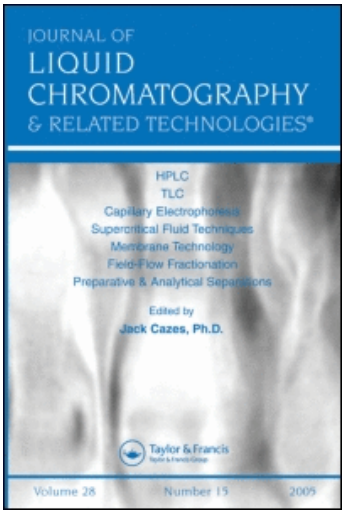

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Chromatographic Isolation of Presumptive Peptide Flavor Principles from Red Meat

Arthur M. Spanier[a]; Judson V. Edwards[b]
[a] Food Flavor Quality 1100 Robert E. Lee Boulevard, New Orleans, Louisiana [b] Microbial/Plant Technology Southern Regional Research Center, New Orleans, Louisiana

To cite this Article Spanier, Arthur M. and Edwards, Judson V.(1987) 'Chromatographic Isolation of Presumptive Peptide Flavor Principles from Red Meat', Journal of Liquid Chromatography & Related Technologies, 10: 12, 2745 — 2758
To link to this Article: DOI: 10.1080/01483918708066823
URL: http://dx.doi.org/10.1080/01483918708066823

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(12), 2745-2758 (1987)

CHROMATOGRAPHIC ISOLATION OF PRESUMPTIVE PEPTIDE FLAVOR PRINCIPLES FROM RED MEAT

Arthur M. Spanier[1] and Judson V. Edwards[2]

[1]*Food Flavor Quality*
[2]*Microbial/Plant Technology*
Southern Regional Research Center
U.S.D.A./A.R.S.
1100 Robert E. Lee Boulevard
New Orleans, Louisiana 70124

ABSTRACT

Top round, bovine semimembranosus and adductor muscle was selected as a model for isolation of presumptive, low molecular mass (M_r) flavor peptides. The isolation and purification of these peptides (<5,000 M_r) from 'cooked' and 'cooked-stored-recooked' meat was developed by combining various chromatographic techniques. Peptide samples were initially made by preparing acetic acid extracts of meat followed by the removal of organic soluble lipids and carbohydrates by phase partition extraction. The lipid-free extracted material was subsequently subjected to size exclusion chromatography using Sephadex G-25 resulting in two major polypeptide groups with M_r of 1500 to 3000. This material was now available for further purification by both semipreparative and analytical reverse phase (RP) - high performance liquid chromatography (HPLC) for separation of hydrophilic and hydrophobic peptides. Separation of the peptides into these two groups is particularly important since the perception of sweet taste is usually associated with hydrophilic peptides while bitter (and often sour) taste is associated with hydrophobic peptides. Semipreparative RP-HPLC of peptides from the low M_r material revealed highly significant differences in the hydropilic and hydrophobic peptide composition of 'cooked' versus 'cooked-stored-recooked' samples i.e., the former appeared to have equal amounts of the two classes of peptides while the latter appeared to contain predominantly hydrophobic peptides. Peptides prepared semipreparatively were readily available for further examination by analytical RP-HPLC and analyzed by diode array detection. The latter method revealed major differences in the hydrophobic peptide components found in the two meat groups.

INTRODUCTION

For most people, meat is an important part of the diet, not only because it is a major source of proteins and amino acids for the body nourishment, but also because most people like the taste or flavor of it. Since meat/muscle tissue is primarily proteinaceous, it represents a remarkable reservoir of peptidic flavor principles. These flavor principles can be formed by various chemical means (e.g., proteolysis, oxidation, heating, etc.) and at many different time points between the animal's slaughter and the serving of the finished product. Since consumer acceptance of any food product is primarily based on the food's flavor characteristics, then the more complete an understanding of a food's flavor biochemistry the better the chances for production of products with high degrees of consumer acceptability.

