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Biochemical and environmental applications of microcolumn liquid and supercritical fluid chromatography*

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Abstract: Recent trends in microcolumn liquid chromatography and capillary supercritical fluid chromatography are reviewed, emphasizing research carried out in the author's laboratory. Current and potential applications of these methods in biochemistry, medicine and environmental science are stressed.

Keywords: *Microcolumn liquid chromatography; capillary supercritical fluid chromatography; complex mixtures; enhanced mass sensitivity; novel detectors; laser fluorescence detection.*

Introduction

Microcolumn liquid chromatography (LC) and capillary supercritical fluid chromatography (SFC) are two relatively young analytical separation methods that share some common instrumental features and scope of application. Both approaches have been gaining in popularity during recent years. Microcolumn LC has evolved as an important development in separation science following the description of microbore columns by Scott and Kucera [1, 2], the development of miniaturized LC components by Ishii *et al.* [3], and the introduction of capillary columns (of both the open-tubular and packed-capillary varieties) by Tsuda and Novotný [4, 5]. With the gradual recognition of the various advantages of miniaturized LC, numerous other laboratories have quickly moved into this general area and enriched the field with new ideas and instrumental technology. The first report on capillary SFC [6] appeared a few years after the introduction of microcolumn LC. Interest in this technique is currently high due to its many unique analytical advantages, as well as the recent surge of general interest in the chemistry and technology of dense gases and supercritical fluids.

While most laboratories working on SFC are currently preoccupied with research into the fundamentals, microcolumn LC has already matured to the point where the initial technical difficulties have been adequately resolved, so that unique applications are becoming feasible. Both separation techniques have borrowed substantially from certain

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column and detection technologies of modern gas chromatography (GC). The rationale behind their further development and application includes: (a) the design of high-efficiency systems; (b) mobile-phase economy; and (c) improvements in detection capabilities. Currently there are three books [7–9] which review the most important aspects of microcolumn LC. This article will briefly discuss the most significant trends in microcolumn LC and SFC, leading to some unique applications in biochemistry and environmental science.

High-resolution Separations

The enormous complexity of most biological materials is now well-documented through the extensive use of capillary GC (cf [10, 11]) and high-resolution electrophoretic techniques [12–14]. With the rapidly increasing emphasis on the multicomponent analysis of non-volatile mixtures, there is a growing concern about the need for similar resolving power in the area of LC. During the last decade, chromatographic profiles of endogenous metabolites have been acquired primarily through the use of capillary gas chromatography because of its extremely high resolving power and capability for both universal and selective detection. However, problems are frequently encountered, as non-volatile mixtures of polar metabolites (such as peptides and amino acids, urinary acids, steroidal conjugates and prostaglandins) are difficult to analyse by gas-phase methods without tedious derivatization techniques. In addition, certain labile biological molecules are difficult to quantitate at trace levels due to irreversible adsorption and decomposition problems encountered with GC. Pending the availability of suitable detection methods and high chromatographic performance, the use of condensed mobile phases (either liquids or supercritical fluids) should be more attractive for the separation and analysis of these non-volatile biological mixtures.

Various compounds of environmental and technological interest are often found in complex sample matrices. Some environmental pollutants are directly encountered in mixtures (e.g. halogenated biphenyls, or various polycyclic aromatic compounds originating from combustion processes). Alternatively, the primary pollutants emitted into air or water systems can undergo numerous chemical reactions which are affected by light, biological transformations, the presence of naturally occurring substances, etc. Such processes may generate additional substances to be monitored and, consequently, lead to the need for more efficient separations. In addition, different sample components occurring in cases of multichemical contamination may span a wide range of molecular weights, polarities, and chemical features [15]. Thus, a comprehensive approach to sample characterization will also necessitate techniques with great resolving power.

With biological and environmental samples being the most representative examples, resolution problems in complex mixtures have received increasing attention lately. From the theoretical point of view, these important questions have been addressed by Davis and Giddings' statistical theory [16], emphasizing further needs for increased separation efficiency and additional retention principles. Likewise, much interest has lately been expressed in the use of multidimensional LC techniques [17], which could significantly augment 'brute force' (i.e. high plate numbers) in the resolution of complex mixtures.

Having outlined the needs for improved component separation, attention will now be given to the current and future role of microcolumn LC and capillary SFC in this regard. The importance of their high separation efficiency was already noted by Scott [18] shortly after the first description of microbore columns, when he stated that, ". . . today, such

efficiencies are novel and are required only in a minority of circumstances. As the technique develops and expands into the field of biological materials, very high efficiency columns will no longer be a luxury but a necessity." However, only recently have the resolution advantages of microcolumns been realized in a variety of practical examples [19–23], using slurry-packed capillaries [24, 25] to achieve efficiencies in excess of 250 000 theoretical plates within a total analysis time of several hours. An example is shown in Fig. 1, which features the resolution of numerous fluorescent components from the aromatic fraction of carbon black [23]. As evidenced by mass-spectral data obtained for the individual fractions, numerous close isomers have been adequately resolved at this high resolution.

