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Role of the Solvent Glycerol in the Maillard Reaction of D-Fructose and L-Alanine

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The volatiles in the headspace above a solution of [$^{13}C_6$]fructose and alanine in glycerol/water, heated in a closed vial at 130 °C for 2 h, were analyzed by solid-phase microextraction in tandem with GC−MS. Carbonyl compounds and pyrazines were among the detected components. The examination of their mass spectra showed that most of the 1-hydroxy-2-propanone and 2,3-pentanedione were $^{13}C_3$-labeled, the majority of the 2-methylpyrazine and 2-ethyl-3-methylpyrazine were $^{13}C_5$-labeled, and 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were mainly $^{13}C_6$-labeled. This is in agreement with the literature, and corresponds to the incorporation of fructose carbons, and in the case of 2,3-pentanedione, 2-ethyl-3-methylpyrazine, and 3-ethyl-2,5-dimethylpyrazine alanine carbons, into the molecules. However, minority fractions of 1-hydroxy-2-propanone (10%) and 2,3-pentanedione (14%) were found unlabeled, 2-methylpyrazine (10%) and 2-ethyl-3-methylpyrazine (11%) only doubly labeled, and 2,5-dimethylpyrazine (20%) and 3-ethyl-2,5-dimethylpyrazine (27%) only triply labeled, suggesting they contain carbons originating from the solvent glycerol. This could be confirmed by reaction of fructose and alanine in [$^{13}C_3$]glycerol/water, which produced the same volatiles, with 11−27% existent in their $^{13}C_3$-labeled form. Hence, glycerol participated not only as a solvent but also as a precursor in the reaction.

KEYWORDS: Maillard reaction; glycerol; fructose; alanine; solid-phase microextraction; mass spectrometry; ^{13}C-labeling

INTRODUCTION

The Maillard reaction is, together with lipid oxidation, without doubt the most important source for aroma compounds generated when food is cooked, baked, or roasted. The flavor industry, too, makes use of the Maillard reaction to produce meatlike, cocoalike, and other process flavors (*1*). Higher temperatures and low water activity favor the formation of pyrazines in the Maillard reaction (*2*), some of which are key aroma compounds and contribute roasted notes to coffee, cocoa, roasted nuts, and other foods (*3*−*5*). The presence of the amino acid alanine in the reaction leads to an increased formation of ethylpyrazines (*6*) as well as of 2,3-pentanedione (*7*), and labeling studies have confirmed the incorporation of alanine carbons into both these compounds (*8*, *9*).

Glycerol is found in all natural oils and fats, and is an important intermediate in the metabolism of all living beings. It is naturally present in many foods, and is used as a food additive, e.g., as a conditioner and moisturizer, as well as a solvent for flavor extracts. Glycerol influences the Maillard reaction of reducing sugars and amino acids. Eichner and Karel (*10*) studied different glycerol/water systems and found that the browning rate decreased with increasing water activity. Mustapha and co-workers (*11*) observed stronger browning of lysine and xylose in glycerol than in an aqueous matrix, even though the reactants were not completely soluble in glycerol. They concluded that the reaction matrix was inert and did not directly take part in the reaction, and that the difference in color formation was due to the different physiochemical environments. Likewise, Jousse and co-workers (*12*), who studied the reaction kinetics of the Maillard reaction between alanine and glucose in glycerol, did not consider the solvent as an active reagent. Even the heating of amino acids in glycerol, in the absence of reducing sugars, gives rise to a certain extent of browning (*13*). It was suggested that glycerol oxidation products are involved. Small amounts of pyrazines have been detected in the reaction between asparagine and glycerol by Koehler and Odell (*14*). Acrolein and glyceraldehyde were thought to be the reactive intermediates derived from glycerol.

The present study was undertaken to study the influence of glycerol on the volatile compounds that are formed in the Maillard reaction between alanine and fructose, particularly with regard to what extent glycerol is actively taking part as a reactant. Isotopically labeled compounds were used to elucidate the origin of the carbons in pyrazines and other reaction products.

