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SUPERCRITICAL-FLUID CHROMATOGRAPHY–MASS SPECTROMETRY OF HIGH-MOLECULAR-WEIGHT BIOPOLYMERS

INSTRUMENTAL CONSIDERATIONS AND RECENT PROGRESS

DOUGLAS M. SHEELEY and VERNON N. REINHOLD*
Division of Biological Sciences, Department of Nutrition, Harvard School of Public Health, Boston, MA 02115 (U.S.A.)

SUMMARY

Interest in supercritical-fluid chromatography (SFC) of biological samples has recently increased. The following is a summary of recent progress in SFC–mass spectrometry interfacing and its application to the analysis of high-molecular-weight biopolymers. The approaches taken in several interface designs and results obtained with each are presented and compared. Areas which are of special concern in working with high mass, thermally labile, or polar analytes include interface heating and flow restrictor design. These topics are discussed in light of previous work, and directions for further study are suggested.

INTRODUCTION

The structural analysis of biopolymers has been advanced considerably in recent years by the introduction of newer instrumental methods for their characterization. One approach of considerable importance has been the mass spectrometer, using a "soft" ionization technique called fast atom bombardment (FAB)[1]. This ionization method has extended structural understanding to samples of greater polarity and higher molecular weight, and application to post-translationally modified peptides has been most important. For these materials and related biopolymers, other soft ionization techniques[2–4], such as direct chemical ionization (DCI)[5] and laser desorption (LD)[6–8], have also contributed to sensitive methods for understanding sample molecular weights and oligomer sequence. To capitalize on this progress, mass spectroscopists have focused considerable effort on the interfacing of existing liquid chromatographic (LC) systems that resolve these polar and/or higher-molecular-weight biopolymers. Most successful in this regard has been the technique of "flow" or "dynamic" FAB[9].

As important as these LC systems are in biological research, their mass spectrometric (MS) interfacing imposes serious instrumental constraints, especially when coupled with FAB desorption. As a method for ion generation, FAB is a very selective technique, a problem very apparent when analyzing samples composed of mixtures. With these samples, product ions are usually a function of surface activity

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not sample concentration, and when compared to other ionization techniques, FAB remains insensitive. For most lipophilic samples, FAB is a very ineffective method of ionization, and frequently unsuccessful. These problems can be related, in large part, to the desorption matrix which causes a biased presentation of the sample. The problems are minimized in "flow FAB" by dynamic surface motion and a lower concentration of matrix[10]. The problem is also diminished in FAB desorption from a polyimide belt[11] which does not require an applied matrix and appears to avoid sample suppression. But this high-performance liquid chromatographic (HPLC) belt interface is expensive, appears to have high mass limitations, and is technically demanding. DCI, as an alternative approach, does not require a desorption matrix and samples are always detected. However, heat-initiated desorption causes pyrolysis of many highermolecular-weight and non-volatile materials, which limits its applicability in these areas.

A separations technique that has received relatively little attention for the analysis of biopolymers has been supercritical-fluid chromatography (SFC). This approach, combined with the low flow-rates of capillary columns[12–14] and the ease of solvent elimination provides features most appropriate for MS interfacing[15–17]. Specifically, these factors are: (a) the utilization of column efficiencies provided by enhanced analyte diffusion coefficients, (b) a smaller volume of mobile phase that is more effectively pumped, (c) the avoidance of high temperatures and thermal limits experienced with gas–liquid chromatography (GLC) and some LC–MS interfaces; and, probably the most important, (d) a possibility for capturing the extreme sensitivities of negative-ion chemical ionization by utilizing derivatization protocols developed in GLC–MS.

Most applications of SFC–MS have been limited to non-polar analytes of low mass. Higher-molecular-weight samples of limited polarity, such as fatty alcohols, ethoxylated alcohols[18,19], and mono- and diglycerides[18,20], however, have provided most interesting results. Unfortunately, the poor solubility of peptides, nucleotides, and oligosaccharides in CO_2 has precluded a direct application of SFC to these important biomolecules. Derivatization has been used in GC to overcome sample polarity, and enhance volatility and thermal stability. Similarly, for SFC, analyte derivatization has served to increase solute miscibility[22]. Using this strategy, researchers have reported success in the study of sucrose polyesters[22], glycosphingolipids[23] and N-linked glycans from glycoproteins[24], as well as SFC–MS of glucose homopolymers in excess of 5 kD[25]. Two cyclic peptides also have been studied by SFC–MS; these samples, however, were not derivatized[26,27].

The study of biopolymers by SFC has been very limited, and derivatization schemes to broaden the application are not usually considered. Thus, applications are limited to those analytes that are miscible in the more convenient mobile phases; a feature constrained by the relatively few gases possessing critical points in a workable temperature-pressure range.

