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Characterization of cellulose acetates according to DS and molar mass using two-dimensional chromatography

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ABSTRACT

A two-dimensional liquid chromatographic method (2D LC) was developed to analyze the heterogeneities of cellulose acetates (CA) in the DS-range DS = 1.5–2.9 with respect to both, molar mass and degree of substitution (DS). The method uses gradient liquid chromatography (HPLC) as the first dimension in order to separate by DS followed by separation of the different fractions by size (SEC) in the second dimension. The 2D experiments revealed different correlations between gradient and SEC elution volume. These correlations might arise from differences in the synthetic conditions. The newly developed 2D LC separation therefore provides new insights into the heterogeneity of CAs.

1. Introduction

Cellulose derivatives are synthetic polymers, which serve different industrial applications in the field of food, pharmacy, medicine, cosmetics and packaging due to their unique chemical and physical properties (Coffey, Bell, & Henderson, 2006; Doelker, 1993; Liu, 2006; Shibata, 2011; Siepmann & Peppas, 2001). The suitability of a given material for a particular application is determined by the chemical structure of the substituents, the chemical composition (i.e. average degree of substitution, DS, and/or average molar substitution, MS) and the molar mass and molar mass distribution (MMD). However, often derivatised celluloses from the same source having the same chemical composition and molar mass vary significantly in performance if applied in a particular application. Thus, the influence of the chemical heterogeneity, i.e. the distribution of the substituents among and along the chains as well as its correlation with molar mass has to be taken into account additionally (Arisz, Kauw, & Boon, 1995; Fischer et al., 2008; Kamide, Okajima, Kowsaka, & Matsui, 1987; Rinaudo, 2004; Takahashi, Fujimoto, Miyamoto, & Inagaki, 1987; Viridén, Wittgren, & Larsson, 2011). Therefore it is essential to characterize these parameters in order to establish reliable relationships between the structure and the properties of cellulose derivatives.

A number of analytical tools have been used to investigate the correlation between chemical composition and molar mass of cellulose derivatives. Cellulose xanthate (CX), carboxymethyl cellulose (CMC) and cellulose acetate (CA) were analyzed using SEC with multi-angle laser light scattering, refractive index and ultraviolet (MALLS/RI/UV) detection (Fischer, Krasselt, Schmidt, & Weightman, 2005; Fischer et al., 2008). These analyses required the presence of UV-active groups that can be detected by a UV-detector as a function of the molar mass determined by MALLS/RI. It was revealed for both CX and CMC that fractions of different molar mass possess a different DS with the lower molar mass fractions being higher substituted than the fractions of high molar mass, while the opposite was observed for CA. However, since SEC separations are based on size rather than on DS, the dependencies of DS on molar mass in these investigations do not represent true separations with respect to DS, but the chemical composition at each SEC elution volume has to be regarded as an average value of coeluting species differing in DS. In addition, since the derivatives like CMCs or CAs do not contain UV-active groups, they have to be modified to useful derivatives for this type of analysis. Thus, the applied modification procedures have to be quantitative to achieve reliable data. Fitzpatrick et al. (2006) investigated the dependence of DS on molecular size for fractions of intact and enzymatically hydrolyzed methyl celluloses (MC) by SEC-MALLS/RI/NMR. These data were correlated with cloud-point measurements. The results revealed no significant dependence of the DS on the molecular size for the fractions of the intact sample and nearly the same cloud curves were obtained

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Table 1
Characterization data of samples used (samples from the industrial source are marked in grey).

Sample name	DS[a]	σ^2 [b]	LS-M_w [c] [g/mol]	Correlation form of molar mass and gradient elution volume[d]
Sample 1	1.53	0.05	57,600	Form 2
Sample 2	1.59	0.06	72,700	Form 2
Sample 3	1.66	0.12	73,500	Form 2
Sample 4	1.72	0.01	44,600	Form 3
Sample 5	1.81	0.11	68,100	Form 2
Sample 6	1.87	0.12	64,900	Form 2
Sample 7	1.92	0.09	63,200	Form 2
Sample 8	1.95	0.12	76,400	Form 2
Sample 9	2.09	0.05	70,700	Form 2
Sample 10	2.16	0.14	71,900	Form 1
Sample 11	2.19	0.11	71,000	Form 1
Sample 12	2.27	0.13	74,400	Form 1
Sample 13	2.42	0.07	64,400	Form 3
Sample 14	2.45	0.06	64,700	Form 3
Sample 15	2.45	0.08	93,300	Form 3
Sample 16	2.60	0.05	69,800	Form 3
Sample 17	2.92	0.0007	175,000	Form 1

[a] Determined by ^{1}H-NMR.
[b] Calculated from DS-distribution peaks.
[c] Determined by SEC-MALLS in N,N-dimethyl acetamide/lithium chloride.
[d] Determined by 2D LC.

for fractions differing in size. However, significant differences in the clouding behaviour were observed for fractions from enzymatic degradation, the DS of which decreased with decreasing size. Thus, the changes in clouding behaviour were explained by differences in the DS and DS-distribution.

