The results in Table I show that the ninhydrin reactor in our system was as much a disadvantage as an advantage with respect to color yields. Although for many compounds the yield relative to norleucine was higher with the reactor (smaller CF value, *e.g.*, hydroxyproline, glutamine, glutamic acid, glycine, half-cystine, cystathionine, methionine, tyrosine, phenylalanine, histidine and the methylhistidines) for almost as many other compounds the yield was lower (larger CF value, *e.g.*, phosphoethanolamine, taurine, sarcosine, alanine, β-alanine, β-aminoisobutyric acid, γ-aminobutyric acid and ammonia). The much poorer reaction for already poor color yielders (such as phosphoethanolamine, taurine, sarcosine, β-alanine, and β-aminoisobutyric acid) is especially serious when analyzing complex samples that contain numerous compounds of interest besides the common protein amino acids.

That the two methods of reducing ninhydrin did not yield equivalent color constants is less surprising than distressing. To avoid potentially gross errors, it appears necessary to determine color constants on every compound of interest for each different method of preparing hydrindantin–ninhydrin reagent. Harassed analysts would welcome more complete elucidation of the basic chemistry of hydrindantin and ninhydrin as related to their reactions with amino acids and other ninhydrin-positive compounds.

Soil and Water Conservation Research Division of the Agricultural Research Service, U.S. Department of Agriculture in cooperation with the Oregon Agricultural Experiment Station, c/o Department of Soils, Oregon State University, Corvallis, Ore. 97331 (U.S.A.)

M. YAMAMOTO
J. L. YOUNG

1 J. L. YOUNG AND M. YAMAMOTO, *J. Chromatogr.*, 53 (1970) 373

Received January 25th, 1971

CHROM. 5228

Sugar borate chromatography using refractometry for monitoring

After the introduction by KHYM AND ZILL[1,2] of the ion-exchange chromatographic separation of neutral sugars as borate complexes, many analytical modifications of this principle have been described[3–15]. The eluates have, as a rule, been analysed colorimetrically. We have found it of interest to try differential refractometry for this purpose. Although less sensitive than the colour reactions, refractometry has advantages in the form of being highly reproducible and easy to run with minimal cost of reagents. Furthermore the components may afterwards be recovered for further identification. The use of refractometry does involve certain difficulties, however, in the form of sensitivity to variations of buffer concentration and lack of specificity. In the present paper we report the successful application of refractometry in the routine analysis of different sugars in SOMOGYI filtrates[16] of food products. The determination of the individual sugars in such products is an essential problem in the food industry.

Experimental

Borate buffer. 5 l of 0.33 *M* borate buffer pH 8.0 (25°) was prepared from H_3BO_3 (70.915 g), $Na_2B_4O_7 \cdot 10H_2O$ (47.671 g) and carbon dioxide-free distilled water (1 ml toluene was added as preservative). The borax was weighed out for half a year's use at a time in order to avoid variations which could arise from losses of water of crystallisation during storage of the hydrate. The freshly prepared buffer should give no peaks when analysed in the chromatographic system, using a short column (see below).

Sugar solution. Standard solutions of 2–10 mg/ml of each component (analytical grade: maltose and lactose corrected for water of crystallisation) in borate buffer or distilled water were stored frozen and allowed to reach room temperature before analysis. The unknown samples to be analysed were dispersed in water and clarified[16] by the addition of zinc sulphate and barium hydroxide in this order.

Anionic resin. AG 1X4 minus 400 mesh (Bio-Rad Laboratories) was fractionated according to particle size[17] with 0.02 *M* NaCl at 20°. Fractions with particle sizes of 30–50 and 30–40 μ (measured by microscopy) were used for the "long" (disaccharide) and "short" (monosaccharide) columns, respectively. Before being packed in the columns the resin was converted to the borate form.

Chromatographic equipment. (See Fig. 1.) Resin was packed in 0.45 × 10 cm ("short column") and 0.6 × 80 cm ("long column") jacketed glass tubes (cross hatched areas) with teflon fittings. The columns were used for the separation of monosaccharides and disaccharides, respectively. The columns were thermostated at 45° by circulating water and connected with teflon tubing (I.D. 0.8 mm). Cheminert 3-way valves (Chromatronix Inc.) were inserted to direct the buffer to the alternative routes 1, 2 or 3. The buffer was stored in a 5-l Mariotte vessel provided with an Ascarite tube in the air inlet, and continuously pumped through the system by a Jeol JLC-P3 pump (Japan Electron Optics Co., Ltd., Japan) at the rate of 18 ml/h.

