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Note

Reversed-phase high-performance liquid chromatography of insulin

Resolution and recovery in relation to column geometry and buffer components*

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A number of reports describing reversed-phase separations of insulin, insulin-related and non-insulin-related substances have recently been published[1–74].

Of the 74 references listed in Table I, all except refs. 7, 8, 9, 41, 46, 47, 49, 58, 59 and 60 used acetonitrile as organic modifier (alone or as principal constituent). The stationary phase used was C_{18} in the majority of reports (all except refs. 6, 7, 10, 16, 18, 19, 20, 55, 57, 63, 64, 70 and 73). pH in the mobile phase was adjusted to 2–5 except in refs. 12, 22, 25, 30, 53, 58, 59, 60 and 69. Only in one of the 74 references was the recovery of insulin peptide measured.

Since recovery and resolution are critical parameters when estimating the content of impurities in insulin preparations, we have investigated the influence of column length and inner diameter upon these parameters by using a single stationary phase eluted with triethylammonium phosphate (TEAP), ammonium sulphate or trifluoroacetic acid (TFA) in acetonitrile.

Column efficiency has been determined for insulin peptide and monodesamidoinsulin (mol.wt. 5700), whereas recovery has been estimated for insulin peptide as well as proinsulin–insulin dimers (mol.wt. 9000–12 000).

Furthermore, column-to-column and batch-to-batch variations for the actual stationary phase have been investigated.

MATERIALS AND METHODS

High-performance liquid chromatographic (HPLC) equipment: Waters M6000

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TABLE I

COMPOSITION OF MOBILE PHASES USED FOR REVERSED-PHASE HPLC OF INSULIN (INSULIN AS ONE AMONG SEVERAL POLYPEPTIDES OR SEPARATION OF INSULIN FROM DIFFERENT SPECIES) AND INSULIN-RELATED SUBSTANCES (DESAMIDOINSULIN, ARGININE INSULIN, ETHYL ESTER INSULIN, PROINSULIN, INSULIN DIMERS, A- AND B-CHAINS AND IODINATED INSULIN DERIVATIVES)

	Reference number	
	Insulin	*Insulin-related substances*
Phosphoric acid	7, 39, 41	23, 50, 52, 53
Acid K/Na phosphates	19, 46, 64	9, 17, 18, 20, 27, 36, 45, 51
Phosphates + additives*	8, 40, 48, 67	3, 5, 6, 15, 27, 31, 43, 54, 57
Other salts**	29, 38	4, 11, 14, 22, 25, 34, 44, 50, 51, 55, 56, 65, 69, 70, 72, 74
Alkylammonium salts	26, 32, 33, 38, 42, 62	1, 12, 24, 53, 56, 59, 60, 65, 69, 74
Alkylammonium salts + additives***	21, 28, 37	2, 10, 13
Trifluoroacetic acid	5, 16, 32, 35, 61, 66, 73	68, 69, 71
Others§	47, 63	30, 49, 58, 59, 60, 71, 72

* Perchlorate or neutral salts.
** Ammonium sulphate, sodium sulphate, ammonium acetate or ammoniumphosphate.
*** Perchlorate.
§ Hexafluoroacetone[30], acetic acid[47], Tris–phosphoric acid[49], Tris[58], perchloric acid[59,60], acetic acid–octanesulphonate[63], formic acid[71], TFA–octanesulphonate[72].

pumps; WISP 710 B, 660 solvent programmer, 720 system controller, 730 data module and 840 chromatography control station; Pye Unicam or Waters λ_{max} 480 UV detectors.

Columns: 50 × 4.0 mm I.D. (LiChrocart), 125 × 4.0 mm I.D. (LiChrocart), 250 × 4.0 mm I.D. (LiChrocart), 250 × 7.0 mm I.D. (HiBar steel column), 250 × 2.0 mm I.D. (steel column). All columns were packed with LiChrosorb RP-18 (7 μm). The 250 × 2.0 mm I.D. column was packed by Mikrolaboratoriet (Aarhus, Denmark). All other columns were supplied by Merck.

Mobile phases: three systems were used.

A	*B*
(1) 0.125 *M* ammonium sulphate (pH 4.0)–22.5% acetonitrile	0.125 *M* ammonium sulphate (pH 4.0)–45% acetonitrile
(2) 0.25 *M* TEAP (pH 3.0)	50% A–50% acetonitrile
(3) 0.05% trifluoroacetic acid	50% A–50% acetonitrile

Gradients were prepared by mixing buffers A and B.

