Food Chemistry **28** (1988) 257–268

Quantitative Analysis of Vitamin C (L-Ascorbic Acid) by Ion Suppression Reversed Phase Chromatography

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(Received 27 April, 1987; revised version received 1 September, 1987;
accepted 1 September, 1987)

ABSTRACT

Ion suppression reversed phase chromatography of vitamin C (L-ascorbic acid) is described using a pH-stable, polymeric adsorbent (PLRP-S), a low pH sodium dihydrogen phosphate buffer as the mobile phase, and spectrophotometric detection. The optimization of the method was performed with reference to the separation of L-ascorbic acid and D-erythorbic acid (D-isoascorbic acid). The quantitative assay is demonstrated by the determination of vitamin C in fruit juice.

INTRODUCTION

Various attempts have been made to use high performance liquid chromatography (HPLC) for the determination of L-ascorbic acid (the biologically important antioxidant, vitamin C). Rouseff (1979) reported a method for the determination of L-ascorbic acid in orange juice which utilized a strong anion-exchange HPLC material. Anion-exchange methods (Williams *et al.*, 1973; Pachla & Kissinger, 1976) are limited by the use of boric acid buffers, pH 8·0, and gradient elution systems, such systems being inappropriate as vitamin C is most stable in acid solutions (van Nickert, 1982). Cation-exchange (Ashoor *et al.*, 1984) utilizes dilute solutions of mineral acids as the eluent after mixing the sample with EDTA. The adsorbents used are the microporous 7% crosslinked poly(styrene/divinylbenzene), fully sulphonated materials which have limited mechanical stability under HPLC conditions. Amino bonded columns (Parviainen *et al.*, 1986; Rose & Nahrwald, 1981), have been used for the analysis of L-ascorbic acid but it has been noted (Rose & Nahrwold, 1981) that duplicate runs are often required. Gradient elution may be necessary with high percentages of acetonitrile in the mobile phase. Finley & Duang (1981) reported a method using paired-ion reversed phase chromatography with a C18 bonded phase. Ion-pairing agents such as quaternary ammonium compounds or tertiary amines are used to control solute retentivity (Sood *et al.*, 1976; Pachla & Kissinger, 1979). The mobile phases required are often complex and reproducibility poor as solute retentivity and selectivity are dependent upon the type and concentration of the ion-pairing agent used. Reversed phase chromatography, using silica based C18 materials (Bianchi & Rose, 1985), has been demonstrated to provide excellent retention of L-ascorbic acid at low pH. However, dissolution of the silica matrix is accelerated under such conditions (Krstulovic & Brown, 1982). Such analytical columns must therefore be protected by C18 silica presaturation columns which can, under some circumstances, result in deposition of silica in the injection valve causing blockages or premature wear.

The purpose of this work was to develop both a qualitative and quantitative reversed-phase analysis of L-ascorbic acid which overcomes the problems inherent in silica-based materials and which does not involve pre-derivatization of the solute, complex mobile phases or gradient elution.

Reversed phase separations utilizing a macroporous copolymer of styrene and divinylbenzene have been reported (Mori, 1978; Lee & Kindsvater, 1980; Lee, 1982). The chemical and pH stability of these materials (Smith, 1984; Dawkins *et al.*, 1986) would enable low pH mobile phases to be used without accelerated column degradation. The very high surface area available for solute stationary phase interaction (Dawkins *et al.*, 1980) should result in the

retention of the hydrophilic L-ascorbic acid from the solvent front without the need for mobile phase additives.

EXPERIMENTAL

Equipment

A modular high performance liquid chromatograph consisting of a Knauer pump model 64 (Knauer, Berlin, FRG), a Rheodyne injection valve model 7125 fitted with a 20 μl loop (supplied by HPLC Technology Ltd, Macclesfield, Cheshire, Great Britain), a Knauer variable wavelength UV detector, model 87, operated at 220 nm and Knauer twin pen strip chart recorder (both obtained from Knauer, Berlin, FRG) were used. A second variable wavelength UV detector, Pye Unicam model LC3, operated at 244 nm, was placed in series as the second detector for the quantitative analysis of vitamin C (Pye Unicam Ltd, Cambridge, Great Britain). The analog detector output was analysed using a Trivector Trilab 2000 computer equipped with an LC/GC data handling programme (Trivector Ltd, Sandy, Bedfordshire, Great Britain).