MATERIALS AND METHODS

Chemicals. Chemicals were of analytical grade. Acetaldehyde, L-alanine, D-fructose, glycerol, 2,3-pentanedione, phosphoric acid, and tripotassium phosphate were from Fluka (Buchs, Switzerland), and

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Table 1. Model Reactions[a]

	amount (mg)			
	A	B	C	D
alanine	6.68	6.68	6.68	6.68
fructose	13.50		13.50	
[$^{13}C_6$]fructose		13.50		
[$^{13}C_6$]glucose				13.50
glycerol	400	400		400
[$^{13}C_3$]glycerol			400	
phosphate buffer (0.5 mol/L, pH 7.00)	100	100	100	100

[a] Reaction at 130 °C (2 h).

[$^{13}C_6$]fructose, [$^{13}C_6$]glucose, and [$^{13}C_3$]glycerol (all 99% enrichment) were from Cambridge Isotope Laboratories (Andover, MA). 2,5-Dimethylpyrazine, 2-ethyl-3-methylpyrazine, and 2-methylpyrazine were from Aldrich (Buchs, Switzerland). The reference compound 3-ethyl-2,5-dimethylpyrazine was from the Firmenich collection.

Reaction. The reactants listed in **Table 1** were dissolved in potassium phosphate buffer (100 mg, 0.5 mol/L, pH 7.00), and glycerol (400 mg) was added. The solutions were filled into 2 mL glass vials and reacted in the septum-closed vials at 130 °C for 2 h in a heated metal block (Reacti-Therm, stirring/heating module, Pierce Chemical Co., Rockford, IL).

Analysis. All samples were analyzed in duplicate by headspace solid-phase microextraction in tandem with gas chromatography coupled to mass spectrometry (HS-SPME/GC−MS). The fiber [divinylbenzene/carboxen/polymethylsiloxane (DVB/CAR/PDMS), coating 50/30 μm, Supelco] was exposed for 5 min at 22 °C to the headspace above the samples. After sampling, the SPME device was placed for 2 min in the GC injector for desorption. The injector was equipped with a 0.75 mm i.d. liner (Supelco) and heated at 250 °C. GC−MS analyses were performed on a GC 6890N coupled to an MSD 5973 (both Agilent, Palo Alto, CA) using an HP-5MS capillary (30 m × 0.25 mm; film thickness 0.25 μm). After insertion of the fiber, the oven temperature was kept at 50 °C for 5 min and then raised by 10 °C/min to 260 °C. The carrier gas flow (helium) was 0.7 mL/min. Mass spectra were generated in the electron impact (EI) mode at 70 eV and at a scan range from *m*/*z* 15 to *m*/*z* 350.

For the calculation of the isotopomer proportions the values were corrected by subtracting the naturally occurring percentages of ^{13}C in $M^+ + 1$. The loss of hydrogen observed with the molecular ion in EI−MS was corrected in the labeled molecular ions by the ratio $(M^+ - 1)/M^+$. For 3-ethyl-2,5-dimethylpyrazine the calculation of isotopomer ratios was based on the ion signal $M^+ - 1$ instead of M^+ because the former was more intense than the molecular ion signal. In this case the values for the labeled $M^{+-}1$ ions were corrected by the ratio $M^+/(M^+ - 1)$.

RESULTS AND DISCUSSION

Alanine and fructose (molar ratio 1/1) were reacted at pH 7 and 130 °C for 4 h in a mixture of phosphate buffer and glycerol (1/4, w/w). The reaction was then repeated, replacing fructose with $^{13}C_6$-labeled fructose. The reaction products were analyzed by SPME with a DVB/CAR/PDMS fiber and an absorption time of 5 min, followed by GC−MS in the electron impact mode. Several carbonyl compounds and pyrazines were among the identified volatiles. Acetaldehyde (**1**), 1-hydroxy-2-propanone (**2**), 2,3-pentanedione (**3**), 2-methylpyrazine (**4**), 2,5-dimethylpyrazine (**5**), 2-ethyl-3-methylpyrazine (**6**), and 3-ethyl-2,5-dimethylpyrazine (**7**) were the major volatiles adsorbed on the fiber and detected by GC−MS. The mass spectra, obtained from the [$^{13}C_6$]fructose/alanine trial, showed M^+ signals which were up to 8 mass units higher compared to those of the spectra obtained with unlabeled fructose and alanine. The reason is the incorporation of ^{13}C atoms originating from [$^{13}C_6$]fructose into the molecules. **Table 2** shows the compounds and the proportion of their ^{13}C-labeled isotope molecules.

