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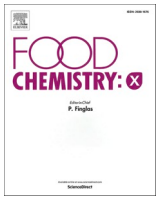

Maturation-induced changes in phenolic forms and their antioxidant activities of walnuts: A dual view from kernel and pellicle

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ABSTRACT

The phenolic profiles and antioxidant activities during walnut maturation are not well understood. This study used UPLC-MS/MS to evaluate phenolic content in walnuts, including free, esterified, and bound forms, at different maturation stages. Findings showed that free phenolics were predominant, comprising 44.57 % in kernels and 56.54 % in pellicles. In vitro assays showed antioxidant capacity decreased with maturation, with IC_{50} values of 0.87–84.43 μg/mL in pellicles and 48.51–712.30 μg/mL in kernels. Most monomeric phenols decreased in concentration as the fruit ripened. OPLS-DA identified 5 and 8 maturity-sensitive phenolics (MSPs) in kernels and pellicles, respectively, with fold changes from 2.32 to 1664.72. Pearson correlation analysis showed a significant correlation between MSPs and antioxidant activity ($r > 0.75$, $p < 0.05$). Bioinformatics analysis elucidated three key metabolic pathways involved in these changes. This research provides insights into walnut phenolic composition, important for optimizing harvest practices and enhancing nutritional value.

1. Introduction

Over the past decade, phenolic compounds, prevalent in the diet, have garnered significant attention due to their diverse biological activities and human health benefits. Phenolics are recognized as essential antioxidants with implications in anti-carcinogenic, anti-inflammatory, antibacterial, anti-aging, anti-fungal, and neuroprotective functions (Bouali et al., 2023). Consequently, increased consumption of phenol-rich foods has been correlated with a reduced risk of various diseases. Walnut (*Juglans regia* L.), a globally cultivated and economically valuable nut, is esteemed for its rich macronutrient content and potential as a dietary source of polyphenols (Wu et al., 2021). With the most diverse phenolic profile and the highest phenolic content among tree nuts, walnuts offer broad prospects for application (Persic, Mikulic-Petkovsek, Slatnar, Solar, & Veberic, 2018). Studies indicated that the skin or pellicle surrounding the walnut kernel contains substantial amounts of phenolic compounds, contributing to its elevated antioxidant activity compared to other nuts (Wang et al., 2022). Current research on walnuts has predominantly focused on fully ripe and often dried walnuts. These studies have demonstrated that variety (Wu et al., 2023), origin (Mo, Zheng, Ni, Shen, & Liu, 2022), and post-harvest processing (Li et al., 2023; Li et al., 2024) significantly influence the phenolic content and antioxidant activity of walnut kernels. However, the biosynthesis and regulatory pathways of phenolic compounds in fresh walnut kernels are still unclear. Preliminary investigations have explored the relationship between total phenols and developmental stages in walnuts. Persic et al. examined phenolic compounds in red-colored pellicle walnuts at various developmental stages, noting a slight decrease in phenolic accumulation during ripening (Persic et al., 2018). In contrast, another study reported a more pronounced reduction in antioxidant capacity and polyphenol content during the maturation process, with decreases ranging from 18.17 %–50.13 % and 9.57 %–67.96 %, respectively (Pycia, Kapusta, Jaworska, & Jankowska, 2019). Previous research on other foods has underscored the importance of

Abbreviations: AC, acetone; ACN, acetonitrile; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ESI, electrospray ionization; HPLC, High-performance liquid chromatography; IS, immature stages; MeOH, methanol; MS, mature stage; MSPs, maturity-sensitive phenolics; KEGG, Kyoto Encyclopedia of Genes and Genomes; OPLS-DA, Orthogonal partial least squares discriminant analysis; TAC, total antioxidant capacity; TPC, total phenol content; VIP, Variable Importance in Projection.

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understanding the changes in phenolic compounds throughout the maturation process to enhance their nutritional value, functional activity, and the development of value-added products (Dong, Hu, Li, & Zhou, 2019; Hou et al., 2021; Ma et al., 2023). To date, the alterations in phenolic profiles and antioxidant potential during walnut fruit ripening remain poorly understood.

Moreover, prior studies have predominantly focused on the free fraction of phenolic compounds, neglecting the bound phenolics in the residues during maturity, which may lead to an underestimation of the actual phenolic content in walnuts (Wu et al., 2023). Phenolics, based on their association with the food matrix, can be categorized into three forms: soluble free, soluble esterified, and insoluble bound (Gruz, Ayaz, Torun, & Strnad, 2011; Zhong et al., 2022). The esterified and bound forms are particularly beneficial to human health due to their antioxidant properties and role in preventing oxidative stress. Our previous work concentrated on phenolic compounds in the pellicles and kernels of mature walnuts, revealing that walnut kernels and pellicles are primarily composed of free phenols, followed by bound and esterified forms (Wu et al., 2021; Wu et al., 2023). This is in stark contrast to fruits and grains, where phenols are predominantly bound. Some studies have also reported changes in the three forms of phenolic compounds during maturation for lemons (Dong et al., 2019), medlar (Gruz et al., 2011), and Rubus chingii Hu (Zhong et al., 2022).

Despite research on changes in total phenolics and antioxidant activities during walnut maturation, the specific forms of individual phenolic compounds and their antioxidant capacities remain unidentified. Therefore, it is imperative to consider the variations in individual phenolic forms during ripening to better comprehend the health benefits of walnuts. The present study aims to systematically investigate the impact of ripening stages on the concentrations of the three phenolic forms and their associated antioxidant capacities. For the first time, this research quantifies the phenolic profiles and related antioxidant capacity of walnut kernels harvested at four distinct maturity stages. The findings could provide valuable insights into the phenolic composition of walnuts, crucial for optimizing harvest practices and enhancing the nutritional value of walnuts for consumer health.

2. Materials and methods

2.1. Collection of walnut samples

Walnut samples were meticulously collected from a specific orchard situated in Dalian City, Liaoning Province, China, encompassing four distinct developmental stages from July to September. These stages were categorized as immature stages 1 to 3 (IS1 - IS3) and the mature Stage (MS). The details of sampling information could be seen in Table S1. Typical sample images for each type of fruit at their respective ripening stages are presented in Fig. S1. All samples (3 kg) were obtained from 15-year-old walnut trees at each stage. At each collection location, we randomly collected 10 walnuts with the same degree of maturity from the same walnut tree. The detailed sampling method can be found in Wu et al. (Wu et al., 2021). The nuts were manually cracked and shelled to obtain the walnut kernels.

2.2. Preparation of samples

To facilitate the separation of the kernels from the pellicles, the samples were subjected to treatment with liquid nitrogen. Subsequently, the samples were freeze-dried for a duration of 15 h to achieve a moisture content below 6 %, which is critical for preserving the integrity of the phenolic compounds and other analytes.

The defatting process, essential for the accurate quantification of phenolic compounds, involved immersion of the kernel in petroleum ether. This step effectively removed lipids that could potentially interfere with subsequent analytical procedures. The processed kernels and pellicles were then stored at −20 °C in a controlled environment, awaiting further analysis.

