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Note

High-performance liquid chromatographic separation of monosaccharides as their peracetylated ketoximes and aldononitriles

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A wide variety of chromatographic methods have been applied to monosaccharides. The most common method is gas chromatography (GC), alone or combined with mass spectrometry (MS). However, this technique presents some problems. On the one hand, sugars must be derivatized to increase their volatility [1–7] and in most instances the derivatization process leads to multiple products (anomeric mixtures, furanose or pyranose forms) that complicate the analysis; to avoid this problem, derivatives such as alditols [8,9] and oximes [10–13] which give rise to acyclic structures have been prepared. On the other hand, GC is generally a destructive method, in which the complete structural elucidation must be made by using reference samples. By the use of GC–MS, it is easy to establish if a compound is a C_6- or an C_5-aldose. However, the only way to distinguish among stereoisomers is to use reference samples and compare their retention times.

An alternative to GC is high-performance liquid chromatography (HPLC) [14,15], in which different none-destructive detection systems can be used. HPLC permits amounts of pure substance to be collected, which can then be studied by use of another analytical technique [16]. HPLC has been applied extensively in carbohydrate chemistry [14,15,17–20]. In spite of the vast literature in which a variety of analytical conditions are described, most of them are of restrictive application and there is none that could separate simultaneously all monosaccharides. With the latter aim, we have studied the HPLC of mixtures of peracetylated aldononitriles and peracetylated ketoximes. The reasons for the choice of those derivatives were (a) both of the two families of sugar derivatives have been the subject of extensive previous GC–MS studies [21–23], making possible rapid control of our work by an alternative method; (b) they are easy to prepare, purify and store, and are reasonably stable for long periods of time; and (c) the intermediate polarity of the peracetylated aldononitrile (PAAN) and peracetylated ketoxime (PAKO) derivatives allows their HPLC analysis by using either polar, intermediate or apolar stationary phases.

In this paper we report the behaviour of PAAN and PAKO sugar derivatives.

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EXPERIMENTAL

Materials

Reagents for derivatization (analytical-reagent grade), solvents used as eluents (HPLC grade) and the following sugars were supplied by Fluka (Buchs, Switzerland) and/or Scharlau-Ferosa (Barcelona, Spain): dihydroxyacetone (**1**), xylose (**2**), lyxose (**3**), ribose (**4**), arabinose (**5**), allose (**6**), altrose (**7**), glucose (**8**), mannose (**9**), gulose (**10**), idose (**11**), talose (**12**), galactose (**13**), fructose (**14**), sorbose (**15**) and D-glycero-D-guloheptose (**16**). Water was obtained by deionization in a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All organic solvents and problem solutions were filtered over Millipore FH filters (0.5 μm) and the water and aqueous samples over Millipore AH filters (0.45 μm).

Equipment

The HPLC studies were performed on a Waters Assoc. (Milford, MA, U.S.A.) equipment consisting of Model M-45 and M-6000A high-pressure pumps, a Model R-401 differential refractometer, a Model 450 variable-wavelength detector, a Model 660 solvent programmer and an integrator data module. The injector was a Rheodyne 7125. The HPLC columns were as follows: Rad-Pak cartridges of 10-μm particle size (Waters Assoc.) (10 cm $\times$ 0.7 cm I.D.), in the two versions, silica gel and C_{18} reversed-phase material; carbohydrate analytical (Waters Assoc.), an amino phase of 10-μm particle size (25 cm $\times$ 0.4 cm I.D.) and reversed-phase μBondapak C_{18} of 5-μm particle size (25 cm $\times$ 0.4 cm I.D.) from E. Merck (Darmstadt, F.R.G.); and Fmoc-L-threonyl-aminopropyl phase of 5-μm particle size (25 cm $\times$ 0.4 cm I.D.), prepared as previously described [24]. The experimental conditions are detailed in each instance in the respective tables.

GC Separations were performed with a Hewlett-Packard system consisting of a Model 5890 gas chromatograph provided with a flame ionization detector and a Model 3390A integrator and equipped with a 5% phenylmethylsilicone cross-linked capillary column (25 m $\times$ 0.25 mm I.D.) (SE-54, Hewlett-Packard). The temperatures of both the injection port and the flame ionization detector were 300°C. The injection volume was 0.5 μl. For further details, see Table I.