Considerable confusion still exists as to why higher efficiencies are obtained with totally packed microcolumns (of ≤1 mm i.d.) compared with conventional (4.6 mm i.d.) packed columns. While the early theoretical predictions concerning the column length

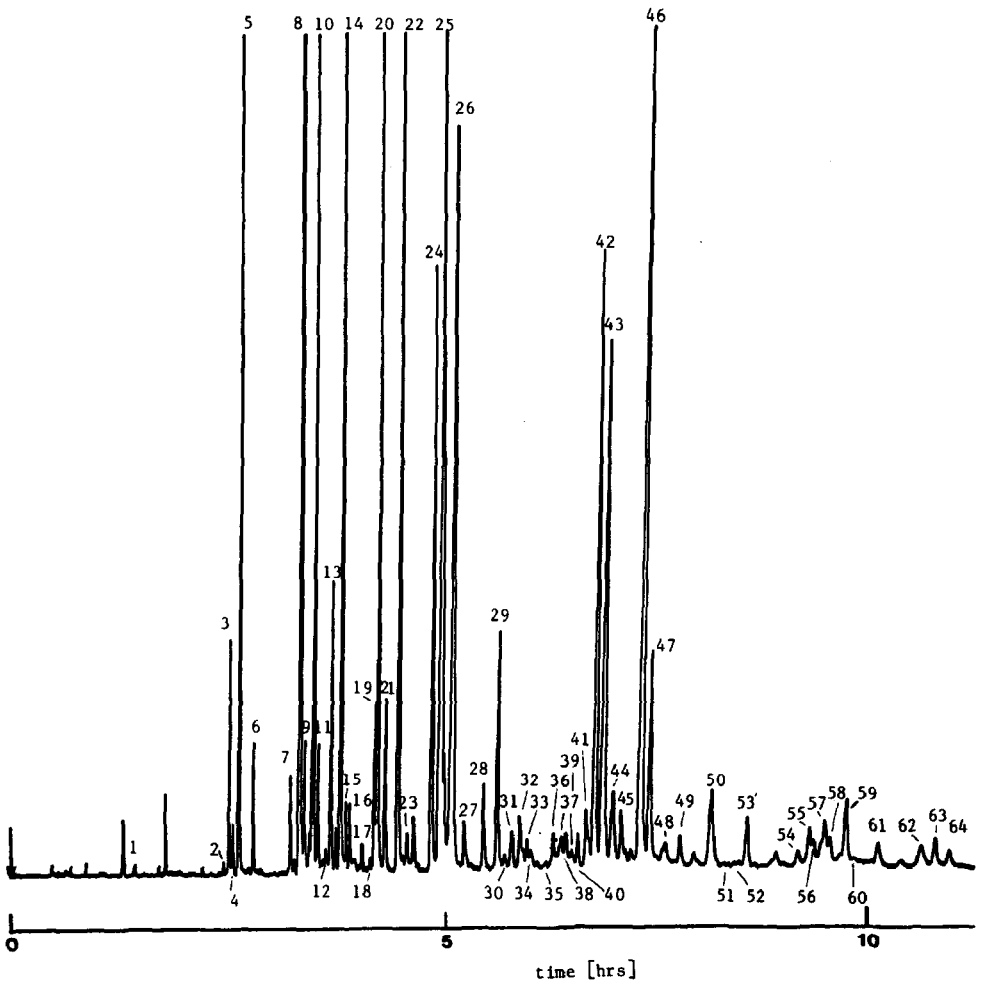

Figure 1
High-resolution chromatogram of heavy polyaromatic compounds (5- through 9-ring structures) extracted from carbon black, using a slurry-packed capillary LC column. Reproduced from [23] by permission of Pergamon Press.

and particle size in LC [26, 27] are approximately valid, and no currently known theoretical reasons directly implicate the column diameter in efficiency considerations, *practical results* obtained with microbore and slurry-packed fused-silica columns clearly show their technological advantages. In simple terms, although all of these column types can be effectively packed to achieve reduced plate-height values close to theoretical limits, microbore and packed fused-silica capillaries can be packed in greater lengths and joined together with greater ease. The column diameter may, however, be critical in work with very small particles, where problems arising from the heat-of-friction [28] may rule against columns of larger diameter.

Open tubular columns of very small radius and semipermeable packed capillaries still remain potential column types for the highly efficient separation of very small samples, even though enormous technological difficulties, as well as sampling and detection problems, currently hinder any progress in this area. It should be noted, however, that gradual advances are being made with both column types [29, 30]. A detailed treatment of LC in columns of capillary dimensions is given elsewhere [31]. Eventually, if developed to their full potential, open tubular columns could provide superior efficiencies, of the order of 10^6 theoretical plates. Meanwhile, the other 'simpler' column types having plate numbers between 100 000 and 500 000, as discussed above, open up numerous exciting prospects for the analysis of complex mixtures.

Once a suitable column type is employed, it is entirely feasible to record complex profiles of biologically or environmentally important substances using miniaturized LC detectors, provided such compounds absorb light in the UV, fluoresce, or are electrochemically active. As this is not the case with many endogenous metabolites, the compounds of interest must often be derivatized to incorporate a detectable moiety into their structures. Various pre-column derivatization techniques are of primary interest, although post-column arrangements may occasionally be feasible for microbore columns [32]. Several simple pre-column derivatization schemes have been considered in the author's laboratory [19–21] for the detection of biologically important compounds such as steroids, bile acids and prostaglandins. An example is shown in Fig. 2, which illustrates two different urinary steroid profiles of interest in biomedical investigations [20]. Although the same samples can also be effectively profiled by capillary GC [33], microcolumn LC is highly complementary in detecting several previously unobserved metabolites.

