ELSEVIER

Journal of Chromatography A, 730 (1996) 75–81

JOURNAL OF CHROMATOGRAPHY A

Exclusion chromatography of polypropylenamine dendrimers

Y. Mengerink, M. Mure, E.M.M. de Brabander, Sj. van der Wal*

DSM Research, P.O. Box 18, 6160 MD Geleen, Netherlands

Abstract

Size-exclusion chromatography of polypropylenamine (POPAM) dendrimers is investigated. The nitrile-terminated half-generations can be analysed on polystyrene-divinylbenzene-based column packings using tetrahydrofuran as a mobile phase. Several basic and acidic aqueous phase systems were compared for exclusion chromatography of POPAM-amine dendrimers. The optimum system consists of a reversed-phase silica stationary phase deactivated by tetraazacyclotetradecane and a mobile phase of 0.25 *M* formic acid at 60°C. Several by-products were identified by thermospray mass spectrometric detection.

Keywords: Polypropylenamine; Dendrimers

1. Introduction

Dendrimers are highly branched and symmetric macromolecules that are synthesized in a cascade of repetitive reactions with branches emanating from a central core [1]. Their stepwise method of synthesis by alternating reactions allows for a high degree of purity of the product in each generation [2].

The branching and concomitant accessibility of the well-defined number of endgroup functionalities on the surface of the molecule as well as the precisely known molecular mass and size ($M_w/M_n \approx 1.001$) are of interest.

Polypropylenamine (POPAM) dendrimers are synthesized via a repetitive double Michael addition of acrylonitrile on a primary amino group followed by a hydrogenation of the nitrile groups to primary amine groups (see Fig. 1). Recently, we reported the development of a process for large-scale production of POPAM dendrimers from inexpensive starting materials [3]. To optimize the production of up to 100-kg batches, analytical methods were necessary.

Since these dendrimers contain the same structural elements in each generation, no discrimination between different isomeric defects can be made by IR or NMR spectrometry, which can only be used to determine the general composition.

In the research reported here we describe exclusion-HPLC methods to determine the composition by generation of dendrimer; elsewhere, we focus on imperfections in the nitrile-terminated half-generations [4].

2. Experimental

2.1. Apparatus

All experiments using UV-absorbance detection were performed on an HP1090 DR5 gradient liquid chromatograph equipped with a

* Corresponding author.

SSDI 0021-9673(95)00868-3

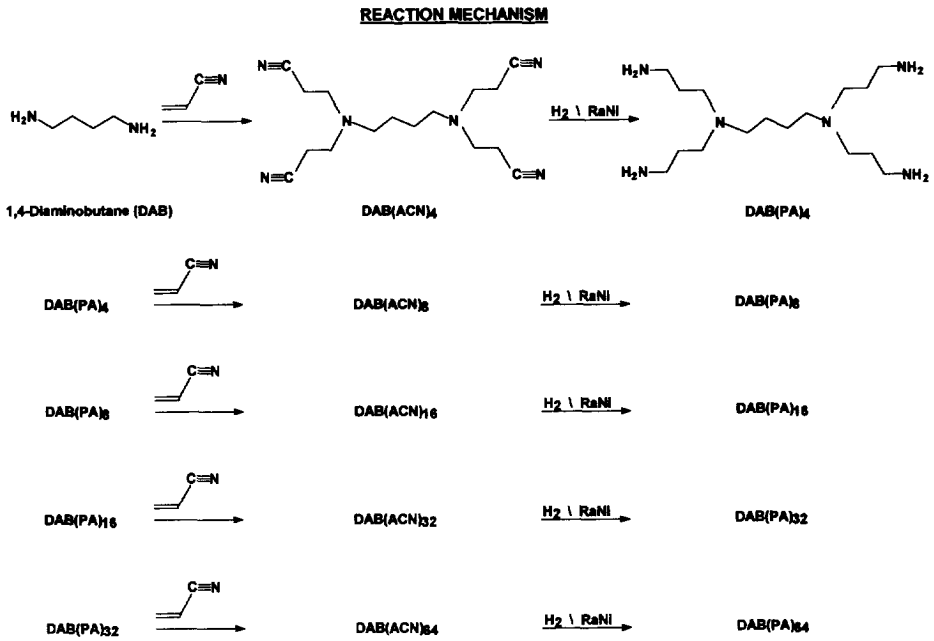

Fig. 1. Reaction sequence for synthesizing POPAM dendrimers.