Derivatization procedure

Sugars or a sugar mixture (50 mg) are weighed into a capped vial, dissolved in pyridine (0.7 ml) and a 0.72 *M* solution of hydroxylamine hydrochloride in pyridine (0.7 ml) is added. The mixture is heated in a bath at 60°C for 10 min. Acetic anhydride (0.25 ml) is added and the mixture is heated at 75°C for a further 10 min. Solvents are removed *in vacuo* and the residue is dissolved in chloroform (3 ml) and washed with water (3 $\times$ 6 ml). The organic layer is dried over anhydrous sodium sulphate, vacuum evaporated and the residue is filtered over silica gel with chloroform as eluent. The oily residues obtained by distillation of the solvent are applied in the HPLC study.

RESULTS AND DISCUSSION

Capillary gas chromatography

We have studied mixtures of PAAN and PAKO derivatives by GC–MS, using a

5% phenylmethylsilicone cross-linked capillary column, which gives shorter retention times and better resolution than the coated columns used by Seymour [21–23]. The temperature programme used by us was 190°C for 10 min, raised at 1°C/min to 202°C, held for 1 min, raised at 4°C/min to 250°C, held for 20 min. The chromatographic results are given in Table I and Fig. 1. These conditions permit good separations among the different chain length PAAN and between isomers. However, some of the separations are not effective enough to obtain baseline recoveries, *e.g.*, with mannose and altrose, the retention times (t_R) of which are 20.52 and 20.75 min, respectively. The relative retention times of PAKO derivatives are greater than those of PAAN derivatives with same chain length, *e.g.*, fructose-PAKO elutes at 34.30 min and sorbose-PAKO at 35.16 min, whereas the C_6-PAAN compounds (**6–13**) elute between 19.21 and 22.63 min (under the experimental conditions given in Table I).

Use of polar HPLC phases: silica and aminopropyl phases

A simple mixture such as that of **1**, **2**, **8** and **9** derivatives can be easily analysed by using a silica or an aminopropyl phase, and a binary mixture of hexane and acetone as eluent; the exact composition of the eluent is not of critical importance. However, under the selected experimental conditions, the best results were achieved with 11 or 12.5% acetone using the silica phase and 14.3% acetone using the aminopropyl phase (Table II).

Mixtures of six or seven derivatives were resolved by using an Fmoc-L-threonyl-aminopropyl phase (Fig. 2), prepared as previously described [24], with a hexane–dioxane (90:10) as eluent and a flow-rate of 1.0 ml/min. Retention times and baseline widths for sixteen compounds are given in Table III.

To summarize, polar phases allow the analysis of simple mixtures but compared with other phases (see below) they are more difficult to manipulate, give poorer baselines and require longer equilibration times.

Use of HPLC reversed-phases

An extensive study of the capabilities of reversed-phases (specifically, octadecyl-silica phases) for HPLC analysis of PAAN and PAKO derivatives was performed. In

TABLE I

RETENTION TIMES OF SELECTED PAAN/PAKO DERIVATIVES, USING AN SE-54 CAPILLARY CROSS-LINKED COLUMN

Carrier gas, helium; flow-rate, 5 psi; injection volume, 0.5 μl. For temperature programme, see text.

Compound	t_R (min)	Compound	t_R (min)
2	10.94	**10**	21.58
3	10.31	**11**	22.17
4	9.87	**12**	20.24
5	10.54	**13**	22.63
6	19.21	**14**	34.30
7	20.75	**15**	35.16
8	21.16	**16**	32.04
9	20.52		

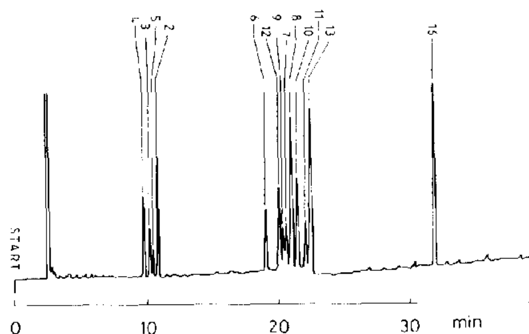

Fig. 1. GC analysis of PAAN derivatives of C_5- and C_6-aldoses and D-*glycero*-D-*gulo*-heptose.

most of the experiments, either Rad-Pak C_{18} cartridges or μBondapak C_{18} metallic columns were used.

