# Enzyme-Assisted Extraction of Shea Fat: Evidence from Light Microscopy on the Degradative Effects of Enzyme Treatment on Cells of Shea Kernel Meal

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**ABSTRACT:** A light-microscopic study on enzyme-treated sliced shea kernels was done to investigate the principles involved in enzyme-assisted shea fat extraction. The raw, ellipsoidally shaped kernels, measuring 1.3 to 2.0 cm, were cross-sectioned into slices of about 2-mm thickness, which were then dissected into four or six pieces. These samples were treated with or without enzymes for predetermined periods, fixed and processed for paraffin sections (5 μm). Sections were stained with periodic acid-Schiff base (PAS) or Coomassie blue (CB) to localize the complex polysaccharides and proteins, respectively. The cells of the enzyme-treated samples showed distinct degradation and a high loss of cellular integrity. PAS reaction and CB staining of these cells confirmed high loss of cellular materials compared to control samples. This was also reflected in the increases of the soluble protein and free sugar contents of the effluent water collected from samples after treatment. The observations indicated that enzyme treatment degrades the oilseed materials during preextraction. They thus support the principle of using enzyme treatments to improve fat yield in solvent extraction systems, as has been reported by various investigators. *JAOCS 73*, 449–453 (1996).

**KEY WORDS:** Enzyme-assisted fat extraction, light microscopy, shea kernels, shea kernels cells.

Reports already published on the subject of enzyme-assisted extraction of shea fat indicate that preextraction treatment of shea kernel meal with a protease and a cellulase/hemicellulase significantly increases the fat yield in an aqueous extraction system (1,2). Similar reports on other oilseeds also have been published by various researchers (3–9). Graile *et al.* (10), reviewing the principles involved in enzyme-assisted fat extractions, noted that enzyme treatment further degrades the cellular structures of the oilseed meal after the mechanical and heat treatments, which usually precede the enzyme treatments. This reduces the extraction losses due to inadequacies of the usual preextraction treatments in rupturing oilseed cells to free the oil for extraction (10).

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As a part of investigations on the principles involved in the aqueous enzyme-assisted extraction of shea fat, structural changes of enzyme-treated shea kernel slices were examined by light microscopy. The findings are reported in this paper.

## MATERIALS AND METHODS

The shea (*Butyrospermun parkii*) kernels used in this study were obtained from Ghana. The typical chemical characteristics were previously reported (1). The enzymes used, Sumizyme-AP (acid protease from *Aspergillus niger*; activity, 50,000–200,000 U/g) and Sumizyme C (cellulase/hemicellulase from *Trichoderma reesei*; activity, 1500 U/g), were obtained from Shin Nihon Chemicals (Anjoh City, Japan).

*Enzyme treatment.* Intact kernels were sliced in cross-sections of about 2-mm thickness and dissected into four to six parts each. Samples of these, about 3 g each, were weighed into 25-mL conical flasks, and 15 to 20 mL water was added (just enough water to completely cover the sample). The enzymes were added, each at a rate of 1% of the sample weight, and stirred to dissolve completely. A drop of toluene was added to inhibit microbial growth. The flasks were incubated in a water-bath shaker at 37°C for 24, 48, or 72 h. At the end of the treatments, the water from each treatment was decanted into a separate flask. The samples were washed with warm water (about 40°C), and the washings were added to that already collected for quantitative soluble protein (SP) and free reducing sugar (RS) estimation. The washed samples were then prepared for light-microscopic examinations. Three control setups were made: (i) samples of the sliced kernels were used for light microscopy without any hydrolysis treatment (control-1); (ii) samples were treated with water only, no enzyme (control-2); and (iii) an enzyme solution of similar concentrations as used in the treatments, similarly incubated and analyzed for SP and free RS contents to be used as blanks to correct the values measured from the effluents of the enzyme-treated samples.

*Preparation of samples for light microscopy.* The samples were fixed in ethanol (70%)/acetic acid/formalin solution (90:5:5, vol/vol/vol) for 48 h. They were dehydrated through

a graded series of alcohol washes, in the order: 50% ethanol, (two times), *tert*-butyl alcohol/95% ethanol/water (1:4:5, 2:5:3, 7:10:3, and 11:9:0), *tert*-butyl alcohol/100% ethanol (3:1), and *tert*-butyl alcohol (two times at 42°C). The dehydrated samples were incubated in *tert*-butyl alcohol/paraffin (1:1) solution at approximately 60°C for 2 h, and then incubated two times in paraffin (approximately 60°C) for 1 h each. They were then embedded in paraffin and sectioned (5 μm in thickness).

