HPLC Determination of Long Chain Saturated Fatty Acids in Tall Oil Rosin

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A method was developed for the analysis of long chain saturated fatty acids or their esters from tall oil or tall oil rosin. The method is specific for 18 through 28 even carbon number acids with a detection limit for each at 0.05% based on rosin. Ultraviolet detection is achieved through phenacyl derivatization. Omission of the saponification step allows selective determination of the free fatty acids.

Analysis and testing methods for tall oil derived rosin and fatty acids have played important roles in developing many of the practical uses for these materials. Improved methods have been reported for analysis of rosin and fatty acids in kraft mill process streams (1), pulp and paper mill effluents (2,3) and pitch deposits in paper mills (4,5).

Although sophisticated capillary gas chromatographic (GC) methods for tall oil analysis offer the high separation capability often needed for complex natural mixtures, the abundance of GC-resolvable components in tall oil can unnecessarily complicate some analyses such as total resin or total fatty acids (6). This appears true for the analysis of long chain saturated fatty acids (C20 through C26) in tall oil rosin (TOR).

A quantitative GC method for the determination of C20 to C26 long chain saturated fatty acids (LCSFAs) in TOR can be inferred from the extensive work of Dorris et al. (5) and Holmbom (7). The difficulties cited for peak overlap and split mode injection discrimination suggest that a need remains for a relatively simple and quantitative LCSFA analysis from tall oil or TOR.

In this paper, a high pressure liquid chromatographic (HPLC) technique for LCFSA analysis in TOR is described. By comparison to alternate methods this technique is simple, quantitative and was developed with three additional objectives: specificity for different chain length fatty acids, detection sensitivity at least to 0.1% on a TOR basis and determination of both free and esterified fatty acids.

EXPERIMENTAL

Equipment. The HPLC method was developed on an isocratic QA-1 Waters model from Waters Chromatography Division, Millipore Corp., Milford, Massachusetts, and that was equipped with an auto sampler, Hewlett Packard 3390A integrator and a fixed wavelength UV detector at 254 nm. The mobile phase used was acetonitrile with a flow rate of 1.0 ml/min. Injection volume was 20 μl from the auto sampler. The column was a Beckman C-8 Ultrasphere, 5-micron, 4.6 mm × 25 cm. The guard column consisted of a CN prepacked minicartridge Guard-PAK from Waters Chromatography. The total elution volume for all chromatograms was 20 ml.

Sample heating and evaporation for derivation was conducted in Pierce Chemical Reacti-Therm, Reacti-Vap and 3-ml Reacti-Vials.

Solutions. The derivatizing reagent was an acetonitrile solution of 0.1M p-bromophenacyl bromide (Aldrich Chemical Co., Milwaukee, Wisconsin and 0.005 molar 18-crown-6 ether (Aldrich). Other solutions used were 0.64M aqueous KOH, 0.64M methanolic KOH and 0.64M methanolic HCl. Standard fatty acids, saturated C18, C20, C22, C24, C26 (Sigma Chemical Co., St. Louis, Missouri) were dissolved in THF at 0.18 mg/ml. External standard containing each fatty acid at 30 μg/ml was prepared from dilutions of freshly made, 0.18 mg/ml solutions. Cholesteryl behenate and ethyl stearate obtained from Sigma were dissolved in THF. Tall oil rosin (Hercules) was dissolved in THF at 6 mg/ml and was used the same day. Fresh samples were used to avoid the formation of a precipitate which would have occurred after several weeks. All solvents were UV grade from Burdick and Jackson, Muskegon, Michigan.

Free acid determination. Each set of samples to be analyzed should include a control. A control consists of a rosin sample to which a standard fatty acid blend was added at 0.5% level. Into each of two Reacti-Vials was transferred 100 μl of fresh rosin solution. To the control vial, 100 μl of the standard fatty acid mixture was also added. To both the control and sample vials was transferred 0.10 ml of aqueous KOH followed by one ml of methanol; the vials were mixed on the vortexer.