Columns

The polymeric, macroreticular HPLC packing was the commercially available PLRP-S, poly(styrene/divinylbenzene) material with 5 μm and 8 μm nominal particle sizes (Polymer Laboratories Ltd, Church Stretton, Shropshire, Great Britain). Two analytical column sizes were used, 150 mm × 4·6 mm inside diameter and 250 mm × 4·6 mm inside diameter, each being fitted with 2 μm porosity sintered stainless steel frits. The same length, approximately 3 in of 0·010 in inside diameter stainless steel tubing was used to connect the columns of both dimensions to the chromatograph.

Chemicals

The mobile phases used for both development of the qualitative analytical method and the subsequent quantitative analysis were prepared from double distilled water. Sodium dihydrogen phosphate, Fisons AR grade, was used as the buffer salt and pH adjustment performed in all cases by the addition of 0·5M-hydrochloric acid, Fisons volumetric grade (Fisons Scientific Equipment Division, Loughborough, Leics., Great Britain). Acetonitrile, HPLC grade (Fisons Scientific Equipment Division, Loughborough, Leics., Great Britain) was used in the optimization of the mobile

phase. Metaphosphoric acid, GPR grade, was used for L-ascorbic acid solution stability determinations (BDH Chemicals Ltd, Atherstone, Warwickshire, Great Britain) as was DL-homocysteine (Sigma Chemical Co., Poole, Dorset, Great Britain). L-Ascorbic acid (Sigma Chemical Co., Poole, Dorset, Great Britain) and D-erythorbic acid (Flurochem Ltd, Glossop, Derbyshire, Great Britain) were used as the reference materials.

RESULTS AND DISCUSSION

Preliminary experiments showed that resolution of L-ascorbic acid and D-erythorbic acid could be achieved using a small pore size, high surface area HPLC adsorbent, PLRP-S 100Å.

Mobile phase optimization

In reversed phase separations of ionic and ionizable solutes both retentivity and selectivity can be influenced by changes in mobile phase pH and ionic strength (Pietrzyk *et al.*, 1978). Retentivity increases as net solute charge decreases. For ion suppression reversed phase chromatography of acids a mobile phase with a pH below the pK_a of the solute is necessary. The pH (over the range pH 1 to 14) and chemical stability of the poly(styrene/divinylbenzene) matrix enables mobile phases with pH less than the pK_a of L-ascorbic acid (pK_a 4·17) (Crawford & Crawford, 1980) and of high ionic strength to be evaluated in development of the qualitative method.

The optimization of the mobile phase was carried out with reference to the separation of L-ascorbic acid and D-erythorbic acid using a 250 × 4·6 mm inside diameter column packed with the 5 μm particle size adsorbent. The flow rate was 0·5 ml/min throughout. Values of solute capacity factors (k') for L-ascorbic acid and D-erythorbic acid were calculated (Majors *et al.*, 1984) from:

$$k' = (t_r - t_0)/t_0$$

where t_r is the retention time of the solute and t_0 is the retention time of an unretained peak which, in this case, was taken to be the solvent front obtained by injecting the solutes prepared in water. The selectivity (α) of the separation was calculated as the ratio of the capacity factors of D-erythorbic acid and L-ascorbic acid:

$$\alpha = k' \text{ D-erythorbic acid}/k' \text{ L-ascorbic acid}$$

It is clear from Table 1 that, at a constant ionic strength (0·2M), decreasing the pH to 2·14 results in an increase in capacity factor for both solutes; below pH 2·00 a further reduction in pH has minimal effect. No effect of ionic

TABLE 1
Influence of pH on the Capacity Factors and Selectivity of L-Ascorbic Acid and D-Erythorbic Acid at Constant Ionic Strength (0·2M Sodium Dihydrogen Phosphate)

pH	*Capacity factors*		*Selectivity*
	k' L-ascorbic acid	*k' D-erythorbic acid*	
3·93	0·22	0·27	1·23
2·83	0·43	0·57	1·33
2·45	0·46	0·62	1·35
2·25	0·49	0·68	1·39
2·14	0·49	0·68	1·39
2·00	0·49	0·65	1·33

strength (within the range 0·4 to 0·02M) on capacity factor for L-ascorbic acid was observed (see Table 2). More variation was obtained in the capacity factor of D-erythorbic acid, resulting in a maximum in the calculated selectivity between 0·2 and 0·3M. The effect of pH at lower ionic strength on the capacity factors and selectivity of the test solutes (Table 3) shows that the solute retentivity is influenced to a greater extent by changes in pH when the ionic strength is less than 0·1M.