*Staining*. Sections were deparaffinized with xylene, and washed successively with 100, 95, 85, 70, and 50% ethanol and water. They were examined by periodic acid-Schiff base (PAS) reaction to localize complex polysaccharides (11), and by Coomassie blue (CB) (Nacalai, Kyoto, Japan) (12) staining to localize proteins. Finally, the sections were dehydrated with 90, 95, and 100% ethanol, cleared with xylene and covered.

*Estimation of SP and RS released*. The water samples collected were made to 100 mL with distilled water and further diluted until concentrations suitable for the analytical methods were obtained. Total SP was measured by the Lowry *et al.* method (13), and total free sugar by the phenol-sulfuric acid method (14).

*Enzyme-assisted shea fat extraction*. In a separate experiment, shea kernel meal samples, 30 g each, were treated with enzymes as previously described (1,2) in a $2^4$ factorial experiment. The factor levels were: enzyme concentration (0 and 1%), treatment time (2 and 6 h), meal/water ratio (1:2 and 1:6), and pH of meal mixture (3.4 and 5.5). The pH was adjusted with citric acid/sodium citrate buffer as previously described (2). The fat yield and the SP and free sugar levels of the separated water (effluent) were determined after adjusting volumes of water to the same level during fat extraction. The fat was extracted as follows. First, the treated meal mixture was centrifuged (5,000 × *g* for 10 min). The supernatant was quantitatively transferred into a separatory funnel with 20 mL hexane. The aqueous layer was drained. The hexane layer was washed three times with warm water (about 40°C) and then collected into a weighed dish. The hexane was evaporated on a boiling water-bath and dried in an air-oven at 100°C for two hours. Yield was expressed as percentage of the Soxhlet-extractable fat in the sample, as previously reported (2).

## RESULTS AND DISCUSSION

The light micrographs (Figs. 1A–3L) demonstrate the structural appearances of the treated and untreated shea kernel samples. Several slides were prepared for each treatment, and the figures presented are just a few of them. Generally, the degradative effects of the enzyme treatment were clearly demonstrated. At the end of the 72-h treatment, some of the enzyme-treated kernel slices had apparently decreased in size, and the treatment water had become deeply pigmented (brownish) and oily. The brownness of the water increased as the length of treatment time increased.

Figure 1 represents the control-1 samples. A distinct, thick, continuous epidermis and clear undegenerated inner cell layer

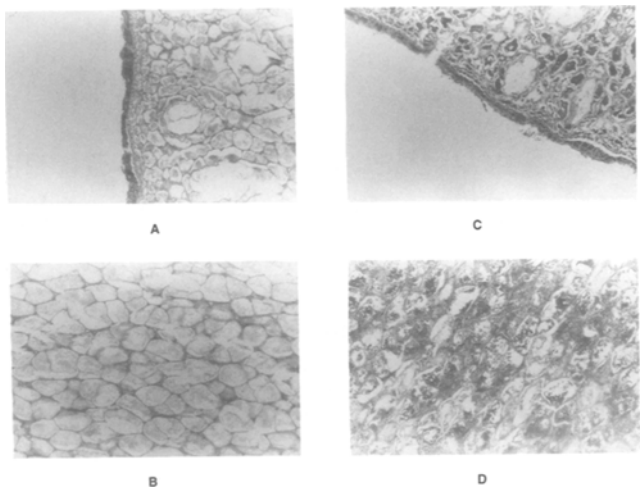


**FIG. 1.** A–D: Light micrographs of the untreated shea kernel (Control-1), (magnification, ×400).[A and B, with periodic acid-Schiff base (PAS) reaction; C and D, with Coomassie blue (CB), CB staining].

was observed. An intensive PAS reaction was observed in the epidermis and cellular wall, and moderate reaction in the cytoplasm. CB-positive substances were highly localized in the medullar cells. These results suggest the retention of a high proportion of the macromolecular (cytoplasmic) components. The samples treated with water only (control-2) showed some degeneration (Fig. 2). Generally, the epidermal cells degenerated, and the cytoplasmic substances positive for both PAS and CB were also less than in control-1 samples. The effect was more apparent in the 72-h treated samples. Figure 3 shows the effects of the enzyme treatment. Cellular degradation was more clearly observed, particularly in the epidermal region. A majority of cells in these treatments even lost their definite shapes or structures (or their integrity), and their components, as indicated by PAS reactions and CB staining, were to a larger extent removed.

