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SPECTRAL PROPERTIES OF TRIFLUOROACETIC ACID–ACETONITRILE GRADIENT SYSTEMS FOR SEPARATION OF PICOMOLE QUANTITIES OF PEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The spectral properties of ternary systems composed of trifluoroacetic acid (TFA), water and various concentrations of acetonitrile were investigated in the low UV wavelengths. Due to the strong solvent dependency of the $\pi \rightarrow \pi^*$ band of TFA, no isosbestic point, as expected for a one-step dissociation equilibrium, was observed, but the spectra meet closely at 215 nm, which seems to be the optimum wavelength for highly sensitive detection of protein and peptide separations by reversed-phase gradient chromatography, using aqueous TFA *versus* acetonitrile. Furthermore, it was shown that a weak mid-gradient hump appearing during elution at 215 nm is caused by an overlap of the $n \rightarrow \pi^*$ transition of the free acid and the $\pi \rightarrow \pi^*$ absorption band of the anion, causing baseline irregularities in the region 212–218 nm.

INTRODUCTION

Since trifluoroacetic acid (TFA) was introduced into reversed-phase high-performance liquid chromatography (HPLC) of proteins and peptides by Bennett *et al.*[1], a great number of authors have reported the successful application of this reagent to various separation problems[2–11]. Doubtless, TFA has some major advantages over other organic or inorganic acids used as mobile phase additives: (1) a low UV cut-off; (2) an excellent solvent for polypeptides; (3) a strong ion-pairing agent (for in-

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teraction mechanism see ref. 12); (4) easy to remove by lyophilization; (5) does not block the amino terminus and (6) is miscible with most common organic modifiers.

The transparency of TFA in the wavelength range of 210–230 nm is of great convenience, allowing the detection of peptides independently of the presence of aromatic amino acids. In addition, low wavelength detection allows very sensitive monitoring of proteins bearing aromatic side chains[13]. Changing the wavelength from 280 to 214 nm usually results in a ten-fold increase in the peak height, which means that a few nanomoles of protein can be recorded at a detector range of greater than 0.5 a.u.f.s. However, modern methods in protein chemistry and the introduction of small diameter silica particles and microbore columns[14] for reversed-phase HPLC have made feassible the separation and analysis of picomoles of polypeptides[15,16]. In order to record separations of such low amounts the detector range has to be lowered to 0.1 a.u.f.s. or less. The use of gradient elution, often inevitable, then raises the problem of baseline drift which limits the sensitivity of detection.

In this paper, we report that optimum setting of the wavelength results in a drastic decrease in the baseline drift and in an increase in the detection sensitivity.

MATERIALS

Acetonitrile was of HPLC grade (J. T. Baker, Deventer, The Netherlands), TFA was of sequenal grade (Pierce, Rockford, IL, U.S.A.). All solutions were made up with water doubly distilled from glass apparatus.

The spectra in Fig. 1 were recorded with a DU-8 spectrophotometer (Beckman, Berkeley, CA, U.S.A.); for all other spectra a spectrophotometer from Perkin Elmer, Hitachi (Tokyo, Japan) was used.

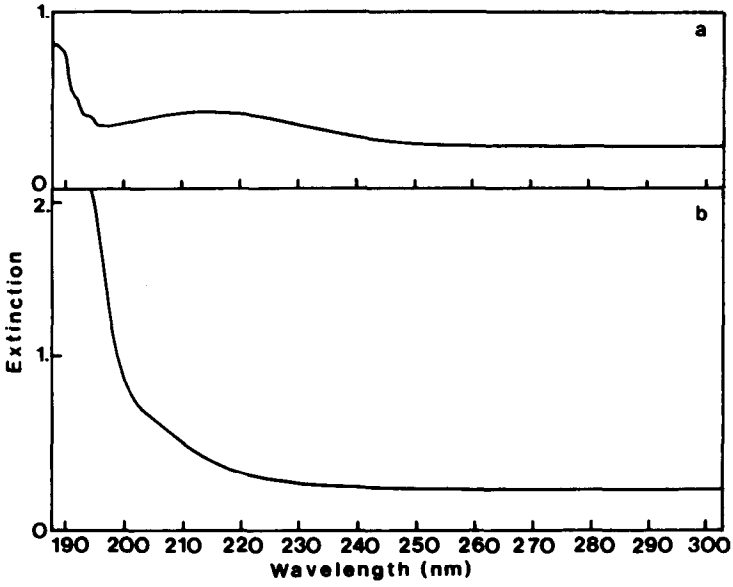

Fig. 1. Electronic spectra of 0.01 *M* TFA in acetonitrile (a) and in water (b).

The HPLC system (Beckman) consisted of two Model 114 pumps, a Model 421 system controller and a Model 460 detector (fixed wavelength). Chromatograms were recorded by a Shimadzu Chromatopac C-R1B recorder.