Table 2. Proportion of Isotopomers from the Reaction between [$^{13}C_6$]Fructose and Alanine in Glycerol

			m/*z*	proportion of labeled carbon atoms in the molecule (%)								
no.	compound[a]	RI[b]	(M^+)	0[c]	1	2	3	4	5	6	7	8
1	acetaldehyde	<500	44	98	0	2						
2	1-hydroxy-2-propanone	662	74	10	1	3	86					
3	2,3-pentanedione	700	100	14	0	3	77	0	6			
4	2-methylpyrazine	826	94	1	0	10	4	4	81			
5	2,5-dimethylpyrazine	913	108	4	0	1	20	0	4	71		
6	2-ethyl-3-methylpyrazine	999	122	1	0	11	6	3	65	14	0	
7	3-ethyl-2,5-dimethylpyrazine	1078	136	6	0	1	27	0	5	60	0	1

[a] Compounds were identified in the corresponding reaction between fructose and alanine on the basis of the comparison of mass spectra and retention times with those of reference compounds. [b] Linear retention index on HP-5. [c] Number of ^{13}C atoms in the molecule.

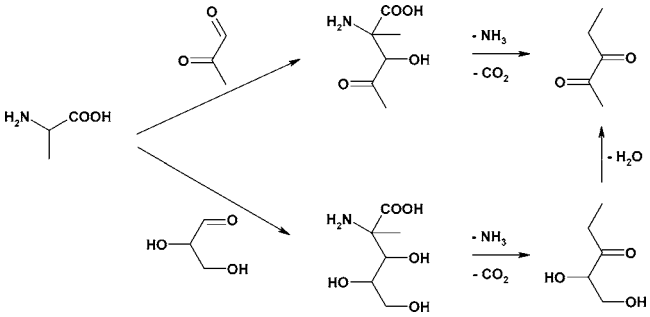

Figure 1. Formation of **3** from alanine and the glucose fragmentation products 2-oxopropanal and glyceraldehyde (adapted from ref *9*).

Virtually all of **1** was found unlabeled, indicating that its carbons do not stem from [$^{13}C_6$]fructose but rather from the amino acid alanine by Strecker degradation. The percentage of compound **2** which was triply labeled (86%) originates from decomposed [$^{13}C_6$]fructose. On the other hand, a proportion (10%) of **2** was unlabeled, and in principle the carbons could derive from either alanine or the solvent glycerol. There is evidence to suggest that it comes from glycerol, because when glycerol is heated by itself under the same reaction conditions, **2** is detected, together with 2-propenal, as the main volatile formed during heating (data not shown). Dehydration of glycerol is supposed to be responsible for its formation.

3 is a well-known Maillard product and is present in many foods. Most of **3** in the reaction with [$^{13}C_6$]fructose was found to be triply labeled. The mass spectra indicate that C-1 to C-3 of the molecule are labeled (data not shown). The unlabeled C-4 and C-5 atoms origin most probably from alanine. The literature indicates that the presence of alanine in the Maillard reaction promotes the generation of **3**, and there is proof that two C atoms of alanine are incorporated in the C_5 skeleton of **3** (*9*, *15*). Yaylayan and Keyhani have proposed a mechanism (*9*) which is based on the reaction between alanine and the sugar degradation products 2-oxopropanal and glyceraldehyde to explain the formation of **3** (cf. **Figure 1**). A different pathway is suggested by Weenen and Apeldoorn (*7*) which involves the Strecker degradation of alanine and the aldol addition of the resulting aldehyde **1** to **2**, followed by dehydration of the resulting 3,4-dihydroxy-2-pentanone to **3**. Hofmann showed earlier that the reaction between **1** and **2** in aqueous solution actually generates **3** (*16*). Since both compounds **1** and **2** have been identified among our volatile reaction products, the pathway to **3** via aldol condensation followed by dehydration is likely. Interestingly, 14% of **3** in the reaction with [$^{13}C_6$]fructose was found unlabeled, and hence, fructose is not associated with