These observations confirm the fact that the enzyme treatment actually causes significant further degradation of the fine meal when treated. This clearly showed up, even though trimming of the sample during the sectioning might have significantly obscured visibility because the more degraded surface layers were probably trimmed-off. Trimming was done until the surface to be cut was leveled and devoid of any paraffin inclusion. Thus, the degradation is expected to be more clearly seen in electron microscopy. The longer treatment time, compared to that in the extraction process (1–9), was chosen because the sample sizes were large and it was desired to make the effects visible. In our previous report on enzyme-assisted aqueous shea fat extraction (2), we considered a treatment time of 6 h for finely milled shea kernel meal to be adequate for an optimum increase in yield, as a further yield increase of only about 1% occurred for a time increase from 6 to 20 h. Comparing hydrolysis times of 3, 6, and 12 h, Sosulski *et al.* (6) observed maximum increases in oil yield when they treated canola with carbohydrases for 12 h at 50°C

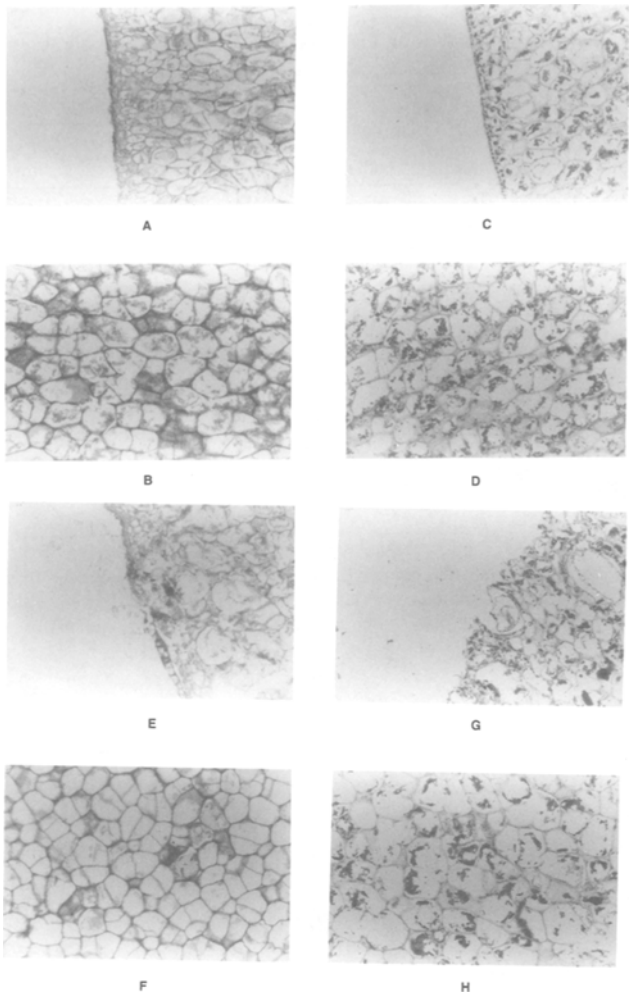


**FIG. 2.** A–H: Light micrographs of the shea kernels treated with water only (control-2), for 24 or 72 h; (magnifications, ×400). A–D, treated for 24 h (A and B, with PAS reaction; C and D, with CB staining); E–H, treated for 72 h (E and F, with PAS reaction; G and H, with CB staining). See Figure 1 for abbreviations.

to enhance oil extraction with hexane. However, with a mixture of papain, amylase, and cellulase, Buenrostro and Lopez-Munguia (4) treated avocado for 1 h at 65°C to obtain optimum yield. Also, in a study to investigate the enzymatic hydrolysis pretreatment for mechanical expelling of soybean, and using the response surface methodology, Smith *et al.* (9) predicted an optimum treatment time of 13.24 h. Thus, in the actual fat extraction process, the treatment time would be shorter than was used in these microscopic studies. The time would invariably depend on a number of factors, such as the raw material characteristics, specific activities of the enzymes, enzyme concentration, and other enzyme activity-dependent factors.

