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SIMPLE, EFFICIENT TERNARY SOLVENT SYSTEM FOR THE SEPARATION OF LUTEINIZING HORMONE-RELEASING HORMONE AND ENKEPHALINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography based on hydrophobic interaction of amino acid side-chains with octyl-, octadecyl-, cyanopropyl-, or phenyl-silica-bonded stationary phases has become the method of choice for the purification and analysis of small- and medium-size peptides. In the ion-pair mode, the organic solvent modifiers commonly used are either acetonitrile or methanol in the presence of suitable counterions. Recently, instruments with ternary, and even quaternary solvent delivery systems have become available. Although these instruments are undoubtedly more powerful and versatile, they are also more complex to handle. Moreover, their high price is a deterrent for many laboratories. In this paper it will be demonstrated that when methanol and acetonitrile are used simultaneously as organic modifying agents, in many cases better separations can be obtained than when using a binary gradient with either acetonitrile or methanol alone. Briefly, gradient elution was used with methanol-water (25:75), containing linearly increasing amounts of acetonitrileewater (80:20) at constant 0.1% trifluoroacetic acid concentration. Five commercially available prepacked columns were compared, namely: Whatman Partisil ODS-3, Knauer LiChrosorb RP-8, Varian MicroPak MCH-5, Waters μBondapak C_{18}, and Vydac 218TP. As reference peptides were used Met-Enk, Leu-Enk, Leu-Enk-Arg-Lys, Leu-Enk-Arg-Arg, dynorphin$_{1-13}$, β-melanotropin, Lys-bradykinin, neurotensin, angiotensin, and luteinizing hormone-releasing hormone (LHRH). This simple, yet efficient gradient system was successfully applied to the separation and purification of *de novo* biosynthesized enkephalins and LHRH in trophoblastic shells of the human placenta.

INTRODUCTION

In recent years, the literature on the analysis of a variety of compounds by high-performance liquid chromatography (HPLC) has grown at an explosive rate[1,2]. For laboratories that specialize in the isolation and characterization of small amounts of biomolecules such as endogenous peptides, HPLC may in fact be the method of

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choice, combining high sensitivity and resolving power with near quantitative recovery of the desired biological material[3]. In reversed-phase HPLC of peptides, use is made of the inherent hydrophobic properties of amino acid side-chains such as Trp, Leu, Phe, Ala, Ile, Met and Pro in aqueous medium[4,5]. To minimize electrostatic interactions caused by the free amino and carboxyl groups, chromatography is carried out with strong acids as eluents at a pH around 2.1, well below the p*I* values of most peptides. At this low pH both groups are present in the fully protondted forms, i.e. as $-NH_3^+$ and COOH, respectively. The ammonium ions are neutralized by the presence of counteranions such as CF_3COO^- or $CF_3CF_2CF_2COO^-$ (refs. 6 and 7), whereas the hydrophobic stationary phase is of the silica-C_8, -C_{18}, or -phenyl type. When as a prerequisite the biological activity of the desired peptides also has to be preserved, the choice of the components of the mobile phase becomes limited. Thus, the use of non-volatile inorganic buffers, such as phosphates, is avoided. In reversed-phase HPLC, the most popular solvents used as organic modifiers are acetonitrile and methanol. Both are volatile and transparent in the 210-220 nm region, the preferred wavelengths for peptide absorption analysis.

Most studies reported so far use either methanol or acetonitrile separately as the organic modifier. In this paper, we report the successful resolution and purification of biologically active peptides by reversed-phase HPLC, based upon the simultaneous use of methanol and acetonitrile as a ternary solvent system in the form of a linear gradient driven by a single, microprocessor-controlled pump,

EXPERIMENTAL

Materials

Reference synthetic peptides were purchased from Peninsula Laboratories, (San Carlos, CA, U.S.A.) and United States Biochemical Corp. (Cleveland, OH, U.S.A.) HPLC-grade methanol and acetonitrile were from Fisher Scientific, HPLC-quality water was prepared in our laboratory by double distillation in an all-glass-and-Teflon apparatus with final removal of trace organic contaminants by gravity filtration through two Sep-Pak C_{18} cartridges (Waters Assoc.). Trifluoroacetic acid (TFA, Aldrich) was distilled before use. The prepacked HPLC columns were purchased directly from the manufacturers. L-[2,3,5,6-^{3}H]Tyrosine (77.6 μCi/mmol) was obtained from Amersham Corp. Fresh human placentae, obtained at birth, were from the University hospital.

