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Impact of Thermal Processing Associated with Essential Oil Recovery on the Phenolic Metabolome and Bioactivity of Lemon, Mandarin, and Orange Peel Wastes

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ABSTRACT: Citrus peel wastes are valuable sources of phenolic compounds (PCs); however, the industrial essential oil extraction exposes the peels to heat, which may significantly affect their concentration, stability, composition, and biological activities. This study aimed to evaluate the impact of thermal processing associated with prior essential oil recovery on the phenolic metabolome and bioactivity of lemon, mandarin, and orange peels. Aqueous-ethanol extracts were prepared from crude peels (CPEs) and heat-treated residues remaining after the essential oil extraction (HPEs) of citrus peel wastes. The molecular-network-assisted LC-MS/MS analysis identified a total of 120 annotated metabolites, predominantly 98 phenolics, and showed a marked reduction in phenolic diversity after heat treatment. In CPEs 59, 55, and 44, phenolics were detected in lemon, mandarin, and orange, whereas phenolics in 43, 49, and 43 were noticed in their corresponding HPEs, respectively. CPEs showed stronger antioxidant activity, with lower IC_{50} values in the DPPH assay (38.72, 60.38, and 70.77 μg/mL) and NO inhibition assay (61.54, 90.32, and 117.50 μg/mL) compared with HPEs. The extracts also exhibited significant anti-inflammatory activity through selective inhibitory effects against the COX-2 enzymes. Overall, thermal treatment during essential oil extraction markedly altered the phenolic composition and reduced the biological activity of citrus peel extracts. These findings demonstrate that processing conditions critically influence the functional value of citrus peel wastes.

KEYWORDS: *waste valorization, thermal processing, citrus peels, GNPS, molecular Network, secondary metabolites*

1. INTRODUCTION

Citrus processing generates large quantities of peel residues, representing an abundant and cost-effective source of natural antioxidants. Citrus peels are particularly rich in phenolic compounds (PCs), including flavonoids and phenolic acids, as well as vitamin C and carotenoids.[1,2] These bioactive constituents are largely responsible for the antioxidant and anti-inflammatory properties attributed to citrus byproducts, highlighting their potential for application in functional foods and nutraceutical formulations.[3−6] Among this bioactive potential, PCs are especially significant because they can be extracted from natural sources and used as natural food additives,[7,8] providing a preferable alternative to synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene.[9] Furthermore, incorporating PCs into food products enhances their nutritional value and health benefits, which are essential to the food industry.[10,11]

Despite this potential, the stability and recovery of PCs remain challenging. Their considerable structural diversity results in marked differences in chemical stability. Whereas certain PCs are relatively stable, others are susceptible to oxidation, volatilization, enzymatic degradation, or thermal decomposition.[12] Consequently, extraction conditions, including temperature, processing method, storage, and matrix composition, strongly influence both the qualitative and quantitative composition of recovered phenolics.[13,14] Therefore, careful selection and optimization of the extraction process are fundamental to prevent chemical degradation or structural modification of the target compounds.[5,15] This consideration is particularly relevant in the citrus processing industry, where steam distillation or hydrodistillation is widely used for essential oil production.[16−20] Although economically advantageous, this process subjects the peels to elevated temperatures and high moisture levels, conditions that may promote the degradation of thermolabile phenolics and consequently alter the biological activity of the residual biomass.[21] Despite this, most studies investigating citrus peel phenolics have focused on fresh or untreated materials,[22−24] with limited attention given to the chemical and functional changes occurring after industrial essential oil extraction.

In parallel, the growing demand for natural additives and functional ingredients has increased interest in valorizing agri-food wastes.[25,26] Upcycling citrus peels into bioactive and

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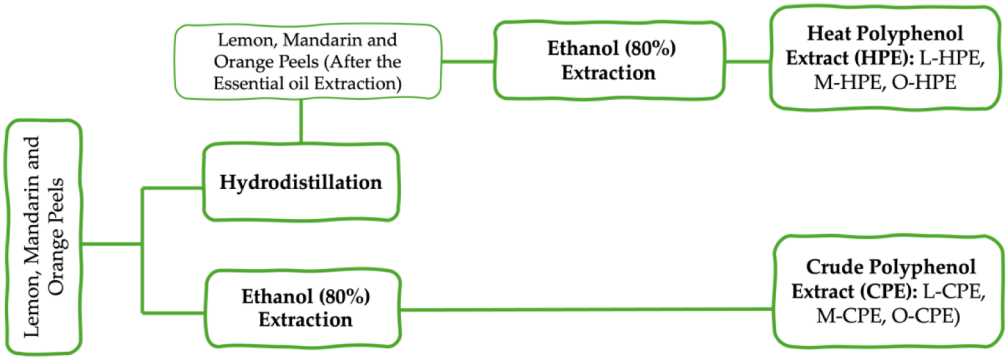

Figure 1. Diagram of heat (HPE) and crude (CPE) polyphenol extraction.

functional extracts aligns with circular economy principles and contributes to sustainable production and consumption. For effective valorization, it is essential to understand how industrial processing affects the chemical composition and biological activity of these residues, particularly the phenolic metabolome.[27] Exploring this potential has become a fundamental topic of interest throughout the food value chain.[28−30] In addition to the significance of upcycling practices, it is crucial to understand how extraction methods and temperatures affect the recovery of these compounds, which is vital for enhancing their valorization. Recent advances in metabolomics provide powerful tools for this purpose. Molecular networking, combined with LC-MS/MS analysis, enables visualization and annotation of structurally related metabolites based on fragmentation similarity, facilitating comprehensive characterization of complex plant extracts and the discovery of potential analogues.[31] Nevertheless, this approach has rarely been applied to compare crude citrus peels and heat-treated residues generated after essential oil extraction.

The present study aims to comprehensively characterize the phenolic constituents of peels from three *Citrus* species: lemon (*Citrus* × *limon* (L.) Osbeck), mandarin (*Citrus reticulata* Blanco), and orange (*Citrus* × *sinensis* (L.) Osbeck), using LC-ESI-MS/MS metabolomic profiling, and to correlate their chemical composition with antioxidant and anti-inflammatory activities. Furthermore, this work explores the effect of industrial heat processing (hydrodistillation residue) on the stability of thermolabile phenolic compounds and their consequent impact on biological efficacy, highlighting the potential value of citrus peel byproducts for nutraceutical and functional applications.

2. MATERIALS AND METHODS

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid (Vitamin C), celecoxib, and Griess reagent were purchased from Sigma-Aldrich, USA. Sodium nitroprusside (s.d. Fine-Chem Ltd., Mumbai), indomethacin, and acetylsalicylic acid were purchased from the Arab Drug Company (ADCO), Egypt. The anti-inflammatory activity was assessed using a Cayman Chemical Co., USA kit.

2.2. Plant Material and Extraction Procedure

Fresh citrus fruits (lemon, mandarin, and orange) were collected in November 2023 from the farm of National Research Center, Agricultural Production and Research Station, El Nubaria Province, El Behira Governorate (Egypt). Fruit waste (peels with little pulp residues) was supplied after extracting the juice from the fresh, healthy fruits using commercial squeezers. A total of 6 kg of fresh lemon, mandarin, and orange peels were separately selected, and this process was independently repeated three times (n = 3). For each repetition (6 kg), the fresh peels were divided equally into two portions. First, 3 kg of each citrus fresh peel were extracted by maceration with 80% ethanol (6 L with a mass/solvent ratio of 1:2 (w/v)) until exhaustion, −ve test for TPC and TFC. Separately, each extract was filtered and dried following the methods by Ibrahim et al. (2024).[29] Each dried crude peel extract (CPE) was stored in a separate dark-colored bottle at 4 °C to obtain lemon, mandarin, and orange CPEs (L-CPE, M-CPE, and O-CPE, respectively; Figure 1). Furthermore, the remaining 3 kg of fresh peels were subjected to heat hydrodistillation in a Clevenger-type apparatus (3 h, 100 °C) to get the essential oils. The obtained data were recently published.[30] Each heat-treated peel residue (HP) left after essential oil extraction was extracted and stored by the same method as that for CPE revealed (L-HPE, M-HPE, and O-HPE) for lemon, mandarin, and orange heat-treated peel residue extracts, respectively (Figure 1).

2.3. Phytochemical Analysis

2.3.1. Total Phenolic Content (TPC). The total phenolic content (TPC) of heat (L-HPE, M-HPE, and O-HPE) and crude polyphenol extracts (L-CPE, M-CPE, and O-CPE) was determined using Folin−Ciocalteu reagent, following the method described by Farid et al. (2022).[32] The standard calibration curve of gallic acid (y = 0.00346x + 0.00076; R^2 = 0.9916) has a linear range of 0−160 μg/mL, with a detection limit (LOD) of 16.62 μg/mL and a quantification limit (LOQ) of 50.38 μg/mL. TPC results were calculated using eq 1, expressed as mg of GAE/g of DE.

$$\text{TPC (mg GAE/g DE)} = C_{\text{gallic acid}} \times V \times m\,M \quad (1)$$

where $C_{\text{gallic acid}}$ is the standard (gallic acid) concentration established from the calibration curve, V is the dilution factor, m is the total extract weight, and M is the dry extract (DE) concentration.

2.3.2. Total Flavonoid Content (TFC). The total flavonoid content (TFC) of the heat (L-HPE, M-HPE, and O-HPE) and crude polyphenol extracts (L-CPE, M-CPE, and O-CPE) was measured using the aluminum chloride reagent, as described by Farid et al. (2022).[32] The calibration curve (y = 0.00087x + 0.00175; R^2 = 0.9985) was constructed using quercetin (QE) as a standard, with a linear range of 0−280 μg/mL, a LOD of 12.59 μg/mL, and a LOQ of 38.14 μg/mL. The results were estimated according to eq 2 and are expressed in mg QE/g DE.

$$\text{TFC (mg QE/g DE)} = C_{\text{quercetin}} \times V\,m \quad (2)$$

where $C_{\text{quercetin}}$ is the standard (quercetin) concentration established from the calibration curve, V is the volume of extract (mL), and m is the total extract weight (g).