Considering the advantages SFC can bring to biopolymer separations, the structural insight provided by SFC–MS, and with the expectation of improved selectivity and sensitivity; we would suggest applications to problems in biomedical research and molcular biology to be well served. In this regard we have discussed some of the critical aspects of SFC and MS interfacing, the limitations, current progress, and perceived advantages, in the hope others may share their problems and progress.

INSTRUMENTAL CONSIDERATIONS

An excellent review discussing the basic principles of SFC–MS interfacing has recently been published[28]. This summary is therefore focused on those features particularly significant to the characterization of high-molecular-weight samples, a focus that relates immediately to the design and fabrication of flow restrictors. In previous MS interfacing an assortment of designs have been used (Fig. 1), varying principally in the shape and length of the restricting region. The simplest restrictors are fabricated from deactivated capillary tubing and coupled with a zero dead volume union to the unmodified column tip. The volume of effluent passing through the restrictor is proportional to its length and internal diameter. The platinium–irridium crimped tube is an example of a simple restrictor which can be effective but is limited with problems of reproducibility. Laser-drilled disks are easy to prepare, but this device is more technically demanding because of the difficulty in mounting and alignment. Considering the several types of restrictors that have been employed, three are most commonly used: the multipath frit[29], the robot-pulled tapered restrictor[22], and the integral restrictor[30]. Both the frit and tapered restrictors are relatively long, while the integral and laser-drilled disk restrictors are short. This design aspect has significant bearing on sample transmission and ionization, for it relates directly to mobile phase density and analyte solubilities.

These features can be appreciated better by considering the principles of hydrolics and the equation of continuity (Fig. 2A). From these relationships it can be expected that as a conduit is restricted, the velocity of a passing fluid must increase to maintain a constant flow. Under such circumstances, Bernoulli's law requires that this increase in flow velocity must result in decreased pressure at this point (Venturi effect, Fig. 2B). This pressure decrease is important because the solvating power of a supercritical fluid is dependent on density and therefore pressure. With pressure decreasing through a finite restrictor, this presents an opportunity for analyte nucleation (*e.g.*, association and/or precipitation), to occur previous to orifice exit and

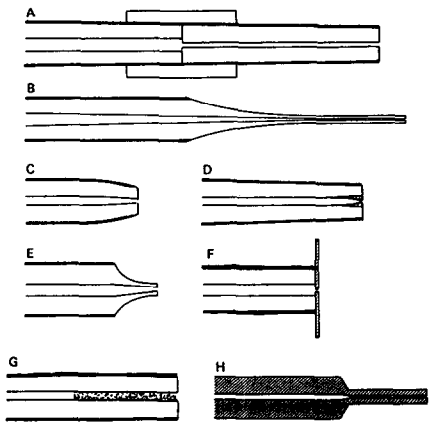

Fig. 1. Examples of flow restrictors used for SFC. (A) Straight capillary, (B) robot-pulled tapered capillary, (C) Guthrie and (D) deposition integral restrictors, (E) quick-drawn capillary, (F) laser-drilled pinhole, (G) multipath frit, (H) pinched tube. Reprinted with permission from ref. 28.

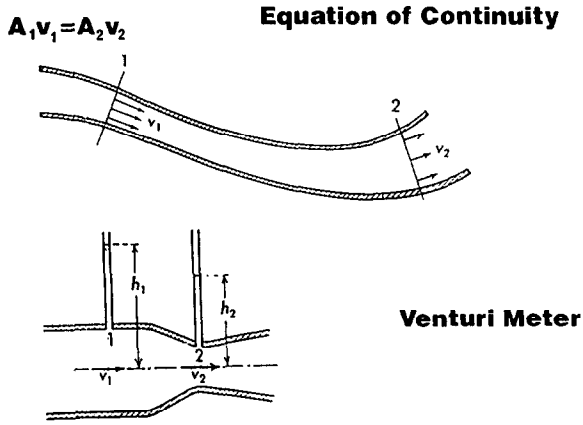

Fig. 2. (A) Schematic of a restricted conduit, showing that as diameter decreases, velocity of the moving fluid must increase. (B) Venturi meter, demonstrating Bernoulli's principle, that as velocity of a fluid increases, its pressure decreases.

analyte desolvation. Analyte solubility, transit time in the restrictor, desolvation characteristics, and the methods used for eluant measurement make this area very difficult to approach on first principles. However, some indications of nucleation may have been observed experimentally by flame ionization detector (FID) spiking when using different restrictors, pressures, and temperatures[31]. The relationship of restrictor design, analyte solubility and nucleation to the observed experimental factors of FID spiking, restrictor plugging, and losses in sensitivity may not be as clear as one would wish. But, from the above, it does suggest that transmission and ionization at high mass may be directly related to mobile phase density, which can change markedly in restrictors.

