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De facto molecular weight distributions of glucans by size-exclusion chromatography combined with mass/molar-detection of fluorescence labeled terminal hemiacetals

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Abstract

A major capability of polysaccharides in aqueous media is their tendency for aggregation and dynamic formation of supermolecular structures. Even extended dissolution processes will not eliminate these structures which dominate many analytical approaches, in particular absolute molecular weight determinations referring to light scattering data. An alternative approach for determination of de facto molecular weight for glucans with free terminal hemiacetal functionality (reducing end group) has been adjusted from carbohydrates for midrange and high-dp glucans: quantitative and stabilized labeling as aminopyridyl-derivatives (AP-glucans) and subsequent analysis of SEC-separated elution profiles based on simultaneously monitored mass and molar fractions by refractive index and fluorescence detection. SEC-DRI/FL of AP-glucans proved as an appropriate approach for determination of de facto molecular weight of constituting glucan molecules even in the presence of supermolecular structures for non-branched (pullulan), branched (dextran), narrow distributed and broad distributed and for mixes of compact and loose packed polymer coils (starch glucan hydrolizate).

Keywords: Pyridylamination of glucans with terminal hemiacetal; SEC-mass/molar; Absolute molecular weight distribution: Mass fractions; Absolute molecular weight distribution; Molar fractions; Narrow distributed non-branched AP-glucans; Broad distributed branched AP-glucans; Mixed type branched AP-glucans

1. Introduction

Polysaccharides are provided by nature as a result of rather complex processes of formation, transformation and modification. Therefore, different to laboratory- or industry-scale polymer syntheses, their molecular weight cannot simply be correlated with well known conditions and controlled mechanisms of a limited number of compounds. However, identical to synthetic polymers, even for polysaccharides material properties are strongly correlated with molecular characteristics. Hence, accurate determination of molecular weight for polysaccharides is an important feature for comprehensive characterization of polysaccharide-based materials and often molecular weight itself is taken as a polymer classification criterion [1–3]. However, even if degree of polymerization, excluded volumina, packing densities, branching characteristics or interactive potentials are listed, molecular weight is required to compute these characteristics from monitored raw data. In general, molecular weight of polymers may be determined either calibrated – comparing with well defined reference materials – or absolute, without reference materials but increased efforts in monitoring and data processing. Well established absolute techniques are dual detection of (light) scattering intensity and mass-fractions of SEC-separated elution profiles (SEC-mass/scattering intensity), and dual detection of viscosity and mass profiles of SEC-separated polymers (SEC-mass/viscosity). Of course, both techniques work for polysaccharides, but they are extremely sensitive

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towards high molecular components, in particular towards supermolecular associates or aggregates, and obtained results are dominated by these fractions, even in the case of minor amounts only. An alternative for materials with pronounced tendency to form aggregates, such as for polysaccharides, would be specific and quantitative labeling and subsequent monitoring of mass and molar elution profiles of SEC-separated derivatives. A possibility for glucans with free terminal hemiacetal functionality (reducing endgroup) would be quantitative and stabilized labeling with a proper chromophor/fluorophor and subsequent dual detection of SEC-separated glucan-derivatives with respect to molar fractions by means of fluorescence or UV–vis and mass-fractions by refractive index (SEC-mass/molar).

Labeling of the terminal hemiacetal functionality of carbohydrates with chromophors/fluorophors for analytical purposes is well established [4–12] for a wide range of fluorophors [4,13–15]. Maybe the most important and best reproducible approach is reductive amination [4,16] with 2-aminopyridyl (AP) forming AP-mono-, AP-di and AP-oligosaccharides [17–19] with sufficient stability to monitor them by means of UV/fluorescence (Fig. 1). AP-derivatives of carbohydrates are applied as well in liquid chromatography (LC), in particular in HPLC, as, due to the charged nature of these compounds, in capillary electrophoresis (CE) [20–24]. Quantitative transformation of carbohydrates into AP-derivatives simply can be achieved by an excess of 2-aminopyridine. Subsequent stabilization of formed AP-derivatives requires instant reduction of formed imins to secondary amines, typically with sodium cyanoborohydride [25] or dimethylamine–borane complex [19]. Analysis by means of CE [6,23] is well established for AP-oligomers with degree of polymerization (dp) up to approx. 30, however, it is no standard procedure for polymers. Reproducible LC-analysis requires the careful elimination of 2-aminopyridin excess and of salts [19] and even is well established for AP-oligomers with dp up to approx. 30. Nevertheless, even polymers already have been investigated, for instance the non-branched type starch glucan amylose which has been transformed into AP-amylose and investigated by means of SEC equipped with dual detection of mass-fractions by refractive index and molar fractions by fluorescence detection with off-line calibration with synthetic AP-amyloses [26]. A similar approach additionally has been applied to the branched type starch glucan amylopectin [27,28].

This paper reports a procedure for quantitative formation of AP-derivatives for oligomer- and polymer-type glucans with free terminal hemiacetals. The procedure is based on established procedures for conversion of carbohydrates into AP-derivatives and was extensively proofed [29,30] for stability of the formed AP-glucans with respect to subsequent determination of absolute molecular weight distributions by means of SEC-mass/molar analysis.

2. Material/methods

2.1. Material

AP-maltose was prepared for calibration of the fluorescence detector by mixing 1 g maltose with 2.7 g 2-aminopyridine (AP), 12 mL H_2O_{dest} and 2.5 mL concentrated acetic acid (pH 6.5) in a 100 mL round bottomed flask. The resulting solution is stirred for approx. 20 h at 60 °C. The solution then is supplemented with 1.5 g sodium cyanoborohydride or with dimethylamine–borane complex and left again for approx. 20 h at 60 °C. The resulting product is precipitated by slowly adding the solution to 150 mL cooled acetone. The precipitate is purified on a G3 frit by washing with approx. 150 mL acetone and eliminating acetone residuals by streaming with nitrogen. The resulting raw product which is dried at 50 °C is achieved with a

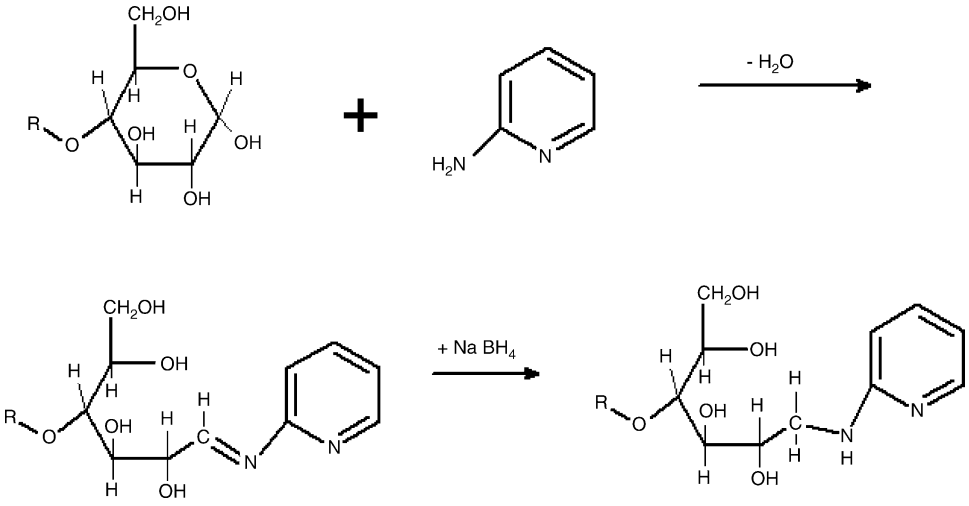

Fig. 1. Reductive pyridyl-amination of glucans at terminal free hemiacetal position (reducing OH-group): glucose (R=H)/glucan (R=Glc_n) and 2-aminopyridin form an intermediate Schiff base by opening the hexose ring; reduction yields the secondary pyridylamino-glucose (R=H)/glucan (R=Glc_n).

yield of approx. 80%. The obtained raw AP-maltose is purified from salts by chromatographic separation on a semi-preparative Fractogel HW 40 (450 mm, i.d. 15 mm)-system with H_2O_{dest} as eluent. Therefore, 1 mL of approx. 10 mg/mL concentrated eluent-dissolved AP-maltose was applied to the separation system and 96% pure AP-maltose was obtained by cutting the fraction according to refractive index and to UV ($E_{239\,nm}$) signals. In order to get a reasonable amount of reference material, AP-maltose fractions of several runs were collected and freeze-dried over P_2O_5 in vacuum.

