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Plant-Wide Target Metabolomics Provides A Novel Interpretation of the Changes in Chemical Ingredients during *Dendrobium officinale* Traditional Processing

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Abstract: The traditional processing of *Dendrobium officinale* (DO) is manufactured through five necessary processing steps: fresh strips, drying at 85 °C, curling, molding, and drying at 35 °C (Fengdou), and the antioxidant activity of DO were increased after processing into Fengdou. To comprehensively analyses the changing functional components, a plant-wide target metabolomics were employed. In total, 739 differential ingredients were identified in five processing treatments, mainly highlighting differences in variation with phenolic acids, flavonoids, lipids, and amino acids and their derivatives, and glycosylation of aglycone result in the up-regulation in flavonoid glycosides levels. Temperature is a key step in DO processing production. In addition, the main enrichment of specific differential ingredients was found in five different metabolic pathways, including glucosinolate biosynthesis, linoleic acid metabolism, flavonoid biosynthesis, phenylpropanoid biosynthesis, ubiquinone and other terpene quinone biosynthesis. Correlation analysis clarified that total phenols and flavonoids showed a significant positive correlation with antioxidant capacity. This study provides new insights into the influence of processing processes on DO quality, which may lead to guidance for the high-quality production of DO.

Keywords: *Dendrobium officinale*; traditional processing; widely-targeted metabolic analysis; chemical ingredients

1. Introduction

Dendrobium officinale (DO) Kimura & Migo is a perennial herb in the *Dendrobium* of the family Orchidaceae, which has been used historically for edible and medicinal purposes for over 2000 years [1]. The types of chemical components contained in the plant mainly include polysaccharides, phenols, alkaloids, and amino acids [2,3], and there is substantial *in vitro* or *in vivo* evidence showing that bioactive compounds present in DO have antioxidants properties [4], which attracted domestic and foreign scholars to conduct in-depth research on DO. Among them, polysaccharides have been reported to reduce insulin resistance and abnormal lipid metabolism in obese mice [5], and inhibit inflammatory factors from alleviating liver metabolic disorders in diabetic mice [6]. While some studies on the immunomodulatory effects of DO alkaloids [7] are also quite well-established. There is growing evidence that phenolic compounds in DO have antioxidant and cytoprotective properties, and bisbenzyl compounds have been widely used in the production of various skin care products and drugs [8].

The moisture content of fresh DO can be up to 80% [9], and though it appears shiny, breed bacteria are easily infected. Thus, it is necessary to control moisture during DO post-harvest storage, transportation, and processing without biological activity reduction. The common drying methods of DO are sun drying, hot air drying, infrared drying and freeze drying, etc. However, *Fengdou* has

always been the popular processing product of DO due to its advantages of small space and good keeping qualities. Our previous studies found that the total flavonoid and total polyphenol content of DO was elevated after processing into *Fengdou* [10], whereas the exact mechanism remains unclear. Several studies have been conducted to compare the chemical components before and after DO processing, such as polysaccharides, total phenols, and total flavonoids [11], etc. However, *Fengdou* is made through various processes such as drying, hooping, and baking, and the mechanisms of compositional changes during this process are largely unknown. Hence, it is critical to systematically compare of the chemical profiles during fresh DO strips into *Fengdou*.

Plant-wide target metabolomics is a new field of metabolomics, which can be effective for high-dimensional complex data obtained from the analysis and help to observe the differences between samples. Significant variations in the metabolic profile of DO have been revealed by metabolomic approaches, which depend on a variety of factors such as species, growth conditions, and different parts [12]. There are also studies that reveal the material basis and mechanisms of disease prevention and treatment through metabolomics, such as the application of DO to gastric mucosal protection [13] and the study of metabolic mechanisms of preventive effects on diabetes [14]. Although the research provides lots of information about DO products under different conditions, the composition changes of DO during processing have not been clarified.

The main objective is to comprehensively study the dynamic changes of compounds during processing through metabolomics and to summarize the key processing steps. It will help to further investigate the accumulation and metabolic regulation mechanisms of active compounds in DO during processing and provide new insights for quality control during functional food of DO production.

2. Materials and methods

2.1. DO samples in different processing stages

In this study, 15 kg of fresh DO was collected on 10 November 2021 from the Longmu *Dendrobium* Department of Longshan County, Longshan town (Yunnan, China), and authenticated by Prof. Pengfei Tu following the features described in Chinese Pharmacopoeia (2020 edition), and processed according to DO local standards for food safety. Briefly, the 15 kg of fresh DO is removed from the debris (clean the residual surface leaves and other debris, excise irregular branches) washed with pure water, controlled the water and then randomly divided 1 kg as sample FA1. The others were placed under the oven at 85 °C for baking, rubbing while drying to facilitate curling, removing residual leaf sheaths and other debris as much as possible during the softening process, removing water until softened, and taking out 1 kg as sample FB2. The remaining part of the baked soft *Dendrobium* strips was twisted into a spiral shape, and 1 kg of the sample was left to name sample FC3. After shaping twice, the sample should be tightly curled, not spread out and have a beautiful, uniform shape, and 1 kg of sample is named sample FD4. Finally, it was placed in the oven at 35 °C, dried at low temperature, and named sample FE5, as shown in Figure 1A. The five samples were used for UPLC-ESI-MS/MS analysis, and three biological replicates were performed for each sample.

