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APPLICATION OF GEL PERMEATION CHROMATOGRAPHIC SYSTEMS TO THE DETERMINATION OF THE MOLECULAR WEIGHT OF INULIN

W. PRAZNIK* and R. H. F. BECK
Universität für Bodenkultur, Institut für Chemie, Gregor Mendelstrasse 33, A-1180 Vienna (Austria)
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SUMMARY

The application of different gel types to the separation of inulin is discussed. A system consisting of two different columns is used for the determination of different inulins of various Compositae. The system is calibrated with glucan oligomers, by comparing the migration behaviour of the glucan series with the inulin series. The molecular weights of inulins of various plants are calculated from the different molecular distributions using the calibrated column system. The general belief that inulin has a chain length between 30 and 35 monomeric units, irrespective of the source, could not be confirmed.

INTRODUCTION

In the past few decades gel permeation chromatography (GPC) has become one of the most important techniques for the separation, preparation and analysis of oligo- and polysaccharides. High-molecular-weight polysaccharides were previously fractionated on porous glass[1,2], dextran[3–6], agarose [4–8] and polyacrylamide[9] gels. The separation of high-molecular-weight polysaccharides especially of starch on dextran and agarose gels, has been described, and the calibration of high-molecular-weight separating gel columns with structurally closely related calibration standards has been proposed[5–7]. In recent times high-molecular-weight polysaccharides have also been fractionated by high-performance gel chromatography[10,11]. Low-molecular-weight polysaccharides and oligosaccharides are usually separated on porous glass[12], dextran[12,13], and polyacrylamide[9,14–17] gels. Nowadays polyacrylamide gels are favoured because of their mechanical stability, high flow-rate and excellent resolution. In the fractionation of maltooligosaccharides and polymaltotrioses on Biogel P-6 a separation up to a degree of polymerisation of 60 is possible[15].

GPC was first applied to the determination of the chain length of fructans by Pollock *et al.*[9]. They found an average chain length of 34 monomeric units for a commercial inulin of the Jerusalem artichoke using a polyacrylamide (Biogel P-150) column. Other authors[9,12,18–29] also found an average chain length for the inulin of Compositae between 30 and 35 monomeric units. This results in a mean molecular weight of *ca.* 5000 to 6000 Dalton. The classical methods for the determination of

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the molecular weight of inulin were cryoscopy and the determination of the glucose content after acidic or enzymatic hydrolysis. These methods gave comparable results indicating a chain length of 30 monosaccharide units. In this article we describe two low pressure GPC systems for the determination of different molecular weight inulins from various plants of the Compositae family. After calibration of the system the mean molecular weight and the average chain length are calculated according to the method of Schulz[30].

EXPERIMENTAL

Preparation of samples

The samples were isolated from the inulin storing organs of the following plants: tubers from Jerusalem artichoke (*Helianthus tuberosus*) and dahlia (*Dahlia variabilis*); roots from chicory (*Cichorium intybus*) and dandelion (*Taraxacum officinale*); blossom disks from artichoke (*Cynara scolymus*).

The portion of the plant material (*ca.* 20 g wet weight) soluble in hot water is deproteinised and freeze-dried and stored dry in the dark. For further purification samples were recristallised from aqueous solution.

Gel permeation chromatography

The columns were filled with Biogel P-6 (200–400 mesh, Bio-Rad Labs.), and Sephacryl S-200 (superfine, Pharmacia). The gels were hydrated and deaerated and slurry-packed into the columns (140 × 1.6 cm I.D. for Biogel P-6, and 90 × 1.6 cm I.D. each for Biogel P-6 and Sephacryl S-200 combined).