The observations also suggest that degradation is initiated by the water, and that the enzymes accelerate it, as expected. Generally, the micrographs suggest that the enzymes' actions progress from the surface of the materials. The extent apparently increases with increasing treatment time. These observations support the idea that for high efficiency in enzyme-

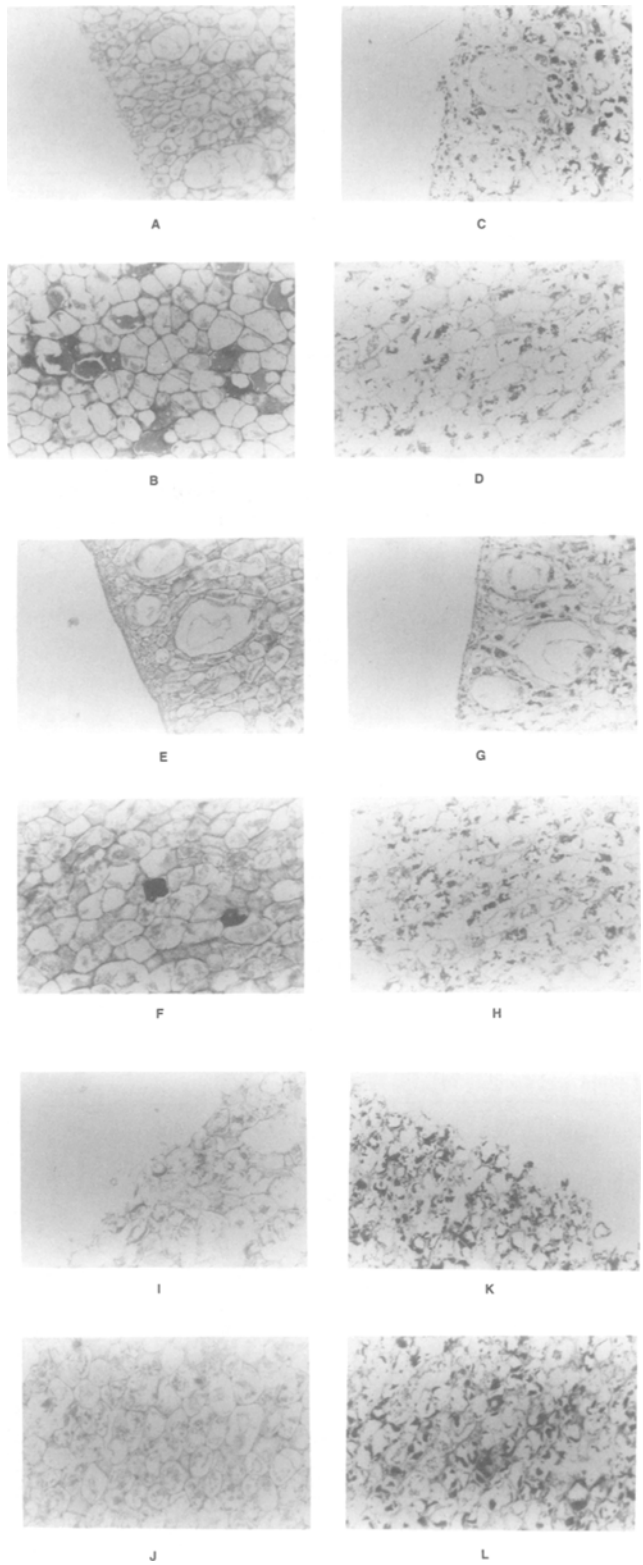


**FIG. 3.** A–L: Light micrographs of shea kernels treated with enzyme, for 24, 48, or 72 h; (magnification, ×400); A–D, treated for 24 h (A and B, with PAS reaction; C and D, with CB staining); E–H, treated for 48 h (E and F, with PAS reaction; G and H, with CB staining); I–L, treated for 72 h (I and J, with PAS reaction; K and L, with CB staining). See Figures 1 and 2 for abbreviations.