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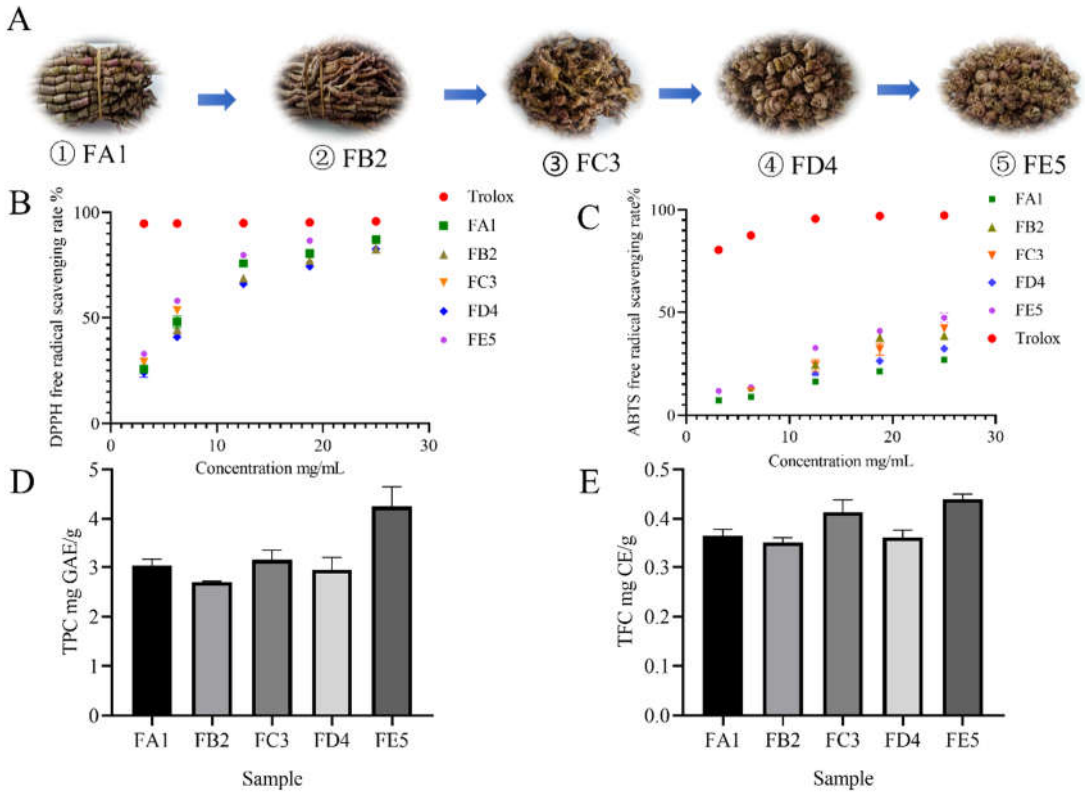

Figure 1. Appearances of DO samples at the end of each processing step. The fresh strip (FA1), drying at 85°C (FB2), curling (FC3), molding (FD4), and drying at 35°C (FE5) **(A)**; The DPPH scavenging capacity **(B)** and ABTS scavenging capacity **(C)** of DO samples in different processes; The contents of total phenols **(D)** and flavonoids **(E)** of DO samples in different processes.

2.2. Sample preparation and extraction

The five type samples are powder that were freeze., which were then ground with a mixer mill (MM400, Retsch) and zirconia bead for 1.5 min at 30 Hz. Lyophilized powder (50 mg) was dissolved with 1.2 mL 70% methanol solution, vortexed every 30 min for 30 sec (6 times in total). The samples were then centrifuged for 10 min at 12000 rpm/min, and the supernatant was taken to detect the activity. Meanwhile, other supernatant filtrated prior to UPLC-ESI-MS/MS analysis. Quality control (QC) samples were then prepared by combining all of the sample extracts. The QC sample was performed every-six samples to assess the repeatability of the measurement process.

2.3. Antioxidant Activity

2.3.1. DPPH radical scavenging activity assay

The experiments were performed as modified on the protocol described by Zhang et al. [26]. Firstly, a proper amount of the extraction filtrate was obtained by the method in section 2.2 to prepare samples with different concentrations. The 2 mL of DO sample solutions with different concentrations were respectively added into 2 mL DPPH free radical stock solution (0.1 mg/mL) that shook well and left in the dark for 30 min. The anhydrous 95% methanol was used instead of the sample extract as the negative control, and Trolox was used as a positive control. Absorbance was measured at 515 nm. Each sample was measured three times. The percentage of inhibition was calculated as [Ac−As/Ac×100], where Ac is the absorbance of the negative control and as is the absorbance of the test samples.

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2.3.2. ABTS radical scavenging activity assay

The antioxidant capacity was evaluated by the 2,2′-azino-bis (3- ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical method reported by Liu et al. [15]. The calculation method was the same as in Section 2.3.1.

2.4. Total Phenolics and total flavonoid Quantification

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method described by Castaldo [16], and the results were expressed as mg of gallic acid equivalent (GAE) per or 100 g of dry matter (DO). In addition, total flavonoid content (TFC) was determined using the colorimetric method described by Luo et al [17], and the results were expressed as mg of rutin equivalent (CE) per or 100 g of DO.

2.5. UHPLC-MS conditions

The analytical conditions were as follows, UPLC: column, Agilent SB-C_{18} (1.8 μm, 2.1 mm×100 mm). The mobile phase consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. Sample measurements were performed with a gradient program that employed the starting conditions of 95% A, and 5% B. Within 9 min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1 min. Subsequently, a composition of 95% A, 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was set as 0.35 mL per minute; The column oven was set as 40 °C; The injection volume was 4 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS. AB4500 Q TRAP UPLC/MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 550 °C; ion spray voltage (IS) 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II(GSII), curtain gas (CUR) was set at 50, 60, and 25 psi, respectively; the collision activated dissociation (CAD) was high.

2.6. Qualitative and statistical analysis of ingredients

Compounds were identified based on the MWDB (metwa database). Substance characterization is performed based on secondary spectral information, and the analysis removes isotopic signals, repeating signals containing K^+, Na^+, NH_4^+, and repeating signals of fragment ions that are themselves other larger molecular weight substances. The quantitative analysis was mainly based on their abundance in the UPLC-ESI-MS/MS analysis. The precursor ions are broken by collision chamber-induced ionization to form many fragment ions are formed after the fragment ions are filtered through the triple quadruple rod to select the desired characteristic fragment ion, eliminating the non-target ion interference and making the quantification more accurate and reproducible. To study the accumulation of differences in metabolite, a multivariate statistical analysis was conducted on the metabolic data of each sample. The PCA was used to display the variables, which was performed using GraphPad Prism v9.01 (GraphPad Software In, La Jolla, CA, USA). An OPLS-DA model was performed using the R software package MetaboAnalystR to compare the composition characteristics of different DO. The variable importance in the projection (VIP) ≥ 1 in the OPLS-DA model and the absolute FC (fold change) ≥ 2 or FC ≤ 0.5 was set for screening differential chemical ingredients. Venn diagrams were generated online and used to show the number of differential chemical ingredients. The KEGG compound database was used to annotate the different chemical ingredients, which were mapped to the KEGG pathway database. The pathways with ingredients that are significantly regulated are subjected to component pathway sets enrichment analysis. *P*-values from hypergeometric tests are used to assess their significance.