In recent years, a number of reports have appeared regarding both synthetic and naturally occuring flavor peptides (1-4). These studies have largely focused on the relation of structure to function in relatively small, linear and cyclic peptides with a common theme being the peptide's close molecular relationship to bitter or sweet taste. Though chemical changes in protein upon cooking have been documented (5), few studies have investigated the role of peptides in determining the flavor of 'cooked' and/or cooked-stored-recooked' meat products.

Proteins of molecular mass (M_r) greater than 10,000 have been isolated from meat products by utilizing reverse phase (RP)-high performance liquid chromatography (HPLC) of heat-sensitive bovine and procine muscle proteins (6). Other workers have studied heat-induced changes in myofibrillar proteins (7) using sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) to separate proteins (>10,000 M_r) with the view of examining the relationship of structure to function. Similar approaches with fish muscle have reported changes in free amino acids as a result of protein denaturation during frozen storage (8). Methods for differentiating among soy, pork, chicken and beef protein (9, 10) in raw and processed meat products utilized different methods for peptide profiling such as ion exchange chromatography (9), HPLC and TLC (10). HPLC has also been applied to the determination of histidine dipeptides in meat (11).

While all of the studies cited above have examined meat (beef, fish, poultry) proteins, peptides and amino acids, none of the studies made attempts to relate the structure of these components with their possible function in flavor. Research on the structure-function relationship of taste-eliciting peptides have shown a definitive correlation between flavor perception and amino acid sequences and polarity characteristics (2,3). We were able to find

only one literature account (and that only in abstract form) which correlated the hydrophobicity of hydrolyzed protein with bitterness (4). While no reports have correlated peptide differences in 'cooked' and 'recooked/rewarmed' food with their possible relation to 'taste', some years ago Zaika, et. al. (12) reported that heating or cooking of water-soluble extracts from beef (i.e., containing primarily amino acids and sugars) elicited good beefy-'aroma' responses. In view of the Zaika report, it is tempting to speculate that upon pyrolysis (the heating of the food during the cooking process) or after reaction with other meat components e.g., sugars, larger 'taste' peptides can form products giving defined beefy-'aromas'.

In an initial approach to addressing some of the above concepts regarding the relation of a peptides structure to its function in flavor perception in natural food systems, we have selected a system of off-flavor development called 'warmed-over flavor' or 'W.O.F.' in meat (13). This model allows us to examine the beef food product for the presence, formation and/or destruction of presumptive peptide flavor principles both during the initial cooking stages and during the cooked-stored-recooking period.

We have chosen to focus attention on low molecular weight peptides which are potentially involved in taste eliciting responses. Peptides in the M_r range of <5,000 represent a class of compounds which might lend themselves more readily to a category of presumptive flavor principles since they more readily fit some of the dimensional characteristics and requirements for ligand-receptor interaction. Furthermore, (i) they are readily formed through proteolysis of other meat proteins, (ii) many of the cooking byproducts of amino acids and peptides with sugars (Maillard reaction products) can survive the heating process as potential flavor principles, and (iii) small peptide analogs are more readily synthesized and natural peptides more readily modified for testing of their efficacy as flavor enhancers. The present study, therefore, outlines an approach for the isolation and purification of low M_r peptides from a W.O.F. model in roasted beef by utilizing a series of extractions and chromatographic techniques.

MATERIALS AND METHODS

Materials:

Top round (semimembranosus and adductor muscles) from Black Angus steers was purchased from a local supermarket chain and utilized as sample material. Extraction solvents included deionized water, acetic acid, a 10mM HEPES buffer

containing 0.25M sucrose, 20mM KCl, and 0.02% NaN_3 (as a bacteriostat) adjusted to a physiological pH of 7.2, and methylene chloride:methanol (2:1). Chromatography solvents included Millipore™ milli-Q water, acetic acid, acetonitrile, and trifluoroacetic acid. Equipment employed included a Tekmar™ Tissuemizer, Sorvall™ RC-5B and RC-70 centrifuges, and a VirTis™ Freezemobile 12 freeze dryer. Chromatographic equipment and columns were as follows: a Pharmacia™ Sephadex gel filtration column (2.5cm x 100cm) coupled to an Isco™ UA-5 ultraviolet detector and Isco Foxy fraction collector, a Waters™ 350 computer controlled HPLC with dual model 510 pumps and a Waters 490 programmable multiwavelength detector, a Dupont™ Zorbax ODS column (9.4mm x 24cm) and a Hewlett Packard™ (HP) model 1090M HPLC workstation mated to an HP diode array detection system.