Liquid interaction chromatography is well suited for analyzing chemical heterogeneity in polymers (Chang, 2003, 2005; Philipsen, 2004; Trathnigg, 2000). However, interaction chromatography ideally separates by chemical composition only. Thus, any information on the molar mass distribution is lost. The opposite is true in size exclusion chromatography. Since the separation parameter in SEC is hydrodynamic size, molecules of different chemical composition having the same hydrodynamic size, will coelute. By coupling interaction chromatography and SEC in a two dimensional setup (2D LC), information on different aspects of molecular heterogeneity and their correlations can be obtained simultaneously (Baumgaertel, Altuntas, & Schubert, 2012; Berek, 2010; Pasch, 2000). Typically gradient HPLC is applied in the first dimension and coupled to SEC in the second dimension (Kilz, Krüger, Much, & Schulz, 1995; Raust, Brüll, Moire, Farcet, & Pasch, 2008; Siewing, Schierholz, Braun, Hellmann, & Pasch, 2001). There are several reasons why this separation order is preferred over the inverse one. Sample capacity in HPLC usually is higher than in SEC, which is rather sensitive for column overloading, especially when analysing high molar mass samples. HPLC allows adjusting more parameters in order to improve separation efficiency. Such optimizations allow obtaining more homogeneous fractions. Longer gradient run times used to optimize the separation do not interfere with the need of a fast second dimension separation for comprehensive sample characterization. In addition, application of SEC as the second dimension eliminates the necessity of second dimensional column equilibration. Finally, SEC as second dimension separation eliminates the danger of breakthrough effects upon transferring fractions from the first into the second dimension. Such breakthrough peaks are often observed in gradient chromatography of polymers when the sample solvent is a strong eluent (Jiang, van der Horst, & Schoenmakers, 2002).

If using HPLC in the first dimension, separation occurs by chemical composition and in the second dimension the compositionally homogeneous fractions elute as a function of decreasing size. Greiderer, Steeneken, Aalbers, Vivó-Truyols, and Schoenmakers (2011) applied 2D chromatography, to hydroxypropylmethylcellulose (HPMC), using a reversed-phase gradient system in the first and an aqueous SEC system in the second dimension. A large impact of column temperature on the retention times in both dimensions was observed. This was attributed to the occurrence of thermal gelation phenomena within the columns, requiring performing the experiments at a well-controlled low temperature. At low temperature, a separation according to molar hydroxypropyl substitution was established in the first dimension. The retention time increased with increasing molar substitution, while a separation according to molar mass in the second dimension was obtained. The separation method provided information about molar mass and chemical heterogeneities of the samples and their correlations. We have recently reported on gradient separations according to DS for CAs in the range DS = 1.5–2.9. However, using this one dimensional separation we were not able to investigate the correlation of DS on molar mass. Therefore it was the aim of the present work to develop a 2D separation for the simultaneous characterization of DS- and molar mass distributions of CAs and to show the advantages of 2D chromatography as compared to the underlying single chromatographic separations.

2. Experimental part

2.1. Solvents and samples

Dichloromethane (DCM), dimethyl sulphoxide (DMSO), 1,4-dioxane and methanol (MeOH) (VWR, Darmstadt, Germany) were of HPLC grade and used as received. Ammonium acetate was purchased from VWR (Haasrode, Belgium).

CA samples with average DS ranging from DS = 1.5–2.9 were used. Five of the samples (sample 4, DS = 1.72; sample 13, DS = 2.42; sample 14, DS = 45; sample 15, DS = 2.45; sample 17, DS = 2.92) were obtained from an industrial source (Rhodia Acetow GmbH, Freiburg im Breisgau, Germany). The others were prepared in our laboratory by partial saponification of a commercial high DS sample (sample 16, DS = 2.60, Acetati, Italy). Information on the average DS, variance (σ^2) of the DS-distribution, weight average molar masses (M_w) of the samples is compiled in Table 1. The details on the synthesis and characterization of the samples with respect to DS, σ^2 and M_w are given elsewhere (Ghareeb & Radke, 2013; Ghareeb, Malz, Kilz, & Radke, 2012).

Table 2
Gradient profile of multi-step gradient from DCM to MeOH.

t (min)	0	10	20	30	40	50	50.01
% MeOH	0	1	4.5	13	35	70	0

2.2. Chromatographic system

CA samples were characterized using gradient HPLC in the first dimension and SEC in the second dimension. A Shimadzu system comprising a DGU-14A degasser, a FCV-10Alvp solvent mixing chamber, a LC-10Advp pump and a SIL 10Advp auto sampler was applied for gradient HPLC and SEC. An Advantec SF 2120 Fraction collector (Advantec MFS, Inc., Dublin, CA, USA) was used to collect fractions eluting in the first dimension. The columns were kept at 35 °C applying a column oven K 4 (Techlab GmbH, Erkerode, Germany). For detection, an evaporative light scattering detector (ELSD, -ELS 1000, Polymer Laboratories, UK) was utilized. The detector was operated at a nebulizer temperature of 170 °C, an evaporation temperature of 295 °C and a gas flow of 1.6 SLM. SEC calibration was performed using PMMA standards (PSS Polymer Standards Service GmbH, Mainz, Germany) having molar masses ranging from 3600 to 1.2×10^6 g/mol. Data collection and processing was performed using 'WinGPC Software version 7.0' (Polymer Standards Service GmbH, Mainz, Germany). Normalized 2D chromatograms were constructed using Origin™ software (OriginLab Corporation, Northampton, USA).

