4. TEXTOS ORIGINALES.

4. 1. Textos pertenecientes a las introducciones del artículo de investigación.

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Bioconversion of solid food wastes to ethanol

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Solid food processing wastes and by-products are cofermented with cheese whey to produce ethanol. The experimental procedure involves the use of an enzymatic process to convert starch and lactose into fermentable sugars. These reducing sugars are then fermented to alcohol by distiller's dried yeast and a high-ethanol tolerant yeast, *Saccharomyces cerevisiae*. Cheese whey is used as a wetting agent and provides macro- and micronutrients for the microorganisms. Cofermenting food processing wastes with cheese whey, in the presence of high- and low-temperature enzymes, induces a 33-36% increase in alcohol yield. This procedure also significantly reduces the fermentation time from 60 to 12 h.

Keywords: *Bioconversion; cofermentation; cheese whey; ethanol; solid wastes; gas chromatography*

Energy and environmental issues take turns driving the development and use of alternative fuels for motor vehicles. As the availability of petroleum-derived fuels and industrial feedstocks decreases owing to depletion and also economic and political developments, renewable sources of organic compounds are tested for their suitability as alternatives to petroleum-based substances. Recent environmental concerns such as ozone non-attainment, solid waste management and control of toxic air pollutants have been other reasons for finding clean-burning alternative fuels.

Ethanol production from agricultural products has been in practice for the past 80 years. Ethanol can be produced from many kinds of raw material that contains starch, sugar or cellulose. Wastes from food processing industries represent a severe pollution problem and need better waste management techniques. Utilization of food processing wastes to produce fuel alcohol with an increased efficiency has been under investigation in our laboratory for the past few years. We were able to develop a novel and highly efficient cofermentation system for food wastes containing starch and lactose.

Fermentation is an anaerobic, energy-releasing transformation of carbohydrates by living organisms. Yeast can ferment a wide variety of sugars and oligosaccharides other than glucose. The D-hexoses and oligosaccharides fermented most often by yeast are glucose, mannose, fructose, galactose, maltose, lactose, melibiose, trehalose and raffinose. The yeast in most widespread use for alcoholic fermentation is *Saccharomyces cerevisiae*. Several studies on ethanol production *via* fermentation and the effects of different factors on the fermentation have been published[1–7] in the past decade. Utilization of cheese whey as the liquid portion of a fermenting corn mash has been investigated by Whalen *et al.*[8] Their work involved the fermentation of lactose/corn mash by the use of a dual yeast inoculum (*Kluyveromyces marxianus* and distillerís yeast). This lactose/glucose cofermentation process took 60–72 h for completion. We investigated the use of whey with bakery products and other starchy waste products by the application of lactose hydrolysis in conjunction with a single yeast inoculum to reduce the fermentation time and an increase in alcohol yield.

The objectives of this work were to study the effect of low- and high-temperature enzymes on hydrolysis of food wastes, to compare the fermentation of bakery products with mixed waste products and to study the cofermentation of cheese whey and starchy food wastes.

Experimental

Materials

Feedstocks

Raw materials for ethanol production include various types of starchy waste products from bakeries and food processors and cheese whey from dairy processing industries. The types of waste samples used were bread, biscuits, buns, cakes, donuts, potato chips and flour. The bakery products (2–3 weeks old) were collected from a local bakery and were stored below 5 °C. Cheese whey was obtained from a local cheese plant and was refrigerated until used. Samples were ground to a fine powder using an electrical blender.

Enzymes

Fungamyl is a purified fungal α-amylase produced from a selected strain of *Aspergillus oryzae*. This enzyme hydrolyzes 1,4-α-glucosidic linkages in amylose and amylopectin. Termamyl is an α-amylase isolated from a soil bacterium, *Bacillus licheniformis*. This enzyme hydrolyzes 1,4-α-glucosidic linkages in starch and possesses a high degree of heat stability. Amyloglucosidase Novo (AMG) is an *exo*-1,4-α-D-glucosidase (glucoamylase) obtained from a selected strain of *Aspergillus niger* by submerged fermentation. This enzyme hydrolyzes both1,4- and 1,6-α-linkages in starch. Lactozym (Novo Industries, Bagsvaerd, Denmark) is a β-galactosidase (lactase) preparation produced by submerged fermentation of a selected strain of the yeast *Kluyveromyces fragillis*. When Lactozym reacts with lactose, a mixture of glucose and galactose is formed. High T is Alltech's brand name for its improved bacterial α-amylase. High T is a carefully selected blend of enzymes derived mainly from *Bacillus subtilis*. Allcoholase II is a carefully selected blend of enzymes derived from *Aspergillus niger* and *Rhizopus niveus*. Allcoholase II is designed to be a complex blend of many enzyme activities including α-amylases, amyloglucosidase, protease and cellulase. In addition, it contains many vitamins and yeast growth factors. Since Allcoholase II works at the same temperature range as yeast, it can be added with the yeast. All the enzymes were bought from Novo Nordisk (Baltimore, MD, USA).

Organism

The yeast we used for fermentation experiments was *Saccharomyces cerevisiae*. Two types of yeast strains were used, distiller's yeast and Alltech Enriched Yeast. Distiller's yeast was obtained from the Redstar Division of Universal Foods (Milwaukee, WI, USA). Alltech Enriched Yeast (AEY) is a high-ethanol tolerant yeast obtained from Alltech (Lexington, KY, USA).

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Speciation as an analytical aid in trace element research in infant nutrition†

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The aim of this work was twofold: to study the binding pattern of trace elements in formulas as compared with breast milk and the relationship between trace elements in breast milk and in maternal dietary intake. To investigate the binding form of trace elements in these nutritive fluids, methods for protein separation were combined with methods for trace element determination in the eluted fractions. HPLC and ICP-AES or ICP-MS were coupled on-line for the simultaneous speciation of elements of nutritional interest, *viz.*, Ca, K, Mg, P, S, Co, Cu, Fe, I, Mn, Mo, Se and Zn, and also the heavy metals Cd and Pb in both human milk whey and formulas. In order to minimize interactions between the labile metal protein complexes and the column material, size-exclusion chromatography was used for protein separation. The binding pattern of trace elements in formulas is significantly different from that in breast milk and depends on its main component (cow's milk or soy), its processing (hydrolysis) and the chemical form (inorganic) of the added compounds. For example, compared with breast-fed infants the iron supply of formula-fed infants is much higher (up to 20-fold); in addition, the binding forms of Fe are very different in the two fluids. This has to be evaluated with respect to interactions with other elements during intestinal uptake. The investigation of breast milk samples from different regions of the world showed comparable shapes for the elution profiles and for Mo and Se a dependence on the regional maternal dietary intake. Speciation studies carried out on breast milk samples as a function of the selenium content showed significant changes in the zinc-binding pattern. In particular, citrate (as a zinc-binding component) was found to decrease with increasing dietary selenium intake of the mother.

Keywords: *Size exclusion chromatography; inductively coupled plasma mass spectrometry; element speciation; human breast milk; infant formula; binding pattern; selenium; citrate*

During the prenatal period, the fetus is supplied with minerals and trace elements *via* maternal circulation and controlled placental transfer. After separation from the mother, the newborn has to develop its own functions and regulatory systems, including respiration, digestion and immune defenses. Trace elements are involved in the form of metalloproteins and enzymes at all stages in the development of these processes. Infancy is further characterized by an extremely high rate of synthesis of tissue cells, which leads to the infant's doubling its birth mass in a period of only 4 months. The infant's trace element requirement is supplied not only by amounts transferred *via* the mother's milk in specific binding forms or by formula, but also from prenatal stores. Special attention must be paid to very low birth mass, premature infants because they are born with lower stores of essential micronutrients. Trace elements must be added to pre-term infants' formulas to satisfy their higher dietary requirements.

In early infancy, breast milk or cow's-milk-based and soy-based formulas are the only dietary source of essential trace elements. The mother's milk provides an adequate supply of all micronutrients for the full-term infant. The concentrations and the fairly well defined binding pattern of the essential trace elements in human milk are therefore used as a reference. On the other hand, the trace elements chromium, copper, zinc, iron, manganese, molybdenum, iodine and, recently, selenium have been added to the formulas as compounds and at concentration levels that are different from those found in breast milk. With the sole exception of selenium, the trace element intake of infants *via* formula is significantly higher than *via* breast milk.[1,2] The iron supply was found to be up to 20 times higher despite the fact that the high hemoglobin of newborns forms a reservoir. In the case of manganese, the supply of the formula-fed infant can be as much as 1000 times higher than that of the breast-fed infant. During the first months of life such high values are critical with respect to known Fe–Zn, Fe–Cu and Mn–Fe interactions. As negative effects of high iron supplementation (>4 mg l^{-1}) significantly lower levels of glutathione peroxidase in serum[3] and superoxide dismutase in erythrocytes[4] have been observed in formula-fed infants. In addition, given the prooxidant effects of excessive amounts of iron in the iron(II) form, the balance between the formation and inactivation of free radicals generated by the rapid growth rate of premature newborns during the first months of life might be disturbed.

In spite of the significantly lower trace element intake of breast-fed infants, their serum concentrations of the essential elements Cu, Fe and Zn are comparable to those of formula-fed infants.[1,5] Further, mass and length gains, as developmental parameters, were comparable for the two groups over a period of 4 months.[5] Because no signs of deficiency were observed in breast-fed infants, the bioavailibility of copper, iron and zinc of the special binding proteins in human milk must be considerably higher than that in cow's milk or soy-based formula.

In the light of these facts, we considered it of importance to investigate the concentration, chemical form and nutritive value of trace elements in both human milk and infant formulas, with our ultimate goal being to obtain as much information as possible about adequate infant nutrition. We therefore carried out speciation studies to determine the binding form of trace elements in these nutritive fluids, combining methods for protein separation with methods for trace element determination in the eluted fractions.

Experimental

Sample collection and preparation

Human milk

The human milk samples were taken from mothers living in three countries (Germany, Spain and Venezuela) in order to

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Analysis of carbonaceous aerosols: interlaboratory comparison

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Carbonaceous aerosols are present in many workplace and environmental settings. Some of these aerosols are known or suspect human carcinogens and have been linked to other adverse health effects. Exposure to diesel exhaust is of particular concern because it has been classified as a probable human carcinogen and use of diesel-powered equipment is widespread in industry. Because previously used methods for monitoring exposures to particulate diesel exhaust lack adequate sensitivity and selectivity, a new method was needed. A carbon analysis technique called the 'thermal–optical method' was evaluated for this purpose. In thermal–optical analysis, carbon in a filter sample is speciated as organic and elemental (OC and EC, respectively). When the thermal–optical method was initially evaluated, only one instrument was available, so interlaboratory variability could not be examined. More recently, additional instruments were constructed and an interlaboratory comparison was completed. Eleven laboratories participated in the study, including four in Europe that employ an alternative thermal technique based on coulometric detection of CO_2. Good agreement (RSDs $\leq$ 15%) between the total carbon results reported by all laboratories was obtained. Reasonable within-method agreement was seen for EC results, but the EC content found by the two methods differed significantly. Disagreement between the OC–EC results found by the two methods was expected because organic and elemental carbon are operationally defined. With all filter samples, results obtained with the coulometric method were positively biased *relative* to thermal–optical results. In addition, the alternative method gave a positive bias in the analysis of two OC standard solutions. About 52% and 70% of the carbon found in two aqueous solutions containing OC only (sucrose and EDTA, respectively) was quantified as elemental, while EC contents of about 1% and 0.1% (respectively) were found by the thermal–optical method. The positive bias in the analysis of the OC standards is attributed largely to inadequate removal of OC during the first part of the analysis; lack of correction for pyrolytically formed carbon (char) also is a factor. Results obtained with a different thermal program having a higher maximum temperature were in better agreement with the thermal–optical method.

Keywords: *Carbon analysis; elemental carbon; black carbon; soot; carbonaceous aerosol; diesel exhaust; diesel emissions; diesel particulate; combustion aerosol*

Many workplace and environmental settings contain aerosols composed primarily of carbon. Carbonaceous aerosols encountered in these settings include asphalt fumes, oil mists, cigarette and wood smokes, carbon black, and diesel exhaust. Some of these aerosols are known or suspect human carcinogens (*e.g.*, cigarette smoke and diesel exhaust, respectively) and have been linked to other adverse health effects (*e.g.*, asthma, heart disease). Exposure to diesel exhaust is of particular concern because it has been classified a probable human carcinogen[1,2] and diesel equipment use is widespread in industry (*e.g.*, trucking, transit, mining, railroads, agriculture). An estimated[2] 1.35 million workers are routinely exposed to diesel engine exhaust, and exposures in some industries are relatively high (*e.g.*, >0.5 mg m^{-3}). Unfortunately, health-based exposure criteria for diesel particulate have not yet been established. A Threshold Limit Value (TLV) of 150 μg/m^{-3} has been proposed[3] but has not yet been adopted.

Particulate diesel exhaust, like fine particulate air pollution in general, also is of concern with respect to noncancer health effects. The US Environmental Protection Agency (EPA) has proposed an inhalation Reference Concentration (RfC) of 5 μg m^{-3} for the noncancer health effects of diesel exhaust,[4] and the State of California Office of Environmental Health Hazard Assessment (OEHHA) has proposed adoption of this value for the chronic inhalation reference exposure level in California.[5] The RfC for a substance is an estimate of a daily exposure of humans, including sensitive subgroups, that is 'likely to be without appreciable risk of deleterious effects during a lifetime of exposure'.[5] Comprehensive reviews of the potential health effects of exposure to diesel exhaust exposure have been recently published.[6,7]

Because diesel exhaust is a chemically complex mixture containing thousands of compounds, some measure of exposure must be selected. Given the high carbon content of diesel particulate, a carbon-based method was investigated. The method, recently published as National Institute for Occupational Safety and Health (NIOSH) Method 5040,[8] is based on an evolved gas analysis technique called the 'thermal–optical method'. With this technique, speciation of organic and elemental carbon (OC and EC, respectively) is accomplished through temperature and atmosphere control and by an optical feature that corrects for pyrolytically generated carbon, or 'char', formed during the analysis of some materials. Although both organic and elemental carbon are determined in the analysis, EC is the superior marker of diesel particulate because it constitutes a large fraction of the particulate mass, it can be quantified at background (*i.e.*, environmental) levels, and its only significant source in most workplaces is the diesel engine. Different approaches can be applied for OC–EC analysis, but a thermal–optical method was selected because the instrumentation has desirable design features not present in other carbon analyzers. An in-depth discussion on Method 5040, including both technical and exposure-related issues, has been published elsewhere.[9]

In a previous study,[10] different methods gave widely varying results in the speciation of organic and elemental carbon. For this reason, OC–EC methods are considered *operational*, in the sense that the method itself defines the analyte. Given its operational nature, it is important to examine interlaboratory variability of the method; however, when the thermal–optical method was initially evaluated, only one instrument was available, so interlaboratory variability could not be examined.

Anexo

More recently, additional instruments were constructed by a commercial laboratory[11] and an interlaboratory comparison was conducted. Seven laboratories that perform thermal–optical analysis participated in the comparison. Six of these used NIOSH Method 5040 (*i.e.*, they used identical instrumentation and thermal program), while the seventh used a variation on the method. Another thermal technique based on coulometric detection of CO_2 is being used in Europe for occupational monitoring of diesel particulate. Four laboratories employing the coulometric method also participated in the interlaboratory comparison, giving a total of eleven laboratories (seven thermal–optical and four coulometric). Discussion of the methods and a summary of the results of the intercomparison are reported in this paper.