2.3.3. LC-MS/MS Analysis, Data Processing, and Metabolites Annotation. The chemical compounds present in the Citrus HPEs and CPEs were analyzed by liquid chromatography-electrospray ionization−tandem mass spectrometry (LC-ESI-MS/MS), using an Exion LC AC system for separation and a SCIEX Triple Quad 5500+ MS/MS system equipped with electrospray ionization (ESI) for

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detection.[33] The analysis was performed using a C-18 column (Ascentis Express 90 Å, 2.1 × 150 mm, 2.7 μm). An injection volume of 5 μL was used with a flow rate set at 0.3 mL/min. The mobile phases consisted of A (ammonium formate at 5 mM, pH 8) and B (acetonitrile, HPLC grade). The chromatographic gradient was programmed as follows: 0−1 min at 5% B, 1−20 min gradually increasing from 5% to 100% B, 20.01−25 min at 100% B, and 25.01−30 min returning to 5% A. MS/MS analysis was carried out in negative ion mode over a mass range of 100 to 1000 Da, using EMS-IDA-EPI for MS1 with parameters including a 25 psi curtain gas, −4500 V ion spray voltage, source temperature at 500 °C, and 45 psi for ion source gases 1 and 2. For MS2, the mass range was set from 50 to 1000 Da, with a declustering potential of −80, a collision energy spread of 15, and a collision energy of −35. Peak detection and spectrum interpretation were performed with PeakView 1.2 software (SCIEX, Framingham, MA, USA). Compound identification was done manually by comparing MS data and retention times with literature references, alongside comparisons with GNPS libraries (Table 2).[33]

2.3.4. Molecular Network Creation and Visualization. A negative molecular network (MN) was created using the online workflow (https://ccms-ucsd.github.io/gnpsdocumentation/) on the GNPS website (http://gnps.ucsd.edu). Raw data were converted into an open-source format. mzml format with the MSConvert tool (ProteoWizard Software Foundation, Version 3.0.19330, USA) and subsequently uploaded to GNPS via WinSCP. To compare the six analyzed extracts, six were analyzed separately. Mzml data sets were processed as a single job and uploaded into six distinct groups. The GNPS analysis used parameters such as precursor ion mass tolerance and MS/MS fragment ion tolerance set to 0. 0.2 and 0.5 Da, respectively. A network was then generated, where edges were filtered to have a cosine score above 0. 0.7 and more than six matched peaks. Additionally, the maximum size of a molecular family was set to 100. Finally, the MS^2 spectra in the generated network were searched against GNPS's spectral libraries, including reference spectra (GNPS-Collections), community spectral libraries (GNPS-community), and other third-party libraries (e.g., MassBank, Respect, and NIST).[31] All matches between MN and library spectra required a score above 0.7 and at least six matched peaks. The resulting network can be accessed through the link https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=48ced393b63345999495322c5ba64eb1, which displays the spectral clusters separately. In this approach, each node represents a spectrum (metabolite), while edges connect metabolites with similar spectral features, reflecting structural or chemical relationships. Details about the corresponding compounds that could be identified through GNPS libraries, such as the library ID and the number of matching fragment ions, were also observed. Visualization of the spectral network was performed using Cytoscape 3.9.1, where each spectrum is depicted as a node and the connections between nodes as edges, indicating structural relationships through MS analysis. Within each node, each color corresponds to a single peel extract, and the areas of these colors reflect the relative abundance or intensity of a specific metabolite across all studied extracts.

Alongside metabolite dereplication through the GNPS platform, this process was performed manually by searching the fragmentation pattern using several literature sources (Table 2) and some natural product databases, including ChemSpider (http://www.chemspider.com/) and PubChem (https://pubchem.ncbi.nlm.nih.gov/).

2.4. Biological Activity

2.4.1. Antioxidant Activity. *2.4.1.1. DPPH Radical Scavenging Assay.* The Citrus CPEs and HPEs were screened for free radical scavenging using a DPPH assay, following the procedure outlined by Ibrahim et al. (2021).[34] DPPH (0.1 mM) was prepared in methanol. Citrus polyphenol-rich extracts and vitamin C were prepared with successive concentrations (31.25−1000 μg/mL) in methanol, with a reaction time of 30 min, and measured at 517 nm using a spectrophotometer. The DPPH radical scavenging activity was assessed using eq 3. The linear modeling of the obtained data was performed, and the results expressed the concentration needed to scavenge 50% of the initial DPPH radicals (IC_{50}). A lower IC_{50} value indicates that the extract is more effective at scavenging DPPH, which suggests more significant antioxidant activity.

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{A0} - \text{Abs}_{\text{sample}}}{\text{Abs}_{A0}} \quad (3)$$

where Abs_{A0} is the absorbance of the control, and Abs_{sample} is the absorbance of the treated sample with the extract at different concentrations.

2.4.1.2. Nitric Oxide (NO) Radical Scavenging Assay. Citrus CPEs and HPEs were screened for free radical scavenging using an NO assay, following the procedure outlined by Ibrahim et al. (2021).[34] The NO radical scavenging activity of the tested material was evaluated using sodium nitroprusside (SNP) and compared to a standard (Vitamin C). Eq 3 was used to calculate the NO radical scavenging activity. The data obtained were linearly modeled, and the results were expressed as the concentration required to scavenge 50% of the initial NO radicals (IC_{50}).

2.4.2. Anti-Inflammatory Activity. The Citrus CPEs and HPEs were tested for in vitro anti-inflammatory activity via the inhibition of cyclooxygenase enzymes (COX1 and COX2). This assay includes both ovine COX1 and human recombinant COX2 enzymes, allowing the user to screen isoenzyme-specific inhibitors, following the procedures outlined by Ibrahim et al. (2024).[29] Indomethacin and celecoxib were used as standards for assessing the anti-inflammatory activity against COX1 and COX2, respectively.

2.5. Statistical Analysis

Statistical analyses were performed using SPSS software (IBM SPSS Statistics, version 25; Chicago, IL, USA). Results are presented as means ± the standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Duncan's post hoc test for multiple comparisons was applied. Statistical significance was considered at $p \leq 0.05$.

2.5.1. Determination of IC_{50} Values. IC_{50} values for each extract were calculated by using SPSS version 25. All assays were conducted in triplicate, and data are expressed as mean ± SEM. Group comparisons were made using one-way ANOVA followed by Duncan's post hoc test, with statistical significance set at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Extraction Yields

The extraction yields of heat (L-HPE, M-HPE, and O-HPE) were 60 ± 1.24 g of DE, 55 ± 0.92 g of DE, and 73 ± 1.52 g of DE, respectively, and the crude polyphenol extracts (L-CPE, M-CPE, and O-CPE) were 100 ± 2.30 g of DE, 95 ± 0.99 g of DE, and 125 ± 2.00 g of DE, respectively.

3.2. Phytochemical Investigation

3.2.1. Total Phenolic (TPC) and Flavonoid (TFC) Contents. Phenolic and flavonoid compounds have various biologically essential functions, such as radical scavenging, antioxidant properties, and other health-promoting properties.[35] Natural resource compounds are recommended as food additives in food processing, and their addition has also been reported to provide nutritional and health benefits.[36]

Table 1 presents the total phenolic contents (TPCs) and total flavonoid contents (TFCs) of crude (L-CPE, M-CPE, and O-CPE) and heat-treated (L-HPE, M-HPE, and O-HPE) polyphenol extracts. The data indicate that the CPEs exhibited the highest total phenolic and flavonoid levels. The decreases in TPC and TFC values for the HPEs could be attributed to the high temperature used during the hydrodistillation of essential oils, as elevated temperatures are commonly associated with the degradation of polyphenols.[37] Additionally, L-CPE showed the highest TPC and TFC compared with

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Table 1. Total Phenolic (TPC) and Flavonoid (TFC) Content of the Studied *Citrus* Species Peel Extracts[a]

Citrus peels extracts		TPC (mg GAE/g DE)	TFC (mg QE/g DE)
Lemon	L-CPE	343 ± 1.99[a]	166 ± 0.87[a]
	L-HPE	55 ± 3.24[d]	26 ± 0.99[d]
Mandarin	M-CPE	244 ± 2.54[b]	74 ± 2.03[c]
	M-HPE	43 ± 2.31[f]	27 ± 1.54[d]
Orange	O-CPE	230 ± 1.65[c]	84 ± 1.84[b]
	O-HPE	48 ± 1.54[e]	24 ± 1.20[d]

[a]Data presented as mean ± SE. One-way ANOVA was used for data analysis (n = 3). Different lowercase letters within the same column designate significant differences (p < 0.05). TPC: total phenolic content; TFC: total flavonoid content; L-CPE: lemon crude peel extract; L-HPE: lemon heat-treated peel residue extract; M-CPE: mandarin crude peel extract; M-HPE: mandarin heat-treated peel residue extract; O-CPE: orange crude peel extract; O-HPE: orange heat-treated peel residue extract.

those of M-CPE and O-CPE, respectively. A recent study by Mateus et al. (2024)[38] evaluates the TPC in citrus extracts derived from Portuguese orange, lemon, and lime byproducts. The highest TPC was found in lime (62.62 mg of GAE/g), followed by lemon (23.77 mg of GAE/g) and orange (14.22 mg of GAE/g). Additionally, the TFC was assessed using epicatechin equivalents (EE), in the same order as TPC: lime (19.63 mg EE/g), lemon (9.44 mg EE/g), and orange (3.00 mg EE/g). This research supports our study, demonstrating that lemon extracts have a higher total phenolic content (TPC) and total flavonoid content (TFC) than orange peel extracts.

3.2.2. HPLC Profiling and MS Analysis. LC-MS/MS analysis in negative ionization mode is a helpful tool for structural investigations of phenolics and flavonoids, as it identifies relevant fragments and relative intensities of specific diagnostic ions. Therefore, the current study aims to assess the metabolome diversity in the negative ionization mode of the aqueous ethanol extracts of three fruit peels of different *Citrus* species before and after essential oil extraction. The base peak chromatogram (BPC) of the CPEs and HPEs showed corresponding changes (Figure 2), suggesting that the biological significance of the two extraction techniques may differ. Furthermore, the analysis of the BPC revealed that the most prevalent metabolites that may be regarded as potential tag candidates, with a broad range of intriguing biological features, are flavonoids and phenolics (Retention time (Rt) 2.00−15.00 min).

