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Characterization of Odor-active Compounds in Pond Apple (*Annona glabra* L.) Fruit

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Abstract

Pond apple (*Annona glabra* L.) is a tree native to the tropical and subtropical regions of America. It has an edible yellowish-orange pulp that can be made into jam and jelly. Volatile constituents of pond apple fruit were isolated by headspace-solid phase microextraction (HS-SPME) and analyzed by gas chromatography with GC-FID and GC-MS. A total of 48 volatile compounds were detected, 46 of them were positively identified in pond apple fruit. This study revealed potent odorants that are responsible for the overall aroma of this fruit by application of HS-SPME-GC-O analysis and odor activity values. A total of twenty-five odorants were identified as potentially contributing to the aroma of the fruit, from which ethyl 3-methylbutanoate, α-pinene, myrcene, methyl 2-hydroxy-4-methylpentanoate, ethyl hexanoate, *p*-cymene, limonene, (*Z*)-β-ocimene and linalool were the most odor-active compounds. The strong agreement between the results of the OAV strategy and HS-SPME-GC-O suggests that the latter has significant potential as a quick and straightforward tool for monitoring and evaluating the aroma quality of pond apple fruit.

Keywords: Pond apple, Annona glabra, HS-SPME, GC-MS, GC-O, odor activity value

1. Introduction

Flavor is a key factor in consumer preference and acceptance of food. It involves taste, aroma, and mouthfeel, all of which are influenced by complex interactions between volatile and non-volatile compounds (Schieberle and Hofmann, 2011). The study of flavor is crucial in the food industry to develop products that meet consumer preferences. Additionally, it plays a key role in the creation of new foods, the improvement of existing products, and quality control. Aroma and flavor are important quality parameters in foods, caused by chemical substances. However, the active compounds responsible for aroma and flavor, which are naturally present in foods, show extreme differences in their olfactory and taste activity, defined as the relationship between concentration and sensory threshold (Kilic-Buyukkurt, 2024).

Numerous volatile compounds have been detected in various food products, and some of them are responsible for giving a food its characteristic aroma. These compounds are known as aroma-active compounds or key odor components (Gou et al., 2021, Cengiz et al., 2023). A fundamental stage in aroma studies involves the isolation and identification of the distinctive aromatic constituents present in the food product. Gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS) are the commonly used techniques for characterizing these compounds (Wang et al., 2021; Kilic-Buyukkurt, 2024).

In contrast to more traditional isolation methods, headspace solid-phase microextraction (HS-SPME) has been extensively used for isolating volatile

compounds from many fruits (Pino and Barzola-Miranda, 2020; Cuevas-Glory et al., 2020; Pino and Trujillo, 2021; Gou et al., 2021; Liu et al., 2022). This technique is rapid, simple, sensitive, and solvent-free. When combined with gas GC-MS and GC-O (HS-SPME-GC-O), it becomes a highly effective method for identifying odor-active compounds.

Annona glabra L. is a fruit native to the tropical and subtropical regions of America and is distributed from southern Florida, Central America, the Antilles, and Caribbean islands to South America, mainly in mangrove areas, but also is found in Asian countries. The common names include pond apple, alligator apple, monkey apple, corkwood (USA), bagá (Cuba) and araticum do brejo, araticum do Rio (Brazil) (Pino, 2010). The fruit is oblong to spherical and apple sized or larger, 7–15 cm long and up to 9 cm in diameter, and falls when it is green or ripening yellow. It has an edible yellowish-orange pulp that can be made into jam and jelly. The aroma is sweet and the taste is reminiscent of ripe honeydew melon.

Although some studies reported the volatile compounds profile of the pond apple (Santos et al., 1998; Pino et al., 2002; Thang et al., 2013), the isolation methods used were based mainly in distillation techniques which can potentially lead to some loss of aroma due to the high-temperature application. In addition, no previous reports were found in the literature (Scopus and Web of science) about the odor-active compounds of this fruit.

Therefore, the aims of this study were to determine the volatile constituents' profile of the pond apple fruit by HS-SPME as well as to determine the odor-active compounds.

2. MATERIALS AND METHOD

2.1. Materials

Undamaged ripe fruits (6 samples) were collected directly from different plants grown in a commercial orchard in the vicinity of Havana. Pulp was separated from skin and embedded seeds, then the whole pulp was passed through a 60-mesh sieve to yield the final pulp.

Standards of chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and some were generously given by Robertet (Grasse, France). A *n*-alkane solution (C_6–C_{32}) from Sigma–Aldrich (St. Louis, MO, USA) was used to determine the linear retention index (LRI) of each compound.

