ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 322 (1999) 128–134

CARBOHYDRATE RESEARCH

High-performance anion-exchange chromatography of sugar and glycerol phosphates on quaternary ammonium resins

Ian C. Schneider, Preston J. Rhamy, Ruth J. Fink-Winter, Peter J. Reilly *

Department of Chemical Engineering, Iowa State University, Ames, IA 50011, USA

Received 20 July 1998; accepted 3 August 1999

Abstract

Twenty-six sugar and glycerol phosphates were separated by high-performance anion-exchange chromatography on an alkyl quaternary ammonium column at pH 6.0 with 0.1 M sodium acetate eluant. Capacity, asymmetry, and response factors of the compounds vary widely. Secondary acidic dissociation constants of 14 of these phosphates were determined in an attempt to explain the separation mechanism. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Dissociation constants; Glycerol phosphates; HPAEC; HPLC; Quaternary ammonium resins; Strong-base resins; Sugar phosphates

1. Introduction

High-performance anion-exchange chromatography (HPAEC) with strong-base resins has been widely used to separate sugars. Early articles described the separation of simple mono-, di-, and oligosaccharides [1–7], homologous oligosaccharide series [7–9], and oligosaccharides with several different monomeric residues [10–15]. These separations usually occurred at very high pH where the slight acidity of carbohydrates allows a proton to be abstracted. Strong-base resins have also separated sugar compounds such as aldonic and uronic acids that are negatively charged at more neutral pH values [6,16–18].

The separation of sugar phosphates, which usually also have a negative charge near neutral pH, has been comparatively neglected. Henderson and Henderson used strong-base HPAEC and a Na formate buffer at pH 3.3 with a europium metal complex to chromatograph 11 sugar mono- and bisphosphates [19]. They later used the same complex with a ion-pair formic acid–tetrabutylammonium hydroxide reagent to separate fructose 6-phosphate and glucose 6-phosphate from each other for the first time and, with different concentrations of buffer and the addition of HCl, to separate fructose 1,6-bisphosphate from fructose 2,6-bisphosphate [20]. Smrcka and Jensen used strong-base HPAEC and a trimesic acid–boric acid eluant with LiOH to separate four sugar monophosphates, three bisphosphates, or several phosphorylated carboxylate compounds [21]. Smith and MacQuarrie used two different strong-base alkanol quaternary ammonium columns, one with carbonate–bicarbonate buffer and the other with 4-cyanophenolate buffer, to separate many

* Corresponding author. Tel.: +1-515-294-5968; fax: +1-515-294-2689.

E-mail address: reilly@iastate.edu (P.J. Reilly)

PII: S0008-6215(99)00210-4

different organic phosphates of different charges [22]. Hull and Montgomery separated two sugar monophosphates, two sugar bisphosphates and two phosphorylated glycerates by strong-base alkanol quaternary ammonium HPAEC with an NaOH–2-propanol gradient [23]. Swezey separated 11 sugar phosphates and other intermediary metabolites using a gradient of decreasing borate and increasing NH_4Cl concentration by HPAEC with a trimethylammonium resin [24]. Generally, however, when sugar phosphate separation has been examined, researchers have concentrated on the inositol polyphosphates [25–34].

We report here the use of HPAEC with an alkyl quaternary ammonium resin at pH 6.0 with 0.1 M sodium acetate (NaOAc) buffer to separate 26 sugar and glycerol phosphates. Except for L-3-glycerophospho-1D-myo-inositol, all have two potentially dissociable hydrogen atoms. Capacity factors of the α-sugar 1-phosphates have been correlated with those of the phosphate-free parent compounds, obtained with 0.1 M NaOH eluant on an almost identical column [7], and secondary acidic dissociation constants of some of the phosphates have been measured to allow comparison of factors that determine capacity factors. We had previously separated some of these phosphates by capillary gas chromatography following their derivatization with hexamethyldisilazane [35].

