



# Formation of phenylacetic acid and benzaldehyde by degradation of phenylalanine in the presence of lipid hydroperoxides: New routes in the amino acid degradation pathways initiated by lipid oxidation products

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## ABSTRACT

Lipid oxidation is a main source of reactive carbonyls, and these compounds have been shown both to degrade amino acids by carbonyl-amine reactions and to produce important food flavors. However, reactive carbonyls are not the only products of the lipid oxidation pathway. Lipid oxidation also produces free radicals. Nevertheless, the contribution of these lipid radicals to the production of food flavors by degradation of amino acid derivatives is mostly unknown. In an attempt to investigate new routes of flavor formation, this study describes the degradation of phenylalanine, phenylpyruvic acid, phenylacetaldehyde, and β-phenylethylamine in the presence of the 13-hydroperoxide of linoleic acid, 4-oxononenal (a reactive carbonyl derived from this hydroperoxide), and the mixture of both of them. The obtained results show the formation of phenylacetic acid and benzaldehyde in these reactions as a consequence of the combined action of carbonyl-amine and free radical reactions for amino acid degradation.

## 1. Introduction

Amino acid catabolism produced by microorganisms in foods is responsible for the formation of both pleasant and unpleasant aroma compounds (Chen & Liu, 2016; Geffroy et al., 2018; Lee, Cheong, Curran, Yu, & Liu, 2016). In addition, this process is also responsible for the production of compounds that may compromise consumers' health, such as biogenic amines (Liu, Xu, Ma, & Guo, 2018; Tomita, Nakamura, & Okada, 2018). All these "good" and "bad" compounds can also be produced by chemical means in foods as a consequence of carbonyl-amine reactions. Thus, the importance of Maillard reaction on the flavor profile of foods has long been known (Chigwedere et al., 2019; Hou et al., 2017). In addition, this reaction has received an increased attention in recent years because of its involvement in the production of vinyl derivatives of amino acids such as acrylamide (Belkova et al., 2018).

A lesser-known pathway for amino acid degradation is that

produced by lipid-derived reactive carbonyls. Lipid oxidation is a main source of carbonyl compounds in foods (Bastos, Costa, & Pereira, 2017), and these compounds have been shown to degrade amino acids in a similar way to that of carbohydrate-derived reactive carbonyls (Hidalgo & Zamora, 2016). In fact, the production of Strecker aldehydes (Hidalgo & Zamora, 2004), α-oxoacids (Zamora, Navarro, Gallardo, & Hidalgo, 2006), amines (Zamora, Delgado, & Hidalgo, 2012), and vinyl derivatives (Zamora & Hidalgo, 2008) by amino acid degradation initiated by lipid-derived reactive carbonyls has been shown.

However, reactive carbonyls are not the only products of the lipid oxidation pathway. In fact, these carbonyls are produced as a consequence of the breakage of the primary lipid oxidation products (lipid hydroperoxides), a reaction that also produces free radicals in addition to other less reactive compounds. Differently to the role of lipid-derived reactive carbonyls on amino acid degradation, very little is known on the ability of free radicals derived from lipid hydroperoxides to degrade amino acids.

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The hypothesis of this study was that the presence of lipid hydroperoxides would produce amino acid degradation, and that some of the produced products might be different to those produced by carbonyl-amine reactions, which were previously described (Hidalgo & Zamora, 2016). Hence, the objective of this research was to study comparatively the degradation of phenylalanine (1), and some of its derivatives, in the presence of the 13-hydroperoxide of linoleic acid (LOOH), 4-oxononanal (ON, a reactive carbonyl derived from this hydroperoxide), and the mixture of both of them. This knowledge would contribute to the understanding of the amino acid degradations produced when free radicals, reactive carbonyls, or both of them are present, and would complete the general scheme previously proposed for amino acid degradation by lipid-derived reactive carbonyls (Hidalgo & Zamora, 2016). As phenylalanine derivatives, phenylpyruvic acid (2), phenylacetaldehyde (3), and  $\beta$ -phenylethylamine (4) were selected because they have been shown to be the first degradation products of phenylalanine in the presence of lipid-derived reactive carbonyls (Hidalgo & Zamora, 2016).

## 2. Materials and methods

### 2.1. Materials

As lipid oxidation products, LOOH and ON were employed as models of a lipid hydroperoxide and a reactive carbonyl derived from it, respectively. LOOH was prepared by oxidation of linoleic acid with lipoxygenase (Zamora, Gallardo, & Hidalgo, 2008), and ON was prepared by ring opening of 2-pentylfuran with *N*-bromosuccinimide (Zamora, Alcon, & Hidalgo, 2012). Methyl 13-hydroxyoctadeca-9,11-dienoate was prepared by reduction of LOOH with sodium borohydride and later esterification with diazomethane as described previously (Zamora et al., 2008). This compound was employed as standard to study the conversion of LOOH into its corresponding alcohol.

As phenylalanine derivatives, phenylalanine (1), phenylpyruvic acid (2), phenylacetaldehyde (3),  $\beta$ -phenylethylamine (4), phenylacetic acid (5), and benzaldehyde (6) were employed. All these compounds, as well as other chemicals employed in these studies were purchased from Sigma-Aldrich (St. Louis, MO), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland), and were of the highest available grade (usually, > 99%).

### 2.2. Degradation of phenylalanine, and its derivatives, in the presence of lipid oxidation products

Solutions of phenylalanine (1, 10  $\mu$ mol), or one of its derivatives (phenylpyruvic acid (2), phenylacetaldehyde (3), or  $\beta$ -phenylethylamine (4), 10  $\mu$ mol), the lipid oxidation product(s) (LOOH, ON, or both of them, 10  $\mu$ mol when a single compound was employed or 10  $\mu$ mol of each when the mixture was added), 100  $\mu$ L of water, 100  $\mu$ L of methanol, and 300  $\mu$ L of 0.3 M sodium phosphate buffer were heated at 100 °C for 1 h. Two pH values (3 and 8) and two reactions atmospheres (air and nitrogen) were assayed. At the end of the heating time, solutions were cooled to room temperature and treated differently depending on the compounds to be determined.

