Applications of Combined Gel Permeation Chromatography and High Speed Liquid Chromatography for the Separation of Complex Flavor Mixtures

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This paper describes the use of combined liquid chromatographic techniques for the characterization of citrus essential oils and a selected alcoholic beverage, rum. These mixtures were separated by both gel permeation and partition modes of liquid chromatography. The techniques described establish the feasibility of this approach for controlling processing variables in the production of these products and determining the contribution of extraction–reaction mechanisms to the aging of alcoholic beverages and describe a method for the purification of individual components for further analysis.

In the characterization of a complex mixture such as an essential oil, a fermentation reaction, or a spice, a single separation technique is usually not adequate to provide all of the desired information from that mixture. This problem of simplifying complex samples such as essential oils, spices, or fermentation products has been approached classically by means of selective extraction, column chromatography, low temperature crystallization, and preparative gas chromatography as discussed by Schmit and Dixon (1968). Martin *et al.* (1963, 1965) and Schmit (1969) have reported on the characterization of alcoholic beverages by gas chromatography through the development of highly selective stationary phases for the separation of low molecular weight constituents and by employing selective extraction followed by derivative formation for higher molecular weight components (Schmit, 1969). All of these methods have helped define the particular mixture under investigation but generally have the disadvantage of being time consuming and not as selective as might be desired.

High speed liquid chromatographic techniques have the advantage that relatively little sample preparation may be necessary and that both volatile and nonvolatile materials can be chromatographed and separated. By combining these new separation methods alone or in combination with previously established methods, the complete characterization of these mixtures can be more nearly realized. It is the purpose of this study to show the broad potential for this method in the separation and characterization of flavor components. For this reason samples of dissimilar chemical nature and origin were selected.

EXPERIMENTAL SECTION

Apparatus. In this study, a DuPont Model 830 liquid chromatograph equipped with dual detectors (uv and refractive index) and a gradient elution accessory were employed. The mass spectrometer used was a DuPont Model 21-110C. Columns for gel permeation chromatography (gpc) were Biobeads SX-2 (Bio-Rad Laboratories, Richmond, Calif.; available from E. I. du Pont de Nemours & Co., Wilmington, Del.). Columns for high speed liquid chromatography (lc) were packed with Permaphase ODS for reversed-phase separations and Permaphase ETH for normal partition separations (E. I. du Pont de Nemours & Co., Instrument Products Division, Wilmington, Del.). The essential features of the instrument and the column materials have been discussed previously in publications by Felton (1969), Kirkland and De Stefano (1970), Kirkland (1968, 1969, 1971), and Byrne *et al.* (1971). The solvents used for the mobile phase were spectral or ACS grade and were not purified further.

E. I. du Pont de Nemours & Co., Inc., Instrument Products Division, Wilmington, Delaware 19898.

Sample Preparation. The juice oils were diluted in chloroform to give 15% solutions for the gpc analysis. For lc analysis, the oils were diluted with tetrahydrofuran to produce a concentration of 0.5–1.0%.

The samples used for the alcoholic beverage study were extracted with chloroform using the following procedure: 75 ml of the alcoholic beverage was extracted twice with 25 ml of chloroform. The chloroform extract was blown to dryness with a stream of dry nitrogen and reconstituted to 1 ml with tetrahydrofuran. The alcoholic beverages were purchased through a commercial outlet.

Chromatography. Gpc separations were carried out using a 1 m × 7.9 mm i.d. column packed with Biobeads SX-2. Elution volumes were recorded every 5 ml using a syphon counter. Gpc fractions were collected using a manually operated fraction collection valve that is standard on the Model 830 liquid chromatograph. Fractions were then blown to dryness with a stream of dry nitrogen and reconstituted to 1 ml with tetrahydrofuran.

Reversed-phase partition liquid chromatography was carried out using a Permaphase ODS column operated at 60° and employing gradient elution. In most cases, linear gradients from water to methanol at rates of 3%/min to 5%/min change in methanol concentration were used.

