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Aqueous size exclusion chromatography of hydroxyethyl-amylopectin

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A modified starch component, hydroxylated amylopectin (HAP), was analysed by a low angle laser light scattering apparatus used with high-performance steric exclusion chromatography. The correct weight-average molecular weight and molecular weight distribution (MWD) have been obtained and compared with calibration methods used in conventional chromatography. The modified amylopectin was first chemically scissored and afterwards fractionated using a low-pressure apparatus with crosslinked agarose gel and with a fraction collector on line. For comparing with conventional methods the calibration curves were obtained from polyethylene oxide and polysaccharides provided by Polymer Laboratories. The HAP fractions were separated using a three-columns system and degased buffered filtered water as the mobile phase. This paper discusses the different valuable methods of calculations leading to the molecular weight and MWD characterisation. Failure in the universal calibration is observed for grafted carbohydrates.

INTRODUCTION

The fast and accurate characterisation of hydroxyethyl starch (HAP) has already been the object of numerous studies (Sommermeyer *et al.*, 1987; Kobayashi *et al.*, 1985; Praznik *et al.*, 1985). At first, low-pressure size exclusion chromatography (SEC) has frequently been used but the analysis required several hours per sample. The developments of high-pressure-resistant phases, either porous glass or synthetic gels have allowed a decrease in the measurement times.

Determination of molecular weight by SEC requires a calibration curve for which a set of standards are usually used.

To resolve the main problem of the calibration, several methods are possible.

- A universal calibration relating the elution volume, V_e, to the hydrodynamic volume, $[\eta] M_w$ of fractionated standards of chemical nature different from that of the sample under study (Benoit *et al.*, 1967).
- A direct calibration obtained with fractions of the same polymer. The fractionation of HAP can be performed by precipitation from aqueous solution with acetone and isopropyl alcohol or by preparative SEC (Sarazin *et al.*, 1992).
- The direct coupling with a mass molecular weight detector such as a low angle laser light scattering (LALLS) (Kaye, 1973; Ouano & Kaye, 1974; Ouano, 1976). This technique has been very useful for the characterisation of various hydrosoluble polymers such as dextrans, poly(ethylene oxide) (PEO) or polysaccharides (PSA) (Jordan, 1980; Kim *et al.*, 1982).

However, the fractionation is a long and difficult process in the absence of commercially available fractions of HAP. On another hand, LALLS apparatus is very expensive and very difficult to use continuously in aqueous medium for current analysis.

The first method remains the most simple and the aim of this paper is to establish its limitations, by systematically comparing results obtained from the

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three methods and also from conventional multi-angle light scattering.

The different processes are at first tested with commercial standards and narrow HAP fractions and then with a primary industrial batch of HAP200.

EXPERIMENTAL

Materials

Depolymerised HAPs were obtained from Roquette Frères SA: HAP1 with a molar substitution of 0·5 (MS) for HAP were used. HAP1 fractionation was performed on a preparative low-pressure SEC using ACA 22 gel from ICS. A set of fractions (HAP1 F1–F6) were studied (Sarazin *et al.*, 1992).

The PEO and linear PSA (pullulan ref: SAC10) were standard samples from Polymer Laboratories (Church Stretton, Shropshire, UK). Dextrans samples were supplied by the authors' laboratories.

Methods

Analytical SEC

The SEC device includes a model LCII Shimadzu pump, a Waters injector and two detectors: a differential refractometer (Waters R410) as a concentration detector and a LALLS apparatus (KMX Chromatix) as molecular mass detector. Three columns from Shodex TSK SW4000 SW3000 PW60 were used.

The data issued were analysed and lead to straightforward absolute molecular weight determinations.

It is assumed that the resolution power of these columns is high enough to minimise the axial diffusion effect as already mentioned by Tung and Runyon (1969) and Hamielec and Meyer (1986). This will be discussed later on.

The solutions prepared for SEC were heated to 70°C for 12 h before injection. The volume of the injector loop was 200 μl. The aqueous mobile phase was a 0·1 M sodium acetate ($NaCH_3CO_2$, at pH6) tridistilled water solution and filtered on millipore 0·5 μm fluoropore.

LALLS

The SEC device is coupled with a LALLS apparatus Chromatix CMX100. The scattered intensity (I_i) measurements are made at a *diffusion* angle θ 5·1–6·1°.

Conventional elastic laser light scattering (MALS)

MALS measurements were performed at 37 ± 0·1° on two home-built automatised photogoniodiffusiometers (Libeyre *et al.*, 1981) equipped with laser sources λ_1 = 632 nm and λ_2 = 488 nm. The angular dependence was measured within the scattering angle range 30°C and 150°.

Polymer was dissolved (2×10^{-4}–2×10^{-3} g/cm^3) in buffered solvents (pH 7 phosphate buffer) containing 400 ppm of sodium azide prepared with a prior tridistilled water. The complete solubilisation is obtained after 24 h stirring and by heating during 4 h till 60°C. The solutions were made clear by centrifugation at 15000 rpm for about 4 h.

