Open Journal of Nutrition and Food Sciences

ISSN 2690-2478

Research Article

Trends of Quality Indices, Volatile Aroma Compounds Accumulation and Biological Capacities along Ripening Stages of Citrus fruits: Measuring Ripeness for Harvest Planning

Myriam Laminea[1], Fatma Zohra Rahalib[2] and Ahmed Mlikia[3]*

[1]*Laboratory of Plant Molecular Physiology, Biotechnology Center of Borj-Cedria, Tunisia*

[2]*Laboratory of Medicinal and Aromatic Plants, Biotechnology Center of Borj-Cedria, Tunisia*

[3]*Laboratory of Wind Energy Management and Waste Energy Recovery, Research and Technology Center of Energy, Tunisia*

Abstract

The aim of this work was to elucidate the effect of maturity and species on taste and aroma profiles, and related of antioxidant and antibacterial activities of three citrus species across three ripening stages. For all species the highest antioxidant activities were reached at the immature stage. Commercial mature stage offers the highest yield of essential oil, highest peel proportion, and high pH values. Among the tested fruit species, *C. limon* and *C. sinensis* semi-mature fruits were found to be the best for harvesting polyphenols, while their ripen fruits were found the best for flavonoids. Ripe fruits were found to be the best for harvesting for the best antibacterial activities. Fruits could be harvested at immature or mature stages where the highest limonene level was reached. The outcomes of this study could be beneficially used by harvesters, consumers, traders or food and nutraceutical industries looking to harness maximum nutraceutical potential.

Keywords: Ripening; Citrus; Aroma profiles; Biological activities; Metabolic markers

Introduction

Citrus flavor is one of the most appreciate flavor worldwide and it is one of the main characteristics of citrus fruits influencing consumer choice, beginning with the visual selection and leading to the consumption of the fruit. The flavor composition is influenced both by genetic and environmental factors, so it is specific to species and variety, and strictly dependent on pre and postharvest handling of fruits [1]. It derives from a complex combination of soluble compounds, principally sugars, acids, flavonoids and Volatile Compounds (VOCs). The overall combination of the volatile compounds that represent the odoriferous portion of the flavor profile is defined as aroma [2]. There are several factors that can affect citrus fruits flavor, and can be divided in two general classes: pre- and post- harvest factors. The aromatic pattern derives from a complex combination of numerous factors. So different species of citrus are characterized by different aromatic patterns showing that there is a genetic control in the expression of the aromatic profile [1,3]. Even often the differences are mainly quantitative, and only a few compounds are variety-specific [4]. The harvest time is strictly related to the content and the composition of fruits. So it is able to affect internal characteristics of the fruits [5]. Therefore, harvesting at the wrong time will lead to undesirable consequence such as fruit rot and post-harvest losses.

For quality assessment, subjective (sensorial analysis) and objective (analytical methods) methods were used. Sensorial analysis can have large sources of variation, low through put, and it can be costly [6]. Accordingly, alternative analytical techniques, since they are non-subjective, highly repeatable, and reproducible, such as separation techniques based on chromatography in tandem with mass spectrometry could be very beneficial.

A few studies have reported the evolution of the chemical composition of the essential oils in citrus fruits during the ripening stage [7,8]. The variation levels of chemical classes of citrus fruits essential oils do not evolve linearly with the ripening stages [7], and lead to drastic variations in essential oils quality [9,10]. Thus, it becomes important to ensure that the essential oils have been produced with fruits collected at the optimum ripening point.

At this point, we should remark that the optimum ripening point may depend on the desired application of the essential oil composition; the optimum ripening condition being different for uses such as antioxidant, antifungal, antimicrobial, anti-inflammatory, antiparasitic, green solvent, etc [7,11-13].

Keeping this in consideration, the present study systematically investigated the accumulation of the volatile profile during the maturation of commercialized citrus fruits and to identify the optimal stage with desirable compounds and biological activities. For the

Citation: Laminea M, Rahalib FZ, Mlikia A. Trends of Quality Indices, Volatile Aroma Compounds Accumulation and Biological Capacities along Ripening Stages of Citrus fruits: Measuring Ripeness for Harvest Planning. Open J Nutr Food Sci. 2021; 3(1): 1017.

Publisher Name: Medtext Publications LLC

Manuscript compiled: Dec 27th, 2021

***Corresponding author:** Myriam Laminea, Laboratory of Plant Molecular Physiology, Biotechnology Center of Borj-Cedria, BP 901, 2050 Hammam-Lif, Tunisia, E-mail: meriam.lamine29@gmail.com

purpose of optimizing the harvesting practices, such information would be useful to harvesters, consumers and traders aiming to maximize the nutraceutical potential of these fruits.

Material and Methods

Fruit sampling and harvest conditions

Fruits of three species i.e. *C. limon*, *C. reticulata* and *C. sinensis,* in the full production phase between 7 and 10 years old, were harvested from farms in the cap bon peninsula (North-Eastern Tunisia; 36.78N; 10.63E). The selected trees were grown under the same pedoclimatic and cultural conditions. These species were chosen based on their commercial importance in the citrus industries and because they are known to have significant difference of bioactive content and metabolome [14]. Date of fruit setting of citrus samples in this study was from middle of October to January. Fruits, randomly collected from various positions on each tree, were manually picked (four plots × ten plants for each species) and sorted into three different maturity stages based on size and color according to local cultivator who has experience with fruit harvesting. Harvest was done from 8 am to 3 pm. During this period, the dew has already dried up and the fruits have already lost their turgor. The infected/damaged fruits were removed from the bulk and remaining was immediately processed for extraction on the same day. Fruit were dried in lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) with a condenser temperature of -55°C and pressure of 7×10^{-2} mbar to constant weight.

Preparation of extract

About 2 gm of the fruit sample was extracted with 20 ml of 80% methanol (having 0.2N HCl concentration) in mortar-pestle and transferred to test tubes. The mixture was heated at 60°C for 60 min in a water bath and cool down to room temperature. The mixture was filtered with Whatman filter paper (No.1) and the filtrate was stored at -20°C during analysis.

Determination of Total Phenolics (TP), Total Flavonoids (TF) and Total Tannins (TT)

The TP, TF and TT content were estimated by Folin-Ciocalteu colorimetric method, aluminium chloride colorimetric method and Folin-Denis method respectively [15-17].

