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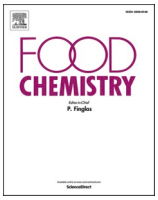

Screening the Citrus Greek National Germplasm Collection for fruit quality and metabolic footprint

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ABSTRACT

Citrus fruits are one of the most important fruits in the global food industry due to their unique taste and nutritional benefits. Herein, we characterize the physicochemical and bioactive attributes of twenty-nine Greek citrus accessions, including oranges, mandarins/clementines, lemons, bergamot, citrons and lime along with twenty-seven highly commercial international cultivars. The assessed genotypes differ in various quality traits including color, ripening, and textural attributes. Several indigenous cultivars displayed desirable organoleptic traits, such as the oranges 'Valencia Oval Porou' (e.g., juice content and ascorbic acid) and 'Sanguine Gouritis' (eg., soluble solids (SSC) and acidity (TA) ratio), the mandarin 'Clementine Porou' (e.g., SSC/TA) and the lemon 'Vakalou' (e.g., firmness, acidity). Differences in primary metabolites, mainly in sugars, organic acids and amino acids were recorded among the tested species and cultivars. In addition, the autochthonous orange cultivars 'Sanguine Gouritsis' and 'Valencia Oval Porou' contained high sucrose levels whereas 'Lainato Chanion' had high hesperidin content. This large-scale analysis supports the ample availability of genetic resources for the development of citrus cultivars with improved nutritional quality traits.

1. Introduction

Citrus (genus Citrus L.) is among the most important cultivated crops in terms of area and production values around the world (Liu et al., 2012). Citrus fruits, like orange, mandarin, lemon, lime, bergamot and citron, are well-accepted by consumers all over the world because of their attractive colors, pleasant flavors and aroma (Zhong and Nicolosi, 2020). In addition to this, *Citrus* fruits are highly nutritious, containing many primary metabolites, that are tightly linked to their unique quality, as well as secondary metabolites, which form an excellent source of bioactive substances (Saini et al., 2022). It has been reported that the main sugars of Citrus fruit are glucose, fructose, and sucrose, and the main acids are citric and malic acid (Lado et al., 2018). It was also documented that hesperidin and β-cryptoxanthin are the main flavonoids and carotenoids, respectively (Lin et al., 2023). The results of numerous epidemiological studies indicated a direct correlation between fresh citrus fruit or juice consumption and low risks of chronic diseases, such as cancer, cardiovascular diseases, and diabetes (Farag et al., 2020; Saini et al., 2022). Moreover, primary and secondary metabolites are significantly different depending on the *Citrus* species and cultivars, leading to different general quality parameters and health benefits (Saini et al., 2022). Nevertheless, comparative analysis of quality characteristics and metabolite profiles according to different fruits *Citrus* species and cultivars has been limited.

Citrus fruit quality traits and metabolic dynamics are generally affected by genetic variability and are influenced by the cultivation environment (Ladaniya, 2007; Ding et al., 2015). The success of *Citrus* crop improvement, therefore, lies in efficiently identifying and

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incorporating genetic diversity from various plant genetic sources including currently cultivated cultivars, newly developed cultivars, landraces and germplasm collections (Rao et al., 2021). Currently, the fruit industry is characterized by the fact that only a few international cultivars are widely grown in most citrus-producing countries, while traditional, locally-adapted cultivars are threatened with extinction (Zhong and Nicolosi, 2020). This trend in citrus cultivation increases the risk that a significant part of the remaining diversity of citrus cultivars will be lost (Vincent et al., 2020), resulting in the 'crop genetic erosion' phenomenon.

The Mediterranean basin is a particularly dense area of autochthonous genotypes of citrus and is known for being a source of healthy foods (Duarte et al., 2016). Within Greece, many traditional citrus cultivars were grown which are well adapted to the environmental conditions of different regions. On this basis, the aims of the present study are to characterize and compare quality parameters and levels of bioactive compounds in fruits from the local germplasm of citrus in Greece, comprising twenty-nine autochthonous oranges, mandarins/clementines, lemons, bergamots, citrons and limes cultivars, using also twenty-seven widespread international cultivars as reference. This is the first wide report focusing on the variations in fruit quality and metabolic traits among autochthonous and international citrus cultivars. The results contribute to an investigation of nutrition in different *Citrus* species/cultivars and provide a scientific basis for breeding citrus aspects.

2. Materials and methods

2.1. Plant material and sampling

Physiological and metabolic data of fifty-six *Citrus* cultivars belonging to nine *Citrus* species (*C.* × *aurantium* var. *sinensis* L., *C.* × *aurantium* var. *deliciosa* ined., *C.* × *aurantium* var. *clementina* ined., *C.* × *limon* var. *limon* (L.) Burm. f., *C.* × *limon* var. *bergamia* ined., *Citrus medica* L *C.* × *aurantifolia* var. *aurantifolia*) were determined at the commercial harvest stage from the germplasm collection of the Institute of Olive Tree, Subtropical Plants and Viticulture (ELGO-DIMITRA, Chania, Crete, Southern Greece). The cultivars were divided into oranges (twenty-five cultivars, *C.* × *aurantium* var. *sinensis* L.), mandarins/clementines (eleven cultivars, *C.* × *aurantium* var. *deliciosa* ined., *C.* × *aurantium* var. *clementina* ined. *C.* × *aurantium* var. *clementina* ined × *(C.* × *aurantium* var. *paradisi.* ined. × *C.* × *aurantium* var. *deliciosa* ined)), lemons (twelve cultivars, *C.* × *limon* var. *limon* (L.) Burm. f.), bergamots (three cultivars, *C.* × *limon* var. *bergamia* ined.), citrons (three cultivars, *Citrus medica* L.), and limes (two cultivars, *C.* × *aurantifolia* var. *aurantifolia*). The examined cultivars are shown in Table S1. The germplasm orchard consisted of 25-year-old trees, planted in the same block, at 4 × 6 m spacing between rows and along the row, all of them being grafted onto *C* × *aurantium* var. *aurantium* L. (sour orange) rootstock, which is the main rootstock in Greece. Trees were grown in open field with standard cultivation tasks, in line with the optimum agricultural practices. The meteorological data during fruit growth and sampling (growing season 2021-22) are given in supplementary information (Table S2).

Fruit of examined *Citrus* cultivars/species were picked at commercial harvest time (details for each cultivar about harvest period are provided in Table S1) and subsequently evaluated for their textural properties, physiological characteristics, and endocarp metabolite traits. Briefly, the sampling was performed from three trees from the inner and outer parts of the canopy, subdivided into four quadrants. About eight fruit *per* tree were collected, while the fruit of each tree was randomly combined to obtain three representative biological replicates. Fresh fruit was hand-harvested, boxed, and transported immediately to the Pomology laboratory (AUTh) for its quality evaluation. The fruit intended for primary and secondary metabolite analysis were separated from the seeds and the endocarp was frozen with liquid nitrogen and stored at −80 °C for further analysis.

2.2. Citrus fruit quality characteristics

2.2.1. Color parameters

The internal color of the endocarp of *Citrus* fruit was measured with a Minolta CR200 colorimeter (Minolta, Osaka, Japan) using the CIE (Commission International de l'Eclairage) parameters referring to Lightness (L^*), Redness (a^*) and Yellowness (b^*). Chroma (C^*) and Hue angle (H°) was calculated using the equations; $C^* = (a^{*2} + b^{*2})^{0.5}$ and H° = arctan (b^*/a^*). Values L^*, a^* and b^* were converted to the *Citrus* Color Index (CCI) via the formula CCI = (a^* 1000)/(b^*L^*) (Singh and Reddy, 2006). Each measurement was performed at the endocarp transverse segment of each fruit and the calculated means are presented in Table S2.