2. Experimental

Materials.—Sugar and glycerol phosphates obtained from Sigma Chemical Co. were used without further purification. They were the following: 2-deoxy-α-D-ribose 1-phosphate, di(monocyclohexylammonium) [$(CHA)_2$] salt, approx. 95%; 2-deoxy-D-ribose 5-phosphate, Na_2 salt, monohydrate, 98%; dihydroxyacetone phosphate, Li_2 salt, approx. 97%; D-erythrose 4-phosphate, Na_2 salt, 61%; D-fructose 1-phosphate, Ba salt, monohydrate, 98%; D-fructose 6-phosphate, Ba salt, 0.5 mol H_2O/mol, 71%; α-L-fucose 1-phosphate, $(CHA)_2$ salt, approx. 99%; β-L-fucose 1-phosphate, $(CHA)_2$ salt, 98% min.; α-D-galactopyranosyl phosphate (α-D-galactose 1-phosphate), K_2 salt, 5.5 mol H_2O/mol, 98%; D-galactose 6-phosphate, Ba salt, 90% min.; α-D-glucose 1-phosphate, K_2 salt, dihydrate, 99%; β-D-glucose 1-phosphate, Na_2 salt, 1.5 mol H_2O/mol; D-glucose 6-phosphate, K_2 salt, dihydrate, 98%; DL-glyceraldehyde 3-phosphate, free acid; *sn*-glycerol 3-phospho-*myo*-inositol (L-3-glycerophospho-1D-*myo*-inositol), K salt, 98%, 5 mg solid/mL MeOH–water (1:1); DL-α-glycerophosphate (DL-glycerol 3-phosphate), Na_2 salt, hexahydrate, approx. 95%; β-glycerophosphate (glycerol 2-phosphate), Na_2 salt, 4.5 mol H_2O/mol; DL-*myo*-inositol 1-monophosphate, $(CHA)_2$ salt, approx. 95%; *myo*-inositol 2-monophosphate, $(CHA)_2$ salt, approx. 95%; α-D-mannopyranosyl phosphate (α-D-mannose 1-phosphate), Na_2 salt, 2.5 mol H_2O/mol, synthetic; D-mannose 6-phosphate, Ba salt, 1.5 mol H_2O/mol, 98%; α-D-ribofuranosyl phosphate (α-D-ribose 1-phosphate), $(CHA)_2$ salt, monohydrate, approx. 90%; D-ribose 5-phosphate, Na_2 salt, 1.5 mol H_2O/mol, 98–100%; D-ribulose 5-phosphate, Na_2 salt, monohydrate, approx. 90%; α-D-xylopyranosyl phosphate (α-D-xylose 1-phosphate), $(CHA)_2$ salt, synthetic; D-xylulose 5-phosphate, Na_2 salt, approx. 80%.

Chromatography.—The HPAEC system had an ISCO 2350 HPLC pump, Erma ERC-3510 degasser, Dionex CarboPac PA (25×3 mm i.d.) precolumn, Dionex CarboPac PA1 (250×4 mm i.d.) column, Knauer 198.00 refractive index detector, Dionex AI-450 chromatography software, and Gateway 2000 PS-100 computer for recording detector output. Both column and precolumn contained polystyrene/divinylbenzene pellicular resin beads with alkyl quaternary ammonium groups.

The eluant was room temperature 0.1 M NaOAc buffer, made with solid NaOAc and deionized water and brought to pH 6.00 with glacial HOAc. The pH was measured with a Fisher Accumet 910 pH meter and Corning 476087 general purpose combination electrode, standardized with Fisher pH 4.00 potassium biphthalate and pH 7.00 K_2HPO_4–NaOH buffers. Sugar and glycerol phosphate samples were dissolved in 0.1 N NaOAc buffer to, with one exception, 2–20 g/L. Injection volumes were 20 μL and eluant flow

was 1 mL/min. Variations in data caused by changes in eluant batches were normalized by use of capacity factors of standards. In general, each sample was chromatographed twice; peak dimensions and asymmetry and response factors were calculated individually and then averaged. Weights and therefore response factors are based on the free acid contents of the pure, dry compounds in the samples.