Essential oil extraction

Samples (500 gm) were suspended in water and subjected to hydrodistillation for 3 h using a Clevenger-type apparatus in accordance with European Pharmacopoeia method (Council of Europe, 1997). The steam pressure was fixed at 3 bars. When the boiling water begun and we got the first drop of EO, we regulate the temperature to a point where there was controlled boiling. The extracted essential oil was dried over anhydrous sodium sulphate (Na_2SO_4) and then stored at 4°C in brown glass vials.

Evaluation of the antioxidant activity

The antioxidant properties were assessed by the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging assay [18]. An aliquot of 2 mL of essential oils, at different concentrations (0.1 µg mL-2000 µgmL 1), in methanol was added to 500 lL of a methanolic solution from DPPH at 0.2 mM. The solution was incubated for 30 min, at room temperature, in the dark and the absorbances at 517 nm were recorded. BHA (Butylated hydroxyanisole) was used as positive control. All samples were analysed in triplicate. The percentage of inhibition was determined using the following equation: DPPH scavenging activity (%)=[(AcAt)/Ac] 9 100; Ac: Absorbance of Control; At: Absorbance of the Test made.

Antimicrobial activity

The antimicrobial activity was evaluated by the disk diffusion method as described by [19]. Bacterial strains were grown on Mueller Hinton (MH) plate for 18 h to 24 h at 30°C preceded by an inoculation onto the nutrient agar. Each sterile plate was prepared with medium and then 100 µL of microbial suspension (1×10^6 CFU mL-1) was spread onto the hardened plate. A sterile filter paper disk (6 mm) impregnated with 6 µL of fruit essential oils was placed on the plates. Then the petri dishes were incubated for 18 h at 37°C (bacteria). Gentamicin (10 µg disk-1) was used as positive control. The antibacterial activity was assessed by measuring the diameter of Inhibition Zone (IZ) around the discs.

GC-MS analysis, data processing and compound identification

As a very sensitive, simple, and fast technique, Gas Chromatography-Mass Spectrometry (GC-MS) was selected for the analysis. Volatile compounds profiling was performed on a GC HP 5890 (II) interfaced with a Hewlett-Packard (HP) 5972 mass spectrometer with electron impact ionisation (70 eV). A HP-5 MS capillary column (30 m 90.25 mm, 0.25 mm film thickness; HP) was used using helium as a carrier gas at a flow rate of 1 mL min 1. The program used was isothermal at 70°C, followed by 50°C-240°C at a rate of 5°C min 1, then held at 240°C for 10 min. The split ratio was 60:1. Scan time and mass range were 1 s and 40 m/z-300 m/z respectively.

Data processing and compound identification were performed [20,21]. Before the alignment step between chromatograms using the MetAlign software [22], the raw data were treated by ChromaTOF software 2.0 (Leco Corp., St Joseph, MI, USA). The mass spectra of the representative masses were used for tentative identification by matching to the spectral NIST08 libraries and by comparison of the retention index calculated using a series of alkanes. Authentic reference standards were used to confirm the identity of the metabolites. Percentage compositions of samples were calculated according to the area of the chromatographic peaks using the total ion current.

Statistical analysis

The experiments were performed using a completely randomized design. For each species and stage, all experiments were performed in at least three independent replicates and results were presented as the mean ± standard deviation values. Analysis of Variance (ANOVA) and Duncan's multiple test was applied to verify significant differences among phytochemicals and antioxidant activities of three ripening stages of citrus fruits at a level of $p<0.05$, using SPSS 23.0 software (SPSS Inc., Chicago, IL). Normalised data were uploaded at MetaboAnalyst 2.0 (http://www.metaboanalyst.ca/MetaboAnalyst/) (Xia & Wishart, 2011). Data integrity check was performed in order to ensure that all the required information was collected. Row-wise normalisation was performed to allow general-purpose adjustment for differences among samples Afterward, Log transformation and auto-scaling were performed to make features more comparable when doing uni and multivariate analysis. A heatmap was employed to display the distribution of all the data and to characterize the relative levels of the metabolites throughout all sample groups. Venn diagrams were also created using online Venny software (Venny 2.1

http://bioinfogp.cnb.csic.es/tools/venny/).

Results and Discussion

Fruit maturity and quality assessments

The three developmental stages showed a wide variability of the observed quality characteristics regardless of species (Table 1). While peel proportions (%) showed an upward trend during citrus fruit ripening for *C. limon* and *C. reticulata*, and a downward for *C. sinensis* fruits; fruit firmness decreased with ripening stages for *C. sinensis* and increased for *C. limon* and *C. reticulara*. At immature stage, *C. limon* and *C. reticulata* exhibited the highest peel proportion; while at commercial mature stage, *C. limon* and *C. sinensis* revealed the highest proportion. The highest firm fruit values were detected at immature stage for *C. limon* and *C. reticulata*.

Variations were observed for the juice parameters between different species and ripening stages. Overall, at immature stage, no significant variations among species were detected and TSS ranged from 8.92°Brix to 9.68°Brix. At commercial mature stage, *C. sinensis* showed the highest TSS content (12°Brix), followed by *C. reticulata* (10.7°Brix) and *C. limon* (8°Brix). Generally, Brix index of the citrus fruits increased with ripening stages, which might indicate changes in the balance of their chemical compositions and quality characteristics, as previously reported for other citrus fruits [23]. Exception for *C. limon* where brix decrease significantly among stages. The pH levels of Citrus species varied between 2.25 (*C. limon*) and 3.14 (*C. sinensis*) at immature stage, while it ranged between 4.74 *(C. limon*, *C. sinensis)* and 4.9 *(C. reticulata)* at commercial mature stage. Remarkably, the pH increased significantly from immature stage to mature stage for all species. In fact, the pH plays an important role in the sensory quality of fruit, affecting the perception of sweetness, with increased pH correlating with increased sweetness [24]. Accordingly, the unripe fruits harvested on October could be mainly used in vinegar production due to their strong acidity, while the ripe fruits collected from January are in demand by various food and beverage industries, or are consumed as fresh fruits.