Experimental

Reagents and materials

All air samples were collected in the field with the exception of a mixed sample of urban particulate and cigarette smoke, where cigarette smoke was collected in the laboratory on 25 mm diameter portions taken from a field sample. In addition to the mixed sample, the sample set included an urban particulate sample collected at a construction site (diesel-powered compressors were used) near a downtown area, a sample collected in a loading dock area (diesel trucks used) of a building, one collected in the bay of a fire engine house, and two wood smoke samples. A high-volume sampler loaded with quartz-fiber filters (8 × 10 inch QM-A, Whatman, Clifton, NJ, USA) was used for sample collection. To remove possible carbon contamination, the filters were precleaned in a muffle furnace at 800 °C for approximately 2 h. After sample collection, rectangular portions of the filters were distributed to the participating laboratories for analysis in triplicate. To ensure matched sample sets, multiple analyses were performed across all filters prior to distribution of the portions. Two aqueous standard solutions containing only OC were also included in the sample sets. Reagent grade sucrose and a 0.05 M calibrated standard solution (both from Aldrich, Milwaukee, WI, USA) of the disodium salt of ethylenediaminetetraacetic acid (EDTA) were used for preparation of the OC standards. These standards provided a check on the accuracy of the total carbon (TC) data as well as a check on the pyrolysis correction feature of the thermal–optical method (both standards char during analysis).

Analytical methods

Six laboratories analyzed samples by a thermal–optical method,[8] (*i.e.*, 5040), one used a similar method[12] (TOR) having an optical correction feature based on filter reflectance rather than transmittance, while four others used an alternative thermal technique[13] with detection based on coulometric titration of CO_2. All Method 5040 laboratories (TOM1 through TOM6) employed the same thermal program (or parameter file), while users of the coulometric technique employed different protocols. The different thermal protocols used by the participating laboratories are listed in Table 1.

One coulometric laboratory (C2) reported two sets of results for each sample. One set (C2A) corresponds to results obtained with the thermal program normally used by the laboratory, while the second set (C2B) corresponds to results obtained by the protocol used by laboratories C3 and C4. This protocol (see Table 1) is specified in an official method[13] currently being used in Germany (Method No. 44) for occupational monitoring of diesel particulate. The two sets of data were obtained simply by reading the integrator counts at different times (see Results and discussion: Thermal treatment).

Briefly, in NIOSH Method 5040,[8] speciation of organic and elemental carbon is accomplished through temperature and atmosphere control, and by an optical feature that corrects for pyrolytically generated 'EC' (or 'char') formed during the analysis of some materials (*e.g.*, cigarette and wood smoke). Light from a pulsed diode laser is passed through the filter to allow continuous monitoring of filter transmittance. The analysis proceeds essentially in two stages. In the first, organic carbon and carbonate carbon (if present) are volatilized from the sample in a pure helium atmosphere as the temperature is stepped to a maximum (about 860 °C in Method 5040). Evolved carbon is catalytically oxidized to CO_2 in a bed of granular MnO_2, reduced to CH_4 in an Ni–firebrick methanator, and quantified as CH_4 by a flame ionization detector (FID). During the second stage of the analysis, a pyrolysis correction (if needed) and the EC measurement are made. The oven temperature is reduced, an oxygen (2%)–helium mix is introduced, and the oven temperature is again raised. As oxygen enters the oven, pyrolytically generated EC is oxidized and a concurrent increase in filter transmittance occurs. Correction for the char contribution to EC is accomplished by identifying the time at which the filter transmittance reaches its initial value. This point is defined as the 'split' between organic and elemental carbon. Carbon evolved prior to the split is considered 'organic' (including carbonate), and carbon volatilized after the split and prior to the peak used for instrument calibration (final peak) is considered 'elemental'. If desired, the presence of carbonate can be verified through analysis of a second portion (punch) of the filter after its exposure to HCl vapor. In the second analysis, the absence or diminished size of the suspect peak (typically the fourth 'OC' peak) is indicative of carbonate in the original sample. Normally, a 1.5 cm^2 rectangular portion (taken with a punch) of the filter deposit is analyzed. Organic and elemental carbon are reported as μg C per cm^2 of deposit area. The total EC and OC on the filter are calculated by multiplying reported values by the sample deposit

Table 1 Thermal Protocols

Laboratory	OC determination	EC determination
TOM1–TOM6*	In helium: 250 °C, 1 min; 500 °C, 1 min; 650 °C, 1 min, 850 °C, 1.5 min; reduce to 650 °C, switch to oxygen mode	In 2% oxygen in helium: 650 °C, 30 s; 750 °C, 30 s; 850 °C, 1 min; 940 °C 2 min (or more)
TOR†	In helium: 120 °C, 4.5 min; 250 °C, 3.5 min; 450 °C, 4–5 min; 550 °C, 8–10 min, switch to oxygen mode	In 2% oxygen in helium: 550 °C, 6–7 min; 720 °C, 2.5 min; 820 °C, 2.5 min
C1	In nitrogen: 800 °C, 10 min	In oxygen: 800 °C, 7 min
C2A‡	In nitrogen: 200 °C, 2 min; 400 °C, 4 min; 560 °C, 6 min (or until stable)	In oxygen: 800 °C, 4.5 min; 1000 °C, 2 min (or until stable)
C2B‡, C3, C4 (Method No. 44)	In nitrogen: 200 °C, 2 min; 400 °C, 2 min; 550 °C, 4 min	In oxygen: 800 °C, 4.5 min

* TOM is thermal-optical method detailed in NMAM 5040.[8] † Times specified for thermal–optical reflectance (TOR) method are estimates based on scale shown in thermograms; different parameter files were used for analysis. ‡ Laboratory reported two sets of data. One set obtained with specified protocol (C2A) and second set (C2B) obtained with Method No. 44[13] protocol.

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High-Precision Conductometric Detector for the Measurement of Atmospheric Carbon Dioxide

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A new, lightweight instrument has been developed for measuring atmospheric mixing ratios of the greenhouse gas carbon dioxide with high precision and accuracy. This modified total organic carbon analyzer uses a bundle of semipermeable hollow fiber membranes to continuously equilibrate CO_2 in air with a recirculated stream of deionized water. Aqueous carbon dioxide hydrolyzes and dissociates to form the ions H_3O^+ and HCO_3^-, thereby increasing the conductivity. The bipolar pulsed method is used to measure the conductivity of the water before and after contact with air. Potential interferences that may also increase the conductivity, such as acids and bases, are removed at the inlet by bicarbonate and bisulfate scrubbers. The detector has been field tested and exhibits a $1/e$ response time of ~30 s and precision (RSD) of 0.1%. Field results for measurements at altitudes in the range of 1700–3600 m MSL exhibited an average deviation of 0.16% from values obtained by flask sampling and laboratory measurement using the well-established method of nondispersive infrared absorption. High accuracy and precision combined with the ability to obtain real-time, continuous data make this detector well suited for evaluating landscape-scale carbon dioxide fluxes from vertical profiles through the convective boundary layer.

The recent increase in atmospheric CO_2 mixing ratio is one of the most significant changes in the trace gas composition of the atmosphere.[1] The observed 30% rise, from 280 to 360 ppmv since the beginning of the industrial revolution, accounts for only ~50% of the CO_2 released into the atmosphere from anthropogenic sources.[2] The remainder of the CO_2 released from fossil fuel burning and deforestation is assumed to have been absorbed by the oceans and terrestrial biosphere. Direct measurements of CO_2 fluxes are needed in order to determine the strengths of these sinks and to close regional and global carbon budgets. In addition, flux measurements are necessary to improve the global circulation models that predict future CO_2 concentrations and climate change.

Currently, CO_2 concentrations are determined either by collecting air in flasks for analysis offsite or by continuous monitoring in the field. Offsite analysis is usually performed by GC/TCD, GC/FID with a methanizer, or nondispersive infrared absorption (NDIR). The disadvantages of batch analysis include sample storage and transport problems, limitation of the number of measurements by the number of available flasks, and a significant time lag between flask sample collection and analysis. For example, in a recent field campaign aimed at measuring the fluxes of greenhouse gases in the Amazon rain forest of Peru, we were limited to six flask samples to characterize each vertical profile through the convective boundary layer.[3] Continuous monitoring is almost exclusively performed by NDIR. The limitations and errors associated with open- and closed-path NDIR analyzers have been extensively discussed by Leuning and Judd.[4] Disadvantages of in situ analysis by NDIR include instrument expense (and therefore limited sampling sites) and the inability to use NDIR from kite or small balloon platforms because of excessive weight and power requirements.

The new technique described here for measurement of CO_2 mixing ratios is based on the increase in conductivity that occurs when deionized water makes contact with air by use of microporous hollow fiber membranes. The detector is sufficiently small and lightweight to be operated from kite and balloon platforms for continuous vertical profiling of the atmosphere and has adequate precision and accuracy to determine landscape-scale fluxes of CO_2 from vertical profile measurements.

There are previous reports of conductometric techniques for measuring gas-phase CO_2.[5–14] Initial designs were cumbersome and slow because they incorporated large amounts of air and water and required time for degassing.[5–10] Van Kempen and Kreuzer[11] and Himpler et al.[12] used microsensors and semipermeable membranes but did not study atmospheric levels of CO_2. Symanski et al.[13,14] designed microsensors for atmospheric CO_2 and were successful at measuring concentrations that would be found in highly polluted air. The instruments measured CO_2 mixing ratios

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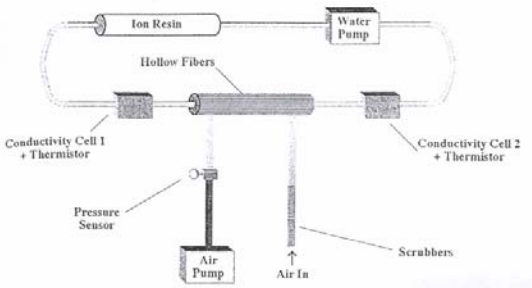

Figure 1. Schematic diagram of the conductometric detector.

in the range 0−3% and were not tested extensively at concentrations characteristic of "clean" air (~350−370 ppmv). Furthermore, the continuous microsensor developed by Symanski et al. exhibited a RSD of ~2%. This precision is adequate for polluted air measurements but does not meet the precision required (~0.1%) for monitoring the small concentration variations that are found in relatively unpolluted air, e.g., in the atmosphere above a forest canopy.

THEORY

Gas-phase CO_2 is transported across a porous membrane and equilibrates with deionized water according to the following reactions:

$$CO_2(g) = CO_2(aq) \qquad K_h$$

$$CO_2(aq) + 2H_2O = HCO_3^-(aq) + H_3O^+(aq) \qquad K_a$$

where K_h is the Henry's law constant and K_a is the apparent dissociation constant. The residence time required for equilibration limits the response time of the detector and was determined experimentally to be ~14 s (see below). Once equilibrium is achieved, the concentration of dissolved CO_2, which by Henry's law is proportional to the gaseous CO_2 partial pressure, is determined from the equation

$$[CO_2(aq)] = \frac{[H^+][HCO_3^-]}{K_a} = \frac{[H^+]^2 - K_w}{K_a}$$

in which K_w, the water dissociation constant, and K_a are corrected for temperature. The concentration of H^+ is determined from the conductivity difference using the equation

$$\Delta\lambda(\mu S/cm) = D\{\lambda_{H^+}[H^+] + \lambda_{OH^-}(K_w/[H^+]) + \lambda_{HCO_3^-}([H^+] - K_w/[H^+])\}_{cell\ 2} - D\{\lambda_{H^+}[H^+] + \lambda_{OH^-}(K_w/[H^+]) + \lambda_{HCO_3^-}([H^+] - K_w/[H^+])\}_{cell\ 1}$$

where D is the temperature-corrected water density and λ_x is the temperature-corrected ionic conductance of species x.

EXPERIMENTAL SECTION

Apparatus. A schematic diagram of the carbon dioxide conductometric detector is shown in Figure 1. This detector is a modification of the Sievers model 800 portable total organic carbon analyzer. A magnetic water pump (Iwaki, model MD-6L) continuously recycles the water through a strong acid and strong base mixed-bed ion-exchange resin. The water then passes through a conductivity cell fitted with a thermistor. The water conductivity is measured by the bipolar pulsed conductivity method that allows for the measurement of current after the double layer has been discharged and when parallel and serial capacitances no longer influence the current.[15] The water flows through the interior of polypropylene microporous hollow fibers (i.d. 280 μm, o.d. 380 μm, pore size 0.2 μm, Akzo Oxyphan PP50/280) that allow for the transport of carbon dioxide from the surrounding airstream. The increased conductivity due to equilibration with CO_2 is measured using a second conductivity cell and thermistor before being recycled through the ion-exchange resin. The airstream is drawn through the system by an ECC Ozonesonde pump (EN-SCI, Inc.) at a flow rate of 70 cm^3/min. The air sample passes through the appropriate scrubbers and is then thermally equilibrated in a water bath before passing around the outside of the hollow fiber membranes. A single-port pressure sensor (Motorola MPX5100AP) is positioned in the air flow pathway between the exchange membranes and the air pump in order to measure the actual air pressure within the gas/liquid exchanger. Teflon or stainless steel tubing (grade 316L) is used for all instrument connections. Conductivity, pressure, and temperature data are acquired in a quickbasic program in which $[CO_2(aq)]/P$ is calculated based on the aforementioned equations. The prototype instrument fits into a case with dimensions 49 cm × 33 cm × 13 cm, and the overall power consumption is 48 W.

Optimization. Several water delivery systems were evaluated, including a gravity drip system, a pressurized bag, and a syringe pump. The effect of the water flow rate was studied using the syringe pump (Figure 2). The conductivity increase due to a CO_2 gas standard with a mixing ratio of 359.1 ppmv was measured while the water flow rate was varied from 0.3 to 13.3 mL/min. The conductivity was constant up to a flow rate of ~1.5 mL/min and fell sharply at higher flow rates. The volume of the hollow fiber bundle was measured gravimetrically and found to be 0.36 mL. Thus, a flow rate of 1.5 mL/min corresponds to a residence time of 14 s. The final design incorporated a low-noise recycling water pump with a fixed flow rate of 1.0 mL/min and a residence time within the hollow fiber exchanger of 22 s.

Two experiments were performed in an attempt to increase the conductivity signal and the rate of response. The transfer module was wrapped with heating tape and brought to 54 °C. The higher temperature increased the conductivity for the dissolved and dissociated CO_2, but this gain was almost entirely offset by a decrease in the solubility of CO_2. All subsequent measurements were made at room temperature since the higher temperatures showed very little net effect on the final conductivity measurements while increasing the noise by a factor of ~4. In another experiment, the enzyme carbonic anhydrase was added to the water supply in an attempt to increase the rate of hydrolysis of CO_2. This protein coated the electrodes and proved to be unusable in this system.

Interferences. Interferences from the increased conductivity due to gas-phase nitric acid and sulfuric acid and gases such as

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Refinement of the Borohydride Reduction Method for Trace Analysis of Dissolved and Particulate Dimethyl Sulfoxide in Marine Water Samples

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A recently reported borohydride reduction method for the trace determination of aqueous dimethyl sulfoxide (DMSO) was adapted for use with a different sample preparation and analytical system, and the adaptation and optimization steps that we followed gave us further insight into the method. Increasing the proportion of reducing agent was critical. A number of compounds with potential for analytical interference were tested, but all proved negative. Water blanks were problematic, with substantial DMSO contamination observed in all but very recently purified water. Preliminary comparison with the highly specific and precise enzyme-linked method gave very good agreement. When DMSO analysis was done sequentially after analysis of dimethyl sulfide and alkali hydrolysis for dimethylsulfoniopropionate, we found that the DMSO concentration was not affected by increasing the length of the hydrolysis step. This allows storage and/or transport of hydrolyzed samples in gastight containers. The adapted method was applied to the novel determination of DMSO on glass fiber filters, and this revealed a significant pool of particulate DMSO in marine particles.