Preparation of Meat Samples:

Forty gram cubes (±0.4g) of top round were cooked by convection baking at 176.7°C (350°F) for periods of 15 minutes each; half of the samples were recooked similarly after 2 days refrigerated storage at 4-5°C (40°F). All samples were weighed, minced finely and then made 20% (w/v) with cold (4°C) 10mM HEPES homogenization buffer described above. The minced sample was then homogenized by three, four-second-bursts at full speed using a Tekmar™ Tissuemizer.

Extraction Procedure:

The homogenized meat samples (20%, w/v) were filtered through gauze (4 layers) and the resulting filtrate diluted to the equivalent of a 10% homogenate (w/v) with water. The resulting solution was made acidic by supplementing with acetic acid to 25%. The 25% acetic acid mixture was gently stirred for 3.0 minutes and centrifugated at 10,880x*g* for 10 minutes at 4°C in a SS-34 rotor in a Sorvall RC-5B centrifuge. The supernatant solution was then centrifuged at 100,900x*g* for 60 minutes at 4°C in a Dupont RC-70 ultracentrifuge using an A841 rotor. The resulting supernatant solution was lyophilized in a Virtis Freezemobile 12 freeze dryer, with the final lyophilizate appearing as a semi-solid oil. Because of the oil-like nature of the lyophilizate, the material was resuspended in 25%-acetic acid (200 ml) and extracted with methylene chloride:methanol (2:1, v:v), in a ratio of 1 part sample to 1 part of extractant. The aqueous portions of the extracts were lyophilized with the resulting material being a fine white fluffy powder. Weight of the powder ranged from 1 - 2 grams from twenty grams of starting material.

CHROMATOGRAPHY:

Sample lyophilizates (200 mg dry weight) taken from 'cooked' and 'cooked-stored-recooked' meat extracts were applied to a Sephadex G-25 gel filtration column (previously calibrated with standards of known M_r). The samples were loaded in a volume of approximately 2.0 ml in column eluant (25%-acetic acid) and the column eluted at a flow rate of about 20.0 ml/hr. Column fractions of 5.0 ml were collected and the eluant was monitored at 254 nm. The resulting chromatographic profiles are shown in Figure 2. Peak fractions from the gel filtration purification were lyophilized. Peaks eluting at approximately 1,800 M_r were prepared for further analysis by reverse phase (RP) high performance liquid chromatography (HPLC).

Samples recovered from the gel filtration columns were prepared for injection by dissolving the lyophilized residues taken from either peak I or II (Figure 2) in a acetonitrile (0.5 ml residue/2.0 ml acetonitrile). The sample was then applied to a Waters™ C-18 reverse-phase Sep-pak cartridge [pre-washed with 4.0 ml of a 80:20 acetonitrile:water (v:v) mixture]. The Sep-pak cartridge was washed again with a 2.0 ml solution of the same ratio of acetonitrile/water. Typically 300 µl of the resulting 4 ml sample was then injected onto a Dupont™ ODS Zorbax column and eluted with a gradient ranging from 5% water (with 0.1% TFA) to 95% acetonitrile (with 0.05% TFA) over a 60 minute period resulting in the separation of various hydrophilic and hydrophobic peaks.

The major separated hydrophobic peak was selected for further examination on an analytical RP-HPLC (Hewlett Packard™). The material (from 5 to 25 µl) was injected into a HP-HPLC system (model 1040M) coupled to a diode array detection system. The samples were run isocratically at 50:50 with a 0.1% aqueous TFA: 0.05% TFA in acetonitrile (v:v). Run time was for 5 minutes at 40°C; the recorded spectral range of the diode array detector was 200-340 nm.

