Journal of Food Composition and Analysis 133 (2024) 106344

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Characterisation of aroma-active compounds in dried *Dendrobium* spp. stems (Shihu) using GC-Olfactometry and a modified NIF-SNIF method

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ARTICLE INFO

Keywords:
Gas chromatography-olfactometry
Odour perception
Methional
1-octen-3-ol
Volatile compounds

ABSTRACT

Dendrobium orchid stems (Chinese name Shihu) are becoming popular ingredients in food and drinks such as wine, teas and desserts, however, its aroma chemistry is not currently understood. Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Olfactometry (GC-O) were used to analyse two *Dendrobium* species. The Nasal Impact Frequency-Surface of Nasal Impact Frequency (NIF-SNIF) method was employed on thirteen untrained panellists using a newly built open-source software ("nif-simple"). An expert annotated odour descriptions and intensities of NIF-SNIF peaks. Twenty-four volatile aroma compounds were found using GC-MS, with 22 shared compounds and 17 reported for the first time. GC-O NIF-SNIF indicated nine aroma compounds were aroma-active in *D. nobile* and *D. officinale*. Five were found in both species suggesting that they are species-independent markers. Aroma-active compounds were characterised as brown, mushroom, metallic, nutty and potato aromas. Methional (NIF 83 %) and 1-octen-3-one (NIF 81 %) were most frequently detected in *D. nobile* and *D. officinale* respectively, and this study proposed aroma formation mechanisms in dried *Dendrobium* stems. Modified Frequencies demonstrated that NIF-SNIF is an effective GC-O method for identifying key aroma-active compounds. Understanding the flavour properties of Shihu could enhance its global appeal and encourage NIF-SNIF to be used in future studies.

1. Introduction

Dendrobium stems are a famous Chinese traditional herb that has been consumed in East Asia since the Han Dynasty, spanning over 1800 years. They have a "bitter and slightly sweet" flavour profile and are commended not only for their traditional medicinal properties (Yuan et al., 2019) but also for their long history as food products. The *Dendrobium* genus belongs to the Orchidaceae (Orchid) family, which are epiphytic and lithophytic in nature, from which it gets its name in Chinese ("Shihu", 'living on the rocks'). In recent years, Shihu has received attention in the Chinese market, with the premium product, Tiepishihu, made from the stems of *Dendrobium officinale*, gaining prominence. Tiepishihu has been featured as an ingredient in nutraceutical beverages, health foods, Chinese tea and cooking (Meng et al., 2019; Kirkwood et al., 2023). Tiepishihu can be eaten fresh, or it is often preserved through freeze-drying or oven-drying (Teoh, 2016). Notably, Shihu is expanding its presence into various food applications, such as stir-fries, sauces, teas, wine, juices and desserts (Cakova et al., 2017), and more recently has been explored as an ingredient in soft candies (Zhenlin et al., 2022) and puffed *D. officinale* (Kim et al., 2020).

The sensory properties of a food product play an important role in initiating consumer interest and acceptance. For instance, the aroma of green tea is crucial for consumer approval as well as its market value (Yang et al., 2013). Volatile compounds are important plant secondary metabolites, which contribute to the aroma of foods and beverages. Volatile compounds are generally lipophilic liquids with low molecular

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https://doi.org/10.1016/j.jfca.2024.106344
Received 19 February 2024; Received in revised form 9 May 2024; Accepted 15 May 2024

mass and high vapour pressure at room temperature (Muhlemann et al., 2014). Plant volatile compounds are categorised according to their biosynthesis pathway: terpenoids (e.g. limonene), phenylpropanoids (e.g. coumarins), fatty acid derivatives (e.g. hexanal), amino acid derivatives (e.g. indole) and carotenoid derivatives (Dudareva et al., 2013). The advancement in analytical techniques has made volatile compounds one of the most extensively studied classes of plant secondary metabolites (Pichersky & Gershenzon, 2002). A few studies have investigated the volatile composition of Tiepishihu and identified the aroma compounds nonanal (*rose, orange*) and furfural (*bready*), which appear to be common across Tiepishihu studies (Chen et al., 2016; Dong et al., 2020; Ma et al., 2018). Overall, more than 250 volatile compounds have been reported in Tiepishihu (Chen et al., 2016; Ma et al., 2018; Dong et al., 2020), but it is unlikely that all will have an aroma. To the best of the authors' knowledge, the aroma-active compounds responsible for the aroma signature of Shihu are unknown.

The predominant methods employed for aroma analysis include Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Olfactometry (GC-O). Compounds that are separated and eluted from GC can be evaluated by an analytical detector (GC-MS) or by human assessors (GC-O). GC-O involves assessors smelling each peak as they elute from the GC column, and this is used to identify which volatile compounds are potentially aroma-active, i.e. those with concentrations higher than respective odour thresholds (OT). GC-O is a powerful tool that has allowed researchers to pinpoint and identify aroma-active volatile compounds within flavours and perfumes.

Three types of GC-O methods are used to identify key aroma-active compounds in flavour science: dilution analysis (e.g. Aroma Extract Dilution Analysis, AEDA), detection frequency (e.g. Nasal Impact Frequency-Surface of Nasal Impact Frequency, NIF-SNIF, and Modified Frequency) and time-intensity (e.g. OSME) (Welke et al., 2021). Dilution analysis involves GC-O analysis of stepwise dilutions of sample extracts until no odours are detected. Detection frequency methods are effective because they are simple and generally not as time-consuming as other methods (Welke et al., 2021). The number of aroma-active compounds identified through detection frequency is comparable to that of dilution analysis: in Sherry vinegar, there were 55 aroma peaks, aligning at the same retention times in both techniques (Callejon et al., 2008). Conceived by Pollien and colleagues at Nestle in 1996, the NIF-SNIF approach remains underutilised compared to the popular AEDA method (Welke et al., 2021). NIF-SNIF corresponds to the percentage of panellists that perceive an odour at a given retention time (% NIF), and NIF along with odour duration is used to calculate odour peak area (SNIF). Whilst AEDA typically uses between 1 and 3 assessors, NIF-SNIF involves 6–12 assessors (Welke et al., 2021; Pollien et al., 1997). By having a greater number of assessors, potential issues with large inter-individual differences in odour perception can be overcome using NIF-SNIF (i.e. specific anosmias, Welke et al., 2021). To the best of the authors' knowledge, the inclusion of SNIF data has only been described by two studies (Capobiango et al., 2015; Bezman et al., 2001) in which a 60 % (NIF) threshold had been applied (Capobiango et al., 2015).

While *Dendrobium* stems (Shihu) are an emerging food ingredient, there exists a gap in understanding which volatile compounds contribute to their aroma. The aim of this study is to characterise the volatile aroma profile of *Dendrobium* stems using GC-MS and GC-O to identify, for the first time, which volatile compounds in Shihu are aroma-active. This study will contribute to a deeper understanding of Shihu and bring the NIF-SNIF method to the forefront as an invaluable tool in characterising aroma compounds in a unique food product.