2.3. Reagents and chemicals

High-purity reference standards (> 98 %) for the targeted 32 phenolic compounds were obtained from Sigma-Aldrich (Shanghai, China), with details provided in Table S2. High-performance liquid chromatography (HPLC)-grade solvents, including methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), acetone (AC) and formic acid (FA), were procured from Merck China (Hangzhou).

2.4. Extraction of phenolic compounds

The methodologies are adapted from previous research (Wu et al., 2021). The samples (kernel, 0.50 g; pellicle, 1.00 g) was extracted three times with 50 mL of a 7:3 (*v*/v) acetone-water mixture. The supernatants were combined and evaporated to remove acetone, after which the pH was adjusted to 2.0 with 2 M HCl. Free phenolics were isolated using ethyl acetate extraction, followed by rapid evaporation and redissolution in MS-grade methanol. The residual hydrolysate was treated with 2 M NaOH for 1 h under an inert atmosphere before being acidified back to pH 2.0 with 2 M HCl, which facilitated the extraction of esterified phenolic compounds using ethyl acetate. Bound phenolic compounds were extracted using similar procedures, with an additional NaOH treatment step.

2.5. Antioxidant activity assessment

The antioxidant potential was assessed using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, with modifications as necessary (Ma et al., 2023). Extracts were diluted and mixed with an ethanolic DPPH solution (1×10^{-4} mol/L), then incubated in the dark at 37 °C for 30 min. Absorbance was measured at 517 nm, and the IC_{50} value, which indicates the concentration required to scavenge half of the DPPH radicals, was calculated; lower IC_{50} values indicate higher antioxidant efficacy.

2.6. Qualitative analysis by UPLC-MS/MS

The analysis was performed on an Agilent 1290 Infinity II UPLC system coupled with a 6460C QQQ triple quadrupole mass spectrometer (Agilent Technologies, CA, USA), equipped with an electrospray ionization (ESI) source. The system operated in both positive and negative dynamic multiple reaction monitoring (DMRM) modes, with a 0.02 s dwell time, using a Poroshell 120 EC-C18 column (100 × 2.1 mm, 1.9 μm). Optimal ionization and fragmentation conditions were determined via full scan MS/MS analysis. Instrument parameters included an ion source temperature of 300 °C, a N2 drying gas flow rate of 11 L/h, and a capillary voltage of 3500 V. The mobile phase consisted of A (0.05 % FA in water) and B (acetonitrile: methanol, 4:1). The elution gradient was as follows: 0–12 min (15 % - 50 % B), 12–15 min (50 % - 60 % B), 15–19 min (60 %–95 % B), with a return to initial conditions by 20 min. The flow rate was maintained at 0.3 mL/min, with a 5 μL injection volume. The typical chromatograms could be seen in Fig. S2.

2.7. Statistical analysis

Statistical analyses were conducted using SPSS PASW Statistics 18.0 (IBM, USA). Following ANOVA, Tukey's test was applied to identify significant differences ($p < 0.05$). Orthogonal partial least squares discriminant analysis (OPLS-DA) was employed for phenolic profiling across different developmental stages. Variable Importance in Projection (VIP), *p*-values, and absolute log2 fold changes were used to determine regulated phenolic substances at each stage. Data visualization was facilitated through Metware Cloud (https://cloud.metware.cn) and Origin2019. Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathway analysis was performed for functional enrichment of differential metabolites using the KEGG database.

3. Results

3.1. Characteristics of phenols during walnut developmental stage

Utilizing the LC-MS/MS methodology established in our previous work (Shen et al., 2021), we systematically identified and quantified 27 distinct monomeric phenolic compounds in walnut kernels and 26 in pellicles at various developmental stages. These phenolics are categorized into four main classes: phenolic acids, flavanols, flavones, and flavonols.

Among these, phenolic acids represented the predominant class in both kernels and pellicles, with their proportion ranging from 45.28 % to 94.69 % in kernel and 31.27 % to 97.40 % in pellicles. The dynamics of phenolic acid concentrations in the kernels showed an initial decline, followed by an increase as the fruit matured, as depicted in Fig. 1-A. Specifically, the level of free phenolic acids in the kernels decreased by 80.67 % from IS1 to IS3, before increasing by 66.08 % from IS3 to MS. A similar trend was observed in pecans, where free phenolic acids initially decreased by 76.93 % and subsequently recovered by 31.96 % during fruit maturation (Bouali et al., 2023). Notably, the variation in phenolic acid content within the pellicles differed significantly from that in the kernels. Peak concentrations of all three forms of phenolic acids in the pellicles were observed at IS2 (Fig. 1-B), with esterified phenolic acids reaching a peak of 2258.59 μg/g, nearly four times higher than in earlier developmental stages. Flavanols, identified as the principal flavonoid subclass in walnuts, predominantly existed in their free form, accounting for 76.49 % in kernel and 56.90 % in pellicles. The concentration of