Recent interest in dimethyl sulfoxide (DMSO) in the marine environment stems from its widespread occurrence in nature and its potential role in the biogeochemical cycle of dimethyl sulfide (DMS), a key species in the global sulfur cycle and the precursor of climatically active sulfur aerosols in the atmosphere.[1] However, relatively few measurements of DMSO levels in natural waters have been made to date,[2,3] essentially because of the scarcity of sufficiently sensitive and selective analytical procedures. During the past few years, five methods for trace analysis of aqueous DMSO have been reported. All involve gas chromatography, either via direct injection of the water aliquot[4,5] or via reduction and subsequent determination of the evolved DMS.[6−9] Simó et al.[9] developed a borohydride reduction method which is relatively simple and performs well at nanomolar concentration levels. When used as part of a sequential protocol, this technique allows analysis of a suite of methylated sulfur compounds, e.g., DMS, methanethiol, dimethylsulfonipropionate (DMSP), and DMSO, in the same water sample.[9] The method has been applied successfully in a number of field studies (refs 10 and 11 and Simó, unpublished work).

In this paper, we report on refinements to the borohydride reduction method for DMSO analysis which resulted from adapting the technique for a different sample preparation and GC analytical system to that described by Simó et al.[9] New insight into the method has been gained, including the need to adjust the proportion of reductant specificity, blank troubleshooting, sample storage, and the first-ever application of the method to analysis of particulate DMSO ($DMSO_p$). This information should be useful for those intending to analyze aqueous DMSO by reduction methods.

EXPERIMENTAL SECTION

Standards and Reagents. Standard solutions of DMSO were prepared by weighing DMSO (99.5%, Sigma, UK) into a volumetric flask and making successive dilutions in Milli-Q water. The stock and the working standards were stored at 4 °C after slight acidification with HCl. DMSP-HCl was obtained from Research Plus (Bayonne, NJ), and standard solutions were prepared as for DMSO. Sodium borohydride (98%, Aldrich Chemical Co., Milwaukee, WI) was used in the form of 0.045- and 0.1-g pellets. Hydrochloric acid (7% w/w) and a 10 M solution of sodium hydroxide in Milli-Q water were used for acidification and alkali hydrolysis.

Analytical Setting. The system for purging and cryotrapping DMS was identical to that described by Turner et al.[12,13] and used by Hatton et al.[8] The analytical system consisted of a Varian 3300 gas chromatograph fitted with a dual-flame photometric detector

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Determination of Cyanide in Whole Blood by Capillary Gas Chromatography with Cryogenic Oven Trapping

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Cyanide, one of the most important toxic substances, has been found measurable with high sensitivity by capillary gas chromatography (GC) with cryogenic oven trapping upon injection of headspace (HS) vapor samples. The entire amount of cyanide in the HS sample could be cryogenically trapped prior to on-line GC analysis. A 0.5-mL volume of blood in the presence or absence of cyanide and propionitrile (internal standard, IS) was added to a vial containing 0.25 mL of distilled water, 0.3 g of Na_2SO_4, 0.2 mL of 50% H_3PO_4, and 0.1 g of ascorbic acid (when needed), and the mixture was heated at 70 °C for 15 min. A 5-mL volume of the HS vapor was introduced into a GC capillary column in the splitless mode at −30 °C oven temperature that was programmed up to 160 °C for GC analysis with nitrogen−phosphorus detection. A sharp peak was obtained for cyanide under the present conditions, and backgrounds were very clean. The extraction efficiencies of cyanide and IS were 2.89−3.22 (100 or 500 ng/mL) and 2.42%, respectively. The calibration curve showed good linearity in the range of 25−1000 ng/mL and the detection limit was ~2 ng/mL. The coefficients of intraday and interday variations were 2.9 and 11.8%, respectively. The mean blood cyanide level measured for actual fire victims was 687 ± 597 ng/mL (mean ± SD, n = 9). Endogenous blood cyanide concentration for healthy subjects was 8.41 ± 3.09 ng/mL (mean ± SD, n = 6).

Cyanide is known as one of the most rapidly acting and powerful poisons; it inhibits cytochrome oxidase of the mitochondrial respiratory chain.[1] Suicidal, accidental, or homicidal death by cyanide salts is frequently experienced in forensic toxicological practice. Several researchers reported that cyanide occasionally played a significant role in the cause of death of fire cases.[2−7]

For analysis of cyanide, the most classical is a colorimetric method with microdiffusion;[8,9] fluorometric methods were also reported.[10−12] Methods using gas chromatography (GC) with electron capture detection (ECD)[13−15] and with nitrogen−phosphorus detection (NPD)[16] and mass spectrometry (MS),[17] after suitable derivatizations, were reported. GC measurements of cyanide with NPD without derivatization were usually made using the headspace (HS) method.[4,18−20] In most of these reports, conventional packed columns, which give relatively low sensitivity and poor separation, were used.[4,13,14,16−19] With wide-bore capillary columns, only a 0.5-mL volume of the HS vapor can be injected;[20] with medium-bore capillary columns, split injection giving less than 5% of efficiency has to be used.[15] Solid-phase microextraction [21] has been applied to analysis of cyanide in human whole blood.[22]

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Recently, a microcomputer-controlled device for cooling oven temperatures below 0 °C has become available for new types of GC instruments. It was originally designed for rapid cooling of the oven to reduce time for analysis. This new device has been applied for determining chloroform and methylene chloride in blood.[23]

In this paper, we have established a new GC technique using the cryogenic oven for measuring cyanide in whole blood without any complicated pretreatment; as much as 5 mL of the HS vapor for cyanide can be introduced without any loss into a medium-bore capillary column by use of a low oven temperature. This means that 10−100 times higher sensitivity can be obtained by this method as compared with that of the previous methods.[4,8−20,22]

EXPERIMENTAL SECTION

Materials. Potassium cyanide and methyl acetate were obtained from Wako Pure Chemical Industries (Osaka, Japan), and propionitrile was from Aldrich (Milwaukee, WI). Other chemicals were of analytical grade. Human whole blood samples were obtained from healthy volunteers or cadavers that were dissected or examined in our department.

Procedure. A stock solution of a mixture of cyanide (5 μg/mL) and propionitrile (internal standard, IS, 8 μg/mL) was prepared in 0.1 N NaOH solution. To a 7.0-mL screw-cap vial containing 0.5 mL of whole blood were added 0.25 mL of distilled water, 0.05 mL of a stock solution of cyanide and propionitrile (250 and 400 ng/vial, respectively), 0.3 g of Na_2SO_4, and 0.2 mL of 50% H_3PO_4 solution. For measurements of very low endogenous cyanide in whole blood, 0.1 g of ascorbic acid should be added to the vial in order to inhibit conversion of a minor amount of thiocyanate into cyanide.[24−26] The vial was rapidly sealed with a Teflon-lined silicone septum cap and then put on an aluminum block heater (Reacti-Therm Heating/Stirring model, Pierce, Rockford, IL) after vortexing for 30 s. After heating at 70 °C for 15 min, a 24-gauge needle of a plastic syringe (10-mL volume) was inserted into the vial; a 5-mL volume of the HS vapor was drawn into the syringe and injected into the GC port in the splitless mode at −30 °C of the oven temperature.

GC Conditions. GC analyses were carried out on an HP 6980 series gas chromatograph equipped with an NPD and with a cryogenic oven temperature device (Hewlett-Packard Co., Wilmington, DE). An electrically operated solenoid valve introduced liquid carbon dioxide at a rate appropriate to cool the oven to desired temperatures. The GC conditions were as follows: column used a Supel-Q PLOT fused-silica capillary (30m × 0.32 mm i.d., Supelco Inc., Bellefonte, CA); column temperature −30 to 160 °C (1-min hold at −30 °C, 10 °C/min from −30 to 160 °C); injection temperature 150 °C; detection temperature 260 °C; and helium flow rate 3 mL/min. The vapor samples were injected in the splitless mode, and the splitter was opened 1 min after completion of the injection.

MS Conditions. To identify endogenous cyanide in human whole blood, we used a quadrupole GC/MS system QP 5050A connected to a GC-17A gas chromatograph (Shimadzu Corp., Kyoto, Japan). MS conditions were as follows: ionization current 60 μA; ion source temperature 200 °C; electron energy 70 eV; and accelerating voltage 1.5 kV. GC conditions were the same as described above. To avoid the interference by nitrogen (m/z 28) coexisting in the HS vapor, the measurements of m/z 27 ion were initiated 5 min after the injection of the HS vapor.

Comparison with the Previous HS-GC Method. To reproduce the previous HS-GC method, we followed the conditions reported by Seto et al.[20] using a GS-Q wide-bore capillary column (30 m × 0.53 mm i.d., J & W Scientific, Folsom, CA); we performed the analyses of cyanide in both split (1:3.7) and splitless modes. To a 0.5-mL volume of whole blood, 100 ng of cyanide was added and analyzed by both our method and the previous method for comparison. A 0.5-mL volume of HS was introduced into GC for the previous method.

Cyanide in Whole Blood of Actual Fire Victims and of Healthy Subjects. Whole blood or cerebrospinal fluid (CSF) samples were obtained from victims of actual fires at forensic autopsies or medical examinations. They were analyzed less than 1 h after the samplings. When immediate analyses were impossible, the samples were stored at −80 °C until analysis. Carboxyhemoglobin (COHb) was measured by the spectrophotometric method of Katsumata et al.[27] For measuring samples at high concentrations of cyanide, 0.5 mL of whole blood diluted 10-fold was used.

For measurements of endogenous cyanide in blood of healthy subjects, six healthy volunteers refrained from smoking for 24 h before samplings. Whole blood (~6 mL) was drawn and subjected to cyanide measurements immediately. In this kind of experiment, the amount of IS was 40 ng/vial; 0.1 g of ascorbic acid was added to the vial.

RESULTS AND DISCUSSION

Analytical Conditions. Various conditions for the HS extraction of cyanide and propionitrile from whole blood were tested. Heating at 70 °C gave more vapor pressure when compared to heating at 50 °C; this was advantageous to draw as much as 5 mL of HS vapor. We heated the vials at 70 °C for 5, 10, 15, and 30 min; peak areas of cyanide and propionitrile (IS) were almost constant after 15 min. Thus, we heated the vials at 70 °C for 15 min.

We have tested various initial oven temperatures for trapping cyanide and propionitrile vapor (Figure 1). At 20 °C, the peak of cyanide (filled arrows) was quite broad and became sharper upon lowering the oven temperature to −30 °C, while the lowering of initial oven temperature did not affect the peaks of IS (open arrows). At −40 °C, the peak area of cyanide was almost the same as those at −30 °C (data not shown). Thus, −30 °C was selected for trapping both compounds.

Identification of Endogenous Cyanide. Figure 2 shows GC profiles for nonextracted authentic cyanide and IS (12.5 and 10 ng on-column, respectively) dissolved in methyl acetate and for HS extracts from 0.5 mL of whole blood in the presence (250 ng

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RP-HPLC Binding Domains of Proteins

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Procedures have been developed to identify the chromatographic binding domains of horse heart cytochrome *c* (Cyt c) and bovine growth hormone (bGH) during their interaction with reversed-phase sorbent materials. The procedure involves adsorption of the protein solute to the chromatographic sorbent, followed by proteolytic cleavage. Comparison of the proteolytic map obtained for Cyt c and bGH in free solution with the corresponding map obtained when these proteins are adsorbed to the chromatographic sorbent revealed significant differences in the digestion pattern. Following characterization of the peptides generated in both maps, the results indicated that specific regions on the surface of both Cyt c and bGH are inaccessible to tryptic cleavage when adsorbed to the hydrophobic surface of both a C-4 and a C-18 sorbent. Based on the assumption that the region of the protein surface that is in contact with the sorbent remains intact and bound to the sorbent during the digestion step, while the protein surface that is exposed to the solvent is accessible to proteolysis, the regions that were inaccessible to tryptic digestion were found to correspond to hydrophobic domains on the protein surface. These results also suggest that the three-dimensional structures of these proteins remain largely intact upon adsorption to the hydrophobic surface.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is now a central technique for the analysis and purification of biological molecules as a result of the high level of reproducibility, selectivity, and sensitivity that can be achieved.[1,2] Due to its ability to monitor subtle changes in molecular conformation, RP-HPLC is also now emerging as a powerful technique for studying the role of lipid-like surfaces in several biorecognition phenomena, such as the action of antimicrobial peptides[3] and the role of hydrophobicity in protein folding.[4] However, further significant progress in the development of RP-HPLC is impeded by the lack of theoretical models which accurately describe the molecular details of peptide and protein interactions in RP-HPLC. The slow development of detailed physicochemical models is largely due to the complex structural equilibria that peptides, and particularly proteins, can undergo in RP-HPLC systems.[3−15]

A full understanding of the chromatographic process requires detailed knowledge of the chemical and physical nature of both the mobile phase and the stationary phase and also information on the types of interactions which occur between the solute and the ligand or the solvent. While little is known about the detailed molecular structure of proteins at the chromatographic surface, experimental data with species variants of proteins, as well as recombinant mutants, indicate that proteins interact with the chromatographic surface in an orientation-specific manner.[16−18] The retention behavior of proteins, which can be described in terms of the affinity and kinetics of the interaction, is therefore determined by the molecular composition of a specific contact region. Although the contact region for small peptides may involve contributions from the total or a large proportion of the molecular surface of the solute,[19,20] for larger polypeptides or proteins, retention data suggest that the contact region represents a relatively small portion of the total solute surface.[16−18] The retention properties of larger polypeptides and proteins are

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therefore determined by the specific contact amino acid residues rather than by the entire amino acid sequence. However, the location and identity of these chromatographic contact regions of proteins cannot be readily established. Without this information, it is not possible to predict the molecular basis of the retention behavior of a protein, and this limitation constrains the further development of RP-HPLC as a technique to study protein–surface interactions.

To address this problem, procedures have been developed in this study to identify the chromatographic contact regions of proteins when adsorbed to reversed-phase sorbents. In particular, proteolytic techniques have been used to probe the surface region of horse heart cytochrome *c* (Cyt c) and bovine growth hormone (bGH) while adsorbed to an *n*-butyl (C-4) and *n*-octadecylsilica (C-18) reversed-phase sorbent. Following proteolytic digestion and characterization of the derived fragments, the results were correlated with the known three-dimensional structure of these two proteins and provide insight into the location of the possible contact regions as well as the orientation of these two proteins at the surface of reversed-phase sorbents.

MATERIALS AND METHODS

Chemicals and Reagents. Water was quartz-distilled and deionized in a Milli-Q system (Millipore, Bedford, MA). Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Paris, KY) and from EM Industries Inc. (Darmstadt, Germany). Trifluoroacetic acid (TFA) was obtained from Auspep (Parkville, Australia). Recombinant bovine growth hormone (bGH) was generously provided by The Upjohn Co. (Kalamazoo, MI), and horse heart cytochrome *c* (Cyt c) and pepsin were obtained from Sigma (St. Louis, MO). Trypsin and chymotrypsin were purchased from Worthington Inc. (Freehold, NY). Ammonium bicarbonate was obtained from Mallinckrodt, and acetone and calcium chloride were from BDH Chemicals (Kilsyth, Australia).