Reaction mixtures assigned to determination of phenylacetaldehyde (3) and benzaldehyde (6) were treated with 30  $\mu$ L of internal standard (a solution of 54.8 mg of methyl heptanoate in 25 mL of methanol) and 500  $\mu$ L of acetonitrile, and were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) using oven temperature program no. 1.

Reaction mixtures assigned to determination of phenylalanine (1), phenylpyruvic acid (2),  $\beta$ -phenylethylamine (4), and phenylacetic acid (5), were treated with 40  $\mu$ L of 1 M NaOH, and 15 mg of sodium borohydride. Mixtures were stirred and kept under dark for 30 min. Then, 200  $\mu$ L of acetone was added to eliminate sodium borohydride excess. Reduced samples were diluted with 100  $\mu$ L of methanol, and 60  $\mu$ L of

1 M NaOH, 35  $\mu$ L of pyridine, and 20  $\mu$ L of methyl chloroformate were added. Samples were then stirred and, after 20 s, other 20  $\mu$ L of methyl chloroformate was added. Samples were newly stirred and, after 2 min, 0.1 M sodium bicarbonate solution (400  $\mu$ L) was added. After new stirring, 30  $\mu$ L of internal standard (a solution of 54.8 mg of methyl heptanoate in 25 mL of methanol) and 700  $\mu$ L of dichloromethane were added. After a final stirring, samples were centrifuged at 2000g for 5 min, and the organic layer was separated and analyzed by GC-MS using oven temperature program no. 2. An analogous procedure was also employed for preparing the derivative of methyl 13-hydroxyoctadeca-9,11-dienoate.

### 2.3. Degradation of phenylpyruvic acid in the presence of *t*-butyl hydroperoxide

To confirm the role of radical reactions in the degradations produced by LOOH and not by ON, the degradation of phenylpyruvic acid (2) in the presence of *t*-butyl hydroperoxide using the same conditions described above was also studied. Briefly, solutions of phenylpyruvic acid (10  $\mu$ mol), *t*-butyl hydroperoxide or ON (10  $\mu$ mol) or the mixture of *t*-butyl hydroperoxide and ON (10  $\mu$ mol of each), 100  $\mu$ L of water, 100  $\mu$ L of methanol, and 300  $\mu$ L of 0.3 M sodium phosphate buffer, pH 8, were heated at 100 °C for 1 h under air. At the end of the heating time, solutions were cooled and treated differently depending on the compounds to be determined as described in Section 2.2.

### 2.4. GC-MS analyses

GC-MS analyses were conducted with an Agilent 7820A gas chromatograph coupled with an Agilent 5977 mass selective detector, quadrupole type (Agilent Technologies, Santa Clara, CA). Compounds were separated on a fused-silica HP-5MS UI capillary column (30 m length, 0.25 mm inner diameter, 0.25  $\mu$ m coating thickness) from Agilent, and 1  $\mu$ L of sample was injected in the pulsed splitless mode. The following working conditions were employed: carrier gas, helium (1 mL/min at constant flow); injector, 250 °C; transfer line to mass selective detector, 280 °C; electron ionization (EI), 70 eV; ion source temperature, 230 °C; and mass range, 28–550 amu. Two different oven temperature programs were employed depending on the compounds to be determined, as indicated above. Oven temperature program no. 1 was from 40 °C (3 min) to 200 °C at 20 °C/min and then held at 200 °C for 1 min. Oven temperature program no. 2 was from 80 °C (1 min) to 150 °C at 10 °C/min, then to 300 °C at 20 °C/min, and finally held at 300 °C for 1 min.

### 2.5. Determination of phenylalanine and its derivatives

Phenylalanine (1), phenylpyruvic acid (2), phenylacetaldehyde (3),  $\beta$ -phenylethylamine (4), phenylacetic acid (5), and benzaldehyde (6) were quantified by preparing standard curves of these compounds and using the same procedures described above (including reduction and derivatization when required). Six concentration levels (0, 2, 4, 6, 8, and 10  $\mu$ mol) were used for each compound. Compound content was directly proportional to compound/internal standard area ratio ( $r > 0.99$ ,  $p < 0.001$ ). Relative standard deviation (RSD) was always < 10%.

### 2.6. Statistical analysis

All data are mean  $\pm$  SD values of, at least, three independent experiments. Because samples had to be treated differently depending on the compounds to be determined, all experiments were repeated at least six times and, then, three samples were employed to determine phenylacetaldehyde (3) and benzaldehyde (6), and another three samples were employed to determine phenylalanine (1), phenylpyruvic acid (2),  $\beta$ -phenylethylamine (4), and phenylacetic acid (5). Statistical

**Table 1**  
Phenylalanine derivative recovered after 1 h heating as a function of reaction pH, reaction atmosphere, and the presence of lipid oxidation products.