The data were analysed by computer using the classical Zimm procedure and a linear polynomial fit to the classical equation:

$$\frac{Kc}{\Delta I_\theta} = \frac{1}{M_w < P(\theta) >} + 2A_2c \qquad (1)$$

where

$$P(\theta) = 1 + \frac{q^2}{3} < R_G^2 > + Bq^4$$

where ΔI_θ is the scattered intensity excess between the solution and solvent intensity; c is the polymer concentration in g/cm^3; M_w is the weight-average molecular weight of the solute; q is the scattering vector $q = (4\pi n \sin(\theta/2))/\lambda$; A_2 is the second virial coefficient; $< R_G^2 >^{1/2}$ is the Z-average radius of gyration; θ is the scattering angle; and K is an optical constant proportional to the square refractive index increment $(dn/dc)^2$ (see eqn (12)).

The data analysis is often made by neglecting the third term of $P(\theta)$ which is justified when the angular dependences are linear.

Refractometry

The dn/dc values which play an important role in the determination of M_w by light scattering, have been determined with two different devices.

The first one, is a classical Brice–Phoenix apparatus operating with red light (λ = 632 nm) at polymer concentrations larger than 5×10^{-3} g/cm^3.

The second one is a homebuilt refractometer based on the same principle with a higher sensitivity photoelectric detection (Sarazin François, 1988). The second apparatus operates at a lower concentration and allows dn/dc measurements on solutions prepared for light scattering experiments ($10^{-4} \leqslant c \leqslant 10^{-3}$ g/cm^3).

The refractometry measurements were carried out at the Donnan equilibrium of the polymers and after dialysis.

The dialysis equilibrium were obtained with a set of home-made cells of equal volume. Each set of two cells of 2 ml containing in one part the solvent separated from the solution by a cellulose dialysis membranes from spectrapor with a molecular cut-off less than 5000. In a second set of experiments, we have calculated the dn/dc values from the integration of the peak given by the on-line differential refractometer. Besides, if the dn/dc value is well known, this method ascertains the

lack of polymer adsorption on the set of columns. Secondly, the measured dn/dc may be the true $(dn/dc)_\mu$ as in osmotic equilibrium. In fact the columns play the role of the dialysis membranes.

For the HAP fractions a value of 0·142 cm³/g has been measured with the differential refractometer on line calibrated with POE fractions of dn/dc = 0·138 cm³/g.

Viscosimetry

An automatic viscosimeter of the Gramain–Libeyre type (Gramain & Libeyre, 1970) regulated at ± 0·1°C and equipped with a 0·43 mm diameter capillary was used. In this type of viscosimeter the shear rate is around 1000 s for dilute aqueous solution. This has been checked by using a low shear rheometer from Contraves (LS 30) that all the solutions are Newtonian within the investigated range of concentration. Intrinsic viscosity is calculated from the extrapolation of the reduced viscosity at $c = 0$ according to

$$\eta_{red} = [\eta] + K'[\eta]^2 c \qquad (2)$$

where c is the polymer concentration (g/cm³) and K' is the Huggins constant.

Osmometry

The number-average molecular weight have been determined by a Mechrolab Model 502 device operating in 0·1 M NaCl at 25°C. The semipermeable membranes from Millipore were made of cellulose fibres. The number-average molecular weight are issued from the following calculations:

$$\lim_{c_p \to 0} \frac{(\Pi/c)}{} = \frac{RT}{M_n} \qquad (3)$$

where Π is the osmotic pressure; c is the solution concentration; R is the gas constant; and T is temperature in Kelvin s.

THEORETICAL

The one-detector gel permeation chromatography (GPC)

In this case, refractometry is the usual technique.

M1

The method based on the molecular weight calibration needs well-characterised fractions of the polymer under study. The calibration curve is usually written as

$$\log (M_{top}) = a V_e + b \qquad (4)$$

where M_{top} is the molecular weight of the fractions with narrow molecular weight distribution (MWD), and the V_e at the top of their peak. By neglecting the possible effects of adsorption and axial dispersion, the partition of the chromatographic peak allows the direct integration of each mean molecular momentum: M_w and M_n.

M2

The Tung and Runyon (1969) method takes into account the axial dispersion effect by introducing the σ_{chr} parameter which characterises the half broadening of the peak and the axial dispersion ratio σ^2/a^2 where the coefficient a is the slope of the molecular weight calibration curve from eqn (4). This ratio is a sum of two terms as follows:

$$\sigma^2_{chr}/a^2 = [\sigma^2/a^2] + \gamma^2 \qquad (5)$$

The first one is due to the axial dispersion, it depends on the column efficiency and can be obtained by using standards of well-known MWD. The second one is due to the polydispersity index of the sample and for a distribution of the Weslau type we get $\gamma^2 = \log [M_w/M_n]$

$$P(\ln [M]) = k \exp [-1/(2\gamma^2)) \ln^2 [M/M_p] \qquad (6)$$

where $P(\ln [M])$ is the concentration of the M molecular weight species, k a constant, M_p the peak molecular weight (zero momentum) obtained from the M1 calibration.

This type of distribution corresponds to symmetrical SEC peaks which is not often the case. On the other hand, this method requires well-characterised fractions of well-known polydispersity for the dispersion ratio determination.

