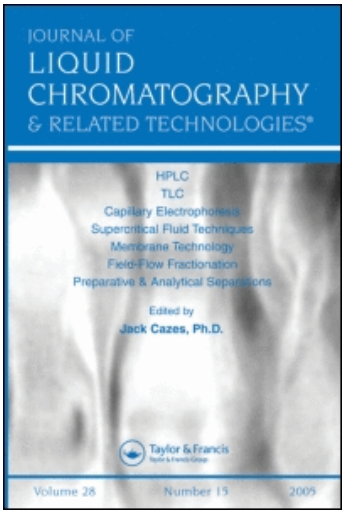

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INVESTIGATION OF 2-METHOXYETHANOL AS A MODIFIER IN ACETONITRILE FOR THE REVERSED PHASE HPLC OF PROTEINS

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ABSTRACT

The results of using 2-methoxyethanol as a mobile phase modifier combined with acetonitrile for the separation of a standard mixture of proteins is described. Selectivity comparisons are made between this system and similar mobile phase systems containing acetonitrile, methanol, n-propanol and iso-propanol.

INTRODUCTION

With widespread use of modifiers such as methanol (MNL) and acetonitrile (ACN) in reversed-phase liquid chromatography (RPLC), little attention has been paid to some of the less familiar modifiers which are available. As a consequence of the rapid expansion of the field of biotechnology, the propanols, both n-propanol (NPA) and iso-propanol (IPA), are becoming increasingly important as HPLC mobile phases,

especially with the analysis of the more hydrophobic biopolymers, particularly proteins and peptides.[1] However, high back-pressure is a problem with the propanols as a result of their increased viscosity relative to solvents like MNL and ACN. Since one is usually dealing with biopolymers with similar structures, especially when analyzing samples such as enzymatic and chemical digests, an additional degree of selectivity over the propanols would also be a desirable trait for a mobile phase component used in the separations.

A group of solvents that would seem to be ideally suited to meet the hydrophobicity requirements of the propanols while possibly demonstrating some unique selectivities are the ethylene glycol ethers, of which one of the best known examples is 2-methoxyethanol (2ME). This could be considered a propanol "model" based on its carbon content but the presence of the methoxy group would be expected to contribute an additional degree of polarity to the molecule. These slight differences could prove useful from a selectivity standpoint. One report has already found the selectivity of 2ME beneficial for the isocratic separation of sheep, beef, pork and human insulins.[2] Since the viscosity of 2ME (1.74 cP)[3] compared to the propanols (2.3 cP, 2.4 cP)[3] is lower, a secondary benefit could be a decrease in overall system back pressure. One would expect the hydrophobicity of the glycol ethers to be similar to that of the propanols, thereby retaining this important property.

We wish to report some of our initial results on the use of 2ME as a modifier in the separation of a standard mixture of proteins and the comparisons between it and some of the other solvents commonly used as mobile phases in protein separations.

EXPERIMENTAL

Instrumentation and Procedures:

Analyses were done on either a Varian Model 5000 liquid chromatograph (Walnut Creek, CA) equipped with a UV-200 detector or a CM4000 liquid chromatograph (LDC, Riviera Beach,

FL) equipped with a SM4000 detector. Data acquisition was accomplished using a Spectra Physics (Santa Clara, CA, USA) model 4270 data system. The column used was a Phenomenex W-Porex C18 (Rancho Palos Verdes, CA, USA), 4.6 mm x 25 cm packed with 5 micron particles. The elution conditions involved a linear gradient from 5%-95%B (B = organic modifier) over a period of 30 minutes at a flow rate of 1 mL/min and detection wavelength of 280 nm (approx. 0.064 AUFS). Injection volumes were 10 microliters and made through either an electric-or air-actuated C6W Valco valve (Houston, TX, USA).

Solvent A = 0.1% trifluoroacetic acid (TFA) in water. Solvent B = 50% (v/v) organic modifier (NPA, IPA, MNL, 2ME) in acetonitrile also containing 0.1% TFA. Chromatographic peaks represent approximately 200-300 micrograms of each protein.

Reagents and Materials

Protein standards were obtained from Sigma (St. Louis, MO, USA) and were used as received. Mobile phase solvents were HPLC grade from Burdick and Jackson (Muskegon, MI, USA). TFA was from Aldrich Chemical (Milwaukee, WI, USA) and distilled prior to use. Uracil was also from Aldrich and used as received. Abbreviations used are as follows: Uracil - Ura; Ovalbumin - Ova; Bovine Insulin - Bl; Ribonuclease A - RNAse; Lysozyme - Lyz; Cytochrome C-CytC; Albumin (egg) - Alb; Bovine Serum Albumin - BSA. Uracil was incorporated into the standard sample in order to estimate the column void volume for K' and related calculations.

