ELSEVIER

Journal of Chromatography A, 744 (1996) 231–240

JOURNAL OF CHROMATOGRAPHY A

Peptide stacking by acetonitrile–salt mixtures for capillary zone electrophoresis

Zakariya K. Shihabi

Pathology Department, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157, USA

Abstract

Many small natural and synthetic peptides can be stacked for capillary zone electrophoresis by dissolving the peptides in a mixture containing acetonitrile and high concentrations of inorganic salts. In many instances one third of the capillary can be loaded with peptides dissolved in a mixture of 2 volumes acetonitrile and 1 volume of 1% sodium chloride leading to about 20-fold enhanced detection. This stacking is dependent on the presence of both salts and acetonitrile. Natural peptides such as enkephalins, angiotensin and insulin chain B in addition to peptides released from the action of proteolytic enzymes on proteins were concentrated by this method. From a practical point of view, the stacking in acetonitrile is more useful since it removes proteins, counteracts the deleterious effects of high concentrations of inorganic ions present in the sample and stops the enzymatic reaction. Furthermore, it allows a larger volume of the sample to be loaded on the capillary increasing the sensitivity of the CE. This stacking produces a greater sample concentration and better resolution than the traditional stacking obtained in aqueous low ionic strength buffers. The mechanism is also different since it is improved by a high concentration of ions in the sample. Furthermore, since proteins are eliminated, the electropherograms are cleaner and the capillary does not require thorough washings between samples, speeding up the analysis and extending the capillary life.

Keywords: Stacking; Peptides; Enkephalins; Insulin; Angiotensin; Acetonitrile; Salts

1. Introduction

Because of their charge and strong absorbance at 214 nm peptides are suited for separation by CZE. However, peptides are usually present in biological samples in low concentrations in the presence of large amounts of proteins and salts. Commercially, pure peptides are very expensive to prepare in high concentrations. In addition, the sensitivity of detection in CZE is very low. Thus, it is desirable to be able to concentrate, as much as possible, the peptides present in the sample directly on the capillary (stacking).

Stacking by the use of dilute aqueous buffers in the sample in CZE has been described by several workers [1–5]. It is is very desirable in CZE because it offers a simple means for concentrating compounds directly on the capillary. This type of stacking is suited for analysis of compounds in a clean matrix devoid of a high concentration of proteins or salts. In practice, it is not very useful for the analysis of low levels of compounds present in biological samples in the presence of high concentrations of ions and proteins.

Acetonitrile offers an effective method to remove high concentrations of proteins. In CZE a unique type of stacking occurs when mixtures of acetonitrile and inorganic salts are present in the sample (not in the buffer) as described recently [6–8]. This stacking is different from that occurring in buffers of low ionic strength in that it occurs in acetonitrile solutions and it is aided by the presence of high

0021-9673/96/$15.00
PII S0021-9673(96)00132-X

concentrations of inorganic ions in the sample. Here we demonstrate that this type of stacking is well suited for analysis of many small synthetic and natural peptides. The acetonitrile has other additional advantages such as counteracting the deleterious effects of ions, removing the proteins present in the sample and terminating the enzymatic reactions. The overall effect is a better sensitivity and improved resolution.

2. Experimental

2.1. Apparatus

A Model 2000 capillary electrophoresis instrument (Beckman Instruments, Fullerton, CA, USA) was set at 214 nm, 30°C and 12 kV. The capillary, 42 cm×50 μm I.D., was filled with the separation buffer, 250 m*M* boric acid (pH 9.2), by pressure injection for 2 min.

2.2. Chemicals

All chemicals were obtained from Sigma (St. Louis, MO, USA).

2.3. Synthetic peptides mixture

Gly–Trp, 0.5 mg/ml; Gly–Gly, 2 mg/ml; Gly–Gly–Gly, 2 mg/ml dissolved in 30 m*M* borate buffer (pH 9.2).

2.4. Natural peptides analysis

Angiotensin 60 mg/l, leucine-enkephalin 120 mg/l, and insulin chain B 160 mg/l were dissolved in a 20 m*M* borate buffer (pH 10.4) containing 0.9% NaCl. One volume of the peptide solution was mixed with two volumes of acetonitrile. The peptides were injected to fill either 1.5 or 30% of the capillary to the detector and electrophoresed in a 200 m*M* borate buffer (pH 10.4) at 12 kV; detection at 214 nm.

2.5. Serum deproteinization with acetonitrile

Serum, 100 μl, was vortex-mixed with acetonitrile, 200 μl and centrifuged at 14 000 *g* for 20 s. The supernatant was introduced into the capillary by pressure injection.

