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Gradient elution isotachophoresis with direct ultraviolet absorption detection for sensitive amino acid analysis

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ABSTRACT

This work demonstrates coupling of the newly described electrophoretic enrichment technique of gradient elution isotachophoresis (GEITP) to a low-cost, conventional ultraviolet absorbance detector to realize sensitive measurements with a universal detector, eliminating the need for fluorescent analytes or derivatization. The effects of various parameters on enrichment were studied, including current density varied by leading electrolyte concentration, current density varied by applied electric field, and counter-flow acceleration across varying capillary inner diameters. Optimized parameters were applied to the enrichment and separation of the amino acids tryptophan (Trp) and tyrosine (Tyr). Limits of detection for Trp and Tyr were 51 and 215 nM, respectively, reflecting sensitivity enhancements of 860- and 1900-fold. Analysis times were less than 6 min, and peak height RSDs were less than 4%. A demonstration of enrichment and separation of these amino acids from artificial cerebrospinal fluid is additionally shown as a first step to realizing biochemical monitoring by GEITP.

1. Introduction

CE is gaining much attention and admiration as a separation technique in a myriad of fields, including chemistry, biology, and the pharmaceutical sciences; CE has truly matured from mainly a research technique to a routine method due to the ability to perform high-resolution separations with small sample requirements. However, in order to perform sensitive analyses, frequently CE requires the use of laser-induced fluorescence (LIF), necessitating either natively fluorescent analytes or derivatization techniques. Universal detectors, such as UV absorption or refractive index, suffer from poor concentration limits of detection (LODs) [1,2]. The detection problem has been exacerbated when translated to microfluidic devices, which can employ channel widths down to the nanometer regime, thick substrates, and substrates with high autofluoresence or UV absorption characteristics [3]. As an alternative to improved detection schemes, pre- or on-line concentration methods can be utilized to enrich analytes above the detection limits of the common detectors used with CE instruments.

Many methods have been developed for sample enrichment in CE (as reviewed in references [4–6]), including: isotachophoresis (ITP); field-amplified sample stacking or injection (FASS or FASI); sweeping; pH junction methods; gradient electrofocusing; and solid-phase extraction (SPE). Electrophoretically based preconcentration, which achieves enrichment through differences in electrophoretic velocities of analytes during separation, can be loosely grouped into two categories. The first, equilibrium electrofocusing, relies on creating a point within the separation region where analytes experience zero net velocity. Velocity gradients can be achieved through pH, as in isoelectric focusing (IEF), electric fields, as in electric field gradient focusing (EFGF), or conductivity, as in conductivity or temperature gradient focusing (CGF and TGF, respectively) [5,7]. While IEF is the most popular of the methods, it is typically limited to proteins and peptides, which have accessible isoelectric points, while the other gradient methods have proven to be more universal, with concentration improvements on the order of 10–10,000-fold.

The second category, dynamic enrichment methods, also utilize velocity gradients but without a null velocity point, and include FASS, FASI, and sweeping methods. Enrichments typically on the order of 10–5000-fold have been noted. While ITP with a counter-flow could be considered an equilibrium method, it is more frequently employed in CE as a transient preconcentration step and has been demonstrated in both single and coupled-column formats, obtaining 100–500,000-fold concentration enhancements [4,8].

Gradient elution moving boundary electrophoresis (GEMBE) is a recently described separation technique which combines continuous sample injection with a hyrdodynamically controlled variable bulk counter-flow in a capillary or microchannel [9]. As the counter-flow is reduced from high to low, ionic analytes are sequentially

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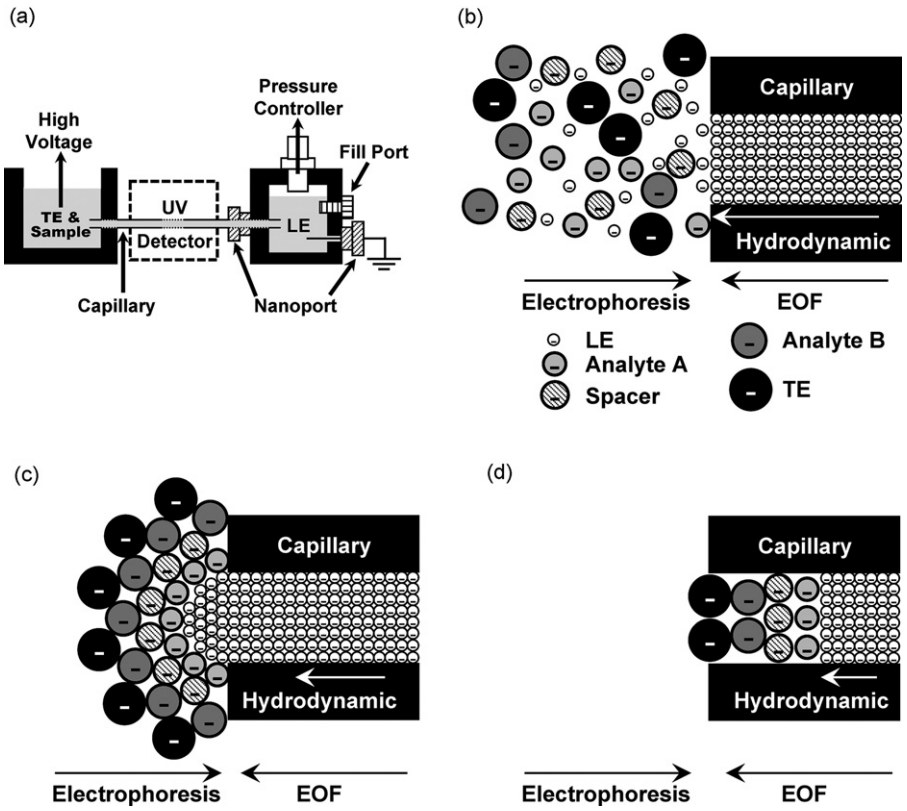