Measurement of acidic dissociation constants.—The negative $\log_{10}$ of the secondary acidic dissociation constant (pK_2) was obtained by titration at 25 °C of salts of a number of the sugar and glycerol phosphates subjected to HPAEC. This was done with the same pH meter but with a Corning 476531 general purpose combination electrode standardized with 50 mM of either Fisher pH 2.00 HCl–KCl or pH 4.00 potassium biphthalate buffer, plus 50 mM Fisher pH 7.00 K_2HPO_4–NaOH and 50 mM Fisher pH 10.00 K_2CO_3–KBO_3–KOH buffers. The titrant was 10 or 100 mM Fisher standardized HCl, except for the free acid of DL-glyceraldehyde 3-phosphate, when it was 10 mM Fisher standardized NaOH, and the initial sample concentration was generally 4–10 mM. In these ranges there is little effect of varying either acid or sample concentration on pK_2, obtained by measuring the pH at 50% of the acid volume needed to reach the equivalence point (Fig. 1) and correcting to infinite dilution by calculating the activity coefficient f_i for each ion i with the Debye–Huckel equation as

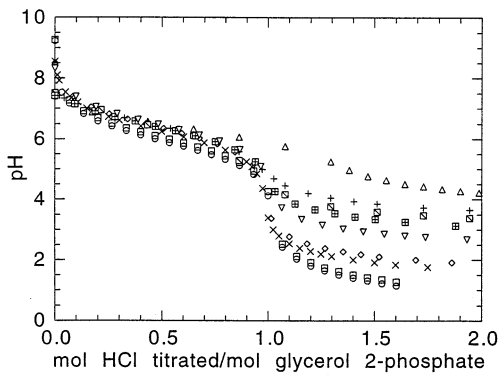

Fig. 1. Effect of HCl concentration and initial sample concentration on the titration of glycerol 2-phosphate. (HCl, mM/glycerol 2-phosphate, mM): △, 1/0.116; +, 1/0.463; ⧅, 10/0.463; ⊞, 10/1.06; ▽, 10/5.19; ◇, 100/29.6; ×, 100/100; □, 1000/300; ○, 1000/600.

modified by Davies [36]:

$$-\log_{10} f_i = 0.5115 \frac{z_i^2 I^{1/2}}{1 + I^{1/2}} - 0.1 z_i^2 I$$

where z_i is the charge and I is the ionic strength. Values of pK_1, which would be obtained from the pH at 1.5 times the acid volume needed to reach the equivalence point, are much more strongly affected by changes of acid and sample concentrations (Fig. 1) [37].

3. Results

HPAEC with strong-base resin and 0.1 M NaOAc buffer eluant at pH 6.0.—Twenty-six sugar and glycerol phosphates, one with one dissociable hydroxyl group and 25 with two, were chromatographed on the CarboPac PA1 column. Their k' values are presented in order of increasing magnitude in Table 1. Neither sugar bisphosphates nor compounds with both phosphate and carboxyl groups exited the column with the present eluant, although presumably they would if a gradient with an increasing concentration of NaOAc buffer were imposed upon the column. Furthermore, no satisfactory peak could be obtained with DL-glyceraldehyde 3-phosphate.

Measurement of acidic dissociation constants.—Values of pK_2 were obtained for 14 of the 26 sugar and glycerol phosphates that had been subjected to HPAEC (Table 1). In addition, values for seven of the chromatographed phosphates had been obtained previously [39]. For five phosphates, values from both the literature and from our work exist. Three agree rather closely but two pK_2 values, those for α-D-galactose 1-phosphate and DL-glycerol 3-phosphate, differ significantly from each other, that obtained here for the former being higher and that for the latter being lower than the corresponding literature values [39].