Initial phenylalanine derivative	Reaction pH	Reaction atmosphere	Lipid oxidation product			
			None	LOOH	ON	LOOH + ON
Phe (1)	3	air	954.32 ± 187.46 a,A	805.62 ± 74.13 a,A	614.51 ± 87.08 a,B	525.41 ± 29.74 a,B
	3	nitrogen	764.44 ± 74.87 a,A	781.92 ± 49.69 a,b,A	467.88 ± 67.89 b,B	458.49 ± 11.42 a,B
	8	air	876.42 ± 49.25 a,A	644.38 ± 64.44b,B	503.53 ± 39.93 a,b,C	542.15 ± 21.24 a,C
	8	nitrogen	767.89 ± 41.74 a,A	726.73 ± 71.49 a,A	496.98 ± 63.14 a,b,C	471.24 ± 58.58 a,B
PPA (2)	3	air	840.35 ± 92.06 a,A	301.68 ± 24.76 a,B	735.42 ± 27.02 a,A	331.11 ± 65.58 a,B
	3	nitrogen	978.50 ± 49.60 a,A	507.88 ± 55.88 b,B	655.93 ± 59.68 a,C	481.95 ± 44.99 b,B
	8	air	816.10 ± 39.26 a,A	11.68 ± 3.57 c,B	202.81 ± 15.63 b,C	47.53 ± 18.25 c,B
	8	nitrogen	885.94 ± 114.12 a,A	15.51 ± 8.16 c,B	252.62 ± 60.18 b,B	80.54 ± 21.99 c,B
PAC (3)	3	air	660.01 ± 42.31 a,A	528.05 ± 6.88 a,B	625.65 ± 36.54 a,A	529.36 ± 33.35 a,B
	3	nitrogen	631.25 ± 46.54 a,A	613.37 ± 43.31 a,A	657.60 ± 54.87 a,A	611.22 ± 14.72b,A
	8	air	381.91 ± 36.16 b,A	414.82 ± 44.18 b,A	398.14 ± 71.78 b,A	387.08 ± 5.77 c,A
	8	nitrogen	351.04 ± 12.23 b,A	367.23 ± 21.61 b,A	365.80 ± 22.95 b,A	346.11 ± 38.65 c,A
PEA (4)	3	air	442.47 ± 44.88 a,A	354.24 ± 33.33 a,A,B	346.11 ± 35.76 a,b,B	205.20 ± 15.82 a,C
	3	nitrogen	459.36 ± 33.32 a,A	354.94 ± 31.23 a,A	446.23 ± 149.05 a,A	257.45 ± 15.37 a,A
	8	air	370.49 ± 93.07 a,A	235.20 ± 23.35 b,B	236.93 ± 10.61 b,B	125.51 ± 30.58 b,B
	8	nitrogen	330.98 ± 71.56 a,A	191.99 ± 9.07 b,B,C	249.95 ± 10.84 a,b,A,B	115.05 ± 14.32 b,C

Values (in  $\mu\text{mol}/\text{initial mmol}$ ) correspond to the phenylalanine derivative recovered at the end of the incubation time (1 h at 100 °C) and are mean  $\pm$  SD for, at least, three independent experiments. For each phenylalanine derivative, means in the same column with a different lower case letter are significantly different ( $p < 0.05$ ). Means in the same row with a different upper case letter are significantly different ( $p < 0.05$ ). Abbreviations: Phe, phenylalanine; PPA, phenylpyruvic acid; PEA,  $\beta$ -phenylethylamine; PAC, phenylacetaldehyde; LOOH, 13-hydroperoxide of linolenic acid; ON, 4-oxo-2-nonenal.

differences among means were evaluated by employing analysis of variance (Snedecor & Cochran, 1980). When  $F$  values were significantly different, group differences were estimated by the Tukey test (Snedecor & Cochran, 1980). Statistical comparisons were carried out using Origin® v. 7.0 (OriginLab Corporation, Northampton, MA). The significance level is  $p < 0.05$  unless otherwise indicated.

### 3. Results

#### 3.1. Degradation of phenylalanine and its derivatives in the presence of LOOH, ON, and the mixture of them

Stability of phenylalanine and its derivatives upon heating in the presence of lipid oxidation products depended on the kind of derivative, the lipid oxidation product involved, and the reaction conditions. Table 1 collects the amount of phenylalanine derivative remaining in the reaction mixture after 1 h at 100 °C.

Phenylalanine (1) was relatively stable in the absence of lipid oxidation products under air and at pH 3, and there were not significant losses when heated under nitrogen or at pH 8. However, LOOH decreased the amount of phenylalanine (1) recovered at pH 8 under air, and ON and the mixture of LOOH and ON decreased the amount of phenylalanine (1) recovered at the two pH values assayed and in the presence and in the absence of oxygen. These results suggested that phenylalanine (1) was more stable in the presence of LOOH than in the presence of ON.

Results were different when phenylpyruvic acid (2) was studied. Thus, although both LOOH and ON decreased the amount of phenylpyruvic acid (2) under the four reaction conditions assayed, phenylpyruvic acid (2) disappearance was significantly ( $p < 0.05$ ) higher in the presence of LOOH than in its absence. Therefore, these results suggested that phenylpyruvic acid (2) was more stable in the presence of ON than in the presence of LOOH.

Phenylacetaldehyde (3) was not very stable under the employed reaction conditions, even in the absence of lipid oxidation products. Thus, most assayed reaction conditions produced a high degradation of this aldehyde, which was higher at pH 8. When lipid oxidation products were present, observed degradation was similar to that observed in the absence of lipid oxidation products with the exception of pH 3 under air and in the presence of LOOH.

$\beta$ -Phenylethylamine (4) also resulted unstable under the employed reaction conditions and more than 50% of the amine disappeared after heating under the different assayed conditions. In addition, it was degraded to a higher extent at pH 8 than at pH 3. In the presence of lipid oxidation products, amine losses increased and the highest losses were observed when LOOH and ON were simultaneously present. In addition, LOOH and ON produced similar degradation.

Observed disappearances of phenylalanine (1), phenylpyruvic acid (2), phenylacetaldehyde (3), and  $\beta$ -phenylethylamine (4) were produced simultaneously to the appearance of other phenylalanine derivatives (Tables 2–4). Among them, the formation of phenylacetic acid (5), phenylacetaldehyde (3) and benzaldehyde (6) in the presence of LOOH, ON, and both of them is described in the next sections. Although formation of both phenylpyruvic acid (2) and  $\beta$ -phenylethylamine (4) was also studied, these compounds were always absent in the analyzed samples, most likely because they were rapidly degraded once produced (see below).

#### 3.2. Formation of phenylacetic acid by degradation of phenylalanine and its derivatives in the presence of LOOH, ON, and the mixture of them

Formation of phenylacetic acid (5) by degradation of phenylalanine (and its derivatives) in the presence of LOOH, ON, and both of them is shown in Table 2. Phenylacetic acid (5) was mostly produced at pH 8 when starting from phenylpyruvic acid (2) and in the presence of LOOH (the conversion of phenylpyruvic acid into phenylacetic acid was almost 100% at pH 8 under air). When ON was also present, the amount of phenylacetic acid (5) produced was significantly reduced. When ON was the only lipid oxidation product present, there was not any significant difference between the amount of phenylacetic acid (5) produced in the presence of ON and that produced by the control without any lipid oxidation product added. This suggests that the conversion of phenylpyruvic acid (2) into phenylacetic acid (5) both needs the presence of free radicals and it is not produced by the reaction with lipid derived reactive carbonyls.