variable volume autosampler and a diode-array detector (Hewlett-Packard, Boblingen, Germany). The other experiments were done with a system assembled from a Marathon autosampler (Spark, Emmen, Netherlands), a Mistral column thermostat (Spark) with a Rheodyne 7010 injection valve (Rheodyne, Cotati, CA, USA), a Varian 9010 ternary gradient solvent delivery system (Varian, Walnut Creek, CA, USA) and a Sedex 45 evaporative light-scattering detector (Sedere, Vitry/Seine, France). Data obtained from a diode-array detector were evaluated on a ChemStation, and those from the light-scattering detector on a HP1000 laboratory data system (Hewlett-Packard).

2.2. Samples, chemicals and columns

The dendrimer samples were produced in batches of up to 100 kg in a multi-purpose plant at DSM and were used without purification. The samples were characterized by infra-red and ^{13}C-NMR spectra before being used.

The tetranitrile dendrimer is a white solid, while the other nitriles and the amines are tan-coloured, viscous liquids. The nitriles are soluble in acetonitrile, acetone and chloroform, the amines in water and methanol. Solubility of the nitriles in water is less than 0.5% (v/v, at 20°C).

Dissociation constants of amine groups were calculated using pKalc 2.0 (Compudrug, Hungary) and are given in Table 1.

All solvents were of p.a. quality (Merck, Darmstadt, Germany) or equivalent.

A 300 × 7.8 mm Ultrastyragel 100 Å column (Waters, Milford, MA, USA) was used for SEC of nitrile-terminated dendrimers in tetrahydrofuran.

For the amine-terminated dendrimers we evaluated five different packing materials using acidic and basic aqueous mobile phases.

We employed a 300 × 7.8 mm TSK G-Oligo-PW column (TosoHaas, Montgomeryville, PA, USA). This 6-μm particle size, cross-linked, hydroxylated polyether material has 125 Å pores, a 0.2 mequiv/ml positive charge (in contrast to most other TSK-G PW columns) and can operate in up to 50% organic modifier and at 80°C. The maximum flow-rate on this column is 1.0 ml/min [5].

Table 1
Calculated pK_a values of the first two POPAM generations

	DAB $(ACN)_4$	DAB $(PA)_4$		DAB $(ACN)_8$	DAB $(PA)_8$
$RRRNH^+$	4.07	6.45		9.30	6.36
	3.07	7.44		8.31	6.39
$NHHH^+$		9.31	$RRRNH^+$	3.24	6.82
		9.73		2.81	7.17
		10.08		2.46	7.35
		10.51		2.04	7.59
			$NHHH^+$		9.01
					9.36
					9.61
					9.81
					10.01
					10.21
					10.45
					10.81

A set of hydroxyethylmethacrylate (HEMA) columns was made up of two 250×4.6 mm HEMA 40 Å columns (Alltech, Deerfield, IL, USA). The 250×4 mm Nucleosil 120-5 C_{18} columns used were obtained directly from Macherey-Nagel (Düren, Germany).

A 304×10 mm HR10/30 column containing 13 μm Superdex 75, a dextran and agarose composite, had a pressure limit of 30 bar, while the packing material itself is limited to a linear flow rate of 0.38 mm/s (Pharmacia LKB, Uppsala, Sweden).

A 300×7 mm column of 10-μm silica support coated with a polyamine polymer (SynChrom, Linden, IN, USA) was also used for comparison.