2.6. Enzymatic hydrolysis

2.6.1. Pepsin hydrolysis

150 μl hemoglobin substrate (6 g/l in 100 m*M* HCl containing 0.9% NaCl) was mixed with 50 μl of pepsin (4450 U/ml). Samples were incubated at 37°C for 10 min. At the end of the incubation period, 500 μl acetonitrile was added to the mixture, vortex-mixed, and centrifuged at 14 000 *g* for 15 s. The capillary was filled to 15% of its volume with the supernatant. The separation buffer was 100 m*M* borate (pH 9.2).

2.6.2. Trypsin hydrolysis

150 μl hemoglobin substrate (6 g/l in 50 m*M* Tris (pH 8.1) containing 0.9% NaCl) was mixed with 50 μl of trypsin (22 000 U/ml). Samples were incubated at 37°C for 10 min. Afterwards, acetonitrile was added and the samples were treated as described for pepsin.

3. Results and discussion

3.1. Sample volume and stacking

In order to achieve a high plate number in CE, the sample size has to be very small, preferably less than 1% of the capillary length [9]. Under such conditions the separation and the sharpness of the peaks for the three peptides (Gly–Trp, Gly–Gly, Gly–Gly–Gly) are very good, whether a weak buffer or an acetonitrile-salt mixture is used Fig. 1. Unfortunately, the sensitivity is very low which makes analysis of peptides by CZE very difficult.

However, when the sample volume is greatly increased to 30% of the capillary volume such that the capillary is overloaded with sample (Fig. 2), the stacking property of the weak buffer is lost (Fig. 2A). Salt addition alone was ineffective (Fig. 2C), whereas acetonitrile alone was more effective but the resolution incomplete (Fig. 2B). On the other hand, the inclusion of both acetonitrile and NaCl was more effective (Fig. 2D). The separation is good and the

