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Odour active aroma compounds of sea fig (*Microcosmus sulcatus*)

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Abstract

Sea fig is an original shellfish appreciated for its powerful "marine, iodized" flavour. Different methods of analysis by gas chromatography, coupled with olfactometry (GC–O), were studied and compared. Subsequently, odour-active aroma compounds of sea fig extract were analyzed by OSME and CHARM analysis. Twenty-nine olfactive areas were observed by OSME, and 18 by CHARM analysis. Volatile compounds of the extract were analyzed by mass spectrometry and specific detectors associated with the GC. Twenty molecules, responsible for these odours were elucidated. Among them, 12 were directly identified by GC–MS, and the remaining 8 only by GC–O and standard sample injection. Moreover, 10 volatile sulfur-containing compounds were revealed as the major olfactive contributors. The two principal character-impact compounds possessed "marine, fresh" and "fishy, crustaceous" odours. The first could not be identified; however, trimethylamine was attributed to the other, and defined as a key compound of sea fig aroma.

Keywords: *Microcosmus sulcatus*; Aroma; Seafood; Odor active compounds; GC–O; CHARM; OSME

1. Introduction

As the most important criterion of marine product acceptability is aroma, industrialists are very concerned with the identification of the flavour compounds in seafood. Consequently, they are widely studied (Shahidi, 1998; Spurvey, Pan, & Shahidi, 1998) and can be described as sweet, distinctly plant-like, often accompanied by metallic and fishy attributes. Aldehydes, ketones, nitrogen and sulfur-containing compounds are considered as the most important contributors to the odour of shellfishes. Aldehydes contribute to the plant-like aroma, and ketones to the sweet floral and fruity flavour of raw crustaceans. Alkyl-pyrazines with roasted notes, and sulfur-containing compounds with cabbage-like or meaty notes, contribute to the overall cooked odour of shellfish (Spurvey et al., 1998).

However, few reports have been published concerning raw shellfishes (De Quiros, Lopez-Hernandez, Gonzalez-Castro, De la Cruz-Garcia, & Simal-Lozano, 2001; Pennarum, Prost, & Demaimay, 2002a; Yasuhara, 1987). Moreover, only the character-impact compounds of fresh oyster have been investigated (Kirn et al., 2000; Pennarum, Prost, & Demaimay, 2002b; Pennarum, Prost, Haure, & Demaimay, 2003; Piveteau et al., 2000). These components: hex-3(*E*)-en-l-ol, decanal, undecan-2-one and nona-3,6(*E,Z*)-dien-1-ol, have been described as representative of freshness and sensory qualities of shells, with fresh and marine odours. Gas chromatography-olfactometry (GC–O), combining GC separating capabilities and human nose sensibility, is a complementary method for the detection of potent

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fragrant molecules present in complex mixtures. Methodologies of GC–O can be classified into detection frequency methods: nasal impact frequency (NIF) (Pollien et al., 1997), intensity methods, odour specific magnitude estimation (OSME) (McDaniel, Miranda-Lopez, Watson, Micheals, & Libbey, 1990) and dilution methods, (Acree, 1993). Among dilution methods, combined hedonics of aromatic response measurement (CHARM) (Acree, Barnard, & Cunningham, 1984) and aroma extract dilution Analysis (AEDA) (Ullrich & Grosch, 1987) are generally used.

The aim of the present work was to apply olfactometric methods to sea fig extract to determine its most potent odourants, and to evaluate their importance among volatile and semi-volatile compounds from this shellfish. Sea fig (*Microcosmus sulcatus*) is a marine organism, an ascidia of the tunicates branch. This shellfish, commonly called "violet" in France, is consumed by Mediterranean people and is much appreciated for its powerful "marine" and "iodized" flavour. To our knowledge, sea fig's volatile compounds have never been studied. The purpose is to determine key components responsible for its specific organoleptic characteristics. These molecules were tentatively detected and identified by specific detectors: atomic emission detector (AED), pulsed flame photometric detector (PFPD), thermoionic specific detector (TSD) and mass spectrometry, associated with the GC. Retention indices on polar and apolar columns allowed us to identify a few of them only perceived by GC–O. Each structure was confirmed by injection of commercial or synthesized standard samples.

