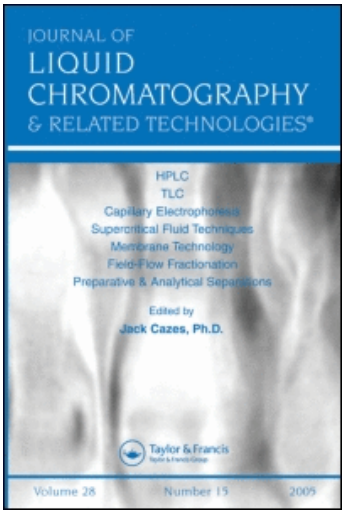

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Preparative Gel Permeation Chromatography

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PREPARATIVE GEL PERMEATION CHROMATOGRAPHY

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I. INTRODUCTION

This Chapter is devoted to a particular mode of liquid chromatography : Gel Permeation Chromatography (G.P.C.). The field of G.P.C. mainly concerns polymers but can also be applied to small organic molecules where, as we will see later, the technique is more closely related to classical liquid chromatography.

The prime difference between polymers and small molecules is that a polymer is not a well-defined specie. It is comprised of a mixture of macromolecules having different lengths (and, sometimes, shapes) and characterized by a molecular weight distribution (M.W.D.). But molecular heterogeneity can be more important when macromolecules are branched : the lengths and the positions of branches are generally random. Finally, in the case of copolymers, even the chemical composition can vary between macromolecules. Accordingly, we generally have complex mixtures that cannot be treated as in classical

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liquid chromatography. Separations will only occur on the basis of average properties. As the mechanism of GPC fractionation directly involves molecular size in solution, the fundamental parameter is the macromolecule hydrodynamic volume that leads to polymer fractionation according to the component sizes.

Historically, it is in the university of Uppsala (Sweden) in 1959 that the technique appeared. Porath and Flodin (1) succeeded in fractionating hydro-soluble polymers using a packing made of cross-linked dextran gel. This is the root of the Sephadex® family (2) and the technique of Gel Filtration Chromatography (G.F.C.) (3), still widely used today by biochemists. These "soft gel" packings only operate under low pressure and characteristically involve very long analysis times. A second step was made in 1962 by the Dow Chemical Company, where Moore (4) performed the separation of organo-soluble polymers on cross-linked polystyrene gels. Gel Permeation Chromatography was born. These semi-rigid packings, partially swelled by organic solvents, withstand a higher pressure, leading to shorter analysis times. Immediatly, Waters Associates, Inc. commercialized this packing under the name of Styragel® (5) and a liquid chromatograph : the GPC 100. The technique spread very quickly in industry as well as in the university laboratories and then, the number of papers devoted to GPC exponentially increased.

In 1966, the first totally rigid porous packing appeared in France. De Vries (6-8) synthetized, in Pechiney-St-Gobain, porous silica beads, commerciallized at once in Europe under the name of Spherosil® and in United States under the name of Porasil®. Unfortunately, the strong interactions between the silica surface and a number of solutes prevented the universal use of this packing which its physical and mechanical properties promised as a result of its compatibility with the quasi-totality of solvents.

Finally, a further step was made in 1974 with the comming of micro-packings (9-11). Waters Associates introduced Microstyra-

gel ® with the same structure of Styragel ® , but with a particle size of 10μm instead of 50μm. The elution time decreased from a few hours to some ten minutes with the high pressure technology simultaneously developped in liquid chromatography. Thus, H.P. (High Performance or High Pressure) was added before the name of this technique to characterize this new technology : H.P.G.P.C. We can note that the name "Size Exclusion Chromatography" (S.E.C.) and H.P. S.E.C. (12) is more commonly used with micro-packings based on silica (13-16). The most recent developments involve surface modifications of porous silica packings by organic grafting (Microbondagel ® (5), TSK Gel SW ® (17), Lichrospher diol ® (18)) or by organic coating (19) to prevent adsorption phenomena.

The preparative aspect of GPC was taken into account from the beginning since, as GPC is a non-destructive technique, it is easy to collect liquids at the outlet of the chromatograph and thus recover the fractionated polymer. We must remember that, before GPC, the only available methods of polymer fractionation involved coacervation methods in solvent-non solvent mixtures or precipitation methods by temperature gradient. They required days or weeks of tedious labor and enormous volumes of solvent to achieve a crude fractionation of a few grams of polymer. Gradient elution chromatography (20) was later used, but some difficulties appeared, mainly with copolymers. Gel Filtration Chromatography turned from the beginning to the preparative mode thanks to the relative simplicity of instrumentation. With the impulse of the Pharmacia Company (2), GFC rapidly reached the industrial level in biochemical applications. Kilograms of enzymes, proteins and natural products are commonly isolated (21-23).

In 1967, Waters Associates (5) manufactured the first preparative GPC instrument, the "Ana Prep" (24), capable of operating under moderate pressure with rigid and semi-rigid packings, enabling the fractionation of several grams of polymer in a single process. Some other systems were later described (25-28). There is no basic difference between analytical and preparative instruments, only the

size is adjusted according to the amount of substance that must be fractionated.

We can thus define the different GPC methods :

- analytical method. Injected quantity about 1mg in an analytical instrument for analytical measurements.
- semi preparative method. Injected quantity about 100mg in an analytical instrument (repetitive or large columns). Allows the characterization of fractions by spectroscopic methods (IR, NMR, etc ...).
- preparative method. Injected quantity 1-10grams in a specialized preparative instrument for the preparation of reference polymers or well-defined materials.
- industrial method. More than1kilogram injected.Only available for industrial production of biological substances and natural products.

But before studying the preparative aspect of GPC, let us examine its basic principle.

II. GPC BACKGROUND

II.1. Principle

Gel Permeation Chromatography is liquid chromatography on a porous packing. The mobile phase inside the column can be considered as separated into two volumes : the void volume V_o, volume of solvent around the packing particles , and the porous volume V_p, volume of solvent inside the packing's pores. Usually, these two volumes have approximately the same value. Many attempts have been made to explain polymer fractionation in GPC (see the reviews of Audebert (29-30) and Hagnauer (31)), but now, the size exclusion mechanism is widely demonstrated and accepted (32-36). It is based on the principle that a macromolecule cannot approach a wall at a

distance smaller than its radius, that defines an accessible volume V_{acc}

$$V_{acc} = k.V_p$$

with $0 < k < 1$ depending of the molecular size (Figure 1). The elution volume is then defined by

$$V_e = V_o + V_{acc} = V_o + k.V_p$$

A very high molecular weight polymer ($k = 0$) is eluted at V_o, whereas elution volume is a function of size for polymer sizes ($k < 1$) smaller than exclusion limit. This function is called "calibration curve". Assuming a proportionallity between size and molecular weight, and using the logarithm of molecular weight versus elution volume, the plot is quasi-linear (Figure 2). The molecular weight calibration curve has the disadvantage of being different from one polymer to the next. This drawback is avoided in Benoit's "universal calibration" (37-38). The universal parameter $[\eta].M$, product of molecular weight by intrinsic viscosity, is proportional to molecular size (hydrodynamic volume) and does not depend upon the nature of a polymer. This leads to the unique "universal calibration curve" for all the polymers (Figure 3)

$$\text{Log } [\eta].M = f(V_e)$$

But the situation can become very complicated when strong interactions occur between the packing and the solute. Adsorption leads to a term proportional to the packing specific surface $K'.S$ and partition a term $K''.V_s$ proportional to stationary phase volume. The resulting elution volume takes the form

$$V_e = V_o + K.V_p + K'.S + K''.V_s$$

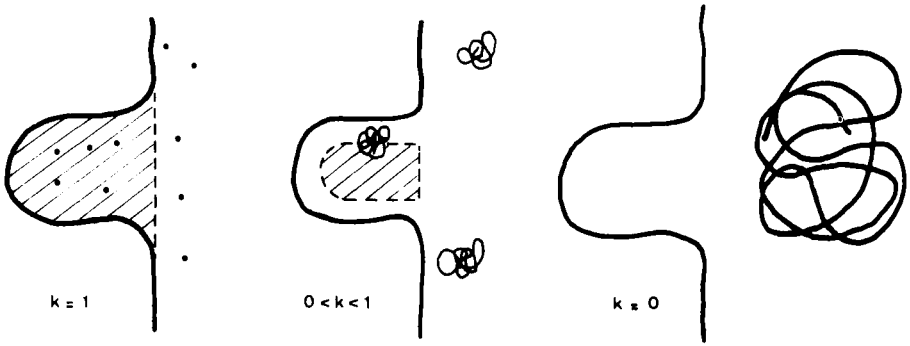

Figure 1 : The accessible volume of pores as a function of molecular size.

