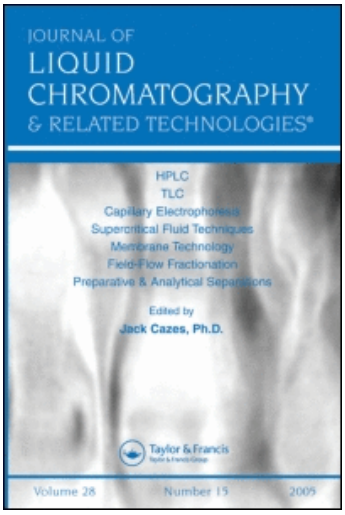

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A Rapid Method for the Estimation of Bile Salts in Complex Tanning Brines by RPHPLC

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ABSTRACT

To preserve bovine hides until they are processed into leather, they are often cured in large raceways of saturated brine. This process prevents the growth of most microorganisms, but under some conditions these hides are still susceptible to decomposition by halophilic archaebacteria. Low amounts of porcine bile added to the brine used for curing, have exhibited control of growth of these primitive microorganisms. Reversed-phase high pressure liquid chromatography (RPHPLC) has been applied to monitor bile concentration in saturated brine mixtures containing contaminants associated with animal hides (bacteria, blood, dirt, fats, hair,

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manure, urine, etc.). With minimal sample preparation, this method can be used by either the hide curing, packing, or tanning industry to assess the need to charge brine solutions with additional porcine bile during the continuous process of hide addition and removal. Calibration curves of porcine bile added to highly contaminated raceway brine have demonstrated linearity of detection between 25–340 ppm bile. The method of standard addition was used to identify and quantify a porcine bile component, glycochenodeoxycholic acid. Reversed-phase high pressure liquid chromatography was also used to determine the relative solubility of porcine vs. bovine bile in solutions of varying salt concentration.

Key Words: Archaebacteria; Bile; Brine; Halophiles; Reversed phase HPLC.

INTRODUCTION

"Red heat" is the term associated with the growth of the halophilic archaebacteria that are sometimes observed on the surfaces of brine-cured bovine hides. The term red is appropriate since these halophilic bacteria form purplish-red colonies that, under certain conditions, digest and ultimately decompose the hides. An immediate drawback of such growth is the degradation of the grain (intricate surface characteristic composed of epidermal keratin) of the hide, thus resulting in the devaluation of infected hides used for the production of leather.[1,2]

Bile salts can inhibit the growth of halophilic archaebacteria via lysis of the bacterial cells,[3] and hog bile was shown to be effective in preventing the growth of halobacteria when added in relatively small (ppm) quantities to saturated "raceway" brine. (Raceway refers to large vats in which bovine hides are typically preserved through salt curing in a continuous process.) The concentration of bile in a commercial raceway must be routinely monitored and adjusted, since continuous processing results in the depletion of the bile by adsorption to hides as they are removed from the raceway. Reversed phase-high pressure liquid chromatographic separation using ion-pairing reagents is an effective and widely used technique for the quantitation of bile salts.[4–6] Ordinarily, a sample clean-up step on ion-exchange resin or reversed phase columns is required prior to the separation to remove contaminants which would interfere with the analysis or damage the column. Because raceway brine typically contains high levels of numerous contaminants (bacteria, blood, dirt, fats, hair, manure, urine, etc.) normally associated with animal hides, this cleanup was expected to be very challenging. However, it was found that a simple centrifugation and filtration step could be used to prepare samples for

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analysis. Using this approach, porcine bile components were detected in raceway brine with a linear range of 25–340 ppm. A primary hog bile constituent, glyco-3α, 7α-dihydroxy-5β-cholanoic acid or glycocheno-deoxycholic acid (GCDC) was used as a marker to quantitate bile concentration.[6] In addition, the HPLC technique was used to qualitatively compare the solubility of porcine vs. bovine bile in sodium chloride solutions of varying concentration.