Results obtained using a mixture of tetrahydrofuran and water as eluent are given in Table IV. It can be observed that, as a rule, PAKO derivatives are eluted faster than PAAN derivatives and that, within each group, retention times increase with increasing molecular weight. Because of the existence of *syn* and *anti* isomers and their frequent overlapping on elution, PAKO derivatives give more irregular peaks than PAAN derivatives.

Mobile phases with high tetrahydrofuran contents tend to give oscillating baselines, which make accurate work difficult. More stable baselines and better results were obtained with methanol–water mixtures as eluents; the solvent strength of these mixtures can be made comparable to that of the tetrahydrofuran–water mixtures by using appropriate proportions [25].

In the methanol–water system, elution of the products follows the order of molecular weights; faster elution of the corresponding PAKO derivatives is no longer observed (Table IV). A higher resolution is obtained for similar elution volumes when the 5-μm particle μBondapak C_{18} column is used instead of the 10-μm Rad-Pak C_{18} cartridge.

TABLE II

CAPACITY FACTORS (k') OF SELECTED PAAN/PAKO DERIVATIVES USING A CARBOHYDRATE ANALYTICAL AMINOPROPYLSILICA PHASE (I) AND A SILICA PHASE (RAD-PAK CARTRIDGES) (II) WITH MIXTURES OF HEXANE AND ACETONE AS ELUENT

Sample injection, 15 μl.

Column	Acetone (%)	Flow-rate (ml/min)	k'			
			1	2	8	9
I	14.3	0.6	4.0	4.5	5.2	3.6
I	14.3	0.8	2.8	3.3	3.6	2.5
II	11.0	1.0	13.2	14.3	16.8	11.9
II	12.5	1.0	11.1	12.2	14.5	10.5
II	14.5	0.8	8.7	9.4	10.9	8.2

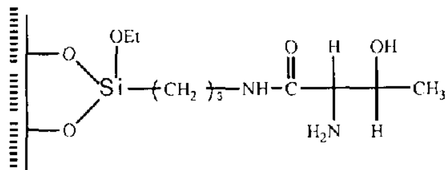

Fig. 2. Fmoc-L-threonyl-aminopropyl HPLC phase.

The acetonitrile water and acetone- water systems give results similar to those with the methanol–water system (Table IV). Ternary systems were also studied, particularly mixtures of water, tetrahydrofuran and methanol. Table V gives some of the more significant results; it can be observed that depending on the relative proportions of the organic solvents, what could be called the "tetrahydrofuran effect" (faster elution of PAKO than PAAN derivatives) is retained. Mixtures in which the proportion of tetrahydrofuran relative to methanol is higher than 40% give rise to unstable baselines. The best ternary system found was water tetrahydrofuran - methanol (65:10:25), with a flow-rate of 0.8 ml/min. The ternary systems water–acetonitrile–methanol and water–acetonitrile–tetrahydrofuran gave poor results; as expected ("tetrahydrofuran effect"), when using the latter PAKO derivatives eluted faster than PAAN derivatives.

When their use is possible, UV detectors have advantages over refractive index detectors; obviously, one is the possibility of using polarity gradients. Another is the intrinsic high sensitivity of UV detection, which makes it suitable for the analysis of minor components. However, PAKO and PAAN derivatives absorb at λ_{max} lower than 207 nm with small absorption coefficients, which seriously limits the choice of organic solvents to be used in eluent mixtures; in practice, only mixtures of water with high-purity methanol and acetonitrile enter into consideration. The detection limits for the investigated derivatized monosaccharides using the UV detector were 3 μg per injection. The data in Table VI were obtained using methanol and acetonitrile as

TABLE III

CAPACITY FACTORS (k') AND BASELINE WIDTHS (w) OF SELECTED PAAN/PAKO DERIVATIVES USING A FMOC-L-THREONYL-AMINOPROPYL PHASE

Eluent, hexane dioxane (9:1); flow-rate, 1.0 ml/min; sample injection, 15 μl.

