Identification of the Oxidative Products and Ozonolysis Pathways of Polyphenols in Peanut Skins

Enjie Diao, Xiangzhen Shen, Zheng Zhang, Ning Ji, Wenwen Ma, Haizhou Dong[*]

College of Food Science and Engineering, Shandong Agricultural university, Taian, People' Republic of China
*Corresponding author: haizhoudong@126.com

Abstract Many studies have proved that ozone can decompose aflatoxins in peanuts, while polyphenols in peanut skins can also be destroyed due to its strong oxidative capacity. The polyphenols and their oxidative products in the peanut skin extracts at different ozonated times were separated and identified by HPLC-Q-TOF/MS. Based on the accurate molecular weight from mass spectrogram, and consulted the reported literatures, nine polyphenols and nine main oxidative products were identified in the peanut skins. According to the oxidative mechanism of phenols and the Criegee mechanism of ozonolysis, the ozonolysis pathways of nine polyphenols were proposed. The structures of oxidative products showed that ozone can destroy the polyphenols, so the effects of ozone treatment on the peanut skins should be considered by processors in the detoxification of aflatoxin-contaminated peanuts.

Keywords: polyphenols, ozone, oxidative product, criegee mechanism, peanut skin

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1. Introduction

Polyphenols are natural components in peanut skins that possess beneficial properties acting as natural antioxidants, colorants, antimicrobials and vasodilators [1,2,3]. In the storage of peanuts, the polyphenol contents in peanut skins are influenced by temperature, oxygen, light, enzyme, metallic ions [4,5].

Peanuts are widely consumed in China owing to their high nutritional values and good taste. However, they are often contaminated by aflatoxins before or after harvest when being exposed to a high humidity and temperature environment. Aflatoxins are associated with various diseases, such as aflatoxicosis and cancer in domestic animals and humans throughout the world [6]. It is not always possible to prevent peanuts from being contaminated by toxigenic fungi, so physical, chemical and biological methods have been developed to remove or degrade aflatoxins in contaminated products [7,8,9].

Ozone as a detoxification agent has been widely used to decompose aflatoxins in foods due to its many advantages, such as high detoxification efficiency, reactivity, penetrability, and spontaneous decomposition into a non-toxic product (oxygen) without forming any hazardous residues on the treated products [10,11,12].

However, ozone is a strong oxidant (2.07 mV) after fluorine [13], which can also destroy polyphenols in foods [2,14]. At present, the oxidative products and ozonolysis pathways of polyphenols in peanut skins have not been studied and reported. So, the objectives of this study were to identify the polyphenols and their oxidative products in

peanut skins by HPLC-Q-TOF/MS, and then deduced the ozonolysis pathways of polyphenols based on their oxidative products.

2. Materials and Methods

2.1. Materials

Unpeeled peanuts (*Arachis hypogaea*, *Baisha* variety) were purchased from local market. Peanut skins were obtained by artificial peeling and freeze-dried (<1% of moisture content), and then stored in a dark at 4°C until analysis.

Methanol (HPLC-grade), was purchased from Shandong Yuwang company, China. Chloroform, ethanol, ethyl acetate, glacial acetic acid were all analytical-grade, and obtained from Tianjin Sitong company. Water was purified using a Aike Lab pure water system (Taiwan, China).

2.2. Methods

2.2.1. Ozone Treatment of Peanut skins

Peanut skins were ozonated according to the procedure provided by Diao et al. [15] using a self-made ozone treatment system (Figure 1). Briefly, 300 g of peanut skins were put into the ozonation column and treated by ozone (50 mg/L of ozone at a flow rate of 5 L/min) for 60 h. 60 g samples were taken from the ozonation column at 12 h intervals, and another 60 g sample without being treated by ozone was taken as the control. The control and

treated samples were packed in black polyethylene bags and stored in the dark at 4°C.

Figure 1. Schematic of ozone treatment system

2.2.2. Separation and Purification of Polyphenols in Peanut skins

The polyphenols were extracted by the method of Yu et al. with minor modification [1]. Briefly, the ozonated peanut skins at different exposure times were milled into fine powder (pass 60 meshes) with a grinder (XW-25, Jiuyang, Shanghai). The milled skins (10.0 g) were extracted with 200 mL 80% (v/v) methanol in water with the help of ultrasound at 25 kHz for 30 min at ambient temperature. The slurry was vacuum-filtered using 0.22 μm filter membrane, and then methanol was evaporated from the filtrate under reduced pressure using a rotary evaporator (R-1001, Greatwall, Zhengzhou) at 35°C. After removing of the methanol, the crude extract was redissolved in 50.0 mL deionized water. 50.0 mL chloroform were added in the crude extract solution to remove lipids using a separatory funnel, and then 50.0 mL ethyl acetate were added in the water phase to separate the phenols from water. The water phase was removed, and the ethyl acetate was evaporated under reduced pressure using the rotary evaporator at 35°C. The extract was redissolved in 50.0 mL methanol and then was vacuum-filtered using 0.22 μm filter membrane. The all resulting extracts were stored in glass containers wrapped in foil at 4°C until used, respectively. The whole processes were completed under dim light to minimize light induced degradation of polyphenols.