**TABLE 1**
**Amounts of Soluble Protein and Reducing Sugars Released in the Treatment of Shea Kernel Meal Slices[a]**

| Treatment time (h) | Soluble proteins (mg/g sample) | | Free sugars (mg/g sample) | |
|---|---|---|---|---|
| | Water-only treatment | Enzyme treatment | Water-only treatment | Enzyme treatment |
| 24 | 6.89 | 8.25 | 10.94 | 15.15 |
| 48 | 7.65 | 9.89 | 12.97 | 20.65 |
| 72 | 7.92 | 10.35 | 13.84 | 18.30 |

[a]Values are means of four determinations on two effluent samples of duplicate setups.

assisted extraction, materials to be enzyme-treated should be milled into fine particles to have higher surface areas for the enzyme.

The treatment water (effluent) samples collected were pigmented, suggesting the release of chemical substances. This corresponded with the decreased PAS- and CB-positive substances (complex polysaccharides and proteins, respectively) in the longer- and/or enzyme-treated samples, and apparently, to the loss of cellular integrity. Measurements of the SP and RS contents of the effluent samples (Table 1) confirmed that some cellular components were actually released into the water during the treatments. Both SP and free sugar levels were higher in water samples from the longer treatments. Increases were more apparent in the enzyme-treated samples than in the water-only (control-2)-treated samples. Levels apparently increased with treatment time.

The data from the enzyme-assisted fat extraction studies (Table 2) also indicate significant increases in SP and sugar levels. Effects due to the enzyme concentration, meal/water ratio, and pH were significant at the 5% significance level for all three indices measured (Table 3). Although the emphasis here is not on fat, it is also worthy to note the significant increases in yield, which were consistent with the previous reports (1,2). The relative increase (about 15%) seemed smaller here than previously reported. This can be attributed to the atypically high yield values for the controls. The aqueous (rural) process, upon which the study sought to improve, yields less than the values for the controls observed here (2). Thus, compared to the rural process, the relative increase would be more substantial. Again, fat yield was significantly dependent on meal/water ratio and pH of the meal–water mixture. It was better at neutral than at acidic pH, and at the 1:2 than at 1:6 meal/water ratio level. The trends in these relationships were previously shown. Data from this experiment confirmed the observations made during the microstructural studies.

**TABLE 2**
**Fat Yield and Soluble Protein and Free Sugar Concentrations[a]**

| Treatment conditions | | | | Fat yield (%) | mg/g sample | |
|---|---|---|---|---|---|---|
| Enzyme concentration (%) | Time (h) | Meal/water | pH | | Proteins | Sugars |
| 0 | 2 | 1:2 | 3.4 | 59.9 | 6.95 | 23.63 |
| 0 | 2 | 1:2 | 5.5 | 60.14 | 7.12 | 25.24 |
| 0 | 2 | 1:6 | 3.4 | 55.66 | 7.05 | 24.17 |
| 0 | 2 | 1:6 | 5.5 | 57.5 | 7.11 | 24.83 |
| 0 | 6 | 1:2 | 3.4 | 58.6 | 7.19 | 26.78 |
| 0 | 6 | 1:2 | 5.5 | 61.5 | 7.90 | 27.46 |
| 0 | 6 | 1:6 | 3.4 | 56.0 | 7.75 | 26.11 |
| 0 | 6 | 1:6 | 5.5 | 57.5 | 8.60 | 26.49 |
| 1 | 2 | 1:2 | 3.4 | 66.8 | 7.46 | 27.83 |
| 1 | 2 | 1:2 | 5.5 | 68.2 | 7.95 | 29.46 |
| 1 | 2 | 1:6 | 3.4 | 67.0 | 7.78 | 29.76 |
| 1 | 2 | 1:6 | 5.5 | 68.2 | 8.16 | 30.79 |
| 1 | 6 | 1:2 | 3.4 | 68.4 | 9.38 | 31.50 |
| 1 | 6 | 1:2 | 5.5 | 69.1 | 9.59 | 33.10 |
| 1 | 6 | 1:6 | 3.4 | 65.0 | 10.04 | 31.68 |
| 1 | 6 | 1:6 | 5.5 | 65.6 | 9.97 | 31.68 |

[a]Measured in the effluent, in an enzyme-assisted shea fat extraction process, with treatment at different conditions. Values are means of four determinations from two batch extractions.

