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COUPLING LIQUID CHROMATOGRAPHY WITH REDOX CHEMILUMINESCENCE DETECTION

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SUMMARY

The post-column oxidation of many chemical species with dilute nitric acid, nitrous acid, or nitrite ion produces nitric oxide, which is subsequently detected by its chemiluminescence reaction with ozone. Organic compounds, such as sugars, react with dilute nitric acid at elevated temperature and pressure to produce a surrogate nitric oxide pulse for measurement on a chromatographically useful time scale. In addition, certain inorganic reducing agents, *e.g.*, iron(II), react under similar conditions and are sensitively detected. Ultraviolet irradiation can initiate the redox reactions of easily oxidized inorganic species with aq. nitric acid at 25°C, and it also enhances the reactivity of analytes at elevated temperatures. Aqueous nitrate ion is not effective as a redox reagent, while nitrous acid and nitrite ion are less potent oxidizing agents than nitric acid. A post-column reaction system is described and the characteristics of redox reactions of potential analytes with aq. nitric acid and other reagents are discussed. Illustrative chromatograms which show the high-performance liquid chromatographic separation and chemiluminescence measurement of sugars at ppm levels are presented.

INTRODUCTION

As the need has arisen for more specialized selective detection methods in both gas and liquid chromatography (LC), the utilization of post-column reactions has become more apparent. Such reaction schemes are the basis for a variety of sensitive and selective detection techniques that can be applied to liquid chromatography. Recently, the use of chemical derivatization, photochemical reactions, and modification of gas chromatographic (GC) detectors as post-column techniques in LC has been reviewed[1–4]. These post-column methods can be used advantageously in the determination of analytes that are difficult to measure by conventional means.

The recently developed redox chemiluminescence detector (RCD) is based upon catalyzed post-column redox chemistry of analytes with nitrogen dioxide or nitric acid to form nitric oxide[5–10]. The surrogate nitric oxide pulse, which is created by selective reaction of analytes, is sensitively detected by chemiluminescence, pro-

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duced in a downstream reaction with ozone. Although gas-phase redox reactions have been applied to a variety of chromatographic analyses, corresponding application of the liquid-phase oxidation of analytes is less well developed.

In this paper we report the development of an LC–RCD interface. A reaction system is described and evaluated for use as a post-column detector in LC. Choice of liquid phase reagents, enhancement of oxidation by catalysis or photochemical means, and methods of removing nitric oxide formed from reactions in the liquid phase are considered. Finally, the performance of a coupled HPLC–RCD system is examined for repetitive separation and quantitation of a mixture of sugars.

EXPERIMENTAL

Instrumentation

Two experimental systems were employed: (i) a flow injection system without a chromatographic column or eluent mixing device, and (ii) an apparatus consisting of the flow injection components along with an HPLC column and mixing tee, coupled to the post-column reactor and RCD. The measurement of nitric oxide, separated from the liquid phase eluent, was performed with a TECO Model 14D NO/NO_x analyzer (Thermo Electron, Waltham, MA, U.S.A.). In the flow injection experiments, liquid reagents were delivered with a Bio-Rad Model 1330 HPLC pump (Bio-Rad Labs., Richmond, CA, U.S.A.). An additional pump supplied the water eluent flow when the analytical separation was performed. Analytes were introduced into the system with a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) fitted with a 20-μl sample loop. For the chromatographic determinations, an analytical column was fabricated from a 4.5-cm length of 6.3 mm O.D. × 4.5 mm I.D. stainless-steel tubing. Zero-dead-volume fittings and 6.3-mm O.D./ 2-μm porosity stainless-steel fritted disks contained the packing within the column. The column was slurry-packed with *ca.* 1.2 g of Macronex CHO-682 lead(II)-loaded cation-exchange polymer (Interaction Chemicals, Mountain View, CA, U.S.A.). Packing was accomplished at a water flow-rate of 0.6 ml/min with a column pressure of *ca.* 1000 p.s.i. for 20 min. The column was maintained at 90°C in an aluminum block heater during the packing procedure. Flexon HP fluoropolymer tubing (1.6 mm O.D. × 0.25 mm I.D.) was used to transfer the 0.16 *M* aq. nitric acid reagent and the column eluent to a low dead-volume Kel-F mixing tee (Alltech Assoc., Deerfield, IL, U.S.A.).