Normal partition separations were carried out using a Permaphase ETH column operated at ambient temperature and employing gradient elution. The gradients employed were hexane to isopropyl alcohol and in most cases were linear at rates of 2%/min and 3%/min change in isopropyl alcohol content.

Separation Mechanism. The separations obtained in this study are a result of several different mechanisms. The gpc separations are based primarily on a difference in molecular size. These columns are calibrated by chromatographing standards of known molecular weight and then plotting log molecular weight *vs.* elution volume. By careful control of chromatographic conditions and periodic checks on the calibration, molecular weight approximations can be obtained and fractions having a narrow molecular weight distribution can be collected for further investigation.

The reversed-phase separations are somewhat more complex. Generally, compounds elute from reversed-phase columns in the order of their decreasing water solubility and therefore it is generally not possible to predict the relative molecular weight of the eluted peaks.

In normal partition separations, compounds generally elute in the order of their increasing affinity for the polar stationary phase or conversely their decreasing solubility in the hydrocarbon mobile phase. It is again invalid to compare retention time and relative molecular weight.

These separation mechanisms should be kept in mind when gpc fractions are chromatographed by liquid chromatographic techniques. It is entirely possible for a higher

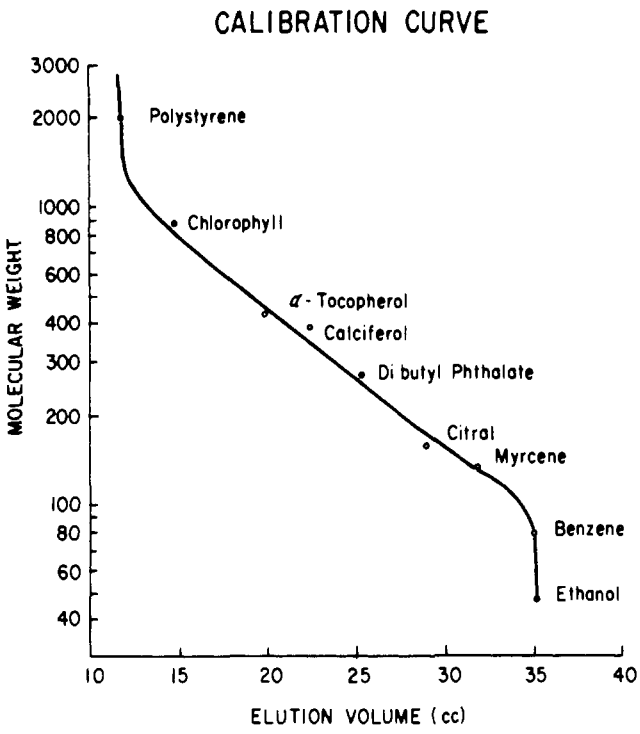

Figure 1. Calibration curve for Biobeads SX-2. Operating conditions: column, 1 m × 3/8 in. i.d.; flow rate, 0.8 ml/min; solvent, chloroform; molecular weight range, 85–1000.

molecular weight fraction to have a shorter retention time on an lc system than a lower molecular weight fraction.

More complete discussions of these separation techniques are found in a recent text by Kirkland (1971a) and by Henry *et al.* (1972) and Schmit *et al.* (1971).

RESULTS AND DISCUSSIONS

Calibration of the Gpc Column. For the purposes of this study, the gpc columns were calibrated with pure standards having different structure and molecular weight. The calibration curve obtained with these standards is shown in Figure 1. These compounds were selected because they represented typical molecular weights and structures of expected constituents in the samples. It should be remembered that the separation mechanism in gpc is by molecular size and not molecular weight so that the calibration curve in Figure 1 should be used only for molecular weight estimates. These estimates appear to be rather accurate, as can be seen from an example discussed later in the study where molecular weight data from a mass spectrometer compared well to that predicted by the calibration.

The essential oils from Valencia orange, tangerine, and grapefruit were compared using gpc techniques. Typical chromatograms are shown in Figures 2 and 4. In these analyses the refractive index detector was particularly sensitive to compounds with molecular weights of 200 and below. Conclusions as to the relative quantity of material in each peak are not valid based on these chromatograms because the detectors (uv or ri) respond to differential uv absorbance and refractive index and not to mass.