EXPERIMENTAL

Materials

Materials used were: GCDC (sodium salt), hog bile, ox bile, sodium chloride, taurochenodeoxycholic acid (sodium salt), and tetrabutyl ammonium phosphate (TBAP; Sigma Chemical Co., St. Louis, MO), used raceway brine (National By-Products, Bellevue, NE), and HPLC grade acetonitrile (Spectrum Quality Products, Inc., New Brunswick, NJ). Hog bile was obtained from a local abattoir. Other chemicals used were of reagent grade.

Apparatus

High pressure liquid chromatography samples were pre-filtered with 0.22 μm regenerated cellulose 25 mm syringe filters (Scientific Resources Inc., Eatontown, NJ). A Vortex-Genie 2 vortexing unit (A. Daigger & Co., Inc., Lincolnshire, IL) was used to mix HPLC samples and sample injection was performed with a 50 μL Hamilton 705 Rheodyne syringe (Chrom Tech, Inc., Apple Valley, MN). The HPLC equipment was composed of the following: a Supelcosil™ LC-18 model 5-8298 HPLC column (25 mm × 4.6 mm 5 μm particle size, Supelco, Inc., Bellefonte, PA), a Series 400 liquid chromatograph with an SEC-4 solvent cabinet (Perkin-Elmer, Norwalk, CT), a Rheodyne 7125-075 manual injection valve (Rheodyne, Inc., Cotati, CA) and a 20 μL sample loop, a flow-through UV–Vis detector model 785A (Applied Biosystems, Inc., Foster City, CA), and an integrator model 3396A (Hewlett-Packard Co., Avondale, PA).

Procedures

All RPHPLC samples were prepared as follows: (1) ~2 mL aliquots of the well-mixed (moderate vortexing) samples were placed into 2 mL polypropylene microcentrifuge tubes; (2) the samples were centrifuged (10,000 × g) for 3 min; (3) the supernatant (sometimes directly below a partial layer of fat) was

removed with a Pasteur pipette; and, (4) the supernatant was filtered through a 0.22 μm syringe filter into a 1.5 mL polypropylene microcentrifuge tube until at least 0.5 mL of filtrate was collected. The samples were then subjected to isocratic RPHPLC as previously described,[5] using a mobile phase consisting of 65 mL water, 35 mL acetonitrile, and 1 mL 0.5 M TBAP, but with the exceptions that strictly C-18 packing material was used as the stationary phase and the detector wavelength was set to 205 nm rather than 214 nm, giving improved sensitivity of response for the peaks of interest (data not shown). The integrator parameters were as follows: −3 attenuation (^2), 0.5 cm/min chart speed, 50 area reject, −5 threshold, and 0.20 peak width.

The effect of salt concentration on bile solubility was determined by dissolving bile in solutions of reagent-grade NaCl in nanopure water.

RESULTS AND DISCUSSION

The chromatogram of 250 ppm hog bile in raceway brine (Fig. 1), displayed in Fig. 1(A), depicts fairly good resolution for several major hog bile components. The peak heights of these components relative to elution time [Fig. 1(B)] were selected for further study of linearity.

Using the method of standard addition, we determined that the peak corresponding to ~42–43 min elution was the hog bile component, GCDC. This is unusual since this component was previously determined[5] to elute at ~30 min under virtually identical conditions. The next peak that eluted downstream of GCDC at ~48 min was identified, also via standard addition, as taurochenodeoxycholate (data not shown); this peak identification does concur with the previous determination.[5] Figure 2(A) shows the chromatograms of 250 ppm hog bile in water and 250 ppm hog bile in water containing an added 150 ppm of GCDC (inset). The response and increase in response (peak height) associated with no added or added GCDC, respectively, were plotted and is displayed in Fig. 2(B). Extrapolation of the resulting line to the x-axis, and taking the absolute value of that value, indicated an initial concentration of 62.7 ppm or 25.1% for GCDC in 250 ppm hog bile.