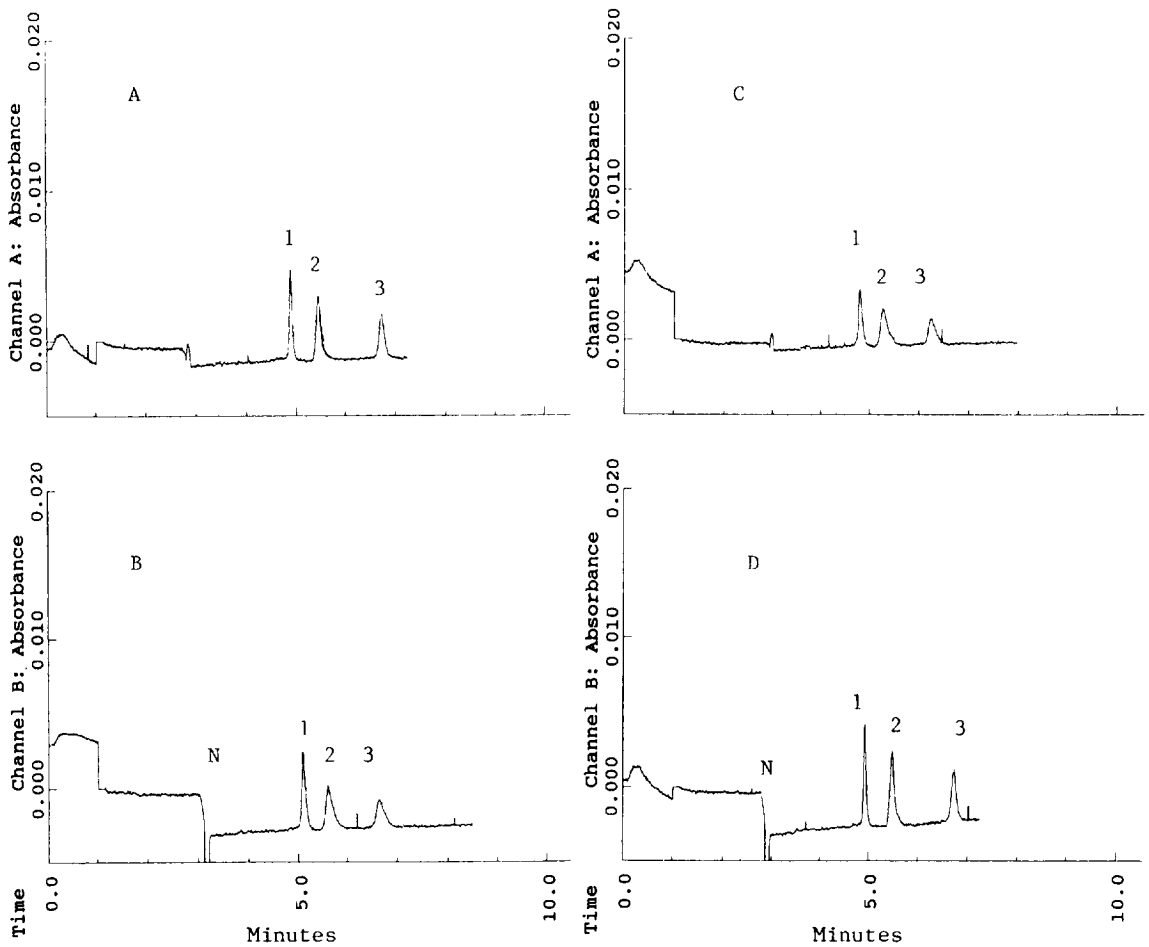

Fig. 1. Electropherogram of Gly–Trp (1), Gly–Gly–Gly (2) and Gly–Gly (3). Sample filled 1.5% of the capillary volume and was prepared in: A, 25 μl peptides+75 μl water+200 μl water; B, 25 μl peptides+75 μl water+200 μl acetonitrile; C, 25 μl peptides+75 μl 1% NaCl+200 μl water; D, 25 μl peptides+75 μl 1% NaCl+200 μl acetonitrile.

peaks are sharp. More importantly, the sensitivity was enhanced by about 20-fold.

Fig. 3 illustrates the fact that increasing the sample volume causes an increase in peak height. This increase deviates slightly from linearity because of a slight loss of plate number and it is different from one peptide to another. For example, this deviation is more pronounced for Gly–Gly than that for Gly–Trp.

3.2. Effect of acetonitrile

A concentration of acetonitrile above 50% is critical for the stacking to take place. However, a peptide like Gly–Trp is more dependent on the concentration than other peptides Fig. 4.

3.3. Stacking and the molarity of the separation buffer

As seen in Fig. 5, strong separation buffers yield better separations and favor stacking, similar to that observed for stacking by low ionic strength in the sample [2,6], but at the expense of an increase in analysis time.

Indirectly, strong buffers, allow a larger volume of the sample to be loaded on the capillary.

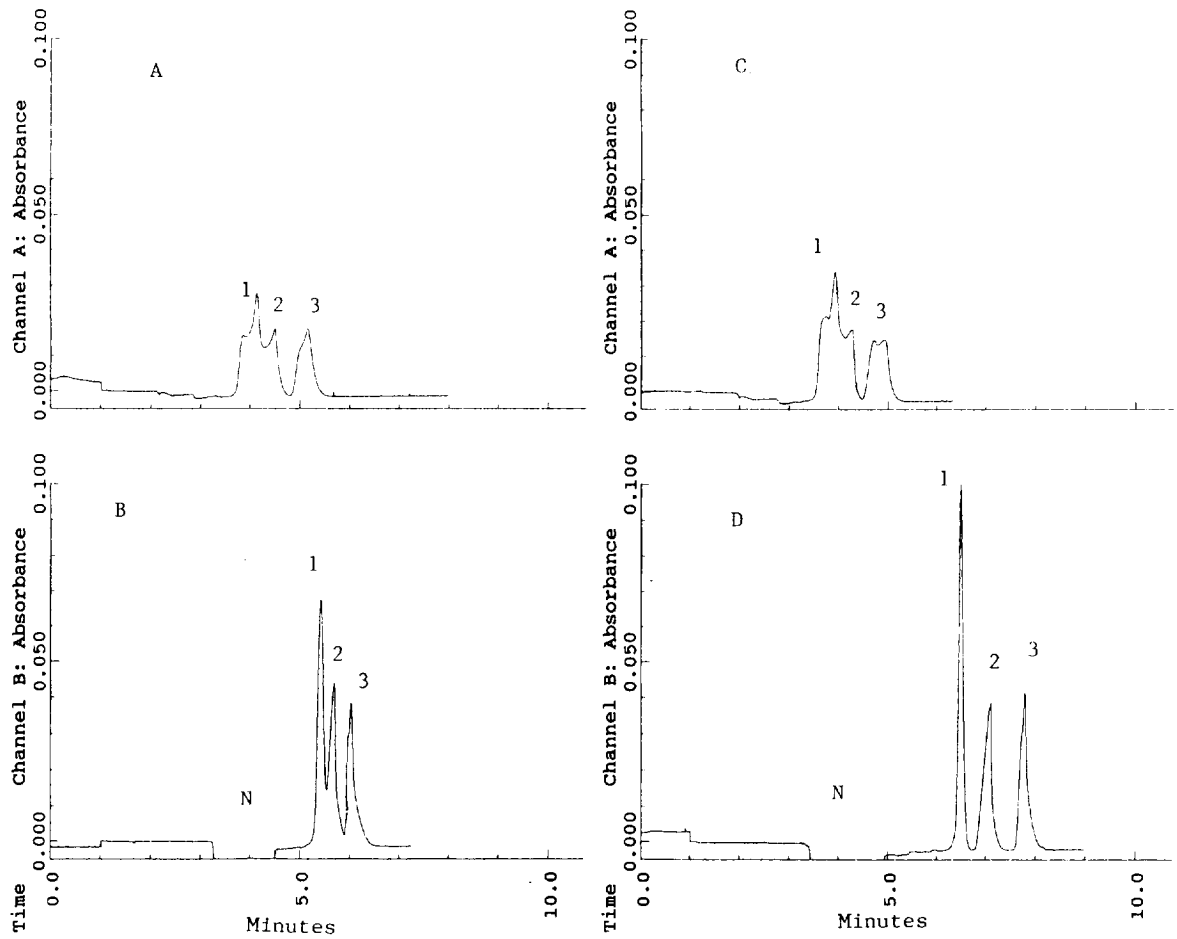

Fig. 2. Same as in Fig. 1 except 30% of the capillary volume was filled with sample.