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3. Results and discussion

3.1. Effect of DO samples on antioxidant activity

To study the effect of processing on the antioxidant capacity of DO, we measured DPPH free radical scavenging activity and ABTS cation free radical scavenging activity (Figure 1B). Samples of FE5 showed the strongest antioxidant activity among all samples either in DPPH or in ABTS assay. The scavenging effect of DPPH radicals was significantly enhanced with the increase of DO extract concentration in the range of 3.125 mg/mL-25.000 mg/mL. At the same bolus, the DPPH radical scavenging effect also gradually increased during the processing of DO. Under the concentration of 3.125 mg/mL, the DPPH radical scavenging rate of sample FA1 and FE5 were 25.33% and 32.99%, respectively. Oxidation resistance of FE5 is better than that of FA1. The same results were also confirmed in the ABTS radical scavenging experiment (Figure 1C). The increase of antioxidant capacity in the making could be due to the formation of Maillard reaction products (MRPs) [18] under high temperature, and the promotion of intracellular secondary metabolite production under the external pressure accelerated.

3.2. Overview of the profile of ingredients

To explore the material basis of antioxidation, the figure showed that the TPC and TFC levels significantly increased after all of process phases, as evidenced in Figure 1D-E. The TPC and TFC observed in the FE5 were quantified at value of 4.25mg GAE/g and 0.44 mg CE/g, respectively. Considering the correlation between composition and activity, it is possible to observe that the polyphenol content affected antioxidation differently. Research shows that there is a strong correlation between polyphenol content and antioxidant capacity of blueberries, fragaria nubicola, apples, and other vegetables or fruits [19–21]. In order to further clarify the material basis of antioxidation, we carried out a widely non-targeted metabolite analysis for comprehensive metabolic profiling of FA1, FB2, FC3, FD4, and FE5 based on the UPLC–MS/MS system. The composition characteristics of each sample were screened and a total of 1182 chemical ingredients (Table S1) were determined, including alkaloids (8.12%), amino acids and derivatives (10.41%), flavonoids (16.50%), lignans and coumarins (3.81%), lipids (13.45%), nucleotides and derivatives (5.24%), organic acids (8.29%), phenolic acids (13.37%), quinones (3.47%), terpenoids (4.23%), and others (13.11%) (Figure 2A). Results showed that flavonoids, phenolic acids, lipids, amino acids and derivatives were the dominant chemical ingredients in DO.

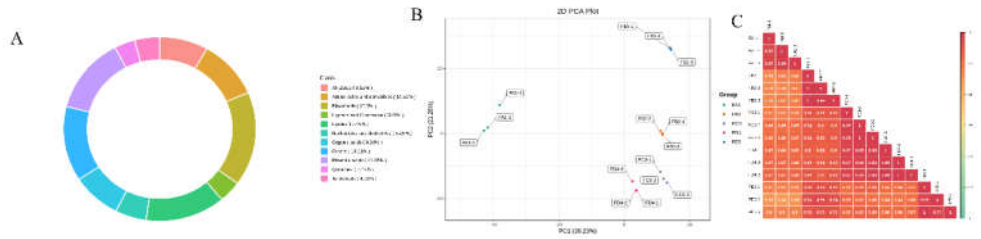

Figure 2. Classification of the 1182 ingredients of DO samples **(A)**; PCA score plot **(B)**. The sampling groups were color-coded as follows: green, FA1; orange, FB2; purple, FC3; pink, FD4; blue FE5; red QC; Correlation diagram of ingredients of different processes of DO **(C)**.

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3.3. Multivariate statistical analysis

Multivariate statistical analysis was performed on the five groups to show differences in their chemical ingredients. Principal component analysis was performed using unsupervised models to analyze the identified chemical ingredients and to assess the overall differences between samples (Figure 2B). In the principal component analysis score plot, it is tentatively suggested that the processing steps from FA1 to FB2 and FD4 to FE5 may be the key stages affecting the compositional changes. The OPLS-DA analysis model can effectively screen for differential chemical ingredients by removing effects that are not relevant to the study. In this experiment, the OPLS-DA score plots of each group (Figure S1) showed that the five samples of different processing processes were separated in pairs, indicating the variability of metabolic profiles among sample groups. Based on the results of the OPLS-DA model, 200 times permutation tests were performed to validate the OPLS-DA model in this experiment (Figure S2). As shown in Figure 2C, the correlation coefficients between the groups of samples with different processing procedures in the processing of DO are all greater than 0.7. It is indicated that baking at 85 °C has a greater effect on the chemical composition of FA1 and FB2. Analyzed in the same way, the effect of curling and sizing on the chemical composition of the samples was inappreciable. It can be seen that DO has significant variability in chemical composition between the raw material and the processed product *Fengdou*, with the highest contribution from baking at 85 °C, followed by drying at 35 °C, and the smaller effect from curling and shaping.

3.3. Identification of differential chemical ingredients

According to the results of the OPLS-DA model, a total of 739 differential chemical ingredients were screened between groups for variable importance (VIP) ≥ 1, FC ≥ 2 or FC ≤ 0.5 in the prediction. Differential chemical ingredients were mainly divided into amino acids and derivatives, phenolic acids, flavonoids, lipids and others, with a large proportion of lipids (17.19%), amino acids and derivatives (14.21%), phenolic acids (13.66%) and flavonoids (10.82%) in that order. As the volcano plots (Figure 3A-D), the smallest number of differential metabolites was found in the FC3 and FD4 group, with 67 (30 up and 37 down). Moreover, there were 481 significantly different chemical ingredients between FA1 and FB2 (271 up and 210 down), 195 ones between FB2 and FC3 (166 up and 29 down), and 362 ones between FD4 and FE5 (51 up and 311 down). From the Figure 3E, it is obviously to see that there are 80 unique differential chemical ingredients in the FA1 *vs.* FB2 group, which also confirms the previous conclusion that temperature is a key factor affecting the changes of chemical ingredients.

