

# Optimization of Headspace Solid-Phase Microextraction Conditions for the Identification of Volatiles Compounds from the Whole Fruit of Lemon, Lime, Mandarin and Orange

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## Abstract

An optimum method has been developed for extracting volatile organic compounds (VOCs) which contribute to the aroma of different species of citrus fruit (orange, lemon, lime, and mandarin). Headspace solid phase microextraction (HS-SPME) combined with gas chromatography (GC) coupled with flame ionization detection (FID) is used as a very simple, efficient and non-destructive extraction method. A three phase 50/30  $\mu\text{m}$  PDV/DVB/CAR fibre was used for the extraction process. The optimal sealing time for volatiles reaching equilibrium from whole fruit in the headspace of the chamber was 20, 16, 8 and 16 hours for lemon, lime, mandarin, and orange respectively. Optimum fibre exposure times for whole fruit were 2, 4, 2 and 2 hours for lemon, lime, mandarin, and orange respectively. Three chamber volumes (500, 1000 and 2000 ml) were evaluated for the collection of VOCs with the 500 ml chamber being selected. The 500ml chamber produced the highest quality peak areas and quantity of extracted volatiles. As a result of fruit respiration, the percentage of oxygen ( $\text{O}_2$ ) of all citrus fruit species in 500 ml chamber decreased from 21.8% to 18.8% in the 20 hours sealing time, while carbon dioxide ( $\text{CO}_2$ ) contents increased to 2.9% also in the 20 hours sealing time. The results of this study showed the feasibility of this technique for identifying VOCs from four of the citrus fruit species and its potential as a routine method for physiological studies on citrus fruit or on other fruit species.

## Keywords

Citrus Fruits, VOCs, HS-SPME/GC-FID, Lemon, Lime, Mandarin, Orange

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## 1. Introduction

Citrus is a genus of flowering trees and shrubs in the family of Rutaceae and is considered among the most important horticultural industries in the world. Citrus fruit have the highest international trade value of all food [1]. The contribution of the citrus industry to the world economy is estimated at more than \$10 billion USD annually [2]. The Australian citrus industry considers as one of the largest fresh fruit industries in Australia, and definitely the largest fresh fruit exporter with an annual average export volume of 170,000 tons and a value of \$190 million AUD [3]. Citrus fruit are used not only for foods and drinks but also in perfumes, cosmetic, soaps, and many other aromatic products. They are widely grown in the world's tropical and subtropical regions and are native to parts of China, India, New Caledonia and northern Australia [4]. Taxonomic identification is confounding because there are many, but citrus can generally be classified into the following categories: sweet oranges (*C. sinensis*), mandarins (*C. unshi*), tangerines (*C. tangerina*, and *reticulata*), and (*C. clementine*), sour/bitter oranges (*C. aurantium*), lemons (*C. lemon*), limes (*C. aurantifolia* and *latifolia*), grapefruit (*C. paradisi*) and pummelos (*C. grandis*), hybrids (e.g., tangelos, tangors, and limequats), and citrons (*C. medica*, which have a rind that is used primarily for confectionary and is only commercially grown in limited areas). All of which are exported to various markets around the world [5].

Citrus species produce essential oils in their fruits which are used in soap, food, perfumes, repellents and others [6]. Citrus consumption depends closely on their aroma and flavour. Chemically, the aroma and flavour are given by the presence of volatile compounds that impress the olfactory receptors. Detailed analysis of the aroma components of citrus fruits is important for citrus industries to ensure the production of quality foods and free of pests and pathogens. Citrus have been considered as emitters of volatile organic compounds (VOCs) in chamber studies under controlled environmental conditions [7]. These VOCs can be extracted by various techniques, such as microwave-assisted hydrodistillation extraction, solvents and recently, solid phase microextraction (SPME) which is used to profile and quantify these compounds. Headspace solid phase microextraction (SPME) nowadays, is considered the method of choice for most of the volatile extraction in flavour chemistry/food [8] [9] and particularly in Citrus [10] [11]. There are many methods for obtaining citrus volatiles, such as citrus oil (essential oil) and these methods have been extensively reviewed [12] [13]. Unfortunately, the aroma of extracted oils rarely represents the delicate natural aroma of citrus, because of uncontrolled temperature during the steam distillation process [14]. Likewise, other extraction methods using ground samples and solvents also frequently fail to capture the natural aromas [15]. To identify VOCs produced by citrus or other fruit, it is necessary to develop easy to operate, repeatable, sensitive, rapid and cost-effective method. Until now, there are no studies about the use of headspace solid phase microextraction (HS-SPME) technique for the whole fruit of citrus species. The SPME method is