2.2. Headspace solid-phase microextraction analysis

A DVB/CAR/PDMS fiber, 1 cm long, provided by Supelco (Bellefonte, PA, USA) was chosen for analyses. This combination allows the fiber to adsorb a wide range of compounds, from non-polar to moderately polar, making it suitable for diverse volatile flavors (Balasubramanian and Panigrahi, 2011). The experimental procedure was similar those reported earlier (Pino and Barzola-Miranda, 2020; Pino and Trujillo, 2021). For each test, pulp (3 g), Milli-Q water (3 mL) and sodium chloride (1 g) were placed into a 15-mL vial sealed with a PTFE-lined screw cap. The extractions were carried out under magnetic stirring at 600 min^{-1} at 40 °C for 30 min, after equilibration of the samples for 10 min at the same temperature. Assays were made by triplicate.

2.3. GC-FID and GC-MS analysis

GC-FID analysis was carried out using a HP-6890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, USA) equipped with a flame ionization detector and a DB-5 MS capillary column (30 m x 0.25 mm x 0.25 µm; J & W Scientific, Folsom, CA). The column oven was initially set to 50 °C for 2 min and then programmed to rise to 250 °C at a rate of 4 °C/min, where it was held for an additional 10 min. Hydrogen served as the carrier gas, flowing at a rate of 1 mL/min. Both the injector and FID temperatures were maintained at 250 °C. Thermal desorption was made at 250 °C in splitless mode for 2 min, using a 0.75 µm inlet liner. Quantitative analysis of the active

odorants was done by calculating their relative quantitative correction factors with the "single-point correction method", which is similar to the standard addition method (Liu et al., 2020). The methodology used was described earlier (Pino and Barzola-Miranda, 2020).

GC-MS analysis was carried out on a using a QP-2010 Ultra (Shimadzu, Japan) system with a similar capillary column and chromatographic parameters as for the GC-FID. The MS worked in electron impact mode at 70 eV ionization energy and in scanned mode from m/z 35 to 350, at 1.3 scan/s. Ion source and interface temperature were 250 °C. Identification of compounds was achieved by matching linear retention indexes and mass spectra with those of chemical standards and commercial libraries (NIST 05, Wiley 6, NBS 75 k and Adams 2001).

2.4. Direct SPME-GC-O

The procedure was performed following the methodology described earlier (Pino and Roncal, 2016). GC-O analyses were performed with a gas chromatograph Konik 4000 A instrument (Konik, Barcelona, Spain). The end of the fused silica capillary was connected to a deactivated Y-shaped glass splitter dividing the effluent into two equal parts, which were transferred via two deactivated fused silica capillaries (25 cm x 0.25 mm) to a sniffing port and FID. The sniffing port consisted of a cylindrically shaped aluminum device with a beveled top and a central drill hole connected to the capillary. Oven temperature was held at 250 °C, carrier gas (hydrogen) flow rate was 1 mL/min and nitrogen (30 mL/min) was used as makeup gas. The SPME fiber was introduced into the GC port (splitless mode for 2 min, injector temperature at 250 °C). Because no chromatographic separation was carried out by the short capillary, volatile compounds arrived simultaneously at the sniffing port. For each SPME extract, three trained assessors perceived and evaluated the resulting global odor. Sensory analysis sessions were performed only after a suitable training: panelists were first familiarized with fresh pond apple and asked to agree on a common list of descriptors. A similarity test was carried out by triplicate on the SPME odors obtained from the same homogenate. Sniffers were asked to smell the reference pulp (3 mL) contained in a plastic cup sealed with a pierced cap at 23 °C. They had to memorize the odor and then describe it using the descriptors list. Then they evaluated with the direct GC-O device the different extracts, rating their similarity to the reference using a 10 cm scale ranging from 0 (close to the reference) to 10 (far from the reference). Panelists had to smell the reference before each sample evaluation.

2.5. Gas chromatography–olfactometry of the SPME profile

The odor active compounds trapped on the fiber were analyzed by GC-O on gas chromatograph Konik 4000 A instrument (Konik, Barcelona, Spain) with the same operating parameters that those above-mentioned for GC-FID analysis. For each odor stimulus, a panelist from a trained panel of three trained assessors recorded the detection time and gave an odor description. GC-O frequency analysis was performed following the methodology described earlier (Pino and Barzola-Miranda, 2020). Detected odors (quality and retention times) were marked in the chromatogram. Each sample was sniffed in duplicate by each panelist. Zones of the chromatogram which were detected with the same descriptor, at least two times, were considered as odor zones.