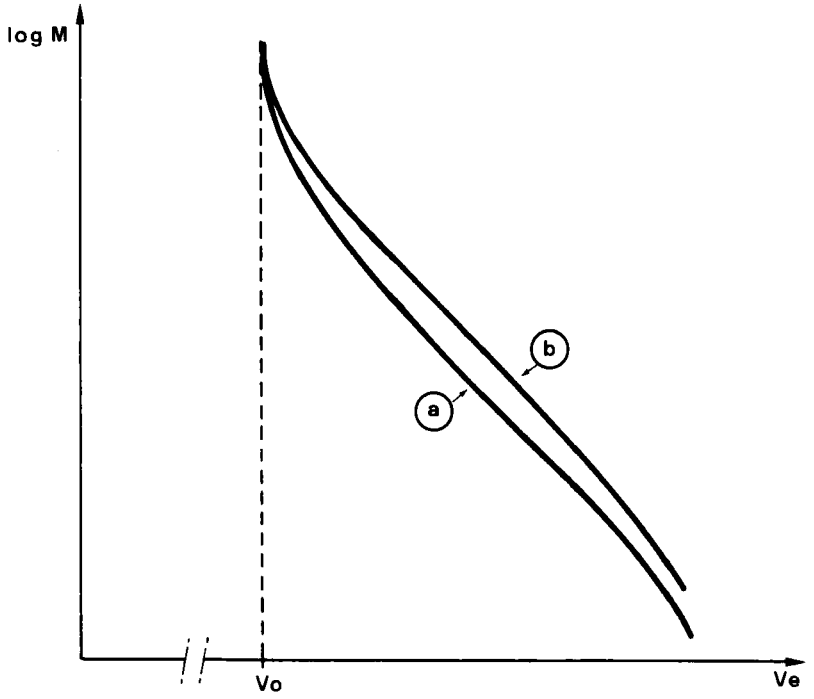

Figure 2 : Calibration curves for two different polymers (a) and (b).

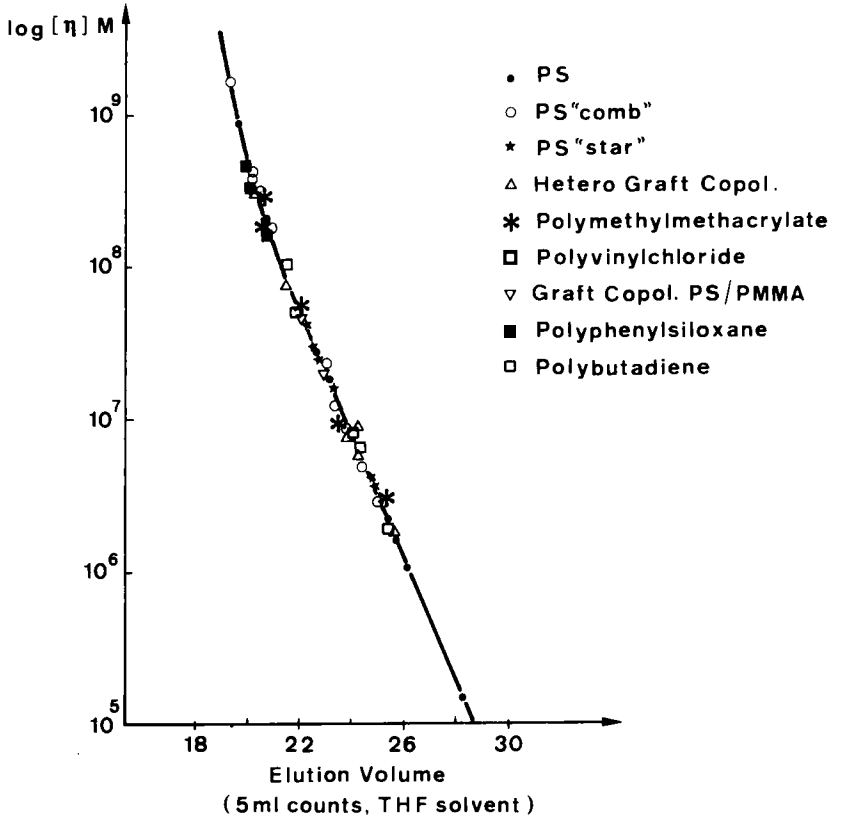

Figure 3 : The universal calibration curve. (Reprinted with permission from ref. 38).

which cannot be represented by a simple relationship. Consequently, the stationary phase, and especially the mobile phase are usually chosen so that interactions do not occur, steric exclusion being the only mechanism of fractionation, allowing the use of universal calibration.

II.2. Instrumentation

GPC equipment is very close to classical HPLC equipment. We will not describe it here but we will only point out the differences.

The pumping system is a classical one, but must deliver the solvent at an extremely constant flow rate so that elution times should be accurately converted into elution volumes for the use of the calibration relationship between molecular weights and elution volumes. Due to the internal volume of GPC columns, sample sizes are often greater than 100µl ; a loop injector is widely used. The intrument can easily be automated by the use of an automatic injector.

In contrast to classical LC, the most common detector is the differential refractometer which measures concentration variations. This detector is not very sensitive, but has the advantage of being non specific (universal). Cases where a polymer is not detected are unusual. The trend, today, is to introduce a second detector, sensitive to molecular weight to improve data processing possibilities. The first mass detector used is the capillary viscometer (39-42). This detector, providing intrinsic viscosity measurements across the molecular weight distribution, permits the use of universal calibration. This detector cannot operate in HPGPC because of the extremely small volume of modern columns. A continuous viscometer, firstly described by Ouano (43) and recently improved (44-47), can be used to generate the required data. Unfortunately, this detector is not commercially available at the present time ; it is represented in Figure 4. Another mass detector has also been used : the low angle laser light scattering (LALLS) photometer (48) represented in Figure 5. It provides information concerning weight-average molecular weight directly, eliminating the need for calibration. It is manufactured by Chromatix, Inc. (49). Finally, other photometric detectors are occasionally coupled to GPC systems. Ultraviolet photometers permit the study of copolymer composition (50-52) and an infrared photometer has been used for polyolefine analysis (53).

II.3. Columns and chemicals

The most widely used mobile phase for organic polymers is tetrahydrofuran (THF) because of its excellent solvation pro-

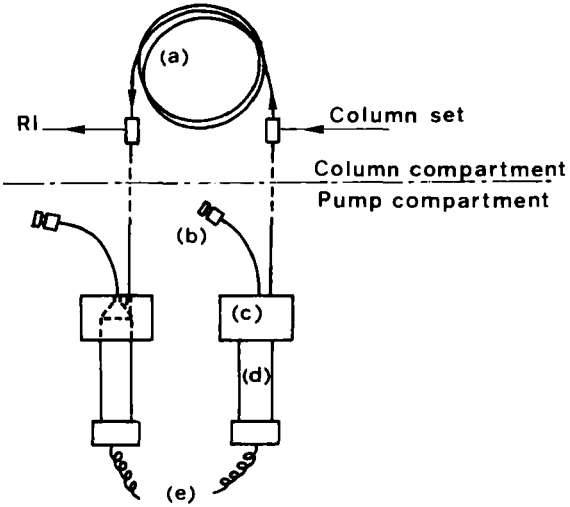

Figure 4 : Scheme of the continuous viscometer. (Reprinted with permission from ref. 46-47).
a) capillary tube ; (b) purge ; (c) transducer holder ; (d) pressure transducer ; (e) electronic units.

perties. Toluene, chloroform and methylene chloride are also routinely employed. When a polymer is insoluble in these solvents, other possibilities are available. Polyolefins are analyzed in trichlorobenzene (TCB) or in o-dichlorobenzene (ODCB) at 135°C, polyamides and polyesters can be chromatographied in hexamethylphosphotriamide (HMPT) or meta-cresol at 100°C or at room temperature in hexafluoroisopropanol (HFIP). Dimethylformamide (DMF) is also used in some particularly difficult cases. Water soluble polymers are, of course, analyzed in aqueous solution, often with addition of a buffer of variable ionic strength to avoid ionic effects which greatly perturb size exclusion properties.

A listing of GPC packings is given in the preparative section. As resolution varies with the square of the particle diameter, high resolution packing diameter are generally between 5 µm and 10µm ; the use of these packings requires high pressure

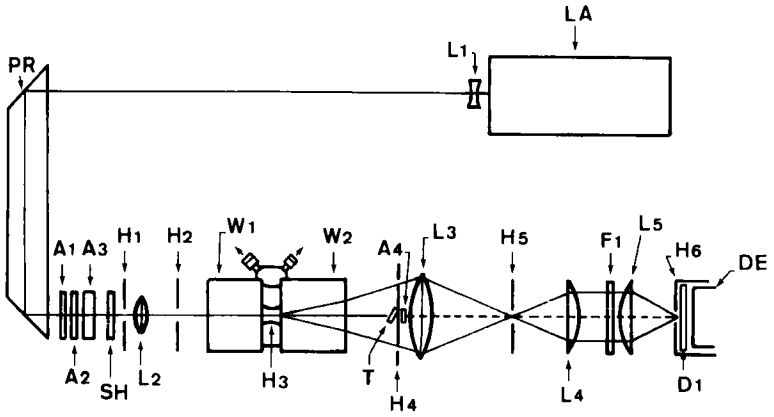

Figure 5 : The LALLS detector. (Reprinted with permission from ref. 48).

