Water-Soluble Flavor Precursors of Beef. Extraction and Fractionation

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The dry material of aqueous meat extract prepared from lyophilized meat after removal of petroleum ether–extractable lipids accounts for 6.5% of the fresh weight. Fractionation of water-soluble flavor precursors by dialysis and gel permeation chromatography on a stacked column of equal heights of esterified Sephadex G-25 fine, G-15, and G-10 resulted in 12 fractions. The fractions exhibiting high meaty aroma intensity amounted to 1.1% of the fresh weight. The procedure developed is sufficiently quantitative to demonstrate differences in precursor contents among different muscles. Evidence suggests that precursor characteristics reside in more than one molecular structure and are correlated with sulfur-containing amino acids present.

Many attempts have been made to isolate and identify the compounds responsible for meat flavor; these have been partially successful. Batzer *et al.* (1960, 1962), Hornstein *et al.* (1960), Hornstein and Crowe (1960), Landmann and Batzer (1962), and Macy *et al.* (1964 a, b) reported that low molecular weight water-soluble compounds present in the diffusate of raw meat aqueous extracts are flavor precursors. Inosine, hypoxanthine, glucose, proline, isoleucine, valine, serine, and α- and β-alanine have been found as constituents of glycoprotein fraction isolated from beef flavor precursors (Batzer *et al.*, 1962). The characteristic color, aroma, and flavor of meat extracts are attributed to Maillard reaction between amino acids and sugars or sugar phosphate (Bender *et al.*, 1958; Wood, 1961).

The heating of cysteine (or of protein hydrolyzates enriched with cysteine) together with a reducing sugar—e.g., ribose—or other aldehydic compound, with the object of producing meat-like flavor, has been the subject of several patents (May, 1960; May and Morton, 1961; Morton *et al.*, 1960). The absence of sulfur-containing amino acids, which are precursors of the important sulfur-containing ingredients of meat aroma, is surprising; perhaps these compounds have been overlooked. Recently, Wasserman and Gray (1965) reported the presence of methionine in the meaty aroma fractions obtained by dialysis and gel permeation chromatography of aqueous raw beef extracts.

From these reports, little is known about the identity of the flavor precursors of beef. The present work was performed to increase the yield of beef flavor precursors, improve the fractionation technique to obtain less complex multicomponent fractions, and identify the sulfur-containing amino acids and sugars present in these fractions.

EXPERIMENTAL

Preparation of Lyophilized Beef Diffusate (Figure 1). U. S. Good grade round of beef of unknown history was purchased from a meat market. *Semimembranosus*, *semitendinosus*, and *gracilis* muscles were dissected and freed from fat and connective tissues. Each trimmed muscle was ground twice in an electric grinder at 5° C. The ground muscles were weighed and blended with an equal weight of cold deionized water (ca. 5° C.) for 2 minutes. The resulting slurry was shell frozen and lyophilized. The volatiles resulting from lyophilization (16 ml. per kg. of beef) were collected in traps maintained at −195° C. for further studies. The lyophilized homogenized muscles were extracted with petroleum ether (b.p. 30° to 60° C.) in a Soxhlet apparatus for 24 hours. The residue after extraction was blended for 4 minutes with tenfold its weight of cold, deionized water (ca. 5° C.) containing 0.1% $CHCl_3$ to inhibit bacterial growth and tissue phospholipases. The slurry was stirred continuously for 3 hours while held at 5° C. The mixture was centrifuged for 20 minutes at 5120 × G at about 5° C. The supernatant was filtered through Whatman filter paper No. 41 and designated first filtrate. The precipitate was blended with an equal weight of water and subjected to the same process, and the resulting extract was lyophilized and named second filtrate. The meat residue was lyophilized.

An Oxford rotating multiple dialyzer capable of handling 16 samples simultaneously was used in this work. Dialysis bags prepared from Union Carbide seamless cellulose dialysis tubing (Viscose process, size 27, 1.312-inch flat width, 0.0010-inch wall thickness, and average bore radius of 24 A.) were thoroughly washed with deionized water to remove glycerol and sulfur compounds. The dialysis bags, each filled with 250 ml. of deionized water, rotated in about 14 liters of the first filtrate for 18 hours at 5° C. The dialysis tubing contents are called diffusate. Two batch dialyses were effected, combined, and designated diffusate I. The nondiffusible fraction was lyophilized, combined with lyophilized second filtrate, dissolved in 12 liters of deionized water, and dialyzed twice. The resultants after lyophilization were designated diffusate II, diffusate III, and nondiffusible fraction, respectively. The lyophilized powders were transferred to brown vials and kept under vacuum in a desiccator above P_2O_5 at about −20° C.