2. Materials & methods

2.1. Dendrobium nobile (DN) and Dendrobium officinale (DO)

Twelve *D. nobile* (DN) plants (Comet King variety) were purchased from Lawrence Hobbs Orchids Limited (Crawley Down, UK), and grown in a glasshouse with natural light between 18 and 22 °C, with weekly watering. Samples were harvested after 8 months, and stems were cut (1.43 kg) from all plants using a scalpel. After sampling, the stems were rinsed with deionised water (3 L) and drained. DN stems were then cut into 2 cm×0.5 cm diagonal pieces in a bucket. DN stems were dried at 70 °C for 48 h in a convection oven (MOV-112, Sanyo, Osaka, Japan), on foil trays before freeze-drying for a week to remove any residual moisture.

Dried Tiepishihu (*D. officinale*, DO) stems were purchased from Guizhou, China, and shipped for analysis. Samples were stored away from light and at room temperature and analysed within six months.

Both DN and DO stems were individually ground using a Ninja chopper (Ninja, UK) for 1.5 min then sieved through 800 μm then 500 μm sieves to obtain a fine powder. The powders were analysed within a month, and stored in a closed cupboard at room temperature (approx. 12–15 °C) away from sunlight.

2.2. Sample preparation

Each sample (in duplicate) of 0.50 g (± 0.01 g) was weighed into GC headspace vials (amber glass, 20 mL, 22.5 mm × 75 mm, Supelco, UK). Ultrapure water (Milli-Q, MQ), 0.50 g (± 0.01 g), was added to the vials, then capped with 18 mm silicone/PTFE septa (Supelco, UK). For GC-MS analysis, an internal standard (ISTD), 20 μl of 3-heptanone (0.001 % v/v) was added to each vial. Samples were all analysed in triplicate.

2.3. "nif-simple" application

The "nif-simple" application, designed by Andy Stringer (Stringer, 2023), was cloned from the GitHub repository, and run from a web server on a laptop. The application has a large green button in the middle of the app with three states: inject, start, and stop. The inject button is used for the analyst to synchronise with the GC retention time, i.e. after the GC has injected the sample. The inject button then changes to "start" and once pressed, it changes to "stop." This application was used for the NIF-SNIF procedure.

2.4. Panellists

2.4.1. Recruitment and introduction

A recruitment email was sent out to the School screening for NIF-SNIF panellists that were: i) ≤ 50 years old, ii) had a self-reported 'normal' sense of smell, and iii) had zero to very little experience using GC-O. Consent was given and this yielded 13 panellists (6 Male, 7 Female) recruited for the study.

NIF-SNIF panellists were given a separate ten-to-fifteen-minute introduction to the GC-O NIF-SNIF technique before the first session, and were shown how to use the "nif-simple" app to record their responses during the sessions. The panellists were not told when the odours might start, which is usual practice for GC-O analysis.

2.4.2. GC-O sessions

GC-O panellists were asked to not wear perfume or strongly scented clothes on the day of the assessment. The panellists were also instructed not to eat strong food or drink two hours prior to the analysis. They were allowed to drink water during the assigned two breaks. The panellists were given inconvenience compensation at the end of each session. After two preliminary sessions (results not shown), it was established that there were two regions of the GC run where no odours were present (9.0–9.5 and 15.0–15.5 min), and these were chosen as water breaks for panellists.

2.4.2.1. NIF-SNIF procedure. The detection frequency method, NIF-SNIF, from Pollien et al. (1997) was modified by asking the panellists to indicate when an odour was detected, without describing the odours, to keep the task simple. Using the “nif-simple” application, panellists recorded data by clicking “start” when they could smell an odour and clicking “stop” when they could no longer smell it. They were asked to repeat this for every odour perceived during a thirty-minute session (5–35 min of GC run). Each NIF-SNIF panellist took part in four sessions, analysing DO and DN, in duplicate over four days (within one month). To minimise panellists anticipating odours from memorising elution time, the order of samples and replicates was randomised.

2.4.2.2. Odour descriptions and intensities. One expert (10+ years of GC-O experience) was recruited for the project. The expert has worked on commercial and academic projects and performed GC-O on a wide range of food sample types. The analyst worked previously in the flavour industry and therefore trained and familiar with compound descriptors. The expert had not conducted GC-O analysis on Shihu before the present study, which lowered the likelihood of the assessor anticipating specific odours. They were tasked with analysing the GC-O odours of DN and DO by 1) verbally describing each odour (using a microphone and Dictaphone, without prompts) and 2) recording each odour intensity from a category ratio scale. The odour intensities were as follows: barely detectable, weak, moderate, strong, very strong, and extremely strong. Odour intensities from the expert were converted into numerical values based on a labelled magnitude scale from Borg (1982): barely detectable = 0.5, weak = 2, moderate = 3, strong = 5, very strong = 7, extremely strong = 10.

2.5. GC-MS and GC-O analysis

2.5.1. SPME-GC-MS of volatile compounds

Samples were analysed using GC-MS with a Trace 1300 Gas Chromatograph hyphenated to an ISQ series Single-Quadrupole Mass Spectrometer (Thermo Fisher, UK). Samples of DO and DN (0.5 g) were weighed into a vial (20 mL) and sample headspace was extracted for 30 min at 50°C using a 50/30 µm DVB/CAR/PDMS SPME fibre (Supelco, Sigma Aldrich, UK). The fibre was desorbed in the GC-MS inlet (splitless for 0.15 min at 250 °C) and compounds analysed on a ZB-WAX column (30 m × 0.25 mm inner diameter × 1 µm film thickness, Phenomenex Inc., Macclesfield, UK) fitted with a guard column (0.5 m × 0.25 mm inner diameter, Phenomenex Inc., UK). GC parameters: Carrier gas, Helium, (5.0 mL/min) at constant pressure (18 psi). GC oven programme: initial temperature 40 °C held for 2 min, increased to 240 °C at 6 °C/min, and held at the top temperature for 5 min. Mass spectrometer parameters: transfer line temperature at 250 °C, energy voltage was 70 eV, scanning range 35–300 *m/z* (0.2 dwell/scan time), and ion source at 200 °C.

Analytical standards isophorone (97 %), 1-octen-3-one (FG, 50 % in 1-octen-3-ol), 4-oxoisophorone (98 %), β-damascenone (1.1 – 1.4 %), octanal (99 %), 1-octen-3-ol (≥99 %), hexanoic acid (≥98 %), methyl octanoate (99 %), and 2,6-dimethylpyrazine (≥98 %, FG), were purchased Sigma Aldrich (Poole, UK). Methional (97 %) was purchased from Alfa Aesar (Thermo Fisher Scientific, Hemel Hempstead, UK). 3-(methylthio)propyl acetate (98 %) was purchased from abcr (Karlsruhe, Germany). Analytical standards were diluted to 0.01 – 0.1 % (v/v) in methanol, and run separately on the same SPME-GC-MS method as the samples.

