

Article

Changes in Physicochemical Characteristics and Bioactive Potential of Corfu ‘Nagami’ Kumquat (*Fortunella margarita* Swingle) During the Harvesting Period

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Highlights

What are the main findings?

- Ripening altered the physicochemical and bioactive profile of Corfu ‘Nagami’ kumquat.
- Late-harvest-stage fruits had higher phenolic content and free-radical scavenging capacity.

What are the implications of the main findings?

- Optimized ultrasound-assisted extraction enabled consistent comparisons across harvest stages.
- Harvest stage is relevant when selecting kumquat fruit for bioactive applications.

Abstract

‘Nagami’ kumquat (*Fortunella margarita* Swingle), a citrus fruit cultivated in Corfu, Greece, contains bioactive constituents whose levels may vary during fruit maturation. This study investigated changes in the physicochemical composition and bioactive potential of Corfu ‘Nagami’ kumquat during the commercial harvesting period (December–March) and optimized ultrasound-assisted extraction conditions for standardized comparisons among harvest stages. Fruits collected during the study period were analyzed for moisture, soluble sugars, proteins, lipids, total phenolic content, and free-radical scavenging capacity. Response surface methodology was used to optimize methanol concentration, extraction temperature, and extraction time, while principal component analysis (PCA) was applied to evaluate patterns associated with fruit maturation. During maturation, moisture and protein contents decreased, whereas lipid content increased. The optimized extraction conditions were 14% methanol, 45 min, and 54 °C. PCA showed clear separation of early- and late-harvest-stage samples, with the first two principal components explaining 97.9% of the total variability. The observed differentiation was primarily associated with DPPH scavenging, moisture, proteins, lipids, soluble sugars, and phenolic content. Late-harvest-stage fruits exhibited the highest free-radical scavenging capacity and phenolic content, with harvest stage related to variations in the bioactive potential of Corfu ‘Nagami’ kumquat.

Keywords: ‘Nagami’ kumquat; *Fortunella margarita*; harvesting-period variation; free-radical scavenging capacity; ultrasound-assisted extraction; phenolic compounds



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1. Introduction

Kumquat (*Fortunella* spp.) is a citrus fruit belonging to the Rutaceae family. Native to southeastern China, kumquats were historically classified within *Citrus* [1–3]. Walter T. Swingle later reclassified them under the genus *Fortunella* [2,3], named after Robert Fortune, who introduced kumquats to Europe [2]. Today, kumquats are cultivated in China, Japan, Southeast Asia, the Mediterranean region, the United States, Australia, South Africa, Argentina, and Brazil [3]. The principal types include ‘Marumi’ (*Fortunella japonica*) and ‘Nagami’ (*Fortunella margarita*), while ‘Meiwa’ (*Fortunella crassifolia*) is also widely grown and was traditionally considered a natural hybrid of the two [1,3]. Despite their broad geographical distribution, kumquats remain a relatively niche citrus crop and are valued as a specialty fruit. Kumquat production statistics remain limited, with published figures often being outdated or restricted to a few individual producing regions. For example, production in Korea was estimated at 3589 t in 2001 [3].

In Greece, kumquat cultivation is closely associated with the island of Corfu, where ‘Nagami’ kumquat (*F. margarita* Swingle) was introduced in 1924 and has since been cultivated commercially. Annual kumquat production on Corfu has been reported at approximately 140 t. Corfu-grown ‘Nagami’ kumquat is recognized as a Greek citrus product with Protected Geographical Indication status, reflecting its regional identity and agricultural importance. This designation provides a rationale for investigating compositional variation in the cultivar under Corfu’s specific production conditions. Kumquat is commonly consumed with the peel, which contributes to its characteristic sweet taste followed by a slightly bitter aftertaste. Its aroma is largely attributed to essential oils accumulated in peel oil glands, with limonene identified as the predominant volatile compound in ‘Nagami’, ‘Marumi’, and ‘Meiwa’ grown under Greek agroclimatic conditions [4].

Kumquat contains dietary fiber, vitamins, minerals, phenolic compounds, flavonoids, carotenoids, and essential oils, several of which have been associated with antioxidant and other biological activities [5–8]. Phenolic and flavonoid compounds generally occur at higher levels in the peel than in the pulp [9], highlighting the contribution of the peel to the nutritional value of the whole fruit. This combination of nutrients and bioactive constituents has increased interest in kumquat as a source of functional ingredients for food, pharmaceutical, and cosmetic applications [5]. As kumquat is commonly consumed whole, changes in the relative proportions of peel and pulp during maturation may also influence the overall concentration of bioactive and nutritional constituents delivered by the edible fruit. The present study did not separately quantify peel-to-pulp ratios or analyze these tissues independently; therefore, the contribution of each anatomical fraction to the observed changes during the harvesting period cannot be established.

Fruit metabolite composition varies among kumquat genotypes and tissues. Comparative metabolomic analysis of ‘Huapi’ and ‘Rongan’ kumquats identified flavonoids and lipids as major differentially accumulated compound classes [10]. Several flavonoid compounds were reported at higher levels in kumquat peel than in seeds, while phenolic-acids displayed cultivar- and tissue-specific patterns [11]. Fruit ripening is likewise associated with dynamic changes in flavonoids, limonoids, alkaloids, phenolic acids, and coumarins, with flavonoid, limonoid, and alkaloid content generally declining as fruit development progresses [12]. At the level of individual flavonoids, compounds such as hesperidin, narirutin, eriocitrin, neohesperidin, poncirin, and kaempferol derivatives have been reported to differ according to cultivar, tissue, geographical origin, and maturity stage [13,14].

These compositional changes during fruit development are accompanied by variation in radical scavenging responses and physicochemical characteristics. In ‘Meiwa’ kumquat, free-radical scavenging capacity increased in the skin toward harvest, whereas it decreased in the flesh, indicating that antioxidant responses may differ between fruit tissues during

ripening [15]. Differences in fruit size, acidity, soluble solids, color, and phenolic characteristics have also been reported across developmental stages, although these parameters may be influenced by cultivar and environmental conditions [16,17]. Phenolic and flavonoid contents have also been reported to vary across fruit maturity stages, with higher levels observed in immature than mature kumquats [9]. Recent compositional analyses of *F. margarita* have further demonstrated variation in sugars, organic acids, phenolic compounds, carotenoids, and volatile constituents, highlighting the combined influence of developmental stage, geographical and climatic conditions, and analytical methodology on the observed chemical profile [18].

Despite this growing body of evidence, information on how these changes translate into variation across successive commercial harvest stages of *F. margarita* under a specific production environment remains limited. In Corfu, Greece, investigations of the fruit's chemical characteristics have been relatively scarce [4,14]. Particularly, physicochemical characteristics, phenolic-related parameters, and antioxidant-related properties, as reflected by free-radical scavenging capacity, have not been comprehensively evaluated throughout the commercial harvesting period. Given the economic importance and established regional identity of this cultivar, addressing this gap is important for understanding compositional changes across successive harvest stages and identifying the variables associated with these changes under local production conditions.

Beyond variation during the harvesting period, the extraction procedure is an important methodological consideration when evaluating the recovery of phytochemical constituents. Ultrasound-assisted extraction has been successfully applied to kumquat peel for essential-oil recovery, resulting in increased oil yield, accelerated extraction kinetics, and enhanced free-radical scavenging capacity. The efficiency of the process, however, depends on the operating conditions, such as the treatment time [19,20]. Consequently, standardized extraction conditions are important when comparing phenolic content and radical scavenging capacity among samples collected at different harvest stages. Optimization of ultrasound-assisted extraction can therefore reduce extraction-related variability and provide a consistent basis for assessing changes in bioactive-related parameters during the harvesting period.

The present study investigated changes in the physicochemical characteristics and bioactive-related parameters of 'Nagami' kumquat from Corfu (*F. margarita* Swingle) throughout the commercial harvesting period (December–March). Ultrasound-assisted extraction conditions were optimized using response surface methodology (RSM) to establish standardized conditions for the determination of total phenolic content and free-radical scavenging capacity. Principal component analysis (PCA) was subsequently applied to explore patterns associated with fruit maturation and harvesting stage. By combining optimized ultrasound-assisted extraction with the systematic evaluation of physicochemical, phenolic, and radical scavenging parameters across successive harvest stages, this study provides an integrated characterization of compositional changes occurring during the commercial harvesting period of Corfu-grown 'Nagami' kumquat.

2. Materials and Methods

2.1. Plant Material and Sampling Procedure

'Nagami' kumquat (*Fortunella margarita* Swingle) fruits were collected from a single cultivated tree located in Kontogialos, Corfu, Greece. The tree was planted directly in the soil and was maintained under open-field conditions at its cultivation site. The tree was approximately 7 years old and 1.7 m in height and was grown on its own roots (non-grafted). It was not treated with pesticides and did not receive controlled irrigation;

water availability therefore depended on natural precipitation and the prevailing field conditions. The tree was managed according to standard local agricultural practices.

Sampling was performed monthly from December to March, covering the commercial harvesting period. A total of 24, 18, 13, and 13 fruits were randomly selected in December, January, February, and March, respectively, with the number of fruits varying according to the peel-color categories represented each month. Three peel-color categories were represented in December, two in January, and one in both February and March. Fruits were collected from different branches, mostly from the northern and western sides and the middle portion of the canopy, at approximately 0.5 m above ground level. At each date, different branches were selected to avoid repeated sampling from the same branches.