2. Materials and methods

2.1. Material

Fresh commercial sea figs (1 kg), *M. sulcatus,* were obtained from the Bay of Sete on the Mediterranean coast of France in November 2003. Extraction was performed immediately after purchasing.

2.2. Obtaining extract

Twenty individual sea figs (1 kg) were shelled and the flesh with internal remaining sea water (300 g) was extracted using dichloromethane (HPLC Grade, 400 g) under ultrasonic treatment (47 kHz, 25 °C, Branson 3510, Branson ultrasonics, Danbury, Connecticut) for 1.5 h. The organic layer was dried over anhydrous sodium sulfate and concentrated to 2 ml using a RapidVap evaporator (Labconco, Kansas City, MO). The obtained extract was filtered by HPLC syringe filter (25 mm, 0.45 μm PVDF, Alltech associate, USA). The extract was stored at low temperature (4 °C) prior to analysis.

2.3. Chemicals

Methanethiol, butane-2,3-dione, dimethyldisulfide, pent-2(*Z*)-enol, butyric acid, hex-3(*Z*)-enol, hept-4(*Z*)-enal, furfurylthiol, oct-l-en-3-one, eucalyptol, *p*-cresol, 2-acetyl-2-thiazoline, nona-2,6(*E*,*Z*)-dienal and nona-2,6(*E*,*Z*)-dienol were purchased from Sigma–Aldrich (Saint Quentin, France), trimethylamine from Merck Eurolab (Briare, France), 3-methylbut-2ene-l-thiol, and 2-methyl-3-furanthiol from Oxford Chemicals (Cleveland, United Kingdom). 2-Methylthiazolidine (Fernandez, Dunach, Fellous, Lizzani-Cuvelier, & Loiseau, 2002) and octa-l,5-dien-3-ol (Lin, Welti, Vera, Fay, & Blank, 1999) were synthesized using published procedures and characterized by GC–MS and ^{1}H, ^{13}C NMR.

2.4. GC–O

GC–O was conducted on a 6890 GC (Agilent Technologies, Massy, France), equipped with a Flame Ionization Detector (FID) and a sniffing port ("Sniffer 9000", Brechbuhler Scientific Analytical Solutions, Grand-Lancy, Swiss) equipped with a HP 7683 Series Injector, using a HP-1 (polymethylsiloxane, J & W Scientific) column (50 m × 0.32 mm; film thickness, 0.52 μm) or an HP-INNOWAX (polyethylene glycol, J & W Scientific) column (60 m × 0.32 mm; film thickness, 0.5 μm). The oven was started from 40 to 130 °C at 2 °C/min, then from 130 to 250 °C at 4 °C/min and held at 250 °C for 25 min for the apolar column, and from 60 to 220 °C at 2 °C/min, then held at 220 °C for 10 min, for the polar column. Conditions were as follows: temperature conditions for injector and detector: 250 °C; gas vector: He; constant flow: 1.5 ml/min. Retention indices were determined by calculation within a range of alkanes used as standards, starting from C5 to C28.

OSME analyses was performed with 8 trained panellists. For CHARM analyses, 5 dilutions with a factor of 3 were applied. For each dilution analysis, two panellists were required, each one replacing the other every twenty minutes.

2.5. GC–MS analysis

GC–MS analysis was carried out using an Agilent 6890/5973A system (Agilent technologies, Massy, France), equipped with a multifunction automatic sampler (Combi-Pal, CTC Analytics, Zwingen, Swiss), using an HP-1 (polymethylsiloxane, J & W Scientific) column (50 m × 0.20 mm; film thickness, 0.5 μm) and an HP-INNOWAX (polyethylene glycol, J & W Scientific) column (60 m × 0.25 mm; film thickness, 0.5 μm). Helium was used as carrier gas at a constant flow rate of 1.5 ml/mm. GC conditions were the same as above for GC–O, respectively, with apolar and polar columns. Mass spectra analyses (electronic impact) were per-

formed at 70 eV and the mass range was 35–350. Identification of constituents was based on comparison of the retention indices with pure references, and on computer matching with commercial mass spectra libraries (Nist98, Nistrep94 Library) and with a home-made library built up from pure substances and the MS literature data (Adams, 1995; BACIS, 1999; Davies, 1990; Jennigs & Shibamoto, 1980).