It was therefore concluded that, to resolve the L-ascorbic acid from both the solvent front and the D-erythorbic acid, a 0·2M sodium dihydrogen phosphate mobile phase with the pH adjusted to 2·14 using hydrochloric acid would be optimum. Under these conditions, small changes in mobile phase ionic strength and pH would have a negligible effect on the solute

TABLE 2
Influence of Ionic Strength on the Capacity Factors and Selectivity of L-Ascorbic Acid and D-Erythorbic Acid at Constant pH (pH 2·14)

Ionic strength (M)	*Capacity factors*		*Selectivity*
	k' L-ascorbic acid	*k' D-erythorbic acid*	
0·40	0·47	0·64	1·36
0·30	0·49	0·68	1·39
0·20	0·49	0·68	1·39
0·10	0·47	0·63	1·34
0·05	0·49	0·65	1·33
0·02	0·49	0·67	1·37

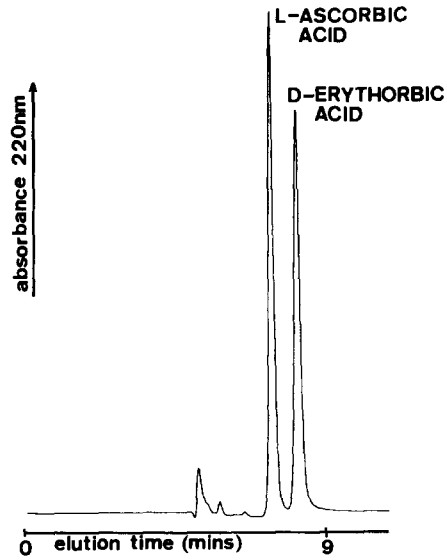

Fig. 1. Separation of L-ascorbic acid and D-erythorbic acid using optimized mobile phase 0·2M sodium dihydrogen phosphate, pH 2·14.

capacity factors and selectivity. Figure 1 illustrates the resolution achieved for L-ascorbic acid and D-erythorbic acid using this mobile phase.

In reversed phase chromatography the addition of an organic modifier, such as methanol or acetonitrile, increases the solvent strength of the mobile phase, so reducing solute capacity factors (Krstulovic & Brown, 1982). The addition of acetonitrile to the mobile phase at low concentrations (1% or 5% v/v) was found to reduce the solute capacity factors for both these

TABLE 3
Influence of Ionic Strength on the Capacity Factors and Selectivity of L-Ascorbic Acid and D-Erythorbic Acid at Low pH

Ionic strength (M)	*pH*	*Capacity factors*		*Selectivity*
		k' L-ascorbic acid	*k' D-erythorbic acid*	
0·20	2·14	0·49	0·68	1·39
0·20	2·25	0·49	0·68	1·39
0·10	2·14	0·47	0·63	1·34
0·10	2·28	0·49	0·67	1·37
0·05	2·14	0·49	0·65	1·33
0·05	2·30	0·46	0·62	1·35
0·02	2·14	0·49	0·67	1·37
0·02	2·35	0·45	0·61	1·36

TABLE 4
Influence of Acetonitrile Concentration (by volume) on the Capacity Factors and Selectivity of L-Ascorbic Acid and D-Erythorbic Acid (0·2M Sodium Dihydrogen Phosphate, pH 2·14, as the Aqueous Component of the Mobile Phase)

% Acetonitrile	*Capacity factors*		*Selectivity*
	k' L-ascorbic acid	*k' D-erythorbic acid*	
0·0	0·49	0·68	1·39
1·0	0·38	0·49	1·29
5·0	0·18	0·23	1·28

hydrophilic water soluble acids (see Table 4) to such an extent that baseline resolution was lost when 1% v/v acetonitrile was present.

Column variables

The requirement for column length and adsorbent particle size was evaluated by comparing the resolution achieved for the separation of L-ascorbic acid and D-erythorbic acid using the standard mobile phase (0·2M sodium dihydrogen phosphate, pH 2·14), the resolution being calculated from:

$$R_s = \text{difference in retention time/mean peak base width}$$

as defined by Bristow (1976).