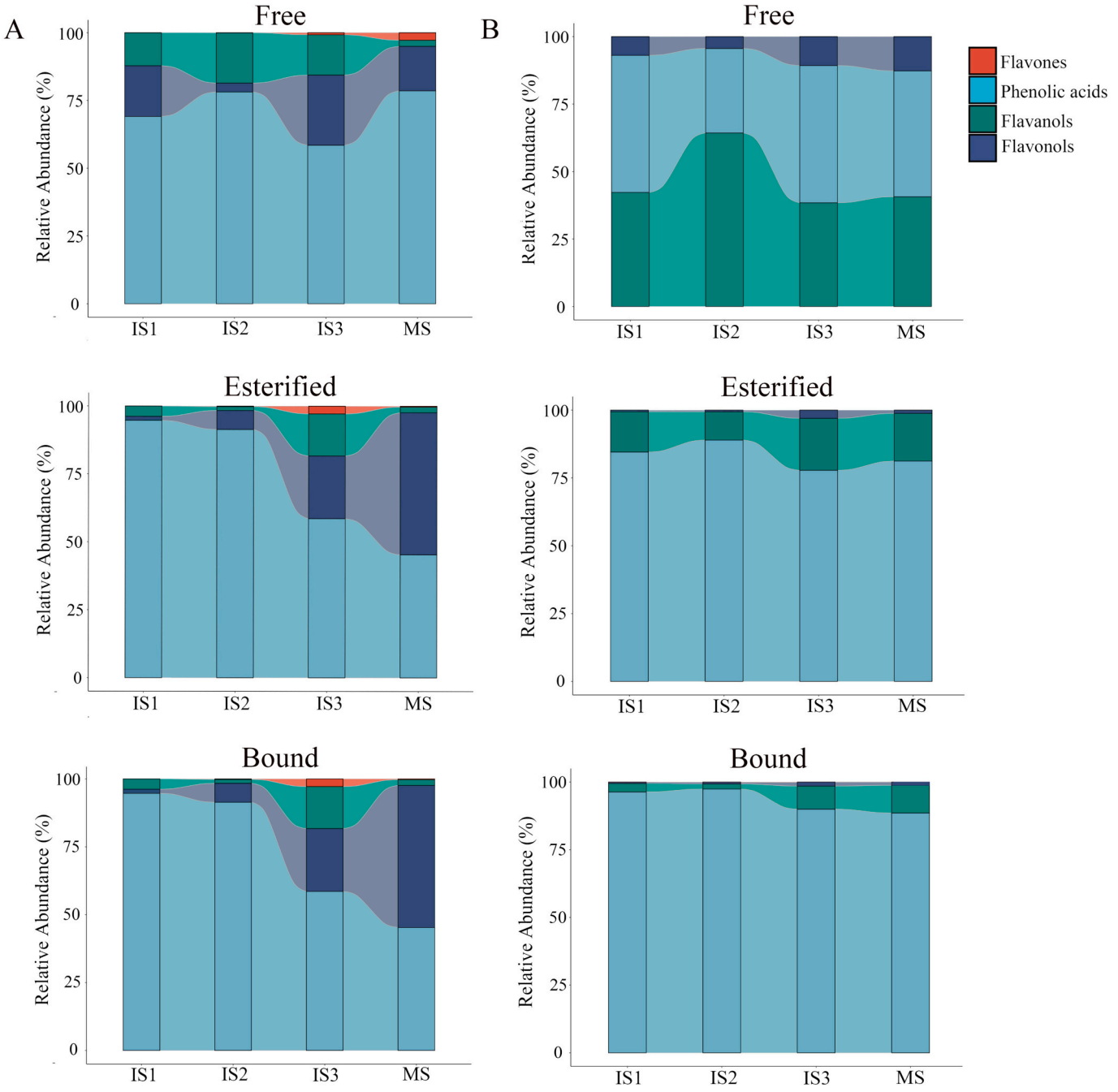

Fig. 1. Distribution of phenolic compounds across ripening stages in walnut kernel and pellicle. A, kernel. B, pellicle.

free flavanols in kernel displayed a linear decrease, whereas the esterified and bound forms followed a biphasic pattern, with a substantial decline of 94.74 % in the free form by MS. In pellicles, flavanols initially showed an increase before decreasing in both free and esterified forms, while the bound form exhibited a sustained upward trend. The highest concentrations of flavanols in kernel occurred at IS1 (free), IS3 (esterified), and MS (bound), whereas the peak levels in pellicles were consistently recorded at IS2 across all forms. Flavones, the least abundant subclass, maintained concentrations below 0.5 μg/g in all samples, with their maximum levels detected at MS in the kernels and IS2 in the pellicles. Studies on other species, such as Rubus chingii (Zhong et al., 2022) and Schisandra sphenathera (Ma et al., 2023), also reported a declining trend in phenolic acids and flavonoids during fruit development.

In terms of total phenol content (TPC), pellicles exhibited significantly higher concentrations of all three forms of phenols compared to kernels. The concentrations were 1249.49 μg/g (free), 869.70 μg/g (esterified), and 624.80 μg/g (bound) in pellicles, compared to 144.39 μg/g (free), 34.65 μg/g (esterified), and 98.98 μg/g (bound) in kernels. Previous research has indicated that the free total phenolic content in mature walnut pellicles is over 20.85 times greater than that found in kernels (Wu et al., 2023). This study corroborates our previous findings that walnut kernels are rich in free phenols, which remain the dominant form throughout walnut development in both pellicles and kernels, with pellicles consistently showing higher phenol concentrations than kernels.

3.2. Monomeric phenols during walnut developmental stage

Our investigation of 25 prevalent monomeric phenols in walnut pellicle and kernel revealed that ellagic acid was the predominant compound across all chemical forms. In the pellicle, the average concentrations were found to be 456.76 μg/g for free forms, 685.81 μg/g for esterified forms, and 328.51 μg/g for bound forms. In contrast, the kernel exhibited lower average concentrations of 68.80 μg/g for free, 15.31 μg/g for esterified, and 53.63 μg/g for bound forms. Consistent with previous research (Wu et al., 2021), our findings confirm the prominence of ellagic acid as the leading free monomeric phenol in walnut kernels, wherein it significantly outstrips other phenolic compounds by a factor ranging from 1.38 to 4688.86 times. Regarding the dynamic changes in catechin, epicatechin, and procyanidin B2 —substances abundant in the pellicle—we observed notable increases in