A differential refractometer, model R4, (Waters Associates Ltd., Stockport,

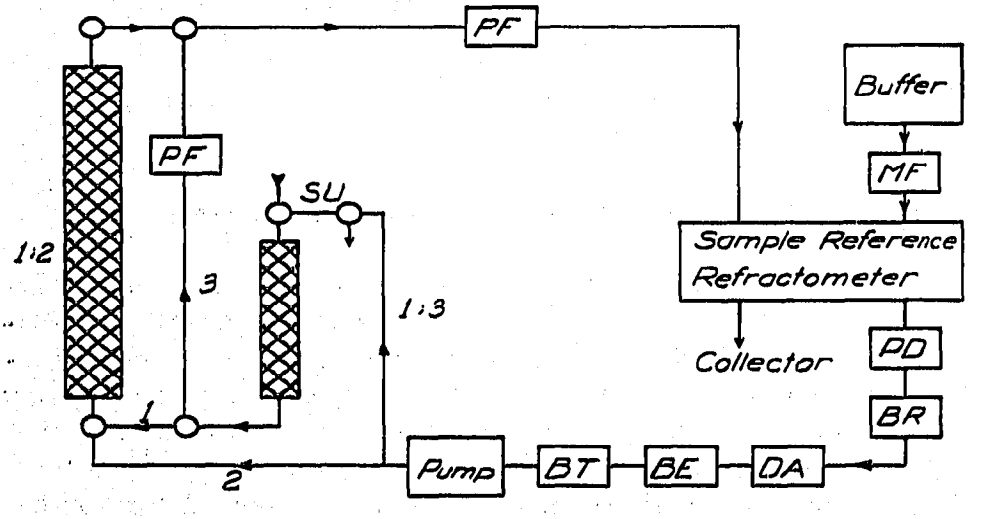

Fig. 1. Flow scheme of the two-column chromatographic system. The buffer passes through a 0.3 μ Gelman membrane filter (MF), the reference cell of the refractometer, the pulsation damper (PD), a buffer refiner (BR) consisting of a 1 × 4 cm column with AG 1 × 4 renewed every second month, a deaerator (DA) in which the buffer is heated to 100°, a buffer equilibrator (BE) consisting of a 500 ml vessel provided with a magnetic stirrer, a bubble trap (BT), the pump, the columns (alternative routes 1, 2 and 3), backpressure filters (PF) and the sample cell of the refractometer to the collector.

Great Britain) thermostated at 30° ± 0.1 was used for monitoring. The refractometer readings were recorded by a Texas Servo-riter II (Texas Instrument Inc., full scale 100 mV).

Analytical procedure. The sample application unit (SU, Fig. 1) was rinsed by gravity with buffer and with at least 1 ml of the sample solution. A 0.2 ml sample (actual volume 208 μl) was trapped in the sample application capillary. The sample was forced into the short column by the buffer flow directed through the short and long columns (route 1, Fig. 1). When the disaccharides had been eluted into the long column (after 35 min in our system) the buffer was passed through the short column only (route 3) to elute the monosaccharides. After 4 h the buffer was directed through the long column (route 2) for elution of the disaccharides. The total time for a complete