2.3. Chromatographic conditions

2.3.1. Gradient HPLC

Gradient separations were carried out on a pure silica stationary phase Nucleosil, 5 μm particle size, 100 Å pore diameter, 250 mm × 4.0 cm L × I.D. (Macherey-Nagel GmbH, Düren, Germany). The injected sample volume was 45 μL at a concentration of 5.8 g/L. The samples were dissolved in DMSO and diluted by DCM DMSO/DCM (58/42, v/v). A multi-step gradient from DCM as poor eluent to MeOH as strong eluent was used as shown in Table 2.

The flow rate of the mobile phase was 1.0 mL/min unless mentioned otherwise. 15 fractions were collected for each gradient run. To get sufficient concentrations of the fractions, the fractionations were repeated 10 times. The solvents (DCM and MeOH) were vaporized in open-air at room temperature and the residues were subsequently re-dissolved in 106 μL DMSO for the SEC experiments.

2.3.2. 2nd dimension SEC

Molar mass characterization of the fractions was carried out using a single PSS-GRAM Linear XL column (10 μm particle size, 300 mm × 8.0 mm L × I.D., PSS Polymer Standards Service GmbH, Mainz, Germany). The injected volume of each fraction was 70 μL. An eluent mixture composed of 45% DMSO and 55% 1,4-dioxane containing 50 mmol/L ammonium acetate at the flow rate of 1.0 mL/min was used.

3. Results and discussion

2D LC analysis of complex polymers requires optimization of a variety of parameters. First, the selected mobile phases have to be compatible to the columns and the detector. The sample load has to be high enough to allow for reliable detection in the second dimension, while at the same time the high concentrations injected into the first dimension should not severely reduce the resolution of the first dimension separation. Finally the flow rates in both dimensions need to be optimized to allow for comprehensive characterization in a reasonable analysis time. Taking advantages of the proper sequence of separation methods, it would be beneficial to use the gradient HPLC (i.e. the multi-step gradient from DCM to MeOH) and SEC methods (i.e. DMAc/LiCl) developed recently in our laboratory as the first and second dimension, respectively (Ghareeb & Radke, 2013; Ghareeb et al., 2012). However, the low refractive index increment of CA in DMAc/LiCl requires injection of at least approx. 0.3 mg material into SEC to obtain a sufficiently intense RI-signal. On the other hand, the maximum injected mass for the gradient HPLC was found to be approx. 0.35 mg. At higher injected masses a breakthrough peak was observed despite considerable effort spent for optimization. At such injected masses, the concentrations of the fractions injected in the second dimension would become too low for suitable RI-detection in on-line 2D experiments. Therefore, RI-detection was not an option for an on-line 2D separation. Due to the higher sensitivity ELSD seemed to be a better choice. However, the use of the non-volatile LiCl in the recently developed SEC method is incompatible with this detector. Therefore attempts were undertaken to develop a SEC method where potential aggregation of the CAs is supressed using a volatile salt. Application of ammonium acetate using DMSO as the SEC eluent was found suitable for this purpose. However, the high boiling solvent resulted in noisy signals and was incompatible with the flow rates required for on-line 2D chromatography. Therefore attempts were undertaken to dilute the high boiling DMSO with a lower boiling solvent in order to achieve better S/N-ratio.

Ultimately, it was found that a mixture composed of 45% DMSO and 55% 1,4-dioxane containing 50 mmol/L ammonium acetate was an appropriate eluent with respect to the detector compatibility and S/N-ratio at optimized detector settings and standard SEC conditions. Addition of ammonium acetate into the eluent mixture was found to suppress potential aggregates. However, injections of 0.35 mg did not result in a sufficient detector S/N-ratio for this new SEC system to be useful for on-line 2D separations. Since we were, despite all efforts, not able to identify suitable conditions for on-line 2D separations, the 2D characterizations were carried out off-line. Using a fraction collector, fractionations were repeatedly performed in the first dimension (gradient HPLC), in order to collect sufficient amounts of each fraction. Easy evaporation of the solvents used in the first dimension (i.e. DCM and MeOH) allowed obtaining high enough concentrations for injection into the second dimension upon redissolution of the material in a small volume of DMSO. Preference of ELSD over RI was given based on the higher sensitivity of the former detector. 45% DMSO and 55% 1,4-dioxane containing 50 mmol/L ammonium acetate was employed as the second dimension eluent.

CAs of different DS were analyzed using the 2D LC method. The resulting normalized SEC chromatograms of 15 consecutive fractions for three samples (sample 9, DS = 2.09; sample 12, DS = 2.27 and sample 16, DS = 2.60) and reproducibility for the last two samples are exemplified in Fig. 1.