It is obvious from these chromatograms that the three essential oils differ considerably in composition. All three essential oils have uv absorbing components above molecular weight 300, which is the usual limit for gas chromatographic separations. Both tangerine and Valencia orange oils have components which are apparently above 500 molecular weight. This is quite different from chromatograms of the juice oils of the same fruits (not shown)

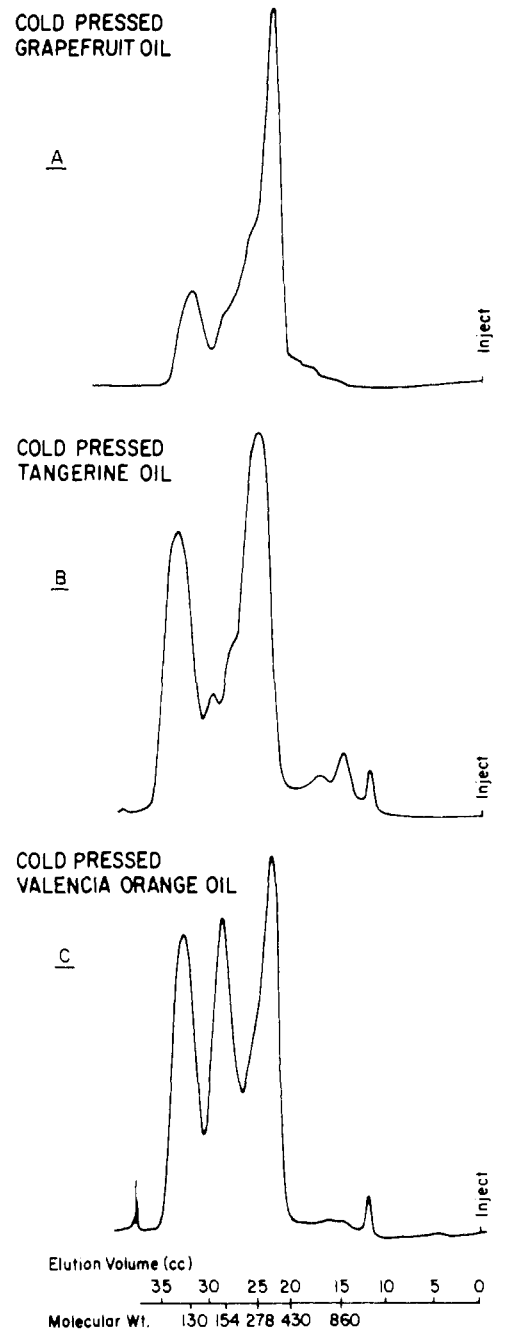

Figure 2. Steric exclusion separations of cold-pressed citrus oils. Operating conditions: column, 1 m × 3/8 in. i.d.; flow rate, 0.8 ml/min; solvent, chloroform; molecular weight range, 85–1000.

which do not contain any components above about 350 molecular weight.

In the case of the cold-pressed Valencia orange oil, samples from different sources were chromatographed and showed substantial differences in the relative magnitudes of the various peaks, although the molecular weight range was similar in all cases. These variations in gpc chromatograms could be due to climate or seasonal variations or even the methods used to produce the oil.

In order to further characterize cold-pressed Valencia orange oil and to demonstrate the use of combined liquid chromatographic techniques, a series of preparative experiments were undertaken. Cold-pressed Valencia orange oil with its broad molecular weight distribution was selected to illustrate this combined approach. A sample of the oil, approximately 5 mg, was separated by gpc and fractions