Pulse-labeling *experiments*

The incorporation of tritiated tyrosine by human placental trophoblast preparations, and the extraction and purification of the resulting peptides were carried out as described earlier*.

High-performance liquid chromatography

The following system was used: a Varian Model 5020 chromatograph, a Valco 200-μl loop injector, a Hitachi-Altex variable-wavelength detector set at 215 nm connected in series to an Altex single-wavelength (280 nm) monitor, a Pharmacia FRAC-100 fraction collector, and a Kipp & Zonen double-channel potentiometric recorder. The analytical column was protected by a silica pre-column and a glass-

packed guard column. The solvents used were filtered, degassed, and their absorbance matched prior to solvent equilibration of the entire HPLC system. Both pre-column and analytical column were kept at 40°C by a microprocessor-controlled heating mantle. Prior to injection into the Valco sample valve, the peptide solutions were filtered through glass-fiber filters in a Swinney adapter. The actual chromatographic parameters are described in the legends and the text.

RESULTS AND DISCUSSION

A comparison of the physical properties of methanol and acetonitrile pertinent to this study is summarized in Table I. At first view, methanol seems to have more undesirable properties than acetonitrile. It is more volatile (therefore more difficult to keep at constant concentration in water), it is more viscous (leading to higher compressibility and column back pressures), and finally it has a higher UV wavelength, cut-off point. On the other hand, methanol is chemically inert, easier to remove from collected peptides, and cheaper. In short, it has advantages and disadvantages. In practice the methanol concentration is kept at a minimum by using it as solvent A.

That this simple gradient system proves to be at least as efficient as a gradient based on acetonitrile alone is shown in Fig. I, where a μBondapak C_{18} column is developed with a linear gradient of either methanol-water (25:75) containing 0.1% TFA (system I), or acetonitrilewater (10:90) containing 0.1% TFA (system II) as the weaker solvent A. In both instances solvent B is acetonitrile-water (80:20) containing 0.1% TFA. Whereas the first four peptides are well resolved with both gradient systems, peptide 7 was completely separated from peptide 8 in system I, but only partially so in system II. Therefore, in this experiment with a μBondapak C_{18} column, the methanol-acetonitrile-water solvent system clearly is better than the acctonitrile-water system. It should be noted that the elution order of the various peptides remains the same in both systems.

A different pattern emerged when the mixture of ten peptides was chromatographed on a Partisil ODS-3 column (Fig. 2). In both systems I and II, peptides 3 and 5, and 7 and 8, were not separated. Judging from the base width of peptide 10, this particular column shows a higher efficiency with the acetonitrileewater solvent

TABLE I

COMPARISON OF SOME PHYSICAL PROPERTIES OF METHANOL AND ACETONITRILE[9]

Property	*Methanol*	*Acetonitrile*
Molecular weight	*32*	41
Boiling point	64.7°C	81.6°C
Refractive index	*1.328 (20°C)*	1.344 (20°C)
Viscosity	0.55 cP (20°C)	0.38 cP (15°C)
Dielectric constant	32.7 (25°C)	37.5 (20°C)
Vapor pressure	125 Torr (25°C)	88.8 Torr (25°C)
Dipole moment	*2.87* D *(20°C)*	3.44 D (20°C)
Flash point	*12°C*	6°C
uv cut-off	*1* A at *205* nm	1A at 190nm