Further searches for suitable pre-column derivatization methods to enhance the detection of isolated classes of biologically important molecules will undoubtedly be stimulated by advances in column performance and sampling modes. A preliminary example from this area is given by a chromatogram of model prostaglandins (Fig. 3), tagged using a newly developed fluorescent derivatization agent [21]. A pre-column (heart-cutting) arrangement [34] has been used here to remove the excess reagent.

By way of comparison, a brief discussion will now be given on the potential of capillary SFC in the analysis of complex mixtures. As pointed out in the initial paper on capillary SFC [6], favourably large solute diffusion coefficients combined with relatively low mobile-phase viscosities and small pressure drops make this technique extremely attractive for high-efficiency modes of operation. A subsequent optimization study [35] indicated that, once again, efficiencies of the order of 10^6 theoretical plates should be feasible. Recent detailed investigations of mass-transfer processes [36–38] in open tubular columns coated with immobilized polymeric layers appear to substantiate these predictions.

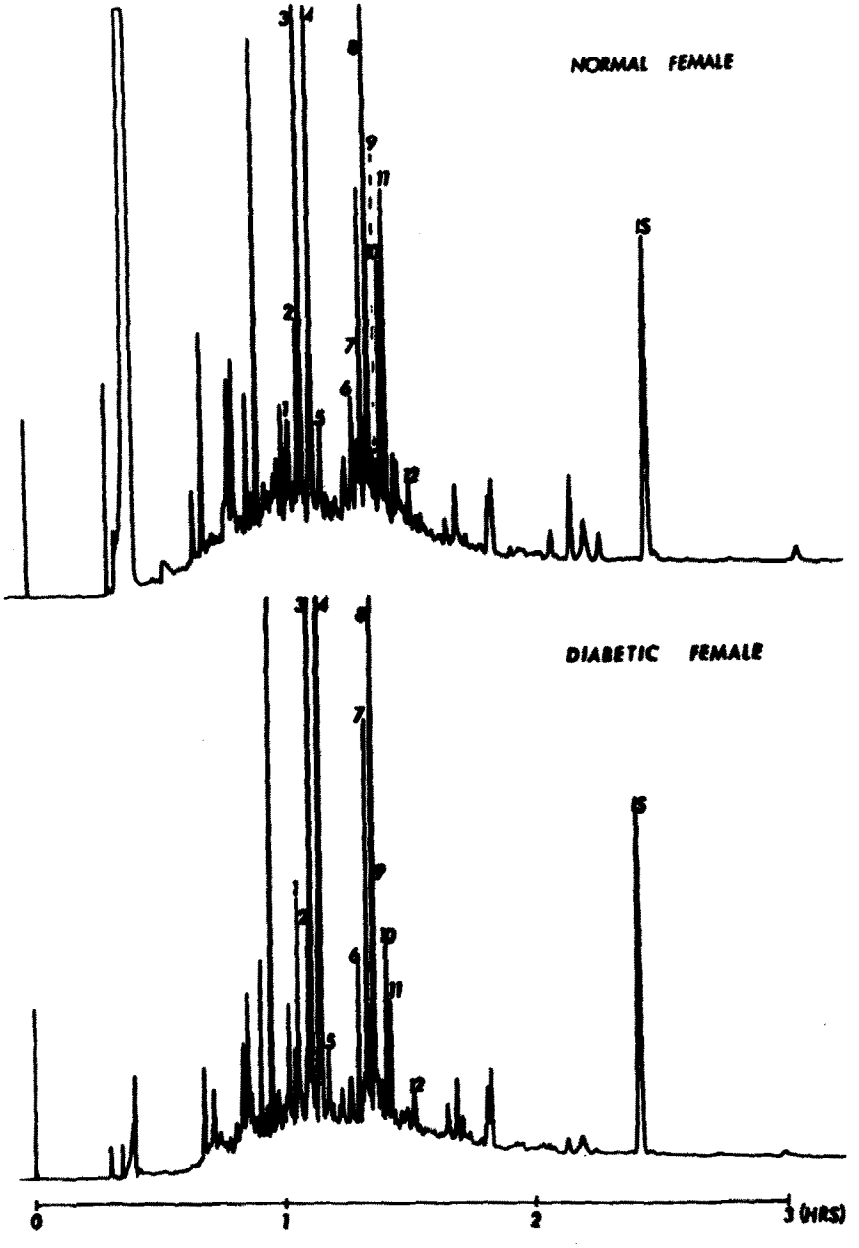

Figure 2
Comparison of the representative metabolic profiles of urinary hydroxy steroids (after sample benzoylation) from a normal and a diabetic human female, obtained by microcolumn LC. Reproduced from [20] by permission of the American Chemical Society.