The comparison of the normalized chromatograms for the fractions of sample 12 and sample 16 revealed a good reproducibility, despite the manual steps involved in our 2D approach. When comparing the SEC elution volumes of the different fractions of a single sample, correlations between SEC elution volume (which correlated with molar mass) and gradient elution volume can be observed. For sample 12 (DS = 2.27) the chromatograms of fraction 1–7 shift to the lower SEC elution volume (i.e. higher molar mass) with increasing fraction number (i.e. gradient elution volume or lower DS, (Ghareeb & Radke, 2013)). At higher gradient elution volume the SEC elution volumes become nearly constant (fraction 7–15). This type of correlation will be termed form 1 in the following. For sample 9 (DS = 2.09) and sample 16 (DS = 2.60) a similar decrease in SEC elution volume with first dimension fraction number can be observed up to fraction 7. However, contrasting to sample 12, the SEC elution volumes continue to decrease for the

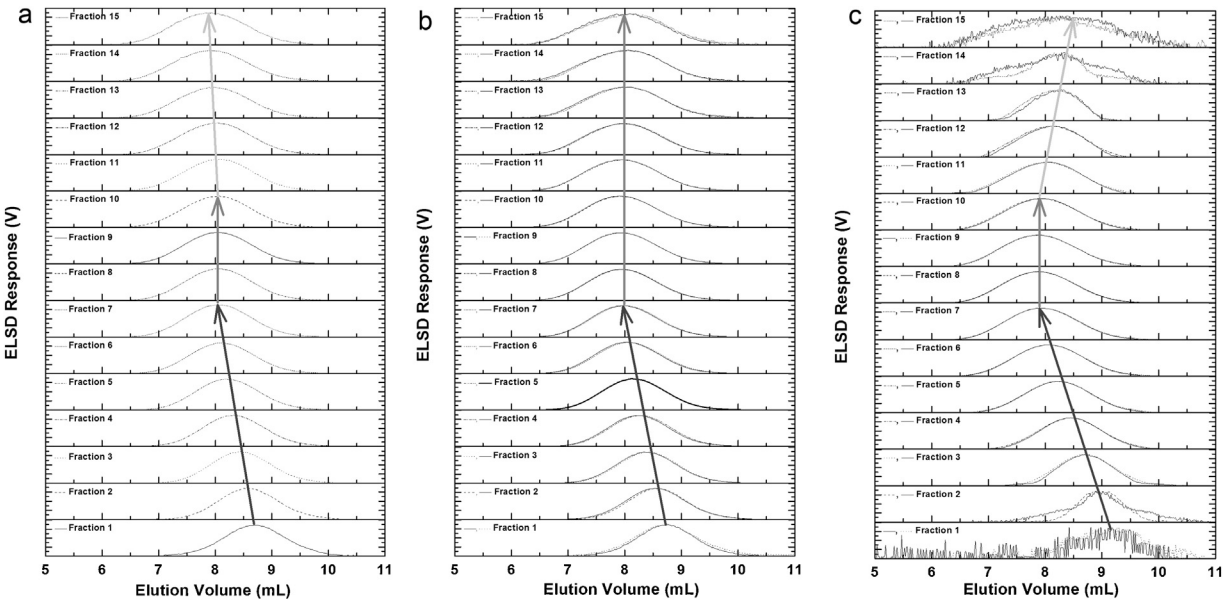

Fig. 1. Normalized SEC chromatograms of the fractions for sample 9, DS = 2.09 (a), sample 12, DS = 2.27 (b) and sample 16, DS = 2.60 (c).

fractions 11 to 15 of sample 9 (form termed form 2) while a clear increase in elution volumes is observed for the fractions 11 to 15 of sample 16 (termed form 3).

In order to understand which correlation might result from a given distribution in terms of DS and molar mass, it is useful to examine the effects of molar mass and DS on the two-dimensional chromatogram. We will first discuss what chromatogram can be expected if the DS distribution is independent of molar mass. Such a distribution should arise if all hydroxyl groups in the cellulose have identical reactivities. We first discuss the dependence of gradient elution volume on molar mass (and thus on SEC elution volume) for a chemically homogeneous (co)polymer. The two-dimensional chromatogram of a chemically heterogeneous copolymer, for which the DS distribution is independent of molar mass, can then obtained as a superposition of chromatograms of chemically homogenous copolymer fractions.