excessively used for the analysis of volatile compounds. The HS-SPME technique is a new, simple, rapid, eco-friendly and solvent-free sample preparation technique for the extraction of volatile compounds [16] [17]. The HS-SPME technique gives simultaneously tens or hundreds of possible volatile compounds and also provides interesting results when gas chromatography (GC) is combined with either Flame Ionization Detector (FID) or mass spectrometric detection (MS), but it must be optimized for the volatiles being targeted [18] [19]. Many factors can affect the optimization of extraction conditions, such as the correct fibre and an appropriate chamber for capturing the VOCs, the temperature used during extraction and the extraction time from the headspace [20]. So far, there has been no systematic work on optimizing extraction conditions for whole fresh citrus fruit. Therefore, this study will determine the optimal conditions of sealing time, extraction time and chamber size for citrus fruit volatile isolation by the headspace solid phase microextraction (HS-SPME) technique with gas chromatography coupled with Flame Ionization Detector GC-FID.

## 2. Materials and Methods

### 2.1. Reagents

An n-hexane 95% was purchased from Sigma-Aldrich Australia, catalogue number 270504-2L. Ethanol was purchased from MERK (Germany) (high-performance liquid chromatography HPLC grade), and the n-Alkane standard (C7-C30) was purchased from Sigma-Aldrich Australia, catalogue number 49451-U.

### 2.2. Apparatus and Equipment

An Agilent Technologies gas chromatograph 7829A (serial number CN14272038) fitted with an HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm, RESTEK, catalogue number 13423) non-polar, with a flame ionization detector (FID) was used. The SPME extractions were carried out in three-phase Divinylbenzene/carboxen/polydimethylsiloxane DVB/CAR/PDMS fibre, 50/30 µm (Sigma-Aldrich Australia, catalog number 57347-U), which was designed for analytes with a broad range of polarities (suitable for C2-C20 range) [21] [22] (Sigma-Aldrich Australia, catalog number 57347-U), attached to a manual SPME holder (Supelco Inc.). The fibres were conditioned as recommended by the manufacturer and the supplier specifications before analyses. A 500, 1000 and 2000 ml Pyrex (Silverlock Packaging; JG2701 FL, JG2879 FL and JG2901 FL respectively) glass jar with a 5 mm port drilled into one side, into which septa (20633 Thermogreen® LB-2 Septa, plug) was placed and was used for collection of citrus fruits VOCs. Aluminum foil 150 m × 44 cm (Vital Packaging Company) was used to cover the glass jar opening and extract volatile organic compounds (VOCs) emitted from fruits. Witt OXYBABY® 6.0 (WIT-Gasetechnik GmbH & Co KG T, Germany) was used for monitoring head space fruit concentration of carbon dioxide, oxygen, and nitrogen from respiring fruits was by inserting the needle through jars septa.

### 2.3. Samples

Fresh samples of orange, lemon, lime and mandarin fruits were purchased from different vendors at local shopping centres. The orange, lemon and mandarin were weighed at approximately 150 g and the lime at approximately 100g. The fruit was checked and washed well with warm water to get rid of the wax, and then conditioned at room temperature ( $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) for 24 h before the experiment was conducted.

### 2.4. Gas Chromatogram Condition

The GC\_FID run time was 45mins the oven column temperature ranged from  $50^{\circ}\text{C} - 250^{\circ}\text{C}$ , programmed at  $5^{\circ}\text{C}/\text{min}$ , with a final hold time of 5 min. Helium (He) was used as the carrier gas at 1.1 mL/min constant flow, and detector (FID) temperatures of  $290^{\circ}\text{C}$ , injection port temperature  $250^{\circ}\text{C}$ , and the GC-FID instrument was operated under the splitless mode.