2.2.2. Internal quality parameters

The juice content (% of total weight), soluble solid concentration (SSC, % Brix, digital refractometer; Atago PR-101, Atago Co. Ltd., Japan), juice pH (Eco Titrator, Metrohm, Switzerland), titratable acidity (TA, as % citric acid, potentiometric titration with 0.1 N NaOH up to pH 8.2, using 1 mL of diluted juice in 100 mL dH_2O.), ripening index (SSC TA^{-1}) were determined according to Meléndez-Martínez et al. (2007). The weight (grams; analytical balance 0.1gr.; KERN, model EMB 6000-1, Germany) of fruit was recorded. Endocarp (without seeds) samples were oven-dried at 65 °C for 48 h to a constant weight to determine dry matter (DM%). Ascorbic acid content was determined using 2,6-dichlorophenolindophenol titration according to Meléndez-Martínez et al. (2007). In all cases, three biological replications of eight fruit *per* replication were used (Table S3).

2.2.3. Endocarp firmness determination

Firmness of endocarp were determined based on the required force to penetrate juice vesicles of eight fruit *per* replication (three biological replicates *per* cultivar), using a Texture Analyzer TA XT2i (Stable Microsystems, Godalming, Surrey, UK) (Goulas et al., 2015). Fruit cut transversal and placed on the 'crisp fracture support ring' then the maximum force of penetration using an 8-mm diameter stainless steel probe were recorded in two segments without seeds. In all measurements, the speed of the arm was 20 mm s^{-1}. Results were expressed in Newtons (N).

2.3. Primary and secondary metabolite profiling in the endocarp of the various Citrus cultivars

2.3.1. Primary metabolite analysis by gas chromatography–mass spectrometry (GC-MS)

Citrus fruit samples (500 mg of frozen grinding endocarp; segments without seeds) at the physiologically mature stage were extracted with 1.4 mL methanol plus 0.1 mL adonitol (0.2 mg ml^{-1}) solution for 10 min at 70 °C with continual agitation. After collecting the supernatant via centrifugation, 750 μL chloroform and 1500 μL dH_2O were added. From the upper polar phase, 0.15 mL was dried (using a vacuum dryer), and derivatized with 40 μL methoxyamine hydrochloride (20 mg mL^{-1}, 120 min, 37 °C), and then with 70 μL *N*-methyl-N-(trimethylsilyl) trifluoroacetamide reagent (MSTFA) for 30 min at 37 °C. Subsequently, 1 μL of primary metabolite extracts were injected into a PerkinElmer Clarus® 590 GC equipped with Clarus® SQ 8 S MS (Perkin Elmer, USA) (Karagiannis et al., 2021). The ratio was established at 20:1. A TR-5MS capillary column dimension 30 m × 0.25 mm × 0.25 μm was used. The injector temperature was set to 220 °C, ion source to 230 °C and the interface to 250 °C. The temperature program was kept at 70 °C for 2 min, then increased to 260 °C with a rate of 8 °C min^{-1}, and remained for 18 min. One mL min^{-1} was set for the carrier gas flow rate. M/Z was recorded in the range of 50–550. Internal standards, NIST11, and the GOLM database were used to identify peaks. The relative response of the internal standard peak of adonitol was utilized to standardize the observed metabolite proportions (Karagiannis et al., 2018). The

normalized values were additionally analyzed by one-way ANOVA followed by the least significant difference (LSD) test ($P \leq 0.05$). The data of the identified metabolites are reported in Table S4. The analysis included three independent biological replications for each cultivar.

2.3.2. Polyphenolic analysis of Citrus cultivars by ultra–performance liquid chromatography – Tandem mass spectrometer (UPLC – MS/MS)

For polyphenolic extraction, frozen fruit grinded tissues were dried in a freeze-dryer (Freeze-dryer Alpha 1–2 LD plus, Christ, Osterode, Germany; in −24 °C) and then converted to a fine powder. Freeze-dried *Citrus* segment tissue (100 mg of endocarp) was mixed with 4 mL methanol (80%) into a 15-mL falcon tube. The mixture was sonicated (20 min), shaken (3 h, 20 °C) and incubated at 4 °C (overnight) in the dark. Secondary metabolite extract was acquired following filtration through a 0.22 μm PFTE membrane into a glass vial.

Targeted ultra-performance liquid chromatography-tandem mass spectrometer (UPLC–MS/MS) was performed on a Waters Acquity system (Milford, MA, USA) consisting of a binary pump, an online vacuum degasser, an autosampler, and a column compartment. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm, kept at 40 °C. The phenolic analysis was performed as previously described (Vrhovsek et al., 2012). Data processing was carried out using the Mass Lynx Target Lynx Application Manager (Waters).

2.3.3. Carotenoid analysis by ultra-performance liquid chromatography method with diode array detection (UPLC-DAD)

Citrus carotenoids were extracted as previously reported by Multari et al. (2020). Briefly, 200 mg of freeze-dried and pulverized samples were mixed with 5 mL of the mixture MeOH/Acetone/Hex, 25/25/50 %, v/v/v, vortexed and mixed on an orbital shaker for 10 min, under dim light conditions. Following, the extraction proceeded into an ultrasound bath for 5 min (10 °C and 59 KHz), the mixtures were centrifuged for 10 min (1800 *g*; 4 °C), and the organic layers were collected to 50 mL falcon tubes. The extraction was repeated three times and the organic layers were combined and dried under reduced pressure in a GeneVac concentrator.

The crude extracts were saponified by adding 4 mL of 15 % KOH in MeOH (w/v) and incubated overnight at room temperature under shaking. After saponification, 4 mL of NaCl (9 %, w/v), and 5 mL of Hex: diethyl ether (3:1, v/v) were added to the samples. The mixtures were placed on an orbital shaker for 10 min at room temperature, vortexed and centrifuged (5 min; 1800 *g*; 4 °C). This process was repeated three times. The organic layers were combined and washed three times with 5 mL of water. The organic layers were dried under reduced pressure in GeneVac concentrator and reconstituted in 0.8 mL of ACN/MTBE/MeOH, 60/20/20 (v/v/v). The extracts were filtered through 0.22 μm PTFE membranes and stored at −80 °C until HPLC analysis.

Carotenoids were analyzed on an Agilent 1200 UHPLC-DAD instrument (Agilent Technologies, Wokingham, UK), as described (Multari et al., 2018). The chromatographic separation was conducted using a YMC C30 Carotenoid column (250 × 2.1 mm; 3 μm particle size) kept at 35 °C. The flow rate was 0.35 mL min^{-1}, the injection volume was 10 μL, and the DAD was set at 350–550 nm. Each Citrus cultivar was extracted and analyzed in triplicate and the results were expressed as mg of an individual carotenoid compound identified per kg of dried tissue using calibration curves of external standards violaxanthin, antheraxanthin, neoxanthin, lutein, zeaxanthin, β-criptoxanthin, α-carotene and β-carotene injected. The cumulative contents of carotenoids were determined by summing up the individual compounds found in the same tissue (Table S5).

2.4. Statistical analysis

The statistical analysis was performed using the SPSS (SPSS v25.0., Chicago, USA) by Least Significant Difference (LSD) test (using three biological replicates) at a 5% level of significance. Hierarchical analysis and clustering heatmaps in primary and secondary metabolites as well as principal component analysis (PCA) among physiological traits and metabolites of citrus cultivars were employed using online Clustvis software (http://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo, 2015).