Moreover, by applying the Tung and Runyon's method for the calculation of the polydispersity, and supposing a Weslau type distribution (Weslau, 1956) for the sample distribution it is easy to recover all the mean molecular momentum of mass:

$$M_w = M_p \exp [\gamma^2/2] \quad M_n = M_p \exp [-\gamma^2/2] \qquad (7)$$

where

$$\gamma^2 = \log [M_w/M_n] \qquad (8)$$

M3

The third classical method is based on the well-known universal calibration. It is indeed rather well established that the driving parameter of the chromatography separation is the hydrodynamical volume which is proportional to $[\eta_i] M_i$.

Equation (4) should be written as follows:

$$\log [[\eta] M] = b' + a' V_e \qquad (9)$$

This calibration led to the development of online viscosimeter (Ouano, 1972; Peyrouset *et al.*, 1975; Lesec & Quivoron, 1976) which allows the determination of the MWD of a given polymer sample from calibration made with chemically different standards. The viscosity of each fraction of the peak is then well determined. (Here, this is called method M3,1.)

The method M3,2 corresponds to the case where no viscosimeter is available on the GPC device, the same result may be obtained if the Mark-Houvink law of the polymer under study is well known:

$$[\eta] = KM^{3\nu - 1} \tag{10}$$

where ν is the excluded volume exponent.

The two-detectors GPC

Refractivity and LALLS apparatus were used.

M4

In order to avoid the hints of a standard calibration, it is more convenient to use an on-line molecular weight detector coupled with the concentration determination. The weight average molecular weight, M_{wi}, at each eluted volume fraction is obtained from the Rayleigh ratio:

$$\Delta R_i = K c_i M_{wi} = \frac{\Delta I_i}{I_0} \Phi Q(n) \tag{11}$$

where ΔI_i is the excess of scattering from the solute, and Φ is a constant, and $Q(n)$ a factor which depends on the refractive index.

The constant K includes the usual optical parameter as the increment refractivity index, the solvent index n and a calibration factor f for the light scattering depending on Φ and $Q(n)$.

$$K = \frac{4\pi^2 n^2}{\lambda^4 N_A} \left(\frac{dn}{dc}\right)^2 f \tag{12}$$

where N_A is the Avogadro number, and λ is the wavelength.

The useful M_{wi} of the sample is then obtained by integrating the whole chromatogram. This calculation only needs to know the concentration C_p of the solution coming out the columns which is given by the solution concentration, the injection loop volume and the peak limits:

$$M_w = \Sigma \Delta R_i / K \Sigma c_i = \Sigma \Delta R_i / K \times C_p \tag{13}$$

So we are able to deduce first the calibrating curve $M_w = f(V_e)$ which can lead to the calculation of the M_n using the concentration values given by the refractivity measurement c_i:

$$M_n = \Sigma c_i / \Sigma (c_i / M_{wi}) \tag{14}$$

All the other molecular weight momentum can also be obtained.

Remarks

(i) The light scattering detector is molecular weight times concentration dependent as $c \times M_w$; so the average molecular weight determination will depend on the response of both detectors at the limits of the peak.

For example, a low concentration of a high-molecular-weight fraction will contribute strongly to the increase of the M_w and slightly to the M_n. Moreover, polydisperse sample with low-molecular-weight limit will give no response in the light scattering detectors and still a high response in the refractivity detector more concentration dependent. This enhances the problem of the volume lag between the two detectors and of the superposition of the molecular weight and concentration signals of each fractions the peak.

(ii) In the case of partial adsorption or high-molecular-weight retention the M_w value obtained by the light scattering detector will be underestimated. In this case it is useful to control the refractometer response by comparing the refractivity increment of the injected polymer with the one obtained by integrating the refractometer signal which is proportional to the concentration.

(iii) The axial dispersion is more likely of great importance in the determination of the polydispersity index when we deal with a sample of large MWD or when the column efficiency is small: neglecting this effect will overestimate the polydispersity index.

M5

The problem addressed here is the impact of the use of polydisperse samples as molecular weight standards and particularly the dextran samples as shown in Table 1. In this case we try a molecular weight calibration (M1 method) by plotting the molecular weight at the top of the peak samples measured by the light scattering detector versus the elution volume. The fraction of the peak top is supposed to be a narrow distribution.

Moreover, the scaling law exponent of the Mark-Houwink expression of the dextran fit better the HAP hydrodynamical dimensions behaviour. This property should give a better reliability to the molecular weight calibration.

RESULTS AND DISCUSSION

Characterisation of the HAP fractions

Conventional light scattering and viscosimetry

We have measured the M_w of dextran and HAP fractions. The results are given in Table 1 and for PSA and PEO the values furnished by the manufacturer are shown. The molecular weight dependences of the intrinsic viscosity are reported in Fig. 1 for the four types of polymers in water.