Total contents of polyphenols, flavonoids and tannins

Higher TPC was found in the semi mature stage than commercial harvest stage, except for *C. reticulata* where the immature stage exhibited the highest contents (Figure 1A). The changes of total phenolic content in fruits, showed different trends during ripening. The TP content was reduced during ripening for *C. sinensis* (10 mg GAE/g DW to 4.23 mg GAE/g DW) and *C. reticulata* (10 mg GAE/g DW to 1.15 mg GAE/g DW). Reports indicated that as the fruits proceed towards maturation their phenols get oxidized and take part in the biosynthesis of anthocyanins which accumulate during fruit ripening [25], thus the phenol concentration gets reduced in ripened fruits. Moreover, a good amount of polyphenols was accumulated in unripened fruits, which justify the phenomenon of fruit protection from various fruit borne diseases during pre-maturation stage [26]. However, phenolic content in *C. limon* fruits increased from 10 mg GAE/g DW to 12.75 mg GAE/g DW at commercial stage.

At immature stage, we noticed that the highest FT was detected in *C. reticulata* fruits, while at CM stage *C. sinensis* exhibited the highest content. Compared with the first stage, the FT content increased during maturation stages for *C. limon* (12.5 mg to 20.76 mg GAE/g DW) and *C. sinensis* (2.59 mg to 21.74 mg GAE/g DW). However, TF content decreased at CM stage for *C. reticulata* (15.5 mg GAE/g DW to 12.68 mg GAE/g DW), respectively (Figure 1B). While some authors reported that the TF content varied according to the studied species and the ripening stages [27], others did not detect any variations [28]. Generally, occurence of high content of phenolics and flavonoids in *C. limon* fruits confirmed their value in nutrition and would be an attractive source of bioactive compounds for further utilization.

Compared with the first stage, tannin contents of Citrus fruits significantly ($P< 0.05$) decreased by 2.1-fold and 2.5-fold for *C. limon* and *C. sinensis*, respectively, during maturation (Figure 1C). The exception is noted for *C. reticulata* fruits where the tannin levels rise during ripening stage (from 0.01 mg QE/g DW to 0.19 mg QE/g DW). The tannin seasonal patterns that show a decreasing trend during citrus fruits ripening was similar to previous studies on plant skins/peels [29]. Generally, the tannins and polyphenols contents were influenced by each other as the fruit proceed towards maturation; phenolics were being oxidized and used to biosynthesis of the flavylium ring, leading to the accumulation of anthocyanins during ripening and *vice versa* [25]. Furthermore, the lower tannin content in ripened fruits provided better acceptability to the humans and frugivorous animals/insects [30]. However, tannins are detriments of astringency or favor in beverages such as wine, tea and fruit juice [31] and their optimum presence in these fruits can be better utilized for preparations of fruit wines, teas, etc.

The citrus fruit at stage one used in this study can be greatly used to produce fruit wines and tea due to high TPC, TFC and flavor of astringency because of high tannin level. In the present study, a decrease in tannin content and TPC and an increase in TSS toward fruit maturation also marked the shift in the taste characteristic of citrus fruit, which means the semi mature citrus fruit are more popular and directly edible.

Total antioxidant activities of citrus extracts

The antioxidant activities of citrus extracts and essential oils were evaluated by DPPH assay and the result are shown in Figure 2. Independent of the species, the highest DPPH values were found at immature stage in all species for both methanolic extracts and essential oils. Compared to the first stage, the IC50 values increased significantly with ripening stages. At immature stage, both methanolic extract and essential oils from *C.reticulata* and *C. sinensis* exhibited the highest antioxidant activities. At maturity, methanolic extract from *C. sinensis* fruits exhibited the highest activity, while essential oil from mature *C. reticulata* fruits had the highest antioxidant potential. Accordingly, strong antioxidant ability in citrus species may guide commercial producers in harvesting fruits with good antioxidant properties.

Antibacterial activity

The antibacterial activity of the essential oils extracted from the different citrus fruits at different harvesting periods was assessed against human pathogenic bacteria (Table S1). Results showed great differences in the activity between citrus species and during ripening stages. In fact, the essential oil extracted from the fruits of the same species exhibited different levels of susceptibilities that were revealed by microorganisms. Among the selected microorganisms, *S. aureus* and *E. coli* were more sensitive than *P. aeruginosa* bacteria. Considering, *E. coli*, as a Gram (-) bacteria, the highest antibacterial activity was detected at IM stage for *C. limon* and *C. reticulata*; and at CM for *C. sinensis*. *C. limon* and *C. sinensis* were effective against *P. aeruginosa* only at maturity while mandarin essential oil remained inactive against this strain. In accordance with our results

[32], reported no activity of the essential oil extracted from mature mandarin fruit against *P. aeruginosa*. Generally, mature fruits seemed to exhibit the highest antibacterial activities against *S. aureus*, where *C. reticulata* fruits were the best active. According to the ripening stage, for the Gram (+) bacteria, the antibacterial activities increased with maturity. The differences on the oils activity found in our experiment between ripening stages may be related to the modifications of the oils composition during fruit maturation. For the four citrus species, the variations of the activity were not associated to that of the major compound level as limonene. This is in agreement with literature where limonene was found to be a weak antibacterial compound [33]. These findings sustain the fact that the antimicrobial activities are due to a large spectrum of metabolites that could play an important role in conferring these biological activities and thus we should consider all compounds and not restrict to the major ones. Moreover, the inhibitory activity of an essential oil is known to result from a complex interaction between its different constituents, which may produce additive, synergistic, or antagonistic effects, even for those present at low concentrations [34,14].