AP-derivatives of several glucans (pullulan, dextran and partially hydrolized starch-glucan) were prepared according to the procedure in Table 1 and investigated by means of SEC-mass/molar with respect to de facto (absolute) molecular weight distributions and molecular weight mean values.

Pullulan 20 (Shodex, Pullulan P-82 kit) and Dextran 25 (Pharmacosmos Dextran standards kit) are commercially available materials with manufacturer specification provided on datasheets. AP-pullulan was applied for testing the reliability of quantitative transformation and stabilization of derivatives for standard-grade narrow distributed non-branched glucans, AP-dextran for slightly broader distributed branched glucans.

Broad distributed Pullulan, a gift of Dr. Beate Pfannemüller (Freiburg/FRG), and Dextran 70, a commercial bulk product of Pharmacia, were applied to test the quantitative and stable formation of AP-glucans with non-standard-grade broad distributed, non-branched and branched materials.

Partially α-amylase-hydrolized waxymaize starch glucans, with ultrafiltration eliminated low molecular components, a gift of Laevosan (Fresenius), Austria, was applied to test the quantitative and stable formation of AP-glucans for a mix of loose and densely packed highly short-chain branched starch glucan (amylopectin).

2.2. Background of glucan labeling

Several requirements have to be fulfilled before AP-glucans may be judged as appropriate to obtain de facto (absolute) molecular weights by subsequent SEC-mass/molar analysis:

- Hemiacetals must be 'chemically' available for pyridylamination: at native conditions polysaccharides typically are heavily aggregated and stabilized as well by inter- as by intra-molecular H-bonds; additionally, immobilized water layers form barriers for fast quantitative substitution. Hence, extended dissolution/reaction periods must be applied to achieve total transformation of hemiacetal groups. An advantage of the suggested procedure is the fact that approx. 5 molar 2-aminopydine is a weak base (pK_a 6.82) and, hence, supports loosening of transformation-hindering H-bonds, optionally hydrolizes esters (phosphates, lipids) and releases mineral cations (Ca^{2+}, Mg^{2+}, K^+, Na^+) and optionally attached proteins.
- Hemiacetal transformation must be quantitative: kinetics of derivatization of hemiacetals with 2-aminopyridine was investigated with Pharmacia-dextran T70. This glucan is a well known polymeric compound which perfectly elutes with all components in the selective separation range of the applied SEC-system. Degree of derivatization of terminal hemiacetals was determined referring to obtained apparent number average of molecular weight (M_n(app)) for increasing periods of derivatizing with 2-aminopyridine according to the procedure presented in Table 1. Although dextran T 70 dissolves easily in aqueous media and hence, terminal hemiacetals are supposed to be very well accessible for substitution with 2-amionpyridine, transformation follows an exponential law with achieved 50% after slightly more than 2 h, and quantitative substitution after approx. 26 h at 60 °C

Table 1
Preparation of quantitative turnover of free hemiacetal groups in glucans and stabilizing of formed AP-glucans

Materials/situation	Action
100 mg of oligo/polysaccharide with terminal hemiacetal + 1000 mg 2-aminopyridin in a locked-up 10 mL Pyrex tube	Vortex mixing/homogenisation
+0.5 mL H_2O_{bidest}	Dissolving at 60 °C, stirring till clear solution is achieved
+2.5 mL 4 M HCl	Neutralization to pH 7
Formation of Schiff-base	Stirring for 20 h @ 60 °C
+0.2 g Na BH_4CN_{solid} → stabilization of AP-glucans by reduction of amides	Stirring for 48 h @ 60 °C
Clear solution of AP-glucans	10 min centrifugation @ 3500 rpm
Precipitating AP-glucans	Adding clear solution dropwise to 50 mL Me OH in a 100 mL Erlenmeyer
Reducing Me OH-dissolved excess of 2-amyinopyridin and salts	Separation of AP-glucan precipitate by centrifugation (50 mL centrifuge tube, 10 min @ 3500 rpm); removing supernatant with a Pasteur-pipette; transfer of precipitate in a 10 mL centrifuge tube filled with Me OH
Eliminating Me OH-dissolved excess of 2-amyinopyridin and salts	Three times: washing of precipitate with 5 mL Me OH in a 10 mL centrifuge tube; 10 min centrifugation @ 3500 rpm; removing the supernatant with a Pasteur-pipette
Elimination of Me OH	Two times: washing with 5 mL acetone in a 10 mL centrifuge tube; 10 min centrifugation @ 3500 rpm; removing the supernatant with a Pasteur-pipette
Approx. 80% yield of AP-glucans as dry fine white powder	Streaming with N_2 @ 50 °C for approx. 4 h

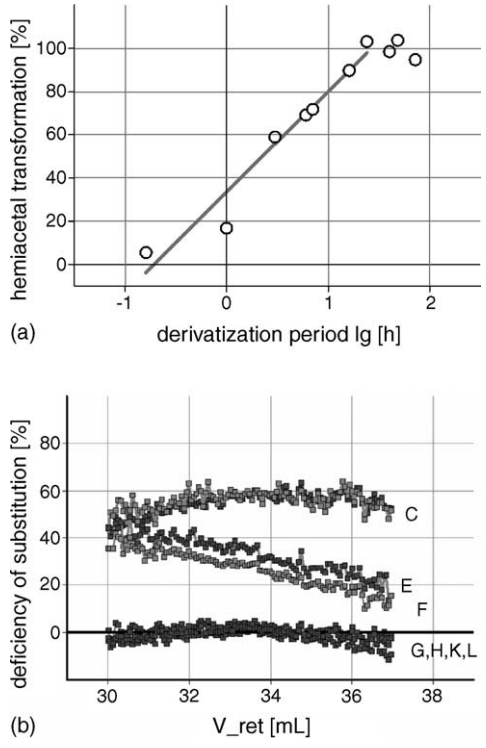

Fig. 2. (a) Transformation rate in logarithmic scaling of derivatization periods; linear approximation intercept = 33.27 and slope = 46.86: 50% transformed after 2.3 h; 100% transformed after 26.5 h; t_% = 33.27 + 46.86.c_g(h). (b) Development of derivatization for increasing periods for individual SEC-separated fractions of Dextran T 70. Deficiency of pyridylamination on Dextran T 70 @ increasing reaction periods according Table 2: C → 3 h, E → 7 h, F → 16 h, G → 24 h, H → 40 h, K → 48 h, L → 72 h.

(Fig. 2(a); Table 2). Detailed investigation of individual glucan fractions of dextran T70 reveals a rather uniform attack of hemiacetals in the initial derivatization status up to transformation of approx. 60%, followed by preferred transformation of low-dp glucans with obvious reciprocal dependence of transformation on degree of polymerization (Fig. 2(b)). Although molecular weight distribution covers approx. the same range for transformation of 60 and 100%, the apparent high-dp fractions decrease significantly with increasing periods of derivatization procedure and the maximum shifts to lower dps. Consequently, computed apparent mean values, particular in terms of number average molecular weight M_n(app), decrease to a stable within experimental error actual M_n-values when quantitative substitution is achieved (Table 2).