Apparatus. Chromatographic analyses of protein digestions were carried out with a Beckman System Gold chromatographic system (Beckman Instruments, Inc., Fullerton, CA), consisting of a dual-pump programmable solvent module 126 and a variable UV detector module 166 and controlled using System Gold software (version 5.0). All chromatographic profiles were monitored at 214 nm. Chromatographic analyses of growth hormone digestions were performed with a Waters Associates (Milford, MA) liquid chromatograph 484 system, consisting of two model 6000A solvent delivery pumps, a U6K universal injector, a WISP model 712B sample processor, and an M660 gradient programmer. The detector used was a Lambda-Max model 484 LC spectrophotometer operating at 215 nm, and the total system was controlled by Maxima 820 operating software.

Chromatographic Procedures. Analyses of protein digests were performed on a Bakerbond Widepore *n*-butylsilica column (J.T. Baker Chemicals, Phillipsburg, NJ) with dimensions of 250 × 4.6 mm i.d., containing sorbents of 5 μm nominal particle size and 30 nm average pore size or a Zorbax 300 SB-C8 reversed-phase column with dimensions of 4.6 mm × 15 cm, with particles 5 μm in diameter and 300 Å average pore size (Rockland Technologies, Newport, DE). Bulk solvents were degassed by sparging with helium. Linear gradient elution was performed from 0.1% (v/v) TFA in deionized water (buffer A) to 0.09% (v/v) TFA with 50% (v/v) aqueous acetonitrile (buffer B) over gradient times of 50 and 150 min for Cyt c and from 0.1% (v/v) TFA in deionized water (buffer A) to 0.09% (v/v) TFA with 80% (v/v) aqueous acetonitrile (buffer B) over gradient times of 30, 60, and 120 min for bGH, both at a flow rate of 1 mL/min. Injection size of peptide solutions varied between 30 and 100 μL in analytical separations and between 0.5 and 1.5 mL in preparative separations.

In Situ Tryptic Cleavage of Proteins. *Binding of Cyt c to RP-HPLC Sorbents in a Batch Procedure.* The sorbents used for all in situ digestion experiments were Vydac C-4 or C-18 silica (The Separations Group, Hesperia, CA). Fifteen milligrams of Vydac C-18 silica (5 μm particle size and 30 nm pore diameter) was washed and vortexed with 1 mL of methanol and then three times with 1 mL of 35% acetonitrile to suspend the sorbent material. After each washing step and centrifugation, the solvent was discarded. The sorbent then was resuspended for 2 h in 1 mL of 0.1% TFA containing 7.5 mg of Cyt c under continuous agitation to allow maximal binding. After centrifugation, the pellet was washed four times with 1 mL of cleavage buffer (50 mM ammonium bicarbonate, 2 mM calcium chloride) to remove all nonbound Cyt c. After each centrifugation, the supernatant was analyzed by RP-HPLC as described above to show the degree of Cyt c removal.

Calculation of Binding Efficiency. To calculate the amount of bound Cyt c, the adsorbed protein was eluted from the C-18 sorbent with 75% acetonitrile/0.1% TFA (2 × 0.25 mL). An aliquot of the eluate was then analyzed by RP-HPLC and the amount of bound Cyt c measured by comparison with a Cyt c standard curve.

Cleavage of Accessible Sites of Cyt c following Binding. The sorbent with bound Cyt c was incubated with TPCK-treated trypsin in cleavage buffer under continuous agitation to cleave accessible cleavage sites. Based on the calculation of the binding efficiency, a protein:trypsin ratio of 10:1 was chosen. The cleavage process was stopped after 18 h with the addition of two drops of 2 M HCl.

Preparation of Cyt c Peptides. After centrifugation of the sorbent, an aliquot of the collected supernatant containing the tryptic peptides which did not bind to the C-4 or the C-18 sorbent (the nonbound fragments) in the presence of the cleavage buffer was analyzed by RP-HPLC under the same chromatographic conditions as a tryptic digest of Cyt c generated in the absence of sorbent material, with the same protein:protease ratio and the same cleavage conditions (the free solution digest). The sorbent was washed four times with 1 mL of cleavage buffer to remove peptides which had not bound to the sorbent. An aliquot of the last supernatant was analyzed by RP-HPLC to confirm the absence of residual amounts of free peptides.

The remaining tryptic peptides which were bound to the sorbent (the bound fragments) were eluted with 75% acetonitrile/0.1% TFA (2 × 0.25 mL). After centrifugation, an aliquot of the combined supernatants was analyzed by RP-HPLC. An in situ experiment was also performed using cleavage buffer without trypsin. In addition, trypsin was incubated in cleavage buffer in the absence of both Cyt c and sorbent, under the same conditions as for the analysis of the free solution digest of Cyt c to distinguish Cyt c fragments from autocatalytic trypsin fragments.

Identical procedures were used for the in situ tryptic digestion of bGH.

Cleavage of Proteins with Chymotrypsin and Pepsin. Proteolytic in situ and solution cleavages of Cyt c and bGH with

Anexo

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Nanoliter Chemistry Combined with Mass Spectrometry for Peptide Mapping of Proteins from Single Mammalian Cell Lysates

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A nanoliter-chemistry station combined with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was developed to characterize proteins at the attomole level. Chemical reactions including protein digestion were carried out in nanoliter or subnanoliter volumes, followed by microspot sample deposition of the digest to a MALDI-TOF mass spectrometer. Accurate mass determination of the peptides from the enzyme digest, in conjunction with protein database searching, allowed the identification of the proteins in the protein database. This method is particularly useful for handling small-volume samples such as in single-cell analysis. The high sensitivity and specificity of this method were demonstrated by peptide mapping and identifying hemoglobin variants of sickle cell disease from a single red blood cell. The approach of combining nanoliter chemistry with highly sensitive mass spectrometric analysis should find general use in characterizing proteins from biological systems where only a limited amount of material is available for interrogation.

At the early development stage of a disease such as cancer, only a small population of normal cells undergoes transformation and a change of the proteome is expected to occur in these tumor cells.[1,2] In cell research, a number of cell lines derived from tumors in in vitro cell culture systems have been used as sources of large numbers of cells of a uniform type and they play an essential role in the process of investigating cell functions. However, because of the difference in the environment of cell growth in the intact organism and the culture, great care must be taken in extrapolating the results of in vitro experiments to the reality in vivo.[1,2] This is particularly true for proteins, whose identity and abundance can vary greatly at different stages of cell development or expressing conditions. Thus, analyzing the primary cells isolated from a tissue, instead of a cultured cell line, is the only way to provide a direct correlation of the change in protein contents and identities with a biological event, such as the progression of a disease, without running into a risk of potential artifacts of cell culture. This requires very sensitive analytical methods, because the number of tumor or other disease cells available for investigation from a tissue is often limited.

At present, several tracer techniques involving radiolabeling, immunoassay, and fluorescence tagging have been used to provide information on the distribution of usually known proteins in a small number of cells or a single cell.[1,2] Miniaturized detection schemes based on electrochemical, laser-induced fluorescence detection and, more recently, mass spectrometry have shown great promise in analyzing cellular components including peptides and proteins in single cells.[3-12] However, unequivocal identification and characterization of trace amounts of unknown or modified proteins in very small volumes associated with tissues, single cells, subcellular compartments, and exocytosis still remain a formidable task. In this report, we describe an analytical approach that combines three rapidly developing techniques, namely, nanoliter or subnanoliter chemistry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and protein database searching, to characterize attomole quantities of proteins from small-volume samples including single cells.

EXPERIMENTAL CONSIDERATION

Rapid advances in MS over the past decade or so have made it a very sensitive tool for the detection of peptides and proteins. High sensitivity in MS is generally attained by reducing the sample presentation volume to improve the sampling efficiency. Low-volume electrospray ionization (ESI)[13,14] and microspot MALDI[9,15-17] have been shown to provide attomole detection of peptides and proteins. As demonstrated in proteome analysis by MS,[18] protein

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Analytical Methods Section

The determination of food colours by HPLC with on-line dialysis for sample preparation

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A simple method is reported for the determination of synthetic dyes in sugar rich foods. Extraction of the dyes is achieved with water at room temperature. The extract is then centrifuged or filtered prior to dialysis using an ASTED system. A portion of the dialysate is transferred directly to the HPLC column without further concentration, where adequate sensitivity is achieved for the determination of dyes in a range of foods. Samples studied include boiled sweets, fruit gums, lemon curd, jelly, blancmange and soft drinks.

INTRODUCTION

Synthetic colours, mainly azo dyes, have been used in a wide range of food products for many years. The sensory perception of colour is an important quality attribute and many processed products have been coloured either to replace natural colours destroyed during processing or to provide colour in products which would otherwise be colourless, as, for example, soft drinks. The current trend is, however, away from the use of such synthetic dyes despite the extensive toxicological screening which they have undergone. The lists of permitted synthetic dyes are progressively being reduced and a number of food processors are relying on the use of natural colours to impart the desired colour to their products. Unfortunately, many of the natural colours (e.g. anthocyanins, carotenoids and betalaines) do not have the same stability under processing conditions as their synthetic counterparts. There will always, therefore, be a tendency (or at least a temptation) for some food processors to include synthetic dyes in their products without the correct label designation.

There is, therefore, a well-defined need for precise and accurate methods for the determination of synthetic dyes in foods, particularly for the following reasons:

(i) to determine whether there are synthetic dyes present in foods and if so, whether they are correctly permitted;
(ii) to determine the levels of such dyes;
(iii) to confirm the absence of added dyes in foods where they are not declared;
(iv) to check on the stability of dyes during processing and storage (Damant *et al.*, 1989).

There are many well-documented methods for the chromatographic separation of synthetic dyes (Saag, 1988). These are either based on ion-exchange methods or now more commonly on ion-pair chromatography under reversed phase conditions. A detailed study of the factors affecting retention under these conditions has recently been published (Damant, 1990). The simplest mobile phase conditions are those based on ammonium acetate buffers. The problem in methods for the quantitative determination of synthetic dyes in foods does not, therefore, lie in their separation, but rather in the means for their quantitative isolation from the food matrix. Traditional methods, such as adsorption on to wool or polyamide powder (Lehmann, 1970) tend not to be quantitative and can lead to dye degradation. A milder means of extraction, either from the food itself (e.g. soft drinks) or from an aqueous extract of the food, would offer considerable advantages and this is the situation encountered with dialysis. This technique has been used as a means of sample preparation for vitamin analysis by HPLC (Nicholson *et al.*, 1984). However, only recently has a fully automated system been made commercially available, which allows considerable flexibility in terms of dialysis conditions, coupled with automated injection of the sample into the HPLC column (Green *et al.*, 1989). The power of the technique is further extended by allowing enrichment of the determinand in the dialysate on a small trace enrich-

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ment cartridge prior to elution to the analytical HPLC column. The combination of dialysis and trace enrichment then leads to a complete sample preparation systems for microconstituents of foods, which is marketed under the acronym ASTED (automated sample treatment through enrichment of dialysates).

MATERIALS AND METHODS

Reagents and standards

All reagents were of analytical grade. Solvents for chromatography were of HPLC grade.

Samples of synthetic dyes (Amaranth, Brown FK, Ponceau 4R and Sunset Yellow FCF) were kindly donated by Mr Stewart Reynolds (MAFF Laboratory, Norwich, UK). Stock solutions of the dyes, either singly or as a mixed standard (500 μg ml^{-1}) were prepared by dissolving the dye (50 mg) in 0·04 M ammonium acetate buffer (pH 7, 100 ml). These were diluted as necessary with the same acetate buffer to form working standards (0–30 μg ml^{-1}).

HPLC method

A Gilson pump (model 303) was used at a flow rate of 1·0 ml min^{-1}. The mobile phase consisted of 0·04 M ammonium acetate buffer (pH 7, 400 ml) and methanol (200 ml) made up to 1 litre with water. The column (150 × 4·6 mm i.d.) was packed with Spherisorb S5 ODS 2 (5 μm particle size) and was used at ambient temperature. Detection was achieved with an Applied Biosystems 757 absorbance detector set at 475 nm (0·005 AUFS).

ASTED conditions

The standard ASTED system (Gilson Medical Electronics, Villiers-le Bel, France) was used without modification. This consisted of a model 231 automatic sampling injector and two dilutors (Model 401). The dialyser (100 μl) was constructed of poly(methyl methacrylate) and fitted with a 15 kD molecular weight cut-off membrane. The dialyser recipient solvent was 0·04 M ammonium acetate (pH 7) and water was used as the priming solvent for dilutor 0. File 181 was configured as shown in Table 1. The method File 151 was prepared as shown in Table 2.

Table 1. File 181 configuration

Prompt	Value	Program variable
Rack code	50	AA0
Process number	2	AA1
Donor volume	100	AA2
Recipient volume	175	AA3
Concurrent	0	AA4

Table 2. File 151 format

Program	Value	Function variable
A0	130	Sample volume (μl)
C2	0	Sample height (mm)
A1	0	Reagent number
A17	5	Air gap volume (μl)
B0	0	Pulse mode
A13	200	Load volume (μl)
A15	3	Dispense speed 0
B1	500	Dialyser volume (μl)
B2	0	Aspirate speed 1
B3	5	Dispense speed 1
C11	200	Wait after injection (s)
A16	2000	Donor purge volume (μl)
B4	2000	Recipient purge volume (μl)
B5	500	Regeneration volume (μl)

Sample preparation

Soft drinks

Samples were degassed by purging with helium for 5 min and then used directly for analysis.

Boiled sweets and fruit gums

A number of sweets (usually a maximum of four) were weighed into a small beaker and dissolved in 0·04 M ammonium acetate buffer (15 ml) with the aid of an ultrasound bath. The solution was allowed to stand for 15 min and the supernatant was decanted into a volumetric flask (20 ml). Any residue was washed with ammonium acetate buffer, and the washings were used to dilute the extract to volume.

Jelly and blancmange

A portion of the sample (*c.* 10 g) was weighed into a small beaker and thoroughly mixed with 0·04 M ammonium acetate (10 ml). This was dispersed with the aid of an ultrasound bath for 20 min. The sample was then filtered into a 15 ml volumetric flask and made up to volume with further washings.

Assay procedure

The system was set-up for Process 2, using the ASTED User's Guide (Gilson, 1988). The HPLC column was equilibrated with mobile phase (*c.* 30 ml). The integration file was set up, followed by selection and running of the Configure file 181 and the Reset file 150. The samples were added to the sample rack (code 50) as described in the Users' Guide. The method file 151 was then selected and run after responding to tube number and number of samples.

RESULTS AND DISCUSSION

The original intention in this study was to exploit the full capabilities of the ASTED system for the quanti-

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Analysis of Serotonin in Whole-Blood Samples — A Novel Fully Automated Method

The authors have developed an on-line, high-pressure, solid-phase extraction (SPE) method for high performance liquid chromatography (HPLC) analysis of serotonin in whole blood and plasma enriched with blood platelets. The article elucidates the advantages of on-line, high-pressure SPE for the analysis of these samples. Experimental results of the developed method and the current method, which is based on perchloric acid deproteinization and centrifugation, show good correspondence. The new method's reproducibility varies from 2 to 5%, depending on the kind of matrix, and the serotonin recovery is 100%. Compared with the current method, the new method is much faster and less tedious.

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For many years, serotonin (5-hydroxytryptamine) has been known as a pharmacological substance. As early as 1948, Rapport (1) described the structure of the compound. Today, serotonin is known generally as a neurotransmitter and neuroregulating compound (2). Serotonin participates in the regulation of important functions, including circadian rhythm (3), temperature regulation (4), aggression control, (5) and sexual function (6). Researchers have observed changes in serotonin metabolism accompanying psychiatric diseases, including forms of depression (7). In cases of migraine attacks, the concentration of serotonin in plasma with high platelet concentrations can increase as much as three times. Furthermore, a correlation exists between the severity of the attack and the serotonin level (8).