There are very big differences between PEO which

Table 1. Initial values of PEO and SAC 10 polysaccharides obtained from the manufacturers

Batch	$< M_w >$	$< M_n >$	PI	Label
DE 2000	$1{\cdot}92 \times 10^{6a}$	$7{\cdot}0 \times 10^5$	2·7	Dextran
DE 506	$3{\cdot}8 \times 10^{5a}$	$3{\cdot}0 \times 10^5$	1·8	Dextran
DE 150	$2{\cdot}6 \times 10^{5a}$	$2{\cdot}1 \times 10^5$	3·2	Dextran
DE 249	$2{\cdot}03 \times 10^{5a}$	$3{\cdot}7 \times 10^5$	5·4	Dextran
HAP f_1	$9{\cdot}3 \times 10^{5a}$	$7{\cdot}15 \times 10^5$	1·3	Hydroxyamylopectin
HAP f_2	$4{\cdot}28 \times 10^{5a}$	$3{\cdot}48 \times 10^5$	1·23	Hydroxyamylopectin
HAP f_3	$3{\cdot}04 \times 10^{5a}$	$1{\cdot}92 \times 10^5$	1·4	Hydroxyamylopectin
HAP f_4	$2{\cdot}09 \times 10^{5a}$	$1{\cdot}92 \times 10^5$	1·4	Hydroxyamylopectin
HAP f_5	$1{\cdot}03 \times 10^{5a}$	$8{\cdot}5 \times 10^{4b}$	1·2	Hydroxyamylopectin
HAP f_6	$6{\cdot}6 \times 10^{4a}$	$5{\cdot}7 \times 10^{4b}$	1·6	Hydroxyamylopectin
20904-1	$5{\cdot}0 \times 10^4$	$4{\cdot}6 \times 10^4$	1·09	PSA
20905-1	$1{\cdot}05 \times 10^5$	$9{\cdot}5 \times 10^4$	1·1	PSA
20906-1	$1{\cdot}97 \times 10^5$	$1{\cdot}74 \times 10^5$	1·13	PSA
20907-1	$4{\cdot}02 \times 10^5$	$3{\cdot}59 \times 10^5$	1·12	PSA
20831-1	$2{\cdot}37 \times 10^4$	$2{\cdot}23 \times 10^4$	1·06	PEO
20835-1	$1{\cdot}08 \times 10^5$	$1{\cdot}02 \times 10^5$	1·06	PEO
20836-3	$1{\cdot}55 \times 10^5$	$1{\cdot}04 \times 10^5$	1·04	PEO
20840-5	$7{\cdot}88 \times 10^5$	$7{\cdot}50 \times 10^5$	1·05	PEO

[a]MALLS measurements (Sarazin, 1992).
[b]From osmometry.

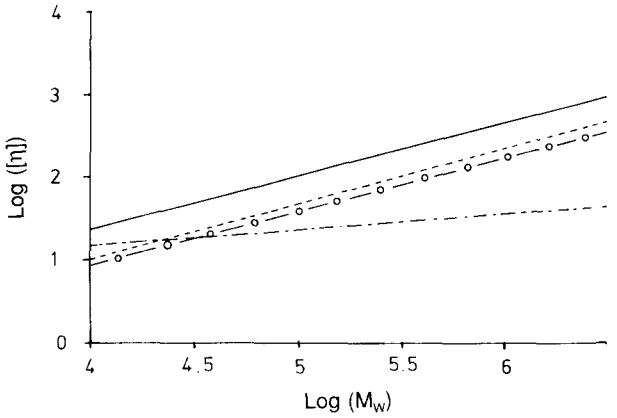

Fig. 1. Plot of the intrinsic viscosity law for hydrosoluble polymers with different hydrodynamic behaviour in good solvent versus their weight-average molecular weights. (——) PEO; (—○—) PSA; (– – –) dextran; and (–·–·–) HAP.

has a conformation of extended coil and HAP: the different curves correspond to the following scaling laws:

PEO (Sarazin *et al.*, 1992) $[\eta] = 5{\cdot}888 \times 10^{-2}$ $M_w^{0{\cdot}65}$ cm³/g $6000 < M_w < 1{\cdot}2 \times 10^6$ (15)

PSA (Sarazin *et al.*, 1992) $[\eta] = 2{\cdot}188 \times 10^{-2}$ $M_w^{0{\cdot}65}$ cm³/g; pH6 (16)

Dextran (Wales *et al.*, 1953) $[\eta] = 1{\cdot}99 \times 10^{-2}$ $M_w^{0{\cdot}675}$ cm³/g (17)

HAP (Sarazin *et al.*, 1992) $[\eta] = 2{\cdot}34 \times 10^{-1}$ $M_w^{0{\cdot}33}$ cm³/g (18)

All these laws were obtained for the respective polymers under good solvent conditions (pH and ionic strength) at room temperature (25°C) with well-defined fractions of relatively low polydispersity. They have been used to establish the Universal calibration for this set-up. The Mark-Houwink-Sakurada law for the HAP intrinsic viscosity was not corrected from the polydispersity which was found to depend on the molecular weight of the fractions (Sarazin *et al.*, 1992). The very low exponent of the HAP scaling law for the intrinsic viscosity is consistent with a globular shape for the macromolecules due to the grafted structure. The branching number g is a decreasing function of the molecular weight (Sarazin *et al.*, 1992). This is to be compared with a highly branched polymer as the dextran which behaves with similar viscosity low exponent (Granath, 1958; Garg & Stivala, 1978). On Fig. 2 are drawn the molecular weight calibration curves for these polymers; they exhibit different behaviours according to the scaling law of their hydrodynamic dimension.