Following each sampling event, fruit-bearing branches were transported under refrigerated conditions by air to the laboratory in Thessaloniki, Greece. Upon arrival, branches with attached fruits were stored under the same refrigerated conditions until analysis. Fruits were detached from the branches, washed with distilled water, gently blotted with paper towels to remove excess surface moisture, and visually classified according to peel color as described in Section 2.4. Sample preparation and analysis were performed within 1–3 days after collection for all sampling dates.

Because all samples were obtained from one tree over a single December–March period, the experiment represents within-harvest-stage variation rather than biological replication across multiple trees or harvesting periods. For physicochemical and bioactive analyses, three independent homogenized preparations were used as analytical replicates and do not constitute independent biological replicates at the tree or orchard level. Therefore, the observed patterns should be interpreted within the context of the sampled material and this sampling period and should not be generalized to the wider ‘Nagami’ kumquat population or production system without further validation across multiple trees and harvesting periods.

2.2. Chemicals and Reagents

Methanol ($\geq 99.8\%$) was provided by Chem-Lab NV (Zedelgem, Belgium). Ethanol ($\geq 99.8\%$), acetone ($\geq 99.8\%$), chloroform ($\geq 99.8\%$) and sulfuric acid ($\geq 95\%$) were purchased from Fisher Scientific (Loughborough, UK). Folin–Ciocalteu reagent was a product of Sordalab (Étampes, France). Gallic acid anhydrous ($\geq 99\%$) and bovine serum albumin (BSA) ($\geq 96\%$) standards were obtained from Glentham Life Sciences (Corsham, UK), while the sucrose ($\geq 99.8\%$) standard was from Penta Chemicals (Praha, Czech Republic). Phenol crystallized (AGR ACS grade) and sodium carbonate anhydrous ($\geq 99.5\%$) were received from Labkem (Barcelona, Spain). Sodium chloride (NaCl, 99.97%) and sodium sulfate anhydrous ($\geq 99.8\%$) were supplied by Lach-Ner (Neratovice, Czech Republic). Phosphate-buffered saline (PBS) solution (10 $\times$, pH 7.4) was obtained from Alfa Aesar (Kandel, Germany). Bradford assay solution and 1,1-diphenyl-2-picrylhydrazyl (DPPH) ($>97\%$) were provided by TCI Europe N.V. (Zwijndrecht, Belgium). Deionized water was produced using a Zalion 600 ion-exchange water purification system (Ionel S.A., Athens, Greece).

2.3. Instrumentation

The analytical instruments used in this study included a UV–Vis spectrophotometer (UV–1600PC, VWR, Radnor, PA, USA), digital angle centrifuge (RS–0506, Auxilab S.L., Beriáin, Spain), ultrasonic bath (JPS10A, Vevor, Frankfurt, Germany; 2 L capacity, 40 kHz frequency, 60 W ultrasonic power, and 100 W heating power), analytical balance (ACN220G, AXIS Sp. z o.o., Gdańsk, Poland), magnetic hot plate stirrer (MSH–300, Biosan, Riga, Latvia),

vortex mixer (V-1 Plus, Biosan, Riga, Latvia), and natural convection oven (JeioTech, Daejeon, Republic of Korea).

2.4. Fruit and Seed Morphological Characterization

Before sample preparation, morphological characteristics of fruits and seeds were recorded to evaluate structural changes associated with maturation. Fruit length, width, and height were measured in cm using a ruler. Length corresponded to the longitudinal axis from the stem end to the blossom end, while width and height represented the maximum transverse dimensions. After sectioning, seed length, width, and height were also measured in cm using the same approach, with length corresponding to the longitudinal axis as well as width and height representing the maximum transverse dimensions. Morphological measurements were performed on 5 fruits per harvest month $\times$ peel color group, apart from the December orange group, for which 9 fruits were available and analyzed. Fruits were then manually sectioned, and seeds were removed. Peel color was visually assessed by both authors at every sampling occasion and classified into three predominant categories based on the overall external appearance: dark green (predominantly deep green), light green (predominantly pale green), and orange (predominantly orange with no substantial green coloration). Both authors applied the same visual criteria consistently throughout all sampling dates. Although peel color was used as a practical indicator of fruit developmental stage, classification was based on visual assessment rather than instrumental colorimetry. Therefore, some degree of observer-dependent variability cannot be excluded. Following morphological evaluation, seeds were discarded, and de-seeded fruit material was crushed using a mortar and pestle and subsequently homogenized with a laboratory blender to obtain a uniform fresh sample for physicochemical and bioactive analyses.

2.5. Preliminary Solvent Screening

A preliminary solvent screening experiment was conducted to identify the most suitable extraction solvent system for ultrasound-assisted recovery of bioactive compounds from 'Nagami' kumquat. Homogenized fresh kumquat samples (0.5 g) were transferred into 15 mL polypropylene centrifuge tubes, and 5 mL of extraction solvent was added. The evaluated solvent systems were: (a) water, (b) methanol, (c) 50% aqueous methanol (*v/v*), (d) ethanol, (e) 50% aqueous ethanol (*v/v*), (f) acetone, and (g) 50% aqueous acetone (*v/v*). Samples were extracted under identical ultrasound-assisted conditions (45 °C for 50 min) using an ultrasonic bath. Following extraction, samples were filtered through 0.45 μ m polytetrafluoroethylene (PTFE) syringe filters, and extracts were analysed for free-radical scavenging capacity using the DPPH assay. Aqueous methanol was selected for subsequent optimization of ultrasound-assisted extraction conditions using RSM, in which methanol concentration was evaluated as an independent variable.

2.6. Ultrasound-Assisted Extraction

Ultrasound-assisted extraction was performed using aqueous methanol as the extraction solvent system selected during preliminary screening. Homogenized fresh kumquat samples (0.5 g) were transferred into 15 mL polypropylene centrifuge tubes, and 5 mL of methanol/water solution was added. Extraction was performed using an ultrasonic bath operating at 40 kHz and 60 W ultrasonic power. Methanol concentration, extraction time and extraction temperature varied according to the RSM experimental design. The temperature values specified in the RSM corresponded to the initial settings of the ultrasonic bath and were not actively maintained throughout sonication. As ultrasound treatment caused progressive heating of the bath and sample, the temperature displayed on the ultrasonic bath was checked during each extraction, and the maximum value reached was recorded. Accordingly, extraction temperature in the RSM was interpreted as the initial ultrasonic

bath setting rather than as a constant sample temperature, and the resulting temperature ranges were considered when interpreting the temperature effect on the extraction response. After extraction, samples were filtered through 0.45 µm PTFE syringe filters, and the resulting extracts were used for the assessment of DPPH radical scavenging capacity and total phenolic content.

2.7. Experimental Design and Optimization of Extraction Conditions

The ultrasound-assisted extraction process was optimized using RSM, with DPPH-based radical scavenging capacity (Y_{DPPH}) selected as the response variable. DPPH was used as a practical chemical indicator of the recovery of radical scavenging constituents under the investigated extraction conditions. The optimization was therefore intended to establish extraction conditions that maximize the measured DPPH response rather than to represent a direct measure of biological antioxidant activity.

A face-centered central composite design was generated using Minitab software (version 22.4, State College, PA, USA) to evaluate the effects of three independent variables: methanol concentration (X_1 , % *v/v*), extraction time (X_2 , min), and extraction temperature (X_3 , °C). The experimental design consisted of 20 randomized runs, including six replicates at the central point to estimate experimental variability and evaluate model adequacy. The investigated factors as well as their coded and actual levels are presented in Table 1.

Table 1. Coded and actual levels of the independent variables used in the response surface methodology experimental design.

Factor	Variables	Levels		
		Coded Values		
		−1	0	1
		Actual Values		
Methanol concentration (% , v/v)	X_1	0	50	100
Extraction time (min)	X_2	10	50	90
Extraction temperature (°C)	X_3	30	45	60

The relationship between independent variables and response was described using a second-order polynomial equation (Equation (1)):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y_{DPPH} is the predicted response; X_1 , X_2 , and X_3 are the independent variables; and β_0 , β_1 , β_2 , β_3 , β_{11} , β_{22} , β_{33} , β_{12} , β_{13} , and β_{23} are the estimated regression coefficients, with β_0 representing the model intercept.

The extraction conditions selected for subsequent analyses were 14% methanol in water (*v/v*), 45 min extraction time, and 54 °C extraction temperature. Extracts obtained under these conditions were subsequently used to evaluate variation during the harvesting period in the bioactive potential of ‘Nagami’ kumquat.

2.8. Determination of Free-Radical Scavenging Capacity

The radical scavenging capacity of the extracts was evaluated using the DPPH assay [21]. Briefly, 100 µL of extract was mixed with 3 mL of freshly prepared DPPH solution (0.1 mM in methanol). The mixture was incubated in the dark for 30 min at room temperature, and absorbance was measured at 517 nm using a UV–Vis spectrophotometer. A solvent control (blank) was prepared by replacing the fruit extract with the corresponding extraction solvent and applying the same assay conditions. The control absorbance was

recorded as A_{Control} . For each extract, a separate blank containing methanol instead of DPPH solution was also prepared for spectrophotometer zero adjustment.

Radical scavenging capacity was first calculated as percentage inhibition according to Equation (2):

$$\text{Radical scavenging capacity} = \left(\frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100\% \quad (2)$$

where A_{Sample} and A_{Control} correspond to the absorbance values of the sample and control solutions, respectively.