3. Results and discussion

Citrus fruits are among the highest-consumption fruits for energy, nutrients, and health supplements (Farag et al., 2020; Rao et al., 2021; Saini et al., 2022). In this study, we examined the endocarp quality and bioactive metabolites of fifty-six cultivars from six *Citrus* species, grouped into oranges (twenty-five), mandarins / clementines (eleven), lemons (twelve), bergamots (three), citrons (three), and limes (two). The studied fruit material includes the *ex-situ* Greek Citrus germplasm collection (Chania, Creta; twenty-nine autochthonous cultivars) and twenty-seven globally popular cultivars (Table S1). Numerous factors, such as cultivar, climate, environment, and ripening time may affect citrus quality and metabolic composition (Ladaniya, 2007; Lux et al., 2019; Vincent et al., 2020). It should be noted that this research used commercially ripe citrus fruits from the same experimental orchard, and therefore, the genotype impact may explain the observed variance in quality and metabolic parameters. This study presents the first thorough large-scale characterization of Citrus accessions, mostly from Greece, to help enhance this species by utilizing current diversity.

3.1. Physiological characteristics of the citrus cultivars

At commercial harvest of each cultivar (Table S1), the analysis of various physiological traits including weight, endocarp color indexes, juice content, firmness, DM, SSC, acidity (TA), juice pH, maturity index (SSC TA^{-1}) and ascorbic acid (AsA) among the evaluated oranges, mandarins/clementines, lemons, bergamots, citrons, and limes revealed extensive variability (Fig. 1, Table S3).

3.1.1. Quality differences among orange and mandarin cultivars

Quality differences among the orange and mandarin cultivars in the quality parameters were identified. For example, the oranges with the highest values were 'Navellina Artas' for the weight (342 g), 'Salustiana' for firmness (10.1 N), 'Tarocco' for juice (58%), 'Matsitiko Chiou' for SSC (15%) and 'W. Navel SRA 100′ for ascorbic acid (74 mg/100 mL) (Fig. 1, Table S3). In addition, endocarp assessment of mandarins/clementines indicated that 'Willowleaf' had the lowest weight (67.6 g), firmness (0.9 N) and the highest ascorbic acid content (82 mg / 100 mL) while the 'Nova' hybrid produced the highest juice content (62%) with high acidity (1%) (Fig. 1, Table S3).

One of the standard parameters for determining fruit quality at harvest is the maturity index, frequently measured in quality control and breeding programs for oranges and mandarins (Garcia Neves et al., 2018). Among the studied common oranges, the Greek accessions 'Plake Artas' and 'Thilikoportokalia' showed rather low maturation indexes (7.2 and 5.1, respectively) (Fig. 1, Table S3), indicating that these cultivars do not fulfill the minimum ratio (8.0) to be considered appropriate for market distribution (Lado et al., 2014). From all studied blood orange, the Greek cultivar 'Sanguine Gouritsis" disclosed the highest maturation index (15.0), due to balanced SSC and TA contents (Fig. 1, Table S3), thus exhibiting superior fruit taste and high-quality juice.

In consistency with previously reported findings (Simón-Grao et al., 2014), our results revealed that the 'Clementine' mandarins group demonstrated the highest mean value of maturation index (19.3), possibly reflecting its high SSC level (Fig. 1, Table S3). Present data (Fig. 1, Table S3) also evidenced that the Greek accession "Clementine Porou" exhibited similar quality characteristics, including fruit weight, juice content, SSC, juice pH, and SSC/TA, to that of 'Clementine SRA 63', which is one of the major cultivated clementine cultivars across the world (Liu et al., 2012). Thus, the indigenous 'Clementine Porou'

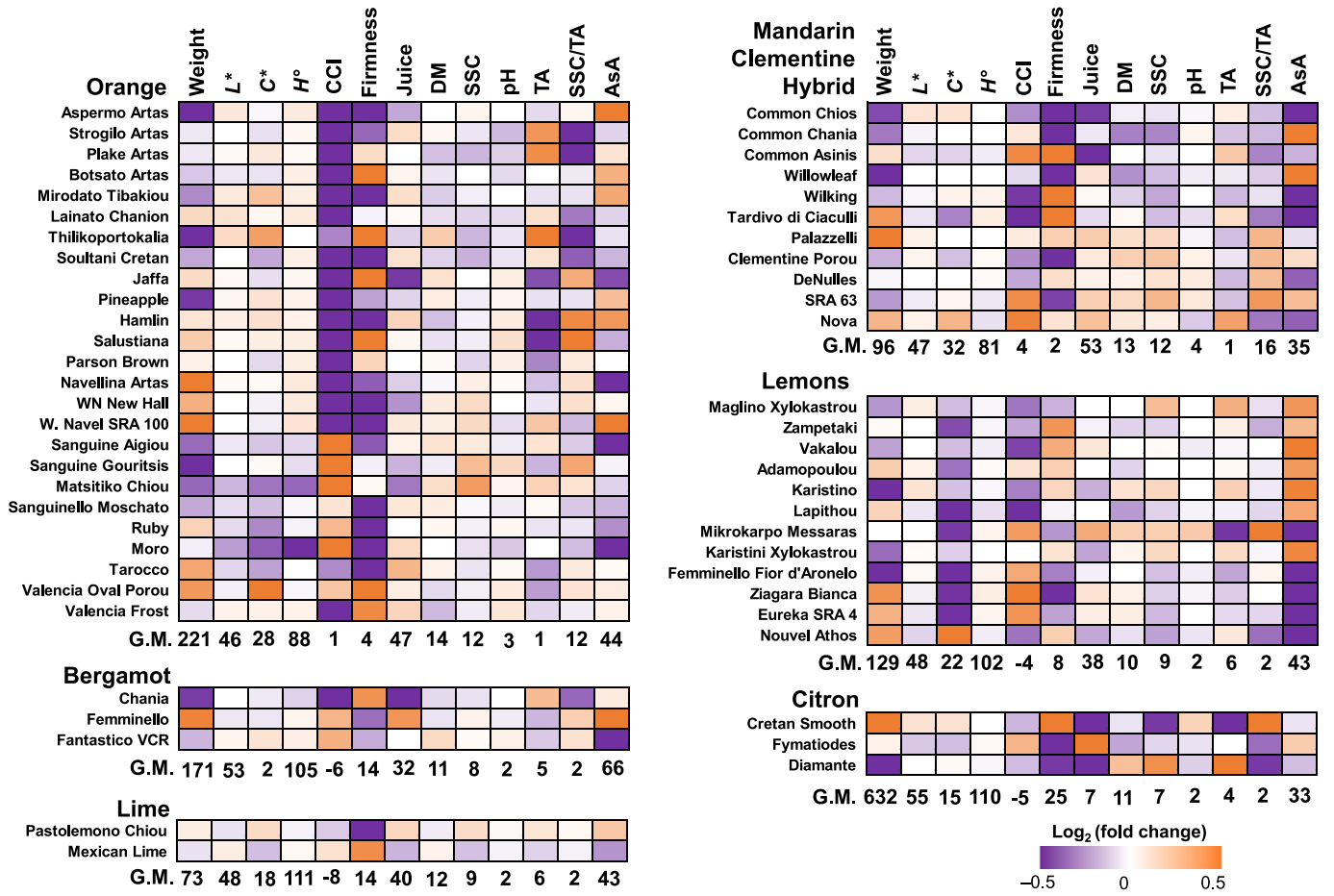

Fig. 1. Heatmap profile of *Citrus* endocarp quality traits (weight (gr.), lightness; *L**, Chroma; *C**, Hue angle; *H°*, CCI, firmness (N), juice content (%), dry matter (DM, %), soluble solid concentration (SSC, % Brix), pH of juice, titratable acidity (TA, % citric acid), ripening index (SSC TA^{-1}), ascorbic acid content (AsA, mg 100 mL^{-1}) of fifty-six *Citrus* cultivars divided into oranges, mandarins and clementines, lemons, bergamots, citrons, and limes, harvested at physiologically mature stage. Orange color indicates an increase and purple a decrease based on the grand mean of each citrus cultivar category (oranges, mandarin clementines, etc.). G.M. indicates the grand mean of each trait. Numeric data are provided in Table S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

accession could be considered an alternative cultivar to 'Clementine SRA 63'. We also note that all the Greek mandarin/clementine cultivars are fulfilling the minimum value of acidity (0.5%) (Fig. 1, Table S3), which is needed for commercialization (Lado et al., 2014).