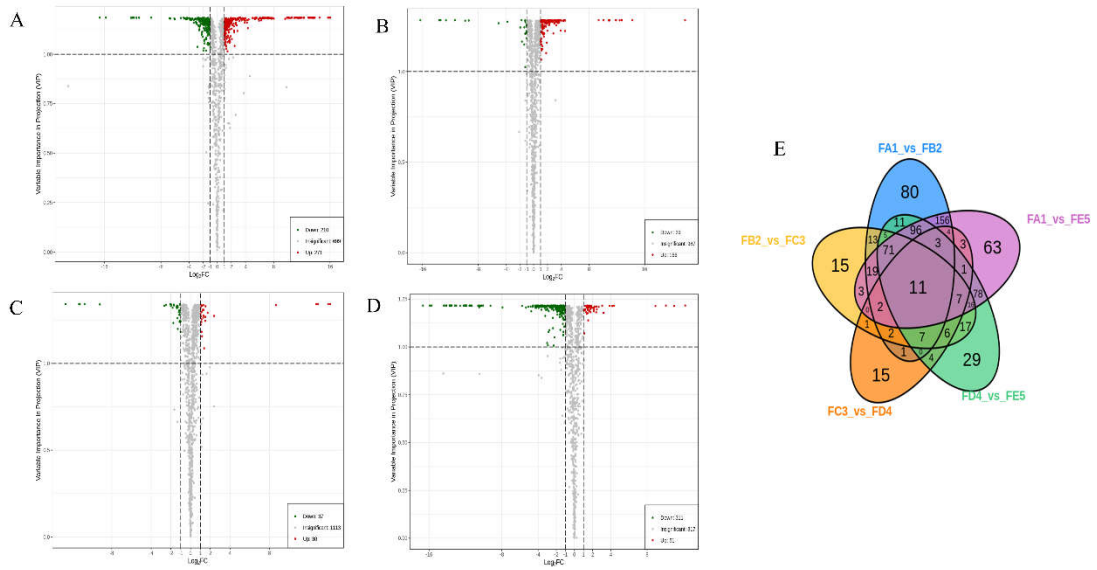

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Figure 3. Volcano plots of the differential ingredients expression levels between FA1 *vs.* FB2 groups **(A)**, the FB2 *vs.* FC3 groups **(B)**, the FC3 *vs.* FD4 group **(C)** and the FD4 *vs.* FE5 group **(D)**; Venn diagram illustrating the overlapping and specific differential ingredients for four comparison groups (FA1 *vs.* FB2, FB2 *vs.* FC3, FC3 *vs.* FD4, FD4 *vs.* FE5) **(E)**.

3.3.1. Changes in Phenolic acids Profile

Phenolic acids are one of the main components of DO, which are common in natural plants. As shown in the correlation heat map (Figure 4), there is a significant correlation between total polyphenols and antioxidant capacity. The 68 phenolic acid differentials chemical ingredients were screened in the DO FA1 *vs.* FE5 group (Figure S3A), with 31 up-regulated and 37 down-regulated. There were 21 unique differential ingredients in the FA1 *vs.* FB2 group during the processing of DO (Table 1). In the FA1 *vs.* FB2 group, 6 differential chemical ingredients such as 3,4,5-trimethoxybenzoic acid were down-regulated, 15 differential chemical ingredients such as 4-hydroxybenzaldehyde were up-regulated. Among them, vanillin (Figure S4A) and 4-hydroxybenzaldehyde were up-regulated after roasting processing at 85 °C, which results in the existence of aromas increasing, and its changes may be closely related to lignans. Some studies found that lignans are widely present in plants, which can be oxidized to form vanillin under high-temperature conditions [22]. In the correlation analysis, vanillin has strong antioxidant capacity that reacted with ABTS radicals *via* a self-dimerization mechanism [23]. In addition, the phenolic acid chemical ingredients such as syringaldehyde-4-*O*-glucosideand 2-*O*-galloyl-D-glucose which contain glycosides were also upregulated after baking at 85 °C. The fold changes (Log_2FC) of some glycosides vary greatly, for example, the 2-*O*-galloyl-D-glucose and syringaldehyde-4-*O*-glucoside, in the order of 15.80 and 13.04, respectively (Figure S4B). Meanwhile, the content of syringaldehyde showed a downward trend from FA1 to FB2 processes. Yu et al. [24] found the presence of glycosylation modifying enzymes and pathways of phenolic acids in poplar, and accordingly the above phenolic glycosides may be formed from phenolic acids modified by glycosides. In the study of glycosides synthesis by Chen et al. [25], temperature was an important factor in enhancing the conversion rate of chemical ingredients. The specific differential metabolites in the FD4 *vs.* FE5 group included seven species of vanillic acid, methyl syringate (Figure S4D), and 4-hydroxybenzoic acid which showed a downward trend. Meanwhile, the relative content of syringic acid and *p*-coumaric acid were increased in FD4 compared with FE5 (Figure S4E). The 35°C of drying has a greater influence on the content of compounds containing carboxyl and ester bonds, which may be the reversible reaction catalyzed by esterase and the decrease of ester content due to high acidity [26]. Phenolic acid specific differential chemical ingredients were not generated in the curling and shaping processes. In addition, coniferyl alcohol was a persistent differential metabolite that was down-regulated in the curling process (Figure S4F). The fixation procedure in this experiment is equivalent to extrusion in usual food processing, and in this experiment, coniferyl alcohol was up-regulated after fixing, which was consist well with the results from extrusing black rice studied by Ti et al. [27].

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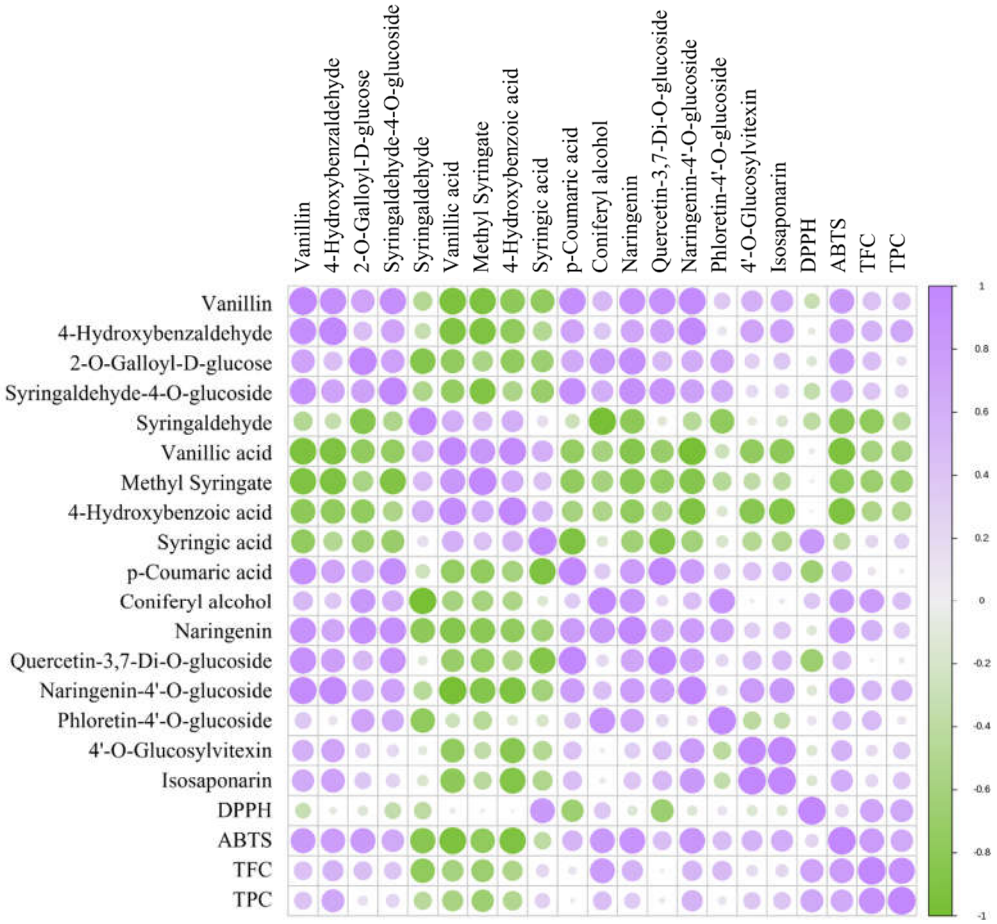