2.5.2. SPME-GC-O of volatile compounds

GC-O configurations: A splitter (Trojan, UK) was installed at the end of a ZB-WAX column on a separate Trace 1300 GC such that the flow was split 1:1 to a detector (data not used for semi-quantification), and a custom-built odour port. The latter consisted of a capillary (1 m: 0.5 m × 0.25 mm), sheathed in a heated transfer line (200 °C) and which terminated in a glass cone for sniffing the eluted volatile compounds. Samples were extracted and analysed using the same method replicated from SPME-GC-MS.

A GC-O “standard” was used to confirm that the column eluants were arriving at the odour port and the detector simultaneously. The SPME method sampled a solution of isoamyl acetate and analysed on the GC-O system. A major peak at LRI 1139, identified on the NIST library to be isoamyl acetate, was detected as ‘banana’ at the odour port simultaneously by an expert. This confirmed that the transfer lines and the flow splitter were set up correctly.

Alkane standards were run every second day to account for any peak drift. The retention times of the alkanes were averaged, and then used to convert NIF times to linear retention indices (LRI).

2.6. Moisture analysis

Powder (0.25 g ± 0.02 g) from each sample was added to pre-dried foil pie tins (around 12.7 cm diameter) and dried in a convection oven at 105 °C for 24 h (Memmert UN10 oven, Memmert GmbH, Schwabach, Germany). Analysis in duplicate. Moisture content was calculated using Eq. 1:

$$\text{Moisture content (expressed as a decimal)} = \frac{\text{Difference in weight (g)}}{\text{Initial weight (g)}} \tag{1}$$

2.7. Data analysis

2.7.1. GC-MS identification of compounds and selection criteria

Data processing was conducted manually by using XCalibur Qual Browser 2.2 (Thermo Fisher, UK). Background subtraction was conducted with XCalibur, using blank raw data files to subtract from sample files.

Compounds were identified by three options 1) comparing the analyte spectra to NIST 2017 and FFNSC3 library spectra (probability match ≥70 %) (minimum criterion), 2) retention indices (Isothermal Kovats) from alkane series C7-C30, Eq. 2, and 3) as well as by running authentic standards.

$$\mathrm{RI} = 100\frac{T_n - T_x}{T_{n+1} - T_n} + 100 \quad n \tag{2}$$

where T_x is the retention time of the analyte of interest, T_n is the retention time of the preceding alkane and T_{n+1} of the proceeding alkane, and n is the number of carbons of the preceding alkane (Rostad & Pereira, 1986).

The peak identities were screened for aroma properties by searching their CAS registry numbers on The Good Scents Company Information System unless otherwise stated. Only compounds with an organoleptic description were included in the selection criteria. Odours from GC-O data that could be identified as an aroma compound were termed ‘aroma-active.’

2.7.2. GC-MS semi-quantification

The quantifier ion of each analyte of interest was noted (Appendix Table 1) and used to express concentrations relative to the internal standard (Eq. 3):

$$\text{Relative concentration (ppb)} = \frac{\text{Peak area of compound}}{\text{Peak area of ISTD}} \times \text{ISTD final concentration in sample (ppb)} \tag{3}$$

Since moisture content can impact the extraction efficiency of aroma compounds with headspace extraction (Ghiasvand et al., 2018), values from technical replicates were averaged and then corrected for moisture content using Eq. 4 ensuring that the data was expressed in a dry weight (DW) basis (Xiao et al., 2014; Shen & Shao, 2005).

$$\text{Corrected relative concentration (ppb, DW)} = \frac{\text{Relative concentration (ppb)}}{1 - \textit{moisture content}} \tag{4}$$

2.7.3. NIF-SNIF data processing

The responses obtained from the ’nif-simple’ app were transformed into binary code, where a value of 1 indicated the presence of an odour, and 0 indicated its absence. This binary data was utilised to generate basic aromagrams, with LRI serving as the x-axis and odour presence/

Table 1
Aroma compounds identified and quantified in two Shihu species (DN and DO) from GC-MS analysis.

	RT	LRI	Compound[a]	CAS	PubChem CID	Odour reference literature[2]	Odour threshold (ppb)[3]	Concentration (ppb, ±SD)[*]		Previously identified in Shihu	Base of identification
								DN	DO		
Aldehydes											
1	3.03	-[#]	2-methylpropanal	78–84–2	6561	Aldehydic, fresh	2.3	115.27 (±3.06)	11.47 (±0.74)	-	MS
2	4.80	929	2-methylbutanal	96–17–3	7284	Cocoa, nutty	1	34.75 (±0.84)	3.82 (±0.58)	-	MS
3	4.90	934	3-methylbutanal	590–86–3	11552	Fruity, dry, green, chocolate	2	246.43 (±6.47)	33.65 (±4.69)	-	Std, MS
4	8.93	1100	Hexanal	66–25–1	6184	Green, fatty, leafy	5	52.94 (±9.82)	160.80 (±54.62)	a	MS, LRI
5	14.53	1311	Octanal	124–13–0	454	Waxy, citrus	0.7	32.35 (±4.10)	13.78 (±1.01)	-	Std, LRI
6	17.20	1416	Nonanal	124–19–6	31289	Rose, orange	1	83.39 (±13.99)	16.05 (±1.58)	a,b	MS, LRI
7	20.76	1566	Benzaldehyde	100–52–7	240	Fruity, bitter almond	3500	207.17 (1 ±7.08)	80.63 (±4.30)	a	MS, LRI
8	22.91	1662	β-Cyclocitral	432–25–7	9895	Saffron, herbal	5	6.48 (±0.67)	7.99 (±0.57)	-	MS
9	19.21	1499	Furfural	98–01–1	7362	Bready	23000	45.98 (±3.08)	10.75 (±0.09)	a,c	MS, LRI
10	19.00	1492	Methional	3268–49–3	18635	Musty, potato, tomato	0.2	9.76 (±0.21)	0.25 (±0.05)	-	Std, LRI
							Sum	834.52	339.19		
Esters											
11	3.29	-[#]	Methyl acetate	79–20–9	6584	Sweet, fruity		290.57 (±58.75)	78.34 (±21.10)	-	MS
12	11.71	1204	Methyl hexanoate	106–70–7	7824	Fruity, pineapply	84	131.36 (±33.38)	307.54 (±89.52)	-	MS
13	14.40	1306	Methyl heptanoate	106–73–0	7826	Fruity, sweet, orris	4	72.84 (±21.52)	52.09 (±17.71)	-	MS
14	17.01	1409	Methyl octanoate	111–11–5	8091	Waxy, greeen	200	1504.22 (±357.32)	169.40 (±58.18)	-	Std, MS, LRI
15	26.36	1826	Methyl salicylate	119–36–8	4133	Minty, wintergreen	40	122.02 (±32.32)	ND	-	MS
							Sum	2121.01	607.37		
Acids											
16	18.66	1476	Acetic acid	64–19–7	176	Sour, vinegar		139.37 (±40.16)	181.68 (±49.96)	c	MS, LRI
17	27.15	1865	Hexanoic acid	142–62–1	8892	Fatty, sweaty	3000	44.96 (±10.85)	269.46 (±26.30)	-	Std, MS, LRI
							Sum	184.33	451.14		
Alcohols											
18	18.37	1464	1-octen-3-ol	3391–86–4	18827	Mushroom, earthy, green	1	26.04 (±2.78)	56.62 (±4.29)	-	Std, MS, LRI
19	28.79	1949	Phenethyl alcohol	60–12–8	6054	Floral, rose	1100	64.09 (±27.18)	ND	-	MS
							Sum	90.13	56.62		
Others											
20	15.83	1362	6-methyl-5-hepten-2-one	110–93–0	9862	Citrus, green	50	34.82 (±2.57)	48.57 (±1.23)	-	MS
21	22.45	1641	Isophorone	78–59–1	6544	Woody, tobacco		38.98 (±6.10)	2.69 (±0.23)	-	MS
22	30.00	2013	2-acetyl pyrrole	1072–83–9	14079	Musty, nutty	170000	8.51 (±2.00)	17.80 (±1.36)	c	MS
23	30.47	2039	β-ionone epoxide	23267–57–4	5352481	Fruity, orris		3.99 (±1.02)	28.28 (±2.80)	-	MS
24	37.43	-[#]	Dihydroactinidiolide	17092–92–1	27209	Fruity, ripe, apricot		5.33 (±1.24)	29.27 (±1.94)	d	MS
							Sum	91.63	126.61		
							Total	3321.62	1580.93		