2.6. GC-AED

GC coupled with AED, monitored on sulfur-selective acquisition was used for the detection of the sulfur-containing compounds. The system consisted of a 6890 GC (Agilent Technologies) equipped with a multifunction automatic sampler (Combi-Pal, CTC Analytics) and coupled to an AED G2350A (Agilent Technologies). The GC was fitted with an HP-INNOWAX (polyethylene glycol, J & W Scientific) column (60 m × 0.25 mm; film thickness, 0.5 μm). GC conditions were the same as above for GC–MS with the polar column. The temperatures of the AED were as follows: inlet, transfer line and cavity block: 250 °C. Element selective chromatograms were obtained for carbon and sulfur-containing compounds (emission wavelengths at 179 and 181 nm, respectively). Helium was used for plasma at 35 ml/min. The reagent gases were: oxygen used at 33.6 ml/min, hydrogen used at 7.2 ml/min, and auxiliary gas (nitrogen/methane mixture) used at 33 ml/min.

2.7. GC-PFPD and GC-TSD

GC-PFPD and GC-TSD analyses were accomplished using a CP 3800 GC (Varian, les Ullis, France) equipped with a multifunction automatic sampler (Combi-Pal, CTC Analytics), using two HP-1 (polymethylsiloxane, J & W Scientific) columns (50 m × 0.20 mm; film thickness, 0.5 μm) connected with PFPD and TSD, respectively. GC conditions were the same as above for GC–MS with an apolar column.

Conditions for PFPD analysis. Injector and detector temperature: 200 °C, gas flow rate to the detector was set to be 14.5 ml/min for hydrogen, 17 ml/min for air 1 and 10 ml/min for air 2.

Conditions for TSD analysis. Injector and detector temperature: 250 °C, current ceramic bead power ranged from 3.2 to 3.5 A. Gas flow rate to the detector was set at 4 ml/min for hydrogen and 170 ml/min for air.

3. Results and discussion

3.1. Preliminary study

Different extraction methods (solvent extraction, vacuum headspace, solid phase micro extraction, using different fibres) were studied. For each extraction method, samples odours were compared with the intact matrix by eight skilled tasters. In the case of solvents and vacuum headspace-generated extracts, static mode evaluation, using smelling strips, was performed. SPME studies were carried out by GC–O, using a 200 °C heated deactivated capillary column (1 m × 0.32 mm) to avoid chromatographic separation. Dichloromethane solvent extraction was found to provide the best odour-representative extracts and was then adopted for this study. Obtained extract is filtered for further analyses. Being a complex mixture of volatile, semi-volatile and non-volatile compounds, extract was difficult to analyse. However, this method led to a good estimation of the character impact compound concentration in sea fig.

A comparison of methods commonly applied for GC–O analyses was already described by Abbott, Etiévant, Issanchou, and Langlois (1993), Guichard, Guichard, Langlois, Issanchou, and Abbott (1995), Le Guen, Prost, and Demaimay (2000) and Serot, Prost, Visan, and Burcea (2001). A comparative study of these methods was also performed in our experiments. Two methods, NIF and OSME, involving 8 panellists, were applied to the sea fig solvent extract. GC–O was interrupted during solvent elution to avoid dichloromethane absorption by operators. Both methods revealed a total of 29 aroma-active compounds. However, we faced some difficulties in discerning the most powerful odourants with the NIF method. This lack of discrimination has already been described by Le Guen et al. (2000). The OSME method brought the most potent aroma notes to the fore: indeed 7 odours represented almost 50% of total areas of the olfactogram. This method is the most accurate according to Guichard et al. (1995), but it needs sensitive assessors, rigorously trained for odourant intensity evaluation.