Flow-rates: 1.0 ml/min (4.0 mm I.D.), 0.25 ml/min (2.0 mm I.D.), 3.1 ml/min (7.0 mm I.D.).

Temperature: 45°C for system 1, ambient for systems 2 and 3.

Samples: (1) crystalline porcine insulin (Nordisk Gentofte A/S Batch G-63) containing *ca.* 75% insulin peptide; (2) highly purified porcine insulin (Nordisk Gen-

tofte A/S batch 297) containing >99% insulin peptide; (3) b-component isolated from sample 1 (Sephadex G-50SF in 3 *M* acetic acid) containing primarily proinsulin and insulin dimers.

Elution schemes:

(1) Isocratic elution: $k' \approx$ 10–15 for insulin peptide. Number of theoretical plates (*N*) was calculated for insulin peptide. Resolution was estimated for insulin peptide and monodesamidoinsulin. Sample: 1.

(2) Gradient elution: $k' \approx$ 10–15 for insulin peptide. System 1: isocratic until elution of insulin peptide followed by a linear acetonitrile gradient (4%) during approximately ten times the mobile phase hold-up time. Systems 2 and 3: the acetonitrile concentration was increased linearly or slightly concave (3–5%) during approximately 25 times the mobile phase hold-up time. Samples: 1, 2 and 3.

(3) Recovery analyses: the area under the UV curve obtained after gradient elution relative to that obtained after injecting the same sample directly into the UV photometer. Samples: 2 and 3.

N was calculated according to the formula $N = 25(t_R/W_{4.4})^2$, t_R being the retention time for insulin peptide and $W_{4.4}$ the peak width measured at 4.4% peak height. Resolution $= 2.5(t''_R - t'_R)/(W''_{4.4} + W'_{4.4})$. Symbols marked ″ indicate monodesamidoinsulin, ′ indicates insulin peptide.

UV detection was performed at 215 nm, except for recovery analyses (280 nm). Estimations of resolution, recovery and plate number were performed in triplicate or more.

RESULTS

Gradient elution of 100 μg of crystalline porcine insulin and 100 μg of b-component in relation to various column lengths and inner diameters are shown in Fig. 1 (system 1) and Fig. 2 (system 2), respectively. The elution positions for the principal components (insulin peptide, monodesamidoinsulin, proinsulin and insulin dimer) are marked on the figures.

Use of shallow acetonitrile gradients in system 3 did not result in any acceptable resolution of insulin peptide and insulin-like components, due to poor peak shape. Acceptable peak shape could only be obtained using steep acetonitrile gradients (0–50% acetonitrile during 60 min), but, under these conditions, selectivity (*i.e.* the ability to separate the numerous, closely related insulin-like components in crystalline insulin) was seriously diminished.

Isocratic elution of 100 μg of crystalline porcine insulin using systems 1, 2 and 3 are shown in Fig. 3. Under these conditions, the use of TFA–acetonitrile resulted in poor peak shape, whereas almost symmetrical peaks were obtained using TEAP and ammonium sulphate in acetonitrile.

Resolution, plate number and recovery for LiChrosorb RP-18 columns in relation to varying column length and inner diameter for systems 1 and 2 are given in Table II. Due to the above-mentioned non-ideal peak shape obtained using system 3, correct estimations of recovery and resolution could not be performed. In addition, the same parameters have been calculated for six individual 250 × 4.0 mm I.D. columns, representing two different batches (Table III).