**TABLE 3**
**Multiple Range Analysis on the Data for Fat Yield and Soluble Protein and Free Sugar Concentrations of the Effluent[a]**

| Factor | Level | Fat yield (%) | Mean values | |
|---|---|---|---|---|
| | | | Protein (mg/g sample) | Sugars (mg/g sample) |
| Enzyme (%) | 0 | 58.35[a] | 7.46[a] | 26.05 |
| | 1 | 67.16[b] | 8.79[b] | 30.72[b] |
| Time (h) | 2 | 62.59[a] | 7.45[a] | 27.29[a] |
| | 6 | 62.92[a] | 8.80[b] | 29.48[b] |
| Meal/water | 1:2 | 61.43[a] | 7.49[a] | 28.04[a] |
| | 1:6 | 64.08[b] | 8.31[b] | 28.73[a] |
| pH | 3.4 | 62.04[a] | 7.95[a] | 27.90[a] |
| | 5.5 | 63.47[b] | 8.30[b] | 28.87[a] |

[a]By enzyme concentration, treatment time, meal/water ratio and pH. Analysis was done with the Statgraphics software (STCC Inc, Rockville, MD), multiple factor analysis of variance, 95% least significant difference intervals. Values with same superscript within columns were not significantly different.

In conclusion, it has been demonstrated that preextraction enzyme digestion increases cellular degradation and significantly increases oil recovery upon extraction. This treatment could be a valuable unit-operation to increase fat yield in solvent-based oilseed extraction processes.

## REFERENCES

1. Tano-Debrah, K., and Y. Ohta, Enzyme-Assisted Aqueous Extraction of Fat from Kernels of the Shea Tree, *Butyrospermum parkii, J. Am. Oil Chem. Soc. 71*:979–983 (1994).
2. Tano-Debrah, K., and Y. Ohta, Enzyme-Assisted Aqueous Extraction of Shea Fat: A Rural Approach, *Ibid. 72*:251–256 (1995).
3. Fullbrook, P.D., The Use of Enzymes in the Processing of Oilseeds, *Ibid. 60*:476–478 (1983).
4. Buenrostro, M., and C.A. Lopez-Munguia, Enzymatic Extraction of Avocado Oil, *Biotechnology Lett. 8*:505–506 (1985).
5. McGlone, O.C., C.A. Lopez-Munguia, and J.C. Carter, Coconut

Oil Extraction by a New Enzymatic Process, *J. Food Sci. 51*:695–697 (1986).
6. Sosulski, E., F.W. Sosulski, and E. Coxworth, Carbohydrase Hydrolysis of Canola to Enhance Oil Extraction with Hexane, *J. Am. Oil Chem. Soc. 65*:357–361 (1988).
7. Cheah, S.C., M.A. Augustin, and L.C.-L. Ooi, Enzymatic Extraction of Palm Oil, *PORIM Bulletin*, Vol. 20, Palm Oil Research Institute of Malaysia, Kuala Lumpur, 1989, pp. 30–36.
8. Sosulski, K., and F.W. Sosulski, Enzyme-Aided vs. Two-stage Processing of Canola: Technology, Product Quality and Cost Evaluation, *J. Am. Oil Chem. Soc. 70*:825–829 (1993).
9. Smith, D.D., Y.C. Agrawal, B.C. Sarkar, and B.P.N. Singh, Enzymatic Hydrolysis Pretreatment for Mechanical Expelling of Soybeans, *Ibid. 70*:885–890 (1993).
10. Graille, J.M., M. Pina, and D. Montet, Biotechnology of Lipids: Some Possible Applications, *Oleagineux 43*:188–190 (1988).
11. Humason, G.L., *Animal Tissue Techniques*, 2nd edn., W.H. Freeman and Co., San Francisco, 1967, pp. 304–308.
12. Saio, K., and M. Monma, Microstructural Approach to Legume Seeds for Food Uses, *Food Microstructure 12*:33–341 (1993).
13. Lowry, O.H., N.J. Rosebrough, A. Farr, and R.J. Randall, Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*:265–275 (1951).
14. Dubois, M., K.A. Gills, J.K. Hamilton, P.A. Rebers, and F. Smith, Colorimetric Method for Determination of Sugars and Related Substances, *Anal. Chem. 28*:350–356 (1956).

[Received May 16, 1995; accepted January 19, 1996]