### 2.5. Optimization of Solid-Phase Microextraction

For optimization of the HS-SPME, the variables chosen were sealing time, extraction time and different volumes of chambers for extraction, while the SPME fibre (DVB/CAR/PDMS), the fibre extraction temperature of  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , the weight of samples were kept constant. In order to optimize the sealing time, extraction times and chambers volumes all factors influencing the equilibrium between the analyses and the fibre were taken into consideration. Different sealing times, different extraction times and different extraction chambers volumes were used for the different species of fresh citrus fruit. The fibres were cleaned between each extraction by placing them into the GC injection port for 15 min at  $250^{\circ}\text{C}$  to ensure the absence of carry-over peaks and contaminants in blanks and next injections to have good repeatability between the injections. All three fibres were calibrated using standard n-alkene C7-C30 after dilution in the ration of 1/10 ml in n-hexane, and then desorbed for one hour at room temperature and this procedure was repeated twice with three replications before analysis. The results presented as the mean values.

#### 2.5.1. Optimization of Sealing Time

To determine the best sealing time; the citrus fruits species individually sealed in 1000 ml glass jars for (2, 4, 8, 12, 16 and 20 h). The extraction efficiency of the six different sealing time was determined by comparing the peak area of the eight compounds from all citrus fruit species under the same extraction time, SPME fibre, desorption time, and GC conditions. The fibres were exposed to the HS of the glass jars for 2 hours. After exposure, the fibres were retrieved and injected into the heated injection port ( $250^{\circ}\text{C}$ ) of a GC-FID and desorbed for 10 min. Each sample was replicated thrice.

#### 2.5.2. Optimization of Extraction Time

Each fibre was exposed to the HS of the 1000 ml glass jar containing individual citrus fruit for 3 different time periods (1, 2 and 4 hours). After exposure, the fi-

bre was retrieved and injected into the heated injection port (250°C) of a GC-FID and desorbed for 10min. Each sample was replicated thrice.

### 2.5.3. Optimization of Chambers Volume

To determine the most efficient extraction method of the VOCs emitted by citrus fruits, a comparison was made between different volumes of glass jars (500, 1000 and 2000 ml). The results showed that 500 ml glass jar volume achieved higher efficiency for VOCs extraction from all citrus fruits samples, so the 500 ml jar was chosen because it was efficient for capturing the VOCs emitted. Individually, citrus fruits were placed into the glass jar and the opening covered with aluminum foil and incubated at 25°C ± 1°C for 8 h sealing time and 2 h extraction time. Each sample was conducted in triplicate.

### 2.6. Gas Composition inside the Glass Jars

An Oxybaby gas analyzer was used for monitoring headspace composition of the respiration of the citrus fruit during the sealing time in 500 ml glass jar. Three replicates were used to determine the gas composition.

### 2.7. Data Analysis

The GC data including retention time and peak area were collected and integrated into the chromatography software Agilent Chemstation, and then exported to Microsoft Excel for further analysis. The repeatability of replicates from the same sample was verified by checking the chromatogram pattern features such as detected peak retention times and peak areas.