4. Discussion

At pH 6.0, the value at which chromatography was conducted, L-3-glycerophospho-1D-*myo*-inositol, the one compound with only

Table 1
Separation of organic phosphates on a Carbopac PA1 quaternary ammonium column eluted with 0.1 M NaOAc buffer at pH 6.0; acidic dissociation constants of many of the same phosphates

Compound	t_R [a] (min)	k'	W (μg)	w_0 (min)	a (min)	AF	H ($mV \times 10^4$)	A ($mV \times min \times 10^4$)	RF ($mV \times min/g$)	pK_1	pK_2
L-3-Glycerophospho-1D-*myo*-inositol	1.61 (2) [b]	0.11	2.2	0.205	0.09	1.3	3.0	0.32	14.9		
α-L-Fucose 1-phosphate	6.52(2)	3.45	152	0.455	0.34	0.34	2.7	0.76	0.50		
2-Deoxy-α-D-ribose 1-phosphate	7.86(2)	4.42	90	0.455	0.22	1.1	5.1	1.18	1.31		
α-D-Galactose 1-phosphate	8.01 (2)	4.56	183	0.775	0.51	0.52	19.2	6.47	3.53	1.00 [c]	6.17 [c], 6.50(2)
β-L-Fucose 1-phosphate	8.10(1)	4.63	176	0.55	0.30	0.83	4.2	1.42	0.81		
D-Galactose 6-phosphate	8.28(2)	4.77	295	0.75	0.29	1.6	17.7	5.98	2.03		6.51(4)
myo-Inositol 1-phosphate	8.35(2)	4.78	60	0.465	0.25	0.86	4.8	1.07	1.78		
D-Mannose 6-phosphate	8.45(2)	4.85	247	0.73	0.26	1.8	17.7	6.45	2.62		6.63(3)
α-D-Glucose 1-phosphate	8.65(1)	4.92	360	1.29	0.33	2.9	13.2	7.66	2.13	1.11 [c]	6.13 [c], 6.26(5)
D-Glucose 6-phosphate	8.58(2)	4.93	318	0.99	0.38	1.6	22.8	10.52	3.31		6.11 [c]
2-Deoxy-D-ribose 5-phosphate	8.56(2)	4.94	226	0.84	0.17	3.9	10.2	4.19	1.85		6.28(3)
DL-Glycerol 3-phosphate	8.68(2)	5.00	203	1.04	0.245	3.2	6.6	3.38	1.66		6.66 [c], 6.44(3)
D-Ribose 5-phosphate	8.73(1)	5.10	409	1.33	0.39	2.4	12.0	6.35	1.55		6.33(7)
α-D-Xylose 1-phosphate	9.00(2)	5.23	196	0.89	0.185	3.8	9.3	4.07	2.08		6.26(2)
α-D-Mannose 1-phosphate	9.17(2)	5.28	59	0.415	0.175	1.4	3.0	0.74	1.15		
D-Fructose 6-phosphate	9.15(2)	5.31	229	0.95	0.31	2.1	13.2	5.98	2.62	0.97 [c]	6.11 [c]
Dihydroxyacetone phosphate	9.58(2)	5.59	107	0.97	0.36	1.7	2.4	1.16	1.07	1.77 [c]	6.45 [c]
D-Fructose 1-phosphate	9.46(2)	5.60	237	1.36	0.28	3.8	9.6	5.83	2.47		6.19(3)
Glycerol 2-phosphate	9.58(2)	5.68	341	1.72	0.47	2.7	6.3	4.93	1.45	1.34 [c]	6.55 [c], 6.55(5)
α-D-Ribose 1-phosphate	9.74(2)	5.69	110	0.935	0.21	3.5	6.6	2.69	2.44		6.28(2)
D-Erythrose 4-phosphate	10.02(2)	5.96	176	0.655	0.235	1.8	1.2	0.45	0.26		
myo-Inositol 2-phosphate	10.45(2)	6.18	158	1.36	0.295	3.6	8.1	5.07	3.21		5.84(2)
D-Ribulose 5-phosphate	11.65(2)	7.06	149	1.36	0.33	3.2	3.0	2.03	1.36		
β-D-Glucose 1-phosphate	11.91(2)	7.21	62	1.77	0.27	6.1	6.1	4.39	7.04		5.89(2)
D-Xylulose 5-phosphate	13.17(2)	8.05	45	1.53	0.425	2.6	4.5	3.75	8.28		
DL-Glyceraldehyde 3-phosphate										1.42 [c]	6.45 [c], 6.41(2)