The resulting percentage inhibition values were converted to gallic acid equivalents using the calibration curve (5–100 mg/L). The corresponding concentration was subsequently corrected for the extraction volume and sample mass and expressed as mg gallic acid equivalents per kg of fresh fruit.

2.9. Determination of Total Phenolic Content

Total phenolic content was determined using the Folin–Ciocalteu colorimetric assay [21]. Briefly, 200 μL of extract was mixed with 500 μL of Folin–Ciocalteu reagent and 5 mL of deionized water. After incubation for 3 min in the dark, 1 mL of sodium carbonate solution (37%, w/v) was added, and the mixture was diluted to a final volume of 10 mL with deionized water. The reaction mixture was incubated in the dark for 60 min, and absorbance was measured at 725 nm using a UV–Vis spectrophotometer. A reagent blank containing extraction solvent instead of extract was prepared under identical conditions. Quantification was performed using a gallic acid calibration curve (25–400 mg/L), and results were expressed as mg gallic acid equivalents per kg fresh fruit.

2.10. Determination of Moisture Content

Moisture content was determined according to AOAC Official Method 930.04 [22]. It was calculated from the difference between initial and final sample masses and expressed as a percentage of fresh fruit mass (% w/w).

2.11. Determination of Total Soluble Sugar Content

Total soluble sugars were extracted from homogenized fresh kumquat samples [11]. An aliquot of 0.5 g homogenate was transferred into a centrifuge tube, and 10 mL of deionized water preheated to 70 $^{\circ}\text{C}$ was added. Samples were vortexed for 1.5 min and incubated at 70 $^{\circ}\text{C}$ for 30 min, with intermittent vortex mixing (10 s every 5 min). Following extraction, samples were centrifuged at 4500 rpm for 10 min, and supernatants were collected for sugar determination.

Total soluble sugar content was determined using the phenol–sulfuric acid method [23]. Briefly, 2 mL of appropriately diluted extract (1:18, v/v) was mixed with 1 mL of phenol solution (1%, w/v), followed by addition of 5 mL concentrated sulfuric acid. Samples were incubated for 30 min at room temperature, with vortex mixing after the first 15 min. Absorbance was measured at 490 nm against a reagent blank. Quantification was performed using a sucrose calibration curve (50–500 mg/L), and results were expressed as g sucrose equivalents per kg fresh fruit.

2.12. Determination of Total Soluble Protein Content

Soluble proteins were extracted from the previously prepared fresh kumquat homogenate [24]. Briefly, 0.5 g of sample was mixed with 4 mL PBS and vortexed for 2 min. Samples were allowed to stand for 15 min at room temperature, with brief vortex mixing

every 5 min to facilitate protein extraction. Following extraction, samples were centrifuged at 4800 rpm for 15 min, and the supernatants were collected for protein determination.

Protein concentration was determined using the Bradford assay [21]. An aliquot of 0.5 mL of protein extract was mixed with 1.5 mL diluted Bradford reagent and incubated for 5 min at room temperature. Absorbance was measured at 595 nm against a blank containing PBS and diluted Bradford reagent. Protein concentration was quantified using a bovine serum albumin (BSA) calibration curve (0.5–4 g/L), and results were expressed as g BSA equivalents per kg fresh fruit.

2.13. Determination of Total Lipid Content

Total lipid content was determined according to the Folch extraction method with modifications [25]. Homogenized fresh kumquat samples (0.5 g) were extracted with 10 mL chloroform:methanol (2:1, *v/v*). Samples were vortexed for 1.5 min and incubated at room temperature for 30 min, with intermittent vortex mixing (10 s every 5 min). Phase separation was induced by adding 2 mL of NaCl solution (0.9%), followed by vortex mixing for 30 s and centrifugation at 4800 rpm for 10 min. The lower chloroform phase containing lipids was collected, dried over anhydrous sodium sulfate, and transferred to a pre-weighed glass vial. Solvent removal was performed at 40 °C until complete evaporation. Lipid content was determined gravimetrically and expressed as percentage of fresh weight (% *w/w*).

2.14. UV–Vis Spectral Analysis

UV–Vis absorption spectroscopy was performed as a qualitative complementary analysis to compare the overall spectral profiles of the extracts across the harvesting period. The analysis was restricted to extracts obtained from orange-colored fruits because orange fruits were the only peel-color category represented in all four sampling months (December–March), thereby allowing a consistent qualitative comparison across the complete harvesting period without introducing differences associated with peel-color category. Extracts were prepared under the optimized ultrasound-assisted extraction conditions described above. Spectral measurements were performed from 200 to 800 nm after appropriate dilution using M.Wave software (version 1.0.23, M.R.C. Laboratory Equipment, Harlow, UK). The analysis was not intended for identification or quantification of individual phenolic, flavonoid, or other UV-absorbing compounds.

2.15. Statistical Analysis

Morphological measurements were performed on individual fruits and seeds from each harvesting month, with fruits classified according to peel color category. For each harvest month $\times$ peel color group, morphological measurements were performed on 5 fruits, except for the December orange group, for which 9 fruits were available and analyzed. Fruit and seed measurements obtained from these specimens were treated as morphological observations, with individual fruits serving as the biological sampling units. Physicochemical and bioactive analyses were conducted using three independent homogenized sample preparations per harvesting month, and results are presented as mean $\pm$ standard deviation. These three preparations represent analytical replicates derived from the sampled fruit material and do not constitute independent biological replicates at the tree or orchard level. Therefore, the statistical comparisons describe variation within the sampled tree and the investigated harvesting period rather than population-level variation across trees or harvesting periods.

Statistical analyses were performed using JASP software (version 0.98.1, Amsterdam, The Netherlands). Homogeneity of variances was assessed using Levene's test before analysis of variance (ANOVA). Differences among harvesting stages were evaluated using one-way ANOVA followed by Tukey's honestly significant difference (HSD) multiple

comparison test when significant differences were detected. Statistical significance was set at $p < 0.05$. PCA was performed using standardized physicochemical and bioactive variables (DPPH radical scavenging capacity, phenolic content, moisture, soluble sugars, proteins, and lipids) to visualize relationships among variables and evaluate sample differentiation according to harvesting month and peel color category. PCA was considered an exploratory multivariate analysis and was interpreted within the scope of the sampled tree and harvesting period.

Optimization of ultrasound-assisted extraction was performed using RSM in Minitab software (version 22.4, State College, PA, USA). Model adequacy was evaluated by ANOVA, including regression coefficient significance, lack-of-fit testing, and adjusted and predicted coefficients of determination (R^2 -adjusted and R^2 -predicted, respectively). Regression terms with $p < 0.05$ were considered statistically significant, and reduced models were generated by retaining significant terms and terms required to satisfy the hierarchical principle. Model coefficients were estimated by minimizing the sum of squared differences between experimental and predicted responses. The optimized extraction conditions were experimentally validated, and measured response values were compared with model predictions.

3. Results

3.1. Morphological Characteristics During Fruit Maturation

Representative images of the cultivated tree and the fruit are displayed in Figure 1. Morphological characteristics of ‘Nagami’ kumquat fruits and seeds were evaluated throughout the commercial harvesting period to characterize structural changes associated with fruit development. Fruit dimensions differed across the sampled harvest stages and peel color categories (Table 2). Orange fruits were generally larger than dark- and light-green fruits, with fruit dimensions increasing markedly during the harvesting period. The most pronounced increase occurred between December and January, followed by a continued increase through March.

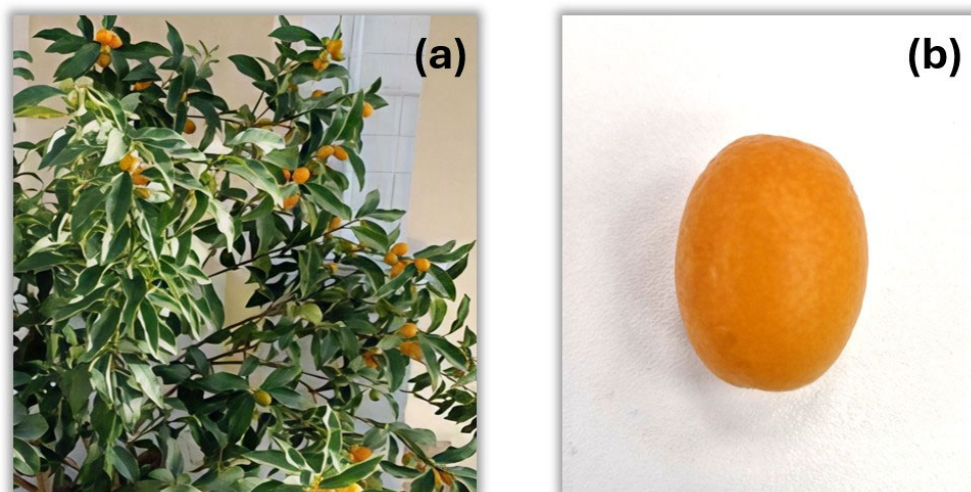


Figure 1. ‘Nagami’ kumquat (*Fortunella margarita* Swingle) plant and fruit material. (a) The sampled tree at its cultivation site in Kontogialos, Corfu, Greece. (b) Orange-colored fruit collected at the end of the commercial harvesting period.