3.1.2. Quality differences among lemon and lime cultivars

Significant variations were also found in the quality traits among lemon and lime cultivars. Particularly, lemons of 'Ziagara Bianca' had the largest weight (173 g) and the lowest endocarp firmness (4.5 N), whilst 'Nouvel Athos' had the lowest SSC (7.6 %) and ripening index (1.3). On average, the highest juice (48%), DM (12%), pH (2.2), and ripening index (2.5) were observed in the indigenous 'Mikrokarpo Messaras', however, this cultivar exhibited the lowest acidity value (4.1%) which is a crucial factor for lemons (Morales Alfaro et al., 2021). On the other hand, the autochthonous cultivar 'Maglino Xylokastrou' marked both the highest SSC (10.6%) and acidity (7%) values, making it an interesting lemon cultivar regarding its sensory properties. In addition to this, we generally found that the Greek lemon cultivars showed high fruit firmness (8.2 N) compared to the foreign ones (6.5 N), demonstrating their potential benefit towards postharvest handling. Another interesting observation made in our study was that all Greek lemon accessions (except 'Mikrokarpo Messaras') had almost three times more ascorbic acid (mean value of 56,9 mg/100 mL) than the studied foreign accessions (21,4 mg/100 mL) (Fig. 1, Table S2). Given that vitamin C contributes to the overall antioxidant activity and nutritional value of citrus (Lado et al., 2018), the above data suggest that the Greek-oriented lemon cultivars are a diverse genetic material accumulating significantly different levels of ascorbic acid, which can be exploited to obtain a vitamin C-rich diet.

3.1.3. Quality differences among citrons and limes cultivars

The present data suggested that the quality features of citrons and limes significantly depended on the cultivar and principally differed compared to the rest *Citrus* species. For instance, citrons have a high weight (632 g) and very low juice content (7%) in contrast to other citrus fruit (weight ranged between 73 and 221 g and juice content from 32 to 53%), showing utilization of the fruit and especially the albedo and flavedo parts, rather than the endocarp (Fig. 1, Table S2). It was also demonstrated that the autochthonous lime 'Pastolemono Chiou' could be considered an excellent choice of commercial Greek lime fruit since its juice content (44.6%) and SSC (9.8%) (Fig. 1, Table S2) were above the minimum requirements of the international market (42 and 6.8%, respectively; Morales Alfaro et al., 2021).

3.2. The proportions of both primary and secondary metabolite in the endocarp of citrus fruit

Using analytical methods, especially gas and liquid chromatographic methods, could compare citrus fruits of different species, as each species would have its unique chemical fingerprint (Goh et al., 2022). In

addition to the physiological characteristics of the endocarp, therefore, an analysis of primary and secondary metabolites was performed (Figs. 3, 4 and 5, Table S3 & S4). In particular, Fig. 2 depicts the proportions (in percentages) of both primary and secondary (polyphenolic and carotenoid content were presented separately) metabolites in fifty-six *Citrus* cultivars with transverse endocarp phenotype. Except for the general citrus division (oranges, mandarins, etc.), the twenty-five orange cultivars were categorized as common (thirteen), navel (three), sanguine (seven), and valencia (two) cultivars (Fig. 2). Data showed that sugars from primary metabolites and hesperidin from polyphenolic compounds were the most abundant among the fifty-six citrus cultivars examined (Fig. 2), confirming previous findings (Lado et al., 2018). Current results documented that specific primary and secondary metabolites do exist in different abundances within the endocarp of *Citrus* species (Fig. 2). Considering the abundance and absolute value of the polyphenolic fracture, for example, it was shown that the abundance of polyphenols in *Citrus* species endocarp (Table S4) following the oranges > mandarins > lemons > lime > bergamot > citrons. These findings support the notion that metabolic hallmarks can be used to facilitate the taxonomy of *Citrus* species (Abad-García et al., 2014).

Regarding the proportion of oranges' primary metabolites (water content excluded), sugars ranged between 76 and 92%, acids from 4 to 9%, and alcohols from 3 to 14%. Hesperidin and narirutin comprised 75 to 85% and 4 to 9%, respectively, of the polyphenolic compounds in oranges, whereas β-carotene and lutein were detected in the endocarp of oranges, apart from Valencia oranges, where violaxanthin was identified as the main compound (51 to 55%) (Fig. 2). Meanwhile, in the endocarp of mandarins and clementines, primary metabolites like sugars ranged between 86 and 90%, acids from 5 to 8%, and alcohols from 4 to 7% (Fig. 2). Hesperidin and narirutin were also the main part of polyphenolic compounds in mandarins/clementines ranging from 71 to 90% and from 2 to 11%, respectively, whereas low abundance of the rest polyphenolics was observed. In mandarins/clementines, β-carotene was the most abundant carotenoid followed by lutein (Fig. 2). However, interesting differences between clementines and the rest mandarins were noticed. For instance, mandarins contained β-carotene while clementines contained violaxanthin in low abundance (Fig. 2).

In the endocarp of lemons, bergamots, citrons and limes, a distinct shift in the primary metabolites was discovered, with an increase in the proportion of acids ranging from 30 to 47% and a decrease in the proportion of sugars ranging from 35 to 62%, while alcohols ranged from 6 to 20% (Fig. 2). The major polyphenolic compounds in lemon and lime were hesperidin and eriocitrin (instead of narirutin) ranging from 33 to 55% and from 19 to 41%, respectively (Fig. 2) which was consistent with previous results (Peterson et al., 2006). In bergamots, the eriocitrin ranged between 26 and 50% while hesperidin ranged between 18 and 42% ('Femminello Fior d'Aronelo' had narirutin at 24%) whereas in citrons the hesperidin ranged between 31 and 70%. Endocarp carotenoids were absent in citrons and mainly consisted of β-carotene in lemons, bergamots and limes followed by violaxanthin in some lemon cultivars (Fig. 2).

3.3. Primary metabolic fingerprint of the various citrus fruit

The citrus grouping based on primary metabolite discrimination at the *Citrus* species and individual cultivar levels could be important indicators for breeding and resource utilization (Lin et al., 2023). Wang et al. (2016) showed that concentrations of many primary metabolites varied greatly among the *Citrus* samples, and they also suggested considering various metabolic components as possible parameters for

Fig. 2. Endocarp phenotype of *Citrus* cultivars in the transverse segment. The internal cycle indicates the ratio (as a percentage) of primary metabolites in endocarp (sugars, acids, alcohols, amino acids, and other compounds); water content excluded, followed by the middle cycle of targeted polyphenolic compounds (hesperidin, narirutin, eriocitrin, and other polyphenolics), and the external cycle indicates the ratio of carotenoids (violaxanthin, β-carotene, α-carotene, and lutein).