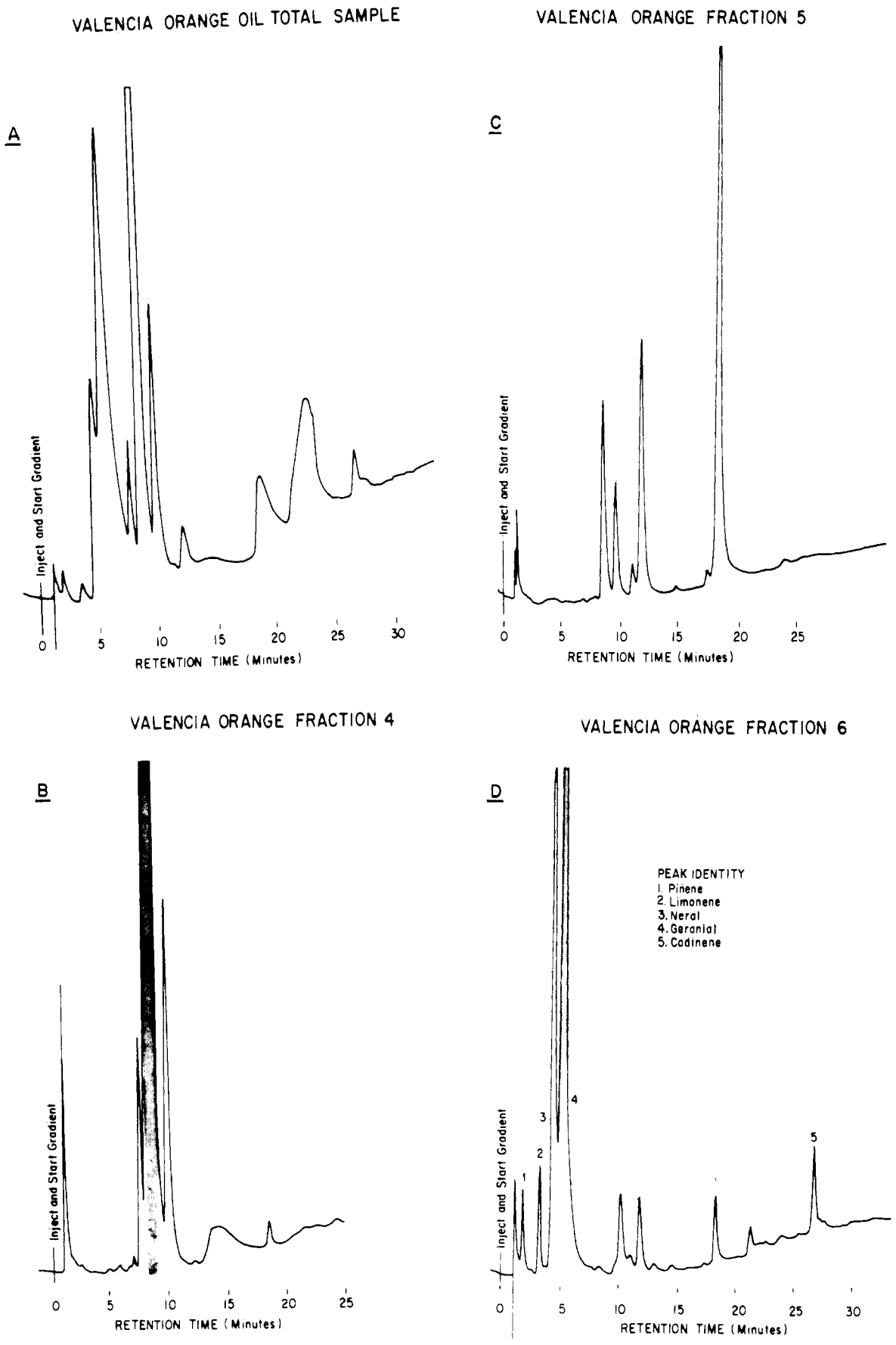

Figure 3. Reversed-phase liquid chromatographic separations of the total cold-pressed oil and collected fractions. Operating conditions: column, 1 m × 2.1 mm; Permaphase ODS mobile phase, linear gradient from 5% MeOH/95% H_2O to 100% MeOH at 3%/min; column temp, 50°; flow rate, 1.5 ml/min; detector, uv photometer.