Seed dimensions showed a similar developmental pattern, particularly for seed length and width, which increased substantially in orange fruits from January onward. In contrast, seed height showed limited variation among the sampled stages. The data indicate progressive fruit enlargement and seed development during maturation, with peel color broadly reflecting the developmental stage.

Table 2. Morphological characteristics of Corfu ‘Nagami’ kumquat fruits and seeds during the commercial harvesting period according to harvest month and peel color category ¹.

	Fruit Length (cm)	Fruit Width (cm)	Fruit Height (cm)	Seed Length (cm)	Seed Width (cm)	Seed Height (cm)
December, Dark green	1.46 ± 0.05 ^c	1.04 ± 0.09 ^c	1.36 ± 0.05 ^d	0.36 ± 0.05 ^d	0.24 ± 0.05 ^b	0.12 ± 0.04 ^a
December, Light green	1.48 ± 0.11 ^c	1.18 ± 0.11 ^c	1.32 ± 0.16 ^d	0.18 ± 0.04 ^e	0.26 ± 0.05 ^b	0.10 ± 0.00 ^a
December, Orange	2.21 ± 0.20 ^b	1.63 ± 0.18 ^b	1.82 ± 0.18 ^{bc}	0.50 ± 0.11 ^c	0.21 ± 0.06 ^b	0.11 ± 0.03 ^a
January, Light green	1.66 ± 0.09 ^c	0.94 ± 0.09 ^c	1.58 ± 0.13 ^{cd}	0.16 ± 0.05 ^e	0.22 ± 0.04 ^b	0.12 ± 0.04 ^a
January, Orange	2.90 ± 0.42 ^a	1.82 ± 0.20 ^b	2.04 ± 0.30 ^b	0.76 ± 0.09 ^b	0.50 ± 0.07 ^a	0.12 ± 0.04 ^a
February, Orange	3.02 ± 0.29 ^a	2.14 ± 0.17 ^{ab}	2.54 ± 0.23 ^a	1.00 ± 0.07 ^a	0.60 ± 0.07 ^a	0.12 ± 0.04 ^a
March, Orange	2.94 ± 0.46 ^a	2.40 ± 0.23 ^a	2.76 ± 0.25 ^a	1.02 ± 0.04 ^a	0.70 ± 0.10 ^a	0.14 ± 0.05 ^a

¹ Values are presented as mean ± standard deviation. Morphological measurements were performed on 5 fruits per harvest month × peel color group, apart from the December orange group, for which 9 fruits were available and analyzed. Different lowercase letters within the same column indicate significant differences among the sampled groups according to one-way analysis of variance followed by Tukey’s honestly significant difference post hoc test ($p < 0.05$).

3.2. Optimization of Ultrasound–Assisted Extraction Conditions

3.2.1. Selection of the Extraction Solvent

The extraction efficiency of different solvent systems was initially evaluated to identify the most suitable medium for obtaining extracts with high DPPH radical scavenging capacity. Significant differences were observed among the tested solvent systems (Table 3). Methanol and 50% aqueous methanol produced the highest responses and did not differ significantly from each other ($p > 0.05$). Ethanol and acetone showed intermediate responses and were likewise comparable, whereas 50% aqueous ethanol produced the lowest response ($p < 0.05$). Water and 50% aqueous acetone showed intermediate-to-low responses. Based on these results, methanol/water mixtures were selected for subsequent RSM optimization, allowing solvent polarity to be systematically varied by changing methanol concentration.

Table 3. Effect of extraction solvent systems on the DPPH radical scavenging capacity of Corfu ‘Nagami’ kumquat fruit extracts. The results are expressed as mg gallic acid equivalents per kg fresh fruit ¹.

Solvent	DPPH Radical Scavenging Capacity (mg/kg)
Water	670.2 ± 6.45 ^c
Methanol	1019.6 ± 7.15 ^a
Methanol 50%	1023.9 ± 7.35 ^a
Ethanol	927.3 ± 6.66 ^b
Ethanol 50%	645.3 ± 5.55 ^d
Acetone	908.2 ± 7.25 ^b
Acetone 50%	673.7 ± 7.75 ^c

¹ Values are presented as mean ± standard deviation ($n = 3$). Different lowercase letters indicate significant differences among solvent treatments according to one-way ANOVA followed by Tukey’s HSD post hoc test ($p < 0.05$).

3.2.2. Response Surface Modelling

The effects of methanol concentration, extraction time, and extraction temperature on DPPH radical scavenging capacity were evaluated using a face-centered central composite design. Although the extraction settings specified in the RSM represented the initial ultrasonic bath settings rather than constant sample conditions, progressive heating occurred during sonication. The maximum value reached was recorded for each run, ranging from approximately 33–37 °C, 49–55 °C, and 64–69 °C for initial bath settings of 30, 45, and 60 °C, respectively. Accordingly, the effect of X_3 was interpreted in relation to the initial bath setting and the corresponding range reached during sonication.

The experimental responses varied substantially across the design space, ranging from 150.9 mg/kg to 1320.4 mg/kg fresh fruit (Table 4). The quadratic model provided a good fit to the experimental data, with R^2 , adjusted R^2 , and predicted R^2 values of 95.22%, 90.91%, and 77.86%, respectively.

Table 4. Experimental runs and measured DPPH radical scavenging capacity obtained from the face-centered central composite design used for ultrasound-assisted extraction optimization.

X_1 (% <i>v/v</i>)	X_2 (min)	X_3 (°C)	Y (mg/kg)
50	50	45	1224.1
0	10	60	1011.2
100	90	30	150.9
0	10	30	754.6
0	90	60	703.3
50	50	45	1095.7
50	50	45	1052.9
50	50	45	1320.4
0	50	45	1226.7
50	50	45	1170.6
100	90	60	420.8
100	50	45	611.9
100	10	60	432.0
100	10	30	252.1
50	10	45	721.2
50	50	30	967.3
50	50	45	1266.9
50	50	60	1063.6
50	90	45	678.4
0	90	30	590.5

ANOVA (Table 5) showed that the fitted quadratic model was statistically significant ($p < 0.001$). Methanol concentration (X_1) was the most influential linear factor ($p < 0.001$), while extraction temperature (X_3) also had a significant linear effect ($p = 0.022$). Extraction time (X_2) was not significant as a linear term ($p = 0.092$). Significant quadratic effects were observed for methanol concentration (X_1^2 ; $p = 0.027$) and extraction time (X_2^2 ; $p < 0.001$), whereas the quadratic temperature term was not significant ($p = 0.298$). None of the two-way interactions were significant ($p > 0.05$). The lack-of-fit test was also non-significant ($p = 0.432$), supporting the adequacy of the fitted model.

The final reduced regression equation (Equation (3)) describing DPPH radical scavenging response was:

$$\Upsilon = -151 + 0.10X_1 + 34.2X_3 - 0.0667X_1^2 - 0.2414X_2^2 \quad (3)$$

where Y represents DPPH radical scavenging capacity expressed as mg gallic acid equivalents per kg fresh fruit (mg/kg), X_1 represents methanol concentration (% v/v), X_2 represents extraction time (min), and X_3 represents extraction temperature ($^{\circ}\text{C}$).

The reduced model was subsequently used to determine optimum extraction conditions.

Table 5. ANOVA for the quadratic response surface model describing DPPH radical scavenging capacity ¹.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	9	2,255,102	250,567	22.11	0.000
Linear	3	708,115	236,038	20.83	0.000
X_1	1	584,963	584,963	51.62	0.000
X_2	1	39,338	39,338	3.47	0.092
X_3	1	83,814	83,814	7.40	0.022
Square	3	1,529,654	509,885	45.00	0.000
X_1^2	1	76,403	76,403	6.74	0.027
X_2^2	1	410,125	410,125	36.19	0.000
X_3^2	1	13,681	13,681	1.21	0.298
2-Way Interaction	3	17,334	5778	0.51	0.684
X_1X_2	1	16,164	16,164	1.43	0.260
X_1X_3	1	808	808	0.07	0.795
X_2X_3	1	362	362	0.03	0.862
Error	10	113,318	11,332		
Lack-of-Fit	5	61,187	12,237	1.17	0.432
Pure Error	5	52,131	10,426		
Total	19	2,368,420			

¹ DF, degrees of freedom; Adj SS, adjusted sum of squares; Adj MS, adjusted mean square; F-Value, F-statistic; p-Value, probability value. A p -value < 0.05 was considered statistically significant.

3.2.3. Influence of Extraction Variables and Optimum Conditions

The relative importance of the extraction factors was further visualized using the standardized Pareto chart (Figure 2). Methanol concentration had the largest standardized effect, followed by the quadratic effect of extraction time. The quadratic effect of methanol concentration and the linear effect of extraction temperature also exceeded the significance threshold.