3.2.3. Molecular Networking and Metabolites Annotation. This study aimed to compare the metabolite composition of CPEs and HPEs across three *Citrus* species with respect to their antioxidant and anti-inflammatory protective properties to enhance their beneficial medicinal effects. For that purpose, HPLC-MS/MS was selected for its sensitivity, selectivity, and robustness.[39,40] A negative molecular network (MN) was constructed to examine the obtained mass data visually. MN classifies mass spectrometric data by comparing spectral similarities in the fragmentation patterns of various structurally related metabolites. The current MN contained 870 nodes comprising 64 clusters (each with a minimum of two connected nodes) and 559 self-looped nodes (Figures 3 and 4); it enabled visual assessment of the various compound groups and analogues, which aided in the recognition of derivatives and isomers. The significant groups were cluster A (A1: lyso-glycerophosphoethanolamine; LGPE and lyso-glycero-3-phosphocholine; LGPC, A2: flavone and flavonol-*O*-diglycosides, A3: methoxylated flavonoid-*O*-diglycosides), cluster B (flavanone-*O*-glycosides), cluster C (polymethoxylated flavonoid aglycones), D (lyso-glycerophosphoinositol; LGPI), cluster E (hydroxycinnamic acid-*O*-glycoside), F (hydroxycinnamic acid-*O*-glycoside and chlorogenic acids), cluster G (flavanone-*C*-glycoside), H (methoxylated flavonoid-*O*-glycoside), I (flavonoid-*C*-glycoside), and J (flavonoid-*C*-diglycosides) (Figure 2). Appropriately, 120 metabolites were annotated, belonging to different classes in descending order as flavonoids > phenolic acids > phospholipids > coumarins > amino and organic acids > fatty acids > terpenoids. They were annotated based on accurate mass values, chemical formulas, retention times, fragmentation patterns, and spectral matches in GNPS libraries, where applicable. Additionally, comparison of their fragmentation data with previously reported literature confirmed their putative identification (Table 2). These results enabled the specific detection of metabolites in peel extracts of three *Citrus* species for the first time.

Across the LC-ESI-MS/MS metabolomic investigation, the phenolics comprised most of the annotated metabolites (98 compounds), accounting for almost 81.7% of the total compounds. These comprised 16 phenolic acids and their derivatives, 79 flavonoids, and 3 coumarins. Flavonoids exhibited remarkable structural diversity, being represented by *O*-glycosides (39 compounds) > aglycones (22 compounds) > *C*-glycosides (15 compounds) > *O*-*C*-glycosides (3 compounds). The predominance of *O*-glycosylated flavonoids is expected for Citrus peels, where flavanone and flavone rutinosides and neohesperidosides constitute the major storage forms. This distribution agrees with previous phytochemical reports of *Citrus japonica* Thunb. fruits and further confirms that Citrus peels preferentially accumulate flavonoids as glycosylated derivatives.[2]

3.2.3.1. Phenolic Compounds. Overall, LC-MS-detected metabolites showed a marked reduction in phenolic diversity after heat treatment. In CPEs 59, 55, and 44, phenolics were detected in lemon, mandarin, and orange, whereas 43, 49, and 43 were noticed in their corresponding HPEs, respectively. The major losses involved hydroxycinnamic acid esters and flavonoid *O*-glycosides, whereas *C*-glycosyl flavonoids remained comparatively stable. This confirms that thermal processing primarily induces hydrolysis and oxidation of thermolabile phenolics rather than general extraction loss.

3.2.3.2. Phenolic Acids and Derivatives. Phenolic acid structures were also annotated, including hydroxycinnamic acid derivatives that are characterized by a fragment ion and represented in clusters D and F (Figure 4). Coumaric acid (5; m/z 163.01 $[M\text{-}H]^-$) was distributed in the six tested extracts, while caffeic acid (8; m/z 179.01 $[M\text{-}H]^-$) was specified for the O-CPE, indicating its high susceptibility to degradation during heating. However, trihydroxycinnamic acid (14; m/z 195.02) and its -*O*-hexoside derivatives (7 and 13; m/z 357.01) were present in both lemon extracts, with the highest concentrations in the L-CPE, and markedly reduced after thermal processing. Similarly, for the *O*-hexoside substituents of coumaric (16; m/z 325.02), sinapic (20; m/z 385.01), ferulic (23; m/z 355.01), and hydroxyferulic (84; m/z 371.05) acids, however, were concentrated in the L-HPE.

Chlorogenic acid derivatives were identified based on the fragment ions $[\text{quinic acid-H}]^-$ at m/z 191 and $[\text{quinic acid-H-H}_2\text{O}]^-$ at m/z 173, corresponding to quinic acid and

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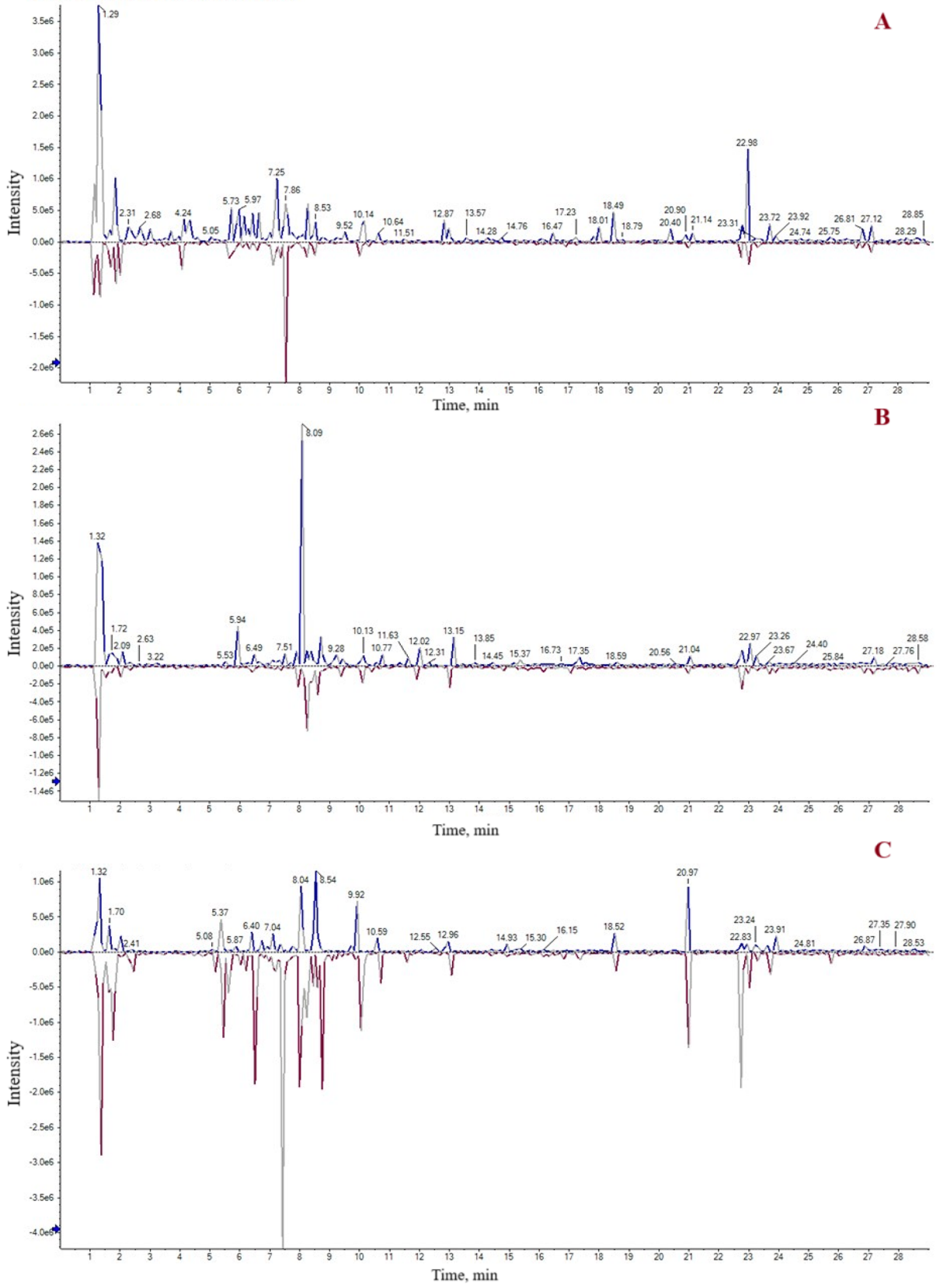

Figure 2. Base peak chromatograms of CPEs (blue line) and HPEs (red line) for sweet orange (A), lemon (B), and mandarin (C) peels.

dehydrated quinic acid, respectively. These included caffeoyl quinic acid (11; *m*/*z* 353.06) and coumaroyl quinic acid isomers (15; *m*/*z* 336.95 and 17; *m*/*z* 337.05). Notably, these esterified phenolic acids were concentrated in CPEs and diminished in HPEs, indicating thermal hydrolysis of ester bonds followed by oxidative degradation (Table 2, Figures 3 and 4). Other coumaroyl derivatives were also observed in the lemon extracts, especially the L-CPE, and represented as the

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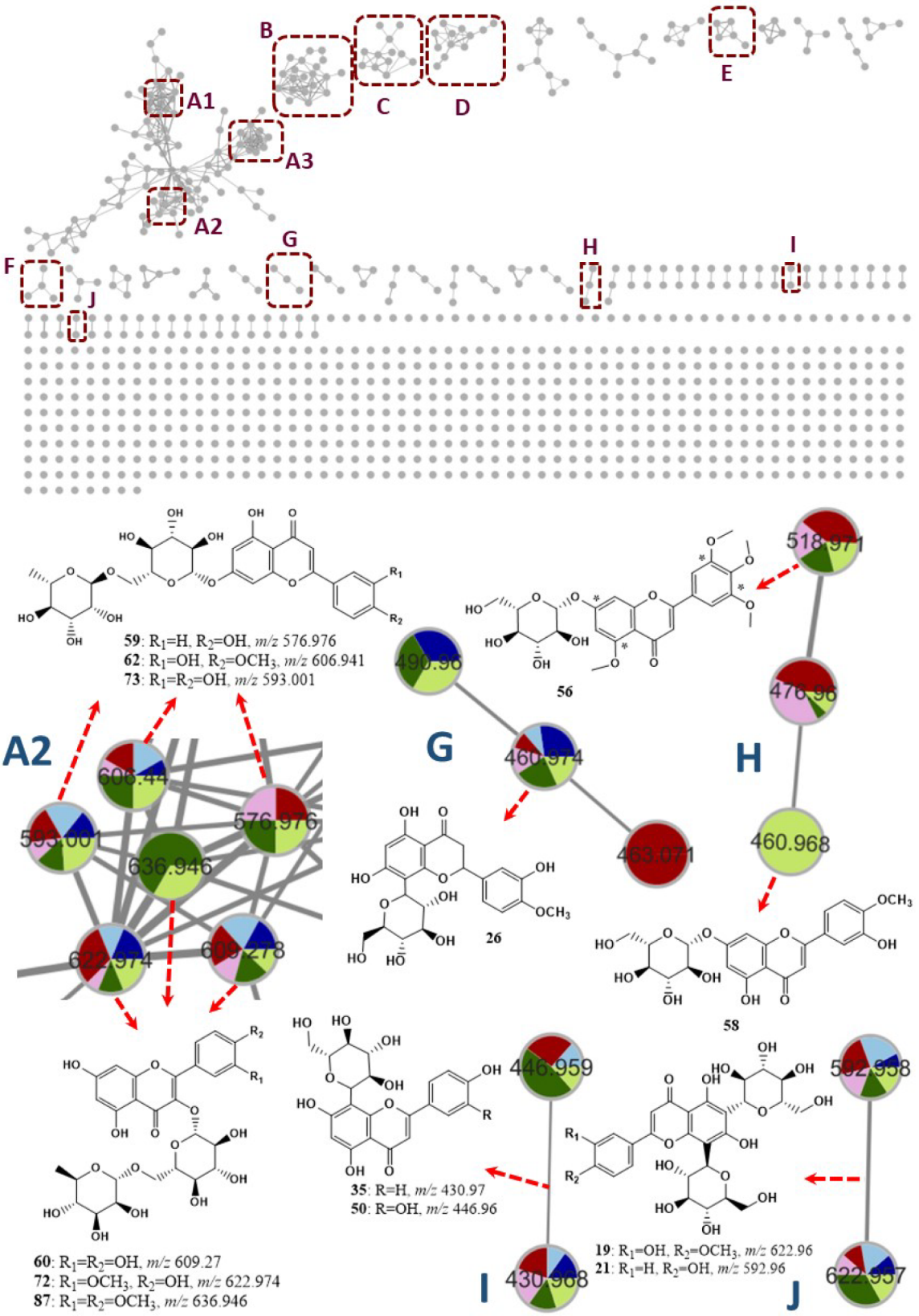