Schematic diagram of the optical system of the LALLS showing the critical parts of the photometer : (LA) laser ; (L1) diverging lens, 500mm focal length ; (PR) folding prism ; (A1-A3) attenuators ; (SH) shutter/ calibrating attenuator ; (H1) condenser aperture ; (L2) condenser lens, 150mm focal length ; (H2) secondary aperture ; (W1, W2) sample cell windows ; (H3) sample aperture; (T) beam trap ; (H4) safety attenuator ; (L3) relay lens, 50mm focal length ; (H5) field stop ; (L4, L5) detector lenses, 40mm focal length ; (F1) narrow-band filter ; (H6) detector aperture ; (D1) diffuser, (DE) photomultiplier.

systems. We can distinguish three different kinds of packings : the soft gels, the semi-rigid, and the rigid gels.

- Soft gels are made of cross-linked polymers, mainly water soluble polymers. They are highly swelled by the solvent and cannot operate under pressure. Consequently, particle size is around 100µm and resolution is poor, leading to long analysis times (often several hours). This is the case of classical Gel Filtration Chromatography packings such as cross-linked dextran or polyacrylamide gels. They are generally packed in glass columns.

- Semi-rigid packings are based on highly cross-linked organic polymers such as styrene-divinylbenzene copolymers. They are moderately swelled by solvents, but exhibit sufficient mechanical strength to be used under pressure. The classical particle diameter is 50µm, but the modern packings having 10µm diameters allow good resolution with analysis durations of 10-15 minutes. They are usually packed in stainless steel tubes of 7-8mm I.D. and 30-50cm length.
- Rigid packings are mainly based on porous silica. They support high pressure and generally have particle diameters between 5µm and 10µm. They can be spherical or irregularly shaped. The silica surface is either untreated, or is modified by organic functionalities to reduce adsorption. They are usually packed in 4mm or 8mm I.D. stainless steel columns, 25 or 30 cm long. It should be noted that column dimensions given are merely indicative of commercially available columns ; other sizes have been routinely employed.

Due to the synthetic methods used to prepare them, GPC packings have pore sizes leading to a limited fractionation range. For broader ranges, it is possible to mix several different packings with different pore size distributions to obtain a "linear" column, covering the desired broad range of molecular weights. But, the most widely used method is to assemble a column "set", with different fractionation-range columns connected in series, and thus to tailor the column set to the separation to be performed.

For analytical applications, the column set has to be calibrated. In organic media, anionically synthetized polystyrene standards with very narrow distributions are the most suitable for establishing a universal calibration. But today other polymer standards are available for molecular weight calibration : polytetrahydrofuran , polymethylmethacrylate, polyvinylacetate, etc ... For aqueous media, dextran, protein or polystyrene sulfonate standards are used. In addition, some mathematical methods

(54-55) exist to calibrate a column set using broad distribution polymer standards (56-61). Finally, the bimodal-pore-size-distribution method (62) is applied to bimodal packings containing two discrete pore sizes with equal pore volumes. All the calibration methods have been recently reviewed by Janca (63).

II.4. Analytical applications

Let us simply consider the GPC chromatogram as a finger print of a polymer. As the elution volume is a quasi-linear function of the logarithm of molecular weight, the chromatogram does not correspond to the distribution curve but to its transform in molecular weight on a logarithmic scale, the intensity being multiplied by molecular weight (64). But the chromatogram can be interpreted more advantageously , since the curve digitization provides a set of data each being characterized by the concentration, C_i, and the molecular weight, M_i, through the calibration curve. Then, it is easy to calculate, by integrating across the whole chromatogram, the different average molecular weights in number, in viscometric, in weight and in z

$$\bar{M}_n = \frac{\Sigma C_i}{\Sigma C_i/M_i} \qquad \bar{M}_v = \left[\frac{\Sigma C_i M_i^a}{\Sigma C_i M_i}\right]^{1/a} \qquad \bar{M}_w = \frac{\Sigma C_i M_i^2}{\Sigma C_i M_i} \qquad \bar{M}_z = \frac{\Sigma C_i M_i^3}{\Sigma C_i M_i^2}$$

where a is the Mark-Houwink exponent.

The polydispersity, $I = \bar{M}_w/\bar{M}_n$, represents the broadness of the molecular weight distribution. Integral and derivative molecular weight distribution curves can also be calculated. When a viscometric detector is used, or when the K and a Mark-Houwink coefficients are known, Benoit's universal calibration can be used (44-46). These calculations are tedious, and the trend in GPC data handling is to automate the chromatograph with a mini- or microcomputer for data acquisition ant treatment. Accuracy of results are greatly increased when taking into account instrumental imperfections such as axial dispersion, concentration effets, etc ...

(65). We will not describe then here. (see references in (31)). We will merely notethat when the polymer is branched, the dual detection refractometer-viscometer (46) approach leads to the determination of branching distribution and frequency in addition to average molecular weights and the molecular weight distribution curve (66).

Modern GPC instrumentation is thus an excellent analytical tool providing, in a relatively few minutes, all the above-described structural parameters of polymers. For applications, see the review of Janca (67).

III. PREPARATIVE METHOD

III.1. General

The purpose of preparative GPC is multiple. It can be the separation of one polymer from an other one on the basis of molecular weight or the purification of a polymer from low molecular weight compounds such as plasticizers, dyes or monomeric residues. One can separate polymer additives for identification or a particular oligomer in an oligomer mixture. Using low porosity gels, small organic molecules can be separated for purification or identification. But the primary application of preparative GPC is the preparation of narrow distribution polymer standards by fractionation of a broad polymer. More simply, this technique is also applied to narrow the polymer distribution by eliminating the highest and lowest molecular weight components and obtain a best-defined material. The technique employed will depend upon the objective of the experiment but especially on the amount of material to be purified.

With classical analytical columns (7mm I.D.), the amount of injected polymer is approximately 1mg. For a semi-preparative operation, 10 to 100mg are usually involved. This is often the

amount required for identification by spectroscopic methods (IR, NMR, etc ...). Repetitive injections/separations are performed on an analytical instrument equipped with analytical columns when 10mg are adequate. This operation can be automated by using an automatic injector and a fraction collector. For more material , the same holds true, but by replacing the analytical column with a semi-preparative, high-performance column, having an internal diameter around 25mm, and increasing the mobile phase flow rate. This can be done in a single operation or as a repetitive one.

For higher loadings (above 1 gram) the analytical GPC instrument is not convenient and a specific preparative installation has to be used with preparative columns, the size of which are dependent upon the required capacity.

III.2. The column

For relatively small amounts of sample, columns of small particle size (10µm) should be used to prepare small amountsof purified material in short times at high resolution. Tubing is around 8mm to 20mm I.D. and about 50cm long. It is equipped with classical end-fittings. For large amounts of sample, larger columns must be used, but small particles are not convenient due to their high cost ; they are replaced by conventional packings (50 or 100µm). But, because of their poor efficiency, and in order to obtain the same resolution, the column length must be dramatically increased, involving long separation times and large amounts of solvent. Preparative columns are usually 2 inches in diameter and 4 feet in length, equipped with distributor end-fittings holding porous metal discs as retainers. As in analytical GPC, several columns are commonly connected in series so as to obtain the required molecular weight separation range for the material that must be fractionated.

The largest columns are used in industrial preparative chromatography, almost exclusively in the Gel Filtration Chroma-

tography (21-22) mode where kilogram scale is exceeded for enzyme purification on 40cm diameter columns (23). Industrial columns have been used up to 2 meters in diameter. This technique is outside the scope of this chapter.

High performance packings are conveniently purchased in pre-packed columns because of the difficulty of packing small columns with very high numbers of plates. The most important ones are listed in Table I. Conventional packings are available either in pre-packed columns or in bulk. The most important ones are given in table II. Table III presents the different manufacturers of these packings.

The filling of preparative columns is easier than for small columns and is generally performed using a slurry method. Dry organic packings are put in suspension in a suitable solvent for swelling. Mixing in an ultrasonic bath is recommended to destroy agregates. The slurry is then pumped into the column, topped with a pre-column, then pushed by the solvent under pressure to settle the packing. The pre-column is then removed and replaced with an end-fitting. Rigid packings are also packed by the slurry method for particle sizes smaller than 30μm. Above this size the slurry method or the dry-packing technique can be applied. For silica packings, it was found (26) that a dry filling with application of axial vibrations to the column (60mm diameter - 122 cm length) could provide efficiencies more than twice that obtained for analytical columns.

In contrast to analytical GPC, column efficiency is not the major parameter in preparative GPC. The trend is to heavily load the column with a sample to recover the largest amount of purified material per run, with the consequent loss of efficiency. A compromise must be found between the sample loading, the degree of purification (efficiency), and the required run time.