3. Results and discussion

3.1. Choice of separation method

Polarity—and ensuing solubility (see Sect. 2)—and functional group basicity are the main parameters to guide the choice of chromatographic separation conditions. The pK_a values of the amine functionalities were calculated (see Sect. 2) and are listed in Table 1.

Polyamines are notorious for frustrating efficient chromatography [6]. However, up to aliphatic pentamines were separated on polystyrene–divinylbenzene based ion-exchangers [7].

An alternative possibility, also serving to improve detectability, may be derivatisation before separation. Pentamines are also the largest polyamines to be analysed semi-quantitatively by prechromatographic derivatisation and gas chromatography followed by mass spectrometry [8]. In the case of POPAM-dendrimers, the errors introduced are expected to be at least as large as the content of error-structures to be determined, unless detection is specific for a functional group unique to the impurity (i.e. a secondary amine in a nitrile half-generation).

Only direct analysis is considered here.

3.2. Nitrile-terminated dendrimers

Size-exclusion chromatography of the POPAM-nitrile half-generations is rather straightforward for the pure components. The tertiary amines are located on the inside of the molecule (cf. Fig. 2) and allow efficient chromatography on Ultrastyragel columns in tetrahydrofuran. Occasionally, strongly adsorbing minor constituents are noticed, but in tetrahydrofuran sensitive detection of trace impurities at a wavelength less than 220 nm is not feasible, and

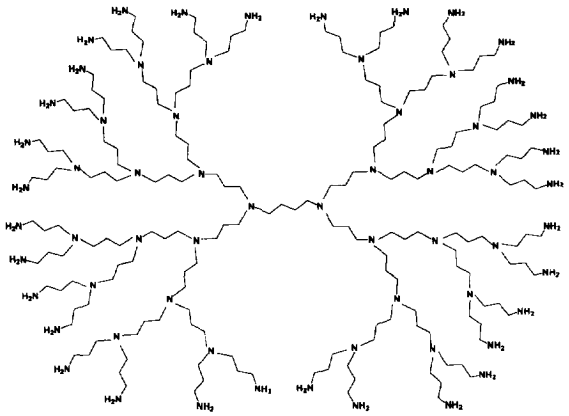

Fig. 2. Molecular architecture of $DAB(PA)_{32}$.

refractive index detection is not sensitive enough either, so the size-exclusion mode for nitrile half-generations has been now all but abandoned for a much more sensitive and selective reversed-phase method [4].

3.3. Amine-terminated dendrimers

At *high pH* the POPAM-amine dendrimers are uncharged polar molecules and can be expected to show little adsorption to neutral packing materials.

Previously, polyether columns eluted with 0.05 *M* phosphate buffer at pH 11 were used [1].

TSK G-Oligo-PW 125 Å pore size packing is best suited for the lower generations of dendrimer. Addition of at least 20% methanol to a 0.05 *M* phosphate buffer, pH 11, at a temperature of 50°C was necessary to suppress adsorption, but at higher methanol concentration selectivity decreases, presumably due to swelling of the packing.

Even higher concentrations of methanol were used by Woerner with hydroxyethylmethacrylate (HEMA) columns [9]. The HEMA packing material is fully compatible with organic eluents, so the content of methanol in the mobile phase was varied to study its influence on elution time and peak shape (see Figs. 3a and 3b, respectively). The results indicate an adsorption minimum at about 85% methanol.