Figure 3. Molecular network (negative mode ionization) outlined the clusters of interest. Clusters A2, H, G, I, and J showed the shared compounds in the three studied *Citrus* species: lemon (red), mandarin (green), and sweet orange (blue). CPEs (dark color) and HPEs (light color).

acetyl and malonyl substituents of the *O*-hexoside derivatives. They could be annotated as coumaric acid *O*-acetyl hexoside (55; m/z 367.03 $[M\text{-}H]^-$, 66; m/z 413.06 $[M\text{-}H+HCOOH]^-$, and 97; m/z 435.02 $[M\text{-}H+HCOONa]^-$), as well as coumaric acid *O*-malonyl hexoside (81; m/z 410.89). The loss of these conjugates suggests cleavage of acyl-glycosidic linkages and

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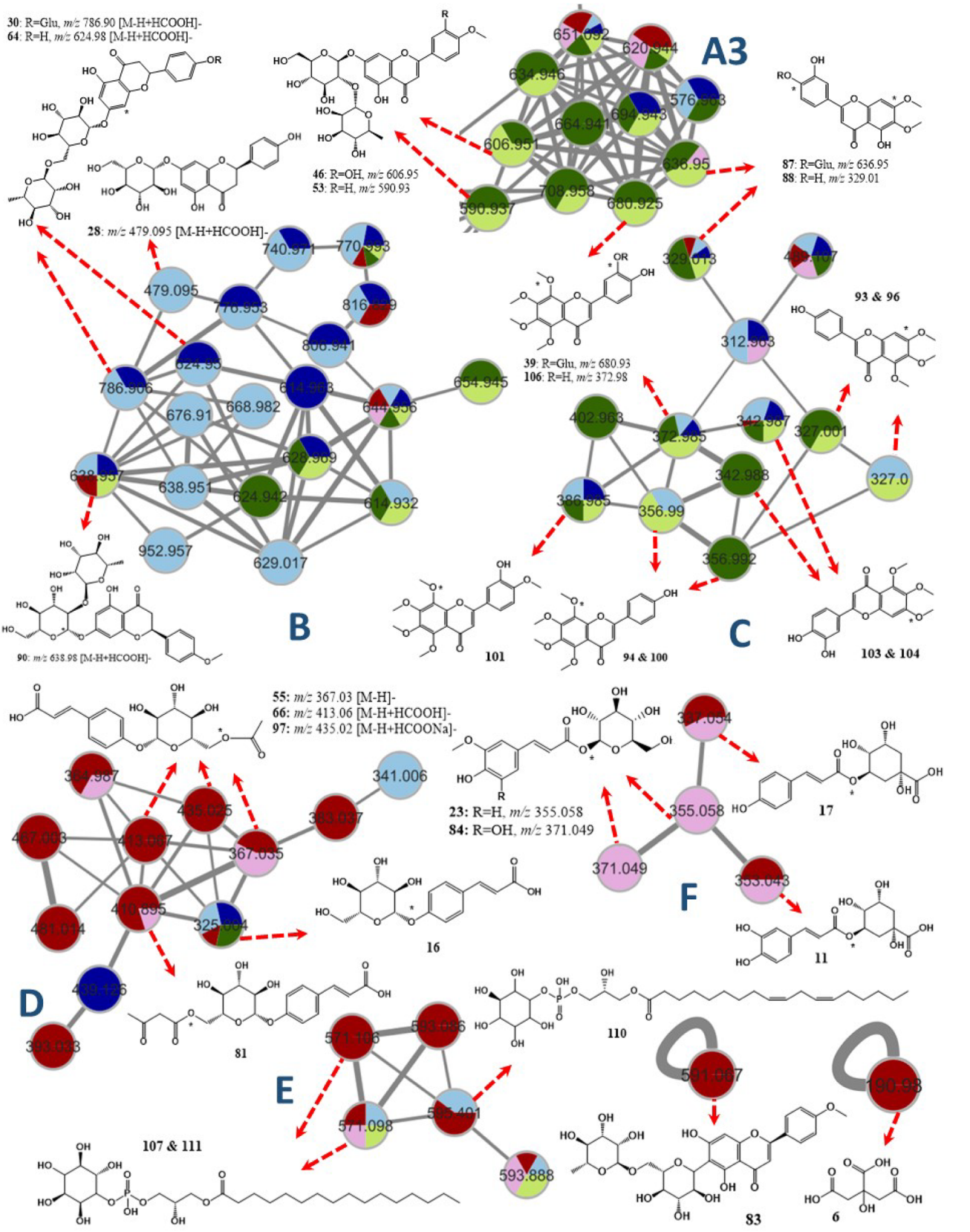

Figure 4. Clusters of interest (A−F). Cluster B: characterized compounds colored sweet orange (blue). Clusters A3 and C: characterized compounds in mandarin (green). Clusters D−F and two self-looped nodes: characterized compounds in lemon (red). CPEs (dark color) and HPEs (light color).

subsequent oxidation. In contrast, simple phenolic hexosides, such as hydroxyferulic acid-*O*-hexoside (84; *m*/*z* 371.05), appeared relatively enriched in L-HPE, suggesting that heat promoted de-esterification reactions converting complex

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Table 2. Tentative Identification of Chemical Compounds Using LC-MS/MS Analysis (Negative Ion Mode) of the Studied *Citrus* Peel Extracts[a]

No.	Tentative identification	RT (min)	$[M-H]^-$	L-CPE	L-HPE	M-CPE	M-HPE	O-CPE	O-HPE	Fragments	Ref.
						Phenolic compounds					
						Phenolic acids and derivatives					
5	Coumaric acid*	1.404	163.01	+	+	+	+	+	+	119	47
7	Trihydroxycinnamic acid-*O*-hexoside	1.489	357.01	+	+	-	-	t	-	195, 177, 151, 136, 121	47
8	Caffeic acid	1.552	179.01	-	-	-	-	+	-	135	48
11	Caffeoyl quinic acid*	1.935	353.05	+	+	-	-	-	-	221, 191,173, 161, 113, 111	32
13	Trihydroxycinnamic acid-*O*-hexoside isomer	2.014	357.01	+++	++	-	-	-	-	195, 177, 151, 136, 121	47
14	Trihydroxycinnamic acid	2.411	195.02	+	-	-	-	-	-	151, 136, 121	47
15	Coumaroyl quinic acid	3.254	336.95	+++	+	-	-	-	-	191, 163, 145, 119	49
16	Coumaroyl-*O*-hexoside	3.536	325.01	+	-	+	-	+	+	163, 145, 119	50
17	Coumaroyl quinic acid isomer	3.605	337.05	+++	++	-	-	-	-	191, 163, 145, 119	33
20	Sinapic acid-*O*-glucoside	4.256	384.98	+	-	+	+	+	+	223, 205, 164	32
23	Feruloyl-*O*-hexoside	4.498	355.01	-	+++	-	-	+	-	193, 175, 161, 134	51
55	Coumaroyl-*O*-acetyl hexoside	7.55	367.03	+	+	-	-	-	-	163, 145, 119, 117	50
66	Coumaroyl-*O*-acetyl hexoside isomer	8.24	413.09#	+	-	-	-	-	-	367, 163, 145, 119, 117	50
81	Coumaroyl-*O*-malonyl hexoside	9.42	410.98	+	-	-	-	-	-	367, 325, 163, 145, 119, 117	50
84	Hydroxyferulic acid-*O*-hexoside	9.658	371.05	-	+	-	-	-	-	209, 179, 161	52
97	Coumaroyl-*O*-acetyl hexoside isomer	11.97	435.03#	+	-	-	-	-	-	367, 163, 145, 119, 117	50
						Flavonoids					
						Flavonoid-*O*-glycoside					
18	Myricetin-*O*-hexoside	3.740	479.01	+	+	+	++	-	-	317, 289, 179, 167, 151, 125	50
25	Isorhamnetin 3, 4'-di-*O*- glucoside*	5.324	638.94	+	+	-	-	-	-	477, 315	32
27	Myricetin-*O*-rutinoside	5.920	625.01	-	-	+	-	-	-	579, 317, 299	50
28	Prunin = Naringenin 7-*O*-glucoside*	6.098	479.09#	-	-	-	-	-	+	433, 271, 151	41
30	Naringenin 7-*O*- neohesperidoside *O*-glucoside	6.162	786.91#	-	-	-	-	+	+++	741, 579, 433, 271	41
33	Isorhamnetin rhamnosyl rutinoside	6.271	768.97	+	+	-	-	-	-	623, 461, 315	53
34	Trihydroxy-dimethoxyflavone *O*-glucoside	6.316	491.01	-	-	+	-	-	-	329, 313, 161, 152	54
39	Dihydroxy-tetramethoxyflavone *O*-rutinoside	6.52	680.93	-	-	+	+	-	-	373, 359	
42	Monohydroxy-tetramethoxyflavone *O*-rutinoside	6.93	664.94	-	-	+	-	-	-	357	41
44	Quercetin *O*-glucosyl rutinoside	7.11	816.89#	+	-	-	-	+	+	771, 609, 463, 301	53
45	Myricetin-*O*-rutinoside isomer	7.13	624.97	+	+	+++	++	+	+	317, 300, 299	55
46	Diosmetin 7-*O*-neohespridoside	7.17	606.95	-	-	+++	+	-	-	299, 285, 271	2
47	Dihydroxy-trimethoxyflavone *O*-rutinoside	7.23	651.09	+	+	+	+	+	+	343, 329	56
48	Apigetrin = Apigenin 7-*O*-glucoside*	7.25	431.01	-	-	+	+	-	+	269	33
49	Quercetin *O*-glucosyl rutinoside isomer	7.26	770.98	+	-	+	+	+	+	609, 463, 301	2
51	Trihydroxy-trimethoxyflavone-*O*-hexoside	7.32	521.03	-	-	-	-	+	-	359, 341, 315, 151	2
53	Fortunellin = Acacetin-7-*O*-neohesperidoside	7.36	590.94	-	-	+	+	-	-	445, 283	2
54	Eriodyctiol 7-*O*-rutinoside*	7.38	594.97	-	-	+	-	+	-	287, 151	2
56	Monohydroxy-tetramethoxyflavone-*O*-hexoside	7.62	518.97	+	+	+	+	-	-	357, 315, 314, 299, 271	
57	Isorhamnetin 3-*O*-glucoside	7.77	476.97	+	+	t	t	-	-	315, 314, 300, 299, 229	2
58	Eridictiol = Diosmetin 7-*O*-glucoside	7.78	460.97	-	-	-	+	-	-	299, 298, 283	2
59	Isorhoifolin = Apigenin 7-*O*-rutinoside*	7.988	576.98	+	+	+	+	-	-	269	
60	Rutin*	8.047	609.01	+	+	+	+	+	+	301	2
61	Narirutin = Naringenin 7-*O*-rutinoside*	8.05	625.01#	-	-	+	t	-	-	579, 459, 271	2