After mixing, the combined eluent flow was introduced into the post-column reactor. The post-column reactor (Fig. 1) was fabricated from 30 m of a 0.32 mm O.D. × 0.23 mm I.D. uncoated fused-silica capillary column (SGE, Austin, TX, U.S.A.) installed in a GC oven to provide a stable heat source over the temperature range from ambient to 300°C. A short length of column, extending from the GC oven, was immersed in a cooling bath to maintain a constant eluent temperature of *ca.* 25°C. A flow restrictor consisting of an approx. 50 cm × 0.32 mm O.D. × 0.05 mm I.D. fused-silica capillary (SGE) was coupled to the reactor coil to induce backpressure in the system. Maintaining the backpressure at *ca.* 1200 p.s.i. permits the redox reactor to be operated at temperatures up to 300°C without vaporizing the eluent. The restrictor capillary was inserted into a gas separator (Fig. 2), which removes nitric oxide from the eluent stream for subsequent chemiluminescent detection.

The flow injection experiments were accomplished by direct connection of the

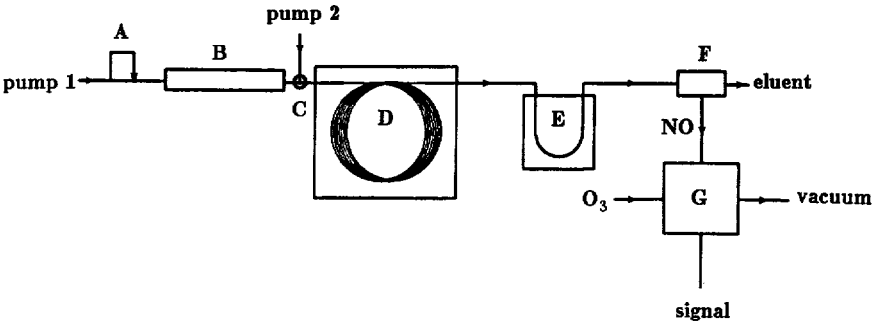

Fig 1. Apparatus used in the chromatographic separation of sugars. Removal of the chromatography column and mixing tee components constitutes the flow injection device for studies of redox reactions in the liquid phase. Pump 1 delivers the water eluent, pump 2 delivers dilute aq. nitric acid. A = 20-μl loop injector, B = 4.5-cm long HPLC column, C = Kel-F mixing tee, D = 30 m fused-silica capillary reactor coil in a GC oven, E = flow restrictor capillary in a cooling bath, F = nitric oxide gas separator, G = nitric oxide chemiluminescence detector.

reaction coil to the loop injector. Analytes were injected directly into a 0.1 *M* aq. nitric acid stream, with subsequent oxidation in the reaction coil. Nitric oxide produced from reaction in the coil was separated and detected as before. In the flow injection experiments with UV irradiation, an air-cooled photochemical reactor, consisting of 1.0 m × 1.6 mm O.D. × 0.3 mm I.D. PTFE tubing (Anspec, Ann Arbor, MI, U.S.A.) coiled around a Pen-Ray UV lamp (UV Products, San Gabriel, CA, U.S.A.) was inserted between the loop injector and capillary reaction coil.