At very *low pH*, when the amine-terminated

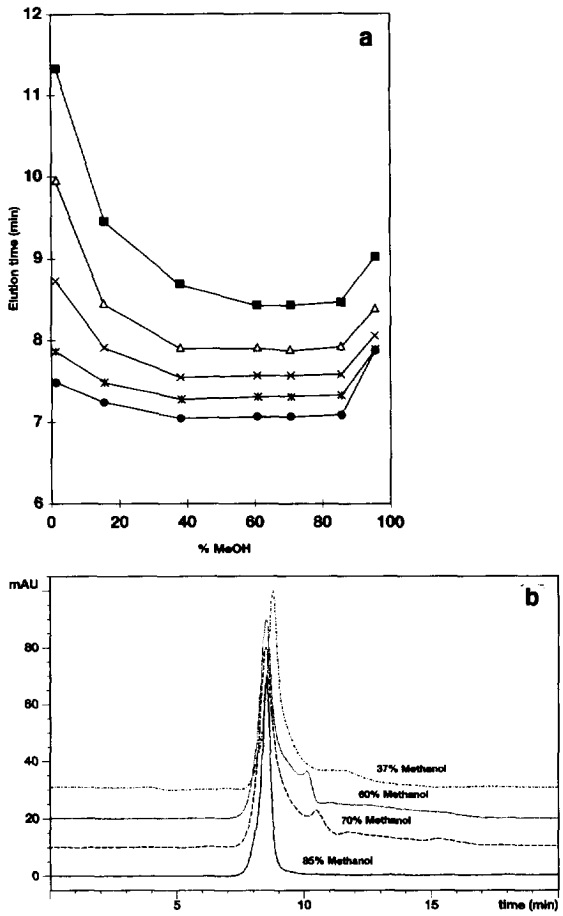

Fig. 3. Influence of methanol on SEC separation of polyamine-terminated dendrimers: (a) elution time, (b) peak shape for $DAB(PA)_4$. Columns: two 250 × 4.6 mm HEMA Bio SEC 40 Å columns. Injection: 2 μl of 5 mg/ml dendrimer. Eluent: 0.1 *M* NaOH (pH 12 with phosphoric acid) with different percentages of methanol. Detection: absorbance at 220 nm. ■ = $DAB(PA)_4$; △ = $DAB(PA)_8$; × = $DAB(PA)_{16}$; * = $DAB(PA)_{32}$; ● = $DAB(PA)_{64}$.

dendrimers as well as the packing material bear a positive charge, exclusion chromatography may be performed also.

A packing material that shows little adsorption for biopolymers and can be operated also at pH 11 is Superdex 75. Selectivity and efficiency in 0.3 *M* Na_2SO_4 + 0.1% trifluoroacetic acid are excellent (see Fig. 4). This material does not tolerate high flow rates (>0.4 mm/s) or more than 30% organic solvent unfortunately and is contained in an inconvenient column format.

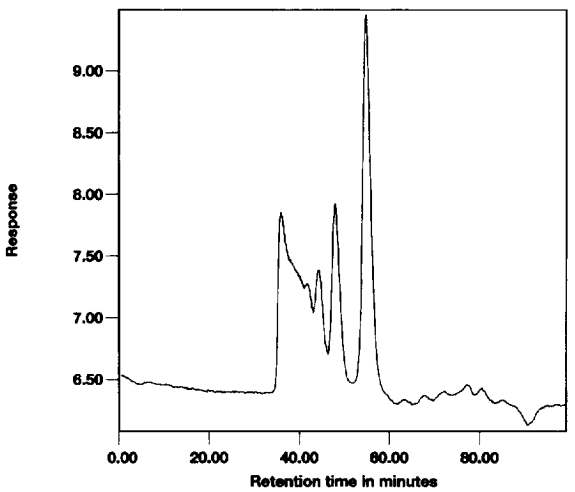

Fig. 4. Analysis of $DAB(PA)_{64}$ oligomers. Column: 304 × 10 mm Superdex 75. Injection: 20 μl of 5 mg/ml $DAB(PA)_{64}$ in eluent. Mobile phase: 0.25 ml/min 0.3 *M* Na_2SO_4 + 0.1% trifluoroacetic acid, 25°C. Pressure: 10 bar. Detection: absorbance at 220 nm.

The selectivity and the apparent efficiency decrease on addition of 20% acetonitrile to a buffer of pH 2.5, possibly indicating the onset of adsorption.