Table I : High performance GPC packings

Packing	Type	Nominal Particle size (µm)	Nominal max.MW	manu-facturer
Organic				
Ultrastyragel	Cross-linked polystyrene	10	4.10^7	1
Microstyragel	" "	10	4.10^7	1
Shodex A 800 (8mm I.D.)	" "	10	5.10^7	2
Shodex H 800 (20mm I.D.)	" "	10	5.10^7	2
TSK - Gel	" "	10	5.10^7	3
PL - Gel (8mm I.D.)	" "	5 ou 10	4.10^7	4
PL - Gel prep. (20mm I.D.)	" "	10	4.10^7	4
Lichrogel PSM	" "	10	2.10^6	5
Aqueous				
Spheron	Poly(hydroxyethyl methacrylate)	10	10^8	6
Shodex OH pak	Methacrylic copolymer	10	5.10^5	2
Shodex Ion pak	Sulfonated cross-linked PS	10-20	3.10^7	2
TSK - Gel PW	Hydroxylated copolymer	10	5.10^6	3
Both organic and aqueous				
Lichrospher Si	Pure silica	10	8.10^6	5
Zorbax PSM	" "	6	6.10^6	7
Lichrospher-Diol	Grafted silica (glycerol-propyl)	10	8.10^6	5
Microbondagel E	" " (polyether)	10	2.10^6	1
Protein I	" " (?)	10	5.10^5	1
TSK Gel SW	" " (?)	10	6.10^5	3

Table II : Conventional GPC packings

Packing	Type	Nominal particle size (μm)	Nominal max. MW	manufacturer
Organic				
Styragel	Cross-linked polystyrene	37-75	5.10^8	1
Merckogel OR-PVA	Vinylacetate copolymer	50	8.10^3	5
Bio beads	Cross-linked polystyrene	37-75	$1.5.10^4$	9
Aqueous				
Sepharose	Agarose	50-200	4.10^6	8
Bio-gel	Polyacrylamide	37-300	5.10^5	9
Ultrogel	Agarose-polyacrylamide	60-140	10^6	10
Sephadex	Cross-linked-dextran	10-300	5.10^5	8
Enzacryl gel	Poly(acryloyl-morpholine)	40-300	10^5	11
Both organic and aqueous				
Porasil	Pure silica	37-75	5.10^6	1
Spherosil	Pure silica	37-200	5.10^6	12
Vit-X	Pure silica	37-50	10^7	13
Fractosil	Pure silica	37-125	very high	5
CPG (controlled pore glass)	Glass	37-150	8.10^8	14-15
Glycophase-CPG	Grafted glass (glyceryl)	37-125	8.10^8	14-15

Table III : Packing Manufacturers

Rfce	Manufacturer	Address	Country	US Supplier
1	Waters Associates Inc.	Milford-Mass.	USA	
2	Showa Denko KK	Tokyo	Japan	Perkin-Elmer Corp.-Norwalk-Conn.
3	Toyo Soda Manufacturing	Tokyo	Japan	Varian Ass.-Palo Alto-Calif.
4	Polymer Laboratories Ltd	Shropshire	England	Polymer Lab. Ltd -Stow-Ohio
5	E. Merck	Darmstadt	Germany	EM Laboratories Inc.-Elmsford-N.Y.
6	Lachema	Brno	Czechoslov.	Pharmacia Fine Chem.-Piscataway-N.J. Pierce Chem. Comp.-Rockford-Ill.
7	Du Pont de Nemours	Wilmington-Del.	USA	
8	Pharmacia Fine Chemicals	Uppsala	Sweden	Pharmacia Fine Chem.-Piscataway-N.J.
9	Bio-Rad Laboratories	Richmond-Calif.	USA	
10	LKB	Bromma	Sweden	LKB Instruments Inc.-Rockville-Maryl.
11	Koch-Light Laboratories Ltd	Bucks	England	Aldrich Chem. Comp.-Milwaukee-Wisc.
12	Rhône-Poulenc	Paris	France	Waters Ass. Inc.-Milford-Mass. Supelco Inc.-Bellefonte-Penn.
13	Perkin-Elmer Corp.	Norwalk-Conn.	USA	
14	Electronucleonics Inc.	Fairfield	USA	
15	Pierce Chemical Company	Rockford-Ill.	USA	

III.3. Instrumentation

As a general rule, the preparative GPC instrument is directly evolved from the analytical one, the size being larger. The pumping system must deliver solvent up to 500ml/mn with a pressure limit about 1000-2000 PSI. It generally consists of a reciprocating pump although other types are used. A loop injector, equipped with a large-volume loop (10-100ml) is commonly used. Detection is achieved either by a specific low-sensitivity detector designed for preparative purposes, or by a classical analytical detector with a stream splitting device, to enable only 1% or less of the mobile phase to flow through the detector.

As it is usual to load a preparative instrument higher than an analytical one, concentrations encountered at the outlet of the column should require a low-sensitivity detector. When using a UV spectrophotometric detector, it is possible to reduce sensitivity by tuning to a wavelength away from a strong adsorbance band. This method still allows detection in a linear mode. At the outlet of the detector, a fraction collector is used for solute collection. It is commonly equipped with a siphon counter, isolating fractions as a function of volume or time. The schematic diagram of the Waters Chromatoprep® is given in Figure 6. Fittings and tubing are not as critical as with analytical GPC, regarding the large internal volume of preparative columns. It is relatively easy to design, in the laboratory, a preparative instrument for a particular purpose, by connecting together the above described components and home-made preparative GPC instruments are often described in the literature.

The first commercial preparative GPC instrument was the Waters "Ana-prep®" (5,24) but it is no longer available. Barlow (25) designed a preparative GPC where columns are placed in an oven at temperatures from ambient to 150°C. Four foot length by 2.4 in. O.D. columns, packed with Styragel® of different poro-

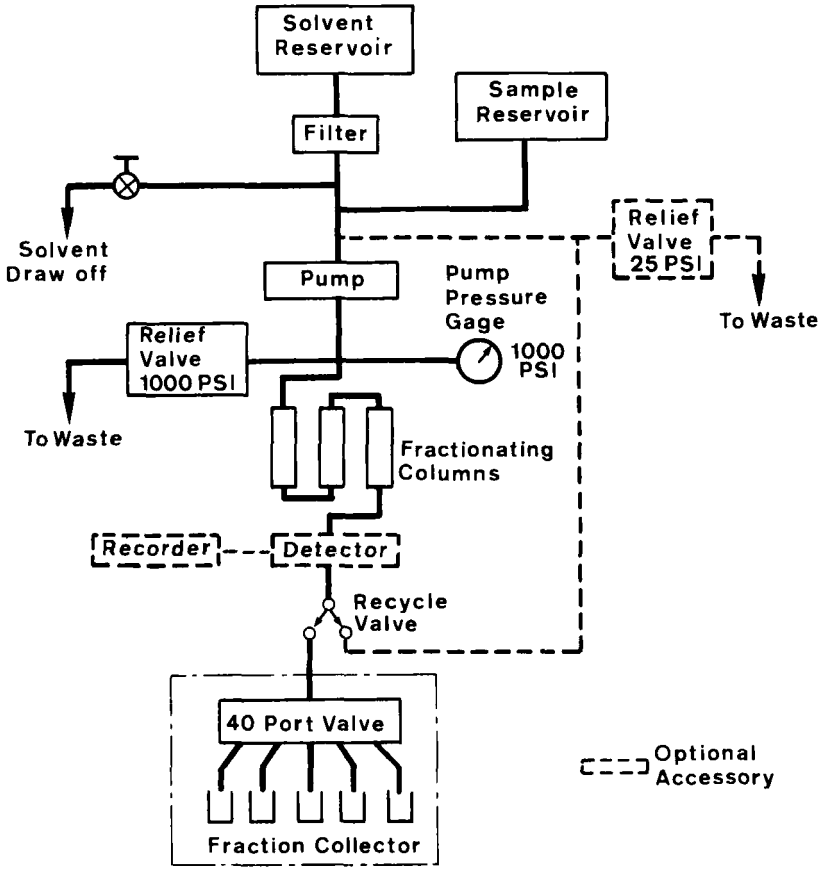

Figure 6 : Schematic diagram of Chromatoprep. (Reprinted with permission from ref. 69).

sities, were used. Styrene-butadiene rubbers were fractionated using benzene at ambient temperature and ethylene-vinylacetate copolymers using xylene at 60°C. One gram of polymer was fractionated at each injection. Peyrouset and Panaris (26) have constructed a preparative chromatograph suitable for fractionating samples of the order of 1 gram per injection. The sample introduction system consists of a 100ml sampling loop and five two-way elec-

trovalves allowing the filling of the loop with the polymer solution and its introduction into the columns. Columns were stainless steel tubes 122cm long and 60mm in diameter. They were packed with a porous silica (Spherosil ®) having particle diameters ranging from 100 to 200µm and of various porosities (100 -4 000 Å). The efficiency was around 3500 plates per column. Using THF as mobile phase, polystyrene, polyvinylchloride and polybutadiene were fractionated into narrow fractions. Increasing the temperature to 150°C with 1,2,4-trichlorobenzene as solvent, the same authors (68) performed the fractionation of a high density polyethylene into narrow fractions for calibration of analytical GPC of polyolefines. Montague (27) describes an apparatus with 4 ft by 1 inch O.D. columns, filled with cross-linked polystyrene gels. A room temperature preparative instrument was commercialized by Waters Assoc. (5) allowing recycle operation (69-70) : the chromatoprep ® (Figure 6). It is equipped with a recycle valve and a 40-port valve fraction collector. This instrument has now been replaced by a preparative liquid chromatograph, the Prep LC 500 A ®, specially designed for radially compressed columns. This technique being inconvenient for semi-rigid GPC packings, Nevertheless, this instrument can be employed with classical preparative columns.

For industrial applications, a continuous chromatography process was described by Barker (71-72) where the movement of the packing is achieved by column switching using the moving port principle (73). Dextran samples were fractionated with this countercurrent chromatography on glass columns (5.1cm I.D.- 70cm long) filled with Spherosil ® XOB 075.