RESULTS AND DISCUSSION

As outlined in Figure 1, a series of extractions and chromatographic steps were employed in an approach designed to isolate peptides from 'cooked' and 'cooked/stored/recooked' meat samples. Homogenized meat samples were initially brought to 25% with acetic acid and centrifuged by differential centrifugation to separate the soluble peptides and proteins from the larger, insoluble material such as myofibrillar fragments, cellular organelles and debris. Samples were made 25% acetic acid to provide for maximal extraction and

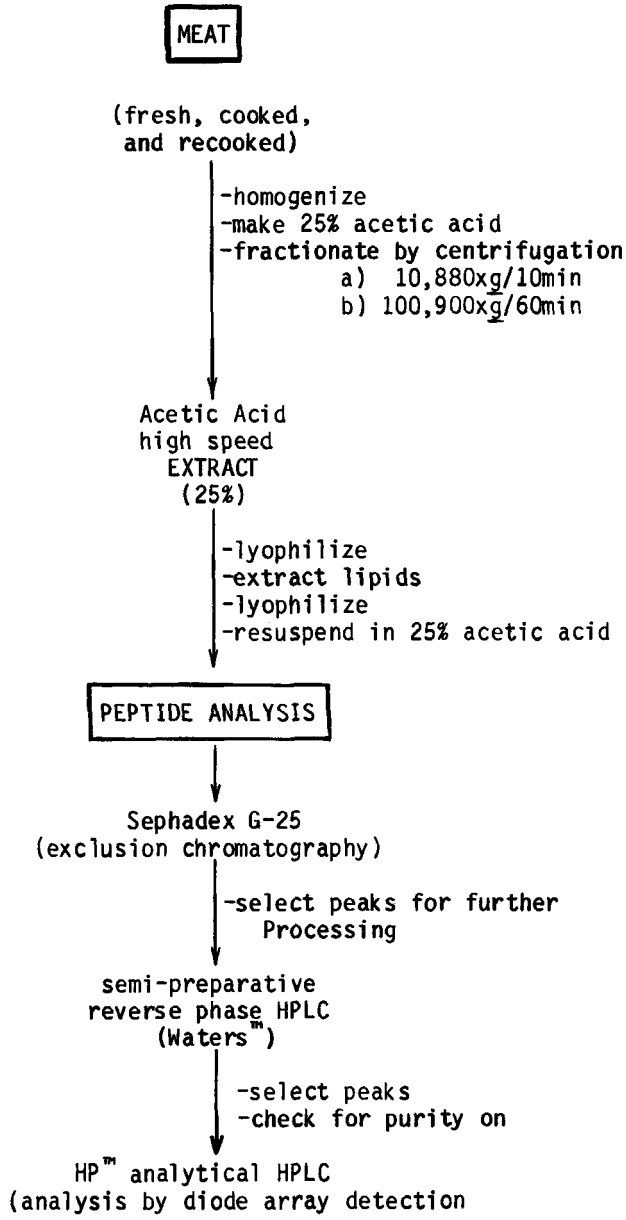

FIGURE 1: Scheme of protocol for isolation and characterization of meat peptides.

dissolution of both hydrophobic and hydrophilic peptides from the homogenate. As with the lower speed centrifugation (10,880xg), centrifugation of the extracts at 100,900xg was performed to enhance the transfer of a clear supernatant solution of peptide-protein extract for further analysis. Following lyophilization of this solution the resulting residue appeared as an oily light-brown semisolid. Extractions of the resulting lyophilizate from a resuspended 25% acetic acid solution with methylene chloride:methanol afforded aqueous and organic extracts. The aqueous extract was lyophilized yielding a white fluffy powder. The organic extact was evaporated in vacuo to yield a clear oil (Note: Organic lipid extractions were perform at this later step rather than after homogenization in an effort to keep working volumes within the most economical processing range). The lyophilizate tested positively for polypeptides in a BCA (bicinchoninic acid, 14) and Lowry (15) determination whereas the oil tested positively for carbohydrates in a phenol-sulfuric acid test (16). Sodium dodecyl sulfate, polyacrylamide gel electrophoresis (data not shown) of the lyophilizate revealed protein and peptide constituents within the M_r range of 5,000 - 200,000 Daltons with the latter representing solubilized myosin.