The hydrodynamic calibration or the so-called universal calibration (Benoit, 1967) recalls all the results on a unique straight line as shown in Fig. 3. Some recent comments and new experiments (Dubin & Principi, 1989) on the universal calibration showed some evident discrepancies on its feasibility when it is applied to polymers of various branching properties. These results can be correlated with the poor fit shown on Fig. 3 by the full line.

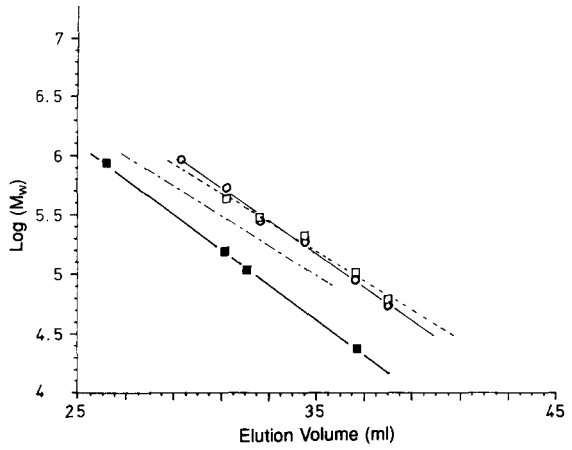

Fig. 2. Weight-average molecular weight calibration versus the elution volume of the following hydrosolubles polymers. (– – –) Dextrans; (□) HAP with MALLS molecular weight determination; (○) HAP with LALLS molecular weight; (–·–·–) linear polysaccharides (PSA); and (——■——) PEO.

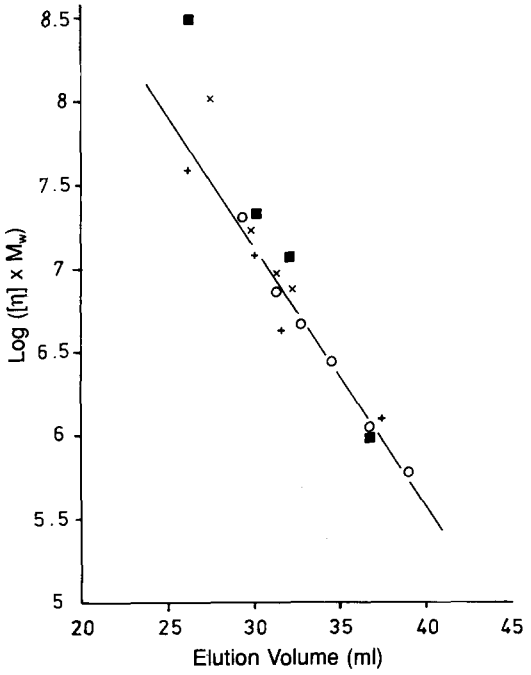

Fig. 3. Universal calibration plot from log ($[\eta] \times M_w$) versus the elution volume of some hydrosoluble polymers as follows: (○) HAP; (×) dextran; (■) PEO; (+) PSA; and (——) linear regression (full line).

The value of dn/dc plays an important role in the determination of M_w by light scattering. The Greenwood & Houston (1975) review of dn/dc values for various PSA systems in aqueous solution shows that their variations with λ, salinity and temperature are very low and may be negligible. We have measured with two refractometers dn/dc values in aqueous solution within the range of 0·138–0·144 cm^3/g which are significantly higher than what were found by other authors (Lederer *et al.*, 1985) who worked on deionised solutions through a mixed bed ion exchange column. With a dialysis purification we obtained a dn/dc value of 0·135 cm^3/g in much better agreement with literature data.

In the course of our light scattering measurements we were able to measure the dn/dc values after each experiment on the same centrifugated buffered solutions with 0·1 M sodium acetate. The dn/dc obtained with dilute solution does not vary very much with concentration and was close to 0·135 cm^3/g which was used afterward.

A typical Zimm plot is shown in Fig. 4 for the HAP2 batch where the experimental data and the calculated points are drawn. The calculated values are obtained from a polynomial regression according to $Kc/I = F\,[1, c_i, q^2, q^4]$.

Comparison between MALLS and LALLS detection (M_w)

The values of the average molecular weight determined by a MALLS (Sarazin & François, 1988) and a LALLS (KMX)-SEC coupling are listed in Table 2. By defining MR as the ration of the M_w values obtained with these two methods:

$$MR = \frac{M_w \text{ KMX}}{M_w \text{ MALLS}} \qquad (19)$$

we can consider as acceptable the molecular weight determination having the MR lying between 0·8 and 1·2.

Usually samples with a polydispersity index lower than 1·1 have the MR ratio very close to 1.