[*] Mean concentration relative to the internal standard (dry weight basis). ppb – parts per billion, SD, standard deviation. LRI – match with retention index of in-house generated data using ZB-Wax column; Std, match with authentic standard using ZB-Wax column.

[#] LRI could not be generated because the analyte was out of the alkane series. a – Chen et al. (2016); b – Ma et al. (2018), c – Dong et al. (2020); d – Hu et al. (2020).

[a] Compounds identified using Mass Spectral (MS) libraries (≥70 % probability). [2]The Good Scents Company (2023); 3 Odour threshold in water from Leffingwell (2023) unless otherwise stated.

absence represented as 0/1 on the y-axis. These aromagrams were first combined and averaged for each panellist and then the aromagrams were summed, averaged and converted to a percentage, representing detection frequency (% NIF). If a signal was recorded by only 1/13 assessors in one replicate, it was excluded from the results to eliminate background noise. Odourants detected at the same LRI by at least 30 % participants were classed as aroma peaks and were then further considered for compound identification.

SNIF area: separate LRI and NIF values were entered for each panellist. Area under the curve was integrated (Excel, Microsoft, USA) using the NIF values for each LRI using the trapezoidal rule, summed, and then averaged across all panellists.

2.7.4. *Modified frequencies*

Modified frequencies (MF) were calculated by using Eq. 6 from Brattoli et al. (2013):

$$\text{MF } (\%) = \sqrt{\text{detection frequency } (\%) \times \text{odour intensity } (\%)} \tag{6}$$

where detection frequency was obtained from NIF (%) and odour intensity (% of maximum intensity, data obtained from expert analyst) (Brattoli et al., 2013).

3. Results and discussion

3.1. *Volatile composition of Dendrobium stems*

The composition of volatiles from *Dendrobium* stem powder (DN and DO) was established using GC-MS. In total, 24 aroma compounds were identified and their mean relative concentrations and odour descriptions from the literature are shown in Table 1. Aldehydes (10 compounds) were the main constituents of the *Dendrobium* powders followed by esters (5), acids (2), alcohols (2) and others (5). Twenty-two aroma compounds were in common between the two species, which showed similarities in their volatile aroma profile. The total concentrations of aroma compounds in DN (3322 ppb) are more than double that of DO (1581 ppb). Among the aldehydes, one sulphur-containing compound, methional (*musty, potato, tomato*) was found; one with an exceptionally low OT (Leffingwell, 2024). Methyl octanoate (1504 ppb) and methyl hexanoate (308 ppb) were found to be the major components in DN and DO, respectively. Compounds hexanal, nonanal, benzaldehyde, furfural, acetic acid, 2-acetylpyrrole and dihydroactinidiolide were qualitatively reported in stems of *D. officinale* (Chen et al., 2016; Dong et al., 2022; Hu et al., 2020; Ma et al., 2018), four of which being aldehydes. Apart from those seven compounds, 17 were found for the first time in the present study (including 1-octen-3-ol and methyl salicylate).

In terms of biosynthesis, a total of 15 aroma compounds are plant volatiles: eight compounds could be originating from fatty acids (hexanal, octanal, nonanal, methyl acetate, methyl hexanoate, methyl octanoate, hexanoic acid, 1-octen-3-ol), five from carotenoids (β-cyclocitral, 6-methyl-5-hepten-2-one, isophorone, β-ionone epoxide, dihydroactinidiolide), and two from amino acids (methional and phenethyl alcohol) (Dudareva et al., 2013). The presence of 2-methylpropanal, methylbutanals, furfural, and 2-acetylpyrrrole suggested the Maillard reaction occurred at some stage in the treatment of the samples (Jousse et al., 2002). This could arise from the drying process or during SPME extraction. The Maillard reaction, an example of non-enzymatic browning, is a complex network of chemical reactions which starts with a reducing sugar (e.g. glucose), condensing with a compound which has a free amino group (e.g. lysine) (Martins et al., 2000). This product, an N-substituted glycosylamine rearranges to form the Amadori rearrangement product, which is degraded into volatile compounds (Martins et al., 2000). DN had higher levels of Maillard reaction-derived aroma compounds than DO, including 2-methylbutanal, 3-methylbutanal, furfural, and methional. These compounds may be formed through thermal processing, in our case the drying at 70 °C (Jousse et al., 2002) and grinding of Shihu. The aroma compounds present in Shihu are the result of biosynthesis or formed through thermal processing. Volatile compounds extracted from Shihu using Solid Phase Microextraction (SPME) at 50 °C for 30 min might have influenced the formation of aroma-active compounds. Thus, future studies could explore extraction techniques at lower temperatures.

3.2. *Aroma-active compounds in Shihu*

3.2.1. *NIF-SNIF*

The NIF-SNIF technique, a well-designed and relatively underutilised GC-O method, was employed to pinpoint aroma compounds in *Dendrobium* stems. Fig. 1 displays NIF-SNIF aromagrams representing odour perceptions recorded from 13 panellists for two samples. There were 9 aroma peaks found in each *Dendrobium* species (GC-O, NIF>30 %). Most aroma-active compounds were ketones in DN and aldehydes in DO. It was possible to determine the chemical identity of most NIF peaks with the help of an expert analyst to include odour descriptions: 2/3-methylbutanal (brown, warm, bready), hexanal (green, waxy, aldehydic), 1-octen-3-one (mushroom, earthy, metallic), 2,3-dimethylpyrazine (cocoa, nutty), 1-octen-3-ol (mushroom, metallic, nutty), methional (brown, potato, amino acid), 4-oxoisophorone (honey) and β-damascenone (plastic, earthy, metallic). This study confirms that the aroma of Shihu is not from a single odorant but rather a combination of aroma-active compounds. NIF-SNIF also helped to identify three aroma-active compounds that were not detected by GC-MS but by odour descriptions and matching authentic standards (β-damascenone, 1-octen-3-one, 2,3-dimethylpyrazine).