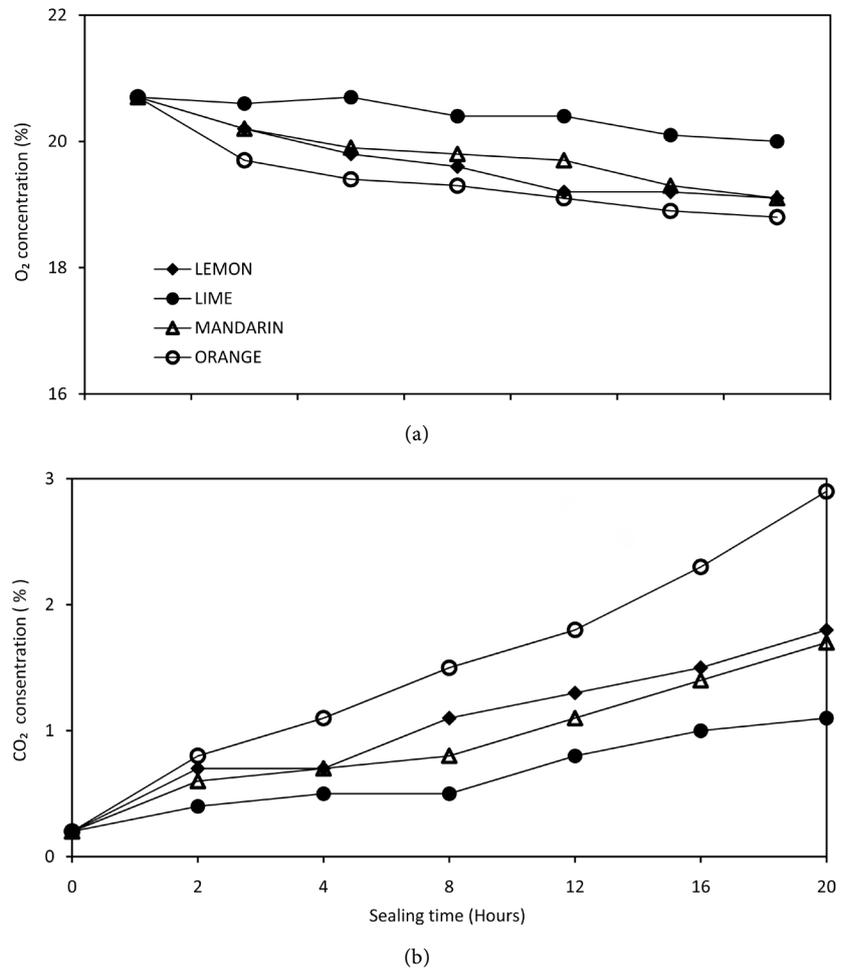
## 3. Results

### 3.1. O<sub>2</sub> and CO<sub>2</sub> Headspace Concentration

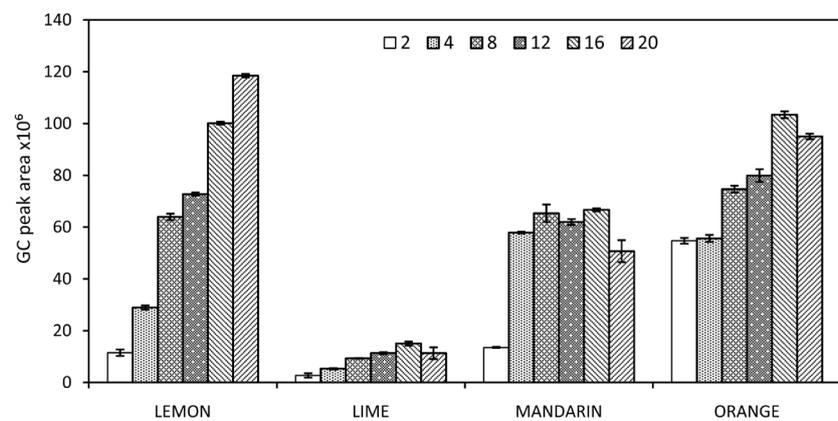
As a result of fruit respiration, the contents of O<sub>2</sub> decreased during the first two hours in varying proportions reaching 19.7% and 20.6% in orange fruit and lime, respectively. As for the lemon and mandarin fruit, oxygen consumption rate was same as control which is 20.2% (**Figure 1(a)**). After 20 hours sealing time, the oxygen level dropped down to 18.8% and 20% for oranges and lime respectively. Carbon dioxide had a lower accumulation in the first 2 hours of sealing time. In 4 hours of sealing time, level CO<sub>2</sub> build up to 0.5% in lime and 1.1% in orange (**Figure 1(b)**). Carbon dioxide gas production continued to build up in all citrus fruit species and reached a level of 2.9%, 1.8%, 1.7% and 1.1% at 20 hours in orange, lemon, mandarin, and lime respectively.