APPLICATIONS

Previous reports of high-molecular-weight SFC–MS transmission have utilized different samples, restrictors, and diverse strategies for interface and restrictor heating. Thus, it is difficult to make direct comparisons and draw solid conclusions about the selective advantages of each. Additional differences lie in the variety of mass spectrometers utilized and the degree of instrumental modification required. In the work summarized below, some of these differences have been mentioned.

In a study of peptide SFC–MS, Huang *et al.*[27], working with a double focusing instrument (Finnigan-MAT Model 8430), have recently presented chemical ionization (CI) data for valinomycin, a cyclic peptide in excess of 1100 a.m.u. (Fig. 3). This spectrum was obtained in the negative-ion mode, using methane as reagent gas. When additional amounts of CO_2 were added to the reagent gas, greater structural detail was obtained due to charge exchange fragmentation[32]. These data were obtained using a multipath frit restrictor, maintained at 350°C, and a source temperature of 280°C. In this interface the high-temperature region (350°C) was limited to the frit portion of the restrictor. A diagram of the SFC–MS interface is presented in Fig. 4.

Additional cyclic peptide data has been presented by Kalinosky *et al.*[26] utilizing

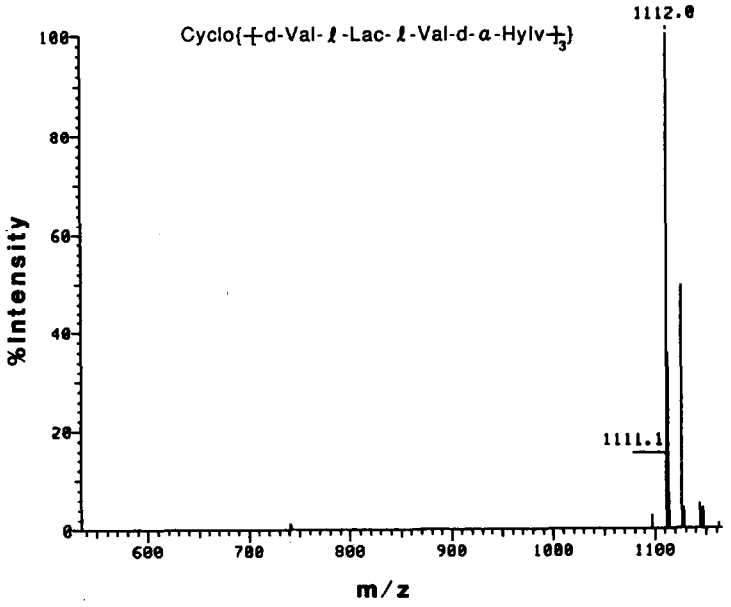

Fig. 3. The methane CI mass spectrum of valinomycin, obtained by SFC–MS, using a multipath frit restrictor maintained at 350°C. Reprinted with permission from ref. 27.

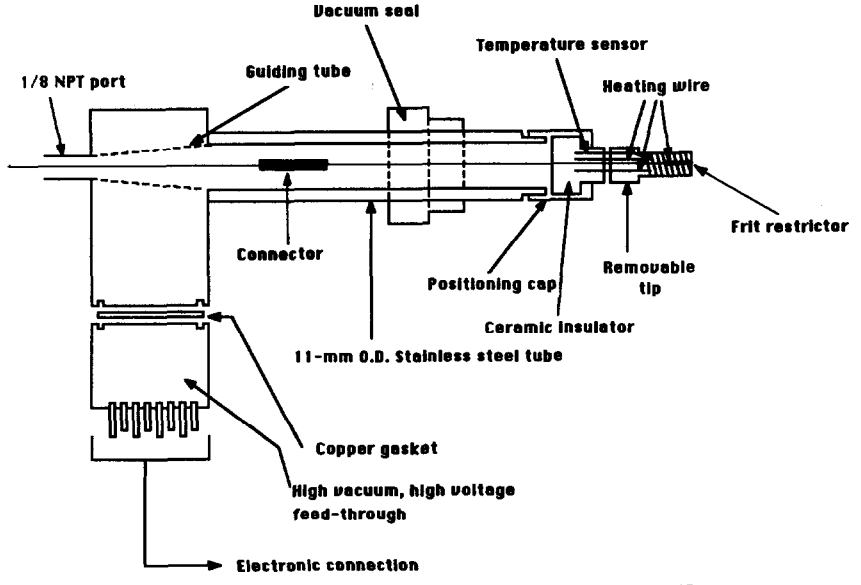

Fig. 4. Schematic diagram of the SFC–MS interface used by Huang *et al*[27]. The column is maintained at oven temperature up to the restrictor region, which is heated separately. Reprinted with permission from ref. 27.