To each vial was added two drops of 0.1% ethanolic phenolphthalein solution from a 100-μl syringe. While mixing on a vortexer each vial was neutralized to a clear endpoint with 0.64M methanolic HCl added drop by drop from a 100-μl syringe. The color was just brought back to pink with 0.64M methanolic KOH.

All vials were carefully concentrated to the 0.1-ml graduation mark using a nitrogen sparge via the Reacti-Vap while maintaining the block temperature at 50-55°C in the Reacti-Therm.

To each vial was transferred 0.5 ml derivatizing reagent (p-bromophenacyl bromide and 18-crown-6) and 1.5 ml acetonitrile. All vials were tightly sealed and heated 30 min at 77 °C to affect the derivatization reaction.

After cooling to room temperature each reaction mixture was filtered through 0.5-micron Millex SR disposable filter units and collected in autosampler vials. The autosampler was loaded and samples were injected when the column was in equilibrium with the mobile phase.

Total fatty acid determination. A sample and control vial containing rosin, potassium hydroxide, methanol and standard fatty acids (control) were prepared as indicated in the above free acid determination. If the free acid determination was also made, the same controls used in that analysis was also used for the total fatty acid determinations. The sample vial was heated to 77°C in the aluminum heating block (Reacti-Therm) for 90 min. The heated vial was cooled to room temperature, and the remaining analysis was carried out as in the free acid determination starting with phenolphthalein addition.

RESULTS AND DISCUSSION

In the present work, the UV detection of chromato-

graphed LCSFA's was enhanced by derivatization with p-bromophenacyl bromide to form phenacyl esters. Phenacyl esters have been reported for the HPLC detection of various biologically important organic acids (8-10) and for a range of fatty acids analyzed at the ng level (11,12). Additionally, 2-naphthacyl esters have been used to enhance UV detection of fatty acids analyzed by HPLC (13).

Crown ethers catalyze the p-bromophenacyl bromide (PBPB) esterification of carboxylic acids through the solid-liquid phase transfer of their potassium carboxylic salt (11). The recommended procedure is to form the salt in methanol or water, then neutralize to a phenolphthalein end point and evaporate to dryness. Crown ether and PBPB, dissolved in an aprotic solvent, are added to the dried solid in a separate step.

Formation of the potassium salts of LCSFAs within the rosin matrix in methanolic KOH gave partially soluble products which, when derivatized and chromatographed, resulted in unacceptably large response factor variations. A systematic examination of solvent blends led to the 1:1:10 ratio of water, THF and methanol which was found to remain homogenous during the saponification and neutralization steps. Considering that the rosin matrix consists of organic carboxylic acids present at over 100 times the concentration of LCSFAs, the homogeneous reaction medium is then essential for precise salt formation and neutralization over reasonable periods of time. These two factors would be expected to affect derivatization yields due to the strong dependence of derivatization yield on reaction medium pH (8).