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Table 2. continued

No.	Tentative identification	RT (min)	[M-H]⁻	L-CPE	L-HPE	M-CPE	M-HPE	O-CPE	O-HPE	Fragments	Ref.
						Flavonoid-*O*-glycoside					
62	Diosmin = Diosmetin 7-*O*-rutinoside*	8.12	606.97	+	+	++	++	+	+	299, 284	2
63	Rhoifolin = Apigenin 7-*O*-neohesperoside*	8.166	576.97	+	+	+	+	-	-	431, 269	2
65	Monohydroxy-tetramethoxyflavone-*O*-hexoside	8.23	519.04	-	-	-	-	+	-	357, 343, 151	2
67	Naringenin 7-*O*-neohesperidoside	8.25	624.95#	-	-	-	-	+	+	579, 271, 177, 151, 119	2
68	Quercetin 7-*O*-glucoside	8.321	462.99	+	+	+	+	+	+	301, 300	2
70	Hesperidin = Hesperetin 7-*O*-rutinoside*	8.71	644.96#	+	+	+	+	+	+	609, 301, 286	
72	Isorhamnetin 3-*O*- rutinoside	8.86	622.97	+	+	+	+	+	+	315, 300	32
73	Luteolin 7-*O*-rutinoside	8.816	593.01	+	+	+	+	+	+	285, 255, 121	33
74	Quercetin *O*-rhamnosylglucoside-rhamnosylglucoside	8.92	916.97	-	-	-	-	-	+	609, 301	53
75	Monohydroxy-pentamethoxyflavone *O*-rutinoside	9.05	694.98	-	-	-	-	-	+	387, 373, 329, 301	41
77	Hexamethoxyflavone *O* rutinoside	9.20	708.96	-	-	+++	+	-	-	401	41
82	Dihydroxy dimethoxy flavanone-*O*-neohesperidoside	9.44	668.98#	-	-	-	-	-	+	623, 315, 301	
85	Isosakuranetin-*O*-rutinoside	9.932	638.98#	+	-	-	+	+	+	593, 285	44
87	Trihydroxy-dimethoxyflavone-*O*-rutinoside	9.992	636.92	-	-	+	+	-	-	329, 315, 193, 145	44
90	Isosakuranetin-7-*O*-neohesperidoside	10.34	638.98#	+	-	-	+	+	+	593, 447, 285	32
						Flavonoid-*C*-glycoside					
19	Diosmetin 6,8 di-*C*-glucoside	4.140	622.97	+	+	+	+	+	+	533, 503, 413, 383	57
21	Apigenin 6,8 di-*C*-glucoside	4.290	592.95	+	+	+	+	+	+	503, 473, 413, 383, 353	57
22	Eriodictyol-*C*-rutinoside	4.452	594.97	-	-	+	-	+	++	449, 359, 329, 151, 135	58
26	Diosmetin 6-*C*-glucoside	5.634	460.94	+	+	+	+	+	+	473, 371, 341, 299	59
35	Vitexin	6.345	430.97	+	+	+	+	+	+	341, 311, 269	58
37	Luteolin *C*-rutinoside	6.414	593.01	-	-	-	-	+	-	503, 473, 447, 371, 341, 323	2
38	Diosmetin *C*-rutinoside	6.424	607.07	+	+	+	+	+	+	517, 487, 461, 443, 323, 294	
40	Naringenin *C*-rutinoside	6.541	579.17	+	+	+	+	++	++	489, 459	
41	Hesperetin 8-*C*-glucoside = Dihydrodiosmetin 8-*C*-glucoside	6.601	463.07	+	-	-	-	-	-	373, 343	2
43	Diosmetin 8-*C*-glucoside	6.958	460.97	+	+	+	+	+	+	371, 341, 299	2
50	Orientin*	7.28	446.96	+	+	+++	+	-	+	357, 327	2
52	Trihydroxy-dimethoxyflavone 8-*C*-glucoside	7.35	491.01	-	-	+	+	+	-	401, 371, 356, 328	2
71	Apigenin-*C*-rutinoside	8.773	577.04	+	+	-	-	-	-	367, 205	2
83	Acacetin-*C*-rutinoside	9.65	591.06	+	-	-	-	-	-	547, 503, 487, 443, 429, 347, 385	41
98	Isosakuranetin 8-*C*-glucoside	12.19	447.01	-	-	+	-	-	-	357, 327, 221	
						Flavonoid*O*-*C*-glycosides					
29	Saponarin = Vitexin 7-*O*-rhamnoside*	6.133	576.96	+	+	-	-	-	-	431, 311, 293	54
31	Vitexin-*O*-pentoside	6.180	562.95	+	+	t	t	+	+	431, 413, 341, 311, 293	60
89	Acacetin 8-*C*-glucoside-*O*-rhamnoside	10.33	591.04	+	-	-	-	-	-	445, 385, 355	61
						Flavonoid aglycones					
64	Diosmetin*	8.19	298.98	+	+	+	+	+	+	284, 209, 183, 167	2
69	Quercetin*	8.54	301.03	+	-	++	++	+	+	283, 255, 183, 157, 151	2
76	Hesperetin = Dihydrodiosmetin*	9.1	300.85	+	+	+	+	+	+	287	2
79	Isorhamnetin	9.22	315.01	+	+	-	-	+	-	300, 269, 272, 151, 129	62
80	Trihydroxy-trimethoxyflavone	9.356	358.98	-	-	+	-	-	-	343, 329, 314, 301, 257, 186, 179	33
86	Isosakuranetin	9.945	285.01	+	-	-	+	+	+	271, 243, 175, 164, 151, 137, 108	63
88	Trihydroxy-dimethoxyflavone	10.18	329.01	+	-	++++	+	+	+	314, 299, 271	33
91	Tetrahydroxy-dimethoxyflavone; Viscidulin III	10.8	344.96	+	+	+	-	-	-	315, 299, 287, 284, 271, 259, 243, 221, 165	64

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Table 2. continued

No.	Tentative identification	RT (min)	[M-H]⁻	L-CPE	L-HPE	M-CPE	M-HPE	O-CPE	O-HPE	Fragments	Ref.
						Flavonoid aglycones					
92	Tetrahydroxy monomethoxyflavone	10.83	314.98	+	+	+	-	-	-	300, 271, 227, 151	64
93	Monohydroxy trimethoxyflavone	10.86	327.01	-	-	-	+	-	+	312, 311, 297, 283, 269, 254, 177, 117	64
94	Monohydroxy-tetramethoxyflavone	11.01	356.99	-	-	+	+++	-	-	327, 312, 299, 284, 207	64
95	Dihydroxy dimethoxyflavone; Cirsimaritin	11.12	312.99	-	+	-	-	++	+	298, 283, 269, 255, 227, 211, 163, 135	64
96	Monohydroxy trimethoxyflavone isomer	11.4	327.00	-	-	+++	+	-	-	312, 311, 297, 283, 269, 254, 177, 117	64
99	Luteolin	12.88	285.02	-	-	-	+	-	-	270, 243, 227, 164,	65
100	Monohydroxy-tetramethoxyflavone isomer	12.02	356.99	-	-	+	-	-	-	327, 312, 299, 284, 207	64
101	Monohydroxy-pentamethoxyflavone	12.31	386.98	-	-	+	+	+	+	373, 357, 343, 329, 327, 312	
103	Dihydroxy-trimethoxyflavone	12.86	342.99	t	-	+	+	+	+	313, 299, 285	64
104	Dihydroxy-trimethoxyflavone isomer	13.43	342.99	-	-	+	-	-	-	313, 299, 285	64
106	Dihydroxy-tetramethoxyflavone	13.75	372.99	-	-	+	++	+	+	358,357,343,329,300	64
108	Dihydroxy-pentamethoxyflavone	14.43	402.96	-	-	+	-	-	-	373,359,329,315,	64
109	Apigenin	14.48	269.03	+	+	-	-	-	-	227, 169, 171, 151, 117	46
112	Naringenin	9.9	270.98	-	-	-	+	-	+	153, 109	61
						Coumarins					
24	Hydroxy methoxy coumarin	4.68	191.04	+	+	-	-	-	-	177, 149	46
32	Trimethoxy coumarin	6.25	235.16	+	+	-	-	-	-	207, 201, 177, 176, 161	
36	Hydroxy methoxy coumarin isomer	6.41	191.08	+	-	-	-	-	-	176, 148, 111	66
						Terpenoids					
78	Ichangin*	9.22	487.01	+	-	-	-	+	+	427, 383, 297, 253, 145	67
102	Limonin	12.76	469.10	+++	+	+	+	+	+	381, 321, 321, 278, 229, 227, 199, 135	67
						Primary metabolites (organic acids, amino acids, lipids, and saccharides)					
1	Quinic acid	1.15	191.01	+	+	+	+	+	+	111	33
2	Arginine	1.161	173.01	+	+	+	+	+	+	155, 137, 111	46
3	Citramalate	1.210	147.02	+	+	-	+	+	-	147, 129, 101	32
4	Sucrose	1.355	341.02	+	-	+	-	+	+	179, 161, 149, 89	32
6	Citric acid	1.429	190.98	+	-	-	-	-	-	127, 111, 109	32
9	Hydroxy proline	1.731	130.09	+	+	+	+	+	+	113, 84	32
10	Phenylalanine	1.865	164.04	+	+	+	+	+	+	147, 120, 119, 101	46
12	Malic acid	1.964	133.01	-	-	+	-	-	-	115, 101, 71	46
105	Octadecatrienoyl glycero-phosphoinositol*	13.58	593.09	+	-	-	-	-	-	413, 315, 277, 259 241, 153	53
107	Hexadecanoyl-glycero-phosphoinositol*	14.09	571.09	+	+	-	+	-	+	391, 333, 315, 259, 241, 223, 153	53
110	Linoleoyl-glycero-phosphoinositol*	14.65	595.41	+++	-	-	-	-	++	415, 315, 279, 259 241, 153	53
111	Hexadecanoyl-glycero-phosphoinositol isomer*	14.8	571.11	+	-	-	-	-	-	391, 315, 259, 241, 153	
113	Hydroxyoctadecatrienoyl-glycero-phosphoethanol amine	15.35	474.11	+	-	-	-	-	-	343, 334, 279, 214, 196, 140	45
114	Hexadecanoyl-*sn*-glycero-3-phospho-glycerol	15.5	483.11	+++	-	-	-	-	+	255, 245, 227, 153	38
115	Docosadienoyl-*sn*-glycero-3-phosphocholine	15.85	450.11	-	-	-	-	-	+	253, 214, 196, 184, 153, 140	38
116	Hydroxyoctadecadienoyl- glycero-phospho-ethanolamine	16.15	476.12	+	+	-	+	-	+	346, 279, 214, 196, 140	38
117	Octadecenoyl-glycero-phosphoethanolamine	17.85	478.15	+	-	-	+	-	+	348, 281, 214, 196, 153	38
118	Linolenic acid	17.13	277. 09	-	+	-	+	++	+	259, 233, 205,127	61
119	Hexadecanoyl-glycero-phosphoethanolamine	17.5	452.12	+	+	+	+	-	+	312, 255, 196, 153	2
120	Linoleoylglycerol	17.35	353.12	+	+	+	+	+	+	337, 261	46