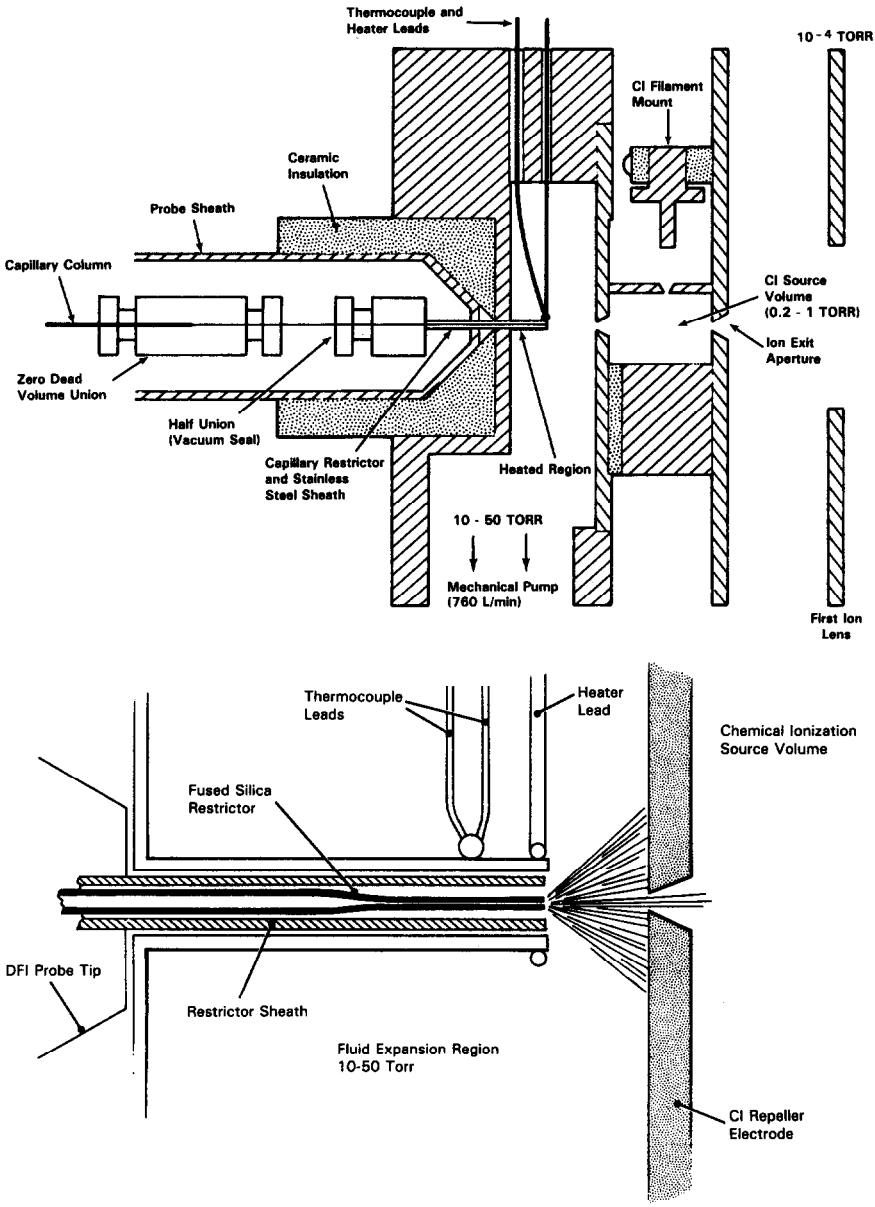

Fig. 5. Schematic of the "high-flow-rate" interface of Smith and Udseth[21]. The source is differentially pumped to accomodate the high flow-rates of packed-column SFC. Restrictor heating occurs in the last 7 mm. Reprinted with permission from ref. 21.

packed SFC columns and a "high flow-rate" interface[21] (Fig. 5). This interface, designed to accomodate the faster flow-rates of packed columns, was combined with an integral restrictor and heated along the last 7 mm of the restrictor to about 145°C. The peptide, cyclosporin, was chromatographed in a CO_2 mobile phase doped with

