Characterization of Volatile Constituents of an N^{α}-Formyl-L-lysine–D-Lactose Browning System

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The volatile constituents of an N^{α}-formyl-L-lysine–D-lactose nonenzymatic browning system have been analyzed by gas–liquid chromatography and mass spectrometry. Twenty-six compounds have been identified. Among them are benzyl formate, δ-valerolactam, 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, and 3,5-dihydroxy-2-methyl-4*H*-pyran-4-one. The results are considered in relation to those obtained from previously studied lactose–casein and other milk-related systems.

The application of gas–liquid chromatography (glc) for the study of complex mixtures in flavor research is always subject to criticism, based on the possibility of formation of artifacts. Our previous study on the volatile constituents of a lactose–casein model system (Ferretti and Flanagan, 1971a; Ferretti *et al.*, 1970) is all the more exposed to this type of criticism for the following reasons. The continuous dichloromethane extraction of the wet browned mixture might have resulted in migration, into the solvent phase, of intermediate products of the Maillard reaction containing a sugar moiety and a small nitrogenous protein fragment. The formation of similar products has been documented by Hough *et al.* (1952) in the case of glucose reacting with ammonia. Injection of such intermediates into the glc column would almost certainly result in the formation of artifacts. Furthermore, in the earlier part of the work (Ferretti *et al.*, 1970), the dichloromethane extract was directly chromatographed, whereas in the second part (Ferretti and Flanagan, 1971a) the oil remaining after solvent evaporation was first subjected to a cold-finger molecular distillation. Under these circumstances, and keeping in mind the possibility of having carbohydrate derivatives in the extract, the issue of full and direct comparability of the two resulting sets of data could reasonably be raised.

The present study was undertaken not only to test the validity of the criticism outlined above but also to ascertain the origin of certain products found in previously studied systems undergoing nonenzymatic browning. The practical relevance of this work is related to a more general study carried out in our laboratory on nonlipid precursors of off-flavors in dairy products.

EXPERIMENTAL SECTION

Gas Chromatographic and Mass Spectrometric Methods. Column A: 8 ft × 0.125 in. o.d. packed with 100–120 mesh Chromosorb W (AW/DMCS treated) coated with 15% Triton X305 plus 2% phosphoric acid. Temperature programming was from 70 to 180° at 4° per min. Column B: 6 ft × 0.125 in. o.d. packed with 100–120 mesh Chromosorb W (AW/DMCS treated) coated with 5% Carbowax 20M plus 1% Versamid 900. Temperature programming was from 60 to 190° at 2° per min. The carrier gas (He) was supplied with a head pressure of 40 psi. Both columns, stainless steel and DMCS treated, were placed in a 5750 Hewlett-Packard gas chromatograph and were coupled to an LKB-9000 mass spectrometer for rapid scanning of the total glc effluent. The mass spectral parameters were the same as previously reported (Ferretti and Flanagan, 1972). Injection size was 3 μl (CH_2Cl_2 solutions).

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Browning Procedure. Three-and-one-half grams of N^{α}-formyl-L-lysine, prepared according to Wolf *et al.* (1952), was added to a solution of 14.5 g of α-lactose monohydrate in 90 ml of water (molar ratio 1:2) and the solution was freeze-dried immediately. The resulting powder was heated at 75° and 75% relative humidity for 22 hr.

Preparation and Analysis of the Dichloromethane Extract. The brown mass was ground in a mortar in the presence of Celite 545 and extracted with CH_2Cl_2 in a Soxhlet for 24 hr. The extract, after careful removal of the solvent as usual, yielded 90 mg of a light brown oily residue. A few milligrams of this was directly chromatographed on column A and the effluent was fast scanned with the mass spectrometer, without prior fractionation.

The remainder of the oil was subjected to cold-finger molecular distillation at a vacuum of 50 μ for 2 hr. The bath temperature was kept constant at 80°, except during the last 3–5 min, when it was allowed to rise to 115°. Twenty milligrams of nearly colorless material was collected. This was chromatographed on column B, and the effluent was continuously scanned as usual.

Reference Compounds. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one used as reference compound was kindly supplied by John E. Hodge, Northern Regional Laboratory, Peoria, Ill. 3,5-Dihydroxy-2-methyl-4*H*-pyran-4-one was a gift from Philip E. Shaw, U. S. Fruit and Vegetable Products Laboratory, Winter Haven, Fla. All other compounds were obtained from reliable commercial sources or prepared as reported in our previous papers. All reference materials were purified by glc before determination of their spectral properties.

RESULTS

The compounds identified in the dichloromethane extract, before and after molecular distillation, are listed in Table I according to their order of elution from column B. The approximate relative concentrations are indicated in a single column, because their variations after molecular distillation were minor. As usual, identification was based on glc retention times and mass spectra. Table II shows the mass spectra (70 eV) of the authentic and unknown compounds identified for the first time in our laboratory in model or milk-related systems.