Figure 4. Correlation heatmap of specific differential ingredients and antioxidant activity. TPC = total phenolic content; TFC = total flavonoid content; DPPH = DPPH· scavenging capacity; ABTS = ABTS·+ scavenging capacity.

Table 1. Unique differential ingredients (VIP ≥ 1) responsible for the chemical ingredient variation caused by the traditional processing of DO.

Number	Compounds	Formula	*m/z*	Factor	Fold Change	VIP	*P*-value
			Phenolic acids				
1	1-*O*-Sinapoyl-*β*-D-glucose	$C_{17}H_{22}O_{10}$	386.3506	FA1 *vs.* FB2	2.89E+00 ↑	1.173	7.30E-03
2	Dihydrocaffeoylglucose	$C_{15}H_{20}O_9$	344.3139	FA1 *vs.* FB2	9.27E+03 ↑	1.185	2.41E-03
3	Syringaldehyde-4-*O*-glucosid	$C_{15}H_{20}O_9$	344.3139	FA1 *vs.* FB2	8.44E+03 ↑	1.185	4.56E-03
4	Sinapinaldehyde	$C_{11}H_{12}O_4$	208.2106	FA1 *vs.* FB2	2.47E+00 ↑	1.172	6.90E-05
5	Isovanillin	$C_8H_8O_3$	152.1473	FA1 *vs.* FB2	4.52E+00 ↑	1.174	9.44E-03
6	1-*O*-Feruloyl-*β*-D-glucose	$C_{16}H_{20}O_9$	356.3246	FA1 *vs.* FB2	1.22E+01 ↑	1.180	4.84E-05
7	2-(Formylamino)benzoic acid	$C_8H_7NO_3$	165.1461	FA1 *vs.* FB2	2.48E+00 ↑	1.167	2.46E-04

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8	Caffeic aldehyde	$C_9H_8O_3$	164.1580	FA1 *vs.* FB2	2.04E+00 ↑	1.106	3.50E-03
9	Vanillin	$C_8H_8O_3$	152.1473	FA1 *vs.* FB2	4.52E+00 ↑	1.181	2.61E-04
10	*p*-Coumaric acid	$C_9H_8O_3$	164.1580	FA1 *vs.* FB2	2.51E+00 ↑	1.178	3.37E-03
11	4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.1213	FA1 *vs.* FB2	2.14E+00 ↑	1.156	2.85E-03
12	Methyl 4-hydroxybenzoate	$C_8H_8O_3$	152.1743	FA1 *vs.* FB2	4.82E+00 ↑	1.181	6.34E-06
13	Gallacetophenone	$C_8H_8O_4$	168.1467	FA1 *vs.* FB2	1.23E+04 ↑	1.185	1.43E-04
14	Cinnamic acid	$C_9H_8O_2$	148.1586	FA1 *vs.* FB2	6.44E+00 ↑	1.168	1.99E-03
15	2-*O*-Galloyl-D-glucose	$C_{13}H_{16}O_{10}$	332.2601	FA1 *vs.* FB2	6.02E+04 ↑	1.185	5.85E-05
16	1-*O*-(3,4-Dihydroxy-5-methoxy-benzoyl)-glucoside	$C_{14}H_{18}O_{10}$	346.2867	FA1 *vs.* FB2	2.65E-01 ↓	1.151	3.58E-02
17	2-Acetyl-3-hydroxyphenyl-1-*O*-glucoside	$C_{15}H_{20}O_7$	312.3151	FA1 *vs.* FB2	3.48E-01 ↓	1.166	1.34E-02
18	Vanillin acetate	$C_{10}H_{10}O_4$	194.1840	FA1 *vs.* FB2	3.26E-01 ↓	1.135	5.10E-02
19	2,5-Dihydroxybenzaldehyde	$C_7H_6O_3$	138.1207	FD4 *vs.* FE5	7.29E-05 ↓	1.193	6.80E-04
20	4-Hydroxybenzoic acid	$C_7H_6O_3$	138.1207	FD4 *vs.* FE5	4.79E-01 ↓	1.211	7.53E-04
21	Ferulic acid methyl ester	$C_{11}H_{12}O_4$	208.2106	FD4 *vs.* FE5	2.45E-01 ↓	1.199	1.77E-03
22	Methyl syringate	$C_{10}H_{12}O_5$	212.1993	FD4 *vs.* FE5	3.90E-01 ↓	1.173	3.03E-02
23	Vanillic acid	$C_8H_8O_4$	168.1467	FD4 *vs.* FE5	4.46E-01 ↓	1.215	4.53E-04
24	4-*O*-Methylgallic Acid	$C_8H_8O_5$	184.1461	FA1 *vs.* FB2	4.09E-01 ↓	1.102	3.26E-03
25	Eudesmic acid	$C_{10}H_{12}O_5$	212.1993	FA1 *vs.* FB2	1.29E-01 ↓	1.159	1.78E-03
26	3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid	$C_9H_{10}O_4$	182.1733	FD4 *vs.* FE5	4.16E-01 ↓	1.145	2.41E-02
27	4-Methylphenol	C_7H_8O	108.1378	FA1 *vs.* FB2	4.55E-02 ↓	1.178	9.79E-03
28	Methyl 3-(4-hydroxyphenyl) propionate	$C_{10}H_{12}O_3$	180.2005	FD4 *vs.* FE5	6.99E-04 ↓	1.208	1.03E-01
		Flavonoids					
29	Isorhamnetin-3-*O*-sophoroside	$C_{28}H_{32}O_{17}$	640.5435	FA1 *vs.* FB2	3.29E+00 ↑	1.148	4.77E-04
30	Butin	$C_{15}H_{12}O_5$	272.2528	FA1 *vs.* FB2	9.44E+00 ↑	1.185	2.00E-04