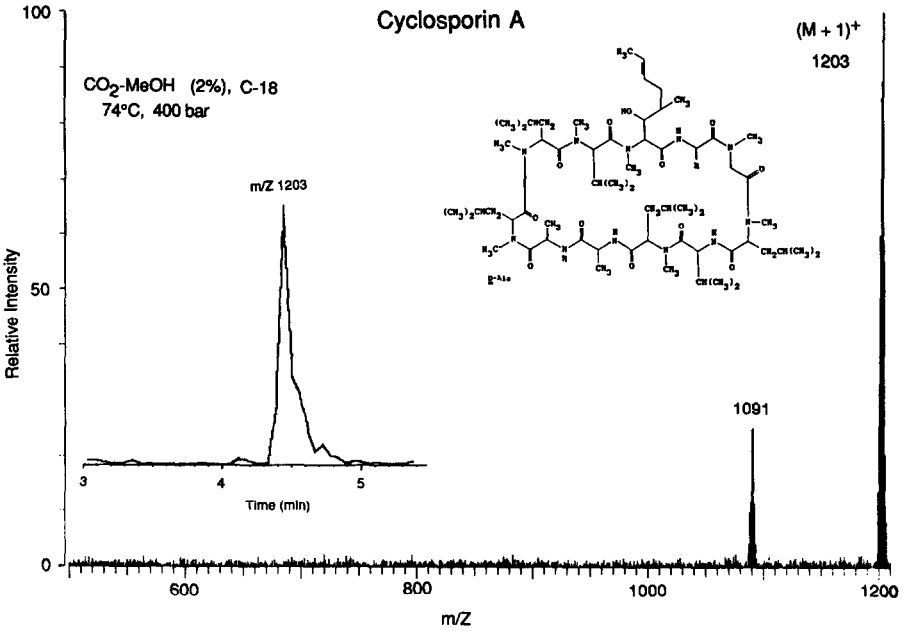

Fig. 6. Selected ion chromatogram and CI mass spectrum of cyclosporin A obtained using the "high-flow-rate" interface with an integral restrictor heated to 145°C. Reprinted with permission from ref. 26.

2% methanol and analyzed by CI-MS. The selected ion chromatogram $(MH)^+$ and the mass spectrum are presented in Fig. 6. A notable difference between this interface, (integral restrictor), and the previous frit restrictor[32] is the lower temperature requirements for molecules of similar structure. Higher flow-rates, however, would be expected to improve transmission and thereby demand less thermal assistance.

Methanol was used as a modifier in the latter case and also served as a convenient CI reagent gas, providing abundant protonated molecular ions, $[M+H]^+$, while still showing adequate fragmentation to study molecular detail.

High-molecular-weight waxes have been analyzed by SFC–MS utilizing an unmodified GC transfer line (Hewlett-Packard Model 5985B GC–MS system)[34]. These authors have demonstrated transmission and characterization of components weighing about 950 dalton. An integral restrictor fabricated directly on the column end was inserted through the transfer line, and heated to one hundred degrees above column temperature, which for the separations demonstrated, was 125°C (interface temperature 225°C). Fig. 7 is the mass spectrum obtained from a component detected in beeswax by methane chemical ionization. The authors have tentatively identified the sample as a dipalmitoyl ester of a C_{24} diol.

Chester and Innis[33] and Pinkston et al.[35] have extended high-molecular-weight studies to carbohydrates using trimethylsilylation to block the polar hydroxyl groups. This relatively simple method of depolarization also insures mobile phase solubility. SFC–MS of a series of glucose polymers prepared in this manner has extended high mass transmission and detection to approximately 3000 Dalton. CI spectra were obtained with a quadrupole (VG 30-250, VG Masslab) instrument using ammonia as

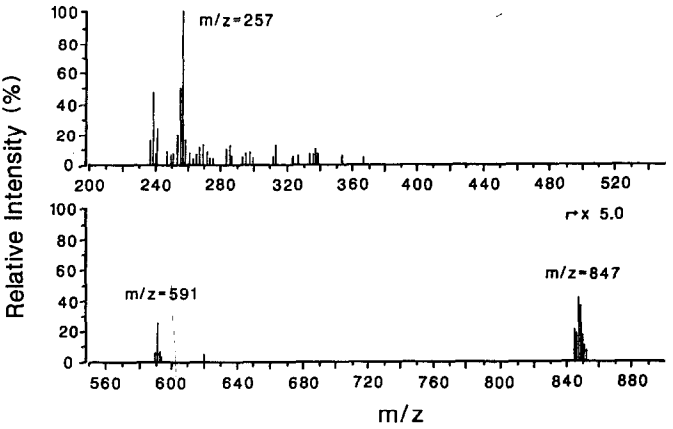

Fig. 7. Mass spectrum of a component from beeswax, obtained using an HP 5985B GC–MS, with an integral restrictor introduced through the normal GC inlet, heated to 225°C. Reprinted with permission from ref. 34.

the reagent gas[35]. The $[M+NH_4]^+$ ions were detected up to *m/z* 2830 which was the expected ion for $(Glc)_7$ of this homopolymer series (Fig. 8). This mass range approximates the detection limits of the quadrupole mass spectrometer and is not a constraint imposed by the interface. For these studies, a robot pulled tapered restrictor, fabricated on site, was used. This interface (Fig. 9), was designed to maintain temperatures equal to that of the column in one zone and heated to 350°C in a second zone for a length of about 4 cm where restriction occurs.