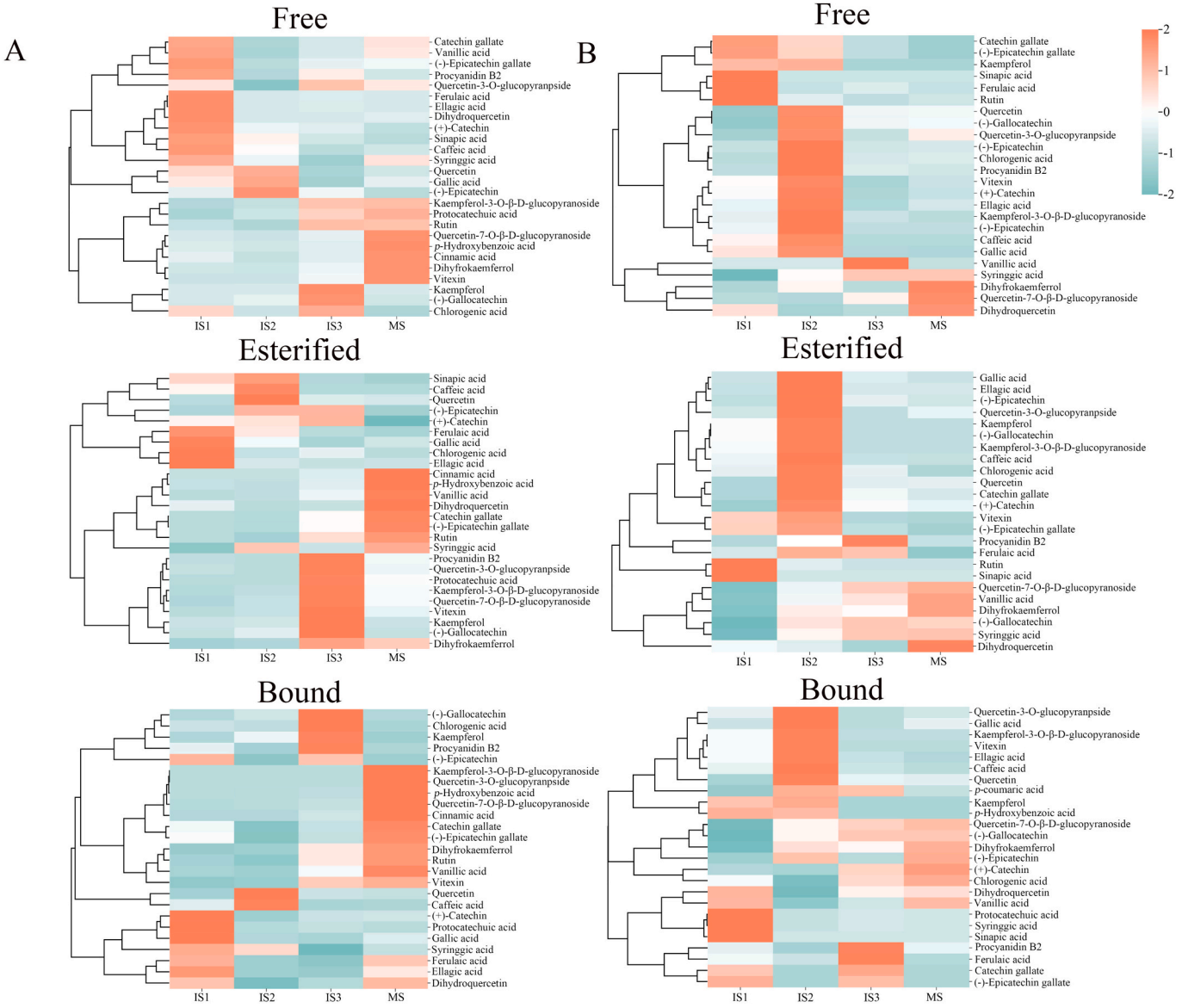

Fig. 2. Heat map of phenolic compounds under different ripening stages. A, kernel; B, pellicle.

their free forms: 120.00 %, 5879.11 %, and 1190.43 %, respectively, from the IS1 to IS2 developmental stages. However, during MS, these concentrations declined to 25.34 %, 10.62 %, and 22.52 % of those observed during IS2. The observed increase in procyanidin B2 levels may be due to the polymerization of catechin, which leads to the formation of this compound (Dibanda, Akdowa, Rani, Tongwa, & Mbofung, 2020). As illustrated in Fig. 2-B, most free and esterified monomeric phenols in the pellicle initially exhibit an upward trend before either decreasing or maintaining a steady pattern. This decline in various phenolic forms could be associated with the gradual depletion of primary metabolites during fruit development, which may limit the substrates essential for phenolic synthesis (Gruz et al., 2011). Notably, certain monomeric phenols, such as syringic acid, *p*-hydroxybenzoic acid, sinapic acid, and kaempferol, experienced a significant reduction exceeding 86 % during the MS phase. Conversely, the kernel displayed a marked increase in monomeric phenols throughout fruit development, particularly in the esterified and bound forms (Fig. 2-A). For instance, the esterified concentrations of vanillic acid, cinnamic acid, and *p*-hydroxybenzoic acid increased by 5202.37, 7523.95, and 47.26 times, respectively, relative to IS1 levels. Similarly, bound concentrations of quercetin-3-O-glucopyranoside, kaempferol-3-O-glucopyranoside, and *p*-hydroxybenzoic acid increased by 17,037.82 %, 9050.94 %, and 3617.03 %, respectively, from IS3 to MS. However, the free forms of ellagic acid, ferulic acid, catechin, and dihydroquercetin, which were initially present in relatively high concentrations, showed a gradual decrease during fruit development, with reductions of 78.98 % for ellagic acid and 99.92 % for catechin. A similar trend of decline in free gallic acid and catechin, along with an increase in free epicatechin, has also been reported in hazelnut kernels (Persic et al., 2018).

The level of free gallic acid in walnut kernel also displayed a gradual

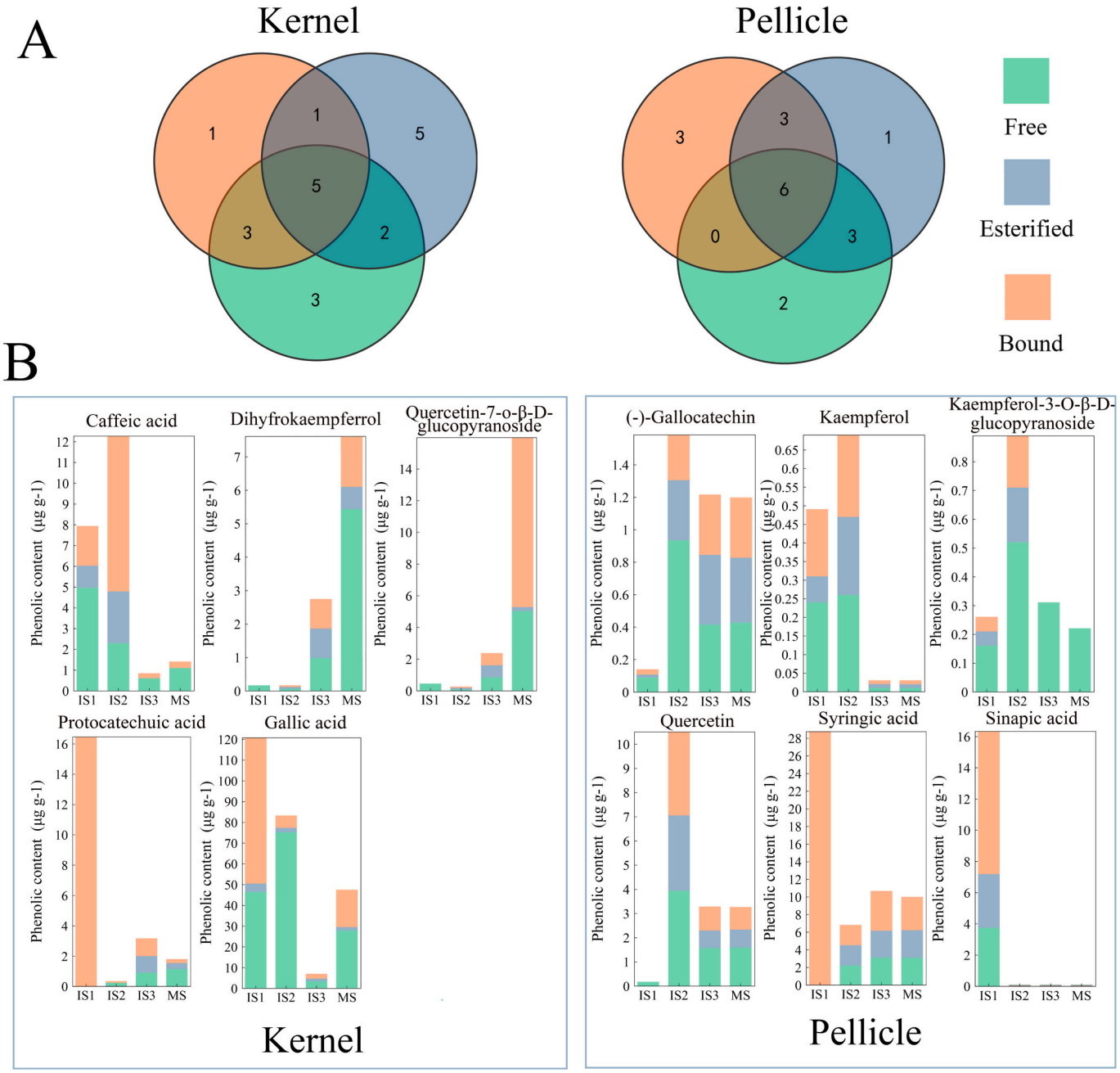

Fig. 3. Differential phenols and their response to developmental stage. A, Venn diagram of three forms of monomer phenols significantly affected by development in walnut. B, the contents of monomer phenol in walnut with three forms were significantly affected by development.