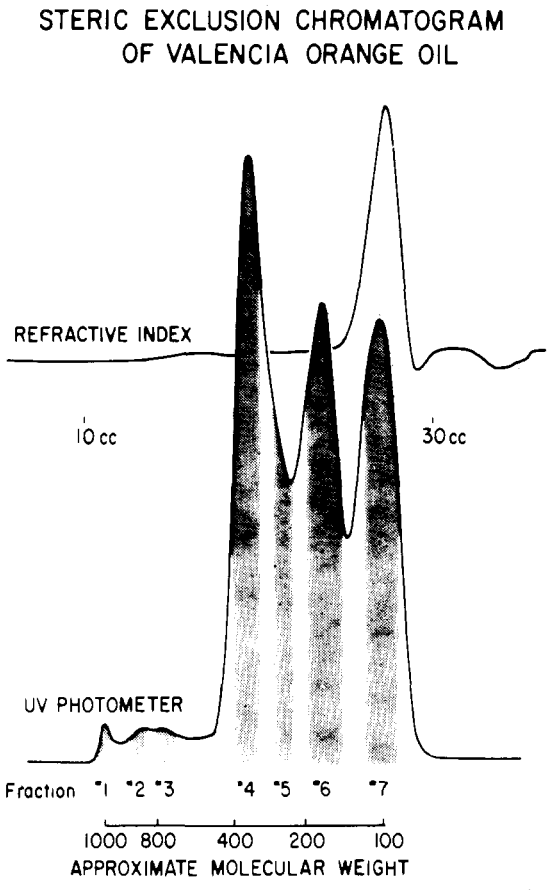

Figure 4. Fractions collected from preparative gpc for characterization by liquid chromatography. Operating conditions: column, 1 m × 3/8 in. i.d.; flow rate, 0.8 ml/min; solvent, chloroform; molecular weight range, 85–1000.

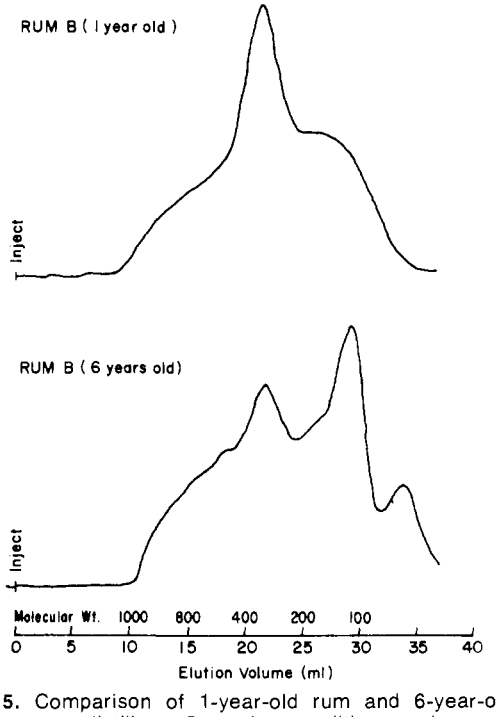

Figure 5. Comparison of 1-year-old rum and 6-year-old rum from the same distillery. Operating conditions: column, 1 m × 3/8 in. i.d.; flow rate, 0.8 ml/min; solvent, chloroform; molecular weight range, 85–1000.

were collected as shown in Figure 3. These fractions were reduced in volume to dryness under a stream of dry nitrogen. The fractions with molecular weights of 200 and below were placed in an ice bath during the evaporation to reduce the loss of the lower molecular weight constituents. The dried fractions were diluted to 200 μl with isopropyl alcohol. Aliquots of these fractions were then chromatographed by high speed reversed-phase liquid chromatography. This approach reduced the overall complexity of the sample by limiting the range of molecular weights in any given fraction.

In preparing these collected samples, it was observed that fractions 4 through 7 all had an orange odor, with fractions 4 and 6 being the most pronounced, and that fraction 2 contained all of the pigmented material.