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31	7-Methoxy-3-[1-(3-pyridyl) methylidene]-4-chromanone	$C_{16}H_{13}NO_3$	267.2793	FA1 *vs.* FB2	2.41E+00	↑ 1.142	4.64E-03
32	Isorhamnetin-3-O-rutinoside-4'-*O*-glucoside	$C_{34}H_{42}O_{21}$	786.6847	FA1 *vs.* FB2	2.50E+00	↑ 1.147	5.82E-03
33	3,4'-Dihydroxyflavone	$C_{15}H_{10}O_4$	254.2375	FA1 *vs.* FB2	1.02E+03	↑ 1.185	1.91E-03
34	6,7,8-Tetrahydroxy-5-methoxyflavone	$C_{16}H_{12}O_6$	300.2629	FA1 *vs.* FB2	2.07E+00	↑ 1.133	1.95E-03
35	Tangeretin	$C_{20}H_{20}O_7$	372.3686	FD4 *vs.* FE5	3.43E+00	↑ 1.215	2.67E-04
36	Naringenin	$C_{15}H_{12}O_5$	272.2528	FA1 *vs.* FB2	9.79E+00	↑ 1.184	1.09E-03
37	3,5,7,3'4'-Pentamethoxyflavone	$C_{20}H_{20}O_7$	372.3686	FD4 *vs.* FE5	2.87E+00	↑ 1.205	3.37E-04
38	Quercetin-3-*O*-(6''-*O*-*p*-coumaroyl) sophoroside-7-*O*-rhamnoside	$C_{42}H_{46}O_{23}$	918.8008	FA1 *vs.* FB2	3.72E+00	↑ 1.175	1.37E-02
39	Apigenin-7,4'-dimethyl ether	$C_{17}H_{14}O_5$	298.2901	FD4 *vs.* FE5	3.11E+00	↑ 1.189	3.72E-03
40	Quercetin-3-*O*-glucosyl (1→4) rhamnoside-7-*O*-rutinoside	$C_{39}H_{50}O_{25}$	918.7993	FA1 *vs.* FB2	3.12E+00	↑ 1.165	1.53E-02
41	5,6,7,3',4',5'-hexanmethoxyflavone	$C_{21}H_{22}O_8$	402.3946	FA1 *vs.* FB2	5.91E+00	↑ 1.181	1.73E-03
42	Eriodictyol-3'-*O*-glucoside	$C_{21}H_{22}O_{11}$	450.3928	FA1 *vs.* FB2	3.88E+00	↑ 1.169	4.17E-03
43	1,8-dihydroxy-2,6-dimethylxanthen-9-one	$C_{15}H_{12}O_4$	256.2534	FA1 *vs.* FB2	1.34E+04	↑ 1.185	1.12E-02
44	Pinocembrin	$C_{15}H_{12}O_4$	256.2534	FA1 *vs.* FB2	4.11E+00	↑ 1.129	1.16E-02
45	Quercetin-3,7-di-*O*-glucoside	$C_{27}H_{30}O_{17}$	626.5169	FA1 *vs.* FB2	2.57E+03	↑ 1.184	1.55E-02
46	Naringenin-4'-*O*-glucoside	$C_{21}H_{22}O_{10}$	434.3934	FA1 *vs.* FB2	2.02E+00	↑ 1.163	1.46E-02
47	Trilobatin	$C_{21}H_{24}O_{10}$	436.4093	FA1 *vs.* FB2	2.43E+00	↑ 1.039	6.96E-02
48	Isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside	$C_{34}H_{42}O_{21}$	786.6847	FA1 *vs.* FB2	4.43E+00	↑ 1.147	8.48E-03

↑ : Fold Change ≥ 2; ↓: Fold Change ≤ 0.5.

3.3.2. Changes in flavonoids Profile

The biosynthetic pathway of flavonoids in plants is complex, andmost of them exist in the form of flavonoid-bound glycosides, while a few ones in free form. The 35 flavonoid differential ingredients were screened in the FA1 *vs.* FE5 group, leading to the fact that 31 compounds were up-regulated and 4 ones were down-regulated. Among them 17 flavonoids that include naringenin, quercetin-3,7-di-*O*-glucoside, naringenin-4'-*O*-glucoside, and phloretin-4'-*O*-glucoside (Figure S4G) were up-regulated as unique differential ingredients in the FA1 *vs.* FB2 group (Table 1).

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Due to the loss of water during baking at 85°C, almost no glycoside hydrolysis reaction exists. It is widely known that enzyme and moisture are two key factors in glycoside hydrolysis, so that the first barking has the effect of killing enzymes and protecting glycosides [28]. The naringenin, quercetin-3,7-di-*O*-glucoside, naringenin-4'-*O*-glucoside, phloretin-4'-*O*-glucoside, and other flavonoid glycoside ingredients are positively correlated with the radical scavenging ability of ABTS in the process from FA1 to FB2. It is recorded that TFC prevented the accumulation of lipofuscin and protein carbonylation, and upregulated the gene expression levels of hsp-16.2, gst-4, etc. [29]. In this experiment, no flavonoid specific differential ingredients were found in the two processes of curling and shaping during the processing of DO. Then the 4'-*O*-glucosylvitexin, isosaponarin were common differential ingredients (Figure S4H-I), some were down-regulated in the process of curling and up-regulated in the shaping step. This phenomenon is consistent with the finding from twin-screw extrusion of Folium Artemisiae Argyi and puffed cereals [30,31].

