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Short Communication

Background noise reduction in post-column continuous-flow analysis combined with RPLC and computer-aided detection for the characterization of peptides*

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Introduction

Combination of LC-diode array with FIA as a post-column reaction system offers several possibilities for enhanced detection capability. Recognition of phenolic compounds can be achieved by a novel technique based on computer-aided photodiode array detection of the pH shifted solutes after post-column continuous-flow analysis [1, 2]. It is proposed that this technique may be used in the characterization of peptides, such as those generated from tryptic digests. Although phenylalanine (Phe). tyrosine (Tyr), and tryptophan (Trp) display characteristic chromophores above 240 nm, the extensive band overlap leads to complex composite profiles in peptide mixtures. Moreover, in samples of biological origin, additional absorbing constituents often contribute spectral interference. A common problem arises from the closely overlapping chromophores of Tyr and Trp between 270–280 nm. Although Trp, being rather more absorptive than Tyr, tends to dominate the spectrum, the labile phenolic group of Tyr permits its spectrum to be shifted to higher wavelength (ca. 293 nm) by raising the solution pH. Since the Trp chromophore is less pH-sensitive, this feature may form the basis for resolving peptides containing varying proportions of these amino acids. Baseline noise is a major limitation in hybrid LC–FIA systems, so it is essential that noise be minimized for LC–FIA to be used at its full potential. A number of options for achieving this have been explored and are discussed.

Experimental

Reagents

Methanol (HPLC grade, Rathburn Chemicals, Walkerburn, UK) was used as received. The 50-mM and 5-mM solutions of potassium dihydrogen phosphate (AnalaR BDH, Poole, UK), and 0.1 M potassium hydroxide solution (Convol BDH, Poole, UK) were prepared with glass-distilled water and filtered by Millipore 0.45-μm filters using an all-glass apparatus. All eluents and FIA carrier streams were degassed for 10 min in an ultrasonic bath under reduced pressure. Glycyl-L-tyrosine (Gly–Tyr) was obtained from Sigma (St Louis, MO, USA). Solutions of Gly–Tyr were prepared in distilled water.

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Apparatus

The chromatographic system used consisted of a Kratos SF 400 pump (Kratos Analytical, Warrington, Cheshire, UK) with a Rheodyne injection valve (Model 7125) provided with a 20-μl loop, together with an ABI 1000S Diode-array detector (Applied Biosystems, Ramsey, NJ, USA) and a BD 40 Chart Recorder (Kipp & Zonen, Delft, Holland). The ABI 1000S was interfaced, via a RS-232C link, to an IBM compatible Personal Computer (Elonex, Bradford, UK), with a Panasonic KX-P1081 printer (supplied by ABI) and a HP-ColorPro Plotter (Hewlett Packard, Cheadle Heath, Stockport, Cheshire, UK). Data acquisition and manipulation was performed using LabCalc software (Galactic Industries Corporation, Salem, NH, USA).

The post-column reaction system consisted of a Gilson (Anachem, Luton, UK) Minipuls 3 peristaltic pump, together with Elkay Accurated tubing (Laboratory Products (UK) Ltd, Basingstoke, UK) and two single-bead string reactors (SBSR) (100 cm × 0.8 mm i.d. and 50 cm × 0.8 mm i.d.). All connections were made using 0.5 mm i.d. PTFE tubing. The FIA carrier stream was combined with the post-column eluent stream using a suitable T-piece (Anachem). In the optimized system configuration, the two carrier streams were combined using a suitable Y-piece (Anachem).

LC–FIA conditions

A stainless-steel column (250 × 4.6 mm i.d.) packed with Techsphere ODS (HPLC Technology Ltd, Macclesfield, UK) was used for a RPLC method previously developed for the separation of aromatic amino acids [3]. The optimized mobile phase, pumped at 1.0 ml min^{-1}, consisted of methanol–5 mM KH_2PO_4 (pH 4) buffer, (10:90, v/v). Detection was effected using the diode-array at 272 nm. The optimized FIA system consisted of 0.03 ml m^{-1} tubing, a pump speed of 10 rpm, delivering 0.05 ml min^{-1} from each tube. 0.1 M potassium hydroxide was used to effect the post-column pH shift.

Results

Previous reports in the literature, have used post-column continuous-flow analysis combined with RPLC to characterize microgram quantities of phenolic plant extracts on column [1, 2]. Peptide analysis requires the recognition of sub-microgram quantities with low absorbance detection levels. Using the system configured as described in ref. 1 (Fig. 1a), an unworkable level of background noise was observed using sub-microgram quantities of amino acids and peptides and various FIA carrier streams: mobile phase, buffer, and potassium hydroxide solution. Experiments initiated to minimize this noise yielded the following observations.