[a] Explanation of symbols: t_R, elapsed time between injection and peak mode; k', capacity factor, $(t_R - t_0)/t_0$, where t_0 is elapsed time between injection and beginning of solvent peak and is between 1.43 and 1.47 min in all cases; W, weight of standard entering detector, based on pure, dry free acid; w_0, peak width at baseline; a, elapsed time between beginning of peak at baseline and peak mode; AF, asymmetry factor, $(w_0 - a)/a$; H, peak height; A, peak area; RF, response factor, A/W [38].
[b] Number of chromatographic runs or titrations in parentheses.
[c] Ref. [39].

one dissociable hydroxyl group, is almost all negatively charged. The bulk of the pK_2 values of the 24 compounds with two dissociable hydroxyl groups are between 6.1 and 6.6. Therefore, at pH 6.0, slightly more than half of the phosphate groups of these compounds have one negative charge, somewhat fewer than half have two negative charges, and a very small fraction have no negative charges. This difference in net charge causes L-3-glycerophospho-1D-*myo*-inositol to be eluted before the other compounds.

Asymmetry factors varied greatly, being much higher for the later-eluting compounds than for the ones that eluted earlier. The tailing of those compounds with higher k' values, which decreases the probability that they can be fully separated, presumably would be ameliorated by using an eluant with an increasing NaOAc concentration gradient. This was not done in this project so that k' values unaffected by changing buffer composition could be obtained.

Response factors also varied greatly. Differences in molecular structure did not seem to explain this. Those compounds having very low response factors, such as α-L-fucose 1-phosphate, β-L-fucose 1-phosphate, and D-erythrose 4-phosphate, were no more likely to have low capacity factors than those compounds with high response factors, such as L-3-glycerophospho-1D-*myo*-inositol, α-D-galactose 1-phosphate, D-glucose 6-phosphate, β-D-glucose 1-phosphate, and D-xylulose 5-phosphate.

Capacity factors of the 24 sugar and glycerol phosphates with two dissociable hydroxyl groups that gave discernable peaks ranged from 3.45 to 8.05, with the bulk falling between 4.5 and 6.0. An increasing NaOAc gradient would reduce this range, but as mentioned above, it would probably increase resolution between most peaks by reducing their asymmetry.

There is a statistically insignificant negative correlation ($R = 0.71$) between k' and pK_2 for the 17 sugar and glycerol phosphates having both values, caused by the relative similarity of the latter values to each other. This suggests that other factors also have a substantial effect on k', unlike the case with sugars and sugar alcohols, where the pK range is much wider and there is a strong correlation between k' and pK for monosaccharides [7].

Correlations between k' values of the same sugar phosphates obtained by us and by others on strong-base columns under different conditions are not possible because not enough compounds in common have been chromatographed. However, several further important observations on our results can be made:

(i) Both α-L-fucose 1-phosphate and α-D-glucose 1-phosphate were eluted before their corresponding β-linked forms. Sterically, it appears easier for equatorial β-1-phosphate groups rather than axial α-1-phosphate groups to bind to the alkyl quaternary ammonium groups on the carrier; in addition, β-D-glucose 1-phosphate has a lower pK_2 than does α-D-glucose 1-phosphate.

(ii) α-D-Mannose 1-phosphate and α-D-ribose 1-phosphate were eluted after D-mannose 6-phosphase and D-ribose 5-phosphate, respectively, but 2-deoxy-α-D-ribose 1-phosphate was eluted before 2-deoxy-D-ribose 5-phosphate. Furthermore, α-D-galactose 1-phosphate and D-galactose 6-phosphate were eluted at about the same time, as were α-D-glucose 1-phosphate and D-glucose 6-phosphate. The inductive effect of the ring oxygen causes high acidity of the hydrogen atom of the anomeric hydroxyl group [40], and by extension to hydrogen atoms of phosphate groups linked to the oxygen atom of that group. This would make k' values of most sugars with α-1-phosphate groups greater than those of the equivalent sugars with phosphate groups linked to other hydroxyl groups.