Lyophilized meat residue after aqueous extraction was extracted in Soxhlet apparatus with $CHCl_3$–MeOH, 50 to 50 (v./v.), and then with $CHCl_3$–MeOH–H_2O, 14:82:4 (v./v./v.), solvents containing 0.05% hydroquinone to prevent oxidation of unsaturated fatty acids. The lipid extracts were designated lipid II and III, respectively. The lipid-free residue was dried under vacuum to ensure complete absence of any traces of organic solvents and moisture.

Gel Permeation Chromatography (GPC). MATERIALS. Bio-Gel P-6, 50- to 150-mesh (Calbiochem, Los Angeles, Calif.) and Sephadex types G-25 fine, G-15, and G-10 (Pharmacia, Uppsala, Sweden) were used in this study. Fine grade Sephadex G-25 (20- to 80-microns) was used

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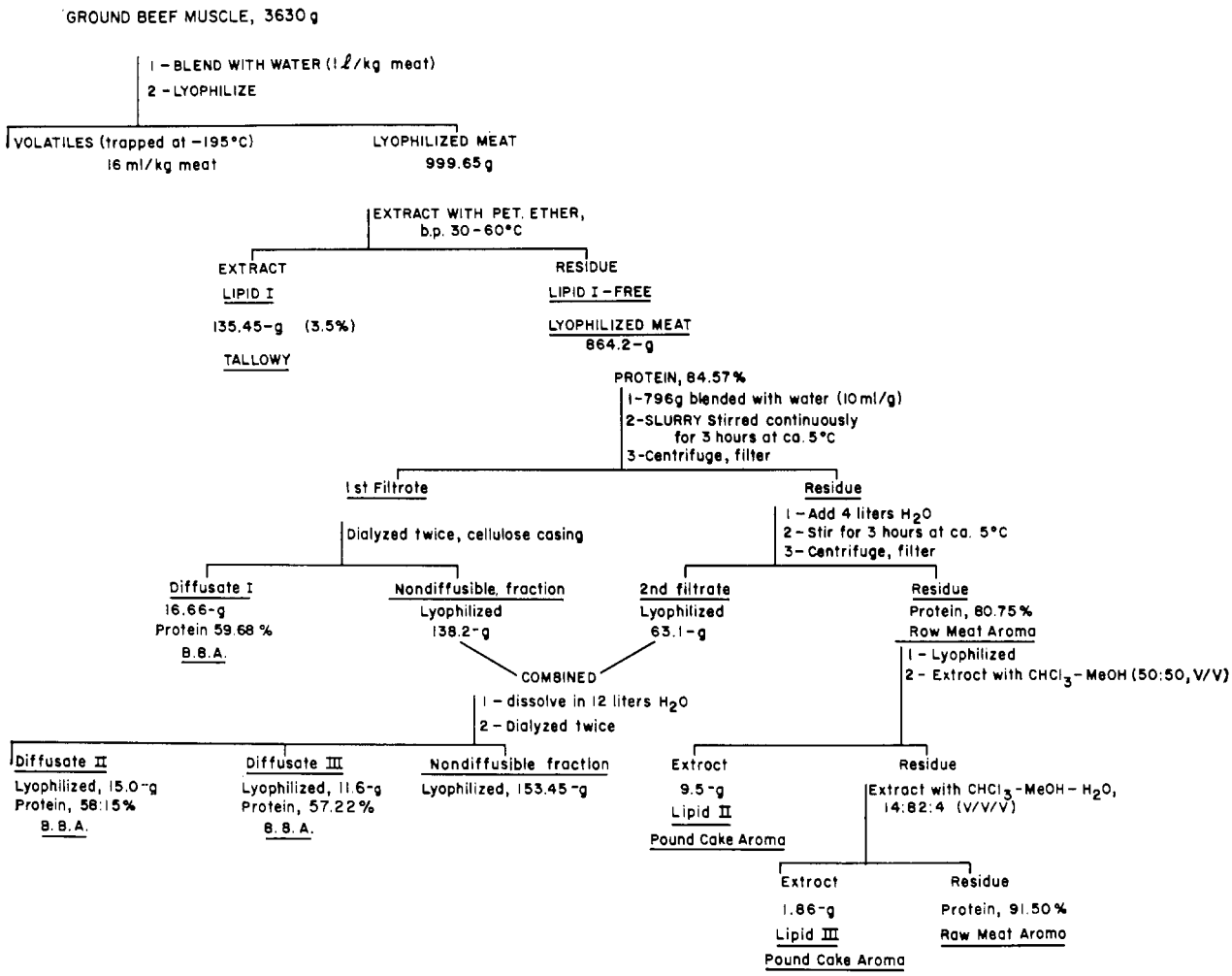