In addition to this major source of phenylacetic acid (5), this compound was also produced, although to a much lower extent, from phenylacetaldehyde (3) in the presence of LOOH and the mixture of LOOH and ON. On the other hand, phenylacetaldehyde (3) in the presence of ON did not produce more phenylacetic acid (5) than that

**Table 2**  
Phenylacetic acid formed after 1 h heating as a function of reaction pH, reaction atmosphere, and the presence of lipid oxidation products.

Initial phenylalanine derivative	Reaction pH	Reaction atmosphere	Lipid oxidation product			
			None	LOOH	ON	LOOH + ON
Phe (1)	3	air	n.d.a,A	2.76 ± 0.80 a,B	2.12 ± 0.14 a,b,B	14.76 ± 0.73 a,b,c,C
	3	nitrogen	n.d. a,A	2.89 ± 0.43 a,B	n.d. b,A	10.81 ± 0.59 a,b,c,C
	8	air	n.d. a,A	3.17 ± 0.44 a,B	8.59 ± 1.42c,C	7.99 ± 0.48 a,c,C
	8	nitrogen	n.d. a,A	1.04 ± 0.20 a,A	1.31 ± 0.76 a,b,A	3.43 ± 0.22 a,B
PPA (2)	3	air	3.74 ± 0.14 a,A	536.31 ± 56.94b,B	5.77 ± 0.02 a,c,A	407.49 ± 16.22 d,C
	3	nitrogen	2.53 ± 1.38 a,A	338.04 ± 24.97c,B	3.88 ± 0.47 a,b,A	254.08 ± 15.63 e,C
	8	air	39.91 ± 7.91b,A	965.40 ± 21.36 d,B	20.24 ± 2.98 d,A	544.10 ± 40.26f,C
	8	nitrogen	13.41 ± 1.28c,A	863.00 ± 93.04 e,B	8.73 ± 1.23c,A	468.51 ± 0.46 g,C
PAC (3)	3	air	21.02 ± 3.49 d,e,A	45.65 ± 10.21 a,B	22.65 ± 4.51 d,A	40.77 ± 0.05c,h,B
	3	nitrogen	23.71 ± 1.49 d,A	46.32 ± 4.83 a,B	24.80 ± 1.68 d,A	44.21 ± 3.83b,h,B
	8	air	16.35 ± 1.95c,e,A	34.15 ± 0.81 a,B	21.68 ± 1.33 d,C	25.42 ± 2.30 a,h,C
	8	nitrogen	15.84 ± 1.96c,e,A	39.51 ± 2.72 a,B	20.59 ± 1.87 d,A	28.86 ± 2.39 a,h,C

Values (in  $\mu\text{mol}/\text{mmol}$  of initial phenylalanine derivative) correspond to the phenylacetic acid produced at the end of the incubation time (1 h at 100 °C) and are mean  $\pm$  SD for, at least, three independent experiments. Means in the same column with a different lower case letter are significantly different ( $p < 0.05$ ). Means in the same row with a different upper case letter are significantly different ( $p < 0.05$ ). Phenylacetic acid was not produced by  $\beta$ -phenylethylamine degradation in the presence of lipid oxidation products. Abbreviations: Phe, phenylalanine; PPA, phenylpyruvic acid; PAC, phenylacetaldehyde; LOOH, 13-hydroperoxide of linolenic acid; n.d., not detected; ON, 4-oxo-2-nonenal.

produced when phenylacetaldehyde (3) was heated alone. Analogously to the above described for phenylpyruvic acid (2), the conversion of phenylacetaldehyde (3) into phenylacetic acid (5) needs the presence of free radicals and its formation is not favored in the presence of lipid carbonyls.

A final source of phenylacetic acid (5) was phenylalanine (1). In this case, the highest amounts of phenylacetic acid (5) were obtained when the amino acid was heated in the presence of the mixture of LOOH and ON at pH 3.

Differently to the above discussed for compounds 1–3,  $\beta$ -phenylethylamine (4) did not produce phenylacetic acid under the assayed reaction conditions (data not shown).

### 3.3. Formation of phenylacetaldehyde by degradation of phenylalanine and its derivatives in the presence of LOOH, ON, and the mixture of them

Formation of phenylacetaldehyde (3) by degradation of phenylalanine (and its derivatives) in the presence of LOOH, ON, and both of them is shown in Table 3. Phenylacetaldehyde (3) was mainly produced by degradation of phenylalanine (1) at pH 3 and in the presence of both air and the mixture of LOOH and ON, although the presence of lipid

oxidation products always promoted the formation of phenylacetaldehyde (3). In addition, the ability of LOOH to degrade the amino acid was similar to that of ON, therefore suggesting that the conversion of phenylalanine (1) into phenylacetaldehyde (3) can occur both by carbonyl-amine reactions (when reaction took place in the presence of ON) and by the involvement of free radicals (when reaction took place in the presence of LOOH).

Differently to the conversion of phenylalanine (1) into phenylacetaldehyde (3), phenylacetaldehyde formation from phenylalanine derivatives 2 and 4 was rather limited under the assayed reaction conditions and the addition of lipid oxidation products usually produced less phenylacetaldehyde (3) than that observed when the amino acid degradation product was heated alone (Table 3).

### 3.4. Formation of benzaldehyde by degradation of phenylalanine and its derivatives in the presence of LOOH, ON, and the mixture of them

Formation of benzaldehyde (6) by degradation of phenylalanine (and its derivatives) in the presence of LOOH, ON, or both of them is shown in Table 4. Benzaldehyde (6) had two main origins: phenylacetaldehyde (3) and phenylpyruvic acid (2).

**Table 3**  
Phenylacetaldehyde formed after 1 h heating as a function of reaction pH, reaction atmosphere, and the presence of lipid oxidation products.