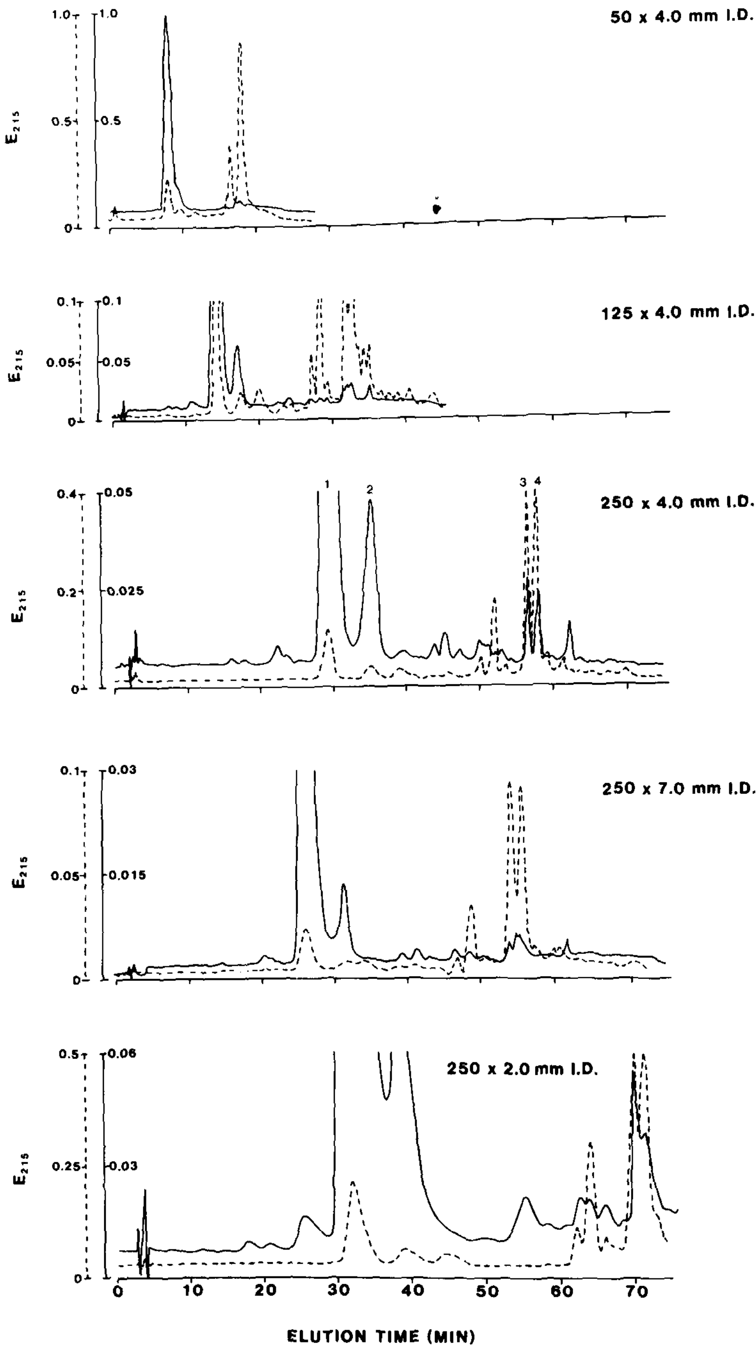

Fig. 1. Gradient elution of 100 μg of crystalline porcine insulin (——) or 100 μg of b-component (---) using LiChrosorb RP-18 columns with varying length and I.D. Mobile phase: system 1. The peaks marked with numbers correspond to insulin peptide (1), monodesamidoinsulin (2), proinsulin (3) and insulin dimer (4).