To minimise the void volume the columns had adaptors (AC 16, Pharmacia) attached. The elution was carried out at room temperature with deaerated, distilled water containing 0.002% sodium azide to prevent the growth of microorganisms. Samples (10–20 mg) dissolved in 1 ml of solvent were injected with a loop valve into the system. Eluted carbohydrates were continuously detected with a Waters RI-403 detector and recorded (Omniscribe, Housten Instrument). The eluate was fractionated and sampled (3 ml), and the carbohydrate concentration was detected by the anthrone method[31]. In the fractionation of low-molecular-weight amylose (Hayashibara), the method of Somogyi-Nelson[32,33] was used to determine the reducing power: v_o was determined with blue dextran (Pharmacia), and v_t was determined with deuterium oxide (Merck). Both substances were added to the dissolved samples before injection as an internal standard that does not influence the separation behaviour of inulins but permits the control of the correct functioning of the column system.

Calibration

For the calibration of the Biogel P-6 column branched polymaltotrioses were prepared by the action of pullulanase (Sigma) on pullulan (Sigma). After incubation the mixture was deproteinised, centrifuged and lyophilised[15]. Polymaltotriose mixture and low-molecular-weight amylose (Hayashibara) were used for calibration. The reducing power of eluted maltooligomers and polymaltotrioseoligomers was determined[31–33], and from that the chain length was calculated. Approximate correspondence in the retention behaviour between these two series of glucanoligomers was established: the relation between the distribution coefficient K_{av}, the log of the molecular weight and the log of the chain length is given in Fig. 1.

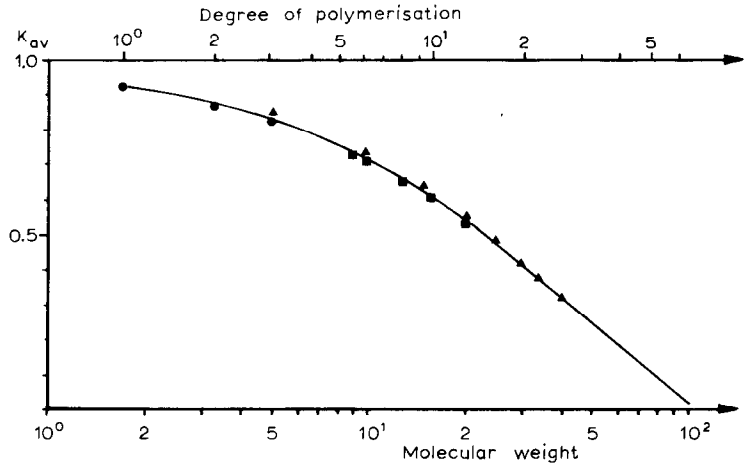

Fig. 1. Calibration curve of the Biogel P-6 column. Relation between the distribution coefficient K_{av} and the molecular weight. Data points: ▲ = partially debranched pullulan; ■ = low-molecular-weight amylose; ● = fructose, sucrose and raffinose.

The Biogel P-6–Sephacryl S-200 column system was calibrated analogously to the Biogel P-6 column with polymaltotrioses and amylose, and in addition with dextran 1000 (Laevosan, Linz, Austria) in the low-molecular-weight range. In the high-molecular-weight range, dextran T 10 and T 40 (Pharmacia) were used for calibration. The distribution of the dextrans was compared with the given distribution (Pharmacia). The integral mass distribution function of the eluogram obtained from the Biogel P-6 Sephacryl S-200 system was adjusted to the given integral mass distribution function by calculation. Fig. 2 shows the relation between K_{av}, the log of the molecular weight and the log of the chain length.