decline, decreasing by over 60 %. Our findings align with previous studies, indicating that free monomeric phenols, which are initially abundant in walnut kernels, tend to diminish as the fruit ripens. Conversely, esterified and bound monomeric phenols do not follow the same downward trajectory; rather, they exhibit a progressive upward trend over time. This increasing trend in bound and esterified phenolic compounds has been similarly noted in research on Rubus chingii (Zhong et al., 2022). These observations suggest that the increase in esterified and bound phenol content may be linked to their conjugation with free phenols and macromolecules in the cell wall, a process that can enhance the strength of the fruit pericarp and its resilience to external stressors (Maffei, Arimura, & Mithoefer, 2012). Moreover, the bound forms of certain monomeric phenols, such as ellagic acid, quercetin-3-O-glucopyranoside, and kaempferol-3-O-glucopyranoside, exceeded their free counterparts at the MS by factors of 1.17, 123.16, and 71.63, respectively. Other fruits have also demonstrated high concentrations of bound phenols, with emerging evidence suggesting that bound phenols may confer distinct health benefits to the human gut, distinct from those of free phenols (Tomas et al., 2020).

3.3. Differential phenols and their response to developmental stage

OPLS-DA was employed to investigate the relationship between individual phenolic compounds and the developmental stages of walnuts. This analysis effectively distinguished the three forms of phenolic compounds across developmental stages, as illustrated in Fig. S3. In both the pellicle and kernel, monomeric phenols that exhibited variable importance in projection (VIP) scores exceeding 1 and a *p*-value below 0.05 were identified as significantly influenced by the developmental period.

In the pellicle, the levels of six monomeric phenols—gallocatechin, kaempferol, kaempferol-3-O-β-D-glucopyranoside, quercetin, syringic acid, and sinapic acid—were markedly affected by developmental stages. Notably, the peak concentrations of these phenols occurred during the initial three stages, followed by an overall downward trend (Fig. 3-A). Sinapic acid demonstrated the highest variability, with a coefficient of variation reaching up to 173 %. In the kernel, three forms of five additional monomeric phenols (caffeic acid, dihydrokaempferol, protocatechuic acid, gallic acid, quercetin-7-O-β-D-glucopyranoside) were also significantly responsive to developmental changes. The specific shifts in phenolic content within walnut pellicle and kernel, evidencing significant differences across developmental stages, were further characterized (Fig. 3-B). Protocatechuic acid showed the most pronounced response, with a coefficient of variation of 119 %. Similar findings have been reported in studies of goji berries (Gruz et al., 2011), passion fruit (da Costa et al., 2023), sweet orange peel (Omoba, Obafaye, Salawu, Boligon, & Athayde, 2015), and lemon peel (Mehmood et al., 2020), where gallic acid, caffeic acid, syringic acid, ferulic acid, and rutin in various forms exhibited significant responses to developmental variation. In the present study of walnut pellicle, ferulic acid, gallic acid, rutin, and caffeic acid were also identified as significantly responsive, with coefficients of variation exceeding 81.77 %.

In the walnut kernel, free phenols such as caffeic acid, gallic acid, and quercetin-7-O-β-D-glucopyranoside have also exhibited significant developmental variability, as seen in studies of blueberries (Xie, Xu, Zhou, Liu, & Zhao, 2019), mangoes (Vithana, Singh, & Johnson, 2019), and navel oranges (Hou et al., 2021). The average coefficients of variation for the identified differential monomer phenols in the pellicle were 92.54 %, 100.40 %, and 94.91 %, while in the kernel, they reached 101.76 %, 104.40 %, and 118.58 %. The significant response of all three forms of monomeric phenols to developmental stages indicates that phenolic compounds in walnuts undergo a complex series of biosynthetic reactions during ripening, potentially involving continual transformations among these forms.

3.4. Assessment of antioxidant activities in walnut pellicle and kernel

The antioxidant activities of phenolic compounds within the walnut pellicle and kernel during four developmental stages were evaluated using the DPPH assay, as outlined in Table 1. The DPPH assay quantifies antioxidant potency through IC_{50} values, with lower values indicating higher antioxidant efficacy.

In the walnut pellicle, free phenols showed relative stability throughout the maturation process, exhibiting minimal variance of 5.44 % and an average IC_{50} value of 0.87 μg/mL. These values were significantly lower than those of esterified and bound phenols, suggesting a superior antioxidant capacity of 3.83 and 74.60 μg/mL, respectively. This finding is consistent with previous research which ascribes the walnut pellicle's strong antioxidant properties to its rich content of free phenols (Li, Shi, Li, Zhang, & Pan, 2022). Conversely, in the walnut kernel, free phenols displayed significant fluctuations. As the fruit matured, the antioxidant activity of the kernel's free phenols declined by 90.93 %, with the IC_{50} value increasing from 11.36 μg/mL at IS1 to 120.45 μg/mL at MS. This significant decrease is supported by studies on walnuts and pecan, which reported a similar decline in antioxidant activity of free phenols in the kernel during development (Amin, Masoodi, Baba, Khan, & Ganie, 2017; Bouali et al., 2023). Comparable patterns have been observed in other fruits such as dates fruits (Shahdadi, Mirzaei, & Garmakhany, 2015), gourds (Singh et al., 2015), and goji berries (Gruz et al., 2011), where the decrease in antioxidant activity is presumed to be due to a reduction in total phenolic and flavonoid content (Papaioannou, Liakopoulou-Kyriakides, & Karabelas, 2016).

Esterified and bound phenols in the kernel also demonstrated antioxidant activities, albeit less prominent than those of free phenols. The average IC_{50} values for both esterified and bound phenols in the kernel were above 470 μg/mL. Notably, the antioxidant activities of esterified and bound phenols in the pellicle exhibited considerable variability, with an initial increase from IS1 to IS2, followed by a decrease as the fruit matured. In the kernel, a gradual decline in antioxidant activities of esterified and bound phenols was observed, with esterified phenols experiencing a drastic reduction of 95.52 %. Contrasting results were found in plums, where the antioxidant activity of bound phenols increased by 72.50 % throughout maturity, indicating that variations in phenolic composition and primary metabolites may account for these differences (Zheng, Wu, Deng, Yi, & Li, 2023). Collectively, the trend in antioxidant activity for both the walnut kernel and pellicle was as follows: free phenols > esterified phenols > bound phenols. This pattern reinforces the significance of free phenols in imparting antioxidant properties to walnuts, highlighting the need for continued exploration of phenolic profiles throughout fruit development to further understand their roles in health benefits and culinary applications.