Figure 1. Procedure for preparation of beef diffusate (B.B.A., broiled beef aroma)

since fine particles gave a high resolution column, and there is a more rapid approach to equilibrium conditions.

The various types of Sephadex contain free carboxyl groups amounting to 10 to 20 μeq. per gram of dry weight (Flodin, 1962; Gelotte, 1964) resulting in very weak ion exchange properties. To eliminate this effect, Sephadex was treated with diazomethane. Sephadex was added to ice-cold ethereal diazomethane solution (ca. 5° C.), stirred for 3 hours, and then left at room temperature (28° C.) overnight to ensure complete esterification of the free carboxyl groups. Sephadex was filtered, washed with diethyl ether, and dried in a rotary evaporator. Esterification under these conditions minimized any side reactions such as acylation, in the broad sense, of the carbonyl groups (Satchell, 1963).

COLUMN PREPARATION. Chromatographic columns (2.5 × 40 and 2.5 × 110 cm.) pretreated with 1% dichlorodimethylsilane in benzene to reduce wall effects were packed, according to Porath (1960) and Gelotte (1964), with swelled gel to a height of 30 and 100 cm., respectively.

PREPARATION OF ESTERIFIED SEPHADEX G-25, G-15, AND G-10 STACKED COLUMN. Esterified Sephadex G-25 suspension in deionized water was packed to one third the height of a 2.5 × 100 cm. glass column. To stabilize the resulting bed, the gel was washed with 1 liter of water. Then, an aqueous suspension of esterified Sephadex G-15 was added to attain a height of two thirds of the column. The resulting bed was stabilized by washing the column with 2 liters of deionized water. The rest of the column was filled with esterified Sephadex G-10 gel, and the column was washed with 3 liters of water. Examining the column for faulty packing with blue dextran 2000 showed no points of imperfection in packing the gel. At the boundaries between Sephadex types in the column, no zone packing was observed.

About 12 mg. of beef diffusate per gram of dry gel was used for loading the column. As resolution capacity appeared to be more dependent on the volume of applied sample than on its concentration (Bennich, 1961), all samples were made in solutions of two tenths of the void volume.

Elution of the beds was performed at a flow rate of about 35 to 40 ml. per hour, independent of the column or type of gel used. The ultraviolet absorbance of the effluent was recorded using Uviscan Model II (Buchler Instruments, Inc., Fort Lee, N. J.) equipped with a V.O.M.-6 Bausch & Lomb recorder.

All GPC experiments were performed at room temperature and the fractions were arbitrarily labeled in numerical order, according to their sequence of elution. The frac-

tions comprising each peak were pooled separately, lyophilized, transferred to brown jars, and kept in a desiccator under vacuum above P_2O_5 at $-20°$ C.

Identification of Fraction Components. The presence of carbohydrate moiety in the various fractions was determined qualitatively by the anthrone test (Toennies and Kolb, 1964). Ninhydrin reagent (Moore and Stein, 1954) was used to test for the presence of amino nitrogen. Phosphorus was detected according to the method of Fiske and Subbarow (1925). Protein content was determined by the Kjeldahl method (Association of Official Agricultural Chemists, 1965). The aromas of the fractions were evaluated by placing a few milligrams of each sample on a cover slide and heating on a melting point apparatus block at 105° C. The ultraviolet spectra of aqueous solutions of the fractions were recorded with a Cary Model 14M recording spectrophotometer.