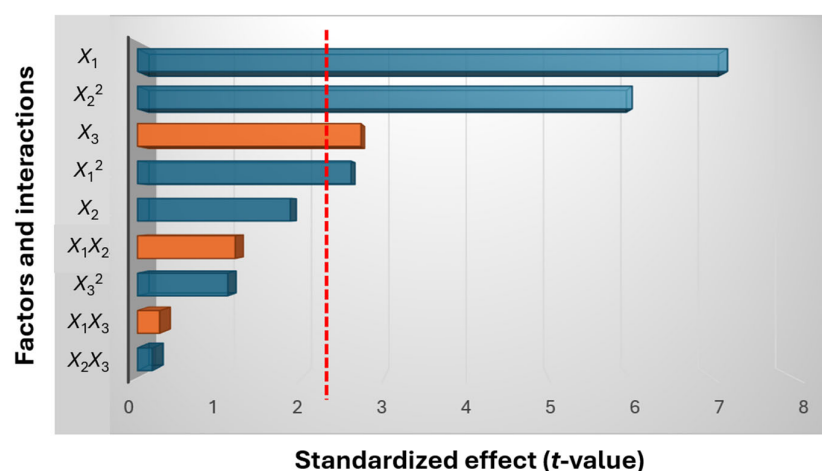


Figure 2. Standardized Pareto chart showing the effects of methanol concentration (X_1 , % v/v), extraction time (X_2 , min), and extraction temperature (X_3 , $^{\circ}\text{C}$) on DPPH radical scavenging capacity (Y_{DPPH}) during ultrasound-assisted extraction optimization. Dark blue and orange bars indicate negative and positive effects, respectively. The vertical reference line corresponds to the significance threshold ($p = 0.05$), with effects extending beyond this line considered statistically significant.

The response surface plots (Figure 3) illustrated that DPPH radical scavenging capacity was highest at relatively low methanol concentrations combined with intermediate extraction times and intermediate-to-high temperatures. At higher methanol concentrations, as well as at very short or prolonged extraction times, the response decreased. Numerical optimization identified 14% methanol in water (*v/v*), 45 min extraction time, and 54 °C extraction temperature as the optimum conditions, with a predicted DPPH response of 1267 mg/kg and a desirability of 0.954. The optimized procedure was subsequently applied to the evaluation of variation during the harvesting period.

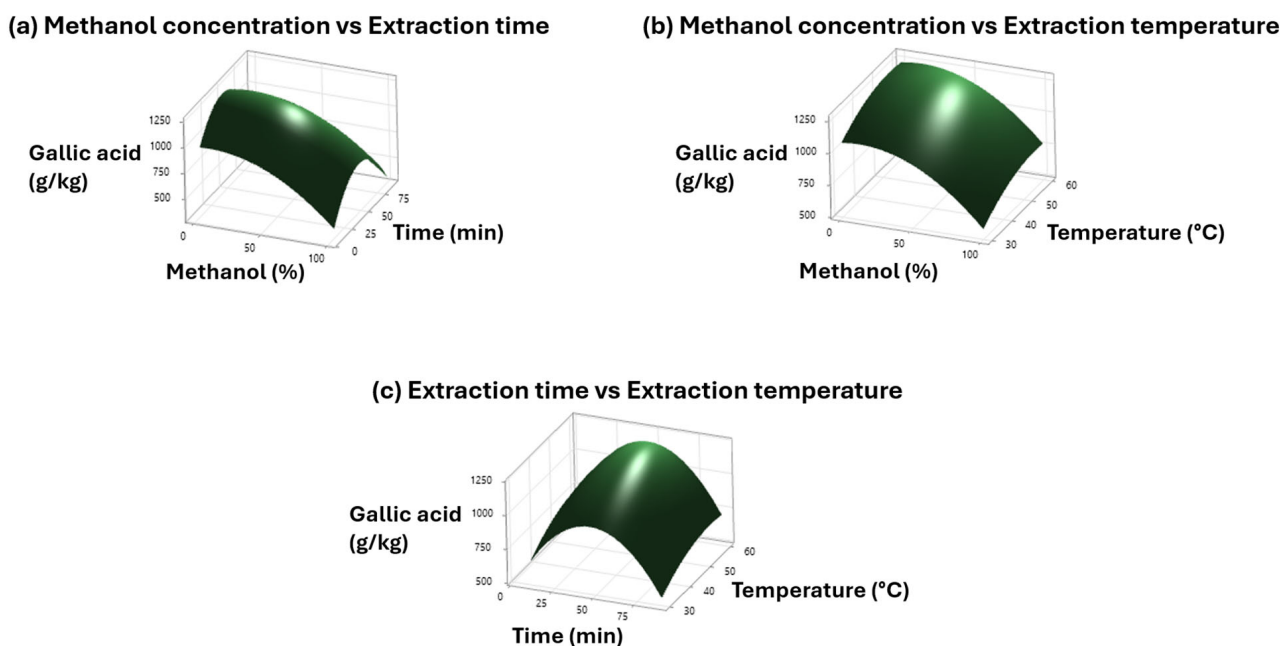


Figure 3. Three-dimensional response surface plots showing the effects of methanol concentration (X_1 , % *v/v*), extraction time (X_2 , min), and extraction temperature (X_3 , °C) on DPPH radical scavenging capacity expressed as mg gallic acid equivalents per kg fresh fruit. (a) Methanol concentration and extraction time; (b) methanol concentration and extraction temperature; (c) extraction time and extraction temperature. In each plot, the third factor was maintained at its central level.

3.3. Changes in Bioactive Potential During the Harvesting Period

3.3.1. Total Phenolic Content

The total phenolic content of ‘Nagami’ kumquat extracts increased progressively across the sampled harvesting stages (Figure 4). The lowest value was observed in December dark-green fruits, whereas the highest occurred in March orange fruits. Phenolic content increased by approximately 90% between these two sampled stages, with the measured values differing significantly between the December dark-green and March orange groups ($p < 0.05$).

3.3.2. DPPH Radical Scavenging Capacity

DPPH radical scavenging capacity followed a similar pattern to total phenolic content across the harvesting period (Figure 5). The lowest and highest values were measured in December dark-green and March orange fruits, respectively, with the measured values differing significantly between the evaluated groups according to Tukey’s HSD test ($p < 0.05$).

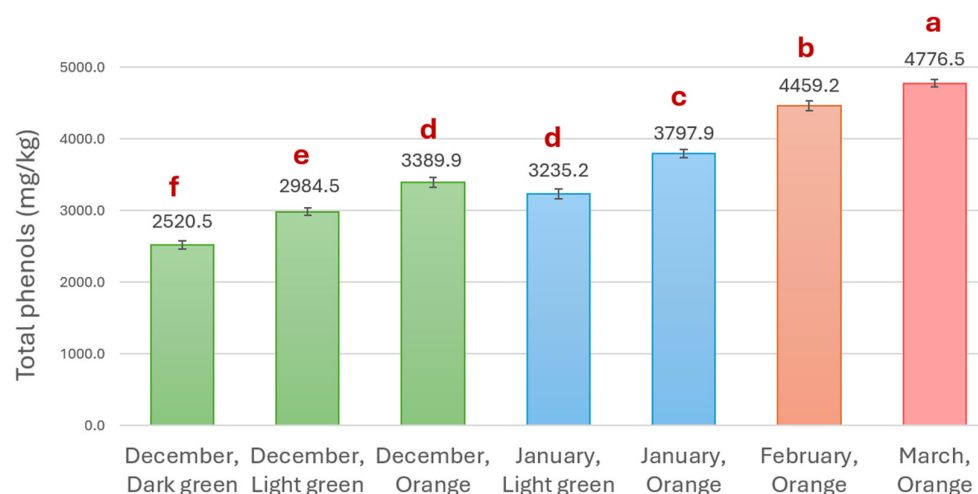


Figure 4. Variation in total phenolic content of Corfu ‘Nagami’ kumquat extracts during the commercial harvesting period (December–March). Values are expressed as mg gallic acid equivalents per kg fresh fruit and presented as mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among the sampled groups according to one-way ANOVA followed by Tukey’s HSD post hoc test ($p < 0.05$).

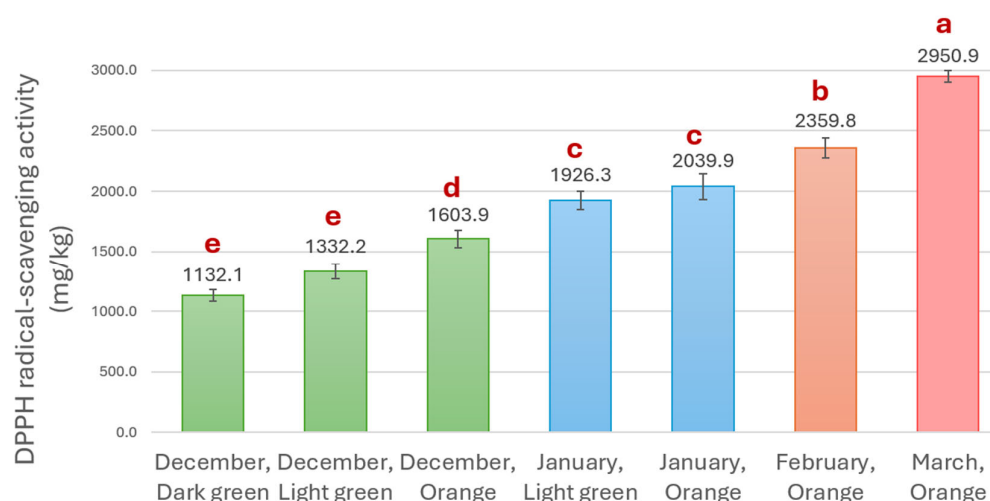


Figure 5. Variation in DPPH radical scavenging capacity of Corfu ‘Nagami’ kumquat extracts during the commercial harvesting period (December–March). The response was expressed as mg gallic acid equivalents per kg fresh fruit. Values represent mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among the sampled groups according to one-way ANOVA followed by Tukey’s HSD post hoc test ($p < 0.05$).