3.5. Identification for maturity-sensitive phenols and their relationship with antioxidant activity

To further explore the relationship between walnut maturity and phenolic compounds, samples were categorized into immature stage (IS) and mature stage (MS). Using OPLS-DA, we identified maturity-sensitive phenolics (MSPs) that distinguish these developmental stages, confirming notable variations in phenolic profiles (Fig. S4). Specifically, we identified eight phenolic compounds in the pellicle and five in the kernel as MSPs (Fig. 4-A). Notably, three forms of *p*-hydroxybenzoic acid in the kernel and three forms of kaempferol in the pellicle were particularly sensitive to maturity variations. The content of kaempferol decreased more than 920-fold, while the content of *p*-hydroxybenzoic acid significantly increased by over 17-fold, underscoring their crucial role in maturity differentiation. Additionally, several free monomeric phenols were identified as MSPs. In the pellicle, three free MSPs were found, with gallic acid and caffeic acid significantly downregulated by 6.77 and 6.20 times, respectively, from the immature to mature stage. Similar downregulation patterns was observed in the free gallic acid content in

Table 1
Antioxidant capacity of walnuts under different developmental stages.

	DPPH assay (IC_{50})							
	pellicle				kernel			
	IS1	IS2	IS3	MS	IS1	IS2	IS3	MS
Free phenolics	0.89 ± 0.31a	0.82 ± 0.18a	0.83 ± 0.16a	0.93 ± 0.17a	11.36 ± 2.49c	8.75 ± 0.78c	53.46 ± 14.81b	120.45 ± 7.6a
Esterified phenolics	4.52 ± 1.74a	2.27 ± 0.28b	3.34 ± 0.38a	3.97 ± 1.22a	48.04 ± 13.94d	610.08 ± 61.61b	231.78 ± 21.85c	991.92 ± 10.02a
Bound phenolics	136.33 ± 45.97a	41.61 ± 8.99b	40.64 ± 6.16b	115.14 ± 39.88a	375.4 ± 112.83b	982.33 ± 2.05a	986.67 ± 4.03a	504.79 ± 334.69b

Note: The IC_{50} value represents the inhibitor concentration after removing half of the DPPH radical; the higher the IC_{50} value, the lower the antioxidant activity.

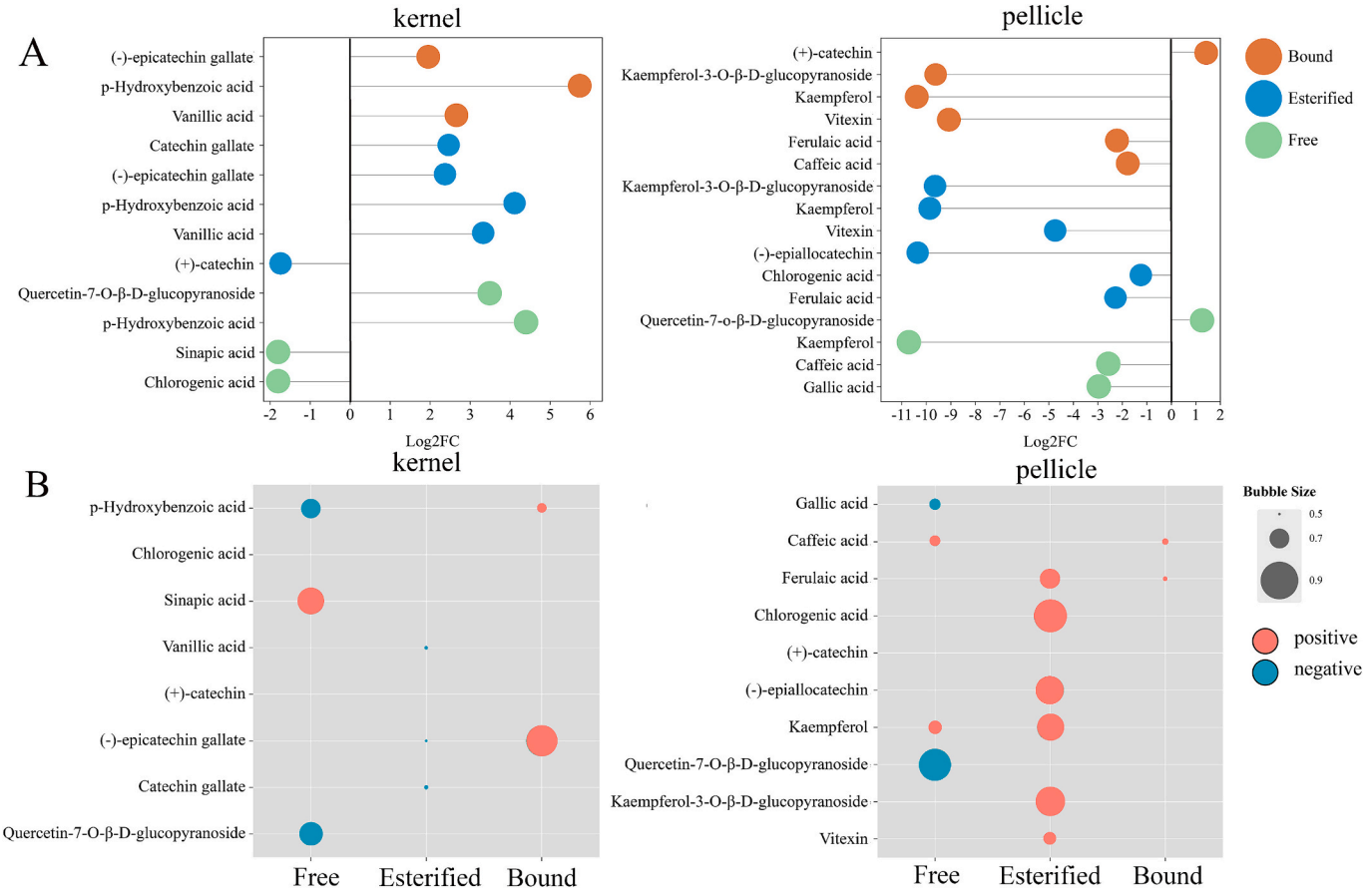

Fig. 4. Identification for maturity-sensitive phenols and their relationship with antioxidant activity. A, log2FC of maturity-sensitive phenols (MSPs) of walnut. B, correlation between maturity-sensitive phenols (MSPs) and antioxidant activity.

lemon peel (Mehmood et al., 2020) and free caffeic acid in grumixama peel (Bonin et al., 2024). In the kernel, three free MSPs were identified, including chlorogenic acid and sinapic acid, which showed significantly downregulation by more than 3.52 times. Previous studies have indicated that other monomeric phenols, such as ferulic acid and caffeic acid in navel oranges, are also significantly affected by maturity and play an important role in distinguishing maturity stages (Hou et al., 2021).