For two samples, MR is significantly higher (Table 3). Their chromatograms are characterised by a strong exclusion peak in the high-molecular-weight region (PSA 20907-1 and DE 150). We may assume that the LALLS (KMX) detector takes into account agregates which disappear by centrifugating solutions for the MALLS measurement. In Fig. 5 it is shown that near exclusion agregates or non-Newtonian flow effect for extremely-high-molecular-weight lead to a bimodal distribution behaviour for the LALLS detector, which does not appear on the refractogram.

In conclusion, the use of a on-line LALLS detector gives a quite reliable M_w for samples which have a normal separation on the high-performance gel chromatography.

Discussion on methods for SEC data calculation

We used the HAP fractions for all the comparisons between results obtained by the above-mentioned methods.

In Table 2 are also listed the results obtained by the methods M4 for the polydispersity index (MWD) and the M_n (coupling SEC-LALLS). The polydispersity index is in that case higher due to the method of M_n determination which gives here a smaller value, for the reasons mentioned above.

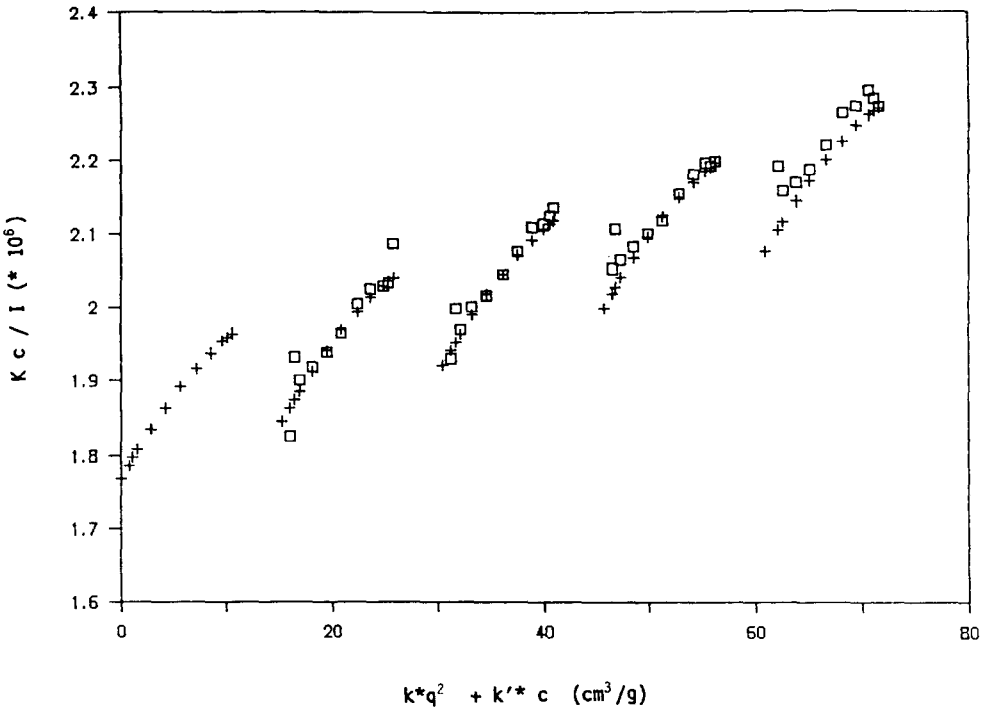

Fig. 4. A typical Zimm plot for HAP2 batch (PI = 6·2) (Sarazin *et al.* 1992) obtained by a MALLS apparatus with a wave length of 633·2 nm.

Table 2. Reports of the MR ratio (MR = M_{wLALLS}/M_{wMALLS})

Batch	$< M_{wMALLS} >$	$< M_{wLALLS} >$	V_E	M_{wLALLS}/M_{wMALLS}	M_w/M_n (M4)
HAP f_1	930 000	—	29·3		1·3
HAP f_2	428 000	526 000	31·25	1·23	1·23
HAP f_3	303 700	280 000	32·64	0·85	1·47
HAP f_4	209 000	193 000	35·53	0·92	1·3
HAP f_5	103 000	89 400	36·61	0·87	1·2
HAP f_6	66 000	—	39·01	—	—
DE 2000 (dextran)	$1·92 \times 10^6$	$2·4 \times 10^6$	27·4	1·09	2·7
DE 506	380 000	540 000	29·6	1·42	1·8
DE 150	260 000	680 000	31·0	2·6	3·2
DE 249	203 000	218 000	32·0	1·07	—
20904-1 (PSA)	50 000		37·4		
2905-1	105 000	119 000	31·5	1·13	1·11
20906-1	197 000	157 000	30·0	0·8	1·15
20907-1	402 000	1 250 000	26·1	3·1	1·34
20831-1 (PEO)	23 700	23 000	36·7	0·97	1·08
20835-1	108 000	108 500	32·1	1·00	1·07
20836-3	155 000	160 000	30·2	1·03	1·06
20840-5	788 000	892 000	26·3	1·00	1·07

In Table 3 are listed the results obtained by the methods M2 and M3, respectively. The discrepancies between values here presented draw the difficulties encountered in the use of chemically different standards for calibration without on line apparatus. In the M2 and M5 procedures, the calibration curves were obtained respectively with PSA and dextran of a higher hydrodynamical volume than the HAP (see Fig. 1). In that case the molecular weight determined either from standard molecular weights for M2 or the LALLS molecular weight at the top of the standard peaks for M5, lead to underestimate the average molecular weight.

