Journal of Chromatography, 357 (1986) 39–48
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 438

ENHANCED PEAK RESPONSES DUE TO SOLVENT INTERACTIONS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

SOLOMON PERLMAN* and JOEL J. KIRSCHBAUM
Squibb Institute for Medical Research, New Brunswick, NJ 08903 (U.S.A.)
(First received October 28th, 1985; revised manuscript received December 23rd, 1985)

SUMMARY

The liquid chromatography of a variety of pure compounds gave larger or smaller peak areas and heights (responses) depending on the injection solvent. The high-performance liquid chromatographic system was unchanged, with the pump, flow-rate, column, column temperatures and detector type and settings invariant for each comparison. Only the solvent for the analyte varied. For reversed-phase columns, the responses of each compound increased with the polarity of the solvent, and only compounds capable of forming intramolecular hydrogen bonds exhibited this effect. The retention time was not affected. Using normal-phase silica columns with steroids and essentially non-aqueous mobile phase, an analogous dependence of response on solvent was also found.

INTRODUCTION

This paper reports details of a phenomenon involving changes in peak appearance due to the solvent. Most discussions of peak anomalies focus on the types caused by voids in the column packing[1], by column overload associated with a overly concentrated sample or a too large injection volume[2] and by a faulty sampling device[3]. Reports of peak broadening associated with sample–solvent interactions can also be found[4–6]. We have found that a diversity of compounds, using an assortment of reversed-phase columns with both buffered and unbuffered mobile phases, and UV or refractive index (RI) detectors yield peaks whose size depends on not only the molar absorptivity but also the injection solvent. The common denominator of all substances that we have found to exhibit this effect is the ability to form intramolecular hydrogen bonds, as described in this paper. In addition, we correctly predicted an analogous effect using normal-phase silica columns with non-aqueous injection solvents for steroids.

* Presented, in part, at the *American Chemical Society National Conference, Washington, DC, U.S.A., September, 1983* and at the *9th International Symposium on Column Liquid Chromatography, Edinburgh, July 1–5, 1985*. The majority of papers presented at this symposium has been published in *Journal of Chromatography*, Volumes 352 and 353.

TABLE I

CHROMATOGRAPHIC PARAMETERS OF THE COMPOUNDS INVESTIGATED

Compound	*Column*	*Mobile phase*	*Flow-rate (ml/min)*	*Detection*	*Exhibits peak enhancement effects (yes or no)*	*Capacity factor (k')*
Captopril	ODS*/PRP-1**/phenyl	Methanol–water-phosphoric acid 50:50:0.05	1.0	214 nm; RI	Y	2.0
Disulfide of captopril	ODS	Methanol–water–phosphoric acid 50:50:0.05	1.0	214 nm	N	*ca.* 4.0
S-Acetyl captopril	ODS	Methanol–water–phosphoric acid 50:50:0.5	1.0	214 nm	N	2.1
S-Benzoyl captopril	ODS	Methanol–0.3 *M* ammonium phosphate 60:40 (pH 3.0)	1.5	214 or 234 nm	N	4.8
Nadolol	ODS/phenyl	Methanol–acetate buffer 40:60	2.0	220 or 270 nm	Y	1.8
Nadolol acetonide	ODS	Methanol–ammonium phosphate 0.1 *M* 50:50	1.0	270 nm	N	1.7
Nadolol derivative I	ODS	Methanol–ammonium phosphate 0.1 *M* 72:28	1.0	215 nm	N	2.1
Hydrochlorothiazide	Phenyl	Methanol–water–phosphoric acid 20:80:0.1	1.0	270 nm	Y	3.4
Bendroflumethiazide	ODS/phenyl	Methanol–acetate buffer, pH 5 40:60	2.0	270 nm	Y	*ca.* 6.0
Disulfonamide of bendro.	Phenyl	Methanol–acetate buffer, pH 5 25:75	1.0	270 nm	Y	2.0
Halcinonide	ODS	Acetonitrile–water 70:30	1.0	238 nm	N	*ca.* 1.3
Triamcinolone acetonide	ODS	70:30	1.0	238 nm	N	1.5
Triamcinolone	ODS	50:50	1.0	238 nm	N	2.0
o-Nitroaniline	ODS	Methanol–water–phosphoric acid 60:40:0.1	1.0	215 nm	Y	2.2
p-Nitroaniline	ODS	60:40:0.1	1.0	215 nm	N	1.6
o-Phenoxybenzoic acid	ODS	75:25:0.1	1.0	215 nm	Y	1.6
p-Aminobenzoic acid	ODS	80:20:0.1	1.0	215 nm	N	2.3
Methylparaben	ODS	60:40:0.1	1.0	215 nm	N	2.2