Table 3. Proportion of Isotopomers from the Reaction between Fructose and Alanine in [$^{13}C_3$]Glycerol

				proportion of labeled carbon atoms in the molecule (%)								
no.	compound[a]	RI[b]	m/z (M+)	0[c]	1	2	3	4	5	6	7	8
1	acetaldehyde	<500	44	97	2	1						
2	1-hydroxy-2-propanone	662	74	85	4	0	11					
3	2,3-pentanedione	700	100	79	2	1	18					
4	2-methylpyrazine	826	94	77	1	7	13	0	2			
5	2,5-dimethylpyrazine	913	108	65	1	1	26	0	0	7		
6	2-ethyl-3-methylpyrazine	999	122	59	17	0	20	2	2	0	0	
7	3-ethyl-2,5-dimethylpyrazine	1078	136	62	0	3	27	0	2	6	0	0

[a] Compounds were identified in the corresponding reaction between fructose and alanine on the basis of the comparison of mass spectra and retention times with those of reference compounds. [b] Linear retention index on HP-5. [c] Number of ^{13}C atoms in the molecule.

Table 4. Proportion of Pyrazine Isotopomers from the Reaction between [$^{13}C_6$]Glucose and Alanine in Glycerol

				proportion of labeled carbon atoms in the molecule (%)								
no.	compound[a]	RI[b]	m/z (M+)	0[b]	1	2	3	4	5	6	7	8
2	1-hydroxy-2-propanone	662	74	8	1	4	87					
3	2,3-pentanedione	700	100	9	0	3	78	1	9			
4	2-methylpyrazine	826	94	4	0	15	7	5	69			
5	2,5-dimethylpyrazine	913	108	13	0	2	37	1	3	44		
6	2-ethyl-3-methylpyrazine	999	122	3	2	21	9	5	51	9	0	
7	3-ethyl-2,5-dimethylpyrazine	1078	136	17	0	2	40	0	5	35	0	1

[a] Compounds were identified in the corresponding reaction between fructose and alanine on the basis of the comparison of mass spectra and retention times with those of reference compounds. [b] Linear retention index on HP-5. [c] Number of ^{13}C atoms in the molecule.

the generation of this part. Possibly the solvent glycerol is involved in its formation, as in the formation of **2**. To verify this assumption, unlabeled fructose and alanine were reacted together in [$^{13}C_3$]glycerol (cf. reaction C in **Table 1**). The results in **Table 3** show that 18% of **3** in this reaction is triply labeled, an unambiguous corroboration for the integration of glycerol carbons into the molecule. Possibly the glycerol dehydration product **2** is reacting with acetaldehyde stemming from alanine. This is supported by the fact that also a considerable part of **2** (11%) is likewise found triply labeled and thus derived from [$^{13}C_3$]glycerol.

In 81% of pyrazine **4** and 71% of **5** from the reaction between alanine and [$^{13}C_6$]fructose, all carbons were labeled (**Table 2**). In 10% of **4** only two carbons and in 20% of **5** only three carbons were labeled, indicating that possibly the remaining three unlabeled carbons in the molecule come from glycerol. The formation of 13% triply labeled **4** and 26% triply labeled **5** from unlabeled alanine and fructose in [$^{13}C_3$]glycerol clearly confirmed the origin of these carbons from the solvent (cf. **Table 3**).

A majority (65%) of the ethyl-substituted pyrazine **6** was found labeled five times, and 60% of **7** was labeled six times in our experiment with alanine and [$^{13}C_6$]fructose (cf. **Table 2**). The remaining two unlabeled carbons are thought to derive from alanine because it is known that the ethyl groups of alkyl-substituted pyrazines consist to a great extent of the C-2 and C-3 atoms of alanine when it is present in the reaction (*6*, *8*). A minority of **6** (11%) was only doubly labeled, and equally a small portion of **7** (27%) was found only triply ^{13}C-labeled. In these cases it was assumed that the ethyl fragment derives from alanine and one C_3 fragment in the molecule stems from glycerol, adding up to five unlabeled carbons. This assumption could be verified by the findings from the reaction in [$^{13}C_3$]glycerol (reaction C, **Table 1**). **Table 3** shows that 20% of **6** and 33% of **7** are labeled three times, confirming that in these cases one glycerol C_3 skeleton was integrated into these molecules.