Preparative scale fractionation of the lyophilizate was accomplished (Figure 2) on a Sephadex™ G-25 column equilibrated with 25% acetic acid in water and previously calibrated with molecular weight standards ranging from 500 to 5,000 M_r. Elution profiles for extracts of 200 mg (dry weight) lyophilizate of uncooked meat were very similar to each other but differed significantly from the uncooked samples. These peptides eluted in the M_r range of 1,500 - 3,000 based on the calibration peptide standards.

The results shown in Figure 2 repetatively demonstrate that the beef peptide extracts principally contain constituents which are proteins and/or polypeptides of M_r greater than 5,000 and two partially resolved fraction series within a M_r range greater than 1,500 but less than 3,000. The first group, or polypeptide peak I, falls towards the higher MW region while the second group, or polypeptide peak II, falls within the lower MW range. The relative amount of protein (% of total A_{254}) found in peak I and peak II is low in the control (group 0 = uncooked meat) and high in the experimental groups (15 = cooked and 15' = 15 minutes cooked, stored 2 days at 4°C, recooked for 15 minutes). Examination of peak I polypeptide material reveals that all experimental groups show nearly identical absorbances at 254 nm with the experimental value being greater than the control or PEAK I: group 0 < 15 = 15'. On the other hand, a marked differences in relative absorbance is seen in peak II polypeptide material, i.e., the 15 minute 'cooked' samples (group 15)

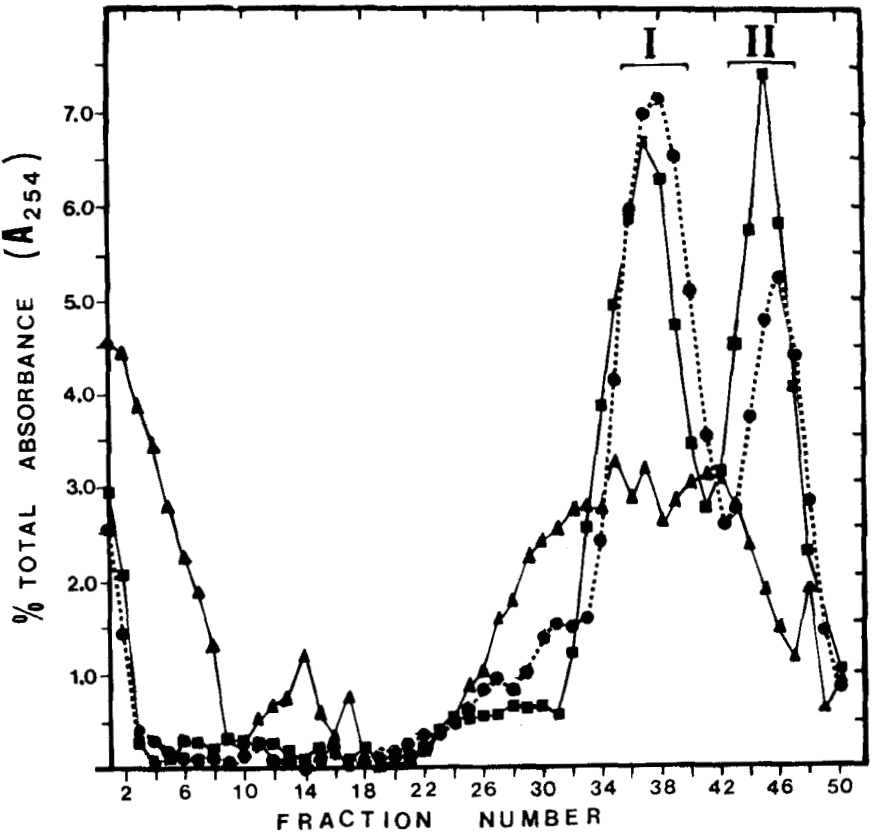

Figure 2: SIZE EXCLUSION CHROMATOGRAPHY OF MEAT PROTEIN/PEPTIDE EXTRACTS.