As an extension of the study by Pinkston *et al.*[35], we wished to evaluate two major points using a high voltage magnetic instrument: (i) were there mass limitations to sample desolvation and ionization; and, (ii) what were the temperature requirements for the interface and restrictor. Imposed on these primary concerns were additional points that for successful application to biopolymers, both processes should be demonstrated on thermally labile and high-molecular-weight samples at sensitivities comparable, at least, to that realized by FAB.

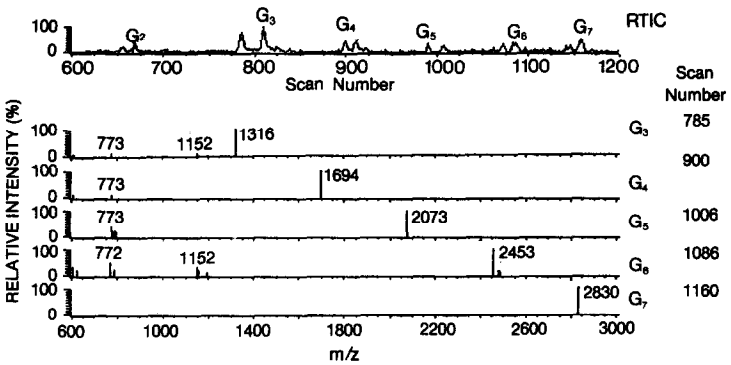

Fig. 8. Total ion chromatogram and selected ammonia CI mass spectra of a pertrimethylsilylated glucose homopolymer mixture. A robot-pulled tapered restrictor was used, at a temperature of 350°C. Reprinted with permission from ref. 35.

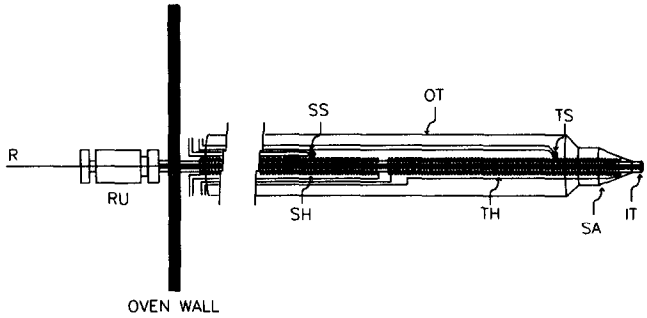

Fig. 9. Diagram of the interface used by Pinkton *et al.*[35]. Reprinted with permission from ref. 35.

For this work[25] a high-performance mass spectrometer was used (VG ZAB-SE, VG Analytical), operating at an ion source potential of 8 kV. Presented in Fig. 10 is a drawing of the standard DCI probe modified with an additional ceramic feed to accept the SFC capillary column. A platinum heating wire of the same type used in the standard probe is wrapped in a coil directly around the column end. Heating was necessary and supplied to the last millimeter of column, corresponding to the length of the integral restrictor fabricated at that point. In this design restrictors may be changed without disruption of the heating wire. The precise temperature at the restrictor tip has been difficult to assess because of the extremely small area of the heated region, but the platinum wire temperature under normal operating conditions was estimated to be 280°C based on its length, resistance, and the current supplied by the DCI control unit. Lower currents resulted in tip plugging and the operating current was set slightly above this point. Over a rather broad range above this temperature, no discernible effect on the eluting spectra was detected, although excessive heating currents were not

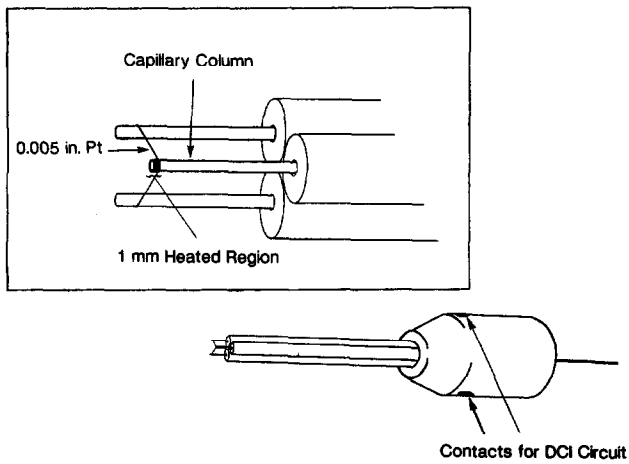

Fig. 10. Diagram of the SFC–MS probe from ref. 25. The standard DCI probe tip is configured with additional ceramic feed to accomodate the SFC column. Contact points privide electrical coupling to tabs on the side of the ion source to control temperature. The inset shows the enlarged tip with the capillary column and heating wire.

