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New Approaches to Carbohydrate Analysis in Food

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Man analyses food with his taste-sense with respect to sweetness, acidity and bitterness. Fundamentally, evolution has truly pointed the way with respect to the future of food analysis in that the response time of man's taste buds is immediate, no extracellular reagents are required and rapid changes can be detected in a self-sampling mode. Perfection is not achieved in three aspects of the analysis. Firstly, it is difficult to distinguish between two compounds that are both sweet such as sucrose and saccharin, particularly in mixtures. The sensing mechanism is thus limited, based on a selective non-specific sensor. Secondly, it is not quantitative in absolute terms but can be made so by comparison with standardised known solutions. Its strength is its ability to distinguish between two solutions of only slightly differing sweetness. Its third weakness is that other compounds that are not sweet may bind to the taste buds and obscure the senses; indeed, this can happen with some very sweet proteins recently discovered. In total, man's senses with respect to food analysis are of a limited number but are self-renewing and mobile.

What then will be the future basis of man's instruments for performing his food analysis? Logically they should be based on a limited number of sensors made specific for each component of food by the addition of a cheap component such as a disc that converts a widely based sensor (e.g. a thermistor) into a specific sensor. Since every component of man's food has been made by a specific enzyme and often can be modified by a series of types of enzymes (e.g. oxidases, hydrolases) in a specific way, it is evident that there exists in nature a vast range of protein enzymes that will interact either absolutely specifically with a food carbohydrate (e.g. glucose oxidase with D-glucose) or less specifically with a related group of compounds, thus permitting both specific or group analysis. Enzymes will act both on small and large substrates. Other proteins exist in nature that will interact specifically with carbohydrate components of food. Many seeds contain special proteins called lectins that interact with carbohydrate components but these, like antibodies, are more applicable to the analysis of carbohydrates in a macromolecular form and further have no catalytic activity themselves. However, they are useful in their immobilised form on

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supports like agarose as tools for separating carbohydrate-containing entities of food prior to analysis by a sensor. In short, our analysis would be more reliable if separation accompanied sensing by a specific enzyme.

Enzymes can now be presented in a formidable number of ways, each one of which may be suitable for a particular mode of analysis. Thus tubes of glucose oxidase immobilised on the inside of nylon tubes can be purchased from one manufacturer (Miles Laboratories) and used as reaction tubes in an Autoanalyser. This saves one reagent line but the Autoanalyser still has an insatiable thirst for other reagents, illustrating all that is bad in the current analytical approach. Another manufacturer (Leeds & Northrup) has immobilised the glucose oxidase on porous glass beads and sensed the resultant hydrogen peroxide produced by this enzyme with glucose by using a three-electrode amperometric cell. This represents a major step forward. The instrument assays 0–50 p.p.m. of glucose and gives 98 per cent. of its response in less than two minutes. The accuracy is ± 2 per cent. of instrument range and reproducibility is $\pm$ 1 per cent. of instrument range. The flow though the column is 5 ml/minute nominal at $35 \pm 0{\cdot}1$°C. Normal maintenance procedures call for flushing the instrument for 15–20 minutes once every 24 hours with 50 p.p.m. of H_2O_2. The instrument remains within calibration specifications for two weeks before there is a drop in response without this maintenance. The upper limit for glucose concentration is set by the solubility of oxygen in the buffer required for oxidation of the glucose to gluconolactone. The concentration of oxygen in equilibrium with air in the buffer is 8 p.p.m.

An alternative approach to sensing an enzyme reaction is the enzyme thermistor developed by Mosbach, Danielsson, Bergerud and Scott[1]. The thermistor is put in direct contact by placing it in the bed of a microcolumn (0·8–1 ml) of immobilised enzyme and letting the reactants flow over the thermistor tip. The technique was applied to the determination of D-glucose, penicillin G and urea, using glass-bound glucose oxidase, penicillinase and urease respectively. Linear relationships between Δt measured and concentration of substrate present were observed. Solutions were pumped through at 60 ml/hr. and the system was allowed to equilibrate for 1–2 min. Reproducibility was ± 2 per cent. and the Δt recorded was as high as 50 per cent. of the theoretical value.

Having combined a sensor with a specific immobilised enzyme, it is now quite feasible[2] to interpose between the two a separation device such as a dialysis membrane. It is even feasible to immobilise the enzyme on the membrane itself, though if the Autoanalyser dialysis membrane is used it is wise to coat this already set up in its holder and attach the requisite enzyme thereto *in situ*. We have attached dextranase to such a membrane for the analysis of dextran, a common, often unsuspected, impurity in commercial sugar. The carbohydrates produced from the dextran are small enough to diffuse through the membrane and there be assayed. Starch impurities can be assayed in a similar way using immobilised glucamylase. In practice, it is often easiest to keep the dialysis membrane and immobilised enzyme separate. A good all-purpose method of coating dialysis membranes can be based on the method described by Gray, Livingstone, James and Barker[3].