Initial phenylalanine derivative	Reaction pH	Reaction atmosphere	Lipid oxidation product			
			None	LOOH	ON	LOOH + ON
Phe (1)	3	air	0.66 ± 1.33 a,b,A	53.29 ± 4.99 a,B	52.29 ± 7.64 a,B	83.19 ± 8.51 a,C
	3	nitrogen	2.65 ± 0.74 a,c,A	31.58 ± 6.09 b,B	58.83 ± 3.26 a,C	68.61 ± 2.68 b,D
	8	air	4.28 ± 1.53 c,A	28.45 ± 7.15 b,B	24.29 ± 1.51 b,B	29.99 ± 4.27 c,B
	8	nitrogen	2.04 ± 0.02 a,b,A	28.78 ± 1.10 b,B	22.41 ± 2.06 b,C	41.13 ± 1.14 d,D
PPA (2)	3	air	2.77 ± 0.28 a,c,A	1.64 ± 0.12 c,B	0.93 ± 0.07 c,C	2.05 ± 0.28 e,B
	3	nitrogen	1.31 ± 0.14 a,b,A	0.95 ± 0.15 c,B	0.31 ± 0.07 c,C	0.44 ± 0.06 e,C
	8	air	0.65 ± 0.05 a,b,A	0.13 ± 0.01 c,B	0.36 ± 0.05 c,C	0.20 ± 0.07 e,B
	8	nitrogen	0.88 ± 0.30 a,d,A	0.17 ± 0.09 c,B	0.39 ± 0.04 c,B	0.21 ± 0.02 e,B
PEA (4)	3	air	1.81 ± 1.49 a,d,A	0.31 ± 0.09 c,A	0.19 ± 0.07 c,A	0.48 ± 0.12 e,A
	3	nitrogen	0.64 ± 0.27 a,d,A,B	0.42 ± 0.07 c,A	0.51 ± 0.09 c,A	0.96 ± 0.15 e,B
	8	air	0.01 ± 0.01 b,d,A	0.01 ± 0.01 c,A	0.02 ± 0.01 c,A	0.02 ± 0.01 e,A
	8	nitrogen	n.d. b,d,A	0.78 ± 0.09 c,B	0.37 ± 0.04 c,C	0.30 ± 0.07 e,C

Values (in  $\mu\text{mol}/\text{mmol}$  of initial phenylalanine derivative) correspond to the phenylacetaldehyde produced at the end of the incubation time (1 h at 100 °C) and are mean  $\pm$  SD for, at least, three independent experiments. Means in the same column with a different lower case letter are significantly different ( $p < 0.05$ ). Means in the same row with a different upper case letter are significantly different ( $p < 0.05$ ). Abbreviations: Phe, phenylalanine; PPA, phenylpyruvic acid; PEA,  $\beta$ -phenylethylamine; LOOH, 13-hydroperoxide of linolenic acid; n.d., not detected; ON, 4-oxo-2-nonenal.

**Table 4**  
Benzaldehyde formed after 1 h heating as a function of reaction pH, reaction atmosphere, and the presence of lipid oxidation products.

Initial phenylalanine derivative	Reaction pH	Reaction atmosphere	Lipid oxidation product			
			None	LOOH	ON	LOOH + ON
Phe (1)	3	air	0.01 ± 0.02 a,A	1.07 ± 0.16 a,B	1.34 ± 0.15 a,C	n.d. a,A
	3	nitrogen	0.10 ± 0.09 a,A	0.44 ± 0.04 a,B	0.86 ± 0.06 a,C	1.26 ± 0.14 a,D
	8	air	0.02 ± 0.03 a,A	1.49 ± 0.26 a,B	0.67 ± 0.14 a,C	0.90 ± 0.08 a,C
	8	nitrogen	n.d. a,A	1.63 ± 0.02 a,B	0.46 ± 0.16 a,C	0.98 ± 0.06 a,D
PPA (2)	3	air	13.03 ± 1.46 b,A	23.62 ± 1.19 b,B	16.29 ± 0.26 b,A	22.31 ± 2.06 b,B
	3	nitrogen	11.99 ± 8.79 b,e,A	15.81 ± 0.56 c,B	17.43 ± 1.36 b,B	17.58 ± 0.87 c,f,B
	8	air	8.79 ± 7.44 c,e,f,A	11.02 ± 0.31 d,f,A	10.41 ± 0.47 c,e,g,A	9.65 ± 2.22 d,A
	8	nitrogen	7.44 ± 0.29 c,f,A	9.03 ± 1.69 d,A	8.10 ± 0.68 c,e,A	9.59 ± 0.63 d,A
PAC (3)	3	air	14.12 ± 1.59 b,A	40.68 ± 2.47 e,B	13.46 ± 1.92 d,f,A	36.11 ± 0.39 e,B
	3	nitrogen	18.92 ± 5.28 d,A	21.72 ± 3.93 b,A	13.59 ± 1.40 d,f,A	21.78 ± 2.95 b,c,A
	8	air	14.82 ± 1.49 b,A	20.05 ± 1.25 b,B	11.83 ± 2.82 f,g,A	16.90 ± 2.03 f,A,B
	8	nitrogen	11.15 ± 1.27 b,f,A	13.44 ± 2.70 c,f,A	9.13 ± 0.97 e,A	11.85 ± 2.54 d,A
PEA (4)	3	air	1.45 ± 0.64 a,A	2.11 ± 0.92 a,A	1.10 ± 0.27 a,A	1.47 ± 0.37 a,A
	3	nitrogen	0.90 ± 0.38 a,A	1.59 ± 0.14 a,B	1.06 ± 0.07 a,A,B	1.58 ± 0.14 a,B
	8	air	0.73 ± 0.30 a,A	1.92 ± 0.45 a,B	0.67 ± 0.12 a,A	1.18 ± 0.29 a,A,B
	8	nitrogen	0.69 ± 0.75 a,A	1.53 ± 0.12 a,A	1.13 ± 0.29 a,A	1.26 ± 0.59 a,A

Values (in  $\mu\text{mol}/\text{mmol}$  of initial phenylalanine derivative) correspond to the benzaldehyde produced at the end of the incubation time (1 h at 100 °C) and are mean  $\pm$  SD for, at least, three independent experiments. Means in the same column with a different lower case letter are significantly different ( $p < 0.05$ ). Means in the same row with a different upper case letter are significantly different ( $p < 0.05$ ). Benzaldehyde was not produced by phenylacetic acid degradation in the presence of lipid oxidation products. Abbreviations: Phe, phenylalanine; PPA, phenylpyruvic acid; PEA,  $\beta$ -phenylethylamine; PAC, phenylacetaldehyde; LOOH, 13-hydroperoxide of linolenic acid; n.d., not detected; ON, 4-oxo-2-nonenal.