Results and Discussion

Our primary emphasis in this work was to quickly uncover any obvious selectivity differences between the various modifiers combined with ACN in the same relative concentration. Although the work reported is meant to be only a superficial study, there are some immediate differences which could possibly be enhanced with further work. Although the results do not indicate that 2ME has unique selectivity effects in and of

itself, the possibility that some unique selectivities in combination with acetonitrile may be present is evident. A wide range of modifier concentrations were not explored in this work to keep the variables simpler. There are probably certain situations when 2ME may yield advantages even though it may also be possible to get the same results with mixtures of the other modifiers. This possibility can be inferred from the work of Power.[4] The use of 2ME as a modifier allows adequate separation of the protein mixture used as is seen in Fig. 1. Besides the protein standards employed, several other peaks appeared either as single, resolved entities or as shoulders.

These are probably either minor impurities or degradation products since the samples were not made up fresh and were stored as solutions in 0.1% aqueous TFA. The presence of these is actually beneficial since we were not trying to identify components as much as separate them. These extra peaks thus give an additional measure of resolution achieved between the different mobile phases used.

When the separation achieved with the 2ME mobile phase (Fig. 1) is compared with those of the other modifiers (Figs. 2 & 3), it can be seen that a slight additional degree of selectivity is evident. Only with the 2ME mobile phases are all of the components well resolved almost to baseline. The separation with IPA and NPA (Fig. 3) are similar in that all 6 major peaks are discernible. However, the resolution factors are slightly less, especially between RNAse and CytC (see Table 1). These two components are unresolved in the corresponding MNL and ACN chromatograms (Fig. 2). It would be far more interesting to observe what the selectivity effects would be on a more homologous mixture of proteins and peptides rather than the varied mixture used in this work. Unfortunately, a sample of this type was not readily available to us to use in the work described. The results of these resolution differences are tabulated in Table 1 based on using the differences in retention times as a measure of selectivity.[5] As can be seen, the elution order of the various components remained unchanged with the different mobile phases.

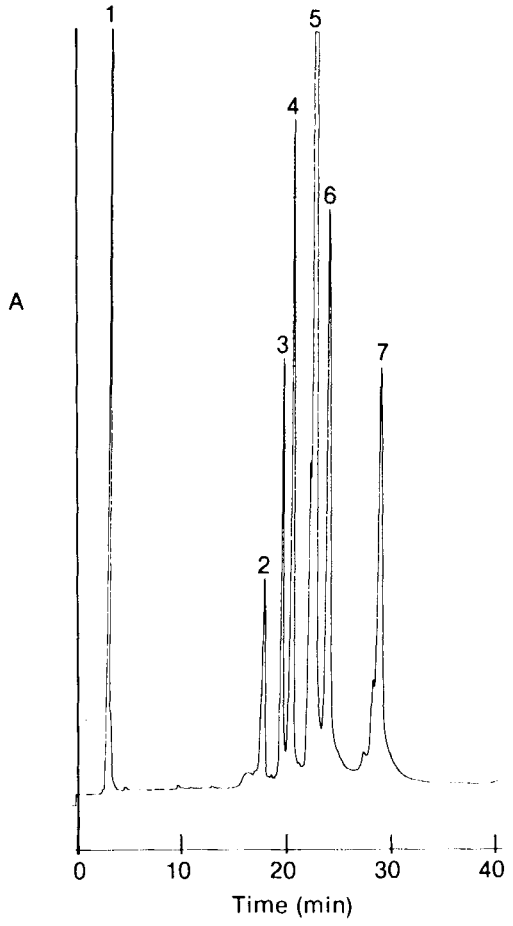

FIGURE 1 - Elution of protein sample with 2ME as mobile phase modifier. See **EXPERIMENTAL** for details.

Peak Identification: 1-Ura, 2-BI, 3-RNAse, 4-CytC, 5-Lyz, 6-BSA, 7-Ova.