Since brine used for the curing of animal hides is maintained at near saturation in commercial raceways, it was considered imperative to assess the effects of sodium chloride concentration on bile solubility. Figure 3(A) and 4(A) show the chromatograms of 250 ppm hog and ox bile, respectively, in water. Only two major ox bile peaks were deemed well resolved using the chromatographic procedure employed for hog bile separation. Major bile component responses (peak heights) vs. salt concentration in which the bile solutions were contained, were plotted for hog bile [Fig. 3(B)] and ox bile [Fig. 4(B)]. Only the hog bile component at 11.6 min elution time

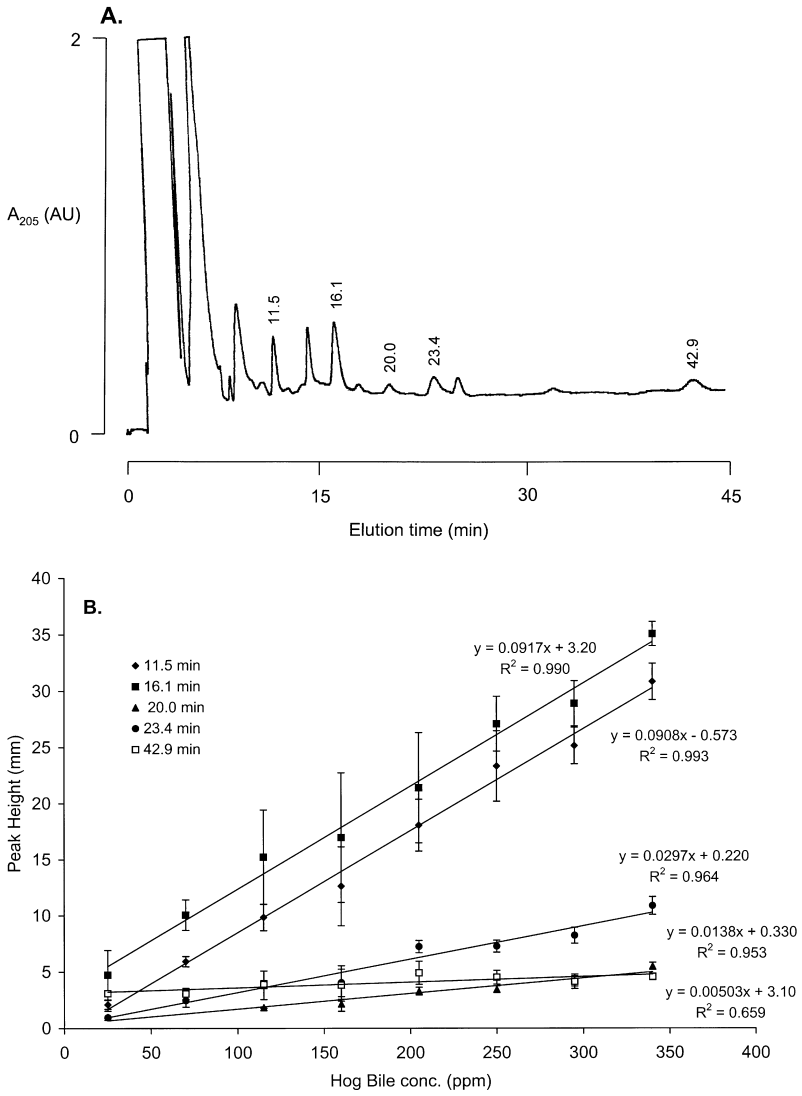

Figure 1. Ion-pair reversed-phase high pressure liquid chromatogram of separated hog bile components (250 ppm) in raceway brine mixture. The chromatogram displayed in (A) indicates the absorbance at 205 nm vs. elution time (relative to injection point) for the sample components. Major peaks, revealed to quantifiably change magnitude upon varying added bile concentration, were labeled (elution time in min) and were plotted (B). The data points in the plot represent average values obtained from three separate experiments performed on three separate days.