3.3.3. Changes in amino acids and derivatives profile

In the current study, A total of 88 amino acids were identified in the FA1 *vs.*FE5 group (Figure S3C), with 69 metabolites down-regulated and only 19 ones up-regulated, showing an overall trend of down-regulation. Amino acids are divided into essential amino acids and non-essential amino acids., and eight of the essential amino acids, L-threonine, L-isoleucine, L-leucine, L-valine, L-phenylalanine, L-methionine, L-lysine, and L-tryptophan have been taking on down-regulation during the processing of DO, which was influenced by baking at 85 °C and drying at 35 °C. Furanosine is a product of the L-lysine melad reaction [32]. When the internal temperature of grilling and frying is lower than 90°C, lysine is mainly synthesized as furanosine [33]. The important condition for significant changes in 13 non-essential amino acids such as γ-glutamyl phenylalanine, L-aspartic acid and *N*-methylglycine during DO processing was 85 °C drying. Meanwhile, 16 non-essential amino acids were significantly down-regulated when drying at 35 °C. During heat processing, the sum of all free amino acids in the heated samples was significantly lower than in the raw sample, it is consistent with the amino acid changes after temperature treatment in this experiment [34].

3.3.4. Changes in Lipids Profile

Based on the dynamic changes of the lipid during the processing of DO, 118 lipid differential ingredients were screened from the FA1 *vs.* FE5 group. Among them, just half of which were up-regulated (Figure S3D). Lipids can be further divided into lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), glycerol ester (GE), free fatty acids (FC), phosphatidylcholine (PC), and 3-Hydroxy-palmitic acid methyl ester. There are 15 FC such as hydroperoxylinoleic acid and 3-hydroxy-palmitic acid methyl ester, 4 LPE such as LPE 17:0 and LPE 16:0, and 11 LPC such as LPC 20:2 and LPC 12:0 in DO processing as the specific differential ingredients in the FA1 *vs.* FB2 group. In the FA1 *vs.* FB2 group, the FC were down-regulated, while LPC and LPE were up-regulated. In addition, there are 13 down-regulated FCs such as γ-linolenic acid and α-hydroxy linoleic acid, which were also present in the FD4 *vs.* FE5 group as specific differential ingredients. The decrease of FCs may be partly due to the inactivation of lipase (LA) and lipoxygenase (LOX) caused by high-temperature processing [35], which catalyzed the hydrolysis of triacylglycerols (TAGs) to release FC [36]. It is worth mentioning that the inactivation of LA and LOX is related to two key factors, namely temperature and time [37], which is consistent with DO 85 °C baking and 35 °C drying in this study. It indicates that heating is detrimental to the retention of FC. Although curling and setting did not occur specific differential ingredients, some GEs were affected by them and showed an upregulation trend. Yang et al. [38] suggested that the increasing trend of the bound lipid of noodles continued during the pressing process, and the comparison of the most important GE of pressed and leached oil tea seed oil, pressed GE exceeds leached GE by 30%. It indicates that extrusion facilitates lipid retention.

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3.4. KEGG function annotation and enrichment analysis

The differential ingredients were analyzed by KEGG metabolic pathways separately. Firstly, the 88 amino acids and their derivatives were annotated into 55 metabolic pathways, of which the 6 essential amino acids were significantly enriched in glucosinolate biosynthesis (ko00966) (Figure 4A). Meanwhile, 118 differential lipids ingredients were annotated to 10 metabolic pathways (Figure 4B), with alpha-Linolenic acid metabolism (ko00592) and Linoleic acid metabolism (ko00591) being the two most significantly enriched pathways (Figure 4C), which were associated with the synthesis of oxylipins in fatty acid metabolism which can promote lipid metabolism. The phenolic acids and flavonoids as secondary ingredients, 68 differential phenolic acids were annotated to 13 metabolic pathways (Figure 4D), with three metabolic pathways more significantly enriched in phenylalanine metabolism (ko00360), phenylpropanoid biosynthesis (ko00940) and ubiquinone and other terpenoid quinone biosynthesis (ko00130).

3.5. Processing-induced chemical transformation mechanisms

Generally, the processing-induced compositional variations of DO are predominantly driven by temperature, water, and external force, which also uncover chemical transformation mechanisms by processing to some extent [39]. With regard to the shift of differential ingredients, many reactions may be involved, such as oxidation, hydrolyze, esterification of small-molecular compounds as well as condensation, glycosylation, etc. According to the metabolomics results, the differential ingredients of flavonoids were taken as an example to briefly describe the changes (Figure 5). First of all, the reaction for glycoside hydrolysis was disrupted by a sharp decrease in moisture content, and the hydrolase enzymes gradually lost their activity which contribute to retaining component. The contents of aglycones such as quercetin and phloretin showed a downward trend in FA1 *vs*. FB2 group. At the same time, glycosides (quercetin-3,7-di-*O*-glucoside and phloretin-4'-*O*-glucoside) were up-regulated in FA1 *vs*. FB2, suggesting that aglycones were converted into glycosides by polysaccharide or monosaccharide modification. It also indicates that the rate of glycosylation was greater than de-glycosylation when baking at 85 °C. It is suggests that a broad range of flavonoid substrates could be glycosylated at their 3- and/or 7-hydrogen sites by the recombinant enzyme, such as quercetin [40]. Besides, some phenolic hydroxyl, and ester, as well as glycosides containing glycosidic bonds, might involve oxidative decomposition, and esterification of alcoholic hydroxyl by processing. It may be the potential chemical mechanism that raw materials and processed DO represent different substances.

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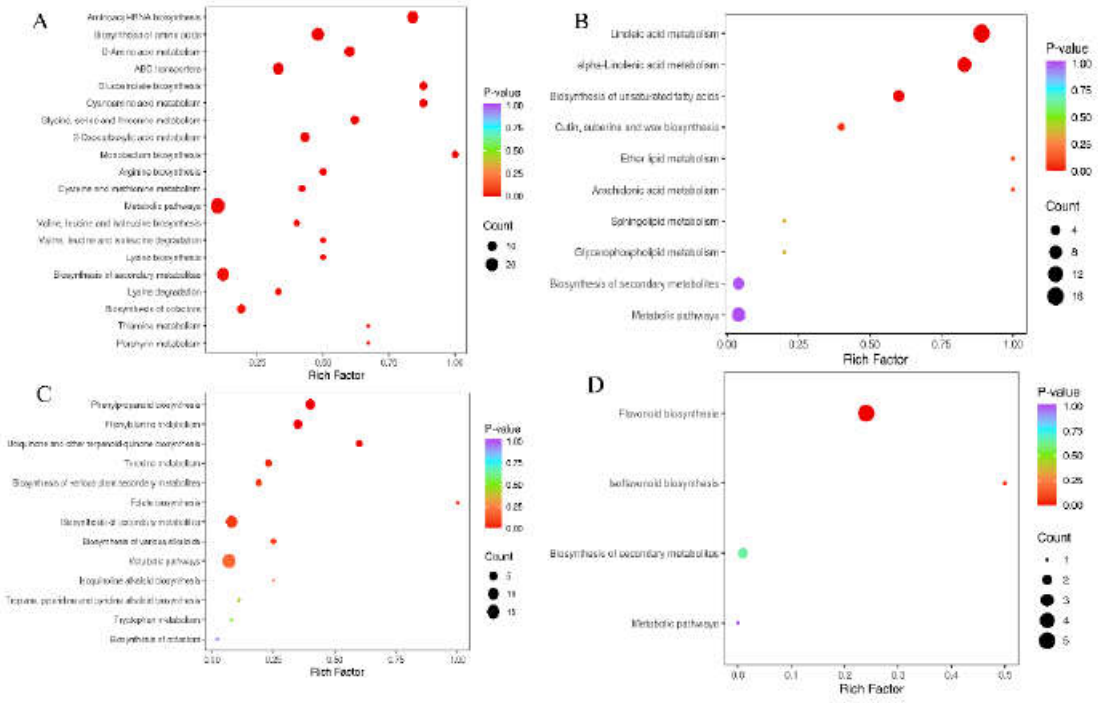

