Table I. Sweetness of 4-Chloro-DL-kynurenine Relative to That of Sucrose

sucrose ref, %	isosweet concn of 4-chloro-DL-kynurenine, %[a]
2	0.025
4	0.05
6	0.08
8	0.11

[a] Concentration at which all judges scored the sweetness of the 4-chloro-DL-kynurenine solution as equal to or greater than that the sucrose solution provided.

Table II. Taste Thresholds for Sucrose and 4-Chloro-DL-kynurenine

stimulus	threshold[a] % by wt
sucrose	0.8
4-chloro-DL-kynurenine	0.01

[a] Concentration judged sweet by all panelists.

references, while its relative sweetness was somewhat dropped at levels above 6% sucrose reference levels. It was noteworthy that **6** did not differ significantly from sucrose in aftertaste and off flavor.

The result of threshold measurement is shown in Table II. The threshold concentration of **6** for sweetness was $^1/_{80}$ of that of sucrose on a weight base. This indicated that **6** was ~80 times as sweet as sucrose.

From the above two sensory analyses, the relative sweetness of **6** is ~80 times sweeter than sucrose. Since **6** is in the racemic form, it is possible that, as with tryptophan, only one optically active isomer may be sweet.

Although the compound **6** has not been tested for safety for use in foods, our data seems useful for structural modification of aromatic amino acids in order to design a new class of intensely sweet compounds.

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Cleanup of Methanolic Extract in High-Pressure Liquid Chromatography of Fructose, Glucose, and Sucrose in Onion Powder

A minicolumn of aluminum oxide could be used to clean up onion extract in 80% buffered methanol. Without this step, the injector and analytical column of the high-pressure liquid chromatograph (LC) became seriously damaged after many injections. Buffered methanol, pH 6.6, prepared with acetate buffer instead of phosphate was suitable for extraction and injection of soluble sugars from onion powder. Phosphate salts in methanol form needles that are dangerous for the analytical column of the high-pressure LC.

After publication of the paper of Gorin (1979), our institute purchased a Waters analytical high-pressure liquid chromatograph (LC) and found that the type of onion extract described in that paper could not be directly injected without damage to the injector and column. Samples had to first be clarified. Furthermore, the use of methanol with phosphate buffer, as in that paper, was dangerous as phosphate salts in methanol tended to crystallize after 1–2 days as needles, whatever the temperature (5 or 20 °C). So we looked for a means of cleaning up the sample and for another buffer instead of phosphate. Gutman (1974) found aqueous acetate satisfactory, even at 100 °C, although theoretically its pK seems inappropriate for buffering in the pH range 6.0–6.6. At lower pH, sucrose is hydrolyzed.

MATERIALS AND METHODS

Onion powder was that prepared Feb. 14, 1977, from bulbs not treated with maleic hydrazide (Gorin, 1979). The subsample was never removed nor opened during storage under nitrogen at –70 to –80 °C.

Buffered methanol was prepared as described before (Gorin, 1979) except for replacement of phosphate buffer by 0.1 M acetate buffer, pH 6.6 (Gutman, 1974), of which 20 mL was mixed with 80 mL of absolute methanol (Merck, 6009).

Reference Solutions of Soluble Sugars. Fructose (Merck, 5323), glucose (Merck, 8342 E), and sucrose (Merck, 7651 E) were separately dissolved in buffered methanol (100 mL), so that the volume of 10 μL injected into the high-pressure LC contained 25, 50, 75, or 100 μg of the sugar.

Preparation of the Minicolumn. This column was based on the work of Dunmire and Otto (1979). Aluminum oxide (Merck, 1097) was activated at 105 °C for 90 min and poured into a Pasteur pipet (Figure 1).

Onion Extract. Of the subsample, 500 mg (in former tests 100 mg) was suspended in buffered methanol (10 mL) at 55 °C and, after 15 min, centrifuged at 35280g for 30 min at 5 °C. The supernatant constituted the extract. Despite the increase in the ratio of powder to buffer, soluble sugars were still completely extracted from the

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Table I. Mass Fraction of Soluble Sugars in Onion Powder (g/100 g) by Liquid Chromatography with Cleanup of the Methanol Extract through Al_2O_3 Minicolumn (Sept 1979-March 1980) with the Waters or Chrompack Columns[a]

	with cleanup				without cleanup, Waters column	
	Chrompack column		Waters column			
sugar	$\bar{x}_1$[b]	s_1	$\bar{x}_2$	s_2	$\bar{x}_3$	s_3
fructose	15.40	0.17	14.99	0.09	13.61	0.24
glucose	11.35	0.17	11.44	0.26	12.31	0.38
sucrose	13.51	0.20	13.12	0.13	13.75	0.15