In general the elution volume in gradient chromatography of polymers is influenced by the chemical composition and the molar mass of the analyte. At low molar masses the elution volume of a chemically homogeneous polymer typically increases with molar mass. However, the molar mass dependence vanishes at high molar masses and the gradient elution volume becomes constant (Bashir & Radke, 2007). For cellulose derivatives a chemically homogeneous polymer corresponds to a sample where all chains have identical DS. Strictly, even the distributions of the different AGUs among and along the polymer chains have to be identical. However, for binary copolymers the effect of copolymer composition on elution volume is more pronounced than the effect of microstructure (Zhu, Ziebarth, Macko, & Wang, 2010). Therefore, the effect of DS on elution volume is expected to be stronger than the effect of the placement of the substituents within and among the AGUs. In other words we regard a CMC as being a binary copolymer composed of AGUs and substituents. The expected dependences of elution volume on molar mass for three different DS values are schematically depicted in Fig. 2. While the inner line can be interpreted as the peak maximum of a hypothetical DS distribution, the outer lines might be assigned to DS values at a specific height of the DS distribution (e.g. half height), which is assumed to be independent of molar mass (see above). Those fractions being of identical chemical composition will be separated by molar mass in the first dimension. Consequently, at low molar masses the peaks in the second dimension (SEC) of a chemically homogenous sample are expected to be narrow. For high molar masses however, the elution volumes in the first dimension become independent of molar mass. Therefore the late eluting fractions of a chemically homogenous polymer are expected to be heterogeneous in molar mass, resulting in broad peaks in the second dimension (SEC) (compare e.g. chromatograms of broadly distributed polystyrenes (Gerber & Radke, 2005a, 2005b)). Contrasting to this expectation, the SEC chromatograms in Fig. 1 are generally broadly distributed. This is a consequence of the heterogeneity with respect to DS. Each CA fraction of a given uniform DS has to be regarded as a chemically homogeneous polymer exhibiting its own molar mass dependence in gradient HPLC, as explained above. Thus, for a heterogeneous sample the fractions at low first dimension elution volumes will contain molecules differing in DS and molar mass, as depicted in Fig. 2. Since the HPLC retention volume increases with decreasing DS at a given molar mass (Ghareeb & Radke, 2013), gradient fractions at lower molar masses are expected to exhibit an increase in DS with decreasing molar mass, when analyzed in the second dimension (SEC). For the high molar mass components the separation in the first dimension is expected to be due to chemical

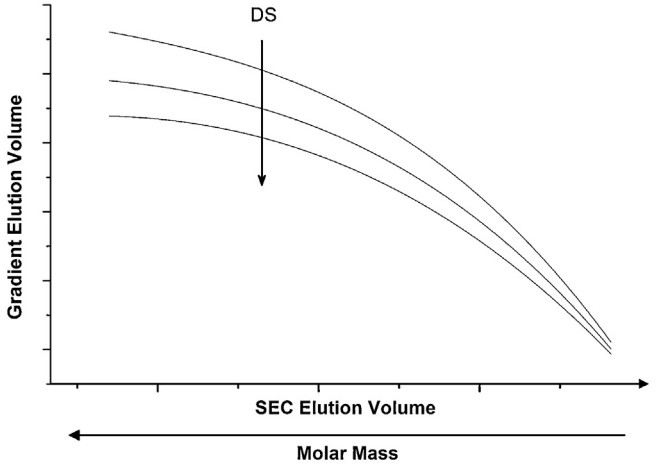

Fig. 2. Schematic representation of influence of molar mass and DS on elution volume in gradient chromatography and SEC.