Five aroma-active compounds were found to be in common between the two species (as shown in Fig. 2): 2/3-methylbutanal, 1-octen-3-one, 1-octen-3-ol, methional and β-damascenone, and so it may be assumed that these aroma compounds are species-independent. As with GC-MS data previously, NIF-SNIF can also help to speculate that the Maillard reaction, lipid oxidation, beta-carotene degradation, and plant biosynthesis are pathways involved in Shihu flavour generation (Dudareva et al., 2013). In our case, there are reactions and therefore precursors (e.g. sugars, amino acids, pigments) in common between DO and DN.

Methional was found to be the most reported aroma-active compound (defined as the highest frequency) in DN (NIF 83 %) followed by 2/3-methylbutanal (71 %), and 1-octen-3-ol (63 %). In the case of DO, the most reported aroma-active compound was 1-octen-3-one (81 %) followed by 1-octen-3-ol (73 %), hexanal (73 %), and methional (69 %).

Methional is considered an essential ’character impact compound’ in potatoes (Ulrich et al., 2000). Methional has a remarkably low OT of 0.2 ppb (Leffingwell, 2023), which was present at relatively low concentrations (9.76 ppb and 0.25 ppb for DN and DO, respectively) and its high odour-activity suggests that minor compounds can play an important role in aromas. The low OT combined with the content in DN is the reason for the high NIF value. Oppositely, abundant compounds from GC-MS such as methyl octanoate and benzaldehyde had no olfactory response.

1-octen-3-one (81 % NIF) was the most reported aroma-active compound in DO, and along with 1-octen-3-ol (73 %), they have mushroom and metallic odours. Many molecules with eight carbon atoms are produced by fungi as an act of defence, and particularly 1-octen-3-ol, is formed by the enzymatic transformation of linoleic acid (Xu et al., 2019). GC-O and omission tests confirmed that both are key aroma compounds in *Volvariella volvacea* mushrooms (Xu et al., 2019). It is likely that the drying conditions impact the content of 1-octen-3-ol and 1-octen-3-one. 1-octen-3-ol is reduced to the fresh mushroom-smelling 1-hydroxy-3-octanone via 1-octen-3-one (Delcros et al., 2023), so this might explain the presence of both compounds in Shihu.

The unique aroma compound for DN, 4-oxoisophorone (honey), was detected by 42 % of panellists in DN. It has also been reported in saffron stigma (Moraga et al., 2009). In another study, the same C_9

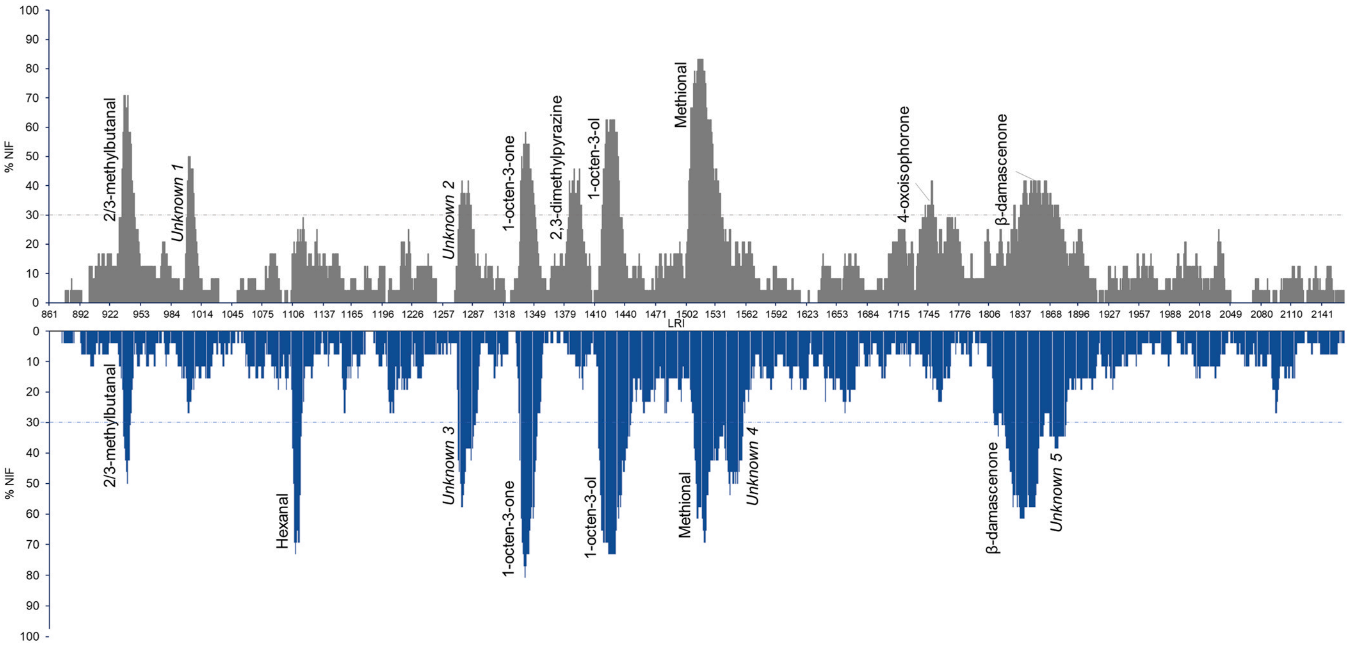
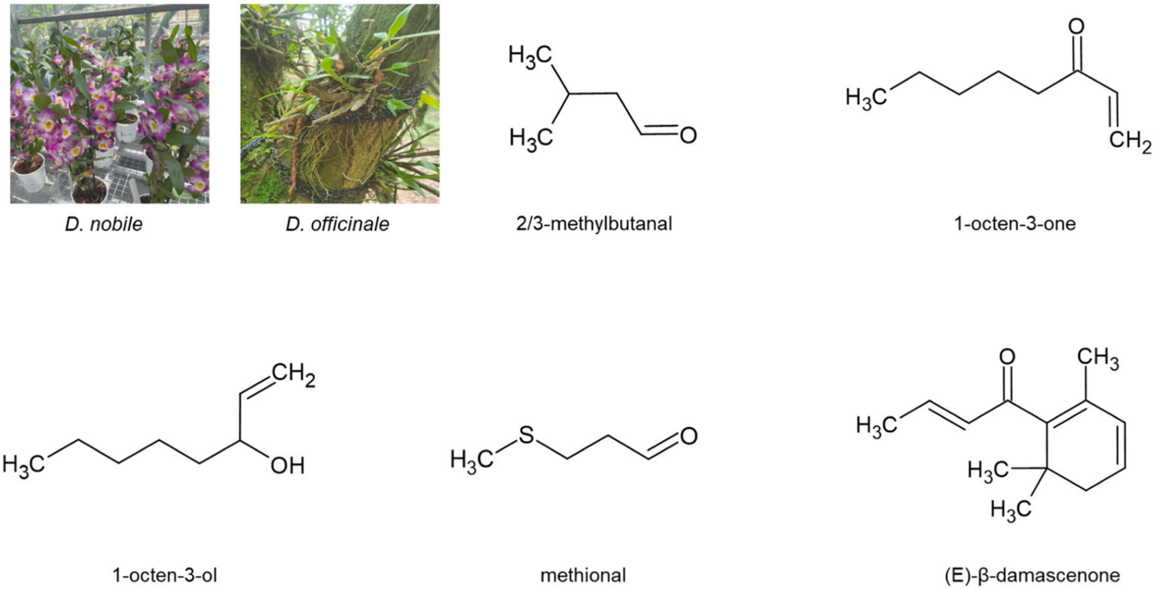