[a] Data without cleanup (Nov-Dec 1977, Waters column) are from Gorin (1979). Powder was prepared from onion cv. Hyduro on Feb 14, 1977. [b] $\bar{x}_1$ is the average of eight replicates. $\bar{x}_2$ and $\bar{x}_3$ are the averages of triplicate analyses. $s_1 = s_2 = s_3 = [\Sigma d^2/(n-1)]^{1/2}$.

powder, according to liquid chromatographic and enzymatic tests. A higher concentration of sugars than before was necessary in order to set the detector at an attenuation of 4× (earlier tests, 2×). The refractometer detector gave excessive background noise at 2×.

Cleanup Procedure. Three portions of extract (each of 1 mL) were successively poured into the minicolumn and allowed to drain through. A fourth portion of 2 mL was then poured on, and 2 mL of the cleaned-up extract was collected and filtered through a Millipore clarification kit (Filter FHL P 01300) as a safety precaution to remove any traces of glass wool derived from the minicolumn. The filtered extract was then treated ultrasonically (Bandelin, Sonorex RK 255, West Germany). It was brough to 20 °C (Colora Kryothermostate WK 5) before injecting it into the high-pressure LC.

External Reference. A reference solution containing the three sugars together, fructose (500 mg), glucose (500 mg), and sucrose (500 mg), in the buffer (100 mL) was submitted to the same cleanup procedure. Thus, the injected volume of 10 μL contained 50 μg of each sugar.

Conditions in the Liquid Chromatograph. The sample (10 μL) was injected into a Waters 6 UK injector. The 6000 A pump (Waters) regulated the flow rate at 1.5 mL min^{-1} with a pressure of ~6.3 MPa (~900 psi) of the solvent with acetonitrile (Baker, 8004) and twice distilled water in the volume ratio 75:25 kept at 25 °C (Labora Mannhein Precitherm PFV).

Already prepared chromatographic columns were used: either carbohydrate (Waters art. 84038) or Lichrosorb 10 NH_2 (Chrompack, 25140). The latter type of column required an adaptor (Chrompack, 24899) but was much cheaper. The columns were kept at 25 °C.

The differential refractometer (Waters, Model R 401) was also kept at 25 °C and set at "Att. 4×", with the power switch always at "on".

RESULTS AND DISCUSSION

In all experiments with reference solutions or with onion powders, the pH was 6.0–6.6.

With the reference solutions of fructose, glucose, and sucrose, there was a rectilinear response between peak height (mm) and amount of the sugar injected.

The regression equations ($y = a_0 + a_1x$) were as follows: for fructose, $a_0 = -0.255$, $a_1 = 0.248$, and $r = 0.998$; for glucose, $a_0 = 0.065$, $a_1 = 0.191$, and $r = 0.999$; for sucrose, $a_0 = 0.165$, $a_1 = 0.167$, and $r = 0.999$. y is peak height in millimeters, a_0 is the y intercept, a_1 is the slope, and r is the coefficient of correlation.

In all subsequent runs, peak heights of the chromatograms were in the range of rectilinear response of 25–100 μg.

Solutions containing known amounts of the three sugars in the buffer were submitted to the same cleanup procedure and gave the following recoveries, calculated from peak height with respect to the external reference solution:

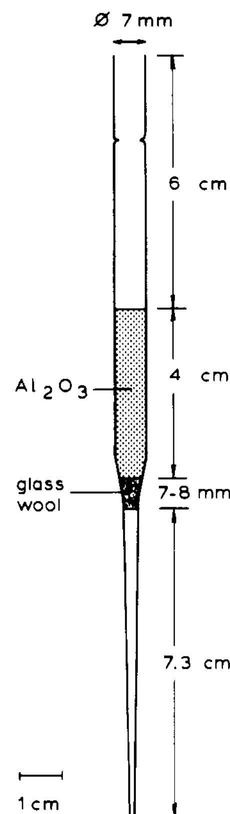

Figure 1. Diagram of the aluminum oxide minicolumn.

fructose, 99–101%; glucose, 97–104%; sucrose, 99–101%. Thus, the minicolumn did not retain the three sugars at all.

The cleanup of the onion extract seemed complete, as two main diffuse yellow bands appeared in the aluminum oxide minicolumn. They were probably flavonol derivatives of quercetin (Trammell and Peterson, 1976) and lipophilic sulfur compounds ("onion oil") (De Wit et al., 1979). The minicolumn Sep-pak (Waters, 51910) was unsatisfactory for this purpose, perhaps retaining the onion oil but allowing the quercetin derivatives to pass through.