2.2.3. Identification of Polyphenols and Oxidative Products by HPLC-Q-TOF/MS

The extracts of peanut skins were analyzed using a LC 1200 high performance liquid chromatography (Agilent, Palo Alto, CA) equipped with an autoinjector and a quaternary HPLC pump. Chromatography was performed on a 4.6 × 250 mm inner diameter, 5 μm, VP-ODS C18 column. The injection volume was 10.0 μL. The mobile phase was 0.5% glacial acetic acid in water (A) and 0.5% glacial acetic acid in methanol (B). The gradient parameters used were as follows: elution rate of 1.0 mL/min, with %(B)=10-20% (0-5 min), 20-50% (6-20 min), 50-80% (21-30 min), 80% (31-35 min), 80-10% (36-40 min), and 10% (41-45 min).

In order to identify the polyphenols and their oxidative products in peanut skins, Q-TOF/MS was used to determine their molecular weights. It was performed on a Q-TOF/MS equipped with a dual electrospray interface (Agilent 6520). TOF/MS analysis was performed in a negative-ion mode using full scan mode and the mass range of *m/z* 100-2000. The optimized conditions were as follows: capillary and fragmentor voltages were 3500 V and 175 V, respectively; the skimmer voltage was 65.0 V; the flow rate of drying gas (N_2) was 12.0 L/min; drying gas temperature was 350°C; nebulizer was 35 psi, and octopole radio frequency was 750 V. The data station operating software used was the Mass Hunter Workstation software (version B.04.00). A reference mass solution containing reference ions 112.9856 and 1033.9881 was used to maintain mass accuracy during the run time.

3. Results and Discussion

3.1. Identification of Polyphenols and Oxidative Products by LC-Q-TOF/MS

LC-Q-TOF-MS has been widely used to analyze and identify organic compounds and their degradation products of food contaminants in many literatures [15,16,17,18]. Qiu et al. [19] and Appeldoorn et al. [20] have identified the polyphenols in peanut skins/shell by LC-ESI/MS, Maldi-TOF/MS, and DPPH-HPLC-DAD-TOF/MS, respectively. In addition, Palafox-Carlos et al. [21], Sarnoski et al. [22], Reed, [23] and Lazarus et al. [24] had used LC-MS to separate, identify and quantify the major phenolic compounds in mango fruit and peanut skins. Lou et al. [25,26] had also identified the A-type and B-type polyphenols from peanut skins using the NMR spectra.

For this experiment, HPLC-Q-TOF/MS was used to identify the polyphenols in the peanut skins. Firstly, LC was used to separate the polyphenols and their oxidative products (OPs) in peanut skin extracts, and the base peak chromatogram of separated polyphenols is presented in Figure 2.