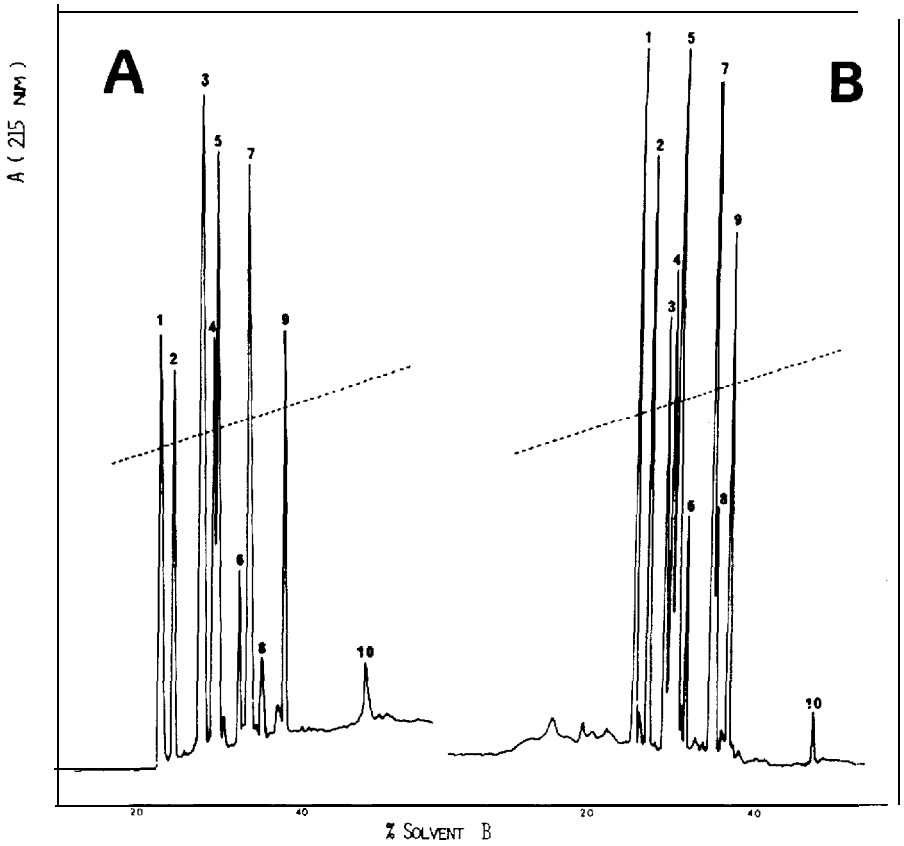


Fig. 1. Separation of a mixture of ten peptides on a μBondapak C_{18} column. Ten μl of a mixture of ten peptides at a concentration of 1 μg/μl in 0.1% TFA was injected and the column was eluted with a linear gradient from 0 to 60% solvent B for 60 min at a flow-rate of 0.8 ml/min. To identify the peaks, each peptide was analyzed separately under identical chromatographic conditions. (A) System I: solvent A, methanol-water (25:75) containing 0.1% TFA; solvent B, acetonitrile-water (80:20) containing 0.1% TFA. (B) System II: solvent A, acetonitrile-water (10:90) containing 0.1% TFA; solvent B, acetonitrile-water (80:20) containing 0.1% TFA. Peak numbers correspond to the peptide numbers in Table II.

system. Since the two columns used were packed with 10-μm silica particles, I next tried a shorter column, packed with 5-μm particles (MCH-5, Fig. 3). On this 15-cm long column, the two solvent systems eluted the peptides in a different order. With solvent system I, peptide 4 was well separated from peptide 8, but the latter was not separated from peptide 6. With system II, peptides 4 and 8 are not resolved, as is peptide 9. With both solvent systems, peptides 5 and 7 are not separated. Overall, system I performs slightly better.

A less hydrophobic, octyl-silica column of 7-μm average particle size was then studied (Fig. 4). In this case, with both solvent systems, peptides 3 and 8 were eluted together. Overall, we can state again that solvent system I achieves the better separations (*cf.* resolution of peptides 4 and 9). Finally, I tested a column packed with 10-μm silica particles containing much larger (300-Å) pores. The results are shown in Fig. 5. On this column, solvent systems I and II gave rise to a very different elution