- The applied derivatization process must not cause glucan degradation and formed AP-derivatives must be sufficiently stable (Table 1). Mild conditions in terms of reaction temperatures in the range 60–65 °C guarantee no thermally caused glucan degradation and, nevertheless, provide reasonable reaction rates. Stabilization of AP-derivatives primarily is achieved by reduction of the intermediate Schiff-base (imin) to the secondary amin at neutral pH 6–7. Neutralization of weak alkaline 2-AP with HCl provides a buffer system aminopyridine chloride/aminopyridine which buffers minor HCl-excess. Similar conditions of the medium is obtained if neutralization is achieved with acetic acid, however, formed AP-acetates and excess of acetic acid hardly can be removed quantitatively in subsequent purification procedures, interfere with AP-glucans in subsequent SEC-analysis with 0.05 M NaCl as eluent, shift into selective separation range of AP-glucans and seriously disturb quantification of AP-glucans.
- Well known low-dp AP-derivatives of carbohydrates must be prepared for evaluating quantification of SEC-analysis.

In a first approach retention characteristics of pure 2-aminopyridine chloride on the applied SEC-system was found to be rather enthalpy than entropy controlled, as due to interaction with the gel matrix retention was observed after approx. two times total permeation volume. Less pronounced, however, similar retention behavior was observed with AP-maltose which finally was applied as internal standard for calibration of fluorescence detector signal. Obviously, hydrophobicity of the pyridine-residue results in a shift of the AP-carbohydrates such as AP-maltose out of the selective SEC-separation range which favors these materials as internal standards as they do not interfere with SEC-separated oligomer/polymer materials and are not superimposed by small compounds such as salts.

With respect to the response factor of the fluorescence signal, pure 2-aminopyridine was far higher than any investigated AP-derivatives. The difference between AP-glucose chloride and AP-maltose chloride, however, only was 2%, and the decrease from AP-maltose chloride to AP-maltotriose chloride was found approx. a magnitude smaller than between AP-glucose and AP-maltose, and hence, negligible within experimental error. These results where confirmed for AP-maltohexose and AP-maltoheptaose. Therefore, not 2-aminopyridine [27] but home made extra pure AP-maltose chloride was applied as calibration material for quantification of fluorescence detector elution profiles (Fig. 3). This approach is favored to reported offline-calibration with synthetic AP-amyloses [26–28] as such calibration constants refer to mean values of molecular weight, and hence, depend as well on availability of such materials as on accuracy and significance of prior molecular weight determination.

2.3. SEC-mass/molar

De facto (absolute) molecular weight distributions and mean values of molecular weight were obtained from refractive-index- and fluorescence-detection of SEC-separated AP-glucans. The utilized SEC-mass/molar system consists of an injector (Autosampler Shimadzu SIL 6B/150 μL injection loop), a separation section (precolumn: Fractogel HW 40: 100 mm × 10 mm + Superose 12: 300 mm × 10 mm + Superose 6: 280 mm × 10 mm + Fractogel HW 40: 290 mm × 10 mm), detection of mass fractions

Table 2
Transformation of terminal hemiacetals into AP-derivative for Dextran T 70 at 60 °C

Status of AP-labeling for Dextran T 70	Period (h)	Transformation (mean %) Fig. 2(a and b)	M_n(app) (g/mol)	M_w(app) (g/mol)	Polydispersity M_w/M_n(app)
Reference		100	M_n(ref): 43,450	M_w(ref): 62,100	M_w/M_n(ref): 1.43
A	0.16	6	–	–	–
B	1	17	–	–	–
C	3	62	69,980	93,730	1.34
D	6	63	63,000	95,390	1.52
E	7	75	58,110	86,840	1.49
F	16	79	55,190	83,870	1.52
G	24	96	45,130	63,350	1.40
H	40	94	46,100	64,410	1.40
K	48	96	45,380	64,180	1.41
L	72	97	44,540	62,080	1.39

Linear correlation for logarithmic scaling of derivatization periods: t_% = 33.27 + 46.86 lg(h)

Transformation (%)	Period lg(h)	Period (h)
10	−0.4966	0.32
20	−0.2832	0.52
30	−0.0698	0.85
40	0.1436	1.39
50	0.3570	2.28
60	0.5704	3.72
70	0.7838	6.08
80	0.9972	9.94
90	1.2106	16.24
100	1.4240	26.55

Mole number (n) of AP-dextran is given by the ratio of mass concentration (c) and number average molar mass (M_n) (n (mol/L) = c (g/mL)/M_n (g/mol)) and any (x) percentage of already formed AP-dextran ($n(x)$%) may be computed as 100 times the ratio of M_n(ref)/M_n(app,x).

(Altex 156 differential refractive index), detection of molar fractions (Jasco FP-1520, fluorimeter, excitation @ $\lambda_{ex=315}$, detection @ $\lambda_{em=380}$), a PC-supported data acquisition system (Windows-based PC-20000 A/D-conversion/Burr-Brown; software: CODAwin32/a.h group), digital data processing (Windows-based CPCwin32/a.h group) and documentation (Microsoft Office). For each experiment 5–6 mg of the sample was dissolved in 1 mL eluent (0.05 M $NaCl_{aqu}$) and spiked with 20 μL raffinose (1 mg/20 μL eluent) for correction of flow rate and adjustment of RI-signal, and 20 μL AP-maltose (0.5 mg/mL dissolved in eluent) for adjustment of fluorescence-signal as internal standards. Flowrate was set to 0.6 mL/min by LKB2150 HPLC-pump. Lag-correction for refractometer- and fluorimeter-profiles was achieved by prior determined retention-shift for methanol.

2.4. Computational approach

As well the SEC-separated non-derivatized as the AP-labeled glucans were monitored with respect to their mass- and molar elution profiles. Raw data elution profiles of mass-fractions (raw_mass) were achieved with a differential refractive index (DRI) detector, molar fractions (raw_FL) with a fluorescence detector (FL: $\lambda_{excitation}$ = 315 nm/$\lambda_{emission}$ = 380 nm).

To get absolute mass-information the DRI-calibration constant was determined with concentration series of maltose, AP-maltose, dextran and AP-dextran. For absolute molar concentrations for the SEC-eluted fractions, the fluorimeter calibration constant was determined with concentration series of AP-maltose and AP-dextran. A slight dependence of UV–vis absorption maimum on the degree of polymerization, shifting from 365 nm (AP-maltose) to 380 nm (AP-dextran 70 as an instance for AP-glucans), was observed. With respect to polymer analysis, calibration constant was determined at 380 nm with excitation wavelength 315 nm (Fig. 3). Absolute molecular weight calibration of the SEC-mass/molar system (raw_MWV) was achieved by computation of the logarithm of the $conc_{mass}$ and $conc_{molar}$ profiles ratio (Eq. (1a)). If raw data indicate a linear correlation, a two-coefficient least squares polynomial fit to the obtained raw data (Eq. (1b)) yields a linear calibration of log(M) versus retention volume (fit_MWV). For the case of obviously non-linear raw data, a least squares fit with four to six Cubic B-splines (Eq. (1c)) is an appropriate and numerically stable opportunity to obtain a corresponding non-linear calibration of log(M) versus retention volume (fit_MWV).

$$\text{raw\_MWV} = \lg\left(\frac{\text{conc}_{\text{mass}}}{\text{conc}_{\text{molar}}}\right) \tag{1a}$$

$$\text{fit\_MWV} \leftarrow (y_{\text{fit\_MWV}})_i = \sum_{n=0}^{1} a_n (x^n_{\text{raw\_MWV}})_i \tag{1b}$$

$$\text{fit\_MWV} \leftarrow (y_{\text{fit\_MWV}})_i = \sum_{n=0}^{4} C_n (x_{\text{raw\_MWV}} - X_n)^3_i \tag{1c}$$