Fig. 1. Nasal Impact Frequency/Surface of Nasal Impact Frequency (NIF-SNIF) aromagrams of DN (top, dried at 70 °C for 48 h) and DO (bottom) using GC-olfactometry. The aromagram depicts the average perception of odours by 13 participants. The height of the peaks corresponds to the percentage of participants (% NIF) who detected the aroma. Duplicate analysis was conducted. Grey and light blue line indicates 30 % NIF threshold. Labelled compounds were identified through the utilisation of Mass Spectral libraries, Retention Indices, and Authentic Standards (more information on identification in Table 2).



D. nobile

D. officinale

2/3-methylbutanal

1-octen-3-one

1-octen-3-ol

methional

(E)-β-damascenone

Fig. 2. Five aroma-active compounds, common to *D. nobile* and *D. officinale*.

carotenoid-derived compound was found to be one of the key aroma-active compounds in *D. officinale* flowers along with 1-octen-3-ol, hexanal, nonanal and phenylacetaldehyde (Yang et al., 2022). The presence of aroma-active compounds β-damascenone and 4-oxoisophorone suggested that β-carotene degradation may be a key mechanism of flavour formation in Shihu. In saffron, it has been shown that safranal, another picrocrocin product, concentrations can be controlled by higher drying temperature and duration (Gregory et al., 2005). It is likely that oven drying increases the aroma of Shihu compared to freeze-drying so this could be useful for enhancing Shihu's flavour.

Using NIF-SNIF, it was possible to find differences in the aroma-active compounds from the two species, for example, 4-oxoisophorone in DN and Hexanal in DO, which show unique aroma compounds may be contributing to the flavour profile of DN. Table 2 supported the GC-MS data in Table 1, showing that the concentration of aroma-active compounds was slightly higher for DO (268.32 ppb) than DN (260.7 ppb). Further work is required to analyse a broader range of species and varieties to determine whether 4-oxoisophorone and hexanal are exclusively unique.

SNIF area was higher for DO (11317) than DN (9875) showed the SNIF area DO (11317, 4.49 min). In both samples, β-damascenone and methional had the highest SNIF area (21.6–24.3 % of total area), owing to their long odour persistence. Methional had the highest SNIF area in vacuum-distilled cheddar cheese extract (O'Riordan and Delahunty,

Table 2
Full list of aroma-active compounds in DN and DO stems.

	Name	LRI Waxa	LRI Wax Lit.	PubChem CID	CAS	Odour reference (literature)b	Odour description[#]	Max Odour intensity[#]	NIF-SNIF		Concentration in ppb (st dev)*	Base of identification	OT (ppb)[c]	Previously found
									% NIF	SNIF area (% area)				
Dendrobium nobile (DN) stems														
1	2/3-methylbutanal	937	950[3]	11552; 7284	590–86–3; 57456–98–1	Fruity, dry, green, chocolate	Brown, warm, bready	5.0	71	936 (9.5 %)	210.84 (±60.41)	O/MS/STD/LRI	1.0–2.0	Table 1
2	*Unknown 1*	998	-	-	-	-	-	-	50	480 (4.9 %)	-	-	-	-
3	*Unknown 2*	1272	-	-	-	-	-	-	42	592 (6.0 %)	-	-	-	-
4	1-octen-3-one	1340	1310[5]	61346	4312–99–6	Metallic, mushroom	Mushroom	3.0	58	888 (9.0 %)	8.05 (±0.69)	O/STD/LRI	0.005	-
5	2,3-dimethylpyrazine	1383		22201	5910–89–4	Nutty, coffee	Cocoa, nutty	5.0	46	718 (7.3 %)	4.31 (±0.54)	O/STD/LRI	35000	-
6	1-octen-3-ol	1427	1472[4]	18827	3391–86–4	Mushroom, earthy, green	Mushroom, metallic, nutty	5.0	63	1185 (12.0 %)	26.31 (±3.15)	O/MS/STD/LRI	1.0	Table 1
7	Methional	1516	1470[3]	18635	3268–49–3	Musty, potato, tomato	Brown, potato, amino acid	7.0	83	2068 (20.9 %)	9.76 (±0.21)	MS/O/STD	0.2	Table 1
8	4-oxoisophorone	1749	1668[2]	62374	1125–21–9	Sweet, tobacco, musty	Honey	2.0	42	611 (6.2 %)	1.43 (±0.27)	O/STD/LRI	25.0[d]	-
9	β-damascenone	1852	1835[3]	5366074	23696–85–7	Sweet, fruity, rose, plum	Plastic	0.5	42	2397 24.3 %)	ND	STD/LRI	0.002	-
							Totals	28.5		**9875 (100 %)**	**260.7**			
Dendrobium nobile (DO) stems														
10	2/3-methylbutanal	940	950[3]	11552; 7284	590–86–3; 57456–98–1	Fruity, dry, green, chocolate	Brown, cheesy	3.0	50	434 (3.8 %)	31.78 (±3.78)	O/MS/STD/LRI	1.0–2.0	Table 1
11	Hexanal	1107	1111[4]	6184	66–25–1	Green, fatty, leafy	Green, waxy, aldehydic	5.0	73	790 (7.0 %)	162.05 (±54.05)	O/MS/LRI	5.0	Table 1
12	*Unknown 3*	1272	-	-	-	-	Green, grassy	2.0	58	843 (7.4 %)	-	-	-	-
13	1-octen-3-one	1335	1310[5]	61346	4312–99–6	Metallic, mushroom	Mushroom, earthy, metallic	5.0	81	1280 (11.3 %)	18.55 (±1.50)	O/STD/LRI	0.005	-
14	1-octen-3-ol	1425	1472[4]	18827	3391–86–4	Mushroom, earthy, green	Mushroom, metallic, nutty	5.0	73	2103 (18.6 %)	55.69 (±3.95)	O/MS/STD/LRI	1.0	Table 1
15	Methional	1510	1470[3]	18635	3268–49–3	Musty, potato, tomato	Brown, potato, amino acid	5.0	69	1615 (14.3 %)	0.25 (±0.05)	O/MS/STD	0.2	-
16	*Unknown 4*	1549	-	-	-	-	Potato	3.0	50	1057 (9.3 %)	-	-	-	-
17	β-damascenone	1818	1835[3]	5366074	23726–93–4	Sweet, fruity, rose, plum	Plastic, earthy, metallic	2.0	62	2442 (21.6 %)	ND	STD/LRI	0.002	-
18	*Unknown 5*	1874	-	-	-	-	-	-	38	753 (6.7 %)	-	-	-	-
							Totals	30.0		**11317 (100 %)**	**268.32**			