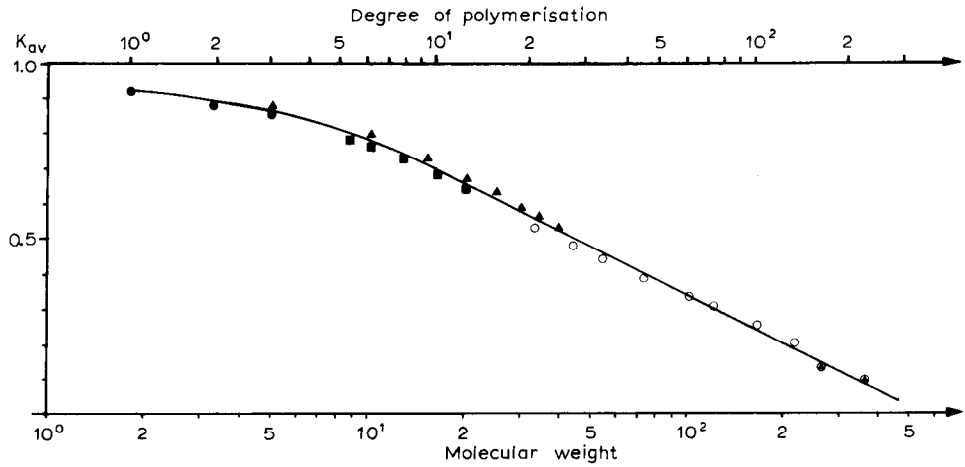

Fig. 2. Calibration curve of the combined Biogel P-6/Sephacryl S-200 column system. Relation between the distribution coefficient K_{av} and the molecular weight. Data points: ▲ = partially debranched pullulan; ■ = low-molecular-weight amylose; ● = fructose, sucrose and raffinose; ○ = dextran T-10; ◐ = dextran T-40.

Calculation

Calculation of the average degree of polymerisation ($\bar{P}_w$ and $\bar{P}_n$) was carried out according to the method of Schulz[30]:

$$\text{weight average: } \bar{P}_w = \sum_{P=1}^{\infty} (P \cdot m_p) \tag{1}$$

$$\text{number average: } \bar{P}_n = \frac{1}{\sum_{P=1}^{\infty} (m_p/P)} \tag{2}$$

m_p is the weight fraction of the polymer of the corresponding degree of polymerisation.

If a polymolecular substance is divided into a sufficient number of pieces, whose weight fractions are m_i and average degrees of polymerisation are P_i, analogous application of eqns. 1 and 2 gives the following:

$$\text{weight average: } \bar{P}_w = \sum_{i=1}^{n} (p_i \cdot m_i) \tag{3}$$

$$\text{number average: } \bar{P}_n = \frac{1}{\sum_{i=1}^{n} (m_i/P_i)} \tag{4}$$

If these equations and the integral mass distribution function $I(P)$ are used, $\bar{P}_w$ and $\bar{P}_n$ can be calculated very easily. $I(P)$ is divided into ten equal segments ("theoretical fractions"), whose weight fractions are all $m_i = 0.1$. The average degree of polymerisation of each "theoretical fraction" is got by determining the appropriate degree of polymerisation for the singel K_{av} values 0.05, 0.15, 0.25, ... 0.95 on the abscisses. So eqns. 3 and 4 are simplified as follows:

$$\text{weight average: } \bar{P}_w = 0.1 \cdot \sum_{i=1}^{10} p_i \tag{5}$$

$$\text{number average: } \bar{P}_n = \frac{10}{\sum_{i=1}^{10} (1/P_i)} \tag{6}$$

The ratio $\bar{P}_w/\bar{P}_n$ indicates the degree of inhomogeneity of polydisperse systems (Table I).

TABLE I

PERCENTAGE DISTRIBUTION, AVERAGE DEGREE OF POLYMERISATION ($\bar{P}_w$, $\bar{P}_n$) AND DISPERSITY FACTOR ($\bar{P}_w/\bar{P}_n$) OF INULINS

Inulin source	*Percentage within a DP range*			$\bar{P}_w$	$\bar{P}_n$	$\bar{M}W_w$	$\bar{M}W_n$	$\bar{P}_w/\bar{P}_n$
	2–19	*20–40*	*>40*					
Jerusalem artichoke native	74	20	6	14	6	2282	990	2.33
Jerusalem artichoke purified	2	24	76	62	49	10100	7990	1.25
Dahlia native	31	21	40	37	20	6000	3260	1.85
Dahlia purified	5	30	65	53	43	8640	7000	1.23
Artichoke native	–	13	87	82	65	13370	10600	1.26
Artichoke purified	–	–	100	105	90	17000	14700	1.17
Chicory native	55	18	17	23	11	3750	1800	2.09
Dandelion native	41	31	28	29	14	4730	2290	2.07