The amounts of fructose, glucose, and sucrose in clarified buffered methanol did not change over several days at room temperature.

Table I shows the mass fraction of soluble sugars after cleanup with aluminum oxide and with the two types of chromatographic columns and without the cleanup in the earlier tests (Gorin, 1979).

A Student's t test ($\alpha = 0.05$) showed the following. There was no significant difference between the averages ($\bar{x}_1$ and $\bar{x}_2$) of the respective sugars with the two types of column. There was a significant difference between the more recent data and the earlier data ($\bar{x}_2$ and $\bar{x}_3$). The earlier and the recent data differed by 5–9%; they were

similar for practical purposes.

The aluminum oxide column offers a simple and cheap way of preparing samples that can be placed in ampules of an automatic injector for continuous analysis during the day and night.

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Automated Method for Determining in Vitro Cholinesterase Inhibition by Experimental Insecticide Candidates

An automated method for the determination of electric eel cholinesterase (ChE) inhibition by experimental insecticide candidates was developed. It was based on the manual procedure of Ellman et al. [Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, 7, 88–95] using acetylthiocholine iodide (ATChI) as the substrate and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as the color reagent. The substrate and color reagent concentrations were individually varied in order to determine optimum reaction conditions. The ChE assay conditions were found to be 1.5 mM ATChI and 0.38 mM DTNB. The reproducibility of the automated method was evaluated by the simultaneous analysis of 10 aliquots from each of three electric eel ChE solutions containing 247, 413, and 1054 units/L ChE. Coefficients of variation (CV) of 5.9–9.0 were obtained for each sample, respectively. Variation between runs was examined by comparing the three electric eel ChE solutions containing 270, 513, and 1247 units/L ChE for 10 different days. A CV of 3.7–7.9% was obtained for each sample, respectively. The test requires an initial 30 min of reagent preparation and thereafter as many as 400 samples/h can be analyzed. The sample volume requirement is as little as 20 mL of enzyme solution.

Screening for insecticidal activity of experimental carbamates and organophosphates may be done by determining the cholinesterase (ChE) inhibition produced by the insecticide candidates. As a ChE enzyme source, housefly head (Hellenbrand, 1967) and bovine erythrocyte (Hastings et al., 1970) are used. Many automated, as well as manual, methods are being used for the assay of ChE activity. However, a centrifugal analyzer, CentrifiChem (Union Carbide Corp.), which is extensively used in hospital laboratories for automated blood clinical chemistries, has not been applied for this purpose. This study reports the application of centrifugal analyzer methods for ChE inhibition studies using the electric eel enzyme preparation. The optimum reaction conditions for ChE assay for the centrifugal analyzer were examined and the reproducibility of the procedure for ChE assay was evaluated. On the basis of this methodology, the I_{50} value (defined as the amount of chemical required to inhibit 50% of the control ChE activity) for thiofanox, 3,3-dimethyl-1-(methylthio)-2-butanone *O*-[(methylamino)carbonyl]oxime, was established.

MATERIALS AND METHODS

Reagents. Electric eel (Sigma Chemical Co.; No. C-3389; 355 units of acetylcholinesterase/mg) was used as the enzyme source for the determination of optimum conditions. One milligram was dissolved in 100 mL of 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4). This yielded a stock solution of 3.55 units/mL. This stock enzyme solution was stable for at least 2 weeks if kept refrigerated when not in use.

The 0.05 M Tris buffer (pH 7.4) was prepared by combining 6.64 g of NaCl, 6.05 g of tris, and 45 mL of 1 N HCl. This was diluted to 900 mL with distilled water. The pH was adjusted to 7.4 with 1 N HCl and the volume was adjusted to 1 L with distilled water. The substrate used was a 38 mM solution of acetylthiocholine iodide (ATChI), made by adding 109 mg of ATChI to 10 mL of distilled water. The color reagent, DTNB [(5,5-dithiobis(2-nitrobenzoic acid)], was prepared by dissolving 100 mg of DTNB in 500 mL of 0.05 M Tris buffer (pH 7.4). Both ATChI solution and color reagents were stable at least for 4 weeks at 4 °C.

Apparatus. The CentrifiChem 400 Analyzer was used for analyses. The CentrifiChem Pipettor was used for delivery of samples, reagents, and diluents.

Basic Reaction Conditions. All determinations were made by using 20 μL of enzyme samples, 50 μL of H_2O, and 350 μL of reagent. An initial absorbance reading was taken at 15 s after the start of the reaction, and the second reading was taken after an additional 15 s. The wavelength used was 405 nm.

In order to find optimal analytical conditions, the analyses were carried out while individually varying the

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