Phenylacetaldehyde (3) was the main origin of benzaldehyde (6), mainly under air at pH 3 and in the presence of either LOOH or the mixture of LOOH and ON. On the other hand, phenylacetaldehyde (3) heated in the presence of ON did not produce more benzaldehyde (6) than the control. These results suggested that, analogously to the formation of phenylacetic acid (5), the formation of benzaldehyde (6) requires the presence of free radicals in the reaction mixture.

A significant amount of benzaldehyde (6) was also produced by degradation of phenylpyruvic acid (2), although to a lower extent than the observed for phenylacetaldehyde (3). Nevertheless, and analogously to that observed for the degradation of phenylacetaldehyde (3), benzaldehyde (6) was mainly produced under air at pH 3 and in the presence of either LOOH or the mixture of LOOH and ON. This also suggested a role of free radicals in the formation of benzaldehyde (6).

Contrarily to phenylacetaldehyde (3) and phenylpyruvic acid (2),  $\beta$ -phenylethylamine (4) only produced very small amounts of benzaldehyde (6). In addition, only LOOH and the mixture of LOOH and ON produced higher amounts of benzaldehyde (6) than the control, therefore confirming previous observations about the role of free radicals in the formation of benzaldehyde.

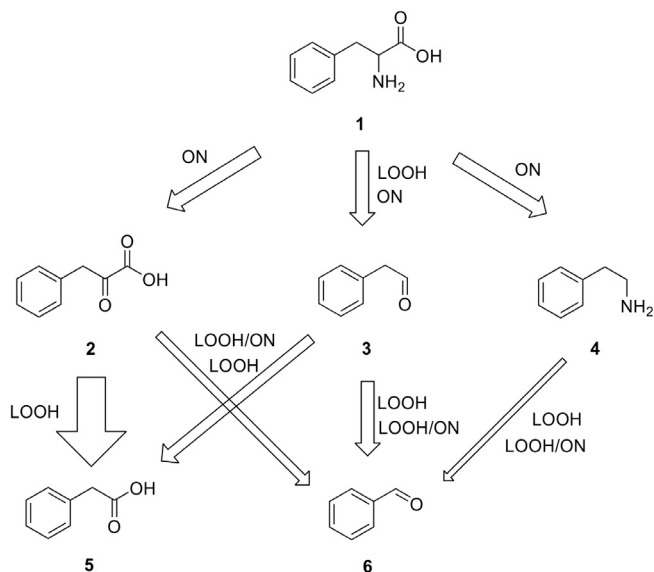
### 3.5. Degradation of phenylpyruvic acid in the presence of *t*-butyl hydroperoxide, ON, and the mixture of them

When LOOH was substituted by *t*-butyl hydroperoxide, which produces free radicals (Patel, Nandwana, Sah, & Kumar, 2018) but cannot form reactive carbonyls, analogous results to those above described were also obtained. Thus, phenylpyruvic acid (2) was converted almost 100% into phenylacetic acid (5) when the reaction was carried out only in the presence of *t*-butyl hydroperoxide at pH 8 under air (data not shown). This yield decreased to 72% when using the mixture of ON and *t*-butyl hydroperoxide, and yields decreased to 2–4% in the presence of only ON or in the absence of lipids, therefore confirming the essential role of free radicals in this reaction.

## 4. Discussion

Previous studies have shown that lipid-derived reactive carbonyls are able to degrade amino acids (Hidalgo & Zamora, 2016). Thus,

phenylalanine (1) has been shown to produce phenylpyruvic acid (2), phenylacetaldehyde (3), and  $\beta$ -phenylethylamine (4) in the presence of lipid-derived reactive carbonyls. However, these compounds are not final degradation products and, for example, the conversion of  $\beta$ -phenylethylamine (4) into styrene in the presence of lipid-derived reactive carbonyls has been shown (Hidalgo & Zamora, 2007). Fig. 1 shows a general scheme for these reactions. The results obtained in this study allow completing this scheme by incorporating reactions in which free radicals are needed. Thus, free radicals derived from LOOH have been shown both to be an additional origin of phenylacetaldehyde (3) and to participate in the degradation of phenylpyruvic acid (2), phenylacetaldehyde (3), and  $\beta$ -phenylethylamine (4). Two phenylalanine



**Fig. 1.** Phenylalanine (1) degradation in the presence of reactive carbonyls (4-oxo-2-nonenal, ON) and lipid hydroperoxides (LOOH). Reactive carbonyls are involved in the formation of phenylpyruvic acid (2), phenylacetaldehyde (3), and  $\beta$ -phenylethylamine (4). Formation of phenylacetic acid (5) and benzaldehyde (6) requires the presence of the free radicals produced in the thermal decomposition of LOOH.

degradation products have been shown to be produced as a consequence of the presence of free radicals: phenylacetic acid (5) and benzaldehyde (6). These compounds were not detected previously when phenylalanine degradations were studied in the presence of reactive carbonyls and in the absence of oxidizing conditions. In addition, the free radical formation of phenylacetaldehyde by lipid hydroperoxides, which was previously hypothesized (Hidalgo & Zamora, 2016), has been shown to be produced to a certain extent in addition to its formation as a consequence of carbonyl-amine reactions (Hidalgo & Zamora, 2004).