2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one undergoes partial dehydrogenation to 3,5-dihydroxy-2-methyl-4*H*-pyran-4-one during gas chromatography–mass spectrometry. Dehydrogenation under similar conditions had previously been observed in the case of 2-furyl hydroxymethyl ketone (Ferretti and Flanagan, 1971b). Dehydrogenation of the dihydro compound occurs also when its acetone solution is allowed to stand in an open vial for several hours.

Four unknowns (having apparent mol wt 138, 111, 126, and 102) could not be identified.

DISCUSSION

All the classes of compounds found previously in the lactose–casein model system (Ferretti and Flanagan, 1971a; Ferretti *et al.*, 1970) are represented in Table I, with the exception of the alkylpyrazines and the pyridines. Compounds found in relatively large amounts in the present study were also major components of the browned lactose–casein mixture.

New compounds, *i.e.*, not previously found in the lactose–casein or milk-related systems studied by us, are benzylformate, δ-valerolactam, 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, and 3,5-dihydroxy-2-methyl-4*H*-pyran-4-one. The last two are known products of nonenzymatic browning, recently identified by Jurch and Tatum (1970) and Shaw *et al.* (1971) as hexose degradation products, and by Mills *et al.* (1970) during a study of thermal decomposition of Amadori compounds.

δ-Valerolactam probably arose from the Strecker degradation of lysine (preceded by hydrolysis of the formyl derivative), followed by oxidation of the resulting aldehyde, and finally lactamization of δ-aminovaleric acid. Benzylformate must have formed by formylation of benzyl alcohol.

Table I. Volatile Compounds Identified in an N^{α}-Formyl-L-lysine–D-Lactose Browning System

Compound	Molecular distillation		Approximate relative amount[a]
	Before	After	
1, 2-Furaldehyde[b,c,d]	+	+	m
2, Acetic acid[b,c,d]	+	+	m
3, 2-Furfuryl formate[b,c]	+	+	s
4, 2-Acetylfuran[b,c,d,e]	+	+	s
5, 2-Furfuryl acetate[b,c]	+	+	s
6, 5-Methyl-2-furaldehyde[b,c]	+	+	m
7, Protoanemonin[b,c]	+	+	s
8, 2,2′-Difurylmethane[b,c]	+	+	m
9, Mol wt, 138[f]	+	+	s
10, γ-Butyrolactone[b,c]	+	+	m
11, Furfuryl alcohol[b,c,e]	+	+	l
12, 5-Methyl-(2-furyl)-(2′-furyl)methane[b,c]	+	+	s
13, Benzyl formate	+	+	s
14, 2-Butenoic acid γ-lactone[b,c]	+	+	l
15, Mol wt, 111[f]	+	+	m
16, Benzyl alcohol[b,e]	+	+	m
17, Benzothiazole[d,g]	−	+	s
18, 2-Acetylpyrrole[b,c,d]	+	+	s
19, Difurfuryl ether[b,c]	+	+	s
20, Maltol[b,c,d,e]	+	+	l
21, *o*-Cresol[d]	+	+	s
22, 5-Methyl-2-formylpyrrole[c]	+	+	s
23, δ-Valerolactam	+	+	m
24, Mol wt, 126[f]	+	+	s
25, Mol wt, 102[f]	−	+	s
26, 2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	+	+	m
27, 3,5-Dihydroxy-2-methyl-4*H*-pyran-4-one	+	+	m
28, Benzoic acid[d,e]	+	+	s
29, 5-Hydroxymethyl-2-furaldehyde[b,c]	+	+	l
30, β-Hydroxy-γ-butyrolactone[b,c]	+	+	l

[a] l = large, m = moderate, and s = small. [b] Compound identified also in a lactose–casein model system (Ferretti *et al.*, 1970; Ferretti and Flanagan, 1971a). [c] Compound identified also in heated whey powder (Ferretti and Flanagan, 1971b). [d] Identified also in stale nonfat dry milk (Ferretti and Flanagan, 1972). [e] Identified also in edible spray-dried whey (Ferretti and Flanagan, 1971c). [f] Unidentified compound. [g] Present as an artifact (see Discussion).

Table II. Mass Spectra of New Compounds Identified in Model Browning or Dairy Systems

δ-Valerolactam (mol wt 99), relative intensity			Benzyl formate (mol wt 136), relative intensity		
m/e	Authentic	Unknown	*m/e*	Authentic	Unknown
99	100	100	136	54	43
98	17	19	108	31	28
71	10	10	107	29	27
70	24	26	91	100	100
56	14	20	90	82	68
55	34	33	89	21	23
43	48	47	79	35	32
42	67	70	77	37	35
41	53	56	65	25	28
39	18	24	63	12	15
30	79	73	51	27	27