3.3.3. UV–Vis Spectral Characteristics

UV–Vis absorption spectroscopy was used as a qualitative complementary approach to compare the overall spectral profiles of extracts across the harvesting period. Because orange-colored fruits were the only peel-color category represented in all four sampling months, only orange-fruit extracts were analyzed to allow a consistent comparison across December–March. The UV–Vis absorption spectra of orange-colored ‘Nagami’ kumquat extracts collected during the harvesting period are presented in Figure 6. Measurements were performed over the wavelength range of 200–800 nm using extracts prepared under the optimized ultrasound-assisted extraction conditions. All extracts showed broad absorption in the UV region, followed by a marked decrease from approximately 290 nm and several narrow peaks between 340 nm and 360 nm. Absorbance remained close to baseline above 400 nm.

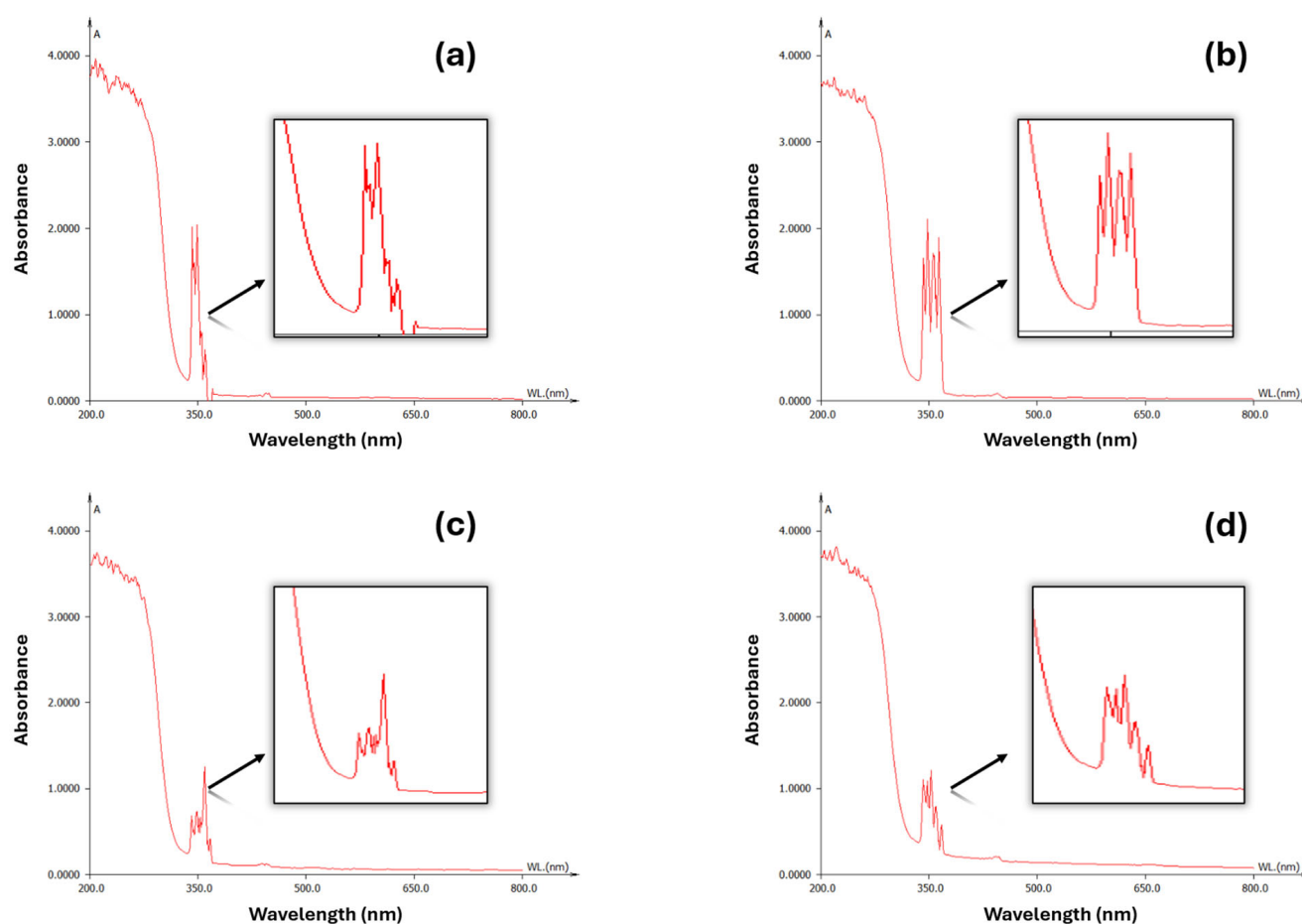


Figure 6. UV–Vis absorption spectra of extracts prepared from orange-colored Corfu ‘Nagami’ kumquat fruits collected during the commercial harvesting period: (a) December, (b) January, (c) February, and (d) March. Samples were obtained under optimized ultrasound-assisted extraction conditions (14% methanol in water (*v/v*), 45 min extraction time, and 54 °C extraction temperature). Absorbance spectra were recorded over the wavelength range of 200–800 nm.

Although the general spectral profiles were similar among harvesting months, differences were observed in the relative intensity and distribution of peaks between 340 nm and 360 nm. December extracts showed two dominant peaks, whereas January exhibited a more complex profile. The dominant peak decreased in intensity in February and March, while neighboring peaks became relatively more pronounced. These differences indicate variation in the overall UV-absorbing profiles of the extracts during the harvesting period. As UV–Vis spectroscopy was used as a qualitative tool, the observed spectral features cannot be definitively attributed to specific phenolic, flavonoid, or other compounds without further compound-specific analysis, such as chromatographic techniques.

3.4. Changes in Physicochemical Characteristics During the Harvesting Period

3.4.1. Moisture Content

Moisture content was highest in December fruits and lowest in March orange fruits (Figure 7). The measured values differed significantly among the sampled harvesting-stage groups according to Tukey’s HSD test ($p < 0.05$). The lower moisture content recorded at the later sampling stage corresponded to a greater relative proportion of dry matter, alongside the higher phenolic and lipid contents measured in these samples.

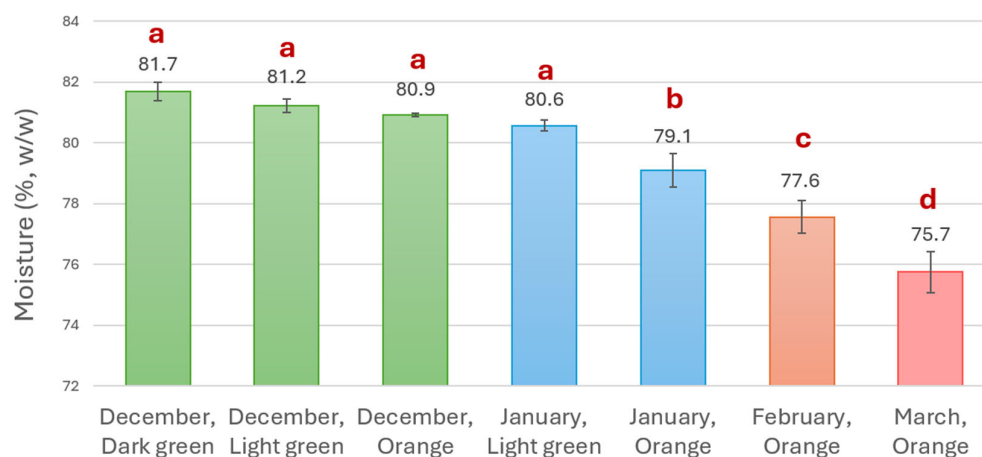


Figure 7. Variation in moisture content of Corfu ‘Nagami’ kumquat fruits during the commercial harvesting period (December–March). Values are expressed as percentage of fresh fruit mass and presented as mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among the sampled groups according to one-way ANOVA followed by Tukey’s HSD post hoc test ($p < 0.05$).

3.4.2. Total Soluble Sugar Content

Total soluble sugar content varied with harvest stage and peel color category (Figure 8), without showing the continuous increase observed for phenolic content. The highest concentration was recorded in December light-green fruits, whereas the lowest occurred in March orange fruits. In orange fruits, sugar concentration increased from January to February before declining in March. Tukey’s HSD test indicated significant differences among the evaluated groups ($p < 0.05$). Unlike phenolic content and DPPH radical scavenging capacity, sugar content showed no consistent increase during the study period.

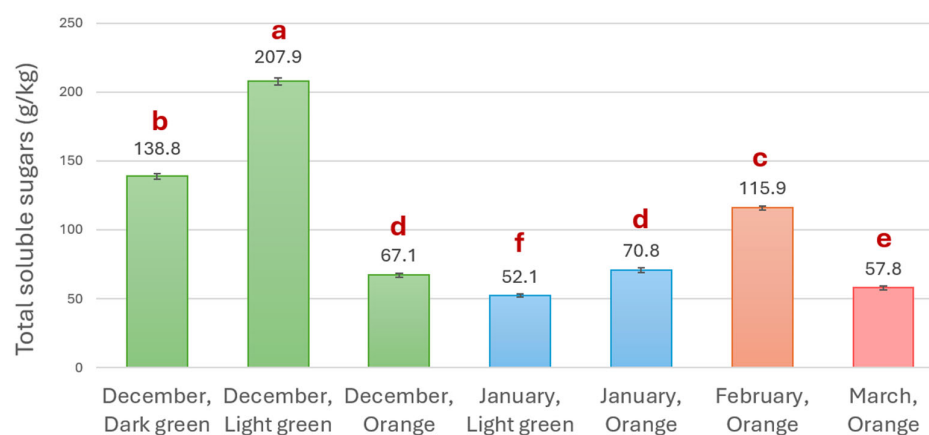


Figure 8. Variation in total soluble sugar content of Corfu ‘Nagami’ kumquat fruits during the commercial harvesting period (December–March). Sugar concentration was determined by the phenol–sulfuric acid method and expressed as g sucrose equivalents per kg fresh fruit. Values represent mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among the sampled groups according to one-way ANOVA followed by Tukey’s HSD post hoc test ($p < 0.05$).