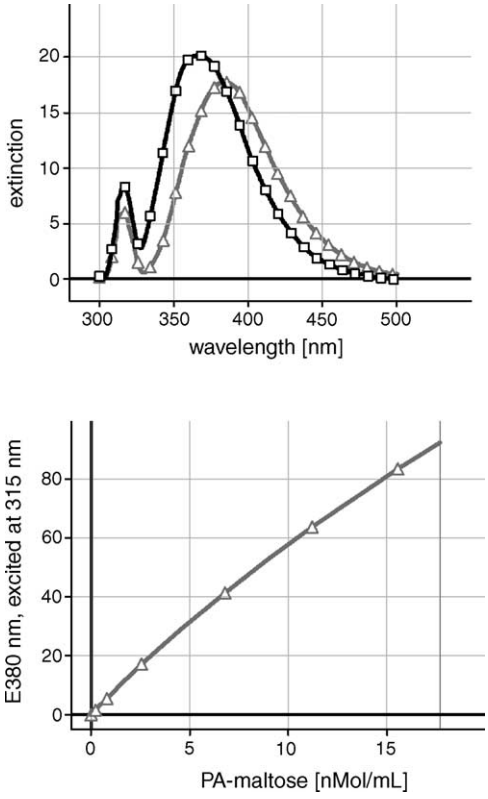

Fig. 3. UV–vis spectra of aminopyridyl (AP)-maltose (□) and aminopyridyl (AP)-dextran (△); calibration of fluorescence detector with a concentration series of AP-maltose @ 380 nm; molecular weight of AP-maltose ($C_{17}H_{28}O_{10}N_2$) 420 g/mol.

Application of linear or non-linear molecular weight calibration function to SEC-elution profiles of mass fractions enables computation of molecular weight distributions (differential mass fractions molecular weight distribution: m_MWD_d (Eq. (2a)); differential molar fractions molecular weight distribution: n_MWD_d (Eq. (2b)), mean values of molecular weight (number average: M_n; weight average: M_w) and polydispersity (PD) as ration M_w/M_n.

$$m(M)_i = \frac{\mathrm{d}m(M)}{\mathrm{d}M} \quad \text{with}$$

$$\int_0^\infty m(M)\,\mathrm{d}M = \text{m\_MWD\_d} = 1.0 \tag{2a}$$

$$n(M)_i = \frac{m(M)_i/M_i}{\int_0^\infty [m(M)_i/M_i]\,\mathrm{d}M}$$

$$\text{with} \quad \int_0^\infty n(M)\,\mathrm{d}M = \text{n\_MWD\_d} = 1.0 \tag{2b}$$

m_MWD_d is the differential molecular weight distribution – mass fractions; n_MWD_d the differential molecular weight distribution – molar fractions; *i* the *i*th component; *n* the molar fraction; *m* the mass fraction and *M* is the molecular weight of a narrow fraction.

3. Results and discussion

Quantitative and stabilized transformation of glucans with free terminal hemiacetal functionality into AP-glucans provides an opportunity to mark individual glucan molecules, and hence, an opportunity of 'counting' molecules, or in more chemical terms, to determine molar concentrations by UV/fluorescence spectroscopy. This approach does not interfere with optional present supermolecular structures, in particular for the case of dynamically formed/disintegrated aggregates and associates. As combined information about molar and mass concentration of sample fractions provides an approach to absolute fraction molecular weight, SEC-mass/molar-analysis of fluorescence-labeled glucans is an alternative to aggregate-sensitive light scattering or viscosity to determine absolute molecular weight distributions. Although molecular weight of aggregates for sure are of interest, de facto molecular weight of constituting glucan molecules might be an even more important parameter, since many material qualities are correlated with molecular weight of constituting molecules and not with apparent molecular weight of aggregates. Practically any prognosis of material characteristics refers to the magnitude of mean molecular weights and shape of molecular weight distribution. Hence, for appropriate classification of material qualities, reliable de facto molecular weight data are desperately needed, in particular for materials with complex background regarding their formation and with pronounced tendency to form supermolecular structures – such as for glucans.

3.1. AP-labeled narrow distributed low-dp standard glucans

The capability of introduced derivatization procedure for absolute molecular weight determination of glucans with terminal hemiacetals by SEC-mass/molar was tested in a first approach with different types of narrow distributed standard glucans: non-branched pullulan 20 from Shodex P-82 kit and branched dextran 25 from Pharmacosmos dextran standard series. Both glucans are bacterial exopolysaccharides, pullulan made by *Aureobasidium pullulans*, dextran for instance by *Pseudomonas mesenteroides*.

Pullulan consists of $\alpha(1\rightarrow 6)$ linked maltotriose residues, a terminal hemiacetal, contains no branches and no substituents. In aqueous media and for moderate concentrations, pullulans only have minor tendencies to form supermolecular structures. Regarding the datasheet, the investigated fraction P20 is rather uniform with respect to degree of polymerization distribution, specified with polydispersity PD 1.07 (Table 3).

Dextran consists of $\alpha(1\rightarrow 6)$-linked and $\alpha(1\rightarrow 3)$-branched glucosylresidues with varying ratios between 50/50 and 95/5 for the content of $\alpha(1\rightarrow 6)$ and $\alpha(1\rightarrow 3)$ linkages, respectively. Dextrans are well soluble in aqueous media, however, primarily due to the branching, they form intramolecular stabilized rather compact coils. The high-dp

Table 3
Datasheet specifications and obtained experimental data from SEC-mass/molar for investigated polysaccharides: Pullulan 20, Dextran 25; Dextran T 70, broad distributed Pullulan and partially hydrolized starch glucan

	Pullulan P-20 (Shodex P-82 standards kit)			Dextran 25 (Pharmacosmos Dextran Standards kit)		
	Manufacturer datasheet	SEC-mass/molar	Δ (%)	Manufacturer datasheet	SEC-mass/molar	Δ (%)
M_p (g/mol)	22,670	21,900	−4	21,400	23,600	+10
M_w (g/mol)	23,200	24,300	+5	23,800	24,130	+1
M_n (g/mol)	21,400	22,490	+5	18,300	18,720	+2
M_w/M_n	1.07	1.09	+2	1.27	1.29	+2

		Dextran T 70	Broad distributed Pullulan	Partially hydrolized starch glucans
Origin/provider		Pharmacia	Gift of Laboratory Prof. Pfannemüller, Freiburg/FRG	Gift of Laevosan (now Fresenius), Linz/Austria
Determined by SEC-mass/molar	M_w (g/mol)	62,100	124,000	89,700
	M_n (g/mol)	43,450	71,000	40,700
	M_w/M_n	1.43	1.75	2.20
	Molecular weight range (g/mol)	$(10–200) \times 10^3$	$(3–400) \times 10^3$	$(1–320) \times 10^3$
Composition		$\alpha(1 \rightarrow 6)$-Glc_n $\alpha(1 \rightarrow 3)$-[$\alpha(1 \rightarrow 6)$-Glc_n] (branches)	$\rightarrow 6)$-[Glc-$\alpha(1 \rightarrow 4)$-Glc-$\alpha(1 \rightarrow 4)$-Glc]-$\alpha(1 \rightarrow$	$\alpha(1 \rightarrow 4)$-Glc_n $\alpha(1 \rightarrow 6)$-[$\alpha(1 \rightarrow 4)$-Glc_n] (branches) elimination of low-dp components by ultracentrifugation
Molecule-conformation @ conditions of SEC-elution: aqueous 0.05 M NaCl		Homogeneous-compact coils	Homogeneous-stiff, flexible rod-shaped structures	Heterogeneous-mix of helical, rod shaped + compact coils
SEC-calibration function		Linear	Linear	Non-linear

dextrans even tend to form supermolecular aggregates, however, with high solubility in aqueous media. According the datasheet, dextran 25 is broad distributed compared to pullulan 20, however, in absolute terms still may be judged as midrange/narrow polydisperse (Table 3).