RESULTS

The freeze-dried extract of mature Jerusalem artichoke tubers harvested in autumn was separated on the Biogel P-6 column. Fig. 3 shows the asymmetric elution profile (curve 1): the low-molecular-weight portion is predominant, although a small part of the sample is excluded. The exclusive limit of the Biogel P-6 is 10000 dalton[15] (Fig. 1), which indicates that polymers with a degree of polymerisation of more than 60 are present in native inulin of Jerusalem artichoke tubers. Curve 2 in Fig. 3 shows the methanol-precipitated portion of Jerusalem artichoke extract, and curve 3 shows the twice recrystallised inulin obtained from methanol-precipitated inulin. Each step of purification leads to enrichment of the higher polymers.

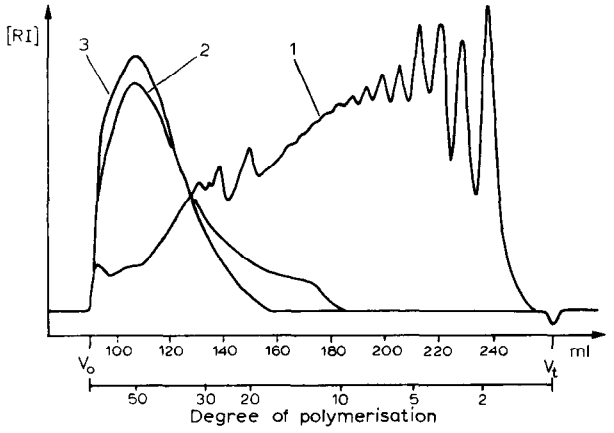

Fig. 3. GPC separation on Biogel P-6 of native (1), methanol-precipitated (2) and recrystallised (3) inulin from Jerusalem artichoke (*Helianthus tuberosus*). Amounts of 20 mg of native (1) and 10 mg of purified (2 and 3) inulin were dissolved in 1 ml of eluent and injected. Bed volume, 140 × 1.6 cm I.D.; flow-rate, 22 ml/h; room temperature, 25°C; solvent, deionised deaerated water containing 0.002% sodium azide; refractive index detector (8 ×); chart speed, 25 mm/h. v_o was determined with blue dextran (v_o = 90 ml); v_t was determined with deuterium oxide (v_t = 260 ml).

Fig. 3 shows that the Biogel P-6 column is not suitable for the separation of all inulins. The high-molecular-weight inulins are situated within a range of the chromatogram that can not be accurately calibrated. To determine such inulins, a chromatographic system that allows a separation in the optimal range of the chromatogram is required. For the separation of native and purified inulins in particular, the whole range from sucrose to polymers with a degree of polymerisation of more than 100 has to be covered. For efficient determination of low- and high-molecular-weight inulins we developed a system consisting of two columns packed with different gels, the first with Biogel P-6 and the second with Sephacryl gel S-200. This combination can effect separations in the range 200–50 000 dalton. Fig. 4 shows the separation of native and crystallised inulins of Jerusalem artichoke (*cf.* Fig. 3).

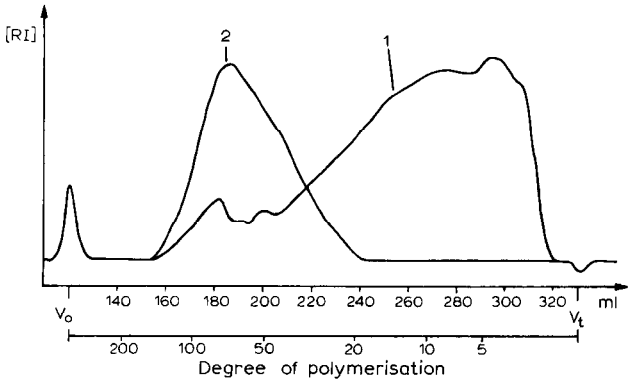

Fig. 4. GPC separation on Biogel P-6/Sephacryl S-200 of native (1) and recrystallised (2) inulin from Jerusalem artichoke (*Helianthus tuberosus*). Amounts of 20 mg of native (1) and 10 mg of recrystallised (2) inulin were dissolved in 1 ml of eluent and injected. Bed volume, both columns 90 × 1.6 cm I.D.; flow-rate, 22 ml/h; room temperature, 25°C; solvent, deionised deaerated water containing 0.002% sodium azide; refractive index detector (8 ×); chart speed, 25 mm/h. v_o was determined with blue dextran (v_o = 120 ml); v_t was determined with deuterium oxide (v_t = 330 ml).