The analysis of serotonin in whole blood is interesting because the compound is deposited in thrombocytes, which resemble some nerve cells. Disturbances in the central nervous system, where serotonin acts, can in some cases be measured indirectly by monitoring the serotonin metabolism in blood. Using thrombocytes as a model system, we can examine the influence of psychotropic agents (9). The normal level of serotonin in blood varies from 70 to 160 ng/mL (10).

The current method for measuring serotonin in whole blood or in platelet-enriched plasma requires three steps: adding perchloric acid to the sample, centrifuging it, and injecting some of the supernatant into a high performance liquid chromatography (HPLC) system (11). If we were able to perform an equally reliable, but less tedious and time-consuming, solid-phase extraction (SPE) method, it would be a step forward. Common off-line SPE does not seem to be the proper choice for analysing serotonin in whole blood. In the past, analysts have reported that SPE cartridges become clogged with whole-blood samples (12), which caused disturbed flow patterns and provided irreproducible results.

On-line, high-pressure SPE is better suited to viscous and complex matrices such as whole blood. In this article, we will describe a method that uses on-line, high-pressure SPE for the automated analysis of serotonin in whole-blood samples.

EXPERIMENTAL

Current serotonin analysis method: The current method for measuring serotonin in whole blood or platelet-enriched plasma uses perchloric acid. To obtain a platelet-enriched plasma sample, whole blood is centrifuged for 20 min at 4 °C and ≤350*g*. Samples are deproteinized by mixing 400 µL of the platelet-enriched plasma with 100 µL of 1.5 M ascorbic acid and then adding 100 µL of 4 N perchloric acid. The mixture is centrifuged for 15 min at 4 °C and 7000*g*. (Serotonin solutions must be stored at 4 °C because degradation is quick at higher temperatures.) Next, 100 µL of the supernatant is injected directly into an HPLC system.

Automated on-line, high-pressure SPE: We used a Prospekt SPE system (Spark Holland, Emmen, The Netherlands) to perform automated on-line, high-pressure SPE. Figure 1 shows the switching configurations of the on-line SPE system. Our first step was activating a 10 mm × 2 mm C18 SPE cartridge (Prospekt, Spark Holland) with methanol, washing it with water, and conditioning it with a sodium chloride solution in preparation for the direct injection of a whole-blood sample. All cells were lysed by mixing one part of whole blood with three parts of 0.3% sodium dodecylsulfate in an isotonic sodium chloride solution. The process yielded a clear red solution that did not block the sieve or the packing material in the SPE cartridge. These blood samples were injected directly onto the SPE cartridge using a Marathon autosampler (Spark Holland), so serotonin or 5-hydroxyindole acetic acid could be analysed by HPLC without any problems.

We used 500 µL of isotonic sodium chloride solution and water to remove plasma proteins from the SPE cartridge after direct injection. We performed the separation and measurement of serotonin under isocratic HPLC conditions. After each analysis, the SPE system automatically replaced the used cartridge with a new one, avoiding carryover and the collection of irreversibly extracted components on the cartridge.

4. 2. Textos pertenecientes a los artículos ‘académicos informales’.

This program has been a good way to provide community service as well as good public relations for Calvin College. It has received attention from the local television and newspapers. At Calvin College, one of the expectations for faculty and staff is active participation in student recruitment and the establishment of good community relationships for the college. This program has been a low-maintenance and invigorating way to participate in these college efforts. The benefits to the directors of the program have been twofold. First, we have become more aware of what is happening in local elementary science education, and second, the enthusiasm and excitement of the elementary students and teachers have been refreshing and rejuvenating.

The program outlined here is not intended to replace hands-on or laboratory-based teaching. Rather, it supplements these teaching methods by promoting enthusiasm for the discipline and by providing an exciting introduction to the language of chemistry.

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Is It Real Gold?*

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On March 12, 1997, Ann Landers advised a writer to believe her boyfriend, who claimed the necklace he had given her for Christmas was "real gold", despite the fact that it kept turning her neck green. She went on to say that, "Some people have an element in their system that does this." What should a chemist make of this exchange?

First of all, what is "real" gold? To a chemist, "real" gold might imply "pure" gold. The gift necklace was surely not "pure" in a chemical sense, because 100%, or 24 carat gold (also spelled "karat", and always marked as "K") is too soft to be practical for use in jewelry. Jewelry is usually made of 18 or 14 carat gold, whose weight fraction of gold is 18/24 or 14/24, respectively (*1*). The "carat" system was invented by the British in about the year 1300 (*2*) to facilitate the use of gold in commerce. In the United States, the lowest allowed carat designation for gold is 10, but a $^1/_2$-carat error is allowed, so that "10K" can be marketed that is only 9.5K, or 39.6% by weight gold. In Britain, items that are only 9K can be sold, but there is no margin for error on the low side; France's lowest carat designation is 18K. The rest of the material in the alloy can be a variety of other metals; those most often used are copper, nickel, or silver. The composition of the alloy is not disclosed in the "carat" marking, and different alloying metals are used to make different colors. For example, notice the three colors of gold in the 19th-century English verge pocket watch illustrated in Figure 1.

*This paper is dedicated to the memory of Arthur A. Harris, watchmaker and jeweler, an honest man.

Figure 1. An early 19th-century English verge pocket watch, with a dial made with three different-colored gold alloys. The case is Hallmarked London, 1819. Photograph courtesy of *Pieces of Time*, 1-7 Davies Mews, London. (A color version of this figure appears on page 147.)

The metals used to make different colors are usually:

Yellow: Au, Cu, Ag, Zn
White: Au, Cu, Ni, Zn
Red: Au, Cu
Green: Au, Ag

The alloy called "green gold" (which is only slightly greenish) is rarely used, so the boyfriend of Ann Landers' correspondent was most likely claiming that the gift necklace was one of the recognized alloys whose minimum gold content has been designated in Britain by Hallmarks and there and elsewhere by the carat system.

The common phrase "acid test" comes from the practice of testing gold alloys with nitric acid. An alloy of less than about 9 or 10 carat is quickly turned green. Compositions up to 18 carat gold alloy can be tested with aqua regia (a mixture of nitric and hydrochloric acid, in roughly equal proportions); the small spot subjected to the acid will immediately become pale yellow (*3*), as the base metals that provide some of the color are dissolved. Instead of risking damage to the piece of jewelry, tests were often done using a "touchstone", a hard, black, slightly abrasive stone on which the object was rubbed fairly firmly, wiping a small amount of metal onto the stone surface. The tests were done on the stone, and the jewelry could easily be repolished to its original condition. It is interesting that so many of the words involved in this testing process have survived to the present time: "Hallmark", "acid test", and "touchstone".

A perceptive chemist will recognize that the carat marking specifies the minimum weight percentage of gold (only), but neither the identity nor the concentration of the other parts of the alloy. This means that an 18 carat gold item could have from zero to 25 weight percent copper, which corresponds to zero to 51 mole percent copper. Mixtures involving nickel and zinc result in about the same mole fraction of the base metals because of the similarity of their average atomic masses to that of copper.

The question of whether it is possible to oxidize a metal, and therefore to produce the possibility of a colored salt, is largely reflected in the standard potential. For the principal elements of the gold alloys, the pertinent numbers are:

$$Au^{3+} + 3e^- \rightarrow Au \qquad E^0 = 1.42\ V$$

$$Ag^+ + e^- \rightarrow Ag \qquad E^0 = 0.80\ V$$

$$Cu^{2+} + 2e^- \rightarrow Cu \qquad E^0 = 0.34\ V$$

$$Ni^{2+} + 2e^- \rightarrow Ni \qquad E^0 = -0.23\ V$$

$$Zn^{2+} + 2e^- \rightarrow Zn \qquad E^0 = -0.76\ V$$

These data suggest why gold is a "noble" metal: the potential required to oxidize it is near the maximum available in aqueous solutions. Consider, for example, combining the half-cells

$$Au(s) = Au^{3+} + 3e^- \qquad E^0 = -1.42\ V$$

or

$$Cu(s) = Cu^{2+} + 2e^- \qquad E^0 = -0.34\ V$$

with the half-cell for a good oxidizer, such as:

$$NO_3^- + 4H_3O^+ + 3\ e^- = NO + 6H_2O \qquad E^0 = 0.96\ V$$

It is obvious that nitric acid will not oxidize gold but will easily oxidize copper. However, the prediction of the conditions under which a metal might be oxidized depends upon more than just the potential for producing the "bare" (or hydrated) metal ion. One must also consider that the metal ion may be stabilized in solution by formation of a complex ion, which is the reason why both the nitric acid oxidant and the hydrochloric acid complexing agent are required when aqua regia (literally, royal water—a phrase coined by the alchemists to designate a solvent for "noble" metals) dissolves gold. When gold is dissolved in aqua regia, the reaction is:

$$Au(s) + 4Cl^- + NO_3^- + 4H_3O^+ = AuCl_4^- + NO(g) + 6H_2O$$

Since the potential for

$$AuCl_4^- + 3\ e^- \rightarrow Au + 4\ Cl^-$$

is 1.00 volt (*4*), the dissolution of gold in aqua regia becomes thermodynamically favorable. Oxidation by ordinary air (or air contaminated by sulfides) can tarnish silver, copper, and nickel, but pure gold is impervious to attack, even by concentrated nitric or hydrochloric acid acting independently. The chloride ion in a person's perspiration can facilitate the oxidation of the base metals in a gold jewelry alloy. But another factor impacting on whether these metals are leached out of necklaces, earrings, or dental work is the fact that mixtures of gold, silver, and copper with other metals are less reactive than one would predict if their alloys were ideal solutions. Greenwood and Earnshaw (*5*) say that these materials "can be thought of as nonstoichiometric intermetallic compounds of definite structural types...."

When people experience an allergic reaction to "real gold" jewelry, it is almost (*6*) always one of the base metals that is the culprit, and nickel is by far the most notorious in this respect. It seems that some people develop an amazingly acute sensitivity to this metal, and this most often occurs after ears are pierced and gold-plated earrings are inserted (*7*). Since the gold plating is usually quite thin and it is often applied on top of a layer of nickel plating, it is not too surprising that the wearer is often exposed to significant amounts of nickel as the gold wears, cracks, and is scratched. What is surprising is that the body "learns" to react to these ions only after it has been sensitized by previous exposure. The precise mechanism of this sensitization is not well understood.

Consider the original question, "was the necklace gold, or not". If it were "real" 14K or 18K gold, it is unlikely that a person who does not sweat aqua regia would develop a green neck. It is much more likely that the boyfriend had passed off a gold-plated necklace as more expensive jewelry. If some misrepresentation occurred in this case, Georgius Agricola reminds us that it was not the fault of the element: "if by means of gold and silver and gems men can overcome the chastity of women, corrupt the honour of many people, bribe the course of justice and commit innumerable wickednesses, it is not the metals which are to be blamed, but the evil passions of men which become inflamed and ignited" (*8*).

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Why Gold and Copper Are Colored but Silver Is Not

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It is well known that 80% of chemical elements are metals. When polished, all metals shine owing to reflection of photons by external valence electrons dynamically forming metallic bonds (*1*). White light reflects on most metals without color absorption or change to the naked eye; but copper and gold are yellow because they absorb "blue" and "red" photons by electron transitions between spectromeric configurations $ns^1(n-1)d^{10} \leftrightarrow ns^2(n-1)d^9$ of external sublevels (*2*).

The next question is why silver, with the same external electronic configuration as copper and gold (group 11, IB), is not yellow. The answer is simple, considering atomic radii, ionization potentials and nuclear charge:

	Cu	Ag	Au
Atomic radius/pm	117.3	133.9	133.6
1st ionization energy/eV	7.725	7.576	9.22
2nd ionization energy/eV	20.29	21.48	20.52
Nuclear charge	25	35	59

All values taken from ref *3*.

The atomic radius of silver is 16.6 pm larger than that of copper, allowing a bigger difference between sublevels s and d, which is sufficient to restrict the transition $s^1d^{10} \leftrightarrow s^2d^9$ to a lower probability. This is equally supported by the first ionization energy: since it is lower in silver, the fact that one external electron is ejected more easily than in copper atoms is justified.

With their higher nuclear charge (35 vs 25) silver atoms also have larger radii (Δ = 16.6 pm), and the distance between external sublevels—both spatial and energetic—is too large to freely allow s $\leftrightarrow$ d transitions. However, the distance is not large enough to prevent the transitions completely, and after several reflections on two parallel silver mirrors, white light becomes pale yellow (*4*).

Now we must face an unexpected problem: why is gold yellow? According to the same line of reasoning, gold would be colorless if it had bigger atoms. But gold atoms are *not* larger than silver; the radii of silver and gold are practically identical owing to lanthanide contraction (*3, 5*). Comparing ionization energies, the value 9.22 eV for gold is about 20% higher than 7.576 eV for silver because gold has a larger nuclear charge (59 vs 35) while its radius is practically the same. Thus, external s and d sublevels are close enough to allow the necessary transition. As a result, the probability of transition between sublevels is similar to that of copper, and gold is again yellow.

We can now perceive the necessary conditions for a metal to be yellow, like copper and gold (*2*):

1. Adequate external electronic configuration $s^1d^{10} \leftrightarrow s^2d^9$ (group 11, IB).
2. Sublevels s and d close enough to allow transition $s^1d^{10} \leftrightarrow s^2d^9$ to occur significantly (Cu, Au).

In contrast, all other metals shine silvery, colorless to the naked eye because they do not possess the necessary electronic external configuration and transition probability to appear colored.

Much work has been undertaken in connection with relativistic effects on metal properties (*6*); however a final question remains: are metals (except for Cu and Au) really colorless? Various tinges are reported, such as yellow for silver and blue for osmium. How many more will be detected when a complete survey is made? What number of atomic layers must be crossed (twice) in metals to produce a definite color? What about the effect of atomic packing, holes, and impurities? But this is another story and we would be very happy if research is aroused and enhanced by our questions.

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Both Nylon and PET Fibers Burn Continuously under Atmospheric Conditions

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We would like to present two series of photographs showing the characteristic burning behaviors of a nylon fiber and a polyethyleneterephthalate (PET) fiber, in order to help people safely handle these fibers in their everyday lives.

In many textbooks, especially on textiles, nylon and PET fibers are classified as flammable but self-extinguishing (*1*). In other references, we have read that nylon and PET give off combustible gases when they are heated above their decomposition temperatures (*2*). According to references, nylon gives propylene (8.8% in volume of total detected gases evolved), cyclopentanone (32.2%), hexamethylenediamine and other methylene amines (22.5%), and others (3), and PET gives ethylene (8.3% in volume of total detected gases evolved), acetaldehyde (10.9%), benzoic acid (37.5%), and other phenyl compounds (*4*).

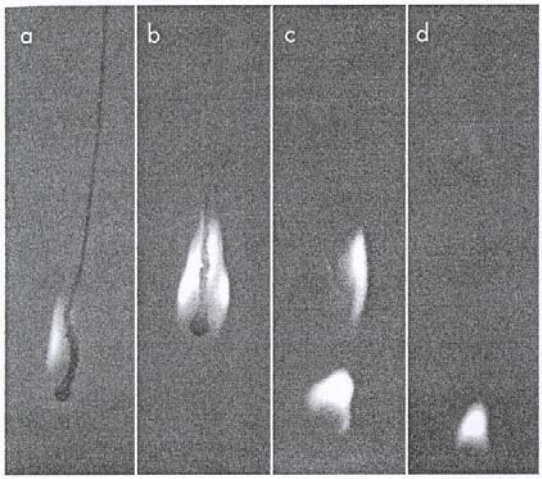

Figure 1. Characteristic burning of nylon threads: (a, b) with mild propagating flame; (c, d) continuous burning even after release of a fire ball.