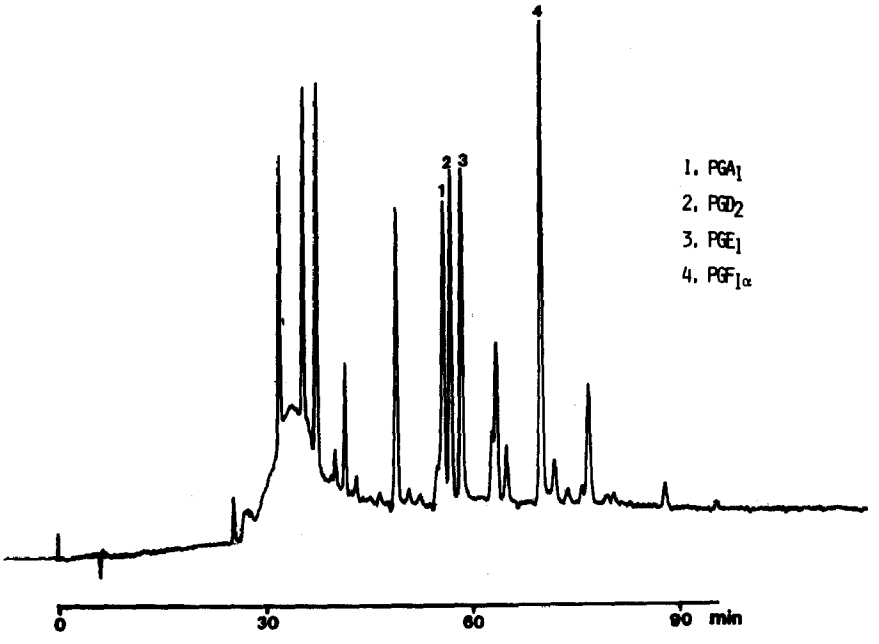

Figure 3
Chromatogram of model prostaglandins, tagged with a new fluorescent reagent [21] and separated with a C_{18} microcolumn.

While capillary SFC, in terms of its practical application, may lag somewhat behind the current achievements of microcolumn LC, projections in this area are highly optimistic. Recently published chromatograms of coal-tar aromatics [39] and carbon black extract [40] demonstrate that complex mixtures can, indeed, be handled using this new technique. While the efficiencies currently obtained are roughly comparable with those of microcolumn LC, as discussed above, there is still much room for improvement in SFC. As discussed below, the ultimate advantages of each of these techniques may not reside in the column efficiency aspects, but rather in their overall instrumental capabilities, including detection possibilities. Both microcolumn LC and SFC, with their extremely low flow-rates, can readily utilize 'exotic' or environmentally offensive mobile phases. However, until the ability to work with more popular phases, such as supercritical ammonia, is fully developed in SFC, the general solute solvation capabilities of LC make the latter technique unquestionably superior for the separation of polar molecules.

While the detection aspects of capillary SFC will be addressed in more detail below, it should be noted that currently available detectors already make numerous applications feasible. As an example of a natural product separation, Fig. 4 shows the capillary SFC separation of some UV-absorbing substances isolated from a culinary spice [41]. An example of an environmental application, a chromatogram of toxaphene [42] recorded on a capillary SFC–mass spectrometry system, is given in Fig. 5.

Improved Detection Mass Sensitivity

As the geometrical column parameters, such as the column diameter and/or the particle size, are decreased, a significant reduction must also occur in the injection and

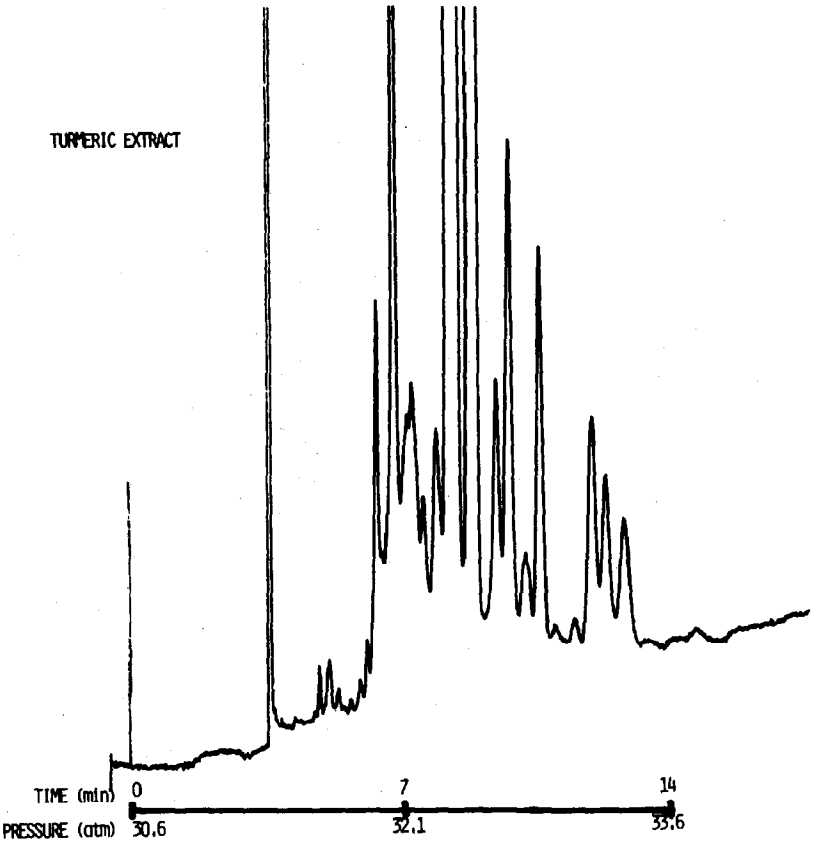

Figure 4
Capillary SFC separation of aromatic compounds from turmeric. Column: 21 m × 100 μm i.d., coated with 1% SE-54; mobile phase, butane. Reproduced from [41] by permission of the author.

detection volumes. In order to preserve the overall system efficiency, these volumes must be of the order of a fraction of a microliter for the 'conventional' microbore columns and about one nanoliter for 'true' capillaries [43, 44]. Such reductions have far-reaching consequences in the design, measurement sensitivity and utilization of LC detectors; this subject is among the most widely discussed and greatly misunderstood matters in the field of microcolumn LC.