Further investigations on the inulin of the well known inulin-containing plants of the Compositae family confirmed the necessity of the Biogel P-6/Sephacryl S-200 system. Artichoke inulin was totally excluded on the Biogel P-6 column. Fig. 5 shows the separation of native and purified inulin of the blossom disks of artichoke (*Cynara scolymus*) on the combined system. In contrast to the Jerusalem artichoke inulin, the artichoke inulin shows an extraordinarily high percentage of extremely high-molecular-weight inulin. Purification leads to an even higher molecular weight (an average chain length of more than 100 monomeric units calculated for $\bar{P}_w$). The calculation of the molecular weight for the native artichoke inulin does not include the low-molecular-weight compounds found in untreated extracts. Also the fraction in the middle of the chromatogram (Fig. 5, curve 1) is not used in the calculation. The area under the elution profile that is limited on its right side by the dotted line is used for calculation of the molecular weight of the native extract. The ratio $\bar{P}_w/\bar{P}_n$ is remarkably low for the native artichoke inulin (Table I).

Native dahlia inulin (Fig. 6) is also a very homogeneous mixture of polymers. In contrast to the inulin from Jerusalem artichoke tubers, the dahlia tuber inulin has

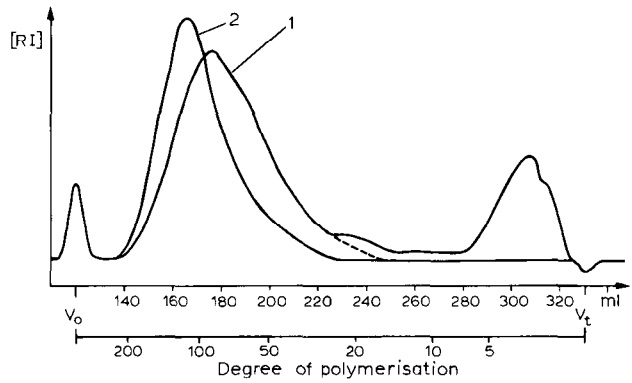

Fig. 5. GPC separation on Biogel P-6/Sephacryl S-200 of native (1) and purified (2) inulin from artichoke (*Cynara scolymus*). Amounts of 20 mg of native (1) and 10 mg of purified (2) inulin were dissolved in 1 ml of eluent and injected. Chromatographic conditions as in Fig. 4.

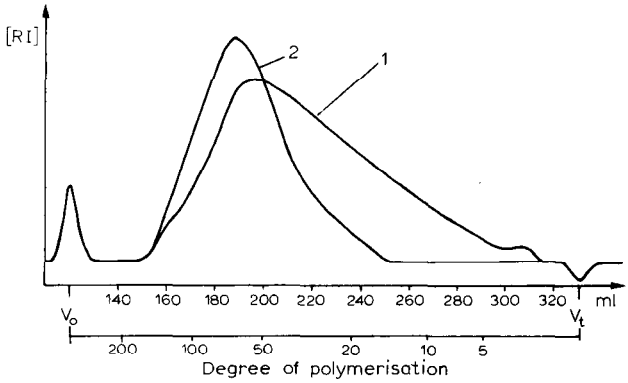

Fig. 6. GPC separation on Biogel P-6/Sephacryl S-200 of native (1) and purified (2) inulin from dahlia (*Dahlia variabilis*). Amounts of 20 mg of native (1) and 15 mg of purified (2) inulin were dissolved in 1 ml of eluent were injected. Chromatographic conditions as in Fig. 4.

a large proportion of high-molecular-weight inulin. Crystallisation leads as before (Figs. 3–5) to an increase in the average molecular weight.

