



# Identification of odour-active compounds of pasteurised orange juice using multidimensional gas chromatography techniques



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## ABSTRACT

Odour-active compounds present in pasteurised orange juice were identified by gas chromatography-olfactometry (GC-O) employing heart-cut multidimensional GC techniques with olfactometry (O) and mass spectrometry (H/C MDGC-O/MS) and comprehensive two-dimensional gas chromatography-accurate mass time-of-flight MS (GC  $\times$  GC-accTOFMS). Headspace solid phase microextraction sampling proved to be qualitatively adequate for the analysis of pasteurised orange juice. The GC-O approach distinguished 13 potent odour regions (detection frequency  $\geq 3$ ) in the orange juice extract, in which 7 regions were then subjected to detailed identification of the compounds that contribute to the odour, by using higher resolution H/C MDGC-O/MS. This analysis permitted the odour-active peaks to be better resolved on the <sup>2</sup>D column, with removal from background matrix, for the seven regions. GC  $\times$  GC-FID and GC  $\times$  GC-accTOFMS reveal the overall complexity of the volatile compounds in the product and assisted in assignment of the isolated peaks of the odour-active compounds, confirming the identification in a number of cases. Four aldehydes (hexanal, heptanal, octanal, citral), 2 esters (ethyl butanoate, methyl hexanoate), and 4 monoterpenes ( $\alpha$ -pinene, D-limonene, linalool,  $\alpha$ -terpineol) were confirmed in accordance with olfactometry assessment in the processed juice. This multi-assessment instrument approach of GC-O, GC  $\times$  GC, and H/C MDGC provided an effective insight into the processed orange juice aroma.

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## 1. Introduction

Orange juice is one of the major traded commodities throughout the world, and the most widely consumed fruit juice. It comprises 46% of the worldwide fruit juice consumption, amounting to 18,449 million L in 2013 (Markstrat, 2013). This can be attributed to the attractive flavour, aroma, and colour of orange juice, as well as its health benefits (Aptekmann & Cesar, 2013; Cesar, Aptekmann, Araujo, Vinagre, & Maranhao, 2010; O'Neil, Nicklas, Rampersaud, & Fulgoni, 2011). The flavour of food, together with its appearance and texture is crucial for the acceptance and consumption of a particular food (van Ruth, 2001). The main challenges for the orange juice industry are to retain the flavour close to that of freshly-squeezed juice, and to guarantee consumers a

consistent flavour throughout the year (Vervoort et al., 2012). In this context, the identification of odour-active compounds in orange juice is of particular interest to the orange juice industry.

Fresh orange juice comprises a complex mixture of volatile compounds. It is very important to identify these compounds because only a portion of them are odour-active, and they collectively contribute to the overall perceived odour (Delahunty, Eyres, & Dufour, 2006; van Ruth, 2001). A number of studies have been published on fresh orange juice aroma during the past eight decades and many volatile compounds have been identified. The aroma of freshly squeezed orange juice has been attributed to aldehydes (mainly acetaldehyde, hexanal, octanal and decanal), esters (especially ethyl butanoate), and terpenes (such as myrcene,  $\alpha$ -pinene, limonene), in addition to some alcohols and ketones (Arena, Garrera, Campisi, & Nicolosi Asmundo, 2006; Bazemore, Goodner, & Rouseff, 1999; Hinterholzer & Schieberle, 1998; Marin, Acree, Hotchkiss, & Nagy, 1992; Rouseff, Goodner, & Naim,

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2001). Thermal processing and subsequent storage can change the desirable fresh orange juice aroma, due to unstable aroma compound losses or formation of off-flavours, which involves complex chemical reactions. However, thermal processing is still the most cost-effective means to prevent microbial proliferation and enzyme activity (Ruiz Perez-Cacho & Rouseff, 2008a,b).

The volatile profile is generally chemically/instrumentally detected, but does not necessarily reflect the aroma profile of the sample (d'Acampora Zellner, Dugo, Dugo, & Mondello, 2008; van Ruth, 2001), making it important to use supplementary methods to identify the odour-active compounds responsible for the overall aroma. GC-olfactometry (GC-O) is a valuable technique to characterise odour-active compounds based on the perception of human assessors (Delahunty et al., 2006). GC-O has been applied to food and beverage, fragrances, and essential oils analysis (Chin, Eyres, & Marriott, 2012a; Dussort et al., 2012; Eyres, Marriott, & Dufour, 2007; Janzantti & Monteiro, 2014; Qiao et al., 2008; Rouseff et al., 2001). Detection frequency (Dussort et al., 2012; Falcão, de Revel, Rosier, & Bordignon-Luiz, 2008), dilution to threshold (Benzo et al., 2007; Kang & Baek, 2014), and direct intensity (Garruti, Franco, da Silva, Janzantti, & Alves, 2003; Pham, Schilling, Yoon, Kamadia, & Marshall, 2008) are the main GC-O techniques. In particular, detection frequency analysis is based on the assumption that the relative number of subjects detecting an odour at any given retention time during a GC-O run reflects the relative importance of that odour compound. The integrated response of a whole panel makes it possible to minimise factors such as inattentive assessors and specific anosmia towards certain compounds on the final aromagram (Debonneville, Orsier, Flament, & Chaintreau, 2002; Falcão et al., 2008; Pollien et al., 1997).