As pointed out quite early by Scott and Kucera [45], the use of miniaturized columns for certain types of analysis can result in a significant enhancement of mass sensitivity if miniaturized concentration-sensitive detectors are utilized. However, in comparing the relative detection response using different chromatographic columns, two factors must be taken into consideration. First, the effect of solute dilution by columns of different dimensions and efficiency must be considered. As the column dimensions are decreased, or the efficiency is increased, the elution volume of a chromatographic peak is simultaneously reduced. While the solute is eluted in a smaller volume with micro-columns compared with conventional columns, detectability may be significantly enhanced under certain circumstances.

The second factor to be considered is the effect of detector miniaturization on measurement sensitivity itself. For example, in the miniaturization of flow-cells for absorbance detection, the optical pathlength is necessarily reduced, and this will

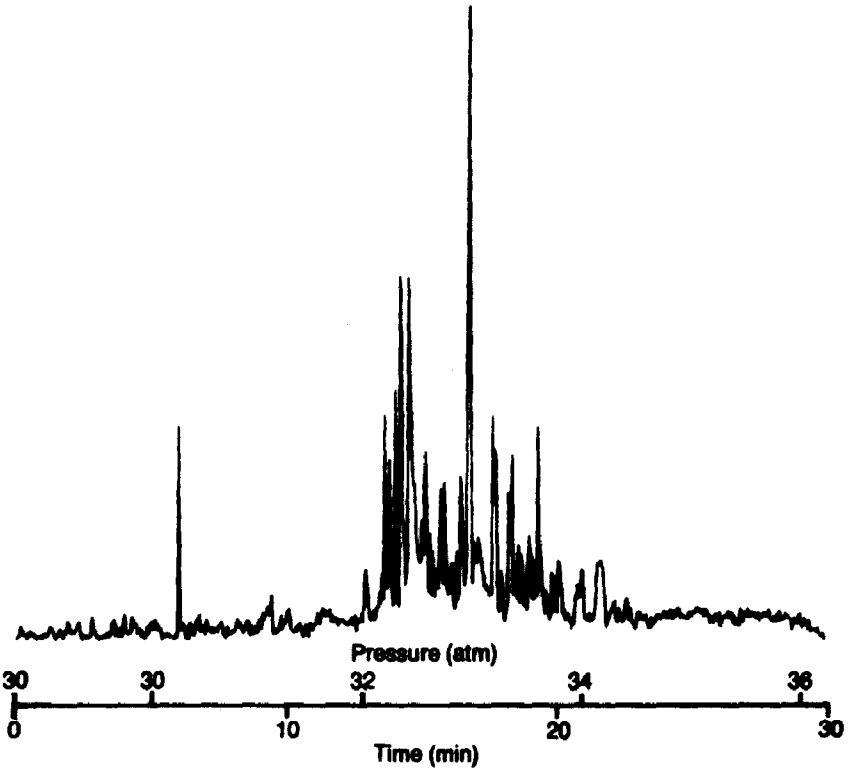

Figure 5
Capillary SFC separation of a toxaphene sample, detected as a total-ion plot using an on-line mass spectrometer. Reproduced from [42] by permission of the American Chemical Society.

decrease the sensitivity in a proportional manner. At this stage, the *detector noise* may also increase, further decreasing the benefits derived from reduced solute elution volumes. However, the situation may be entirely different with certain electrochemical detection techniques, where the noise level is significantly *reduced* with electrode miniaturization. The important point is that generalizations in this area, if made without carefully considering the specifics of a given detector, are often counterproductive. As shown in a recent review [31], the effect of column and detector miniaturization must be treated differently for mass- and concentration-sensitive detectors, and the relative responses for different column types must be compared under two practical limiting conditions: (a) sample-limited cases; and (b) capacity-limited cases. Together, these two conditions represent a practical, impartial comparison of detectability with conventional columns and microcolumns. Naturally, similar considerations are valid in microcolumn SFC.

A few more specific cases which pertain to biochemical and environmental applications will now be discussed. The above-mentioned increase in mass sensitivity due to column and detector miniaturization is valuable *only* if a sample concentration step (either before sample introduction or at the column inlet) occurs. It is thus essential to recognize at the outset whether it is concentration sensitivity *or* mass sensitivity that is important for a given application; under ordinary circumstances, microcolumns tolerate only a fraction of the sample volume which can be injected onto a conventional column. If the total amount of sample is limited, as is often the case in biological or medical

applications, the use of microcolumns will undoubtedly have rewards of its own. While such sensitivity enhancement is particularly attractive with truly high-sensitivity detectors (e.g. spectrofluorimetric or electrochemical measurements), interestingly enough, there is a small practical gain even with miniaturized UV-absorbance detectors [20, 31, 45], in spite of the very obvious 'miniaturization penalty' due to the reduced optical pathlength.