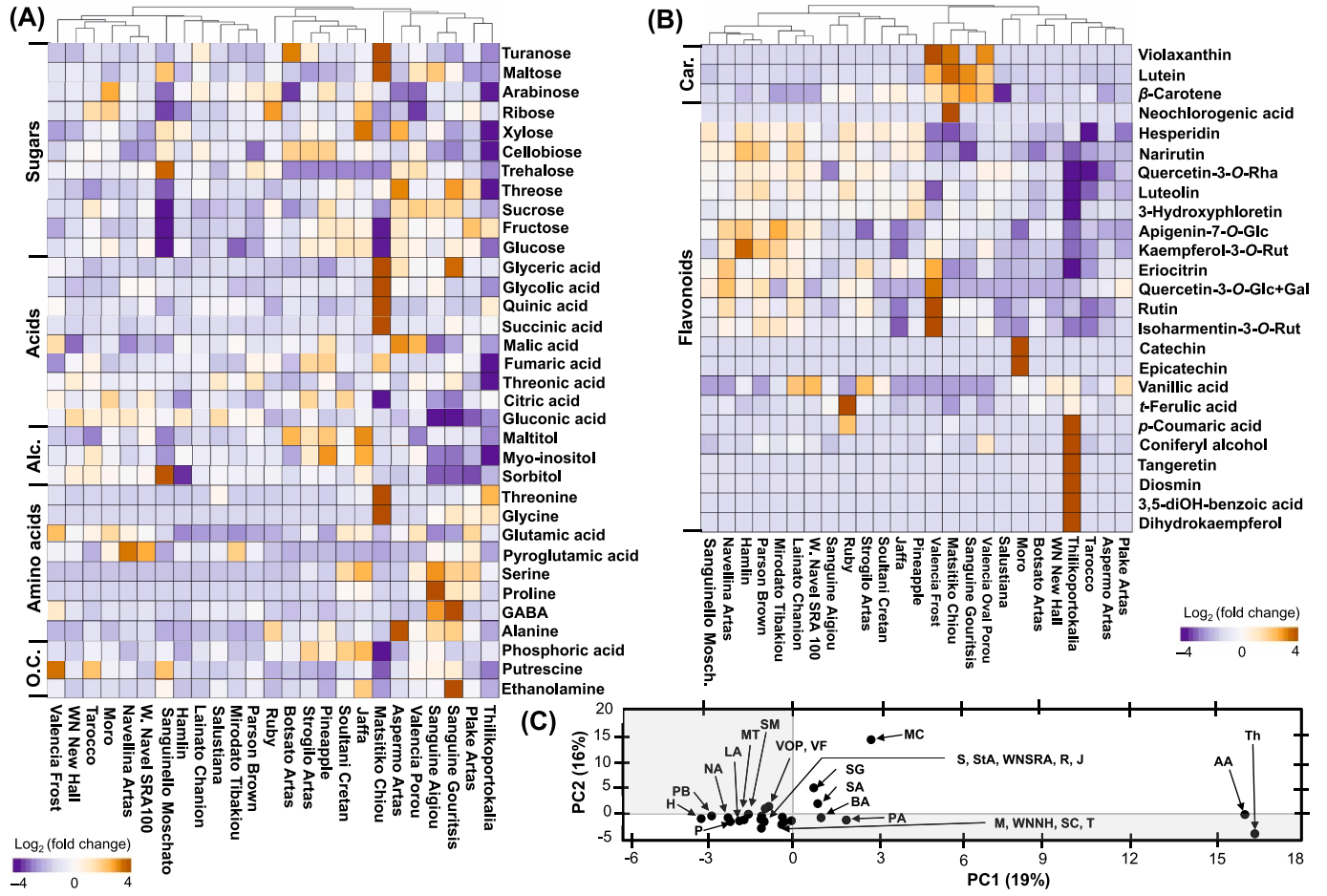

Fig. 3. Primary (A) and secondary (B) metabolites of the evaluated orange cultivars presented as a heat map profile along with hierarchical cluster analysis. A color scale that is proportional to the ratio of each identified metabolite depicts the fold change among oranges. Principal component analysis (PCA) among the physiological data and identified metabolites were constructed to obtain an overview of orange discrimination (C). Data for each primary and secondary metabolite means are provided in Table S4 & S5. Abbreviation list; Alcohols (Alc.), Other compounds (O.C.), Carotenoids (Car.), Pineapple (P), Hamlin (H), Parson Brown (PB), Navellina Artas (NA), Lainato Chanion (LA), Mirodato Tibakiou (MT), Sanguinello Moschato (SM), Valencia Oval Porou (VOP), Valencia Frost (VF), Matsitiko Chiou (MC), Sanguine Gouritsis (SG), Sanguine Aigiou (SA), Strogilo Artas (StA), Botsato Artas (BA), Plake Artas (PA), Salustiana (S), W. Navel SRA 100 (WNSRA), Ruby (R), Jaffa (J), Moro (M), WN New Hall (WNNH), Soultani Cretan (SC), Tarocco (T), Aspermo Artas (AA), Thilikoportokalia (Th). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the classification of *Citrus*. However, the complexity of the morphology of the *Citrus* genus and the tendency to hybridize between genera complicated the citrus classification based on their central metabolism (Lin et al., 2023). Here, primary metabolites analysis along with PCA assay and hierarchical clustering revealed differences in the compositions and contents of numerous primary metabolites among the different *Citrus* species and cultivars (Figs. 3–5) that may link to the observed quality changes among the examined genotypes (Fig. 1).

3.3.1. *Examples of changes in primary metabolites among Citrus species*

It has been previously reported that among *Citrus* species and even cultivars, there is a variability concerning the acidity of the endocarp. Lado et al. (2018) found that citric acid was the dominant acid in the pulp of all *Citrus* species (70–90%) followed by malic acid in oranges and mandarins. In this study, this fact is verified since citric acid was the most abundant acid in the *Citrus* species tested, while malic acid was the second most abundant acid in mandarins, lemons, limes, bergamot and citrons (Figs. 4, 5 & Table S4). In our work, apart from citric and malic acid, minor quantities of other acids like, fumaric, threonic, quinic, glyceric, glyconic and succinic were detected (Figs. 3–5 & Table S4), which is also in line with the results reported by Lado et al. (2018). In addition to this, we found the highest free amino acid content in lemon fruit with the dominant amino acid being serine followed by threonine and proline (Figs. 3–5 & Table S4). Wang et al. (2016) reported that lemons have the highest content of free amino acids, followed by mandarins, while oranges are rather poor. The current analysis further showed that in mandarins the dominant amino acid was serine followed by equally low amounts of alanine and threonine, while in oranges only traces of threonine and serine could be detected; in contrast, we found that proline was more abundant in lemon fruit (Fig. 4). Given that amino acids can be referred to as the building blocks of human proteins and precursors of a wide range of secondary metabolites (Tzin and Galili, 2010) these above results represent a valuable resource for exploring *Citrus* germplasm for improved nutritional quality traits.

3.3.2. *Primary metabolism in the orange and mandarin cultivars*

Concerning specific *Citrus* species, thirty-four primary metabolites were identified in orange cultivars that are corresponding to eleven sugars, nine acids, three alcohols, eight amino acids and three other compounds. Sucrose, glucose, fructose, and citric acid were identified as the predominant main metabolites in oranges (Table S4), consistent with the findings published by Lado et al. (2018). Especially, the indigenous 'Soultani Cretan' exhibited the highest levels of citric acid and glucose while the highest levels of the detected fructose and sucrose were calculated in the autochthonous cultivars 'Plake Artas' and 'Valencia Oval Porou', respectively (Table S4). Notable low levels of