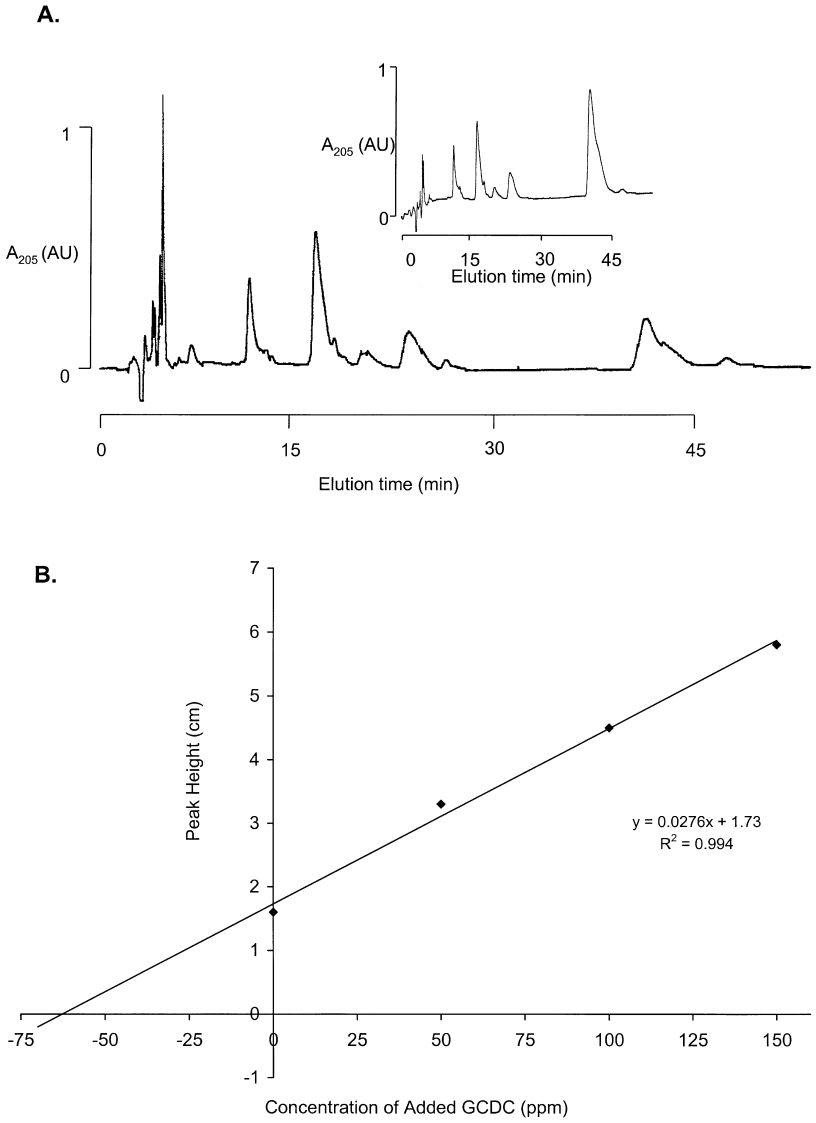

Figure 2. Standard addition of GCDC to hog bile in water. The method of standard addition was employed to identify the GCDC elution time and quantify the bile component. The chromatogram in (A) represents 250 ppm hog bile in water, whereas, the inset shows the profile of 250 ppm hog bile plus 150 ppm of GCDC. (B) shows the standard addition plot of the GCDC response (height of peak at ~42–43 min elution) vs. added GCDC.

