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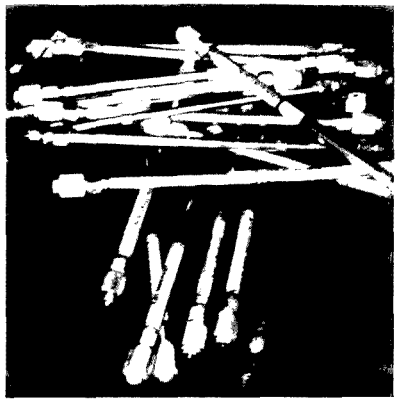

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August 1983

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July, 1983 448 pages, illustrated

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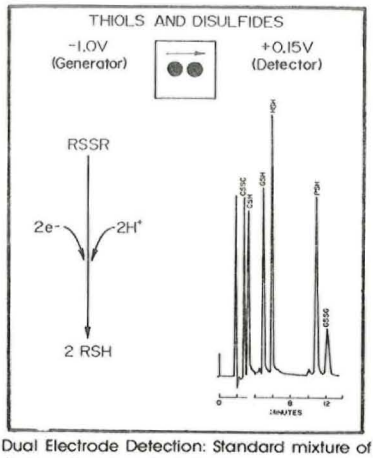

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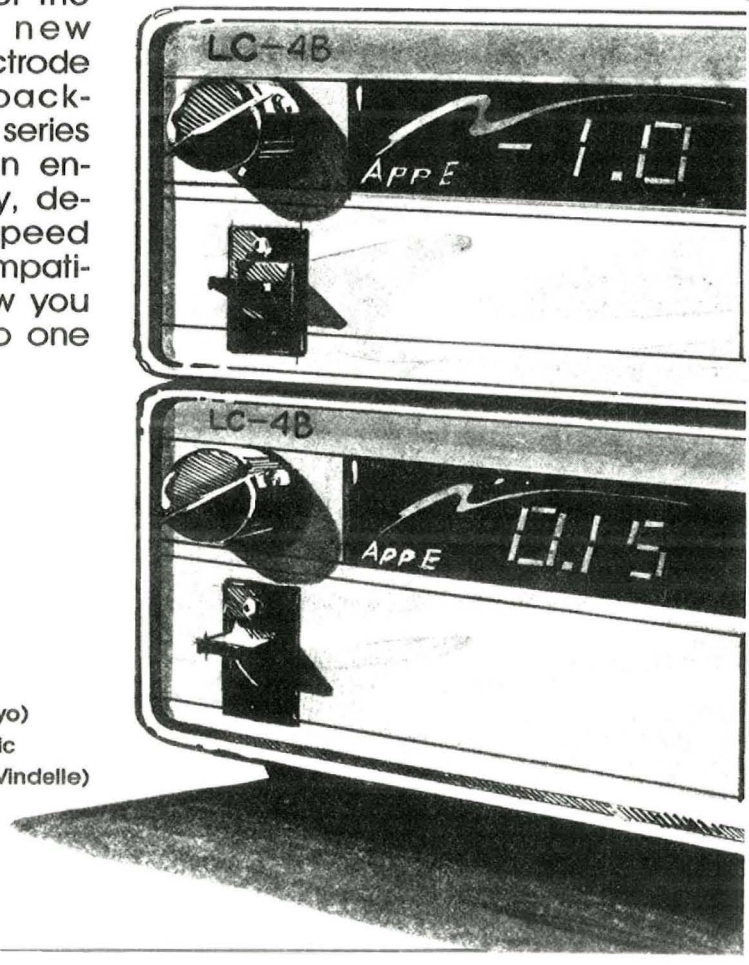

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SOLID PHASE REACTIONS FOR DERIVATIZATION IN HPLC (HPLC-SPR)

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I. INTRODUCTION

As in all other forms of chromatography, high performance liquid chromatography (HPLC) has come to rely quite heavily on derivatization of individual analytes for improved qualitative and quantitative identifications (1-13). Virtually all of this work has involved the use of homogeneous type derivatizations, wherein the sample solution to be injected, in the pre-column approach, or the HPLC effluent, in the post-column approach, are fully mixed with the derivatization reagents in solution. Clearly, homogeneous type derivatizations can be done off-line or on-line, in either the pre- or post-column modes, but in general, pre-column methods have been done off-line, and post-column approaches have been done both on-line and off-line. On-line type derivatizations, in either the pre- or post-column modes, appear to offer some very significant advantages. Major among these is the ability to perform derivatization-separation-detection or separation-derivatization-detection following injection of the sample mixture. The other off-line approach requires an initial derivatization off-line, then injection-separation-detection, in the pre-column mode. Off-line, post-column methods require injection-separation-derivatization off-line, then detection. Ideally, for any derivatization method, be this pre- or post-, on-line or off-line, one would like to be able to avoid any additional sample pre-treatment before injection, and any extra sample/analyte handling after the point of injection. Unfortunate-

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ly, on-line, pre-column derivatizations are very rare in the existing literature, and this has to do with the major problem of using solvents for the derivatization step that will be compatible with the separation needed via the HPLC step. Most recently, various instrument manufacturers have attempted to automate off-line, pre-column derivatizations with automated sample injections-separation-detection of a large number of samples. Automated on-line, post-column derivatizations have also now become available, wherein a homogeneous reagent solution is mixed, after the analytical column, with the HPLC effluent, in a small dead volume, high mixing efficiency chamber before the elevated temperature reaction coil. The final, derivatized eluent solution with unreacted derivatizing reagent are then passed into the detector for the final analyte detection/identification.

There are a number of disadvantages possible via homogeneous type derivatizations, although, by far, these are the most commonly employed approaches for HPLC derivatizations. This approach will generally require the use of additional instrumentation, HPLC pumps, mixing chamber, reaction chamber, heating arrangements, connectors, plumbing lines, etc., depending on whether the pre- or post-column modes are to be utilized. Additional extra dead volume is often encountered, which can/will adversely affect the overall HPLC performance, total apparent plate count, resolution, peak shape, peak heights, and minimum detection limits. In general, this homogeneous approach also increases the overall time for each analysis, along with the corresponding cost per analysis. The addition of extra reagent solutions introduces another possible source of contamination, thus reducing the qualitative validity of the overall analysis. Finally, homogeneous derivatizations often require additional sample handling and manipulations, especially in the off-line form. Despite these overall problems, many, if not most, analytical chemists who use HPLC in one or another form, have used and will continue to use some sort of homogeneous derivatization for their analyses. Derivatizations of any sort are extremely useful, especially wherein the analyte of interest cannot be detected with then available HPLC detectors. The derivatives formed and derivatization reactions employed should be designed to provide a final analyte entering the detector which will provide practical, low levels of detection with high analyte specificity. Thus, in general, any type of derivatization should improve analyte qualitative and quantitative determinations, as well as improving the overall specificity/selectivity of the analysis employed.

Within the past few years, certain investigators have described more novel approaches for performing on-line or off-line, pre- or post-column derivatizations that employ some sort of solid based reactions. We prefer to refer to this field as solid phase reactions or solid phase reactors,

hence the abbreviation of SPR or HPLC-SPR to indicate that such solid phase approaches are used in combination with HPLC. We have recently reviewed, in part, the area of solid phase reactors in HPLC, although this was somewhat limited to just those papers that had actually used HPLC-SPR methods (14). In the present review, we have attempted to describe virtually all literature reports related to HPLC-SPR, and to present/discuss various solid phase reactions that have been reported for synthetic organic applications, but may not, as yet, have been utilized in HPLC applications/analyses. Solid phase reactions would appear to provide a large number of rather significant advantages with regard to improved HPLC operations. In general, the type and amount of additional instrumentation, hardware, mixing chambers, reaction chambers, etc., will be less in SPR approaches than in homogeneous methods of derivatization. What is required in HPLC-SPR are the solid phase reactor itself, a dummy reactor column, various end fittings, and perhaps a low-cost, low dead volume, on-line switching valve. The method does not introduce any additional extra-column dead volume other than that normally introduced with any conventional HPLC guard column. The time of analysis with SPR derivatizations usually will not increase per analysis. There is a slight increase in the overall cost per analysis, but this is always less than that incurred *via* homogeneous derivatizations. With regard to contamination, there will be very little, if any, contamination introduced by the SPR, and this can be eliminated or prevented by careful pre-washing and suitable treatment before it is put on-line. SPRs can be used both on-line and off-line, with somewhat different advantages in the off-line mode. There is no excess derivatizing reagent present in the mobile phase with the SPR, and thus the background noise level should be the same with or without the SPR on-line. Detection limits should always be maximally improved *via* on-line HPLC-SPR approaches. When used in the pre-column mode, Figure 1, then difference chromatography can be employed to improve analyte identification. Such difference chromatography employs two separate injections of the sample mixture, one with a dummy column and the other with the SPR on-line. In the post-column mode, Figure 2, difference chromatography cannot be employed, since retention times cannot be affected *via* derivatization after the separations, but detector responses between the initial analyte and its derivative can be used for improved analyte/compound identification. With HPLC-SPR, there is no additional sample handling or manipulation as compared with analysis in the absence of derivatization. The only additional requirement is for a separate analysis *via* the dummy column together with the analysis with the SPR on-line. In general, SPRs are compatible with a wide variety of HPLC solvents, but this is not always the case that all SPRs will be useful and/or usable with both normal phase and reversed phase type solvents. Many

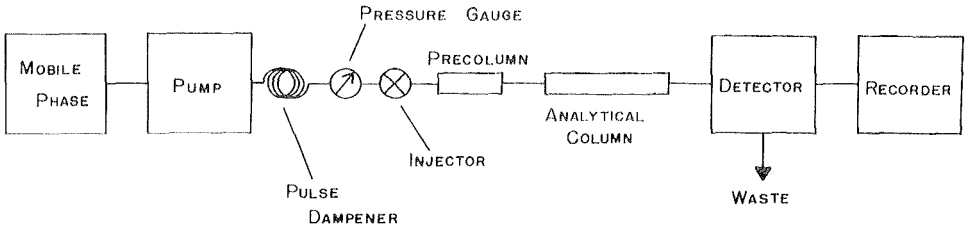


Figure 1. HPLC-SPR with the solid phase reactor in the pre-column mode of operation, before the analytical column.

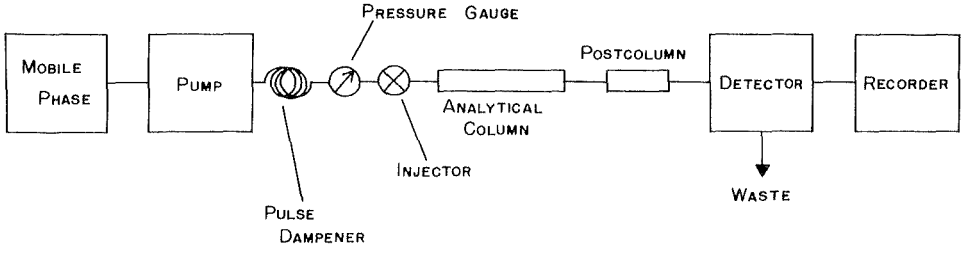


Figure 2. HPLC-SPR with the solid phase reactor in the post-column mode of operation, after the analytical column.

SPR initiated derivatization reactions will occur in real-time, with no increased analyte/sample hold-up within the SPR prior to the separation-detection steps. However, it is always possible to allow the sample solution to remain in contact with the SPR, at ambient or elevated temperatures, before or after the analytical column, in order to increase the percent conversion to the desired derivative. Most SPR methods will lead to derivatization at or slightly above room temperature, in real-time or hold-up time. SPRs can be held at elevated temperatures *via* the use of a constant temperature water bath or similar column heater device already commercially available *via* many suppliers. In many instances, derivatization reactions are more selective and specific than with the corresponding reactions/reagents in homogeneous solution approaches. Thus, from the analytical point of view, quite often improved analyte identification can result *via* the use of more selective/specific derivatizations using the solid phase approach. Since solid phase derivatizations can be designed to improve detection *via* virtually any HPLC detector, these approaches are not limited to improved overall analysis for a particular detec-

tion method. It is entirely possible that one particular SPR will lead to improved detection via ultraviolet-visible (UV-VIS), fluorescence (FL), or electrochemical (EC) methods. Other SPRs may provide enhanced detectability for more than one particular detection method (UV/FL, UV/EC, etc.). The deciding factors are the nature of the functional group within the analyte being modified, and the nature of the derivatization reaction leading to an altered chemical structure. Some SPR reactions may just change one particular functional group with the analyte, such as oxidizing an aldehyde to a carboxylic acid or reducing an aldehyde to an alcohol. Still other derivatization methods may tag an analyte, by the addition of a specific chromophore/fluorophore/electrophore to the original compound's structure. In general, derivatization via compound structure alteration, as opposed to tagging, will provide for improved qualitative identification/specificity, but not necessarily for improved detection limits. Tagging reactions, with or without SPRs, are designed to improve the detectability of the final product, so that overall detection limits will be reduced/improved.

Affinity chromatography has long employed enzyme bound/modified solid supports for improved separation of biological materials, but, in general, these have not involved chemical alterations of their structures. However, this use of enzyme bound supports has encouraged various individuals to employ similar solid phase reagents, employing enzymes, for SPR type derivatizations in HPLC. Clearly, this is an area for vast development over the coming years. Solid phase reactions can most easily be broken down into two general fields or areas, those which utilize solid supported reagents and reactions, and those which use polymer bound/attached reagents and reactions. We term these two approaches as solid supported and polymeric SPRs, with the understanding at the start that the nature of the solid support or polymeric backbone can vary from one type of SPR to another. Most solid supported SPRs will be compatible with normal phase type separations and organic mobile phases. They will not, in general, be at all compatible with reversed phase type conditions in HPLC. The polymeric SPRs will or can be compatible with both normal phase and reversed phase type separations, depending entirely on the nature of the reagent/reactions employed. The very nature of the inorganic or organic reagent on the solid support or polymeric backbone can often dictate whether normal or reversed phase HPLC can be used with a particular SPR. This is perhaps one of the most important considerations to be met in all of HPLC-SPR, what is the compatibility of the HPLC mobile phase with the solid supported or polymeric SPR? That is, there may very well be times when the solvents needed for successful SPR derivatizations will just not be those ideal for the desired separations. There may be other times when the solvents necessary for

a given separation are not at all compatible with the SPR needed for that particular derivatization. There will yet be other times when the solvent conditions for the SPR and those for the HPLC separations can be met by the same solvent or solvent mixture. Some amount of solvent screening or evaluation may very well be called for in the development or perfection of any new HPLC-SPR system, and this can require a considerable amount of time and effort. However, this is not necessarily any more time or effort than what is needed for the development and optimization of a new homogeneous derivatization method.

In many instances, the lifetime of SPRscan be vastly greater than that of a derivatization solution that must be prepared fresh for each application. It is entirely possible to store an SPR in an inert atmosphere, or under an appropriate inert solvent, and to have it remain active for several hundred analyses lasting over many months. Thus, an individual SPR can and should be usable over and over, especially wherein the percent loading of the reagent(s) can be intentionally designed to be quite high (mg/g). The loading of the reagent on the solid or polymeric support can be varied, by appropriate preparation designs, the size of the SPR column itself can also be varied, just as is now done with a commonly used guard column before the analytical column. It is also possible to utilize more than one type of SPR, either in series or parallel, on-line, in HPLC, so as to improve analyte identification *via* a number of related compound/analyte modifications or derivatizations. With the use of appropriate switching valves on-line, it is entirely possible to vary the nature of the analyte modification, just by passing the injected solution to one or another of existing, on-line SPRs, pre- or post-column. This would be the parallel mode of SPR operation. In the case of series operations, a series of appropriate SPRs could be placed one after the other, again either pre- or post-column, so that the initial analyte would be modified, modified again, perhaps modified again, each time selectively, so that perhaps only one particular analyte/compound could possibly undergo the reactions desired to produce a known, finally detectible product.

SPRs are not entirely new HPLC detectors, they employ currently known and available detection methods, but they are designed to improve analyte identification and overall selectivity. Clearly, SPRs can be utilized in an off-line as well as an on-line fashion, as discussed above. In the case of off-line derivatizations *via* SPRs, although of less significance than for on-line approaches, there are still serious advantages as compared with homogeneous, off-line derivatizations. It is entirely conceivable that SPRs can be developed for off-line approaches, wherein the sample mixture is simply injected onto the SPR, this is heated for a given period of time, cooled to room temperature, eluted with a mobile phase compatible solvent

or the mobile phase itself, and an aliquot of this final eluate is then injected onto the HPLC. Such SPR columns for off-line derivatizations could be used over and over again, moved from one laboratory to another, and could form a bank of readily accessible and available derivatization systems that could be used at a moment's notice. There could, in fact, be a bank of SPRs present within any given laboratory, from which individual SPRs could be drawn by an analyst, utilized off-line or on-line, and then returned to the SPR bank for future use by others. Such SPR approaches to derivatization could readily be automated, so that samples could automatically be derivatized by passage, off-line, through a particular SPR set in a laboratory rack, and the effluent would then be collected for subsequent work-up, separation, detection, or related analytical steps. Thus, it would appear that the future of SPRs in analytical chemistry, and especially within HPLC applications, appears very bright, and that these somewhat newer methods of derivatization should find much more widespread acceptance and utilization in the coming years.

II. SOLID SUPPORTED REAGENTS IN HPLC (NON-POLYMERIC)

We define solid supported reagents as those organic or inorganic reagents that are physically or chemically adsorbed onto an inorganic type GC or HPLC solid support, such as silica gel, alumina, clay, Florisil, etc., wherein such supports are not synthetic organic/inorganic polymers. In general, most of the solid supported reagents already described in the synthetic organic literature are adsorbed onto the support, rather than being covalently attached/bonded. It is also possible to use silica based ion exchange materials for the ionic attachment of organic/inorganic reagents, and to then utilize such solid supported, but really ionically attached, reagents for synthetic or HPLC applications. However, as of yet, very few such silica based ion exchange packings have indeed been utilized for any type of HPLC derivatizations. There is some interest at the present time in demonstrating the potential usefulness of this approach for on-line or off-line HPLC reactions. In general, solid supported reagents have some distinct advantages when compared with polymeric type supported materials, especially with regard to the type of mobile phases that are compatible with this type of support. Usually, HPLC applications with silica supported reagents will be limited to normal phase type solvents, hydrocarbons, chlorinated organics, etc. It may prove possible to utilize silica based ion exchange packings for HPLC-SPR, wherein the pH of the mobile phase does not exceed 2-9 in reversed phase approaches. Polymeric based SPRs appear to be more compatible with reversed phase needs, assuming that the chemical reaction to be conducted is also compatible with this type of solvent mixture (MeOH/HOH, EtOH/HOH, ACN/HOH, etc.). In solid

supported reagents, there are at least two important criteria that must be met in HPLC-SPR work: 1) that the solid support be stable and compatible with the HPLC mobile phase; and 2) that the reagents/reactions being employed are also compatible with the mobile phase. If either of these requirements cannot be met with a particular HPLC solvent, then that HPLC-SPR approach is doomed to failure. Alternative SPR methods would have to be investigated for that HPLC mobile phase, or an alternative HPLC approach would have to be found which is compatible with the SPR to be employed.

In the synthetic organic literature, there has evolved considerable interest in the use of reagents supported on insoluble inorganic materials for performing various types of reactions (15, 16). There are several possible reasons for the widespread use of these supported reagents, many of which have become commercially available, such as: 1) the increase in the effective surface area whereon the reaction can take place; 2) the presence of various sized pores in the matrix which can hold the two reacting species in the same general proximity, thus lowering the energy of activation for a given reaction; 3) the selectivity of the reaction is often enhanced; and 4) the reactions are often much cleaner with fewer side products. Despite a well developed and recognized literature in synthetic organic chemistry for solid supported reagents, the use of HPLC-SPR with such reagents/supports is not very well developed nor recognized at this particular time. There are some obstacles, as suggested above, that must be overcome before this technique can readily be applied to HPLC applications/problems. Some of these requirements are: 1) the inorganic support and the supported reagent must be stable under the given HPLC operating conditions; 2) the ideal solvent for the derivatization may not be the best mobile phase for separating the derivatized analytes, thus often a compromise is needed; 3) if the reaction is to be carried out on-line, in real time, it must be fast at ambient or slightly above ambient temperatures; 4) the reaction should quantitatively convert all of the starting compound into a single, known product, or at least react a determined, reproducible percentage of the starting compound to the product; 5) by-products of the reaction should either remain adsorbed on the support or not interfere with either the separation or detection of the desired product. Reactions that will form products that are either gases or insoluble precipitates are clearly incompatible with HPLC-SPR requirements for continued/continuous operation.

Despite the above requirements, which are clearly not insurmountable, there are still many, already described, supported reagents that are currently being used almost exclusively for organic synthesis that might be well applied in HPLC-SPR. Solid supported reagents can be either adsorbed onto, intercalated, dispersed in the inorganic support, or ionically/covalently

attached/bonded. Examples of some inorganic supports that could be used in HPLC-SPR include: silica gel, alumina, Celite, Florisil, graphite, clay, and molecular sieves. The types of chemical reactions that could be used in HPLC-SPR are almost limitless. The supported reagents described and discussed below were only chosen to illustrate those SPRs that can be most directly applicable to HPLC-SPR. However, they do not, by far, represent all of the literature reports on solid supported reagents.

II.A. HPLC-SPR CHEMICAL REDUCTIONS VIA NON-POLYMERIC, SOLID SUPPORTED REAGENTS

By and large, the vast majority of solid supported reagents in synthetic chemistry have to do with either oxidation or reduction type reactions. Remember that we have arbitrarily divided derivatizations in HPLC into two major types or categories: 1) those reactions that will convert one or more functional groups into another type of functional group(s), perhaps best termed functional group conversions; and 2) those reactions that will tag an analyte by adding to the initial compound's structre another compound or part of a derivatizing reagent that provides enhanced UV, FL, and/or EC detectability. Either of these approaches to HPLC derivatization will, in effect, improve analyte identification and/or detection limits. A very large number of organic functional groups are amenable to solid supported reductions, and many papers have already appeared describing a wide variety of suitable organic/inorganic reducing agents on solid supports (17-21).

Quite recently, Krull *et al.* have described the use of sodium borohydride precipitated onto silica gel ($NaBH_3/SiO_2$) as an on-line, pre-column or post-column derivatizing agent in HPLC (17). Difference chromatography was used here to monitor the overall reaction on the SPR for various carbonyl compounds. Standard compounds were injected onto a combination of a dummy (no reagent) pre-column plus analytical column, and then onto a combination of the solid phase reactor (SPR) pre-column plus analytical column. The HPLC-UV chromatogram of the SPR plus analytical column shows a decrease or complete disappearance, depending on the particular analyte, of the starting compound's peak height/area, with a concomitant increase or appearance of the product peak (Figure 3). This $NaBH_4/SiO_2$ type SPR was found to be quite reactive towards most aldehydes, ketones, and some acid chlorides. The SPR was unreactive towards esters, nitro, N-nitroso, alkyl/aryl halides, or amides. The percent reductions of various aldehydes and ketones was monitored as a function of temperature, and this could then be used to differentiate between these two classes of compounds. Products of these reductions, the alcohols, have longer retention times in normal phase HPLC, and these had to be eluted with flow programming. The borohydride reagent was stable with mobile phases

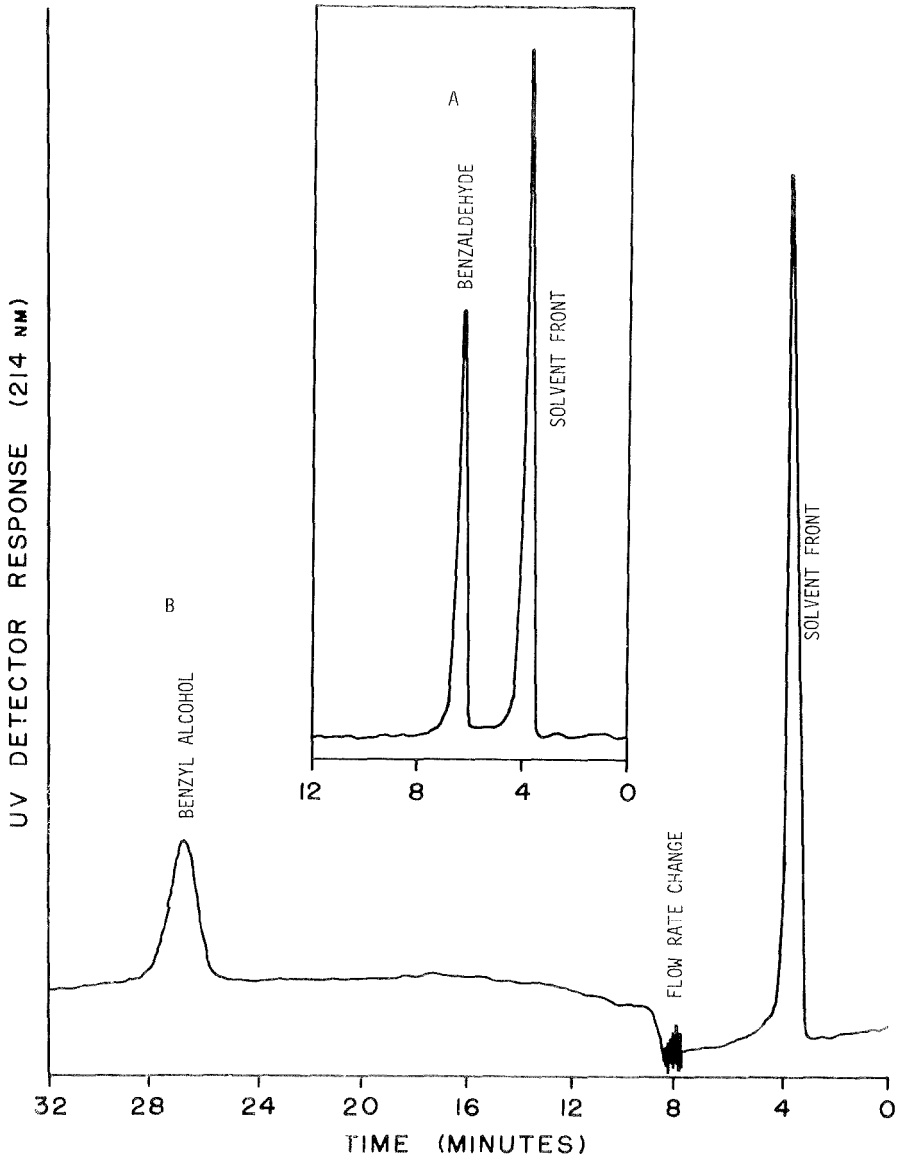


Figure 3. (A) HPLC-UV chromatogram of benzaldehyde using dummy column and silica gel analytical column with 0.3% IPA/hexane at 1 ml/min; (B) HPLC-UV chromatogram of benzaldehyde injected onto reduction column and silica gel analytical column with 0.3% IPA/hexane at 1 ml/min to 8 mins, and then flow rate of 4.3 ml/min to 32 mins. Pre-column mode of HPLC-SPR operation.

having as much as 15% isopropyl alcohol in hexane, and therefore gradient elution could have been used in order to reduce the overall time of analysis via HPLC-SPR for the alcohol reduction products. The long retention time of the product is not necessarily a disadvantage and may actually prove useful in certain applications. If, for example, an aldehyde or ketone eluted at the same time as another component of interest, then a $NaBH_4/SiO_2$ pre-column would/could eliminate the peak and thus serve to "clean-up", in an on-line manner, the overall chromatogram and improve final selectivity. This same approach could easily be used to monitor impurities in commercial preparations of aldehydes or ketones. Analysis of the aldehyde would only show the impurities initially present, assuming that these have not been derivatized, together with the product alcohol peak coming from the original aldehyde. Krull et al. have used this $NaBH_4/SiO_2$ approach to detect cinnamaldehyde in a commercial food product, cinnamon, as well as in a commercial mouthwash product (17). This same HPLC-SPR approach was used to monitor for vitamins K_1 and K_3, although not in actual samples or food products.

Judging from the existing synthetic organic literature, there are many other reports of supported reducing reagents that could be directly applicable for HPLC-SPR analyses/purposes. Some examples of these reagents would be: 1) $NaBH_4$/alumina; 2) $NaBH_4$/Celite; and 3) potassium or other active metals/reagents intercalated into graphite. However, whichever supported reagents are to be considered for HPLC-SPR utilization, clearly such materials must be pressure stable, so that there is no bed collapse in the pre-column mode during actual operations. All of the other requirements of SPRs in HPLC must also be met, as summarized above.

II.B. HPLC-SPR CHEMICAL OXIDATIONS VIA NON-POLYMERIC, SOLID SUPPORTED REAGENTS

The synthetic organic literature is replete with references to the use of solid supported oxidizing reagents. Each specific combination of solid support and chemical reagent has different selectivities and reactivities. The most general and widespread use of supported oxidizing reagents has been to convert alcohols to the corresponding aldehyde, ketone, or lactone derivative. Nef type oxidations have also been described, wherein primary and/or secondary nitro compounds are converted to their aldehydes or ketones (15). Oxidative rearrangements are also possible using solid supported oxidizing agents (22).

Regen and Koteel have described the use of activated potassium permanganate ($KMnO_4$) impregnated onto organic supports, such as molecular sieves, silica gel, and clays, for the oxidation of various alcohols (23). Such oxidations were carried out in benzene, although other nonpolar solvents of

more routine HPLC use (e.g., hexane), might also be effective here. In general, high yields of ketones and modest yields of aldehydes were obtained. Thus, supported permanganate should be ideally suited for normal phase HPLC-SPR, because it is virtually insoluble in most organic solvents. In addition, it is a powerful oxidizing agent and should react very quickly with alcohols in organic mobile phases. The oxidation products, viz., aldehydes/ketones, will not be irreversibly bound to the solid support, the reactions are generally very clean, and the reduced reagent, manganese dioxide (MnO_2), should also remain bound to the support and not interfere with either the separation or detection of oxidation products. Such approaches have not, as yet, been described for HPLC-SPR purposes, although work is now underway in the authors' laboratory related to the use of permanganate supported polymeric reagents for organic oxidations.

Santaniello, Ponti, and Manzocchi have reported that chromic acid (H_2CrO_4) adsorbed onto silica instantaneously oxidizes hydroxyl compounds in ether to their corresponding carbonyl derivatives (24). Pyridinium chromate on silica gel can oxidize allylic and benzylic alcohols, as well as saturated primary or secondary alcohols, even when these contain other acid sensitive groups (25). Pyridinium chromate on silica is a stable reagent and showed no deterioration even after one year of storage at room temperature.

One of the most thoroughly investigated supported reagents has been silver carbonate precipitated onto Celite (15). Ag_2CO_3/Celite is a very versatile oxidizing agent capable of oxidizing primary and secondary alcohols, diols, triols, hydroquinones, phenols, and amines. Reaction times vary depending on the ease of oxidation, but these can be as fast as one minute. The most common solvent used for such oxidations has been benzene, although heptane, toluene, and dichloromethane can also be used.

Lalancette et al. have reported that chromic anhydride incorporated into clay is a specific oxidizing agent for the conversion of primary alcohols to the corresponding aldehydes (26). Sensitive structures, such as terpenes and allylic systems can be oxidized with this type of a reagent, but secondary and tertiary alcohols are unreactive. Some other supported oxidizing agents that can oxidize alcohols to their corresponding carbonyl compounds are chromyl chloride chemisorbed onto silica/alumina (27) and manganese dioxide on carbon, but this latter example may not be stable to high back pressures in HPLC (28).

Oxidative rearrangements, as opposed to simple oxidations, are also possible using supported reagents, and these might be ideal for HPLC-SPR purposes. Taylor and Chiang have described such oxidative rearrangements for alkyl aryl ketones by Thallium (III) nitrate (TTN) adsorbed on montmorillo-

nite K-10 clay (22). The final product in this case was a different alkyl aryl ester. This particular reaction is rapid, selective, and can be carried out in heptane, methylene chloride, carbon tetrachloride, toluene, or dioxane. Oxidations of simple olefins, such as cyclohexene, are very rapid. In non-polar solvents, both Thallium (III) nitrate on the support and Thallium (I) nitrate which is generated during the reaction are tightly bound to the support throughout the reaction.

II.C. HPLC-SPR CHEMICAL DERIVATIZATIONS OF A MISCELLANEOUS NATURE VIA NON-POLYMERIC, SOLID SUPPORTED REAGENTS

Ribhood and Ruthven report that bromine adsorbed on molecular sieves is a selective reagent for the bromination of terminal double bonds (29). This type of an SPR, in the post-column mode, could be used to improve the response of an electron capture detector (ECD) in HPLC-SPR. By choosing a molecular sieve with a pore diameter that is only large enough to admit straight chain hydrocarbons, the brominating agent is then capable of differentiating between a double bond located in a sterically unhindered side chain and one in an accessible position in an alicylic ring. A molecular sieve with a larger pore size would be a more general brominating reagent for HPLC-SPR.

II.D. HPLC-SPR CATALYTIC REACTIONS VIA NON-POLYMERIC, SOLID SUPPORTED REAGENTS

Crown ethers have been shown useful in organic synthesis because of their ability to selectively chelate metal ions from dilute solutions. They have been used to concentrate ionic species/reagents by immobilization on a solid support. Silica gel immobilized crown ethers could also have an important impact on metal ion chromatography and on-line catalytic reactions. Dibenzo-18-crown-6 is a typical crown ether that has been covalently bonded to silica gel, Figure 4, and has been found to concentrate potassium ions from dilute aqueous solutions of potassium chloride (30). Crown ethers of differing ring size could/would selectively concentrate other metal ions and could conceivably catalyze different reactions.