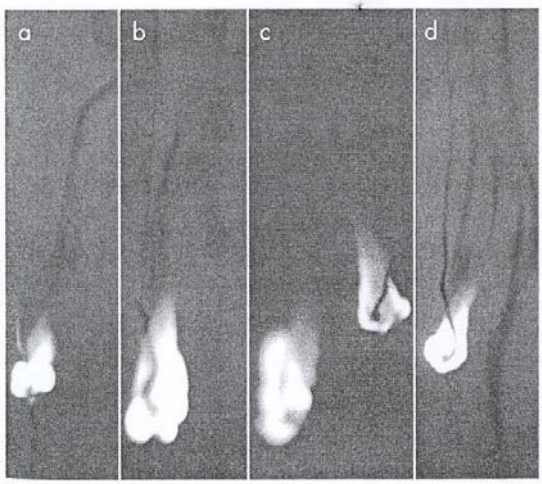

Figure 2. Characteristic burning of PET threads: (a) with violent flame giving black smoke and black solidified residues; (b) a spherical molten part of the thread evolving combustible gases to give rise to a vigorous flame; (c) a fire ball is just leaving from the flame; (d) thread still burns vigorously even after releasing a fire ball.

On the basis of these pyrolysis data, we were doubtful about the flammable but self-extinguishing classification for nylon and PET. So we very carefully performed experiments to see what would happen when fibers caught fire. We selected typical sewing threads for sewing machine (supplied by Teijin Co., Ltd., and Asahi Chemicals Co., Ltd.) for testing.

Thread samples about 50 cm long were hung up just in front of a focused camera and then ignited at the bottom end with a tiny flame from a cigarette lighter. A tiny flame was used because hot air ascended from a big flame and perturbed the thread. Once a part of the terminal end was ignited, it burned continuously, as shown in the series of photographs in this paper, in contrast to the descriptions in textbooks stating that it "burns slowly but if the sample is removed from the flame it self-extinguishes" (*2*).

In nylon thread, as seen in Figure 1, the flame propagates slowly. In PET thread, shown in Figure 2, the flame propagates more quickly and is accompanied by black smoke. Unlike natural fibers such as cellulose, these materials first melt, then give off combustible gases when the temperature exceeds the decomposition temperature of the polymers in the presence of about 21% of oxygen (i.e., under atmospheric conditions). When the ignition flame was removed, the threads continued to burn. During the course of the burning, pictures were taken of the small spherical fire balls composed of a molten polymer. A shutter speed of one one-thousandth of a second and a highly sensitive film (ASA 800) were used. Because the fire ball is changing rapidly, the photographs show scenes that cannot be seen by the naked eye.

CAUTION: We urge you to remember that these small fire balls are composed of viscous molten polymer. They have specific heats that are not only high enough to burn skin but also high enough to cause a big fire. If you want to do this type of experiment, you should wear a glove made of nonflammable fibers so your hand will not be burned.

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Applications and Analogies

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A Chromatographic Parable

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In thirty years of teaching separations courses, I have often searched for an apt allegory to illustrate the fundamentals of chromatographic processes. The following is one version of such a tale that students seem to find interesting and perhaps even informative.

In a small Southern town (it must be a Southern town or the story doesn't work), the people are planning a Fourth of July race from one end of town to the other. The townsfolk have the commonly observed characteristics that most of them are either Saints or Sinners; however, some of the folks are neither Saints nor Sinners (The Agnostic-Teetotalers) and others are both Saints and Sinners (we'll call this group the Hypocrites). The race will be conducted along the main street of town, and, as in most Southern towns, the street is lined with a suitable collection of churches and bars.

During the race the town folks all run at the same speed, but the Saints cannot pass a church without entering to pray for a while, and the Sinners cannot possibly pass by a bar without pausing for a refreshing beer. The immediate question then is who will win the 4th of July race? Most people want the Saints to win the race, but this is not probable because, while they are in church, the Agnostic-Teetotalers are still running. It is fairly obvious, even to college students, that the Agnostic-Teetotalers will win the race, and, quite deservedly, the Hypocrites will come in last. But what about the Saints and Sinners? Who will come in second or third? And finally, what can be done by the City Fathers to alter the outcome of the race next year?

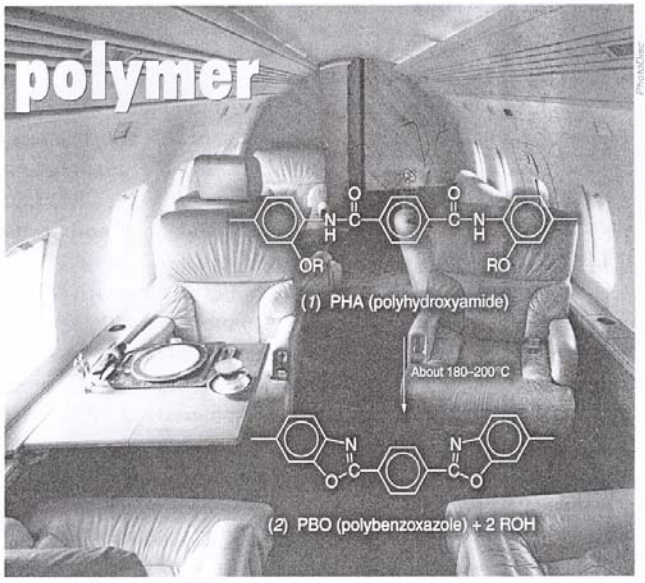
So, what will determine the results of the Saints–Sinners race? Let's say there are ten churches, but only three bars, along the main street. Under these conditions, the Sinners will win the race. Right? Watch out! What if it takes longer to drink a beer than it does to say a prayer?

The point of the exercise is to illustrate the concept that *the results of this particular race are determined by the amount of time the participants spend not racing*, that is, drinking or praying as the case may be. The analogy to chromatographic retention times is obvious if somewhat colloquial. Unfortunately, the analogy between the chromatographic stationary phase and a church or bar is perhaps less exemplary.

A secondary effect is possible if not all the racers run at exactly the same speed, if some Saints pray longer than others, or if some Sinners have more than one beer. In this case, not all the Sinners will reach the finish line at the same time. It is even possible that some very fast Saints could reach the finish line (elute) before some of the more tipsy Sinners or vice versa. Thus, there would be a distribution of individuals within a group of townsfolk and possible overlap of Saints and Sinners at the finish line. In chromatographic terms, the distribution is known as dispersion (described by the universally dreaded van Deemter equation) and overlap results in poor resolution. Both effects lead to diminished results for a chromatographic separation. In the 4th of July race analogy, it is possible that all the townsfolk (Saints, Sinners, Agnostics, and Hypocrites alike) would finish the race at the same time. In my experience, this is the most probable outcome for most Southern towns, as well as most chromatographic experiments.

Issue No 58 July '99

High flying polymer



A new type of fire-resistant polymer could improve your chances of survival in a plane crash, according to Phillip Westmoreland, professor of chemical engineering at the University of Massachusetts Amherst in the US.

Much of today's aircraft interiors are made of polymers because they are lightweight and versatile – they can be dyed different colours and formed into many shapes. They are used in seats, windows, wall panels, floor carpets, wiring, insulation, 'just about everything except the metal chair supports', says Westmoreland.

When a plane crashes and catches fire, polymers decompose from the heat, releasing combustible gases, which in turn also catch fire. According to Westmoreland's co-researcher Richard Lyon, Federal Aviation Authority (FAA), programme manager for fire research and fire safety, 40 per cent of the fatalities that occur in impact survivable air accidents are a result of fire. Fire-resistant polymers are therefore an important target.

Westmoreland and his team focused on polyhydroxyamide (PHA, *1*) as a potential candidate for a fire-resistant polymer. The backbone structure of PHA meant that it could be a useful thermoplastic (softens on heating) for forming into films and fibres. Also at temperatures of *ca* 180–200 °C, PHA converts with very little mass loss to water and a different polymer, *ie* the rigid high-strength polybenzoxazole (PBO, *2*), which decomposes only at very high temperatures (*ca* 600 °C). 'PBO has the best non-flammability of any material we know of, but you just can't use the stuff', commented Westmoreland. PBO is too hard to form into useful products, such as fabrics or panels.

Researchers at the University of Massachusetts synthesised several structural variants of PHA, from the simplest form (R=H), to phosphate-containing R-groups, to see which had the lowest flammability. At the same time, a team at the FAA developed a new microcalorimeter that could evaluate the polymers' ability to burn in milligram quantities, a method with advantages over conventional tests which involve much larger samples – *eg* 'taking an aircraft seat and setting fire to it'. The results revealed that all forms of PHA had low flammability, but the best polymer was the simplest – *ie* when R=H. In tests, this form of PHA gave passengers *ca* 10 times longer to get out of an aircraft than the best existing polymer.

Prize anagram winner

Answers

1. anode; 2. litmus; 3. ester; 4. rubidium; 5. solute; 6. ammonia; 7. octet; 8. absolute zero; 9. ketone; 10. bauxite.

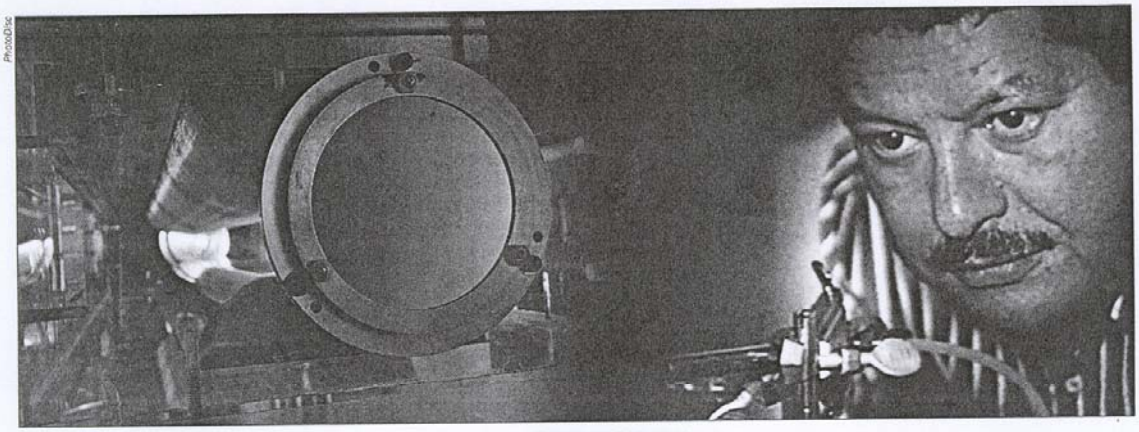
The anagram letters are: alersaoakb.

The solution is: koala bears.

First out of the hat with the correct answers was: Lisa M. Scott from Uplands School, Dorset.

Congratulations Lisa!

Anexo



Flash of inspiration wins Nobel prize for chemistry

CHEMIST AHMED ZEWAIL OF THE CALIFORNIA Institute of Technology (Caltech) (pictured top right) was the recipient of the 1999 Nobel prize for chemistry for a flash of inspiration that is revolutionising our understanding of chemical reactions. Using brief bursts of light from lasers, he developed a way to take 'snapshots' of individual molecules as they change during a chemical reaction.

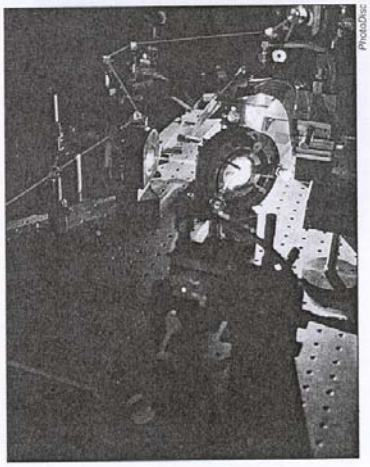
Modern lasers can produce a very short burst of light, lasting a few femtoseconds *ie* a million-billionth of a second. Like a fast camera flash that freezes a dancer in motion, the laser beam can illuminate a molecule as it is transformed from one shape and structure to another during a chemical reaction – its transition state. This transition state, which exists between the reactant and the product, lasts for only femtoseconds, so observing it before it disappears was, until Zewail's experiments, almost impossible. Being able to observe this state is helping chemists find out exactly how particular reactions work and allowing them to predict the outcome of other related reactions as well as the complex interactions of, for example, a drug molecule with a biological receptor.

The earliest attempt to look at reactions as they happen was by H. Hartridge and F. J. Roughton in the 1920s. They used a spectrophotometer to observe what happens when two compounds are mixed and saw chemical reactions taking place in a thousandth of a second. In the 1960s, Ronald Norrish and George Porter came up with the idea of using a flashlamp to freeze the reactions – the shorter the flash, the more transient the reactions they could see. They observed chemistry on the millisecond and microsecond timescales – a thousand times shorter than that possible in the 1920s. Porter and Norrish shared the 1967 Nobel prize with the German chemist Manfred Eigen, who used heat and pressure shock methods to trigger a reaction and observe 'almost' the instant at which it was happening (Eigen was also working at the milli- to microsecond timescale).

During the early 1980s, Dudley Herschbach, Yuan Lee and John Polanyi had improved the ability to observe chemical reactions down to the picosecond scale using vacuum collision experiments – for this work they received the 1986 Nobel prize for chemistry. With shorter and shorter timescales, chemists began to reveal the intermediate chemical species in reactions – not, the transition states, they were still too fleeting, but the structures either side that lasted just long enough for them to record. Once chemists had reached the picoscale, they only needed to take one step further to reach the femto timescale. The femtosecond – 10^{-15} s – represents the frequency at which molecules vibrate, without which there would be no interaction and no chemical change. If chemists could watch molecular vibrations they would have reached the limit of observation.

Lasers make light work

Zewail realised that to observe molecules at this level his flashlamp would have to be very fast, a pulsing laser that flashes once every femtosecond, he reasoned, would do the job. For their simplest experiment, Zewail and his colleagues chose a unimolecular reaction, *ie* where a single substance changes into another without the involvement of a second chemical, and formed a molecular beam in a vacuum chamber. By blasting this beam with a 'pump pulse' of laser light they excited the molecules and triggered a change. Then, by applying a weaker, 'probe pulse' from a laser lasting a few femtoseconds – at a frequency to coincide with the absorption frequency of the suspected transition state of the substance – Zewail and his team obtained a characteristic spectrum from the light emitted by the transition state. They had frozen the reaction.



The chemists compared the characteristic spectrum with the theoretical pattern obtained by using the methods of last year's Nobel chemists John Pople and Walter Kohn (*Educ. Chem.*, 1999, **36**(1), 7) who provided them with the means to predict molecular structure and so their characteristic spectra. Zewail's first unimolecular reaction – the one that started the whole femtochemistry field – was the dissociation of iodine cyanide (ICN), which takes just 200 femtoseconds. His results were published in 1987 in the *Journal of physical chemistry* and showed the transition state just as the carbon–iodine bond in the molecule is about to break to form the cyano radical and an iodine atom.

Zewail and his colleagues then moved on to bimolecular reactions, which involve two interacting chemical species. They studied the reaction of hydrogen with carbon dioxide, which produces carbon monoxide and hydroxy radicals. Zewail's flash revealed that the reaction passes through a transitionary HOCO molecule, which exists fleetingly for a mere picosecond (1000 fs). His team also began to look at a puzzle that had occupied chemical minds for some time – *ie* would two seemingly identical bonds in a molecule break simultaneously in, for instance, a dissociation reaction. For the dissociation of tetrafluorodiiodoethane it turns out that the 'equivalent' C–I bonds do not break at the same time – there is a delay of 200 fs following the splitting of the first.