Lyophilized acetic acid extracts of meat were layered onto a packed column of Sephadex G-25 (packed dimensions 85cm x 2.54cm) as described in text. Flow rate with eluant (25% acetic acid) was 1 drop/6 seconds and fractions of 5 ml/tube were collected for a total of 140 tubes. The data is presented as the "% total absorbance A_{254}" in an effort to normalize the data between sample groups. Data is not presented past the 50th tube since all data beyond this point was at or near background levels. The plots are initiated at the void volume peak tube and represents a M_r of 5,000 while peak I and II represent M_r of 2,500 and 1,800, respectively. The graphic symbols for the "uncooked control", "15 minute cooked", and for the "15 minute cooked-stored-recooked" groups are represented by a triangle, square, and circle, respectively.

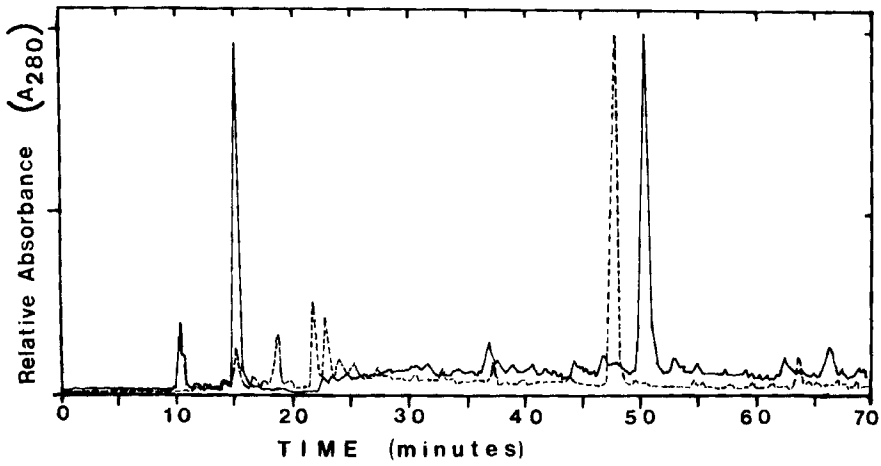

Figure 3: SEMIPREPARATIVE REVERSE PHASE (RP) HIGH PERFORANCE LIQUID CHROMATOGRAPHY (HPLC) OF PROTEIN/PEPTIDE EXTRACTS.

RP-HPLC separations using a Waters™ model 350 with dual model 510 pumps were performed on peak II (1,800 M_r) material previously isolated from meat protein/peptide extracts by gel filtration chromatography on Sephadex™ G-25 (Figure 2). Samples were either from meat cooked for 15 minutes at 350°F, (group 15 = solid line) or from meat cooked for 15 minutes at 350°F, stored for 2 days at 40°F, and recooked for 15 minutes at 350°F, (group 15' = dashed line). The solvent of the elution system was a linear gradient of 5% to 95% in water (0.1% TFA) to acetonitrile (0.05% TFA). Hydrophilic peptides elute from the column during the first 1/2 of the elution period (peaks at 10-20 minutes) while hydrophobic peptides elute from the column during the second 1/2 of the run (peaks at 45-55 minutes)

show a marked increase in the proportion of peak II material while the 'cooked-stored-recooked' samples (group 15') show a smaller increase in this peak yet still greater than the control or PEAK II: group 0 < 15' < 15.

Subsequent purification of the fractions isolated from meat extracts by gel filtration was performed utilizing RP-HPLC. Figure 3 shows the results of a RP-HPLC gradient of samples taken from peak II of 'cooked' (group 15) and 'cooked-stored-recooked' meat (group 15'). Elution of these components of similar molecular mass (M_r) reveals wide differences in column retention times for the major peaks. This suggests that the peptides extracted from the meat samples are distributed into two major peptide groups, i.e. hydrophilic and hydrophobic.