III. POLYMER SUPPORTED DERIVATIZATION REACTIONS IN HPLC

A very large number of polymeric reagents have been described in recent years, and many of these have been summarized in a recent book (31). There are a very large number of possible polymers that could be used as polymeric reagents, and a large number of reagents that could be attached to any given polymer. Hence, the total possible combinations of polymers and reagents to form polymeric reagents are very large, and many of these could/should be compatible with HPLC-SPR requirements/needs. Although most polymeric reagents

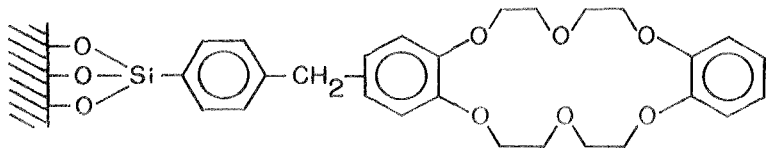


Figure 4. Dibenzo-18-crown-6 covalently bonded to silica gel support (30).

that have already been utilized in HPLC-SPR are organic polymers, there is no reason why inorganic polymers or silica gel based, ionically attached reagents could not be used in HPLC-SPR. These would be sufficiently different from silica gel supported reagents, as described above, to fall into the class of polymer supported/attached reagents, rather than silica or solid supported reagents for HPLC-SPR. Polymer supported reagents, organic polymers, have certain significant advantages as compared with solid supported reagents, not the least of which should be their general compatibility with reversed phase type solvents. Another advantage is that polymeric reagents can often be regenerated by a single synthetic step, and in some instances, this might be accomplished on the HPLC system, merely by passing the appropriate regenerating solution through the spent SPR. Polymeric reagents, ionically or covalently attached, should be usable in a batch process or on-line in HPLC-SPR. Several polymer attached reagents have been described that incorporate an immobilized enzyme on the surface of the polymer beads. This type of polymeric reagent acts as a biological catalyst for certain deconjugation reactions, for example, wherein the catalyst is not consumed, and such SPRs could be used for many separate analyses and/or sample preparations, on-line. The idea of using polymer attached enzymes for on-line catalytic reactions has been used but rarely, although it should have substantial potentials in much of HPLC work. Other polymeric catalysts have already been described in the synthetic organic literature, but very few of these, if any, have ever been utilized in HPLC-SPR type work.

Polymeric reagents can also be used to modify a particular functional group within an analyte's structure, and thereby convert it to a suitable derivative for improved identification, as above for solid supported reagents. Again, a very large number of polymeric supported/attached reagents have been described, many of which should be immediately applicable to HPLC-SPR.

Another type of polymeric reagent consists of a ligand or molecule that is covalently or ionically attached to the polymeric backbone, which can be transferred to a suitable substrate, thereby forming a new derivative that

incorporates all or most of the initial analyte and the tagging molecule. Several of these potential tagging polymeric reagents have also been described in the synthetic organic literature, although very few of these have ever been used in HPLC-SPR applications.

Polymers provide some very useful features, as opposed to solid supports, such as being able to be custom designed so as to provide a micro-environment for specific/selective reactions of the pendant groups. Thus, special electronic and steric conditions can be created in close proximity of the reacting species, leading to enhanced rates of reaction and improved reagent specificity. The chemical and steric structure of the polymer can affect its overall polarity, and this may further influence the overall reactivity of the attached organic/inorganic reagents. The specific steric requirements of the channels and pores of a cross-linked polymer may impart size and structure selectivity on certain reactions of reagents or pendant groups attached to the polymer or diffused into its pores/channels. This area of polymeric reagents in HPLC has recently been reviewed (14).

III.A. POLYMER SUPPORTED REDUCTIONS FOR DERIVATIZATION IN HPLC-SPR

The synthetic organic literature is replete with descriptions of various polymeric reducing reagents, selective for the conversion of aldehydes, ketones, and other carbonyl compounds into the expected alcohol product (31). Borohydride, cyanoborohydride, and related reducing agents have all been used as polymeric reagents, and polymeric borohydride resins are even used commercially for removing traces of aldehydes from process streams of various alcohols being produced commercially (32, 33). Since borohydride is a stronger anion than either chloride or borate, when a solution of sodium borohydride is stirred in the presence of a suitable anion exchange resin, this resin is converted to the borohydride form, as below.

$$P\text{-}\overset{+}{N}(CH_3)_3Cl^- + NaBH_4 \longrightarrow P\text{-}\overset{+}{N}(CH_3)_3BH_4^- + NaCl$$

Krull et al. have recently described the utilization of this type of a solid phase reactor for performing on-line, pre-column derivatizations/reductions of aldehydes under reversed phase HPLC separation conditions (34). Various classes of carbonyl derivatives were evaluated as potential substrates for these reactions, using either MeOH/HOH or ACN/HOH as the mobile phases, including: aldehydes, ketones, amides, acid chlorides, aryl halides, and N-nitroso derivatives. Only the aldehydes could be reduced, under real time, ambient temperatures, using reversed phase type solvents. Identification of an

aldehyde was based on disappearance of the starting substrate on the SPR plus analytical column, along with the formation of the expected, known reduction product (alcohol). Retention times and difference chromatography were obtained using an analysis on the dummy column plus analytical column together with the same analysis of the aldehyde on the SPR plus analytical column. No work has been done, thus far, with this polymeric borohydride SPR in the post-column mode, nor has it been evaluated as a possible SPR for normal phase HPLC. Analysis of the borohydride loading on any given polymeric borohydride SPR was accomplished *via* elemental boron analysis (ICP) and in-house titrations for borohydride by established literature methods (34). A number of applications of this selective method of analyte identification have been described, including the analysis for cinnamaldehyde in both cinnamon spice and a commercial mouthwash sample. Because of the heavy loading of borohydride possible on the anion exchange resins utilized in this study, the final SPRs had very long lifetimes and chemical reactivities, extending over several months and several hundred individual anlayses. Figure 5 illustrates the utilization of this polymeric borohydride SPR in the analysis for cinnamaldehyde from a hexane extract of cinnamon (34). This figure indicates the initially present peak for cinnamaldehyde in this extract on the dummy plus analytical column, followed by the complete disappearance (reduction) of this same peak and the appearance of the expected peak for cinnamyl alcohol on the SPR plus analytical column.

Sodium cyanoborohydride ($Na^+CNBH_3^-$) is known to be a much milder and more selective reducing agent than sodium borohydride. An anion exchange resin incorporating sodium cyanoborohydride has also been described in the literature, and this is also more selective than the polymeric borohydride resin indicated above. There is a commercially available polymeric borane complex, sold by Aldrich Chemical Co., that is also somewhat selective for aldehydes and ketones (35). This particular polymeric reducing agent was prepared by treating poly(2-vinylpyridine) with a borane-methyl sulfide complex in tetrahydrofuran (THF). The final polymeric reagent is capable of reducing both aldehydes and ketones into the expected alcohols at room temperature, but only in the presence of boron trifluoride etherate. Some of the above polymeric reducing agent might find direct applications in HPLC-SPR work.

III.B. POLYMER SUPPORTED OXIDATIONS FOR DERIVATIZATION IN HPLC-SPR

A number of polymeric oxidizing reagents have been described in the existing literature, and these have shown significant advantages when compared with homogeneous oxidation type reactions (31, 36). Cainelli *et al.* have described the preparation and utilization of a macroreticular anion exchange resin incorporating $HCrO_4^-$ as the oxidizing agent. This was shown effective for

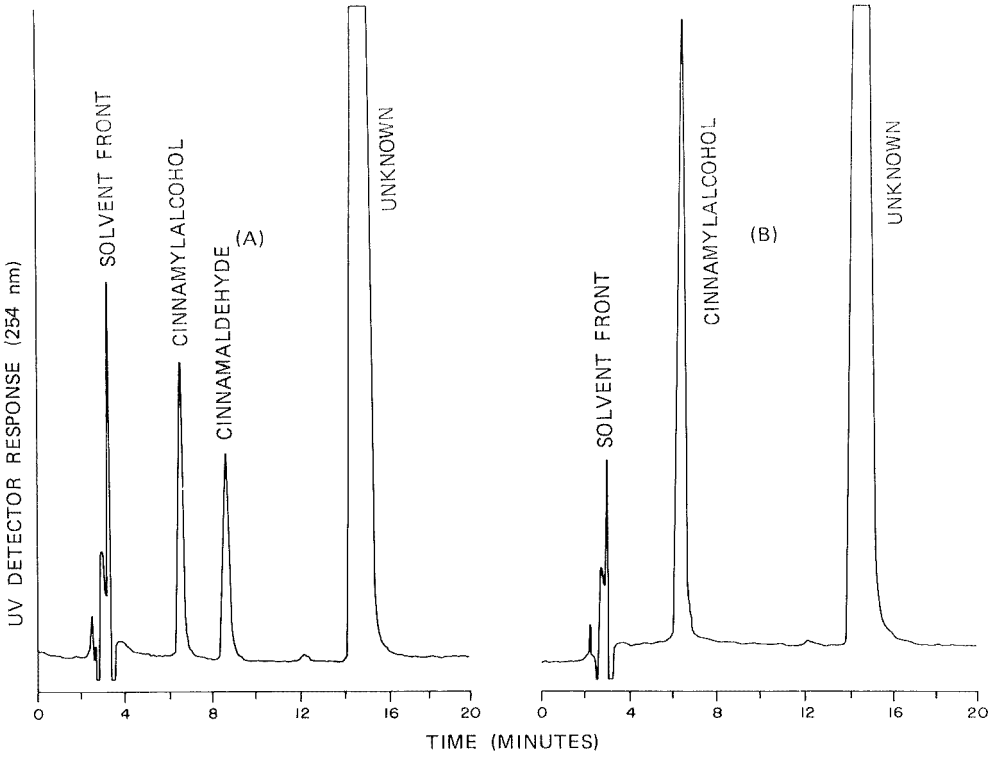
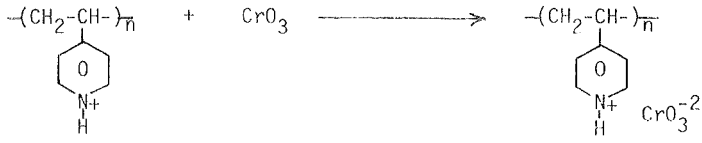


Figure 5. HPLC-UV chromatograms of methanolic extract of commercial sample of cinnamon for determination of cinnamaldehyde. HPLC conditions used a C-18 reversed phase column with mobile phase of 50% ACN/HOH at 1.0 ml/min flow rate, UV detection at 254nm. (A) polymeric dummy column in-line with analytical column; (B) polymeric borohydride reducing column in-line before analytical column.

the complete oxidation of primary and secondary alcohols to the expected aldehydes and ketones. The nature of the solvent used is crucial for effective oxidations, and the most effective ones were: hydrocarbons, chlorinated hydrocarbons, benzene, and ethers. However, acetonitrile with water in various ratios was not as effective as the organic solvents. The rate of the reaction depends both on the structure of the alcohol and on the substrate:resin ratio, this rate being faster for the allylic and benzylic alcohols. Higher ratios of resin:substrate, as expected, also leads to greater overall conversions for such

reactions. Regeneration of the spent resin can be readily accomplished via simple treatment with 2N chromic acid.

An alternate, non-acidic polymeric reagent, poly(vinylpyridinium) dichromate (PVPDC), can be easily prepared by treatment of a poly(vinylpyridine) resin with a slight excess of chromium trioxide (CrO_3) in water at room temperature. After suitable washing, the final polymer can be utilized directly. Frechet et al. have recently described this polymeric reagent, pyridinium dichromate, as an effective oxidizing reagent for alcohols, as below (37, 38). PVDPC is quite stable to prolonged storage. Best oxidation results



are obtained by using the wet reagent in a nonpolar solvent, such as cyclohexane at elevated temperatures, as expected. It is yet possible that this type of a polymeric oxidizing reagent will be compatible with reversed phase HPLC. Recycling of this reagent can be done by using a simple washing reactivation procedure, in which the spent reagent is washed with acid to remove the spent chromium salts. After regeneration of the poly(vinylpyridinium) resin, addition of CrO_3 and water reactivates the PVPDC.

Krull et al. have recently developed a polymeric permanganate oxidizing resin, which has been used in HPLC-SPR for the selective oxidation of both alcohols and aldehydes (39). This material can be used on-line, pre-column fashion, at room temperature and above, for the successful oxidation of various alcohols and aldehydes. Derivatizations can be accomplished using reversed phase, ACN/HOH, separation conditions, as indicated in Figure 6. This is a typical HPLC-SPR application of the polymeric permanganate oxidizing column, here for the oxidation of p-nitrobenzyl alcohol to p-nitrobenzaldehyde, using 50% HOH/ACN as the mobile phase. The maximum percent oxidation for this particular alcohol, at about 46°C, in real-time, on-line with the HPLC, has been about 60%. Other alcohols can be oxidized in amounts/efficiencies ranging from 10% to 50%, very much dependent on the particular structures. Aldehydes, in most cases thus far studied, are oxidized to much greater extents, usually about 100% at elevated temperatures, again on-line, in real-time via HPLC-SPR. Normal phase applications of this SPR have yet to be determined and/or evaluated fully. Clearly, there are a very large number of possible applications for both alcohols and aldehydes via this particular HPLC-SPR approach, and it is possible that other classes of compounds, such as catechols and catecholamines, might be suitable substrates for these derivatization methods (39).

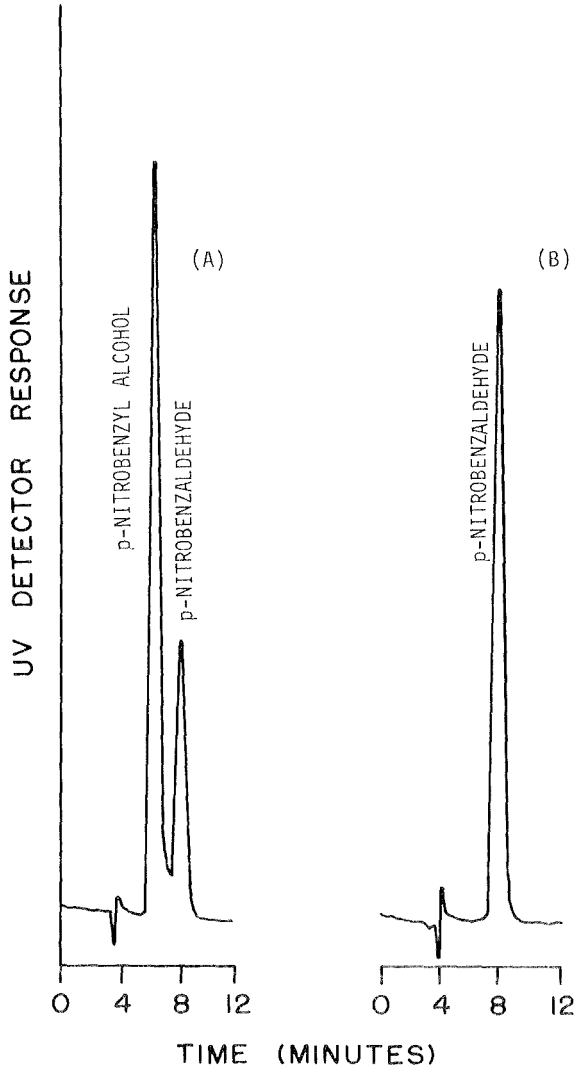


Figure 6. HPLC-UV chromatograms for the polymeric permanganate oxidation of p-nitrobenzyl alcohol to p-nitrobenzaldehyde using C-18 RP-HPLC with 50% HOH/ACN at 0.8 ml/min flow rate, SPR at 46^{o}C on-line, pre-column mode.

III.C. POLYMER SUPPORTED TAGGING REACTIONS FOR DERIVATIZATION IN HPLC-SPR

Despite the fact that several polymeric reagents have already been described for tagging specific classes of analytes, none of these have, as yet, been described for HPLC-SPR derivatizations (31, 40-42). Gelbard and Colonna and Iversen and Johansson have described the preparation and utilization of certain polymeric resins containing phenoxide, p-nitrophenoxide, and naphthoxide anions. These will undergo SN_2 type derivatization reactions with a wide variety of suitable substrates, such as alkyl halides, allyl halides, benzylic halides, and related compounds, at room temperature or above in ethanol or benzene as the solvent. The products in all cases have been aromatic ethers, which has suggested the possible use of these polymeric reagents for HPLC-SPR derivatizations under either reversed phase or normal phase conditions. Clearly, by suitable modification of the aromatic phenoxide or naphthoxide moiety attached to the anion exchange resin, in this case Amberlyst A-26 or Amberlite IRA 900, the final aromatic ethers could improve detection limits for suitable substrates *via* UV, FL, or EC detection. These remain areas for future development in HPLC-SPR derivatizations, with either normal phase or reversed phase conditions. Derivatizations *via* polymeric tagging reactions in on-line HPLC-SPR have the advantage of converting, in one fast, clean reaction, the non-UV absorbing substrate into a suitable tagged derivative with pre-tailored UV absorbing characteristics. This is, in many ways, the ideal approach to take for improved specificity and selectivity in HPLC analyses.

Another approach to on-line derivatizations in HPLC has been described by Werkhoven-Goewie *et al.* (42). However, this particular method has not yet involved a polymeric reagent, although in the future such a modified approach is quite possible. In the work reported here, calcein, a strongly fluorescent compound, is complexed with various divalent metal species, such as palladium, copper, and nickel. These complexes are non-fluorescent, but once the calcein is released, it can then be detected at trace levels *via* FL methods in HPLC. If the calcein-Pd complex is reacted, off-line or on-line, with thiols, thioethers, thioketones, and disulphides, these will quantitatively release the calcein prior to the FL detector. Thus, this derivatization approach does not actually tag the analyte with a fluorophore, but rather it releases a fluorophore from another complex in the presence of the analyte of interest. Detection of the released fluorophore is then an indirect method of analyzing for the initial, sulphur containing analyte of interest. Clearly, such methods are eminently adaptable to polymeric reactions in HPLC-SPR.

III.D. POLYMER SUPPORTED ENZYME CATALYZED DERIVATIZATIONS IN HPLC-SPR

By treating reactive, insoluble polymeric carriers with enzymes, it is possible to prepare covalently bonded, insoluble, and immobilized

enzymes that retain their biological activity (43-46). Immobilization by covalent attachment of the enzyme to the solid support or polymer is the most important method of immobilization. For this purpose, insoluble polymers that swell only slightly in water are required, together with reactive groups that will covalently bond to the enzyme under mild reaction conditions. If this polymeric binding does not occur at the biologically active center of the enzyme, then the catalytic activity of the final bound enzyme should be retained, perhaps to a slightly reduced extent. Some enzyme bound polymers have utilized cellulose or Sephadex as the backbone using a cyanogen bromide coupling method. Poly(aminostyrene) has also been converted into an enzyme carrier by diazotization of the amine followed by reaction with the enzyme, or treatment of the aniline portion of the polymer with thiophosgene to form the thiocyanate and reaction of this with the enzyme (47, 48).

Some work has already been described with regard to the on-line or off-line use of polymer supported/attached enzymes in HPLC-SPR, and the work of most recent importance is that of Bowers and Johnson, although others have also utilized similar approaches (49-52). In general, enzyme catalyzed reactions in HPLC-SPR will release an analyte of interest from its biological conjugate, on-line, and often provide a more simple and convenient sample clean-up procedure for direct analysis of the released analytes. However, it is very difficult to imagine an enzyme supported reagent tagging an analyte for improved overall detection. Other chemical reactions, enzyme initiated, are another possibility, other than deconjugation. Bowers and Johnson have described the use of an immobilized enzyme, beta-glucuronidase, as an on-line, pre-column modification reagent in the HPLC-SPR analysis of certain steroids and steroidal-glucuronide conjugates. In the trace analysis for the free steroid, it is often required to cleave either the glucuronide or sulfate conjugates initially, prior to extraction, derivatization, and/or pre-concentration steps in the sample preparation. The reaction catalyzed here is the enzymatic hydrolysis of the glucuronide conjugates of the analytes of interest, here estriol and estradiol. These steroids, once released, can then be separated and detected using standard reversed phase HPLC conditions and procedures. The beta-glucuronidase was immobilized on controlled pore glass or polyacrylamide. The release of the steroids from their conjugates does not chemically alter/change the structures of the steroid molecules, but only their initial attachment to the glucuronide moiety is altered. Difference HPLC would provide information regarding the relative ratio of bound vs free steroids present in the initial sample matrix, using SPR to provide chromatographic differences. Thus, analysis of the sample without any initial enzymatic cleavage of conjugates would indicate how much free steroids are initially present in the sample. Analysis of the same sample

with enzymatic hydrolysis of the conjugates, followed by analysis for the newly formed/released steroids, would then provide the amount of initially conjugated steroids present in the same sample.

In a typical operation of the enzyme bound SPR, a sample is introduced onto the SPR in one mobile phase that has a low methanol content, and there is then complete conversion of the glucuronide conjugates before the sample travels 1% of the reactor length/bed. The excess enzyme present in the reactor assures a long operational lifetime for the reactor. With the initial mobile phase of 0.05M phosphate buffer, pH 6.8, the cleaved steroids are immediately transferred to the top of the C_{18} analytical column. Once several column volumes of this first mobile phase have passed through the SPR, the reactor bypass valve is turned, removing the SPR from the analytical system. Now a gradient elution mobile phase pattern is started, and this eventually reaches 100% of a strong solvent consisting of 42.5/57.5 phosphate buffer/methanol. As each deconjugated steroid is eluted from the analytical column and reaches the UV detector, it is detected using a wavelength of 280 nm. The immobilized enzyme cannot tolerate a mobile phase consisting of more than 15% methanol, for above this level, it becomes permanently denatured and useless for further reactions. Difference chromatograms for the analysis of estriol and estradiol from their conjugates in biological matrices have been presented by Bowers and Johnson in the literature (49, 50). This is an excellent example of a solid phase reactor system used in the pre-column mode, together with conventional reversed phase HPLC-UV detection. Immobilized enzyme technology has developed to the point where a very large number of commercially available enzymes could be used in this type of SPR for improved HPLC qualitative analyses.

III.E. POLYMER SUPPORTED TRANSESTERIFICATION OR DISULFIDE INTERCHANGE TYPE REACTIONS FOR DERIVATIZATION IN HPLC-SPR

In principle, polymeric transesterification reactions should be quite similar to polymeric disulfide interchange type reactions, both of which have already been described in synthetic organic chemistry (31). In principle, a transesterification reaction can tag a carboxylic or sulfonic acid substrate with an alcohol initially bound to the polymeric ester, wherein the ester was bound to the polymer *via* the carboxylic (sulfonic) acid portion. If the ester is bound to the polymer *via* the alcohol end, then a transesterification reaction with another alcohol would release the carboxylic acid portion in the form of a free ester with the substrate alcohol. Another possible scenario might be wherein an ester bound to the polymer *via* the carboxylic acid portion is reacted with an alcohol, this alcohol substrate undergoes transesterification, displaces the initial alcohol bound to the polymer, and this released

alcohol is then detected after the HPLC column. In one type of transesterification reaction, the initial substrate is tagged, while in the other type of reaction, one portion of the initial ester is displaced and the final compound to be detected is released from its initial attachment to the polymeric SPR. This same type of a scenario is also possible with disulfide interchange type reactions, which involve the replacement of one thiol group in the initial disulfide by another thiol, or the tagging of an initial thiol by another thiol initially on a polymer bound disulfide, leading to a new disulfide which is then detected. All of these various sequences are indeed theoretically possible, but very few of them have ever been utilized in HPLC-SPR type derivatizations. This is despite the fact that a very large number of polymeric transesterification type reactions have already been described in the synthetic organic literature, especially by Patchornik et al. (53-55). In view of the relatively mild conditions often needed for transesterification type reactions, we would expect that polymer supported reactions of this nature in HPLC-SPR applications will shortly be described (56).

In the area of disulfide interchange reactions, some work has been described with regard to HPLC-SPR interfacing, most notably that of Studebaker et al. (57, 58). This approach has proven useful for the detection of thiols, disulfides, and proteolytic enzymes in HPLC eluates. In each case, the analytes of interest release a chromophoric reagent from the polymeric SPR after the analytical column (post-column mode). This once released chromophoric reagent is then detected with a conventional UV-VIS detector in HPLC, at 412 nm for thiols and disulfides or 520 nm for certain enzymes.

These SPRs contain Sepharose polymers with covalently bound thiol groups. Such thiol-Sepharose groups can then form mixed disulfides with a strong chromophore such as m-dinitrophenylcysteine (DNP-cysteine). Clearly, a fluorophore or electrophore could just as readily be incorporated within the mixed disulfide attached to the Sepharose backbone. Wherein this mixed disulfide SPR is placed after the analytical column, individually eluting thiols, the analytes in this case, will undergo, as a function of pH, a rapid and efficient disulfide interchange reaction with the polymer bound disulfide. The eluting thiol thereby forms a new mixed disulfide with the DNP-cysteine moiety, which is now released from the polymeric backbone. The newly formed disulfides, all of which will now have the same chromophoric moiety, then elute from the SPR and enter the UV-VIS detector. This is the basic method for the analysis and identification of thiols by disulfide interchange, but it may also be possible to utilize this same approach for alcohols/phenols.

For the application of this SPR approach to disulfide analysis, one modification of the above described system must be made, on-line. A separate

column of Sepharose-thiol is placed on-line before the Sepharose-disulfide SPR, after the HPLC separation column and before the detector. In operation, as a disulfide elutes from the HPLC column, it first enters the polymeric thiol SPR. Here, the eluting disulfide undergoes a disulfide interchange reaction with the polymer bound thiol. This forms a polymer bound disulfide and a newly released thiolate anion from the initially eluting disulfide. From this point forward, the reactions already described for the direct analysis of a thiol apply, as above. That is, the newly formed thiolate anion now enters the second SPR, containing the DNP-cysteine bound chromophore that will undergo another disulfide interchange reaction with the formed thiolate anion. A new disulfide is then formed, containing the thiolate anion initially released from the analyte disulfide together with the DNP-cysteine initially polymer bound to the disulfide SPR. This new UV active disulfide then elutes from the second SPR and is detected, as above.

One final application or modification of the above polymer bound disulfide interchange reactions has been described by Studebaker *et al.*, and this allows for the analysis of hydrolytic enzymes *via* HPLC-SPR. If we have at the start a hydrolyzable substrate, UV active, bound to a solvent insoluble support, then this could be cleaved by an appropriate enzyme in solution. The overall sensitivity of this particular method would depend on how many chromophoric substrate fragments are cleaved by an enzyme molecule during its lifetime within the SPR. In the application described, Azocoll, a strong vis-absorbing dye molecule is attached to a polymer *via* an amide linkage (-CO-NHRAz, Az = Azocoll). Since this is basically a peptide bond, a suitable peptidase enzyme could hydrolyze this bond, thus releasing a protonated amine-dye molecule (NH_3^+RAz). The amine-dye molecule then enters the optical detector and its response is recorded at a particular wavelength. The formation and detection of the amine-dye molecule thus indicates, in an indirect manner, an initial presence of the correct enzyme in the initial sample solution. Since the enzyme itself has not been consumed in this reaction, it elutes along with the amine-dye molecule, and it can therefore be analyzed by an alternative method, if desired (57, 58). It should be possible to utilize an analogous approach for the HPLC-UV-VIS analysis of other suitable enzymes, assuming that they can also release a detector-active moiety from the SPR.

This entire area of transesterification or disulfide interchange type reactions has been rarely utilized in HPLC-SPR, despite the very large number of possible reactions that have already been utilized on a polymeric support. One would hope that with the wide availability of polymeric and silica bound ion exchange packing materials available today, some enterprising analytical/

organic chemists will creatively utilize such materials for solid phase reactions and derivatizations in improved HPLC qualitative and quantitative analyses.

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V. REFERENCES

1. Lawrence, J.F. and Frei, R.W., Chemical Derivatization in Liquid Chromatography, Elsevier Scientific Publ. Co., Amsterdam, Holland, 1976.

2. Lawrence, J.F., Organic Trace Analysis, Academic Press, Inc., New York, 1981, Chapter 7.

3. Frei, R.W. and Lawrence, J.F., eds., Chemical Derivatization in Analytical Chemistry. Volume 1: Chromatography, Plenum Press, Inc., New York, 1981. Chapters 1, 3, and 4.

4. Knapp, D.R., Handbook of Analytical Derivatization Reagents, J. Wiley & Sons, Inc., New York, 1979.

5. Blau, K. and King, G., eds., Handbook of Derivatives for Chromatography, Heyden & Son, Ltd., London, 1977.

6. Snyder, L.R. and Kirkland, J.J., Introduction to Modern Liquid Chromatography, Second Edition, J. Wiley & Sons, Inc., New York, 1979. Chapter 17.

7. Stewart, J.T., Trends in Analytical Chemistry (TRAC), 1, 170 (1982).

8. Clark, C.R. and Wells, M.M., J. Chrom. Sci., 16, 332 (1978).

9. Lawrence, J.F., J. Chrom. Sci., 17, 147 (1979).

10. Ahuja, S., J. Chrom. Sci., 17, 168 (1979).

11. Gfeller, J.C., Huen, J.M., and Thevenin, J.P., Chromatographia, 12, 368 (1979).

12. Schwedt, G., Ang. Chem. Int. Ed., English, 18, 180 (1979).

13. Ross, M.S.F., J. Chromatogr., 141, 107 (1977).

14. Krull, I.S. and Lankmayr, E.P., American Laboratory, 18 (May, 1982).

15. McKillop, A. and Young, D.W., Synthesis, 401, 481 (1979).

16. Posner, G.H., Ang. Chem. Int. Ed., English, 17, 487 (1978).

17. Krull, I.S., Colgan, S., Xie, K-H., Neue, U., King, R., and Bidlingmeyer, B., J. Liquid Chrom., in press (1983).

18. Santaniello, E., Ponti, F., and Manzocchi, A., Synthesis, 891 (1978).

19. Santaniello, E., Farachi, C., and Manzocchi, A., Synthesis, 912 (1979).

20. Schwartz, D.P. and Reynolds, R., Microchem. J., 20, 50 (1975).

21. Fung, N.Y.M., de Mayo, P., Schauble, J.H., and Weedon, A.C., J. Org. Chem., 43, 3977 (1978).

22. Taylor, E.C., Chiang, C-S., McKillop, A., and White, J.F., J. Amer. Chem. Soc., 98, 6750 (1976).

23. Regen, S.L. and Koteel, C., J. Amer. Chem. Soc., 99, 3837 (1977).

24. Santaniello, E., Ponti, F., and Manzocchi, A., Synthesis, 534 (1978).

25. Singh, R.P., Subbarao, H.N., and Dev, S., Tetrahedron, 35, 1789 (1979).

26. Lalancette, J.M., Rollin, G., and Dumas, P., Can. J. Chem., 50, 3058 (1972).

27. Filippo, S.S., Jr. and Chern, C-I., J. Org. Chem., 42, 2182 (1977).

28. McKillop, A. and Young, D.W., Synthesis, 481 (1979).

29. Risbood, P.A. and Ruthven, D.M., J. Amer. Chem. Soc., 100, 4919 (1978).

30. Leyden, D.E., J. Org. Chem., 46, 2406 (1981).

31. Mathur, N.K., Narang, C.K., and Williams, R.E., Polymers as Aids in Organic Chemistry, Academic Press, New York, 1980.

32. Demko, P.R., Wagner, S.E., Mikulski, R.A., and Clements, J.G., in Ions in Polymers, ed. by Eisenberg, A., Advances in Chemistry Series No. 187, American Chemical Society, Washington, D.C., 1980. Chapter 13.

33. Ventron Corporation Technical Bulletin No. TB478. Resin-borohydride developmental polymeric reducing agent. Ventron Division, Thiokol Corp., Danvers, Mass.

34. Krull, I.S., Xie, K-H., Colgan, S., Neue, U., Izod, T., King, R., and Bidlingmeyer, B., J. Liquid Chrom., in press (1983).

35. Menger, F.M., Shinozaki, H., and Lee, H-C., J. Org. Chem., 45, 2724 (1980).

36. Cainelli, G., Cardillo, G., Orena, M., and Sandri, S., J. Amer. Chem. Soc., 98, 6737 (1976).

37. Frechet, J.M.J., Darling, P., and Farrall, M.J., J. Org. Chem., 46, 1728 (1981).

38. Frechet, J.M.J., Warnock, J., and Farrall, M.J., J. Org. Chem., 43, 2618 (1978).

39. Krull, I.S., Xie, K-H., Colgan, S., Santasania, C., Neue, U., King, R., Bidlingmeyer, B., and Newhart, A., J. Liquid Chrom., in preparation (1983).

40. Gelbard, G. and Colonna, S., Synthesis, 113 (1977).

41. Iversen, T. and Johansson, R., Synthesis, 823 (1979).

42. Werkhoven-Goewie, C.E., Niessen, W.M.A., Brinkman, U.A.Th., and Frei, R.W., J. Chromatogr., 203, 165 (1981).

43. Manecke, G., Chimia, 28, 9 (1974).

44. Zaborsky, O., Immobilized Enzymes, CRC Press, Inc., Chemical Rubber Company, Inc., Boca Raton, Florida, 1973.

45. Weetall, H.H., Immobilized Enzymes, Antigens, Antibodies, and Peptides, Volume 1, Marcel Dekker, New York, 1975.

46. Mosbach, K. FEBS Lett., 62 (Suppl. 1), E80 (1976).

47. Hartley, F.R. and Vezey, P.N., Adv. Organometal. Chem., 15, 189 (1977).

48. Grubbs, R.H., Chemtech, 7, 512 (1977).

49. Bowers, L.D. and Johnson, P.R., Anal. Biochem., 116, 111 (1981).

50. Johnson, P.R. and Bowers, L.D., Anal. Chem., 54, 2247 (1982).

51. Ogren, L., Cisky, I., Risinger, L., Nilsson, L.O., and Johansson, G., Anal. Chim. Acta, 117, 71 (1980).

52. Okuyama, S., Kokubun, N., Higashidate, S., Uemura, D., and Hirata, Y., Chem. Lett., 12, 1443 (1979).

53. Kraus, M.A. and Patchornik, A., J. Polymer Sci.: Macromol. Revs., 15, 55 (1980).

54. Kraus, M. and Patchornik, A., Chemtech, 118 (February, 1979).

55. Kraus, M.A. and Patchornik, A., J. Polymer Sci., 47, 11 (1974).

56. Krull, I.S., LaCourse, W., Neue, U., Bidlingmeyer, B., and Newhart, A., unpublished results (1983).

57. Studebaker, J.F., J. Chromatogr., 185, 497 (1979).

58. Studebaker, J.F., Slocum, S.A., and Lewis, E.L., Anal. Chem., 50, 1500 (1978).

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN OENOLOGY

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INTRODUCTION

In this review I have included every reference I have been able to find in which h.p.l.c. has been used in analysing wines and musts. Clearly an article such as this would be incomplete if allied fields like brewing, food chemistry, and phytochemistry were ignored. I have therefore used my discretion in selecting from the huge number of publications in these areas those papers which seem to me likely to be of value to oenologists.