In the M5 method the fraction at the top of the dextran peaks used in the calibration cannot be considered as similar as a narrow distribution reference.

Table 3. Emphasis of usual calculations on molecular mass characterisations of the HAP fractions using different useful methods

Batch	M2 method (PSA)		M5 method (dextran)		M3 method (PSA)	
	M_w	PI	M_w	PI	M_w	PI
HAP f_1	342 000	1·3	—	—	1 090 000	1·3
HAP f_2	266 000	1·21	267 000	1·62	457 000	1·21
HAP f_3	173 000	1·21	145 000	1·99	300 000	1·21
HAP f_4	105 000	1·26	63 000	2·17	259 000	1·26
HAP f_5	67 000	1·41	25 000	2·87	146 000	1·41
HAP f_6	34 000	1·63	—	—	57 500	1·63

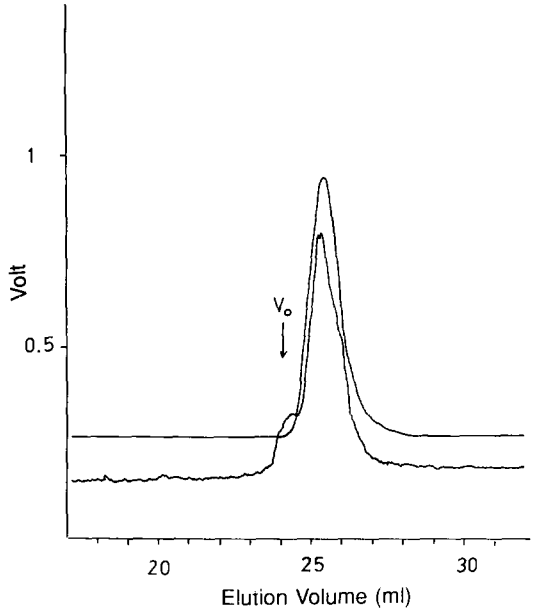

Fig. 5. Typical SEC/LALLS response from DRI (upper line) and LALLS (lower line) detectors for PSA 20907-1.

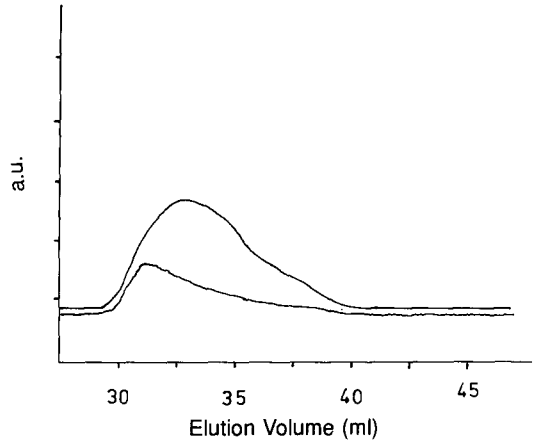

Fig. 6. Typical SEC/LALLS response for the polydisperse HAP200 batch. DRI (upper line) and LALLS (lowest line).

The calculation using this assumption leads indeed to increase the polydispersity of the samples. In the M3 procedure the universal calibration established with the PSA standards tends to overestimate and underestimate the M_w for the higher and the lower fractions, respectively, if the values are compared to those listed in Table 1 and determined by the light scattering techniques. This may be explained by a slight interaction of the PSA standards with the bead packing of the column or by failure from the universal calibration law put forward in Fig. 8 (see later).

In Table 4 are listed the values of a polydisperse HAP from the batch leading to the HAP f_i standards and characterised by the underlined methods. The chromatogram of the batch is shown on Fig. 6 and is ranging over a very large volume scale. The M1 and M4 methods which are not standard dependent give here the most reliable results for M_w by comparison with the MALLS determination. In fact the M_n is perhaps slightly underestimated by LALLS because of the low-molecular-weight fractions eluated till the salt exclusion of the columns. It is also obvious that M2 method using PSA which are not appropriated standards is useless. The M3, 1 batch-method requires the universal calibration law shown in Fig. 3 and the intrinsic viscosity measured on the batch with a capillary viscosimeter. The distribution is then deduced from the

Table 4. The HAP batch molecular mass determination and the problem of a large hump signal on the SEC methods

Method	Calibration type	M_w	M_n	M_w/M_n
MALLS	Static measurements	230 000	—	—
M1	Log (M) (HAP)	269 000	184 600	1·46
M2	Log (M) (PSA)	76 000	15 000	5·06
M3,1	$[\eta]_{BATCH} \times M$	195 000	35 000	5·57
M3,2	Universal calibration (Fig. 3)	220 000	21 600	10·0
M4	LALLS on-line	243 000	167 000	1·46