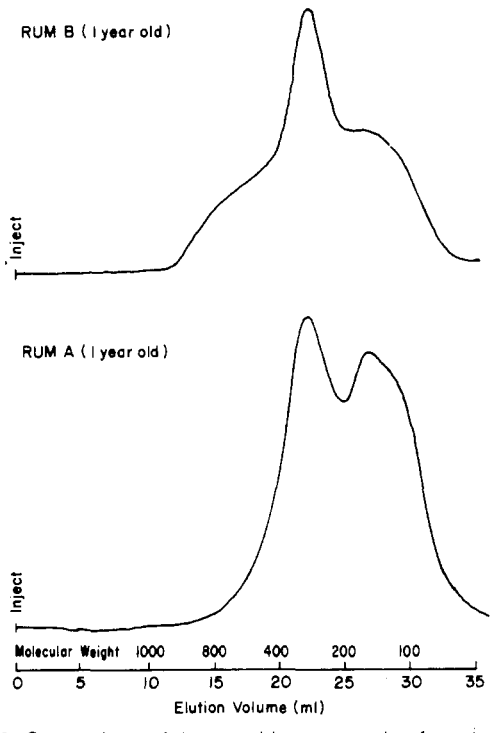

Figure 6. Comparison of 1-year-old rum samples from two distilleries. Operating conditions: column, 1 m × 3/8 in. i.d.; flow rate, 0.8 ml/min; solvent, chloroform; molecular weight range, 85–1000.

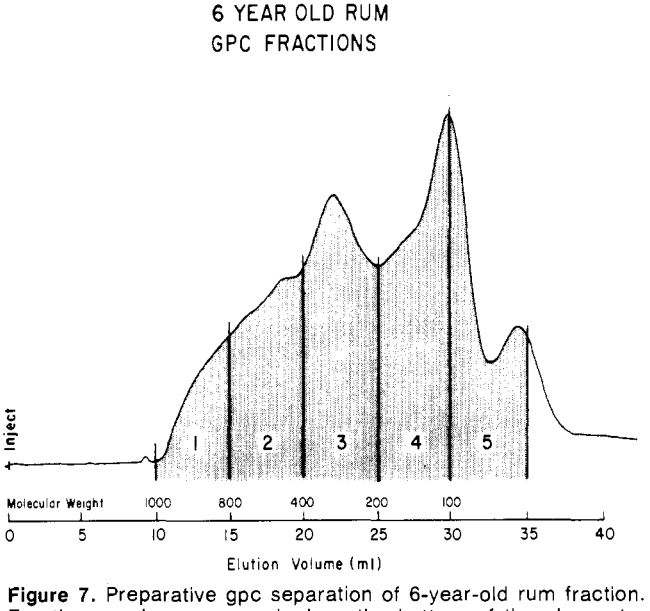

Figure 7. Preparative gpc separation of 6-year-old rum fraction. Fraction numbers are marked on the bottom of the chromatogram. Operating conditions: column, 1 m × 3/8 in. i.d.; flow rate, 0.8 ml/min; solvent, chloroform; molecular weight range, 85–1000.