Although single column (one dimension) GC (<sup>1</sup>D GC) is widely used for volatile compound analysis, multidimensional GC (MDGC) offers high peak capacity and separation enhancement, therefore improving identification of the odour-active compound and its perceived odour (Eyres et al., 2007; Herrero, Ibanez, Cifuentes, & Bernal, 2009; Mayadunne, Nguyen, & Marriott, 2005). MDGC and GC-O techniques are frequently used in food flavour analysis (Cajka, Hajslová, Cochran, Holadová, & Klimánková, 2007; Chin & Marriott, 2015; Chin et al., 2012a; Gogus, Ozel, & Lewis, 2006; Komura, 2006; Williams, Ryan, Olarte Guasca, Marriott, & Pang, 2005); in this context, to the best of our knowledge, this is the first study that has combined GC-O, comprehensive two-dimensional gas chromatography (GC × GC), and heart-cutting MDGC (H/C MDGC) together with accurate mass spectrometry detection (accTOFMS) for analysis of orange juice aroma. Rather than profiling the whole sample, the aim of this work was to identify the compounds of major importance for the aroma of orange juice, to evaluate the occurrence of co-elution, and implement methods for improved separation of these compounds.

## 2. Material and methods

### 2.1. Orange juice samples

Two litres of pasteurised (92 °C for 20 s) orange juice (12.5°Brix) was supplied by a local orange juice manufacturer (Berri Fruit Juice, Lion Pty Ltd, Sydney, Australia) from the 2014 harvest of Valencia orange produce. The juice was packaged in plastic bottles and frozen at -4 °C until analysed.

### 2.2. Chemicals

SPME fibres and holders for manual sampling (Supelco) and concentrated alkane standards (C<sub>8</sub>-C<sub>20</sub>) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride (NaCl) for salting-out was from Merck Chemical Co. (Merck KGaA, Darmstadt, Germany). Analytical reagents used were: hexanal (Vetec, Brazil), linalool, α-terpineol, limonene (Aldrich, Darmstadt, Germany), α-pinene, and methyl hexanoate

(Fluka, Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q purification system (Millipore, Milford, MA).

### 2.3. SPME sampling

Headspace solid phase microextraction (HS-SPME) was performed using a manual holder with a 20 mm length DVB/CAR/PDMS (DCP) 50/30 μm fibre (Supelco, Bellefonte, PA, USA). Eight millilitres of juice as supplied (or diluted 1:1 with water in the case of GC × GC-accTOFMS) was transferred to a 20 mL vial containing 2.0 g of NaCl and a magnetic stir bar (8 mm). The fibre was previously conditioned following the manufacturer's instructions, then it was exposed to the sample headspace for 25 min under vigorous stirring (800 rpm) at 37 °C.

### 2.4. GC-O/FID analysis

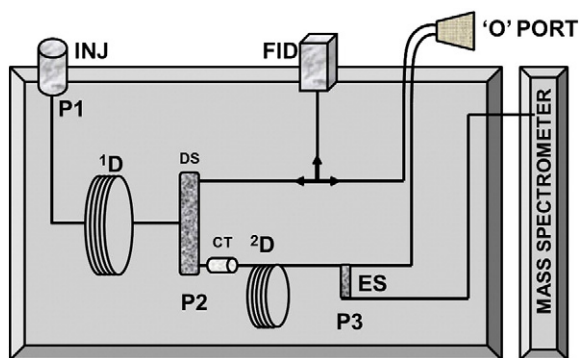
An Agilent 6890 GC (Agilent Technologies, Nunawading, Australia) retrofitted with a Gerstel olfactory port (ODP 2 model, Gerstel Inc., Baltimore, MA) was used for the GC-O/FID analysis. This was the only equipment installed with Gerstel detection frequency software. The capillary column was a SolGel-WAX phase (30 m × 0.32 mm i.d. × 0.50 μm film thickness; SGE Analytical Science, Ringwood, Australia). The column inlet was connected to a split/splitless injector, and the effluent from the column outlet was split using a Y-union to both FID (0.5 m × 0.10 mm i.d.) and olfactory port (1 m × 0.18 mm i.d.) via deactivated fused silica (DFS) capillaries. After using the Y-union connection, the split ratio of effluent to FID and olfactory port was measured to be 1:10 respectively. The GC inlet was set at 240 °C with splitless sampling time of 2 min. The hydrogen carrier flow rate was 3 mL min<sup>-1</sup>. The oven was programmed at 40 °C (hold 3 min), ramp of 3 °C min<sup>-1</sup> to 80 °C; then 10 °C min<sup>-1</sup> to 230 °C (hold 2 min). The FID detector temperature was 250 °C, operated at 20 Hz. The GC-O transfer line temperature was 200 °C and the olfactory port was supplied with a 15 mL min<sup>-1</sup> constant flow of humidified air.

The detection frequency (DF) technique was used to evaluate odour-active compounds (Pollien et al., 1997). Prior to GC-O analysis, six panellists (4 females, 2 males) were trained by sniffing standard mixtures and samples. Panellists were screened and selected using aroma recognition tests, describing various perceived odours, and correctly identifying at least 70%. Triplicate analysis in a randomised order of samples was sniffed during GC analyses (25 min per sniffing session). The odour described by each panellist was recorded and computed as detection frequency (Charles et al., 2000). An odour was considered significant when peaks were detected by three or more panellists (i.e. DF ≥ 3 or ≥ 50% of panellists). A series of alkanes (C<sub>8</sub>-C<sub>20</sub>) was employed to calculate retention indices of analytical peaks according to the procedure of Van den Dool and Kratz (1963).