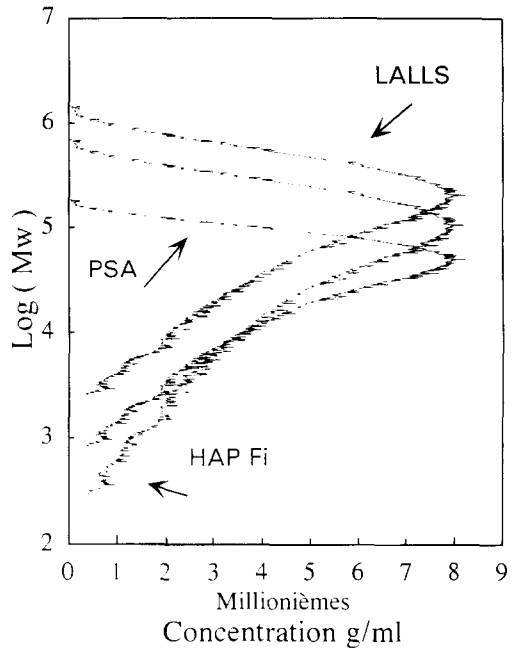

Fig. 7. The universal calibration effect on the molecular weight distribution of the HAP polydisperse batch using polysaccharides or HAP standards.

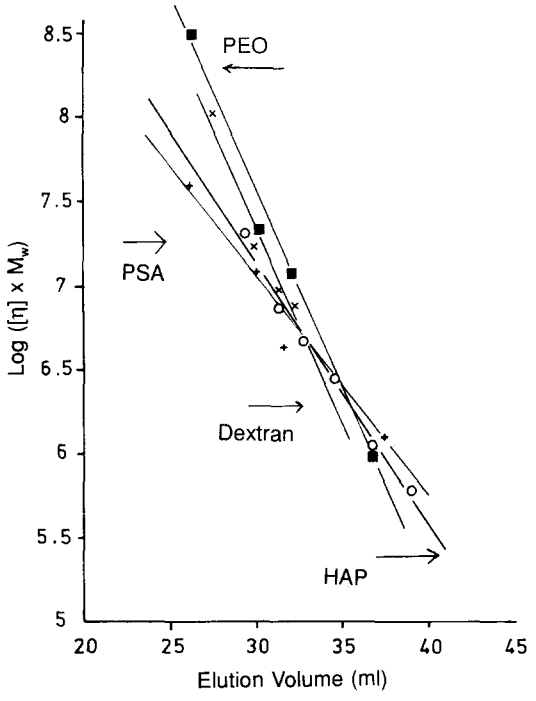

Fig. 8. Linear regression plotted for the different flexible hydrosoluble polymers in the case of specific universal calibration laws. Each polymer is identified with the arrows and drawn separately with symbols of Fig. 3.

Tung and Runyon (1969) calculation and the peak molecular weight.

The M3,2 method also requires the regression line of the universal calibration law shown in Fig. 3 and the scaling law for the intrinsic viscosity of the HAP.

M2 and M3 both give a rather good MWD but different polydispersity. It is obvious that a good characterisation is almost impossible for such large distribution as shown in Figs 6 and 7. In Fig. 7 are drawn the results obtained by the M3,2 and M4 methods, respectively. The shift in the MWD calculated with a universal calibration and with a LALLS measurement confirms the failure of the universal calibration applied to polymers of too different conformation.

In fact, as shown in Fig. 8, good regression lines can be drawn for each type of polymer but these lines intersect. For a same hydrodynamical volume, the first eluated species are PSA and HAP fractions, in the high-molecular-weight range while the inverse tendency is observed for the lower molecular weights. Thus, the PEO characterised by a great chain flexibility may be more sensitive to the shear rate effect which decreases the viscosity than the highly grafted polymers such as HAP. Moreover, the universal calibration implies that there is no specific interaction between polymers and chromatographic material.

These procedures put also the stress on the valuable use of an on-line viscosimeter detector which avoid the hydrodynamic calibration (so-called universal calibration). In fact, the main feature of the on-line viscosimeter especially for hydrosolubles polymers is to operate at a lower concentration than the usual capillary viscosimeter which avoid mainly the aggregation effects.

CONCLUSION

The characterisation by SEC of hydroxyethyl-amylopectin used as plasma expanders and which have a very dense conformation needs to take into account the hydrodynamic volume effects on the molecular weight measurements. The best results are obtained through the SEC-LALLS coupling. Nevertheless, some uncertainties about the polydispersity index arise from the differences in the refractometric and LALLS detections at the beginning and at the end of the peak. The weight-average molecular weight determinations through hydrodynamical calibration are tricky because our results seem to show that the universal calibration fails to explain the elution of polymers of too different macromolecular conformation. For large molecular weight distribution where the base line determination leads to enhance the polydispersity index (M5) by an underestimated number-average molecular weight, the use of an on-line osmometer may be useful (Yau, 1991). Moreover, an important feature of a chromatographic separation may be overlooked in considering only the polydispersity index and molecular weight determination by LALLS using only a two on-line detectors. A viscosimetric

detectors may also give more information on the hydrodynamical separation efficiency for grafted polymers. Besides, the solvent should be chosen very carefully to avoid slight hydrophobic interactions and reduce the enhancement of the polydispersity sample.

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