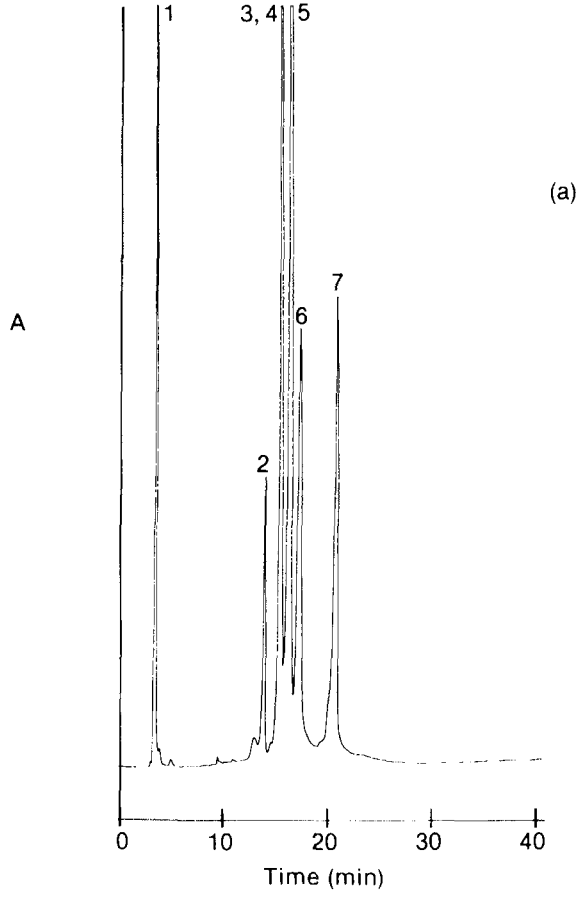

FIGURE 2 - Elution of protein sample with (a) ACN or (b) MNL as mobile phase modifier. See **EXPERIMENTAL** for details.

Peak Identification: See FIGURE 1.

(continued)

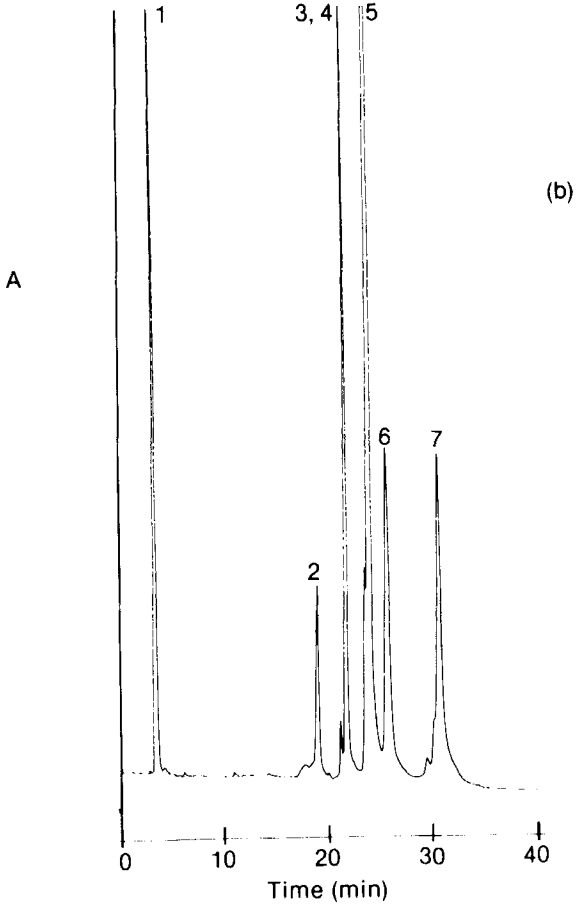

FIGURE 2 (continued)

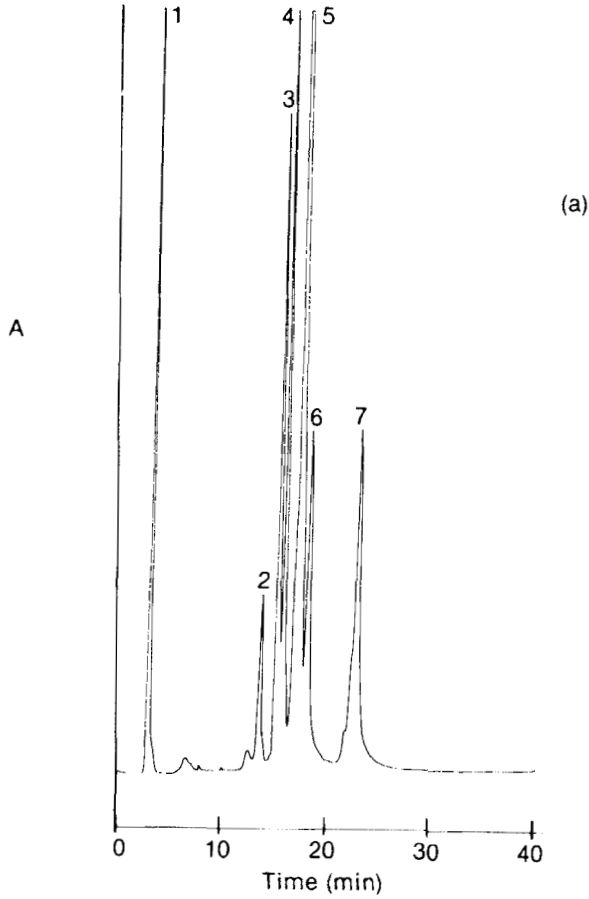

FIGURE 3 - Elution of protein sample with (a) IPA or (b) NPA as mobile phase modifier. See **EXPERIMENTAL** for details.