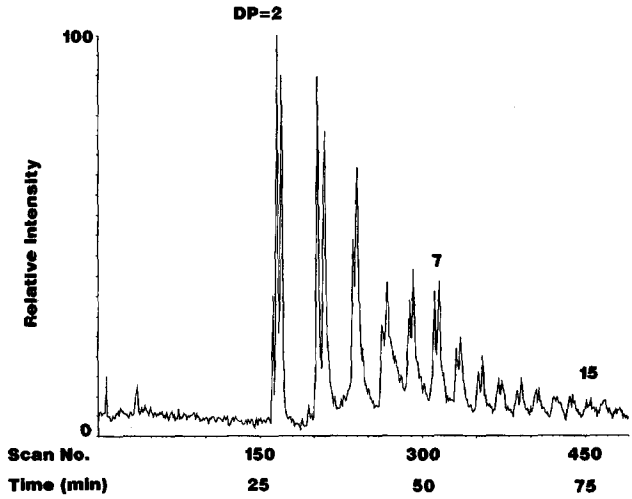

Fig. 11. Total ionization plot of pertrimethylsilylated glucose polymers. Reprinted with permission from ref. 25.

evaluated. The temperature experienced by analytes on passing the restrictor zone must be somewhat lower than that calculated above, due to insulation by the column wall and tip cooling contributed by mobile phase vaporization.

To assess the effectiveness of this restrictor for analyte transmission and ionization, the same glucose homopolymer described previously[35] was prepared and analyzed by SFC and SFC–MS (Fig. 11). A comparison of total ionization plots with FID signal suggests that sample transmission and ionization efficiency is maintained from the low to high mass oligomers. Profiles of individual products in the homopolymer series, $[M + NH_4]^+$, for this sample are presented in Fig. 12 which shows normalized adduct ions from DP2 to DP15. The chromatographic fidelity is very apparent with anomer separation still observable at DP15. Fig. 13 is the mass spectrum for DP15.

The overall sensitivity of this interface was studied by use of known concentrations of cyclodextrin which have been permethylated previous to analysis. Shown at

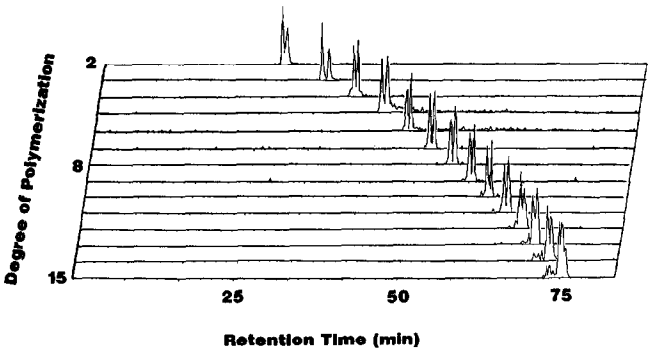

Fig. 12. Selected ion profile of trimethylsilylated glucose polymers. Reprinted with permission from ref. 25.

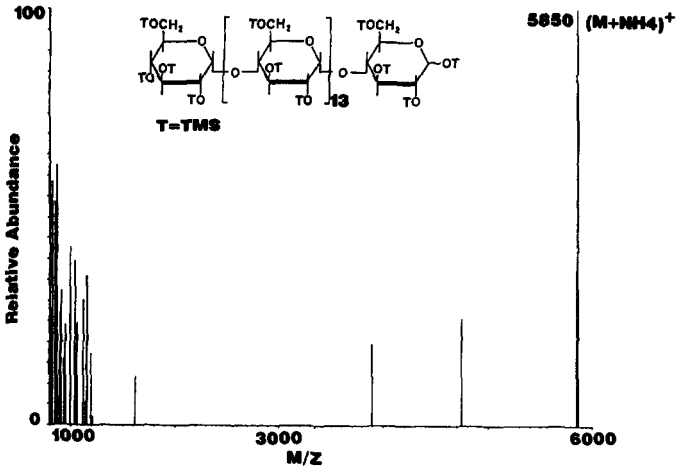

Fig. 13. Mass spectrum of trimethylsilylated glucose oligomer, DP15. Reprinted with permission from ref. 25.

the top of Fig. 14 is the total ion profile (TIP) of 100 ρmol of cyclodextrin. Panel 14B is the ammonium adduct ion profile, m/z 1446 $[M + NH_4]^+$, for that sample. The limits of detectability appear to approach 2 picomol. At this concentration no peaks were detected in the TIP although the ion was easily detected at this concentration (Fig. 14C).