ORGANIC ACIDS

A review of analysis of organic acids in wine has appeared[1]. Palmer and List[2] separated organic acids on Aminex A25 resin in the formate form, eluting with aqueous sodium formate and using R.I. detection. The acids were trapped on a short column of the same resin which was then washed with water prior to elution of the acids onto the separation column. Rapp and Ziegler[3] described separation of citric, malic, tartaric, and succinic acids on Aminex A6 resin eluting with an isobutyl acetate-isobutyl alcohol-water mixture and using R.I. detection;

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with wines enrichment with ion exchange resin was necessary. The same authors[4] used an anion exchange resin to split wine into acid and neutral fractions, then analysed each fraction with a cation exchange resin column eluting with aqueous methanol and using R.I. detection. The neutral fraction contained sugars, ethanol, glycerol, and butane 2,3 - diols and the acid fraction the wine acids. Rajakkyla[5] separated organic acids, including the major wine acids, on Aminex HPX - 87 resin at 65° eluting with 0.01N H_3PO_4 and employing detection at 210nm. The author has seen chromatograms of wines and of standard mixtures of acids using essentially the same conditions as Rajakkyla, separations were excellent and most of the other U.V. absorbing materials from the wine eluted before the acids. The wines were simply filtered before injection and several hundred samples were run without deterioration of the colum performance[6].

Lee[7] separated wine acids on a column of macroporous poly (styrene-divinylbenzene) resin eluting with pH 2.6 buffer and detecting at 210nm. Separation of a standard mixture was excellent but a chromatogram of a Californian wine was congested in the region around tartaric acid. Gump and Kupina[8] investigated analyses of gluconic and other wine acids both on a C-18 column and on Aminex 50W x 4, with R.I. detection. Schneyder and Flak[9] concentrated acids from wine on Lewatit M5020 resin then separated them on a C18 column eluting with a phosphate buffer and using R.I. detection. Jeuring et al[10] separated malic and citric acids by direct injection of filtered apple juice onto a C-8 column, eluting with phosphate buffer and employing detection at 220nm. Bush et al[11] similarly separated C_2 - C_5 fatty acids on a C18 column using a phosphate buffer containing 10% methanol with detection at 210nm. Droz and Tanner[12] separated acids in fruit juices and wine on a C18 column

eluting with an aqueous buffer containing KH_2PO_4, $Bu_4N\ PO_4$ and H_3PO_4 and employing detection at 225nm. Gonnet and Marichy[13] investigated the chromatography of wine acids using a variety of reverse phase columns and solvents but decided that the best separation they obtained was unsatisfactory. Stahl and Laub[14] determined acids in wines and fruit juices on a column of microcrystalline cellulose eluting with ethyl acetate-n-propanol-water and using conductivity, U.V., R.I., and pH detectors in series. Stahl et al[15] compared this method with official methods of analysis and obtained good agreement. Israelian[16] discussed briefly the application of h.p.l.c. to wine analysis and separated wine acids on an ion exchange column. Friberg[17] separated low m.wt. acids (as well as saccharinic acids, aldonic acids, and monosacharides) in various systems including wines and discussed potential applications in the food industry with special reference to winemaking.

The above methods all use R.I. or short wavelength U.V. detection. In the authors experience R.I. detection can give solvent and other peaks which may interfere with the peaks of interest. For example when using a Waters Rad-Pak C-18 column with a phosphate buffer of pH 2.6, water gives a negative peak very close to tartaric acid, reducing the height of the latter peak. Short wavelength U.V. is absorbed by many wine components and broad peaks eluting after the acids can delay injection of the next sample.

A number of other methods of detection have been described which may merit investigation for wine acids. Farinotti et al[18] separated various short chain acids on a C18 column with aqueous acetonitrile, the effluent was mixed with bromocresol purple and the absorbance at 425nm was monitored, it is claimed that this method is ten times more sensitive than using U.V. absorbance at 205nm. Cochrane and Hillman[19] separated anions

on a Vydoc 302 column, eluting with .005M potassium hydrogen phthalate, the eluate was monitored at 302nm and the ions were seen as peaks of decreased U.V. absorbance. Buytenhuis[20] used a similar principle, eluting anions from anion exchange resin with mobile phases of high refractive index to increase the difference in R.I. between the eluant and the eluted ions.

Organic acids in wines can be separated with Dionex Ion Chromatographs[21], these are very costly however. Molnar et al[22] described a similar system in which the suppressor column can be omitted, though at some loss of baseline stability. Both these systems use conductivity detection.

Organic acids can also be analysed after derivatisation and much excellent work has been published using this approach. Methylanthracene derivatives[23] for fluorescence detection and napthyl derivatives[24] for U.V. detection may be prepared by reaction of acids with diazo derivatising agents. Napthacyl[25] and phenacyl[26-31] derivatives have been described, these have very high U.V. absorbance and are readily separated by normal and reverse phase chromatography. Crown ether catalysts[28,29] enable dibasic acids to be esterified and such a catalyst has been used by Gonnet and Marichy[32] as a basis for analysing acids in wines by reverse phase chromatography. Potassium fluoride, which is cheaper than crown ethers, can also be used to catalyse phenacyl ester formation[30,31] however it does not bring about esterification of tartaric, malic, and succinic acids under conditions which esterify acetic and benzoic acids[33].

The author[33] has found that ω -tosyloxy acetophenone (prepared from styrene glycol by tosylation and oxidation) is a useful alternative to phenacyl bromide as it is widely separated from the latter compound in

reverse phase chromatography. This is advantageous where the phenacyl ester of interest and phenacyl bromide chromatograph close together. Also the tosyl compound is not lachrimatory.

Organic acids may also be derivatised with 4-bromomethyl-7-methoxycoumarin[34-36] and the derivatives can be separated by reverse phase chromatography with fluorescence detection. Again the use of crown ether catalysts[36] makes possible derivatisation of dicarboxylic acids.

Shimazu and Watanabe[37] separated organic acids in wines and musts by a new h.p.l.c. analyser using 'a highly specific and highly sensitive detection method'.

Monk et al[38] have separated wine acids by low pressure chromatography on Dowex 50W x 2 eluting with dilute butyric acid and using conductivity detection.

BENZOIC, SORBIC, ASCORBIC, AND ISOASCORBIC ACID

McCalla et al[39] analysed sorbic acid in wine on an anion exchange column eluting with a borate buffer and employing detection at 254nm. Eisenbeiss et al[40] and Clasodonte et al[41] determined sorbic acid in wine in the presence of benzoic acid. Froehlich[42] optimised reverse phase separation of benzoic acid and sorbic acid from each other and from the matrix in wine and in cherry and sauerkraut juices. Kubota[43] determined sorbic acid on an anion exchange column with a phosphate buffer and U.V. detection.

Bui-Nguyen[44] determined ascorbic and isoascorbic acids on a LiChrosorb NH_2 column using aqueous acetonitrile containing KH_2PO_4. Dennison et al[45] employed a similar system with methanol in place of acetonitrile to determine ascorbic acid and deoxy ascorbic acid. The

latter compound was reduced by DL homocysteine to ascorbic acid before analysis. Geigert et al[46] analysed ascorbic and isoascorbic acids on a Waters Carbohydrate analysis column eluting with aqueous acetonitrile containing phosphate buffer.

SUGARS

Palmer and Brandes[47] separated sugars in deionised grape juice on Aminex Q150S K^+ at 60° eluting with water. Rapp et al[48] analysed wine for sucrose, glucose, fructose, glycerol and ethanol by direct injection onto a column of Aminex A6 resin and eluting with water. Rapp et al[49] used this method to study the inversion of sucrose in grapes.

Shimazu et al[50] analysed sugars, polyols and ethanol in wine and must on a Diaion CK08S (Ca^{2+}) resin column. Schmidt et al[51] determined mono and oligosaccharides in beer and wort on Aminex HPX-42 resin at 85° eluting with water. Dunsmire and Otto[52] described a general procedure for analysing sugars in a variety of matrices using a μBondapak/Carbohydrate column eluting with aqueous acetonitrile. Wong-Chong and Martin[53] used a similar method with flow programming to speed analysis, these authors[54] also examined ion exchange resins for sugar separations and obtained excellent resolution on Aminex Q150S(K^+) at 60°, eluting with water. Czerny et al[55] determined sugars and polyols in foods with a μBondapak/Carbohydrate column eluting with aqueous acetonitrile. Palla[56] separated sugars on μBondapak C18 eluting with water. Flak[57] removed acids from wines with ion exchange resin in the acetate form then separated the sugars on either a μBondapak/Carbohydrate column or an amino bonded column. Muller and Siepe[58] separated sugars on

LiChrosorb NH_2 and recommended ethyl acetate-acetone-water as a less toxic alternative to aqueous acetonitrile. Aitzetmuller[59] described a method for the analysis of sugars and glycerol on silica, eluting with aqueous acetonitrile containing an amine modifier, this is claimed to show advantages over a bonded NH_2 packing whilst giving similar separations. Johncock and Wagstaffe[61] used Aitzetmullers' method and discussed the sources of baseline instability in R.I. detection and the need for thermostatting the various parts of the chromatograph to obtain increased sensitivity.

Goiffon et al[62] analysed glycerol and sugars in wines on an amino bonded column eluting with aqueous acetonitrile. Stahl et al[63] using a column of microcrystalline cellulose, a mobile phase of ethyl acetate-n-propyl alcohol-water, and R.I. and microadsorbtion detectors analysed wines for polyhydric alcohols. The same column could be employed to determine C_1-C_5 monohydric alcohols using benzene as the eluent. Iverson and Bueno[64] using a method similar to that of Palmer and Brandes[47] compared h.p.l.c. and g.l.c. for analysis of sugars and concluded that h.p.l.c. was both quicker and more accurate.

All the methods in this section used R.I. detection. It should be noted that sugars can be detected also with short wavelength U.V, although the problems mentioned in the section on acids would apply with sugars also.

BIOGENIC AMINES

Subden et al[65] described a method for determining histamine in wine as its o-phthaldialdehyde derivative on a μBondapak C18 column

eluting with a phosphate buffer in aqueous acetonitrile and with detection at 200nm and 220nm. The same group reported[66] results obtained with Canadian wines. Buteau et al[67] looked at the stability of the o-phthaldialdehyde complex and showed that after derivatisation, acidification or extraction into ethyl acetate was desirable to prevent a rapid decrease in absorbance. Battaglia and Froehlich[68] determined histamine in wine by dansylation, separation on Spherisorb 5SW and fluorimetric detection. This method was later extended[69] to incorporate a step gradient, this separated more than 20 biogenic amines. Woidich et al[70] analysed biogenic amines in wines with a Liquimat 11 amino acid analyser.

CARBONYL COMPOUNDS

Okamato et al[71] analysed acetaldehyde in wines as its lutidine derivative on either a 3-aminopropyltriethoxysilane treated silica column or a bonded amino column, eluting with ethanol-hexane and with U.V. or fluorescence detection. Suzuki and Maruyama[72] determined aldehydes by injecting them in aqueous solution into a heated column of 2,4-dinitrophenylhydrazine on celite which was connected to the h.p.l.c. column, wines analysed this way showed acetaldehyde levels of 24-100mg/l.

Numerous publications have dealt with determination of carbonyl compounds as their 2,4-dinitrophenylhydrazones. Selim[73] showed that the reaction between carbonyl compounds and 2,4-dinitrophenylhydrazine is an equilibrium which on a macroscale is driven to completion by precipitation of the product. On a microscale the reaction may not go to completion due to the hydrazone remaining in solution. The reaction may be driven to

completion by addition of an immiscible organic phase to extract the product. Selim's method appears quite suitable for determination of carbonyl compounds in wines. 2,4-Dinitrophenylhydrazones are readily separated by reversed phase chromatography[73-76].

Carbonyl compounds in beer have been detected down to .1 ug/l by low pressure distillation and trapping in 2,4-dinitrophenylhydrazine solution followed by reverse phase chromatography, and also by a combination of steam distillation and liquid-liquid extraction followed by reaction with o-nitrobenzylhydroxylamine and reverse phase chromatography. The latter reagent is claimed to be superior to 2,4-dinitrophenylhydrazine as a derivatising reagent.

PHENOLIC COMPOUNDS

Anthocyanidins and Anthocyanins

Manley and Shubiak[79] separated the 3-glucosides of malvidin, petunidin, and peonidin on Pellidon (polyamide bonded onto glass beads) eluting with chloroform-methanol. In all later reports of anthocyan(id)in separations reverse phase chromatography has been the method of choice. Adamovics and Stermitz[80] separated delphinidin, cyanidin and pelargonidin (and also the 3-O-rutinosides of quercetin, kaempferol, and isorhamnetin) on a C18 column eluting with aqueous methanolic acetic acid. Wilkinson et al[81] separated the anthocyanidins on a μBondapak C18 column also using aqueous methanolic acetic acid. Akavia and Strack[82] reported that this separation could not be achieved on other reverse phase columns and recommended the use of aqueous acetonitrile containing 1.5% H_3PO_4

as the mobile phase. Akavia et al[83] also described a method for identifying anthocyanins by following the appearance and disappearance of the products of graded acidic hydrolysis. Williams et al[84] separated anthocyanidin 3-glucosides, 3,5-diglucosides, and their p-courmaryl derivatives on a μBondapak C18 column. Wulf and Nagel[85] obtained excellent separations of anthocyanidin 3-glucosides and their acetate, coumarate, and caffeoate derivatives on a octadecylsilyl column, they also examined the products of alkaline hydrolysis and degradation to aid in identification of the pigments. McCloskey and Yenqoyan[86] studied wine colour during aging but the reference[87] given for their chromatographic method is not available to the author. Pergiovanni and Volonterio[88] separated anthocyanins with a formic acid-water-methanol gradient on a μBondapak C18 column or a LiChrosorb RP18 column, and the aglycones with methanol-water-acetic acid on the μBondapak C18 column. Bertrand et al[89] used essentially the same conditions to follow changes in anthocyanin levels during fermentations of Cabernet Sauvignon and Merlot grapes. Preston and Timberlake[90] separated the flavilium and chalcone forms of malvidin 3-glucoside and 3,5-diglucoside on a column of Spherisorb hexyl eluting with a gradient of aqueous methanol containing .6% of perchloric acid. Israelian[16] analysed anthocyanins on a C18 column.

Other Phenolics

Wulf and Nagel[91] separated phenolic acids and D-catechin on a C18 column with acetic acid-methanol-water eluants. They also chromatographed flavanoids and flavanoid glycosides but complete resolution was not possible as some groups of compounds, e.g. aglycones and monoglucosides, overlapped. Ong and Nagel[92] separated caffeoyl, p-

coumaryl, and feruoyl esters of tartaric acid on a C18 column with water-acetonitrile-H_3PO_4 mixtures and also described[93] changes in levels of these compounds during maturation of White Riesling grapes. Nagel et al[94] used a similar method to examine hydroxycinnamic esters of tartaric acid in grapes and wines from the Pacific Northwest of the U.S.A. Nagel and Wulf[95] followed changes in the concentrations of various phenolics during the aging of Cabernet Sauvignon and Merlot wines. Using a C18 column hydroxycinnamic esters were eluted with water-acetonitrile-acetic acid, anthocyanins and polymeric pigments with water-acetone-acetic acid, and ethyl acetate extractable phenolics with water-acetonitrile-acetic acid adjusted to pH4. At pH4 the hydroxycinnamic esters eluted rapidly and did not interfere with examination of other phenolics in the extract. Wulf and Nagel[96] used preparative reverse phase chromatography with recycling to isolate flavanoids from Cabernet Sauvignon and Merlot wines, and Baranowski and Nagel[97] similarly isolated hydroxycinnamic esters from White Riesling grapes.

Symonds[98] partly decolourised wine with 50W x 8 resin, then analysed the organic acids by the method of Palmer and List[2], whilst the phenolic acids were either extracted with ether or into acetone after saturating the wine with salt, then analysed by reverse phase chromatography. Okamura and Watanabe[99,100] looked at phenolic constituents of wine and must on a C18 column eluting with a methanolic aqueous phosphate buffer, the effect of phenolic acid esters of tartaric acid on wine quality was investigated. Nickenig and Pfeilsticker[101] fractionated oxidised wine on Sephadex G25 and examined the fractions for phenolics with a water-methanol-acetic acid gradient on a Nucleosil-7 C8 column, using an esterase to cleave ethyl hydroxycinnamates. On the same

column dicarboxylic acids were separated by elution with .3% phosphoric acid and detection at 195 and 220 nm.

Dumont[102] determined chlorogenic acid in grape juice and wines, inter alia, by enzymic cleavage followed by chromatography of the liberated caffeic and quinic acids on a LiChrosorb RP18 column. Slinkard[103] used h.p.l.c. among other techniques to study phenolics of grape skins.

Villeneuve et al[104] described a general procedure for analysing plant phenolics by reverse phase chromatography with methanol-water-acetic acid mixtures, changes in phenolic acids in grape juice during storage were followed. Murphy and Stutte[105] separated various substituted benzoic and cinnamic acids on a μ Bondapak C18 column using gradient elution with mixtures of n-butanol-methanol-water and acetic acid containing ammonium acetate. The latter compound inhibited intramolecular bonding and made possible separation of all 13 acids examined. Krause and Strack[106] separated a range of hydroxycinnamic acid derivatives on an RP8 column with gradient elution using aqueous methanolic acetic acid or citrate buffer in place of acetic acid, (but note that Waters Associates state[107] that citrate buffers should not be used with their reverse phase columns). It was found that varying the proportion of acetic acid in the solvent produced marked changes in retention times and selectivity which can be used to optimize separations.

The separations described above all used alkylsilyl bonded column packings but other bonded materials can also be utilised. Vanhaelen and Vanhaelen-Fastré[108] used an alkylphenyl column with a gradient of water,

ethanol, and acetic acid to resolve various aromatic acids and phenolics. Also some flavonoids were separated with isocratic elution. The authors note that ethanol gives better separations than does methanol. Nagels et al[109] chromatographed glucose- and quinic acid-cinnamates and benzoates on both RP8 and diol columns. The diol column gave separations similar to adsorbtion chromatography but without the sensitivity to water shown by alumina and silica columns. The elution order was not simply the reverse of that given by the RP8 column, thus offering a different selectivity. A preliminary separation on ECTEOLA cellulose (separation based on charge differences at pH7) was a useful adjunct to the hplc separations. Becker et al[110] used an aqueous acetonitrile gradient on a LiChrosorb NH_2 column to separate the polar flavonoids isoorientin 3'-O- and 4'-O-glucosides which were not resolved on LiChrosorb RP8.

McMurrough[111] analysed phenolics from hops and barley on a μBondapak C18 column and found that for separating flavonol glycosides tetrahydrofuran as the organic modifier gives better resolution than acetonitrile although the latter solvent gives better resolution than methanol.

In one of the few reports of h.p.l.c. of phenolics which did not rely on U.V. detection Roston and Kissinger[112] used electrochemical detection and showed that the current-potential responses of eluted compounds can be used as an aid in identification. Similarly Sontag et al[113] examined electrochemical detection for methyl and ethyl esters of various hydroxycinnamic acids, the esters of different acids are oxidised at different potentials giving scope for selective detection. The methyl and ethyl esters of each acid are oxidised at the same potential. Galensa

and Herrmann[114,115] separated flavones, flavanols, and flavanones by acetylation followed by chromatography on LiChrosorb Si 60, eluting with various mixtures of organic solvents. Of interest in connection with this method is the observation of Coutts et al[116] that phenols may be acetylated in aqueous solution with quantitative recoveries.

There has been an increased interest recently in the role of procyanidins in wine[117,118] and it is appropriate to note here h.p.l.c. of these compounds although most publications have been in non-oenological areas. Lea[119] separated cider procyanidins by reverse phase chromatography using aqueous methanol containing perchloric acid as the mobile phase. The same author subsequently reported[120] the use of Snyder's procedures to optimise separation of procyanidin oligomers showing that shallow gradients are required and that a sharp increase in solvent strength elutes oxidised materials as a fairly sharp band. This may be useful in studying these intractable materials. Lea also suggested[120] that Snyder's procedures could be used to estimate molecular weights of procyanidin polymers.

Jerumanis[121] separated polyphenol oligomers using a gradient of acetic acid in water and a C18 column. Oligomers isolated from beer showed very rapid depolymerisation. Mulkay et al[122] used Jerumanis' method to isolate polymers the structures of which were then elucidated by acetylation and n.m.r. spectroscopy. Kirby and Wheeler[123] analysed beer polyphenols on Spherisorb S50DS with a water-methanol-KH_2PO_4 gradient and found only monomers and dimers. Jende-Strid and Møller[124] separated barley procyanidins with an aqueous acetic acid gradient on a μBondapak phenyl column. Wilson[125] analysed phenols from apple juice

ranging from dimeric to heptameric procyanidins by normal phase chromatography on a bonded CN column. The procyanidins were separated into groups according to their degree of polymerisation in this way. The isomers in each group were then resolved with a C18 column using methanol-water-KH_2PO_4.

AFLATOXINS

Takahashi[126,127] described analysis of wine for aflatoxins, normal or reverse phase chromatography were suitable but reverse phase was preferred, fluorescence and U.V. absorbance detection were both employed. Takahashi and Beebe[128] developed a general method for aflatoxins in foods and beveages including wines. Wei and Chang[129] analysed wines on a uPorasil column after clean up on silica gel and by thin layer chromatography. Sripathomswat and Thasnakorn[130] used h.p.l.c. in studies of aflatoxin producing fungi in a range of foodstuffs including wine.

AMINO ACIDS

Ishida et al[131] separated amino acids in grape juice on a strong acid resin column with fluorescence detection after reaction with sodium hypochlorite then with 2-mercaptoethanol and o-phthaldialdehyde. Casoli and Colagrande[132,133] determined amino acids in sparkling wines by dansylation followed by reverse phase chromatography using a complex acetonitrile-water-phosphoric acid-acetic acid gradient and fluorescence detection. Schuster[134] analysed free amino acids and several vitamins in beverages (and intravenous solutions) on an amino bonded column eluting with an aqueous acetonitrile phosphate buffer and detecting the amino

acids at 200nm. Martin et al[135] determined amino acids in wines and musts as their dansyl derivatives which were separated on a radially compressed reverse phase column using gradient elution and fluorescence detection.

MISCELLANEOUS

Vialle et al[136] measured betaine in wine and beet sugar on a bonded NH_2 column eluting with aqueous acetonitrile and using R.I. detection. Initial sample cleanup was carried out on two ion exchange columns. Tyson et al[137] determined soluble protein in wine and must using a size exclusion column. Protein can however be determined far more cheaply by low pressure chromatography[138,139]. Tenenbaum and Martin[140] used h.p.l.c. to determine saccharin in alcoholic beverages.

Rhys-Williams and Slavin[141] determined methyl anthranilate in grape beverages by reverse phase chromatography with fluorescence detection. Qureschi et al[142] described a method of analysing beverages for purines, pyrimidines, nucleosides, nucleotides, phenolics, and pyrazines. It was found that addition of 1.5% acetic acid to the mobile phase sharpened the peaks but larger amounts impaired the resolution.

Micali et al[143] analysed alcoholic and non alcoholic beverages for β asarone by steam distillation and extraction followed by reverse phase chromatography with fluorimetric detection.

Steuerle[144] determined artificial dyes on a LiChrosorb NH_2 column. An aqueous solution of the dyes was pumped through the column, this concentrated the dyes in a narrow band at the top of the column. The

unadsorbed materials were washed off with acetonitrile-water-acetic acid then the dyes were eluted with a pH gradient. Recovery of L-Red-12 from a spiked wine was quantitative. Martin et al[145] analysed acid fast dyes, including those often found in wines, by reverse phase chromatography and gradient elution after preliminary separation by adsorption onto wool.

Jeuring et al[146] and Frishkorn et al[147] determined furfural and hydroxymethyl furfural in spirits by reverse phase chromatography.

Sponholz and Lamberty[148] determined styrene in wine by direct injection onto a LiChrosorb RP8 column, elution with aqueous methanol and detection at 254nm. Jakob and Schaefer[149] extracted styrene into cychohexane then chromatographed the extract on an MY Porasil column, eluting with cyclohexane and monitoring at 254nm.

Lazzarini et al[150] examined wines for imidazolidine-2-thione (a degradation product of some fungicides) by extraction with methylene chloride from salt saturated wine and chromatography of the extract on alumina. In a later publication[151] from the same group reverse phase chromatography was used. Caccialanza et al[152] also determined imidazolidine-2-thione by reverse phase chromatography.

Toussaint and Walker[153] used liquid chromatography to clean up cyclohexane extracts of wine, eluting polycyclic aromatic hydrocarbons as a group for subsequent analysis by gas liquid chromatography.

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REFERENCES.

1. J. Calmet. Tec. Lab., 47, 496-505, 1976; Chem. Abstr. 85, 107411s, 1972.

2. J. K. Palmer and D. M. List. J. Agric. Food Chem., 21, 903-906, 1973.

3. A. Rapp and A. Ziegler. Chromatographia, 9, 148-150, 1976.

4. A. Rapp and A. Ziegler. Dtsch. Lebensmittel. Rundsch., 75, 396-398, 1979.

5. E. Rajakyla. J. Chromatogr., 218, 695-701, 1982.

6. D. McWilliam. Unpublished results.

7. D. P. Lee. J. Chromatogr. Sci., 20, 203-208, 1982.

8. B. H. Gump and S. A. Kupina. Liquid Chromatographic Analysis of Foods and Beverages. Volume 2, 331-352.

9. J. Schneyder and W. Flak. Mitt. Klosterneuberg, 31, 57-61, 1981.

10. H. J. Jeuring, A Brands, and P. van Doorninck. Z. Lebensm. Unters. Forsch., 168, 185-187, 1979.

11. K. J. Bush, R. W. Russell, and J. W. Young. J. Liquid Chromatogr., 2, 1367-1374, 1979.

12. C. Droz and H. Tanner. Schweiz. Z. Obst. Weinbau., 118, 434-438, 1982.

13. C. Gonnet and M. Marichy. Recent Developments in Food Analysis: Proceedings of the 1 Europ. Conference on Food Chemistry (Euro. Food Chem. 1), 17-20 Feb. 1981. Ed. W. Baltes. Verlag Chemie, Weinheim, 47-50, 1982.

14. E. Stahl and E. Laub. Z. Lebensm. Unters. Forsch., 152, 280-286, 1973.

15. E. Stahl, E. Laub, and R. Woller. Z. Lebensm. Unters. Forsch., 156, 321-328, 1974

16. C. Israelian. Vignes et Vins, 267, 50-52, 1978.

17. T. S. Friberg. Diss. Abstr. Int. B, 39, 693-694, 1978.

18. R. Farinotti, M. Caude, G. Mahuzier, and R. Rosset. Analusis, 7, 449-453, 1979.

19. R. A. Cocherane and D. E. Hillman. J. Chromatogr., 241, 392-394, 1982

20. F. A. Buytenhuys. J. Chromatogr., 218, 57-64, 1981.

21. Dionex Corp. Application Note No. 21, 6/79.

22. I. Molnar, H. Knauer, and D. Wilk. J. Chromatogr., 201, 225-240, 1980

23. S. A. Barker, J. A. Monti, S. T. Christian, F. Benington, and R. D. Morin. Anal. Biochem., 107, 116-123, 1980.

24. D. P. Matthees and W. C. Purdy. Anal. Chim. Acta., 109, 61-66, 1979.

25. M. J. Cooper and M. W. Anders. Anal. Chem., 46, 1849-1852, 1974.

26. Y. L'Emeillat, J. F. Menez, F. Berthou, and L. Bardou. J. Chromatogr., 206, 89-100, 1981.

27. R. L. Patience and J. D. Thomas. J. Chromatogr., 234, 225-230, 1982.

28. E. Grushka, H. D. Durst, and E. J. Kitka Jr. J. Chromatogr., 112, 673-678, 1975.

29. H. D. Durst, M. Milano, E. J. Kitka Jr., S. A. Connelly, and E. Grushka. Anal. Chem., 47, 1797-1801, 1975.

30. J. H. Clark and J. M. Miller. Tetrahedron Lett., 599-602, 1977.

31. J. M. Miller, I. D. Brindle, S. R. Cater, K-H. So, and J. H. Clark. Anal. Chem., 52, 2430-2432, 1980.

32. C. Gonnet and M. Marichy. Analusis, 7, 370-375, 1981; quoted in ref. 13.

33. M. E. Evans. Unpublished results.

34. W. Dunges. Anal. Chem., 49, 442-445, 1977.

35. S. Lam and E. Grushka. J. Chromatogr., 158, 207-214, 1978.

36. E. Grushka, S. Lam, and J. Chassin. Anal. Chem., 50, 1398-1399, 1978.

37. Y. Shimazu and M. Watanabe. Nippon Jozo Kyokai Zasshi, 76, 418-423, 1981; Chem. Abstr., 96, 4869v, 1982.

38. P. R. Monk and P. Iland. Food Technol. Aust., In press.

39. M. A. McCalla, F. G. Mark, and W. H. Kipp. J. Assoc. Off. Anal. Chem., 60, 71-72, 1977.

40. F. Eisenbeiss, M. Weber, and S. Ehlerding. Chromatographia, 10, 262-264, 1977; Chem. Abstr., 87, 20526a, 1977.

41. M. T. Clasodonte, L. Ciraolo, and R. Calapaj. Rass. Chim., 29, 233-236, 1977; Anal. Abstr., 36, 6 F 59, 1979.

42. D. H. Froehlich. J. High Resol. Chromat. and Chromat. Commun., 5, 158-160, 1982.

43. S. Kubota. Yamanashi-kenritsu Eisei Kogai Kenkyosho Nempo., 23, 39-41, 1980; Chem. Abstr., 94, 63692p, 1981.

44. M. H. Bui-Nguyen. J. Chromatogr., 196, 163-165, 1980.

45. D. B. Dennison, T. G. Brawley, and G. L. K. Hunter. J. Agric. Food Chem., 29, 927-929, 1981.

46. J. Geigert, D. S. Hirano, and S. L. Neidelman. J. Chromatogr., 206, 396-399, 1981.

47. J. K. Palmer and W. B. Brandes. J. Agric. Food Chem., 22, 709-712, 1974

48. A. Rapp, O. Bachmann, and A. Zieqler. Dtsch. Lebensmittel. Rundsch., 71, 345-348, 1975.

49. A. Rapp, A. Zieqler, H. Steffan. Vitis, 16, 112-118, 1977.

50. Y. Shimazu, M. Uehara, and M. Watanabe. Nippon Jozo Kyokai Zasshi, 75, 327-331, 1980; Chem. Abstr., 93, 43846t, 1980.

51. J. Schmidt, M. John, H-J. Niefind, and H. Moonen. Brauwissenschaft, 34, 114-117, 1981.

52. D. L. Dunmire and S. E. Otto. J. Assoc. Off. Anal. Chem., 62, 176-185, 1979.

53. J. Wong-Chong and F. A. Martin. J. Agric. Food Chem., 27, 927-929, 1979..

54. J. Wong-Chong and F. A. Martin. J. Agric. Food Chem., 27, 929-932, 1979.

55. M. Cerny, A. Blumenthal, and E. Taube. Mitt. Geb. Lebensmittelunters. Hyg., 71, 141-149, 1980.

56. G. Palla. Anal. Chem., 53, 1966-1967, 1981.

57. W. Flak. Mitt. Klosterneuberg, 31, 204-208, 1981.

58. H. Muller and V. Siepe. Dtsch. Lebensmittel. Rundsch., 76, 156-161, 1980.

59. K. Aitzetmueller. Chromatographia, 13, 432-436, 1980; Chem. Abstr. 93, 130728z, 1980.

60. K. Aitzetmueller. J. Chromatogr., 156, 354-358, 1978.

61. S. I. M. Johncock and P. J. Wagstaffe. Analyst (London), 105, 581-588, 1980.

62. J-P. Goiffon, A. Blachere, J-L. Perez, and E. Portal. Ann. Falsif. Expert. Chim., 73, 17-23, 1980.

63. E. Stahl, E. Laub, and R. Woller. Fresenius' Z. Anal. Chem., 275, 257-260, 1975.

64. J. L. Iverson and M. P. Bueno. J. Assoc. Off. Anal. Chem., 64, 139-143, 1981.

65. R. E. Subden, R. G. Brown, and A. C. Noble. J. Chromatogr., 166, 310-312, 1978.

66. R. E. Subden, C. Duitschaever, K. Kaiser, and A. C. Noble. Amer. J. Enol. Vitic., 30, 19-21, 1979.

67. C. Buteau, C. L. Duitschaever, and G. C. Ashton. J. Chromatogr., 212, 23-27, 1981.

68. R. Battaglia and D. Froelich. J. High Resol. Chrom. and Chrom. Commun., 2, 100-101, 1978.

69. D. Froehlich and R. Battaglia. Mitt. Geb. Lebensmittelunters. Hyg., 71, 38-44, 1980.

70. H. Woidich, W. Pfannhauser, G. Blaicher, and U. Pechanek. Mitt. Klosterneuberg, 30, 27-31, 1980.

71. M. Okamoto, K. Ohtsuka, J. Imai, and F. Yamada. J. Chromatogr., 219, 175-178, 1981.

72. Y. Suzuki and M. Maruyama, Japan Analyst (Bunseki Kagaku), 28, 671-675, 1979; Chem. Abstr., 92, 87589v, 1980.

73. S. Selim. J. Chromatogr., 136, 271-277, 1977.

74. P. R. Demko. J. Chromatogr., 179, 361-364, 1979.

75. M. P. Maskarinec, D. L. Manning, and P. Oldham. J. Liq. Chromatogr., 4, 31-39, 1981.

76. K. Fung and D. Grosjean. Anal. Chem., 53, 168-171, 1981.

77. K. Greenhof and R. E. Wheeler. J. Inst. Brewing, 86, 35-41, 1981.

78. A. Piendel, H. Westner, and E. Geiger. Brauwissenschaft, 34, 62-70, 117-131, 159-166, 301-307, 1981.

79. C. H. Manley and P. Shubiak. J. Inst. Can. Sci. Technol. Aliment., 8, 35-39, 1975.

80. J. Adamovics and F. R. Stermitz. J. Chromatogr., 129, 464-465, 1976.

81. M. Wilkinson, J. G. Sweeny, and G. A. Iacobucci. J. Chromatogr., 132, 349-351, 1977.

82. N. Akavia and D. Strack. Z. Naturforsch., 35c, 16-19, 1980.

83. D. Strack, N. Akavia, and H. Reznik. Z. Naturforsch., 35c, 533-538, 1980.

84. M. Williams, G. Hradzina, M. M. Wilkinson, J. G. Sweeny, and G. A. Iacobucci. J. Chromatogr., 155, 389-398, 1978.

85. L. W. Wulf and C. W. Nagel. Amer. J. Enol. Vitic., 29, 42-50, 1978.

86. L. P. McCloskey and L. S. Yengoyan. Amer. J. Enol. Vitic., 32, 257-261, 1981.

87. L. P. McCloskey and L. S. Yengoyan. J. Agric. Food Chem. Ref. given in ref. 86.

88. L. Pergiovanni and G. Volonterio. Riv. Vit. Enol., 33, 289-308, 1980.

89. A. Bertrand, M. Gauthier, M-H. Salagoity-Auguste. Conn. Vigne Vin., 16, 145-148, 1982.

90. N. W. Preston and C. F. Timberlake. J. Chromatogr., 214, 222-228, 1981.

91. L. W. Wulf and C. W. Nagel. J. Chromatogr., 116, 271-279, 1976.

92. B. Y. Ong and C. W. Nagel. J. Chromatogr., 157, 345-355, 1978.

93. B. Y. Ong and C. W. Nagel. Amer. J. Enol. Vitic., 29, 277-281, 1978.

94. C. W. Nagel, J. D. Baranowski, L. W. Wulf, and J. R. Powers. Amer. J. Enol. Vitic. 30, 198-201, 1979..

95. L. W. Wulf and C. W. Nagel. J. Food. Sci., 45, 479-484, 1980.

96. C. W. Nagel and L. W. Wulf. Amer. J. Enol. Vitic., 30, 111-116, 1980.

97. J. D. Baranowski and C. W. Nagel. Amer. J. Enol. Vitic., 32, 5-13, 1981.

98. P. Symonds. Ann. Nutr. Aliment., 32, 957-968, 1978; Anal. Abstr. 38, 1 F 30, 1980.

99. S. Okamura and M. Watanabe. J. Agric. Chem. Soc. Japan. (Nippon Nogei Kagaku Kaishi), 53, 165-171, 1979; Chem. Abstr., 91, 8946la, 1979.