Seen from Figure 2 A, nine polyphenols were separated from the control peanut skin extract. At 12 h of ozonation time, the peak areas of most polyphenols separated were decreased due to the oxidative role of ozone excepted for polyphenols 5, 7, and 8 (Figure 3 A), which were increased probably due to the release of phenolic compounds ozone-induced previously bound within the various cellular components of peanut skins, or the degradation of larger phenolic compounds into smaller ones [1,27,28,29,30]. During the whole ozonation process, nine OPs were separated from ozonated peanut skin extracts, and their peak areas were increased generally, especially the OP7, which increased greater than those of other OPs (Figure 3 B). Seen from Figure 3, the peak areas of polyphenols were decreased with the increase of ozonation time, while those of OPs were increased firstly and then decreased due to the further ozonolysis of OPs. The polyphenols 2 and 6 were completely decomposed at 36 h and 48 h of ozonation times, respectively, and the OP3, OP8, and OP9 were completely degraded at 60 h and 48 h of ozonated times, respectively. Therefore, ozone treatment can severely destroy the polyphenols in peanut skins, and their OPs can be further decomposed completely by ozone within enough time.
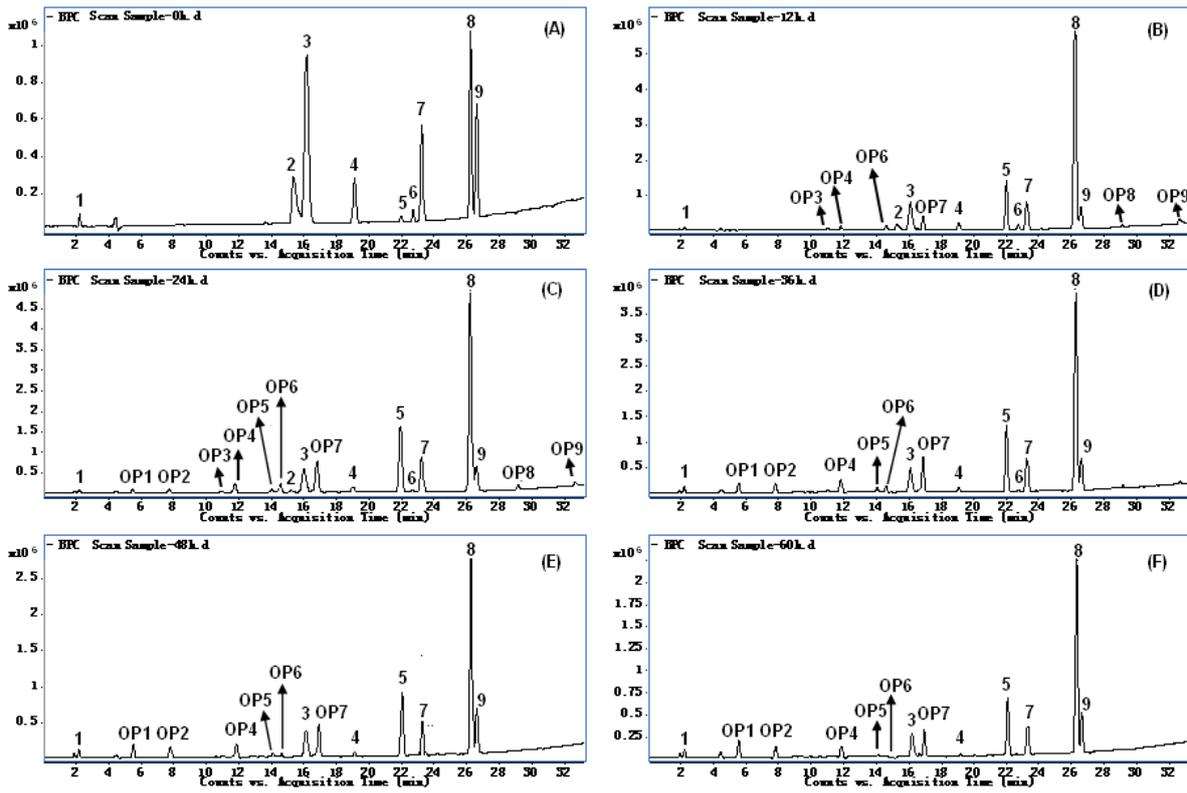
Figure 2. Base peak chromatogram of polyphenols and their oxidative products in the peanut skins at 0 h (A), 12 h (B), 24 h (C), 36 h (D), 48 h (E), and 60 h (F) of ozonation times
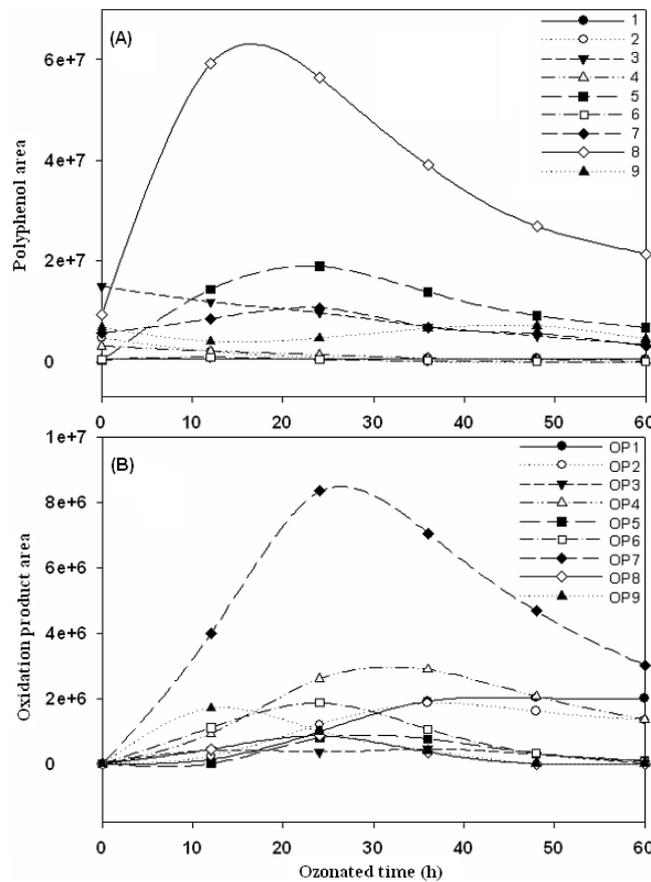