* ODS is octadecylsilane

** PRP-1 is poly(styrene-divinylbenzene).

EXPERIMENTAL

Apparatus

A modular HPLC system consisting of an Altex 110A or Perkin-Elmer Series 4 pump, a Rheodyne 70-10 precision loop-injector containing a 20-μl loop, or a Perkin-Elmer ISS-100 autosampler with either a Perkin-Elmer LC85 or Schoeffel 770 or Kratos 783 variable-wavelength detector or an Erma refractive index detector was used. Data reduction was performed by a Hewlett-Packard 3357 Laboratory Automation System, as a Kipp and Zonen recorder monitored the chromatography. Spectra were obtained using a Hewlett-Packard Model 1040A diode array detector.

Reagents

Water was double-distilled and stored in glass. Methanol and ethanol were HPLC grade (Baker). Sodium acetate, glacial acetic acid, sodium chloride, sodium phosphate and 85% phosphoric acid were all commercially obtained (Fisher or Mallinckrodt). Captopril, nadolol, bendroflumethiazide, hydrochlorothiazide, the disulfonamides of bendroflumethiazide and hydrochlorothiazide, halcinonide, triamcinolone acetonide and triamcinolone were Squibb reference standards. *Ortho-* and *para*-nitroaniline, *o*-phenoxybenzoic acid, *p*-aminobenzoic acid and methylparaben were obtained commercially (Aldrich or Eastman).

Procedures

Table I lists the chromatographic parameters for the compounds tested. All substances, unless stated otherwise, were dissolved in water, methanol and ethanol and injected in duplicate into the equilibrated system.

RESULTS AND DISCUSSION

Typical peak response effects are shown in Fig. 1 for captopril using UV detection at 214 nm and an RI detector. The mobile phase, column and other chromatographic parameters remained unchanged. The peak heights and areas, using both detectors, increased with increasing polarity of the solvent from water to methanol and ethanol. The retention time, however, remained unchanged. That this is not an isolated phenomenon is demonstrated by similar peak response effects being exhibited by hydrochlorothiazide (Fig. 2) and 2,4-disulfamyl-5-trifluoromethylaniline (the disulfonamide of bendroflumethiazide, Fig. 3).

All compounds shown in Table I exhibiting the effect had changes in the peak responses and shapes when dissolved in the different solvents. Table II lists quantitative data for some of the compounds depicted in the figures. That the effect is due to intramolecular hydrogen bonding is apparent since both nadolol derivatives (Fig. 4) and compounds related to captopril with blocked mercapto groups (like S-benzoyl and S-acetyl captopril, Table I), in which such bonding cannot occur, do not exhibit this effect. (Note that the "internally-hydrogen bonded" structure shown in Fig. 5 for nadolol and derivatives does not exist because of steric hindrance demonstrated using molecular modeling.) The presumed configuration of nadolol is shown in Fig. 4. The connection between internal hydrogen bonding and this effect is further confirmed by the traditional hydrogen bond model compounds *o*-nitroaniline and its

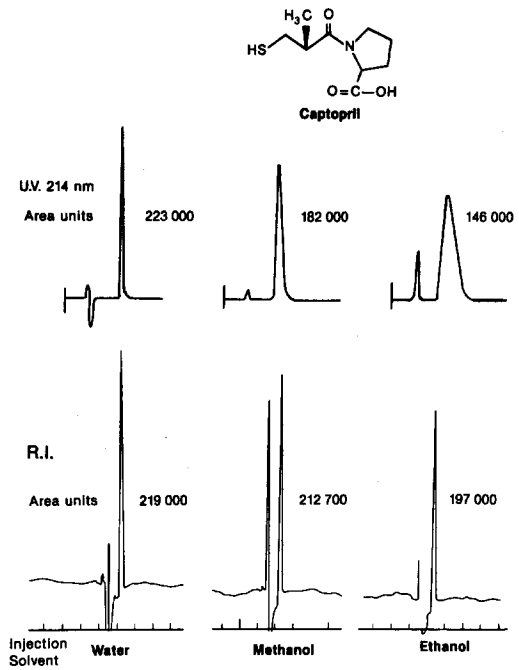


Fig. 1. Peak enhancement effect for captopril dissolved in various solvents, injected into the identical mobile phase and detected using UV and RI detectors. An octadecysilane column was used with a mobile phase of methanol–water–phosphoric acid (50:50:0.05). Detection was at 214 nm.