100. S. Okamura and M. Watanabe. Agric. Biol. Chem., 45, 2063-2070, 1981.

101. R. Nickenig and K. Pfeilsticker. Dtsch. Lebensmittel. Rundsch.,76, 115-119, 1980.

102. E. Dumont. Fresenius' Z. Anal. Chem., 297, 420, 1979.

103. K. W. Slinkard. Diss. Abs.int. B., 41, 938, 1980.

104. F. Villeneuve, G. Abravenel, M. Moutounet, and G. Alibert. J. Chromatogr.,234, 131-140, 1982.

105. J. B. Murphy and C. A. Stutte. Anal. Biochem., 86, 220-228, 1978.

106. J. Krause and D. Strack. J. Chromatogr., 176, 465-469, 1979.

107.Waters Associates. Literature supplied with μBondapak C18 column.

108.M. Vanhaelen and R. Vanhaelen-Fastré. J. Chromatogr., 187, 255-260, 1980.

109.L. Nagels, W. Van Dongen, J. DeBrucker, and H. DePooter. J. Chromatogr.,187, 181-187, 1980.

110.H. Becker, G. Wilking, and K. Hostettmann. J. Chromatogr., 136, 174-175, 1977.

111.I. McMurrough. J. Chromatogr., 218, 683-693, 1981.

112.D. A. Roston and P. A. Kissinger. Anal. Chem., 53, 1695-1699, 1981.

113.G. Sontag, F. I. Schafers, and K. Herrmann. Recent developments in food analysis: proceedings of the 1 Europ. Conference on Food Chemistry (Euro Food Chem. 1), 17-20 Feb. 1981. Ed W. Baltes. Verlag Chemie 1982, Weinheim. Pages41-46.

114.R. Galensa and K. Herrmann. Z. Lebensm. Unters. Forsch., 166, 355-358, 1978.

115.R. Galensa and K. Herrmann. J. Chromatogr., 189, 217-224, 1980.

116.R. T. Coutts, E. E. Hargesheimer, and F. M. Pasutto. J. Chromatogr., 179, 291-299, 1979.

117.E. Haslam. Phytochemistry, 19, 2577-2582, 1980.

118.T. C. Somers. Paper presented at the 62nd General Assembly of the O. I. V. V., Paris, August 1982.

119.A. G. H. Lea. J. Sci. Food Agric., 30, 833-838, 1979.

120.A. G. H. Lea. J. Chromatogr., 194, 62-68, 1980.

121.J. Jerumanis. Europ. Brew. Conv. Proc. Cong. (Berlin), 309-319, 1979.

122.P. Mulkay, R. Touillaux, and J. Jerumanis. J. Chromatogr., 208, 419-423, 1981.

123.W. Kirby and R. E. Wheeler. J. Inst. Brew., 86, 15-17, 1980.

124.B. Jende-Strid and B. L. Møller. Carlsberg. Res. Comm., 46, 53-64, 1981.

125.E. L. Wilson. J. Sci. Food Agric., 32, 257-264, 1981.

126.D. M. Takahashi. J. Assoc. Off. Anal. Chem., 60, 799-804, 1977.

127.D. M. Takahashi. J. Chromatogr., 131, 147-156, 1977.

128.D. M. Takahashi and R. M. Beebe. Liquid Chromatographic Analysis of Foods and Beverages. Volume 1, 99-128.

129.R-D. Wei and S. C. Chang. K'o Hsueh Fa Chan Yueh K'an, 8, 599-603, 1980; Chem. Abstr., 93, 148011v, 1980.

130.N. Sripathomswat and P. Thasnakorn. Mycopathologia, 73, 83-88, 1981.

131.Y. Ishida, T.Fujita, and K. Asai. J. Chromatogr., 204, 143-148, 1981.

132.A. Casoli and O. Colagrande. Amer. J. Enol. Vitic., 33, 135-139, 1982.

133.A. Casoli and O. Colagrande. Industrie della Bevande, 29-34, 1982; FSTA., 224192, 82-08-h1281.

134.R. Schuster. Anal. Chem., 52, 617-620, 1980.

135.P. Martin, A. Suarez, C. Polo, D. Cabezodo, and M. V. Dabrio. An. Bromatol., 32, 289-294, 1980.

136.J. Vialle, M. Kolosky, and J. L. Rocca. J. Chromatogr., 204, 429-435, 1981.

137.P. J. Tyson, E. S. Luis, W. R. Day, B. Walker, and T. H. Lee. Amer. J. Enol. Vitic., 32, 241-243, 1981.

138.T. C. Somers and G. Ziemelis. Amer. J. Enol. Vitic., 24, 47-50, 1973.

139.T. C. Somers and G. Ziemelis. Austral. Grapegrower and Winemaker, 66-67, April 1981.

140.M. Tenenbaum and G. E. Martin. J. Assoc. Off. Anal. Chem., 60, 1321-1323, 1977.

141.A. T. Rhys-Williams and W. Slavin. J. Agric. Food Chem., 25, 756-759, 1977.

142.A. A. Qureshi, N. Prentice, and W. C. Burger. J. Chromatogr., 170, 343-353, 1979.

143.G. Micali, P. Curro, and G. Calabro. J. Chromatogr., 194, 245-250, 1980.

144.H. Steuerle. Z. Lebensm. Unters. Forsch., 169, 429-434, 1979.

145.G. E. Martin, M. Tenenbaum, F. Alonso, and R. H. Dyer. J. Assoc. Off. Anal. Chem., 61, 908-910, 1978.

146.H. J. Jeuring and F. J. E. M. Kuppers. J. Assoc. Off. Anal. Chem., 63, 1215-1218, 1980.

147.H. E. Frischkorn, M. Wanderley-Casado, and C. G. B. Frischkorn. Z. Lebensm. Unters. Forsch., 174, 117-121, 1982.

148.W. R. Sponholz and P. Lamberty. Z. Lebensm. Unters. Forsch., 171, 451-452, 1980.

149.L. Jakob and R. Schaefer. Weinwirtschaft, 117, 904-906, 1981; FSTA., 219684, 82-05-h0703.

150.C. Lazzarini, E. Rossi, and A. Del Re. Chim. Ind. (Milan), 62, 923-926, 1980; Anal. Abstr. 42, 1 F 72, 1982.

151.E. Rossi, A. Lazzarini, A. Del Re, and G. Cerruti.Technologie Alimentari, 3, 27-28, 1980; FSTA., 194657, 80-12-h1803.

152.G. Caccialanza, C. Gandini, C. Roggi, and E. Zecca. Farmaco, Ed. Prat., 35, 449-454, 1980; Chem. Abstr., 93, 219362y, 1980: Farmaco, Ed. Prat., 36, 73-77, 1981; Chem. Abstr., 94, 137938x, 1981.

153.G. Toussaint and E. A. Walker. J. Chromatogr., 171, 448-452, 1979.

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HYDRODYNAMIC AND SIZE EXCLUSION CHROMATOGRAPHY OF PARTICLE SUSPENSIONS - AN UPDATE*

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ABSTRACT

The chromatographic separation of particle suspensions using packed beds has attracted considerable attention in recent years. It has the potential to provide accurate measurement of particle size and size distribution for spherical particles in the submicron range. There are two complementary approaches to the use of chromatography to separate particle suspensions according to size. Size exclusion chromatography (SEC) utilizes porous packing and relies mainly on steric exclusion from the pores of the packing. Hydrodynamic chromatography (HDC) utilizes non-porous packing and relies mainly on the velocity profile in the interstitial regions for size separation. In this paper, the developments in the understanding of these processes are critically examined and shortcomings of present theory are pointed out. Signal detection and chromatogram interpretation methods are reviewed.

INTRODUCTION

For over a decade, chromatographic methods using packed beds have been successfully used for the separation according to size of colloidal

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dispersions. Though vast strides have been made in the understanding of the separation process, some basic problems still remain, pertaining particularly to the holdup of colloid particles in the packed beds and the excessive broadening of colloid peaks (axial dispersion). While earlier research was mainly qualitative, adequate theory now exists to enable quantitative particle size measurement. A number of applications has been reported which demonstrates the considerable ease, rapidity and reliability of chromatographic techniques. These applications include detection of particle agglomeration [1,2] and swelling effects [2], measurement of particle growth kinetics [1,3,4,5], calculation of particle size distribution [6,7,8], etc.

In this paper, we critically review the theoretical and experimental developments concerning colloidal separations in packed columns. New insight is provided and new ideas are suggested for future research which will help to resolve present problems.

REVIEW OF EXPERIMENTAL INVESTIGATIONS

Chromatography has until recently been concerned exclusively with the separation of matter at the molecular level. One result of this restriction to the molecular domain was that, from a practical point of view, chromatographic methods invariably dealt with species in solution. Recently, however, chromatographic separations have been reported where the materials resolved were in suspension rather than in solution. Four major areas of particle chromatography have evolved; non-porous packed systems (HDC), porous packed systems (SEC), capillary chromatography [9] (CPC) and field-flow fractionation [10,11] (FFF). Here, we will be dealing with the first two.

Hydrodynamic chromatography (HDC) is a technique for separating small particles by flow through a packed bed of nonporous particles. This technique was invented by Small [12]. Similar developments using porous beds appeared through an independent study by Krebs and Wunderlich [13]. Studies on HDC have since been actively pursued by Stoisits et al [14], McHugh and co-workers [15,16,17], Nagy et al [18,19,20,21] and McGowan and Langhorst [6], whose major contribution is the development of a theory to explain colloid migration and then the application of this theoretical background in practical cases.

The object of using porous packing in size exclusion chromatography (SEC) has been to improve resolution over the non-porous HDC system by superimposing a steric exclusion effect on the flow separation. Particles smaller than the pore diameter can diffuse into the pores giving a second and more efficient mechanism of retardation and size separation.

Of course, there are relative advantages and disadvantages and the choice between HDC and SEC always depends on the physical system and the final objectives of the researcher. We now discuss briefly the experimental developments in HDC and SEC. To keep generality as much as possible, the principal results of these investigations are summarized at the end of the discussion.

A. Hydrodynamic Chromatography

When colloidal materials are carried in suspension through non-porous packed beds, it has been observed [1,2,22] that the rate of transport of the colloidal particles depends on such factors as the size of the colloid, the size of the particulate material that constitutes the packed column and the flowrate and ionic composition of the eluant.

The rate of migration (transport) of a colloid may be conveniently expressed by a dimensionless quantity, the R_F number, where:

$$R_F = \frac{\text{rate of transport of colloid through the bed}}{\text{rate of transport of the eluant}} \quad (1)$$

R_F gives the rate of migration of a colloid peak relative to a marker species.

In general, particle transport may be governed by one or a combination of the following effects: the hydrodynamic effect [1,2], the ionic effect [1,2] and the Van der Waals effect [1,2]. Figure 1 shows data [1] on the rate of transport of polystyrene latices through ion exchange beds of different diameter $\bar{D}$ (packing diameter). The fact that R_F clearly increases with increasing particle diameter of the latex, D, provides the basis for a chromatographic size separation. As the packing diameter is reduced, R_F increases. Furthermore, the slope of the R_F vs. D plot increases as the size of the packing is reduced, thereby resulting in improved resolution of different particle sizes. Most significantly, R_F is always greater than unity or in other words, the latex particles move more rapidly through the bed than either the carrier fluid or low molecular weight soluble species.

The dependence of R_F on the ionic strength of the eluant [1] is shown in Figure 2. Depending on the ionic strength, the colloidal forces can either enhance or hinder the average velocity of the particle. Decreasing the ionic strength, increases the volume of the relatively slow moving fluid in the interstitial regions from which particles are effectively excluded. Obviously, larger particles are excluded to a greater extent. Consequently, the mean velocity of the particle exceeds that of the fluid; the factor increases with the ratio of particle size to packing diameter. At high ionic strength, Van der

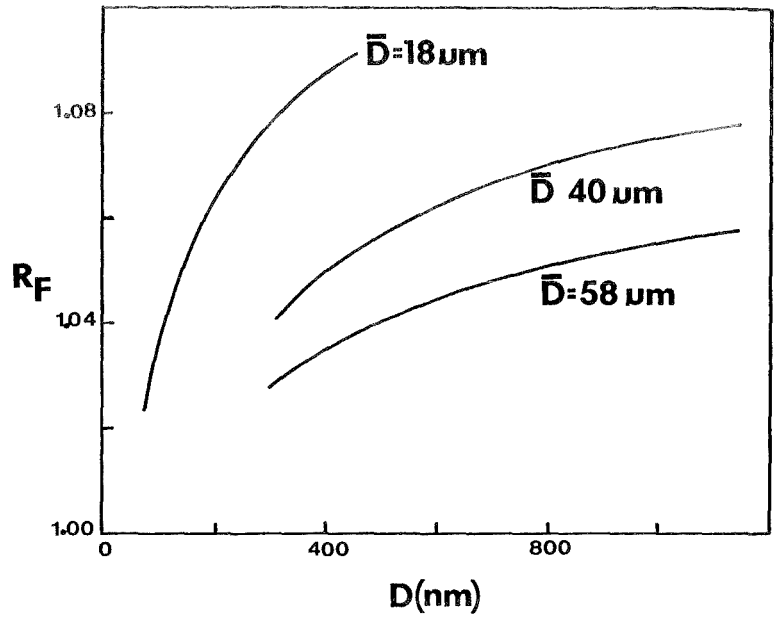

FIGURE 1: The dependence of R_F on latex particle diameter and packing diameter $\bar{D}$.

Waals forces cause the larger particles to spend a greater fraction of their time in the sluggish interstitial regions, so that the R_F dependence on particle size may reverse.

Two approaches have been taken to model the role of the colloidal forces in HDC. The capillary model [1,14,15,16,23] considers the interstitial space as a system of interconnecting parallel capillaries of equal size. In the second approach [24], the speed of the chromatographic transients are calculated from the behaviour of a colloidal suspension in equilibrium in the vicinity of a plane interface. Expressed in this form, the theory is independent of the geometry of the particulate material that constitutes the HDC column. The capillary model approach tends to be more universally accepted.

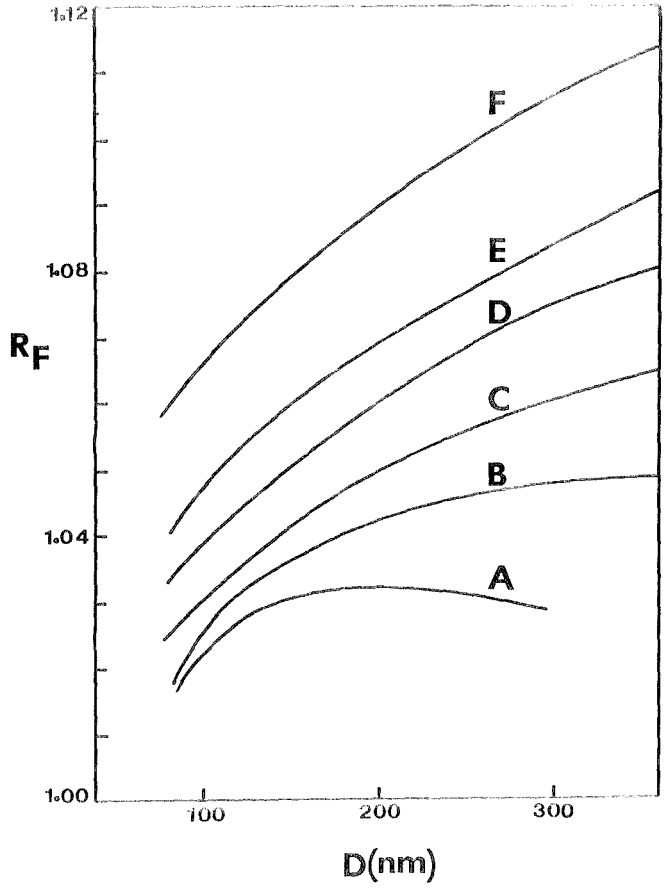

FIGURE 2: The effect of the ionic strength of the eluant on the R_F of polystyrene latices. Eluant concentration moles per liter of NaCl. A, 1.76×10^{-1}; B, $9. \times 10^{-2}$; C, 2.96×10^{-2}; D, 4.6×10^{-3}; E, 1.7×10^{-3}; F, 4.25×10^{-4}.

The experimental observations of Small [1] were subsequently confirmed and further extended by McHugh et al [15] and Nagy [18]. Recently, Nagy et al [19,20] reported a method for improving signal resolution in latex particle size analysis by HDC. Then, in a companion article [21], they presented a comparison of the column resolution characteristics of porous and non-porous packing systems. More recently, McGowan and Langhorst [6] reported an improved technique for the practice of HDC, which utilizes columns of higher efficiency and resolving power, thereby reducing the analysis time from 1.5 hr to 6 min. The integrated, computerized HDC they described, calculates the actual particle size distribution of the sample from molecular size to greater than 1 μm from the chromatogram in an additional 3-5 min. Several examples and experimental applications of their improved technique were discussed concerning HDC size distribution determination of a butyl acrylate/ butadiene latex and a polybutadiene latex reacted with methyl methacrylate.

B. Size Exclusion Chromatography

Krebs and Wunderlich [13] were the first to report a separation of polymethyl methacrylate and polystyrene latices using silica gel having very large pores (500 - 50,000 Å). This was followed by the work of Gaylor and James [25] who fractionated polymeric latices and inorganic colloidal silica, using columns packed with porous glass and water compatible polymeric porous gels. Coll et al [26] and Coll and Fague [27] experimenting with porous glass packing (CPG, 500 - 3,000 Å pore size), found it necessary to add electrolyte as well as surfactant to the aqueous eluant. In the absence of electrolyte, the colloids could not sample the pore volume. Peak broadening was observed to be more

extensive than in size exclusion chromatography of polymer molecules. They also observed that in SEC there is no limit as to how small the particle to be separated can be. The upper limit is a result of a greatly reduced diffusion coefficient for large particles and is probably about 4,000 Å. On the other hand, there appears to be a practical lower limit for HDC[28]. Singh and Hamielec [3], Hamielec and Singh [29] and Singh [30] presented the first comprehensive theoretical and experimental investigation of SEC. Using porous glass and silica packing (100 - 30,000 Å pore size), they established at low ionic strength the universality of the particle diameter-retention volume calibration curve. The slope of the calibration curve was essentially independent of the eluant flowrate; however, it became smaller (corresponding to a better resolution) with a reduction in packing size. The effects of the mobile phase flowrate and latex particle size on peak variance are shown in Table 1. Analytical expressions were derived to correct measured diameter averages for imperfect resolution. They concluded that SEC is sufficiently rapid for the off-line monitoring of latex particle growth in emulsion polymerization. With some modification it could be used in an on-line mode as a sensor for latex reactor control (8,31). However, the present state of SEC does require the development of a proper method for the complete extraction of a PSD from a chromatograph peak profile.

Nagy [18] and Nagy et al [21,32] investigated the chromatography of polystyrene latices using porous glass packing materials (CPG, 500 - 10,000 Å and Fractosil, 25,000 Å pore size). In a distinct departure from previous practice, only emulsifier (anionic) was added to the aqueous eluant, resulting in significantly reduced material loss within the packed bed.

TABLE 1

Peak Broadening Data for Polystyrene Latices Measured by Hamielec and Singh [29]

Eluant Flowrate (mℓ/min)	Chromatogram Variance ($m\ell^2$) PS 1000 Å	PS 2340 Å	PS 3120 Å
0.94	23.09	20.66	19.39
2.58	29.75	28.69	27.88
7.50	34.47	32.89	29.34

Johnston et al [33] reported the feasibility of chromatographing polystyrene latices using porous CPG columns (100 and 3,000 Å pore size). A reduction in packing size caused a small increase in R_F, while, significantly increasing sample loss. In general, the peak variance increased with particle size, attained a maximum and then started to decrease, analogous to the behaviour of polymer molecules (Table 1 gives this decreasing trend). An attempt was made to correlate the statistical properties of the chromatograms of narrow distribution latices with their mean retention volumes. Such an attempt is valid, provided the latices are sufficiently narrow to permit equating their spreading functions with the corresponding measured chromatograms. This condition is not fulfilled for the Dow latices used, as it was easily demonstrated theoretically [34].

Husain [34] and Husain et al [35] also examined the chromatography of polystyrene latices using porous CPG columns (1,000, 2,000 and 3,000 Å pore size). They advocated the merits of calibrating columns individually to weed out those with inadequate peak resolution and significant particle holdup. Common with previous observations, the

extent of skewing in the chromatograms of narrow distribution latices was observed to increase with particle size.

Finally, Kirkland [36] investigated the properties of small porous silica microspheres (less than 10 μ compared with approximately 35 μ CPG packing size, pore size less than 75 nm) and superficially porous particles (solid core, porous crust, packing size less than 25 μ) for characterizing inorganic silica sols in the range 1 - 50 nm. Columns using both types of packing materials exhibited high resolution because of rapid equilibration of slowly diffusing colloids with the pores. The effect of flowrate on peak broadening and the role of ionic strength in colloid separation were observed to be similar to those in earlier studies.

C. Summary of Main Results

A brief summary of the main results of the above investigations is now given under three classifications, namely peak separation, peak broadening and material loss:

Peak Separation

1. The particle diameter-retention volume calibration curve, in general, is composed of two linear segments: a segment at low retention volumes, beyond the exclusion limit of the porous packing, corresponds to HDC size separation, while, a segment at high retention volumes corresponds to SEC size separation.
2. The calibration curve is insensitive to flowrate variations.
3. Increasing the ionic strength of the aqueous eluant causes a shift in the calibration curve to high retention volumes due to increased accessibility of the column voids. At low ionic strength, a universal calibration is obtained.

4. Reducing the packing size improves peak separation.
5. SEC is not limited by a minimum particle size. However, the effectiveness of separation in HDC decreases as the ratio of particle to capillary diameter approaches zero.

Peak Broadening

1. In general with SEC the peak variance increases with colloid size, reaches a maximum and then starts to decrease as the exclusion limit of the porous column is approached. In HDC, it decreases with increasing particle size.
2. An increase in flowrate causes increased peak broadening in SEC. In HDC, the effect is not known.
3. In SEC, dispersion increases at higher ionic strength due to increased pore permeability. A similar effect may be expected in HDC; however, no experimental data have been reported.
4. The chromatograms of narrow distribution particle standards are generally skewed.
5. A well designed packing can significantly reduce dispersion.

Material Loss

1. Increasing the electrolyte concentration of the eluant decreases sample recovery. However, if the ionic strength is adjusted by addition of an ionic emulsifier (within limits), material loss is reduced.
2. Sample loss increases with colloid size.
3. Reduction in the size of the packing, enhances material loss.

While factors governing peak separation are fairly well understood, those that affect peak broadening and particularly sample recovery are

not. A systematic study to determine the roles of colloid composition, packing type, pore size, operating temperature etc. is required to acquire a better understanding of these phenomena.

DETECTION OF COLLOIDAL PARTICLES

Light transmission has been a standard method for the measurement of size of colloidal spherical particles for many years. The fundamental theory was developed by Mie Heller and his coworkers [37,38,39] outlined the theory which gives size distribution curves in heterodisperse systems of nonabsorbing colloidal spheres from turbidity spectra. The assumption was made that the unknown PSD followed a log normal distribution, a distributional form commonly found in latex systems.

Two of the most commonly used modes of colloidal particle detection, namely turbidimetric and differential refractometry detection, are now briefly examined.

A. Turbidimetric Detection

The turbidity for very small particles which behave as Rayleigh scatterers is proportional to the sixth power of the particle diameter. For larger particles obeying Mie scattering theory, the corresponding dependence is lower. As a consequence of the above, the small particle signal is comparatively weak, though it can be augmented by using shorter wavelengths. However, for obtaining particle size distributions, the relative signal is of greater importance than the absolute signal. Calculations by Silebi and McHugh [17] indicate that a change of wavelength or refractive index has a small influence on the relative

signal for nonabsorbing particles. However, the relative signal is improved for absorbing particles due to a significant enhancement of the extinction coefficient of the smaller particles. These theoretical observations were confirmed by Nagy [18] and Nagy et al [32] who chromatographed mixtures of polystyrene latices at 220 and 254 nm (controversy exists as to whether particles absorb at 254 nm; at 220 nm, however, strong absorption occurs). One of Nagy's [18] results, shown in Figure 3, demonstrates the dramatic improvement in the 88 nm peak measured at a wavelength of 220 nm compared to that at 254 nm.

As Heller and Tabibian [40] indicated, appreciable error may result if instruments which are perfectly suitable for ordinary absorption measurements are used for turbidity measurements without proper modifications and precautions. There are three principle sources of error in turbidity measurements [40]: (1) interference of laterally scattered light (2) the corona effect and (3) the effect of the solid angle. While such errors were believed to be negligible by earlier workers [17], their existence was unequivocally demonstrated by Husain et al in a series of papers [34,41,42,43]. They compared the detector response to a suspension of polystyrene spheres with the response to a solution of sodium dichromate. Furthermore, they showed that impurities (such as residual styrene monomer in polystyrene particles) and additives (such as emulsifier) may cause the measured extinction coefficient to differ from theoretical calculations based on Mie theory. The discrepancy may theoretically be accounted for by employing an effective imaginary refractive index ratio (colloid to medium) [18,32].

Maron et al [44] applied turbidimetric techniques to measure the size distribution of polydisperse polybutadiene-styrene latices. Gledhill [45] described a method for constructing a graphical

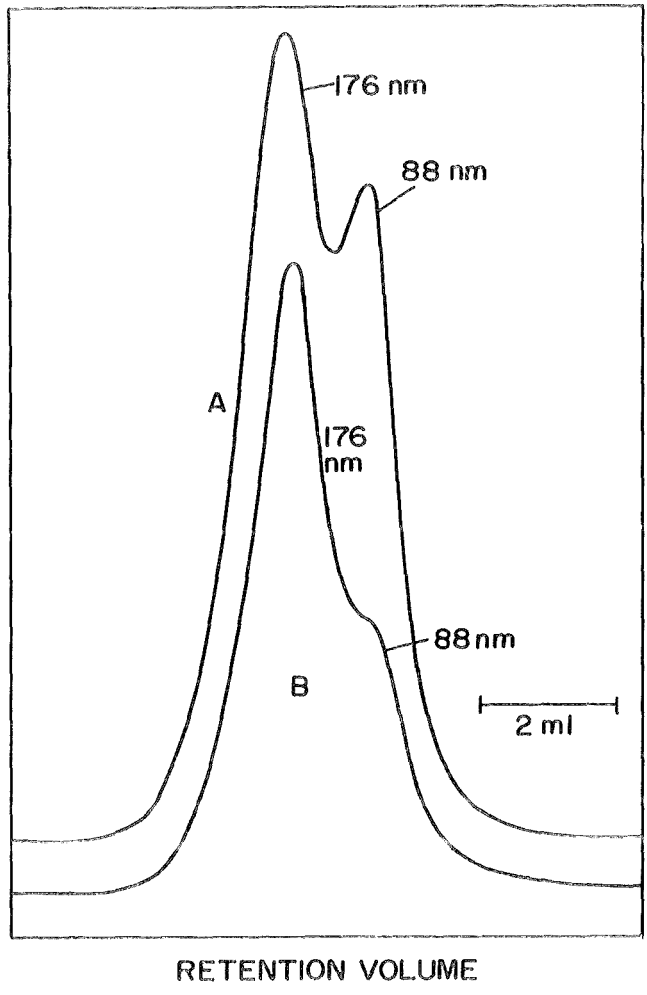

FIGURE 3: HCD separation of a bimodal mixture of 88 nm and 176 nm polystyrene latices. A, response at 200 nm; B, response at 254 nm.

calibration grid for a system of known optical constants and known distributional form, from which the weight mean diameter and standard deviation of the distribution corresponding to observed turbidity measurements could be read directly. However, as Maxim et al [46] indicated in their publication, the turbidity spectra analysis, though very attractive because of the simplicity of the experimental technique, should not be used alone for particle size analysis. This was further discussed in Kiparissides et al [5]. Recently, Nagy et al [20] reported a method for improving signal resolution in latex particle size analysis and data for the specific extinction coefficient for polystyrene indicates that improvement can be obtained for the small particle end of broad size distribution by using turbidity detection at wavelengths less than 254 nm.

B. Differential Refractometry Detection

Zimm and Dandliker [47] derived a general refractive index expression based on the Mie theory. Their expression for the dispersion refractive index, n_s, is given by:

$$\frac{dn_s}{dc} = \frac{3n_m}{2a^3\rho_p} \; \mathrm{Re}\left[\sum_{n=1}^{\infty} \frac{2n+1}{2n(n+1)} (\alpha_n - b_n) \right] , \qquad (2)$$

where c is the weight concentration in gr/cm^3, ρ_p is the particle density, a is a dimensionless size parameter ($a = \pi D/\lambda$, where D and λ are respectively the particle diameter and the wavelength in the medium), n_m and n_s are the refractive indices of the medium and the dispersion, respectively, and α_n and b_n are functions of a and m (m is the refractive index ratio of particle to medium). The above equation (2) does not contain the restriction that a be small and allows calculation of the effect of light scattering on the refractive index of a

colloidal dispersion. In the limit, as a goes to zero, equation (2) reduces to:

$$\frac{dn_s}{dc} = \frac{3n_m}{2\rho_p} \frac{(m^2-1)}{(m^2+2)} \tag{3}$$

a result derivable from Heller's [48] equation. In accordance with equations (2) and (3), dn_s/dc is expected to be independent of c and at small values of a, independent of a as well.

Nakagaki and Heller [49] confirmed the validity of equation (2) for particle diameters as large as 500 nm. Measurements by Silebi and McHugh [17] show a surprising agreement of measured data with equation (3) for polystyrene latices as large as 350 nm. Both measurements were made with polystyrene at a wavelength of 546.1 nm. Subsequent data measured by Nagy [18] from the same laboratory indicates that dn_s/dc reverses in sign with increasing particle size; its implication, therefore, is that the signal is null for some intermediate particle size. Coll and Fague [27] observed that dn_s/dc was independent of c for a given latex, though its value increased linearly with particle diameter. Neither Nagy [18] nor Coll and Fague [27] were at the time able to explain their results satisfactorily. Interpretation of their data is complicated due to the use of a broad wavelength source.

Husain's [34] opinion is that the above seemingly conflicting data is in fact consistent with the Zimm and Dandliker [47] equation. Calculations [47,49] indicate that, depending on the values of m and a, dn_s/dc may either increase with particle size or decrease and eventually change sign.

Differential refractometry shows a less dramatic dependence on particle size (third order) than turbidimetry of nonabsorbing particles (sixth order). This advantage of differential refractometry is,

however, counterbalanced by the requirement of a higher sample concentration compared to the amount necessary for a photometric detection due to the limited sensitivity of available differential refractometers. Of course, with the advent of more sensitive detectors this drawback will likely be overcome.

THEORETICAL ANALYSIS OF PEAK SEPARATION

The passage of an injected sample through the columns and detector generates an output trace on the recorder, the chromatogram. For several reasons, a chromatogram can never fully represent the distribution of colloid sizes in the injected sample. Instrumental spreading (or axial dispersion) causes elution of a single species to occur over a range of retention volumes. The chromatogram of the sample is the superposition of these distributions. When the number of species is small, one might obtain a chromatogram involving many obvious but overlapping peaks. However, with a large number of species, the peaks of individual species are not evident; one usually obtains a unimodal chromatogram and sometimes a more complex one. Interpretation of a chromatogram must, therefore, account for this superposition and involve an evaluation of instrumental spreading and correction of the detector response to obtain the true concentrations of the component species.

While theory adequately predicts peak separation in HDC, a similar comprehensive treatment is lacking for SEC. Attempts [18] to predict peak separation in SEC have not been very successful. We now briefly examine the theories proposed to explain peak separation in HDC and SEC.

A. Hydrodynamic Chromatography

As it was mentioned earlier, two approaches have been taken to model HDC:

(a) The capillary model: A solute particle does not spend the same fraction of its total residence time at each radial position. If interactions between particles are negligible, the residence time distribution for the solute during a transient will be the same as in the case where the solute is continuously injected. From an analysis of the particle continuity equation in the presence of a radial force field, for the case of continuous injection, it can be shown that the radial concentration distribution is a Boltzmann one, given by:

$$C(r) \propto \exp\,[-\phi(r)/kT]\ , \tag{4}$$

where $\phi(r)$, the particle-wall total interaction energy is given by the superposition of the repulsive potentials arising from the double layer and Born repulsive forces and Van der Waals attractive potentials, as

$$\phi(r) = \phi_{DL} + \phi_{B} + \phi_{VW} \tag{5}$$

Then, the average particle velocity can be calculated by weighting the local particle velocity $V_{pz}(r)$ at a given radial position with the concentration at that position, to obtain

$$\bar{v}_p = \frac{\int_o^{R-\delta} v_{pz}(r)\ \exp\,[-\phi(r)/kT]rdr}{\int_o^{R-\delta} \exp\,[-\phi(r)/kT]\ rdr}\ , \tag{6}$$

where the upper integration limit accounts for the inability of a particle to approach the capillary wall closer than its radius, δ. $v_{pz}(r)$ is given by a modified Poiseuille equation which takes the wall effect into account.