For maximum resolution a high efficiency 5 μm particle size is required, packed in a 250 mm × 4·6 mm inside diameter column and operated at a flow rate of 0·5 ml/min (see Table 5). However, this degree of resolution may not

TABLE 5
Resolution Dependency for L-Ascorbic Acid and D-Erythorbic Acid on Adsorbent Particle Size. Column Dimensions and Flow Rate. (Mobile Phase 0·2M Sodium Dihydrogen Phosphate, pH 2·14)

Column dimensions (mm)	*Particle size (μm)*	*Flow rate (ml/min)*	*Resolution*
150 × 4·6	8	1·0	0·64
150 × 4·6	8	0·5	0·86
150 × 4·6	5	1·0	0·86
150 × 4·6	5	0·5	0·95
250 × 4·6	5	0·5	1·20

be necessary for all applications. The normal criterion for a separation is to have an R_s value of 1 (Bristow, 1976), which is considered to be sufficient resolution for most purposes. The 250 mm × 4·6 mm inside diameter PLRP-S 100Å 5 μm column has an R_s value of 1·2 when operated at a flow rate of 0·5 ml/min. As the object of this work was the development of a quantitative method for the determination of vitamin C in complex mixtures, e.g. fruit juices, this was the column chosen for all subsequent work operated at either 0·5 or 1·0 ml/min.

L-Ascorbic acid solution stability

The solution stability of L-ascorbic acid was evaluated using a flow rate of 1·0 ml/min for the standard mobile phase with L-ascorbic acid solutions prepared in:

(a) double distilled water, pH 7·0.
(b) mobile phase (0·2M sodium dihydrogen phosphate, pH 2·14).
(c) 10% metaphosphoric acid.
(d) 5% metaphosphoric acid.
(e) 1 mM DL-homocysteine.

The sample solutions were stored in clear glass bottles at ambient temperature throughout the experiment. No attempt was made to eliminate oxygen or light.

L-Ascorbic acid solution prepared in distilled water at pH 7·0 is unstable, being rapidly oxidized (van Kiekert, 1982) (see Fig. 2). However, this rate of oxidation is dramatically reduced when the L-ascorbic acid is prepared in low pH, high salt concentration solutions such as the mobile phase. When metaphosphoric acid (10% or 5%), a stabilizing agent (van Niekert, 1982), is

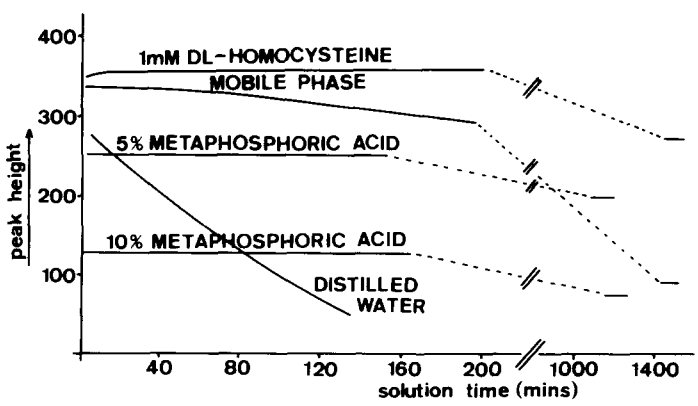

Fig. 2. L-Ascorbic acid solution stability: Plot of solution time versus peak height.

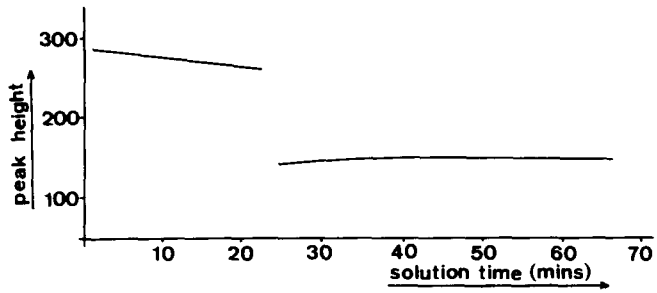

Fig. 3. L-Ascorbic acid solution stability before and after the addition of 1 mM DL-homocysteine (1:1 dilution).

used to prepare the sample solution, oxidation is retarded for a period in excess of 150 min, but after about 20 h some degradation occurs. The presence of 1 mM DL-homocysteine, a reducing agent, in the sample solution, results in an increase in the L-ascorbic acid concentration over the first 20 min due to the initial reduction (Rose & Nahrwold, 1981) of oxidised L-ascorbic acid present in the sample. Evaluation of the 1 mM DL-homocysteine sample solution after 24 h revealed that some degradation had occurred but the solution was stable up to a period in excess of 4 h. To investigate further the influence of 1 mM DL-homocysteine on L-ascorbic acid measurements, an aged sample (25 min) of L-ascorbic acid in mobile phase was diluted (1:1) with 1 mM DL-homocysteine. L-Ascorbic acid peak height measurements were performed over a period of 40 min. The measured peak height increased over the first 20 min as the dehydroascorbic acid was reduced to L-ascorbic acid but then no further increase in peak height was evident (see Fig. 3).