RESULTS AND DISCUSSION

Electronic spectra of TFA in water and in acetonitrile were recorded between 185 and 300 nm (Fig. 1). The spectrum in the organic solvent is in good accord with that of the free acid in the vapour state[17]. Two absorption bands are observed, a medium strong $\pi \rightarrow \pi^*$ band below 190 nm and a weaker $n \rightarrow \pi^*$ band with a maximum at 216 nm. The corresponding anion shows strong absorption below 195 nm and a weak shoulder in the region 200–220 nm. A continuous change in the solvent composition from 0 to 100% acetonitrile in water results in a transition between these two spectra. As shown in Fig. 2, the formation of the shoulder in the

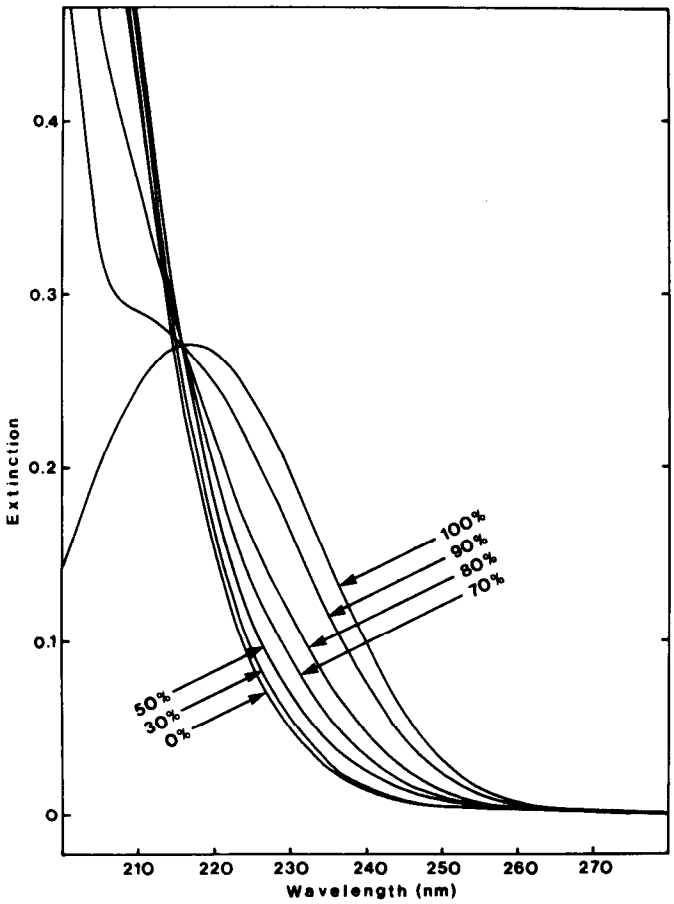

Fig. 2. Electronic spectra of 0.01 *M* TFA in different mixtures of water and acetonitrile. The concentration of the organic solvent is indicated.

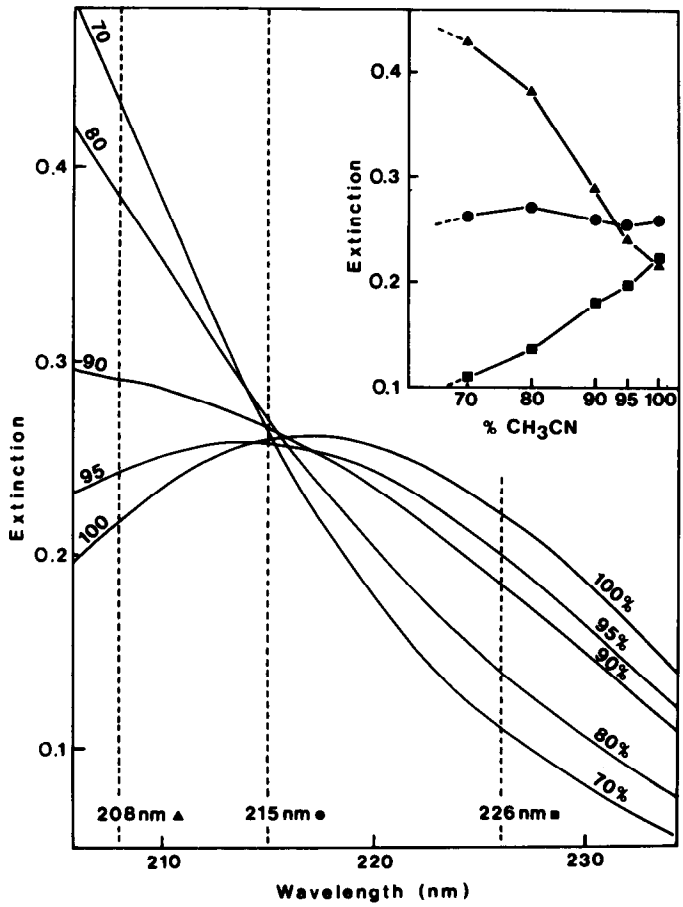

Fig. 3. Detail of Fig. 2. Hypothetical baselines at three different wavelengths (208, 215, 226 nm) were obtained by plotting the absorbance values *versus* the concentration of the organic solvent (insert).