Finally, let us mention the industrial large scale gel filtration units (21-23) where kilograms are treated in a single operation. They are equipped with wide Sephadex ® columns and are mainly devoted to natural product purification.

III.4. Experimental

One of the most critical parameters in preparative GPC is the sample loading because one always tries to obtain the maximum amount of purified substance in a single experiment. This has been recently discussed by Vaughan (72). The effect of sample concentration on the GPC hehaviour of polymers has been widely studied (75-80). Moore (81) introduced the idea of "viscous fingering" that describes peak deformation when severe over-loading occurs. This phenomenon, caused by the difference of viscosity between the solvent zone and the solute zone is accompanied by macromolecular compression where polymer coils do not interpenetrate or intermingle with each other, but shrink (82). In addition, hydrodynamic volumes of solvated polymer molecules are dependent on their concentration (83-84) which can be described by theoretical models (85-87). The viscosity of polymer solutions is responsible for variations in elution volumes (76,88-89), the higher the concentration, the greater the elution volume, especially as the molecular weight increases. Loss of resolution is also observed.

Cooper (90-92) studied experimental variables in preparative scale GPC. He reported that, at higher concentrations, the polydispersities of the high molecular weight fractions are not affected, whereas the tow molecular weight fractions have increasingly higher polydispersities as the sample concentration increases. In order to obtain resolution comparable to analytical experiments, low loadings were used by Kato (93) for the fractionation of a certified material, polystyrene NBS SRM 706 with TSK-gel® columns using a θ solvent (methanol/butanore). (Figure 7). He showed that polydispersities of fractions are much better (1.01-1.03) under these conditions than on Spherosil® or Styragel® columns with THF as solvent (1.17-1.30). When increasing the amount of injected polymer, there is a critical sample size beyond which resolution falls below its analytical level. Kato (94-95) showed that, for polystyrene on polystyrene columns, when using a θ sol-

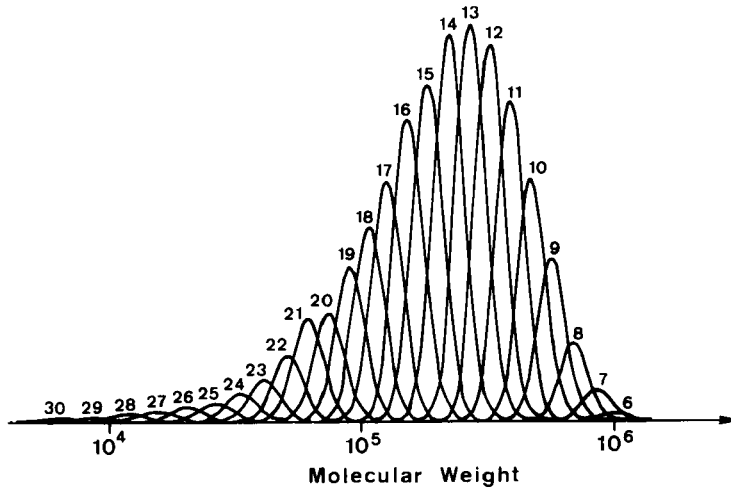

Figure 7 : Molecular weight distribution curves of fractions from polystyrene NBS 706. (Reprinted with permission from ref. 93).

vent, this critical sample size is larger. The polydispersity of fractions rises from 1.02 to 1.15-1.20, then does not vary significantly when increasing sample size further. Consequently, only small amounts of isolated fractions, with very low polydispersity, can be obtained by preparative GPC on a preparative column with the extrapolated analytical conditions. But, when very sharp distributions are not required, it is possible to obtain materials with less narrow distribution (1.1-1.2) in a much higher quantity by considerably increasing the sample size.

The injection volume is another important parameter of preparative GPC (96). Basically, increasing injection volume will produce a significant loss of resolution, but when high loads are used, viscous effects can predominate. It was found (93) that, at a given sample amount, the polydispersity is greater with an injection volume of 10ml than one of 20ml.

Obviously, this effect becomes more significant the higher the molecular weight. As a general rule, it is better to inject low-concentration solutions, even though the injected volume is large, than to increase concentration in a small sample size. Usually, injection volume can be as high as 1 to 5% of the total volume of the columns.

The flow rate is not a determinant parameter in preparative GPC. It is well-known that increasing the flow rate will produce a moderate loss of resolution. Bombaugh (70) found a quasi-linear decrease of resolution between two monodispersed polystyrene standards when increasing the flow rate. On the contrary, increasing the flow rate will save time. Unfortunately, the pressure at the column inlet will rise to a high value, requiring a high pressure instrument. The maximum pressure of the installation is probably the limiting factor when considering an increase of flow rate if we consider that increasing the pressure in large columns could be very hazardous because of the solvent compressibility and the risk of damaging the packing. Cooper (92) uses the same linear flow rate velocity as in analytical experiments and increases throughput by increasing up to three times the flow rate normally used.

An other disadvantage of increasing flow rate is the risk of polymer degradation, particularly inconvenient in preparative GPC. Rooney and Ver Strate (97) found that polymer degradation occurs at flow rates as low as 0.3ml/mn in high performance columns for high molecular weight polymers. This effect was confirmed by Huber and Lederer (98) with high molecular weight polyisobutene at even lower flow rates.

When repetitive injections, followed by accumulation of cut fractions are achieved, injection parameters (sample size, concentration) and flow rate must be severely controlled and kept constant to avoid chromatogram shift and combining of non-identical fractions.

Finally, an important problem in preparative GPC is in handling large volumes of the mobile phase. When handling 10 to 50 liters of solvent, one is faced with the subsequent problem of safety, particularly when the solvent is inflammable. After collection, a classical technique for removing polymers from solution involves precipitation by addition of a miscible non-solvent. The polymer is then filtered and dried. But the mixed solvents cannot be recycled. This is disadvantageous when working with large quantities. One generally prefers to remove the solvent by distillation. In this case, the solvent must be previously purified since, when it contains non-volatile impurities, they are concentrated in the fractions during this operation. In order to obtain the fractionated polymer with suitable physical characteristics, further purification is required. The dry polymer is redissolved in a small amount of another solvent, the solution is filtered, then freeze dried. Polymer recovery is excellent and usually exceeds 90-95%.

IV. APPLICATIONS TO POLYMERS

IV.1. Synthetic polymers

The main purpose of preparative GPC of synthetic polymers is the production of narrow distribution materials, since except in the particular case of anionic synthesis, broad molecular weight distributions are obtained. Polystyrene has often been fractionated for testing the efficiency of the preparative method (26-27,70,92,99-102). Using the reference polystyrene NBS 706, Kato (93) obtained 25 fractions of different molecular weights with polydispersities smaller than 1.03 (Figure 7). Narrow fractions were also prepared with polyvinylchloride (26,102-103), polydimethylsiloxane (104), polymethylmethacrylate (101), polybutadiene (26,102) and polytetramethyleneglycol (28) using THF as solvent. The particular conditions required for the GPC of polyolefines (GPC at 135°C-150°C in trichlorobenzene or o-di-

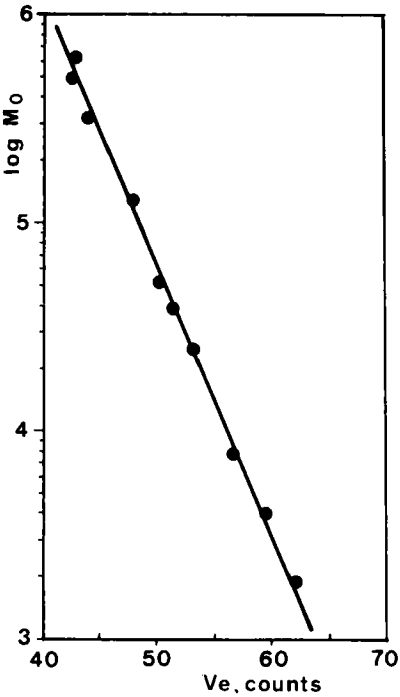

Figure 8 : Calibration curve for narrow fractions of polyethylene. (Reprinted with permission from ref. 68).

chlorobenzene) led an industrial laboratory to produce reference materials to be used as standards in analytical GPC. By fractionating a broad high density polyethylene sample prepared by the Philips procedure, Peyrouset (68) obtained 9 different polyethylene fractionswith molecular weights from 2,000 to 700,000 and polydispersities from 1.06 to 1.20 (Figure 8). The production was achieved with a home-made instrument (26) using TCB at 150°C and large columns packed with Spherosil®. Using a Waters Ana-Prep®, equipped with Styragel® columns in 1,2-dichlorobenzene, Vaughan (101-105) performed the preparative fractionation of polypropylene and produced 11 reference samples covering the molecular weight range of 10,000-600,000. The polydispersities of these materials

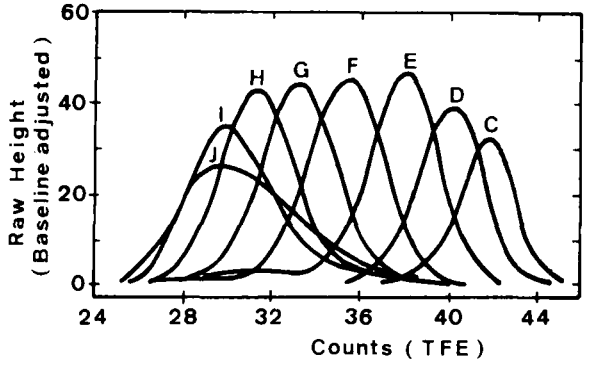

Figure 9 : Chromatograms in TFE of PMMA fractions. (Reprinted with permission from ref. 106-107).

although approximately 1.5, increase with molecular weight, a reflection of the increasing difficulty of fractionating higher molecular weight materials. Finally, in order to calibrate the analytical GPC for polyamides and polyacrylates in 2,2,2-trifluoroethanol (TFE), Provder (106-107) obtained 8 fractions of polymethylmethacrylate in the molecular weight range of 16,000-1,370,000 (Figure 9). Polymers, prepared by routine free-radical bulk and solution polymerization methods to cover a wide molecular weight range, were fractionated with a Waters Ana-Prep ® equipped with a preparative column of Styragel ® 10^4 Å in THF.