Liquid phase studies

The flow injection system previously described was generally operated at an eluent flow-rate of 1.0 ml/min in most experiments. Eluent solutions of 0.1 *M* nitric

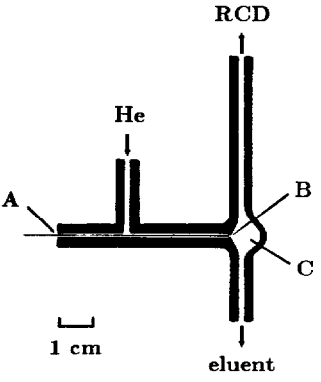

Fig 2. Nebulization device constructed largely from 6.3 mm O.D. × 2.0 mm I.D. borosilicate glass tubing. A = insertion point for a 0.32 mm O.D. × 0.05 mm I.D. fused-silica capillary. The capillary is adjustable within a 6.3 mm O.D. × 0.4 mm I.D. glass tube for optimizing the nebulization. B = *ca.* 0.4-mm outlet orifice. C = *ca.* 500-μl nebulization chamber. During operation, the eluent is nebulized with a 30 ml/min helium flow. The nitric oxide gas is transferred to the redox chemiluminescence detector, and waste liquid is removed by vacuum aspiration.

acid and sodium nitrite were prepared from appropriate reagent grade stock chemicals, with filtered distilled water. A 0.16 *M* nitric acid reagent solution for use in the chromatography experiments was similarly prepared. The 0.1 *M* nitrous acid eluent was prepared by mixing stoichiometric amounts of barium nitrite and sulfuric acid stock solutions. The nitrous acid solution was then decanted, after the insoluble barium sulfate precipitate had settled. All prepared eluent solutions were purged with a helium flow of 10 ml/min for 30 min to remove traces of gaseous nitrogen oxides prior to use in the flow system. Nitric oxide-saturated water was prepared in a glass-frit bubbler, designed to preclude the introduction of atmospheric oxygen. Pure nitric oxide (Scientific Gas Products, South Plainfield, NJ, U.S.A.) was passed at 10 ml/min for 30 min through 250 ml of distilled water which had been previously purged for 0.5 h with helium. An in-line Ascarite trap was used to remove trace levels of nitrogen dioxide from the nitric oxide stream. After allowing a few hours for equilibration, an aliquot of the aq. nitric oxide solution was diluted 50:1 in distilled water as an evaluation standard for the gas separator. Solutions of organic analytes for injection were prepared gravimetrically and by serial dilution to yield standards at *ca.* 1, 5, 10 and 25 ppm concentration levels. Inorganic analyte standards containing anions and cations were similarly prepared from their respective sodium or chloride salts. The reaction coil was operated from 25 to 300°C, depending upon the experiment performed, the temperature being monitored by a thermocouple within the GC oven. The eluent containing nitric oxide in solution was introduced into the gas separator and nebulized by sweeping it through a small orifice with a pressurized helium flow of 30 ml/min. The combined effect of gas flow in this region and the vacuum in the chamber assisted the nebulization process. Effective nebulization was obtained with the generation of a small and uniform droplet size, as this increases the surface area of the gas–liquid interface. The separated nitric oxide was drawn from the upper arm of the nebulization chamber and transferred through a 1.0 m × 1.6 mm O.D. × 0.3 mm I.D. PTFE tubing to the chemiluminescence detector. The detector chamber operating pressure was *ca.* 7 Torr when using a combined dry gas flow of 30 ml/min and did not change appreciably during operation with the gas separator. Installation of a molecular sieve trap between the separator and the detector did not produce a measurable change in nitric oxide response. Thus, quenching of the chemiluminescence by water vapor was assumed to be minimal. The liquid droplets, which were stripped of nitric oxide gas, coalesced and flowed into the lower arm of the separator for continuous removal by vacuum aspiration. A valving arrangement was used to balance the eluent flux to maintain a liquid level that filled approximately half the volume of the lower arm of the separator. This resulted in a separator chamber volume of *ca.* 500 μl, from which nitric oxide is swept by the helium carrier to the detector.