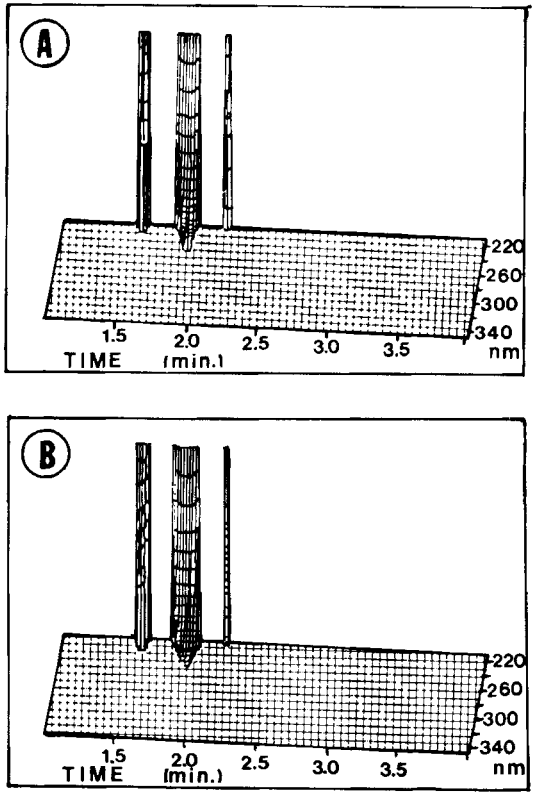

Figure 4: ISOCRATIC, ANALYTICAL RP-HPLC OF HYDROPHOBIC PEPTIDE PEAKS PREVIOUSLY SEPARATED BY PREPARATIVE HPLC.

RP-HPLC was performed using a Hewlett Packard TM model 1090M HPLC unit mated to a diode array detection system. The resolving solvent was a 50:50 isocratic system of water (0.1% TFA) : acetonitrile (0.05% TFA). All data is presented as the ratiogram with the x,y, and z axis representing the elution time, ratiogram-of-absorbance-intensities, and the wavelength, respectively. Chromatograms have a swivel of 355° with a tilt of 25°. The top, middle, and bottom graphs represent (A), solvent blank, (B), the hydrophobic peak of the semi-preparative HPLC (Figure 3) of group 15 ('cooked meat'), and (C) the hydrophobic peak of the semi-preparative HPLC (Figure 3) of group 15' ('cooked/stored/recooked'), respectively.

(continued)

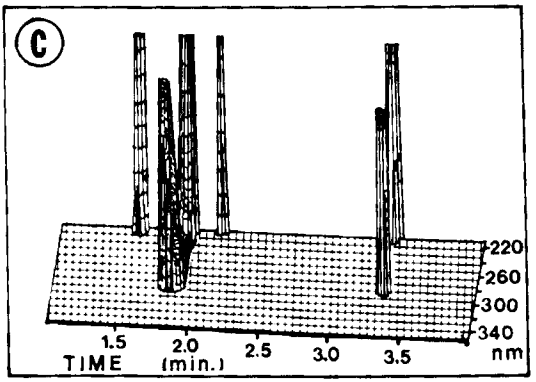

Figure 4 (continued)

In the case of the 'cooked' meat extracts (group 15) we observed nearly equivalent amounts of hydrophilic and hydrophobic constituents (early and late eluting peaks, respectively). On the other hand, group 15' ('cooked-stored-recooked') meat extracts showed an increased proportion of material (% of total A_{280}) in the late eluting, hydrophobic peptide peaks with a marked decrease in the relative amount of material of early eluting, hydrophilic peaks.

The RP-HPLC peaks with retention times of 47 to 50 minutes (Figure 3) were subjected to further separation and analysis utilizing a Hewlett Packard™ analytical HPLC system. Separation was performed in an isocratic solvent system as seen in Figure 4; such a procedure proved to separate components which were not readily resolved in the semipreparative gradient RP-HPLC system described in Figure 3. The sample from the 15 minute 'cooked' group eluted within the solvent front. However, the sample from group 15' (the 'cooked-stored-recooked' meat) showed (i) one component copurifying with the solvent but having an absorption maximum at 280 nm, and (ii) two components eluting later than the solvent all of high purity based on the flat appearance of the ratiogram peaks.