[a]Peaks arranged according to retention time (Rt). L-CPE: lemon crude peel extract; L-HPE: lemon heat-treated peel residue extract; M-CPE: mandarin crude peel extract; M-HPE: mandarin heat-treated peel residue extract; O-CPE: orange crude peel extract; O-HPE: orange heat-treated peel residue extract; *: compounds matched with GNPS libraries; #: [M-H+FA]⁻; -: absence; t: trace; +: present; ++: strong; +++: very strong.

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conjugates into simpler, less bioactive phenolics. Collectively, these findings demonstrate that heat treatment did not merely reduce the extract yield but also altered the phenolic profile through hydrolysis, decarboxylation, and oxidation. Because hydroxycinnamic acids are primary hydrogen-donating antioxidants, their disappearance explains the significant reduction in antioxidant and anti-inflammatory activities observed for HPEs.

3.2.3.3. Flavonoids. The MN analysis grouped flavonoids into *O*-diglycosides (cluster A), flavanone *O*-diglycosides (cluster B), polymethoxylated flavone aglycones (cluster C), flavone *O*-diglycosides (cluster H), and *C*-glycosyl flavonoids (clusters G, I, and J) (Figure 3). Other identified flavonoids appeared as self-looped nodes within the MN. Several studies have dispensed with the variable flavonoid structures present in *Citrus* fruits and emphasized their comparative distribution in the fruit tissues (pulp and peel), seeds, and juice using different extraction methods.[2] *Citrus* fruits are recognized for the occurrence of flavone and flavonol aglycones (apigenin, luteolin, and quercetin) together with their *O*-methylated aglycones (acacetin, diosmetin, and isorhamnetin). In addition to the dihydro-type structures, dihydroapigenin (naringenin), dihydroacacetin (isosakuranetin), dihydroluteolin (eriodictyol), and dihydrodiosmetin (hesperetin) were recurrently present in the *O*-glycosylated form in all fruit parts and different kinds of extracts, mainly as 7-*O*-rutinose substitutions of dihydro-type structures.[2] They are accountable for giving the fruits their bitter flavor and enhancing their exceptional sensory quality.[41] Furthermore, the glycosylation of the flavone aglycones (apigenin, acacetin, and luteolin) is probably with a neohesperidoside moiety at the C-6/8 or 7-*O* positions. Based on the peel extraction procedure, the CPEs are specified for the prominent flavonoid contents with a reduced amount in the HPEs (Table 2). The commonly characterized flavonoid aglycones for the *Citrus* species represented flavone and flavanone types, which appeared as self-looping nodes in MN with a variable distribution within the studied species.

Interestingly, several polymethoxylated flavone aglycones were relatively more detectable after heating. Their fragmentation patterns, including successive losses of CH_3, CO, and H_2O, support demethylation and structural rearrangement reactions. Diosmetin (64; m/z = 298.98) and hesperetin (76; m/z = 300.85) were distributed in all extracts. Moreover, isosakuranetin (86; m/z 285.01) was accumulated in orange and mandarin extracts, luteolin (99; m/z 285.01) in mandarin, and apigenin (109; m/z 269.03) in lemon, in both CPEs and HPEs. Moreover, 12 polymethoxylated flavonoid aglycones were annotated and chiefly characterized for mandarin with marked observation in the CPEs (Figure 2 and Table 2). Their annotation matched with the base peaks of $[M\text{-}H\text{-}14\ (CH_2)]^-$, $[M\text{-}H\text{-}15\ (CH_3)]^-$, $[M\text{-}H\text{-}18\ (H_2O)]^-$, and/or $[M\text{-}H\text{-}28\ (CO)]^-$. These aglycone structures have been characterized before for *Citrus* peels.[2] In the present study, CPEs exhibited a markedly richer flavonoid glycoside composition than HPEs. *O*-mono glycosylated flavonoids, including the 7-*O*-glucoside of naringenin (prunin 28; m/z 433.01), apigenin (apigetrin 48; m/z 431.01), diosmetin (eridictiol 58; m/z 460.97), and the diglycosides as *O*-rhamnosylglucoside substituted derivatives, i.e., rutinoside or neohesperidoside, were abundant in crude extracts but decreased after heating. These compounds contain labile glycosidic bonds that undergo thermal hydrolysis, generating unstable aglycones which may further oxidize or polymerize.[42]

In addition to the reported *Citrus*-characterized 7-*O*-neohesperidoside derivatives of acacetin (fortunellin 53; m/z 590.94 $[M\text{-}H]^-$) and apigenin (rhoifolin 63; m/z 576.97 $[M\text{-}H]^-$), which are present in the peels of three investigated species and unchanged with the extraction methods, other flavonoid-*O*-neohesperidosides were also detected, but with variable distribution (Table 2). They were identified as diosmetin 7-*O*-neohesperidoside (46; m/z 606.95 $[M\text{-}H]^-$), which is characterized for orange, as well as naringenin 7-*O*-neohesperidoside (67; m/z 624.95 $[M\text{-}H+FA]^-$ and isosakuranetin 7-*O*-neohesperidoside (90; m/z 638.98 $[M\text{-}H+FA]^-$) that accumulated in orange and mandarin peel extracts (Figure 2). Their fragmentation sequence proceeds with product ions of their corresponding aglycones after loss of a deprotonated rhamnosyl glucoside moiety (−308 Da) together with low-intensity fragment ions $[M\text{-}H\text{-}146]^-$ and $[M\text{-}H\text{-}180]^-$.[43] In contrast, the rutinoside substitution is characterized by only a fragment ion $[M\text{-}H\text{-}308]^-$ and is represented as a mandarin-specific derivative of naringenin (narirutin 61; m/z 625.01 $[M\text{-}H+FA]^-$). In addition, isorhoifolin (57; m/z 576.98 $[M\text{-}H]^-$) is characterized for lemon and mandarin, whereas dihydroxy-trimethoxyflavone-*O*-rutinoside (47; m/z 651.09 $[M\text{-}H]^-$), diosmin (62; m/z 606.97 $[M\text{-}H]^-$), and hesperidin (70; m/z 644.96 $[M\text{-}H+FA]^-$) have been accumulated in the three investigated species and are unaffected by the extraction behaviors. A limited amount of a flavanone tri-*O*-glycoside that is rarely reported in the genus *Citrus* is characterized for orange extracts and annotated as glucosyl narirutin (30; 786.906 $[M\text{-}H+FA]^-$) (Figure 4).

Alongside the predominant flavone and flavanone structures, flavonol types were scarcely reported in the present study, as noted before in some *Citrus* species.[44] The flavonols aglycones, quercetin (69; m/z 301.03 $[M\text{-}H]^-$), were distributed in the three *Citrus* species peel extracts, while isorhamnetin (79; m/z 315.01 $[M\text{-}H]^-$) was accumulated in the peels of orange and lemon. The annotated mono-*O*-glycosides established that isorhamnetin 3-*O*-glucoside (57; m/z 476.97 $[M\text{-}H]^-$) was restricted to the lemon extracts, and quercetin 7-*O*-glucoside (68; m/z 462.99 $[M\text{-}H]^-$) was found in all extracts. They have been seen earlier in the fruit parts of the kumquat.[2] Similarly, the diglycosyl flavonols, isorhamnetin 3,4′-di-*O*-glucoside (25; m/z 638.94 $[M\text{-}H]^-$), were restricted to the lemon extracts, while a mandarin-specific structure of myricetin-*O*-rutinoside (27; m/z 625.01 $[M\text{-}H]^-$), as well as the 3-*O*-rutinoside of quercetin (rutin 60; m/z 609.27 $[M\text{-}H]^-$) and isorhamnetin (72; m/z 622.97 $[M\text{-}H]^-$), were distributed among all extracts.