para isomer. Only the ortho compound with its internal hydrogen bond exhibits this effect, as do *o*-phenoxybenzoic acid and salicylic acid. Enhanced peak responses occur whether or not buffers are present in the mobile phase and occur on both sili-

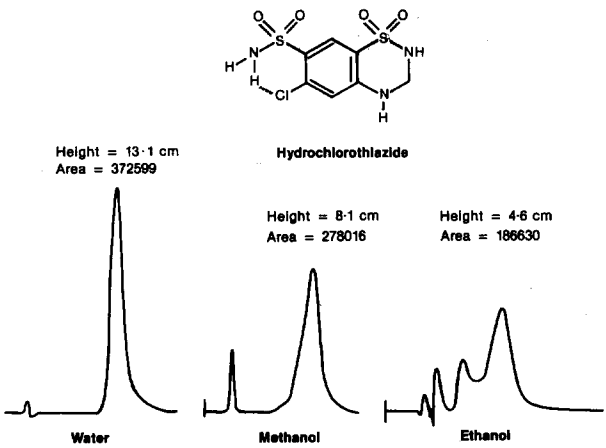


Fig. 2. Variation in peak heights and areas for hydrochlorothiazide dissolved in water, methanol and ethanol prior to injection. An octadecysilane column was used with a mobile phase of methanol–water–phosphoric acid (20:80:0.1). The peak prior to hydrochlorothiazide in ethanol appears to be ethanol. Detection was at 270 nm.

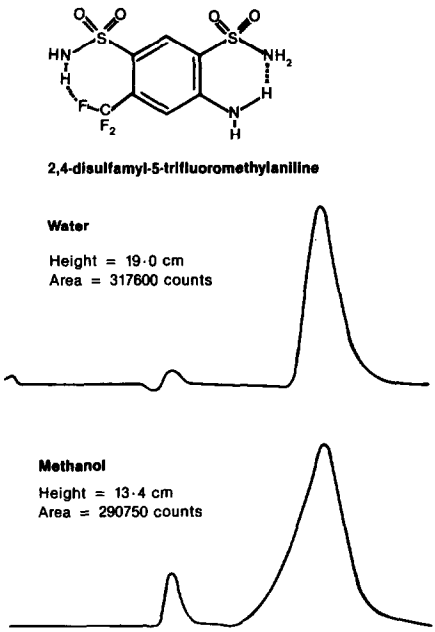


Fig. 3. Differences in peak areas and heights using water, and methanol as injection solvents, for the disulfonamide of bendroflumethiazide (2,4-disulfamyl-5-trifluoromethylaniline). Chromatograms were obtained with the aid of a laboratory computer system. A phenyl column was used with a mobile phase of methanol–acetate buffer, pH 5 (1:3). Detection was at 270 nm.

ca-based reversed-phase and poly(styrene–divinylbenzene) gels; *cf.* Table I. Use of injector loop sizes of 5, 10, 20, 50 and 100 μl gave responses for captopril in water, methanol and ethanol proportionally similar to the UV responses shown in Fig. 1. Fig. 6 demonstrates that the peak enhancement effect in various solvents is linear with concentration from 50 to 1000 μg using a 20-μl loop. The respective correlation coefficients were 0.99983 in water, 0.99993 in methanol and 0.99968 in ethanol. Aqueous buffers at pH 2, 3, 4, and 5 as solvents for captopril gave similar peak responses (within a 1.5% experimental error), showing that this effect is independent of hydrogen ion concentrations over four orders of magnitude for an acidic compound.

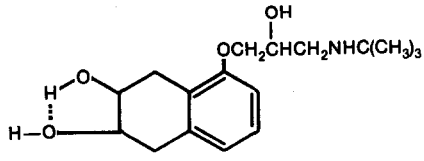
The behaviour of captopril was also investigated in mixtures of methanol and water and ethanol and water (Fig. 7). Peak responses in various solvents at different temperatures has been described briefly elsewhere by us[6]. Altogether, the data also support the hypothesis of intramolecular hydrogen bonding since peak responses were always greater in methanol–water mixtures than in the corresponding ethanol–water mixture, and the peak responses increased with increasing temperature.