Size-exclusion chromatography on a silica-based material with a polycationic layer (CAT-SEC 100 Å) was optimized with respect to ionic strength, temperature and organic modifier. A mobile phase of 0.3 *M* Na_2SO_4 + 0.1% trifluoroacetic acid appears to give the best combination of spectral properties and suppression of adsorption to the packing material.

Addition of 20% acetonitrile increases adsorption. Efficiency improves when adding 60% methanol and using a temperature of 50°C, but adsorption causes additional retention. Because of its additional selectivity, this phase system may be useful for determination of impurities within a generation.

The most convenient phase system for exclusion chromatography is a reversed-phase silica packing (Nucleosil 120-5 C_{18}), deactivated by 1,4,8,11-tetraazacyclotetradecane [10] and eluted with 0.25 *M* formic acid at 60°C. The cyclic amine presumably shields the silanol adsorption sites and renders the column more stable: less interfering silica passes the evaporative light-scattering detector.

3.4. Comparison of phase systems

The resolutions between POPAM-$amine_{32}$ and POPAM-$amine_8$ of the different phase systems used under dissimilar conditions were compared.

In a size-exclusion system the resolution in the linear part of the calibration curve, $R_{2,1}$, is given by [11]:

$$R_{2,1} = \sqrt{N} \cdot p \cdot \log M_{r,1}/M_{r,2} \quad (1)$$

in which: N = average number of theoretical plates for component 1 and 2; $M_{r,1}$, $M_{r,2}$ = molecular mass of components 1 and 2, respectively; p = selectivity, defined as the slope of the calibration curve, or:

$$p = \Delta t_R/t_0 \cdot \Delta \log M_r \quad (2)$$

where t_0 = dead volume of the column.

For practical reasons the baseline disturbance due to the injection solvent was used for t_0.

In size-exclusion chromatography all components should elute before t_0, which thus is the time needed for separation.

With the approximations:

$$H = au + b \quad (3)$$

and

$$u = cP/L \quad (4)$$

in which H = theoretical plate height, u = linear velocity of the mobile phase, P = pressure difference over the columns, L = total column length, and a,b,c = proportionality constants, equations for the separation time, t_0, and resolution become:

$$t_0 = L^2/cP \quad (5)$$

$$R_{2,1} = \sqrt{t_0 cP}\, p \Delta \log M_r (acP + b\sqrt{t_0 cP})^{-0.5} \quad (6)$$

The constants a, b and c and the selectivities are given in Table 2. In Fig. 5 the resolution as a function of separation time is shown for a system pressure of 350 bar (30 bar for Superdex 75 packed in HR10/30 columns). The lower resolution in the HEMA system is caused by a

Table 2
Values of proportionality constants determined (see Sect. 2) and used in Eq. (6)

	a (1/s)	*b* (m)	*c* (m^2/bar · s)	*p*	*u* (mm/s)
TSK G-Oligo-PW	0.135	0.000135	0.0000040	0.189	0.13–0.8
HEMA 40 Å	0.148	0.000256	0.0000101	0.117	0.4 –2.4
Nucleosil 120-5 C_{18}	0.0196	0.000052	0.0000095	0.190	0.5 –3.1
Superdex 75	0.029	0.000039	0.0000043	0.186	0.06–0.26

lower selectivity. This indicates that its 40 Å pore size was too small: a 100 Å pore size is more appropriate for separating the dendrimers. The polyether packing, but especially the agarose-dextran composite, are soft packings that cannot be used under high-shear conditions.

It is clear that the acidic reversed-phase silica system is superior in performance irrespective of separation time. It is more rugged than the other systems and can be built at about a fifth of the expense in columns.

3.5. Analysis and identification

POPAM-amine$_4$ is detectable by post-column reaction with *o*-phthalaldehyde and mercapto-propionic acid; the higher generations are not fluorescent under the same conditions, but they can be detected by UV absorbance at 190 nm. As a detection scheme, a combination of 190-nm absorbance detection followed by post-column reaction with fluorometric detection is feasible.

A simpler alternative is to use a volatile mobile phase and evaporative light-scattering or, for identification, mass spectrometric detection.