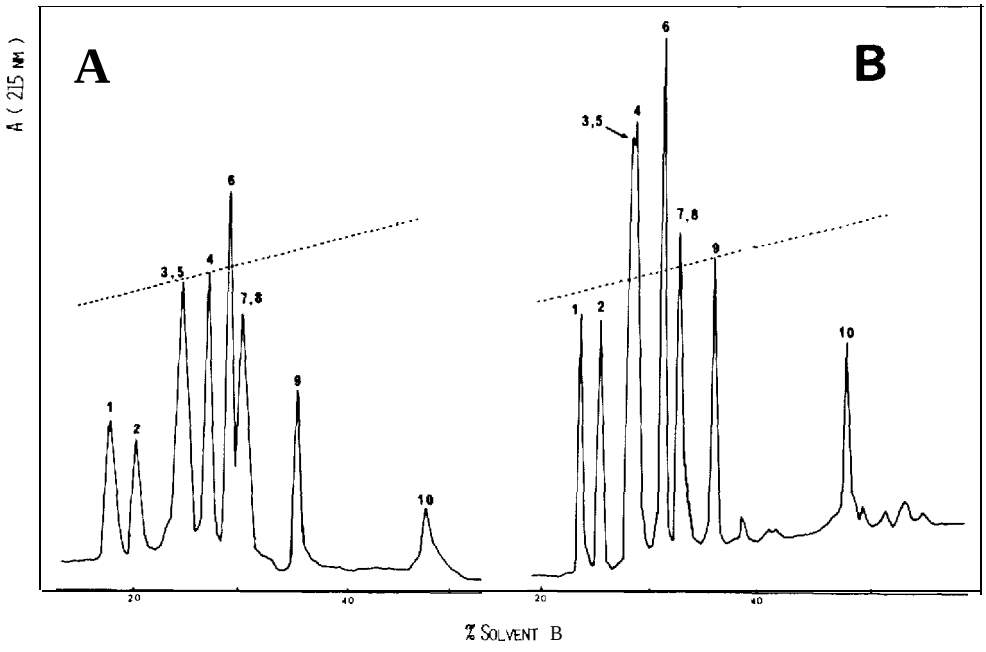


Fig. 2. Separation of the ten-peptide mixture on a Partisil ODS-3 column. The chromatographic parameters are as in Fig. 1.

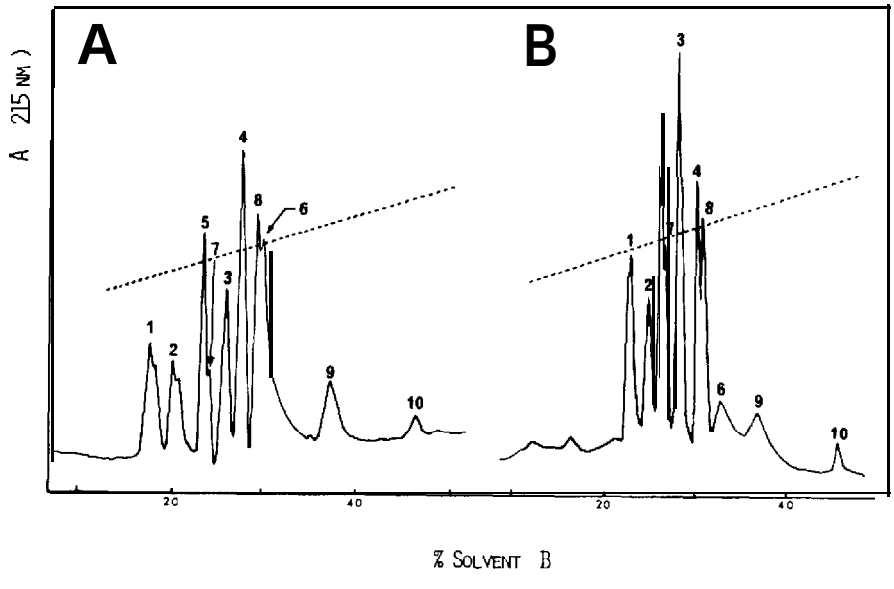
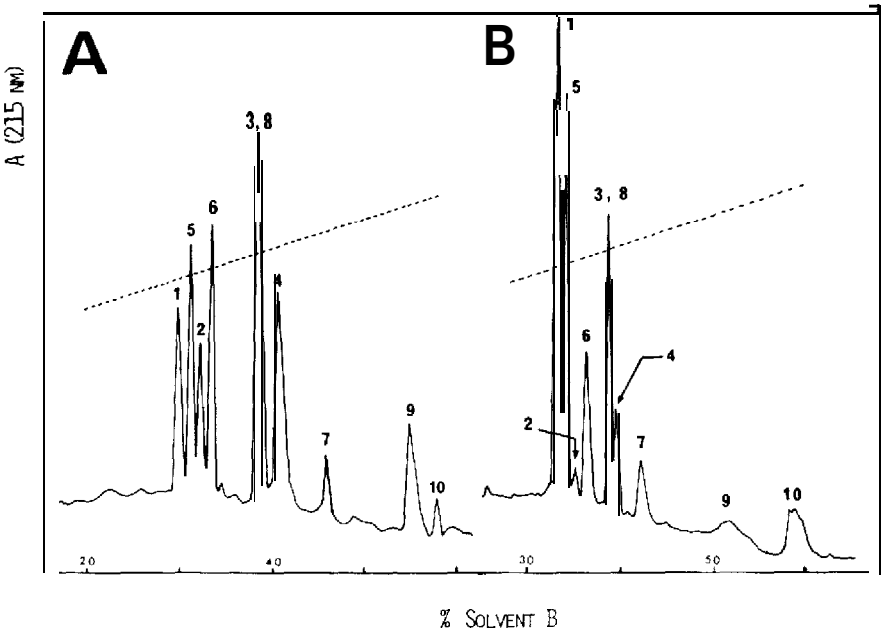