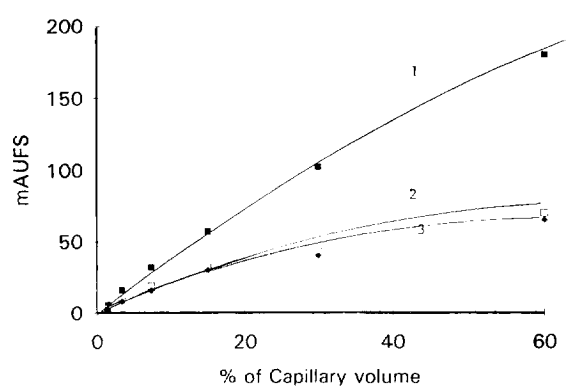

Fig. 3. Effect of sample size as percentage of the capillary volume on peak height (stacking). Peptides as in Fig. 1.

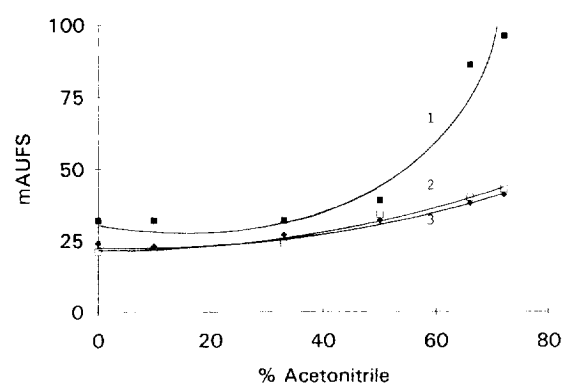

Fig. 4. Effect of acetonitrile concentration on the stacking at 30% sample loading. Peptides as in Fig. 1.