In another experiment alanine was reacted with [$^{13}C_6$]glucose instead of [$^{13}C_6$]fructose in glycerol/water. The isotopomer distribution of the pyrazines (**Table 4**) changes markedly as illustrated in **Figure 2**. Smaller percentages of **4** and **6** labeled five times and of **5** and **7** labeled six times were found in the reaction with [$^{13}C_6$]glucose. This indicates that in the reaction with glucose a higher proportion of glycerol carbons is used in the formation of the pyrazines relative to sugar carbons. We propose that with glucose a lower amount of reactive C_3 fragment intermediates is formed as compared with that formed with fructose. Then a lower amount of reactive intermediates from [$^{13}C_6$]glucose competes with the same proportion of C_3 fragments from glycerol in the reaction with alanine. The result is a relatively lower proportion of pyrazines ^{13}C-labeled five and six times as with [$^{13}C_6$]fructose.

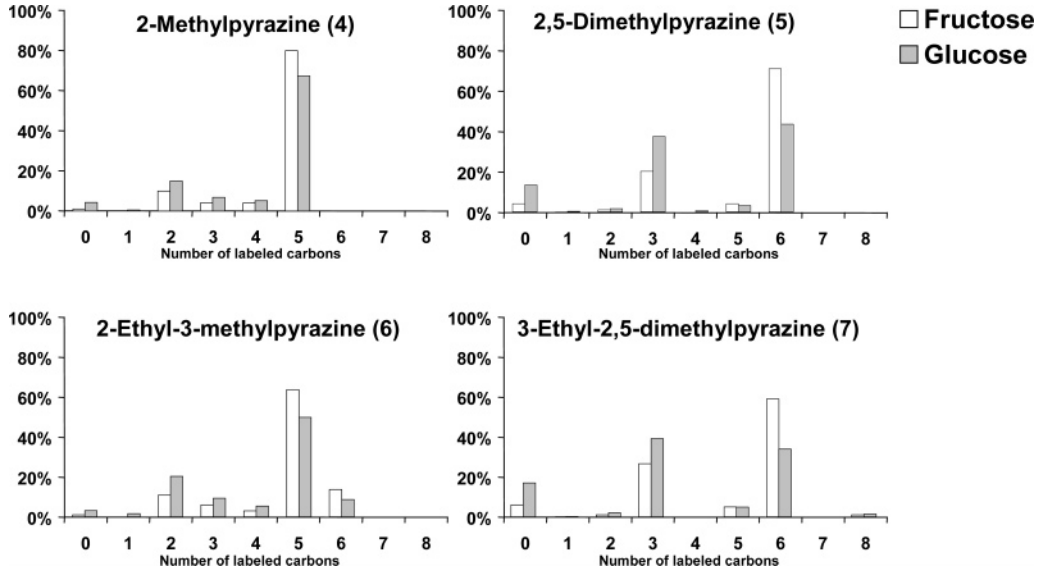

Figure 2. Effect of the kind of hexose on the isotopomer proportion of pyrazines in the reaction between [$^{13}C_6$]hexose and alanine.

The present study has shown that glycerol influences the Maillard reaction not only by influencing the water activity and the physiochemical environment of the reaction matrix but also by acting as a precursor. The volatiles **2**−**7** that were formed in the reaction of alanine and fructose in ^{13}C-labeled glycerol were between 11% and 27% triply labeled, thus proving unambiguously the inclusion of glycerol carbons in the molecules, presumably via **2** or its oxidation product 2-oxopropanal as an intermediate.

ABBREVIATIONS USED

DVB/CAR/PDMS, divinylbenzene/carboxen/polymethylsiloxane; GC−MS, gas chromatography−mass spectrometry; SPME, solid-phase microextraction.

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