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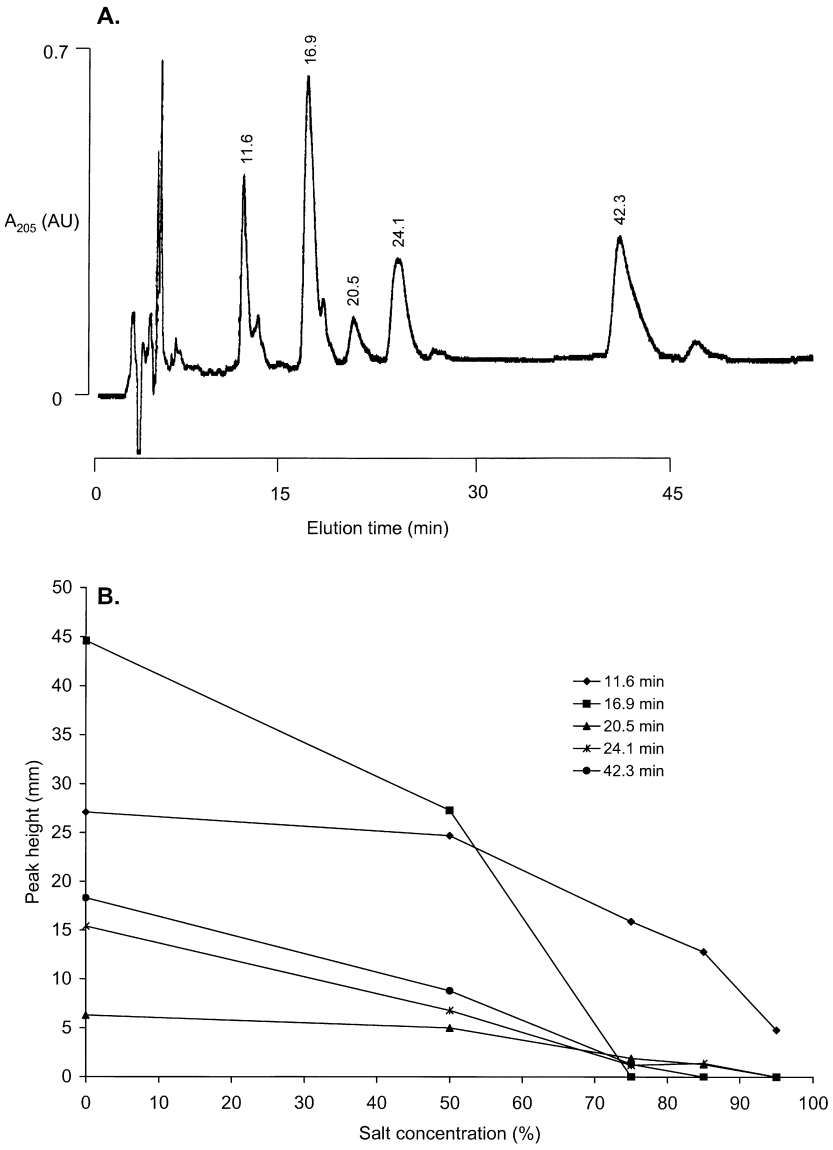

Figure 3. Effect of hog bile solubility upon varying sodium chloride concentration. (A) shows a chromatogram of 250 ppm hog bile in water. The decrease in the amount of major hog bile components is represented in (B) as major peak heights vs. added salt concentration.