Figure 5. KEGG pathway analysis of differential ingredients for four Class (phenolic acids, flavonoids, amino acids and their derivatives, lipids). KEGG pathway for amino acids and their derivatives **(A)**, the lipids **(B)**, the phenolic acids **(C)**, the flavonoids **(D)**. In the plot, each bubble (which represents a metabolic pathway) and abscissa indicate the size of the factors affecting the pathway (bigger bubbles represent bigger impacts). Bubble color indicates the p-value of the enrichment analysis. Lighter colors indicate lower enrichment.

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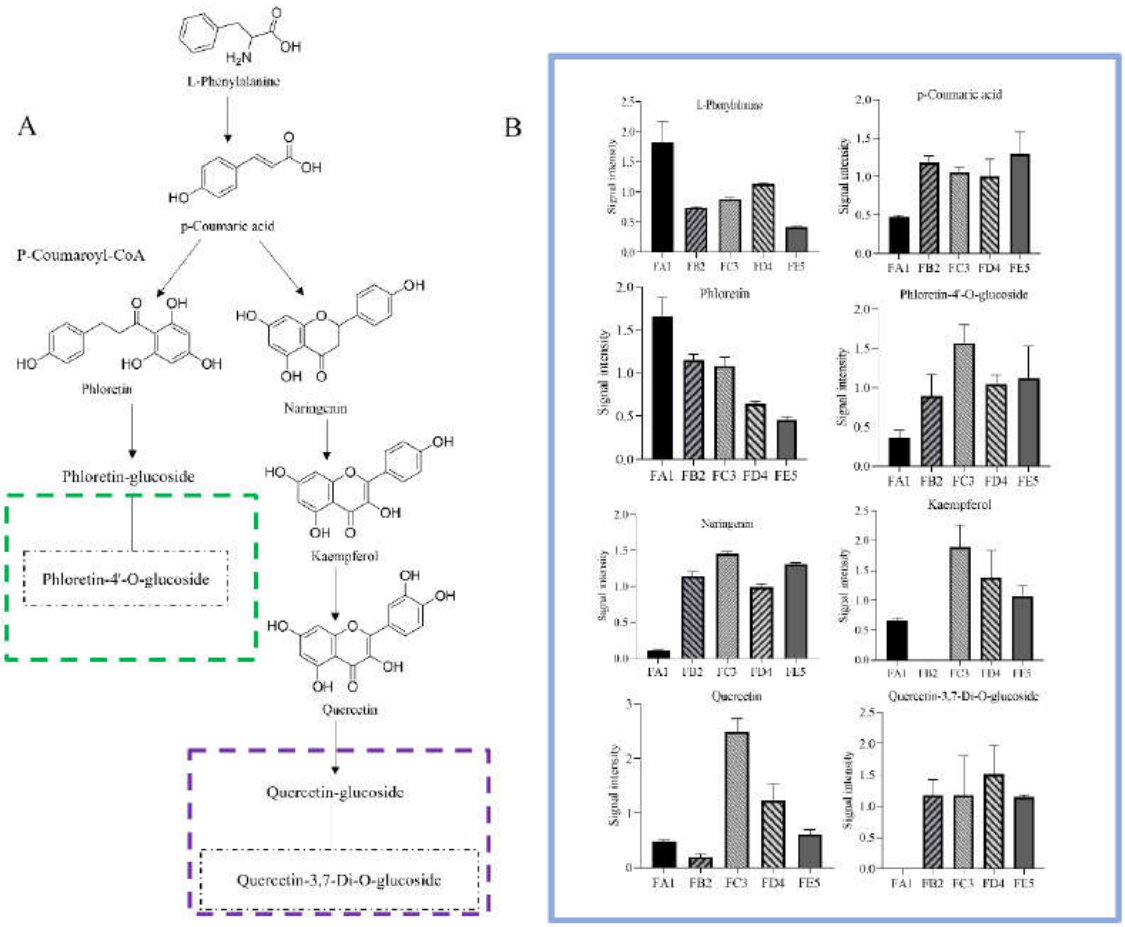

Figure 6. The alteration of the flavonoid pathway **(A)** and relative amount **(B)** during the processing of DO.

4. Conclusions

In this study, the antioxidant activity was the best from FE5 during the different procedures of DO traditional processing. Liquid mass spectrometry-based metabolomics techniques were used to identify changes in ingredients during the traditional processing of fresh strips to DO. The main factors leading to the change of composition during the processing were baking at 85 °C and drying at 35 °C, with less effect of curling and setting. Phenolic acids, flavonoids, lipids and amino acids and their derivatives changed most significantly during the baking at 85 °C and drying at 35 °C. Phenolic acids, flavonoids and lipids produced 28, 20 and 45 unique differential ingredients, respectively, and 8 essential amino acids, which were affected by the baking at 85 °C and drying at 35 °C, and the changes in their contents may affect the quality and activity of DO. *P*-coumaric acid, a unique differential metabolite and a natural antioxidant, connected glucosinolate biosynthesis, flavonoid biosynthesis, phenylpropanoid biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis and other terpenoid-quinone metabolic pathways. By precisely regulating the key ingredients in the pathway, the material basis for the antioxidant capacity of DO was established. This study provides a reference for the quality control and enhancement of active ingredients during DO processing.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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