In addition to flavonoid-*O*-glycosides, flavonoid-*C*-glycosides were found in the negative MN (Clusters G, I, and J) as substitutions of flavones (apigenin, acacetin, luteolin, diosmetin, and trihydroxy-dimethoxyflavone) and flavanones (naringenin, eriodyctiol, isosakuranetin, and hesperetin) (Figure 2). Their fragmentation pattern corresponded to the well-known pattern of losing m/z 60, 90, and/or 120 Da; 6-*C*-glycosides substitution exhibited additional loss of a H_2O molecule (18 Da). The annotated mono-*C*-glycosides were identified as flavone derivatives and detected as diosmetin 6-*C*-glucoside (26; m/z 460.94 $[M\text{-}H]^-$), vitexin (35; m/z 430.97 $M\text{-}H]^-$), diosmetin 8-*C*-glucoside (43; m/z 460.97 $[M\text{-}H]^-$), and orientin (50; m/z 446.96 $M\text{-}H]^-$) which were found in the six investigated extracts, but trihydroxy-dimethoxyflavone 8-*C*-glucoside (52; m/z 491.01 $M\text{-}H]^-$) was restricted to both mandarin extracts and O-CPE. In contrast, flavanones derivatives, hesperetin 8-*C*-glucoside (41; m/z 463.07 M-

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Table 3. DPPH Scavenging Activity (% Inhibition and IC_{50}) of the Studied *Citrus* Species Peels[a]

Tested sample		% Inhibition						
		Concentration (μg/mL)						
		31.25	62.5	125	250	500	1000	IC_{50} (μg/mL)
Lemon	L-CPE	37.54 ± 0.14^{f}	53.16 ± 0.26^{e}	68.94 ± 0.42^{d}	73.09 ± 0.29^{c}	87.46 ± 0.34^{b}	98.57 ± 0.92^{a}	38.72 ± 0.63^{g}
	L-HPE	15.82 ± 0.54^{f}	19.96 ± 1.44^{e}	33.53 ± 1.21^{d}	46.50 ± 1.75^{c}	57.33 ± 0.95^{b}	70.48 ± 1.32^{a}	480.00 ± 0.99^{c}
Mandarin	M-CPE	39.06 ± 0.32^{f}	51.80 ± 0.21^{e}	65.42 ± 0.24^{d}	83.22 ± 0.32^{c}	92.40 ± 0.50^{b}	99.00 ± 0.77^{a}	60.38 ± 0.12^{f}
	M-HPE	12.37 ± 0.55^{f}	24.50 ± 0.68^{e}	30.20 ± 0.87^{d}	36.74 ± 1.12^{c}	39.08 ± 0.63^{b}	55.78 ± 0.22^{a}	792.00 ± 0.81^{a}
Orange	O-CPE	35.70 ± 0.40^{f}	46.96 ± 0.33^{e}	67.26 ± 0.14^{d}	81.53 ± 0.52^{c}	89.13 ± 0.09^{b}	96.66 ± 0.33^{a}	70.77 ± 0.52^{e}
	O-HPE	13.95 ± 0.18^{f}	17.80 ± 0.18^{e}	25.43 ± 0.66^{d}	33.96 ± 0.90^{c}	43.67 ± 0.92^{b}	62.07 ± 0.77^{a}	696.00 ± 0.71^{b}
Standard	**Vit. C**	23.13 ± 0.34^{f}	40.95± 0.49^{e}	64.71 ± 0.33^{d}	72.11 ± 0.17^{c}	85.60 ± 0.27^{b}	96.03 ± 0.39^{a}	120.00 ± 0.25^{d}

[a]Data presented as mean ± SE. One-way ANOVA was used for data analysis (n = 3). Different lowercase letters within the same row designate significant differences (p < 0.05). L-CPE: lemon crude peel extract; L-HPE: lemon heat-treated peel residue extract; M-CPE: mandarin crude peel extract; M-HPE: mandarin heat-treated peel residue extract; O-CPE: orange crude peel extract; O-HPE: orange heat-treated peel residue extract.

Table 4. NO Scavenging Activity (% Inhibition and IC_{50}) of the Studied *Citrus* Species Peel Extracts[a]

Tested sample		% Inhibition						
		Concentration (μg/mL)						
		31.25	62.5	125	250	500	1000	IC_{50} (μg/mL)
Lemon	L-CPE	38.00 ± 1.15^{f}	50.83 ± 1.93^{e}	70.83 ± 1.93^{d}	80.67 ± 0.47^{c}	91.66 ± 0.81^{b}	97.1 ± 0.45^{a}	61.54 ± 0.82^{h}
	L-HPE	4.89^{e} ± 0.57^{e}	5.12 ± 1.43^{e}	21.63 ± 0.74^{d}	46.13 ± 0.71^{c}	61.53 ± 0.61^{b}	69.00 ± 0.37^{a}	465.43 ± 0.54^{c}
Mandarin	M-CPE	39.45 ± 1.00^{g}	47.67 ± 1.20^{f}	67.32 ± 1.16^{e}	81.67 ± 0.33^{dc}	93.57 ± 0.09^{c}	99.30 ± 0.15^{a}	90.32 ± 0.58^{g}
	M-HPE	4.42 ± 0.98^{f}	5.69 ± 0.46^{e}	8.03 ± 0.54^{d}	23.87 ± 0.49^{c}	39.26 ± 0.14^{b}	54.93 ± 0.72^{a}	832.44 ± 0.87^{a}
Orange	O-CPE	25.63 ± 0.40^{g}	47.22 ± 0.52^{f}	57.7 ± 0.36^{e}	65.01 ± 0.12^{d}	78.03 ± 0.43^{c}	93.49 ± 0.59^{a}	117.5 ± 0.69^{d}
	O-HPE	5.24 ± 0.69^{f}	10.72 ± 0.89^{e}	19.29 ± 0.21^{d}	35.53 ± 0.66^{c}	44.66 ± 0.73^{b}	61.06 ± 0.96^{a}	702.11 ± 0.98^{b}
Standard	**Vit. C**	25.32 ± 0.32^{f}	43.61 ± 0.42^{e}	57.83 ± 0.51^{d}	75.25 ± 0.32^{c}	88.12 ± 0.64^{b}	94.89 ± 0.78^{a}	107.61 ± 0.73^{e}

[a]Data presented as mean ± SE. One-way ANOVA was used for data analysis (n = 3). Different lowercase letters within the same row designate significant differences (p < 0.05). L-CPE: lemon crude peel extract; L-HPE: lemon heat-treated peel residue extract; M-CPE: mandarin crude peel extract; M-HPE: mandarin heat-treated peel residue extract; O-CPE: orange crude peel extract; O-HPE: orange heat-treated peel residue extract.

H]), were characterized for the L-CPE, whereas isosakuranetin 8-*C*-glucoside (98; m/z 447.01 M-H]$^{-}$) was categorized for the M-CPE. Similarly, di-*C*-glycosidic forms were found in the six extracts as 6,8-di-*C*-glucoside of diosmetin (19; m/z 622.97 [M-H]$^{-}$) and apigenin (21; m/z 592.95 [M-H]$^{-}$).

Collectively, the *C*-glycosyl flavonoids were detected in most extracts and showed greater stability. This is expected because the *C*−*C* glycosidic bond is resistant to thermal hydrolysis compared to *O*-glycosidic bonds. Therefore, the persistence of *C*-glycosides in HPEs further supports the idea that heat specifically degraded *O*-glycosylated phenolics rather than causing general extraction loss.

Besides the flavonoid-*C*-glycosides, the *O*-*C*-glycosylated flavonoids were also detected, as reported before, in various fruit parts of the *Citrus* species. The *C*-rutinoside of luteolin (37; m/z 593.01 [M-H]$^{-}$) was characterized for orange, of apigenin (71; m/z 577.04 [M-H]$^{-}$) and acacetin (83; m/z 591.06 [M-H]$^{-}$) for L-CPE, while that of diosmetin (38; m/z 607.07 [M-H]$^{-}$) was accumulated in the six extracts. On the other hand, the *O*-rhamnosyl derivatives of vitexin (saponarin 29; m/z 576.09 [M-H]$^{-}$) and acacetin 8-*C*-glucoside (89; m/z 591.04 [M-H]$^{-}$) were accumulated in the L-HPE and L-CPE, respectively, while the *O*-pentosyl derivative (31; m/z 562.95 [M-H]$^{-}$) was probably present in the peels of three *Citrus* species. Furthermore, the *O*-*C*-glycosyl flavanones; eriodyctiol *C*- rutinoside (22; m/z 594.97 [M-H]$^{-}$) was present in the peel extracts of orange and mandarin, while naringenin *C*-rutinoside (40; m/z 579.17 [M-H]$^{-}$) was distributed in all extracts.

3.2.3.4. Coumarins. The fragmentation pattern of compounds 24 and 36 (m/z 191.04 and 191.05) and 32 (m/z 235.16) is in good agreement with the predicted coumarin structures as hydroxy methoxy coumarin isomers and trimethoxy coumarin, respectively, all characterized for the lemon extracts.

3.2.3.5. Terpenoids. One cyclic monoterpene was annotated as limonin (102; m/z = 469.10), and as expected, it was distributed throughout all studied extracts but concentrated in L-CPE. Additionally, ichangin (78; m/z = 487.01), a sesquiterpenoid, was detected in lemon and orange extracts.

3.2.3.6. Phospholipids. Along with the previously addressed flavonoids and phenolics, four classes of lysoglycerophospholipids, encompassing ten metabolites, were discovered in the current species for the first time. They feature a specific structure comprising hydrophobic acyl chain(s) linked to the glycerol backbone at the sn1- and/or sn2-positions, along with a hydrophilic phosphate headgroup at the sn3-position. They are cellular membrane structural components that may also function as signaling markers in various physiological and biological aspects.[45] Various lysoglycerophospholipids were identified based on the substituted hydrophilic phosphate headgroup. This phosphate group is substituted with inositol, ethanolamine, glycerol, or choline to produce their lysoglycerophospho derivatives in the present study. Four lysoglycerophosphoinositols are described based on the existence of inositol-1,2-cyclic phosphate (m/z 241) and inositol monophosphate (m/z 259) fragments; consequently, they are annotated as the octadecatrienoyl- (105; m/z 593.09), hexadecanoyl- (107 and 111; m/z 571.1), and linoleoyl-

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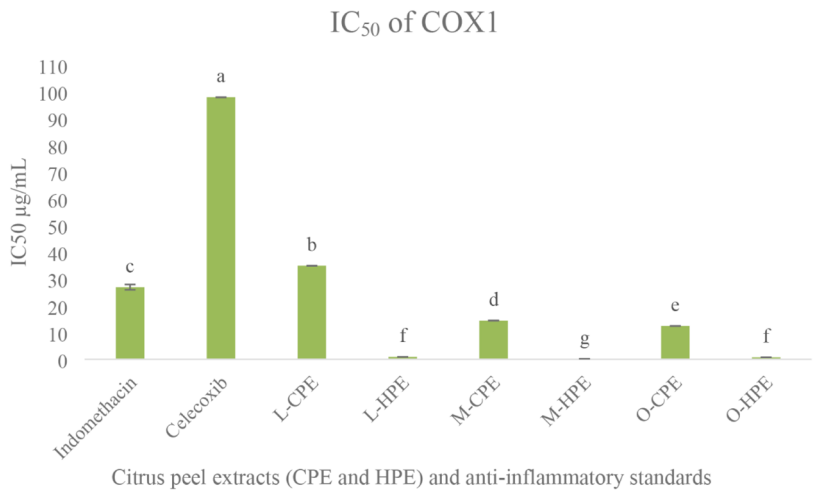

Figure 5. IC_{50} of anti-inflammatory *in vitro* biomarkers COX1 of different citrus extracts compared with different standards. L-CPE: lemon crude peel extract, L-HPE: lemon heat-treated peel residue extract, M-CPE: mandarin crude peel extract, M-HPE: mandarin heat-treated peel residue extract, O-CPE: orange crude peel extract, O-HPE: orange heat-treated peel residue extract. Data presented as mean ± SE. ANOVA one-way was used for data analysis (n = 3). Different lowercase letters within the same group designate significant differences (p < 0.05).