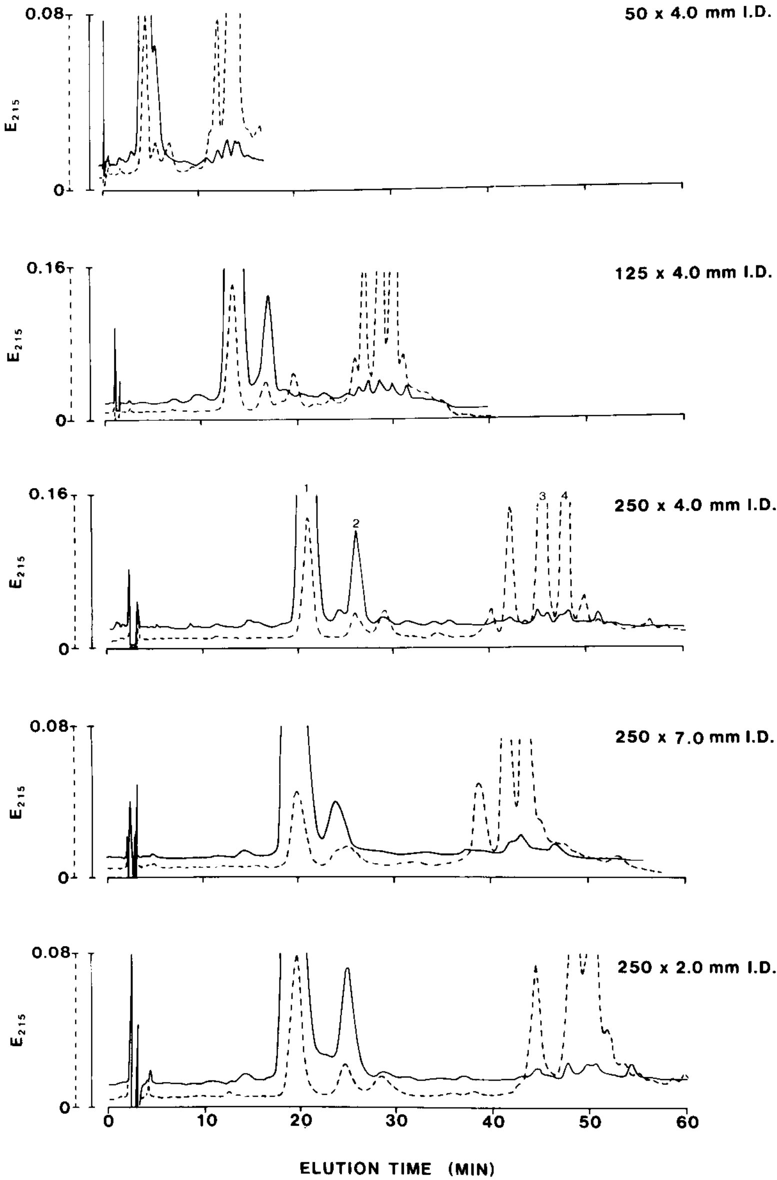

Fig. 2. Gradient elution of 100 μg of crystalline porcine insulin (——) or 100 μg of b-component (---) using LiChrosorb RP-18 columns with varying length and I.D. Mobile phase: system 2. The peaks marked with numbers correspond to insulin peptide (1), monodesamidoinsulin (2), proinsulin (3) and insulin dimer (4).

DISCUSSION

Reversed-phase HPLC analyses of crystalline insulin using LiChrosorb RP-18 (7 μm) as stationary phase and TEAP or ammonium sulphate in acetonitrile as mobile

TABLE II

RESOLUTION, PLATE NUMBER AND RECOVERY FOR LICHROSORB RP-18 COLUMNS WITH VARYING LENGTH AND I.D., ELUTED WITH BUFFER SYSTEM 1, 2 OR 3

The 250 × 4.0 mm I.D. columns used were batch No. 509083 (system 1) and 509266 (systems 2 and 3); n.d. = not determined.

	l (*mm*)	*I.D.* (*mm*)	% *Acetonitrile*	*Resolution*	*Theoretical plates/m*		*l* (*mm*)	*I.D.* (*mm*)	% *Acetonitrile*	*Recovery (%)*	
										Insulin peptide	*b-Component*
TEAP	50	4.0	27	0.85	4800	TEAP	50	4.0	27–30	98.8	52.3
$(NH_4)_2SO_4$	50	4.0	28	0.84	6400	$(NH_4)_2SO_4$	50	4.0	28–32	100.2	59.2
TFA	50	4.0	31	n.d.	200	TFA	50	4.0	31–36	n.d.	n.d.
TEAP	125	4.0	26	1.97	6800	TEAP	125	4.0	26–29	93.5	80.4
$(NH_4)_2SO_4$	125	4.0	28	1.46	9600	$(NH_4)_2SO_4$	125	4.0	28–32	100.8	79.1
TFA	125	4.0	31	n.d.	200	TFA	125	4.0	31–34	n.d.	n.d.
TEAP	250	4.0	26	2.75	6800	TEAP	250	4.0	26–30	100.7	68.6
$(NH_4)_2SO_4$	250	4.0	28	2.03	10000	$(NH_4)_2SO_4$	250	4.0	28–32	100.8	69.4
TFA	250	4.0	33	n.d.	250	TFA	250	4.0	33–38	n.d.	n.d.
TEAP	250	7.0	27	1.70	4500	TEAP	250	7.0	27–30	93.8	66.0
$(NH_4)_2SO_4$	250	7.0	28	1.58	4600	$(NH_4)_2SO_4$	250	7.0	28–32	99.6	65.5
TFA	250	7.0	36	n.d.	280	TFA	250	7.0	35–40	n.d.	n.d.
TEAP	250	2.0	26	2.50	5200	TEAP	250	2.0	27–30	80.7	51.4
$(NH_4)_2SO_4$	250	2.0	28	1.46	3000	$(NH_4)_2SO_4$	250	2.0	28–32	n.d.	n.d.
TFA	250	2.0	36	n.d.	n.d.	TFA	250	2.0	38–43	n.d.	n.d.