Liquid chromatography studies

An aqueous solution, containing *ca.* 10 ppm each of sucrose, fructose, and ribose was prepared to evaluate the separation efficiency, sensitivity, and reproducibility of a sugar analysis. Repetitively injected samples were separated, and the individual sugars were quantitated in the apparatus shown in Fig. 1. The analysis was performed at a flow-rate of 0.4 ml/min with distilled water as the eluent at 90°C. The 0.16 *M* nitric acid reagent was introduced at 0.6 ml/min into the mixing tee, along

with the column eluent, to yield a combined flow-rate of 0.1 *M* nitric acid at 1.0 ml/min. The residence time for analytes in the heated zone of the reaction capillary was *ca.* 60 s.

RESULTS AND DISCUSSION

The oxidation of many compounds by nitric acid under relatively mild conditions up to refluxing temperature is reported extensively in the literature[11]. These reactions were observed to be rather sluggish, with a characteristic induction period before onset of oxidation. The induction period could be shortened and the oxidation enhanced by the addition of nitrogen dioxide or nitrites. The reaction of nitric acid with analytes is characterized by production of aqueous NO_x species, which act as initiators for further oxidation. Interestingly, ordinary radical inhibitors, such as hydroquinones, act as initiators in nitric acid oxidation, since they are readily oxidized by nitric acid to give nitrogen dioxide. The first step in the oxidation of compounds is believed to be an electrophilic attack or hydrogen abstraction by nitrogen dioxide or related radical species generated in solution.

Previous work in our laboratory on oxidation of alcohols, phenols, and sugars indicated that reaction of these substrates with nitric acid at temperatures below 150°C would be too slow for application to post-column detection. It was also discovered that gold or palladium catalysts enhanced the production of nitric oxide[12]. With an early post-column reactor design, consisting of a short glass tube, packed with gold filings, and with 1.0 *M* nitric acid as an eluent at 90°C, only easily oxidized compounds produced enough nitric oxide to be detected. Sensitivity was poor and peak response was very irreproducible in repeated analyses.

Oxidations with nitric acid at elevated temperature and pressure have been used in industry to produce oxygenated compounds from hydrocarbon feedstocks[13–15]. The rates of oxidation with nitric acid under these conditions are greatly accelerated at temperatures above 125°C, and do not exhibit an appreciable induction period prior to reaction. Utilization of high temperature and pressure, along with confinement of the gaseous reactants so that they are not removed or vaporized, greatly enhances the oxidation process. A free-radical mechanism has been suggested to explain the autocatalytic nature of these reactions[14]. Such reactions at relatively high temperature and pressure are the basis of the post-column reactor with chemiluminescence detection.

A related system coupling liquid chromatography and the thermal energy analyzer (LC–TEA), has some of the characteristics needed for an LC–RCD interface[16–20], but the TEA detects only species containing nitrogen that produce nitric oxide upon high-temperature pyrolysis. However, in the RCD interface, optimal detection requires keeping the thermal conversion of nitric acid or other nitrogen-containing reagents to a minimum in order to limit background nitric oxide interference. In addition, the large amount of water vapor produced in the TEA pyrolysis reactor, which degrades chemiluminescence detector performance, should be avoided in designing a similar LC–RCD interface[21]. The ability to maintain the flowing stream in the liquid state throughout the reactor system alleviates many of the problems associated with LC–TEA. Furthermore, by providing a nitrogen-containing reagent precursor for nitric oxide, one can detect a much broader range of organic analytes

in LC–RCD. Additional flexibility can be gained by adjustment of reaction conditions, which allows the redox chemiluminescence detector to be operated either as a selective or as a more general detector.

The ability of analytes to react rapidly with reagents in a post-column system is crucial for application to chromatographic detectors. Under dynamic-flow conditions, the LC–RCD reactor must provide sufficient nitric oxide conversion efficiencies to allow sensitive detection of oxidizable species. The low intrinsic solubility of nitric oxide in aqueous solution at room temperature (0.04 ml nitric oxide/ml water) enables efficient separation of the nitric oxide from water via nebulization or purging. For example, purging techniques have been used to determine very low levels of nitric oxide ($< 10^{-13}$ *M*) in water samples by chemiluminescence detection[22]. These factors and other considerations have provided a basis for development of an LC–RCD system.

