was checked for accuracy by adding it singly to a sample of D-limonene and was again approximately 2.2.

Except for our earlier study on Persian lime oil (Shaw *et al.*, 1971), the percentage of material considered too high boiling to be eluted from a glc column had not been considered in quantitative glc analyses of citrus oils (Bernhard, 1960; Ziegler, 1971). We determined by distillation that approximately 1% of cold-pressed orange oil would remain on the column (nonvolatiles) at the end of a normal glc run. Persian lime oil contained a much higher percentage (7.5%) of nonvolatiles than did orange oil (Shaw *et al.*, 1971).

A panel evaluated the aroma of the synthetic mixture of 15 compounds prepared in approximate proportions to that found in orange oil. The synthetic mixture was judged to have an aroma that was different from late-season orange oil sample A, but it was citrus-like in character. This synthetic mixture is being subjected to more extensive and thorough flavor and aroma evaluations.

The quantitative analytical glc procedure described herein should prove useful for monitoring cold-pressed orange oils for changes in main components. Since late-season orange oil is used at a level of about 0.020% in orange juice as a primary flavoring ingredient, the quantity of each of the 17 main oil components in orange juice can be approximated from these results. However, allowance should be made for those components such as valencene which are already present in juice oil (Hunter and Brogden, 1965) before peel oil addition.

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Free Amino Acids, Sugars, and Organic Acids in Aqueous Beef Extract

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The identified free amino acids, peptides, amines, sugars, sugar amines, sugar phosphates, and organic acids account for 0.55% of the fresh weight of bovine *semimembranosus* muscle. Of 27 ninhydrin-positive compounds, 23 were identified and quantified (mg/100 g fresh weight): phosphoserine, 1.84; taurine, 0.09; aspartic acid, 0.95; threonine, 3.17; serine, 3.60; glutamic acid, 11.60; proline, 0.94; glycine, 3.10; α-alanine, 18.17; cystine, 0.60; valine, 6.91; methionine and methionine sulfoxide (as methionine), 6.48; isoleucine, 5.07; leucine, 9.73; tyrosine, 5.44; phenylalanine, 6.04; β-alanine, 0.96; glucosamine, 3.04; hydroxylysine, 0.59; lysine, 2.40; anserine, 29.43; and arginine, 1.53. In the sugar fraction, glucose, fructose, ribose, inositol, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, and adenosine monophosphate were quantified. Lactic acid accounted for 44.5% of the organic acid fraction; succinic acid was a minor component.

Studies on post-mortem changes in beef (Bodwell *et al.*, 1965; Colombo and Gervasini, 1955; Fredholm, 1960; Grau *et al.*, 1960; Niewiarowicz, 1956; Pavlovskii, 1965; Sharp and Rolfe, 1958) have identified amino acids, peptides, amines, sugars, and sugar phosphates in beef muscle. These compounds contribute to flavor through the nonenzymatic browning reaction (Hodge, 1967; Reynolds, 1965). Their relationship to tenderness of beef has also been considered (Field and Chang, 1969; Locker, 1960; Ma *et al.*, 1961; Parrish *et al.*, 1969). Moreover, these same water soluble, low molecular weight compounds have been reported in studies on an index of incipient spoilage, the effects of heat and irradiation on proteolysis, and the variation of composition among different tissues from the same animal (Gardner and Stewart, 1966; Thompson *et al.*, 1961; Walker, 1952). Numerous other studies have indicated that these soluble compounds may have important effects on meat flavor and palatability (Disney *et al.*, 1967; Dryden *et al.*, 1969; Gunther and Schweiger, 1966; Macy *et al.*, 1964a,b; Tonsbeek *et al.*, 1969; Wasserman and Gray, 1965; Wood, 1956, 1961; Wood and Bender, 1957).

Most of the research cited above pertained to studies with aims somewhat different from the present work; therefore, some of the techniques used were not suitable for this study. The use of heat during extraction or later in the separation is evident in the procedures of Gardner and Stewart (1966), Ma *et al.* (1961), Pavlovskii (1965), Sharp and Rolfe (1958), Walker (1952), Wood (1956), and Wood and Bender (1957). It was shown by Macy *et al.* (1964b) that heat caused substantial losses of sugars and amino compounds in meat extracts. Some studies reported only qualitative results (Locker, 1960; Tonsbeek *et al.*, 1969; Wood, 1961). Other studies quantitated only specific compounds (Bodwell *et al.*, 1965; Disney *et al.*,