Peak Identification: See FIGURE 1.

(continued)

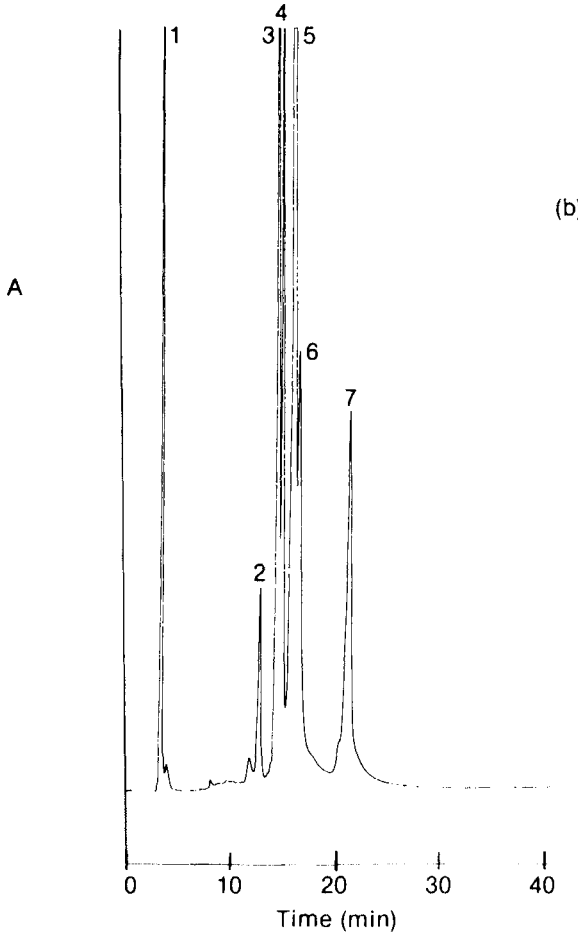

FIGURE 3 (continued)

Table 1

Relative Retention Times of Proteins*

Mobile Phase	BI/RNAse	RNAse/CytC	CytC/Lyz	Lyz/BSA	BSA/Ova
ACN	1.48	0.93	--	--	3.34
IPA	1.50	0.54	1.29	1.03	4.68
NPA	1.43	0.49	1.01	0.59	4.63
MNL	1.91	--	--	1.80	4.21
2ME	1.58	0.91	1.94	1.39	4.74

* -- = unresolved; RT in minutes based on average of 3 runs.

Some of the results in this study were slightly unexpected with regard to anticipated selectivity patterns. On the basis of hydrophobicity, one would expect 2ME to behave more like the propanols. As can be seen from Fig. 1-3, the elution pattern obtained with 2ME resembles that with MNL more so than the propanols. On the other hand, the results with ACN look similar to the propanols. It could be that, in the present results, the solvent polarity aspects may be more important than the hydrophobicity or possibly some intermediate effect is operating. Perhaps some of the other ethylene and propylene glycol ethers may show more hydrophobic tendencies.

One of the parameters we had hoped to reduce was the overall system back pressure. Some eluent combinations leading to lower viscosity mobile phases have been described[6], but 2ME was not included in that study. Our results on a qualitative basis indicate that no significant advantage is realized using 2ME instead of the propanols with respect to decreased overall system back pressure.

Some questions that have not been dealt with here are the degree of denaturation of sample proteins and loss of biological activity. Although we made no attempt to determine the degree of biological activity for the proteins recovered

from the various mobile phases, this is probably something that should be done. This could potentially give an additional advantage to the 2ME mobile phases. However, under the present conditions, the amount of ACN in the mobile phases along with the TFA would certainly favor denaturation and, thus, decreased recovery of biological activity of most proteins. The possibility of using only 2ME as the mobile phase could conceivably avoid this ACN effect, but the resulting compromise would be an increase in overall system back pressure. However, some of the selectivity effects could be utilized and combined with low, flow rates, as described by Glajch[7] to give even better results with possibly lower back pressures as a result of the lower flow rates.

Although the focus in this work has been on proteins, there are undoubtedly numerous other sample systems using both normal- and reversed-phase separation modes where enhanced selectivity effects of other ethylene and propylene glycol ethers might be a fruitful approach for uncovering unique properties useful to the liquid chromatographer for improving separations. Investigation of these aspects is ongoing in our labs and we hope to have additional work to report in the future.

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