Fig. 3. Separation of the ten-peptide mixture on a Varian MicroPak MCH-5 column. The chromatographic parameters are as in Fig. 1.



Fig, 4. Separation of the ten-peptide mixture on a LiChrosorb RP-8 column. The chromatographic parameters are as in Fig. 1.

pattern. With system I, peptides 3 and 6, and 4 and 8 were eluted together, but peptide 7 is completely resolved. In contrast, with system II, peptides 7 and 8 were eluted together, whereas peptides 3 and 6 are barely resolved. Neither solvent system gave adequate separations of the small peptides used in this study. This is to be expected since the larger-pore Vydac TP column is intended for the separation of much larger molecules. A summary of the retention times obtained is shown in Table Il.

Having thus ascertained that the ternary solvent System I gave at least equal, if not better separations on all five columns, I applied it to the purification of a biological extract obtained in a pulse-labeling experiment from fresh preparations of human placental trophoblasts (see ref. 10).

Abundant literature already exists on the separation of enkephalins, LHRH, and other biologically active peptides by reversed-phase HPLC[11–22]. Among the mobile phases used were methanol, acetonitrile, 1-propanol and isopropanol in the presence of TFA or various buffers, such as tetraethylammonium phosphate (or formate), tetrabutylammonium phosphate, pyridine formate, and the like. However, none of the separations was achieved by the simple solvent system described in this paper.

A major advantage of methanol over acetonitrile is its chemical stability in acid medium. This is shown in Table III. An acetonitrile solution, containing 0.1% TFA, shows increasing absorbance at 215 nm as a function of time, concomitant with a decrease in pH, indicating steady hydrolysis to acetamide and acetic acid. In contrast, the absorbance and pH of a methanol–water (25:75) solution, containing 0.1% TFA, remained virtually unchanged. This is very convenient as only solvent B needs to be prepared fresh every day in amounts of 300 ml or less.