Removal of nitric oxide from the liquid phase

The efficiency of the gas nebulization device depicted in Fig. 2 for removing nitric oxide from aqueous solution was determined under dynamic-flow conditions. Repetitive 20-μl injections of the dilute aqueous nitric oxide standard were introduced into a flow injection system with a water flow-rate of 1.0 ml/min at 225°C. The peak area response of a known amount of nitric oxide, injected into a dry carrier stream, was compared with that of the aqueous injection to calculate the nitric oxide removal efficiency. The nitric oxide removal efficiency at 225°C was 28 ± 1%. For a given operating temperature, the efficiency of nitric oxide removal was constant and reproducible over several days. Changing the operating temperature of the coil strongly influenced the observed nitric oxide response. Near ambient temperature, less than 5% of available nitric oxide was removed, while at 275°C removal efficiency was nearly 40%. Concurrent with an increase in reactor operating temperature was a steady drop in coil pressure. Decreased gas solubility at high temperature and the change in resistance to liquid flow, which is due to changes in the eluent viscosity, may explain differences in nitric oxide stripping efficiency.

Nitric acid oxidations

Several species were oxidized by dilute nitric acid to give a chemiluminescence response. When 1–5 ng of iron(II), iodide, thiocyanate, thiosulfate, or nitrite ion were injected into a 0.1 *M* aq. nitric acid eluent at 225°C, an nitric oxide response was readily detected. The nitric oxide response from compounds such as hydroxylamine, ascorbic acid, and saccharides was observed at comparable sensitivity. With injections containing 10–25 ng amounts of chromium(III), tin(II), hydrazine, cyanide, azide, citrate, or malate ion, a nitric oxide signal was detectable. Many species were not detected with the flow injection apparatus operating under the conditions specified. Species such as iron(III), cobalt(II), nickel(II), manganese(II), lead(II), mercury(I), copper(I), sodium, potassium, ammonium, nitrate, sulfate, phosphate, phosphite, arsenite, fluoride, chloride, bromide, hypochlorite, acetate, phthalate ion, sodium dodecyl sulfate, and hydrogen peroxide gave no detectable signal when microgram amounts were injected. Not included in this listing are a large number of organic compounds examined which exhibited varying degrees of reactivity. Compounds possessing easily oxidized hydroxyl groups or unsaturated bonds were readily detected.

For many inorganic species, the degree of nitric oxide response appears to be related to their inherent reducing power. Very active reducing compounds as well as a variety of ions in reduced oxidation states react rapidly in the LC–RCD system. The differences in response factors of as much as 10^5 that are observed for certain ions may allow selective detection of strong reductants in complex matrices. For example, the determination of iron(II) and chromium(III) in the presence of iron(III) and other transition metal cations may be possible. Similarly, iodide ion could be selectively detected in a matrix containing other halogen anions. The lack of reactivity for certain buffer anions, such as phthalate ion may allow their use in eluents in ion chromatography.

The reactivity of analytes in the LC–RCD is dependent upon the characteristics of the species being oxidized, as well as the conditions under which reaction occurs. The initial onset of production of nitric oxide is observed at a fairly distinct temperature, characteristic for a given analyte. The response for some compounds varied by several orders of magnitude dependent upon reaction temperature. For example, easily oxidized compounds such as ascorbic acid were detected at *ca.* 150°C, while more refractory species such as tetrahydrofuran required more vigorous reaction conditions of >200°C for appreciable conversion.