LOOH produced the almost quantitative conversion of phenylpyruvic acid (2) into phenylacetic acid (5) at pH 8 and in the presence of air. This compound, a honey-like GRAS flavoring substance (Adams et al., 2005), is a key aroma compound found in fermented beverages (Corsini, Castro, Barroso, & Duran-Guerrero, 2019; Dongmo, Sacher, Kollmannberger, & Becker, 2017) as well as truffles (Schmidberger & Schieberle, 2017). To the best of our knowledge, this is the first time that the formation of this substance by chemical means has been reported. Having into account that  $\alpha$ -oxo acids have been found in foods in which lipid oxidation plays a major role (Hidalgo, Navarro, Delgado, & Zamora, 2013), presence of phenylacetic acid in these foods should not be discarded. In addition, free radical oxidation of phenylacetaldehyde (3) has been shown to be an alternative, although less efficient, route for the production of phenylacetic acid (5).

Formation of phenylpyruvic acid (2) has been shown to occur by means of reactive carbonyls (Zamora et al., 2006). However, its formation by free radicals does not seem to take place to a high extent because phenylacetic acid (5) is produced to a reduced extent from phenylalanine (1) when only LOOH is present (Table 2). Nevertheless, phenylacetic acid (5) yield increased considerably in the presence of both LOOH and ON. Therefore, the formation of phenylacetic acid (5) from phenylalanine (1) should mainly take in two steps. The first one is the conversion of the amino acid into either phenylpyruvic acid (2) or phenylacetaldehyde (3) by means of reactive carbonyls (although free radicals seem also to induce phenylacetaldehyde (3) formation to a certain extent). Once compounds 2 or 3 are formed, free radicals are required for the conversion of these compounds 2 and 3 into phenylacetic acid (5). A reaction pathway that schematizes all these reactions is proposed in Fig. 2.

As indicated in the figure, in the presence of reactive carbonyls (such as the shown 4-oxo-2-alkenal) phenylalanine (1) is converted either into phenylpyruvic acid (2) or into phenylacetaldehyde (3) or into both of them depending on the reaction conditions (Zamora et al., 2006). Once they have been formed, phenylpyruvic acid (2) can be decarboxylated to produce the benzylcarbonyl radical (8) in the presence of free radicals. This radical has been observed in the photolysis of dibenzylketone (Grissom, 1995), and similar radicals are produced from aldehydes during their free radical oxidation (Hendriks, van Beek, & Heertjes, 1977). The benzylcarbonyl radical (8) would then react with oxygen to produce the corresponding peroxyacid radical (9) and later the peroxyacid (10). A disproportionation reaction of this peroxyacid would be the origin of phenylacetic acid (5). A proof of this mechanism was the identification of the corresponding 13-hydroxy derivative of linoleic acid when starting from 13-hydroperoxide of linoleic acid. This compound was identified by matching the retention index and mass spectrum with the standard prepared by derivatization of methyl 13-hydroxyoctadeca-9,11-dienoate with methylchloroformate (data not shown).

The second derivative produced to a significant extent in these reactions as a consequence of lipid radicals is benzaldehyde (6). This compound, a fruity-like GRAS flavoring substance (Andersen, 2006), has been found in the volatile fractions of almonds (Xiao et al., 2014), cherry (Wen et al., 2014), and tea leaves (Wang et al., 2019), among many other food products. As indicated in Fig. 1, benzaldehyde (6) was produced from phenylacetaldehyde (3), also from phenylpyruvic acid (2) but to a lower extent, and also from  $\beta$ -phenylethylamine (4) but to a

much lower extent. As shown in Table 4, benzaldehyde formation was favored in the presence of air and LOOH at pH 3. Reaction pathways for the conversion of phenylacetaldehyde (3), phenylpyruvic acid (2), and  $\beta$ -phenylethylamine (4) into benzaldehyde (6) are shown in Figs. S-1 to S-3 of the Supplementary Material, respectively.

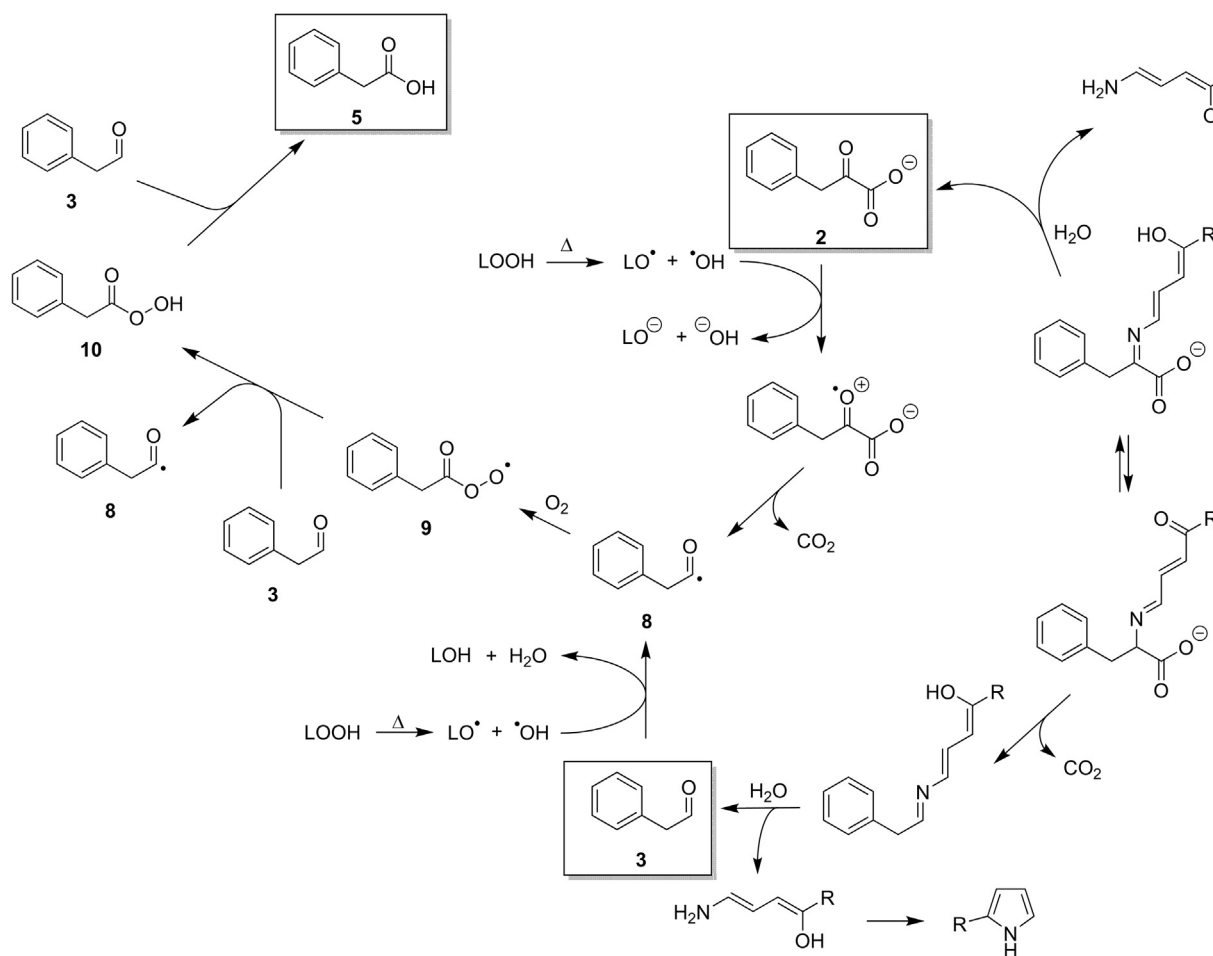
When starting from phenylacetaldehyde (3), the initial steps of the proposed pathway are similar to those above proposed for the formation of phenylacetic acid (5) (Fig. S-1 of the Supplementary Material). However, the produced peroxyacid radical (9), in the place of evolving into the corresponding peroxyacid (10), can produce the corresponding cyclic peroxide radical (11) and, then, the cyclic peroxide (12). The later breakage of this cyclic peroxide would be the origin of benzaldehyde. A similar breakage was proposed by Chu and Yaylayan (2008) for the oxygen-induced formation of benzaldehyde from phenylacetaldehyde observed by oxidative-pyrolysis GC-MS.