Figure 3. Peak areas of polyphenols (A) and their oxidative products (B) in the peanut skins at different ozonation times

3.2. Structural Identification of Polyphenols and Oxidative Products

To confirm the structures of polyphenols and their OPs, it is important to further analyze their accurate masses. Q-TOF/MS of the polyphenols and their OPs were performed to pass to the collision cell using the ion-filtering function of Q-TOF. The accurate masses and molecular formulas of these compound ions (Table 1) were obtained by using the mass calculator and formula calculator, two functions of Agilent Mass Hunter qualitative analysis software. Based on the accurate masses of the compound ions and referred to the reported literatures, the structures of polyphenols and their OPs were deduced preliminary and presented in Figure 4.

Seen from the Figure 4, the peanut skin extracts contain some smaller polyphenols, such as 1,2,3,5-tetrahydroxybenzene (No. 1), shikimic acid (No. 5), 1,4-dihydroxynaphthalene (No. 6), and 5-hydroxy-2-methyl-1,4-naphtoquinone (No. 8), and some larger ones, such as dimer (No. 3 and No. 4) or trimer (No. 2) of flavan-3-ols and flavanone trimer (No. 9). No. 7 is a conjugate of epigallocatechin gallate and gallic acid [31]. Most OPs of polyphenols are ketonic acids (OP1, OP2, OP4, and OP7), phenolic acids (OP3), quinones (OP5 and OP6), and mixed acids (OP8 and OP9). They could be further oxidized to the final products CO_2 and H_2O within an enough ozonation time [32,33]. In addition, some intermediate products were produced in the ozonation process, which could not be detected due to their lower contents or were rapidly oxidized to other products by ozone.

3.3. Ozonolysis Pathways of Polyphenols in Peanut Skins

According to the structural identification of polyphenols and their OPs in the peanut skin extracts, the ozonolysis pathways of polyphenols present in peanut skins are proposed. The ozonolysis pathways of 1,2,3,5-tetrahydroxybenzene (No. 1), procyanidin trimer (No. 2) and procyanidin dimer (No. 3 and No. 4) were presented in Figure 5. The 1,2,3,5-tetrahydroxybenzene was firstly oxidized to cyclohexanehexaone by ozone based on the oxidative mechanism of phenols, and then it was decomposed to 2,3-diketo-succinic acid (OP4) and mesoxalic acid (OP1).

Table 1. Identification of the polyphenols and their oxidative products in the peanut skins during ozone treatment[a]

Peak No.	Retention time (min)	Molecular formula	Observed mass[b] (*m/z*)	Calculated mass[b] (*m/z*)	Mass difference (ppm)	Score[c] (%)	Proposed Products [2,19-26,31]
1	2.23	$C_6H_6O_4$	141.0165	141.0163	-1.42	100	1,2,3,5-Tetrahydroxybenzene
OP1	5.51	$C_3H_2O_5$	117.0194	117.0190	-3.42	100	Mesoxalic acid
OP2	7.76	$C_4H_4O_5$	131.0352	131.0346	-4.58	100	Ketosuccinic acid
OP3	10.94	$C_7H_6O_4$	153.0192	153.0193	0.65	100	2,4-Dihydroxybenzoic acid
OP4	11.74	$C_4H_2O_6$	145.0506	145.0509	2.07	100	2,3-Diketo-succinic acid
OP5	14.08	$C_{11}H_6O_4$	201.0761	201.0763	0.99	100	2-Methyl-5,8-diketo-1,4-naphtoquinone
OP6	14.60	$C_{11}H_6O_4$	201.0761	201.0756	3.48	100	2-Methyl-5,6-diketo-1,4-naphtoquinone
2	15.35	$C_{45}H_{36}O_{18}$	863.1836	863.1829	-0.81	100	Procyanidin trimer[E(C)-A-E(C)-B-E(C)]
3	16.16	$C_{30}H_{24}O_{12}$	575.1216	575.1195	-3.65	100	Procyanidin dimer[E(C)-A-E(C)]
OP7	16.81	$C_5H_4O_6$	159.0663	159.0655	-5.03	100	2,3-Diketoglutaric acid
4	18.99	$C_{30}H_{24}O_{12}$	575.1209	575.1195	-2.43	100	Procyanidin dimer[E(C)-A-E(C)]
5	21.93	$C_7H_{10}O_5$	173.0845	173.0855	5.78	100	Shikimic acid
6	22.66	$C_{10}H_8O_2$	159.1044	159.1052	5.03	100	1,4- Dihydroxynaphthalene
7	23.24	$C_{29}H_{24}O_{16}$	627.0991	627.0992	0.16	100	[Epigallocatechin gallate-gallic acid]
8	26.21	$C_{11}H_8O_3$	187.0401	187.0401	0.00	100	5-Hydroxy-2-methyl-1,4-naphtoquinone
9	26.57	$C_{45}H_{28}O_{18}$	855.1212	855.1203	-1.05	100	3',4',5,7-Tetrahydroxy-flavanone A-type trimer
OP8	29.15	$C_7H_{10}O_8$	201.0315	201.0303	-5.97	100	3,4,5-Trihydroxyl-2-keto-pimelic acid
OP9	32.62	$C_{10}H_8O_6$	223.0252	223.0248	-1.79	100	5,7-Dihydroxy-benzopyran-4-one-2-carboxylic acid