For an ionic marker, ϕ_B and ϕ_{VW} are negligible and its average velocity, $\bar{v}_M$, is obtained by taking the limits of the above integrals in (6) as the particle radius δ tends to zero. R_F is then given by definition as:

$$R_F = \bar{v}_p / \bar{v}_M \tag{7}$$

(b) The equilibrium model: The corresponding expression for R_F is given by:

$$R_F = \frac{V}{A} \frac{1}{\int_0^\infty \exp\left[-\phi(h)/kT\right] dh} , \tag{8}$$

where V is the volume of the mobile phase and A is the surface area of the packing. The R_F dependence on packing diameter is manifested by the presence of A.

Both models adequately predict the variation of R_F with particle diameter over a wide range of ionic strength. Unlike the capillary model, Equation (8) predicts an increase in R_F with hydraulic radius V/A or packing diameter, contrary to the observed dependence. Therefore, the capillary model seems to be more powerful, as mentioned already.

B. Size Exclusion Chromatography

In addition to the factors governing the separation of colloids in HDC, the use of porous packing introduces the possibility of size separation due to steric exclusion from the pores. Due to the complex flow patterns in porous packed beds, the difficulty in predicting the migration of a colloid peak is obvious. Nagy [18] and Nagy et al [32] attempted to simplify this problem by using very large pores relative to the size of the colloids being separated (they used a porous column with a mean pore size of 2.5 μ). Their analysis, therefore, assumes that all particles enter the pores and accordingly, describes one extreme of SEC where permeation by all species occurs. No electrolyte was used and the ionic strength was varied by using surfactant alone, at concentrations below and above the critical micelle concentration. The qualitative

features of their data were similar to those observed in HDC, though, the R_F values were larger due to partial penetration of the pores by the particles.

Nagy [18] modelled his data using a flow-through bank model as shown in Figure 4. The large tubes in a given bank represent the totality of interstitial regions at the same level in the column, while, the small tubes represent the totality of the pores within the packing at the same level. The spaces between the banks have zero volume and serve as a mixing region for altering particle trajectories. The probability of a particle entering a tube at the start of a bank is assumed equal to the ratio of flow through all such tubes to the total flowrate through all tubes in the bank. The principal result of their analysis predicts that:

$$\frac{1}{R_F} = \frac{(V_{pc}/V)}{R_{F,pc}} + \frac{(V_{ic}/V)}{R_{F,ic}} \tag{9}$$

where $R_{F,ic}$ and $R_{F,pc}$, the separation factors corresponding to the interstices and pores respectively, are calculated as before. V_{pc}, V_{ic} and V represent the pore volume, interstitial volume and total void volume, respectively.

Now, it is shown that the result in equation (9) may be obtained using a simpler model, which regards the column voids as a system of parallel capillaries of the interstitial and pore type (no mixing region is considered and capillaries are continuous across the length of the column). The peak retention volume of the colloid peak, V_p, is given by:

$$V_p = n_{pc}\, Q_{pc}\, \bar{t}_{pc} + n_{ic}\, Q_{ic}\, \bar{t}_{ic} \tag{10}$$

where n, Q and $\bar{t}$ denote tube number, flowrate and average residence time, respectively. It follows, therefore, that:

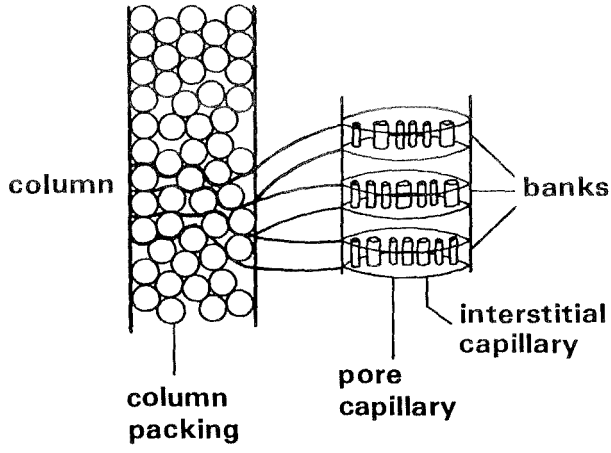

FIGURE 4: A bank model of a SEC column.

$$1/R_F = (n_{pc}\, Q_{pc}\, \bar{t}_{pc} + n_{ic}\, Q_{ic}\, \bar{t}_{ic})/V_m \tag{11}$$

where V_m, the retention volume of a marker peak, is equal to V. If the length of the column is L and the cross-sectional area of a capillary, A, then:

$$\begin{aligned} 1/R_F &= L/V_m\, [n_{pc}\, Q_{pc}/(\bar{v}_p)_{pc} + n_{ic}\, Q_{ic}/(\bar{v}_p)_{ic}] \\ &= L/V_m\, [n_{pc}\, A_{pc}\, (\bar{v}_m/\bar{v}_p)_{pc} + n_{ic}\, A_{ic}\, (\bar{v}_m/\bar{v}_p)_{ic}] \\ &= (n_{pc}\, A_{pc}\, L/V_m)/R_{F,pc} + (n_{ic}\, A_{ic}\, L/V_m)/R_{F,ic} \\ &= (V_{pc}/V)/R_{F,pc} + (V_{ic}/V)/R_{F,ic} \end{aligned} \tag{12}$$

The derived result is identical to equation (9) [18]. The apparent equivalence of the two models is a direct consequence of assigning, in the bank model, the probability that a particle travels through a given tube as equal to the ratio of flow through all such tubes to the total flowrate through all tubes. Therefore, it is not surprising that calculations based on equation (9) agree rather poorly with experimental data since, the equivalent model (which allows no fluid intermixing)

considered here, is hardly representative of the flow process in a packed column.

Nagy [18] cites several reasons, chief among which is the slow diffusion coefficient of colloids, to justify the use of a flow model as opposed to a diffusion model. As pointed out by Small [22], if a bank model is considered, separation by flow would seem unlikely, since very little fluid would flow through the extremely fine pores of the packing when the much less restricted pathway around the particles is available to it. It is beyond the scope of this paper to consider alternate models for SEC. It is, however, suggested [34] that, since the equilibrium theory for HDC is essentially independent of the complex flow geometry, it may be possible to extend the treatment to predict colloid behaviour in SEC. Further work in this direction would undoubtedly be facilitated by a critical review by Casassa [50] who has examined the various models proposed to explain peak migration in SEC.

CALCULATION OF PARTICLE SIZE DISTRIBUTIONS

As we have mentioned before, the axial dispersion phenomenon is a serious imperfection in the chromatography of particle suspensions. The input sample W(y) is distorted as a result, so that the diameter frequency distribution calculated based on the measured response F(v) may be significantly in error.

All rigorous methods of correcting detector response for peak broadening (or axial dispersion) use the following integral equation as the basis:

$$F(v) = \int_0^\infty W(y)\, G(v,y)\, dy \quad , \tag{13}$$

where F(v) is the detector response at retention volume v (i.e. it is related to the true chromatogram W(y) by equation (13)) and G(v,y) is the normalized detector response or spreading function for a particle of diameter D(y) or for a species with mean retention volume y. G(v,y) is often called the instrumental spreading function and is frequently considered to be uniform, i.e.

$$G(v,y) = G(v-y) \quad , \tag{14}$$

which considerably simplifies the mathematical treatment of equation (13). W(y)dy is the area under the detector response due to particles of diameter D(y). W(y) is called the detector response corrected for dispersion. Equation (13) is a Fredholme integral equation of the first kind and has been used extensively in various science and engineering applications. When detection is turbidimetric, both F(v) and W(y) represent turbidities while, in the case of differential refractometry, they represent refractive index increments.

The response for a general detector is given by:

$$F(v) = \int_0^\infty W(v,y)dy \quad , \tag{15}$$

where:

$$W(v,y) \propto N(v,y)\, D^{\gamma}(y) \tag{16}$$

for the Rayleigh scattering regime (γ=3 for refractive index and γ=6 for turbidity detector) and

$$W(v,y) \propto N(v,y)\, D^{2}(y)\, K(y) \tag{17}$$

for the Mie scattering regime, where K(y) is the extinction coefficient for particles of diameter D(y). In both (16) and (17), N(v,y) gives number of particles.

Comparing equations (13) and (15), it is clear that:

$$W(v,y) = W(y)\, G(v,y) \tag{18}$$

and

$$W(y) = \int_0^\infty W(v,y)dv \tag{19}$$

Equations (18) and (19) can be used to derive correction equations for dispersion in the detector cell itself.

Equation (13) may be solved both numerically and analytically. Numerically, it is solved either for $G(v-y)$[41], when $F(v)$ and $W(y)$ are known, or, as is usally the case, the integral equation is solved for $W(y)$, when $F(v)$ and $G(v-y)$ are known, which may then be converted into a particle size distribution. In contrast, analytical solutions enable the direct calculation of moments of the size distribution function; the PSD itself is not obtained. Both methods of solution will be discussed later in what follows.

Forms of the Spreading Function

(a) Uniform spreading function: In other words, its shape parameters are independent of retention volume, i.e. the shape parameters are the same for particles of different diameter. This limiting form should be valid for samples with relatively narrow particle size distributions. For this case, equation (13) becomes [51,52]:

$$F(v) = \frac{1}{\sqrt{2\pi\sigma^2}} \int_0^\infty W(y)\ \exp(-(v-y)^2/2\sigma^2)dy \tag{20}$$

where σ^2, the variance of the uniform Gaussian spreading function is independent of retention volume.

(b) Non-uniform Gaussian spreading function: The integral equation (13) now takes the form:

$$F(v) = \int_0^\infty W(y)\ \frac{1}{\sqrt{2\pi\sigma^2(y)}}\ \exp(-(v-y)^2/2\sigma^2(y))dy \tag{21}$$

(c) General spreading function: Provder and Rosen [52[have proposed the use of a general statistical shape function to account for deviations of the spreading fuention from the Gaussian shape. It has the form:

$$G(x) = \phi(x) + \sum_{n=3}^{\infty} (-1)^n A_n \phi^n(x)/n! \qquad (22)$$

where

$$\phi(x) = \frac{1}{\sqrt{2\pi}} \exp(-x^2/2) \qquad (23)$$

$$x = \frac{v - y}{\sigma}$$

and $\phi^n(x)$ denotes n^{th}-order derivative. The coefficients A_n are functions of μ_n, the n^{th}-order moments about the mean retention volume, μ, of the normalized detector response for a single species.

Numerical Solution of the Integral Equation

Several numerical methods have been reported for the solution of the integral equation. These have been reviewed by Friis and Hamielec [53] and evaluated by Silebi and McHugh [17] for their application to particle chromatography. They conclude that the method of Ishige et al [54] performs better than other available methods. A noteworthy undesirable feature of the method, however, is its tendency to overestimate the number of small particles in a polydispersed sample. Modifications of Ishige's algorithm fail to overcome this defect [53]. Unless a more effective numerical method is developed for solving for the corrected detector response, W(y), it is recommended that analytical methods be used to calculate particle diameter averages.

Analytical Solution of the Integral Equation

Three analytical methods for solving the integral equation have been reported [29,42,55]. Their main features are compared in Table 2.

TABLE 2

A Comparison of the Analytical Methods for Solving the Integral Equation

Attributes	Method* 1 Hamielec and Singh[29]	2 Hussain et al.[42]	3 Hussain et al.[55]
1. Calibration curve	Linear	Nonlinear	Nonlinear
2. Spreading function	Eqn.(25) or Provder and Rosen's[52] shape function	Eqn.(25)	Eqn.(25) or Provder and Rosen's[52] shape function
3. Light scattering theory which may by applied	Rayleigh	Mie	Mie
4. Chemical absorption may be present in tubidimetric detection.	No	Yes	Yes
5. Diameter averages are calculated as a function of retention volume	No	Yes	No

* The refractive index detector (dn_s/dc = constant) can be treated using all the above methods. However, Method 3 is most general.

It is important to realize that, the solution derived for a Gaussian spreading fuention $G_o(v-y)$ is equally applicable to a whole family of functions of the form:

$$G(v,y) = G_o(v-y)\ \Psi(y) \tag{25}$$

where $\Psi(y)$ is an unspecified function of y. This considerably extends [55] the applicability of the solution for a Gaussian spreading function to an infinite set of non-Gaussian, non-uniform functions.

The first solutions of this kind were based on the use of bilateral Laplace transformations and uniform instrumental spreading functions

51,52] and they were applied to the SEC of polymer molecules. The first application to the chromatography of spherical suspensions was made by Hamielec and Singh [29] and Husain et al [55]. Yau et al [56] obtained similar solutions for the case of a uniform spreading function and a linear molecular weight calibration curve (equivalent to a linear particle diameter-retention volume calibration curve in this context). Yau et al [56] focussed on dispersion in the detector cell as did Hamielec [57] and Hamielec et al [58] in accounting for a non-uniform Gaussian spreading function and a nonlinear calibration curve.

The case of a non-uniform spreading function and a nonlinear particle diameter-retention volume calibration curve has been treated by Husain et al [35,42]. A novel method for identifying and estimating the parameters of the instrumental spreading function for column chromatography has been developed and applied to the SEC of particle suspensions [41]. This has revealed that for SEC, the spreading function of polystyrene latex standards in the size range 85-312 nm is skewed towards longer retention volumes. The Provder and Rosen [52] general spreading function gives reasonable fit to experimentally measured spreading functions for particles in the size range 85-220 nm. This is clearly demonstrated in Figures 5, 6 and 7. In these three figures [34], F(v) represents the experimental chromatogram and $G(v-v_p)$ the estimated spreading function. Figure 8 gives the change of the variance σ^2 with particle diameter, and Figure 9 shows a plot of the coefficient A_3 in equation (22) versus particle diameter, as they were used in fitting the general spreading function given by equation (22) to experimentally measured spreading functions. As it is easily understood from Figures 8 and 9, the change of σ^2 and A_3 with particle diameter is consistent with experimental data[34], which showed an increase in

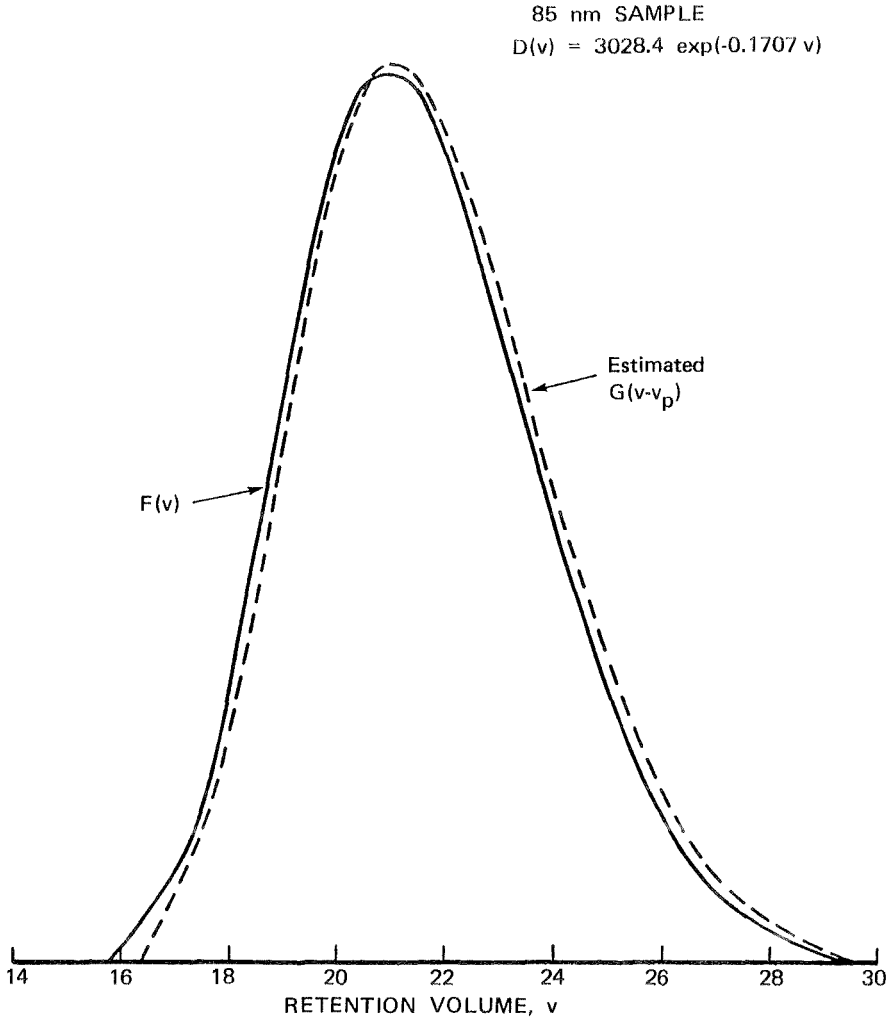

FIGURE 5: Estimation of the spreading function from experimental chromatogram.

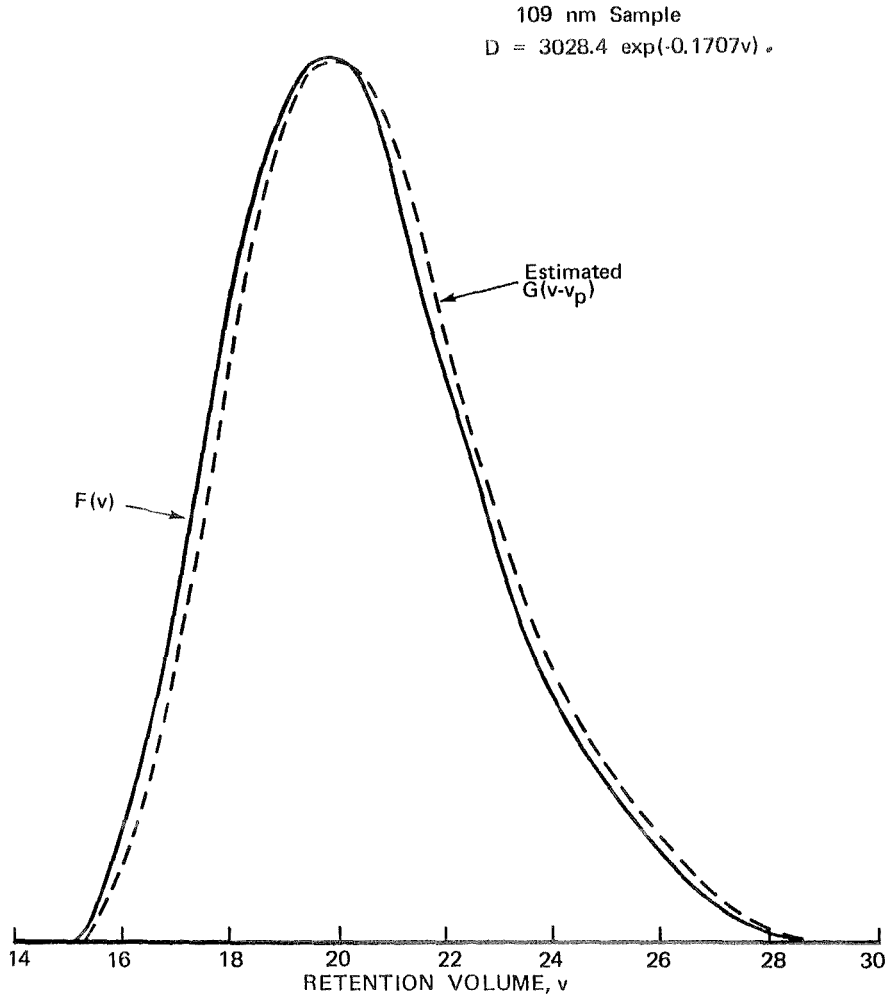

FIGURE 6: Estimation of the spreading function from experimental chromatogram.

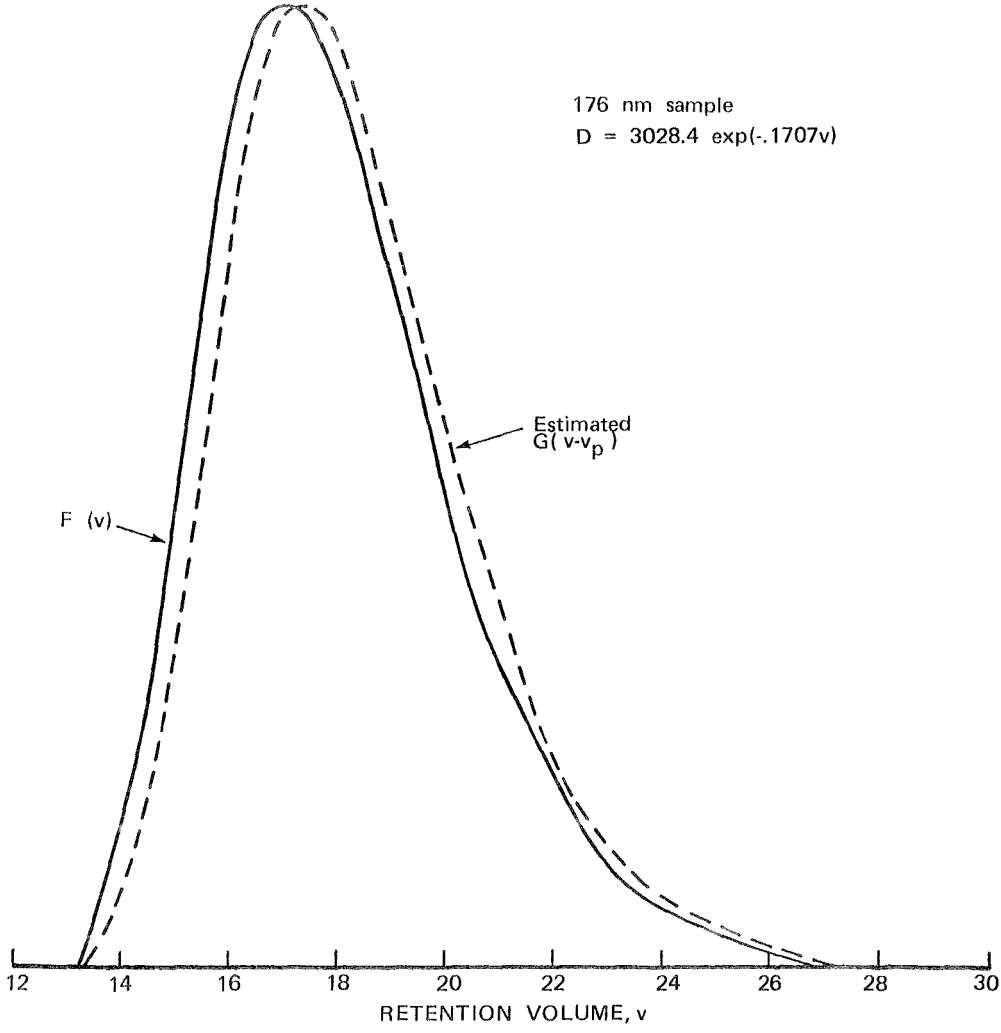

FIGURE 7: Estimation of the spreading function from experimental chromatogram.

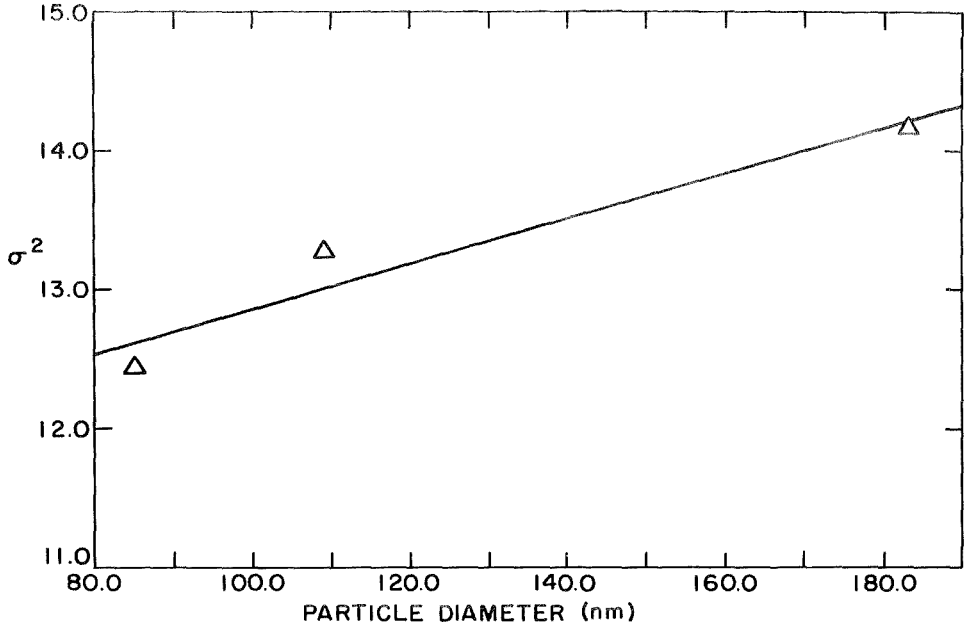

FIGURE 8: Variance versus particle diameter.

skewness of the chromatograms with an increase in particle diameter of the sample. For the 312 nm standard, the fit was poor. It would be of interest to compare experimental G(v,y) with the spreading function predicted by the plug flow dispersion model. It appears that for the HDC or SEC of particles, a skewed instrumental spreading function should be used to properly account for dispersion.

Instrumental Correction for Dispersion

An HDC or SEC operating with normal resolution should provide unimodal and relatively narrow frequency distributions of particle size in the detector cell across the chromatogram of a whole sample. Therefore, a detector system which can provide, say, two moments of the frequency distribution and the particle concentration of the detector

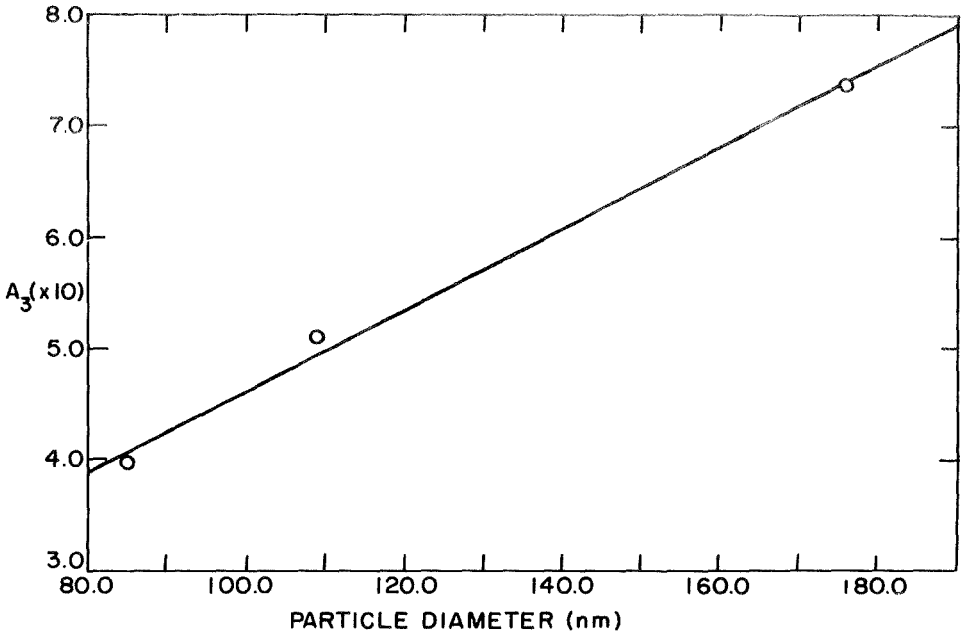

FIGURE 9: Coefficient A_3 in equation (22) versus particle diameter.

cell contents should in principle provide a measure of the frequency distribution of the whole sample and this measure should be largely independent of the resolution of the chromatograph.

To date, the use of a detector system to this end has not been reported. There are, however, at least two detector systems based on turbidity-spectra 43,59] and quasi-elastic light scattering (photon correlation spectroscopy [60,61,62,63,64]) which seem to have the potential for this task.

Fitting the Plug-Flow Dispersion Model

Discussing previously the different forms of the spreading function G(v,y), we have seen that a general statistical shape function

can account for deviations from the Gaussian shape [52]. If we consider equation (22) again and especially the coefficients denoted by A_n, we can see that the first two coefficients are of direct statistical significance and also represent the most useful terms in the infinite series for applications in chromatography.

$$A_3 = \mu_3/\mu_2^{3/2} \tag{26}$$

$$A_4 = (\mu_4/\mu_2^2 - 3) \tag{27}$$

μ_2 is the variance and is equivalent to σ^2. The coefficient A_3 provides an absolute statistical measure of skewness (when μ_3=0, the spreading function is symmetrical about the mean retention volume μ_1 or y. When $\mu_3 > 0$, skewing is towards longer retention volumes). The coefficient A_4 provides a statistical measure of flattening or kurtosis. When $A_4 > 0$, the shape function is taller and slimmer than a Gaussian and so forth. Tung and Runyon [66] used a simpler form to fit skewed detector responses in the SEC of polymer molecules. Silebi [67] has recently shown that skewed instrumental spreading functions derived from the plug-flow dispersion model [68] adequately fit data for particle separations by HDC. This spreading function has the form:

$$G(v,y) = \frac{1}{2\sqrt{\pi Pe^{-1}(v/y)}} \exp\left(-(v-y)^2/4\, Pe^{-1}(v\, y)\right) \tag{28}$$

where Pe = (uL/D) is the Peclet number, u is the superficial velocity in the column, L is the length of the packed bed and D is a dispersion coefficient. The plug-flow dispersion model predicts symmetrical broadening in the packed-bed; however, when dispersion is large, the detector gives a response which is skewed towards larger retention volumes. For small dispersion, Pe > 100 and G(v,y) reduces to a Gaussian shape.

Efforts by the authors to use equation (28) to fit data for particle separations by SEC were proven unsuccessful. It seems that

equation (28) should be used with HDC data and it is the intention of the authors to check the validity of the plug-flow dispersion model in the near future using vinyl acetate latices from their continuous emulsion reactors.

RECOMMENDATIONS

Some recommendations for future work with HDC/SEC can now be made:

(1) An extensive evaluation of various packing materials with differing pore geometry is required. This is necessary to minimize dispersion and particle loss in the columns. The optimum packing particle is probably one with a solid core and superficial surface pores.

(2) Factors affecting particle loss such as (a) ionic strength of eluant, (b) use of ionic surfactant alone as opposed to a mixture of surfactant and electrolyte, (c) effect of glass transition temperature of particles, (d) effect of packing type, (e) effect of column temperature, etc., need to be better understood.

(3) A multiple wavelength UV/Visible light turbidity detector has been theoretically evaluated and found to have reasonable potential as an analytical tool for particle size measurement [43]. Likewise, IR detection has been shown to have some useful features [18]. An experimental investigation of both these detectors is desirable. Also, more sensitive refractometers with a monochromatic light source should be evaluated.

(4) The theory of HDC should be extended to investigate the possibility of predicting the chromatogram shape. The corresponding development of a theory of SEC, to adequately predice peak separation as well as peak shape, may be facilitated by the use of model porous spheres.

(5) The numerical treatment of chromatographic data is rather inadequate. It is desirable to develop new improved methods for recovery of W(y).

REFERENCES

[1] Small, H., "Hydrodynamic Chromatography: A Technique for Size Analysis of Colloidal Particles", J. Coll. Int. Sci., 48(1), 147 (1974).

[2] Small, H., Saunders, F.L. and Solc, J., "Hydrodynamic Chromatography: A New Approach to Particle Size Analysis", Adv. Coll. Int. Sci., 6, 237 (1976).

[3] Singh, S. and Hamielec, A.E., "Liquid Exclusion Chromatography: A Technique for Monitoring the Growth of Polymer Particles in Emulsion Polymerization", J. Appl. Polym. Sci., 22, 577 (1978).

[4] Kiparissides, C., MacGregor, J.F. and Hamielec, A.E., "Characterization of Size Distribution During Continuous Emulsion Polymerization: Oscillations in Vinyl Acetate Polymerization", Polymer Colloids II, Ed. Fitch, R.M., Plenum press (1980).

[5] Kiparissides, C., MacGregor, J.F., Singh, S. and Hamielec, A.E., "Detection of Reactor Performance by Turbidity-Spectra and Liquid Exclusion Chromatography", CJCHE, 58, 65 (1980).

[6] McGowan, G.R. and Langhorst, M.A., "Development and Application of an Integrated, High-Speed, Computerized Hydrodynamic Chromatograph", J. Coll. Int. Sci., 89(1), 94 (1982).

[7] Pollock, M., Ph.D. Thesis, McMaster University (1983).

[8] MacGregor, J.F., Hamielec, A.E., Pollock, M. and Penlidis, A., "Control of Continuous Emulsion Polymerization Reactors", to appear in 1983.

[9] Noel, R.J., Gooding, K.M., Regnier, F.E., Ball, D.M., Orr, C. and Mullins, M.E., "Capillary Hydrodynamic Chromatography", J. Chromat., 16, 373 (1978).

[10] Giddings, J.C. and Myers, M.N., "Steric Field-Flow Fractionation: A New Method for Separating 1 to 100 nm Particles", Sep. Sci. Tech., 13, 637 (1978).

[11] Giddings, J.C., Myers, M.N. and Moellmer, J.F., "Fine-Particle Separation and Characterization by Field-Flow Fractionation", J. Chrom., 14, 501 (1978).

[12] Small, H., U.S. Patent 3,865,717 (1975).

[13] Krebs, V.K.F. and Wunderlich, W., "Die Ermittlung der Teilchengro Benverteilung von Polymer-Dispersionen durch Gelchromatographie", Die Ang. Makrom. Chem., 20(308), 203 (1971).

[14] Stoisits, F.R., Poehlein, G.W. and Vanderhoff, J.W., "Mathematical Modelling of Hydrodynamic Chromatography", J. Coll. Int. Sci., 57, 2(1976).

[15] McHugh, A.J., Silebi, C., Poehlein, G.W. and Vanderhoff, J.W., "Hydrodynamic Chromatography of Latex Particles", J. Coll. Int. Sci., 4, 549 (1976).

[16] Silebi, C.A. and McHugh, A.J., "An Analysis of Flow Separation in Hydrodynamic Chromatography of Polymer Latexes", AIChE J., 24(2), 204 (1978).

[17] Silebi, C.A. and McHugh, A.J., "The Determination of Particle Size Distributions by Hydrodynamic Chromatography: An Analysis of Dispersion and Methods for Improved Signal Resolution", J. Appl. Polym. Sci., 23, 1699 (1979).

[18] Nagy, D.J., "Column Chromatography of Polymer Latexes", Ph.D. Thesis, Lehigh U. (1979).

[19] Nagy, D.J., Silebi, C.A. and McHugh, A.J., "Hydrodynamic Chromatography: An Evaluation of Several Features", J. Coll. Int. Sci., 79(1), 264 (1981).

[20] Nagy, D.J., Silebi, C.A. and McHugh, A.J., "Resolution in Column Chromatography of Polymer Latexes. I. The Light Scattering Behavior of Polystyrene and its Effects on Signal Resolution in HDC", J. Appl. Polym. Sci., 26, 1555 (1981).