### 2.5. Heart-cut multidimensional gas chromatography (H/C MDGC-O/MS)

The MDGC separation was performed on a Bruker SCION TQ GC-MS with a single oven (Bruker Corporation, Preston, Australia) equipped with two capillary columns with different stationary phases. A polar HP-FFAP column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent) and a DB-5ms column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent) were used as the first dimension column (<sup>1</sup>D) and second dimension column (<sup>2</sup>D), respectively. The schematic diagram in Fig. 1 shows the H/C MDGC-O/MS instrument, which consisted of a liquid carbon dioxide CT device (SGE Analytical Science), an SGE olfactory port (Model ODO II), an Agilent G2855A Deans switch (DS) device, and an Agilent G3180B 2-way effluent splitter (ES) (Chin, Eyres, & Marriott, 2012b).

The split/splitless injector (pressure applied P1) was connected to the <sup>1</sup>D column inlet, and operated in splitless mode. Both the <sup>1</sup>D outlet and <sup>2</sup>D inlet were connected to the DS (pressure applied P2) that



**Fig. 1.** Schematic diagram of the integrated H/C MDGC-O/MS instrument. P1: column head pressure; P2: Deans switch auxiliary pressure; P3: effluent splitter pressure; DS: Deans switch; ES: effluent splitter; <sup>1</sup>D: first dimension column; <sup>2</sup>D: second dimension column; CT: cryotrap; FID: flame ionisation detector; 'O' PORT: olfactometry port.

controlled the flow from the <sup>1</sup>D column to the FID detector or the <sup>2</sup>D column. The effluent from the transfer line (1 m × 0.1 mm i.d.) was split using a Y-union approximately 1:1 to both FID (1 m × 0.10 mm i.d.) and olfactory port (0.5 m × 0.1 mm i.d.) via deactivated fused silica (DFS) capillaries. At the same time, the effluent from the <sup>2</sup>D column outlet was split by the ES device (pressure applied P3) with an estimated ratio of 1:1 directed to the mass spectrometer detector (MS) via a transfer line (0.5 m × 0.1 mm i.d.) heated at 250 °C, and the olfactory port (the same as above) via another transfer line (0.55 × 0.1 mm i.d.). The olfactory port was heated to 150 °C and supplied with a 15 mL min<sup>-1</sup> constant flow of humidified air (Fig. 1) (Chin et al., 2012b).

The MDGC system was operated under constant pressure. Inlet P1 was initially 51.5 psi and then decreased to 5 psi at 30 psi min<sup>-1</sup> immediately after the region being heart-cut was completely eluted (this operation permits backflushing). P2 and P3 remained constant. The GC inlet was set at 250 °C, with splitless sampling time of 2 min. The oven was programmed at 40 °C (hold 3 min), with a ramp of 3 °C min<sup>-1</sup> to 80 °C; then 10 °C min<sup>-1</sup> to 230 °C (hold 2 min). The FID was set at 250 °C and 20 Hz. The MS electron ionisation energy was 70 eV at 230 °C with a mass range of 45–400 m/z. NIST MS library version 2.0 was used for library searching.

In the H/C MDGC-O/MS system the sample was sniffed by two trained and selected panellists from the same panel used above, to locate the odour-active peaks in either <sup>1</sup>D or <sup>2</sup>D and to confirm the odour description when the compound was perceived. The chromatogram and aromagram of GC-O/FID analyses were aligned with <sup>1</sup>D peak positions (using a series of alkanes) and odour descriptions in the MDGC analysis. The series of alkanes (C<sub>8</sub>–C<sub>20</sub>) was employed to compare retention indices between both systems, in order to determine target region retention times for the H/C events. Cooling of the SGE CT started 1.5 min prior to transfer of the H/C event of the targeted region to cryofocus compounds. The oven was then cooled down immediately after the H/C event and a new temperature program was used to analyse the H/C fraction. Cryogen supply was then closed to permit remobilisation of the trapped solutes into <sup>2</sup>D. The column was programmed from 40 °C (hold 1 min) to 260 °C (20 °C min<sup>-1</sup>; 2 min hold) for the first two H/C, or from 40 °C (hold 1 min), to 180 °C (10 °C min<sup>-1</sup>; hold 2 min); and then up to 240 °C (20 °C min<sup>-1</sup>) for the other H/C fractions.

The DS allowed target regions to be selectively heart-cut from the <sup>1</sup>D column to the <sup>2</sup>D column to resolve co-eluting regions. Two experienced panellists performed olfactory analysis of the target regions to identify odour-active compounds. The Bruker SCION workstation software (version 2.0) was used for data processing. Peaks were tentatively identified by comparing their mass spectra to the NIST library database records (NIST 08 Mass Spectrum Library) and/or with pure standards. A series of alkanes (C<sub>8</sub>–C<sub>20</sub>) was employed to establish the <sup>2</sup>D retention indices for both samples and pure standards (van Den Dool & Kratz,

1963). The alkane standard was injected into the <sup>1</sup>D column, each alkane was heart-cut, such that all were cryotrapped together; the alkanes were then eluted following the same procedure described above for the heart-cut regions. Odour descriptions and retention indices were compared with reference data such as provided in Flavornet (<http://www.flavornet.org/flavornet.html>).

## 2.6. Comprehensive two-dimensional gas chromatography analysis (GC × GC–FID)

An Agilent model 7890 GC system with FID and retrofitted with a longitudinally modulated cryogenic system (LMCS; Chromatography Concepts, Doncaster, Australia) was used. The LMCS was operated at a modulation period of 7.5 s and cryotrap temperature of –20 °C. The FID was operated at 250 °C at an acquisition rate of 100 Hz. A HP-FFAP capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent) and an Rxi-5sil MS column (1 m × 0.18 mm i.d. × 0.81 μm film thickness; Restek, Bellefonte, PA, USA) were used for <sup>1</sup>D and <sup>2</sup>D, respectively. Hydrogen flow rate was 1.5 mL min<sup>-1</sup>. The GC inlet was set at 250 °C in splitless mode (2 min). The oven was programmed at 40 °C (hold 2 min), ramp of 3 °C min<sup>-1</sup> to 240 °C. A series of alkanes (C<sub>8</sub>–C<sub>20</sub>) was used to establish the <sup>1</sup>D retention indices for each region.