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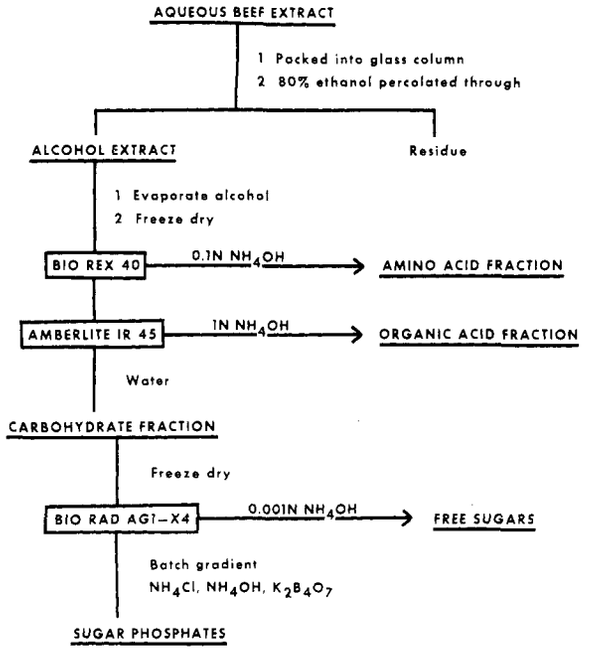

Figure 1. Extraction and fractionation of low molecular weight components in aqueous beef extract.

1967; Dryden *et al.*, 1969; Fredholm, 1960; Gunther and Schweiger, 1966).

The extraction procedure of Mabrouk *et al.* (1969) was shown to provide an increased yield of aqueous extract, and the present study was performed to identify quantitatively the low molecular weight components of the aqueous beef extract obtained using these techniques.

EXPERIMENTAL SECTION

Preparation of 80% Ethanol Extract. A sample of 95.19 g of lyophilized aqueous beef extract (*semimembranosus* muscle, U.S. Good of unknown history) prepared according to the method of Mabrouk *et al.* (1969) was packed into a jacketed glass column (2.5 × 120 cm). Six liters of 80% ethanol containing about 0.1% toluene was percolated through the column while tap water (*ca.* 15°) circulated through the jacket (Figure 1). The alcohol was removed from the extract at room temperature under aspirator vacuum in a rotary evaporator. The residual aqueous solution was shell frozen and freeze dried. The dry ethanol extract was transferred to a Nalgene bottle and stored in a vacuum desiccator over P_2O_5 at −30°.

Fractionation of Lyophilized Ethanol Extract. Separation of the alcohol extract into an amino acid fraction, a carbohydrate fraction, and an organic acid fraction was achieved according to the technique used by Cruickshank (1957). Two glass columns (2.5 × 100 cm) were arranged in series. The first column was filled with Bio-Rex 40, hydrogen form (Calbiochem, Los Angeles, Calif.) and the second contained Amberlite IR-45, hydroxide form (Mallinckrodt Chemical Works, St. Louis, Mo.). The lyophilized ethanol extract (20 g) was dissolved in about 100 ml of deionized water and applied to the Bio-Rex 40 column. After the sample passed completely through the surface of the resin bed in the first column, washing with deionized water began. Washing was stopped when the effluent spotted on filter paper was negative to a spray containing equal volumes of 0.1 *N* silver nitrate and 5 *N* ammonium hydroxide (Partridge and Westall, 1948). The aqueous washings were shell frozen and freeze dried, yielding the carbohydrate fraction. The columns were then separated and the free nitrogenous compounds were displaced from the Bio-Rex 40 column with 0.1 *N* ammonium hydroxide. The organic acids were eluted from the Amberlite IR-45 column with 1 *N* ammonium hydroxide. Each effluent was concentrated under aspirator vacuum in a rotary evaporator at room temperature. The concentrates were shell frozen and freeze dried. All three fractions (free nitrogenous compounds, carbohydrates, and organic acids) were transferred to Nalgene bottles and kept in a vacuum desiccator over P_2O_5 at −30°.