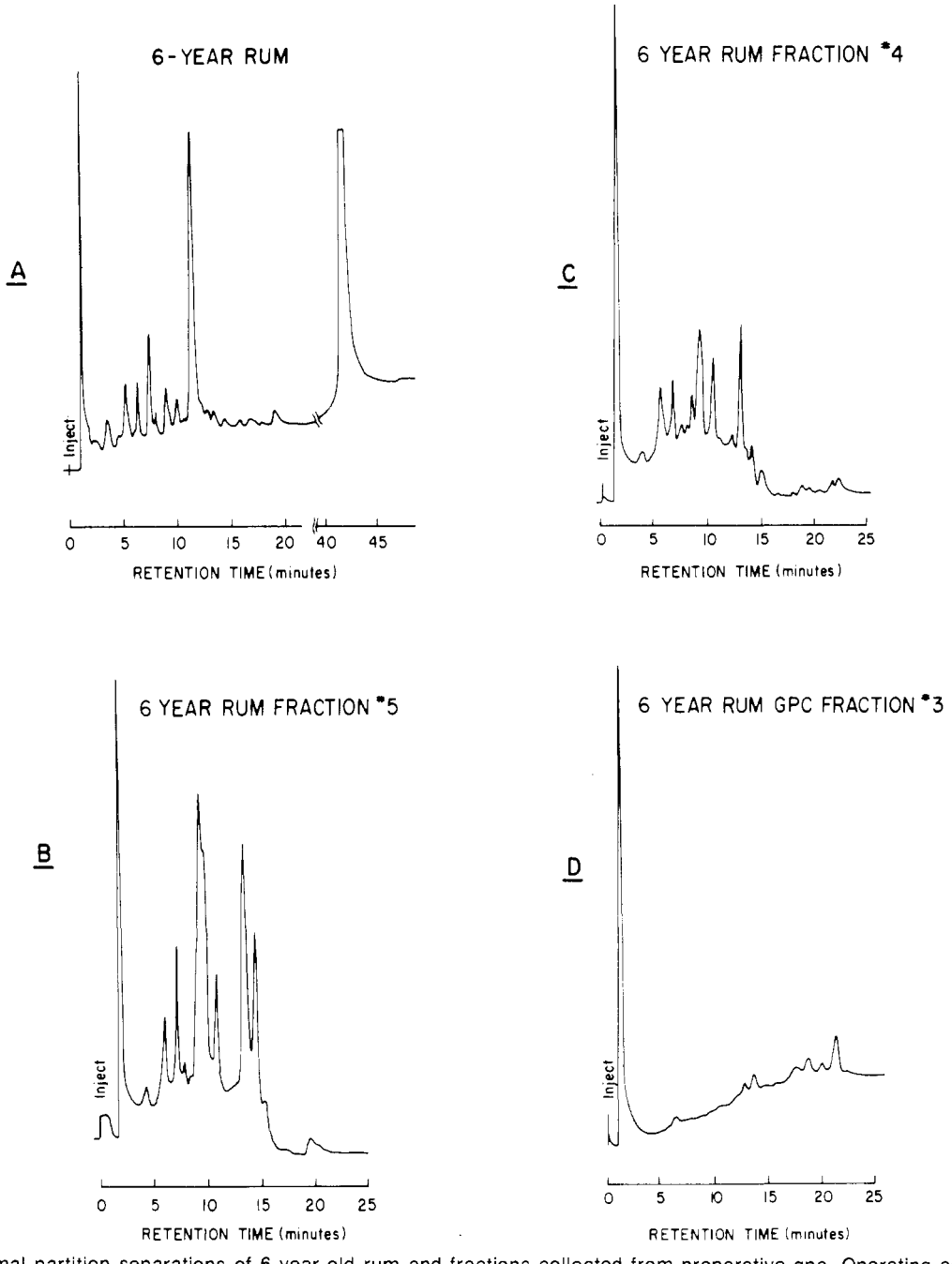

Figure 8. Normal partition separations of 6-year-old rum and fractions collected from preparative gpc. Operating conditions: column, 1 m × 3/8 in. i.d.; flow rate, 0.8 ml/min; solvent, chloroform; molecular weight range, 85–1000.

The reversed-phase separations of typical individual fractions, as well as a total oil sample, are shown in Figure 5. The compounds in fraction 7 were extremely polar and did not chromatograph well on the reversed-phase system. These components were assumed to be acidic, a conclusion that was substantiated by their retention on an anion exchange column where they gave symmetrical well-defined peaks.