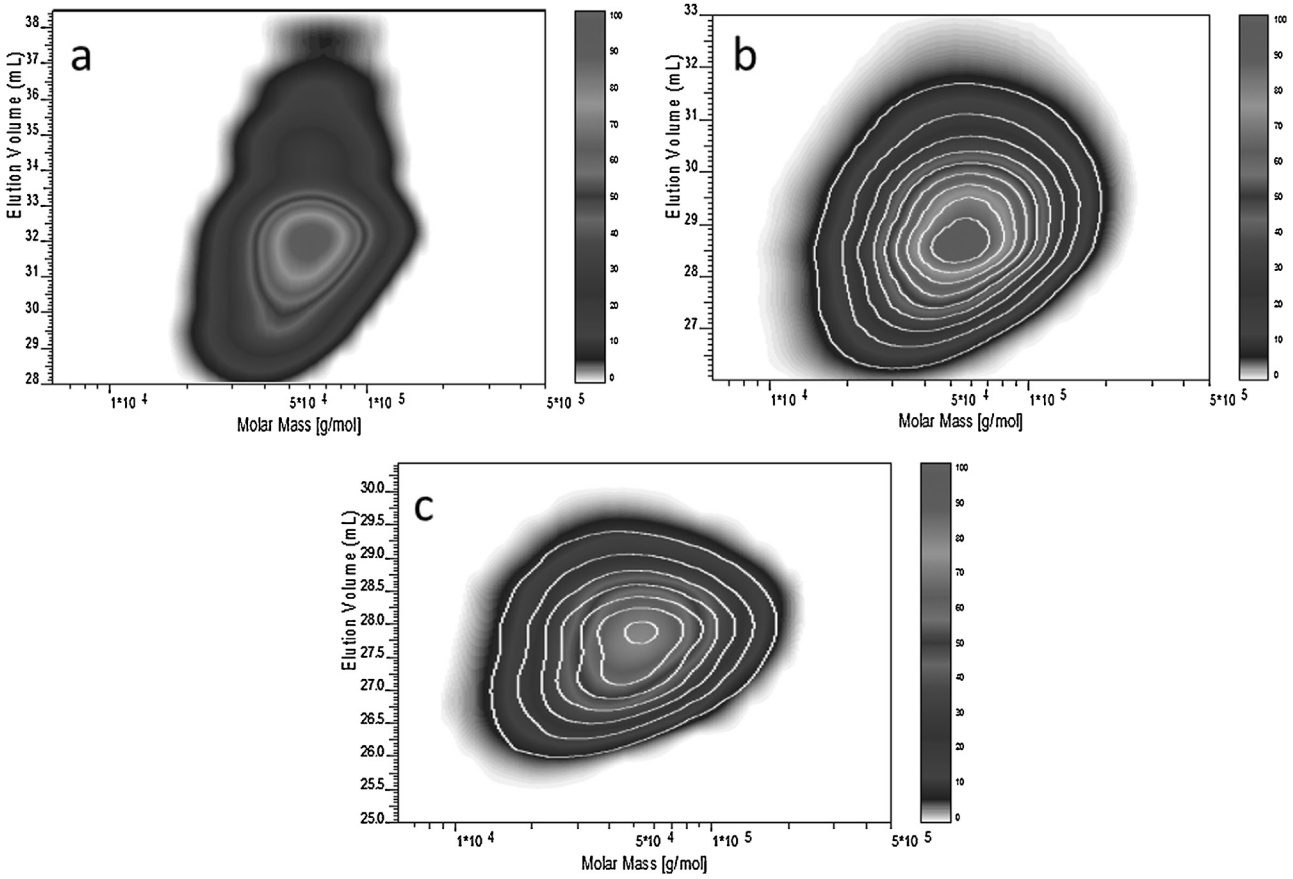

Fig. 3. 2D LC contour plots of sample 9, DS = 2.09 (a), sample 12, DS = 2.27 (b) and sample 16, DS = 2.60 (c). The white isolines in the overlaid contour plots of (b) and (c) indicate the reproducibility of the experiments.

composition (DS) only, and the peak width in SEC is solely due to the molar mass heterogeneity of the chemically homogenous first dimension fraction. The above scenario is therefore in agreement with the observed correlations of form1 and form 2.

The second scenario to be discussed involves the assumption that the molar mass distribution is independent of chemical composition. In such a case, for a given SEC-elution volume (molar mass), the first dimension separation is due to DS only, and the peak maxima should be parallel to the ordinate. Thus, this scenario cannot explain the observed correlations. While the correlations of form 1 and form 2 are in agreement with the first scenario, none of the discussed scenarios can account for the correlation of form 3 of sample 16, indicating that especially the correlations of type 3 must be due to a specific type of heterogeneity, which is most probably due to the process parameters. Nevertheless, all samples could be assigned to one of the above mentioned three correlation forms between molar mass and gradient elution volume. The assignments for the CA samples are given in Table 1.

From the correlation forms it becomes clear that the differences in the correlations arise from fraction 11 to fraction 15. Interestingly, the industrial samples (marked in grey in Table 1) except sample 17 (DS = 2.92) and the precursor (sample 16, DS = 2.60) display the same correlation form (form 3) while the laboratory synthesized samples exhibit either correlations of form 1 or form 2. For the laboratory samples those of larger DS (DS = 2.1–2.3) exhibit correlations of form 1 while those with an average DS < 2.1 show correlations of form 2. The reason for the variations is yet unknown, but one might speculate that it results from differences in the microstructure (i.e. arrangements of differently substituted anhydroglucose units, AGU, along the CA chains). To obtain more information on the origin of the differences in correlations it might be interesting to use a chemical sensitive detector in the second dimension.

To determine the molar masses of the 2D fractions, the SEC was calibrated with a set of well-defined PMMA standards under the same chromatographic conditions. Due to the difference in hydrodynamic volume between PMMA and CA, the PMMA calibration curve was converted into a CA calibration curve using the appropriate correction factors A and B of the relation $M_{CA} = A \times M_{PMMA}^{B}$. The correction factors for the present mobile phase/stationary phase system ($A = 6.031$ and $B = 0.757$) were determined in analogy to the correction factors in DMAc/LiCl (Ghareeb et al., 2012).

The information on DS and molar mass data can be represented in a contour plot. The y-axis of the contour plot corresponds to the elution volume of the first dimension (gradient HPLC) which separates mainly according to DS. On the x-axis the molar masses corresponding to the elution volumes in the second dimension (SEC) are given. The contour plots of the three samples (sample 9, DS = 2.09; sample 12, DS = 2.27 and sample 16, DS = 2.60) and reproducibility for the last two samples are represented in Fig. 3.