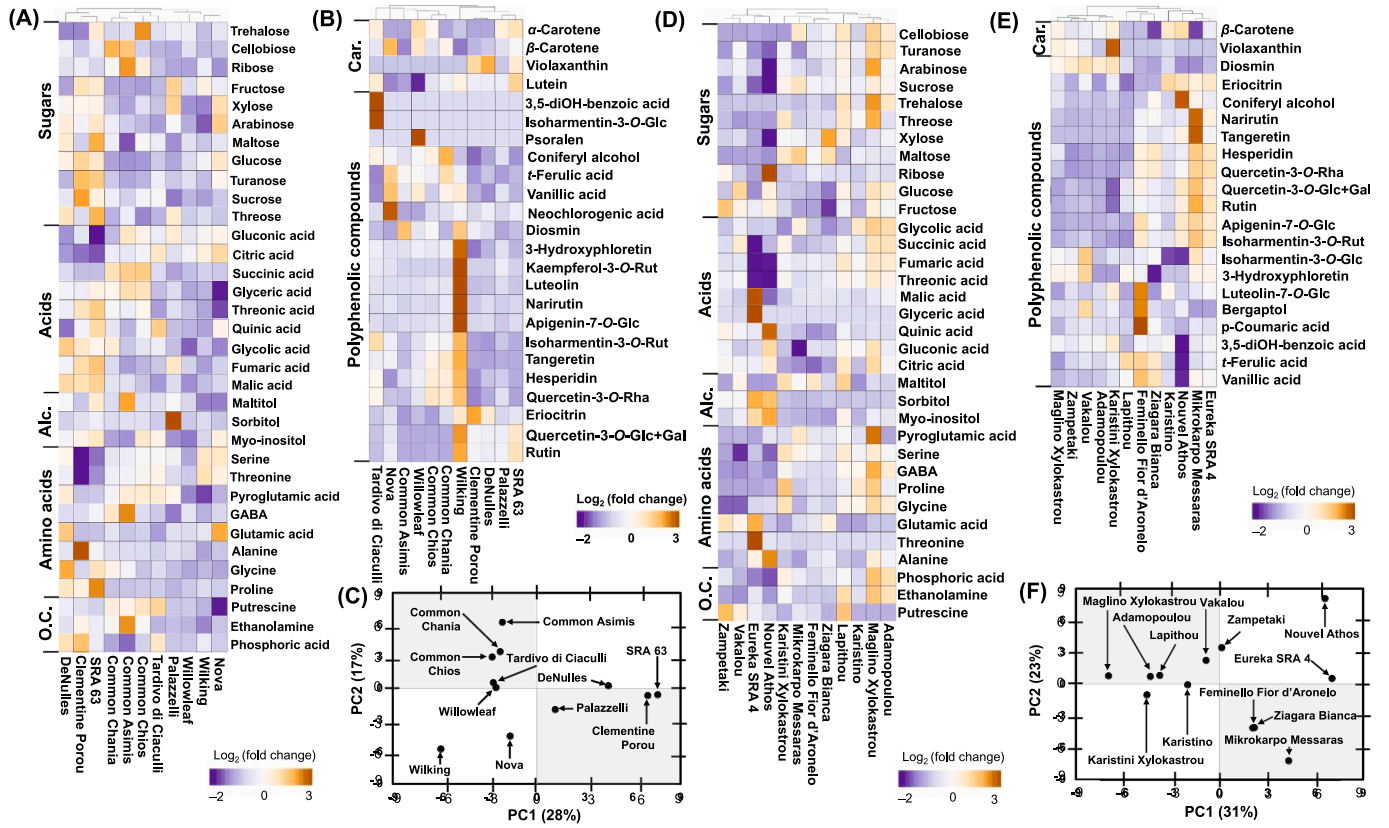

Fig. 4. Primary (A, D) and secondary (B, E) metabolites of the evaluated mandarins/clementines (A, B) and lemons (D, E) cultivars were presented as heat map and hierarchical cluster analysis. A color scale that is proportional to the ratio of each identified metabolite depicts the fold change among mandarins/clementines and lemons. Principal component analysis (PCA) among the physiological data and identified metabolites were constructed to obtain an overview of mandarin/clementine and lemon discrimination (C, E). Data for each primary and secondary metabolite means are provided in Table S4 & S5. Abbreviation list; Alcohols (Alc.), Other compounds (O.C.), Carotenoids (Car.).

citric acid were recorded in the indigenous 'Matsitiko Chiou', while 'Sanquinello Moschato' had the lowest concentration of glucose, fructose, and sucrose (Table S4), which clearly affects the balance between the sweet and sour levels of the evaluated autochthonous genotypes (Fig. 1).

Like oranges, thirty-four primary metabolites were identified in mandarins and clementines (Fig. 4A). The most prevalent sugars in mandarins/clementines were sucrose, glucose and fructose, whereas the most abundant acids were citric and malic acids (Table S4), a fact which is in line with the data presented by other scientific groups regarding sugar and acid fraction in the endocarp of mandarins cultivars (Ladaniya, 2008). Mandarins/clementines have been divided into three clusters based on their primary metabolites (Fig. 4A). The three clementines have been grouped together with the native cultivars ('Common Asinis', 'Common Chios', 'Common Chania') and 'Tardivo di Ciaculli' (Fig. 4A). A higher abundance of citric acid was determined in mandarins than clementines (the highest in 'Nova') whereas malic acid was more abundant in clementines than mandarins (the highest in 'Clementine SRA 63'). Also, the indigenous 'Clementine Porou' reported the highest levels of sugars, particularly sucrose, whereas 'Palazzelli' contained the lowest concentration of sugars (Table S4). The current study also demonstrates that distinct indigenous varieties of oranges and mandarins exhibit variations in their sugar content. The blood orange cultivar 'Sanguine Gouritsis' and the clementine cultivar 'Porou' exhibited distinct characteristics in comparison to other Greek orange and mandarin cultivars, specifically in terms of sucrose content. This finding highlights the potential utility of this collection of germplasm for the cultivation of citrus fruits with an elevated sweetness profile.

3.3.3. *Primary metabolism in the lemon, bergamot, citron and lime cultivars*

As shown in Fig. 4D, eleven sugars, nine acids, three alcohols, eight amino acids, and three other compounds were detected in lemons. The most prevalent sugars in lemons were sucrose, glucose, and fructose whereas citric acid was by far the most abundant acid followed by malic acid (Table S4), as also pointed out by Sun et al. (2019). Based on primary metabolites, lemons were clustered into three groups of them including the native cultivars 'Adamopoulou', 'Karistino', 'Maglino Xylokastrou' and 'Lapithou' (Fig. 4D). Concerning the level of organic acids among lemon cultivars, citric acid was most abundant in the autochthonous 'Maglino Xylokastrou', and malic acid was most abundant in 'Eureka SRA 4' whereas citric and malic acid were measured in considerably lower amounts in 'Femminelo Fior d'Aronelo' and 'Nouvel Athos', respectively (Table S4). Regarding sugars, sucrose, glucose and fructose were highly detected in the indigenous lemon cultivars 'Mikrokarpo Messaras', 'Vakalou' and 'Zampetaki', respectively. In contrast, 'Nouvel Athos' had the lowest level of sucrose while 'Ziagara Bianca' had the lowest content of both glucose and fructose (Table S3).