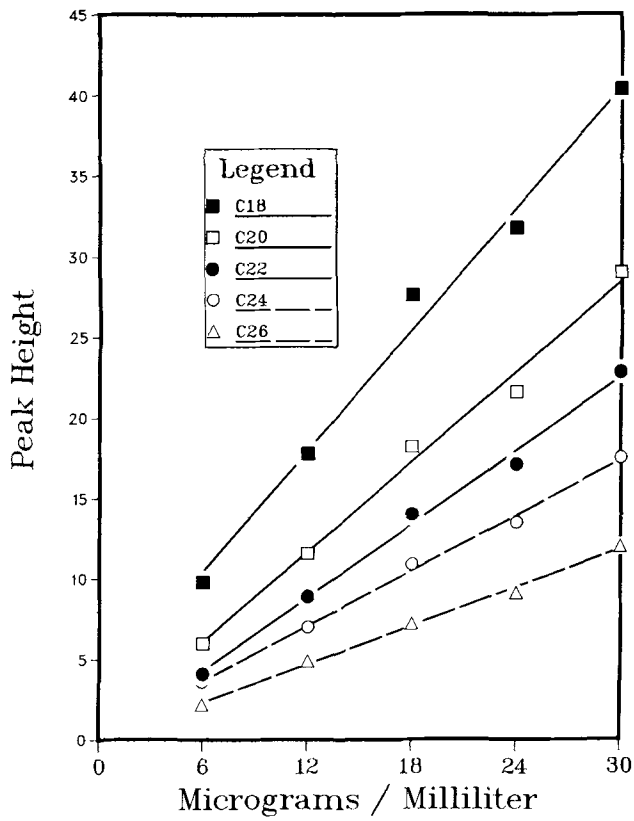

FIG. 2. Peak response for C18-C 26 fatty acids assayed from standard solutions at 6 to 30 μg/ml.

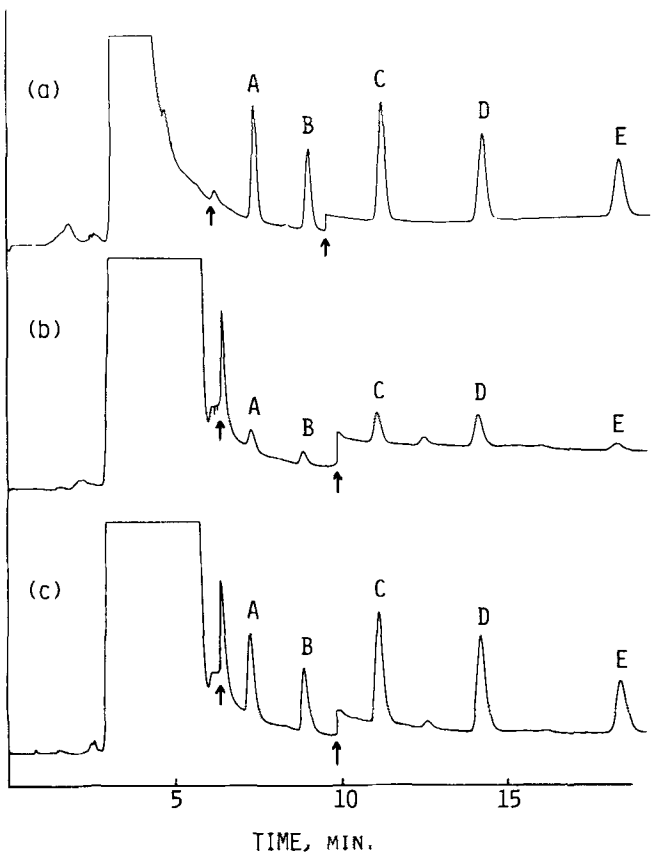

FIG. 1. Typical chromatograms for analysis of (a), a mixture of fatty acids at 100 μg/ml each; (b), tall oil rosin at 6 mg/ml, and (c), rosin spiked with standard fatty acid mixture, 0.5% each. A, C18; B, C20; C, C22; D, C24; E, C26. Arrows indicate each 1/2 attenuation of peak heights. Chromatograms (a) attenuated at 6 and 5 multiples, (b) and (c) attenuated at 5 and 4 multiples.

By maintaining reaction homogeneity during the room temperature salt formation of the free acids, it was possible to avoid saponification of the esters in this step during the heated evaporation step following neutralization. Thus the free acid determination, in the presence of their esters, was achieved by simply omitting the heated saponification step.

For a total LCSFA analysis, the total ester hydrolysis was ensured by use of an extended heating period before proceeding with the derivative esterification reaction. The difference between values for total fatty acid and free fatty acid was equal to the amount of fatty acid bound as esters.