For dilution methods, two assessors were involved, and a dilution factor of 3 was chosen. Despite the fact that AEDA and CHARM, applied to our extract, indicated only 18 potent odourants, these methods, compared with those mentioned above, led clearly to the detection of 2 odours which were very representative of sea fig aroma. However, the CHARM method was preferred to AEDA analysis because it also takes into account the compound elution time period. Thus, for this paper, we have chosen to present and discuss OSME and CHARM results.

3.2. Sea fig extract analysis

3.2.1. Detection of odourants

The dichloromethane sea fig extract was injected into a GC–MS system. Two hundred volatile compounds were identified: alcohols were the major chemical class detected, with 37 volatile compounds. The sea fig extract also contained 21 aldehydes, 28 ketones and 22 acids.

Table 1
GC–O analysis of sea fig (*Microcosmus sulcatus*) extract

Peak number	RI[a]	Odour description[b]	Compound	OSME value[c]	CHARM value[d]
1	< 500/640	Sulfury, rotten	Methanethiol	3.1	0.1
2	502/845	Fishy, crustaceous	Trimethylamine	11.8	24.9
3	554/742	Fresh, marine	NI	9.2	22.5
4	594/990	Buttery	Butane-2,3-dione	0.3	–
5	650/–	Sulfury, gaseous	NI	3.8	1.3
6	733/1089	Sulfury, rubber	Dimethyldisulfide	6.6	2.1
7	759/1321	Green, metallic	Pent-2(*E*)-enol	'2.0	1.6
8	804/1639	Cheese-like	Butyric acid	2.5	0.5
9	807/1122	Sulfury, cured meat	3-Methylbut-2-ene-l-thiol[e]	5.1	7.2
10	816/–	Toasted	NI	0.8	–
11	843/1388	Green, grass	Hex-3(*Z*)-enol	1.9	–
12	852/1284	Meaty, nutty	2-Methyl-3-furanthiol[e]	7.3	7.9
13	885/1262	Metallic, boiled potatoes	Hept-4(*Z*)-enal[e]	2.8	1.7
14	890/1450	Toasted	Furfurylthiol[e]	0.9	–
15	900/1361	Grilled, rice	2-Methylthiazolidine	3.4	0.2
16	959/1315	Mushroom	Oct-l-en-3-one[e]	3.3	1.0
17	965/1390	Metallic	Octa-l,5-dien-3-ol[e]	5.2	14.9
18	1020/1220	Mint	Eucalyptol	0.8	–
19	1028/–	Rubber, sulfury	Sulfur compound[f]	1.7	–
20	1056/–	Grilled, phenolic	*p*-Cresol	3.8	6.1
21	1065/1780	Grilled	2-Acetyl-2-thiazoline[e]	1.5	–
22	1073/–	Roasted pork	NI	7.1	1.7
23	1095/2206	Fresh, marine	Sulfur compound[f]	2.1	1.2
24	1127/1589	Green, fatty, melon	Nona-2,6(*E*,*Z*)-dienal[e]	0.6	–
25	1153/1750	Green, fruity, water melon	Nona2,6(*E*,*Z*)-dienol	0.4	–
26	1161/–	Fresh	NI	0.9	–
27	1167/–	Herbaceous, mint	NI	1.4	–
28	1230/–	Green, fishy, fresh	NI	4.1	1.9
29	1247/2302	Meaty	5-(2-Hydroxyethyl)-4-methyl-thiazole	5.6	3

NI, Compound responsible for odour description not identified.
[a] RI, retention indices on HP-1 column/HP-Innowax columns using C_5–C_{22} alkanes.
[b] Odour description assigned during GC/O analysis.
[c] OSME and CHARM value expressed in area % of peaks olfactogram.
[d] OSME and CHARM value expressed in area % of peaks olfactogram.
[e] Only identified by GC–O and standard sample injection.
[f] Sulfur compound detected by specific detector (GC-AED, GC-PFPD).

Moreover, 17 sulfur and 18 nitrogen components, targeted by specific detectors PFPD, TSD and AED, were identified.