[1]Chen et al. (2016); [2]Yang et al. (2022);[3] Piornos et al., 2017; [4] Chen et al. (2019); [5] Pozo-Bayón et al., 2007. ND= Not detected.

[a]LRI based on apex SNIF peak,

[b]Odour descriptions from The Good Scents Company (2023). [b]LRI based on apex SNIF peak. [c]Leffingwell (2023) – in water - using the higher threshold if a range was provided. [d]Yang et al. (2022) - detected in 5 g/l tartaric acid containing 12 % ethanol.

[#]n=1. O – odour port; MS – mass spectral match; LRI – linear retention index match; STD – match using authentic standard; ppb – parts per billion; OT, odour threshold; SD, standard deviation.

Quantifier ions are listed in Supplementary Table 1.

* Mean concentration relative to the internal standard (dry weight basis).

2001) and fermented bamboo shoots (Fu et al., 2002). In terms of SNIF values, Varming et al. (2004) noted that their SNIF values in blackcurrant juice generally corresponded to the GC-MS peak area. We found that one aroma-active compound, β-damascenone, could not be quantified, so SNIF values did not correspond to the peak area. In this instance, the human nose was able to detect β-damascenone with a higher sensitivity than the MS. β-damascenone is valued by flavourists for its ability to add a ripe berry character to fruit flavours, which was difficult before β-damascenone was commercially available (Wright, 2009).

In Table 2, we found that higher concentrations in one sample meant that NIF and odour intensity were higher. Delahunty et al. (2006) showed that increasing concentrations may not increase detection frequency but rather odour intensity. In the present study, higher concentrations in one sample seemed to have higher SNIF areas, which was also found by Bezman et al. (2001) in orange juice, so the peak area may be more quantitative than just NIF. Odour duration is included in the aromagram by Capobiango et al. (2015) but peak areas were not listed or discussed anywhere.

NIF-SNIF is more representative of the human population because of the number of panellists (NIF-SNIF with 6–12 assessors versus AEDA with 1–3 assessors, Welke et al., 2021; Pollien et al., 1997). Unlike expert-trained panels, NIF-SNIF allowed untrained panellists to detect odours, potentially providing data more closely aligned with consumer perception. This reduces the risk of missing out important aroma compounds, which may occur through techniques that use 1–3 panellists. Knowledge of aroma-active compounds can help select species/varieties, environmental and processing conditions that can enhance the aroma of Shihu. GC-O has been used previously to differentiate Madeira wines and was able to assess and guide the production of wines (Campo et al., 2006).

Overall, the aroma signature of Shihu may be attributed to the compounds mentioned above and GC-MS identities were comparable to that of NIF-SNIF. The results suggest that drying DN at 70 °C (for 48 h) was sufficient for developing Maillard and carotenoid-derived aroma-active compounds. It must be acknowledged that different extraction methods could give differing GC-O results – a study reported methyl dihydrojasmonate and isoeugenol as aroma-active using Stir Bar Sorptive Extraction but not from liquid-liquid extraction (Wijaya et al., 2005). In this study, a small sample size was employed due to the nature of GC-Olfactometry, requiring 13 participants to each attend four sessions over two samples, so the aroma compounds reported are specific to the analysed samples. Further work can be done to replicate the GC-MS analysis with other *Dendrobium* stems and incorporate process (drying) replicates. Furthermore, aroma compounds that have a long after-odour may be masking NIF-SNIF responses of other co-eluting compounds. Future work could include slower GC temperature ramps and splitting each panellist session into two parts (to assess both the start and the end of the chromatogram) (Delahunty et al., 2006). Although this would make the technique have a lower throughput, it would improve odour resolution (Delahunty et al., 2006). Sensory studies (using Chinese panellists) would also be required to ascertain whether the aroma-active compounds are positive or negative attributes to the flavour of Shihu.

3.2.2. *Unknown aroma-active compounds in Shihu*

Since this is the first time for GC-O analysis to be applied to the stems of *Dendrobium*, there remain some unknowns that contribute to the aroma of Shihu (three peaks remain unidentified in DO). Unknown 4 in DO, described as "potato", on the shoulder of methional has not been attributed to any compound. Unknown 4 could be carry over from methional, and the odour would explain this. It may also be another compound with a similar odour description to methional. Similarly, in the case of Unknown 5, this could be from β-damascenone carryover. By a process of elimination, Unknown 4 is unlikely to be 3-(methylthio) propyl acetate (methionyl acetate) due to a difference in LRI (1640, standard). In almonds, there were three aroma-active peaks in a row on a Stabilwax-DA column with potato attributes: methional (LRI 1474), 2-ethyl-3,5-dimethylpyrazine (LRI 1475) and 2,3-diethyl-5-methylpyrazine (LRI 1509) (Erten & Cadwallader, 2017). In the present study, this corresponds to a peak with the following mass fragmentation (*m/z* and abundance): 135 (100 %), 136 (65 %), 97 (17 %), 121 (16 %), 112 (13 %), 108 (12 %), 43 (10 %), 107 (9 %), 42 (9 %), 111 (8 %). This has a similar fragmentation to 2,3-dimethyl-5-ethylpyrazine; ions 136 and 135 are likely to be from fragments C8H12N2 and C8H11N2, respectively.