[a] Ozone conditions: concentration, 50 mg/L; flow rate, 5 L/min; exposure time, 0-60 h.

[b] The *m/z* in the table is the *m/z* of [M-H]⁺.

[c] Score: An overall score is 0-100% , the score closer to 100% is better.
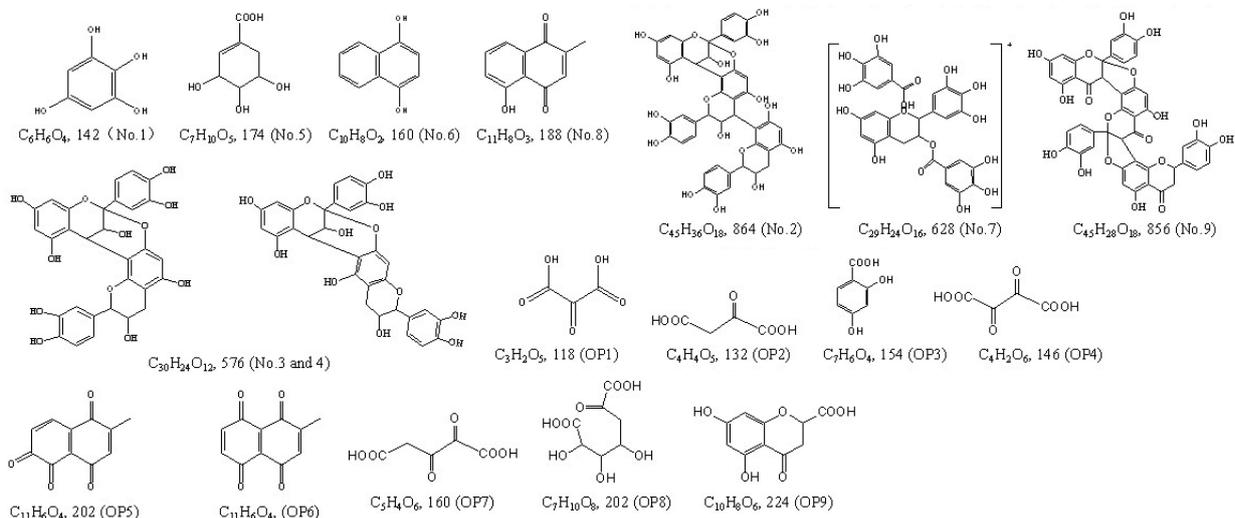