Twenty-nine aroma notes were detected by OSME analysis (Table 1). Two main odour groups could be distinguished: one with marine and fresh notes and another one with meaty and sulfury notes. Two marine odours, described as ''fishy, crustaceous'' (peak 2) and ''fresh, marine'' (peak 3) presented the highest values with, respectively, 11.8% and 9.2%, and can be considered as the most potent character-impact compounds of sea fig extract. Six odourant areas had an OSME value between 5% and 8%: peak numbers 6, 9, 12, 17, 22, and 29. Among them, 5 sulfury odours were described: ''sulfury, rubber'' (peak 6), ''sulfury, cured meat'' (peak 9), ''meaty, nutty'' (peak 2), ''roasted pork'' (peak 22) and ''meaty'' (peak 29). In addition, an aroma note was described as ''metallic'' (peak 17) and characteristic of seafood. The components responsible for these 6 odours are considered as important contributors to the aroma. Ten odourants with OSME value between 2% and 5% were defined as medium character-impact compounds: peaks 1, 5, 7, 8, 13, 15, 16, 20, 23, and 28. They gave some additional organoleptic characteristics with ''green, cheese-like, boiled potatoes, rice, mushroom, phenolic'' notes. Finally, the odours with an OSME value below 2% had more subtile influences on the aroma, with ''butter, mint and melon'' notes (peaks 4, 10, 11, 14, 18, 19, 21, 24–27).

CHARM analysis confirmed these results with a better discrimination of odourants than OSME. The 2 most potent character-impact compounds were the same, with important CHARM values, respectively 24.9% (peak 2) and 22.5% (peak 3). Additionally, a typical fresh shellfish note, described as ''metallic'', was revealed as an important odour-active component of sea figs (peak 17) and represented 14.9% of total olfactive area. Three other odourants possessed a significant CHARM value above 5%: peaks 9, 12 and 20, described as ''sulfury, meaty and grilled''. OSME and CHARM analyses did

not show the same classification and discrimination of the medium character-impact compounds. Indeed, CHARM is based on the individual threshold of each isolated molecule, while OSME translates the individual sensitivity of each panellist to the component. Thus, CHARM analysis did not detect all odourants presenting an OSME value below 2%. Nevertheless, these products could contribute significantly to title overall flavour of sea fig, and may be essential to flavorists in formulating a well-balanced seafood aroma.

3.2.2. Identification of odourants

Among character-impact compounds only detected by OSME analysis, odourants 11, 18, 24–27, brought a vegetable touch with ''green'' notes. Hex-3(*Z*)-enol (peak 11), eucalyptol (peak 18) and nona-2,6(*E*,*Z*)-dienol (peak 25) were directly identified by GC–MS. Unlike hex-3(*Z*)-enol and nona-2,6(*E*,*Z*)-dienol (peak 25), which were identified in seafoods (Josephson, 1991; Spurvey et al., 1998), no study has reported eucalyptol in marine products. Nona-2,6(*E*,*Z*)-dienal (peak 24) was only detected by olfactometry on polar and apolar columns and confirmed by standard sample injection. Indeed, its nasal detection could be explained by a very low odour threshold, 0.02 ppb (Darriet et al., 2002). Peaks 26 and 27 remained unknown. The ''meaty'' touch was brought by odourants 10, 14, 19 and 21. Sulfur compounds with low threshold are often responsible for this kind of odours Specific detectors (PFPD and AED) showed the presence of sulfur compounds at retention time of peaks 10 and 19, but none of them could be identified. However, furfurylthiol (peak 14) and 2-acetyl-2-thiazoline (peak 21) were indicated by standard sample injection on 2 columns of different polarities. Both were described as ''grilled'' notes in meat (Gasser & Grosch, 1990) and popcorn (Schieberle, 1991). 2-Acetyl-2-thiazoline was also reported in cooked clam (Sekiwa, Kubota, & Kobayashi, 1997), but no publications about seafood revealed furfurylthiol as a potent odourant. Finally, OSME led to the identification of butane-2,3-dione (peak 4), described as ''buttery'', which was reported in many shellfish studies, in raw oysters (Kirn et al., 2000; Piveteau et al., 2000), in cooked mussels (Le Guen, 2000), and in cooked crustaceans (Back & Cadwallader, 1997).