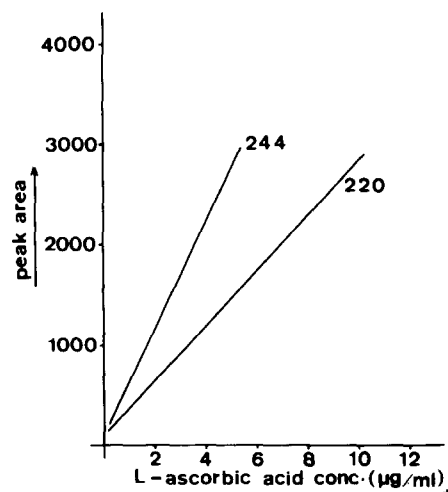

Fig. 4. Calibration curve of L-ascorbic acid at 244 nm and 220 nm.

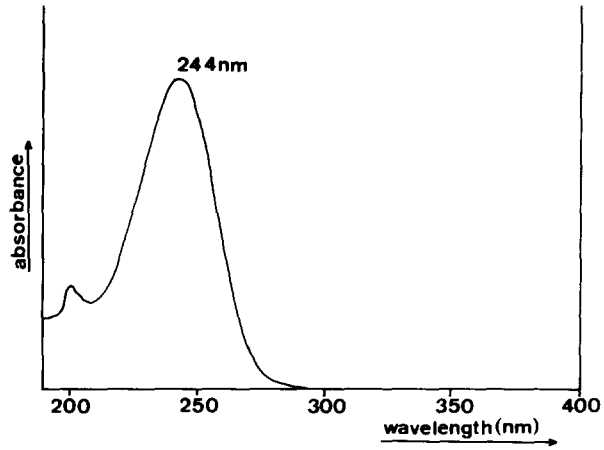

Fig. 5. UV absorbance spectrum of L-ascorbic acid when dissolved in mobile phase, 0·2M sodium dihydrogen phosphate, pH 2·14.

Quantitative analysis of L-ascorbic acid

The 250 mm × 4·6 mm inside diameter column packed with PLRP-S 100 Å 5 μm material was used for the development of the quantitative analysis of L-ascorbic acid. The mobile phase was 0·2M sodium dihydrogen phosphate, pH 2·14, and the flow rate 0·5 ml/min. The calibration graph (see Fig. 4) relates peak area (or height) to L-ascorbic acid concentration using standard L-ascorbic acid solutions (20 μl full loop injections) prepared in 1 mM DL-homocysteine to eliminate oxidative degradation over the period of the calibration. A stock solution was diluted serially with 1 mM DL-homocysteine to produce the lower L-ascorbic acid concentrations. The calibration curves were produced at two wavelengths, 244 nm, the wavelength for maximum absorbance for L-ascorbic acid under the HPLC analysis conditions used (see Fig. 5) and 220 nm by operating the two variable wavelength UV detectors in series. A linear relationship is demonstrated over the range evaluated (0·02 to 5·13 μg/ml at 244 nm and 0·02 to 10·20 μg/ml at 220 nm).

Quantitative determination of vitamin C in fruit juices

The same experimental conditions were used for the analysis of fruit juices as for the production of L-ascorbic acid concentration versus detector response (peak area) calibration curve. Spectrophotometric detection was accomplished with a single variable wavelength UV detector operated at 220 nm.

The fruit juice was extracted from the fresh fruit and immediately diluted with mobile phase, 0·2M sodium dihydrogen phosphate, pH 2·14, in the ratio

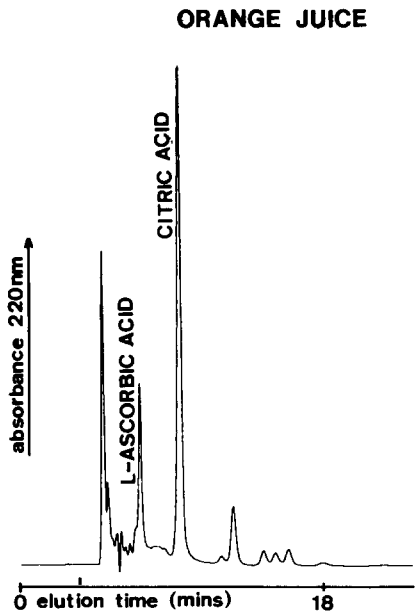

Fig. 6. Elution profile of fresh fruit juice run using standard L-ascorbic acid experimental conditions.