Carbohydrates were obtained by hydrolyzing separately fractions 4 to 10 (Table IV) with 6*N* HCl in hydrolyzing tubes under vacuum at 100° C. for 24 hours (Block *et al.*, 1958). The hydrolyzates of fractions 5 to 10 were light tan, indicating the formation of humin, while fraction 4 was practically clear. To separate the carbohydrate components from the amino acids, aqueous solutions of lyophilized hydrolyzates were passed through Dowex 50, H^+-form 200- to 400-mesh, 8 × linkage. Carbohydrates passed through the column with water. The amino acids were recovered by eluting with 2*N* ammonium hydroxide solution. The amino acids and carbohydrate fractions were lyophilized, and their contents were converted to trimethylsilyl derivatives (Smith and Sheppard, 1965; Sweeley *et al.*, 1963). To determine the sulfur-containing amino acids, samples of fractions 4 to 10 were oxidized with performic acid (Toennies and Homiller, 1942) before hydrolysis.

Gas Chromatography. Programmed temperature gas chromatographic analyses of the trimethylsilyl derivatives of sugars and amino acids were carried out using a Beckman GC-4 gas chromatograph equipped with dual hydrogen flame detector and two 6-foot × 1/8-inch o.d. stainless steel columns packed with 3% w./w. SE-30 on Chromosorb W. The conditions were: inlet temperature, 200° C.; column temperature, programmed at 5° C. per minute from 100° to 200° C. and held until completion of analysis; carrier gas, helium; flow rate, 70 cc. per minute with constant inlet pressure throughout the run.

RESULTS AND DISCUSSION

Upon lyophilization of aqueous extract—i.e., first and second filtrates—of trimmed beef muscle, prepared according to the scheme outlined in Figure 1, a light grayish powder which amounted to 6.5% of the fresh weight of beef was obtained. The powder extract gave a pronounced broiled meat aroma when heated dry and a broth-like aroma when boiled in water. The yield obtained with our procedure is higher than the 3% reported by Hornstein *et al.* (1960) and the 4.8% by Bender *et al.* (1958). This high yield may be attributed to extraction of lyophilized beef with petroleum ether before water extraction. This treatment eliminated the interference of the lipids during filtration and rendered the tissues more permeable to water employed in subsequent extraction. Also, removal of 84% of the lipids present (Table II) reduced emulsion formation during preparation of aqueous meat extract.

The three diffusates obtained, according to the scheme outlined in Figure 1, were grayish white powders. Each diffusate gave positive results when tested with anthrone, ninhydrin, and molybdenum reagents. The three diffusates totaled 1.42% of the fresh weight of meat and gave broiled beef aroma upon heating, while the nondiffusible fraction did not.

Total solids content, petroleum ether–extractable lipids, and total diffusates of the three muscles are listed in Table I.

The total diffusate content of *gracilis* muscle is about 40% of that of either *semimembranosus* or *semitendinosus*. Although there are quantitative differences between the muscles in their diffusate content, qualitative similarities in the meaty aromas of the diffusates are observed.

After water, $CHCl_3$–MeOH, and $CHCl_3$–MeOH–H_2O extractions, beef muscle residue was devoid of flavor but had a slight serumy odor.

Lipid I had a tallowy aroma, while lipids II and III exhibited a "pound cake" aroma. Work is in progress to identify the compounds responsible for the aroma of each fraction. Table II summarizes the content of each lipid fraction of the *semimembranosus* muscle.

Several trial runs involving elution with aqueous 0.05*M* ammonium acetate, 0.05*M* ammonium formate solutions, and deionized water were completed initially to determine optimal conditions for fractionation of beef diffusate on Sephadex. The same elution pattern and analytical data were obtained with the three eluents, but the elution peaks

Table I. Composition of Round Beef Muscles

	Semimembranosus		Semitendinosus		Gracilis	
Total solids, %	27.1	28.0	27.2	26.6	26.4	27.6
Petroleum ether extract, %[a]	3.5	3.9	3.6	3.8	2.9	3.4
Total diffusate, %[a]	1.42		1.42		0.61	

[a] Fresh weight.

Table II. *Semimembranosus* Extractable Lipids

Lipid	Solvent	Wt. %, Fresh Meat	Wt. %, Total Lipids	Aroma
1	Petroleum ether, b.p. 30°–60° C.	3.5	83.54	Tallowy
2	$CHCl_3$–MeOH, 50 to 50 (v./v.)	0.57	13.60	Pound cake
3	$CHCl_3$-MeOH-H_2O, 14:82:4 (v./v./v.)	0.12	2.86	Pound cake

were more symmetrical with water in comparison with ammonium formate and ammonium acetate solutions. Therefore, water was used as the eluent.

A typical elution pattern of diffusate I on Sephadex G-25 is shown in Figure 2.

Average volumes of fractions, lyophilized powder weight, and analysis are shown in Table III.