Both glucan standards were investigated by SEC-mass/molar before and after AP-labeling. In both cases insignificant differences in the elution profiles of mass fractions (Figs. 4(a and b) and 5(a and b)) and, as expected, major differences in fluorescence-detected molar fractions were observed. Whereas normalized DRI-detected elution profiles (mass_ev: area = 1.0 within selective SEC-separation range) of mass fractions remain identical before and after AP-labeling, only for the AP-derivatives molar elution profiles could be obtained (Figs. 4(c) and 5(c)).

Obtained DRI and fluorescence detected SEC-elution profiles are transformed into absolute mass ($conc_{mass} \rightarrow$ mg/mL) and molar ($conc_{molar} \rightarrow$ mol/L) profiles (Figs. 4(d) and 5(d)) for computation of absolute SEC-molecular weight calibration function as the logarithm of profiles ratio (Figs. 4(e) and 5(e)). A linear fit (fit_MWV) to the obtained logarithmic raw-data (raw_MWV) in the well defined midrange section is reasonable as SEC-separation is controlled by an exponential entropy-term ($\exp^{S/R}$) and for the case of quasi-homologous polymers, such as pullulan or in a first approach even for dextran, simplifies to an exponential separation according to molecular weight (degree of polymerization) – or a linear separation according to the logarithm of molecular weight (degree of polymerization). However, linear correlations have different slopes and intercepts for different polymer coil packing densities, and hence, become increasingly complicated – and non-linear – for the case of mixes which, for instance, may be due to variations in branching patterns depending on degree of polymerization.

Absolute molecular weight distributions for pullulan 20 and dextran 25 are obtained by application of the absolute calibration functions (fit_MWV) and result in a dual kind of result: normalized (area = 1.0) molecular weight distribution of mass fractions in differential form (m_MWD_d) and normalized (area = 1.0) molecular weight distribution of molar fractions in differential form (n_MWD_d) (Figs. 4(f) and 5(f)). Mean values in terms of weight average molecular weight (M_w) and number average molecular weight (M_n) simply are computed as the moments of obtained molecular weight distributions. As well for pullulan 20 as for dextran 25 differences for M_w, M_n and M_w/M_n between manufacturer specifications and obtained results from SEC-mass/molar are within experimental error (Table 3).

Molecular weight distribution of molar fractions (n_MWD_d) may be obtained from SEC-mass/molar twofold: directly by applying the absolute calibration function (fit_MWV) on the fluorescence-detected SEC-profile, or indirectly by computation from m_MWD_d, obtained from application of absolute calibration function on the DRI-detected SEC-profile. Both results match sufficiently well and may be regarded as identical within experimental error (Figs. 4(g) and 5(g)). The directly determined n_MWD_d-functions are less noisy in the low-dp range which easily may

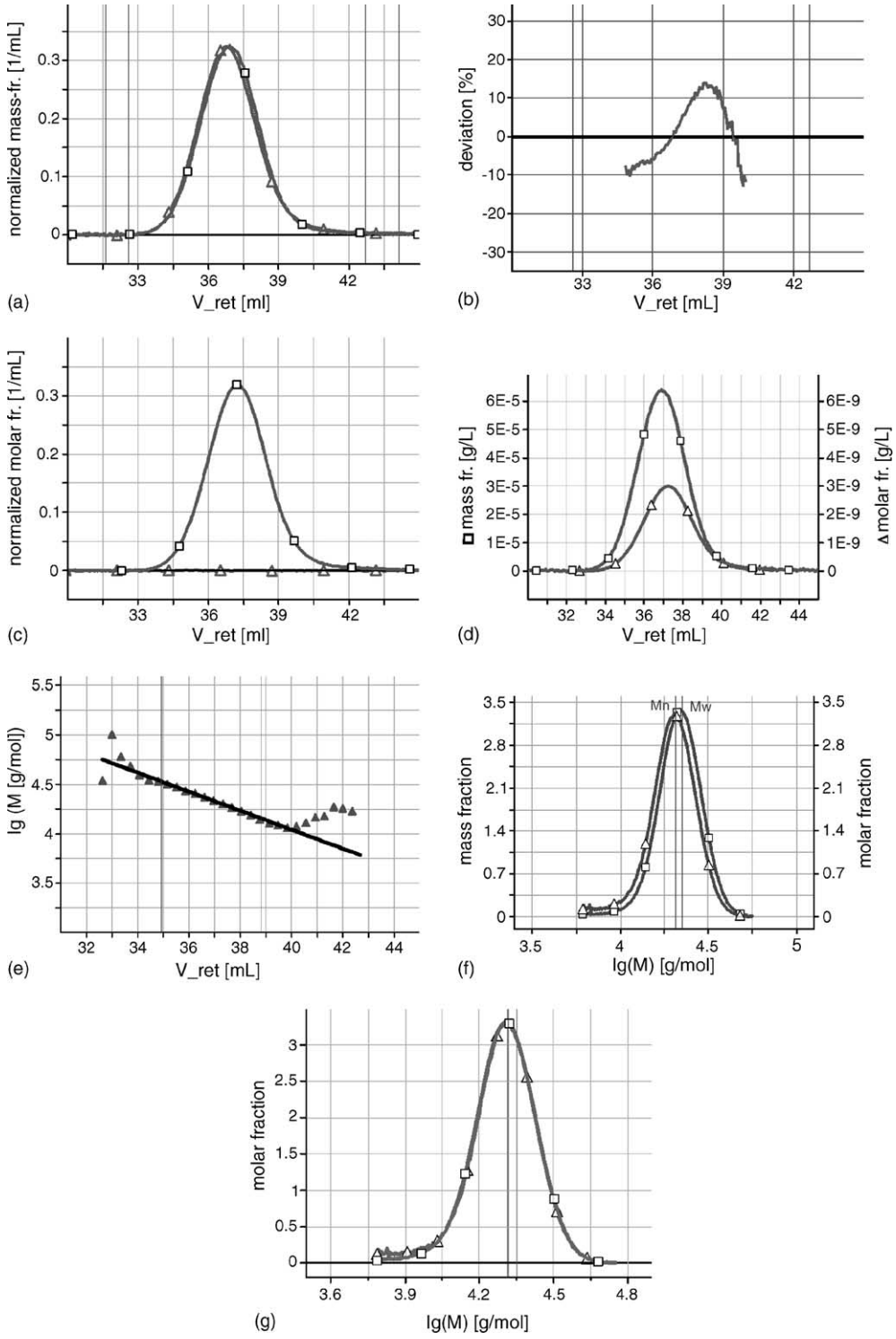

Fig. 4. (a) Pullulan 20 before/after AP-labeling; normalized SEC-elution profile of mass fractions: Pullulan 20 (△), AP-Pullulan 20 (□). (b) Pullulan 20 before/after AP-labeling; differences in the normalized SEC-elution profiles computed as [(AP-Pullulan 20/Pullulan 20) − 1.0] × 100; slightly decreased polarity of AP-derivatives may have caused a slight V_ret-shift to higher values, the resulting mass profile however, is identical with initial Pullulan 20 within experimental error. (c) Pullulan 20 before/after AP-labeling; normalized SEC-elution profile of molar fractions @ λ 380 nm: Pullulan 20 (△) no signal within experimental error; AP-Pullulan 20 (□). (d) AP-Pullulan 20: SEC-elution profiles of absolute mass ($conc_{mass}$, g/L, □) and molar ($conc_{molar}$, mol/L, △) concentrations. (e) AP-Pullulan 20: absolute molecular weight calibration of applied SEC-system; raw data: raw_MWV (△ △ △) and linear least squares fit to well defined linear middle section: fit_MWV (—). (f) AP-Pullulan 20: molecular weight distributions from SEC-mass/molar: distribution of mass fractions (m_MWD_d, □), distribution of molar fractions (n_MWD_d, △); weight average molecular weight M_w = 24,300 g/mol (|), number average molecular weight M_n = 22,490 g/mol (|); polydispersity M_w/M_n = 1.09 and (g) AP-Pullulan 20: distribution of molar fractions (n_MWD_d): directly obtained from fluorescence-detected SEC-profile (□), transformed from DRI-detected SEC-profile (△).