Figure 4. Structures of polyphenols and their oxidative products in the peanut skins
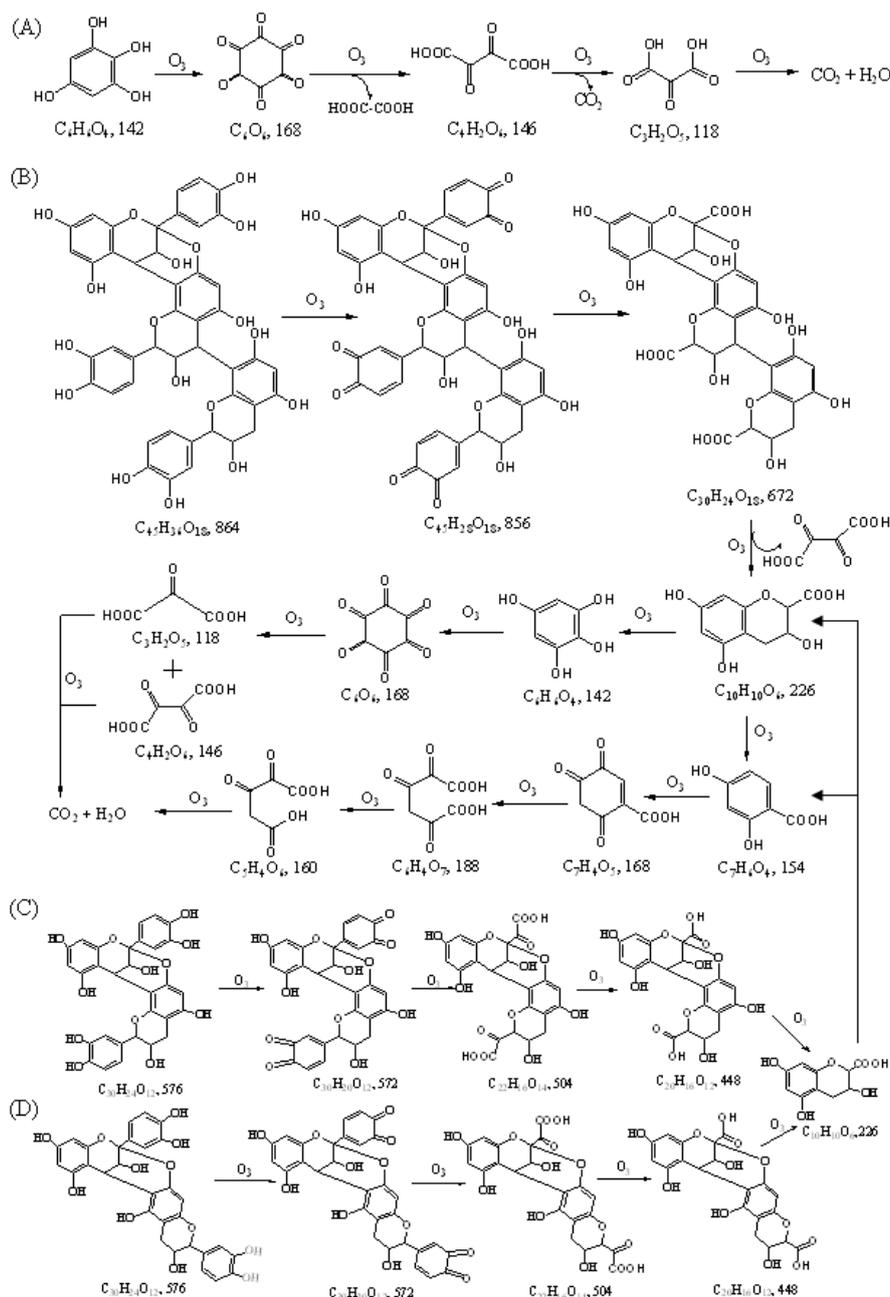
Figure 5. Ozonolysis pathways of polyphenols 1 (A), 2 (B), 3 (C) and 4 (D) in the peanut skins

The final oxidative products were CO_2 and H_2O within enough ozonation time (Figure 5 A).

The procyanidin trimer (No. 2) is a polymer of catechin (C) or epicatechin (E) with a linkage of E (C)-A-E (C)-B-E (C) (Figure 4) [22,23]. During the ozonation process, the two hydroxyls on the B ring of catechin/epicatechin were firstly oxidized to *o*-quinone according to the oxidative mechanism of phenols [33,34]. According to the Criegee mechanism of ozonolysis [35], i.e. the alkene double bonds were cleavaged by reacting with ozone, so B rings on the catechin/epicatechin were oxidized to the carboxyls or carbonyls, and formed the trimer of 3,5,7-trihydroxy-benzopyran-2-carboxylic acid by releasing the OP4. The newly formed trimer was further decomposed to its monomer. The monomer of 3,5,7-trihydroxy-benzopyran-2-carboxylic acid formed undergone two oxidation pathways. The one is that it was oxidized to the 1,2,3,5-tetrahydroxybenzene (No. 1), which was consistent with the peak area change of No. 1, i.e. it was

slightly increased and then slowly decreased in peak area during the ozonation process (Figure 3 A). The followed oxidation steps were the same as those of the 1,2,3,5-tetrahydroxybenzene (Figure 5 A). The other is that it was oxidized to the 2,4-dihydroxy-benzoic acid (OP3). The OP3 is a phenolic acid, which was rapidly oxidized to the corresponding ketonic acid. The ketonic acid was cleavaged by reacting with ozone based on the Criegee mechanism, and transformed to the 2,3,5-triketohexanedioic acid, which could be cleavaged to the 2, 3-diketoglutaric acid (OP7). The final products were also CO_2 and H_2O within an enough ozonation time.