GPC with Sephadex G-25 bed 2.5 × 30 cm. gave five main groups of compounds, two of which contain phosphorus-positive material (peaks 1 and 2). The first was eluted with a volume very close to the void volume (70 ml). Fractions 1 and 2, which amounted to 96.7% of the total diffusate, exhibited strong broiled beef aroma when heated, while the other fractions did not. These results differ from those reported by Batzer *et al.* (1960, 1962) and Wasserman and Gray (1965) because of differences in the fractionation procedure used.

A gel bed with a ratio of 40 to 1 between height and diameter gave somewhat higher resolution between peaks 1 and 2 than a gel bed 12.5 to 1. The peaks obtained with

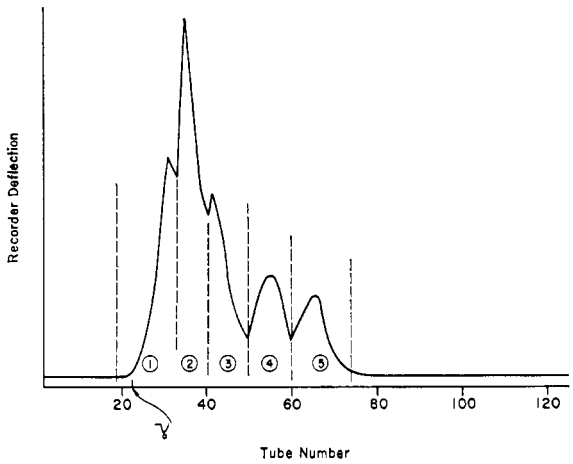

Figure 2. GPC of beef diffusate I on Sephadex G-25 fine, column 2.5 × 30 cm.

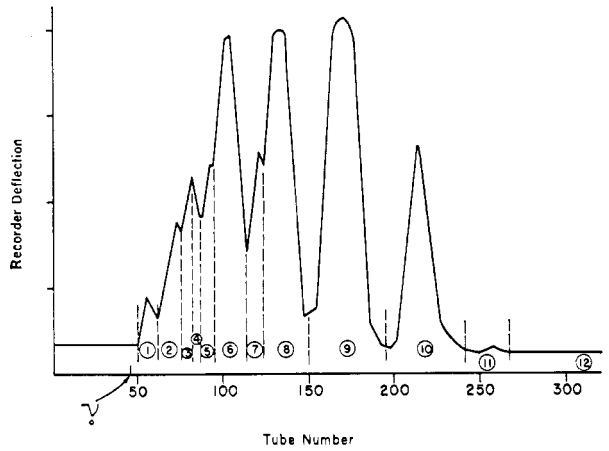

Figure 3. GPC of beef diffusate I on stacked column of esterified G-25 fine, G-15, and G-10, column 2.5 × 100 cm.

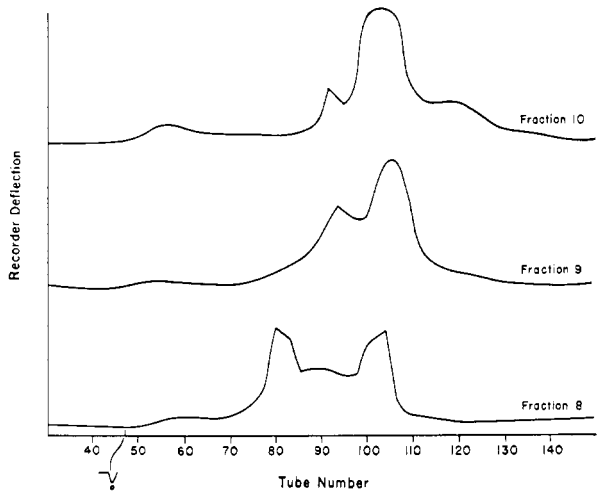

Figure 4. GPC of fractions 8, 9, and 10 of beef diffusate I on stacked column of esterified G-25 fine, G-15, and G-10, column 2.5 × 100 cm.

Table III. Composition of GPC Fractions of *Semimembranosus* Diffusate I[a]

	Fraction				
	1	2	3	4	5
Av. vol. fraction, ml.	51	18	24	33	27
Av. wt. % fresh meat	0.419	0.955	0.027	0.012	0.008
Av. wt. % diffusate	29.49	67.18	1.92	0.82	0.59
Ninhydrin test	+	+	+	+	+
Anthrone test	+	+	−	−	−
Phosphate test	+	+	−	−	−
Aroma	+	+	−	−	−

[a] Sephadex G-25 fine column 2.5 × 30 cm.