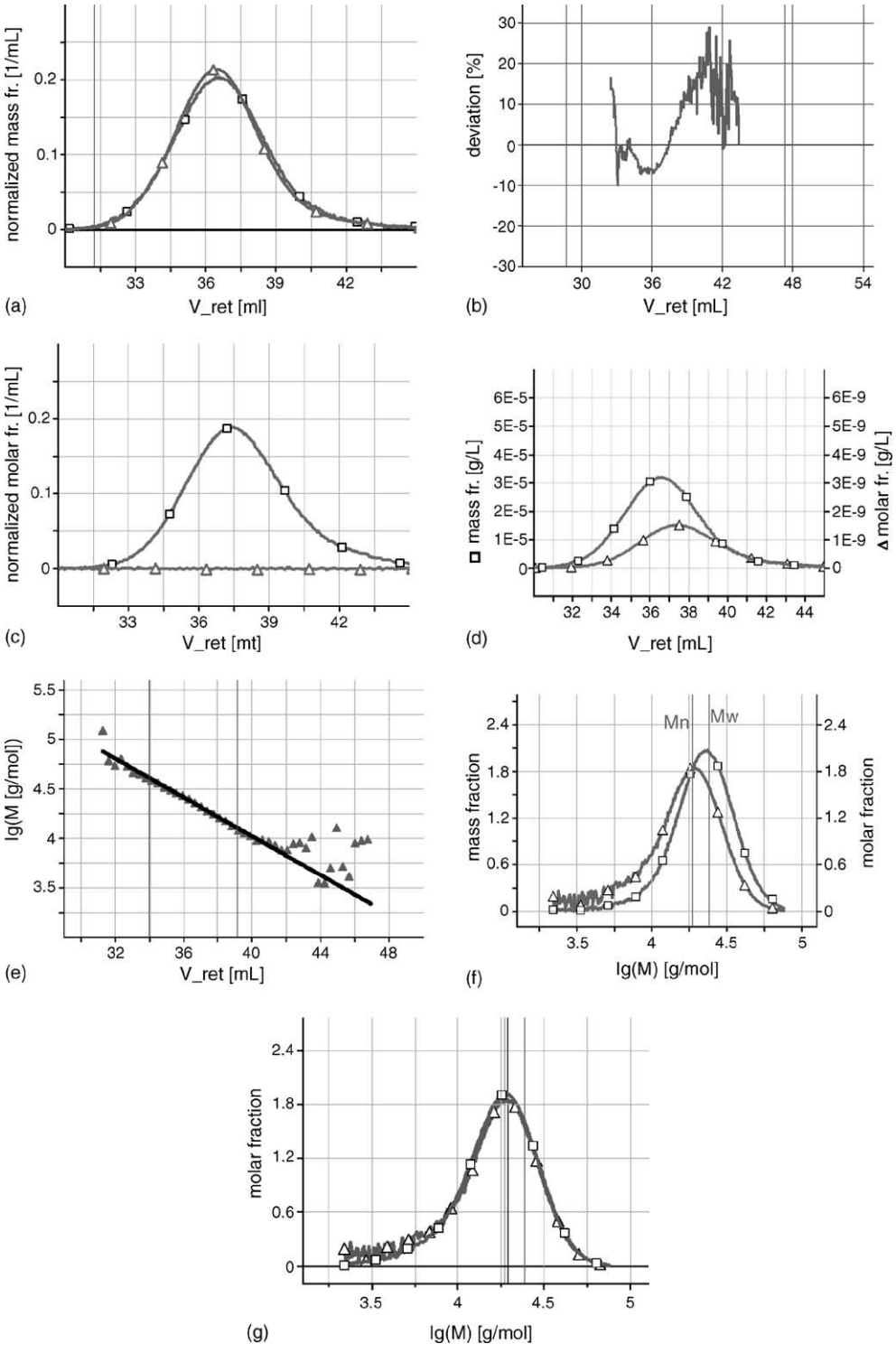

Fig. 5. (a) Dextran 25 before/after AP-labeling; normalized SEC-elution profile of mass fractions: Dextran 25 (△), AP-Dextran 25 (□). (b) Dextran 25 before/after AP-labeling; differences in the normalized SEC-elution profiles computed as [(AP-Dextran 25/Dextran 25) − 1.0] × 100; slightly decreased polarity of AP-derivatives may have caused a slight V_ret-shift to higher values, the resulting mass profile however, is identical with initial Dextran 25 within experimental error. (c) Dextran 25 before/after AP-labeling; normalized SEC-elution profile of molar fractions @ λ 380 nm: Dextran 25 (△) no signal within experimental error; AP- Dextran 25 (□). (d) AP-Dextran 25: SEC-elution profiles of absolute mass ($conc_{mass}$, g/L, □) and molar ($conc_{molar}$, mol/L, △) concentrations. (e) AP-Dextran 25: absolute molecular weight calibration of applied SEC-system; raw data: raw_MWV (△ △ △) and linear least squares fit to well defined linear middle section: fit_MWV (—). (f) AP-Dextran 25: molecular weight distributions from SEC-mass/molar: distribution of mass fractions (m_MWD_d, □), distribution of molar fractions (n_MWD_d, △); weight average molecular weight M_w = 24,130 g/mol (|), number average molecular weight M_n = 18,720 g/mol (|); polydispersity M_w/M_n = 1.29 and (g) AP-Dextran 25: distribution of molar fractions (n_MWD_d): directly obtained from fluorescence-detected SEC-profile (□), transformed from DRI-detected SEC-profile (△).

be explained by the better signal to noise ratio of molar detection for low-dp components compared to mass-detection.

Obviously, quantitative and stabilized AP-labeling of terminal hemiacetals could be achieved for narrow distributed low-dp glucans pullulan 20 and dextran 25. Degradation was observed neither for pullulan 20 nor for dextran 25 and obtained molecular weight distributions and mean values of molecular weight match perfectly with manufacturer specifications.

3.2. AP-labeled broad distributed non-standard-grade glucans

Degree of polymerization of several hundreds and increased heterogeneity in terms of width of molecular weight distribution was investigated with dextran T 70 and a broad distributed pullulan sample. As already demonstrated, quantitative AP-labeling primarily is controlled by accessibility of hemiacetals of the most-high-dp components which needs processing periods of at least 24 h. However, as well for dextran T 70 as for broad distributed pullulan, quantitative labeling could be achieved without hydrolizing glycosidic linkages or any other degradation phenomena, however, stabilizing formed AP-derivatives.

Achieved absolute SEC-elution profiles of mass and molar fractions of dextran T 70 (Fig. 6(a)) enabled computation of a strictly linear absolute SEC-calibration function (Fig. 6(b)) which indicates a rather homogeneous composition in terms of conformation of high- and low-dp molecules. Obtained absolute molecular weight distributions (Fig. 6(c)) range from (10 to 200) $\times 10^3$ g/mol with weight average molecular weight 62,100 g/mol. The slightly lower values for obtained mean molecular weights than specified by the manufacturer might indicate the presence of minor amounts of aggregates in the sample after usual dissolution periods of several minutes.