2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (mol wt 144), relative intensity			3,5-Dihydroxy-2-methyl-4*H*-pyran-4-one (mol wt 142), relative intensity		
m/e	Authentic	Unknown	*m/e*	Authentic	Unknown
144	49	29	142	100	100
115	4	3	113	10	12
101	41	25	87	6	12
73	24	16	85	13	20
72	22	19	71	11	15
55	22	22	68	20	30
45	23	20	67	9	14
44	65	54	57	7	11
43	100	100	55	14	14
42	7	10	43	26	26

γ-Butyrolactone, benzyl alcohol, *o*-cresol, and benzoic acid, which were also found in lactose–casein or/and milk-related systems (see footnotes to Table I), and whose rationalization as products of nonenzymatic browning was considered questionable, indeed appear to be products of the Maillard reaction. γ-Butyrolactone and benzoic acid were identified also as base-catalyzed fructose degradation products (Shaw *et al.*, 1968).

Benzothiazole seems to be a nearly ubiquitous constituent of flavor extracts. Its presence has been reported in numerous food systems, including cocoa concentrate (Flament *et al.*, 1967), cocoa powder (van der Wal *et al.*, 1971), popcorn (Walradt *et al.*, 1970), roasted peanuts (Walradt *et al.*, 1971), soybean milk (Wilkens and Lin, 1970), shallow-fried beef (Watanabe and Sato, 1972), stored casein (Ramshaw and Dunstone, 1969), heated milk (Scanlan *et al.*, 1968), and sterilized concentrated milk (Arnold *et al.*, 1966). Its formation has never been rationalized. The authors recently identified benzothiazole in a vacuum steam distillate of stale nonfat dry milk (Ferretti and Flanagan, 1972) and concluded, mainly based on its low flavor threshold in skim milk, that it might be one of the products more likely to contribute to the stale flavor. The presence of benzothiazole, however, is impossible to rationalize in a model system like the one studied in this work. Suspicion that it might originate from short sections of rubber tubing used to connect parts of the vacuum system prompted us to conduct a control experiment as follows. A Dry Ice condenser was filled with acetone and CO_2, fitted with a 5-ml flask, and the system was pumped for 1 hr, using the same pump and attachments used for the molecular distillation of the dichloromethane extract. After pumping was suspended, the finger of the condenser was washed with CH_2Cl_2. After almost complete evaporation, the residual solvent was analyzed (ms) and found to contain benzothiazole. It is prob-

ably difficult to avoid contamination even if the rubber connections in the vacuum system are short and far removed from the condenser. The authors feel sure that benzothiazole found in stale skim milk powder is also a contaminant.

The pyrazines are increasingly recognized as significant flavor contributors in various manufactured food products. The knowledge of the factors that affect their formation may be important. It is generally accepted that the pyrazines arise from the reaction of α,β-dicarbonyl compounds (dehydroreductones), formed from the degradation of carbohydrates (Hodge, 1953), and amino compounds, with concomitant transamination. The amino group does not need to be the α-amino group of an amino acid, and a Strecker degradation-type of mechanism is not the only one theoretically possible (Dawes and Edwards, 1966; Koehler and Odell, 1970; Koehler *et al.*, 1969; Wang *et al.*, 1969). The α-amino-β-keto compounds resulting from the transamination would condense to give dihydropyrazines and, finally, pyrazines.

Alkylpyrazines have been found in a lactose–casein browning system (Ferretti and Flanagan, 1971a; Ferretti *et al.*, 1970), in spray-dried whey (Ferretti and Flanagan, 1971c), and in stale nonfat dry milk (Ferretti and Flanagan, 1972), but not in heated whey powder (Ferretti and Flanagan, 1971b) nor in the present study. Such lack of uniformity is contrasted by the presence of furans, pyrroles, phenols, and certain lactones in all of the abovementioned systems. The reasons for such lack of uniformity in the case of the pyrazines are difficult to pin down in the absence of direct experimental proofs. One can only speculate that reasons could be found in the factors that might influence the evolution of the Maillard reaction. These factors include the absolute temperature (of heating or storage), moisture content, time, and pH (Ellis, 1959), as well as the molar ratio of lactose to potentially available amino compounds and their physicochemical state within the system.

CONCLUSION

Table I shows that the volatile compounds found in an N^{α}-formyl-L-lysine–D-lactose browning system before molecular distillation are identical to those found after molecular distillation, with the only exception being the unknown having a molecular weight of 102 (benzothiazole proved to be an artifact). From this finding, we can conclude that the molecular distillation did not result in creation of artifacts, and that when the dichloromethane extract was injected directly into the gas chromatograph, additional compounds (artifacts) were not formed. These statements must be considered together; they are either both true or both false. If they are both false, then the alternative is that in both cases the same artifacts are formed. The latter possibility is obviously much less likely.

The logical extension of this reasoning to the data obtained in our previous study of a lactose–casein browning system (Ferretti and Flanagan, 1971a; Ferretti *et al.*, 1970) allows us to answer the criticism outlined in the introductory paragraphs by concluding that the two sets of data obtained in that study are fully and directly comparable.

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