Determining the required ester hydrolysis conditions for the total fatty acid analysis was achieved by considering the nature of the esters to be hydrolized. The type of fatty acid esters likely present in tall oil or its fractionation products are those based on sitosterol or resin alcohols. Sitosterol and resin alcohols combined account for more than half of the tall oil neutrals (14,15). As will be shown below, the likely fatty acid esters in TOR are not easily hydrolyzed, and without complete hydrolysis the total LCSFA analysis would be in error.

Chromatograms resulting from analysis of solutions containing the mixture of standard fatty acids, a typical

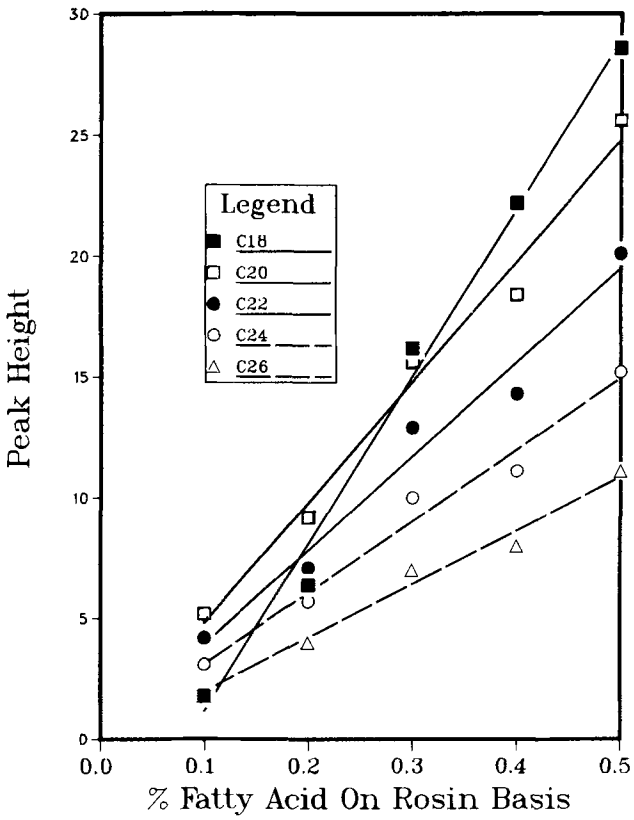

FIG. 3. Response for C18-C26 fatty acids assayed from spike rosin at 0.1 to 0.5 wt % based on rosin.

tall oil rosin solution and tall oil rosin spiked with 0.5% of each standard fatty acid are shown in Figure 1. The linear correlation of analyzed peak height with fatty acid level is shown for both the mixture of standard fatty acids and a rosin solution spiked with the mixture of standard fatty acids, in Figures 2 and 3, respectively. Peak heights were measured by hand. At the lower level of fatty acid analysis, 0.1% based on rosin, the intergrator-determined peak heights and peak areas were much less precise than those determined manually. From the scatter about the regression line, an estimate for the precision of the method was obtained at ± 0.05% based on rosin.

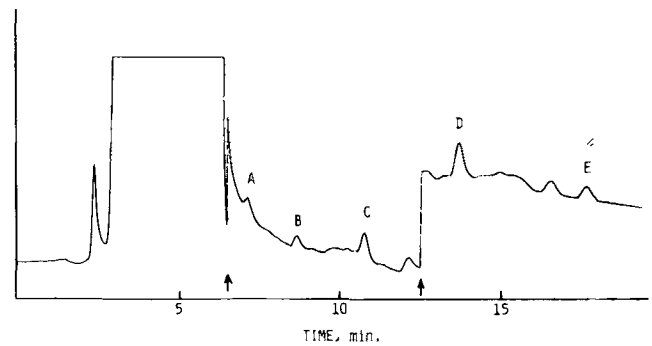

FIG. 4. Typical rosin analysis at attenuation multiple 4 with 0.06% C18, 0.07% C20, 0.17% C22, 0.35% C24 and 0.19% C26. Arrows show baseline shifts.