The phenol compounds 3 and 4 are A-type dimers (2β-O-7, 4β-6, or 2β-O-7, 4β-8) of catechin or epicatechin [22,25]. Their oxidation processes are similar to each other, and the 3,5,7-trihydroxy-benzopyran-2-carboxylic acid is their common intermediate product (Figure 5 C and D). From the beginning of the intermediate product,

the followed oxidation steps are the same as those of the procyanidin trimer (Figure 5 B).

The shikimic acid (No. 5) could be oxidized by ozone with two pathways. The first one is that the three hydroxyls on the hexatomic ring were oxidized to three keto-groups, i.e. 3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid was transformed to 3,4,5-triketo-1-

cyclohexene -carboxylic acid [36], and then it further reacted with ozone based on the Criegee mechanism to form 2,3,4,6-tetraketo-pimelic acid. The carboxys on the both ends of 2,3,4,6-tetraketo-pimelic acid were cleavaged and formed the 2, 3-diketo-glutaric acid (OP7), which was further oxidized to the OP2 by ozone. The final oxidation products were also CO_2 and H_2O (Figure 6 A).
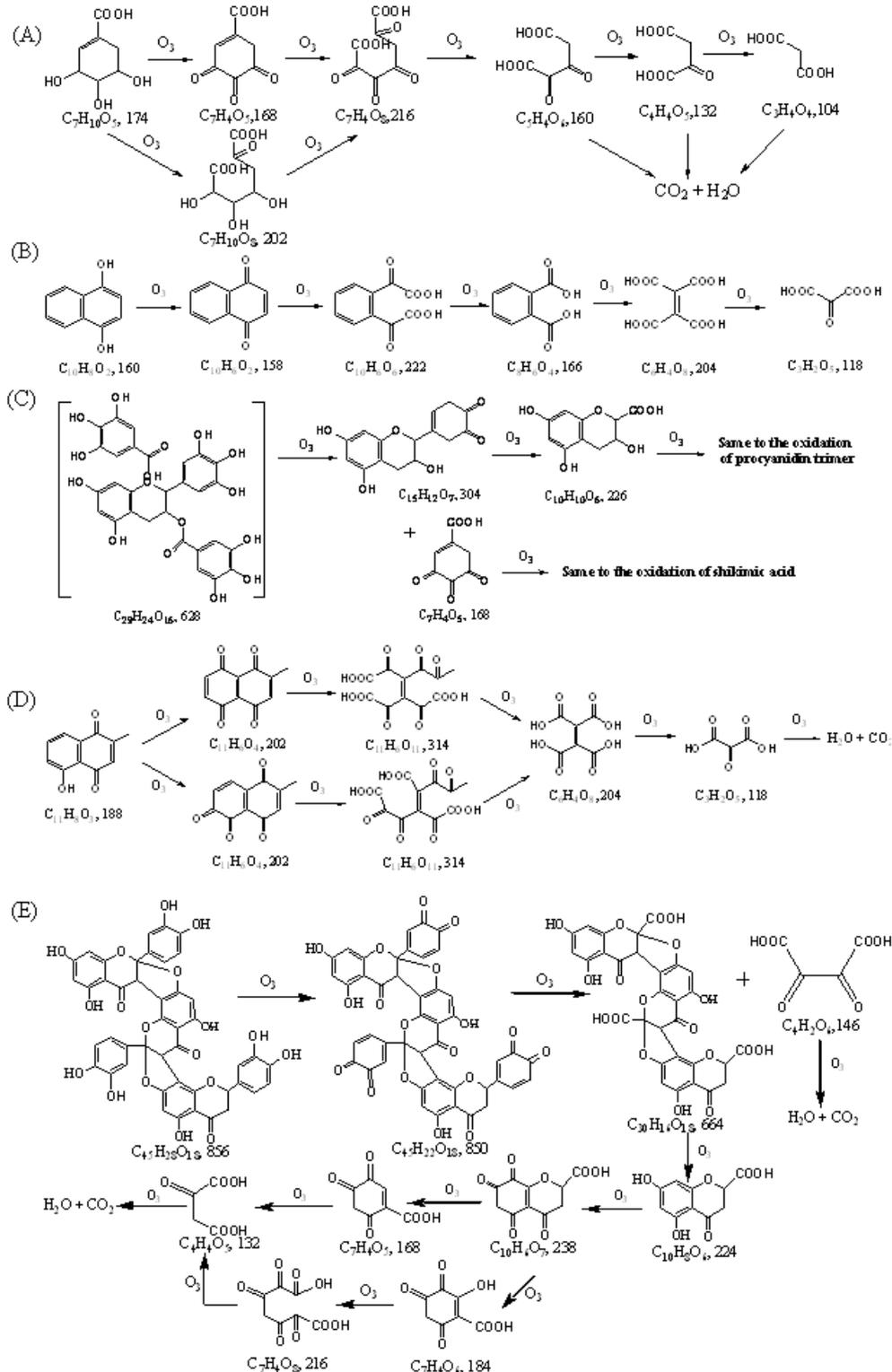