The identity of the peaks in fraction 6 was established by comparison of retention times with standards on both liquid and gas chromatographic systems. The identity of these peaks was further confirmed by mass spectroscopy. Fraction 5, molecular weight 250–300, had a major component, three sizable additional peaks, and a number of minor components. Fraction 4, molecular weight 300–500, was of particular interest in this study in that it had a molecular weight above that commonly encountered in gas chromatographic analysis of Valencia orange oil; it was predominantly a single peak and it had a pronounced orange odor. This fraction was chromatographed and the eluted peak collected for analysis by mass spectroscopy. The results of this analysis showed this peak to be composed of two components of approximately equal concentration. The mass of the parent ions for these compounds was found to be 432.1451 and 417.1205, while the closest empirical formulas were $C_{25}H_{22}NO_6$ and $C_{24}H_{19}NO_6$, respectively. Two other ions of less intensity were also present at 30 mass units less than the parent ions, possibly due to the loss of an NO group upon fractionation.

In the examination of the gpc fractions, some 80 compounds were separated. In the evaluation of these separations, it must be remembered that the detector employed responds only to compounds that absorb in the ultraviolet and that these 80 compounds do not necessarily represent the total number of constituents in the sample.

Alcoholic Beverages. An alcoholic beverage, rum, was

selected for a survey study by the combined gpc–lc technique. Figure 5 shows chromatograms of 1- and 6-year-old rum samples from the same distillery. There is an obvious increase in the concentration of lower molecular weight uv absorbing components in the older rum, while the main peak at about 350 molecular weight remains relatively constant. These differences may be due to variables in the aging process, including the age and history of the barrel, as well as the temperature of the aging area. One possible explanation for these differences could be that the main peak of the 1-year rum at about 350 molecular weight could come primarily from extraction from the barrel, while the lower molecular weight components in the aged rums come from slow extraction and side reactions. To complicate matters, the age of the samples can be misleading if it is not realized that the stated age on the bottle reflects the youngest rum used and not necessarily the age of the entire sample. A 1-year rum, then, could contain various amounts of older rums blended together to give a particular desired taste.

The chromatograms in Figure 6 show 1-year rum samples from different distilleries. The differences in the chromatograms apparently represent the differences in the aging and blending processes.

The 6-year-old rum sample was selected for further characterization by preparative gpc and subsequent liquid chromatography. Fractions were collected in 5-ml volumes as shown in Figure 7. These fractions were blown to dryness with a stream of dry nitrogen and reconstituted to 100 μl. A 5-μl aliquot was then analyzed by partition chromatography. Chromatograms of these fractions and the total rum sample are shown in Figure 8. Fraction 3 with an upper molecular weight of approximately 500 was the highest molecular weight fraction that could be chromatographed under these conditions. By using combined gpc–lc methods, some 60 peaks have been separated from the 6-year-old rum. Similar results have been obtained for aged bourbon samples. Once the relationship between the gpc profile and the aging process has been established, liquid chromatography can be used to isolate those specific compounds of primary importance in the aging process.

CONCLUSIONS

The use of combined gpc–lc for the characterization of complex flavor mixtures has been established. By this technique it should be possible to better determine the identity of those compounds responsible for the odor and flavor of essential oil. In addition, it should be possible to determine the contribution of various climatic and geographic variables to the characteristics of the oil should this be possible. These techniques should also prove valuable in determining the effect of various processing steps on the quality of the final product.

In the analysis of alcoholic beverages, the data presented would suggest that this combined liquid chromatographic approach would prove useful in determining such factors as the influence of barrel age, barrel history, temperature of the aging area, blending, and other variables on the flavor of the final product.

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Automated Colorimetric Measurement of Free Arginine in Peanuts as a Means to Evaluate Maturity and Flavor

Clyde T. Young

This paper includes a review of published information and presents new information relative to the adaptation of the Sakaguchi reaction for measuring free arginine content of raw (green and cured) and roasted peanuts. Emphasis is on the automation of the method for research and industrial use in the peanut industry so that several hundred samples can be analyzed daily. The role of free arginine in flavor development and its relationship to maturity are discussed.

Based upon the work of Newell (1967), Mason *et al.* (1968, 1969) discussed the nonvolatile components of peanuts and their importance in typical and atypical roasted flavor. They pointed out that changes in free arginine were very dramatic and that its concentration was inversely correlated with maturity.

Their work was accomplished with ion-exchange chromatography which is excellent for identification and quantitation of amino acids but too time-consuming for

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