Determination of Free Nitrogenous Compounds. Analysis of the free nitrogenous compounds was performed on a Model K-8000C, automatic recording amino acid analyzer (Phoenix Precision Instrument Co., Philadelphia, Pa.). Three ion exchange columns were used. Specifications and operating conditions for the neutral and acidic amino acids column and for the standard column for basic amino acids are given in the instrument operation manual. The third column (0.9 × 24 cm), Aminex Q-15 S cation exchange resin (Bio-Rad Labs, Richmond, Calif.), was used to achieve a better separation between anserine and lysine. This column was operated at 52° and a buffer of pH 5.28 was used. The identities of the amino acids and related compounds were established by comparison of their elution times with those of pure standards and by coinjection. With the exception of proline, the concentration of each compound was determined by integration of the area under the peak using a Model CRS-110A electronic integrator (Infotronics Corp., Houston, Tex.). The calculation method described in the analyzer manual was used for proline. The concentration of each compound represents the average of from three to five determinations. Nitrogen content was determined by the Kjeldahl method (AOAC, 1970, 24.010, p 393).

Separation of Carbohydrates. An aqueous solution of the carbohydrate fraction was fractionated on a column (1.2 × 26 cm) of AG1-X4, chloride form (Bio-Rad Labs, Richmond, Calif.), according to the method of Khym and Cohn (1953).

Determination of Carbohydrates. Isothermal gas chromatography of the trimethylsilyl derivatives of the free sugars (Sweeley *et al.*, 1963) was performed using a GC-4 gas chromatograph (Beckman Instruments, Fullerton, Calif.). The chromatograph was equipped with dual hydrogen flame ionization detector (FID) and two 6 ft × 1/8 in. o.d. stainless steel columns packed with 3% w/w SE-30 on 80–100 Chromosorb W (acid washed, DMCS treated). Operating conditions were: inlet temperature, 220°; column temperature, 170°; detector temperature, 250°; helium (carrier gas) flow at 20 ml/min with constant inlet pressure throughout the run. Identifications were made by comparing retention times with those of standards and by coinjection. Confirmation of the identity of the compounds was achieved by coinjection with pure standards on dual 6 ft × 3/16 in. o.d. stainless steel columns packed with 3.3% neopentyl glycol succinate on 80–100 Gas Chrom Q. The concentrations of the free sugars were determined from the straight-line correlations between concentrations of standard sugars and the responses of the FID as measured by an Infotronics Model CRS-110A electronic integrator. The quantities calculated for these free sugars represent the average of at least three determinations. The sugar phosphates were quantified colorimetrically (AOAC, 1970, 22.040, p 374).

Determination of Organic Acids. The methyl esters of the organic acids were prepared using ion exchanger as the catalyst according to the method described by Vogel (1956) with the addition of 2,2-dimethoxypropane (Fieser and Fieser, 1967; Lorette and Brown, 1959) as water scavenger. The methyl esters were separated and identified on a Beckman GC-4 gas chromatograph equipped with dual FID and 8 ft × 1/8 in. o.d. stainless steel columns packed with 10% DEGS on 80–100 Chromosorb P. Operating conditions were: inlet temperature, 220°; column temperature, 130°; detector temperature, 250°; and helium carrier gas, 24 ml/min. To confirm the identities of the esters,

Table I. Ninhydrin-Positive Compounds in Aqueous Beef Extract

Compound	mg/100 g fresh wt
Phosphoserine	1.84
Unknown no. 1	(0.2; 0.49)[a]
Taurine	0.09
Unknown no. 2	(1.2; 0.77)[a]
Aspartic acid	0.95
Threonine	3.17
Serine	3.60
Glutamic acid	11.60
Proline	0.94
Glycine	3.10
α-Alanine	18.17
Cystine	0.60
Valine	6.91
Unknown no. 3	(0.3; 2.58)[a]
Methionine and methionine sulfoxide[c]	6.48
Isoleucine	5.07
Leucine	9.73
Tyrosine	5.44
Phenylalanine	6.04
β-Alanine	0.96
Glucosamine	3.04
Hydroxylysine	0.59
Lysine	2.40
Anserine	29.43
Unknown no. 4	(8.7; 1.18)[b]
Arginine	1.53
Total	121.68

[a] Peak area integrator counts and elution time relative to those of aspartic acid. [b] Peak area integrator counts and elution time relative to those of lysine. [c] Calculated as methionine.

coinjection with standards on dual 6 ft × 3/16 in. o.d. stainless steel columns packed with 3.3% neopentyl glycol succinate was performed. Operating conditions were similar to those for the DEGS columns. The calculations used to obtain the concentrations of the organic acids were performed by the procedure outlined for the free sugars.