(110; m/z 595.41) substituted structures. Moreover, a lyso glycerophosphocholine (115; m/z 450.11) is characterized by a production at m/z 184 corresponding to the phosphocholine headgroup, whereas the neutral loss of an ester group to create a $[M\text{-}H\text{-}140]^-$ ion is characteristic of lysoglycerophosphoethanolamines (113; m/z 474.11, 116; m/z 476.2, 117; m/z 478.15, and 119; m/z 452.12). Similarly, product ions with m/z 153 and 245 corresponding to monodehydrated glycerophosphate and glycerophosphoglycerol, respectively, proved the presence of lysophosphatidylglycerol (114; m/z 483.11). They were most likely dispersed differently in each of the extracts studied; however, L-CPE had the highest concentration.

3.2.3.7. Other Primary Metabolites. Additional minor primary metabolites were also detected based on available literature.[33,46] Three amino acids (2, 9, and 10) were present in all extracts, four organic acids (1, 3, 6, and 12) were present in varying amounts across the extracts, and one saccharide (4) was present in varying amounts across the extracts (Table 2).

3.3. Biological Activity

3.3.1. Antioxidant Activity Evaluation. The antioxidant activity of the studied extracts was evaluated using two *in vitro* screening methods (DPPH and NO free-radical scavenging) and compared with that of ascorbic acid (Vitamin C) as a reference standard. All extracts showed concentration-dependent activity; however, clear differences were observed between CPEs and HPEs (Tables 3 and 4). At 1000 µg/mL, the L-CPE, M-CPE, and O-CPE exhibited strong inhibition in both assays, approaching the activity of the reference antioxidant ascorbic acid (96.03 ± 0.39% for DPPH and 94.89 ± 0.78% for NO). The IC_{50} values further confirmed this trend, with L-CPE showing the highest activity (DPPH: 38.72 ± 0.63 µg/mL; NO: 61.54 ± 0.82 µg/mL), followed by M-CPE (DPPH: 60.38 ± 0.12 µg/mL; NO: 90.32 ± 0.58 µg/mL) and O-CPE (DPPH: 70.77 ± 0.52 µg/mL; NO: 117.5 ± 0.69 µg/mL), compared with Vitamin C (DPPH: 120.00 ± 0.25 µg/mL; NO: 107.61 ± 0.73 µg/mL), all significantly lower than those of Vitamin C (p < 0.05). The superior performance of L-CPE correlates with its higher total phenolic and flavonoid contents, indicating that antioxidant capacity is largely governed by phenolic composition. Furthermore, the metabolomic profiling provides a direct chemical explanation for these results. CPEs were particularly rich in hydroxycinnamic acid derivatives (e.g., caffeoyl-quinic and coumaroyl-quinic acids) and flavonoid glycosides such as rutin, hesperidin, diosmin, and rhoifolin. These compounds possess multiple hydroxyl groups capable of donating hydrogen atoms or electrons to neutralize free radicals and terminate radical chain reactions. After heat treatment, several of these thermolabile constituents markedly decreased or disappeared, especially esterified phenolic acids and *O*-glycosylated flavonoids. Thermal processing likely induced hydrolysis of ester and glycosidic bonds followed by oxidation or decarboxylation, converting active phenolics into simpler or methoxylated forms with lower radical-scavenging efficiency. Consequently, HPEs displayed reduced antioxidant activity compared with the corresponding crude extracts.

Research conducted by Mateus et al. (2024)[38] assessed the antioxidant capacity of three citrus extracts using DPPH and ABTS scavenging assays. For DPPH, lemon exhibited the highest activity (11.88 mg of TE/g), followed by lime (8.83 mg of TE/g) and orange, which had approximately half the concentration of lemon extracts (5.00 mg of TE/g). In this study, the authors did not evaluate the nitric oxide assay. However, the NO scavenging results further support this interpretation. Excessive NO production is associated with inflammatory disorders, carcinogenesis, and oxidative stress-related pathologies.[68] The ability of CPEs to scavenge NO suggests that these extracts may suppress reactive nitrogen species formation and interrupt peroxynitrite-mediated oxidative cascades. The lower NO-scavenging capacity of HPEs is therefore attributable to the thermal degradation of phenolic acids and flavonoid glycosides, which are known to interact with reactive nitrogen intermediates.

Overall, the results demonstrate that CPEs are strongly dependent on the integrity of phenolic metabolites, such as flavonoids, phenolic acids, and coumarins[37,69] and the decline observed in HPEs can be attributed to heat-induced degradation of thermolabile compounds rather than differences in extraction yield.

3.3.2. Anti-Inflammatory Evaluation. Inflammation is crucial to the body's healing process, occurring when

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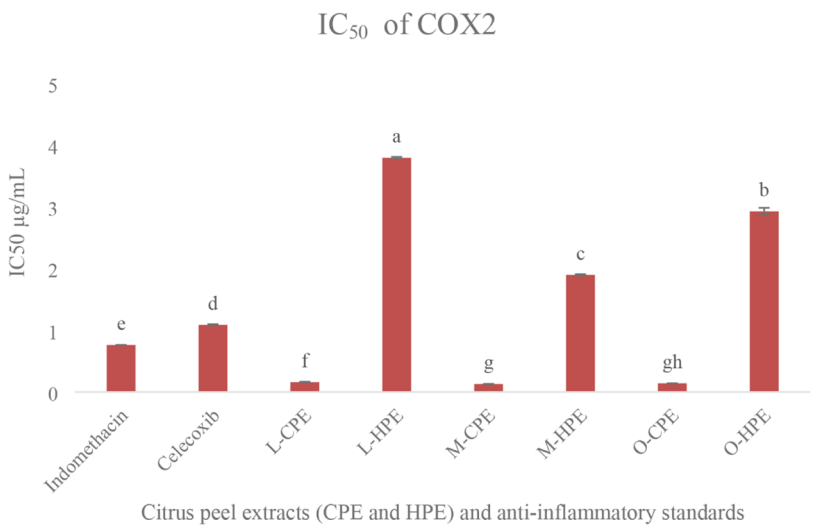

Figure 6. IC_{50} of anti-inflammatory *in vitro* biomarkers COX2 of different citrus extracts compared with different standards. L-CPE: lemon crude peel extract, L-HPE: lemon heat-treated peel residue extract, M-CPE: mandarin crude peel extract, M-HPE: mandarin heat-treated peel residue extract, O-CPE: orange crude peel extract, O-HPE: orange heat-treated peel residue extract. Data presented as mean ± SE. ANOVA one-way was used for data analysis (n = 3). Different lowercase letters within the same group designate significant differences ($p < 0.05$).

inflammatory cells migrate to an injury site or foreign entities such as bacteria.[70] Cyclooxygenase (COX), or prostaglandin H synthase, is crucial for inducing pain and inflammation through the synthesis of prostaglandins (PGs). COX occurs in two isoforms: COX1 is responsible for producing PGs that have homeostatic functions in tissues such as the stomach, kidneys, and platelets, whereas the production of proinflammatory PGs relies on COX2.[71] It is now considered that COX1 is responsible for the initial prostanoid response to inflammatory stimuli, whereas COX2 emerges as the primary contributor to prostanoid synthesis as inflammation progresses.[72] The core chemical classes of anti-inflammatory agents have been reported in natural sources, including polyphenols, flavonoids, alkaloids, terpenoids, saponins, steroids, and tannins.[73] These phytochemicals act by inhibiting mediators that play a key role in preventing inflammation, as inflammatory cytokines induce COX2 and prostaglandin E2 synthesis, which are critical in the pathogenesis of inflammatory diseases.[74] Flavonoids exhibit anti-inflammatory effects through various mechanisms, including inhibition of enzymes such as phospholipase. In contrast, others downregulate proinflammatory cytokines, including TNF-α, which plays a key role in chronic inflammation.

Figures 5 and 6 showed significant anti-inflammatory effects of L-CPE, M-CPE, and O-CPE on biomarkers COX1 and COX2. These extracts exhibited activities with IC_{50} (μg/mL) values of 35 ± 0.2, 14.5 ± 0.3, and 12.5 ± 0.2 for COX1 as well as 0.16 ± 0.0, 0.13 ± 0.0, and 0.14 ± 0.0 for COX2, respectively, compared to those of the reference standards (celecoxib and indomethacin), (98 ± 0.5 and 27 ± 0.1) for COX1, as well as (1.1 ± 0.0 and 0.8 ± 0.0) for COX2, with significant differences ($p < 0.05$). Furthermore, these results are consistent with the LC-MS/MS findings presented in Table 2, which identified several flavonoid compounds, such as naringenin, hesperidin, and narirutin, known for their anti-inflammatory activity, as previously reported by Ibrahim et al. (2024).[29] In contrast, the extracts of the heat-treated peels (L-HPE, O-HPE, and M-HPE) exhibited weak anti-inflammatory activities. This reduction in activity may be attributed to thermal degradation or structural alteration of key bioactive compounds, particularly flavonoids and phenolic acids, which are known to contribute to anti-inflammatory effects. Heat treatment may also reduce the extractability or stability of these compounds, thereby diminishing their biological efficacy.

These findings suggest that the processing method plays a critical role in preserving the anti-inflammatory potential of citrus peel extracts. Furthermore, as shown in Figures 5 and 6, the IC_{50} values for COX1 were consistently higher than those for COX2 across the crude extracts. This suggests that the crude extracts preferentially inhibit COX2, the isoform primarily responsible for inflammatory responses, while sparing COX1, which has protective functions. By selective inhibition of COX2, the extracts can reduce inflammation without significantly affecting the protective roles of COX1. This selectivity profile is desirable in the development of safer anti-inflammatory agents. It may be due to the presence of specific phenolic compounds, as confirmed by LC-MS/MS analysis. Our results are in accordance with the study conducted by Karthikeyan A. (2021), which evaluated the anti-inflammatory activity of *Citrus unshiu* peel flavonoids. The study demonstrated that treatment with these flavonoids reduced the production and expression of key inflammatory mediators and proinflammatory cytokines such as NO, PGE2, TNF-α, IL-1β, iNOS, and COX2 in the LPS-stimulated RAW 264.7 macrophages.[75]

■ ASSOCIATED CONTENT

Data Availability Statement

Data will be available upon request.

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Notes

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The authors declare no competing financial interest.

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