1:50. Dilution with low pH, high ionic strength mobile phase was performed in preference to distilled water to retard vitamin C degradation as determined from the L-ascorbic acid solution stability evaluation. The diluted sample was then filtered and analysed immediately using a 20 μl full loop injection. No other sample preparation was necessary. Figure 6 is a typical elution profile obtained for a sample of fresh fruit juice. The resolution achieved for the complex sample matrix with no pretreatment other than filtration is sufficient to allow quantitation of the vitamin C present in the extracted juice. Table 6 summarizes the vitamin C concentrations determined for orange, grapefruit and lemon juices taken from fruits which have been subject to ageing on storing, but which were otherwise intact.

TABLE 6
L-Ascorbic Acid (Vitamin C) Content of Fresh Fruit Juice

Sample	*Vitamin C concentration in undiluted juice (mg/litre)*
Grapefruit	440
Lemon	594
Orange	727

CONCLUSION

The objective of developing a technique utilizing an isocratic HPLC system with ultraviolet detection and simple mobile phase is achieved through the use of ion suppression chromatography performed with a polymeric, PLRP-S 100 Å 5 μm reversed phase adsorbent.

The optimized system is able to resolve L-ascorbic acid from D-erythorbic acid (D-isoascorbic acid) in 9 min at a flow rate of 0·5 ml/min.

The HPLC method for the quantification of vitamin C requires no derivatization of the ascorbic acid and involves minimal sample preparation. In the case of the determination of vitamin C in fruit juice, dilution and filtration are the only requirements. Determinations of vitamin C at concentration levels down to 0·02 μg/ml are possible when the analyses are carried out at 244 nm, the wavelength of maximum absorbance for L-ascorbic acid in the mobile phase.

REFERENCES

Ashoor, S. H., Monte, W. C. & Welty, J. (1984). *J. Assoc. Off. Anal. Chem.*, **67**, 78.
Bianchi, J. & Rose, R. C. (1985). *J. Micronutrient Anal.*, **1**, 3.
Bristow, P. A. (1976). *LC in practice*, HETP, Wilmslow, UK.
Crawford, T. C. & Crawford, S. A. (1980). *Adv. Carb. Chem.*, **37**, 79.
Dawkins, J. V., Lloyd, L. L. & Warner, F. P. (1986). *J. Chromatogr.*, **352**, 157.
Finley, J. W. & Duang, E. (1981). *J. Chromatogr.*, **207**, 449.
Krstulovic, A. M. & Brown, P. R. (1982). *Reversed-phase high performance liquid chromatography*, Wiley-Interscience, New York.
Lee, D. P. (1982). *J. Chromatogr. Sci.*, **20**, 203.
Lee, D. P. & Kindsvater, J. H. (1980). *Anal. Chem.*, **52**, 2425.
Majors, R. E., Barth, H. G. & Lochmuller, C. M. (1984). *Anal. Chem.*, **56**, 300R.
Mori, S. (1978). *Anal. Chem.*, **50**, 745.
van Niekert, P. J. (1982). *HPLC in food analysis.* (Macrae, R. (Ed.)), Academic Press, London, 187.
Pachla, L. A. & Kissinger, P. T. (1976). *Anal. Chem.*, **48**, 364.
Pachla, L. A. & Kissinger, P. T. (1979). *Methods Enzymol.*, **62**, 15.
Parviainen, N. T., Nyyssonen, K., Penttila, I. M., Seppanen, K., Rauramaa, R., Salonen, J. T. S. & Gref, C. G. (1986). *J. Liq. Chromatogr.*, **9**(10), 2185.
Pietrzyk, D. J., Kroeff, E. P. & Rotsch, T. D. (1978). *Anal. Chem.*, **50**, 497.
Rose, R. C. & Nahrwold, D. L. (1981). *Ann. Biochem.*, **114**, 140.
Rouseff, R. (1979). *Liquid chromatographic analysis of food and beverages*, Vol. 1. (Charalambous, G. (Ed.)), Academic Press, New York and London, 161.
Smith, R. M. (1984). *J. Chromatogr.*, **291**, 372.
Sood, S. P., Sartori, L. E., Wittmer, D. P. & Haney, W. G. (1976). *Anal. Chem.*, **48**, 796.
Williams, R. C., Baker, D. R. & Schmit, J. A. (1973). *J. Chrom. Sci.*, **14**, 618.