Compound	k'	w (min)	Compound	k'	w (min)
2	6.0	1.7	**10**	6.8	1.6
3	3.9	1.3	**11**	10.3	1.8
4	3.6	1.0	**12**	5.0	1.8
5	3.9	1.2	**13**	5.0	1.6
6	4.7	1.3	**14**[a]	8.0	2.7
7	6.0	1.8		9.0	2.3
8	6.3	2.0	**15**[a]	8.4	2.3
9	3.8	1.1		12.3	4.2
			16	7.0	1.8

[a] Two peaks corresponding to the *syn* and *anti* isomers of the PAKO derivatives.

TABLE IV

CAPACITY FACTORS (k') FOR SOME SELECTED PAAN/PAKO DERIVATIVES USING C_{18} REVERSED-PHASES WITH AQUEOUS BINARY SOLVENT MIXTURES AS ELUENTS AND A FLOW-RATE OF 1.0 ml/min

Sample injection, 15 μl.

Compound	Eluent[a]				
	I[b]	II[b]	III[b]	IV[b]	V[c]
1	0.5	1.4	1.3	0.9	2.5
2	2.2	2.3	3.7	3.3	9.5
4	2.5	3.0	—	-	10.9
8	3.0	4.1	4.9	4.6	—
9	3.6	–	5.5	5.5	12.7
14[d]	1.3	4.1		—	10.9
	1.3	4.1	—	—	12.8
15[d]	1.2	3.5	—	—	11.7
	1.2	3.9	—	—	11.7

[a] I, Tetrahydrofuran–water (40:60); II, methanol–water (45:55); III, acetonitrile–water (40:60); IV, acetone–water (40:60); V, acetonitrile–water (25:75).

[b] Rad-Pak C_{18} cartridge.

[c] μBondapak C_{18} column.

[d] Two peaks corresponding to the *syn* and *anti* isomers of the PAKO derivatives.

TABLE V

CAPACITY FACTORS (k') FOR SOME SELECTED PAAN/PAKO DERIVATIVES USING A μBONDAPAK C_{18} REVERSED-PHASE COLUMN WITH WATER–TETRAHYDROFURAN–METHANOL MIXTURES AS ELUENTS

Sample injection, 15 μl.

Compound	Eluent[a]			
	I	II	III	IV
1	1.2	0.9	0.8	2.8
2	4.0	3.8	2.8	6.8
4	5.3	4.9	3.3	8.5
8	7.0	6.3	4.2	10.9
9	10.4	8.8	6.1	14.4
14[b]	3.3	3.0	2.4	6.0
	3.8	3.4	2.6	6.6
15[b]	3.6	3.2	2.4	6.6
	3.6	3.2	2.5	6.6

[a] Water–tetrahydrofuran methanol: I, 70:10:20 (0.8 ml/min); II, 70:12:18 (0.7 ml/min); III, 65:10:25 (0.8 ml/min); IV, 65:10:25 (0.5 ml/min).

[b] Two peaks corresponding to the *syn* and *anti* isomers of the PAKO derivatives.

TABLE VI

CAPACITY FACTORS (k') AND BASELINE WIDTHS (w) OF SELECTED PAAN/PAKO DERIVATIVES WITH μBONDAPAK C_{18} COLUMN WITH NON-ISOCRATIC CONDITIONS, A FLOW-RATE OF 1.0 ml/min AND UV DETECTION AT 207 nm

Sample injection, 5 μl.

Compound	A[a]		B[b]		C[c]	
	k'	w (min)	k'	w (min)	k'	w (min)
2	6.0	0.7	3.1	0.3	5.3	0.8
3	6.4	0.7	3.3	0.4	6.3	0.7
4	6.5	0.7	3.4	0.3	6.2	0.7
5	6.6	0.7	3.4	0.3	6.2	0.7
6	8.7	0.7	4.3	0.4	8.4	0.8
7	8.8	0.8	4.3	0.4	8.5	0.6
8	7.8	0.8	3.9	0.3	7.2	0.6
9	8.8	0.7	4.2	0.5	9.7	0.8
10	8.3	0.8	4.0	0.4	7.1	0.8
11	7.8	0.8	3.8	0.5	6.2	0.6
12	8.6	0.8	4.8	0.4	8.4	0.8
13	8.6	0.8	4.2	0.5	8.8	0.8
14[d]	6.2	0.8	3.0	0.5	7.3	0.8
	6.4	0.8	3.2	0.5	7.3	0.8