Regarding the primary metabolites in bergamots, citrons, and limes cultivars, thirty-two, thirty and thirty-two substances were detected, respectively (Fig. 5A & Table S4). Significant differences among these cultivars in terms of primary metabolites, especially sugars and acids were found. For example, the most abundant sugars were sucrose, glucose and fructose in tested bergamots, citrons and limes cultivars while citric acid followed by malic acid was the most present organic acid (Table S4). PCA analysis of bergamots, citrons and limes using metabolic and physiological data reveals a distinct separation between limes and citrons, while the three bergamot cultivars were also

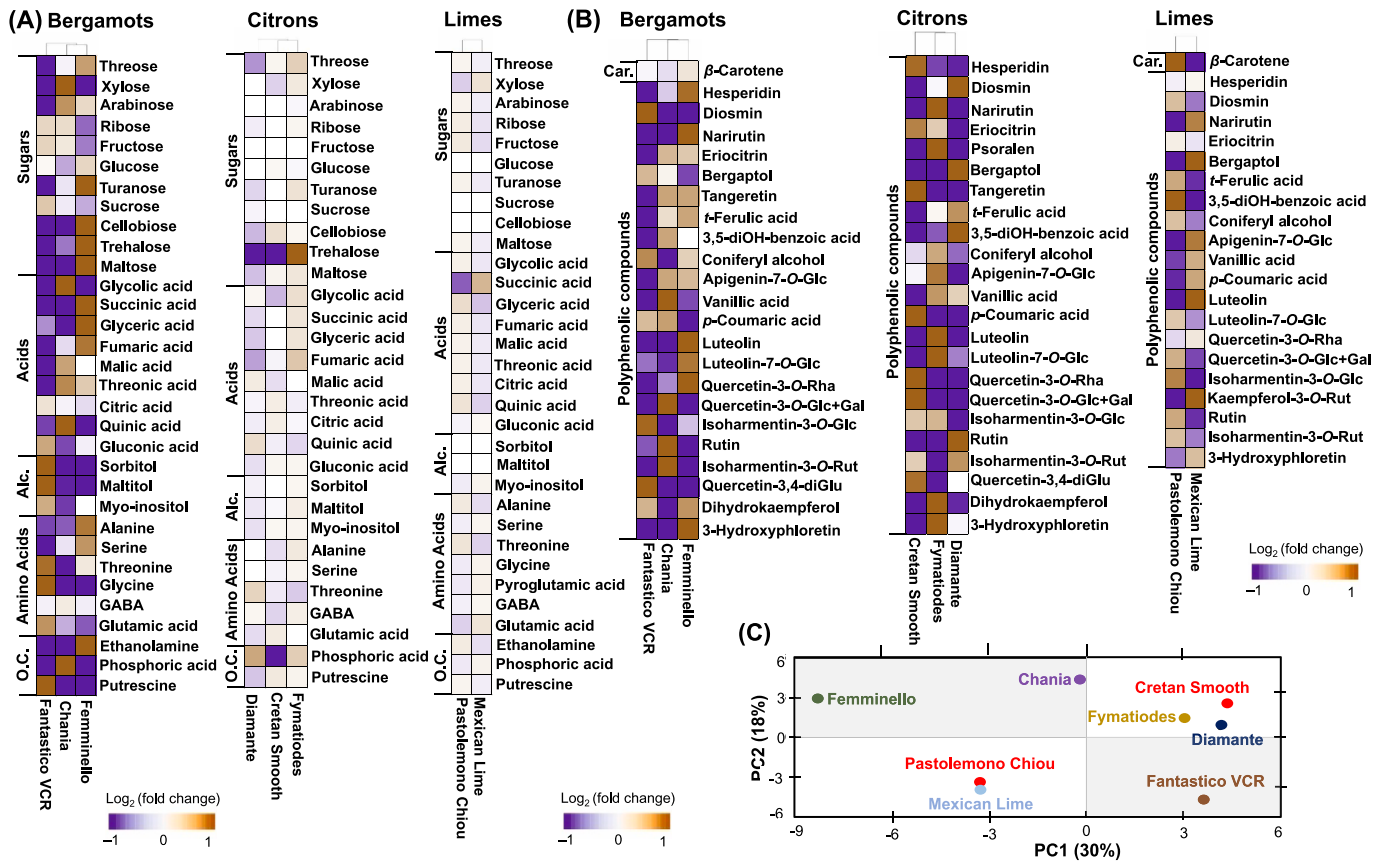

Fig. 5. Primary (A) and secondary (B) metabolites of the evaluated bergamots, citrons and limes cultivars were presented as heat map profiles along with hierarchical cluster analysis. A color scale that is proportional to the ratio of each identified metabolite depicts the fold change among bergamots, citrons, and limes. Principal component analysis (PCA) among the physiological data and identified metabolites were constructed to obtain an overview of bergamot, citron, and lime discrimination (C). Data for each primary and secondary metabolite means are given in Tables S4 & S5. Abbreviation list; Alcohols (Alc.), Other compounds (O.C.), Carotenoids (Car.).

separated (Fig. 5C).

3.4. Secondary metabolic dynamic of citrus fruit

Citrus endocarp contains valuable phytochemicals and is an excellent source of bioactive ingredients (Saini et al., 2022) that these may be influenced by both genotype and environment (Dhuique-Mayer et al., 2009). Thus, the polyphenols and carotenoid components of citrus endocarp were thoroughly analyzed in this study.

3.4.1. Identification of key secondary metabolites in various Citrus species

UPLC–MS/MS analysis reveals that the cumulative flavonoid fractions in the juice of the most cultivated citrus was around 55–62 mg/100 mL in oranges and grapefruit, 36 mg/100 mL in mandarins, and around 32 mg/100 mL in lemons (Figs. 3, 4, 5 & Table S5), that is in line with previous reports (Shorbagi et al., 2022). Orange juice is recognized as a key dietary source of flavanones, a subclass of flavonoids, and hesperetin-7-*O*-rutinoside (hesperidin) and naringenin-7-*O*-rutinoside (narirutin) are the main citrus flavanone components (Gil-Izquierdo et al., 2001). As mentioned above, the flavanone hesperidin was the most abundant flavonoid in all tested orange and mandarin cultivars followed by a minor amount of narirutin. These data are in accordance with Nogata et al. (2006), who highlighted that hesperidin was the dominant flavonoid in oranges and mandarins. We also noticed that the group of common mandarin accessions (*C.* × *aurantium* var. *deliciosa* ined.) had higher polyphenolic content compared to the clementine group and 'Nova' mandarin (Fig. 4B & Table S5), a fact which is in line with the work of Shorbagi et al. (2022). As indicated elsewhere (Nogata et al., 2006; Peterson et al., 2006; Lado et al., 2018) and documented here (Fig. 4E, 5B & Table S5), hesperidin was the major flavonoid in lemons and limes, but in significantly lower concentration compared to that of oranges and mandarins. Hesperidin yield may be affected by the polyphenols extraction method as highlighted by Iglesias-Carres et al. (2019). For instance, in 'Navelina' oranges we determined an amount of hesperidin between 9 and 11 mg g^{-1} (Table S5) which is the half amount of the aforementioned study, and probably this difference is due to the extraction method. The most abundant flavonoid in citrons endocarp was hesperidin while the major polyphenolic fraction in bergamot accession was eriocitrin followed by hesperidin (Fig. 5B & Table S5). This observation may support that eriocitrin can be used as a metabolic tool to verify the authenticity of bergamot-derived products (Tsiokanos et al., 2021).

3.4.2. Comprehensive analysis of carotenoids in different Citrus species and cultivars

Carotenoids contribute to the development of the characteristic color of the peel and pulp of citrus fruit and several differences have been reported among species/cultivars, having a direct effect on consumer choice (Seminara et al., 2023). Initially, carotenoid biosynthesis starts with phytoene and ends in violaxanthin, α- and β- carotenes are found in the middle of the pathway while lutein is found in green flavedo tissues (Rodrigo et al., 2013; Tadeo et al., 2020). In our work, four out of eight carotenoids that were found in Citrus samples were abundant in mandarins and clementines, followed by oranges; in addition, carotenoids were rather poor in lemons, lime and bergamot while being absent in citrons (Fig. 3B, 4, 5B & Table S5). A similar distribution of carotenoids

in various *Citrus* species was reported earlier (Lato et al., 2018). As in previous studies (Lu et al., 2017; Lato et al., 2018), we also observed that in the endocarp of common, navel and sanguine oranges, the dominant carotenoid was β-carotene followed by lutein while violaxanthin was the main carotenoid in Valencia cultivars ('Frost' and 'Oval Porou') (Fig. 3B & Table S4), as also observed by Kato, (2012). The β-carotene and lutein were the major carotenoids in the endocarp of common mandarins/clementines while clementines were rich in violaxanthin (Fig. 4B & Table S5). Especially, lutein has been mentioned as the second most abundant carotenoid in mandarin pulp by Agócs et al. (2007).