However, the lack of a 2-OH in 2-deoxy-α-D-ribose 1-phosphate eliminates the possibility of hydrogen bonding between it and the phosphate group, decreasing the acidity of the latter and making k' of 2-deoxy-α-D-ribose 1-phosphate lower than that of 2-deoxy-D-ribose 5-phosphate.

(iii) There is a rough correlation ($R = 0.88$) between k' values for seven sugars eluted with 0.1 N NaOH from a Dionex HPIC-AS6 column [7] and those of corresponding sugars with α-1-phosphate groups eluted with 0.1 M NaOAc buffer from a very similar Dionex Carbopac PA1 column (Fig. 2). As noted

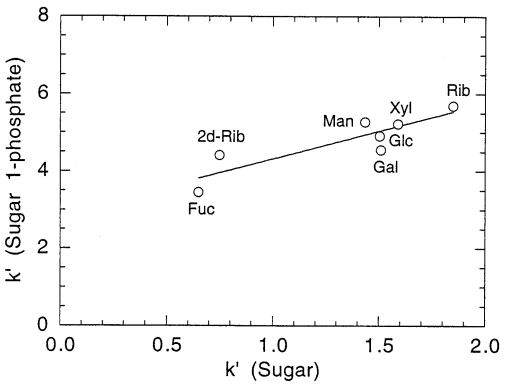

Fig. 2. Correlation of capacity factors of parent sugars eluted from a Dionex HPIC-AS6 column with 0.1 N NaOH with their corresponding α-l-phosphates eluted from a Dionex CarboPac PA1 column with 0.1 M NaOAc buffer at pH 6.0.

above, the strong acidities of anomeric sugar hydroxyl groups should carry over to the corresponding α-1-sugar phosphates.

(iv) There is a statistically insignificant positive correlation ($R = 0.61$) between k' values of parent sugars and those of the corresponding sugars with phosphate groups elsewhere on the molecule other than the reducing oxygen atom.

Sugar and glycerol phosphates are important physiological agents and have potential industrial uses. Their analytical and preparative separation is of value. The technique described here allows that possibility.

Acknowledgements

The authors are grateful for the support of the US Department of Agriculture through the Biotechnology Byproducts Consortium, a partnership of Iowa State University, the University of Iowa, and the City of Cedar Rapids, Iowa. They also thank Charles Glatz and his group for the use of the pH meter and Walter Trahanovsky for helpful discussions.