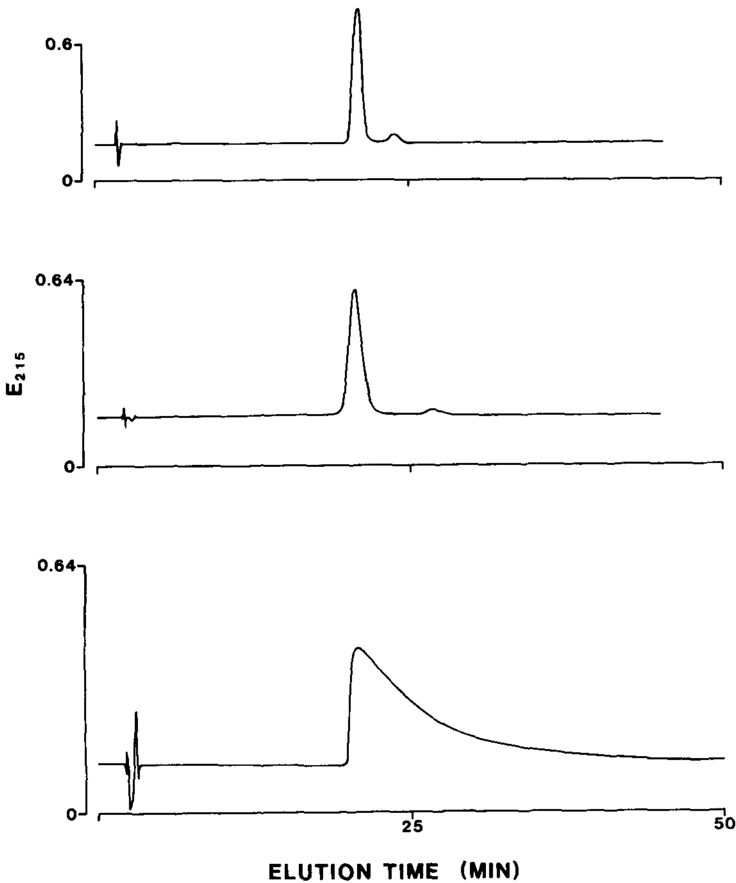

Fig. 3. Isocratic elution of 100 μg of crystalline porcine insulin using a 250 × 4.0 mm I.D. LiChrosorb RP-18 column eluted with buffer system 1 (upper panel), system 2 (middle panel) or system 3 (lower panel).

phase, are comparable in the main: all columns tested separated crystalline insulin into two fractions, one being insulin peptide and insulin-like compounds (mol.wt. 5700), the other proinsulin–insulin dimers (mol.wt. 9000–12 000). However, minor differences between the two buffer systems can be observed: the separation in the proinsulin–insulin dimer region was better using buffer system 1, whereas insulin peptide–monodesamidoinsulin was better resolved in system 2 (see Figs. 1 and 2).

It is common for both systems that satisfactory separation between insulin peptide and monodesamidoinsulin requires a 125-mm column length, and optimal separation of all components present in crystalline insulin (including proinsulin and insulin dimers) requires a 250-mm column length. This emphasizes the need for maximum separation capacity when closely related polypeptides are analysed (long columns, shallow gradients).