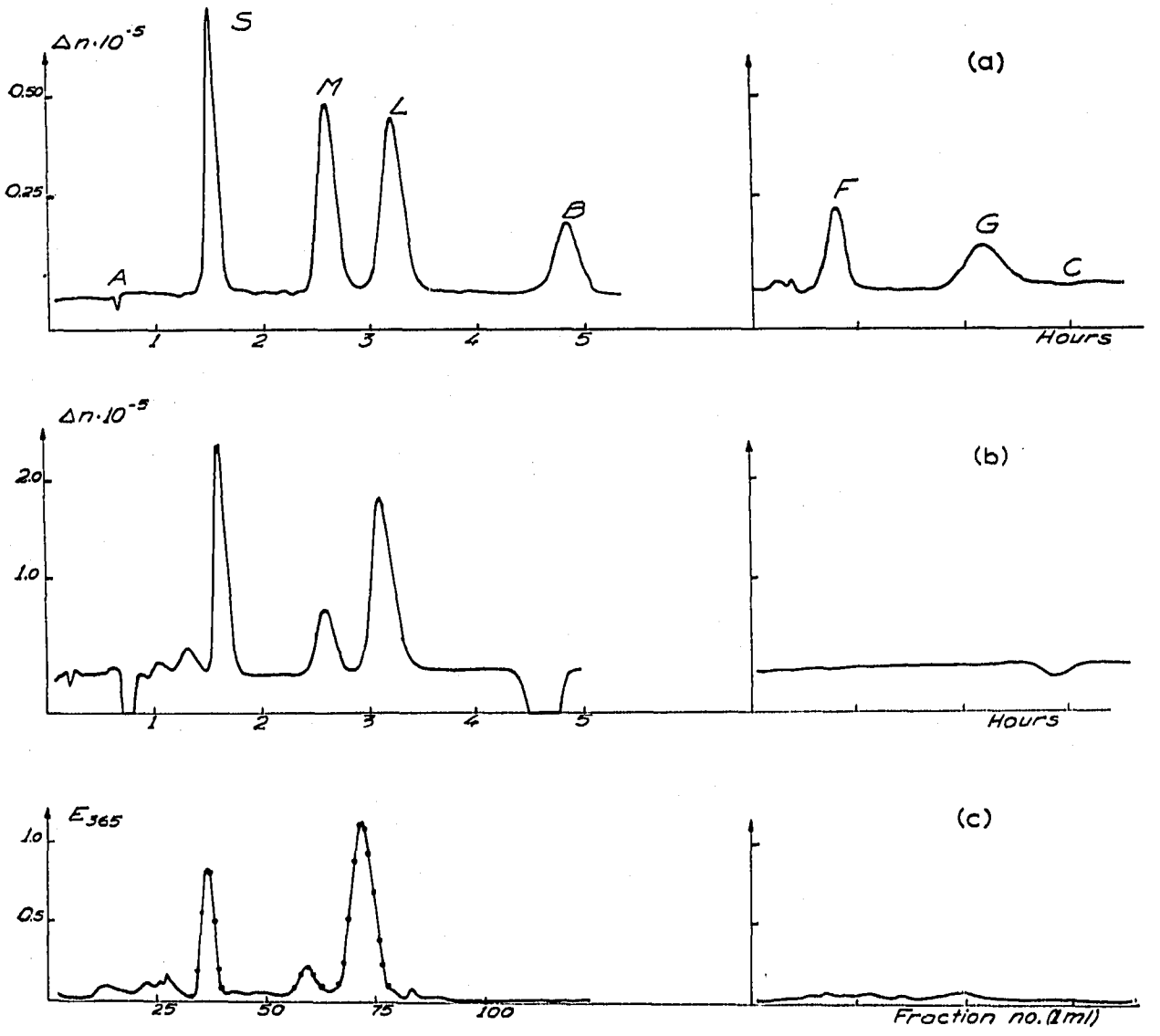

Fig. 2. Chromatograms with sugar samples. (a) 0.3 mg each of sucrose (S), maltose (M), lactose (L), fructose (F) and glucose (G) recorded with the highest sensitivity ("1 ×") of the refractometer. (b) SOMOGYI filtrate[16] of a cereal baby food (sensitivity "4 ×"). (c) same as (b), but analysis performed colorimetrically[18] after dilution 1:5.

analysis was 10 h. The next analysis could be started immediately after a run as no further equilibration was needed.

The sugars were recorded as positive peaks (Fig. 2). The peak area was determined by multiplying the height of the peak by the width at half height.

Results and discussion

It has been indicated[12–15,19] that *e.g.* maltose and lactose cannot be eluted quantitatively with alkaline borate buffers at elevated temperatures because of epimerisation and degradation. We found evidence for such reactions at 45° and pH 8.5, but not at our final conditions of 45° and pH 8.0. The recoveries of sucrose, maltose, lactose, fructose and glucose as analysed colorimetrically[18] were 95–100 % under these conditions.

The sugars gave rise to positive peaks (Fig. 2). As shown in Fig. 3 there is a linear relationship between the amount of sugars analysed and the respective refractometer response areas within the error of the measurements (± 0.02 mg in the 0.5–1.0 mg range). Deviations from this, which are especially pronounced for fructose, may be ascribed to distortion of the peak at the highest concentrations due to overloading of the column. It is evident that the refractometer response to eluted borate complexes of the monosaccharides were about half the values of those of the disaccharides on a weight basis. By contrast, a less pronounced difference was observed between refractive indices of mono- and disaccharides in solution. The lower response of monosaccharides in the column eluate may be ascribed to a more effective displacement of borate ions from the column.

False peaks were encountered at positions A, B and C as indicated in Fig. 2. When diluted buffer was applied as sample, negative peaks (*i.e.* below the base line) at positions A and B were recorded, whereas a buffer sample of higher concentration than that of the eluent gave positive peaks at these positions. It seems reasonable to assume that position A represents the front of the chromatogram and position B represents the