3.4.3. Differences in the compositions and contents of secondary metabolites in orange and mandarin cultivars

Another important point of this study was that there were significant differences in the compositions and contents of secondary metabolites among cultivars of the same *Citrus* species. In orange cultivars, the highest level of hesperidin and narirutin was found in 'Hamlin', while the polyphenolic compounds 3.5-diOH-benzoic acid and dihydrokaempferol were detected only in the indigenous cultivar 'Thilikoportokalia' (Fig. 3B & Table S5). In addition, catechin and epicatechin were identified only in 'Moro' whereas the lowest abundance of hesperidin and narirutin was recorded in 'Tarocco' and 'Sanguine Gouritsis' respectively (Table S5). These observations suggest that, besides the presence of common phenolic compounds, the abundance of these compounds can differ greatly among genotypes, creating a unique chemical profile and thus a unique taste for each orange cultivar. Interestingly, clear discrimination has been observed based on PC1 of the indigenous oranges 'Thilikoportokalia', 'Aspermo Artas', 'Matsitiko Chiou', 'Botsato Artas', 'Plake Artas', 'Sanguine Aigiou', and 'Sanguine Gouritsis' (Fig. 3C). In addition, oranges belonging to the sanguine type ('Matsitiko Chiou', 'Sanguine Aigiou', 'Sanguine Gouritsis') and two Valencia-type oranges ('Valencia Frost', and 'Valencia Oval Porou') have been also clearly separated from other cultivars based on PC2 (Fig. 3C).

The present analysis identified twenty polyphenolic compounds and four out of eight carotenoids in mandarin/clementines samples (Fig. 4 & Table S5). Based on secondary metabolites, mandarins/clementines were classified into two clusters, with 'Wilking' having the highest concentration of hesperidin, narirutin, and other polyphenolic compounds. A cluster has been formed by the three clementines and 'Palazzelli' while a second cluster includes the rest of the mandarin cultivars (Fig. 4B). The lowest abundance of hesperidin and narirutin was found in 'Nova' and 'Clementine Porou', respectively. Interestingly, 'Nova' accession presented the highest content carotenoid fraction of all studied mandarin cultivars (Fig. 4B & Table S5), possibly reflecting its high antioxidant potential (Simón-Grao et al., 2014). Furthermore, as indicated by the PCA analysis of physio-metabolic data (Fig. 4C), a clear separation of clementines and mandarins based on PC1 while PC2 is responsible for the discrimination of 'Nova' and 'Wilking' with the rest mandarin cultivars.

3.4.4. Differences in the compositions and contents of secondary metabolites in lemon, bergamots, citrons, and limes

PCA analysis of physio-metabolic data reveals a clear discrimination of seven out of eight native lemon cultivars (excluding 'Mikrokarpo Messaras') with the four non-native cultivars based on PC1 which accounts for 31% of the total variation. On the other hand, PC2 is mainly responsible for the separation of 'Mikrokarpo Messaras' and 'Nouvel Athos' explaining 21% of the total variance (Fig. 4F). Eventually, the analysis of secondary metabolites resulted in the grouping of lemons into three clusters, herein the indigenous cultivars 'Adamopoulou', 'Karstini Xylokastrou', 'Maglino Xylokastrou', 'Vakalou' and 'Zampetaki' was grouped together (Fig. 4E). This observation along with the clear secondary metabolites-based discrimination of the native oranges observed above (Fig. 3C), indicates that the secondary metabolites not only give each orange cultivar its unique flavor profile but also can serve as an indicator of its origins.

Among polyphenolic compounds, hesperidin and eriocitrin were the most abundant compounds in lemons whilst carotenoids were determined in low content (Fig. 4E). Remarkably, the indigenous cultivars 'Mikrokarpo Messaras' and 'Karistino' had the highest content of hesperidin and eriocitrin, respectively (Fig. 4E). On the contrary, the native 'Lapithou' had the lowest content of both hesperidin and eriocitrin (Table S5). An interesting fact was that some Greek accessions (Maglino Xylokastrou, Zampetaki, Vakalou, Karistino and Karistino Xylokastrou) exhibited a considerable amount of violaxanthin (Fig. 4E & Table S5), supporting the demand to safeguard the autochthonous biodiversity and as a result supporting consumers' health with diversified and superior quality citrus fruit products.

Although bergamots, citrons, and limes fruits are rich sources of bioactive compounds (Nogata et al., 2006; Tsiokanos et al., 2021), no comparative studies investigating the secondary metabolism of these *Citrus* species have been performed so far. In this study, twenty-two, twenty-three, and twenty-one polyphenolic compounds in bergamots, citrons, and limes, respectively as well as one carotenoid compound in bergamots and limes were determined (Fig. 5B & Table S5). Hesperidin and eriocitrin were the most abundant compounds in bergamots and limes, whilst in citrons only hesperidin was determined (Table S5). PCA analysis of bergamots, citrons and lime using physiological as well as metabolic data of endocarp reveals a distinct separation between limes and citrons while the three bergamots were also separated (Fig. 5C), thereby enabling our ability to discriminate citrus using metabolic-driven data.

4. Conclusion

Physiological and metabolite profiling of twenty-nine Greek *Citrus* accession, including thirteen oranges, four mandarins/clementines, eight lemons, one bergamot, two citrons, and one lime, along with twenty-seven highly commercial international cultivars revealed species- and cultivar-specific variations in quality traits as well as primary and secondary metabolic hallmarks. This study highlights those numerous indigenous genotypes, some of them nowadays that are forgotten and underutilized, displayed desirable organoleptic quality attributes and phytochemical levels. Among others, 'Valencia Oval Porou' orange exhibited the most acceptable combined quality traits based on weight, firmness, juice content and ascorbic acid whereas 'Sanguine Gouritis' orange demonstrated the highest ratio of SSC/TA. For mandarins, the Greek-oriented cultivar 'Clementine Porou' presented the best taste intensity based on SSC/TA ratio, an acceptable level of vitamin C and above-average juice content. The indigenous lemon 'Vakalou' sum up the most acceptable quality traits in terms of fruit firmness, above-average juice content, high TA, and the highest vitamin C content. Significant differences in numerous primary metabolites, particularly in sugars, organic acids and amino acids were found in the different *Citrus* species and cultivars. Among the Greek accessions, the blood orange cultivar 'Sanguine Gouritsis' and 'Valencia Oval Porou' could be recognized as being rich in antioxidants since they presented the highest sum of polyphenols and carotenoids. The complementary analytical platforms applied herein have more potential to be considered in further studies investigating the effect of other factors i.e., developmental stage, geographical origin and storage conditions on the metabolism of better-performing cultivars. Overall, our findings filled the data gap for quality and metabolic characterization of autochthonous *Citrus* germplasm which could provide data references for *Citrus* resource utilization, selection and breeding of superior cultivars.

CRediT authorship contribution statement

Michail Michailidis: Writing – original draft, Data curation, Investigation, Formal analysis, Visualization. **Vasileios Ziogas:** Writing – original draft, Funding acquisition, Investigation, Conceptualization.

Eirini Sarrou: Writing – review & editing, Data curation, Investigation, Formal analysis. **Elpida Nasiopoulou:** Data curation, Investigation, Formal analysis. **Vaia Styliani Titeli:** Data curation, Investigation, Formal analysis. **Christina Skodra:** Data curation, Investigation. **Georgia Tanou:** Writing – review & editing, Investigation, Validation, Resources. **Ioannis Ganopoulos:** Writing – review & editing, Funding acquisition, Resources. **Stefan Martens:** Writing – review & editing, Supervision, Validation, Resources. **Athanassios Molassiotis:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137573.

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