A multistage enzyme process using up to four enzymes in sequence to produce

a product that can be assayed is quite achievable. Thus, we have immobilised the four enzymes required to convert glycogen to fructose so that they will all operate simultaneously at the same pH and temperature. It was necessary to immobilise the enzymes in their separate micro-environments which acted as insoluble buffering agents permitting remarkable changes in the pH optimum of the enzymes by up to two pH units. Indeed, we have thus defined the limitation of the applicability of immobilised enzymes separately or in sensors as that of the physiological range of pH and temperature, which fortunately covers the extremes to be encountered in different foods from vinegar to alkaline media. In order to achieve the multistage sequence from glycogen to fructose it was necessary to use membranes to keep the products and substrates of some of the enzymes apart. One we found very suitable for separating ionic (e.g. sugar phosphates) and non-ionic species (e.g. sugars) was a conventional dialysis membrane on which polyacrylic acid had been grafted by giving the membrane a dose of radiation in the dry state to initiate radical formation and then immersing it in acrylic acid. The conversion of glycogen or starch to fructose used (a) phosphorylase in the presence of phosphate to convert these polymers to glucose-1-phosphate, (b) phosphoglucomutase to catalyse the conversion of the latter to glucose-6-phosphate which then became the substrate of (c) phosphoglucoseisomerase to yield fructose-6-phosphate which is broken down by (d) alkaline phosphatase to fructose. Such high technology should now be replaceable in some cases by the use of immobilised cells (see Gist-Brocades[4]) which can even be obtained in a state where they respire and operate like cells in suspension. It would be necessary to operate the immobilised cells with an inhibitor to cut out the metabolic pathway required. We are very near now to man's use of his own taste-buds in the form of whole cells—membranes, concerted reaction sequences and a sensing mechanism.

The wide-ranging sensor required for food analysis of carbohydrates is an oxygen electrode mated with a specific immobilised carbohydrate oxidase (e.g. glucose oxidase) in a true enzyme electrode. The first commercial instrument of this type (Yellow Springs Instrument Co., U.S.A.) became available this year. Although it can be done, it is quite unnecessary to fix the enzyme to the polythene membrane of the oxygen electrode; close proximity (merely contact) will suffice. It is essential, however, for fast response times to have easy open access between substrate glucose, enzyme glucose oxidase and polythene membrane. We have patented a disc of woven glass fibres (curtain material is ideal) to which is chelated the enzyme. Our "cement" is wholly inorganic (Emery, Novais and Barker[5]) and can be based on prior treatment of the glass with titanium tetrachloride, drying, followed by a water wash and mere contact with the enzyme which then chelates to the glass via a permitted food additive! A linear response to glucose is given by our enzyme electrode and the "magic" disc can be used repeatedly for up to 18 months, although in practice this would never be attempted. By the same chelating procedure we can simultaneously immobilise both invertase and glucose oxidase so that now we have a specific sensor for sucrose, as the product of the first enzyme becomes the substrate of the second. Again there is a linear response to sucrose and, remarkably, the response time is almost the same as the two enzymes gear together. In the same manner, by

immobilising glucamylase and glucose oxidase simultaneously on the one disc, we have made a starch sensor. Since there are at least 50 known enzymes that oxidise substrates with consumption of oxygen that can be sensed by the oxygen electrode and hundreds of enzymes that can be coupled with the fifty, the whole field of analysis of food is opened up. Much of this can be done by buying a cheap robust oxygen electrode and making similar discs by the many methods for immobilising enzymes now available.

The "Tomorrow's World" of food analysis, depicted above, in reality only covers half the area of food analysis. Such methods are excellent for beverages, homogenates, jams and extracts just as current methods are in clinical chemistry for serum and urine. New methods for examining food cellular material, preferably of a quantitative nature, are urgently required. The methods of quantitative histochemistry may well be the answer, particularly in the use of "guided" enzymes, conjugates of a direction-finder protein (Bowen and Barker[6]) such as concanavalin attached to marker enzyme. Similar conjugates based on antibody-enzyme conjugates would also be applicable. Films of food material immobilised by our titanium chelation method would immobilise the cellular matter in a surface film that thereafter could be treated with the conjugate which would adhere very specifically to particular macromolecular components. After suitable treatment the "immobilised" enzyme in the surface film could be assayed.

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