The explanation of this peak enhancement phenomenon cannot be something trivial such as variation in solvent viscosities (because the retention times often are identical in the various injection solvents), non-equilibration of the column with the solvent (because we can cycle back and forth between the injection solvents), column

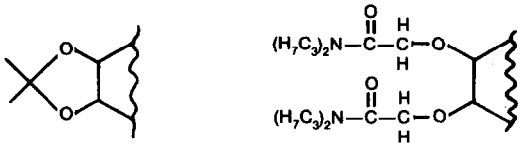
TABLE II

QUANTITATIVE DATA

Compound	*Solvent*	*Height (cm)*	
		UV	*RI*
Captopril	Water	22.3	22.0
	Methanol	18.2	21.3
	Ethanol	14.6	19.7
Hydrochlorothiazide	Water	12.7	
	Methanol	8.0	
	Acetonitrile	8.3	
	Ethanol	5.8	
Bendroflumethiazide	Methanol	12.0	
	Acetonitrile	9.75	
	Ethanol	8.0	
2,4-Disulfonamide-5-trifluoromethylaniline (disulfonamide of bendroflumethiazide)	Water	19.0	
	Dimethyl sulphoxide	17.4	
	Acetonitrile	12.4	
	Methanol	13.4	
	Ethanol	12.5	
o-Nitroaniline	Water	23.0	
	Dimethyl sulphoxide	20.5	
	Acetonitrile	21.0	
	Methanol	13.4	
	Ethanol	6.7	



NADOLOL



Nadolol acetonide

Fig. 4. Structure of nadolol, which shows peak enhancement effect of larger peak height and area in some injection solvents, and two derivatives of the *cis*-hydroxyl groups which lack the effect, *i.e.* gave similar area and height responses under the identical conditions of solvent dissolution and HPLC.

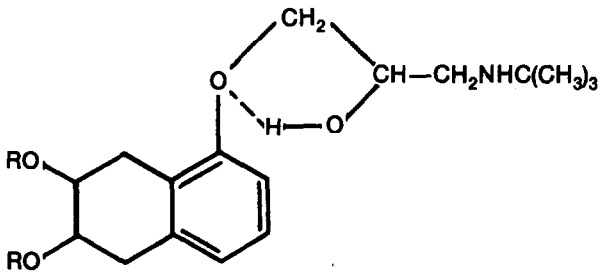


Fig. 5. Sterically forbidden, internal hydrogen-bonded form of nadolol.

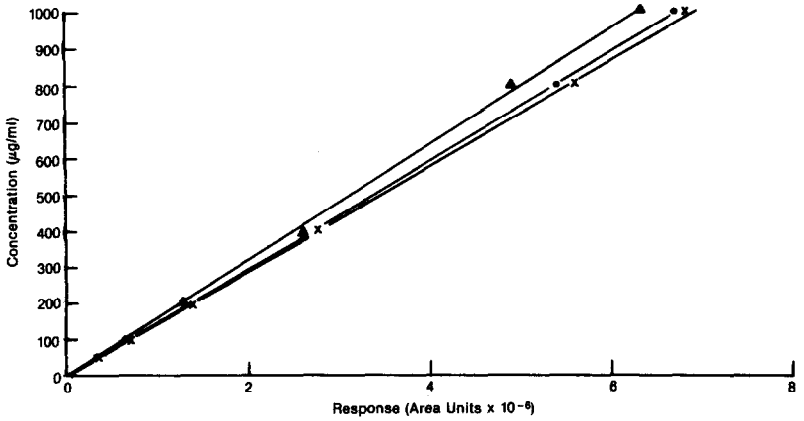


Fig. 6. Linearity of responses of various concentrations of captopril dissolved in water (×), methanol (●), and ethanol (▲).