Quite good reproducibility can be seen for sample 12, DS = 2.27 and sample 16, DS = 2.60 (Fig. 3b and c, respectively). For all samples the molar mass of the fractions ranged from about 20,000 to 200,000 g/mol indicating nearly similar heterogeneity with respect to molar mass. Weight average molar masses of M_w = 60,000, 66,000 and 70,000 g/mol for sample 9, 12 and 16, respectively, were obtained from the contour plots. These values are slightly lower than the absolute molar masses derived from light scattering (see Table 1). This can be explained by a non-linear response of the ELSD with concentration in the 2D experiments. Furthermore, the ELSD response might vary with molar mass which will result in different average molar masses depending

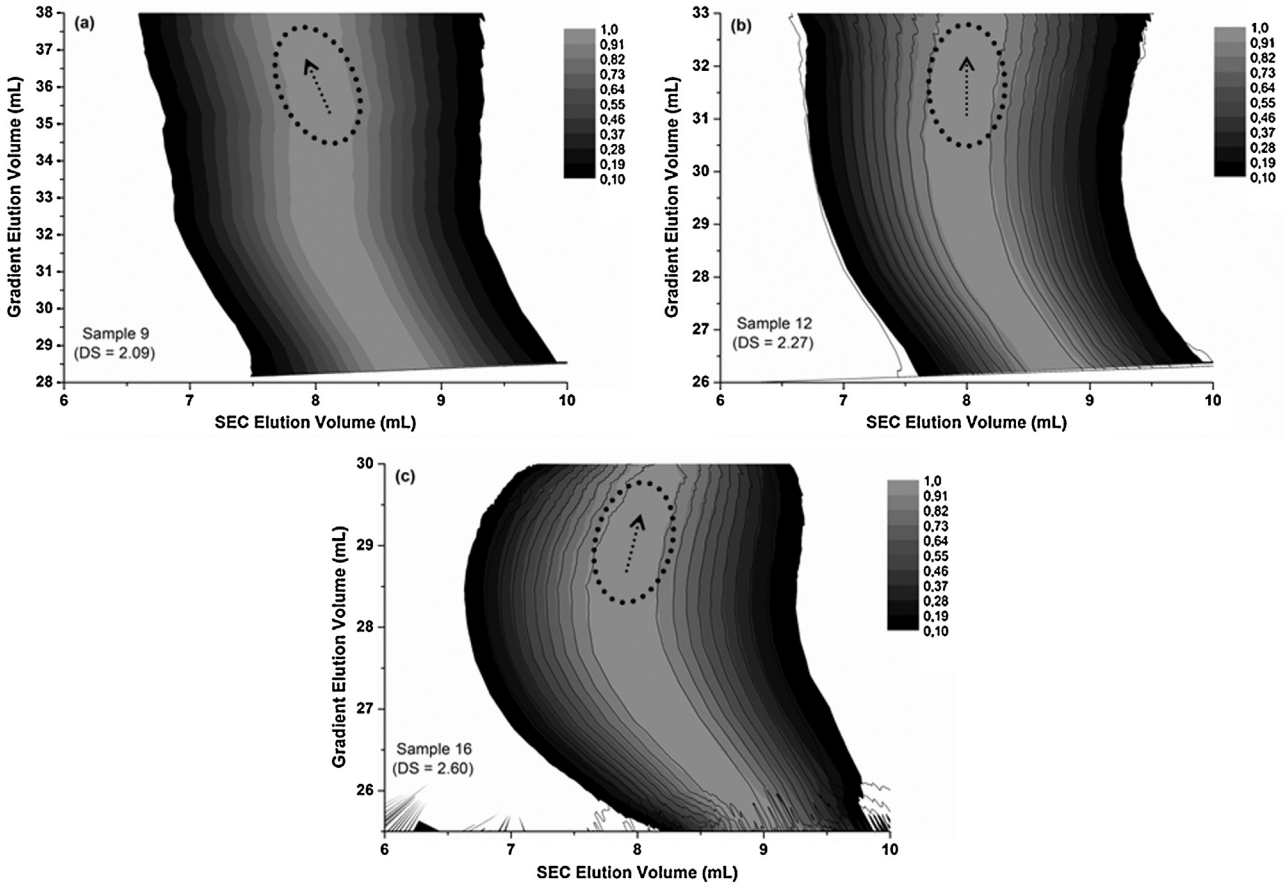

Fig. 4. Normalized 2D LC contour plots of CAs of different DS. The isolines in the overlaid contour plots of b and c indicate the reproducibility of the experiments.

on the detector applied (ELSD or RI). Application of RI-detection would overcome this problem. However, as described above, the low refractive index increment and the low concentrations in the second prevented using RI-detection, despite the repeated fractionations.