Whereas dextran T 70 is branched and supposed to form rather compact coils which are intra-molecular stabilized by hydrogen-bonds, the broad distributed non-branched pullulan forms a rather stiff zigzag-sequence of $\alpha(1 \rightarrow 6)$-linked maltotriose residues resulting in a rather stiff, however, flexible rod-like conformation of pullulan-molecules. The $\alpha(1 \rightarrow 6)$-linkage in pullulan avoids pronounced intermolecular alignment, stabilization and precipitation, finally. Hence, pullulan is well soluble in aqueous media, the excluded volume however, is much higher than that of compact coil forming dextran. As a result, the absolute SEC-calibration function (Fig. 7(b)) obtained from mass/molar-detection of pullulan (Fig. 7(a)), although linear like the one for dextran, shows up with rather different intercept and slope. Due to different molecule conformation in the aqueous eluent, molecular weight at identical positions of retention from

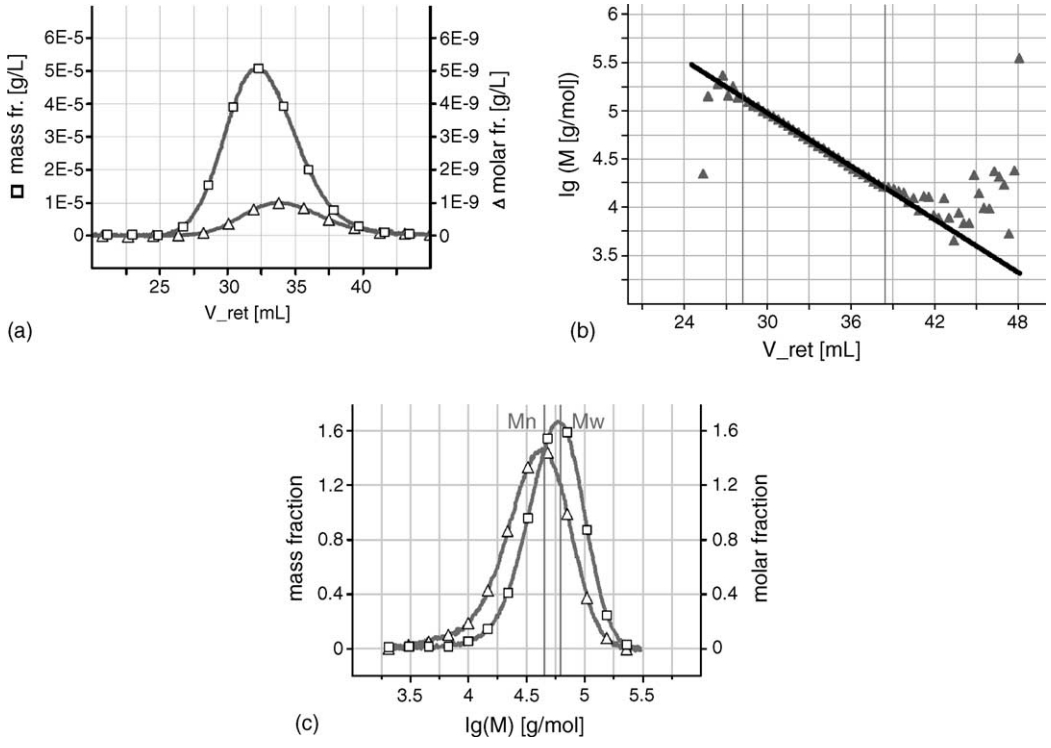

Fig. 6. (a) AP-Dextran 70: SEC-elution profiles of absolute mass ($conc_{mass}$, g/L, □) and molar ($conc_{molar}$, mol/L, △) concentrations. (b) AP-Dextran 70: absolute molecular weight calibration of applied SEC-system; raw data: raw_MWV (△ △ △) and linear least squares fit to well defined linear middle section: fit_MWV (—). (c) AP-Dextran 70: molecular weight distributions from SEC-mass/molar: distribution of mass fractions (m_MWD_d, □), distribution of molar fractions (n_MWD_d, △); weight average molecular weight M_w = 62,100 g/mol (|), number average molecular weight M_n = 43,450 g/mol (|); polydispersity M_w/M_n = 1.43.

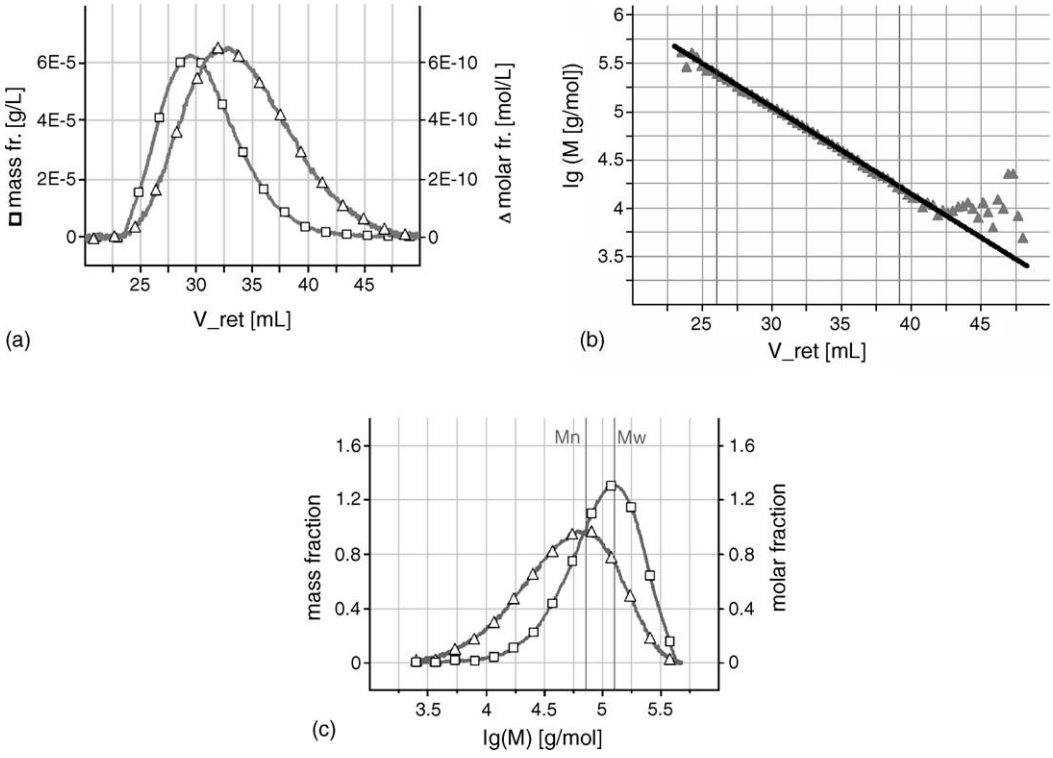

Fig. 7. (a) AP-Pullulan, broad distributed: SEC-elution profiles of absolute mass ($conc_{mass}$, g/L, □) and molar ($conc_{molar}$, mol/L, △) concentrations. (b) AP-Pullulan, broad distributed: absolute molecular weight calibration of applied SEC-system; raw data: raw_MWV (△ △ △) and linear least squares fit to well defined linear middle section: fit_MWV (—). (c) AP-Pullulan, broad distributed: molecular weight distributions from SEC-mass/molar: distribution of mass fractions (m_MWD_d, □), distribution of molar fractions (n_MWD_d, △); weight average molecular weight M_w = 124,000 g/mol (|), number average molecular weight M_n = 71,000 g/mol (|); polydispersity M_w/M_n = 1.75.

SEC separation is significantly lower for pullulan than for dextran. Obtained molecular weight distributions of the investigated pullulan sample (Fig. 7(c)) covers an even wider range than that of dextran and is approx. double of dextran with respect to weight average molecular weight M_w.