Miniaturization benefits have perhaps been most clearly demonstrated with some recent versions of electrochemical detectors. Manz and Simon recently published [46] a version of an ion-selective electrode detector which utilizes a liquid membrane positioned directly at the exit of an open tubular column to yield a total detection volume of less than 1 nl, as shown in Fig. 6. Under favourable conditions, sensitivities in the femtomole range were achieved with monovalent ions. Additional versions of very sensitive miniaturized electrochemical detectors have been reported by Šlais and Krejči [47] and Knecht *et al.* [29].

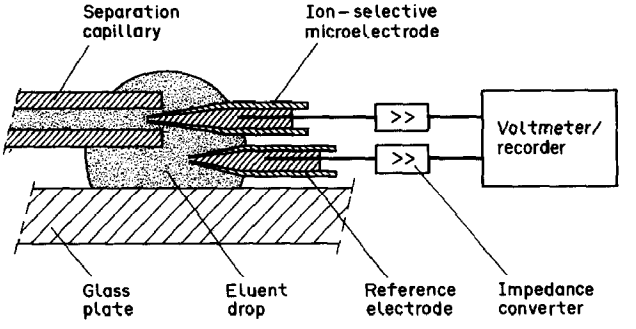

Figure 6
An ion-selective electrode detection system used in conjunction with a small-bore open tubular LC column. Reproduced from [46] by permission of Preston Publications, Inc.

To realize the optimum performance of highly sensitive detectors and miniaturized columns, improvements in techniques used for sample concentration and introduction will become a high priority in biomedical applications. Indeed, sample micromanipulations have already been vital to certain directions in modern medical research [48]. While some of the microcolumn separations research may appear impractical and even esoteric today, the time may be rapidly approaching when the profile of substances released from a single cell can be reliably analysed. There is no denying the importance of such a situation.

New Detection Modes

The extremely low flow rates encountered in microcolumn LC and SFC provide some very unusual detection opportunities, including both the adaptation of some GC detection principles and the possibility for new detection technologies.

LC microcolumns with mobile-phase flow rates of a few μl/min present a unique possibility to nebulize the entire column eluent into a flame detector. Such detectors can work on the principle of flame emission [49], the thermionic effect [50–52], or even flame ionization [53]. Indeed, additional modes of operation are also potentially feasible. Single- and double-flame detector versions have been reported from this laboratory [49–52] in the construction of both phosphorus- and nitrogen-sensitive LC detectors, the sensitivities of which are generally 1–3 orders of magnitude lower than normally

encountered with similar GC devices. A highly valuable feature of these detectors is their extremely small dead volume. Although nebulization problems have so far limited such LC flame detectors to the measurement of relatively small molecules, further developments in this area are eminently worthwhile because of the need for element-specific detection in modern LC.

Using the flame detector in capillary SFC may prove increasingly popular. Importantly, such mobile phases as carbon dioxide and nitrous oxide yield little background signal in the flame ionization detector and thus allow the detection of many carbon-containing solutes. This may be seen as one of the more promising substitutes for the so far elusive universal LC detector. In addition to the SFC flame ionization detector, the remaining element-specific flame or plasma detection principles can also be employed. Once again, effective nebulization at the column outlet has so far limited SFC flame detectors to relatively small molecules. Nevertheless, the approach is successful in analysing thermally-labile solutes, as demonstrated by the example in Fig. 7 [54].

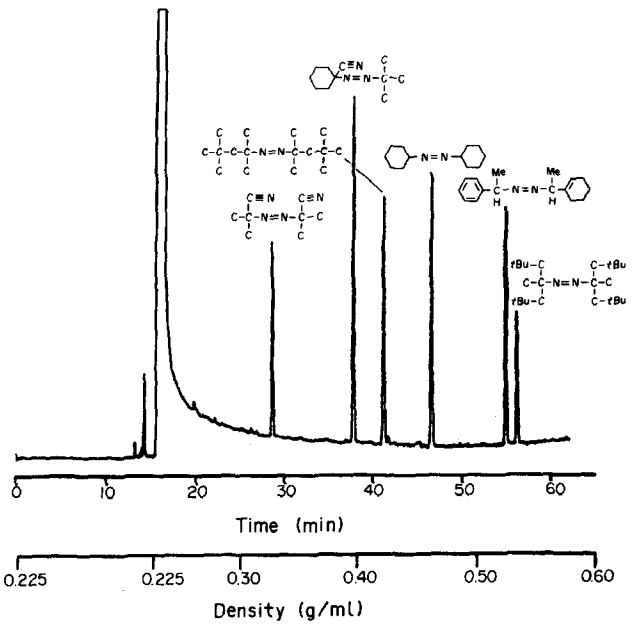

Figure 7
Capillary SFC separation of thermally labile azo compounds, detected with a flame detector. Reproduced from [54] by permission of Elsevier Publishing Company.

Laser-based detection devices can uniquely combine with a variety of microcolumn techniques. Various spectroscopic detectors based on laser technologies have recently been reviewed by Yeung [55], who has also emphasized the advantages of highly collimated laser beams for microcolumn LC [56].