## 2.7. Comprehensive two-dimensional gas chromatography–mass spectrometry analysis (GC × GC–accTOFMS)

An Agilent model 7890A GC coupled with an Agilent 7200 AccQTOFMS was retrofitted with a longitudinally modulated cryogenic system. The LMCS was operated at a modulation period of 7.5 s and cryotrap temperature of –20 °C. A SolGel–WAX (30 m × 0.25 mm i.d. × 0.25 μm film thickness; SGE Analytical Science) and a Rxi-5sil MS (0.8 m × 0.1 mm i.d. × 0.1 μm film thickness; Restek) were used as <sup>1</sup>D column and <sup>2</sup>D column, respectively. The GC inlet was set at 250 °C, in splitless mode and sampling time of 0.1 min. The oven was programmed at 40 °C (hold 2 min), ramp at 3 °C min<sup>-1</sup> to 180 °C then at 15 °C min<sup>-1</sup> to 240 °C. A transfer line was connected at the outlet of the <sup>2</sup>D column to the TOFMS, held at 250 °C. Spectra (mass range 40–450 m/z) were acquired at a nominal acquisition rate of 50 Hz, and a source temperature of 280 °C. Qualitative analysis software data processing was used, with tentative identification performed by matching mass spectra to NIST MS library version 2.0 as well as by comparing with standard compounds, if available. A series of alkanes (C<sub>8</sub>–C<sub>20</sub>) was used to establish the <sup>1</sup>D retention indices for each peak in the H/C regions found in MDGC analysis (Van den Dool & Kratz, 1963).

## 3. Results and discussion

### 3.1. GC–O/FID and heart-cut multidimensional (H/C MDGC–O/MS) analysis

Ideally to a first approximation, volatile compounds absorbed onto the SPME fibre should represent the sample according to sorption efficiency; and if they are effectively sampled, be perceptible by the human nose if above the odour-threshold (d'Acampora Zellner et al., 2008). HS-SPME is a commonly employed sampling method for identification and analysis of volatile essential oils and aroma compounds in general (Toci et al., 2012), provided that the target compounds exhibit acceptable partitioning to the fibre. Hence, HS-SPME was chosen to study aroma-impact compounds in orange juice.

GC–O/FID allows potent odour-active regions to be located, and determination of the corresponding odour description, notwithstanding the possibility of compound overlap. Regions detected by three or more assessors (NIF ≥ 50%) were selected as significant for further identification using H/C MDGC–O/MS. The term “regions” is used rather than “peaks” because at this stage co-elution cannot be ruled out, and even if apparently a single peak was present in the GC trace where an odour was perceived, this does not indicate that only a single compound is

**Table 1**  
Odour-active compounds in pasteurised orange juice identified using GC–O/FID, H/C MDGC–O/MS and GC × GC–TOFMS.

Region no.	Odour description	Detection frequency	H/C (min)	<sup>a</sup> <i>I</i> <sub>polar</sub> , <sup>g</sup> <i>I</i> <sub>polar</sub> SolGel–WAX	<sup>b</sup> <i>I</i> <sub>polar</sub> FFAP	<sup>c</sup> <i>I</i> <sub>reference</sub> Wax or FFAP	Identified compounds	<sup>d</sup> SI	<sup>e</sup> <i>I</i> <sub>apolar</sub> DB-5	<sup>f</sup> <i>I</i> <sub>apolar</sub> DB-5	<sup>c</sup> <i>I</i> <sub>reference</sub> DB-5	Molecular formula	Accurate mass ± error (ppm) <sup>1</sup>
1	Grass, plastic	4	7.3–7.7	1008, 986	1013	1032	α-Pinene	952	913	921	939	C <sub>10</sub> H <sub>16</sub>	136.1252 ± 4.03
2	Fruity, sweet	4	7.9–8.3	1028, 1005	1033	1028	Ethyl butanoate	935	910	<sup>2</sup>	804	C <sub>6</sub> H <sub>12</sub> O <sub>2</sub>	116.0833 ± 1.02
3	Grass, green	4	9.5–9.9	1072, 1044	1078	1084	Hexanal	912	795	793	801	C <sub>6</sub> H <sub>12</sub> O	ND
4	Citric, mint, sweet	5	14.1–14.5	1174, 1146	1196	1178	(A) Heptanal (B) Methyl hexanoate (C) D-limonene	867 884 940	918 942 1075	<sup>2</sup> 944 1068	903 1000 1030	(A) C <sub>7</sub> H <sub>14</sub> O (B) C <sub>7</sub> H <sub>14</sub> O <sub>2</sub> (C) C <sub>10</sub> H <sub>16</sub>	(A) ND (B) ND (C) 136.1246 ± 0.38
5	Fresh, fruity	4	---	1230, -									
6	Lemon, green, fat, bug	5	17.9–18.4	1271, 1241	1282	1280	Octanal	975	1036	<sup>2</sup>	1006	C <sub>8</sub> H <sub>16</sub> O	ND
7	Mushroom, fatty	3	---	1282, -									
8	Green, spicy	5	---	1442, -									
9	Spicy, wood	5	---	1493, -									
10	Flower, lemon	6	24.2–24.6	1538, 1514	1554	1537	Linalool	925	1133	1140	1100	C <sub>10</sub> H <sub>18</sub> O	ND
11	Cheese, fat	4	---	1672, -									
12	Mint, green, fruity, citrus	3	26.6–27.0	1702, 1674	1712	1688	(A) α-Terpineol (B) Citral	946 876	1233 1273	1233 1266	1195 1254	(A) C <sub>10</sub> H <sub>18</sub> O (B) C <sub>10</sub> H <sub>16</sub> O	(A) ND (B) 152.1198 ± 1.53
13	Caramel	3	---	2030, -									

A, B and C: peak correspondence in Fig. 3.