[21] Nagy, D.J., Silebi, C.A. and McHugh, A.J., "Resolution in Column Chromatography of Polymer Latexes. II. A Comparison of Porous and Nonporous Packing Systems", J. Appl. Polym. Sci., 26, 1567 (1981).

[22] Small, H., "Hydrodynamic Chromatography and Flow-Induced Separations", Adv. in Chromat., 15, 113 (1977).

[23] Prieve, D.C. and Hoysan, P.M., "Role of Colloidal Forces in Hydrodynamic Chromatography", J. Coll. Int. Sci., 64, 201 (1978).

[24] Buffham, B.A., "Model-Independent Aspects of Hydrodynamic Chromatography Theory", J. Coll. Int. Sci., 67(1), 154 (1978).

[25] Gaylor, V.F. and James, H.L., Preprints, Pittsburg Conference on Analytical Chemistry, Cleveland, March 1975.

[26] Coll, H., Fague, G.R. and Robillard, K.A., "Exclusion Chromatography of Colloidal Dispersion", Eastman Kodac Co., Rochester, N.Y. (1975).

[27] Coll, H. and Fague, G.R., "Liquid Exclusion Chromatography of Colloidal Dispersion", J. Coll. Int. Sci., 76(1), 116 (1980).

[28] Mori, S., Porter, R.S. and Johnson, J.F., Analyt. Chem., 46(11), 1599 (1974).

[29] Hamielec, A.E. and Singh, S., "Chromatography of Suspensions: An Experimental and Theoretical Investigation of Axial Dispersion Phenomena", J. Liq. Chromat., 1(2), 187 (1978).

[30] Singh, S., "Measurement of Particle Size in the Submicron Range: Evaluation of Liquid Exclusion Chromatography (LEC)", M.Eng., Thesis, McMaster University (1977).

[31] Kiparissides, C., "Modelling and Experimental Studies of a Continuous Emulsion Polymerization Reactor", Ph.D. Thesis, McMaster University (1978).

[32] Nagy, D.J., Silebi, C.A. and McHugh, A.J., "Latex Particle Size Analysis by Chromatographic Methods: Porous Packed Systems and Detection of Polystyrene", Polymer Colloids II, Ed. Fitch, R.M., Plenum Press (1980).

[33] Johnston, J.E., Cowherd, C.L. and MacRury, T.B., "Size Exclusion Chromatography of Model Latices: A Feasibility Study", ACS Symp. Series, 138, 27 (1980).

[34] Husain, A., "Chromatography of Particle Suspensions", Ph.D. Thesis, McMaster University (1980).

[35] Husain, A., Hamielec, A.E. and Vlachopoulos, J., "Particle Size Analysis Using Size Exclusion Chromatography", ACS Symp. Series, 138, 47 (1980).

[36] Kirkland, J.J., "High Performance Size Exclusion Liquid Chromatography of Inorganic Colloids", J. Chromat., 185, 273 (1979).

[37] Heller, W. and Pangonis, W.J., J. Chem. Phys., 26(3), 498 (1957).

[38] Wallach, M.L. and Heller, W., J. Chem. Phys., 34(5), 1796 (1961).

[39] Stevenson, A.F., Heller, W. and Wallach, M.L., J. Chem. Phys., 34(5), 1789 (1961).

[40] Heller, W. and Tabibian, R.M., "Experimental Investigations on the Light Scattering of Colloidal Spheres II. Sources of Error in Turbidity Measurements", J. Coll. Sci., 12, 25 (1957).

[41] Husain, A., Hamielec, A.E. and Vlachopoulos, J., "A New Method for Identifying and Estimating the Parameters of the Instrumental Spreading Function in Size Exclusion Chromatography: Application to Particle Size Analysis", J. Liq. Chromat., 4(3), 459 (1981).

[42] Husain, A., Hamielec, A.E. and Vlachopoulos, J., "An Analysis of Imperfect Resolution in the Chromatography of Particle Suspensions", J. Liq. Chromat., 4(3), 425 (1981).

[43] Husain, A., Vlachopoulos, J. and Hamielec, A.E., "Chromatography of Particle Suspensions: An Absolute Particle Size Detector Based on

Turbidity Spectra Analysis – A Simulation Study", J. Liq. Chromat., 2(4), 517 (1979).

[44] Maron, S.H., Pierce, P.E. and Ulevitch, I.N., J. Coll. Sci., 18, 470 (1963).

[45] Gledhill, R.J., J. Phys. Chem., 66, 458 (1961).

[46] Maxim, L.D., Klein, A., Meyer, M.E. and Kuist, C.H., J. Polym. Sci., Part C, 27, 195 (1969).

[47] Zimm, H.B. and Dandliker, W.B., "Theory of Light Scattering and Refractive Index of Solutions of Large Colloidal Particles", J. Phy. Chem., 58, 644 (1954).

[48] Heller, W., "Remarks on Refractive Index Mixture Rules", J. Phys. Chem., 69(4), 1123 (1965).

[49] Nakagaki, M. and Heller, W., "Effect of Light Scattering Upon the Refractive Index of Dispersed Colloidal Spheres", J. Appl. Phys., 27, 9 (1956).

[50] Casassa, E.F., "Theoretical Models for Peak Migration in Gel Permeation Chromatography", J. Phys. Chem., 75, 3929 (1972).

[51] Hamielec, A.E. and Ray, W.H., J. Appl. Polym. Sci., 13, 1319 (1969).

[52] Provder, T. and Rosen, E.M., "The Instrument Spreading Correction in GPC", Sep. Sci., 5(4), 437 (1970); 5(4), 485 (1970).

[53] Friis, N. and Hamielec, A.E., "Gel Permeation Chromatography: A Review of Axial Dispersion Phenomena, Their Detection and Correction", Adv. Chromat., 13, 41 (1975).

[54] T., Lee, S.I. and Hamielec, A.E., "Solution of Tung's Axial Dispersion Equation by Numerical Techniques", J. App.. Poly. Sci., 15, 1607 (1971).

[55] Husain, A., Vlachopoulos, J. and Hamielec, A.E., "Chromatography of Particle Suspensions: Analytical Corrections for Axial Dispersion", J. Liq. Chromat., 2(2), 193 (1979).

[56] Yau, W.W., Kirkland, J.J., Bly, D.D. and Stoklosa, H.J., J. Chromat., 125, 219 (1976).

[57] Hamielec, A.E., J. Liq. Chromat., 3, 381 (1980).

[58] Hamielec, A.E., Ederer, H.J. and Ebert, K.H., J. Liq. Chromat., 4, 1697 (1981).

[59] Hamielec, A.E., J. Liq. Chromat., 1, 555 (1978).

[60] Chu, B., Ann. Rev. Phys. Chem., 21, 145 (1970).

[61] Chu, B., "Laser Light Scattering", Academic Press, New York, NY (1974).

[62] Berne, B.J. and Pecora, R., "Dynamic Light Scattering", John Wiley and Sons, Inc., New York, NY (1976).

[63] Derderian, E.J. and MacRury, T.B., J. Dispersion Sci. and Tech., 2, 345 (1981).

[64] Zellers, R.L., J. Dispersion Sci. and Tech., 2, 331 (1981).

[65] Brow, J.C., Pusey, P.N. and Dietz, R., J. Chem. Phys., 62, 1136 (1975).

[66] Tung, L.H. and Runyon, F.R., J. Appl. Polym. Sci., 13, 2397 (1969).

[67] Silebi, C.A., "A Theoretical and Experimental Examination of Hydrodynamic Chromatography", Particle Size Analysis II, FACSS Symp., Philadelphia, PA (1981).

[68] Hess, M. and Kratz, R.J., J. Polym. Sci., A-2, 4, 731 (1966).

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Flow Rate Dependence of Elution Volumes in Size Exclusion Chromatography: A Review

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Abstract

This review summarizes experimental data which indicate that elution volumes can change with flow rate in size exclusion chromatography experiments. The mechanisms resulting in flow rate dependent elution volumes are discussed. They can be roughly divided into two classes: 1) anomalous effects, and 2) flow rate dependence of the partitioning of molecules into the micropores of the column packing. The partitioning can depend on flow rate if the partition coefficient is concentration dependent, if viscous fingering occurs, or if molecular migration phenomena are important.

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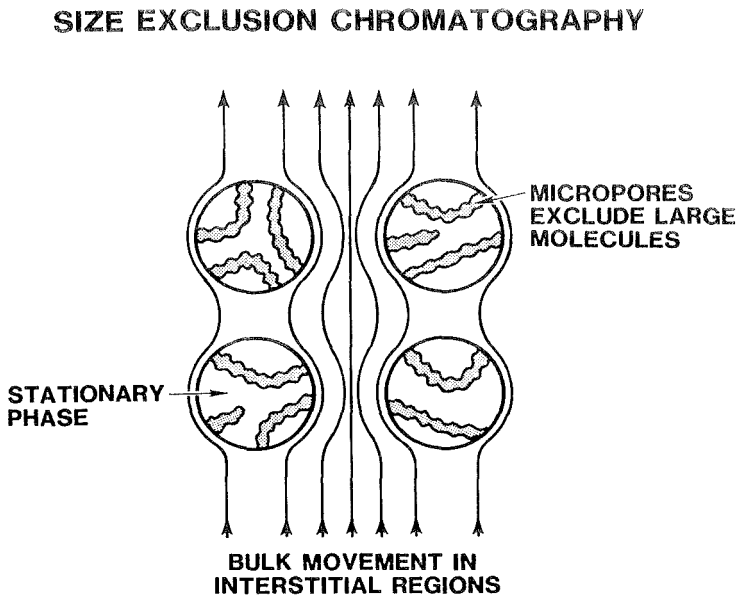
1. Introduction

Size exclusion chromatography (SEC) is a widely used experimental technique which separates macromolecules on the basis of size. The technique is often used to determine the molecular weight distribution of synthetic and natural polymers. Size exclusion chromatogaphy has also been called gel permeation chromatography (GPC) or gel filtration for aqueous solutions; however, size exclusion is the most appropriate name because separation occurs as a result of larger molecules being excluded from the stagnant pores of a chromatographic column to a greater extent than smaller molecules.

In a chromatographic experiment, solvent is pumped continuously through the SEC column. At the desired time, a small sample of a macromolecular solution is injected at the top of the column as a pulse. As the macromolecules flow past the micropores, which are inside of the column packing particles, they partition between the micropores and the interstitial volume, (Fig. 1), i.e., between the stationary and the mobile phases. The partitioning depends upon the pore size and type and the macromolecular size and type. In the absence of adsorption, larger molecules are excluded from the pores to a greater extent than smaller molecules. Since the micropores are relatively stagnant, and the interstitial volume is not, larger molecules move through the column faster than smaller molecules and elute first. Hence, an SEC column separates macromolecules on the basis of size.

The first applications of SEC occurred in biochemistry, where biological macromolecules were separated. Porath[1] used dextran gels in a chromatographic column and separated proteins, peptides, amino acids, and some of their derivatives.



(1) The transport of macromolecules through an SEC column is dependent on the partitioning into the micropores of the column packing.

Based upon the experimental results, Porath speculated that the mechanism of separation was the exclusion of large molecules from the gel. Shortly thereafter, Porath[2] proposed a theory of SEC based upon equilibrium partitioning of macromolecules between unbounded solutions and conical pores. Good qualitative agreement was found between the predictions of this theory and experimental data on low molecular weight dextran fractions obtained by Granath and Flodin.[3] More detailed theoretical descriptions of the partitioning in SEC followed. Squire[4] extended the theoretical development by determining the partitioning of spherical macromolecules in gels modeled as a combination of cones, cylinders, and crevices. Laurent and

Killander[5] determined the partition coefficient of spherical macromolecules in gels modeled as a three-dimensional network of fibers. These theories made SEC nearly unique among types of chromatography, since its behavior could be predicted reasonably accurately.

Moore[6] was the first to apply SEC to synthetic polymers, by separating polystyrenes of narrow molecular weight fractions in a chromatographic column packed with beads, which were made by crosslinkng a polystyrene gel in the presence of diluents. The fractions were shown to be efficiently separated, and this demonstrated the usefulness of SEC in characterizing synthetic polymers in order to aid in their manufacture and use. However, the fact that SEC is the useful technique it is today owes much to the work of Grubisic, Rempp, and Benoit.[7]

In order to use SEC to determine the molecular weight distribution of an unknown sample, one must calibrate the SEC columns used. Calibration consists of determining the elution volumes for polymers of known molecular weight and is usually compiled as a plot of the logarithm of molecular weight versus elution volume. By measuring the elution volume of the unknown sample, its molecular weight can be determined by comparison to the calibration curve. This type of calibration depends upon the polymer/solvent system used in the columns. Grubisic, Rempp and Benoit[7] discovered a nearly universal calibration method for SEC; i.e., a calibration technique for an SEC column which is independent of the solvent or polymer used. A plot of the logarithm of the intrinsic viscosity and molecular weight product ([n]M), versus elution volume, for a particular column, was found to yield a characteristic curve which was independent of the polymer/solvent system. They

deduced that the important macromolecular size in SEC is the hydrodynamic volume, since the intrinsic viscosity and molecular weight product is proportional to this.

Theoretical explanations for the above followed in the work of Casassa,[8,9] Casassa and Tagami,[10] and Giddings et al.[11] These workers calculated the equilibrium partition coefficients for macromolecular models partitioning between an unbounded solution and micropores of simple geometric shape. If the elution of a macromolecular solution through a chromatographic column is sufficiently slow so that equilibrium is maintained between the solute in the flowing stream adjacent to the pore mouth and all of the pore volume, then these theoretical results could be used to predict the elution characteristics of a macromolecule of known size and structure. The relationship between the elution volume and the equilibrium partition coefficient is predicted to be[12]

$$V_e = V_O + K_D V_I \ , \qquad (1)$$

where V_e is the measured elution volume for the macromolecule, V_I is the total volume of the micropores, V_O is the interstitial volume in the column, and K_D is the equilibrium partition coefficient. If equilibrium is not maintained between the solution near a pore mouth and the entire pore volume, then diffusion of the macromolecules could be important. The significance of macromolecular diffusion in SEC was not thoroughly understood until the work of Hermans.[13]

If the equilibrium assumption in SEC is valid, then the elution volume should be independent of flow rate, as predicted

by Eq. (1). The majority of earlier workers concluded that elution volmes were independent of flow rate within the usual flow rate ranges.[14] However, a number of studies have shown that elution volumes can either increase or decrease with flow rate, depending upon the experimental conditions.[15-21] Some of these observations have been rationalized, but others remain unexplained. Some of the possible explanations for these observations of flow rate dependent elution volumes include nonequilibrium effects, molecular structure changes with flow rate, stationary phase changes with flow rate, concentration/flow rate effects, instrumental anomalies, and molecular migration effects. In this review we will summarize, in a mechanistic manner, experimental data which indicate a flow rate dependence of elution volume and some thoughts on the most likely causes for each of the observations.

2. Nonequilibrium Effects

Nonequilibrium effects in SEC can occur in two ways. The first is when equilibrium is not maintained at the micropore mouth between the solution in the interstitial volume and the solution within the micropore. The second is when equilibrium is maintained at the micropore mouth but the solution in the interior of the micropore is not at equilibrium with the solution in the interstitial volume. In this case diffusion of the solute into the micropore could be important. Nonequilibrium effects have been used by a number of authors[15,17,26,27] to explain flow rate dependent elution volumes in SEC.

2.1. Hermans' Model of SEC

Hermans[13] proposed a mathematical model of the chromatographic process in order to understand and assess the role of

diffusion in SEC. Hermans assumed the following about the column and packing:

i) The packing consists of rigid spherical particles with a uniform diameter. Most modern SEC packings are very uniform and many consist of spherical particles.

ii) The column is spatially homogeneous; hence, in any axial cross section of the column the concentration of solute is independent of angular or radial position. Most SEC columns are packed carefully to insure that this assumption is satisfied, since the column resolution diminishes if it is not. Also, the flow through the column is assumed to be plug flow, with no angular, radial, or axial variation in the flow rate.

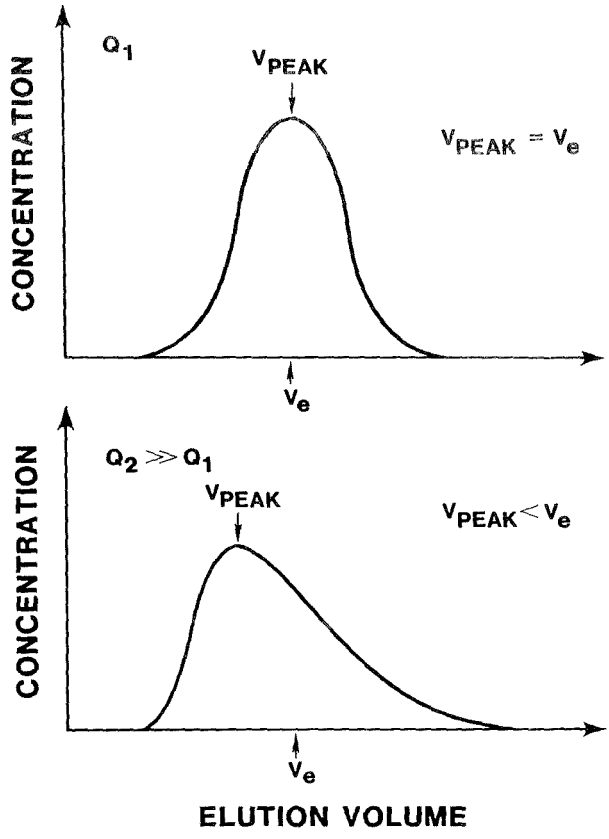
iii) No dispersion of the solute occurs in the mobile phase due to diffusion; i.e., convection dominates the transport of solute in the mobile phase. At the flow rates typically used in SEC, this assumption is valid, since the diffusion coefficients of high molecular weight species are extremely low.

iv) Equilibrium between the solute concentration at the packing surface (but not within the packing particle) and the solute concentration in the mobile phase adjacent to each packing particle is maintained at all times. This assumption has been experimentally verified by a number of independent workers[22-24] whose work is summarized below.

The important diffusion process in the model occurs in the transport of solute from the surface of the packing particles to the interior of the particles.

Hermans solved the mathematical problem with Laplace transforms and obtained exact expressions for the moments (in time) of the solute concentration leaving the end of the column. For the first moment (the mean) an expression identical to Eq. (1) is obtained. This is an extremely important result. Even in the absence of equilibrium between the mobile phase solute concentration and the solute concentration in the pore volume, Eq. (1) predicts the mean elution volume of the solute if equilibrium is maintained at the packing surface. Hence, the theoretical results obtained by Casassa,[8,9] Casassa and Tagami,[10], and Giddings et al.[11] can be used to predict the elution volume of a macromolecular solute, even when diffusion into the packing is slow enough to be significant. Although the first moment is independent of the diffusion of solute into the pores, higher moments do depend on the diffusion rate. Therefore, the peak width and the shape of the elution curve depend on the flow rate through the column and the diffusion of the solute.[5,12,13]

At low flow rates the elution curve is Gaussian in shape, and therefore symmetrical, and the peak position is equal to the first moment. At higher flow rates, however, the elution curve can become skewed when the diffusion rate into the micropores is sufficiently slow,[13] (Fig. 2). If the first moment is independent of flow rate, as predicted by Hermans' theory, and the elution curve becomes more skewed with increasing flow rate, then the peak position of the elution curve should decrease as the flow rate increases. Hence, when observing flow rate dependent elution volumes, one must be careful to measure the position of the first moment, rather than the peak position. This may be an explanation as to why some

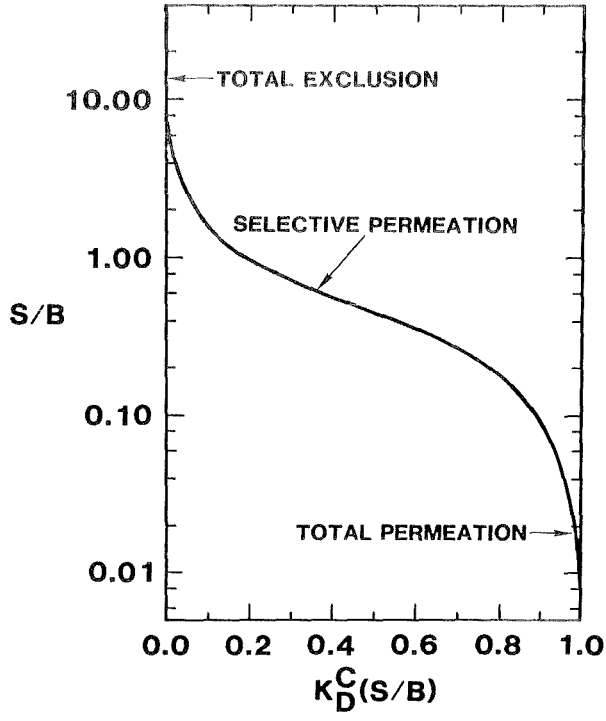


(2) The qualitative effect of flow rate on the skewing of an elution curve and the peak position.

experimentalists have observed elution peaks decreasing with increasing flow rates, even after other corrections had been made to the elution curve.(15)

2.2. Predicted Elution Curves

With the results of Hermans, the elution characteristics of a macromolecular solute can be predicted from a molecular theory for the equilibrium partition coefficient of



(3) SEC calibration curve predicted for the elastic dumbbell model in a chromatographic column. The chromatographic column is assumed to consist of a stationary phase containing uniform capillary micropores.

that macromolecule. Only the weak assumption, that equilibrium is maintained at the surface of the stationary phase, is necessary. One example of this is shown in Fig. 3, in which the macromolecules are modeled with the Rouse model and the micropores are approximated as capillaries.(25) The predicted equilibrium partition coefficient, K_D, is plotted against the ratio of the radius of gyration of the macromolecule to the capillary pore radius, (S/B). This calibration plot is

similar to the universal calibration proposed by Grubisic, Rempp, and Benoit[7] since K_D is linearly related to the elution volume in a chromatographic column, Eq. (1), and the logarithm of the radius of gyration is approximately proportional to the logarithm of the hydrodynamic volume. The similarity in shape of predicted calibration curves to experimentally observed ones gives support to the assumption that equilibrium is maintained at the surface of the stationary phase.

2.3. Comparison with Experiment

Although the shape of experimental SEC calibration curves is the same as that predicted theoretically with any of the equilibrium molecular theories[8-11,25] some questions have remained as to whether equilibrium is always maintained at the stationary phase surface at all flow rates and operating conditions. At high enough flow rates equilibrium may no longer exist, and, in fact, a number of authors have attributed flow rate dependent elution volumes to nonequilibrium effects.[15,17,26,27]

The importance of nonequilibrium at the packing particle surfaces can be estimated by comparing two time scales. The first is the time necessary for a macromolecule to enter a micropore in the stationary phase (or to equilibrate with the pore mouth), and the second is the time for the solute band to pass a micropore. Since the size of the micropores is comparable to the macromolecular size, the first time is essentially the primary relaxation time of the macromolecule, τ. The second time is relted to the flow rate, Q, and the column's loading or sample size, V_ℓ.

$$t = V_\ell / Q$$

Typical values of these parameters in a modern chromatographic experiment are $Q = 1\ cm^3/min$ and $V_\ell = 50\ \mu l$, which result in 3 seconds as an estimate of the time for the solute band to pass any point in the column. Even a very high molecular weight polymer has a relaxation time at least four orders of magnitude smaller than this estimate of τ, and lower molecular weight species have even a smaller relaxation time. Hence, the ratio τ/t is so small that nonequilibrium effects should never be importnt in SEC. Van Kreveld and Van Den Hoed[28] arrived at this same conclusion in a more rigorous manner.

Yau, Malone, and Fleming [22] compared SEC partition coefficients, (obtained from Eq. (1) in a chromatographic experiment), to equilibrium partition coefficients for polystyrene in chloroform partitioning into both a porous glass packing and a polystyrene gel packing. They concluded that equilibrium was attained at the stationary phase surface in all of their experiments, which involved a range of flow rates. The same conclusion was reached in a similar study by Grubisic-Gallot and Benoit[23] and later by Aubert and Tirrell.[24] Hence, we can conclude that flow rate dependent elution volumes, although experimentally observed at times, are not due to nonequilibrium effects.

3. Molecular Structure Changes with Flow Rate

3.1 Molecular Degradation

A number of experimentalists have demonstrated that high molecular weight molecules can be degraded in a chromatographic column at high flow rates.[29-32] These studies have involved a number of different polymers and a number of different instruments. If high molecular weight species are degraded

into lower molecular weight species during flow through a chromatographic column, then their elution volume will increase. Hence, molecular degradation with increasing flow rate results in an apparent increase in elution volumes with increasing flow rates.

3.3. Configurational Changes

When macromolecules are subject to large rates of deformation, their configurations can change by stretching and aligning with the flow streamlines.[33] Such a situation can occur at sufficiently high flow rates in a chromatographic column. Some workers have suggested that this could change the partition coefficient of the macromolecule and, therefore, its elution volume could change with flow rate. These workers have concluded, however, that this effect is of no significance in SEC.[16] This observation is corroborated by molecular theories,[34] which show that the dilute solution partition coefficient of a macromolecule depends only upon the number density of macromolecules outside of the pore, the environment inside of the pore, and on equilibrium being maintained at the pore mouth.

4. Stationary Phase Changes with Flow Rate

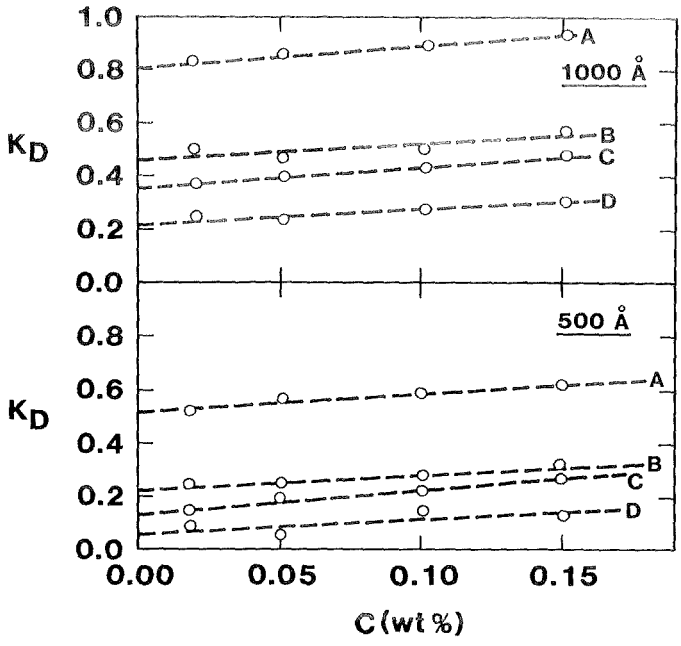
A possibility exists that flow rate dependent elution volumes could arise from changes in the pore volume or pore size with a change in flow rate and a corresponding change in the column's pressure drop. Either of these effects would result in the elution volume decreasing as the flow rate increases. This is a possible mechanism of flow rate dependent elution volumes in columns packed with nonrigid packings,[17] although most authors have concluded that this is not a significant effect in the range of flow rates and pressure drops typically used.[15]

Modern, high-pressure SEC exclusively uses rigid packings which are not affected by variations in the pressure drop. This has been experimentally verified for one rigid commercially available column packing.[35]

5. Concentration/Flow Rate Effects

Elution volumes have been observed to increase as the solute concentration is increased[16-20,36] and also as the volume injected is increased.[16,17,20] These observations suggest that, if equilibrium is maintained at the pore mouths, then the equilibrium partition coefficient must increase with increasing solute concentration. Such an effect has been experimentally shown to be true for both rigid and flexible macromolecules.

Brannon and Anderson[37] measured the equilibrium partition coefficients of three dextrans and bovine serum albumin partitioning into controlled-pore glass beads by a batch mass balance technique. The partition coefficients were measured as a function of concentration and, at low concentrations, were found to increase linearly with concentration. Satterfield et al.[38] had previously performed similar experiments on flexible polystyrene molecules and also found that the partition coefficients increased linearly with concentraton at low concentrations. Aubert and Tirrell[39] measured the partition coefficients of polystyre in a chromatographic packing as a function of concentration and molecular weight. These results are summarized in Fig. 4, and show the same linear dependence on concentration as does the elution volume in a chromatographic experiment[16-20,36] and also show the same qualitative dependence on molecular weight. A number of molecular theories



(4) Experimental partition coefficients as a function of concentration for two different pore sizes, 500Å and 1000Å. A: $\bar{M}_N = 1.1 \times 10^5$; B: $\bar{M}_N = 3.0 \times 10^5$; C: $\bar{M}_N = 9.0 \times 10^5$; D: $\bar{M}_N = 1.8 \times 10^6$.

predict that the partition coefficient should increase linearly with concentration at low concentrations.(40,41,24)

As the flow rate through a chromatographic column increases, the width of the elution peak increases,(12,13) and as a consequence the average concentration of the solute band decreases. Since equilibrium partition coefficients generally decrease as the solute concentration is decreased, the measured elution volume should decrease as the flow rate is increased, for samples of sufficiently high initial concentration.

Another concentration effect, one that is independent of the above, has to do with the viscosity of the injected sample. It has been shown that if the injected solution viscosity differs greatly from the solvent viscosity, then viscous fingering can occur in flow through the column. The amount of viscous fingering depends upon the flow rate and should decrease as the flow rate is increased. Hence, viscous fingering should result in elution volumes changing with flow rate.[16,17]

6. Instrumental Anomalies

Some experimentalists have shown that apparent flow rate dependent elution volumes can result from instrumental anomalies; i.e. the performance of the instrument depending upon the flow rate of solvent through it. Two such anomalies were documented by Yau, Suchan, and Malone[15] on instruments which use a siphon for the purpose of monitoring the flow and elution volume. The first anomaly was due to the fact that solvent would continue to flow into the siphon as it was discharging. This anomaly results in apparent elution volumes appearing lower than they actually are and in apparent elution volumes decreasing with increasing flow rate. The second anomaly was due to the fact that solvent could evaporate before the siphon discharged. At slow flow rates a significant portion of the solvent in the siphon could evaporate before the siphon discharged. This would also result in apparent elution volumes that were lower than the true elution volumes. However, since the evaporation effect decreases as the flow rate increases, the apparent elution volume would increase with increasing flow rate.

When both of these anomalies are operative, the apparent elution volume increases with flow rate at low flow rates and

then decreases with flow rate at high flow rates. A plot of apparent elution volume versus flow rate would show a maximum for a given molecular species. Yau, Suchan, and Malone[15] demonstrated that these two anomalies were the major contribution to their observed flow rate dependent elution volumes. With these instrumental anomalies accounted for, Little et al[42] found no flow rate dependence of elution volumes on an instrument that was similar to that used by Yau, Suchan, and Malone.

7. Molecular Migration Effects

Numerous studies have been done on the flow rate dependence of elution volumes in SEC utilizing nonrigid packings. The results of these studies have been very dependent upon the instrumentation, especially the column packing, and also upon the polymer/solvent system. Different authors have reported elution volumes increasing with flow rate, decreasing with flow rate, or being independent of flow rate.[14] Some of the results have been shown to be directly attributable to instrumental anomalies.[15,42] Other postulated causes of flow rate dependent elution volumes include dispersion effects and concentration/flow rate effects. Nonequilibrium has been shown to be of no significance in SEC. Modern SEC is run only at high pressure and usually only with rigid packings. In this section, some experimental results on flow rate dependent elution volumes occurring in columns with rigid packings are summarized. In addition one possible mechanism for this dependence is discussed.

Gudzinowicz and Alden[18] ran SEC experiments on narrow molecular weight distribution polystyrenes (PS) dissolved in tetrahydrofuran (THF) and measured the peak elution volumes at a number of flow rates. Their SEC column was packed with

44-50 μm diameter rigid, porous glass beads containing micropores of known sizes. Peak elution volumes were measured at a number of flow rates for various PS molecular weights. For low molecular weights (and in particular for benzene) there was little flow rate dependence of elution volumes. At higher molecular weights the flow rate dependence of the elution volume increased significantly. For the highest molecular weight studied ($\bar{M}_N$ = 1.8 x 10^6) the elution volume increased more than 8% as the flow rate doubled. Their results are summarized in Table I.

An experimental study of the flow rate dependence of elution volumes was also undertaken by Aubert and Tirrell,[21,24,35,43] who used a DuPont 830 high pressure liquid chromatograph. Narrow molecular weight distribution PS, obtained from Pressure Chemical Co., was used after dissolving in THF, (Table II). The column packing consisted of 6um diameter silica spheres with 1000Å diameter micropores.[21,3] Figure 5 displays the superimposed elution peaks for some of the molecular weights studied at two different flow rates, 1 cm^3/min and 3 cm^3/min. It is clear that the peak elution volumes increased with increased flow rate. The peak widths also increased with flow rate, as expected from Hermans equilibrium theory.[13] For the highest molecular weight ($\bar{M}_N$ = 2.85 x 10^6) the first moment of the elutant peak could be calculated, since it was displaced far enough from the benzene peak so that no overlap of the peak bases occurred. The elution volume of the first moment increased slightly more with flow rate than the peak elution volume for this high molecular weight sample. The position of the first moment differed only slightly from the peak position since the skewing of the curves was minimal. At

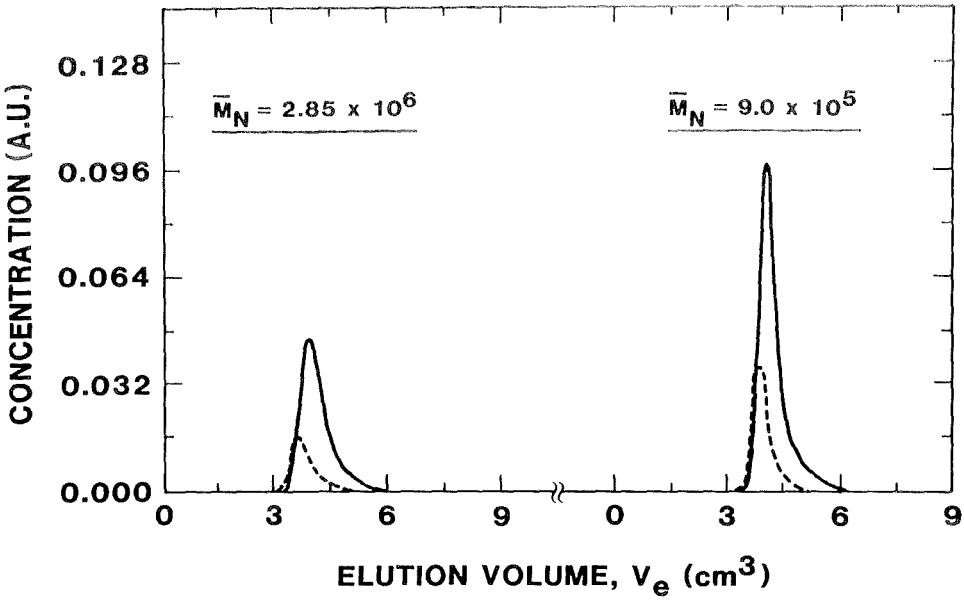
Table I Data of Gudzinowicz and Alden for flow rate dependent elution volumes for PS in THF in porous silica bead columns.