Particularly sensitive techniques are based on laser-induced fluorescence measurements, as emphasized in recent years by Diebold and Zare [57], Hershberger *et al.* [58], and Folestad and co-workers [59]. While femtogram sensitivities have been achieved using this approach, the author's laboratory has recently emphasized [21, 60] the importance of chemical derivatization to enhance the detection capabilities of such laser-based detectors. An example of a laser fluorescence detection system [60] is shown in Fig. 8, while Fig. 9 amply demonstrates its current capabilities. This chromatogram was

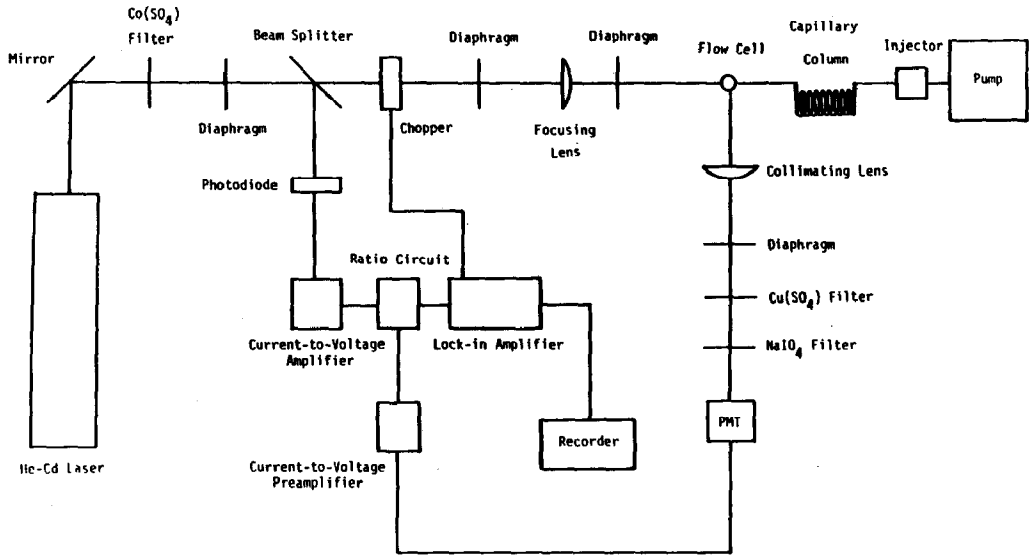

Figure 8
Schematic diagram of a miniaturized chromatographic system, utilizing laser-induced fluorescence detection, with associated equipment. Reproduced from [60] by permission of Elsevier Publishing Company.

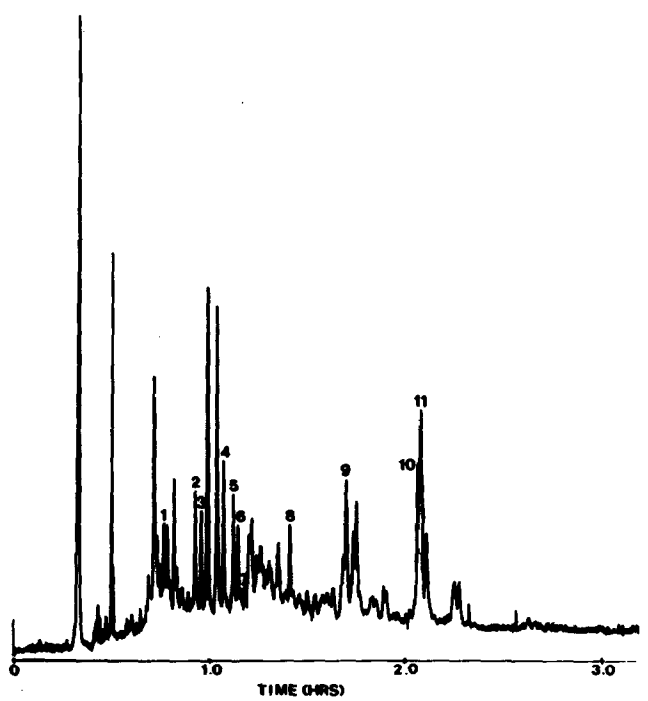

Figure 9
Chromatogram and picogram-level detection of solvolysable plasma steroids on a 2.2-m slurry-packed (5-μm ODS) microcolumn. Reproduced from [60] by permission of Elsevier Publishing Company.