Table IV. Comparison of Fractions Obtained by GPC of *Semimembranosus* Diffusate[a]

	Fraction											
	1	2	3	4	5	6	7	8	9	10	11	12
Aroma	−	+	++	++	+++	+++	++	+	−	−	−	−
Ninhydrin test	+	+	++	+++	+++	+++	++	++	+	+	++	+
Anthrone test	−	−	++	++	++	+++	−	−	−	−	−	−
Phosphate test	−	−	−	++	++	++	−	−	−	−	−	−
Wt. % fresh meat	0.005	0.017	0.087	0.166	0.399	0.710	0.002	0.020	0.0003	0.002	0.001	0.002
Absolute max., mμ	253[b]	274	273	238	250	248	250	249	268	271	268	215, 272, 280, 288

[a] Esterified Sephadex G-25 fine, G-15, G-10 stacked column 2.5 × 100 cm.
[b] No maximum absorption.

Table V. Carbohydrates and Sulfur-Containing Amino Acids Present in Fractions Obtained by GPC of *Semimembranosus* Diffusate

Fraction	Ribose	2-Deoxy-D-ribose	α-Glucose	β-Glucose	Mannose	Galactose + Sorbose	Methionine	Cysteic Acid
4	−	+	−	−	−	−	−	−
5	−	+	−	−	−	−	−	+
6	−	+	−	−	−	−	+	+
7	+	+	+	+	−	−	+	+
8	−	+	−	−	−	−	+	+
9	−	+	+	+	+	+	+	−
10	−	+	+	−	−	−	+	−

Bio-Gel P-6 were neither as symmetrical nor as well resolved as those obtained with Sephadex G-25 column of the same height and diameter.

Instead of coupling three columns of Sephadex G-25, G-15, and G-10 in series, one column (2.5 × 100 cm.) containing equal heights of the three gels was constructed to eliminate the mixing taking place in the eluate tubes from one column to the next. The diffusate was separated into 12 fractions, compared to five fractions obtained with a column of the same dimensions packed with esterified Sephadex G-25. Figure 3 shows the elution curve of diffusate I on G-25, G-15, and G-10 column. Reproducibility on the same column was excellent, as the chromatograms of six runs showed a high degree of similarity with respect to number of fractions separated, the effluent volumes required for their elution, and their chemical analyses. Total recovery after fractionation was 87 to 94%, which is comparable with recovery data reported on GPC.

Seven of these fractions (Table IV) exhibited broiled beef aroma of different intensity. Fractions 5 and 6, which exhibited high aroma intensity, accounted for 78% of the diffusate, while those of lesser intensity (Fractions 3, 4, and 7) amounted to 18%. Fractions 2 and 8 represented 2.6% of the diffusate and exhibited the least meaty aroma intensity.

The chromatograms obtained upon GPC of diffusates 1, 2, and 3 show a high degree of similarity with respect to number, position of fractions separated, and the effluent volumes required for their elution. Comparable fractions gave the same response to ninhydrin, anthrone, and molybdenum reagents and aroma evaluation. However, there were slight differences between the weights of some of the corresponding fractions (±2 to 5%).

Fractions 8, 9, and 10 were refractionated on the same stacked column of esterified Sephadex G-25, G-15, and G-10 (Figure 4). Each gave several peaks, none of which has a retention value as in the initial separation, indicating each is not homogeneous. This confirms the assumption that the fractions obtained by GPC under these experimental conditions are not pure compounds, but mixtures. Therefore, GPC offers a simple method of separating the beef diffusate into fractions of less complexity.

Sulfur-containing amino acids and sugars present in fractions 4 to 10 are listed in Table V.

This work confirms that the major portion of beef flavor precursors are low molecular weight, water-soluble compounds. Qualitative gas chromatographic data indicate the presence of methionine and/or cysteic acid (cystine + cysteine) in the GPC fractions that exhibited meaty aroma. Work is in progress to determine the complete composition of all the isolated fractions.

An appropriate procedure capable of increasing the yield of aqueous beef extract and its flavor precursors has been developed and should be of immense value in future meat flavor investigations. Using an esterified stacked column of three types of Sephadex offered the possiblity of separating beef flavor precursors to several fractions of various degrees of meat intensity.

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