From the figures, it can further be concluded that a 4.0-mm inner diameter is superior to 2.0-mm and 7.0-mm inner diameters, indicating potential problems in the packing procedure for these columns. In the case of the 2.0 mm I.D. column, wall

TABLE III

RESOLUTION PLATE NUMBER AND RECOVERY FOR SIX INDIVIDUAL 250 × 4.0 mm I.D. LICHROSORB RP-18 COLUMNS ELUTED WITH BUFFER SYSTEMS 1, 2 OR 3

Column No.		*l (mm)*	*I.D. (mm)*	*% Acetonitrile*	*Resolution*	*Theoretical plates/m*	V_{Ins} *(min)*
509083	TEAP	250	4.0	26	2.56	6200	18.7
	$(NH_4)_2SO_4$	250	4.0	28	2.03	10000	30.6
	TFA	250	4.0	33	n.d.	350	22.3
509222	TEAP	250	4.0	26	2.38	4800	18.1
	$(NH_4)_2SO_4$	250	4.0	28	2.03	10000	29.2
	TFA	250	4.0	33	n.d.	330	20.2
509266	TEAP	250	4.0	26	2.51	6300	20.1
	$(NH_4)_2SO_4$	250	4.0	28	2.36	7800	30.0
	TFA	250	4.0	33	n.d.	310	19.5
508242	TEAP	250	4.0	26	2.50	5900	19.1
	$(NH_4)_2SO_4$	250	4.0	28	2.36	10700	30.4
	TFA	250	4.0	33	n.d.	350	18.3
509226	TEAP	250	4.0	26	2.51	4500	17.5
	$(NH_4)_2SO_4$	250	4.0	28	2.20	9900	30.4
	TFA	250	4.0	33	n.d.	350	21.1
419289	TEAP	250	4.0	26	2.30	6100	22.9
	$(NH_4)_2SO_4$	250	4.0	28	2.25	8500	39.0
	TFA	250	4.0	33	n.d.	610	13.1

effects may explain some of the reduced separation effectivity. Contradictory to these separations, TFA was found inapplicable for this type of analysis. This probably reflects that the LiChrosorb stationary phase is a non-end-capped material which needs shielding buffer additives in order to minimize non-specific adsorbtion. This is fulfilled by TEAP and ammonium sulphate, whereas the diluted TFA is highly ineffective as cover for free silanol groups.

Except for the 2.0 mm I.D. column, recovery for insulin peptide is close to 100%, independent of column geometry and buffer system. Recovery of b-component was found to be substantially lower (60–80%), see Table II. Since the recovery of insulin peptide and b-component were remarkably similar for the two buffer systems, it may be concluded that the reduced recovery figures found for the 2.0 mm I.D. column can be referred to the deviating packing procedure. Recovery figures for six individual 250 × 4.0 mm I.D. columns were identical. No column-to-column variations (identical batch no.) were found with respect to plate number and hydrophobicity (t_R for insulin peptide under identical acetonitrile concentration), whereas batch-to-batch variations in hydrophobicity were observed.

As can be seen in Table I, the majority of reversed-phase separations of insulin and insulin-related compounds can be divided into two major groups, according to

Column No.		*l (mm)*	*I.D. (mm)*	*% Acetonitrile*	*Recovery (%)*	
					Insulin peptide	*b-Component*
509083	TEAP	250	4.0	26–30	92.2	n.d.
	$(NH_4)_2SO_4$	250	4.0	28–32	100.8	69.4
	TFA	250	4.0	32–37	n.d.	n.d.
509222	TEAP	250	4.0	26–30	91.8	n.d.
	$(NH_4)_2SO_4$	250	4.0	28–32	99.9	60.1
	TFA	250	4.0	32–37	n.d.	n.d.
509266	TEAP	250	4.0	26–30	97.0	n.d.
	$(NH_4)_2SO_4$	250	4.0	28–32	100.6	72.5
	TFA	250	4.0	32–37	n.d.	n.d.
508242	TEAP	250	4.0	26–30	90.7	n.d.
	$(NH_4)_2SO_4$	250	4.0	28–32	101.4	71.2
	TFA	250	4.0	32–37	n.d.	n.d.
509226	TEAP	250	4.0	26–30	93.9	n.d.
	$(NH_4)_2SO_4$	250	4.0	28–32	100.6	68.1
	TFA	250	4.0	32–37	n.d.	n.d.
419289	TEAP	250	4.0	26–30	92.4	n.d.
	$(NH_4)_2SO_4$	250	4.0	28–32	98.6	67.1
	TFA	250	4.0	32–37	n.d.	n.d.

the buffer substances used: one based upon acid or neutral salts and another employing ion-pairing substances (TEAP, TFA). The present work demonstrates that identical results (with respect to separation capacity and recovery) can be obtained when a single stationary phase is eluted with an ion-pairing buffer system or a salt buffer.

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