Trends of aroma volatiles and fruit ripening

At immature stage, *C.limon* exhibited the highest essential oil yields, while at semi immature and mature stages, *C. reticulata* exhibited the highest values. Compared to the first stage, for *C. limon* fruits, essential oil yield decrease with ripening stages, while for the other two species, the essential oil yields increase with maturity (Table 1). Analysis of citrus fruit essential oils composition showed 35 identified compounds presenting fluctuations during ripening (Figure 3). The chromatographic analysis of the volatile fraction extracted by GC-MS in relation to the three ripening stages of the *C. limon* fruits resulted in the identification of 19 components in the three maturation stages. The identified components, their respective retention indices and mean areas at each ripening stage are shown in (Table S2). The volatile compounds found in the *C. limon* fruits belong to different classes, and most relevant were monoterpenes and terpenic alcohols. Although the total percentage of monoterpenes did not change during ripening (83.72%, 83.67% and 85.55%, respectively for IM, SM and CM), there was increase in the levels of terpenic alcohols during the ripening of fruits, where the maximum values were reach at SM stage. Immature fruit presented a limonene/E-B-ocimene/ α-terpineol chemotype since it constituted the predominant compound with percentages of 68.08%/9.84%/1.7% (Figure 3). However, at SM stage, a limonene/sabinene/α-pinene chemotype was detected, where the level of limonene decreased by 2.3 folds while sabinene level increased almost by 30 folds and E-B-Ocimene decreased by 4 folds. At CM stage, the ripe fruits had a limonene/γ-terpinene/borneol chemotype. *C. limon* essential oil was characterized by several compounds that show opposite behavior during ripening; as γ-terpinene and E-B-ocimene. Compared to the first stage, when E-B-ocimene level decreased at CM stage, the γ-terpinene level reached its maximum at maturity. this is in fact in accordance with their biosynthetic pathway, in which γ-terpinene is a precursor of p-cymene [35].

Fruits like *C. sinensis* harvested at three stages of maturity was mainly dominated by monoterpenes, ester acetate and terpenic alcohols (Figure 3). Globally, these essential oil classes, increased significantly with ripening stages. Monoterpenes, as for terpenic alcohols reached maximum values at SM stage, while ester acetate was highest at commercial harvest. Unripe fruits *C. sinensis* exhibited a limonene/B-pinene/linalyl acetate chemotype with 86.43%, 1.54% and 0.79%, respectively. At SM stage, while limonene level decreased, a significant increase of camphor and B-pinene was noted, and accordingly the chemotype changed to limonene/camphor/B-pinene. With the increase of these two compounds, an activation of the related terpene synthases which catalyze their formation from the critical intermediates pinyl and bornyl cation is suggested [36]. At commercial harvest, butyl acetate and B-pinene were identified as the major compounds. Compared to the first stage, ripe fruits were characterized by a significant increase of the terpenic alcohols (α-terpineol and terpinene-4-ol) and acetate esters (butyl acetate). Considering the monoterpenes; α-terpinene, γ-terpinene and sabinene increased with maturity.

Similarly, monoterpenes predominated the *C. reticulata* fruits essential oil in the three harvesting periods (Figure 3). This essential oil class decreased significantly at SM stage then reinstate at maturity. A reverse behavior was detected with terpenic alcohols, which increased by 27 folds at SM and then decreased at maturity. Unripe fruits *C. reticulata* were characterized by the predominance limonene (65%), γ-terpinene (12%) and myrcene (1.6%). At semi mature stage, limonene and γ-terpinene levels were reduced. This reduction was simultaneously switched by an enhancement of 1,8-cineole and E-β-ocimene levels. Shimada et al. [37] reported that the expression of 1,8-cineole and E-β-ocimene genes and their related products in Satsuma mandarin was high in flower and then decreased toward mandarin fruit development. Accordingly, at maturity, 1,8-cineole and E-β-ocimene levels decreased. The essential oil chemotype of ripe fruits becomes limonene (69%)/γ-terpinene (14%)/spathulenol (2.5%). Lota et al. [38] who reported limonene and γ-terpinene as the main compounds of mature mandarins peel oils. Compared to the first stage, the levels of majority of the compounds raised with ripening stages.

Differential volatile signatures from ripening stage and species

Identification of the key-aroma compounds one of the objectives of the present work was to identify a "marker compound/predictive compound" that can be used to determine the botanical origin and authentication of the essential oil and to predict about the maturity stage. According to Radovic et al. [39], a compound is considered as a marker only when its presence or absence was confirmed in all samples collected from the same geographical and pedoclimatic conditions. Accordingly, a list of compounds is proposed as marker compounds in the fruit essential oils of the different Citrus species and for the different ripening stages (Table S3). A comparison between the GC-MS metabolites spectra in the different matrices was performed

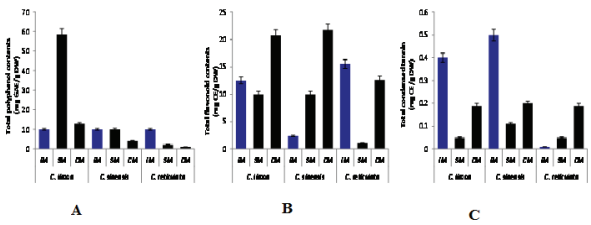


Figure 1: Total phenolic contents (mg GAE/g DW) A, Total flavonoid contents (mg CE/g DW) B, and Total tannin contents (mg CE/g DW) C, in the three citrus species during fruit maturation. Values are presented as mean ± SD (n=3). Vertical bars represent the SD of the means.

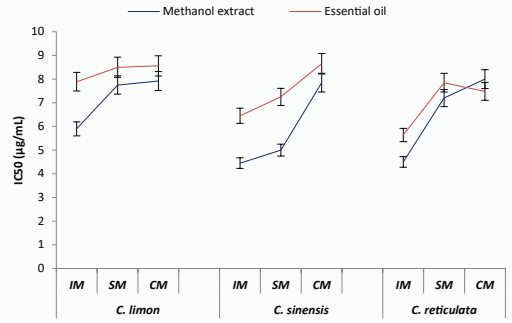


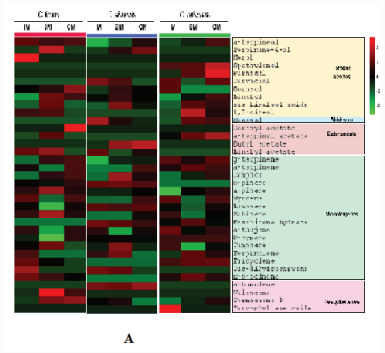
Figure 2: Antioxidant activities detected by DPPH (IC50) of the methanolic extracts and the essential oils in different ripening stage of fruits from the three citrus species (mean ± SD, n=3). Vertical bars represent the SD of the means.

to identify metabolites that are ubiquitously present in all samples and those specific to a single species and as for the ripening stages.