— No suitable heart-cut region could be identified due to lack of sensory response or no identifiable MS response in the MDGC set-up.

<sup>a</sup>*I*: Retention index calculated for <sup>1</sup>D GC–FID/O in SolGel–WAX column.

<sup>b</sup>*I*: Retention index calculated for <sup>1</sup>D in MDGC–MS/O in HP–FFAP column.

<sup>c</sup>*I*: Retention index reference values reported in Flavornet database (<http://www.flavornet.org/flavornet.html>).

<sup>d</sup>SI = MS similarity matching with NIST library (v. 11).

<sup>e</sup>*I*: Retention index calculated for <sup>2</sup>D in MDGC–O/MS in DB-5ms column.

<sup>f</sup>*I*: Retention index calculated for <sup>2</sup>D in MDGC–O/MS in DB-5ms column with pure standard.

<sup>g</sup>*I*: Retention index calculated for <sup>1</sup>D in GC × GC–TOFMS. Since no peak was determined by MDGC analysis for some regions, no entry is given for <sup>g</sup>*I*<sub>polar</sub> in these cases.

<sup>1</sup> Details for accurate mass acquired by using GC × GC–acTOFMS; ND indicates that the molecular mass ion is not distinguishable in electron ionisation mass spectrometry operation.

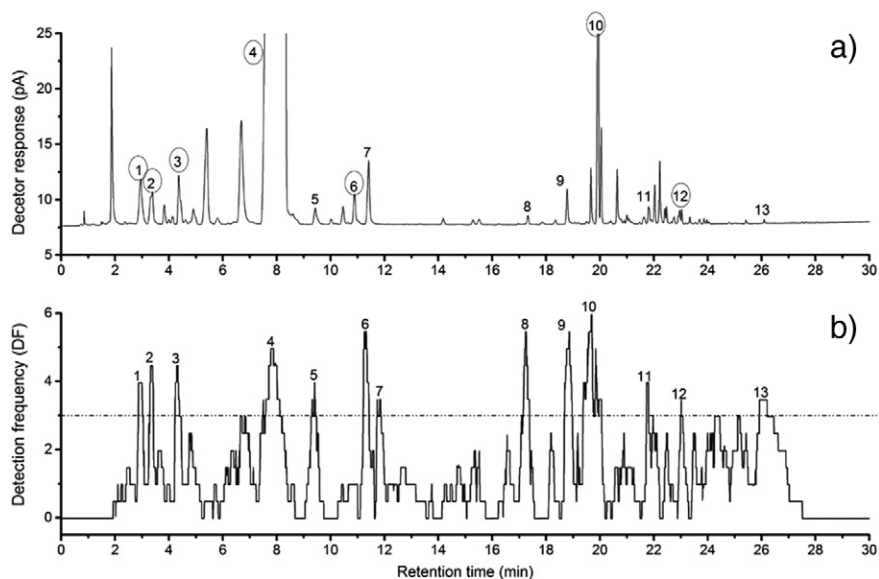
<sup>2</sup> Tentatively identified (based on NIST MS library match without pure standard confirmation).

present. The 13 odour-active regions, numbered from 1 to 13, are reported in Table 1 and shown in Fig. 2.

Fig. 2 shows the GC–FID chromatogram and corresponding aromagram of orange juice. Region 10, described as “flower, lemon”, showed the highest detection frequency, having a value of 6. Region 4 (“citric, mint, sweet”), region 6 (“lemon, green, fat, bug”, region 8 (“green, spicy”), and region 9 (“spicy, wood”), all had a detection frequency of 5. Although region 4 was correlated with a peak with the highest area in the chromatogram, one assessor did not perceive this

aroma. The result confirms that peak area cannot be correlated with high odour intensities; i.e. odour perception depends on compound odour threshold. Due to the complexity of the profile of volatile compounds in orange juice, co-elution is likely on the <sup>1</sup>D column; therefore further investigation by H/C MDGC–O/MS was conducted over the 13 regions, in order to better resolve peaks and tentatively identify the most odour-active compounds.

In the H/C MDGC–O/MS system the sample was sniffed by two selected panellists; the regions previously located and described were



**Fig. 2.** GC–FID chromatogram of orange juice (a) with the selected regions (represented by numbers) corresponding to the aromagram shown in (b). Dashed line indicates a level deemed to be ‘noise’ for the ‘O’ detector, with insufficient confirmation by panellists according to the detection frequency method as positive aroma regions. Positively identified peaks above the line with DF ≥ 3 are reported in Table 1.



found by matching odour descriptions and retention indices (Table 1). It is worth noting that some of the 13 regions located by GC–O/FID could not be successfully identified because, presumably their MS response was too low or their sensory response was not perceived by the two panellists. Since no peak was determined by MDGC analysis for these regions, no entry is given for  $^{87}I_{\text{polar}}$  in Table 1. The remaining 7 regions were positively detected and in the pasteurised orange juice. Four aldehydes (hexanal, heptanal, octanal, citral), 2 esters (ethyl butanoate, methyl hexanoate), 2 terpene hydrocarbons ( $\alpha$ -pinene, D-limonene), and 2 terpene alcohols (linalool,  $\alpha$ -terpineol) were present with response above odour threshold, and with acceptable MS identity (Table 1). All compounds have been reported in previous work, and

odour descriptions match those of the compounds in Table 1, apart from methyl hexanoate (Arena et al., 2006; Bazemore et al., 1999; Buettner & Schieberle, 2001; Hinterholzer & Schieberle, 1998; Marin et al., 1992; Rouseff et al., 2001).