[a] Acetonitrile–water, linear gradient from 30:70 to 40:60 in 20 min.
[b] Acetonitrile–water, linear gradient from 35:75 to 50:50 in 15 min.
[c] Methanol–water, linear gradient from 40:60 to 50:50 in 10 min.
[d] Two peaks corresponding to the *syn* and *anti* isomers of the PAKO derivatives.

solvents and a UV detector. The chromatograms are better when polarity gradients are used. The shapes of the different peaks are more similar and symmetric as a result of diminishing diffusion effects and a greater number of sugars can be separated; they give signals with small and homogeneous baseline widths. The optimum conditions were achieved with methanol–water as eluent with a linear gradient for 10 min from 40:60 (initial state) to 50:50 (final state).

If detection is performed at three different wavelengths, specifically 195, 207 and 215 nm, it can be immediately established if the eluted compound is a PAAN or a PAKO derivative. With PAKO derivatives the largest absorption coefficient corresponds to the peak at 195 nm whereas with PAAN derivatives it corresponds to the peak at 207 nm. Fig. 3 shows the absorption maxima of the PAAN derivative of lyxose and of the PAKO derivative of fructose.

CONCLUSION

Peracetylated aldononitriles and ketoximes are convenient derivatives for application in HPLC. Mixtures of six or seven such sugar derivatives can be well resolved, and the best chromatographic conditions were attained with a C_{18} reversed-phase column using methanol–water and acetonitrile–water as eluents with polarity gradients or water–tetrahydrofuran–methanol (65:10:25) at a flow-rate of 0.8 ml/min.