Pulsation could be reduced by mixing two identical FIA carrier streams prior to mixing with the LC stream (Fig. 2). This resulted in the system being re-configured, as shown in Fig. 1(b).

Because potassium hydroxide carrier solution absorbs more strongly than mobile phase carrier solution at low UV wavelengths, greater noise results on adding alkali post-

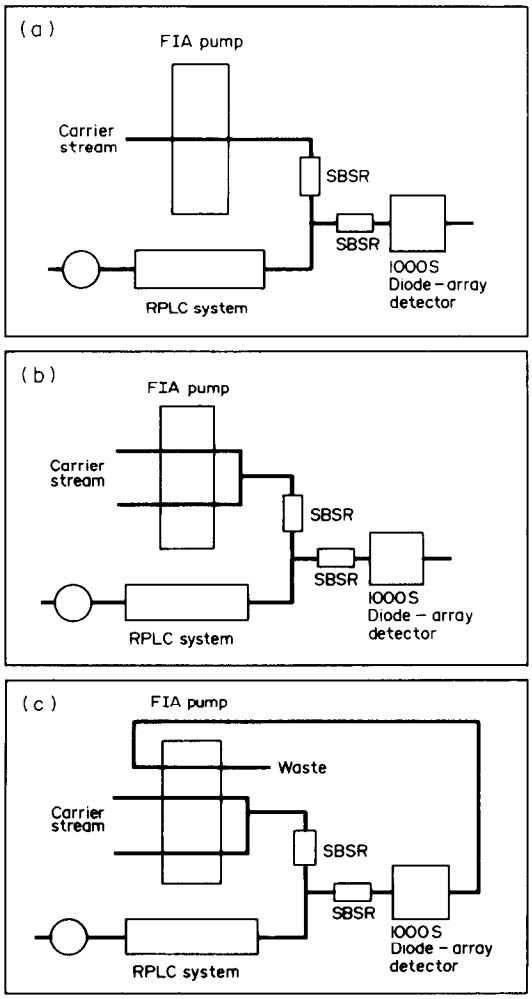

Figure 1
Post-column continuous-flow analysis combined with RPLC; system configuration.

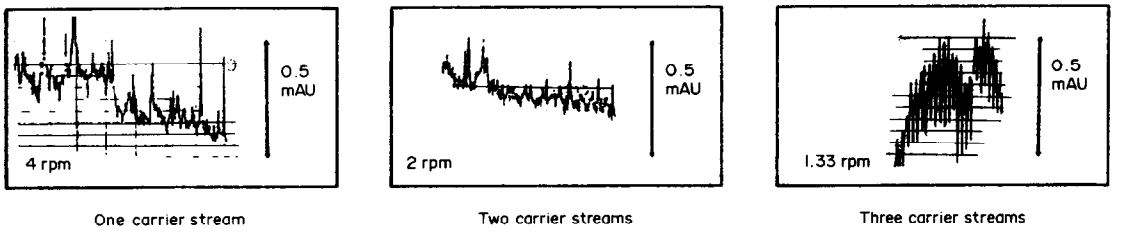

Figure 2
The variation of background noise with increase in the number of FIA carrier streams. Addition of equal amounts of mobile phase post-column using the FIA pump and 0.8 ml m^{-1} tubing.

column. To minimize spectral differences between the mobile phase and the alkali, the concentration of potassium hydroxide was reduced to an optimum of 0.1 M (Fig. 3). The buffering capacity of the phosphate buffer was similarly reduced to facilitate pH-shifting using low volumes of alkali.

Variation in the FIA pump rotation speed and tube diameter were investigated using 0.1 M potassium hydroxide. Baseline noise (primarily pump pulsation) was reduced by reducing the tube diameter; with larger diameter tubing it could be reduced by increasing the pump speed (Fig. 4). Repetition of these experiments revealed large day-to-day variations in noise levels. This was minimized by using small diameter tubing, where the maximum variation in noise observed (with 0.03 ml m^{-1} tubing) was approximately two-fold (Fig. 5). The cause of this variation has not been elucidated, but may arise from slight conformational differences in the peristaltic tubing when clamped onto the pump, since such variation has not been observed within 1 day.

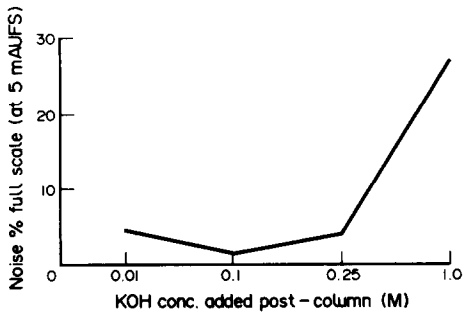

Figure 3
The variation of background noise with potassium hydroxide concentration, added post-column.