Furthermore, the present work confirms orange juice as a complex food matrix, as can be seen in the original chromatographic and odour response (Fig. 2) and the chromatograms of the heart-cut regions (Fig. 3) which often comprise additional low intensity compounds; region 2 comprised two major peaks of similar response. Regions previously considered to be single peaks often appeared as two or more peaks. Although some peaks could not be identified, the majority of them were tentatively identified. Essentially only a major single peak

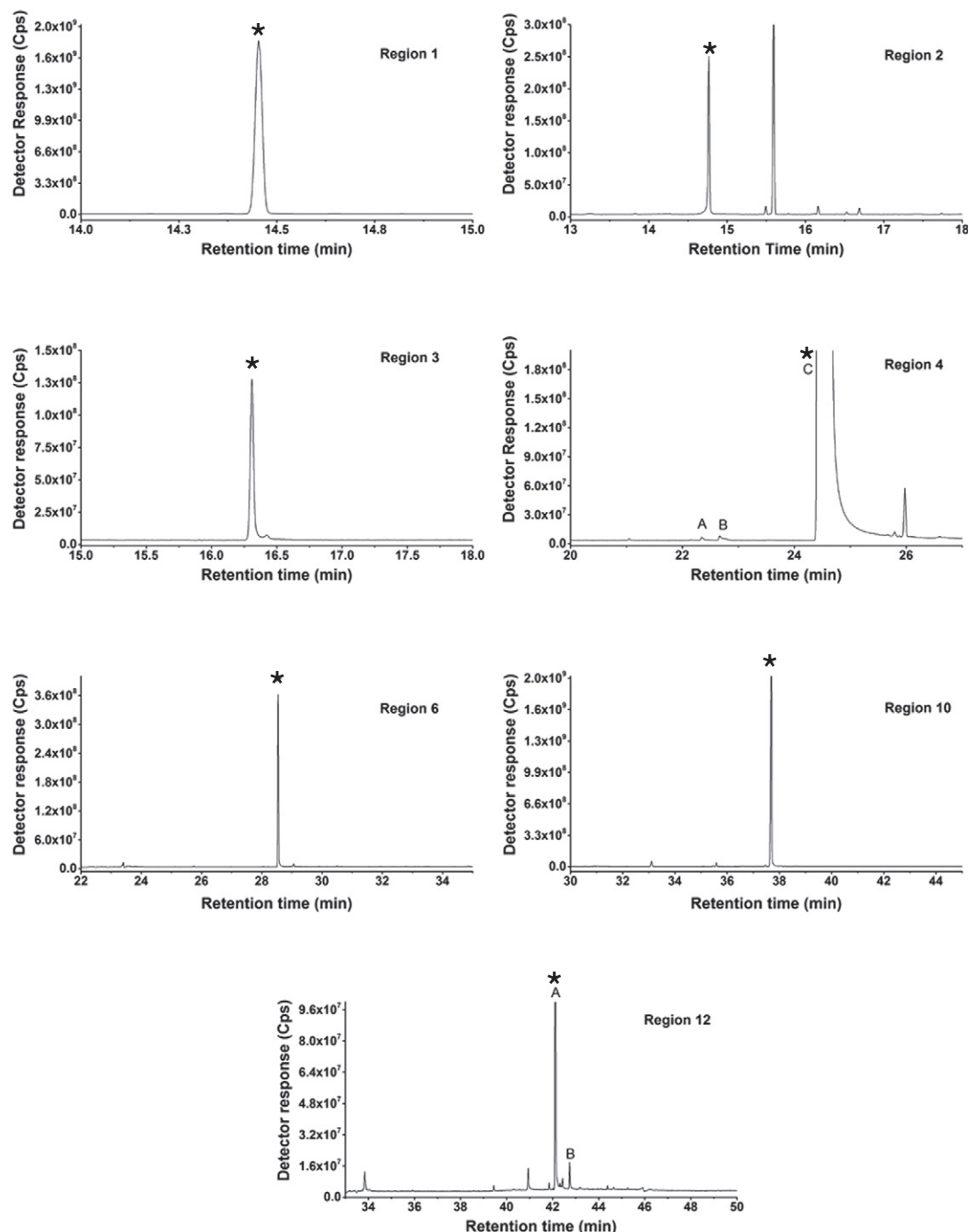


Fig. 3. Gas chromatograms of the  $^{2D}$  analysis of the regions analysed by H/C MDGC–O/MS. Peaks marked by an asterisk are responsible for odour activity.

was identified in regions 1, 3, 6, and 10, corresponding to  $\alpha$ -pinene, hexanal, octanal and linalool, respectively. Aldehydes play an important role for orange juice flavour. Hexanal is formed from 9- and 13-hydroperoxides of linoleate and from other unsaturated aldehydes formed during the oxidation of linoleate (Shahidi, 2000). Previous studies show that hexanal may not have an important contribution to orange juice aroma except for some green notes (Nisperos-Carriedo & Shaw, 1990). Our results showed a strong contribution (DF of 4) for hexanal, and confirmed its descriptor as “green, grass”. Octanal can be related to industrial juice extraction, when relatively high levels of peel oil are introduced into the processed orange juice (Ruiz Perez-Cacho & Rouseff, 2008b). Linalool is the most potent alcohol in orange juice, accompanying a distinctive sweet floral odour (“flower, lemon”; Table 1), and it can be formed by the reduction of aldehydes formed from the oxidation of unsaturated fatty acids (Ruiz Perez-Cacho & Rouseff, 2008b).