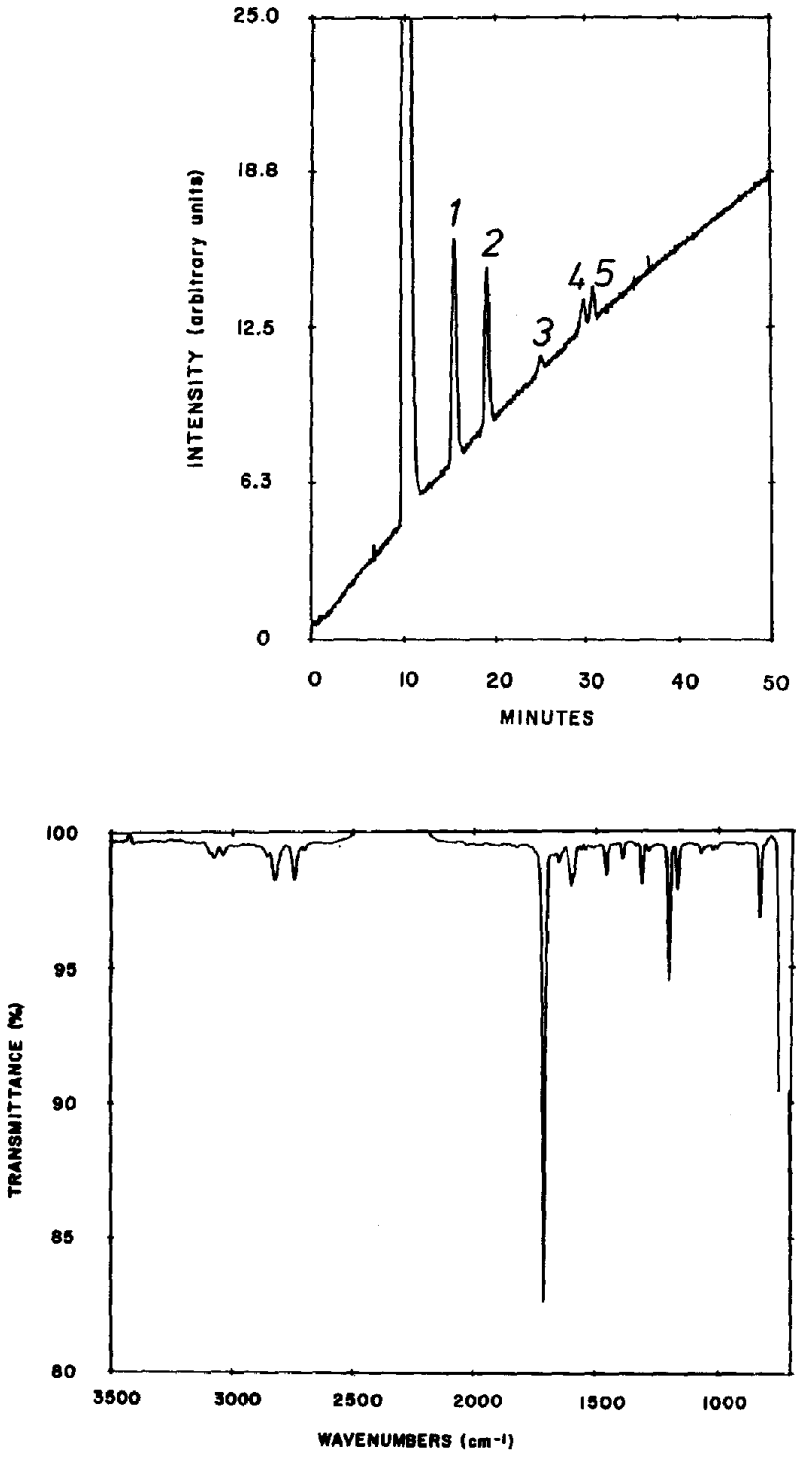

Figure 10
(a) Gram-Schmidt real-time chromatogram of a model mixture: 1, benzaldehyde; 2, *o*-chlorobenzaldehyde; 3, 2,6-di-*t*-butylphenol; 4, 2-naphthol; 5, benzophenone; 2 μg of each injected. (b) IR spectrum (in transmittance mode) of benzaldehyde from the above mixture. Reproduced from [62] by permission of Pergamon Press.

obtained from a fraction of plasma steroids after derivatization with a novel fluorescent reagent [21]. Thus, highly efficient chromatographic separation is here effectively combined with an extremely sensitive detection technique, giving low femtogram detection limits. Such capabilities appear very much in tune with the current needs of biomedical science.

Finally, various ancillary techniques of microcolumn chromatography will be briefly mentioned. As solute identification often completes or supplements the overall analytical task initiated by efficient separation and detection, miniaturized ancillary techniques are an important area of investigation. A recent demonstration [61] that some absorbance imaging detectors can be effectively miniaturized to work with packed-capillary columns will undoubtedly be followed by additional designs applicable to both LC and SFC.

The optical transparency of certain supercritical fluids is a distinct advantage of SFC techniques. This appears particularly attractive with certain fluids exhibiting minimum absorbance in the infrared region (supercritical carbon dioxide and xenon). The author has recently combined capillary SFC with a Fourier transform IR spectrometer [62] and achieved sub-microgram sensitivities. A typical result from this work is shown in Fig. 10, which demonstrates a Gram–Schmidt (total absorbance) chromatogram of model compounds together with an infra-red spectrum of 2 μg of benzaldehyde. While further improvements in this combination are highly desirable, these initial results are considerably better than any previously reported using LC in combination with IR spectroscopy.

Mass spectrometry (MS) has been among the most powerful structural techniques to be used in combination with chromatography. Investigations on the LC–MS combination have been numerous world-wide. In certain LC–MS coupling modes, microcolumns have the beneficial effect of very low flow rates, although the opposite seems to be true for a recently popular thermospray approach [63]. Various aspects of microcolumn use in LC–MS have been reviewed [64, 65], indicating that while there is much room for future improvements, the field of LC–MS is rapidly approaching an era of wide practical utility.

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