### 3.2. Analysis of Volatiles Organic Compounds in Citrus Fruit Species with Different Sealing Time

Total peak areas from the different samples sealed for 2, 4, 8, 12, 16 and 20 hours are compared in **Figure 2**. The amount of volatile compounds was significantly different between those collected at different sealing times. This result showed that 20, 16, 8 and 16 hours sealing period achieved higher efficiency for VOCs



**Figure 1.** Effect of different sealing time on headspace gas composition O<sub>2</sub> (a) and CO<sub>2</sub> (b) of different citrus fruits species.



**Figure 2.** Peaks of volatiles organic compounds (units) produced by different citrus fruit species with 2, 4, 8, 12, 16 and 20 hours sealing time in sample preparation. Error bars are LSD at 5% (n = 3).

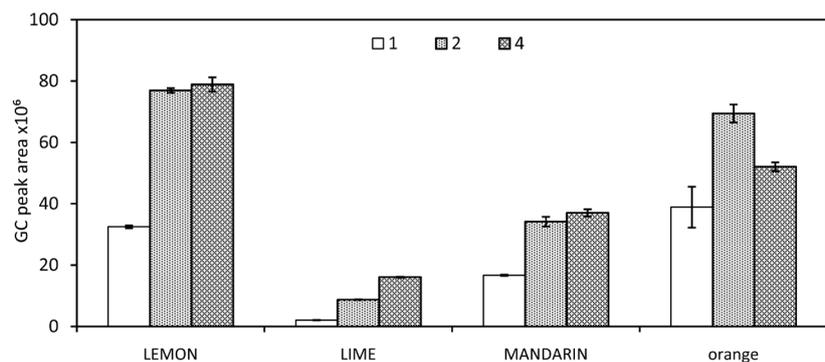
extraction from lemon, lime, mandarin and orange samples respectively. Therefore, the 20, 16, 8 and 16 hours sealing time for lemon, lime, mandarin, and orange respectively were selected for subsequent studies.

### 3.3. Analysis of Volatiles Organic Compounds in Citrus Fruit Species with Different Fibre Extraction Times

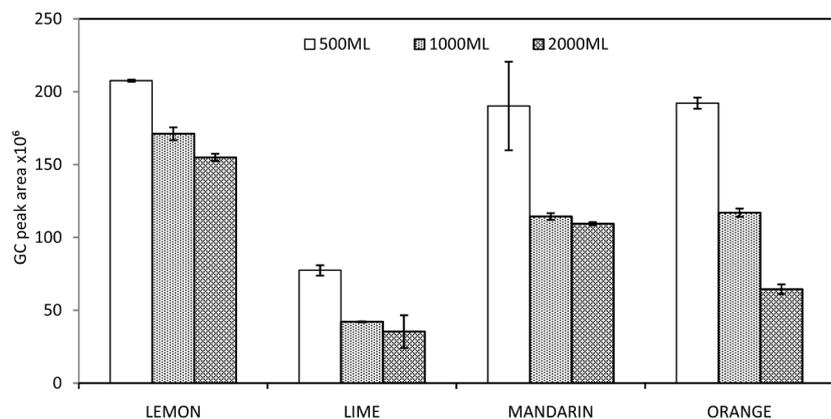
The amount of the volatile compounds did not differ significantly between those collected at 2 and 4 hours from lemon and mandarin, while there were significant differences between them and those collected at 1 hour (**Figure 3**). Therefore, the 2 hours was selected for subsequent studies for both species of lemon and mandarin. In contrast, there were significant differences in the amounts of VOCs produced at the different extraction times from lime and orange fruit (**Figure 3**). Therefore, 2 and 4 hours were selected for best extraction time to absorb the VOCs emitted from the lime and orange respectively.

### 3.4. Selection of Chamber Volume

The extraction efficiency of the three different chambers volumes (500, 1000 and 2000 ml glass jar) was evaluated by comparing the peak area of the eight compounds from all citrus fruit species under the same extraction time, SPME fibre, desorption time, and GC conditions. There were significant differences between the three different chambers size, so the 500 ml jar was chosen because it was optimum for capturing the released VOCs (**Figure 4**).



**Figure 3.** Effects of extraction time with citrus fruit species on the peaks area of volatiles organic compounds at 1, 2 and 4 hours. Error bars were LSD at 5% (n = 3).



