GC-MS methods. LOD = limit of detection.

Sample no.	Sample	HPLC-PCD-FLD (μg/L)	HS-SPME-GC-MS (μg/L)
1	Sparkling sake	LOD	44 ± 5 (11.4)
2	Sparkling sake	LOD	28 ± 7 (25.0)
3	Sparkling sake	584 ± 19 (3.3)	591 ± 56 (9.5)
4	Sparkling sake	2517 ± 41 (1.6)	2411 ± 435 (18.0)
5	Sparkling sake	373 ± 11 (2.9)	334 ± 151 (45.2)
6	Sparkling sake	LOD	46 ± 44 (95.7)
7	Sparkling sake	33 ± 10 (30.3) *	74 ± 16 (21.6)
8	Sparkling sake	68 ± 9 (13.2) *	78 ± 7 (9.0)
9	Sparkling sake	LOD	15 ± 3 (20.0)
10	Sparkling sake	LOD	23 ± 1 (4.3)
11	Sparkling sake	153 ± 3 (2.0)	41 ± 2 (4.9)
12	Sparkling sake	54 ± 8 (14.8) *	74 ± 2 (2.7)
13	Sparkling sake	LOD	41 ± 2 (4.9)
14	Sparkling sake	910 ± 36 (4.0)	797 ± 104 (13.0)
18	Sparkling sake	LOD	LOD
21	Sparkling sake	LOD	30 ± 4 (13.3)
22	Sparkling sake	37 ± 3 (8.1) *	73 ± 6 (8.2)
23	Sparkling sake	52 ± 10 (19.2) *	74 ± 7 (9.5)
24	Sparkling sake	LOD	15 ± 2 (13.3)
25	Sake	1603 ± 13 (0.8)	1249 ± 128 (10.2)
26	Sake	1772 ± 7 (0.4)	1597 ± 157 (9.8)
27	Sake	1582 ± 58 (3.7)	1291 ± 195 (15.1)
28	Sake	1614 ± 21 (1.3)	1555 ± 310 (19.9)
29	Sake	1685 ± 25 (1.5)	1469 ± 103 (7.0)
30	Sake	207 ± 14 (6.8)	156 ± 6 (3.8)
31	Sake	446 ± 13 (2.9)	339 ± 67 (19.8)

Figure 2.

(A) Isovaleraldehyde analysis by HPLC-PCD-FLD and HS-SPME-GC-MS. Circles: sake. Triangles: sparkling sake. Blue dashed line: linear regression line. Grey area: 95% confidence interval. **(B) The relationship between the isovaleraldehyde concentration and relative standard deviation**. Red dashed line: The LOQ of HPLC-PCD-FLD. Blue dashed line: LOQ of HS-SPME-GC-MS. Red circles: analysis of sake by HPLC-PCD-FLD method. Blue circles: analysis of sake by HS-SPME-GC-MS. Red triangles: analysis of sparkling sake by HPLC-PCD-FLD. Blue triangles: analysis of sparkling sake by HS-SPME-GC-MS. Blue area: 95% CI for HS-SPME-GC-MS. Red area; 95% CI for HPLC-PCD-FLD.



costly carrier gas, or lengthy processing time. Indeed, the combined cost of the derivatisation reagent and mobile phase used for HPLC-PCD-FLD is approximately half of that of the carrier gas required for HS-SPME-GC-MS analysis. Further, the method requires minimal sample preparation and the analysis using HPLC-PCD-FLD is achieved in less than a third of the time required by the HS-SPME-GC-MS method. The sample volume required for HPLC-PCD-FLD analysis of about 100 µL, compares favourably with the 10 mL required for the HS-SPME-GC-MS method (Osafune et al. 2020).

The HPLC-PCD-FLD method is well suited for the high throughput screening of isovaleraldehyde in unpasteurised sake and sparkling sake. It also has value for evaluating the impact of manufacturing processes on the concentration of isovaleraldehyde, and the determination of optimal storage conditions under retail conditions. In contrast, the reference HS-SPME-GC-MS method exhibits greater sensitivity (Osafune et al. 2020; Piergiovanni et al. 2023) and can detect isovaleraldehyde concentrations that are one-sixth of that detected by the HPLC-PCD-FLD method. Accordingly, the HS-SPME-GC-MS method adds value in analysing low isovaleraldehyde concentrations in sake samples. However, this approach is time intensive and comes with substantial operational costs amid concern about the shortage of helium. Consequently, it is recommended to select the appropriate method – either HS-SPME-GC-MS and HPLC-PCD-FLD – according to the analytical challenge.

Conclusions

This study presents a rapid, comparatively simple method without the need for pre-treatment using HPLC-PCD-FLD to quantify the concentration of isovaleraldehyde in sake and sparkling sake. The method can determine isovaleraldehyde above the reported threshold level from sensory evaluation. The new approach removes the need for sample preparation (e.g. adjustment of ethanol concentration), internal standard, or manual derivatisation. However, for isovaleraldehyde

levels in sake and sparkling sake below the best estimated threshold (120 µg/L), the HS-SPME-GC-MS technique remains the preferred choice due to its higher sensitivity. However, in a broader context, the newly developed method provides advantages over the HS-SPME-GC-MS method. It requires smaller sample volumes (ca. 100 µL v 10 mL) and analysis is quicker (< 33% of the time required by HS-SPME-GC-MS). These benefits enhance the efficiency but also improve the cost effectiveness of measuring isovaleraldehyde concentration in sake and sparkling sake. Consequently, the adoption of the HPLC-PCD-FLD method could expedite research into controlling isovaleraldehyde levels during the production and storage of both sake and sparkling sake.

Author contributions

Masayuki Takahashi: Methodology, investigation, project administration, visualisation, writing (original draft).
Fumikazu Akamatsu: Conceptualisation, investigation, writing (original draft), supervision.
Atsuko Isogai: Investigation, writing (review and editing).
Che-Chung Lin: Investigation, writing (review and editing).
Maki Kamimoto: Resources, writing (review and editing).
Akiko Fujita: Investigation, resources, writing (review and editing).

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Conflict of interest

The authors declare there are no conflicts of interest.

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