The results described above outline an approach for the isolation and purification of hydrophilic and hydrophobic peptides from meat products using preparative-scale extraction technique, preparative gel filtration and semipreparative and analytical RP-HPLC. The results indicate that the relative levels of hydrophilic and hydrophobic peptides are different when comparing

'cooked' with 'cooked-stored-recooked' meat. Previous findings by others correlating differences in hydrophobicity with taste (1,3) imply that the peptides isolated in these current chromatography studies may be related to differences in perceived flavors in 'cooked' versus 'cooked-stored-recooked' beef. Thus, the results of this investigation suggest that further studies are merited to explore the relation of these presumptive flavor peptides to the model of warmed-over flavor in meat.

REFERENCES

1. Goodman M. and Temussi, A. P. Structure-activity relationship of a bitter diketopiperazine revisited. Biopolymers, 24:1629-1633 (1985).

2. Rodriquez, M., Bland, J. M., Tang, J. W., and Goodman, M. Peptide sweeteners 8. Synthesis and structure-taste relationship studies of L-aspartyl-D-alanyl tripeptides. J. Med. Chem. 28:1527-1529 (1985).

3. Otageri, K., Nosho, Y., Shinoda, I. Fukui, H., and Okai, H. Studies on a model of bitter peptides including arginine, proline, and phenylalanine residues. I. Bitter taste of di- and tripeptides and bitterness increase of the model peptides by extension of the peptide chain. Agric. Biol. Chem. 49:1019-1025 (1985).

4. Nissen, J. A. The relationship of structure to taste of peptides and peptide mixtures 190th ACS National Meeting, Chicago, IL Sept. 1958 Abstract 21 AGFD.

5. Dutson, T. R. and Orcult, M. W. Chemical changes in proteins produced by thermal processing. J. Chem. Ed. 61:303-308 (1984).

6. Davis, C. E. and Anderson, J. B. Size exclusion/HPLC of heated water soluble bovine and procine muscle proteins. J. Food Sci. 49:598-602 (1984).

7. Cheng, C. S. and Parrish, F. C. Heat-induced changes in myofibrillar proteins of bovine longissimus muscle. J. Food Sci. 44:22-24 (1979).

8. Jiany, S.-T. and Lee, T. C. Changes in free amino acids and protein denaturation of fish muscle during frozen storage. J. Agric. Food Chem. 33:839-844 (1985).

9. Agater, I. B., Briant, K. J., Llwellyn, J. W., Sawyer, R., Bailey, F. J., and Hitchcock, H. S. The determination of soya and meat protein in raw and processed meat products by specific peptide analysis. An evaluation. J. Sci. Food. Agric. 37:317-331 (1986).

10. Medina, M. B. and Phillips, J. G. Investigations on trypsin-hydrolyzed peptides for protein identification. J. Agric. Food Chem. 30:1250-1253 (1982).

11. Carnegie, P. R., Ilic, M. Z., Etheridge, M. O., and Collins, M. G. Improved high-performance liquid chromatographic method for analysis of histidine dipeptides, amerine, carnosine and balimine present in fresh meat. J. Chromatogr. 261:153-157 (1983).

12. Zaika, L. L., Wasserman, A. E., Monk, C. A. Jr., and Salay, J. Meat Flavor. 2. Procedures for the separation of water-soluble beef aroma precursors. J. Food Sci. 33:53-58 (1968).

13. Johnsen, P. B. and Civille, G. V. A standardized lexicon of meat warmed-over flavor descriptors. J. Sensory Studies 1:99-104 (1986).

14. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K. Gartner, G. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85 (1985).

15. Lowry, O. H., Rosenbrough, H. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275 (1985).

16. Dubois, M. Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:250-356 (1956).