2.6. Odor detection threshold determination

For certain compounds, the orthonasal odor detection thresholds were determined using a previously described multiple paired comparison test (Pino and Mesa, 2006). In summary, 40 unscreened and untrained assessors participated in finding these thresholds. For each concentration tested, at least 100 responses were collected. The test

involved presenting assessors with multiple samples, alongside an aqueous reference solution. Each sample was individually compared to the reference by smell to detect any potential differences. The statistical analysis used to determine the odor detection thresholds calculated the concentration corresponding to 50% positive responses.

2.7. Determination of odor activity value (OAV)

The OAVs for some odorants were calculated by the ratio of the compound content in the pulp and its odor detection threshold.

3. RESULTS AND DISCUSSION

Given that this study aimed to identify the odor-active compounds using HS-SPME-GC-O to analyze the volatiles from the fruit, the overall aroma of the captured volatiles was assessed at the sniffing port (direct SPME-GC-O). The three experts noted a high similarity (global odor 8.9 ± 0.5) to the original pulp, so the DVB/CAR/PDMS fiber for considered adequate to characterize the volatile compounds.

Forty-eight volatile compounds were detected and forty-six of them were positively identified (Table 1). The major chemical families were terpenes, aliphatic esters, aldehydes, and alcohols. Quantitatively, the chemical composition was dominated by terpenes hydrocarbons, mainly myrcene, (*Z*)-β-ocimene, limonene and α-pinene. In general, similar composition were found using steam distillation (Santos et al., 1998; Pino et al., 2002), but in the most recent report the sesquiterpenes β-caryophyllene, germacrene D, α-cadinol and β-elemene were the major ones (Thang et al., 2013).

Classical GC-O was applied to the compounds trapped in the SPME fiber to find odorant zones in the olfactograms and then identify the compounds potentially responsible for these odors. The odors detected by the panelists, together with the compounds tentatively identified as responsible for those odor impressions are given in Table 2. According to the detection frequency, only 22 compounds were found with values ≥ 4 (67% of coincidence among the assessors). Among them, ethyl 3-methylbutanoate, α-pinene, myrcene, methyl 2-hydroxy-4-methylpentanoate, ethyl-hexanoate, limonene and (*Z*)-*β*-ocimene were found with the higher detection frequency.

The application of HS-SPME-GC-O does not provide unrestricted information about the contribution of single odorants to the overall aroma. Since the volatiles are completely vaporized during GC-O, this method is based on odor thresholds in air (Schmitt et al., 2016). Therefore, to evaluate the contribution of volatile compounds to the aroma of pond apple fruit, they were quantified in the pulp, and the data was used to calculate the OAVs (Table 2). A total of 25 compounds were detected as potentially contributing to the overall fruit aroma (OAV ≥ 1). Among them, myrcene, α-pinene, limonene, methyl 2-hydroxy-4-methylpentanoate, (*Z*)-*β*-ocimene, ethyl 3-methylbutanoate and ethyl hexanoate were the most important. The potentially important odorants identified using the odor activity approach represent a refinement over those identified by HS-SPME-GC-O, addressing and correcting some of the limitations of this technique.

Table 1. Volatile compounds identified in pond apple fruit

Compound	LRI	Area %	Compound	LRI	Area %
Ethyl acetate	612	0.69	Nonan-2-one	1090	0.25
3-Methylbutanal	657	0.03	Nonan-2-ol	1098	0.01
Pentan-2-one	688	0.38	Ethyl 3-hydroxyhexanoate	1130	0.05
3-Methylbutan-2-ol	693	0.43	Methyl octanoate	1127	0.24
Hexanal	802	0.01	Ethyl octanoate	1197	0.04
Ethyl butanoate	805	0.01	Linalool	1097	0.58
Butyl acetate	811	0.01	Terpinen-4-ol	1177	0.53
2-Furfural	836	0.03	α-Terpineol	1189	0.25
(*E*)-2-Hexenal	856	0.03	Ethyl decanoate	1391	0.03
Ethyl 3-methylbutanoate	859	0.01	Germacrene D	1485	0.03
Methyl hexanoate	927	0.39	Geraniol	1253	0.04
α-Pinene	939	11.89	Carvacrol	1299	0.01
Methyl 2-hydroxy-3-methylpentanoate[a]	972	0.15	Methyl eugenol	1406	0.04
Sabinene	975	0.41	Methyl-3-hydroxydecanoate	1430	0.06
β-Pinene	979	0.14	(*E*)-Methyl isoeugenol	1492	0.11
Octan-3-ol	991	0.01	Bicyclogermacrene	1500	0.11
Myrcene	994	41.27	Elemicin	1557	0.05
Methyl 2-hydroxy-4-methylpentanoate	996	1.42	Spathulenol	1578	0.09
Ethyl hexanoate	998	0.48	(*Z*)-Methyl jasmonate	1649	0.03
α-Phellandrene	1003	3.53	α-Cadinol	1654	0.05
p-Cymene	1025	0.85	α-Bisabolol[a]	1686	0.06
Limonene	1029	14.05	(*E,E*)-Farnesol	1725	0.01
β-Phellandrene	1030	0.69	Methyl 3-hydroxytetradecanoate	1862	0.04
(*Z*)-β-Ocimene	1037	20.32	Hexadecanoic acid	1962	0.03