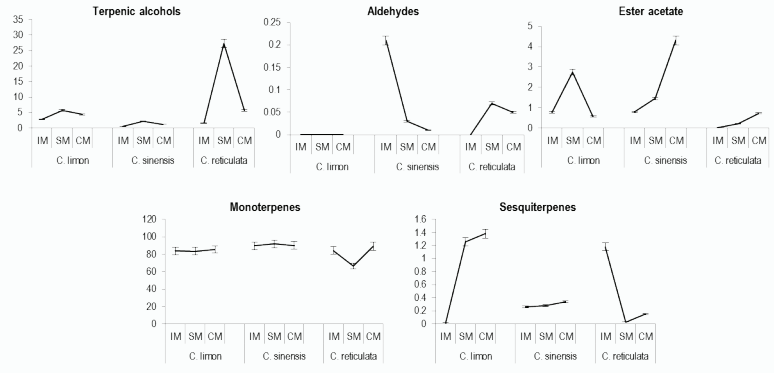
Taking account each single ripening stage, we tended to identify metabolites that are specific or common to the studied species. Accordingly, at immature stage, a total of 7 volatile compounds identified (borneol, camphene, limonene, α-thujene, α-pinene, terpinolene and p-cymene) were common for all of the three species under investigation. At this stage, the largest number of overlapped metabolites (7 shared metabolites; α-terpineol, γ-terpinene, α-terpinene, tricyclene, myrcene, E-β-ocimene and sabinene) was found between *C. limon* and *C. reticulata* (Figure 2 and Table S2). Three fruit-specific metabolites were detected for *C. sinensis* (nonanal, α-Humulene, linalool), 3 for *C. reticulata* (terpinyl acetate, caryophyllene oxide, spathulenol) and only 2 for *C. limon* (1,8-cineole, nerol) (Figure 4 and Table S3).

At semi-mature stage, the three species shared 9 common metabolites (α-terpineol, borneol, camphor, germacrene D, γ-terpinene, limonene, α-pinene, linalool, cis-linalool oxide). Four fruit-specific metabolites were identified in *C. reticulata* (α-terpinene, farnesol, spathulenol, α-thujene), 4 in *C. sinensis* (butyl acetate, α-humulene, cis-dihydrocarvone, carvacrol) and 3 in *C. limon* (linalyl acetate, valencene, sabinene) species. Semi mature fruits from *C. reticulata* shared four metabolites with *C. sinensis*, 4 metabolites with *C. limon* and 3 metabolites with *C. limon* (Figure 4 and Table S3).

The ripe fruits from citrus species shared the highest number of common metabolites (13; α-terpineol, camphor, γ-terpinene, α-thujene, sabinene, p-cymene, borneol, α-terpinene, camphene, limonene, myrcene, α-pinene, linalool). The highest number of specific metabolites was identified in mature fruits of *C. reticulata* (6; terpinyl acetate, spathulenol 1,8-cineol, farnesol, terpinolene, carvacrol), followed by *C. sinensis* (4; terpinene-4-ol, linalyl acetate, α-humulene, butyl acetate) and *C. limon* (2; valencene, geranyl acetate). As for the above mentioned stages, *C. limon* and *C. reticulata* mature fruits shared the highest number of metabolites. Moreover three metabolites were shared between *C. sinensis* and *C. reticulata* and one between *C. sinensis* and *C. limon*.



A)



B)

Figure 3: Volatile evolution patterns in the three citrus species time-course series. A) The heatmap and cluster analysis of volatile compounds in the tthree time-course series (three replicates per stage are shown). Data are expressed as log2 of a ratio (sample/common reference). Red boxes indicate those groups of compounds that increase or decrease during ripening. B) The evolution patterns for volatiles that change during ripening. For each maturity stage (IM to CM) and the mean of the samples (n=3) is shown. Data are expressed as fold changes in relation to IM (of each species) on the log2 scale.

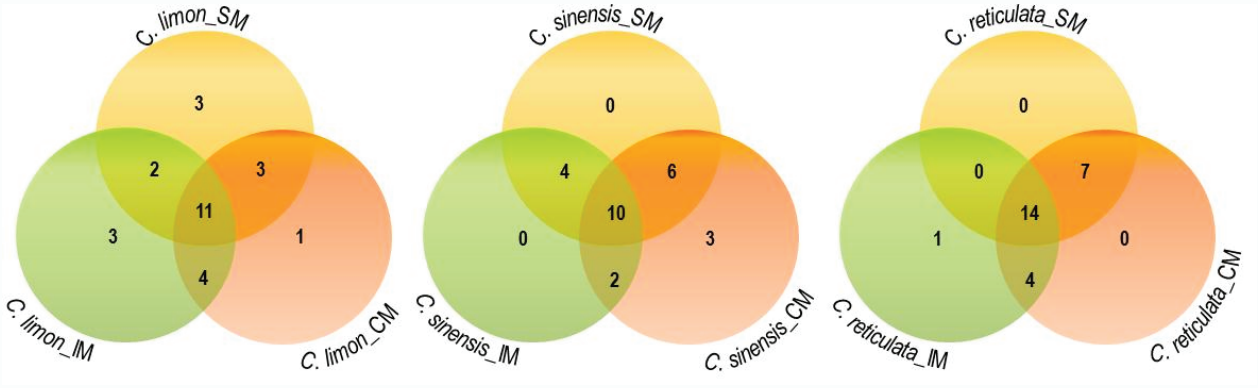


Figure 4: Venn diagram representingmetabolic similarities and specificities of the three studied citrusmatrixes based on fruit volatiles across the three ripening stages. Each number represents a zone of intersectionthat refers to the table presented in Supplementary Table S3.

At species level, eleven metabolites (α-terpineol, borneol, germacrene D, camphene, limonene, γ-terpinene, tricyclene, β-pinene, α-pinene, E-β-ocimene, sabinene) were commonly identified for the *C. limon* fruits harvested from the three stages (Figure 4 and Table S3). Three metabolites were specifically identified at immature stages (cis-dihydrocarvone, terpinolene, nerol), 3 specific to semi mature fruits (terpinyl acetate, cis-linalool oxide, terpinene-4-ol) and one specific to ripe fruits (geranyl acetate). Similarly, high number of shared metabolites between the three stages were detected for *C. reticulata* fruits (terpinyl acetate, borneol, α-terpineol, α-terpinene, limonene, γ-terpinene E-sabinene hydrate, tricyclene spathulenol, α-pinene, α-thujene, E-β-ocimene, terpinolene, p-cymene). On the other hand, for this species, only one compound was exclusively detected at immature stage (caryophyllene oxide). Ten common metabolites were shared between the three ripening stages of *C. sinensis* species (borneol, nonanal, α-humulene, camphene, limonene, E-sabinene hydrate, β-pinene, α-pinene, linalool, p-cymene). *C. sinensis* ripe fruits were characterized by the presence of three unique metabolites (α-terpinene, myrcene, sabinene). Besides the identification of limonene, borneol and α-pinene as conserved metabolic markers (identified in all species and ripening stages), the identified stage-specific and species-specific metabolites, provided important insights into the key molecular processes that determine the quality characteristics of citrus fruits.