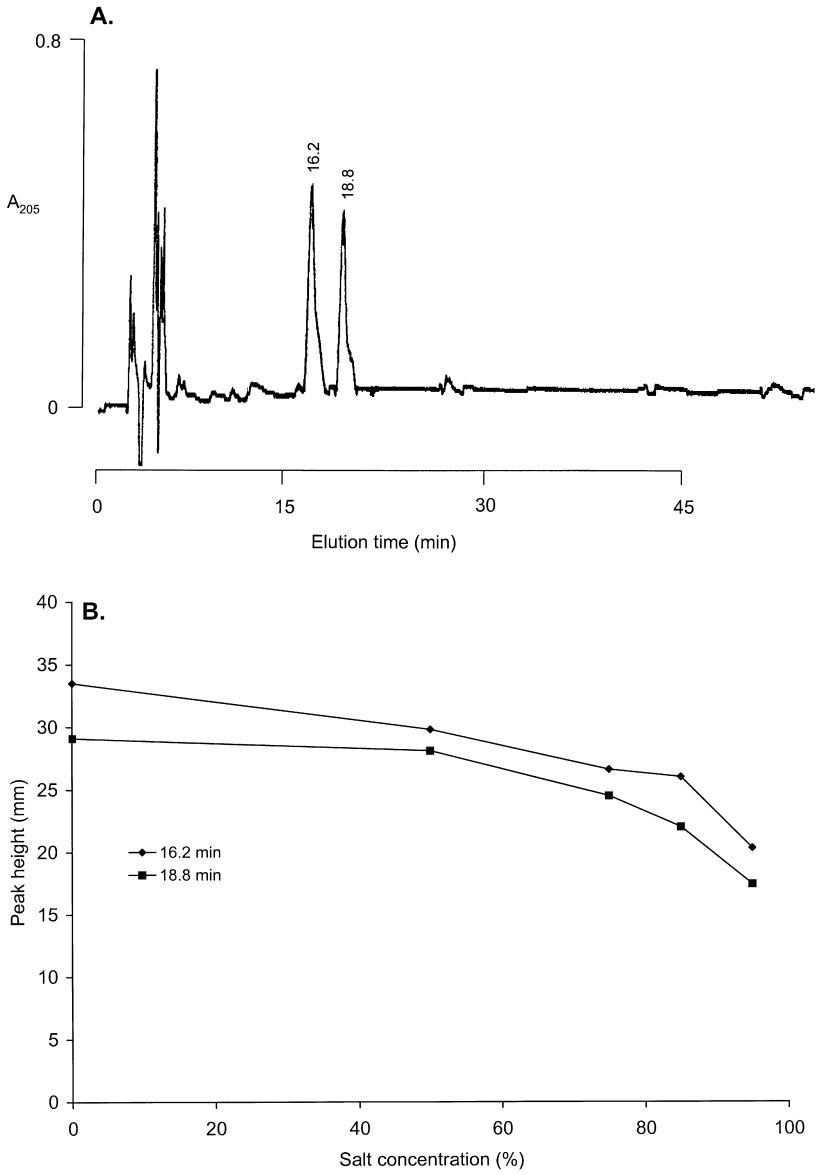

Figure 4. Effect of ox bile solubility upon varying sodium chloride concentration. (A) shows a chromatogram of 250 ppm ox bile in water. The decrease in the amount of major ox bile components is represented in (B) as major peak heights vs. added salt concentration.

maintained significant solubility at the highest salt concentrations tested. However, both ox bile components retained significant solubility, relative to hog bile, at all tested salt concentrations.

CONCLUSION

Control of the growth of halophilic microorganisms and, hence, the occurrence of red heat on salt-cured bovine hides would be an advantage to the US packing, hide curing, and/or tanning industries. Hog bile has been demonstrated to be a candidate for such control. Therefore, in anticipation of monitoring the existing or residual bile content of bile-modified raceway brine, we developed an analytical protocol for the determination of hog bile concentration (in the ppm range). This method was successful in the preparation of calibration curves for hog bile components in raceway brine that resulted in linear responses from 25–340 ppm. Surveying the generated calibration curves, two bile component peaks (11.5 min and 16.1 min, Fig. 1) were considered superior candidates for the monitoring of total bile concentration in raceway brine. We assigned GCDC to a downstream peak and quantified it to be ~25% of total hog bile content using the method of standard addition.

The solubility of hog vs. ox bile in solutions of increasing sodium chloride concentration was determined. Ox bile only exhibited two major components that were well resolved, however, it was expected that the bile composition of bovine (herbivorous) species would be remarkably different from that of porcine (omnivorous) species.[7] Both bile solutions exhibited lower solubilities at higher salt concentrations, although the effect was less dramatic for that from the ox source. Decreased solubility was attributed to the "salting-out" effect and precipitated bile was most likely removed in either the centrifugation or syringe filtration sample preparation step employed prior to HPLC separation. Provided that these HPLC-resolved ox bile components are relatively bioactive with respect to halobacterial lysis, the solubility results indicate that ox bile might present a superior candidate for the purpose of halobacteria control in raceway brine.

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ABBREVIATIONS

GCDC glycochenodeoxycholic acid
RPHPLC reversed-phase high pressure liquid chromatography

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