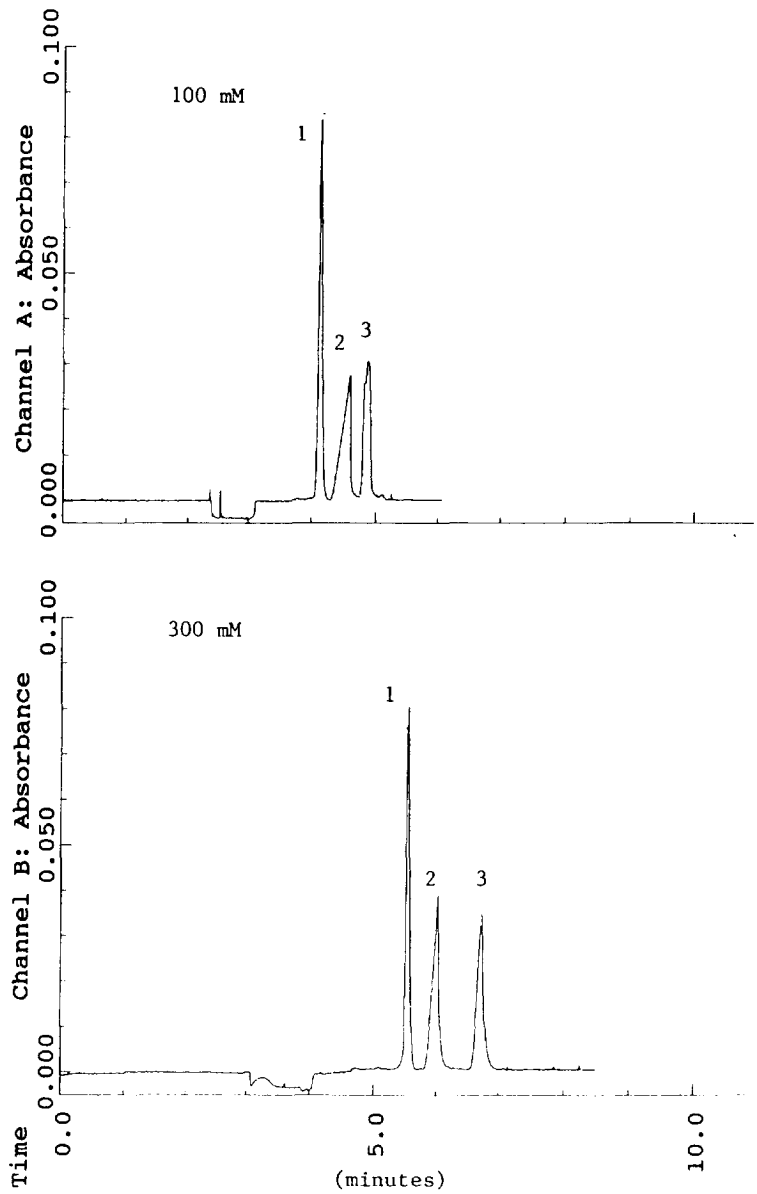

Fig. 5. Effect of the separation buffer molarity (100 vs. 300 m*M* borate buffer, pH 9.2) on the stacking. The capillary was filled to 22% of its volume with sample.

3.4. Practical consideration

Concentration of peptides on the capillary (stacking) is important in at least two areas, protein hydrolysis and analysis of peptides in biological fluids as discussed in the following examples.

3.4.1. Stacking of peptides released from protein hydrolysis

Peptides released by the action of proteolytic enzymes such as pepsin (Fig. 6) and trypsin (Fig. 7) can be concentrated by this method. Here, the addition of acetonitrile at the end of the incubation

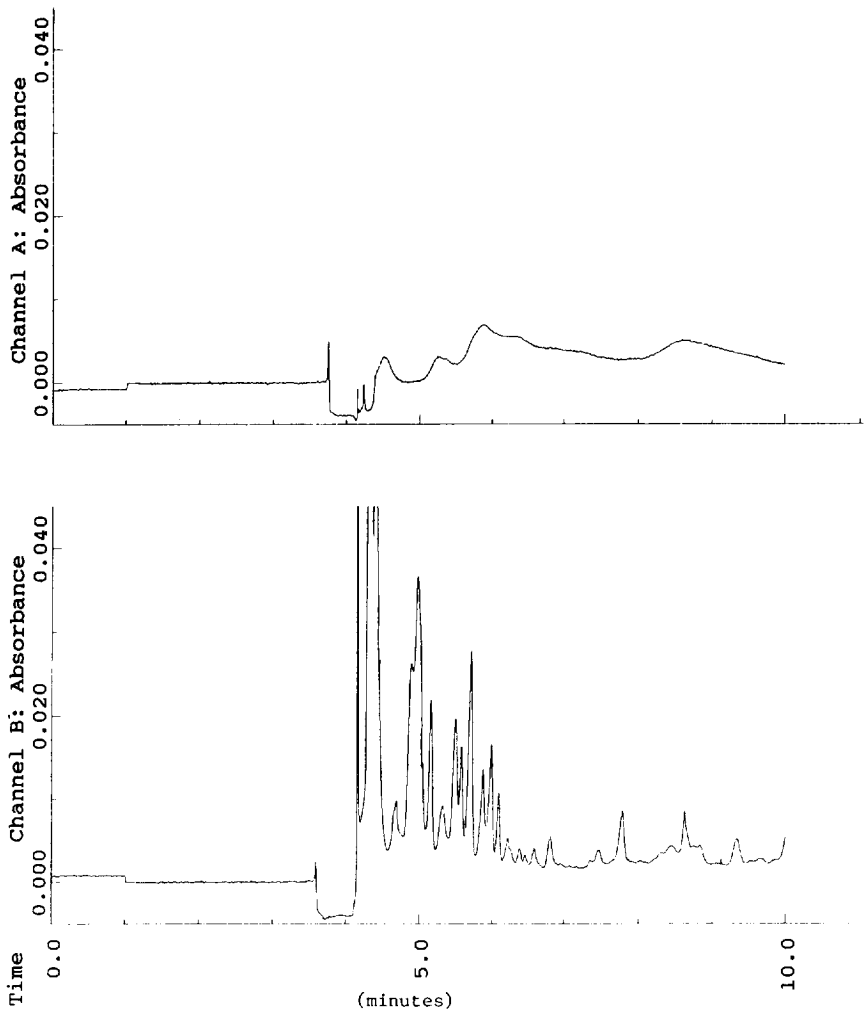

Fig. 6. Stacking of peptides from the enzymatic hydrolysis of pepsin on hemoglobin. Top at 0 and bottom after 10 min of hydrolysis at 37°C; sample loading 15%.

period stops the reaction. After loading the capillary to 15% of its volume with the peptides the peaks are sharp and the separation is good (Figs. 6 and 7). It is difficult to detect most of the peaks with the usual loading of 1% of the capillary volume, especially if the sample contains high level of unhydrolyzed proteins. In this type of analysis, acetonitrile has several advantages: it (1) stops the reaction (2), precipitates the unhydrolyzed proteins and (3) concentrates the peptide on the capillary.