Conclusion

In this study, impact of species and ripening stages on the aromatic profile, phytochemical profile and biological activities of citrus fruits was assessed. Using the information on fruit ripening and nutraceutical compound accumulation, the individual compounds could be better harvested and processed for nutraceutical products formulation. Among the targeted fruit species, *C. limon* and *C. sinensis* semi-mature fruits were found to be the best for harvesting polyphenolics, while their ripen fruits were found the best for flavonoid contents. The accumulation of aroma volatile compounds during ripening stages of citrus fruits showed varying trends. For all the citrus species, in order to obtain essential oil with high limonene content, fruits could be harvested at immature or mature stages where the highest limonene level was reached. The fluctuations in physical and sensory characteristics were in accordance with the changes in individual compounds and biological properties of citrus fruits during ripening. Taking account of the great economic importance of citrus and derived essential oils, this study can help the citriculture industry in determining when to harvest for optimum quality and health promoting properties.

References

1. El Hadi MAM, Zhang FJ, Wu FF, Zhou CH, Tao J. Advances in fruit aroma volatile research. Molecules. 2013;18(7):8200-29.
2. De Rovira D. Dictionary of flavor.Wiley-Blackwell publication. 2nd ed. 2008.
3. Dharmawan J, Stefan Kasapis, Philip Curran. Characterization of volatile compounds in selected Citrus fruits from Asia-Part II: Peel Oil. 2008;20(1):21-4.
4. González-Mas MC, Rambla JL, Alamar MC, Gutiérrez A, Granell A. Comparative Analysis of the Volatile Fraction of Fruit Juice from Different Citrus Species. PLos One. 2011;6(7):e22016.
5. Rekha C, Poornima G, Manasa M, Abhipsa V, Pavithra Devi J, Vijay Kumar HT, et al. Ascorbic acid, total phenol content and antioxidant activity of fresh juices of four ripe and unripe citrus fruits. Chem Sci Trans. 2012;1(2):303-10.
6. Smyth H, Cozzolino D. Instrumental Methods (Spectroscopy, Electronic Nose, and Tongue) as Tools to Predict Taste and Aroma in Beverages: Advantages and Limitations. Chem Rev. 2013;113(3):1429-40.
7. Bourgou S, Rahali FZ, Ourghemmi I, Saïdani Tounsi M. Changes of Peel Essential Oil Composition of Four Tunisian Citrus during Fruit Maturation. Sci World J. 2012;2012:528593.
8. Rowshan V, Najafian S. Changes of Peel Essential Oil Composition of Citrus aurantium L. During Fruit Maturation in Iran. J Essent Oil Bear Plants. 2015;18(4):1006-12.
9. Russo M, Serra D, Suraci F, Postorino S. Effectiveness of electronic nose systems to detect bergamot (Citrus bergamiaRissoetPoiteau) essential oil quality and genuineness. J Essent Oil Res. 2012;24(2):137-51.
10. Do TKT, Hadji-Minaglou F, Antoniotti S, Fernandez X. Authenticity of essential oils. TrAC Trends Anal Chem. 2015;66:146-57.
11. Sarrou E, Chatzopoulou P, Dimassi-Theriou K, Therios I. Volatile Constituents and Antioxidant Activity of Peel, Flowers and Leaf Oils of Citrus aurantium L. Growing in Greece. Molecules. 2013;18(9):10639-47.
12. Wei A, Shibamoto T. Antioxidant/Lipoxygenase Inhibitory Activities and Chemical Compositions of Selected Essential Oils. J Agric Food Chem. 2010;58(12):7218-25.
13. Virot M, Tomao V, Ginies C, Visinoni F, Chemat F. Green procedure with a green solvent for fats and oils' determination. Microwave-integrated Soxhlet using limonene followed by microwave Clevenger distillation. J Chromatogr A. 2008;1196-7:147-52.
14. Lamine M, Rahali FZ, Hammami M, Mliki A. Correlative metabolite profiling approach to understand antioxidant and antimicrobial activities from citrus essential oils. Int J Food Sci Technol. 2019;54(8):2615-23.
15. Singleton VL, Rossi JA. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am J Enol Vitic. 1965;16:144-58.

16. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total favonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2002;10(3):178-82.

17. Ram PR, Mehrotra BN. 1993. Compendium of Indian Medicinal Plants, (Drug Research Preparative: A CDRI Series), Central Drug Research Institute, Lucknow, 2.

18. Hanato T, Kagawa H, Yasuhara T, Okuda T. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chem Pharm Bull (Tokyo). 1998;36(6):2090-7.

19. Rıos JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol. 2005;100(1-2):80-4.

20. Ribeiro PR, Fernandez LG, de Castro RD, Ligterink W, Hilhorst HWM. Physiological and biochemical responses of Ricinus communis seedlings to different temperatures: a metabolomics approach. BMC Plant Biol. 2014;14:223.

21. Ribeiro PR, Willems LAJ, Mudde E, Fernandez LG, Ligterink W, Hilhorst HWM. Metabolite profiling of the oilseed crop Ricinus communis during early seed imbibition reveals a specific metabolic signature in response to temperature. Ind Crops Prod. 2015;67:305-09.

22. Lommen A. MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. Anal Chem. 2009;81(8):3079-86.

23. Rapisarda P, Lo Bianco M, Pannuzzo P, Timpanaro N. Effect of cold storage on vitamin C, phenolics and antioxidant activity of five orange genotypes [Citrus sinensis (L.)Osbeck]. Postharvest Biol Technol. 2008;49(3):348-54.

24. Gunness P, Kravchuk O, Nottingham SM, D'Arcy BR, Gidley MJ. Sensory analysis of individual strawberry fruit and comparison with instrumental analysis. Postharvest Biol Technol. 2009;52(2):164-72.