3.3. AP-labeled broad distributed starch-glucan hydrolizate

Finally, AP-derivatives of a partially – by means of technical grade thermophilic α-amylase – hydrolyzed waxy maize starch glucan were prepared for absolute molecular weight analysis. Waxy maize glucans are highly short-chain branched and form compact and intramolecular stabilized coils. This sample contains a wide range of less compact coils of scb-glucans with tendencies for intermolecular interactions (formation of loose aggregates) and more stiff supermolecular structures formed and stabilized by helix–helix interaction may be the most important classes. As well due to the heterogeneity of possible conformations as due to the expected capability of forming supermolecular structures, determination of molecular weight distribution from SEC-analysis is a challenge.

AP-labeling of waxy-maize starch glucans at least is a promising approach to overcome the problem of supermolecular structures. Provided that quantitative transformation can be achieved with the procedure described in Table 1 within approx. 24 h, aggregation/disintegration-dynamics still may influence SEC-separation and connected detection, in particular light scattering detection, but will not fake (extremely) high molecular weights if molar concentrations are monitored by fluorescence-detection. Combined information of absolute mass concentration profile by DRI-detection and molar concentration profile of SEC-separated waxy maize AP-glucans (Fig. 8(a)) yields an obviously non-linear absolute SEC-calibration function (Fig. 8(b)). The expected mix of different molecule conformations results in a superimposed combination of conformation-specific slopes and intercepts. Each fraction is homogeneous with respect to excluded volume, however, a mix with respect to molecular weights: the identical excluded volume for a loose coil conformation glucan, for instance, will be much lower in molecular weight than a compact coil. Nevertheless, although even a non-linear fit to the raw-data SEC-calibration will not match heterogeneity of conformation within each narrow SEC-separated fraction, it is a first approach to actual molecular weight distribution. For the present experimental data a fit with five equidistantly spaced cubic B-splines provides an appropriate and numerically stable analytical form of the initial raw-data and enables computation of molecular weight distributions and mean values of molecular weight (Fig. 8(c)). However, detailed analysis of the conformation mixes within each

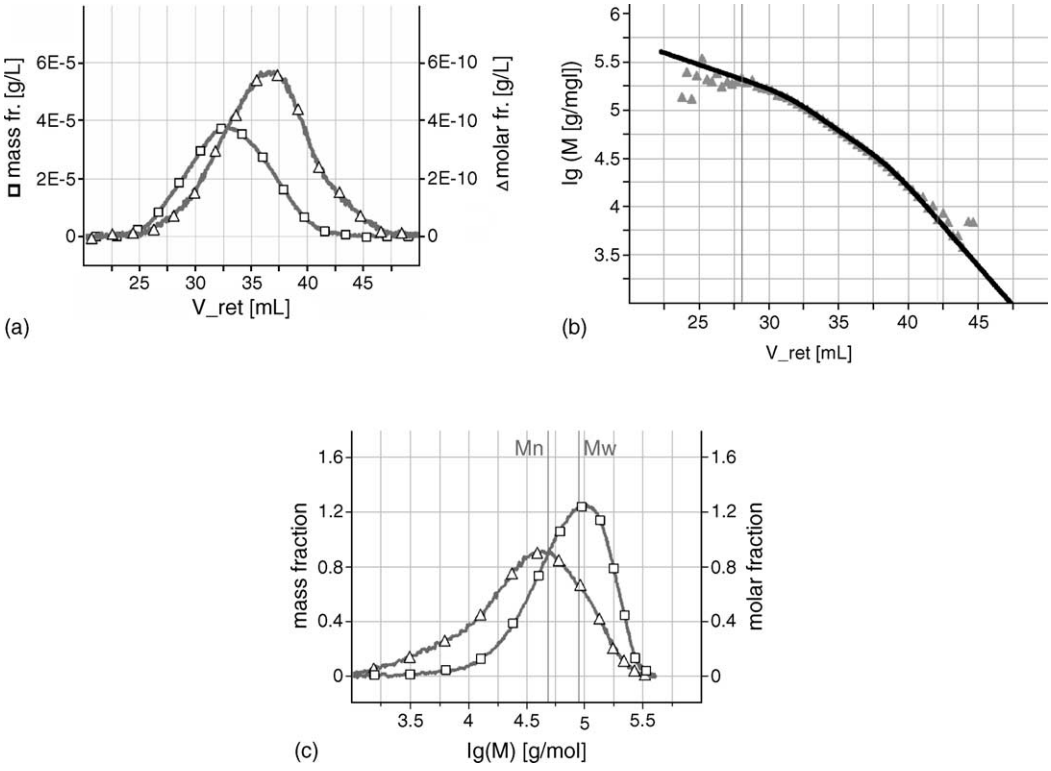

Fig. 8. (a) AP-derivatives of partially hydrolized waxy maize starch: SEC-elution profiles of absolute mass ($conc_{mass}$, g/L, □) and molar ($conc_{molar}$, mol/L, △) concentrations. (b) AP-derivatives of partially hydrolized waxy maize starch: absolute molecular weight calibration of applied SEC-system; raw data: raw_MWV (△ △ △) and linear least squares fit Cubic B-splines to well defined non-linear middle section: fit_MWV (—). (c) AP-derivatives of partially hydrolized waxy maize starch: molecular weight distributions from SEC-mass/molar: distribution of mass fractions (m_MWD_d, □), distribution of molar fractions (n_MWD_d, △); weight average molecular weight M_w = 89,700 g/mol (|), number average molecular weight M_n = 40,700 g/mol (|); polydispersity M_w/M_n = 2.20.

SEC-eluted fraction, and hence, even 'more real' molecular weight analysis would require complementary branching analysis of individual fractions. From point of view of mass fractions, the obtained results verify a rather successful process of elimination of low-dp glucans, from the point of view of molar fractions, however, illustrates that still a non-negligible percentage of these components is present.

4. Conclusion

Determination of de facto molecular weights for materials such with pronounced tendency to form supermolecular structures such as glucans, even with sophisticated experimental equipment still is a challenge. Well established techniques such as SEC-mass/LS and SEC-mass/viscosity are preferentially sensitive to the presence of aggregates and, hence, typically provide far too high figures as mean molecular weights. For glucans with terminal hemiacetals, such as for instance starch glucans, quantitative and stabilized labeling of these terminals opens a chance for an optional analytical approach by means of SEC-mass/molar which has proved not to interfere with supermolecular structures.

A procedure for quantitative and stabilized labeling of terminal hemiacetals of glucans from different sources and with variations in branching characteristics and width of molecular weight distribution has been successfully adapted from carbohydrate chemistry to polymeric compounds.

Quantitative monitoring of mass- and molar elution profiles of SEC-separated AP-glucans was achieved by calibration of DRI (mass) and fluorescence (molar) detector responses with an concentration series of AP-maltose. Different to AP-glucose, the response factors for AP-maltose proved to be insignificantly different to oligomer/polymer compounds.

The potential of AP-labeling combined with subsequent SEC-DRI/FL-analysis for determination of de facto molecular weight distributions was tested with standard-grade commercial low molecular weight glucans. Obtained molecular weight distributions and molecular weight mean values matched perfectly within experimental error with manufacturer specifications. The influence of increased and broader distributed degree of polymerization on obtained results from SEC-DRI/FL was investigated for a non-standard grade broad distributed pullulan and Pharmacia dextran T 70. As well for the loose packed pullulan coils as for the more compact dextran coils obtained values for molecular weight distributions proved the validity of the applied labeling procedure and the analytical approach.

Finally, AP-labeling was applied to a partially hydrolized highly short-chain branched broad distributed mix of waxy

maize starch glucans with tendency to form supermolecular structures. This 'close-to-reality' sample contains a variety of polymer coil packing densities which results in a non-linear SEC-calibration. Hence, the labeling procedure and the SEC-DRI/FL-analysis proved to work even for complex glucan matrices and is supposed to provide de facto molecular weight of constituting glucan molecules even in the presence of supermolecular aggregates.

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