3.4.2. Stacking natural peptides

Many natural peptides such as Leu–enkaphalin, angiotensin and insulin chain B can be concentrated by this techniques as in Fig. 8. With the usual 1% loading, peptides are difficult to distinguish from noise, while increasing the capillary sample load 20-fold makes these peptides very easy to detect (Fig. 8).

3.4.3. Stacking of compounds in biological fluids

The increase in peak height and the good separation obtained by this type of stacking is achievable not only in pure solutions, but also can be achieved for peptides added to serum Fig. 9. As much as 30% of the capillary volume can be loaded with sample with a good peak height and good separation. The high protein concentration of serum (~7000 mg/l)

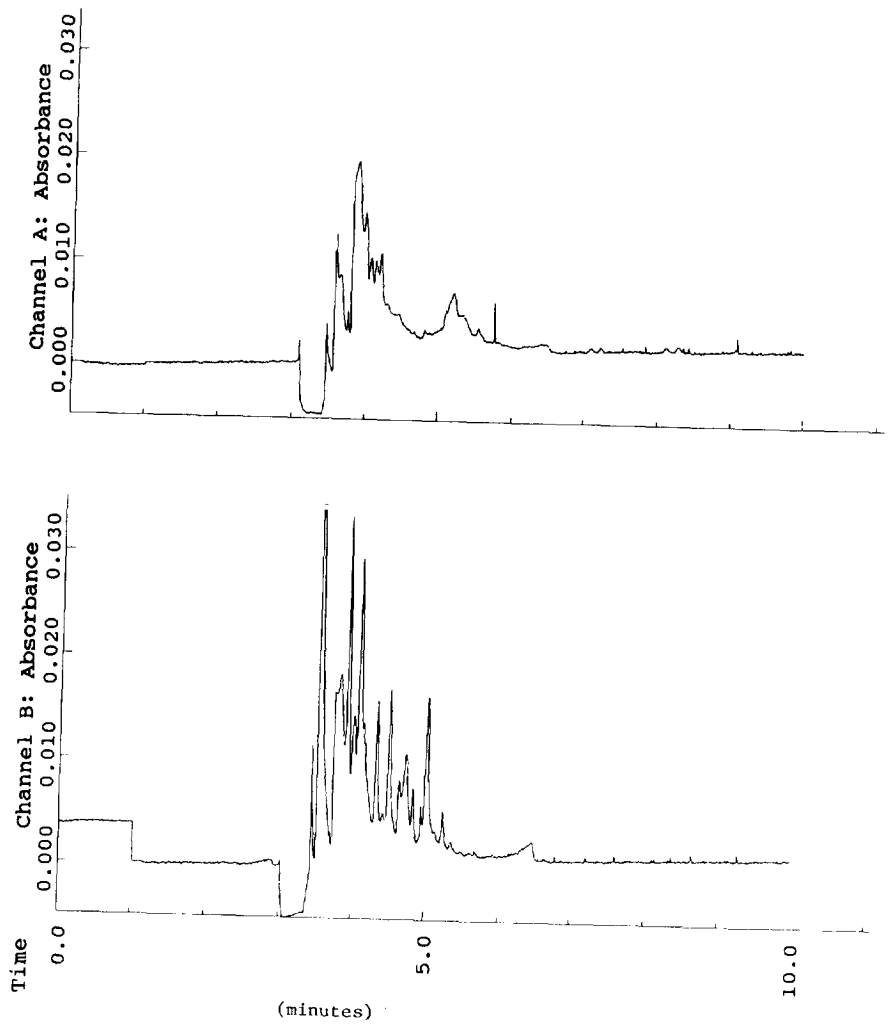

Fig. 7. Stacking of peptides from the enzymatic hydrolysis of trypsin on hydrolysis of trypsin on hemoglobin. Top at 0 and bottom after 10 min at 37°C; sample loading 15%.

prohibits direct loading of a large volume of sample without pretreatment. Serum proteins mask the peptides and foul the capillary walls.

4. Conclusions

The presence of both acetonitrile and salts brings about sample stacking for peptides as well as many other small molecules. The two main factors in producing this type of stacking are the limited solubility of inorganic ions in acetonitrile and the low conductivity of acetonitrile itself. In the absence of salts a limited stacking occurs due to the low conductivity of acetonitrile (Fig. 2). However, when salts are added to the sample a further stacking occurs due to a different and more complex mechanism. Because of the limited solubility of inorganic ions in acetonitrile, they migrate rapidly with some water, leaving behind a more concentrated and narrower segment of acetonitrile. Weakly ionized organic compounds migrate, briefly, behind in the acetonitrile zone, a reminiscence of the "salting effect" in liquid extraction. From another point of view, the peptides in the sample are applied in a