A similar reaction pathway can also be proposed for phenylpyruvic acid (2) because it can also produce the same benzylcarbonyl radical (8) that phenylacetaldehyde as shown in Fig. 2. This reaction pathway is shown in Fig. S-2 of the Supplementary Material. Finally, an analogous reaction pathway can also be suggested for  $\beta$ -phenylethylamine (4), although the produced cyclic peroxide should be slightly different (Fig. S-3 of the Supplementary Material).

In spite of the similar initial steps suggested in Fig. 2 on one hand, and in Figs S-1 to S-3 on the other, the products obtained and the reaction yields are different according to the results shown in Tables 2 and 4. This can be a consequence of both, the different difficulty of producing the benzylcarbonyl radical (8) depending on the starting material, and the promotion of either the peracid (10) or the cyclic peroxide (12) as a function of reaction conditions. Thus, phenylacetic acid (5) is produced at pH 8 to a higher extent than at pH 3 when starting from phenylpyruvic acid (2). This means that the carboxylate ion (2 in Fig. 2) may be the favored species at this pH and this species can be hypothesized to be converted into the benzylcarbonyl radical (8) with preference to the acid form of phenylpyruvic acid (2 in Fig. S-2 of the Supplementary Material). On the other hand, pH 3 and air are the preferred conditions to produce benzaldehyde. It can be hypothesized that these conditions should favor the formation of the cyclic peroxide (12). In relation to the effect of the presence of oxygen, phenylacetic acid (5) was produced to a higher extent in the presence of air when starting from either phenylpyruvic acid (2) or phenylacetaldehyde (3). The reason is likely that oxygen is required in the reaction pathway shown in Fig. 2. On the other hand, this effect was not so clear when starting from phenylalanine (1) because this is a two-step reaction and the formation of the intermediates has different requirements that the formation of phenylacetic acid from phenylpyruvic acid (Hidalgo & Zamora, 2016). Something similar also occurred for benzaldehyde formation (Table 4), although differences were not always significant ( $p < 0.05$ ). Differently to phenylpyruvic acid and benzaldehyde, the effect of oxygen in phenylacetaldehyde formation was not so clear. These results can be interpreted more difficultly because phenylacetaldehyde is decomposed once produced and formation and decomposition reactions can have different requirements.

## 5. Conclusions

Food flavors play a major role in the acceptance of foods by consumers. These compounds are produced by many different routes and, frequently, biochemical pathways alternate with chemical pathways in their formation. This study shows, for the first time, that phenylacetic acid (5) and benzaldehyde (6) can be produced by chemical routes as a consequence of lipid oxidation. Their formation is complex and requires a carbonyl-amine reaction in a first step, to produce phenylpyruvic acid (2), phenylacetaldehyde (3), or  $\beta$ -phenylethylamine (4), which would be later degraded by the free radicals produced in the decomposition of lipid hydroperoxides to form phenylacetic acid (5) and benzaldehyde (6). To the best of our knowledge, this is the first time that a combined



**Fig. 2.** Possible pathways for the coordinate action of reactive carbonyls and free radicals derived from LOOH in the conversion of phenylalanine (1) to phenylacetic acid (5). Reactive carbonyls (4-oxo-2-alkenal in the figure) are responsible for the production of phenylpyruvic acid (2) and phenylacetaldehyde (3). Free radicals degrade these compounds through the formation of the benzylcarbonyl radical (8), and its later conversion into phenylperacetic acid radical (9), phenylperacetic acid (10) and phenylacetic acid (5), respectively.

action of carbonyl-amine and free radical reactions for the production of food flavors as a consequence of lipid oxidation is shown. Because carbonyl-amine (or Maillard) reactions are widely produced in foods by non-lipid-derived carbonyl compounds, the contribution of sugars and quinones, among other carbonyls, to these reactions should not be discarded, as shown previously in non-enzymatic browning reactions (Zamora & Hidalgo, 2005).

#### Declaration of Competing Interest

The authors declare no conflicts of interest.

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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.2019.100037>.

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