References

[1] R.D. Rocklin, C.A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577–1590.
[2] D.C. Johnson, *Nature*, 321 (1986) 451–452.
[3] W.T. Edwards, C.A. Pohl, R. Rubin, *Tappi J.*, 70 (1987) 138–140.
[4] M.R. Hardy, R.R. Townsend, Y.C. Lee, *Anal. Biochem.*, 170 (1988) 54–62.
[5] N.J. Chatterton, P.A. Harrison, W.R. Thornley, J.H. Bennett, *Plant Physiol. Biochem.*, 27 (1989) 289–295.
[6] D.A. Martens, W.T. Frankenberger, *Chromatographia*, 29 (1990) 7–12.
[7] T.J. Paskach, H.-P. Lieker, P.J. Reilly, K. Thielecke, *Carbohydr. Res.*, 215 (1991) 1–14.
[8] K. Koizumi, Y. Kubota, T. Tanimoto, Y. Okada, *J. Chromatogr.*, 454 (1988) 303–310.
[9] K. Koizumi, Y. Kubota, T. Tanimoto, Y. Okada, *J. Chromatogr.*, 464 (1989) 365–373.
[10] M.R. Hardy, R.R. Townsend, *Proc. Nat. Acad. Sci. USA*, 85 (1988) 3289–3293.
[11] R.R. Townsend, M.R. Hardy, O. Hindsgaul, Y.C. Lee, *Anal. Biochem.*, 174 (1988) 459–470.
[12] L.-M. Chen, M.-G. Let, M.-C. Shao, *FASEB J.*, 2 (1988) 2817–2824.
[13] M.R. Hardy, R.R. Townsend, *Carbohydr. Res.*, 188 (1989) 1–7.
[14] R.R. Townsend, M.R. Hardy, D.A. Cumming, J.P. Carver, B. Bendiak, *Anal. Biochem.*, 182 (1989) 1–8.
[15] W.T. Wang, D. Zopf, *Carbohydr. Res.*, 189 (1989) 1–11.
[16] A.T. Hotchkiss Jr., K.B. Hicks, *Anal. Biochem.*, 184 (1990) 200–206.
[17] M. O'Neill, P. Albersheim, A. Darvill, *Methods Plant Biochem.*, 2 (1990) 415–441.
[18] H.-P. Lieker, K. Thielecke, K. Buchholz, P.J. Reilly, *Carbohydr. Res.*, 238 (1993) 307–311.
[19] S.K. Henderson, D.E. Henderson, *J. Chromatogr. Sci.*, 23 (1985) 222–226.
[20] S.K. Henderson, D.E. Henderson, *J. Chromatogr. Sci.*, 24 (1986) 198–203.
[21] A.V. Smrcka, R.G. Jensen, *Plant Physiol.*, 86 (1988) 615–618.
[22] R.E. Smith, R.A. MacQuarrie, *Anal. Biochem.*, 170 (1988) 308–315.
[23] S.R. Hull, R. Montgomery, *Anal. Biochem.*, 222 (1994) 49–54.
[24] R. Swezey, *J. Chromatogr. B*, 669 (1995) 171–176.
[25] R.B. Ellis, T. Galliard, J.N. Hawthorne, *Biochem. J.*, 88 (1963) 125–131.
[26] M.J. Berridge, R.M.C. Dawson, C.P. Downes, J.P. Heslop, R.F. Irvine, *Biochem. J.*, 212 (1983) 473–482.
[27] R.F. Irvine, E.E. Ånggård, A.J. Letcher, C.P. Downes, *Biochem. J.*, 229 (1985) 505–511.
[28] H. Binder, P.C. Weber, W. Siess, *Anal. Biochem.*, 148 (1985) 220–227.
[29] D.B. Wilson, T.M. Connolly, T.E. Bross, P.W. Majerus, W.R. Sherman, A.N. Tyler, L.J. Rubin, J.E. Brown, *J. Biol. Chem.*, 260 (1985) 13496–13501.
[30] A.-S. Sandberg, R. Ahderinne, *J. Food Sci.*, 51 (1986) 547–550.
[31] N.M. Dean, J.D. Moyer, *Biochem. J.*, 242 (1987) 361–366.
[32] G.W. Mayr, *Biochem. J.*, 254 (1988) 585–591.
[33] T.-N. Lin, G.Y. Sun, N. Premkumar, R.A. MacQuarrie, S.R. Carter, *Biochem. Biophys. Res. Commun.*, 167 (1990) 1294–1301.
[34] R.J. Barnaby, *Anal. Biochem.*, 199 (1991) 75–80.
[35] S.L. Johansen, A. Sivasothy, M.K. Dowd, P.J. Reilly, E.G. Hammond, *J. Am. Oil Chem. Soc.*, 73 (1996) 1275–1286.
[36] C.W. Davies, *J. Chem. Soc.*, (1938) 2093-2098.

[37] D.A. Skoog, D.M. West, and F.J. Holler, *Fundamentals of Analytical Chemistry*, 6th edn, Saunders College Publishing, Fort Worth, 1992, pp. 227–228.
[38] N. Dyson, *Chromatographic Integration Methods*, 2nd edn, Royal Society of Chemistry, Cambridge, 1998, pp. 1–33.
[39] R.M.C. Dawson, D.C. Elliott, W.H. Elliott, K.M. Jones, *Data for Biochemical Research*, 3rd edn, Oxford Science Publications, Oxford, 1986, pp. 55–73.
[40] J.A. Rendelman, *Adv. Chem. Ser.*, 117 (1971) 51–69.