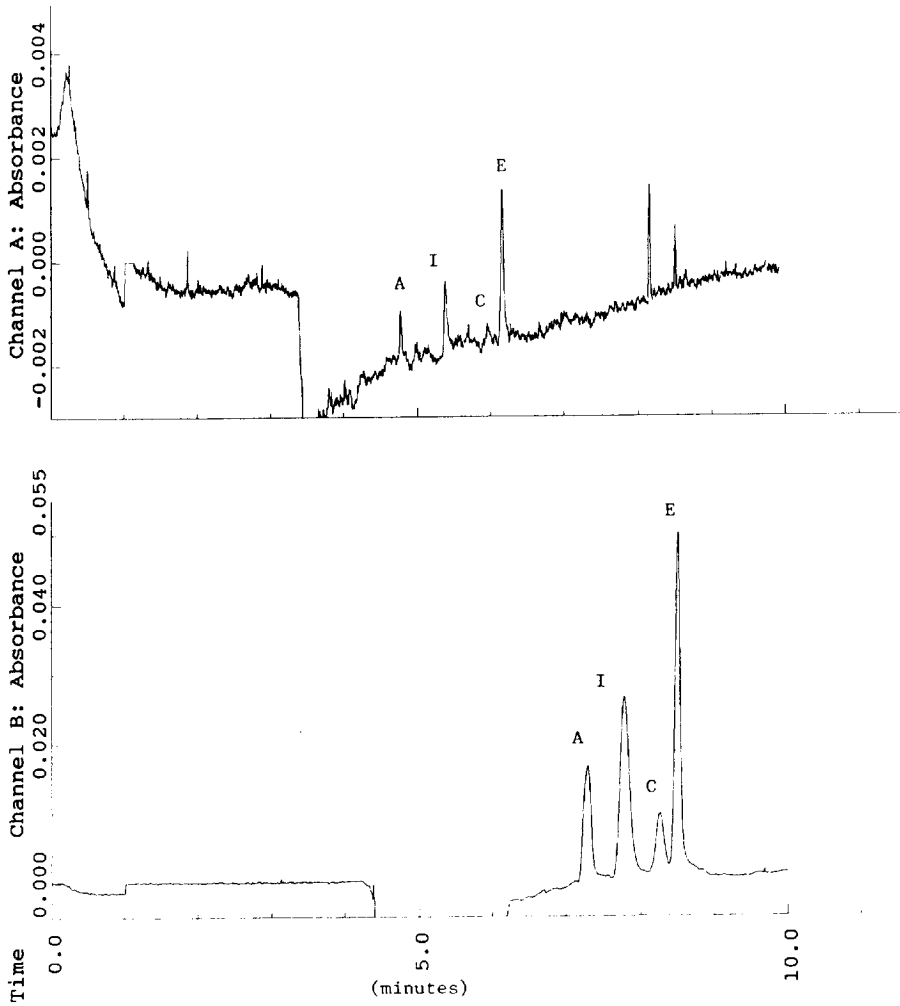

Fig. 8. Stacking of some natural peptides (A=angiotensin, I=insulin B chain, C=impurity in the insulin B chain and E=Leu-enkephalin. Top at 1.5% loading of the capillary and bottom at 30%.

wide zone about 100 mm long which eventually sharpens to a band of just a few mm as evident in Fig. 2D and Fig. 9, where the neutral molecules zone is about 10–20 times the width of the peaks. In order for this to be achieved the two edges (front and back) of the peptide zone have to migrate at different rates. This can occur because of the difference in the field strength between the salt and the acetonitrile zones. As the potential is applied, the inorganic ions migrate rapidly (within the sample zone). The edge of the peptide band within the inorganic ions slows down due to the lower field strength, while the other edge in the acetonitrile experiences a higher field strength and migrates faster. Thus, acetonitrile, ameliorates the mobility and concentration of the sample ions so that conditions similar to a transient isotachophoretic step can prevail [10,11].