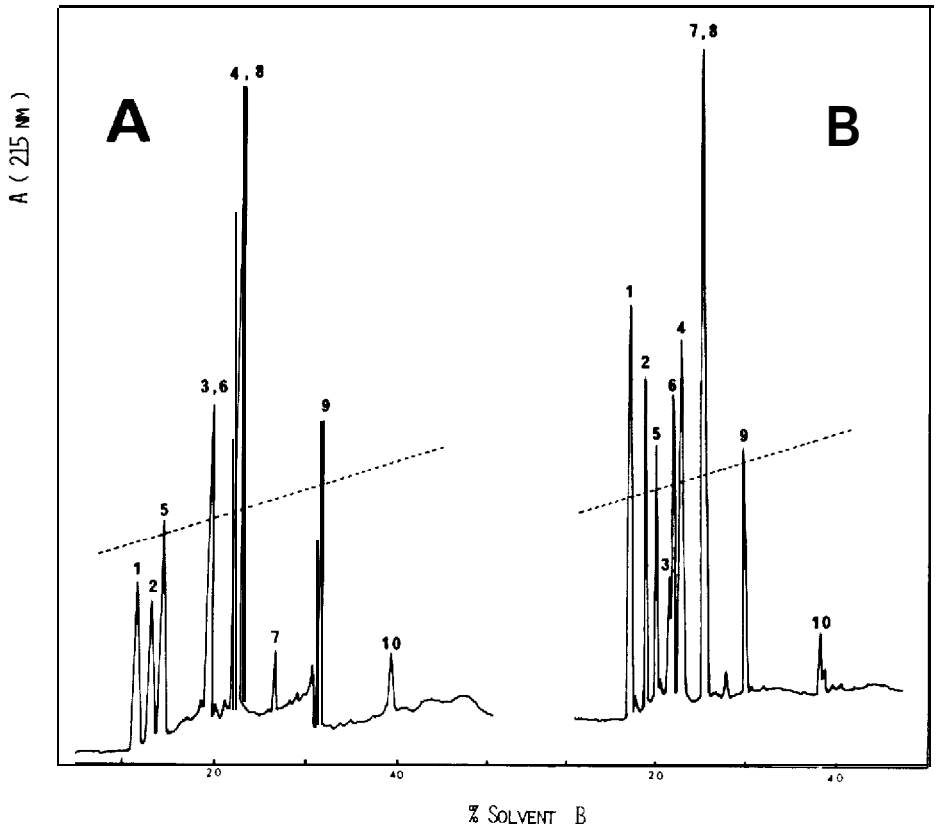


Fig. 5. Separation of the ten-peptide mixture on a Vydac 218TP protein column. The chromatographic parameters are as in Fig. 1.

TABLE II

COMPARISON OF THE RETENTION TIMES OF THE TEN PEPTIDES USED IN THIS STUDY

The chromatographic conditions on all five columns were identical. Flow-rates, 0.8 ml/min; Temperature, 40°C; linear gradient from 0 to 60% solvent B in 60 min. System I: solvent A, methanollwater (25:75) containing 0.1% TFA; solvent B = acetonitrile-water (80:20) containing 0.1% TFA. System II: solvent A, acetonitrile-water (10:90) containing 0.1% TFA; solvent B = acetonitrile-water (80:20) containing 0.1% TFA.

Peptide number assigned	*Peptide (no. of residues in brackets)*	*µBondapak*		*Partisil ODS-3*		*LiChrosorb RP-8*		*MCH-5*		*Vydac TP*	
		I	*II*	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*
1	Leu-Enk-Arg-Lys (7)	*25.2*	*27.6*	*19.3*	*27.4*	*32.7*	*36.9*	*20.2*	*24.7*	*14.2*	*20.0*
2	Leu-Enk-Arg-Arg (7)	*26.8*	*29.1*	*22.3*	*29.2*	*35.2*	*39.0*	*22.7*	*26.9*	*15.9*	*21.5*
3	Lys-bradykinin (10)	30.0	31.1	27.1	31.4	41.2	42.6	27.6	30.0	22.0	24.2
4	β-Melanotropin (17)	31.3	31.8	29.8	31.8	43.3	45.9	30.8	32.7	25.1	25.5
5	Met-enkephalin (5)	31.8	32.4	26.7	31.3	33.9	37.9	25.1	28.1	17.1	22.9
6	LHRH (10)	34.3	32.6	31.2	34.8	36.5	40.1	25.9	28.6	22.2	24.7
7	Dynorphin (13)	35.4	33.7	32.5	34.7	50.4	48.6	31.5	34.8	29.0	28.0
8	Leu-enkephalin (5)	39.0	36.1	33.2	35.7	41.9	43.3	29.1	32.1	25.1	27.1
9	Angiotensin (IO)	39.8	38.7	37.6	39.8	57.3	55.5	38.8	39.0	34.0	32.4
10	Neurotensin (13)	49.6	49.0	49.8	51.3	60.4	62.0	48.2	47.5	41.9	41.2

TABLE III

COMPARISON OF THE CHEMICAL STABILITY OF METHANOL AND ACETONITRILE IN WATER CONTAINING 0.1% OF TRIFLUOROACETIC ACID AS A FUNCTION OF TIME

Time (days)	25% Methanol		10% Acetonitrile		80% Acetonitrile	
	pH	Abs.	pH	Abs.	pH	Abs.
0	2.3	0.294	2.3	0.227	2.2	0.221
1	2.3	0.294	2.3	0.229	2.2	0.239
1.5	2.3	0.294	2.3	0.231	2.2	0.241
2	2.3	0.294	2.3	0.233	2.1	0.247
3	2.3	0.296	2.2	0.244	2.0	0.268
6	2.3	0.308	2.1	0.259	1.8	0.295

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