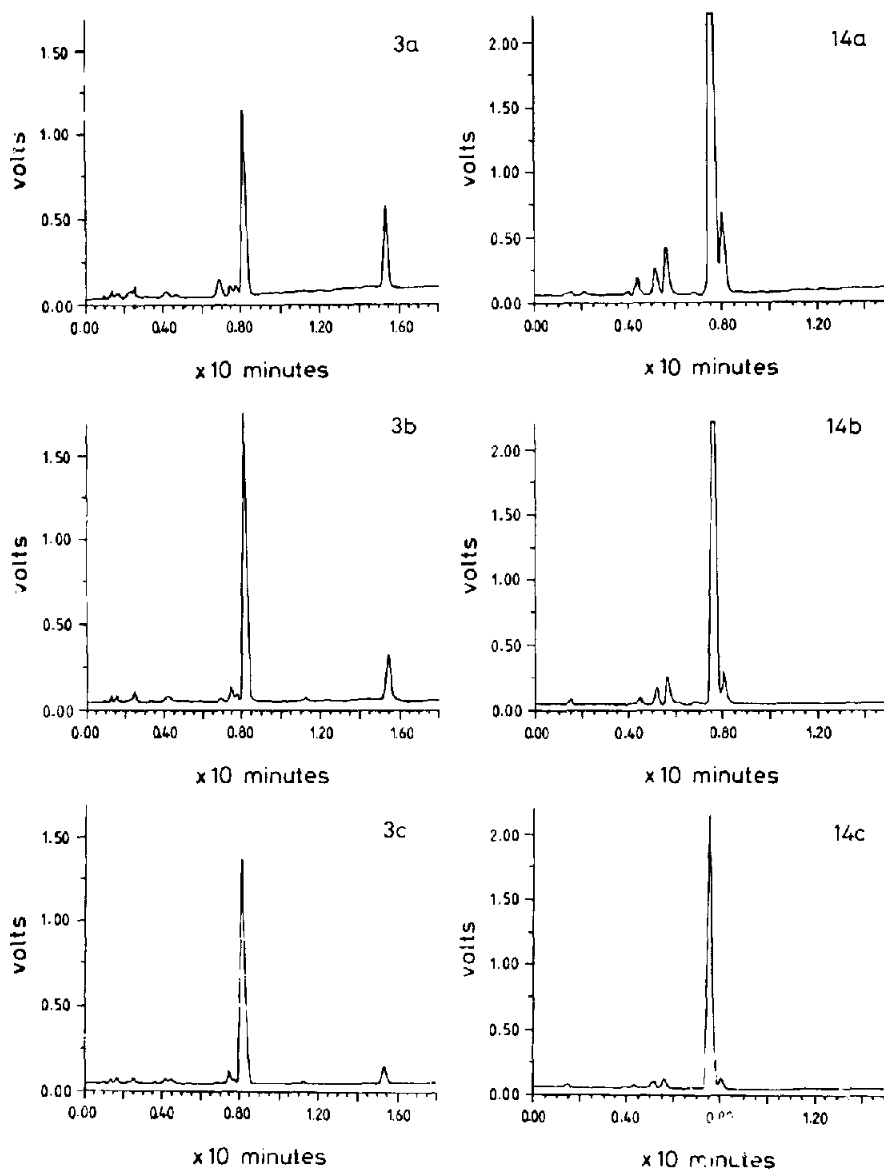

Fig. 3. HPLC with elution gradient. See experimental conditions B in Table VI. **3** = PAAN-lyxose; **14** = PAKO-fructose. UV absorption at (a) 195 nm, (b) 207 nm and (c) 215 nm. Sample injection, 5 μl.

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REFERENCES

1 D. C. DeJongh, *Anal. Chem.*, 42 (1970) 169R–205R.
2 K. Heyns, H. F. Grützmacher, H. Scharmann and D. Müller, *Fortschr. Chem. Forsch.*, 5 (1966) 448–490.
3 R. D. Partridgen, A. H. Weiss and D. Todd, *Carbohydr. Res.*, 24 (1972) 29–44.
4 S. Morgenlie, *Carbohydr. Res.*, 80 (1980) 215–222.
5 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.
6 O. S. Chizhow, N. V. Molodtsov and N. K. Kochetkov, *Carbohydr. Res.*, 4 (1967) 273–276.
7 T. Okuda, S. Saito and M. Hayashi, *Carbohydr. Res.*, 68 (1979) 1–13.
8 R. J. Neeser, *Carbohydr. Res.*, 138 (1985) 189–198.
9 T. Kiho, S. Ukai and C. Hara, *J. Chromatogr.*, 369 (1986) 415–418.
10 D. E. Willis, *J. Chromatogr. Sci.*, 21 (1983) 132–138.
11 G. Petersson, *Carbohydr. Res.*, 33 (1974) 47–61.
12 D. Anderle, J. Königstein and V. Kovácik, *Anal. Chem.*, 49 (1977) 137–139.
13 D. G. Lance and J. K. N. Jones, *Can. J. Chem.*, 45 (1967) 1995–1998.
14 S. Honda, *Anal. Biochem.*, 140 (1984) 1–47.
15 A. Meunier, M. Caude and R. Rosset, *Analusis*, 14 (1986) 363–377.
16 D. Velasco, J. Castells, C. Jaime and F. López-Calahorra, *J. Org. Chem.*, 55 (1990) 3526–3530.
17 L. A. Th. Verhaar and B. F. M. Kuster, *J. Chromatogr.*, 220 (1981) 313–328.
18 C. Churms, in E. Heftmann (Editor), *Chromatography, Part B* (*Journal of Chromatography Library*, Vol. 22B), Elsevier, Amsterdam, 1983, pp. 223–286.
19 R. M. Thompson, *J. Chromatogr.*, 166 (1978) 201–212.
20 J. Thiem, J. Schventner, H. Karl, A. Sievers and J. Reimer, *J. Chromatogr.*, 155 (1978) 107–118.
21 F. R. Seymour, E. C. M. Chen and S. H. Bishop, *Carbohydr. Res.*, 73 (1979) 19–45.
22 F. R. Seymour, E. C. M. Chen and J. E. Stouffer, *Carbohydr. Res.*, 83 (1980) 201–242.
23 F. R. Seymour, S. L. Unruh and D. A. Nehlich, *Carbohydr. Res.*, 191 (1989) 175–189.
24 J. Castells, J. M. Dagà, F. López-Calahorra and D. Velasco, *React. Polym.*, 6 (1987) 207–211.
25 J. W. Dolan, *LC·GC Int.*, 1 (1987) 18–22.