Chicory and dandelion are also well known as inulin-storing plants. Fig. 7 shows the two root-inulins separated on the Biogel P-6/Sephacryl S-200 system. Both inulins show a broad distribution, and a significantly higher proportion of low-molecular-weight compounds than the dahlia tuber-inulin.

Fig. 8 shows the integral mass distribution function $I(P)$ of the inulins so far described by their differential mass distribution functions (Figs. 4–7). It is clear that purification of the native Jerusalem artichoke inulin and the higher-molecular-weight dahlia inulin leads to almost the same distribution of polymers. The same result was obtained with purified chicory and dandelion inulin (not shown). The molecular weight ranges between $\overline{MW}_w$ 8500 and 10000 dalton for purified inulins. Purification procedures that do not depend on the source will always lead to an inulin with a

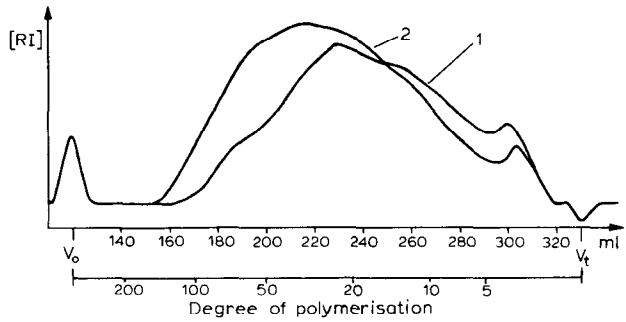

Fig. 7. GPC separation on Biogel P-6/Sephacryl S-200 of native (1) chicory (*Cichorium intybus*) and native (2) dandelion (*Taraxacum officinalis*) inulin. Amounts of 20 mg of chicory (1) inulin and 20 mg of dandelion (2) inulin were dissolved in 1 ml of eluent and injected. Chromatographic conditions as in Fig. 4.

narrower distribution, a higher degree of homogeneity and a higher and a similar molecular weight[23] (Table I).

Table I gives a numerical synopsis of the integral mass distribution functions $I(P)$ (Fig. 8) of the inulins described. The percentage mass distribution below certain chain-length ranges is also listed. The weight average $\bar{P}_w$, the number average $\bar{P}_n$, and the ratio $\bar{P}_w/\bar{P}_n$ are also listed. It is evident that we cannot agree with the commonly believed statement that purified inulin has a molecular weight between 5000 and 6000, corresponding to a chain length of 30–35 monomeric units. Berner[34–37] showed in the early thirties that small amounts of impurities have a large influence on the results of classical determinations of the molecular weight.

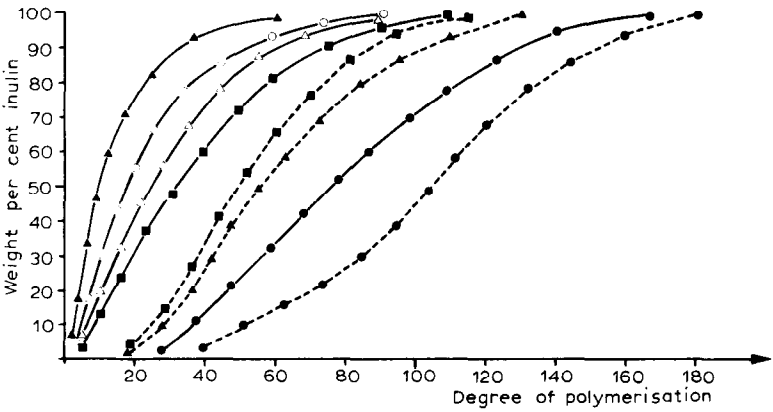

Fig. 8. Integral mass distribution functions of the inulins described in Figs. 4–7. Curves: ▲—▲ = native Jerusalem artichoke inulin; ▲---▲ = purified Jerusalem artichoke inulin; ■—■ = native dahlia inulin; ■---■ = purified dahlia inulin; ●—● = native artichoke inulin; ●---● = purified artichoke inulin; ○—○ = native chicory inulin; △—△ = native dandelion inulin.