25. Shwartz E, Glazer I, Matityahu I, Holland D, Amir R, Bar-llan I, et al. Changes in chemical constituents during the maturation and ripening of two commercially important pomegranate accessions. Food Chem. 2009;115(3): 965-73.

26. Lattanzio V, Kroon PA, Quideau S, Treutter D. Plant phenolics-secondary metabolites with diverse functions. Recent Advances in Polyphenol Research. 2008;1.

27. Wang S, Tu H, Wan J, Chen W, Liu X, Luo J, et al. Spatio-temporal distribution and natural variation of metabolites in citrus fruits. Food Chem. 2016;199:8-17.

28. Moulehi I, Bourgou S, Ourghemmi I, Tounsi MS. Variety and ripening impact on phenolic composition and antioxidant activity of mandarin (Citrus reticulate Blanco) and bitter orange (Citrus aurantium L.) seeds extracts. Ind Crops Prod. 2012;39:74-80.

29. Kyraleou M, Kallithraka S, Theodorou N, Teissedre P, Kotseridis Y, Koundouras S. Changes in tannin composition of Syrah grape skins and seeds during fruit ripening under contrasting water conditions. Molecules. 2017;22(9):1453.

30. Smallwood PD, Steele MA, Faeth SH. The Ultimate Basis of the Caching Preferences of Rodents, and the Oak-Dispersal Syndrome: Tannins, Insects, and Seed Germination. American Zoologist. 2001;41(4):840-51.

31. Dixon RA, Xie DY, Sharma SB. Proanthocyanidins --a final frontier in flavonoid research? New Phytol. 2005; 165(1):9-28.

32. Espina L, Somolinos M, Loran S, Conchello P, Garcia D, Pagan R. Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. Food Control. 2011;22(6):896-902.

33. Burt S. Essential oils: their antibacterial properties and potential applications in foods--a review. Int J Food Microbiol. 2004;94(3):223-53.

34. Perez MJ, Falque E, Domınguez H. Antimicrobial Action of Compounds from Marine Seaweed. Mar Drugs. 2016;14(3):52.

35. Poulose AJ, Croteau R. Biosynthesis of aromatic monoterpenes: conversion of gamma-terpinene to p-cymene and thymol in Thymus vulgaris L. Arch Biochem Biophys. 1978;187(2):307-14.

36. Degenhardt J, Kollner TG, Gershenzon J. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochem. 2009;70(15-16):1621-37.

37. Shimada T, Endo T, Fujii H, Hara M, Omura M. Isolation and characterization of (E)-beta-ocimene and 1,8 cineole synthases in Citrus unshiu Marc. Plant Sci. 2005;168(4):987-95.

38. Lota ML, De Rocca Serra D, Tomi F, Jacquemond C, Casanova J. Volatile components of peel and leaf oils of lemon and lime species. J Agric Food Chem. 2002;50(4):796-805.

39. Radovic BS, Careri M, Mangia A, Musci M, Gerboles M, Anklam E. Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. Food Chem. 2001;72(4):511-20.

Table S1: Antibacterial activity of fruit essential oils of three citrus species at different ripening stages.

Bacteria strains	Ripening stage	Inhibition zone (mm)			
		C. limon	*C. sinensis*	*C. reticulata*	*Gentamicin*
E. coli	IM	27 ± 0.00	na	27 ± 0.00	27
	SM	na	12.5 ± 0.10	2 ± 0.00	
	CM	18 ± 0.02	18 ± 0.12	17 ± 0.00	
S. aureus	IM	4 ± 0.05	na	2.5 ± 0.05	22
	SM	na	2.5 ± 0.05	2.05 ± 0.00	
	CM	17 ± 0.05	14 ± 0.02	21 ± 0.21	
P. aeruginosa	IM	na	na	na	16
	SM	na	na	na	
	CM	14 ± 0.00	12 ± 0.01	na	

Values represent mean of three replicates ± SD; Na: Not Active

Table S2: Phytochemical composition (%) and identification of chemical components of fruit essential oil of the three citrus species at three development stages.