In addition to the differences in the temperature of the onset of oxidation, the rate of change of nitric oxide response for organic compounds varies with increasing temperature. In general, the curve for nitric oxide response *versus* temperature begins at a characteristic onset temperature, and increases gradually before a more rapid conversion occurs and the attainment of a maximum peak signal is achieved. Thus, a plot of the temperature dependence of oxidation for different organic compounds would appear as a series of S-shaped curves, after adjustment for the variation in nitric oxide separator efficiency with temperature. The response, and thus the overall shape of the temperature dependence curve for the nitric oxide signal observed from an individual organic compound, is highly dependent upon the inherent reactivity of the analyte. Strong reducing agents, such as iron(II), exhibit similar temperature-dependent behavior, but are more completely oxidized at lower temperature and over a narrower temperature range. In no case, however, was a strong nitric oxide signal detected when heating the nitric acid reagent at a reactor temperature below 125°C, and peak responses for all analytes decreased rapidly below 150°C. In addition, as the reactor temperature was increased from *ca.* 125°C, the onset of a background nitric oxide signal was also observed, the magnitude of which steadily increased as the reaction coil was heated.

Changes in the extent of the thermal decomposition of aq. nitric acid to form nitrogen dioxide at these operating temperatures affects both the background nitric oxide signal and the nitric oxide produced from reaction of analytes. Nitric acid thermally decomposes in the liquid phase to produce nitrogen dioxide[23]. The nitrogen dioxide intermediate may then oxidize analytes present to produce nitrous acid and nitric oxide or undergo a reversible hydrolysis reaction in aqueous solution to produce nitric acid and nitrous acid. Any nitrous acid produced in these reactions is unstable above *ca.* 65°C and thermally decomposes to give nitric oxide. The relative rates of these two competing reactions involving nitrogen dioxide may be the primary determinants of the signal to noise level in this detection scheme. The complexity of reversible equilibria involving aqueous NO_x species at the high temperatures and

pressures present in the reaction coil makes direct experimental interpretation difficult.

The effects of changes in reaction time were evaluated by varying the eluent flow-rate and by use of reactor coils of different lengths. It was observed that the more easily oxidized species exhibited an approximately linear response with changes in reactor residence time, indicating that reactions are far from complete under the conditions tested so far. Certain refractory compounds, which exhibited minimal or no response under normal operating conditions, were much more easily detected when longer reaction times were used.

As an example of the ability of analytes with easily oxidized functionalities to undergo rapid reaction at elevated temperature, nanogram quantities of glucose were oxidized to yield an easily detectable nitric oxide signal, when the flow injection apparatus was operated at 225°C with 0.1 *M* aq. nitric acid. Linearity of response has been determined to exist over nearly three orders of magnitude.

Just as heating the dilute nitric acid generates intermediate nitrogen species that oxidize analytes to produce nitric oxide, irradiation of the aq. nitric acid eluent stream at 254 nm may also result in the net production of nitric oxide from certain analytes. When analytes, such as iron(II) and other strong reducing agents were injected into the irradiated aq. nitric acid stream, a response was noted even at 25°C. However, at this temperature, organic compounds did not react, indicating the need for additional heating to oxidize these compounds in the short times required for detection of chromatographic peaks. A small increase in background nitric oxide signal was observed when the nitric acid was irradiated. Because nitrogen dioxide is formed as an intermediate in the photolysis of nitric acid, it is assumed to be the reactive species in these oxidations, as well as the source of background nitric oxide from any nitrous acid that is produced by the hydrolysis of nitrogen dioxide. The nitrous acid created in all of these processes is readily dissociated to nitric oxide by UV radiation at 254 nm. When the stream was irradiated and subsequently heated, an increase was noted in the nitric oxide response of all analytes relative to that produced by heating alone. The additional availability of reactive nitrogen dioxide or the influence of other reaction processes may change analyte responses. Urea readily reacts with nitrous acid to yield carbon dioxide, nitrogen, and water, which are not detected by the redox chemiluminescence detector. In preliminary experiments, the addition of urea suppressed the analyte signals, but surprisingly had no detectable effect on the background nitric oxide signal observed.