RESULTS AND DISCUSSION

Approximately 1.8% of the fresh weight of beef is composed of low molecular weight compounds that are extractable from lyophilized aqueous extract with 80% ethanol. Upon fractionation of the alcohol extract according to Figure 1, three fractions were obtained: free nitrogenous compounds, 0.6%; carbohydrates, 0.2%; and organic acids, 0.8%, by weight of fresh beef. The total nitrogen contents of aqueous beef extract, 80% ethanol extract, and the free nitrogenous fraction are 11.74, 7.54, and 19.83%, respectively. In aqueous beef extract, peptides, amino acids, amines, sugar amines, nucleotides, and their derivatives account for the nitrogen content (11.74%). Upon extraction with 80% ethanol, amino acids, amines, sugar amines, short-chain peptides, sugars, sugar phosphates, organic acids, and nucleotides are recovered in the extract. Much of the polypeptide material was not extracted; that accounts for the lower nitrogen content of the alcohol extract (7.54%). Upon separation with ion exchangers, 87.7% of the nitrogen in the ethanol extract was recovered in the free nitrogenous fraction (19.83% N).

In Table I, the concentrations of ninhydrin-positive compounds separated by the amino acid analyzer are recorded. The identified compounds account for about 20% of the free nitrogenous fraction. Twenty-three compounds were identified and quantified. Attempts to establish the identity of the four unknown compounds were unsuccessful. Comparison with retention values of pure standards and coinjection proved that the unknowns were none of the following compounds: phosphoethanolamine, hydroxyproline, sarcosine, asparagine, α-aminobutyric acid, tryptophan, ornithine, glutathione, creatine, carnosine, histidine, 1-methylhistidine, and 3-methylhistidine. Quantitative and qualitative differences among the literature data and the present data are anticipated since beef composition is affected by numerous intrinsic and extrinsic factors (Lawrie, 1965, 1970). However, the relatively good agreement considering the number of variables involved should be noted. The data in Table I agree broadly with those of Ma *et al.* (1961), Macy *et al.* (1964a,b), Walker (1952), Wasserman and Gray (1965), Wasserman and Spinelli (1970), Zaika (1969), and Zaika *et al.* (1968). In a comparative study on the composition of the water-soluble diffusates of beef, pork, and lamb, Macy *et al.* (1964a) found that cysteic acid and ornithine are present only in pork and lamb and glutathione is present in lamb alone. The present investigation confirms the absence of free glutathione, cysteic acid, and ornithine in aqueous beef extracts. The difference between the results of this work and those of Macy *et al.* (1964b) is the absence of phosphoethanolamine, glycerophosphoethanolamine, histidine, and 1-methylhistidine from the extract used in this study and the absence of proline, β-alanine, glucosamine, hydroxylysine, and arginine from the extract used by Macy *et al.* The concentrations of most of the ninhydrin-positive compounds in this investigation are higher than those reported by Macy and coworkers (1964b). This work also confirms the results of Macy *et al.* (1964a,b) and Ma *et al.* (1961) concerning the absence of free tryptophan, and disagrees on this point with Wasserman and Gray (1965).

Table II. Carbohydrates and Organic Acids in Aqueous Beef Extract

Compound	mg/100 g fresh wt
Free sugars	
α-Glucose	17.23
β-Glucose	22.83
Fructose	24.72
Inositol	1.55
Ribose	1
Total	67.35
Sugar phosphates	
Glucose 6-phosphate	0.24
Fructose 6-phosphate	0.27
Fructose 1,6-diphosphate	0.76
Adenosine monophosphate	0.40
Total	1.67
Organic acids	
Lactic acid	356
Succinic acid	2.8
Total	358.8

Free sugars, sugar phosphates, and organic acids identified in aqueous beef extract along with their concentrations are recorded in Table II. The identified carbohydrates and organic acids account for about 34 and 45%, respectively, of the carbohydrate and organic acid fractions. The carbohydrates reported in the present study confirm the findings of Tonsbeek *et al.* (1969) who reported the presence of glucose, fructose, ribose, inositol, and sugar phosphates in aqueous beef extract. While the quantities of glucose and ribose found in this investigation are comparable to those reported by Macy *et al.* (1964b), our data on fructose content are about six times higher. α- and β-glucose account for 58% of the identified carbohydrates in fresh beef and fructose is about 36%. Inositol, ribose, and sugar phosphates are 2.2, 1.4, and 2.4% of the identified carbohydrates, respectively. Our data confirm the occurrence of sugar phosphates in beef which was demonstrated by Wood (1961). Our results concerning the presence of lactic and succinic acids in aqueous beef extract are in agreement with those of Tonsbeek *et al.* (1969). Lactic acid was also reported by Dryden *et al.* (1969), and succinic acid was similarly found by Wasserman and Gray (1965).