Fig. 1. Instrumentation and concept of GEITP. (a) Schematic of instrument used for performing GEITP with UV detection. A 15-cm capillary connected a pressure controlled and grounded reservoir containing 750 μL of leading electrolyte solution to a 100-μL sample reservoir maintained at high voltage. Detection was performed 6 cm from sample inlet. (b) In GEITP the initial bulk flow (hydrodynamic and electroosmotic, EOF) is high enough that LE is pushed into the sample reservoir. (c) It is hypothesized that the LE creates an ionic interface upon which analytes and trailing electrolyte (TE) are enriched as the counter-flow is reduced. (d) As the hydrodynamic flow is further reduced, the interface and analyte zones are pulled into the capillary forming enriched zones based on order of electrophoretic mobility where they will be detected on-capillary. Zone resolution can be achieved using non-detectable spacer ions.

eluted onto the column and detected as boundary interfaces. GEMBE exhibits the advantages of using short separation lengths (typically 1 cm or less) and the elimination of the need to form an injection plug, making it highly amenable to high-throughput and high-density microdevices. GEMBE was further improved by combining an ITP enrichment step with gradient elution (GEITP). In GEITP, leading electrolyte (LE) is introduced into the counter-flow buffer and terminating electrolyte (TE) into the sample matrix [10]. Enrichment can begin outside the capillary at a counter-flow rate great enough to push LE into the sample reservoir, which forms an ionic interface near the capillary inlet. Analytes form ITP-enriched zones at the discontinuous buffer interface. As the counter-flow is reduced, the enriched analytes are introduced onto the column for detection. Analyte resolution can be achieved through the use of non-detectable spacing ions (Fig. 1) [11,12].

GEITP achieves both short length separations (a 30-μm separation has been demonstrated) without defining a discrete injection and rapid enrichment (up to 100,000-fold in 8 min) for trace analyses. Additionally, as compared with other ITP methods, GEITP does not necessitate any buffer or polarity switching, yielding a more reliable and automatable system. The initial description of GEITP studied various parameters' effects on sensitivity enhancement using fluorescence microscopy, including initial counter-flow velocity, counter-flow acceleration, electric field strength, and LE concentration. In this work we have coupled a low-cost single wavelength UV detector to GEITP to demonstrate the applicability of the method with universal detection. Further systematic studies were performed to optimize the enrichment process. Specifically, the effect of current density varied by LE concentration, current density varied by electric field, and counter-flow acceleration were compared across varying capillary inner diameters. Two significant deviations from the previous work's methodology were undertaken. First, the parametric study was constrained to maintaining a constant enrichment time; the prior study did not normalize enrichment to equivalent enrichment times, yielding ambiguous results. Second, lower mobility analytes, tryptophan (Trp; $\mu_{ep} = -25.4 \times 10^{-9}\ m^2/(V\,s)$) and tyrosine (Tyr; $\mu_{ep} = -40.0 \times 10^{-9}\ m^2/(V\,s)$) [13] were utilized, as compared with the high mobility, doubly charged carboxyfluorescein anion; use of slower analytes allowed for more realistic limits of the method, as compared with the use of ideal, highly mobile analytes.

Trp, an essential amino acid, and Tyr, a non-essential amino acid, have been extensively studied due to their prevalence in biological fluids, such as cerebrospinal fluid (CSF) and serum, and in common food and beverages. Current methods to measure Trp and Tyr by CE-based separations include: LIF with fluorescence derivatization, with Trp LOD = 33 nM [14]; direct LIF, Trp LOD = 0.15 nM and Tyr LOD = 50 nM [15]; contactless conductivity, 3–50 μM LODs [16,17]; and electrochemical methods, 150–610 nM LODs [18,19]. Dankova et al. described an ITP-CE method for Trp enantiomer measurements at 220 nm by UV [20]; while noting a 10 nM LOD, the method required an hour of analysis time to load a large sample volume. Recently, Qu et al. developed an amino acid enrichment technique using the interaction of zwitterions with etched fused-silica capillary surfaces; analysis times were on the order of 10 min with 1 min of sample injection [21]. The method achieved a 40-nM LOD for Trp but required multiple buffer and voltage switching steps to load and separate mixtures and exhibited relatively poor peak height