Narrow fractions can also be semi-preparatively isolated for further analysis. Copolymer composition, along the MW distribution, was studied by Barlow (25) for ethylene-vinyl acetate copolymers and styrene-butadiene rubbers. The fractions were analyzed by IR spectroscopy for the chemical composition, by analytical GPC for their molecular weight and by viscosity measurement for the determination of the degree of branching ; this allows for a complete characterization as a function of molecular weight (Figure 10). In the same way, Mirabella (108) determined the copolymer composition as a function of molecular weight by preparative

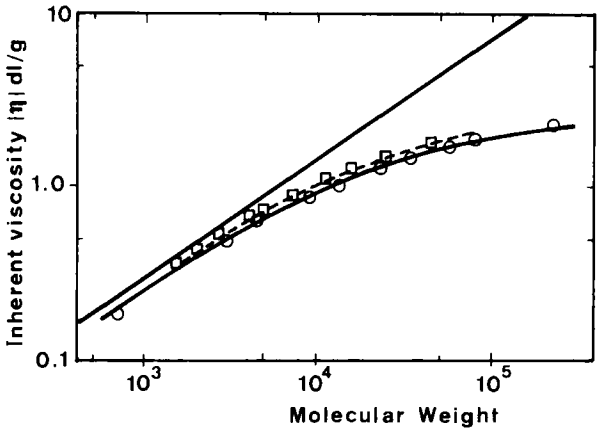

Figure 10 : Viscosity-molecular weight relationship for vinyl-acetate copolymers obtained by fraction analysis. ——• linear polymer ; o—o copolymer A ; □----□ copolymer B. (Reprinted with permission from ref. 25).

GPC of poly(styrene-co-vinylstearate) and IR spectroscopy of the isolated fractions. Using the same technique, Mirabella (109) studied the 1,2, 1,4-cis and 1,4 trans bond contents in a polybutadiene sample and deduced the polymer microstructure units. Low molecular weight carboxy-polybutadiene liquid polymerswere fractionated by Law (110) and analyzed by infrared and chemical analysis. In addition, these narrow fractions were characterized by Vapor Pressure Osmometry and have been used as calibration standards for analytical GPC. Polymer tacticity can also be studied by preparative GPC of poly(2-methoxy-ethyl-methacrylate) (111), NMR and GPC analysis determine the isotactic and syndiotactic components.

When a very sharp distribution is not necessary, a crude procedure, but with a moderate loss of sample is advantageously applied. Heart-cut technique (or shaving technique), described in Figure 11 consists in isolating the heart of the

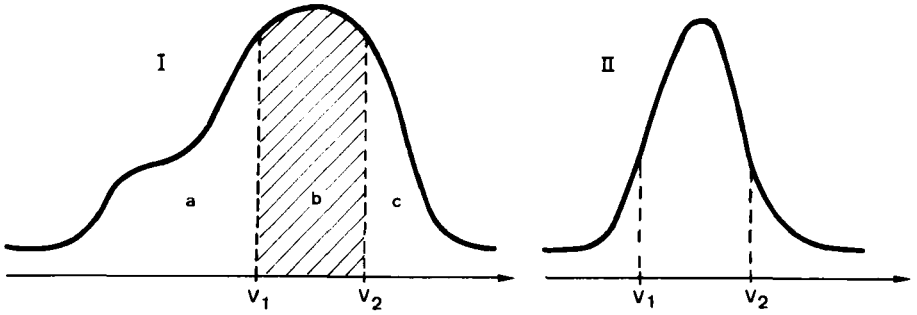

Figure 11 : The heart-cut technique. I. Whole polymer cut into 3 fractions. II. Re-injection of fraction b.

distribution by "shaving"its wings. The wider the cut, the better the yield but the higher the polydispersity of the purified polymer.

Because of its simplicity, preparative GPC also appears to be a very attractive sample clean up prodecure, associated with analytical techniques such as HPLC. A polymer can be isolated from its low molecular weight contaminants or high molecular weight impurities by taking a wide heart-cut (Figure 12). Conversely, one may be interested only in the components contaminating the polymer, such as additives (plasticizers, dyes, stabilizers, etc ...) for their further analysis or quantitation. Since additives are generally low molecular weight compounds, prior to their HPLC analysis, a rough preparative fractionation of the crude polymer is achieved by GPC. In this case, good resolution is not required since small molecules are easily separated from the polymer ; high loads are conveniently injected for the preparative purification (Figure 12). A typical application is given by the preparative separation of pesticides from polymeric natural media such as lipids (112) or cheese and milk proteins (113) for their further HPLC or gas chromatographic quantitation. The

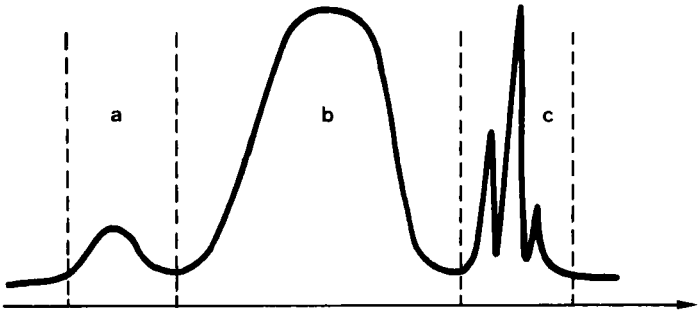

Figure 12 : Clean up procedure of a crude polymer into 3 fractions : a = high molecular weight impurities, b = polymer, c = additives.

METHOD OF OPERATION	
Recycle to collect sequence (V2 in recycle pos.)	Collect to recycle sequence (V2 in collect pos.)
1. Turn pump and recorder off	1. Turn pump and recorder off
2. Flush with approximately 15mls of solvent by opening & closing V1	2. Turn V2 to "recycle" position
3. Turn V2 to "collect" position	3. Flush as "recycle to collect' sequence
4. Turn pump and recorder on	4. Turn pump and recorder on

separation is usually performed on Biobeads gel using cyclohexane as mobile phase (114). The pesticide isolation technique has been recently improved by the use of Styragel® columns with diisopropylether by Tillier and Devaux (115). In the same way, complex flavor mixtures have been separated and analyzed by combined GPC and HPLC (116).

IV.2. Biopolymers

The situation for biopolymers is appreciably different from other polymers. The only applicable mobile phase is water and biopolymers fairly often present a narrow distribution, that brings their GPC analysis nearer to classical liquid chromatogra-

phy. Historically, Gel Filtration has been a method of choice for biopolymer chromatography (117-118). A very significant segment of literature is devoted to the purification of proteins, enzymes, polypeptides, snake venoms, virus, sugars, etc ... The purpose is generally preparative and can sometimes approach the industrial level. Biochemists favor this method, using a soft-gel column which does not need sophisticated and expensive instruments. However, the gels do not withstand the high pressure required for high speed analysis, and very low flow rates should be applied; this leads to long experimental times. Nowadays, the trend is to replace Gel Filtration by GPC using modern aqueous packings. The developments of aqueous GPC were reviewed by Cooper (119) and Barth (120). Adsorption of protein molecules occurs on mineral packings ; controlled-pore glass beads grafted with glycerol were developped to prevent this adsorption (121-124). Engelhart (125) produced chemically bonded stationary phases based on porous silica grafted with various functions. Preparative separations of proteins and enzymes were performed in the mean molecular weight range of 10,000-100,000 on Lichrosorb-diol® packing by Roumeliotis and Unger (126) using 1 inch I.D. and 25cm long columns at a flow rate of 20ml/mn in a buffered eluent of pH 7.5 at a relatively high ionic strength. The separation is achieved within 6 minutes whereas the same separation requires several hours with a gel filtration column. Moreover, the 30mg of separated protein is eluted in only 20ml whereas Gel Chromatography provides highly diluted solutions. Kato (127) studied the performances of the grafted silica TSK gel SW® which is very efficient for protein separation (128-129). An organic cross-linked gel (TSK gel PW®) is described by Hashimoto (130) for protein separations. With a 600 x 21.5cm I.D. TSK G 5000 PW® preparative high performance column, Himmel and Squire (131) reported the characterization of proteins, viruses, ribosomes and polysaccharides such a southern bean mosaic virus, tomato bushy stunt virus, tobacco mosaic virus, turnip yellow mosaic virus, bovine heart myoglobin, human hemoglobin, sea worm chlorocruorin, cytochrome c, apoferritin, pig

thyroglobulin and bovine serum albumin. The solvent used was 10mM phosphate pH 7 buffer in 100mM KCL.