In Figure 3, the response curve for spiked rosin solution was determined by first analyzing rosin for its naturally occurring fatty acid peak heights. These natural peak heights were then subtracted from the corresponding peaks in the spiked rosin chromatograms. Both the analysis of straight rosin and the fatty acid spiked rosin were performed without the heated hydrolysis step and thus reflect only three fatty acid levels. The C18 ordinate intercept for the extrapolated spiked rosin response curve is further from the origin than are the other curves. This is attributed to the poorer separation of C18 from rosin (6.5 min) using acetonitrile mobile phase. The acetonitrile mobile phase optimizes the separation of higher molecular weight fatty acids which are more difficult to analyze by gas chromatography.

Determination of C18 or C20 saturated fatty acids at levels appraoching 0.1%, on a rosin basis, required a lower attenuation for the entire chromatogram, as shown in Figure 4. Baseline shifts were needed to keep the recorder on scale. The response curves shown in Figures 2 and 3 were determined at this lower attenuation setting. As can be seen in Figure 4, quantitation below 0.1% will likely be imprecise due to variability in establishing a precise baseline for the weak fatty acid signals among unidentified peaks in some tall oil rosin samples. A detection limit of 0.05% seems a practical limit due to baseline noise from these rosin peaks.

Table 1 illustrates that selective derivatization of the free acids in the presence of their esters depends both on

TABLE 1

Effect of 77°C Saponification Time on Analysis of Fatty Acid Mixture and Two Fatty Acid Ester Solutions[a]

Heated hydrolysis time (min)	Cholesteryl behenate	Ethyl stearate	Peak heights[b] Free fatty acid blend C18	C20	C22	C24	C26
0	0	1	98	98	99	100	100
30	58	80	98	98	99	99	100
60	-	99	100	101	99	100	100
70	92	98	105	103	101	100	100
90	100	98	98	101	101	100	100
120	100	-	100	98	99	100	100
150	107	-	-	-	-	-	-

[a]Ester molar concentrations equivalent to their corresponding free fatty acid concentrations in the free acid samples, 30 μg/ml.
[b]Peak heights are expressed as percent of their average corresponding free acid values.

the heated hydrolysis time and the ester type. The samples listed with zero hydrolysis heating time were neutralized without heating their alkaline solutions. In the case of the esters, the lack of peak detection indicates that esters were not assayed by this room temperature exposure to the alkaline medium. The ester samples showed derivative fatty acid peak heights which were proportional to their hydrolysis heating times, but the free fatty acid samples showed relatively constant derivatized peak heights.

Even ethyl stearate was not derivatized when the heated saponification step was omitted. Thus, it can be concluded that neutralization to a phenolphthalein endpoint will prevent further ester hydrolysis during the heated evaporation step and derivatization during the heated derivatization step. (This conclusion was also supported by the free acid determination from a sample containing both ester and free acid, but not shown here.) The relatively constant peak heights with heated hydrolysis time for analysis of the free acids indicates that hydrolysis conditions did not affect assay of the free fatty acids. Consequently, the complete hydrolysis procedure allows determination of the total fatty acid from which the ester-bound fatty acids can be calculated.

Cholesterol ester was chosen as a model for the resinous and sitosterol type esters of fatty acids that would likely be formed in tall oil processing. Table 1 indicates that the time for complete hydrolysis of cholesteryl ester was significantly longer than that needed for hydrolysis of ethyl ester. Thus, conditions optimized for complete hydrolysis of cholesteryl ester, i.e., 90 min, should be sufficient for hydrolysis of the type of esters formed in tall oil.

The unsaturated low mol wt fatty acids which make up a large fraction of tall oil were found to elute before the stearic acid peak, and chromatograms for tall oil analysis were quite similar to those for tall oil rosin.

Mobile phase modification with small amounts of tetrahydrofuran will bring the C28 fatty acid onto a 20-ml elution volume chromatogram but at the expense of C18 resolution from rosin.

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