Five peaks were detected in region 2, but the odour “fruity, sweet” was perceived just in the asterisked peak (ethyl butanoate). Esters are considered to be important volatile compounds present in many fruits, including orange. Ethyl butanoate was associated with “fruity, sweet” perception (region 2) and has been reported as the most intense odourant in orange juice, and the most important and abundant ester – exceeding the abundance of all other esters combined (Arena et al., 2006; Bazemore et al., 1999; Buettner & Schieberle, 2001; Mahattanatawee, Rouseff, Valim, & Naim, 2005; Marin et al., 1992; Rega, Fournier, & Guichard, 2003; Tonder, Petersen, Poll, & Olsen, 1998).

Region 4 odour (“citric, mint, sweet”) had a strong persistence; panellists reported they could sense it for more than 1 min. This can be explained by the high concentration of D-limonene (peak C), the most abundant terpene in orange juice, present at 400 times its threshold level in water (Ruiz Perez-Cacho & Rouseff, 2008b), but its odour contribution is controversial. Today, the role of D-limonene for orange juice aroma is still unclear, although in modelling studies limonene has been shown to be an important ingredient to simulate orange juice aroma (Buettner & Schieberle, 2001). Three peaks, corresponding to heptanal (A), methyl hexanoate (B) and D-limonene (C) were identified in region 4, with peak areas of (A) and (B) lower than that of D-limonene. Methyl hexanoate was described as “sweet” and D-limonene as “citric, mint”. The presence of methyl hexanoate in the same region as D-limonene might explain the sweet character of this odoriferous region perceived by most panellists in the  $^1D$  GC analysis. Discovery of methyl hexanoate in region 4 became feasible only using H/C MDGC (Fig. 3, Region 4). This ester has not been reported previously in processed orange juice (Ruiz Perez-Cacho & Rouseff, 2008a). It was well separated from D-limonene and heptanal in region 4 as confirmed

by the library match, authentic standard analysis, and retention index. In order to further improve separation and find possible co-elutions in region 4, repeat analyses employing 3 heart-cuts of much narrower windows were conducted within the original 14.1–14.5 min H/C window. Heptanal could not be detected by any panellist.

Seven peaks were observed in region 12, and two of them ( $\alpha$ -terpineol and citral) were successfully identified by MS matching, adequate MS sensitivity, and suitable retention metrics, although peak A was the one described as having “mint, green, fruity, citrus” odour (Table 1 and Fig. 3).  $\alpha$ -Terpineol is formed from acid-catalysed hydration of terpenes, the heating of which increases the rate of reaction (Ruiz Perez-Cacho & Rouseff, 2008b). Citral (3,7-dimethyl-2,6-octadienal) is a mixture of two isomeric monoterpenic aldehydes, geranial and neral. They could be derived from orange peel oil and are believed to be important contributors to orange juice flavour, when present above their threshold level in water (Ahmed, Dennison, Dougherty, & Shaw, 1978).

### 3.2. Comprehensive two-dimensional gas chromatography analysis (GC $\times$ GC-accTOFMS and GC $\times$ GC-FID)

Comprehensive two-dimensional gas chromatography methods using both accurate mass TOFMS and FID (GC  $\times$  GC-accTOFMS and GC  $\times$  GC-FID) were further used for orange juice aroma analysis. GC  $\times$  GC-FID was used as an initial screening technique to characterise the complexity of the volatile compound profile and to develop the general GC  $\times$  GC approach – selection of modulation periods, modulation temperatures, and column sets. Combining GC  $\times$  GC and accTOFMS is promising because both high resolution and good sensitivity with accurate mass detection is possible. Additionally, the volatile compound profile and odour-active compounds identified using GC-O and H/C MDGC may be reliably correlated with GC  $\times$  GC, providing additional confirmation of the identification, a broader overview of the total volatile composition with indicative identification, and further understanding of orange juice aroma.

The effects of different GC  $\times$  GC modulation periods, modulation temperatures, and choice of columns on the 2D profile were first evaluated using GC  $\times$  GC-FID. Odour-impact regions from 0.5 min to 50 min could be located by matching the retention indices with those from GC-O/FID analysis. In the 2D contour plot (Fig. 4), each dot or zone should ideally represent a different compound; however no identification is provided with the FID detector. Thus identification was performed using GC  $\times$  GC-accTOFMS. Where authentic standards are available, and provided they have the same 2D coordinates within the separation