[a]*Tentative identification (only by matching LRI and mass spectra from libraries). tr: < 0.1%.*

Table 2. Aroma active compounds of pound apple fruit

Compound	Descriptor	Content (µg/kg)	ODT[a] (µg/kg)	DF[b]	OAV[c]
3-Methylbutanal	Malty	20	0.5[d]	5	40
Pentan-2-one	Ethereal-fruity	300	10[e]	5	30
Hexanal	Fatty-green	340	2.4[d]	4	4
Ethyl butanoate	Fruity	10	0.8[d]	4	12
(*E*)-2-Hexenal	Green, leaf	20	10[e]	3	2
Ethyl 3-methylbutanoate	Fruity	10	0.023[d]	6	435
Methyl hexanoate	Ethereal-fruity	310	70[f]	4	4
α-Pinene	Pine	9370	6[g]	6	1562
Methyl 2-hydroxy-3-methylpentanoate	Fruity	120	2.4[h]	5	50
Sabinene	Woody	320	37[e]	4	9
β-Pinene	Woody	110	82[e]	3	1
Myrcene	Sweet-balsamic	32520	1.2[d]	6	27100
Methyl 2-hydroxy-4-methylpentanoate	Sweet-musty	1120	2	6	560
Ethyl hexanoate	Fruity	380	1[f]	6	380
α-Phellandrene	Fresh	2780	200[e]	4	14
p-Cymene	Carrot-like	670	6.2[e]	5	108
Limonene	Lemon-like	11070	10[g]	6	1107
β-Phellandrene	Herbaceous	540	40	4	14
(*Z*)-β-Ocimene	Herbaceous	16010	34	6	471
Nonan-2-one	Green, fruity	200	5[e]	5	40
Methyl octanoate	Winy	190	200[g]	3	1
Ethyl octanoate	Fruity	30	5[e]	4	6
Linalool	Flowery	460	6[f]	5	77
Terpinen-4-ol	Lilac	420	130[e]	4	3
Geraniol	Rose-like	30	1.1[d]	5	27

[a]Odor detection threshold; [b]GC-O detection frequency; [c]Odor activity values calculated by concentration/odor detection threshold ratio; [d]from Czerny et al. (2008); [e]Van Gemert (2011); [f]Pino and Mesa (2006); [g]Leffingwell & Assoc. (2011); [h]Steinhaus et al. (2009).

In terms of similarities between HS-SPME-GC-O and OAV strategies, HS-SPME-GC-O has proven to be highly effective, as it was able to identify 22 of the 25 most important odorants based on OAV criteria with minimal effort. To fully understand the role of these odorants, different reconstitution techniques and sensory evaluations will need to be conducted, which will allow for a more accurate assessment of the discrepancies observed between the two ranking methods.

4. CONCLUSIONS

A total of 48 volatile compounds were detected, 46 of them were positively identified in pond apple fruit. This study revealed potent odorants that are responsible for the overall aroma of this fruit by application of classical HS-SPME-GC-O analysis and odor activity values. A total of twenty-five odorants were identified as potentially contributing to the aroma of the fruit, from which myrcene, α-pinene, limonene, methyl 2-hydroxy-4-methylpentanoate, *(Z)*-β-ocimene, ethyl 3-methylbutanoate and ethyl hexanoate were the most odor-active compounds. The strong agreement between the results of the OAV strategy and HS-SPME-GC-O suggests that the latter has significant potential as a quick and straightforward tool for monitoring and evaluating the aroma quality of pond apple fruit.

Declaration of conflicting interests

No potential conflict of interest was reported by the author.

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