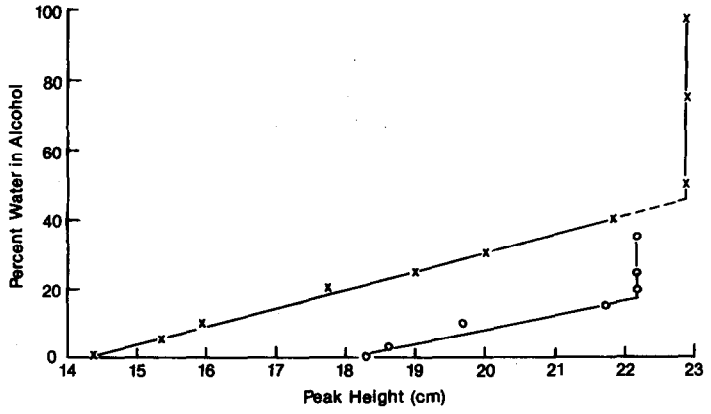


Fig. 7. Peak heights of captopril in various concentrations of methanol–water (○) and ethanol–water (×) mixtures.

overloading (because of the wide linear range) or a HPLC version of the GC "solvent trapping effect" (ref. 8; not likely due to generally similar retention times). Different UV responses of the same compound in different injection solvents was eliminated as an explanation since both captopril and nadolol in water, methanol or ethanol gave identical normalized spectra using a scanning diode-array detector. In addition, captopril showed this peak enhancement effect using an RI detector without exhibiting peak splitting or retention time changes.

While the theoretical argument of Ng and Ng[6] and their experimental results also exhibit peak sharpening corresponding to increasing solvent polarity, no mention is made, and there is nothing in their argument to exclude compounds that do not form internal hydrogen bonds. Since we have experimentally shown this to be the case, the explanation for the phenomenon we observe must be connected with hydrogen bonding.

Any working hypothesis to explain this phenomenon must be constrained by the observations that: (1) many compounds exhibit this effect; (2) peak areas and heights increase in the order ethanol < methanol < water (the peak splitting occasionally seen with ethanol as injection solvent may be related to solubility since retention times differ from the new smaller peak); (3) the retention times are unchanged in water and methanol injection solvents and frequently unaffected by ethanol; (4) only compounds which can form intramolecular hydrogen bonds show this effect; (5) reversed-phase phenylsilane- and octadecylsilane-coated silica and non-silica poly(styrene–divinylbenzene) columns yield differing peak responses that are dependent on injection solvents; (6) the phenomenon is independent of pH in the range 2–5 for at least one compound; (7) diode array scans of the peak for captopril show enhancement of the response without peak shifts (*i.e.* the spectra may be superimposed); and (8) both UV and RI detection reveal this effect.

Because analogues which cannot form hydrogen bonds intramolecularly fail to exhibit the peak enhancement effect, non-covalent hydrogen bonds are considered the most likely cause of the peak enhancement effect.

A prediction of this non-covalent, hydrogen bond hypothesis led us to search for the analogous hydrophobic bonding peak response phenomenon using a silica column, mostly non-aqueous mobile phases and hydrophobic steroids. Such adsorption (normal phase) chromatography of steroids on silica with a mobile phase of methylene chloride–methanol–water (960:38.8:1.2) produced not only the hoped for differences in peak responses but even more surprising results. In some cases, the expected changes of the peak heights and areas occurred, but also extra peaks appeared and the retention times changed when the steroid was dissolved in different solvents (Fig. 8). For example, the behavior of dihydrotriamcinolone acetonide is summarized in Table III. These retention times differences were accentuated with increased temperature, as shown in Table III for triamcinolone. The explanation for the differences in peak area and height may be hydrophobic bonding, analogous to the reversed-phase,hydrogen-bonding situation discussed previously.

We suspect that an internally hydrogen-bonded solute dissolved in a strong (organic) solvent in a reversed-phase system will change its conformation as the surrounding environment changes from solvent to aqueous mobile phase and internal hydrogen bonds are broken. This could well lead to peak splitting or broadening. The extent of the broadening or splitting would depend on the strength of the internal