Since Zewail's pioneering studies in the 1980s and 1990s, many other research teams have begun to use femtochemistry to look at diverse reactions – watching them happen in real-time.

David Bradley is a science writer. He can be contacted via his website: http://www.camsoft.com/elemental/

Pressure to change solvents

Decent decaffeinated coffee has been around since the 1960s, when chemist Kurt Zosel found an alternative to using the toxic and unpleasant tasting benzene to extract the caffeine. He discovered that a 19th century chemical curiosity, known as a supercritical fluid (SCF), could dissolve out the caffeine but leave no solvent residue. Supercritical fluids while still curious are now being used to destroy toxic waste, make industrial chemicals without toxic and highly flammable volatile organic compounds (VOCs) and are even making it easier to take your medicine. So what are these strange materials and why are they so supercritical?

All down to a squeeze

If you apply enough pressure to some gases while heating them they liquefy but keep their gaseous energy. Conversely, heating some liquids while you apply pressure gives them gaseous energy but without losing their density. These fluids are caught between the liquid and gas phase above a certain critical temperature and pressure – they are supercritical fluids, see *Fig 1*. Many common chemicals can become supercritical, from carbon dioxide and water to the noble gas xenon.

Water, for instance, becomes a supercritical fluid when it is heated above 374 °C and put under a pressure of 218 atmos. The fluid looks like a liquid but strangely, on the one hand can be mixed with oil but on the other will no longer dissolve ordinary table salt. These effects can be explained by the changes in the bonds between water molecules which, in the supercritical state, become weaker than normal. So, oily molecules can squeeze in between them but they are too weak to hold the sodium and chloride ions from salt. Amazingly, oxygen dissolved in supercritical water supports 'flameless' combustion. Scientists at Sandia National Laboratories in New Mexico are using this property to destroy industrial and domestic waste without the need for conventional incineration. Dissolved salts and metals come out of the solution and can be recycled or disposed of safely, while the organic content is broken down into carbon dioxide and water by the oxidation process. The process works at lower temperatures than incineration, so there are no nitrogen oxide pollutants produced.

Organic chemists from the University of Leeds have

Will supercritical fluids reduce the volume of domestic waste?

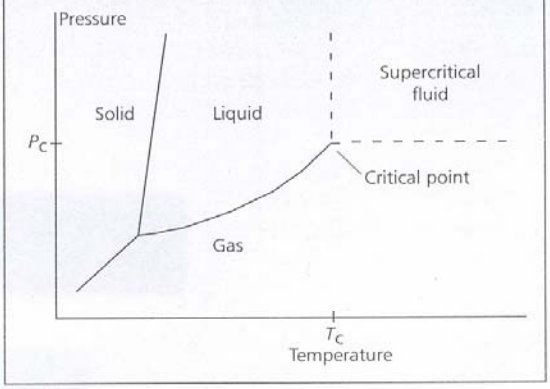
also been quick to latch on to Zosel's early discovery and have been using SCFs to extract natural products from plants and other organic materials for years. Natural flavour molecules, such as vanilla, for instance, can be cleanly extracted from the pod using an SCF. More recently, though, chemists have turned to SCFs to dissolve reactants that usually need a toxic and flammable VOC or do not dissolve at all.

Synthetic chemists are using SCFs in the manufacture of new types of polymer and other molecules that could function as industrial catalysts, thus avoiding the use of harmful solvents. Joseph DeSimone's group at the University of North Carolina in Chapel Hill, for example, is using supercritical carbon dioxide to make new types of fluorine-containing polymer. Adding fluorine atoms to a polymer chain is used to make some tough, smooth and chemically inert materials. Polytetrafluoroethene (PTFE or Teflon) was one of the earliest fluoropolymers, and is still used to coat non-stick frying pans! Modern fluoropolymers have more high-tech applications, such as acting as 'dry' lubricating layers for the moving parts in computers, eg hard drives, where a drop of oil would wreck the electronics. The problem with making these new fluoropolymers, however, is that fluorine atoms have a residual negative charge, which makes them polar so they dissolve best in water. This makes it difficult to process them further because any other chemicals added will usually be soluble only in organic solvents.

DeSimone's team has got around this problem by using supercritical carbon dioxide instead. The chemists can now control the length of the polymer chains and their precise chemical structure. This leads to consistent materials for high-tech aerospace and electronics applications.

Martyn Poliakoff and his team at the University of Nottingham, meanwhile, are exploring how SCFs can help them make new industrial catalysts. They have discovered that they can make organometallic compounds such as metal carbonyls, many of which are too unstable to prepare by conventional methods. Metal carbonyls are used in various industrial reactions as catalysts for speeding up the production of simple materials such as formic acid and formaldehyde and more complex compounds, like pharmaceuticals and polymers. Carbonyl compounds in which nitrogen or hydrogen molecules have been substituted for a carbonyl group can catalyse more complex reactions still. For example, novel piano-stool shaped manganese carbonyls with an attached dihydrogen might be a useful polymerisation catalyst. The problem in making them is that hydrogen and nitrogen gases do not dissolve well in conventional organic solvents at room temperature so it is hard to add the atoms to the starting molecule. The Nottingham

Fig 1 *Phase diagram for an SCF*



team, however, has found that hydrogen mixes very well with supercritical carbon dioxide at 80–100 atmos, allowing the reaction to add hydrogen or nitrogen atoms as needed to the carbonyl compound.

Once the reaction is over, the SCF can be quickly recycled by releasing the pressure and trapping the carbon dioxide gas that escapes. This is one of the major advantages of SCFs over other solvents. VOCs, for instance, become contaminated during a reaction and it is expensive and wasteful to purify them. SCFs avoid this problem because once they become a gas again they leave behind any impurities.

SCFs – not so thick

SCFs are also much less viscous than liquid solvents, so they flow more easily through a reaction system. They can also get into the smallest of crevices and pits inside the reactor system. By flushing the system with an SCF once a reaction is complete any impurities can be washed out, leaving the system pristine and ready to be used again.

But, what about SCFs making it easier to take medicines? Scientists are now using SCFs to help them make drugs that normally have to be injected work when taken by mouth instead. A collaborative team from the US, Canada and Norway has found they can make sub-microscopic particles of the immunosuppressant drug cyclosporin, which is used to prevent transplanted organ rejection, by preparing it in supercritical carbon dioxide and then blasting it into normal water by releasing the pressure. The blast makes billions upon billions of tiny drug particles just fractions of a micrometre in size. These particles are so small that the researchers hope they will be absorbable by the gut so that patients avoid getting the needle.

Amazing what a little warmth and a squeeze will do!

David Bradley

A healthy spread

Cholesterol, an essential constituent of all cell membranes, forms part of the casing that protects nerve fibres and is a precursor in the production of vitamin D, steroid hormones and bile salts. However, too much cholesterol in the blood is associated with heart disease. While reducing elevated cholesterol levels cannot guarantee a healthy heart, scientists and doctors agree that it can reduce the risk of problems. Here we consider how this can be done through dietary considerations, by reducing the use of food components that raise cholesterol and by adding cholesterol-lowering ingredients – *ie* functional foods or 'nutraceuticals'.

Introducing cholesterol

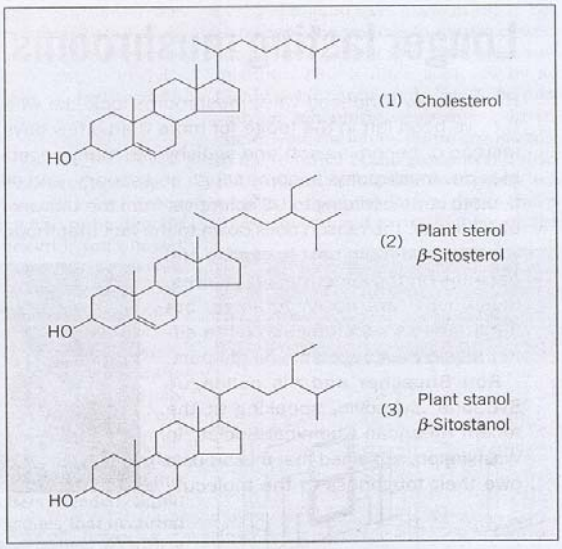
Most of the cholesterol (*1*) we need is manufactured in our liver, *ca* 600 mg day^{-1}. Research suggests that if a healthy adult absorbs *ca* 80 mg day^{-1} of cholesterol from foods such as animal products and eggs, the liver synthesises *ca* nine times as much (*ca* 720 mg day^{-1}). Reducing cholesterol in our diet therefore has only a modest effect on lowering blood cholesterol levels. Scientists therefore considered which other components in food have a significant effect on cholesterol levels.

Cholesterol is insoluble in water and has to be carried around the blood stream as lipoproteins (*ie* all the insoluble lipid molecules in the body, attatched to proteins). Different combinations of lipids and proteins produce complexes of different densities. Low density lipoproteins (LDLs), for example, supply cholesterol to cells, increased levels of which are associated with atherosclerosis – *ie* an accumulation of lipids in plaques on artery walls, which narrows the arteries and restricts the blood flow to the heart (ischaemia) and brain (stroke). In contrast, high density lipoproteins (HDLs) transport cholesterol away from artery walls and therefore act as cardio-protectors. To reduce the risk of heart disease, people therefore need to lower both their total cholesterol levels and their LDL-cholesterol levels in the plasma.

Dietary fats

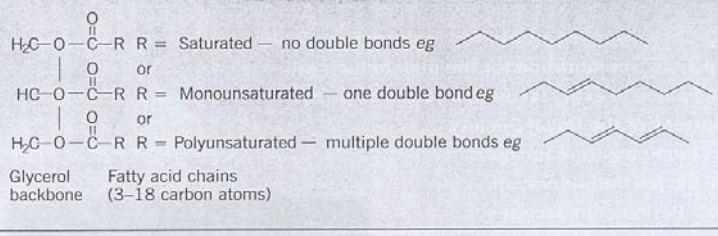
Dietary fats, both animal and vegetable, are made up of a mixture of triglycerides (see Box 1). They are the major food constituents known to have a significant effect on cholesterol levels. Animal fats, in butter for example, consist of a relatively high proportion of saturated fatty acids, some of which according to Judy Donnelly, nutritional biochemist at Trinity and All Saints University College Leeds, 'increase the proportion of LDL-cholesterol in the blood, compared with HDL-cholesterol. Cutting down on the amount of saturated fatty acids we eat could therefore lower our risk of heart disease'. In contrast, vegetable oils, such as those found in margarines, consist of long-chain polyunsaturated and monounsaturated fatty acids, which are associated with lowering LDL-cholesterol levels.

As people become more conscious of the benefits of cutting down excess intake of fats, especially saturated fats, spreads that contain <80 per cent fat are gaining in popularity. It is the saturated fatty acid content that makes butters and margarines solid so we can spread them. In lower fat spreads, fat substitutes are sometimes added to achieve the desired consistency and attributes. Sometimes the substitute is water (in buttermilk and skimmed milk with added salts and preserva-



tives), but it may be that starch molecules or whey proteins, which have been processed to give the particles a uniform size and thus a smooth feel in the mouth, are added. Many of the resulting spreads, however, are not as popular with consumers because, for example, they lack the saturated fatty acids that give butter its distinctive flavour. To improve the acceptability of low fat spreads, researchers are investigating synthetic replacements to animal fats, or 'structural fatitutes'. Such compounds provide many similar properties, such as taste and texture, but they are not digested or absorbed from the gut into the blood and therefore cannot raise LDL-cholesterol levels. They are used in the US in crisps and savoury products, but have not yet been added to fat spreads.

Box 1 *Structure of triglycerides in dietary fats*

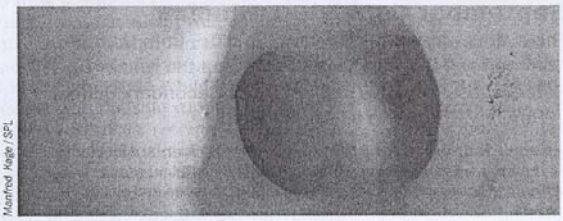


Functional foods

In the past few years the focus of research has shifted to adding ingredients (nutraceuticals) to food to reduce LDL-cholesterol levels. Since the early 1950s scientists have known that plant sterols (2), and their hydrogenated counterparts, stanols (3), have cholesterol-lowering properties. Unfortunately, these compounds are not naturally abundant in the foods we eat. Over the years, scientists have come to realise that these compounds are very effective at lowering LDL-cholesterol levels when sufficient is eaten, for example in rich fat spreads. Such products have recently been developed by esterifying the compounds with fatty acids to increase their fat solubility.

Two fat spreads – Benecol and Flora Proactive – are currently on the market for reducing LDL-cholesterol levels. Benecol contains plant stanol esters (sitostanol esters), and Flora Proactive contains sterol esters. Clinical trials, on people with elevated cholesterol levels, have shown that these products reduce total plasma cholesterol levels and LDL-cholesterol levels by 8–13 per cent, without effecting HDL levels. Both products appear to have no adverse health effects and are non-toxic even in high doses, though a few people with the rare condition, phytosterolaemia cannot metabolise sterols and should avoid them.

Clear arteries – could nutraceuticals be the answer?



Manfred Kage / SPL

According to Donnelly, there are two mechanisms by which these compounds are thought to lower cholesterol levels. 'Cholesterol is not very soluble in the gut and its absorption is slow. Since you have other fats also being absorbed from the gut, cholesterol is one of the last to go through', she explained. 'Plant sterols and stanols have similar structures to cholesterol so they also get left behind. As the concentration of sterols/stanols increases, a threshold level is reached when the cholesterol molecules and the sterols/stanols coprecipitate into a solid crystalline form which cannot be absorbed by the gut'. According to Donnelly, another possibility focuses on micelles, which are clusters of molecules that transport fats across the gut membrane. There is limited capacity for carrying cholesterol, and the plant sterols and stanols compete with cholesterol to get into the micelles, which limits the amount of cholesterol that can be absorbed. 'These mechanisms do not just reduce the absorption of dietary cholesterol', said Donnelly, 'but they also hinder reabsorption of some of the cholesterol produced by the body, which has been used in producing bile salts'. Bile salts are used in the intestine to breakdown the fatty acids that we eat. Normally, the cholesterol in the bile salts would be recycled by re-absorption in the gut, but in this case they are excreted. Essentially more of the cholesterol produced has to go in to producing more bile salts, reducing the amounts in the blood plasma.

Cholesterol-lowering spreads are some of the first functional foods on the market, but scientists are continually identifying ingredients that have potential health benefits. As new advances in food technology allow their incorporation into products, we will see a lot more on the supermarket shelves. Although these products can be beneficial, Donnelly says that she hopes 'people do not begin to rely on them because they are not miracle cures and there are many other factors involved in heart disease, which these products do not address'.

Joanna Gibson

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Apatite for destruction

The industrial revolution of the 18th and 19th centuries brought great prosperity to the UK, but not without a price. The Environment Agency estimates that 300 000 hectares of the UK is contaminated as a result of industrial pollution, for example cadmium and lead contamination associated with the iron, steel and paints industries. Now with the increasing demand for housing, which places pressure on the countryside, the Government requires that 60 per cent of all new housing should be built on reclaimed sites. Using current techniques of remediation – 'dig and dump' and 'soil washing' – the cost of reclaiming this land is estimated at £20 billion. However, scientists at the Natural History Museum believe they have found a cost-effective solution to treating heavy metal pollution by using bone-meal. Their method, presented by Dr Eugenia Valsami-Jones, at the BA festival of science, in London in September, involves 'immobilising' polluting metals as insoluble phosphates. The work is sponsored by the BOC Foundation and the Environment Agency.