**Figure 4.** Peaks of volatiles organic compounds (units) produced by different citrus fruit species extracted by three sizes of chambers (500, 1000 and 2000 ml). Error bars are LSD at 5% (n = 3).

## 4. Discussion

Volatile production is an important quality characteristic of many fruits, which has been extensively studied [23] [24]. The concentration of the CO<sub>2</sub> and O<sub>2</sub> into glass jars is the main factor affecting the production of some volatile organic compounds related to the fruit's aroma. Our results support previous findings in the literature which showed that O<sub>2</sub> consumption was directly related to CO<sub>2</sub> production [25]. Extremely low O<sub>2</sub> levels (0.5 kPa) decreased the emission of straight-chain esters related to the aroma of 'Royal Gala' apples [26], while Barker (1928) found that carbon dioxide injury on oranges took the form of an unpleasant bitter flavour [27].

Most of our knowledge about citrus volatiles has been obtained from studies of processed juices and the peel essential oils, essence oils, and aqueous essences used to flavor juice products [10] [28]. On the contrary, optimization and extraction studies on aroma volatiles in fresh citrus fruit have not much been reported. Optimization of isolation conditions was carried out using a 1 L glass jar with two factors: time needed to reach equilibrium in the headspace and the fiber exposure time. Samples were analyzed by GC-FID. The criteria were a higher number of peaks and greater total area of the chromatogram. The determination of the optimum time of sealing is essential to obtain maximum efficiency of the SPME fibers for particular VOCs. The equilibrium between the citrus fruit species and its volatiles within the glass jar had an impact on the final volatile extraction by the SPME fibre. Normally if there is no significant difference between the sealing time less sealing time is preferred, which agrees with the study by [29] who isolated a number of high-quality volatiles from the headspace of whole banana using 140 min sealing time compared with 15 min for banana pulp, but in this study significant difference was observed between different sealing times so, long sealing time for fresh citrus fruits was selected to isolate high-quality volatiles.

Extraction temperature and time are significant parameters in HS-SPME since both have an effect on the equilibrium during extraction of volatile compounds [30]. In this study, optimal extraction time from the fibre for all citrus fruit species was 2 h, except for lime reaching 4 h which is longer than the time used by [31] who reported 40 min as the optimum extraction time to extract the volatiles compounds produced by some species of citrus fruit juice. The best conditions for isolating volatiles from the headspace of whole banana fruits were 120 min fiber exposure, while for the banana pulp the best conditions were 60 min for exposure times [29]. This difference is most likely due to the difference in fruit extraction part, head space volume and extraction temperature, since, the extraction time depends on the chemical nature of the compounds present, the distribution constant, the fibre polymeric phase, and to the size of the molecular mass (e.g., polyunsaturated fatty acids and other compounds are expected to require longer extraction times depending on their lower partitioning and diffusion coefficient). In the present study, the results indicated that there were differences between all citrus fruit species by total peak area. Apparently, more

volatiles are emitted in 2 h extraction time from orange fruit, and there were no significant differences in 2 and 4 h extraction time from lemon and mandarin, while total peak area from limes fruit reaches high level from 4 h extraction time (**Figure 3**), and this is because different species have different types and amounts of compounds. In general, all plants have the ability to emit VOCs, and the content and composition of these VOCs will depend on the plant species and plant organ.

There were significant differences between the three volumes of glass jars used to capture VOCs. However, since the 500 ml glass jar proved to be the best in capturing the VOCs emitted from different citrus species, which agrees with the study by [32] who used a 2000 ml glass jar to extract a number of high-quality VOCs from 1500 g of peach and pear fruit.

## 5. Conclusion

This study concluded that headspace solid-phase microextraction combined with gas chromatography and flame ionization detection can be used to detect VOC/s from whole citrus fruit species without cutting or extracting juice and essential oil and the optimum condition for sealing and extraction time, were 20, 16, 8 and 16 hours headspace equilibrium and 2, 4, 2 and 2 hours fiber exposure time for lemon, lime, mandarin, and orange respectively.

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