Figure 6. Ozonolysis pathways of polyphenols 5 (A), 6 (B), 7 (C), 8 (D) and 9 (E) in the peanut skins

The 1,4-dihydroxynaphalene (No. 6) was firstly oxidized to 1,4-naphthaquinone based on the oxidation mechanism of phenols, and then the new formed double

bond on the benzene ring was cleavaged by ozone based on the Criegee mechanism to form benzene-1,2-diformyl-carboxylic acid. The two carboxyls formed in the

benzene-1,2-diformyl-carboxylic acid were cleavaged and transformed to phthalandione. For the phthalandione, the double bonds on the 3 and 5 sites of benzene ring were oxidative-cleavaged to form the 2,3-dicarboxy-2-butene diacid, and the final oxidative product was the OP1 (Figure 6 B).

The conjugate of epigallocatechin gallate and gallic acid (No. 7) was dissociated to epigallocatechin and gallic acid by ozone, which were rapidly oxidized to the corresponding ketones (Figure 6 C). The three hydroxyls on the B ring of the epigallocatechin were oxidized to triketone of the epigallocatechin, and then transformed to 3,5,7-trihydroxy-benzopyran-2-carboxylic acid. The following ozonation processes were the same as those of the procyanidin trimer (No. 2).

The 5-hydroxy-2-methyl-1,4-naphtoquinone (No. 8) was firstly oxidized to much 2-methyl-1,4,5,8-naphtoquinone and few 2-methyl-1,4,5,6-naphtoquinone, and then they were oxidized and cleavaged at the sites of the double bond based on the Criegee mechanism, and then formed the corresponding ketonic acids. The common intermediate product was the 2,3-dicarboxy-2-butene diacid, and the final oxidative product was the OP1 (Figure 6 D).

The polyphenol 9 is an A-type trimer of flavanone with a linkage of [2β-O-7, 3β-8]. The two hydroxyls on the B ring of flavanone were firstly oxidized to *o*-quinone based on the oxidation mechanism of phenols (Figure 6 E). The 5,7-dihydroxy-benzopyrone-2-carboxylic acid formed in the oxidation process is an intermediate product, which is different from the intermediate product (3,5,7-trihydroxy-benzopyran-2-carboxylic acid) in the oxidation process of polyphenols 2, 3, 4, and 7. It was further decomposed through two pathways. The first pathway is that it was oxidized to 6-alkenyl-2,4,5-triketo-heptanaphthenic acid and then the OP2 was formed at the role of ozone. The second one is that it was oxidized to 1-alkenyl-2-hydroxy-3,4,6-triketo-heptanaphthenic acid, which was further reacted with ozone based on the Criegee mechanism, and formed the product 2,3,5,6-tetraketo-pimelic acid. The keto-pimelic acid was decomposed to the OP2 by ozone, and the final products were CO_2 and H_2O.

4. Conclusions

Our study shows that ozone, as a strong oxidation agent, can severely destroy the polyphenols in peanut skins. Many studies have verified that the antioxidant capacity of peanut skins is closely correlated to the contents of polyphenols. Thus, the destruction of polyphenols in peanut skins decreases their antioxidant capacity. HPLC can separate efficiently the polyphenols in peanut skin extracts, and Q-TOF/MS can measure accurately their molecular weights. Based on the results of HPLC-Q-TOF/MS, and referred to the reported literatures, nine polyphenols were separated and identified. Most intermediate products of polyphenols were ketonic acids, and the final products were CO_2 and H_2O. The oxidation mechanism of phenols and the Criegee mechanism of ozonolysis can be used to interpret the ozonolysis pathways of polyphenols in the peanut skin extracts.

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