range 220–260 nm proceeds discontinuously, which is due to the high sensitivity of the one-step dissociation equilibrium of TFA to the solvent polarity. In acetonitrile mixtures with high contents of water, TFA dissociates nearly completely due to its low pK_a (< 1), and the spectra are similar to those of the anion. In mixtures of lower polarity, more than 50% of acetonitrile, the dissociation equilibrium is significantly changed, and in mixtures with high amounts of acetonitrile the spectrum of the pure acid dominates. In the case of such an one-step dissociation equilibrium an isosbestic point should be recorded. However, in addition the $\pi \rightarrow \pi^*$ band of TFA shows a pronounced solvent dependency which interferes with the transition of the UV spectra, caused by the shift in the dissociation equilibrium. The combination of these two independent mechanisms precludes the observation of an isosbestic point. Nevertheless the spectra intersect in a small range at approximately 215 nm, and as shown in Fig. 2 the baseline of a reversed-phase gradient separation is largely descending when recorded below 215 nm and largely ascending when recorded above 215 nm. Thus

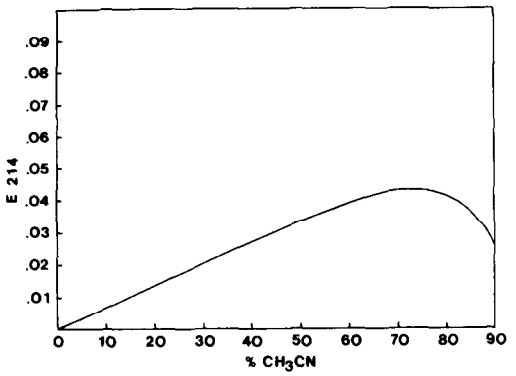

Fig. 4. Baseline of a linear solvent gradient using 0.01 *M* aqueous TFA *versus* 0.01 *M* TFA in acetonitrile, recorded at 214 nm.

when using UV for highly sensitive detection of peptides a wavelength close to 215 nm is recommended.

In Fig. 3 the most dispersing region for 70–100% acetonitrile is shown in detail between 200 and 230 nm. Three intersecting lines were drawn, at 208, 215 nm and 226 nm, and the resulting absorbance values were plotted *versus* the concentration of organic solvent (Fig. 3, insert). As already mentioned, the two baselines recorded below and above 215 nm have divergent and large slope parameters, and in both cases a low detector range setting is impossible. At 215 nm the baseline is nearly flat, except for a weak mid-gradient hump which is due to the fact that the spectra do not meet exactly in one point. This effect is especially pronounced at 214 nm, commonly used with zinc lamps in fixed wavelength detectors, and Fig. 4 shows a typical baseline

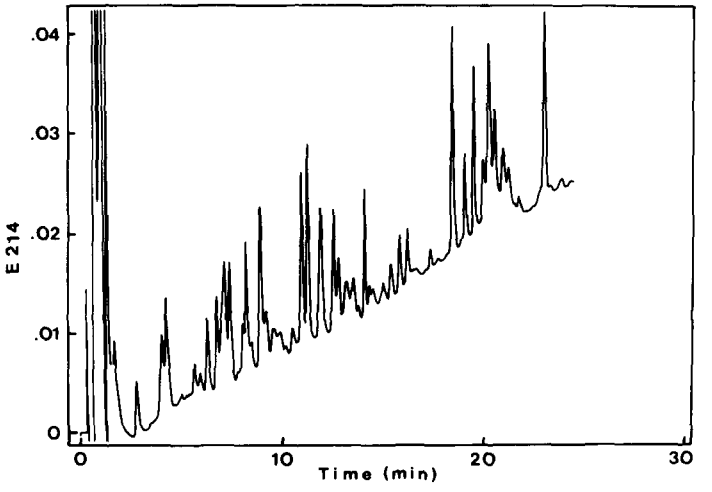

Fig. 5. Reversed-phase gradient separation of peptides, derived by tryptic digestion of 100 pmol of tick-borne encephalitis virus glycoprotein, which was isolated and purified as described in ref. 13. Column: 75 × 4.0 mm I.D. Stationary phase: Polygosil C_8 (Macherey-Nagel, Düren, F.R.G.). Mobile phase: solvent A, 0.01 *M* TFA; solvent B 0.01 *M* TFA in acetonitrile. Gradient: 0 to 90% B in 50 min, linear. Flow-rate: 2 ml/min. Wavelength: 214 nm.

at that wavelength. Nevertheless a minimum detector range setting of 0.04 a.u.f.s. (10 mm path length) is possible, which allows the detection of peptides in the picomole range (Fig. 5).

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