Bone-meal, widely used as a garden fertiliser, is sterilised, crushed animal bone comprising two main components. There is an organic component, *ie* a fibrous protein (collagen) and an inorganic component, ie the crystalline mineral hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$). It is the hydroxyapatite, with phosphate ions locked in its crystal structure, that allows bone-meal to trap heavy metals. The treatment of contaminated land with bone-meal is based on two reactions. First, on mixing with soil, bone-meal dissolves in the pore/rain water, releasing phosphate ions from the crystal structure, along with calcium ions and some hydroxide ions. Secondly, free phosphate ions react with the metal pollutant, forming insoluble metal phosphates. This reaction locks the polluting metal into a rigid mineral structure, thus acting as a 'micro barrier' between the pollutant and the environment. Lab trials of bone-meal as a treatment for heavy metal pollution at the Natural History Museum using Scanning Electron Microscopy (SEM) confirm the formation of metal phosphate minerals with aluminium, copper, zinc, cadmium, nickel, lead and uranium.

In the short-term future the team hopes to demonstrate that the method will work at a variety of contaminated sites, thus establishing the long-term stability of the remediated metals. 'In the future, we hope to see the method being used and contributing to the improvement of the lives of people affected by heavy metal pollution', said Dr Valsami-Jones. ●

Longer lasting mushrooms

Have you ever noticed what mushrooms look like when they've been left in the fridge for more than a few days? Instead of becoming soft and squishy like many vegetables do, mushrooms become tough and spongy, and difficult to cut. According to US scientists from the University of Arkansas, the reason boils down to the fact that though you may find them next to carrots and parsnips on the supermarket shelves, mushrooms are not vegetables, but fungi, and as such degrade quite differently to their vegetable neighbours.

Ron Buescher and his colleague Svetlana Zivanovic, speaking at the recent American Chemical Society in Washington, explained that mushrooms owe their toughness to the molecule chitin, which forms part of its structural walls. (Chitin is also found in the hard exterior casings of shellfish and insects.) As they grow, mushrooms derive all of the chemicals they need to make chitin from the underlying soil. However, the researchers found that chitin levels continue to increase even after the mushrooms are picked, implying that they are able to get the necessary molecules to make chitin themselves.

As for the spongy texture of the degrading mushrooms, the researchers have shown that this is down to the action of protease enzymes, which break down proteins in the fungal cell walls. The degradation of these structural proteins causes the cells to stretch, opening up bigger spaces between them, with the result that the caps open and become flattened.

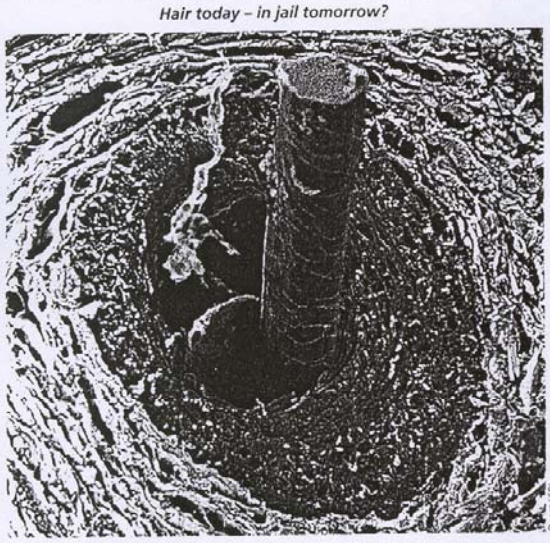
The researchers are currently trying to find a way of inhibiting proteolysis, to extend the shelf-life of mushrooms.

Perspectives

Hair-raising ideas

Hair could tell other people a lot more about you than you might want them to know. Two new methods of hair analyses presented at the American Chemical Society meeting in Washington in August both use supercritical fluid technologies to identify the perpetrators of crime. Typically, hair samples collected at crime scenes are inspected under the microscope to determine colour, thickness and morphology (straightness). But, without resorting to DNA analysis, this frequently gives a profile that is far from unique.

Hair today – in jail tomorrow?



Quest / SPL

At the US National Institute for Standards and Technology, Bruce Benner has come up with an analytical technique based on supercritical fluid (SF) extraction combined with GC–MS that can provide a more reliable chemical hair profile. By exploiting the powerful solubilising ability of $SFCO_2$, Benner is able to strip away from the hair a much greater proportion of the surrounding lipids and other ingredients, including several hormones and other proteins. Recent analyses of a variety of hair samples using the approach have revealed that the technique is highly reproducible, so criminals won't simply be able to disguise themselves by changing the shampoo or conditioner they use.

The external composition of hair also depends on a variety of other factors, Benner says, including what you eat, your gender and ethnic type, as well as your general health and well-being. In fact, looking at the general lipid composition of hair may even be a good way of detecting different illnesses, he adds.

Getting deeper inside the hair shaft can be even more revealing, according to Janet Morrison and Alison Rada at Trinity College, Connecticut. Here, researchers are interested in looking for signs of drug abuse by the sample provider – in particular to detect the illicit use of amphetamines, which includes increasingly common drugs such as MDMA (Ecstacy). Conventional procedures for detecting these drugs in blood and urine samples are notoriously time-consuming and involve a two step process that involves liquid–liquid or solid-phase extraction followed by lengthy derivatisation of the drugs to make analogues suitable for GC–MS analysis. Although even the $SFCO_2$ used for this new extraction process is not powerful enough to dissolve the amphetamines directly, the researchers are able to speed up this process enormously by incorporating the derivatising reagents in this extraction solvent.

By performing both extraction and derivatisation in one step, the researchers are able to reduce the time needed to carry out this detective work from several days to just over an hour. Morrison has already applied a similar technique for cocaine analyses in hair, but both methods will need to be validated by the courts before they can become routinely adopted by toxicologists. Not only do they promise to catch culprits more quickly, but hair greatly expands the time window for drug detection compared with urine and blood. Knowing that hair grows by 1 cm per month, it is possible to obtain an accurate date for when the abuse took place. □

Scrap makes super steel

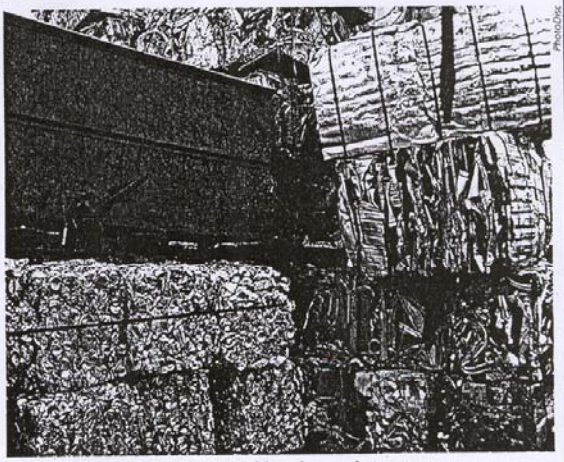
Researchers in the department of materials at Leeds University have discovered how to transform steel scrap that has been contaminated with tin and copper from tin-plated cans and electrical wiring into a high-strength, high-value alloy – their solution is to mix aluminium into the steel. Because aluminium is available in large quantities from scrap cans, this process offers the alluring prospect of upgrading a recycled product while at the same time helping to solve the problem of accumulating scrap metals.

Environmental and economic requirements have meant that 40 per cent of the steel produced in modern steel plants today has been recycled at some stage in its life. Tin-plated steel cans – often (incorrectly) referred to as tin cans – are present in enormous quantities in domestic refuse. European Union directives now require that an additional 250 000 t of tin-coated steel should be recycled annually by 2001.

If tin cannot be removed from the steel scrap, then its presence, together with that of copper, makes the recycled steel prone to cracking, because tin separates out along the boundaries between grains of steel. This happens at concentrations of tin and copper as low as 0.25 and 0.04 per cent respectively.

Tin can be removed from scrap by shredding cans and treating them chemically, but this produces large amounts of effluent that have to be disposed of. Other methods for removing tin from scrap are expensive and limited in efficacy.

But research led by Bob Cochrane, British Steel professor of metallurgy at Leeds, has now shown that when aluminium is added to steel contaminated with tin, this prevents the tin separating out along grain boundaries and reduces grain size. This produces a valuable alloy with increased strength and reduced tendency to crack.



PhotoDisc

Aluminium can(s) make steel stronger

These findings are potentially of great value to steel makers in poorer countries because they offer a way to make high value products out of cheaply available scrap.

The Leeds discovery also makes it possible to make more precise predictions about the properties of alloys. Cochrane says that if these predictions work for other alloy systems then they too may be used in the same way 'to turn scrap into a noble material'. *John Newell*

4. 3. Texto utilizado como ejemplo.

Report

Sol–Gel Encapsulation Methods for Biosensors

Proteins trapped in a sol–gel prepared silicate glass matrix retain their biological function and can be used in optical biosensors

Bakul C. Dave
Bruce Dunn
Joan Selverstone Valentine
Jeffrey I. Zink
University of California, Los Angeles

By nature, many biological macromolecules are highly efficient at recognizing specific analytes or catalyzing reactions in aqueous biological media. These characteristics make biomolecules desirable reagents, but the aqueous medium that is almost always necessary for biomolecular reactions limits their commercial viability. Drastic changes in the preferred buffered aqueous medium often lead to partial or total denaturation and loss of reactivity.

Efforts are being made to harness the utility of these reagents in biosensors by immobilizing them in alternative environments that stabilize them and preserve their reactivities (*1*). Currently, biosensors are being used primarily in clinical testing, but the potential application of biosensors as in situ probes in industrial process monitoring and control appears promising. The quintessential biosensor consists of an immobilized biomolecule that binds or otherwise reacts with a specific analyte, coupled with some type of signal transducer (*2*). Optimum biosensor design requires maximum retention of biomolecular reactivity as well as efficient, cost-effective signal transduction (*3*).

One general area of biosensor research is the immobilization of enzymes or other proteins. The major advantages of protein immobilization are close control of the reaction medium and conditions, prevention of bacterial and chemical degradation, cost-effective reusability of the protein, and enhanced biomolecular stability. However, proteins often fail to retain their native stabilities and reactivities upon immobilization, a flaw that results in low stabilities or altered functional responses of biosensors incorporating them.

Conventional methods of enzyme immobilization include covalent binding, physical adsorption, or cross-linking to a suitable carrier matrix. Alternatively, enzymes can be physically entrapped and microencapsulated in polymeric matrices (*1*). For optimum biostability and reaction efficiency, the preferred host matrix appears to be one that isolates the biomolecule, protecting it from self-aggregation and microbial attack, while providing essentially the same local aqueous microenvironment as in biological media.

Recent research has demonstrated that silicate glasses obtained by the sol–gel method can provide such a host matrix and that biomolecules immobilized by this method retain their functional characteristics to a large extent (*4–6*). Moreover, these functionalized glasses can be prepared so that they are optically transparent, permitting optical monitoring of the spectroscopic properties of the encapsulated biomolecules (*5*). These biofunctional glasses make it possible to retain the specificity and reactivity of biological molecules in the solid state and provide morphological and structural control that is not available when the biological molecules are simply dissolved in aqueous media. Furthermore, the amorphous nature of the glassy material does not impart a geometric order to the entrapped molecules; many of the characteristics of the liquid state are retained despite the fact that the molecule is trapped in a solid ma-

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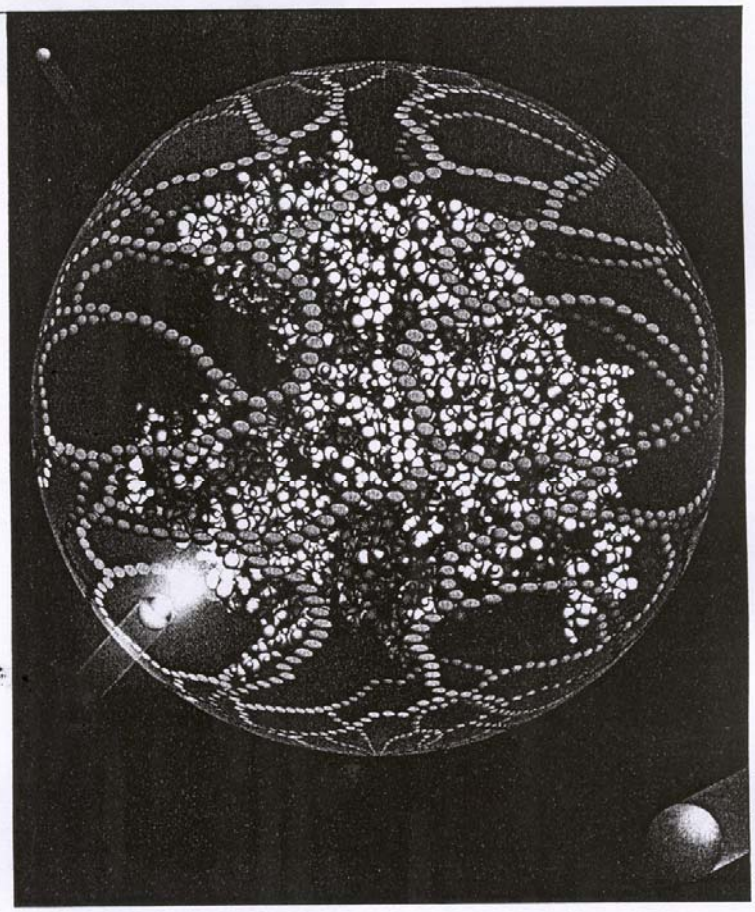

terial. In this article we review recent advances in sol–gel entrapment of biorecognition molecules in these transparent porous silicate matrices and discuss their potential use as optically based sensor elements.

The sol–gel method

Glass prepared by traditional melting of silica is not a feasible host matrix for most organic dopants because of the extreme temperatures required. By contrast, the sol–gel process involves low-temperature hydrolysis of suitable monomeric precursors and is highly suitable for microencapsulation of a variety of molecules that cannot withstand high temperatures (*7*). The silicate matrix is usually formed by hydrolysis of an alkoxide precursor followed by condensation to yield a polymeric oxo-bridged SiO_2 network. In the process, molecules of the corresponding alcohol are liberated.

Controlling matrix characteristics. The initial hydrolysis and polycondensation reactions in a localized region lead to formation of colloidal particles. (A suspension containing these colloidal particles is called a sol.) As the interconnection between these particles increases, the viscosity of the sol starts to increase and leads to the formation of a solid gel. Although the nature of individual events is random and the geometry and pore-size distribution of the product gel are difficult to determine, the nature of the final polymeric gel can be regulated to a certain extent by controlling the rates of the individual steps.

Because protons or hydroxide ions are required for catalysis in silica gel formation, the pH of the reaction medium is an important factor that affects the stoichiometry of the final gel. The presence of free protons in the medium allows protonation of the ligated alkoxide and favors its dissociation. An acidic medium, however, hinders the formation of oxo bridges resulting from the loss of protons from water or hydroxide ions. Acidic catalysis tends to increase the rate of hydrolysis and disfavors condensation reactions, whereas base hydrolysis produces rapid condensation.

Aging. Even after the gelation point, the structure and properties of the gel continue to change as long as solvent remains in its pores and the gel is not allowed to dry. One reason for these changes is that polycondensation reactions are still taking place in the solid amorphous phase, and as a result, cross-linking continues. Spontaneous shrinkage of the gel and the resulting expulsion of pore liquid also occurs. This expulsion is caused primarily by