3.4.3. Total Soluble Protein Content

Total soluble protein content showed a decreasing pattern across the sampled stages (Figure 9). Protein concentration was highest in December fruits and decreased toward the end of the harvesting period, reaching its lowest level in March orange fruits. The measured protein concentrations differed significantly for most pairwise group compar-

isons according to Tukey's HSD test ($p < 0.05$), although the December orange and January light-green groups were comparable.

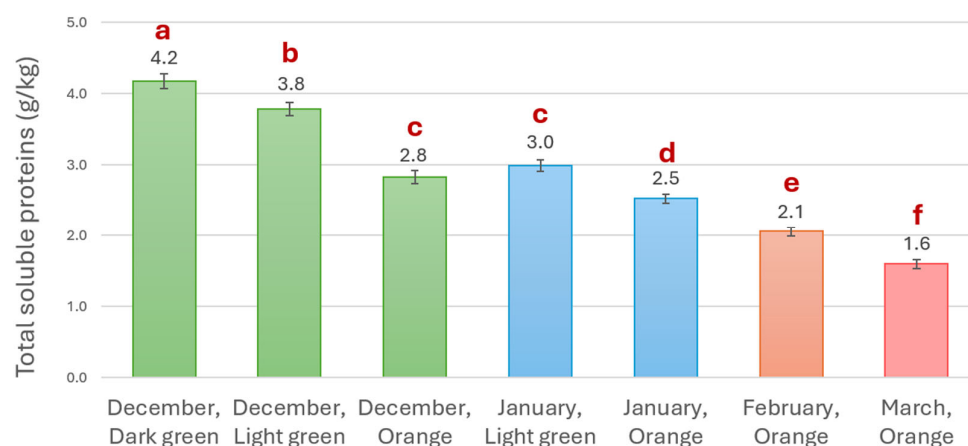


Figure 9. Variation in total soluble protein content of Corfu 'Nagami' kumquat fruits during the commercial harvesting period (December–March). Protein concentration was determined using the Bradford assay and expressed as g bovine serum albumin equivalents per kg fresh fruit. Values represent mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among the sampled groups according to one-way ANOVA followed by Tukey's HSD post hoc test ($p < 0.05$).

3.4.4. Total Lipid Content

In contrast to protein content, lipid concentration was higher at the later sampling stages (Figure 10). The lowest value was recorded in December dark-green fruits, whereas the highest was observed in March orange fruits. The measured lipid concentrations differed significantly among most of the sampled groups according to one-way ANOVA followed by Tukey's HSD post hoc test ($p < 0.05$). No significant difference was observed between the December orange and January light-green groups.

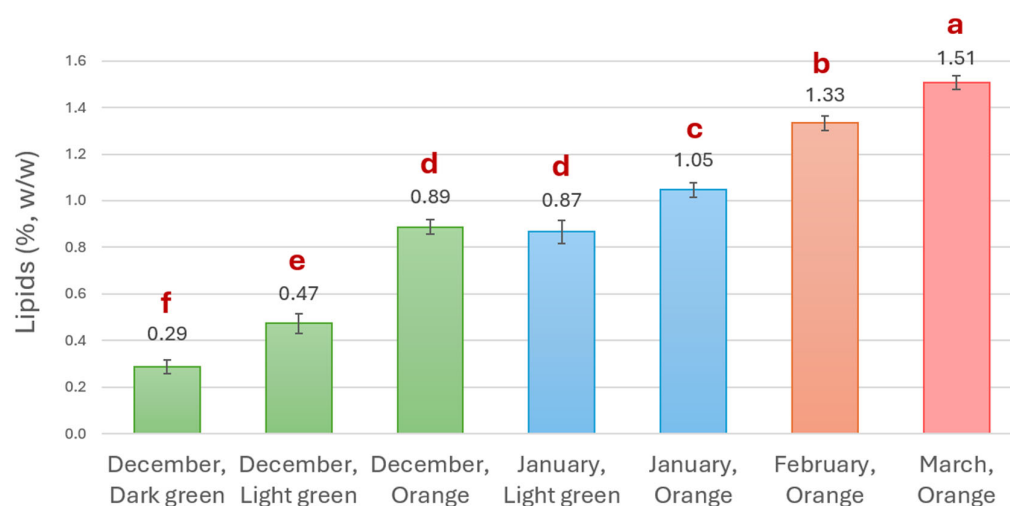


Figure 10. Variation in total lipid content of Corfu 'Nagami' kumquat fruits during the commercial harvesting period (December–March). Lipid concentration was determined gravimetrically following Folch extraction and expressed as percentage of fresh fruit mass (% w/w). Values represent mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among the sampled groups according to one-way ANOVA followed by Tukey's HSD post hoc test ($p < 0.05$).

3.5. Principal Component Analysis of Variation During the Harvesting Period

PCA was performed to investigate the multivariate relationships among the physicochemical characteristics and bioactive properties of Corfu ‘Nagami’ kumquat throughout the December–March harvesting period (Figure 11). The first two principal components (PCs) explained 97.9% of the total variability, with principal component 1 (PC1) and principal component 2 (PC2) accounting for 86.1% and 11.8%, respectively.

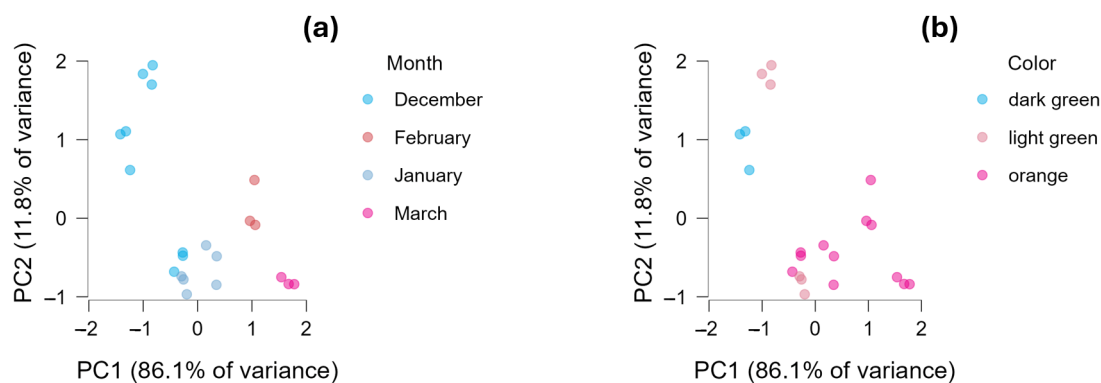


Figure 11. Principal component analysis (PCA) of physicochemical and bioactive characteristics of Corfu ‘Nagami’ kumquat samples. (a) PCA score plot showing sample distribution according to harvesting month (December–March). (b) PCA score plot showing sample distribution according to peel color category (dark green, light green, and orange). PC1 and PC2 represent principal components 1 and 2, respectively.

PCA indicated a clear pattern of sample distribution according to harvesting stage within the sampled tree and harvesting period, with December and March samples positioned primarily at opposite ends of PC1, while January and February samples occupied intermediate positions. Variables associated with DPPH radical scavenging capacity, total phenolic content, and lipid content were positively related to the maturation-related component, whereas moisture and protein contents showed negative associations. Soluble sugar content contributed primarily to the second rotated component. These patterns indicate that the sampled harvesting stages were associated with coordinated variation in physicochemical and bioactive characteristics.

A promax-rotated solution was applied to further interpret component contributions. The first rotated component (RC1) showed strong associations with phenolic content (rotated loading = 1.042), DPPH radical scavenging capacity (0.940), and lipid content (0.907), whereas moisture (−1.058) and protein content (−0.886) exhibited negative associations with RC1. The second rotated component (RC2) was mainly associated with soluble sugar content (rotated loading = 1.015).

4. Discussion

The present study identified notable differences in morphological, physicochemical, and bioactive characteristics across the sampled harvesting stages of ‘Nagami’ kumquat. Fruit enlargement and seed development primarily occurred from December to March, with the most significant growth between December and January. This pattern reflects active maturation under open-field conditions in Corfu. Former controlled-environment research indicated that temperature significantly affects ‘Nagami’ kumquat fruit growth, with higher temperatures suppressing fruit enlargement and lower temperatures prolonging the maturation period [26]. The dimensional increases observed under the open-field conditions of the current study are consistent with these findings, although direct comparisons should be made cautiously due to the differing experimental contexts.

Seed development paralleled overall fruit growth, with marked increases in length and width but limited change in seed height, suggesting uneven development across dimensions. These morphological changes imply ongoing developmental processes during harvest, beyond mere external color transitions. Consequently, classifying fruits into dark-green, light-green, and orange stages offers a practical framework for interpreting associated biochemical variations.