Chromatograms of the successive generations are shown in Fig. 6. To visualize by-products we had to overload the phase system with the lower

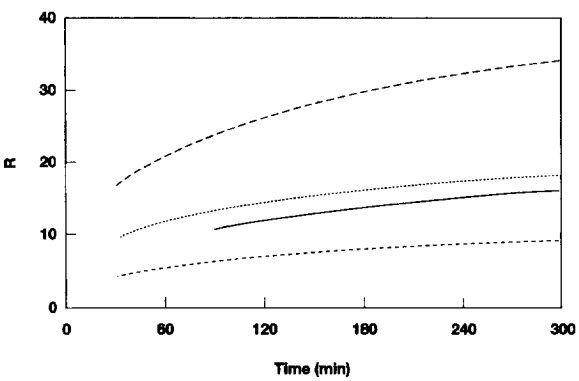

Fig. 5. Performance of different phase systems at 350 bar (30 bar for Superdex 75). The resolution of DAB(PA)$_{32}$ and DAB(PA)$_8$, *R*, is given as a function of separation time: HEMA (short dashes), polyether (solid line), agarose-dextran (dotted line), silica (long dashes).

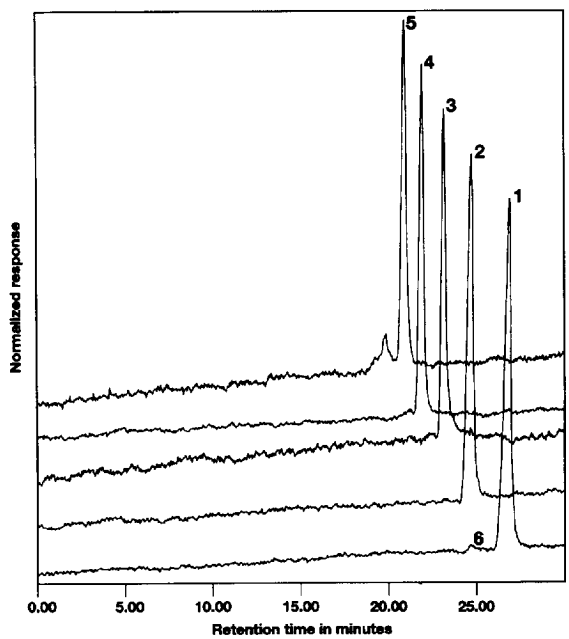

Fig. 6. Analysis of successive generations of POPAM-amine dendrimers on an optimized system. Columns: eight 250 × 4 mm Nucleosil 120-5 C_{18} columns in series. Injection: 20 μl of 0.5 mg/ml amino-terminated dendrimer in eluent. Eluent: 0.5 ml/min, 1% formic acid in water, 60°C. Pressure: 210 bar. 1 = DAB(PA)$_4$; 2 = DAB(PA)$_8$; 3 = DAB(PA)$_{16}$; 4 = DAB(PA)$_{32}$; 5 = DAB(PA)$_{64}$; 6 = 'dimer' of DAB(PA)$_4$.