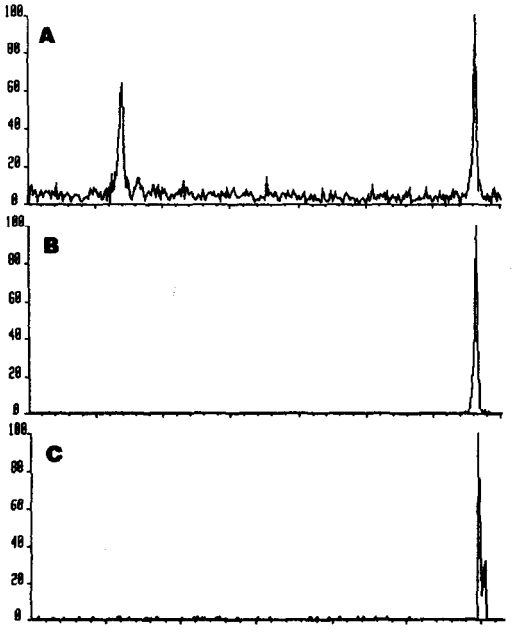

Fig. 14. SFC–MS: (A) total ionization plot for an injection of 150 ng permethylated β-cyclodextrin; (B) Selected ion chromatogram for molecular ion adduct $[M + NH_4]^+$, m/z 1446, for the above injection; (C) selected ion plot of molecular ion adduct $[M + NH_4]^+$ for a 3-ng injection. Reprinted with permission from ref. 25.

DISCUSSION

A practical observation of MS interface operation, that increased restrictor temperatures improve sample detection (up to a limit), suggests that nucleation is occuring. For those compounds that are thermally stable, heating would enhance analyte volatility and thus restrictor transmission. Extensive restrictor heating, however, circumvents one of the advantages offered by SFC, and more importantly, would be self-limiting with thermally labile and/or polar materials which potentially could be chromatographed in more polar mobile phases. In the current context, it would seem that the lower temperature requirements of the integral or disk restrictors, (the shortest of the three), bears out this reasoning. Since abrupt restriction is desirable, (in order to minimize solute precipitation and the necessity for extensive heating), the laser drilled disk would appear to be an ideal restrictor[36], although difficult to fabricate. Subsequently, we have made extensive use of the integral restrictor as the "next best thing" for high mass transmission during SFC–MS interfacing.

Much of the earlier work in SFC–MS has been carried out with low-voltage quadrupole instruments[25,35,36] and from these reports it was not clear whether there were high-mass limitations to detection. Another concern related to high-molecular-weight studies and SFC interfacing was the use of high-voltage sector instruments and the possibility of extensive arcing of the ion source. Two recent reports of interfaces with sector instruments, one operating at 8 kV[25,27] have provided some assurance that this is not a problem.

One of the most obvious features of CI spectra obtained by SFC–MS is their strong molecular ions, with little or no fragmentation. There are a variety of alternatives for developing structural information from these systems, and one's choice would depend largely on its appropriateness for particular classes of compounds. Charge exchange from CO_2 has already been mentioned as a useful tool for obtaining structural detail, and seems to parallel electron impact ionization in the fragmentations it induces[37]. Use of other appropriate CI reagents has potential for providing greater structural detail as well. Work in this laboratory with glycosphingolipids has indicated that while ammonia CI provides molecular ions, with little fragmentation, use of a mixture of CO_2 and methanol as CI reagent can induce very informative fragmentations in this particular class of molecules. Certainly there is much to be done in this area.

Another approach to eliciting structural detail from SFC–MS is through collisionally induced dissociation (CID) of molecular ions. In this case, fragmentation in the ion source is not desirable, because a strong signal for the parent ion is necessary for CID. Here an interesting question is whether CID can be performed on transient CI product ions, and whether collisions in a low-voltage cell would be more desirable than collisions at high energy. Addressing the first question, Hurst[38] has performed MS–MS experiments on SFC–CI-MS generated ions using a TSQ 70 quadrupole, and in preliminary experiments in this laboratory, using direct chemical ionization, metastable ion kinetic energy spectra (MIKES) have been obtained for ammoniated molecular ions of 3500 daltons[39]. The second question, about the informativeness of various collision processes, is more complex, but there are certainly advantages to use of lower collision energies and even chemically reactive reagents in the collision cell.

Obviously, SFC–MS has not caught up with GC–MS or even LC–MS, but it has great potential in its application to biological research problems; an area in great need of all the analytical assistance it can receive. There appear to be no significant mass limitations, at least up to 6000 dalton, and most current difficulties are purely technical and will, more than likely, be resolved. One important area in need of work is in the design of an effective restrictor, which should require far less heating. This could improve SFC–MS both by making the interface design simpler, and by providing broader applicability to materials of biological interest.

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