Run	Solvent Flowrate (ml/min)	$\bar{M}_N$ 1.8×10^6	1.6×10^5	Elution Volumes 5.1×10^4	1.0×10^4	Benzene
A	0.728	12.32	15.50	17.83	20.10	22.33
B	0.907	12.53	15.58	17.90	20.23	22.40
C	1.100	13.15	15.50	17.87	20.15	22.40
D	1.287	13.29	15.70	18.00	20.38	22.55
E	1.470	13.38	15.60	18.00	20.35	22.55

Table II

Polystyrene standards used from Pressure Chemical Co. and their polydispersity

Molecular weight $\bar{M}_n$ (gm/gm-mole)	Polydispersity $\bar{M}_w/\bar{M}_n$
5.0×10^4	< 1.06
2.0×10^5	< 1.06
3.9×10^5	< 1.10
9.0×10^5	< 1.10
2.85×10^6	< 1.30



(5) A comparison of the elution peaks measured in SEC experiments run at 1 cm^3/min (----) and 3 cm^3/min (——). Polystyrene ($\bar{M}_N$ = 2.85 x 10^6 and $\bar{M}_N$ = 9.0 x 10^5) in THF run in the DuPont SE-1000A column.

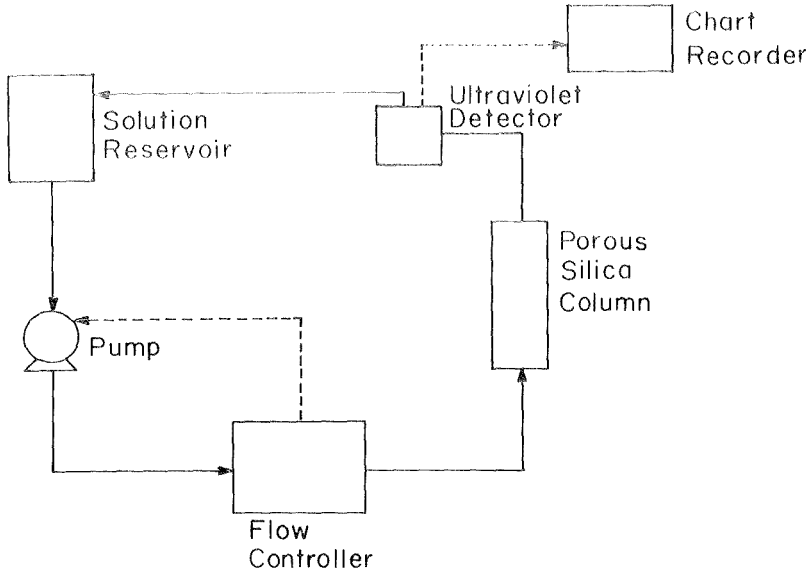
a flow rate of 1 cm^3/min the peak elution volume was 3.57cm^3 and the elution volume of the first moment was 3.79cm^3, while at 3 cm^3/min the peak elution volume was 3.90cm^3 and the elution volume of the first moment was 4.15cm^3. Hence, the elution volumes were found to increase with flow rate. These results and the results of Gudzinowicz and Alden cannot be explained by any of the mechanisms discussed so far, since all of these mechanisms result in elution volumes decreasing with increasing flow rate.

At the same time that these chromatography measurements were made at different flow rates, steady state measure-

ments (independent of time and diffusion rates) were made on the partition coefficient with the same columns and instrumentation, the same polymer/solvent system, and the same range of flow rates.[43] The purpose of these experiments was to determine the origin of flow rate dependent elution volumes in these systems.

These experiments involved pumping polymer solutions continuously through one of the SEC columns and monitoring the effluent concentration (Fig. 6). At a predetermined time the flow rate through the column was changed instantly and the effluent concentration monitored. A typical response of the detector and recorder to a step flow rate change is shown in Fig. 7. The solution used in this example was 0.053 wt % PS ($\bar{M}_N$ = 1.8 x 10^6) in THF. The flow rate was changed first from 3 to 1 cm^3/min and, at a later time increased back to 3 cm^3/min. After the flow rate decrease the effluent concentration was higher, which indicated that the column retention was lower at the lower flow rate. The opposite occurred when the flow rate was again increased. These results indicate the partition coefficient (for partitioning between the interstitial volume in the column and the micropores of the stationary phase) was dependent on the flow rate through the column and increased as the flow rate increased. Similar observations have been made by Chauveteau and Kohler[44] and Willhite and Dominquez in other types of porous media.

The results of these experiments were compiled as the changed column retention, Γ_{ij}, for a given flow rate change from i to j for various molecular weights. These results are summarized in Fig. 8. The apparent dependence of the partition coefficient K_D, on flow rate can be determined from these experimental results by

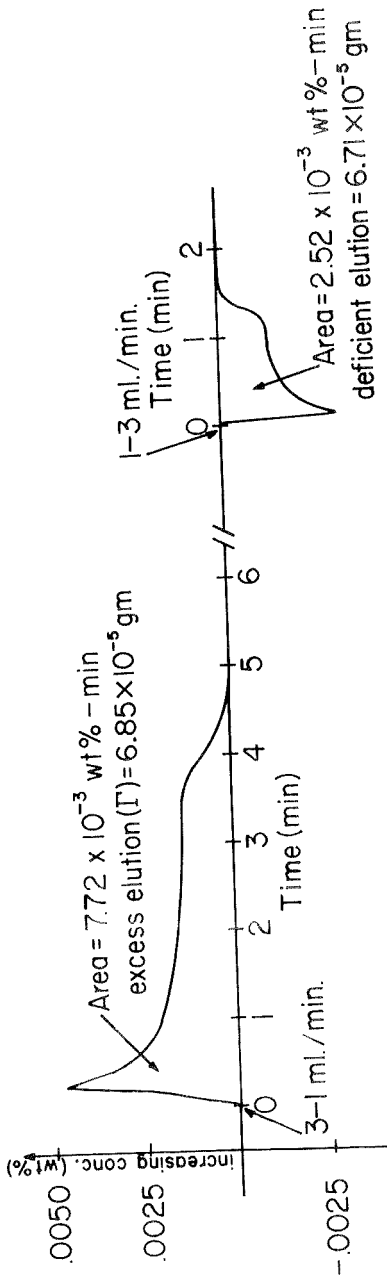


(6) Schmatic of the high pressure liquid chromatograph flow path. Solid lines are flow paths and dashed lines are information paths.

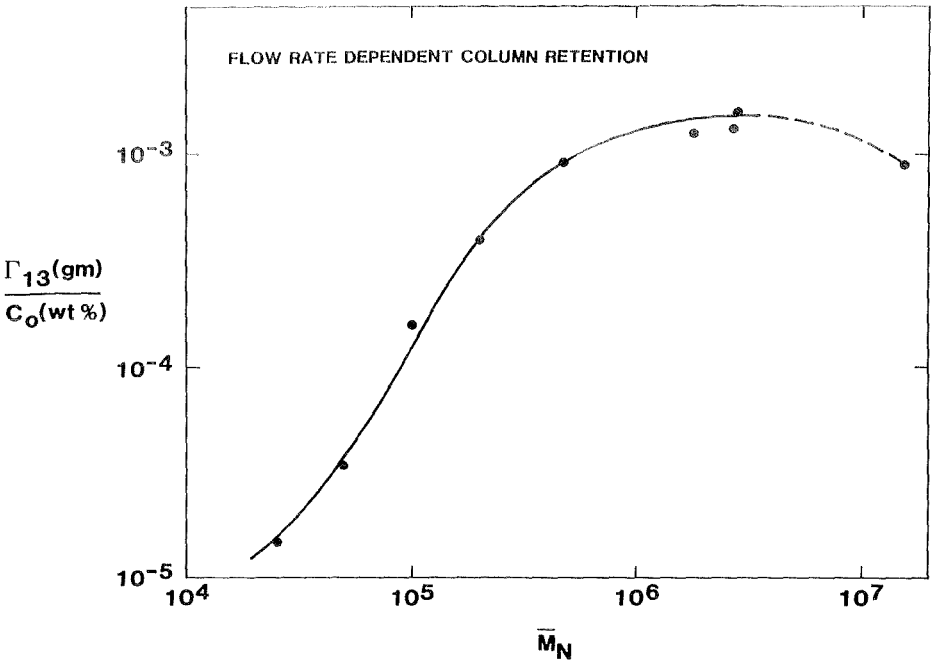
$$K_D(Q_j) = K_D(Q_i) + \frac{\Gamma_{ij}}{C_O V_I} \qquad (3)$$

where C_O is the concentration being fed to the column and V_I is the total volume of micropores. The flow rate dependent partition coefficients predicted from Eq. (3) agree well with those measured in dynamic chromatography experiments,[21,35] (Eq. 1). Table III shows this comparison.

The mechanism causing the apparent flow rate dependence of the equilibrium partition coefficient has not been conclusively determined. One possible mechanism has been proposed which is in good qualitative agreement with all of the experimental observations.[46] The basis of the mechanism is the migration



(7) Typical detector responses to step flow rate changes, (3-1 cm^3/min and 1-3 cm^3/min), for PS ($\bar{M}_N$ = 1.8 x 10^6) in THF in the 1000Å column.



(8) Plot of log (Γ_{13}/C_o) versus long (M_N) $\bar{}$for PS in THF in the 1000Å column. The flow rate changes were between 1 and 3 cm^3/min.

of macromolecules which may occur in the nonhomogeneous and curvilinear velocity field of the interstitial volume. The magnitude and the origin of this macromolecular migration has been described for the Rouse model of a macromolecule and can be summarized by the following equation:

$$\underline{v}^{drift} = \frac{1}{8} \langle \underline{R}\ \underline{R} \rangle : \nabla\nabla\underline{v} + \cdots \qquad (4)$$

The time average migration, or drift velicity of the macromolecule's center of mass, relative to the time averaged

Table III. Measured and predicted dependence of elution volume on flow rate in the DuPont silica columns.

Measured Quantities					Predicted Quantities	
$\bar{M}_n$	Elution Volume, ml		K_D	Γ_i/C_o	K_D	Elution Volume
	Q=1	Q=3	Q=1	1-3ml/min	Q=3	Q=3ml/min
2.85×10^6	3.57	3.90	0.022	1.67×10^{-3}	0.076	3.74
9.0×10^5	3.82	4.02	0.102	1.20×10^{-3}	0.141	3.94
3.9×10^5	4.35	4.47	0.272	7.80×10^{-4}	0.297	4.43
2.0×10^5	4.85	4.92	0.431	4.00×10^{-4}	0.444	4.89
5.0×10^4	5.60	5.67	0.671	5.30×10^{-5}	0.673	5.61
Benzene	6.58	6.63	1.0	0.0	1.0	6.58

solvent velocity, $\underline{v}$, is $\underline{v}^{drift}$, and $\langle \underline{R}\ \underline{R} \rangle$ is the configurational average of the dyadic product of the macromolecules' end-to-end vector. Recently a similar result has been obtained for the Zimm model by incorporating hydrodynamic interactions between different parts of the macromolecule.(47) In a non-homogeneous and curvilinear flow field, such as in a porous media flow, Eq. (4) predicts that macromolecules would migrate to the concave side of the streamlines or toward the surface of the stationary phase in a chromatographic column.(46) This would tend to build up the macromolecular concentration at the stationary phase surface. Since the migration velocity is flow rate dependent, increasing with flow rate, the surface concentration increases with flow rate also.

If equilibrium partitioning occurs between the surface concentration and the micropore concentration, then the micropore concentration is also flow rate dependent. This is equivalent to the equilibrium partition coefficient having an apparent flow rate dependence. This has been the only mechanism proposed to explain the observation of retentive volumes increasing with flow rate in a chromatographic column. Although this mechanism has not been proven to be a cause of flow rate dependent retention, it is consistent with all of the experimental observations.(46)

8. Conclusions

In this review we have summarized, in a mechanistic way, experimental data which show that elution volumes can depend upon the flow rate through an SEC column. The flow rate dependence of elution volumes can be caused either by anomalous effects or physical effects which result in a change in the

distribution of molecules between the interstitial volume in the column and the micropores of the stationary phase. Nonequilibrium effects (i.e. equilibrium not being maintained at the surface of the stationary phase) have been shown both theoretically[28] and experimentally[22-24] to be of no significance in SEC.

A number of anomalous effects have been documented which result in apparent flow rate dependent elution volumes. These include the following:

1) Errors can be caused by estimating the first moment of the elution curve by the position of the peak. These differ if the elution curve is skewed, and the amount of skewing depends upon the flow rate through the column.
2) High molecular weight species can be degraded in the columns if the deformation rates are high enough. The amount of degradation increases with flow rate, which can result in an apparent increase in elution volumes with flow rate.
3) Instrumental anomalies can result in apparent flow rate dependent elution volumes if the instrumental performance depends upon flow rate. The best example of this involves the use of a siphon to collect the effluent and measure the flow rate. The siphon performance depends upon flow rate due to solvent evaporation in the discharge chamber and to the fact that flow continues into the siphon as it is discharging.

A number of physical effects operate in a chromatograph which can result in a change, with flow rate, in the

distribution of molecules between the interstitial volume in the column and the micropores. These include the following:

1) The possibility exists that the stationary phase of an SEC column which is packed with a nonrigid packing can change with flow rate and pressure drop through the column. This change could involve either the micropore shape or volume decreasing as the pressure drop increases, which would result in elution volumes decreasing with increasing flow rate.

2) Concentration/flow rate effects can result in flow rate dependent elution volumes in two ways. The first way is because the equilibrium partition coefficient depends upon cencentration and the average concentration in the solute band depends upon flow rate. At high flow rates the width of the solute band is greater and hence the average concentration in the band is slower. This effect results in elution volumes decreasing as the flow rate is increased. The second way that flow rate dependent elution volumes can occur is if viscous fingering happens in flow through the column. The amount of viscous fingering is flow rate dependent, and the elution volume, and also the shape of the elution curve, depends upon viscous fingering. This effect is of no significance unless the viscosity of the injected solution differs substantially from the solvent viscosity.

3) Molecular migration phenomena can result in the surface of the stationary phase being exposed to an enhanced concentration of solute as the solute band passes.

The enchanced concentration results in greater partitioning into the micropores and a greater elution volume. Since the migration phenomena increases with flow rate, this effect results in elution volumes increasing as the flow rate increases. This mechanism has not been totally justified, but it results in predictions that are in qualitative agreement with data obtained or flow rate dependent elution volumes in column with rigid packings.

REFERENCES

(1) J. Porath, Biochem. Biophys. Acta, 39, 193(1960).

(2) J. Porath, Pure Appl. Chem., 6, 233(1963).

(3) K. A. Granath and P. Flodin, Macromol. Chem.,48, 160(1961).

(4) P. G. Squire, Arch. Biochem. Biophys., 107, 471(1964).

(5) T. C. Laurent and J. Killander, J. Chromatog., 14, 317(1964).

(6) J. C. Moore, J. Polym. Sci., Part A, 2, 835(1964).

(7) R. Grubisic, P. Rempp, and H. Benoit, Polym. Letters, 5, 753(1967).

(8) E. F. Casassa, Polym. Letters, 5, 773(1967).

(9) E. F. Casassa, Macromolecules, 9, 182(1976).

(10) E. F. Casassa and Y. Tagami, Macromolecules, 2, 14(1969).

(11) J. C. Giddings, E. Kucera, C. P. Russel, and M. N. Meyers, J. Phys. Chem., 72, 4397(1968).

(12) L. R. Snyder and J. J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, N.Y. (1974).

(13) J. J. Hermans, J. Polym. Sci., Part A-2, 6, 1217(1968).

(14) T. H. Gouw, ed., Guide to Modern Methods of Instrumental Analysis, Wiley, N.Y., p. 142, (1972).

(15) W. W. Yau, H. L. Suchan, and C. P. Malone, J. Polym. Sci.: Part A-2, 6, 1349(1968).

(16) K. A. Boni, F. A. Sliemers, and P. B. Stickney, J. Polym. Sci.: Part A-2, 6, 1567(1968).

(17) J. H. Duerksen and A. E. Hamielec, J. Appl. Polym. Sci., 12, 2225(1968).

(18) B. J. Gudzinowicz and K. Alden, J. Chromatographic Sci., 9, 65(1971).

(19) A. L. Spatorico, J. Appl. Polym. Sci., 19, 1601(1975).

(20) S. Mori, J. Appl. Polym. Sci., 21, 1921(1977).

(21) J. H. Aubert and M. Tirrell, Sep. Sci. Tech., 15, 123(1980).

(22) W. W. Yau, C. P. Malone, and S. W. Fleming, Polym. Letters, 6, 803(1968).

(23) Z. Grubisic-Gallot and H. Benoit, J. Chromat. Sci., 9, 262(1971).

(24) J. H. Aubert, PhD. thesis, University of Minnesota, (1982).

(25) J. H. Aubert and M. Tirrell, J. Chem. Phys., 77, 553(1982).

(26) W. B. Smith and A. Kollmansberger, J. Phys. Chem., 69, 4157(1965).

(27) W. W. Yau and C. P. Malone, Polym. Letters, 5, 663(1967).

(28) M. E. Van Kreveld and N. Van Den Hoed, J. Chromatography, 149, 71(1978).

(29) E. L. Slagowski, L. J. Fetters, and D. McIntyre, Macromolecules, 7, 394(1974).

(30) J. J. Kirkland, J. Chromatogr., 125, 231(1976).

(31) C. Huber and K. H. Lederer, J. Polym. Sci.: Polym. Letters Ed., 18, 535(1980).

(32) W. G. Rand and A. K. Mukherji, J. Polym. Sci.: Polym. Letters Ed., 20, 501(1982).

(33) J. V. Champion and T. D. Davis, J. Chem. Phys., 52, 381(1970).

(34) J. H. Aubert, S. Prager and M. Tirrell, J. Chem. Phys., 73, 4103(1980).

(35) J. H. Aubert, M.S. thesis, University of Minnesota, (1979).

(36) J. -Y. Chuang and J. F. Johnson, Sep. Sci., 10, 161(1975).

(37) J. H. Brannon and J. L. Anderson, J. Polym. Sci.: Polym. Phys. Ed., 20, 857(1982).

(38) C. N. Satterfield, C. K. Colton, B. D. Turckheim, and T. M. Copeland, AIChE J., 24, 937(1978).

(39) J. H. Aubert and M. Tirrell, "Macromolecules in Narrow Channels," presented at IUPAC meeting, Strasbourg, France, July (1981).

(40) J. L. Anderson and J. H. Brannon, J. Polym. Sci.: Polym. Phys. Ed., 19, 405(1981).

(41) E. Glandt, J. Colloid Interface Sci., 77, 512(1980).

(42) J. N. Little, J. L. Waters, K. J. Rombaugh, and W. J. Pauplis, J. Polym. Sci.: Part A-2, 7, 1775(1969).

(43) J. H. Aubert and M. Tirrell, Rheol. Acta, 19 452(1980).

(44) G. Chauveteau and N. Kohler, Paper SPE 4745 presented at the Improved Oil Recovery Symposium of SPE-AIME, Tulsa, OK (1974).

(45) G. P.Willhite and J. G. Dominguez, in: Improved Oil Recovery by Surfactant and Polymer Flooding, D. O. Shah, R. S. Schehter, eds., Academic Press, N.Y. (1977).

(46) J. H. Aubert and M. Tirrell, submitted to Rheol. Acta, (1983).

(47) G. Sekhon, R. C. Armstrong and M. S. Jhon, J. Polym. Sci., Polym. Phys. Ed., 20, 947(1982).

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR ANALYSES OF ANTIBIOTICS IN BIOLOGICAL FLUIDS.

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INTRODUCTION

The discovery of penicillin and subsequently other antibiotics represents one of the great progresses in modern medicine. Newer agents, such as the aminoglycosides and the cephalosporins, have also contributed significantly to man's struggle against bacterial and fungal infections, and antiviral agents are expected to emerge in the next decades. The evergrowing number of antibiotics have, however, also given rise to several problems. The emergence of bacterial strains resistant to antibiotics is a matter of increasing concern to the medical profession and necessitates the continuing search for new antimicrobial agents and judicious therapy with the ones already in use. Many antibiotics exert undesired toxic side effects when given in excessive doses: examples are the oto- and nephrotoxicity of aminoglycosides, the bone-marrow depressant action of

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chloramphenicol and the nephrotoxic effects of some of the early cephalosporins. Even a comparatively nontoxic drug such as penicillin causes coma and convulsions at high levels in the cerebrospinal fluid. To avoid such undesired effects it is of vital importance to the clinician to have a clear understanding of the pharmacological behaviour of an antibiotic. Usually, abundant data are available on the pharmacokinetics of these drugs in healthy, young volunteers but these do not represent the clinically difficult cases: the sick, often older, patient many times suffering from reduced renal or hepatic function and the very small child with immature function of liver and kidneys. When treating these patients, the clinician must be able to monitor serum levels of antibiotics to ensure safe and effective therapy. Therefore, when a new drug is introduced, it is necessary to perform extensive investigations of pharmacokinetic behaviour not only in healthy volunteers but also in patients with and without disturbances of metabolic pathways and elimination processes in order to make adequate dosage recommendations.

Assays for concentrations of antibiotics in body fluids have, by tradition, been performed in the microbiological laboratory by testing the samples against microbes. The capacity of the biological sample to inhibit growth of a susceptible bacterium is measured and

the results are compared to those obtained with standard samples of known concentrations (1,2). These assays require a rather long time; typically assay plates or tubes must be incubated for about 18 hours before results can be obtained. The demand on the technician for highly accurate laboratory work is great and standardization between different laboratories is difficult. These problems have been emphasized for some of the aminoglycosides (3) and are of continuous concern to the microbiologist and the clinician. A further disadvantage from the pharmacokinetic point of view is that the technique has no inherent potential for detection and quantitation of metabolic degradation products.

These considerations have prompted the development of other methods for assay of antibiotics. Several chemical procedures have been reported: spectrophotometric and fluorimetric measurements which, however, for specificity have to rely on complicated extraction procedures (4-10); gas-liquid chromatographic techniques which are also cumbersome because of the need for derivatization of the drugs to volatile compounds (11-15). A more attractive approach is the introduction of radioimmunoassays and radioenzymatic methods (16-24); these meet the requirements for specificity and sensitivity, but method development is quite demanding, relying on the production of specific antibodies of con-

TABLE 1. Published Assays for Analysis of Antibiotics in Biological Fluids

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
β -lactam antibiotics				
ampicillin	perchloric acid	reverse-phase	UV absorption	26
"	trichloroacetic acid	"	postcol. deriv. UV absorption	27
mecillinam	trichloroacetic acid	"	"	27
"	dilution	"	"	28
amoxycillin	perchloric acid	"	"	26
"	a.dichloroethane+perchloric acid b.n-amylalcohol+dichloroethane	"	postcol. deriv. UV absorption	29
isoxazolyl penicillins*	a.methanol+perchloric acid b.methylene chloride	"	UV absorption	30
"	acetonitrile+methylene chloride	"	"	31
clavulanic acid	none	"	"	32

moxalactam	SEP-Pak	PIC	UV absorption	33
"	a.methanol b.dilution	reverse-phase PIC	"	34
"	ammonium sulphate	reverse-phase	"	35
cephalothin*	ion-pair extraction	ion exchange	"	36
" *	dilution	"	"	37
"	trichloroacetic acid	reverse-phase	"	38
" *	dimethyl formamide	"	"	39
"	chloroform+pentanol	"	"	40
cephazolin	trichloroacetic acid	"	"	38
"	"	"	"	41
"	chloroform+pentanol	"	"	40
cephalexin	a.dilution b.none	"	"	42

*assay includes determination of metabolite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
cephalexin	a.methanol b.none	reverse-phase	UV absorption	43
"	methanol	"	"	44
"	a.phosphoric acid+methanol b.trichloroacetic acid	"	"	45
cephaloridin	trichloroacetic acid	"	"	46
cephradine	a.dilution b.none	"	"	42
cephroxadin	trichloroacetic acid	"	"	45
cefatrizine	"	"	"	47
"	"	"	fluorimetry postcol. deriv.	48
cefuroxime	dimethyl formamide	"	UV absorption	49
"	perchloric acid	"	"	50
"	chloroform+pentanol	"	"	40

cefuroxime	phosphoric acid+methanol	reverse-phase	UV absorption	45
"	a.methanol b.dilution	"	"	51
cefamandole	chloroform+pentanol	"	"	40
"	a.phosphoric acid+methanol b.trichloroacetic acid	"	"	45
cefotiam	dilution	ion exchange	"	37
cefoxitin*	trichloroacetic acid +methanol	reverse-phase	"	52
"	chloroform+pentanol	"	"	40
"	acetonitrile	"	"	53
"	phosphoric acid+methanol	"	"	45
cefsulodin	acetonitrile	"	"	54
cefotaxime*	a.acetonitrile b. dilution	PIC	"	55
" *				

*assay includes determination of metabolite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
cefotaxime*	trichloroacetic acid	reverse-phase	UV absorption	56
" *	chloroform+acetone	"	"	57
" *	perchloric acid	"	"	58
"	chloroform+pentanol	"	"	40
" *	acetonitrile	"	"	59
" *	phosphoric acid+methanol	"	"	45
cephaloglycin*	none	"	"	60
ceftizoxime	a.acetonitrile+buffer b. " +acetic acid	PIC reverse-phase	"	61
cefaclor	methanol	"	"	62
"	not stated	"	"	63
ceftriaxone	methanol	PIC	"	64
cefmenoxime	ultrafiltration	reverse-phase	"	65

Aminoglycosides				
gentamicin	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	66
"	adsorption chrom.	reverse-phase	fluorimetry precol. deriv.	67
"	acetonitrile+methylene chloride	"	"	68
"	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	69
"	acetonitrile+methylene chloride+ethyl acetate	reverse-phase	fluorimetry precol. deriv.	70
"	acetonitrile+methylene chloride	"	UV absorption precol. deriv.	71
"	acetonitrile	"	"	72
"	acetonitrile-methylene chloride	ion exchange	fluorimetry postcol. deriv.	73

*assay includes determination of metabolite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
tobramycin	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	69
"	adsorption chrom.	reverse-phase	fluorimetry precol. deriv.	74
"	acetonitrile+methylene chloride+ethyl acetate	"	"	70
"	acetonitrile+methylene chloride+2-propanol	"	"	75
netilmicin	acetonitrile+methylene achloride+ethyl acetate	"	"	76
"	"	"	"	70
amikacin	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	69
"	adsorption chrom.	reverse-phase	fluorimetry precol. deriv.	77
"	a.methanol+chloroform b.methanol+diethyl ether	normal phase reverse-phase	"	78

sisomicin		acetonitrile	reverse-phase	UV absorption precol. deriv.	72
Chloramphenicol		ethyl acetate	"	UV absorption	79
"	**	methanol	"	"	80
"		acetonitrile	"	"	81
"		ether	"	"	82
"		ethyl acetate	"	"	83
"		acetonitrile	"	"	84
"		chloroform+isopropanol	"	"	85
"	**	ethyl acetate	"	"	86
"	**	trichloroacetic acid	"	"	87
"	**	diethyl ether	"	"	88
"		"	"	"	89

**assay includes determination of pro-drug

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
chloramphenicol**	acetonitrile	reverse-phase	UV absorption	90
" **	ethyl acetate	"	"	91
thiamphenicol	methanol	"	"	92
Sulphonamides				
trisulphapyrimidines sulphadiazine sulphamerazine sulphamethazine	trichloroacetic acid	"	"	93
salicylazosulphapyridine*	a.isoamylacetate b.4-methyl-2-pentane	"	"	94
sulphisoxazole*	methanol	"	"	95
sulphametrole*	perchloric acid	"	"	96
sulphamethoxazole*	"	"	"	97
" *	acetonitrile+ethyl acetate	"	"	98

sulphamethoxazole*	acetonitrile+ultracentrif.	reverse-phase	UV absorption	99
sulphadiazine*	ethyl acetate	normal phase	"	100
Trimethoprim	perchloric acid	reverse-phase	"	97
"	chloroform	normal phase	"	101
"	not stated	PIC	"	102
"	ethyl acetate	normal phase	"	100
"	acetonitrile+ultracentrif.	reverse-phase	"	99
Tetracyclines				
tetracycline	trichloroacetic acid +methanol	reverse-phase	"	103
"	ethyl acetate+phosphoric acid	"	"	104
"	"	"	"	105
" *	a.acetonitrile+phosphoric acid b.acetonitrile+ethyl acetate	"	"	106

*assay includes determination of metabolite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
oxytetracycline	ethyl acetate+phosphoric acid	reverse-phase	UV absorption	104
chlortetracycline	"	"	"	104
doxycycline	diethyl ether+ethyl acetate	"	"	106
"	a.phosphoric acid+acetonitrile b.acetonitrile+ethyl acetate	"	"	107
"	ethyl acetate	"	"	108
Nitroimidazoles				
metronidazole*	ammonium sulphate+methyl ethyl ketone	"	"	109
"	ethanol	"	"	110
" *	methanol+acetonitrile+ potassiumdihydrogen phosphate	"	"	111
"	ether+methylene chloride	"	"	112
" *	acetonitrile	"	"	113

metronidazole*	perchloric acid	reverse-phase	UV absorption	114
tinidazole	chloroform	normal phase	"	115
"	ether+methylene chloride	reverse-phase	"	112
"	perchloric acid	"	"	114
ketaconazole*	ethyl acetate	"	"	116
"	SEP-Pak	"	"	117
"	diethyl ether	"	fluorimetry	118
econazole	"	"	UV absorption	119
thiabendazole*	acetonitrile	"	fluorimetry	120
Rifampicin*	chloroform	"	UV absorption	121
" *	isooctane+dichloromethane	normal phase	"	122
" *	ethyl acetate+n-heptane	reverse-phase	"	123
Nitrofurantoin	methanol	"	"	124

*assay includes determination of metabilite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
Nalidixic acid*	a.chloroform+sodium hydroxide b.sodium hydroxide+ion exchange	reverse-phase	UV absorption	125
Erythromycin**	diethyl ether	"	fluorimetry	126
Amphotericin B	methanol	"	UV absorption	127
5-fluorocytosine	none	ion exchange	"	128
"	ultrafiltration	"	"	129
"	trichloroacetic acid	reverse-phase	"	130
"	acetonitrile	PIC	"	131
Griseofulvin	dichloromethane	reverse-phase	"	132
"	acetonitrile	"	fluorimetry	133
"	diethyl ether	"	"	134
Acyclovir	a.aluminium sulphate+barium hydroxide b.dilution	PIC	UV absorption	135

Adenine arabinoside*	ultrafiltration	ion exchange	"	136
Antimalarial agents				
chloroquine*	a.ethylene dichloride b.trichloroacetic acid+ methanol	PIC	UV absorption or fluorimetry	137
"	hexane	"	UV absorption	138
" *	diethyl ether	normal phase	fluorimetry	139
" *	heptane	PIC	UV absorption	140
mefloquine	ethyl acetate	a.normal phase b.reverse-phase	"	141
dapsone	dichloroethane	normal phase	"	142
primaquine*	none	reverse-phase	"	143
pyrimethamine	dichloroethane	normal phase	UV absorption	142
"	butyl chloride+dichloro-methane	"	fluorimetry	144

*assay includes determination of metabolite(s)

(continued)

stant quality. Also, no potential for metabolite detection lies inherent in these techniques.

In the last decade high-performance liquid chromatography (HPLC) has undergone rapid technical progress and proved itself as a powerful analytical tool (25). The emergence of very small uniform particle packing materials with different physico-chemical properties, sophisticated high pressure pumps and very sensitive flow detectors has made this technique extremely versatile. The short time required for analysis and the obvious potential for separation and detection of metabolic products have held great appeal to investigators of the pharmacological aspects of antibiotics and, indeed, the literature is now, as shown in table I, replete with reports on techniques for determination of various antimicrobial drugs in biological fluids.

PRINCIPLES OF ANTIBIOTIC ASSAY BY HPLC

Basically, all reported methods for assay of antibiotics with use of HPLC employ the same methodological approach. The biological sample is treated with chemical procedures which usually aim at depleting the sample of proteins and/or extract the compound(s) of interest quantitatively. Subtances in the resulting solution are then separated by HPLC and eluting compounds are detected by spectrophotometry or fluorimetry. Recor-

ded peaks are then quantitated against known standards which have been chromatographed with the same separation system.

Sample Treatment

Samples of biological materials usually undergo pretreatment prior to chromatography. The objectives are mainly to rid the samples of protein (pertinent for samples of high protein concentration, such as plasma and serum) and to achieve a gross separation of substances not of interest in the assay while quantitatively solubilizing the compound under assay. Most assay procedures reported employ some pretreatment step (table I); the direct injection of biological samples, such as serum, with considerable protein content is not satisfactory since the analytical column will be obstructed and its performance will rapidly deteriorate. If such an approach is chosen, it is wise to use a guard column before the analytical column and to check column performance frequently (128). Samples of very low protein content can be injected directly or after dilution; however, with urine samples the many endogenous compounds present in high concentrations may produce such a multitude of chromatographic peaks that resolution of the compound(s) under analysis becomes a problem.

Protein removal

This approach is one of the two most commonly used procedures for treatment of samples prior to chromatography. Its objectives are two: first, to deproteinize the sample and second, to solubilize the antibiotic in order to ensure quantitative recovery from the sample. In general, there are four principle methods to precipitate proteins: acid and ammonium sulphate treatment, organic solvent and metal cation precipitation. As shown recently by Blanchard (145) these methods are all quite effective in precipitating proteins; the most popular in liquid chromatographic assays are acid precipitation and organic solvent treatment. Trichloroacetic and perchloric acid are very effective precipitants and are also, like solvents such as acetonitrile and methanol, quite compatible with subsequent chromatography in the reversed phase partition mode.