A key finding was the progressive rise in total phenolic content and DPPH radical-scavenging capacity from early dark-green to late-harvest-stage orange fruits. The parallel trends suggest that phenolic compounds may contribute substantially to the antioxidant capacity of the extracts. This interpretation is supported by previously reported positive correlations in kumquat between total phenolic content and DPPH radical-scavenging capacity ($r = 0.7376$, $p < 0.001$), as well as between total flavonoid content and DPPH response ($r = 0.7352$, $p < 0.001$) [9]. Nonetheless, these *in vitro* findings should be interpreted as measures of the antioxidant capacity of the extracts rather than as direct evidence of physiological antioxidant effects *in vivo*. Further research integrating mechanistic and evidence-based approaches is required to establish the biological relevance of food-derived bioactive compounds [27].

It is important to recognize that the overall increase in total phenolic content does not imply a uniform rise in all individual bioactive compounds during development. Previous investigations have indicated that flavonoid and total phenolic contents are higher in immature kumquats than in mature fruits, with flavonoid content generally decreasing during fruit maturation [13]. Modern metabolite profiling studies have further demonstrated that individual flavonoid and phenolic-acid compounds vary in abundance and distribution across kumquat varieties, developmental stages, and fruit tissues [11,12]. Consequently, the higher total phenolic levels observed in the current samples likely reflect an increase in the combined abundance of these compounds rather than a simultaneous increase in individual constituents during maturation.

Environmental factors may also contribute to variation in these biochemical measurements. Recent research on ‘Nagami’ kumquat showed that water stress, together with salicylic acid and kaolin treatments, influenced phenolic and flavonoid contents and DPPH radical-scavenging capacity [28]. Similarly, cultivation environment has been shown to affect the accumulation of phenolic and flavonoid compounds in kumquat fruits, with open-field cultivation resulting in higher total phenolic and flavonoid contents than greenhouse cultivation [29]. The progressive increase observed in the Corfu samples may therefore reflect the combined effects of developmental stage and the environmental conditions experienced during the study period, rather than a universal maturation-related response across all ‘Nagami’ kumquats.

The variation in phenolic content across the harvesting period was accompanied by observable changes in the overall UV–Vis spectral profiles, especially within the 340–360 nm region. This region falls within the near-UV range in which various plant-derived UV-absorbing constituents may contribute to spectral features [30]. Although the overall spectral pattern remained similar among the different harvesting stages, differences in peak intensity may indicate variations in the concentration and/or composition of UV-absorbing compounds. However, UV–Vis spectroscopy was employed here as a qualitative complementary tool rather than for compound identification or quantification. Therefore, the observed spectral features cannot be attributed definitively to specific phenolic or flavonoid compounds without further compound-specific analysis, such as chromatographic profiling.

In contrast to phenolics and antioxidant capacity, soluble sugars did not show a consistent increase during maturation. The highest sugar concentrations were recorded in December’s light-green fruits, followed by fluctuating levels among the orange fruits,

with a decline observed in March. This pattern indicates that carbohydrate accumulation did not follow the same trajectory as phenolic compounds and antioxidant capacity across the harvesting period. In kumquat, soluble sugar accumulation involves the regulation of sucrose, glucose, and fructose metabolism, including processes related to sugar synthesis, transport, and degradation [31]. External factors, particularly temperature, also influence sugar accumulation. In kumquat shoots, lower temperatures were associated with higher soluble sugar concentrations, whereas prolonged exposure to high temperatures resulted in lower soluble sugar levels, likely due to increased respiratory consumption and altered carbohydrate metabolism [26]. The non-linear pattern observed in the Corfu fruits could reflect the combined effects of developmental stage and environmental conditions during the study period rather than maturation alone.

Protein concentration declined progressively towards March, while lipid content showed an opposite trend, increasing over the same period. This decrease in protein concentration is consistent with previous findings in citrus, where protein content decreased with fruit maturity when expressed on a dry-weight basis [32]. The observed increase in lipid content indicates a higher lipid concentration during the later stages of development. Prior research has shown that kumquat fruits contain diverse lipids and fatty acids, with lipid profiles varying among different species and genotypes [33]. The observed rise in lipid content should therefore be interpreted as a change in the measured concentration, rather than necessarily indicating enhanced biosynthesis. The concurrent decrease in moisture content could also contribute to this apparent change, particularly when values are expressed on a fresh-weight basis. These results reveal changes in extractable protein and lipid levels during maturation, but do not provide information on the specific proteins, fatty acids, or lipid classes involved.

An important consideration is the contribution of peel tissue to the whole-fruit composition. The peel accounts for approximately 29.5% of fruit mass in 'Nagami' kumquat and has higher soluble solids and a higher soluble solids-to-acidity ratio than the pulp [26]. Changes in fruit size and development may alter the relative contribution of peel and pulp to whole-fruit composition. Because 'Nagami' kumquats are commonly consumed whole, changes in the relative proportion of peel and pulp may consequently influence the overall intake of phenolic compounds and other bioactive constituents from the fruit. Furthermore, the distinct phytochemical composition of kumquat peel and pulp, with quercetin and luteolinidin detected in the peel and naringenin and hesperidin in the pulp, demonstrates the chemical differentiation between fruit tissues [18]. The essential oil of Greek-grown 'Nagami' kumquat was also characterized by a high proportion of limonene, which accounted for 84.85% of the total volatiles [4]. Since this study did not separately quantify peel and pulp at each harvest stage, changes in tissue proportions should be considered when interpreting differences among harvest stages in whole-fruit composition. Therefore, an increase in fruit mass does not necessarily equate to a proportional increase in specific constituents available from the entire fruit.

Regarding health implications, the higher phenolic content and DPPH capacity observed in late-harvest-stage fruits should not be interpreted as evidence of proportionally greater health benefits. The biological relevance of food-derived bioactive compounds depends on complex interactions and processes beyond their concentration and chemical antioxidant capacity, including interactions with the gut microbiota, microbial biotransformation, metabolic signaling, and subsequent biological effects [27]. Oral bioavailability is also an important consideration when evaluating the potential health relevance of natural plant compounds, because the amount present in a food does not necessarily correspond to the amount available to exert biological effects in the body [27,34]. Consequently, the increased phenolic content observed in late-harvest-stage Corfu 'Nagami' fruits may indi-

cate greater potential for biological activity, but whether these compositional differences translate into meaningful physiological effects or differences in oral bioavailability remains uncertain. Further studies addressing the stability, absorption, bioavailability, and in vivo degradation and metabolism of these compounds are therefore needed to better establish their biological and health relevance [34].

PCA revealed that samples from the early and late stages of the harvesting period were separated mainly along PC1, which was strongly associated with phenolics, DPPH capacity, and lipids, and inversely related to moisture and protein content. This distribution indicates that the primary variation observed among the sampled harvesting stages involved simultaneous changes in multiple compositional characteristics. Soluble sugars contributed primarily to PC2, reflecting their more variable pattern across the harvesting period and weaker association with the main maturation-related changes.

Several limitations should be acknowledged. The study was conducted on a single 'Nagami' kumquat tree during one harvesting period under the specific open-field conditions of Corfu. As a result, the observed responses may not be representative of the broader population or different cultivation environments. Environmental factors such as temperature fluctuations, rainfall, irrigation practices, and crop load can influence fruit development and biochemical composition. Additionally, biological variability was not assessed through multiple trees—limiting the generalizability of the findings. Future studies involving multiple trees, across different harvest stages, and under contrasting cultivation conditions are necessary to validate and extend these results.

To sum up, maturation of Corfu 'Nagami' kumquat involves progressive increases in fruit and seed size, decreases in moisture and protein content, and increases in lipids, phenolics, and antioxidant capacity, with soluble sugars showing less consistent variation during the harvesting period. The late-harvest-stage rise in phenolic compounds and DPPH capacity suggests an elevated antioxidant potential during this period. These compositional changes provide a foundation for understanding variation during the harvesting period in 'Nagami' kumquat, especially regarding phenolic, lipid, and antioxidant characteristics. However, the extent to which these biochemical shifts translate into health benefits remains to be elucidated through targeted compound-specific analyses and biological studies that consider bioavailability and physiological effects.

5. Conclusions

This study identified differences in the physicochemical composition and bioactive potential of Corfu 'Nagami' kumquat across the sampled harvesting stages. Ultrasound-assisted extraction optimized by response surface methodology established reproducible conditions (14% methanol, 45 min, and 54 °C) for the standardized evaluation of radical-scavenging capacity throughout the investigated harvesting period. Under these conditions, late-harvest-stage fruits exhibited increased total phenolic content and DPPH radical scavenging capacity, accompanied by higher lipid levels and reduced moisture and soluble protein contents, whereas soluble sugars followed a more variable pattern across the harvesting period. PCA further indicated that harvesting stage was the main factor associated with differentiation of the investigated samples. Given the specific Corfu 'Nagami' material, single-tree source, and December–March harvesting period examined here, these results indicate that harvest stage was associated with variation in the measured nutritional and functional characteristics. However, the findings should not be generalized to 'Nagami' kumquat production as a whole without further validation across multiple trees, growing harvesting periods, and cultivation conditions. The optimized extraction procedure provides a useful methodological basis for future studies investigating variation across harvest stages and quality attributes of 'Nagami' kumquat.

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