Table III. Classification of Low Molecular Weight Beef Components

Compound	mg/100 g of fresh beef	% of group total
Ninhydrin positive		
Basic	33.95	28.6
Acidic	12.55	10.6
Aromatic	11.48	9.7
Sulfur	7.17	6.0
Neutral	53.49	45.1
Total	118.64	100.0
Carbohydrates		
Free sugars	67.35	93.5
Sugar amine	3.04	4.2
Sugar phosphates	1.67	2.3
Total	72.06	100.0
Organic acids		
Lactic	356	99.2
Succinic	2.8	0.8
Total	358.8	100.0

The low molecular weight compounds present in beef are grouped into classes and subclasses in Table III to facilitate discussion of their role in beef flavor. In 1968, Solms classified meat aroma into four groups: flavor substances, flavor intensifiers, products of the nonenzymatic browning reaction, and carbonyl compounds resulting from heating fat. It is known that the so-called nonenzymatic browning reaction between carbonyl compounds and proteins or amino compounds gives rise to products which vary in their aroma depending upon the reacting materials and conditions (Hodge, 1967; Reynolds, 1965). Some of these reaction products are unpleasant while others are acceptable and are used to produce cooked-meat-like aroma. Amino acids and sugars are implicated in the production of meat-like aroma (Broderick and Marcus, 1970; Giacino, 1970; Kitada *et al.*, 1970; May, 1970; Morton *et al.*, 1960; Soeters, 1970). Sulfur-containing amino acids which account for about 6% of the identified ninhydrin-positive compounds are implicated in the production of meaty aroma and are reported in most patents concerning meat-like flavor, *i.e.*, those listed above. The basic amino compounds amount to about 29% of the identified amino acid fraction from beef. The contribution of these compounds to flavor *per se* is limited, but upon cooking they contribute to meaty flavor (Shewan, 1955). Smearing the surface of beef with arginine before roasting or boiling generally improves tenderness, aroma, and flavor (Miyake *et al.*, 1971). In many patents concerning the production of meat flavor, individual basic amino acids are required among the ingredients to produce the meat-like aroma. Glutamic acid accounts for 10% of the identified nitrogenous compounds present in beef. Monosodium glutamate *per se* accentuates or enhances the flavor notes of foods and, in the patent literature, it is often cited as one of the amino acids used to produce meaty flavor. Pyrrolidonecarboxylic acid, probably produced from glutamic acid during heating, reacts with ribose 5-phosphate to produce 4-hydroxy-5-methyl-3(2*H*)-furanone, a flavor component of beef broth (Tonsbeek *et al.*, 1969). Another route shown to produce the same compound is the reaction between taurine and ribose 5-phosphate.

Losses in individual amino acids and sugars during heating aqueous beef extract were studied by Macy *et al.* (1964b). The losses of taurine, alanine, and anserine–carnosine were 56, 45, and 58% of the initial concentration, respectively. Ribose appeared to be the most labile sugar to heating followed by glucose, and fructose was most stable. The presence of glucose 6-phosphate and fructose 6-phosphate and the absence of ribose 5-phosphate in aqueous beef extract are in complete agreement with Wood's findings (1961). Wood (1961) demonstrated that in spite of the fact that the individual components of aqueous ox muscle extract possess no flavor with the exception of creatine and inosinic acid, upon heating with glucose for 4 hr at 100° they developed a typical meat extract flavor. Recently, Kirimura *et al.* (1969) reported that in addition to taking part in the Maillard reaction, some amino acids may contribute directly to the taste of food, others accentuate the taste, some contribute to the mouthfeel; furthermore, the buffer action of amino acids may contribute to flavor. In beef extract, no specific amino acid characterizes the taste, but rather all the free amino acids contribute to the complex taste sensation (Kirimura *et al.*, 1969).

The nonenzymatic browning involving mixtures of amino acids and sugars is enhanced by nucleotides (Broderick and Marcus, 1970) and/or organic acids (lactic, succinic, tartaric, fumaric, malic, citric, gluconic, pyruvic, and α-ketoglutaric) (Ohara *et al.*, 1970). Dryden *et al.* (1969) found no consistent association between beef flavor and lactic acid content; however, Hornstein and Crowe (1960) reported that lactic acid may be important in developing meat flavor during cooking. Furthermore, Pearson (1968) indicated that lactic acid *per se* has an important influence on taste.