Several studies have examined the correlation between the structure of monomeric phenols and their antioxidant activity (Xie et al., 2019). For instance, free ferulic acid, caffeic acid, *p*-coumaric acid, vanillic acid, and p-hydroxybenzoic acid were found to exhibit positive correlations with antioxidant activity in oranges (Hou et al., 2021). In black cranberry beans (*Phaseolus vulgaris* L.), free catechin ($r = 0.98$) and bound *p*-hydroxybenzoic acid ($r = 0.92$) were recognized as having strong positive correlations with antioxidant activity (Chen et al., 2015). In this work, Pearson correlation analysis was employed to investigate the relationship between MSPs and antioxidant activity. Interestingly, a greater number of correlations were observed in the pellicle, particularly among the esterified forms of MSPs (Fig. 4-B). Except for quercetin-7-O-β-D-glucopyranoside and luteolin, the esterified forms of six MSPs demonstrated correlation coefficients exceeding 0.75 ($p < 0.05$) with antioxidant activity. Both the free and bound form of caffeic acid also exhibited notable positive correlation with antioxidant activity, with correlation coefficients of 0.60 and 0.53, respectively. In contrast, within the kernel, only three positive correlations were established: free sinapic acid, bound epicatechin gallate, and bound *p*-hydroxybenzoic acid, with correlation coefficients greater than 0.66 ($p < 0.05$).

It is noteworthy that some monomeric phenols were identified as negatively correlated with antioxidant activity. Free quercetin-7-O-β-D-glucopyranoside in both pellicle and kernel, along with free *p*-hydroxybenzoic acid in the kernel, showed significant negative correlations with antioxidant activity, with correlation coefficients above 0.75. This

inverse relationship has been reported in other fruits, although previous studies have predominantly focused on the positive correlations between certain phenolic acids and antioxidant activity. For example, free gallic acid in cherries (Wang, Jiang, Wang, Jiang, & Feng, 2017) and free ellagic acid in red-fleshed kiwifruit (Chen et al., 2023) were found to exhibit significantly negatively correlated with antioxidant activity. Additionally, caffeic acid, *p*-coumaric acid, and ellagic acid were found to be significantly negatively correlated with antioxidant activity in red raspberries (Zhong et al., 2022).

3.6. Factors affecting walnut phenolic composition and forms

The phytochemical composition in walnuts varies significantly due to intrinsic (genetic) and extrinsic (environmental and post-harvest) factors. These influences are crucial in determining TPC, specific phenolic compounds, and total antioxidant capacity (TAC). For example, TPC varied up to 3.57-fold among 11 walnut cultivars grown in Liaoning, China (Wu et al., 2020), and up to 2.52-fold among 11 cultivars in Poland (Pycia et al., 2019). Mo et al. observed a 1.69-fold variation in TPC among walnut kernels from five regions in China (Mo et al., 2022), and up to a 2.08-fold variation among nine orchards in the USA (Wu et al., 2020). However, no significant differences in total phenolics and flavonoids were noted in Romanian walnut plantations (Trandafir, Cosmulescu, Botu, & Nour, 2016). Individual phenolic compounds tend to display even greater variability based on cultivar and geographic origin. For example, Hungarian walnut cultivars exhibited remarkably higher levels of vanillic acid, rutin, and syringic acid—8.2, 7.0, and 8.5 times greater, respectively— when compared to cultivars from other regions. Some studies have reported TAC variations up to 3.04-fold among different cultivars. Drying methods and fruit maturity also substantially affect walnut phenolic content, with different drying methods resulting in variations of 20.08-fold for TPC and 13.69-fold for TAC. Our findings revealed that maturation influenced TPC in kernels and pellicles by up to 8.92-fold and 10.53-fold, respectively, with antioxidant capacity varying by 1.13-fold in pellicles and 13.77-fold in kernels. Notably, individual phenolic types exhibited pronounced variability; for instance, variations in gallic acid, *p*-hydroxybenzoic acid, and protocatechuic acid reached 41.89, 30.64, and 24.06 times, respectively.

Currently, literature on the impact of these factors on the forms and composition of phenolics is limited. A study reported that the variations in the free form of TPC in the pellicles and kernels of different walnut cultivars were significantly higher than those in the esterified and bound forms (Wang et al., 2022). Drying processes significantly affect both the free and esterified forms of TAC in kernels, resulting in variations of 13.69 and 4.74 times, while no notable differences were found for the bound forms. The existing research indicated that these factors exert both type- and form-dependent effects on individual phenolics. Drying caused variations in *p*-hydroxybenzoic acid of 35.28 (free), 4.22 (esterified), and 1.9 (bound) times in kernels, while pellicles showed variation of less than 3.5-fold across all forms. Our study identified *p*-hydroxybenzoic acid as an MSP, noting that maturity considerably affected its levels, resulting in variations of 43.34 (free), 361.39 (esterified), and 418.8 (bound) times in the kernel. Other MSPs, including kaempferol (free and bound) and epicatechin (esterified), exhibited variations of less than 4 times in previous studies (Li et al., 2023; Wang et al., 2022). In contrast, our study found that maturity had a much greater impact, with variations ranging from 346 to 367 times. These findings suggest that maturity might have a more significant impact on the phenolics and their forms in both the kernel and the pellicle. Similar findings have been reported in other crops such as goji berries (Gruz et al., 2011), Rubus chingii Hu (Zhong et al., 2022), and navel orange (Hou et al., 2021). Additional comprehensive studies are warranted to further elucidate the influence of these factors on walnut phenolic profiles.

3.7. Differential expression of phenolics metabolism pathways during maturity stages and related mechanism

To elucidate the molecular mechanisms underlying the differential expression of phenolic compounds during walnut maturation, we conducted a KEGG pathway analysis to identify all pertinent metabolic pathways (Fig. 5-A). Our analysis revealed a significant enrichment of flavonoid biosynthesis and phenylpropanoid biosynthesis pathway in both the walnut pellicle and kernel. Notably, the pellicle also exhibited significant enrichment of flavone and flavonol biosynthesis pathways. Utilizing these enriched phenolic metabolic pathways and supported by evidence from the literature (Li et al., 2023; Li et al., 2024), we delineated a comprehensive phenolic metabolism network (Fig. 5-B).

Cinnamic acid, a pivotal precursor in both the phenylpropanoid and flavonoid biosynthesis pathways, exerts dual regulatory effects on downstream phenolic molecules. It significantly modulated the levels of caffeic and ferulic acids within the phenylpropanoid pathway, as evidenced by a 6.20-fold reduction in the concentration of free caffeic acid in the pellicle. In contrast, these phenolics exhibited relative stability in the kernel, with the downstream metabolite sinapic acid undergoing a significant decrease. In the flavonoid biosynthesis pathway, cinnamic acid regulates the synthesis of cinnamoyl-CoA via the enzymatic action of *p*-coumarate-CoA ligase, thus influencing the levels of the subsequent phenolic compounds such as catechin, epicatechin, gallocatechin, and epigallocatechin. Catechin, identified as a key MSP in both the pellicle and kernel, displayed a significant increase in the pellicle from immature to mature stages, while levels of catechin in the kernel diminished sharply. Epigallocatechin, another prominent MSP in the pellicle, was significantly downregulated, exhibiting a substantial decrease exceeding 300-fold.