Another very simple and therefore attractive means of obtaining protein-free samples for chromatography is ultrafiltration (129,136) through filters with molecular cut-offs of 25,000 or 50,000 MW. However, one must remember that these filters let through only non-protein bound drug, giving a measure of the free fraction in serum. Also, there is a definite need for determining whether drug (or internal standard) is absorbed to the membrane material. If the losses in the filter are

small and constant, it is possible to correct for this in the final calculations. One way of getting around these problems is to use standards prepared in the same medium as the sample (for example, pooled serum); one must, in this case, be sure that protein binding is virtually identical in sample and serum pool. Ideally, such a procedure would use patient's sera obtained before therapy for the preparation of standards.

Extraction procedures

The second popular approach to cleaning the biological sample and recovering the drug for analysis with HPLC is extraction into organic solvents of low polarity. The most widely used are ethyl acetate, ethyl ether, methylene chloride and chloroform. These procedures work extremely well with lipofilic substances, for example chloramphenicol and nitroimidazoles (see table I). Generally, before chromatography, the organic extract is evaporated to dryness and the remaining residue redissolved in mobile phase; this adds another step in the procedure and, of course, some complexity and time. Sometimes, several extraction steps are necessary and the pretreatment procedure becomes quite timeconsuming and cumbersome (30,51,126,141).

In the case of aminoglycosides several reported methods use non-polar solvents to extract derivatives

of these compounds from an aqueous phase after attachement of chromophores to the molecules usually following initial protein precipitation and extraction of other non-polar substances (68,70,71,73,76,78). These antibiotics are extremely polar substances but the derivatives are of much lower polarity and readily dissolve in, for example, ethyl acetate. Therefore, when precolumn derivatization is used, this kind of pretreatment gives quantitative recovery and quite clean samples for chromatography. Another approach is the use of disposable small columns for initial separation by either absorption or ion exchange (66,67,69,74,77). The aminoglycosides are then eluted off the silica gel with methanol after derivatization or underivatized with an alkaline eluent from CM-Sephadex gel. The same method for initial separation of the biological sample is represented by commercially available disposable columns (Sep-Pak cartridges, Water's Ass.) which have been used for assay of moxalactam and a nitroimidazole (33,116).

One of the earliest methods for liquid chromatographic analysis of an antibiotic in body fluids was reported by Cooper et al (36). Cephalothin and desacetylcephalothin were extracted from serum into ethyl acetate as ion pairs with tetraheptyl ammonium ions. Although theoretically this is a quite feasible and rather elegant technique, no further reports on its use in this field have appeared.

Separation Systems

Since many antibiotics exist in solution as ions, a logical choice of separation mode would be ion exchange. Methods employing this approach have been reported (37,63,128,136) but most chromatographers are hesitant to use ion exchange due to the many problems with these systems: strict control of pH, ionic strength and temperature is necessary, column life is rather short since the matrix tends to bleed off from the solid support. From a practical point of view, bonded phase liquid partition is the most appealing chromatographic mode because of the stability and durability of the columns and the more predictable effects of changes in the mobile phase composition. The overwhelming majority of antibiotic assays described in the literature use reverse-phase partition on octadecylsilane chemically bound to a small particle silica matrix (table I); eluting solvents consist of mixtures of buffer and methanol or acetonitrile. In some cases, especially with compounds that are highly ionized or extremely polar, separations by this mode are not satisfactory. Badly tailing peaks may indicate ionization problems; difficulty to get any retention on a reverse-phase column may be encountered, as, for example, with the aminoglycosides. In these cases, paired ion chromatography (PIC) on reverse-phase columns is a very useful technique with

all the practical ease of bonded phase chromatography and none of the difficulties of ion exchange. For the analyses of aminoglycosides, another approach has also been feasible: derivatization of the primary amino groups with chromophores yields less polar compounds which then very readily are separated by reverse-phase partition (67,68,70-78). When post-column derivatization is preferred, however, the paired ion technique is the method of choice (66,69).

Normal phase separations have been successfully employed for determination of antimalarial agents (139, 141,144), trimethoprim (100,101), rifampicin (122) and tinidazole (115). Several of these compounds separate well in reverse-phase systems as well; tinidazole seems, indeed, to be better suited for reverse-phase separation, since published chromatograms in the normal phase mode show broad, tailing peaks.

Detection

Most antibiotics absorb ultraviolet or visible light with extinction coefficients large enough to allow spectrophotometric detection at therapeutic concentrations. The wavelength of detection is determined from the wavelenght of maximum absorption of the compound and, if need be, after consideration of potential interference by other compounds in the sample that may

co-elute from the chromatographic column. In the latter case, a second maximal absorption band for the antibiotic may be chosen where no interference is noted (103). Commercially available flow spectrophotometers for HPLC operate at either fixed or continuously variable wavelengths; naturally, greater flexibility is obtained with variable detectors, but for most of the methods reported, the fixed wavelength detectors are adequate. In some instances, spectrophotometry is not feasible for detection and quantitation. Sometimes, the sensitivity obtained does not suffice (48,133,134); fluorimetry can then be employed to increase the sensitivity of the assay. Another aspect is that fluorescence is a more specific phenomenon than light absorption requiring a certain wavelength for excitation and emitting at another. Therefore, it may be assumed, that less rigorous pretreatment procedures are necessary and specificity is still maintained (118).

Fluorimetry has been of special importance for the analysis of aminoglycosides. These clinically very important compounds show extremely little absorption in ultraviolet or visible light; spectophotometry cannot be used for detecting therapeutic concentrations in body fluids. It was realized by several investigators, that derivatization of these molecules was a necessary step in a chromatographic assay. The attachment of

fluorophores to the primary amino groups of the aminoglycosides was a logical and theoretically simple approach. Dansyl chloride (68,76), fluorescamine (65,66, 69) and orto-phtalaldehyde (67,70,74,75,77) can be used successfully and the reported assays with fluorimetric detection are sensitive enough for therapeutic monitoring and pharmacokinetic studies. More recently, three groups of investigators have reported precolumn derivatization with benzene sulphonyl chloride (71) and fluorodinitrobenzene (72,78) with subsequent spectrophotometric detection.

Derivatization with chromophores can be performed either as a precolumn (preceding chromatographic separation) or as a postcolumn procedure. While it cannot be stated that one method is superior to the other (146), there are some practical aspects to be considered. Postcolumn derivatization requires a more complicated chromatographic system involving a separate pump for the derivatizing reagent and a reaction coil. Precolumn derivatization is technically simpler and, when part of the sample pretreatment procedure, probably results in cleaner extracts of biological fluids. As mentioned above, this approach also facilitates the separation allowing the use of simple reverse-phase partition systems.

Quantitation

The concentration of antibiotic in a sample is calculated by comparing the size of the recorded peak for the specific compound with the peak size obtained from a standard sample of known concentration. There are two ways of measuring peak size: one is to determine the height of the peak, the other is to calculate the area of the peak (25).

Peak height measurement is usually done manually and is the simpler of the two methods. To obtain good accuracy with this approach, column performance must be stable and reproducible and peaks should be symmetrical. It is necessary to run standard samples quite often to check the column during a series of analyses.

Peak area is a more complicated measurement but has a definite advantage over height determinations: it is less influenced by changes in column performance or instrumental parameters. It is, furthermore, not so dependent on peak symmetry. Several methods are available for measurement of peak area (25); highest precision is obtained with use of an electronic digital integrator or a computer.

Another, perhaps more controversial, question is whether to use an internal standard for quantitation (25). In general, for assays with more complex pretreatment procedures or variable recovery of extraction

the addition of a suitable internal standard to the original sample will increase precision of the assay. However, for this statement to be valid, the internal standard must behave equivalently to the compound under analysis throughout the procedure. Furthermore, it must elute close to the substance under analysis but in a vacant spot in the chromatogram. These requirements often make it difficult to find a suitable internal standard; indeed, very often it is not clear in reported methods whether the demands are fulfilled. There exists a definite risk of increasing, instead of decreasing, the degree of imprecision with use of an improper internal standard. For assays employing simple pretreatment procedures, the method of external standard calibration is probably at least as precise, especially if sample injection is done with a sampling microvalve or an automatic injector, since the main source of assay imprecision is variation in the injected volume.

PHARMACOKINETIC IMPLICATIONS

As is evident from table I, many of the reported assays include the detection and quantitation of metabolites or precursors of several antibiotics. This is a unique feature of the HPLC technique as compared to microbiological and radioimmunological methods and opens up new possibilities for the study of pharmacoki-

netics of antibiotics. In some instances, unknown metabolites have been discovered (55,60,140), and it is feasible to isolate and by, for example, mass spectrometry identify such metabolites.

Several pharmacokinetic studies have been done on the cephalosporines; investigations with HPLC as assay method started already in 1973 when Cooper et al (36) showed that approximately one fifth of a cephalothin dose was excreted in urine of healthy volunteers as the metabolite desacetylcephalothin. A later study of the same drug in severely uremic patients gave evidence for an additional route of excretion or metabolism in these patients since only about 50% of a given dose was found in urine, most of it accounted for by the metabolite (147). Other cephalosporins have been studied in patients and volunteers: the kinetics of cefotaxime and its metabolites have been elucidated by several authors (55,56,59); Haginaka showed that extensive and complex metabolization occurs with cephaloglycin, only 0.5% of the dose being excreted in urine as the intact compound (60); a three-compartment pharmacokinetic model has been indicated for cefamandole (51); the perorally administered cephalexin was studied by Nakagawa et al and found to fit a two-compartment model with almost complete urinary recovery and no indication of metabolic degradation (43). Cefaclor, another peroral

cephalosporin, shows first-order absorption and evidence of a substantial non-renal clearance route (63). The excretion routes of some cephalosporins have been extensively investigated by Arvidsson who found that renal elimination of cephapirin and cephaloridin was dependent on drug concentration, that biliary excretion of ceftrixone was dependant on individual variability in the secretion of endogenous biliary lipids and correlated to effects of the drug on the intestinal microflora (148). Stoeckel et al (149) demonstrated concentration-dependant plasma protein binding of ceftriaxone and the kinetics of this drug in infants and young children have been investigated by Schaad et al (150). The plasma protein binding and kinetic behaviour of the two epimers of moxalaxtam in volunteers have been calculated (151) and a study of the clearance of this drug from serum of patients with varying degrees of renal insufficiency has led to a suggestion for dosage regimens with regard to renal function (35).

To date, published investigation of penicillins and their metabolites have been rather scarce; however, Thijssen et al have developed a method for analysis of isoxazolyl penicillins and their 5-hydroxymethyl metabolites and penicilloic acids and estimated the pharmacokinetic parameters of these compounds after oral administration of cloxacillin and flucloxacillin to a healthy volunteer (30).

The majority of HPLC assays for chloramphenicol includes the determination of its succinate ester, the substance used for intravenous administration of the drug. Great interindividual variability has been found in the renal clearance of the succinate ester both in children and adults, with no correlation to renal function (152-154). This variable kinetic behaviour of the pro-drug affects the levels of active chloramphenicol in serum, a factor which needs to be considered in clinical practice.

The pharmacokinetics of metronidazole, a drug which has come into frequent use owing to its excellent activity against anaerobic bacteria, has attracted increasing attention over the past years. The main metabolites of metronidazole can be easily detected and quantitated by HPLC (109,114). The parent drug undergoes extensive break-kown and only about 8% of administered metronidazole is excreted in urine as unchanged drug (114,155). Low concentrations of the hydroxy and acetic acid metabolites were found in healthy volunteers (114,155,156) whereas in patients with renal failure these metabolites reached considerably higher serum levels (157).

The kinetics of combinations of trimethoprim and different sulphonamides have been studied by the HPLC technique (97,100,102) as well as the conversion of

sulfametrole to the N_4-acetyl metabolite in man and the subsequent renal excretion of these two compounds (96).

The in vivo degradation of nalidixic acid and the behaviour of its two major metabolites have been studied by HPLC (125) and the hydrolysis of erythromycin ethyl succinate after peroral administration has been estimated by Tsuji who also detected metabolites of ethyl succinate and of erythromycin itself in serum by this technique (126).

Drugs used for therapy or prophylaxis of malaria exhibit complex pharmacokinetic patterns. It appears that HPLC will be a valuable tool for elucidation of the metabolism and kinetics of several of these compounds; to date, studies have appeared on dapsone with several metabolites (142), pyrimethamine (142) and primaquine (143). The latter study was performed with rats; the authors showed the existence of a new mammalian metabolite of primaquine and verified its presence and structuré by HPLC and mass spectrometry. The complex pharmacokinetics and metabolism of chloroquine are under study in several laboratories (137,139,140).

THE ROLE OF HPLC IN THE LABORATORY

Despite the abundant evidence that the HPLC technique is well suited for analysis of antimicrobial drugs in biological fluids and compares favorably with other

methods in current use, there exists one serious shortcoming of this technology: its capacity is limited. Separations usually take about 5-10 minutes and, with one chromatographic set-up, this means an assay capacity of 6-12 samples per hour. Furthermore, switching from one assay to another involves additional time spent in changing solvents and equilibrating the analytical column. Clearly, traditional microbiological assays and radioimmunological methods are superior with regard to the amount of samples that can be analyzed simultaneously, and, in large microbiological laboratories with large series of samples, routine clinical determinations of antibiotic concentrations are performed with these techniques. Some laboratories use both HPLC and microbiological or radioimmunological techniques routinely: the majority of routine analyses in microbiological laboratories consists of aminoglycoside determinations and with careful technique these can be carried out with bioassays in a satisfactory manner (3); analyses of other agents are done by HPLC (158).

Moreover, HPLC is an alternative method in special situations: interference in the biological technique caused by antimicrobial combination therapy is usually, because of the separative step involved, easily resolved with HPLC; assays of som agents, for example amphotericin B and 5-fluorocytosine, are more sensitively and

accurately done with HPLC; analyses of antibiotics which undergo metabolic breakdown are better performed with a technique that measures metabolites separately, especially in the problem cases represented by patients with hepatic and/or renal insufficiency.

For smaller hospitals without a microbiological laboratory HPLC provides an attractive alternative for routine assays by clinical chemists. Many hospitals use this method for analysis of other drugs and endogenous substances and are well suited to perform antibiotic assays also. It may well be clinically and economically advantageous to use HPLC for routine antibiotic assays in such instances instead of sending samples to a microbiological laboratory for analysis.

For laboratories engaged in pharmacokinetic research with antibiotics, the technique, as described above, offers definite advantages and is rapidly becoming indispensable.

REFERENCES

1. Grove, D.C. and Randall, W.A., Assay Methods of Antibiotics: A Laboratory Manual, Medical Encyclopedia Inc., New York, 1958.

2. Kavanagh, F., Analytical Microbiology, Academic Press, New York, 1967.

3. Reeves, D.S. and Bywater, M.J., J. Antimicrob. Chemother., 1, 103, (1975).

4. Glazko, A.J., Wolf, L.M. and Dill, W.A., Arch. Biochem., 23, 411, (1949).

5. Kohn, K.W., Anal. Chem., 33, 862, (1961).

6. Kakemi, K., Arita, T. and Ohashi, S., Yakugaku Zasshi, 82, 342, (1962).

7. Kelly, R.G. and Hoyt, K.D., Z. Klin. Chem. u. Klin. Biochem., 7, 152, (1969).

8. Lever, M., Biochem. Med., 6, 216, (1972).

9. Richardson, R.A., Clin. Chim. Acta, 63, 109, (1975).

10. Tserng, K-Y. and Wagner, J.G., Anal. Chem., 48, 348, (1976).

11. Resnick, G.L., Corbin, D. and Sandberg, H., Anal. Chem., 38, 582, (1966).

12. Midha, K.K., McGilveray, I.J. and Cooper, J.K., J. Chromatogr., 87, 491, (1973).

13. Mullen, P.W., Mawer, G.E. and Tooth, J.A., Res. Comm. Chem. Pathol. Pharmacol., 7, 85, (1974).

14. Wood, N.F., J. Pharm. Sci., 64, 1048, (1975).

15. Least, Jr., C.J., Wiegands, N.J., Johnson, G.F. and Solomon, H.M., Clin. Chem., 23, 220, (1977).

16. Haas, M.J. and Davies, J., Antimicrob. Agents Chemother., 4, 497, (1973).

17. Mahon, W.A., Ezer, J. and Wilson, T.W., Antimicrob. Agents Chemother., 3, 585, (1973).

18. Schwenk, T., Kelly, K., Tse, K.S. and Sehon, A.H., Clin. Chem., 21, 1059, (1975).

19. Stevens, P., Young, L.S. and Hewitt, W.L., Antimicrob. Agents Chemother., 7, 374, (1975).

20. Broughton, A. and Strong, J.E., Clin. Chim. Acta, 66, 125, (1976).

21. Broughton, A., Strong, J.E. and Bodey, G.P., Antimicrob. Agents Chemother., 9, 247, (1976).

22. Gilbertson, T.J. and Stryd, R.P., Clin. Chem., 22, 828, (1976).

23. Lietman, P.S., White, T.J. and Shaw, W.V., Antimicrob. Agents Chemother., 10, 347, (1976).

24. Stevens, P., Young, L.S. and Hewitt, W.L., Antimicrob. Agents Chemother., 11, 768, (1977).

25. Snyder, L.R. and Kirkland, J.J., Introduction to Modern Liquid Chromatography, John Wiley and Sons, Inc., New York, 1979.

26. Vree, T.B., Hekster, Y.A., Baars, A.M. and van der Kleijn, E., J. Chromatogr., 145, 496, (1978).

27. Westerlund, D., Carlqvist, J. and Theodorsen, A., Acta Pharm. Suecica 16, 187, (1979).

28. Strojny, N. and de Silva, J.A.F., J. Chromatogr., 181, 272, (1980).

29. Carlqvist, J. and Westerlund, D., J. Chromatogr., 164, 373, (1979).

30. Thijssen, H.H.W., J. Chromatogr., 183, 339, (1980).

31. Rudrik, J.T. and Bawdon, R.E., J. Liquid Chromatogr., 4, 1525, (1981).

32. Haginaka, J., Nakagawa, T., Nighino, Y. and Uno, T., J. Antibiotics, 34, 1189, (1981).

33. Konaka, R., Kuruma, K., Nishimura, R., Kimura, Y. and Yoshida, T., J. Chromatogr., 225, 169, (1981).

34. Miner, D.J., Coleman, D.L., Shepherd, A.M.M. and Hardin, R., Antimicrob. Agents Chemother., 20, 252, (1981).

35. Wright, N., Wills, P.J. and Wise, R., J. Antimicrob. Chemother., 8, 395, (1981).

36. Cooper, M.J., Anders, M.W. and Mirkin, B.L., Drug. Metab. Dispos., 1, 659, (1973).

37. Buhs, R.P., Maxim, T.E., Allen, N., Jacob, T.A. and Wolf, F.J., J. Chromatogr., 99, 609, (1974).

38. Wold, J.S. and Turnipseed, S.A., Clin. Chim. Acta, 78, 203, (1977).

39. Nilsson-Ehle, I., Yoshikawa, T.T., Schotz, M.C. and Guze, L.B., Antimicrob. Agents Chemother., 13, 221, (1978).

40. Brisson, A.M. and Fourtillan, J.B., J. Chromatogr., 334, 393, (1981).

41. Wold, J.S., Antimicrob. Agents Chemother., 22, 105, (1977).

42. Carroll, M.A., White, E.R., Jancsik, Z. and Zarembo, J.E., J. Antibiotics, 30, 397, (1977).

43. Nakagawa, T., Haginaka, J., Yamaoka, K. and Uno, T., J. Antibiotics, 31, 769, (1978).

44. Nahata, M.C., J. Chromatogr., 228, 429, (1982).

45. LeCaillon, J.B., Rouan, M.C., Souppart, C., Febvre, N. and Juge, F., J. Chromatogr., 228, 657, (1982).

46. Wold, J.S. and Turnipseed, S.A., J. Chromatogr., 136, 170, (1977).

47. Crombez, E., van der Weken, G., van den Bossche, W. and de Moerloose, P., J. Chromatogr., 173, 165, (1979).

48. Crombez, E., van der Weken, G., van den Bossche, W. and de Moerloose, P., J. Chromatogr., 177, 323, (1979).

49. Nilsson-Ehle, I. and Nilsson-Ehle, P., Clin. Chem., 24, 365, (1978).

50. Hekster, Y.A., Baars, A.M., Vree, T.B., van Klingeren, B. and Rutgers, A., J. Antimicrob. Chemother., 6, 65, (1980).

51. Aziz, N.S., Gameroglio, J.G., Lin, E.T., Gausz, H. and Benet, L.Z., J. Pharm. Biopharm., 6, 153, (1978).

52. Wheeler, L.A., de Meo, M., Kirby, B.D., Jerauld, R.S. and Finegold, S.M., J. Chromatogr., 183, 357, (1980).

53. White, L.O. and Reeves, D.S., Biological/Biomedical Applications of Liquid Chromatography IV, Hawk, G.L., ed., Marcel Dekker, Inc., New York, 1982, p. 185.

54. Borner, K. and Lode, H., J. Clin. Chem. Clin. Biochem., 18, 719, (1980).

55. Reeves, D.S., White, L.O., Holt, H.A., Bahari, D., Bywater, M.J. and Bax, R.P., J. Antimicrob. Chemother., 6, (Suppl. A), 93, (1980).

56. Bergan, T. and Solberg, R., Chemother., 27, 155, (1981).

57. Dell, D., Chamberlain, J. and Coppin, F., J. Chromatogr., 226, 431, (1981).

58. Kees, F., Strehl, E., Seeger, K., Seidel, G., Dominiak, P. and Grobecker, H., Arzneim.-Forsch., 31, 362, (1981).

59. Wise, R., Wright, N. and Wills, P.J., Antimicrob. Agents Chemother., 19, 526, (1981).

60. Haginaka, J., Nakagawa, T. and Uno, T., J. Antibiotics, 33, 236, (1980).

61. Suzuki, A., Noda, K. and Noguchi, H., J. Chromatogr., 182, 448, (1980).

62. Nahata, M.C., J. Chromatogr., 225, 532, (1981).

63. Rothschafer, J.C., Crossley, K.B., Lesar, T.S., Zaske, D. and Miller, K., Antimicrob. Agents Chemother., 21, 170, (1982).

64. Trautmann, K.H. and Haefelfinger, P., J. High Res. Chrom. Chrom. Comm., 4, 54, (1981).

65. Granneman, G.R. and Sennello, L.T., J. Chromatogr., 229, 149, (1982).

66. Anhalt, J.P., Antimicrob. Agents Chemother., 11, 651, (1977).

67. Maitra, S.K., Yoshikawa, T.T., Hansen, J.L., Nilsson-Ehle, I., Palin, W.J., Schotz, M.C. and Guze, L.B., Clin. Chem., 23, 2275, (1977).

68. Peng, G.W., Gadalla, M.A.F., Peng, A., Smith, V. and Chiou, W.L., Clin. Chem., 23, 1638, (1977).

69. Anhalt, J.P. and Brown, S.D., Clin. Chem., 24, 1940, (1978).

70. Bäck, S-E., Nilsson-Ehle, I. and Nilsson-Ehle, P., Clin. Chem., 25, (1979).

71. Larsen, N-E., Marinelli, K. and Heilesen, A.M., J. Chromatogr., 221, 182, (1980).

72. Barends, D.M., Zwaan, C.L. and Hulshoff, A., J. Chromatogr., 222, 316, (1981).

73. Walker, S.E. and Coates, P.E., J. Chromatogr., 223, 131, (1981).

74. Maitra, S.K., Yoshikawa, T.T., Hansen, J.L., Schotz, M.C. and Guze, L.B., Am. J. Clin. Pathol., 71, 428, (1979).

75. Haughey, D.B., Janicke, D.M., Adelman, M. and Schentag, J.J., Antimicrob. Agents Chemother., 17, 649, (1980).

76. Peng, G.W., Jackson, G.G. and Chiou, W.L., Antimicrob. Agents Chemother., 12, 707, (1977).

77. Maitra, S.K., Yoshikawa, T.T., Steyn, C.M., Guze, L.B. and Schotz, M.C., Antimicrob. Agents Chemother., 14, 880, (1978).

78. Wong, L.T., Beaubien, A.R. and Pakuts, A.P., J. Chromatogr., 231, 145, (1982).

79. Koup, J.R., Brodsky, B., Lau, A. and Beam, Jr., T.R., Antimicrob. Agents Chemother., 14, 439, (1978).

80. Nilsson-Ehle, I., Kahlmeter, G. and Nilsson-Ehle, P., J. Antimicrob. Chemother., 4, 169, (1978).

81. Peng, G.W., Gadalla, M.A.F. and Chiou, W.L., J. Pharm. Sci., 67, 1036, (1978).

82. Thies, R.L. and Fischer, L.J., Clin. Chem., 24, 778, (1978).

83. Crechiolo, J. and Hill, R.E., J. Chromatogr., 162, 480, (1979).

84. Petersdorf, S.H., Raisys, V.A. and Opheim, K.E., Clin. Chem., 25, 1300, (1979).

85. Sample, R.H., Glick, M.R., Kleiman, M.B., Smith, J.W. and Oei, T.O., Antimicrob. Agents Chemother., 15, 491, (1979).

86. Aravind, M.K., Miceli, J.N., Kauffman, R.E., Strebel, L.E. and Done, A.K., J. Chromatogr., 221, 176, (1980).

87. Burke, T., Wargin, W.A. and Blum, M.R., J. Pharm. Sci., 69, 909, (1980).

88. Oseekey, K.B., Rowse, K.L. and Kostenbauder, H.B., J. Chromatogr., 182, 459, (1980).

89. Ferrell, W.J., Szuba, M.P., Miluk, P.R. and McClatchey, K.D., J. Liquid Chromatogr., 4, 171, (1981).

90. Nahata, M.C. and Powell, D.A., J. Chromatogr., 223, 247, (1981).

91. Velagapudi, R., Smith, R.V., Ludden, T.M. and Sagraves, R., J. Chromatogr., 228, 423, (1982).

92. Meulemans, A., Manuel, C., Mohler, J., Roncoroni, A. and Vulpillat, M., J. Liquid Chromatogr., 4, 145, (1981).

93. Goehl, T.J., Mathur, L.K., Strum, J.D., Jaffe, J.M., Pitlick, W.H., Shah, V.P., Poust, R.I. and Colaizzi, J.L., J. Pharm. Sci., 67, 404, (1978).

94. Lanbeck, K. and Lindström, B., J. Chromatogr., 154, 321, (1978).

95. Jung, D. and Öie, S., Clin. Chem., 26, 51, (1980).

96. Hekster, Y., Vree, T.B., Damsma, J.E. and Friesen, W.T., J. Antimicrob. Chemother., 8, 133, (1981).

97. Vree, T.B., Hekster, Y.A., Baars, A.M., Damsma, J.E. and van der Kleijn, E., J. Chromatogr., 146, 103, (1978).

98. Gochin, R., Kanfer, I. and Haigh, J.M., J. Chromatogr., 223, 139, (1981).

99. Essers, L. and Korte, H., Chemother., 28, 247, (1982).

100. Ascalone, V., J. Chromatogr., 224, 59, (1981).

101. Weinfeld, R.E. and Macasieb, T.C., J. Chromatogr., 164, 73, (1979).

102. Reeves, D.S., Bywater, M.J., Bullock, D.W. and Holt, H.A., J. Antimicrob. Chemother., 6, 647, (1980).

103. Nilsson-Ehle, I., Yoshikawa, T.T., Schotz, M.C. and Guze, L.B., Antimicrob. Agents Chemother., 9, 754, (1976).

104. Sharma, J.P., Perkins, L.G. and Bevill, R.F., J. Chromatogr., 134, 441, (1977).

105. Sharma, J.P. and Bevill, R.F., J. Chromatogr., 166, 213, (1978).

106. Böcker, R. and Estler, C-J., Arzneim.-Forsch., 29, 1690, (1979).

107. Nelis, H.J.C.F. and de Leenheer, A.P., Clin. Chim. Acta, 103, 209, (1980).

108. de Leenheer, A.P. and Nelis, H.J.C.F., J. Pharm. Sci., 68, 999, (1979).

109. Gulaid, A., Goughton, G.W., Lewllen, O.R.W., Smith, J. and Thorne, P.S., Br. J. clin. Pharmac., 6, 430, (1978).

110. Marques, R.A., Stafford, B., Flynn. N. and Sadée, W., J. Chromatogr., 146, 163, (1978).

111. Wheeler, L.A., de Meo, M., Halula, M., George, L. and Heseltine, P., Antimicrob. Agents Chemother., 13, 205, (1978).

112. Lanbeck, K. and Lindström, B., J. Chromatogr., 162, 117, (1979).

113. Kaye, C.M., Sankey, M.G. and Thomas, L.A., Br. J. clin. Pharmac., 9, 528, (1980).

114. Nilsson-Ehle, I., Ursing, B. and Nilsson-Ehle, P., Antimicrob. Agents Chemother., 19, 754, (1981).

115. Nachbaur, J. and Joly, H., J. Chromatogr., 145, 325, (1978).

116. Alton, K.B., J. Chromatogr., 221, 337, (1980).

117. Andrews, F.A., Peterson, L.R., Beggs, W.H., Crankshaw, D. and Sarosi, G.A., Antimicrob. Agents Chemother., 19, 110, (1981).

118. Swezey, S.F., Giacomini, K.M., Abang, A., Brass, C., Stevens, D.A. and Blaschke, T.F., J. Chromatogr., 227, 510, (1982).

119. Brodie, R.R., Shasseaud, L.F. and Walmsley, L.M., J. Chromatogr., 155, 209, (1978).

120. Watts, M.T. and Raisys, V.A., J. Chromatogr., 230, 79, (1982).

121. Murray, Jr., J.F., Gordon, G.R. and Peters, J.H., Chemother., 3, 266, (1975).

122. LeCaillon, J.B., Febvre, N., Metayer, J.P. and Souppart, C., J. Chromatogr., 145, 319, (1978).

123. Ratti, B., Parenty, R.R., Toselli, A. and Zerilli, L.F., J. Chromatogr., 225, 526, (1981).

124. Aufrère, M.B., Hoener, B. and Vore, M.E., Clin. Chem., 23, 2207, (1977).

125. Cuisinaud, G., Ferry, N., Seccia, M., Bernard, N. and Sassard, J., J. Chromatogr., 181, 399, (1980).

126. Tsuji, K., J. Chromatogr., 158, 337, (1978).

127. Nilsson-Ehle, I., Yoshikawa, T.T., Edwards, J.E., Schotz, M.C. and Guze, L.B., J. Infect. Dis., 135, 414, (1977).

128. Blair, A.D., Forrey, A.W., Meijsen, B.T. and Cutler, R.E., J. Pharm. Sci., 64, 1334, (1975).

129. Diasio, R.B., Wilburn, M.E., Shadomy, S. and Espinel-Ingroff, A., Antimicrob. Agents Chemother., 13, 500, (1978).

130. Miners, J.O., Foenander, T. and Birkett, D.J., Clin. Chem., 26, 117, (1980).

131. Warnock, D.W. and Turner, A., J. Antimicrob. Chemother., 7, 363, (1981).

132. Hackett, L.P. and Dusci, L.J., J. Chromatogr., 155, 206, (1978).

133. Nation, R.L., Peng, G.W., Smith, V. and Chiou, W.L., J. Pharm. Sci., 67, 805, (1978).

134. Kreuzig, F., J. Chromatogr., 163, 322, (1979).

135. Land, G. and Bye, A., H. Chromatogr., 224, 51, (1981).

136. Schneider, H.G. and Glazko, A.J., J. Chromatogr., 139, 370, (1977).

137. Bergqvist, Y. and Frisk-Holmberg, M., J. Chromatogr., 221, 119, (1980).

138. Staiger, M.A., Nguyen-Dihn, P. and Churchill, F.C., J. Chromatogr., 225, 139, (1981).

139. Alván, G., Ekman, L. and Lindström, B., J. Chromatogr., 229, 241, (1982).

140. Brown, N.D., Poon, B.T. and Chulay, J.D., J. Chromatogr., 229, 248, (1982).

141. Grindel, J.M., Tilton, P.F. and Shaffer, R.D., J. Pharm. Sci., 66, 834, (1977).

142. Jones, C.R. and Ovenell, S.M., J. Chromatogr., 163, 179, (1979).

143. Baker, J.K., McChesney, J.D., Hufford, C.D. and Clark, A.M., J. Chromatogr., 230, 69, (1982).

144. Timm, U. and Weidekamm, E., J. Chromatogr., 230, 107, (1982).

145. Blanchard, J., J. Chromatogr., 226, 455, (1981).

146. Lawrence, J.F. and Frei, R.W., Chemical Derivatization in Liquid Chromatography, Elsevier Scintific Publishing Co., Amsterdam, 1976.

147. Nilsson-Ehle, I. and Nilsson-Ehle, P., J. Infect. Dis., 139, 712, (1979).

148. Arvidsson, A., Renal and Biliary Excretion of Cephalosporins in Man, Doctoral Thesis, Stockholm, 1982.

149. Stoeckel, K., McNamara, P.J., Brandt, R., Plozza-Nottebrock, H. and Ziegler, W.H., Clin. Pharmacol. Ther., 29, 650, (1981).

150. Schaad, U.B. and Stoeckel, K., Antimicrob. Agents Chemother., 21, 248, (1982).

151. Yamada, H., Ichihashi, T., Hirano, K. and Kinoshita, H., J. Pharm. Sci., 70, 112, (1981).

152. Slaughter, R.L., Pieper, J.A., Cerra, F.B., Brodsky, B. and Koup, J.R., Clin. Pharmacol. Ther., 28, 69, (1980).

153. Kaufmann, R.E., Miceli, J.N., Strebel, L., Buckley, J.A., Done, A.K. and Dajani, A.S., J. Pediatrics, 98, 315, (1981).

154. Sack, C.M., Koup, J.R., Opheim, K.E., Neeley, N. and Smith, A.L., Ped. Pharmac., 2, 93, (1982).

155. Houghton, G.W., Thorne, P.S., Smith, J., Templeton, R. and Collier, J., Br. J. Clin. Pharmac., 8, 337, (1979).

156. Houghton, G.W., Thorne, P.S., Smith, J., Templeton, R., Collier, J., Moesgaard, F. and Lukkegaard-Nielsen, M., Metronidazole, Royal Society of Medicine International Congress and Symposium Series No. 18, Phillips, I. and Collier, J., eds., The Royal Society of Medicine, London, Academic Press, London, Grune and Stratton, New York, 1979, p. 35.

157. Gabriel, R., Page, C.M., Weller, I.V.D., Collier, J., Houghton, G.W., Templeton, R. and Thorne, P.S., Metronidazole, Royal Society of Medicine International Congress and Symposium Series No. 18, Phillips, I. and Collier, J., eds., The Royal Society of Medicine, London, Academic Press, London, Grune and Stratton, New York, 1979, p.49.

158. White, L.O., J. Antimicrob. Chemother., 8, 1, (1981).

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