This investigation has provided quantitative data regarding the free amino acids, amines, sugars, sugar phosphates, sugar amines, and organic acids present in aqueous beef extract from a single sample. These data should contribute to the continuing effort to define beef flavor precursors.

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Detection of Maillard Browning Reaction Products as Trimethylsilyl Derivatives by Gas–Liquid Chromatography

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D-Glucosylamines and their Amadori rearrangement products, 1-deoxy-1-(N-substituted)amino-D-fructoses, formed in the Maillard browning reaction of D-glucose with various amines, are shown to be readily detectable, together with the reactants, by gas–liquid chromatography of the trimethylsilylated reaction mixture. The procedure affords a convenient method for monitoring the course and extent of the browning reaction. A mixture of *N,O*-bis(trimethylsilyl)acetamide, *N*-(trimethylsilyl)imidazole, and chlorotrimethylsilane was used for trimethylsilylation.

The detection of carbohydrate intermediates in the nonenzymic browning reaction has been achieved mainly by colorimetric methods (Hodge, 1955; Wolfrom and Rooney, 1953; Wolfrom *et al.*, 1955; Talley and Porter, 1968) and by paper chromatography (Kato, 1962; Anet, 1960a,b; Borsook *et al.*, 1955). The unstable dicarbonyl sugar intermediates have been converted into their crystalline osazones (Anet, 1960a,b; Kato, 1960; Machell and Richards, 1960). The gas–liquid chromatographic technique has had limited success in application to this problem because of involatility and instability of the initial sugar–amine derivatives and their subsequent transformation products (Kadunce, 1967). The development of newer reagents for trimethylsilylation (Pierce, 1968) aroused our interest in conducting a reinvestigation on the detection of the Maillard browning reaction products by gas–liquid chromatography (glc) of their trimethylsilyl derivatives. A mixture of *N,O*-bis(trimethylsilyl)acetamide (Klebe *et al.*, 1966), *N*-(trimethylsilyl)imidazole (Horning *et al.*, 1967), and chlorotrimethylsilane was found satisfactory for derivatizing D-glucosylamines, the Amadori products, and other compounds formed in the reaction of D-glucose with *p*-toluidine, 4-aminobutyric acid, and various amino acids, at ambient or at elevated temperatures. The derivatives of the products and initial reactants produce detectable and reproducible peaks on glc. A brief preliminary report of this work has been presented (Wolfrom and Kashimura, 1969). The technique has been applied to freeze-dried mixtures of sugars and amino acids, a model system for such dried food products as orange juice powder, the flavor deterioration of which is considered to be partly due to the nonenzymic browning reaction of reducing sugars and amino compounds (Hodge, 1953). This convenient method for simultaneous analysis of the initial reactants and the transformation products should be of value both for fundamental studies on the browning reaction and also in applied work as a quantitative monitor for product deterioration in stored or processed food products arising from the initial stages of the nonenzymic browning reaction.

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EXPERIMENTAL SECTION

Materials. *p*-Toluidine (mp 44–45°) was recrystallized twice from ether. *N*-*p*-Tolyl-D-glucopyranosylamine [mp 108–114°, $[\alpha]^{23}D$ −117° (*c* 1.0, pyridine)] was prepared by the method of Ellis and Honeyman (1952). 1-Deoxy-1-*p*-toluidino-D-fructose [mp 155–157°, $[\alpha]^{23}D$ −21.0° (*c* 1.0, pyridine)] was prepared by the method of Weygand (1940). *N,N*-Bis(1-deoxy-D-fructos-1-yl)glycine ("difructoseglycine") [amorphous powder, $R_{glucose}$ 0.12 (4:1:1 (v/v) butyl alcohol–acetic acid–water, silver nitrate–sodium hydroxide detection), N 3.67%, ν_{max}^{KBr} 1610–1630 cm^{-1}] and 3-deoxy-D-*erythro*-hexosulose [$R_{glucose}$ 2.6–2.9 (principal component, minor contaminants $R_{glucose}$ 1.20 and 1.75)] were prepared and purified by the method of Anet (1960b). Other carbohydrates used as internal standards for glc had physical constants in good agreement with literature values. Amino acids (Mann Research Laboratories) were used without further purification.

Nonenzymic Browning Reaction. *Reaction of D-Glucose with p-Toluidine.* A mixture of D-glucose (180 mg),