The pellicle uniquely and substantially enriched in the flavone and flavonol biosynthesis pathways. Among the identified MSPs, quercetin, regulated by the common phenolic compound apigenin from the flavonoid biosynthesis pathway, showed a significant decrease from the immature to mature stages. Kaempferol, another shared phenolic molecule in the flavonoid biosynthesis route, also underwent a dramatic decline throughout maturation, culminating in the downregulation of kaempferol-3-O-β-D-glucopyranoside in the flavone and flavonol biosynthesis pathways.

In summary, the intricate dynamics of phenolic compound production during walnut maturation are closely related to the modulation of specific metabolic pathways. The phenylpropanoid and flavonoid biosynthesis pathways, in particular, are pivotal to the synthesis and regulation of key phenolics. Unraveling these processes and their regulatory mechanisms is fundamental for elucidating walnut maturation and the biosynthesis of health-promoting phenolics.

4. Conclusion

This study provides the first comprehensive analysis of the maturation-induced changes in walnut phenolics and their antioxidant activities. We confirm that free phenols dominate throughout development, with higher concentrations in the pellicle than in the kernel. Monomeric phenols peak during the immature stage and decline as fruits mature, which is accompanied by reduced antioxidant capacity. OPLS-DA identified eight and five maturity-sensitive phenolics (MSPs) in the kernel and pellicle, respectively, with *p*-hydroxybenzoic acid and kaempferol notable MSPs in all forms. Strong correlations between MSPs and antioxidant activities highlight their critical roles in walnut nutrition. Pathway enrichment analysis revealed that the flavone, flavonol, and phenylpropanoid biosynthesis pathways are crucial for walnut phenolic development. These findings are vital for optimizing harvest strategies and enhancing walnuts' the nutritional value and health benefits.

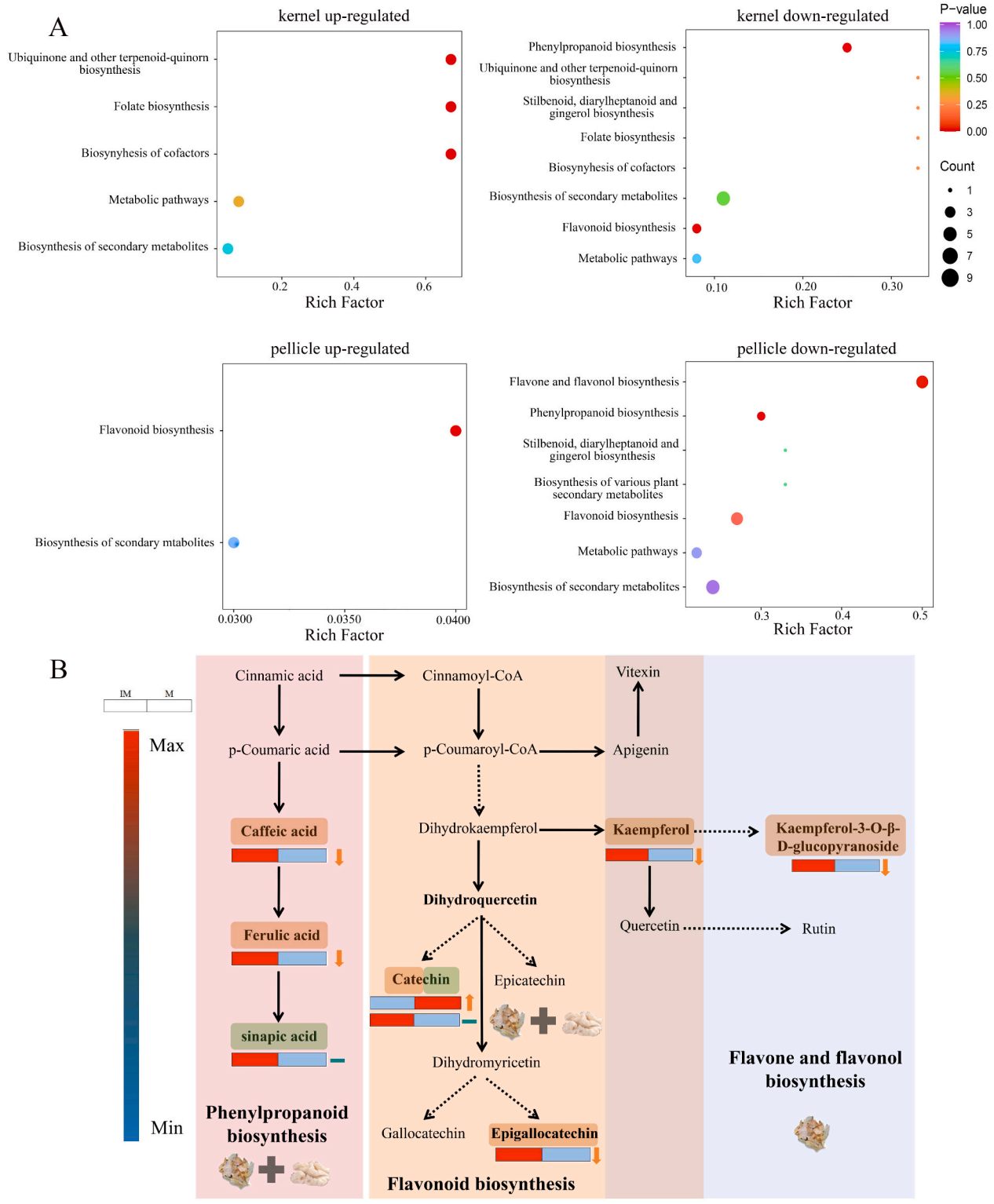

Fig. 5. Metabolic pathways of walnut under maturity. A, Diagram of up-regulated and down-regulated metabolic pathways reflecting walnut maturity differences.B, the different background colors represent different metabolic pathways. Solid arrows indicate direct connections between metabolites, while dashed lines represent indirect connections. The maturity-sensitive phenols (MSPs)in pellicle are marked in red, The maturity-sensitive phenols (MSPs) in kernel are marked in green. Orange arrows up or down indicate up- or down-regulation of the metabolite in pellicle. Green plus and minus indicates that the metabolite is up- or down-regulated in kernel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CRediT authorship contribution statement

Maokai Cui: Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Runhong Mo:** Writing – original draft, Visualization, Software. **Qingyang Li:** Visualization, Formal analysis, Data curation. **Ruohui Wang:** Validation, Methodology, Investigation. **Danyu Shen:** Validation, Methodology, Investigation. **Fubin Tang:** Validation, Methodology. **Yihua Liu:** Writing – review & editing, Visualization, Software, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101792.

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