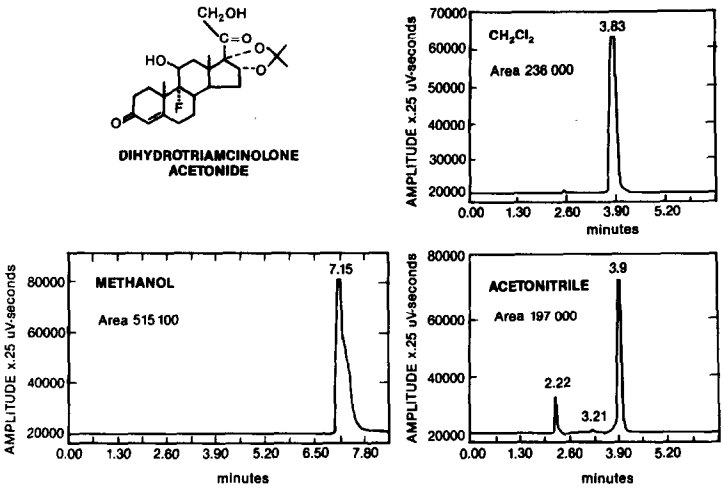


Fig. 8. Normal-phase LC counterpart to reversed-phase HPLC. The steroid dihydrotriamcinolone acetonide dissolved in different solvents shows the peak enhancement effect as well as shifts in retention time using a non-polar mobile phase with a silica column and a mobile phase of methylene chloride–methanol–water (960:38.8:1.2).

hydrogen bond, the flow-rate, the column length and the composition (aqueous content) of the mobile phase.

Presently, we are probing the role of mobile phase ionic strength, and a wider range of pH, and different hydrocarbon-coated silica columns to test for the possibility of selective interactions of the solute with the liquid layer hypothesized[9] to surround the stationary phase. We are also investigating the occasional peak splitting noted with several compounds in some solvents. This should enable us to refine our hypothesis of non-covalent bonding as the cause of different peak responses for solute

TABLE III

DIFFERING PEAK RESPONSES OF STEROIDS IN VARIOUS INJECTION SOLVENTS USING NORMAL-PHASE HPLC

Steroid	*Solvent*	*T (°C)*	*Area (area counts)*	*Retention time (min)*
Dihydrotriamcinolone acetonide	Methanol	Ambient	515 100	7.15
	Acetonitrile		197 000	3.92
	Methylene chloride		236 000	3.83
Triamcinolone	Methanol	Ambient	69 200	9.21
	Acetonitrile		141 300	8.78
	Methylene chloride		56 200	9.78
	Methanol	35	–	7.78
	Acetonitrile		–	7.48
	Methylene chloride		–	9.02

injected in various solvents, and eventually, explain and experimentally prove the cause of this phenomenon.

In conclusion, a new phenomenon in HPLC involving different peak responses due to the injection solvent has been discussed. To date, either hydrogen-bonding-forming compounds in aqueous mobile phases on non-polar columns or hydrophobic-bond-forming compounds in organic solvents on polar columns show this peak enhancement effect. This phenomenon is of theoretical importance since it can provide testable hypotheses regarding the nature of the interactions between solute, solvent and stationary phase. The practical consequences are firstly, that sharper peaks, *i.e.* better resolution, can be induced by merely changing the injection solvent, and, secondly, the necessity to match sample and standard solvents when performing quantitative analyses, was demonstrated.

ACKNOWLEDGEMENTS

The authors gratefully thank the following: Ms. Diana Steele of Colgate University and Ms's. Judith Waidlich, Ulla Schlate, Janine Laurencot, Vanita Kamath, Sharon Gandhi Singh and Roxanne Downing who, as temporary externs from Douglass College, Rutgers University, performed some of the experimental work, Drs. Berry Kline, John Adamovics and Glenn Brewer for constructive suggestions and comments, Mr. Jose Alcantara for illustrating, and Ms. Dawn Antonides for typing this manuscript.

REFERENCES

1 G. Guiochon and H. Colin, in P. Kucera (Editor), *Microcolumn High-Performance Liquid Chromatography*, Elsevier, Amsterdam, 1984, p. 35.
2 P. A. Bristow, *Liquid Chromatography in Practice*, Laboratory Data Control, Riviera Beach, FL, 1976, p. 170.
3 L. F. Simpson, *Proc. Anal. Chem. Soc.*, 16 (1979) 222.
4 P. K. Tseng and L. B. Rogers, *J. Chromatogr. Sci.*, 16 (1978) 436.
5 K. J. Williams, A. Li Wan Po and W. J. Irwin, *J. Chromatogr.*, 194 (1980) 217.
6 T.-L. Ng and S. Ng, *J. Chromatogr.*, 329 (1985) 13.
7 J. Kirschbaum, S. Perlman and R. B. Poet, *J. Chromatogr. Sci.*, 20 (1982) 336.
8 K. Grob, Jr., *J. Chromatogr.*, 253 (1982) 17.
9 P. E. Antle, A. P. Goldberg and L. R. Snyder, *J. Chromatogr.*, 321 (1985) 1.