Compounds	Ri[a]	*C. limon*			*C. sinensis*			*C. reticulata*			Im[b]	*f. value*	*p.value*	-log10(p)	FDR
		IM	SM	CM	IM	SM	CM	IM	SM	CM					
α-terpineol	_	1.7	0.93	1.22	_	0.04	0.52	0.05	0.11	1.25	1,2	1.28E+31	4.57E-123	122.34	6.51E -123
Terpinene-4-ol	1159	_	1.28	_	_	0.05	0.26	_	_	_	1,2	2.93E +32	1.68E -128	127.77	3.11E -127
Nerol	1143	0.24	_	_	_	_	_	_	_	_	1,2	7.43E +30	4.09E -122	121.39	4.45E -122
Spathulenol	1011	_	_	_	_	_	_	0.17	0.21	2.5	1,2	8.43E +30	2.46E -122	121.61	3.14E -122
Farnesol	1058	_	_	_	_	_	_	_	0.06	0.79	1,2	1.83E +32	1.1E -127	126.96	1.02E -126
Carvacrol	_	_	_	_	0.22	0.1	_	0.12	_	0.14	1,2	1.01E +31	1.21E -122	121.92	1.6E -122
Borneol	1031	0.33	0.65	2.54	0.14	0.7	0.3	1.22	0.04	0.04	1,2	5.04E +31	1.93E -125	124.71	6.78E -125
Linalol	1276	_	1.59	0.65	0.12	0.54	0.13	_	0.1	0.67	1,2	1.31E +31	4.25E -123	122.37	6.28E -123
cis-linalool oxide	1139	_	0.49	_	_	0.82	0.03	_	0.36	0.14	1,2	1.61E +31	1.85E -123	122.73	2.85E -123
1,8-cineol	1654	0.51	0.82	_	_	_	_	_	26.43	0.18	1,2	7.66E +30	3.62E -122	121.44	4.06E -122
Nonanal	1165	_	_	_	0.21	0.03	0.01	_	0.07	0.05	1,2	1.63E +32	1.77E -127	126.75	1.31E -126
Geranyl acetate	1189	_	_	0.56	_	_	_	_	_	_	1,2	6.62E +31	6.48E -126	125.19	3.43E -125
α-terpinyl acetate	1752	_	0.15	_	_	_	_	0.02	0.2	0.72	1,2	8.17E +30	2.79E -122	121.55	3.42E -122
Butyl acetate	1230	_	_	_	_	1.45	4.21	_	_	_	1,2	1.05E +31	1.03E -122	121.99	1.41E -122
Linalyl acetate	1697	0.78	2.61	_	0.79	_	0.1	_	_	_	1,2	3.41E +31	9.19E -125	124.04	2.13E -124
γ-terpinene	1564	0.04	5.12	9.96	_	0.34	0.43	12.44	2.53	14.06	1,2	1.27E +32	4.84E -127	126.31	2.99E -126
α-terpinene	1178	0.24	_	1.05	_	_	0.93	0.3	1.52	0.73	1,2	8.06E +30	2.96E -122	121.53	3.42E -122
Camphor	1172	_	0.32	0.58	_	4.81	0.17	_	0.04	0.35	1,2	5E +30	2E -121	120.7	2.11E -121
β-pinene	928	0.11	0.07	0.38	1.54	1.8	0.97	_	_	_	1,2	4.63E +31	2.71E -125	124.57	8.05E -125
α-pinene	939	1.29	5.9	1.14	0.41	0.44	0.7	0.03	0.7	1.25	1,2	2.87E +31	1.83E -124	123.74	3.97E -124
Myrcene	1285	1.54	_	0.99	_	_	0.71	1.59	_	1	1,2	2.27E +31	4.72E -124	123.33	8.31E -124
Limonene	1238	68.08	37.63	69.71	86.43	81.52	85.35	65.37	51.81	69	1,2	2.36E +31	4.03E -124	123.39	7.45E -124
Sabinene	1376	1.06	31.49	0.63	_	_	0.36	1.31	_	0.18	1,2	2.77E +31	2.12E -124	123.67	4.36E -124
E-sabinene hydrate	1383	_	_	_	0.37	0.26	0.14	0.04	0.24	0.04	1,2	2.51E +31	3.16E -124	123.5	6.15E -124
α-thujene	_	0.38	_	0.34	0.37	_	0.2	1.31	0.12	0.39	1,2	4.98E +31	2.02E -125	124.7	6.78E -125
P-cymene		0.14	_	0.5	0.25	0.27	0.21	0.63	0.68	0.7	1,2	8.17E +30	2.79E -122	121.55	3.42E -122
Camphene		0.13	0.9	0.03	0.07	1.22	0.06	0.39	_	0.1	1,2	4.5E +31	3.04E -125	124.52	8.05E -125
Terpinolene	928	0.42	_	_	0.25	1.07	_	0.02	0.7	0.17	1,2	4.52E +31	2.97E -125	124.53	8.05E -125
Tricyclene	939	0.16	0.03	0.01	_	_	_	0.09	0.14	0.15	1,2	1.64E +30	1.72E -119	118.76	1.72E -119
Cis-dihydrocarvone	1018	0.29	_	_	0.13	0.1	_	_	_	_	1,2	4.46E +30	3.16E -121	120.5	3.24E -121
E-β-ocimene	1092	9.84	2.21	0.23	_	_	_	0.77	7.93	1.05	1,2	1.9E +31	9.51E -124	123.02	1.53E -123
α-humulene	928	_	_	_	0.19	0.16	0.34	_	_	_	1,2	3.94E +32	5.17E -129	128.29	1.91E -127
Valencene	_	_	0.37	0.03	_	_	_	_	_	_	1,2	2.16E +32	5.7E -128	127.24	7.03E -127
Germacrene D	991	0.02	0.89	1.35	0.07	0.12	_	_	0.03	0.15	1,2	8.17E +30	2.79E -122	121.55	3.42E -122
Caryophyllene oxide	980	_	_	_	_	_	_	1.18	_	_	1,2	8.07E +30	2.93E -122	121.53	3.42E -122

[a]Retention indices on the HP 5MS column. [b]IM: Identification Method: 1 –comparison of retention times; 2– comparison of mass spectra with MS libraries _: not detected. Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multi-comparison Tukey's test. ***p<.05.*

Table S3: Shared and specific metabolites detected within the four citrus species based on fruit tissue across the three development stages.

Names	Total	Elements
C. limon.CM /C. limon.IM /C. limon. SM	11	a-terpineol Borneol Germacrene D Camphene Limonene g-terpinene Tricyclene b-pinene a-pinene E-Î²-Ocimene Sabinene
C. limon.IM /C. limon.SM	2	Acetate de linalyl 1,8-Cineol
C. limon.CM /C. limon.IM	4	a-terpinene Myrcene a-thujene P-Cymene
C. limon.CM /C. limon.SM	3	Valencene Camphor Linalol
C. limon.IM	3	Cis -dihydrocarvone Terpinolene Nerol
C. limon.SM	3	Acetate d'a-terpinyl Oxyde de cis-linalol Terpinene-4-ol
C. limon.CM	1	Acetate de geranyl
Names	**Total**	**Elements**

C. sinensis.CM /C. sinensis.IM /C. sinensis.SM	10	Borneol Nonanal a-Humulene Camphene Limonene E Sabinene hydrate b pinene a-pinene Linalol P-Cymene
C. sinensis.IM/ C. sinensis.SM	4	Germacrene D Cis-dihydrocarvone Terpinolene Carvacrol
C. sinensis.CM /C. sinensis.IM	2	Acetate de linalyl a-thujene
C. sinensis.CM /C. sinensis.SM	6	a-terpineol Camphor Butyla cetate g-terpinene Oxyde de cis-linalol Terpinene-4-ol
C. sinensis.CM	3	a-terpinene Myrcene Sabinene
Names	**Total**	**Elements**
C. reticulata.CM/C. reticulata.IM /C. reticulata.SM	14	Acetate d'a-terpinyl Borneol a-terpineol a-terpinene Limonene g-terpinene E Sabinene hydrate Tricyclene Spathulenol a-pinene a-thujene E-Î²-Ocimene Terpinolene P-Cymene
C. reticulata.CM/C. reticulata.IM	4	Camphene Myrcene Sabinene Carvacrol
C. reticulata.CM /C. reticulata.SM	7	Nonanal Camphor Germacrene D Farnesol Linalol 1,8-Cineol Oxyde de cis-linalol
C. reticulata.IM	1	Oxyde de Caryophyllene