With regard to the broad range of applications of this new generation of packings and as a result of the time saved during separations, we can predict that high performance rigid packings will gradually replace soft gels in Gel Filtration Chromatography of biopolymers and other natural products.

A good example is given by the purification of influenza virus, performed on an industrial scale in the Institut Pasteur in Paris (132). The column, 10cm in diameter and 120cm in length, is packed with Spherosil® XOB 030 (100-200µm), the flow rate is 150ml/mn and the injection volume is 500ml. This installation is automated and achieves the purification of 12 liters of influenza virus concentrated solution per day.

V. APPLICATION TO SMALL MOLECULES

Since its beginning, GPC has been applied to small molecule separations (133-137). It was shown that, as for polymers, low molecular weight substances were separated on the basis of their molecular volume but their elution behaviour was more complicated than for polymers. Factors affecting the GPC of small molecules, mainly solvent-solute interactions caused by hydrogen bonding (138-139) and adsorption of aromatic compounds on alkylated dextran gels, have been mentioned (140-143). In fact, solvent-solute-gel interactions cannot be neglected in these systems and steric exclusion sometimes is not the only separation mechanism. The solute dissolution into the gel or its adsorption onto the surface can occur and lead to abnormal elution volumes (29,30,144-149). The calibration curve is rarely universal, but nevertheless can be constructed for a family of homologous compounds for molecular weight determination.

For these reasons, the technique of GPC, applied to small molecules is closer to HPLC than to the GPC of polymers. Because of its operational simplicity, GPC lends itself as an efficient method for the isolation of traces or larger quantities of substances from chemically complex mixtures for subsequent analysis or identification. In addition, organic cross-linked gels have a greater capacity than inorganics ; this is very advantageous for preparative work. Sample volume and concentration should be adjusted to prevent undesirable overloading which can destroy resolution. Classical microcolumns such as Micro-Styragel® (5) packed into wide bore tubes (7.8mm) are well suited for sample sizes of less than 1 gram (150-151). Another advantage of organic gels with regard to inorganic ones, is their selectivity in the low molecular weight region because of their smaller pore volume available. Small organic molecules can be separated in organic liquids on conventional gels such as Bio-Beads® or Sephadex® LH 20 (152) or on high speed gels (10-11,151,153), but also oligomers (152,155-157) and polymer additives (157-160).

Because of the high concentration of sample in the eluent, sample clean up and fractionation of complex samples for further analysis can be performed. Shoemaker (154) isolated a contaminant material from a polymer for infrared identification.

The use of porous silica microspheres in high performance size exclusion chromatography of small molecules was described by Kirkland (161). The particles used were made with small pores (< 100 Å) to optimize separations, but the injected quantities are smaller than for organic packings and consequently, preparative work is more difficult.

To increase efficiency, Heitz (162) recycled the mobile phase and obtained good resolution in the separation of oligomers of styrene, butyl-methacrylate and ethylene oxide. A special design for automatic recycling was described by Bombaugh (163-164). (Fi-

gure 13). The recycling device consisted of a 6-port valve connecting the outlet of the detector to the inlet of the pump. When the valve is in the "recycle" position, the eluate flowing from the detector is continuously reintroduced into the column via the pump. In the "collect" position, the eluate passes directly into a glass siphon counter, which delivers constant volumes into a fraction collector. This system provides a very high resolution without increasing the number of columns and accordingly, the solvent volume to be used and the pressure in the pump. The solute passing through the detector and the pumping system at each cycle, the internal volume of the pump must be carefully minimized, but the chromatogram is recorded at each cycle as a display of the progressive status of the separation. There is no practical limit to the increase of resolution by adding columns and recycling except the time. The maximum resolution is obtained when the fastest-moving peak overlaps the slowest with resultant remixing. In this case, if the separation is not completely achieved, it is possible to add columns and increase the number of cycles and, if the pressure increases too much, the flow rate must be set lower with the subsequent increase of the separation time . In the case of two components, Lesec (165) established that with the relative difference between elution volumes $\delta = \Delta V/V$, the plate number N of the column set to be used is given by

$$N \geq 32\ R^2/\delta$$

where R is the required resolution ($R \simeq 1.5$). The subsequent number of cycles to be run is given by

$$n \simeq 16\ R^2/N\delta^2$$

that gives the optimal number of cycles

$$n_{opt} = 0.5/\delta$$

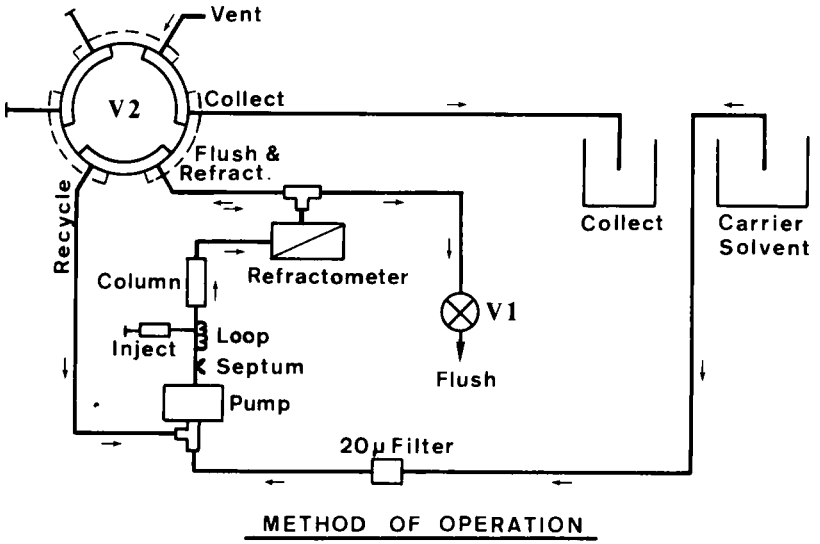

Figure 13 : Schematic diagram of recycle operation. (Reprinted with permission from ref. 69).

also calculated by Kalasz (166).

Oligomers (70,164,167-168) and organic small molecules (163-166) have been separated using this recycling technique. Conroe (169) used a 25mm I.D. and 91cm long column filled with Styragel® 70 Å (37-75mμ). The sample was 1g octanoic acid and 1g nonanedioc acid dissolved in 10ml of T.H.F. It took about 2 hours at a flow rate of 9.9ml/mn for the complete separation.

With very high resolution, it becomes possible to separate small organic molecules with very similar structures, such as isomers. Acetal-alcohol diastereoisomers were successfully separated at a gram scale by Lesec (170-171) for further spectroscopic analysis. It was found that hydrogen bonding between solutes and solvent participated to the separation, and when THF was replaced with di-isopropylether, better resolution was obtained.

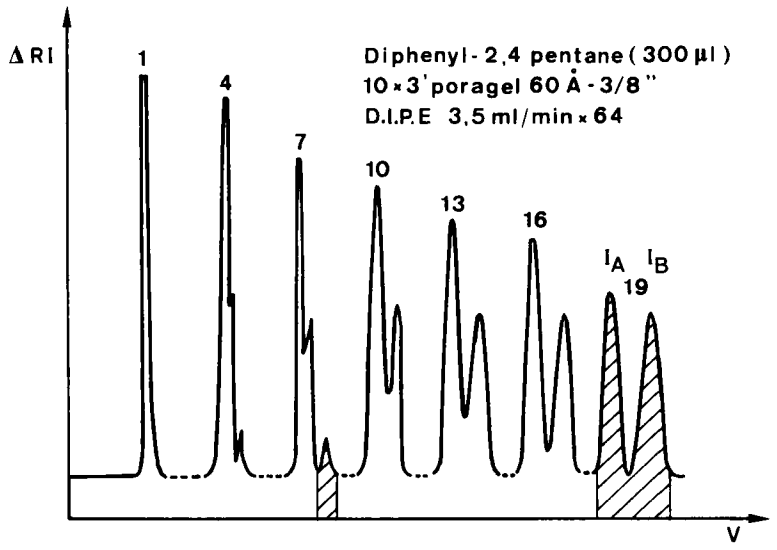

Figure 14 : Recycle separation of configurational isomers of 2,4-diphenylpentane. (Reprinted with permission from ref. 172).

Using the same chromatographic system, configurational isomers were also resolved (172-173) on a gram scale (Figure 14). The molecular structure of oligomers of polystyrene and polyacrylophenone are similar to that of the cross-linked polystyrene gel. The separation was explained by the participation of interactions between the solutes and the gel, enhanced by the use of di-isopropylether as mobile phase which is a poor solvent for polystyrene.