This stacking, as described here, is unique and

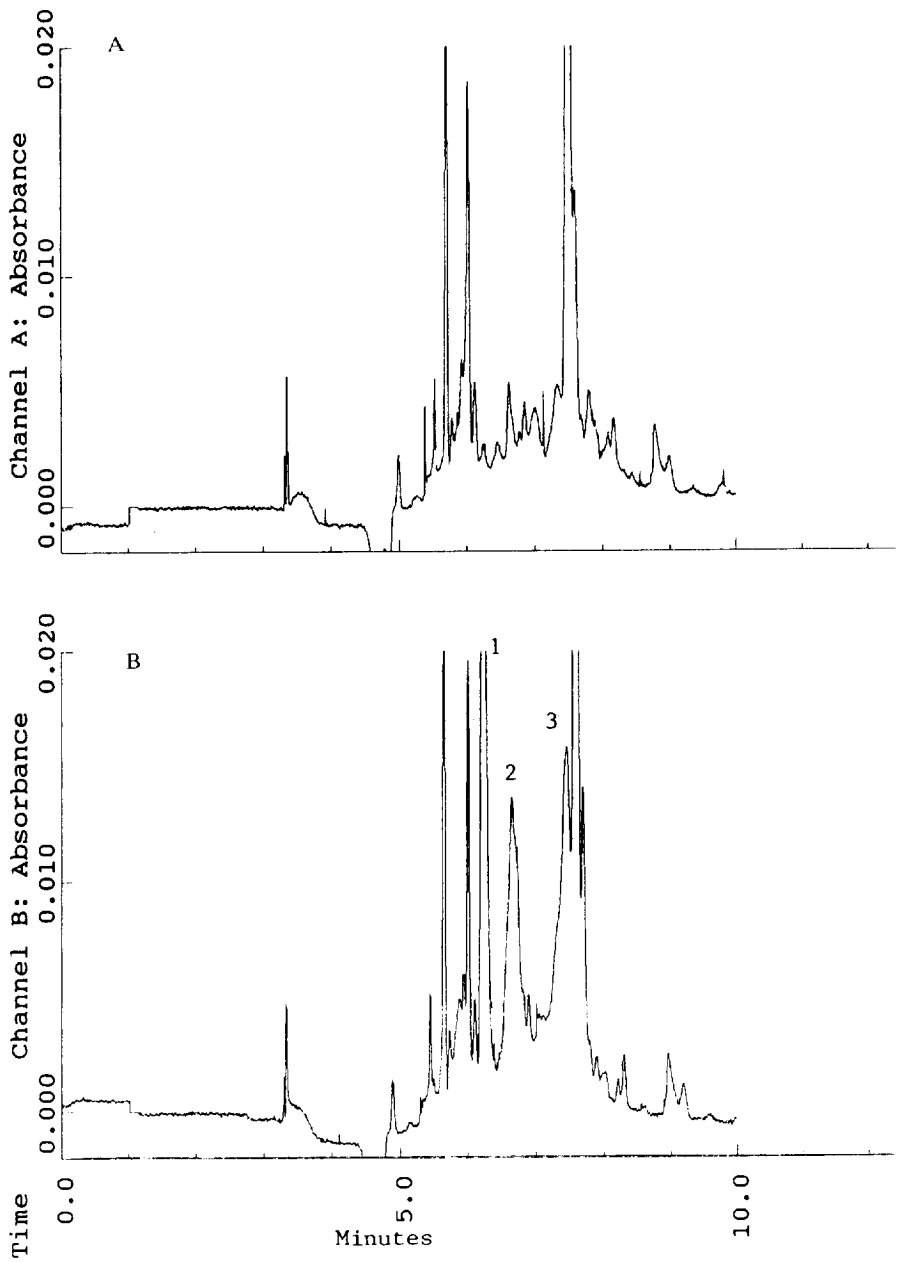

Fig. 9. Electropherogram of a patient serum (A) before and (B) after adding a peptide mixture: Gly–Trp, 50 mg/l (1), Gly–Gly–Gly 200 mg/l (2) and Gly–Gly, 200 mg/l (3). Serum treated as described in Experimental. Sample loading was 30% of the capillary volume.

intriguing from the theoretical aspects, yet, it has great practical importance. Peptides varying in molecular mass from 132 to 3495 Da were concentrated by this method. Peptides resulting from enzymatic hydrolysis by pepsin and trypsin as well as other small molecules [6] were also concentrated by this method. As a result of this stacking, concentrations of up to 20-fold on the capillary can be achieved.

References

[1] F.E.P. Mikkers, F.M. Everaerts and Th.P.E.M. Verheggen, J. Chromatogr., 169 (1979) 11.
[2] A. Vinther and H. Soeberg, J. Chromatogr., 559 (1991) 3.
[3] R.L. Chien and J.C. Helmer, Anal. Chem., 163 (1991) 1354.
[4] D.S. Burgi and R.L. Chien, Anal. Chem., 63 (1991) 2042.
[5] R.L. Chien and D.S. Burgi, Anal. Chem., 64 (1992) 1046.
[6] Z.K. Shihabi, J. Cap, Electrophoresis, 2 (1995) 267.
[7] Z.K. Shihabi, J. Chromatogr. A, 652 (1993) 471.
[8] Z.K. Shihabi and M.S. Constantinescu, Clin. Chem., 38 (1992) 2117.
[9] Z.K. Shihabi and L.L. Garcia, in J. Landers (Editor), Handbook of Electrophoresis, CRC Press, Boca Raton. FL, 1994, pp. 537–548.
[10] P. Gebauer, W. Thormann and P. Boček, J. Chromatogr., 608 (1992) 47.
[11] P. Gebauer, W. Thormann and P. Boček, Electrophoresis, 16 (1995) 2039.