Other reagents for redox reactions

Nitrate ion in neutral aqueous solution was ineffective as an oxidizing agent for all compounds studied, whether used in the thermal or photochemical mode. This observation lends credence to the hypothesis of the formation of a reactive intermediate such as nitrogen dioxide or another active nitrogen species from undissociated aq. nitric acid in solution. Both dilute sodium nitrite and nitrous acid were less active oxidizing agents for the test analytes than nitric acid, as indicated by less formation of nitric oxide. An eluent of 0.1 *M* sodium nitrite reacted with strong reducing agents in the absence of either external heating or irradiation, while heating the sodium nitrite reagent caused an increased nitric oxide background that saturated the nitric oxide detector below 100°C. The background may be due to nitric oxide,

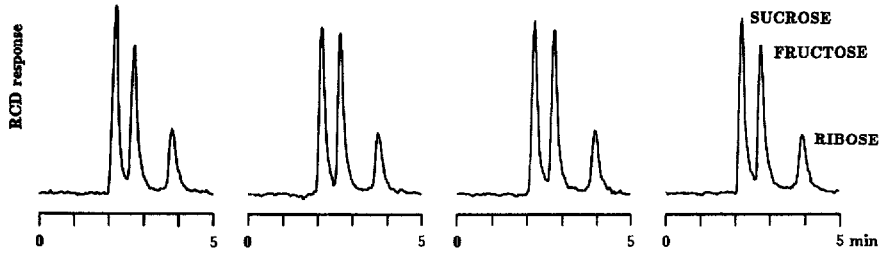

Fig. 3. The nitric oxide response from a series of 20-μl injections containing a mixture of sucrose, fructose, and ribose in water at concentrations of 10.8, 11.1, and 11.2 ppm respectively. Column, reactor, and detector conditions are given in the text.

produced by thermal decomposition of trace levels of nitrous acid in solution. Use of the more dilute 10 ppm sodium nitrite as an eluent reduced background nitric oxide. However, analyte sensitivity was also decreased dramatically. The use of 0.1 *M* aq. nitrous acid eluent was not possible at temperatures above *ca.* 50°C due to the ease of conversion of nitrous acid to nitric oxide. The presence of the large nitric oxide interference at elevated temperatures from these eluents precluded their use as oxidizing agents for the organic compounds tested. Only a few reducing ions [iron(II), thiosulfate and iodide] reacted with nitrous acid at room temperature to give a response. However, irradiation of aq. nitric acid under similar temperature conditions resulted in the reaction of a greater number of inorganic reducing agents. If nitrous acid were the only reactive intermediate formed in the photochemical decomposition of aq. nitric acid, a greater similarity in reactivity with these reductants would have been expected. Consequently, the active intermediate in the formation of nitric oxide from photolytically initiated reactions of nitric acid cannot be nitrous acid alone.

HPLC separation and quantitation of sugars

Chromatograms shown in Fig. 3 indicate the sensitivity and reproducibility in peak shapes for the separation of a mixture of sugars in the LC–RCD detection system. The samples were 20-μl aliquots of a mixture containing *ca.* 10 ppm each of sucrose, fructose, and ribose and were chromatographed using the prepared cation-exchange polymer column. The relative standard deviation of the nitric oxide peak areas for four repetitive injections of *ca.* 200 ng each of sucrose, fructose, and ribose were 3.4%, 5.6%, and 5.7% respectively. Through the use of a longer analytical column, several saccharides that were not efficiently resolved on the short column may be separated. It is interesting to note that no prior derivatization is required for the detection of the sugars and that the sensitivity of this apparatus is comparable to many techniques currently in use for quantitating saccharides. The redox method appears to be especially useful in detection of analytes which lack chromophores.

CONCLUSIONS

Conversion of analytes is known to be dependent on the temperature, the reaction time, contact with catalysts, and the strength of the oxidizing agent used. The reactivity of reagents might be increased by incorporating a catalyst, such as

gold or palladium into the reaction coil, by photolysis, or by a combination of these and other means in future studies. In addition, removal efficiencies for nitric oxide from aqueous streams may be improved with a more effective separator design to increase the sensitivity of detection. Experiments are in progress to enhance analyte sensitivity and to modify the LC–RCD system so that it may be coupled to other LC separation techniques.

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