Another reason for the use of di-isopropylether is a practical one. After their chromatographic separation, compounds are collected in a very dilute solution and the solvent must be evaporated off. THF cannot be handled at asufficient degree of purity because it generally contains non volatile impurities that become concentrated in the collected fractions and interfere with further spectroscopic studies. Di-isopropylether does not have

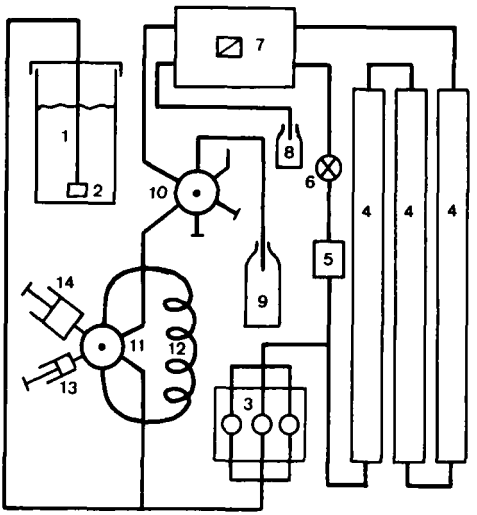

Figure 15 : Preparative instrument. (Reprinted with permission from ref. 165).

1. Solvent reservoir - 2. Filter - 3. Pumping system - 4. Columns - 5. Pressure transducer - 6. Valve - 7. Differential refractometer - 8. Waste - 9. Fraction collector - 10. Recycle 6-port valve - 11. Injection 6-port valve - 12. 100ml loop - 13. Sample syringe - 14. Solvent syringe.

this drawback, but as it swells polystyrene gels less than THF (about 15% less), columns have to be directly packed in this solvent by the slurry technique.

A preparative instrument was designed (165) on the same basis, but at a higher loading scale (Figure 15). Three columns (150cm length and 2.6cm I.D.) were packed with Styragel® 100 Å, particle size 15-25µm, in di-isopropylether. The yield in grams per minute was checked as a function of difficulty of the separation. (Figure 16). For easy separations (α>2) yield is greater than a few grams per minute, but quickly decreases as difficulty increases. It is only 0.4g/mn for α = 1.25 and becomes very low for very difficult separations (0.04g/mn for

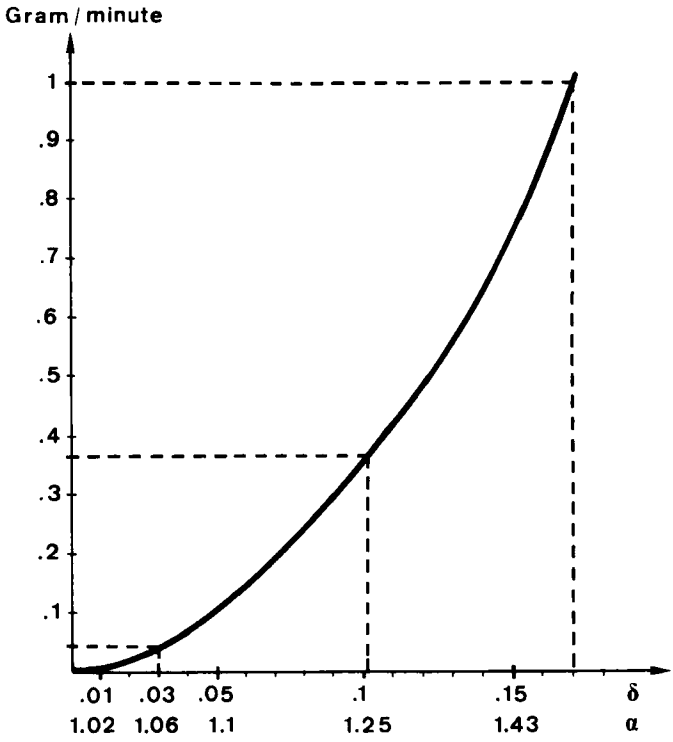

Figure 16 : Yield of preparative GPC as a function of separation difficulty. (Reprinted with permission from ref. 165).

α = 1.06). The sample size can reach 100grams for very easy separations.

The scope of applicability of this instrument is very broad since it combines size exclusion and partition. The advantages are :

- only one volatile solvent (di-isopropylether), convenient for sample recovery.
- a high capacity gel, very efficient in the low molecular weight region.

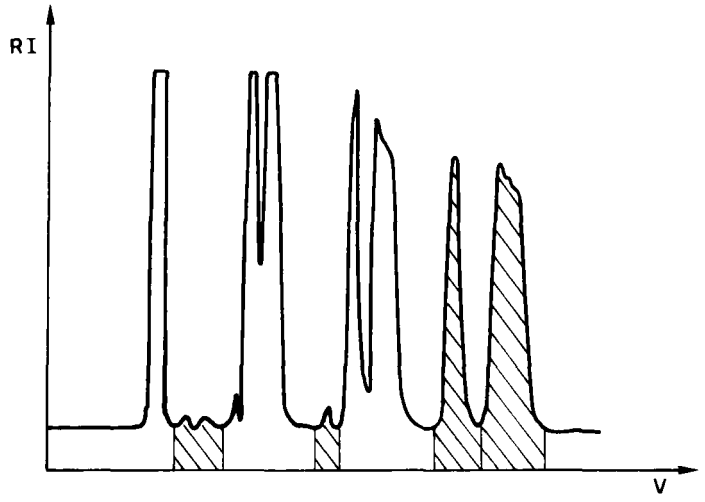

Figure 17 : Recycle chromatogram of DVB-EVB mixture. Flow rate 40ml/mn. Sample size : 30 grams. (Reprinted with permission from ref. 165).

- a moderate quantity of mobile phase because of recycle capability.
- a yield of few grams per minutes with loadings up to 100grams for easy separations.

The disadvantages are :

- no commercially Styragel® column is packed in this solvent and columns must be home-filled.
- the solute must be soluble in di-isopropylether.

Many isomeric mixtures (diastereoisomers, configurational isomers, etc ...) were fractionated. A good example is given by the purification of crude divinylbenzene (D.V.B.), a mixture of divinylbenzene and ethylvinylbenzene (E.V.B.). At low efficiency, a sample loading of 30grams leads to the complete

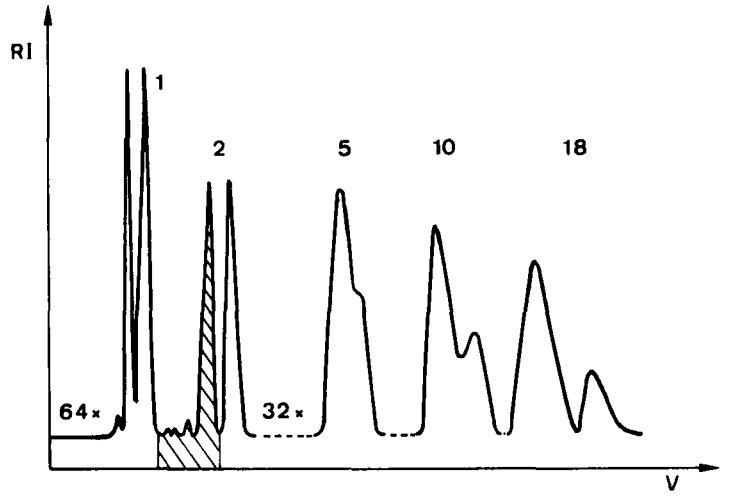

Figure 18 : Recycle chromatogram of DVB-EVB mixture. Flow rate 40ml/mn. Sample size : 5 grams. (Reprinted with permission from ref. 165).

separation of the two components with a yield of 0.19g/mn. (Figure 17). But at high resolution with only 5grams injected, 18 cycles provide the separation of meta and para isomers of DVB at only 7mg/mn (Figure 18). The EVB peak was collected at the 2nd cycle.

Finally, let us mention an alternate approach for recycling. The alternate pumping principle (167-168 ,174) uses two columns and a 6-port valve for switching the columns (Figure 19). When eluting from one column, the solute is introduced into the other column via the valve without passing through the pump. Following elution from the second column, the valve is switched and sample introduced again into the first column. This procedure is repeated to recycle sample alternately through the two columns. The advantage of this procedure is that dead volumes are reduced to a minimum, preserving the complete resolution of columns ; but, two column sets are required instead of one and an automatic switching device must be installed for convenient operation.

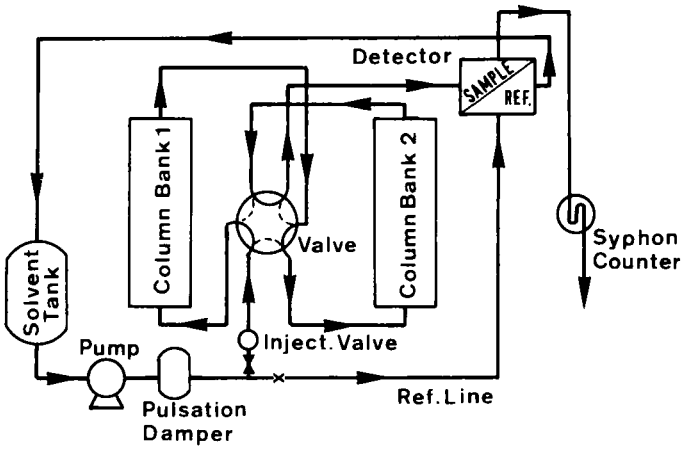

Figure 19 : Schematic diagram of the alternate pumping recycle system. (Reprinted with permission from ref. 168).

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