DISCUSSION

For the separation of inulins from various plants several types of gel were tested. On dextran gels, Sephadex G-25 and G-50, the elution profiles showed only an indifferent distribution of the inulin polymers over the entire profile[38]. Such a separation allowed only a scanty statement of the quantitative composition of the inulin polymers. Other authors[14–17] have proposed highly cross-linked polyacrylamide gels, Biogel P-2, P-4, P-6, for the separation of xylo- and maltooligomers. It occurred to us that the Biogel P-6 would be a possible way to separate native low-molecular-weight inulin (Fig. 3). Biogel P-10 and P-100 were tested for their application to high-molecular-weight inulins. But these two weakly cross-linked polyacrylamide gels did not show such good results as the highly cross-linked polyacrylamide gel. The Biogel P-100 also showed lesser mechanical stability and tended to collapse at higher flow-rates[38].

For the separation of high-molecular-weight inulin a combination of Biogel P-6 with Sephacryl gel S-200 turned out to be the best. Sephacryl gel is a covalently cross-linked allyl dextran that gives the matrix high stability and allows high flow-rates to be used. Because of the N,N′-methylenebisacrylamide cross-linkage, this type of gel has similar qualities to the polyacrylamide gel for the separation of carbohydrates.

Several authors have proposed a calibration with maltooligosaccharides[14] and polymaltotrioses[15] for their systems. However, this calibration method covers only the low-molecular-weight range of GPC systems. Calibration in the high-molecular-weight range is incomparably more difficult. Molecular defined dextrans (Pharmacia) are often used for calibration[4–6,9,11], irrespective of the structure of the polysaccharide to be examined. However, the influence of the polysaccharide structure on the migration behaviour is considerable[7], so we investigated the separation behaviour of several polymer series on our gel systems. Polymaltotrioses, low-molecular-weight amyloses and dextrans showed no significant differences in their retention times, because the spherical arrangement, the secondary and the tertiary structure, does not influence the migration behaviour of low-molecular-weight polymers as much as that of high-molecular-weight polymers, *e.g.* starch polymers[7]. The structure of low-molecular-weight polymers is not the separation criterion, rather the molecular weight and the particle size.

The elution profiles of the inulins were compared with the elution profiles of the calibrating glucan series, and almost complete agreement of the K_{av} values was established. Therefore, the molecular weights of inulins can be calculated with the glucan series calibration. Peak broadening was not calculated because of the asymmetric distribution of native extracts, the heterogeneity of samples and the lack of monodisperse calibration standards in the high-molecular-weight range. The influence on the final results of the determination of the molecular weight is because of the heterogeneity of the samples negligible.

In contrast to previous opinions, it seems that the average chain length of inulin is not 30–35 monomeric units. The source of the inulin and the purification grade both influence, and even determine, the average chain length and the average molecular weight. However, we point out that the determined molecular weights are only static pictures from a dynamic process of the development of plants. This picture

as reflected in the samples is influenced by a lot of factors. We consider the most important to be the degree of maturity[38–41], the climate and the environment, which directly influence the molecular development and in consequence the chain length and the molecular weight of native inulin.

CONCLUSION

GPC can be used not only to determine an average value of the molecular weight but also to describe the distribution of native and purified inulins from various sources. The method of Schulz[30] can be used to calculate the weight average and the number average. The ratio $\bar{P}_w/\bar{P}_n$, which expresses the molecular dispersity of a polymer mixture, can be calculated easily. All these calculations can be done with only one analytical run on a GPC system. One disadvantage is the lack of suitable calibration standards in the high-molecular-weight range. Monodisperse non-distributed standards would simplify this method very much. This calibration problem can be solved by mathematically transferring the integral mass distribution and the differential mass distribution of defined, distributed, heterodisperse standards, *e.g.* linear dextrans (Pharmacia) or synthetic amyloses[7], to their own system.

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