While the molar masses of the samples prepared in our laboratory do not significantly vary, in agreement with our SEC investigation on the molar masses of the same samples (Ghareeb et al., 2012), the DS-distributions vary among the samples (Ghareeb & Radke, 2013). First of all, as expected, every sample elutes within a gradient elution range comparable to that of the single gradient run. The sample with the lowest DS (i.e. sample 9, DS = 2.09) elutes within an approx. 10 mL gradient elution range (Fig. 3a) whereas the sample with the highest DS (i.e. sample 16, DS = 2.60) covers an approx. 5 mL range (Fig. 3c), indicating a higher chemical heterogeneity for the former sample, in agreement with the results on the variance in Table 1. It should be noted that a direct correlation of chemical heterogeneity and peak width is only possible for a linear relation between chemical composition and elution volume. All the samples in Fig. 3 show only one peak. The shape of the contour plot of sample 12(b) and sample 16(c) resembles to each other while the shape of the contour plot of sample 9 appears to be different. In addition, the contour plots show only weak correlations between molar mass and DS. By increasing molar mass the gradient elution volume slightly increases, as expected for the separation of chemically homogeneous samples. However, this weak correlation does not allow clarifying whether the gradient separation is governed by DS only, or whether there is an effect of molar mass as well. In order to definitely clarify this point it would be necessary to have some samples of identical DS but largely different molar masses, which were not available. Alternatively, a chemically sensitive detection in the 2nd dimension could be applied.

At a first look, the contour plots do not reveal the dependences of molar mass on gradient elution volume identified in Fig. 1. To check whether this is a real loss of information, or merely a question of the representation, the elugrams corresponding to a particular gradient elution volume were normalized to peak maximum and a contour plot was constructed from the normalized chromatograms. The resulting plots are given in Fig. 4.

As can be seen, the SEC elution volume remains nearly unchanged for the last 5 fractions (fraction 11–15) of sample 12 (Fig. 4b) whereas it decreases (i.e. molar mass increases) for sample 9 (Fig. 4a) and it increases (i.e. molar mass decreases) for sample 16 (Fig. 4c) similar to Fig. 1. Thus, the informational content of Fig. 1 is blurred in Fig. 3 by the different intensities due to the concentration variations in the non-normalized plots.

To show the potential of 2D chromatography for the characterization of complex CA mixtures, a mixture of two samples differing in DS was analyzed by 2D LC. The 2D contour plot of a 1:1 (wt/wt) mixture of sample 4 (DS = 1.72) and sample 15 (DS = 2.45) is depicted in Fig. 5.

As expected, two peaks are appeared in the plot. Peak 1 eluting from 25 to 29 mL belongs to the high DS sample (i.e. sample 15, DS = 2.45) while peak 2 in the elution range 32–38 mL corresponds to the one of lower DS (i.e. sample 4, DS = 1.72). The heterogeneity with respect to molar mass for both samples is nearly identical, similar to the single SEC run. The M_w values were 63,000 g/mol for sample 15 and 50,000 g/mol for sample 4. If only a separation by size (SEC) would be performed, the resulting chromatograms of sample 4 and sample 15 would overlap, without indication of chemical

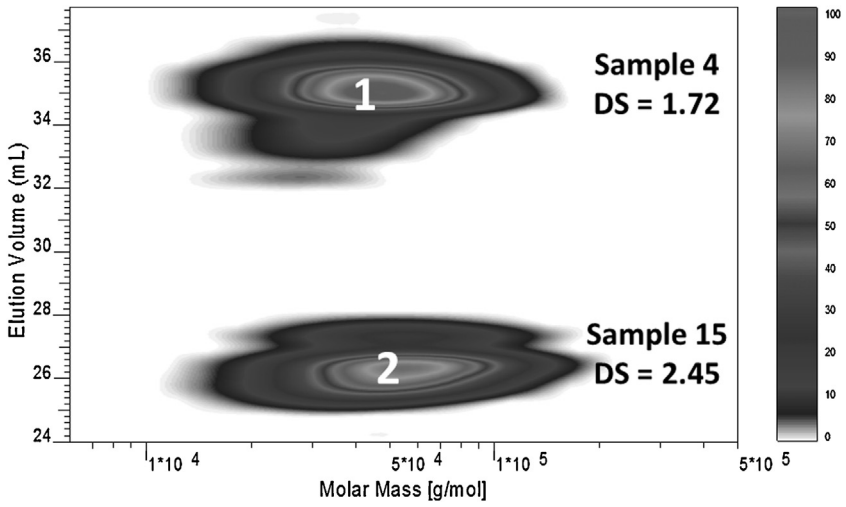

Fig. 5. 2D LC contour plot of a mixture composed of sample 4 and sample 15 [1:1].

heterogeneity. Similarly, a separation by gradient chromatography would reveal two distinct peaks, however without indication of the heterogeneity with respect to molar mass. Thus, only the 2D approach can reveal the heterogeneity of CAs in both dimensions.

4. Conclusions

The strategy of combining both gradient HPLC and SEC proved to be promising in resolving the molecular heterogeneity of CAs. It has been shown that 2D chromatography is capable to separate complex CA mixtures into their individual components. The 2D LC interpretations on individual samples revealed some unexpected features that could not be observed in a single chromatographic run, neither by gradient HPLC nor by SEC. These unexpected features indicated that apart from molar mass and DS, other molecular characteristics such as second order heterogeneities (i.e. arrangements of differently substituted AGUs along the CA chains) or distribution of substituents within AGUs influence the elution behaviour of CAs.

It was also shown that data representation as non-normalized 2D chromatograms might result in a loss of information on correlations between molar mass and chemical composition. The representation as normalized 2D plot eliminates the data blurring due to concentration effects and might be a valuable software feature.

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