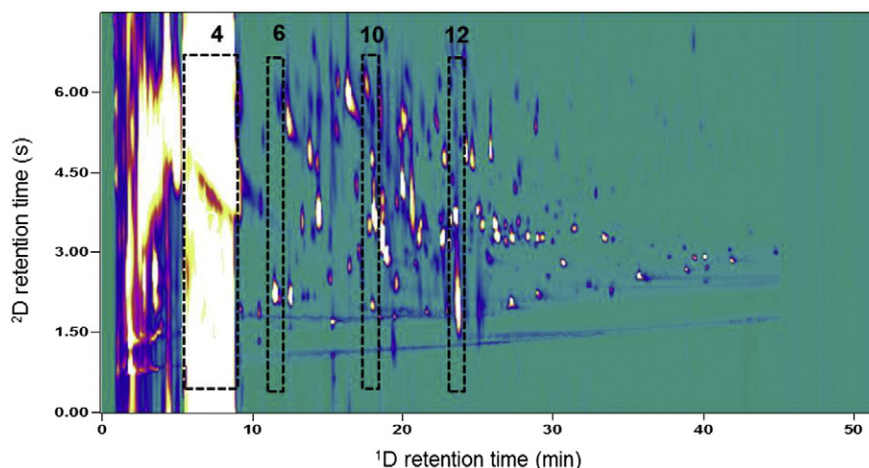


Fig. 4. GC  $\times$  GC-FID contour plot for the analysis of orange juice. Dotted boxes correspond to some odour regions analysed by H/C-MDGC-O/MS.

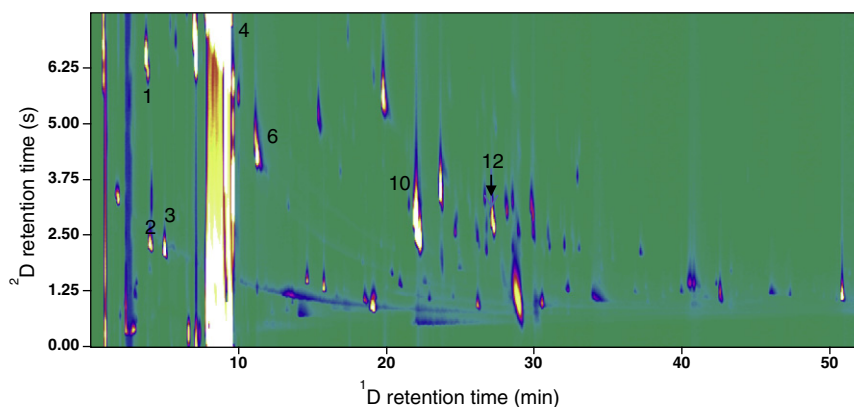


Fig. 5. GC  $\times$  GC-accTOFMS contour plot for the analysis of orange juice. Numbers correspond to odour regions analysed by H/C-MDGC-O/MS.

space plus the same mass spectrum, identity is assured. Without these 3 matching criteria, identification is considered tentative.

Fig. 5 shows the GC  $\times$  GC-TOFMS contour plot for orange juice. When compared to the GC  $\times$  GC-FID (Fig. 4) fewer compounds were detected using TOFMS (Fig. 5) because of additional sample dilution and reduced splitless sampling time, which was chosen in order to give narrower peaks for some of the early-eluting target compounds previously analysed by H/C MDGC. Qualitatively, the FID and TOFMS results should be similar, but exact correspondence is unlikely due to different conditions (e.g. outlet pressure) of the experiments. For best quality confirmation of MS data, the MDGC system would be implemented in the TOFMS instrument, but this was not available here. The GC  $\times$  GC-TOFMS experiment was therefore limited to confirming that compounds proposed in target regions could be suitably identified by accurate mass MS results. This was the case for compounds reported in this section. In Fig. 5, numbers represent the odour-active regions, rather than individual compounds. Note that  $\alpha$ -pinene, ethyl butanoate, hexanal, octanal and linalool from regions 1, 2, 3, 6 and 10, respectively were successfully identified.  $\alpha$ -Terpineol was identified, but co-elution with citral could not be confirmed using GC  $\times$  GC-accTOFMS (Fig. 5, Table 1).

In regard to region 4, only D-limonene was identified in the GC  $\times$  GC-TOFMS analysis. Heptanal and methyl hexanoate, which closely elute with D-limonene on the first column as indicated by all three being transferred to  $^2$ D during the H/C MDGC method, were neither independently detected nor identified using GC  $\times$  GC-accTOFMS, the likely reason being the sample dilution and reduced splitless sampling time. Presumably due to the very large D-limonene peak which leads to peak broadening and overloading in the narrow-bore  $^2$ D column, separate peaks for heptanal and methyl hexanoate are prevented from being distinguished from the limonene peak in the GC  $\times$  GC method. As indicated above, both peaks were reliably detected with H/C MDGC, making the combination of both techniques a valuable combination to analyse orange juice.

#### 4. Conclusion

This study demonstrated an efficient analytical approach using multidimensional techniques of MDGC and GC  $\times$  GC coupled with olfactometry and MS to assess pasteurised orange juice aroma. The GC-O DF technique allowed successful screening of the most significant odour impact zones (according to DF  $\geq$  3) for volatile composition in pasteurised orange juice. Four aldehydes (hexanal, heptanal, octanal, citral), 2 esters (ethyl butanoate, methyl hexanoate), and 4 monoterpenes ( $\alpha$ -pinene, D-limonene, linalool,  $\alpha$ -terpineol) were confirmed in accordance with olfactometry assessment in the processed juice. H/C MDGC-O/MS analysis permitted odour-active regions to be further resolved on the  $^2$ D column, and separation of odour compounds from background matrix. GC  $\times$  GC-accTOFMS confirmed the identification

in a number of cases. Further investigation may be proposed to study aroma changes of the juice caused by packaging, storage, and microbial contamination by application of the GC  $\times$  GC/MDGC-MS/O approach to extend the knowledge of orange juice flavour chemistry in processed products.

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