In the case of medium impact compounds, various olfactory notes were present. Methanethiol (peak 1), pent-2(*E*)-enol (peak 7), butyric acid (peak 8), 2-methylthiazolidine (peak 5) and *p*-cresol (peak 20) describing respectively, ''sulfury, rotten'', ''green, metallic'', ''cheese-like'', ''grilled, rice'' and ''grilled, phenolic'', were directly identified by GC–MS. Except for 2-methylthiazolidine, they have been reported in seafood products (Chung, Yung, & Kirn, 2001; Lee, Suriyaphan, & Cadwallader, 2001; Pennarum et al., 2003). Hept-4(*Z*)-enal and oct-l-en-3-one have been, respectively, attributed to peak 13 with metallic, boiled potatoes'' odour and peak 16 with ''mushroom'' odour, based on standard samples injection. These molecules present very low olfactive thresholds, below 0.1 ppb (Josephson, 1991; Leffingwell & Leffingwell, 1991). Odours ''sulfury, gaseous'' (peak 5), ''fresh, marine'' (peak 23) and ''green, fishy, fresh'' (peak 28) were not elucidated. Indeed, among identified compounds by GC–MS in this retention time area, no one presents the same organoleplic characteristics. However, the marine odour could be attributed to a sulfur molecule, confirmed by PFPD and AED on apolar and polar columns, respectively, at RI 1095 and 2206.

Meaty and sulfury notes characterized the potent odourants: peaks 6, 9, 12, 29 and 22. The compounds responsible for these important flavours were: dimethyldisulfide, 3-methylbut-2-ene-l-thiol, 2-methyl-3-furanthiol, 5-(2-hydroxyethyl)-4-methyl-thiazole and a non-elucidated volatile component. These data revealed that sulfur-containing molecules were the major contributors to the aroma of sea fig, with 10 compounds identified, and a total OSME value of 37.3%. Dimethyldisulfide and 2-methyl-3-furanthiol had already been described in crustaceans (Back & Cadwallader, 1997; Lee et al., 2001). However, 3-methylbut-2-en-l-thiol and 5-(2-hydroxyethyl)-4-methyl-thiazole have not been reported in seafood studies. odourant 17 was also a potent odourant with a typical ''metallic'' note of fresh shellfish. The compound, octan-l,5-dien-3-ol, was directly identified by GC–MS and confirmed by standard sample injection. Moreover, it possessed a low odour threshold of 10 ppb (Whitfield, Freeman, Last, Bannister, & Kennet, 1982) and has been described in several publications about seafoods (Josephson, 1991; Milo & Grosch, 1995).

The first of the two most potent odourants was easily identified by GC–MS and was trimethylamine (peak 2), with a fishy and crustaceous odour. However, the other one with a fresh and marine note (peak 3) was not identified, and no sulfur or nitrogen component was detected by specific detectors at this retention time area. Both can be considered as key compounds, because they present an odour characteristic of sea fig, and a low olfactive threshold (below 1 ppb for trimethylamine (Leffingwell & Leffingwell, 1991)). The nitrogen molecule, confirmed by TSD, has been identified as a character-impact compound in many natural seafood extracts, in particular in crustaceans (Back & Cadwallader, 1997; Cadwallader, Tan, Chen, & Meyers, 1995; Chung & Cadwallader, 1994; Lee et al., 2001).

Direct integration of FID response showed that character-impact compounds among volatile and semi-volatile components represented only a very small part of total ion current: below 0.1% for each of these compounds except for butyric acid (0.3%). Concerning non-identified components, this difference was for two

main reasons. First, some odour were detected at a retention index at which no molecule was identified, probably because the compound was present in minute amount. Second, in some cases, a volatile was identified at a retention index close to that of aroma notes, but the odour description differed from that of the corresponding pure molecule at the sniffing port. These results show the potential of GC–O, which also targeted flavour research on key compounds with very low odour thresholds. Remaining unidentified important character-impact compounds are still under investigation, and an analytical study of the chemical composition of sea fig extract will be published in the future.

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