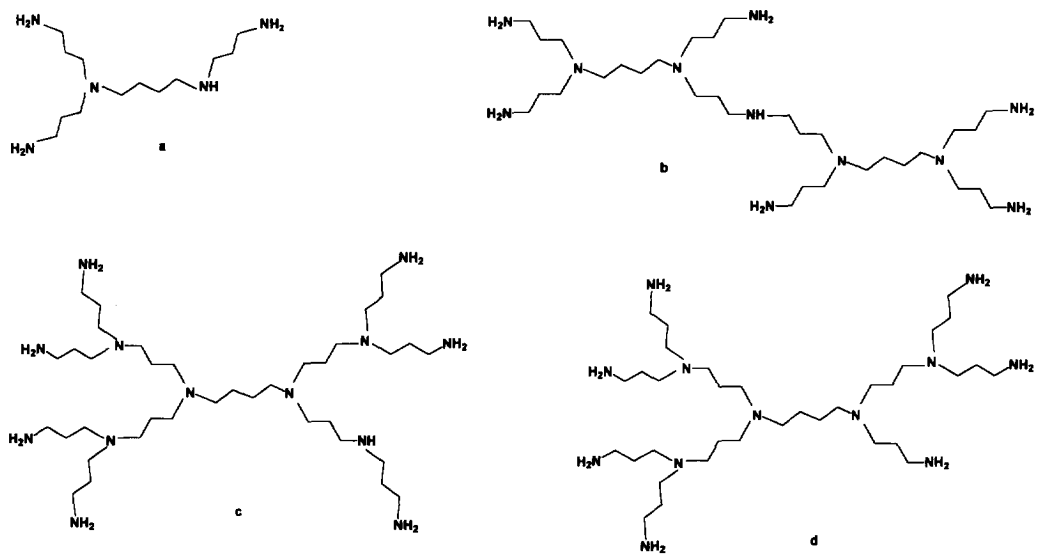

Fig. 7. Proposed structure of several side-products.

generations. Usually only a small peak eluting approximately at the retention time of the next generation is detected.

To identify side-products off-spec samples of POPAM-amine$_4$ and POPAM-amine$_8$ were analysed by coupling the system via a thermospray interface to a quadrupole mass spectrometer. Eluting later than and just separated from POPAM-amine$_4$ ($MH^+/z = 317$) is a component with $MH^+/z = 260$. Its proposed structure is given in Fig. 7a. The peak with the retention time of POPAM-amine$_8$ in the POPAM-amine$_4$ sample shows $MH^+/z = 617$, which is in agreement with the compound in Fig. 7b.

The low resolution for compounds of like size makes the analysis of by-products in POPAM-amine$_8$ problematic. In the mass spectrum of the peak of POPAM-amine$_8$, besides the intact molecule ($MH^+/z = 774$), error products are also present at $MH^+/z = 717$ from the second generation (Fig. 7c) and $MH^+/z = 659$, the product of an error in the first generation (Fig. 7d).

Since we were not able to reduce the level of intensity of these ions to below 1%, and the relative detection efficiency of the different ions is not known, it cannot be excluded that this is at least in part a detection artifact, unless separation is obtained.

Research is being focused now on better resolution of POPAM-amines by mixed-mode HPLC and capillary electrophoresis and, for the known by-products, on functional group-specific derivatisation.

References

[1] D.A. Tomalia, H. Baker, J. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder and P. Smith, Polymer J., 17 (1985) 117–132.

[2] G.J. Kallos, D.A. Tomalia, D.M. Hedstrand, S. Lewis and J. Zhou, Rapid Commun. Mass Spectrom., 5 (1991) 383–386.

[3] E.M.M. de Brabander, A. Nijenhuis, M. Mure, J. Keulen, R. Reintjens, F. Vandenbooren, B. Bosman, R. de Raat, T. Frijns, Sj. van der Wal, M. Castelijns, J. Put and E.W. Meijer, Macromol. Symp. 77 (1994) 51–62.

[4] Y. Mengerink, M. Mure, E.M.M. de Brabander and Sj. van der Wal, manuscript in preparation.

[5] TosoHaas, The separations catalogue, 1994, p. 40.

[6] N. Seiler, J. Chromatogr., 379 (1986) 157–176.

[7] K. Hamana, H. Hamana, M. Niitsu, K. Samejima and S. Matsuzaki, J. Gen. Appl. Microbiol., 38 (1992) 575–584.

[8] M. Niitsu, K. Samejima, S. Matsuzaki and K. Hamana, J. Chromatogr., 641 (1993) 115–123.

[9] C. Woerner, Thesis, Albert-Ludwigs University, Freiburg, 1992.

[10] P.C. Sadek and P.W. Carr, J. Chromatogr. Sci., 21 (1983) 314.

[11] Sj. van der Wal, LC–GC Int., 5 (1992) 36–42.