Re-routing the eluent back through the peristaltic pump is commonly used in FIA systems to reduce background noise. The implementation of this technique in combined RPLC–FIA systems was investigated using the system configured as illustrated in Fig. 1(c). It was observed that there was a slight reduction in background noise (Fig. 5). Since the day-to-day variation in noise was far greater, the technique of re-routing the eluent was found to be non-beneficial.

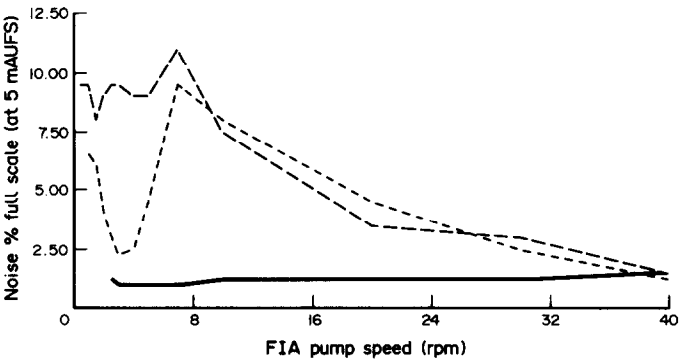

Figure 4
The variation of background noise with pump speed and tube diameter. ——, 0.1 ml m^{-1}; ---, 0.32 ml m^{-1}; – –, 0.8 ml m^{-1} tubing.

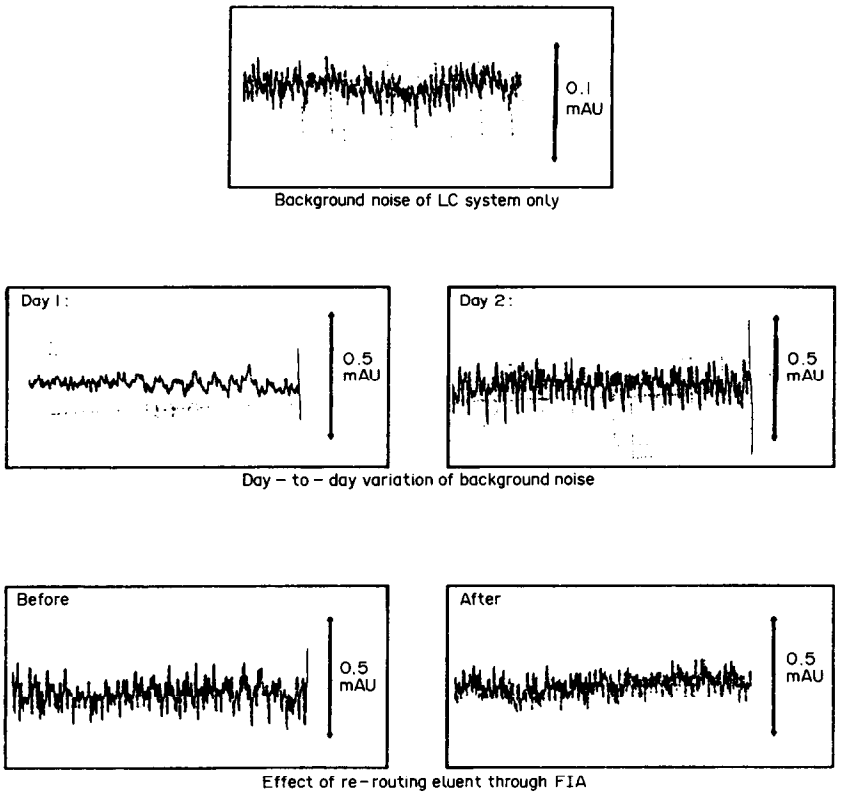

Figure 5
Background noise of the optimized system, illustrating day-to-day variation and the effect of re-routing the eluent through the peristaltic pump.

Discussion

The use of hybrid LC–FIA systems offers an inexpensive and flexible methodology for enhanced detection capability. The experiments described briefly above have defined the limitations of this technology with regard to minimizing the baseline noise ensuring maximum sensitivity.

Using the optimum conditions at 272 nm as described above, the typical detection limit (allowing spectral manipulation) of a peptide containing a tyrosyl residue was found to be 50 ng on column (Gly–Tyr).

The utility and relevance of post-column pH shifting to generate difference spectra for the characterization of oligopeptides (e.g. in tryptic mapping) is currently being explored.

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