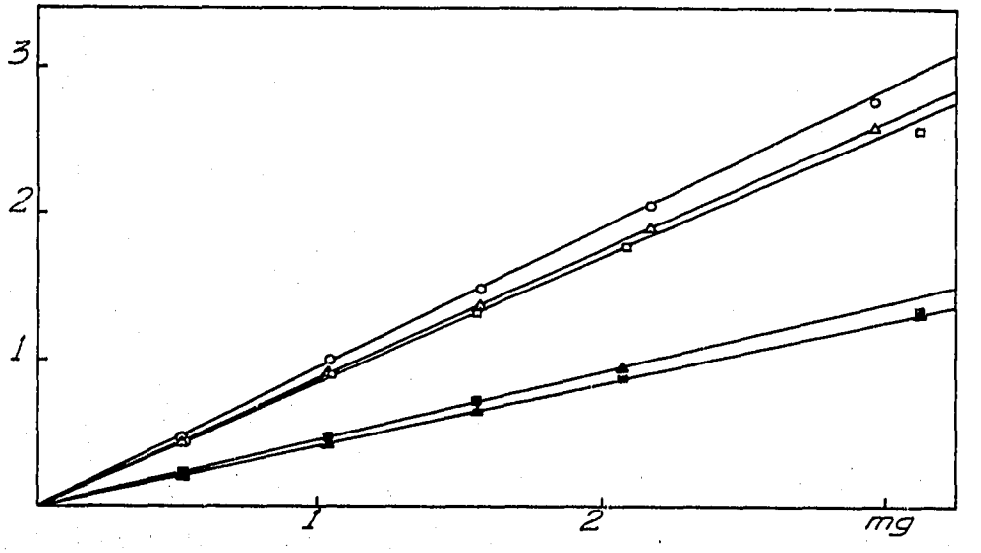

Fig. 3. Relationship between the amount of sugars and refractometric peak areas ($mm^2 \cdot 10^3$) (based on refractometric sensitivity "4×"). ○ = lactose; △ = maltose; □ = sucrose; ■ = fructose; ▲ = glucose.

location of the borate ion. Chloride was recorded as a negative peak at position C (Fig. 2). Apparently the displaced borate rather than the chloride itself was recorded.

Sugar alcohols were also recorded due to the nonspecific nature of the detector. For instance, glycerol can be conveniently analysed by this technique[20], appearing as a peak between sucrose and maltose. Sorbitol and mannitol were eluted among the monosaccharides.

Amino acids and other anions may be recorded by the present technique. Our products did not contain interfering substances in significant amounts. If this would be the case the clarification step should include their removal by ion-exchange resins in order to increase the specificity of the method.

We are greatly indebted to P. G. ANDERSSON, T. C. LAURENT, M. MALM and A. VESTERMARK for valuable discussions, to M. BAMFORD and K.-Å. BENGTSSON for technical assistance, and to M. W. VINCENT for revising the English text.

Marabou Research Laboratory,
172 85 Sundbyberg (Sweden)
JAAKKO J. LILJAMAA

Department of Medical Chemistry,
University of Uppsala (Sweden)
ANUND A. HALLÉN

1 J. X. KHYM AND L. P. ZILL, *J. Amer. Chem. Soc.*, 73 (1951) 2399; 74 (1952) 2090.
2 L. P. ZILL, J. X. KHYM AND G. M. CHENIAE, *J. Amer. Chem. Soc.*, 75 (1953) 1339.
3 A. HALLÉN, *Acta Chem. Scand.*, 14 (1960) 2249.
4 R. SYAMANDA, R. C. STAPLES AND R. J. BLOCK, *Contrib. Boyce Thompson Inst.*, 21 (1962) 363.
5 J. G. GREEN, *Nat. Cancer Inst. Monogr.*, 21 (1966) 447.
6 R. B. KESLER, *Anal. Chem.*, 39 (1967) 1416.
7 G. N. CATRAVAS, *Technicon Symp.*, 1 (1966) 397.
8 P. WEBER, I. BORNSTEIN AND R. J. WINZLER, *Anal. Biochem.*, 14 (1966) 100.
9 J. I. OHMS, J. ZEC, J. V. BENSON AND J. A. PATTERSON, *Anal. Biochem.*, 20 (1967) 51.
10 R. L. JOLLEY AND M. L. FREEMAN, *Clin. Chem.*, 14 (1968) 538.
11 Y. C. LEE, J. F. MCKELVY AND D. LANG, *Anal. Biochem.*, 27 (1969) 567.
12 E. F. WALBORG, JR., L. CHRISTENSSON AND S. GARDELL, *Anal. Biochem.*, 13 (1965) 177.
13 E. F. WALBORG, JR. AND R. S. LANTZ, *Anal. Biochem.*, 22 (1968) 123.
14 E. F. WALBORG, JR., D. B. RAY AND L. ÖHRBERG, *Anal. Biochem.*, 29 (1969) 433.
15 E. F. WALBORG, JR. AND L. E. KONDO, *Anal. Biochem.*, 37 (1970) 320.
16 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 69.
17 B. VASSILIOU AND R. KUNIN, *Anal. Chem.*, 35 (1963) 1328.
18 E. F. WALBORG, JR. AND L. CHRISTENSSON, *Anal. Biochem.*, 13 (1965) 186.
19 R. CARUBELLI, *Carbohyd. Res.*, 2 (1966) 480.
20 S. E. ZAGER AND T. C. DOODY, *Ind. Eng. Chem.*, 43 (1951) 1070.

Received December 30th, 1970