3.2.3. *Modified frequencies*

A drawback of NIF analysis, and other detection frequency techniques, is the assumption that NIF is related to odour intensity (Delahunty et al., 2006). When aroma compound concentrations exist above a population's detectable odour threshold, increasing concentrations do not necessarily lead to an increase in detection frequencies (Delahunty et al., 2006). Taking these considerations into account, the modified frequencies (MF) formula, which includes detection frequencies and

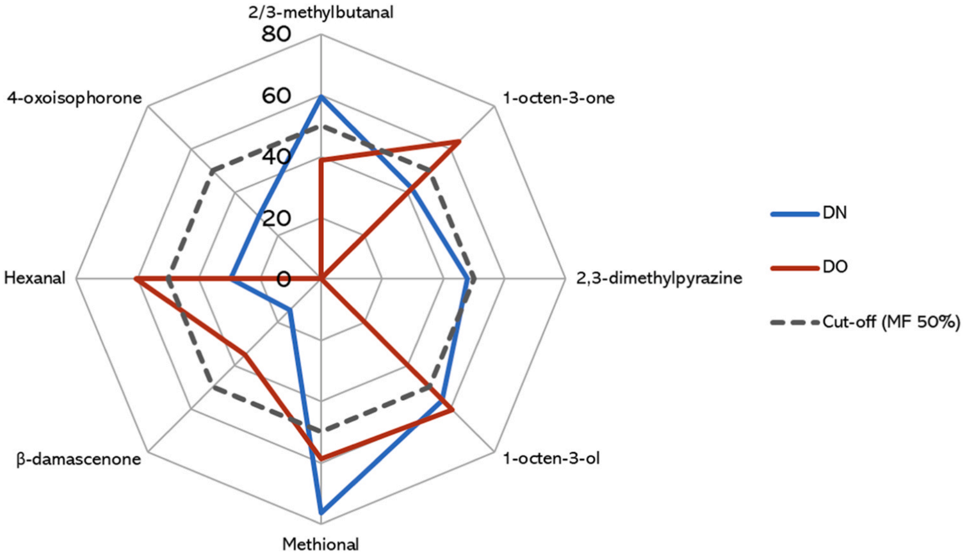


Fig. 3. Modified frequency (MF) (%) values for DN and DO samples. Calculated as the square root of % NIF × Intensity (% of maximum). A threshold cut-off at MF 50 % is displayed as the dotted line.

odour intensities was applied to Shihu in order for “key” aroma-active compounds to be determined. In MF, compounds with values higher than 50 % represent key compounds (Bueno et al., 2011; Brattoli et al., 2013). MF is calculated as the square root of % NIF × odour intensity. This has been used successfully by Lester et al. (2021) and Bueno et al. (2011) to study age-related hyposmia and aroma compounds in lamb meat. In this study, by applying a cut-off threshold of 50 %, the most important key aroma-active compound in DN was methional (76 %) followed by 2/3-methylbutanal (60 %) (Fig. 3). In DO, the most important key aroma-active compound was 1-octen-3-one (64 %) followed by 1-octen-3-ol, methional and hexanal (all at 60 %).

The results from MF suggest that odour intensities must follow a similar trend to detection frequency. These results support that NIF-SNIF can identify the most important aroma-active compounds, but modified frequency might underestimate the importance of compounds such as β-damascenone, 62 % NIF in DO, as it was below the threshold for MF. The downfall of using MF is that, just like in Odour Active Values, it assumes that an aroma compound concentration is linear with intensity, which is certainly not the case for all flavour compounds. For example, β-damascenone, shown previously by Pet’ka et al., 2005, where concentrations exceeding 5 ppm cause a decrease in odour intensity. SNIF may be more appropriate for determining the importance of compounds with unusual slopes of psychophysical function like β-damascenone. β-damascenone is reported to have varied olfactory thresholds in individuals. In a study by Plotto et al. (2006), panellists who could not perceive beta-damascenone (45+ years) within a range found in orange juice had orthonasal thresholds 692 times higher than those who could perceive it. This supports the use of NIF-SNIF on a panel of thirteen panellists to reduce inter-individual variation.

In this study, it was not possible to annotate some compounds such as Unknown 5 because the expert did not report an odour at the corresponding LRI. It is certain that an untrained panel could be used to generate odour descriptions, but this should be done on separate runs focusing only on assessing odour descriptors (Delahunty et al., 2006) so the task is not made more difficult or tiring for panellists.

4. Conclusion

This study successfully determined the aroma-active compounds in an emerging food product, *Dendrobium* stems. Known as "Shihu" in Chinese, they are becoming a popular ingredient in modern Chinese cooking and within the Chinese food industry. This study found NIF-SNIF to be an effective GC-O method to determine aroma-active compounds in Shihu. Nine aroma peaks were defined as aroma-active using NIF-SNIF, including 1-octen-3-one and 4-oxoisophorone. Analysis showed that methional and 1-octen-3-one were the most frequently detected aroma compounds in two *Dendrobium* species used in this study. Our findings contribute to the existing research on *Dendrobium*: using GC-MS this study identified and semi-quantified 17 volatile compounds that were previously unreported (i.e. ionone epoxide). NIF-SNIF helped to identify three aroma-active compounds (β-damascenone, 1-octen-3-one, and 2,3-dimethylpyrazine), which were not detected by GC-MS but by odour descriptions and matching authentic standards. The GC-O aroma-active compounds were characterised as brown, mushroom, metallic, nutty and potato. The research not only gives a further understanding of the aroma-active compounds responsible for the flavour of Shihu but also supports the use of the NIF-SNIF. To improve the assignment of aroma-active compounds, this article recommends including odour descriptions (using separate runs) in future studies. The findings of this study will contribute to enhancing the international recognition and popularity of Shihu.

Funding statement

This work was supported by GoldenKeys High-Tech Materials Co., Ltd. (贵州金之键高科技材料股份有限公司,91520900MA6DL1ER7N, 黔石科合[2019007]), and the Biotechnology and Biological Sciences Research Council (BBSRC) [grant number BB/V017284/1].

Ethical statement

All participants took part voluntarily and received a small compensation. Subjects provided written consent to participate. The study was approved by the Research Ethics Committee, Faculty of Medicine and Health Sciences, University of Nottingham (FMHS 21-0622).

CRediT authorship contribution statement

Ni Yang: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Yingjian Xu:** Writing – review & editing, Supervision, Conceptualization. **Ian Fisk:** Writing – review & editing, Supervision, Conceptualization. **Aidan Kirkwood:** Writing – original draft, Visualization, Project administration, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data that support the findings of this study are available on the Nottingham Research Data Management Repository at https://rdmc.nottingham.ac.uk/handle/internal/11422.

Acknowledgments

The research group would like to thank Guizhou Forestry Bureau (贵州省林业局), Guizhou Academy of Forestry (贵州省林业科学研究院), Research & Development Center of Guizhou Dendrobium Industry (贵州石斛产业发展研究中心), Department of Science and Technology of Guizhou Province (贵州省科技厅), Guizhou Service Center For Academician Expert (贵州省院士专家服务中心) for their support on the resource of natural products and basic related knowledge. The authors would also like to thank Andy Stringer for creating the “nif-simple” application, Joseph Godrich and Prof Andy Taylor for proof-reading, Avinash Kant for his advice on GC-O analysis, Lawrence Hobbs Orchids Limited for advice on the upkeep of Dendrobium plants, and all participants involved in the study.

Appendix A. Supporting information

Supplementary data associated with this article can be found on this article at https://doi.org/10.1016/j.jfca.2024.106344.

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