JOURNAL OF SEPARATION SCIENCE

J. Sep. Sci. 2002, *25*, 1346–1350

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On-column pre-concentration of alcohol dehydrogenase in capillary electrophoresis

The analysis of alcohol dehydrogenase (ADH) at low concentration using capillary electrophoresis is described. Several simple and effective ways to improve detection limits and sensitivity are investigated. These include large volume sample stacking, head column field amplified sample stacking, and sweeping. Results indicate that by using a combination of head-column field amplified sample stacking and sweeping, fluorescently labelled alcohol dehydrogenase can be pre-concentrated online by dissolving samples in water or other low conductivity matrices, and injecting into a high conductivity micellar buffer. The abrupt changes in conductivity cause narrowing of the analyte length and thus enhance the detection sensitivity. Combination of this approach with laser induced fluorescence detection yields a limit of detection of 5×10^{-13} M. Both qualitative and quantitative aspects of this method are investigated.

Key Words: Sweeping; Stacking; Capillary electrophoresis; Alcohol dehydrogenase; Pre-concentration

Received: May 29, 2002; accepted: July 3, 2002

1 Introduction

Capillary electrophoresis (CE) methods afford high-speed and high-efficiency separations, utilise relatively inexpensive and long lasting capillary columns, and consume small volumes of sample and reagent. Since sample can be introduced into the capillary via electrokinetic mechanisms extremely small volumes (pL–nL) can be injected easily. This results in excellent component resolution and also a high mass sensitivity. Unfortunately, conventional absorbance detection methods provide relatively poor concentration detection limits when compared to high performance liquid chromatography (HPLC) methods due to the small injection volumes and short optical pathlengths (25–75 μm) encountered in most systems. This prohibits the use of CE for trace-level analysis.Laser induced fluorescence (LIF) methods have become increasingly popular recently due to the availability of a diversity of highly stable, low cost laser sources [1]. These provide for highly sensitive detection of many analytes at low concentration.

Alcohol dehydrogenase (ADH) is an important protein found in a wide range of organs such as the liver and the lining of the stomach. ADH is primarily responsible for the dehydrogenation of alcohols to aldehydes, whilst also making important modifications to retinol, steroids, and fatty acids [2, 3]. Moreover, larger forms of ADH are found in yeast and many bacteria and commonly used for alcohol fermentation [4, 5]. ADH is a relatively stable protein over a wide range of temperature and pH, with well-characterised activity [6, 7]. In recent studies, it has also been shown that the addition of a fluorescent moiety (for example Cy5, Amersham Pharmacia Biotech UK Ltd.) [8, 9] to surface amines leads to no significant changes in activity [10]. Consequently, through use of derivatisation methods, CE can be employed to analyse ADH with good reproducibility and detection limits.

To further increase detection sensitivity, on-column preconcentration methods are an attractive option. Field-amplified sample stacking (FASS), first demonstrated by Burgi and co-workers results from the differential movement of sample ions at the boundary between low and high conductivity zones [11, 12]. Briefly, sample is initially contained within a low conductivity solution whilst the background buffer region is of high conductivity. Upon application of a voltage, the low conductivity region experiences a higher electric field in relation to the background buffer region. Consequently, sample ions move more quickly in the low conductivity region than in the high conductivity region. The abrupt change in sample ion velocity across the concentration boundary results in a reduction of sample zone length and therefore sample concentration. There are two primary modes of FASS; large volume sample stacking (LVSS) and head column sample stacking (HCFASS). In LVSS, samples are introduced into the capillary hydrodynamically and stacked

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Abbreviations: Alcohol dehydrogenase, ADH; large volume sample stacking, LVSS; field amplified sample stacking. FASS; head column field amplified sample stacking, HCFASS; pseudostationary phases, PSPs.

1615-9306/2002/1812–1346$17.50+.50/0

electrokinetically [13–16]. HCFASS, first described by Zhang et al., involves both injection and stacking of sample by the application of an electric field [17–19]. HCFASS allows the addition of a large number of analyte molecules without introduction of a significant amount of solvent. Cations can be introduced into the capillary using positive polarity whilst anions are added under negative polarity [20].

Recently, a new and highly efficient method for sample pre-concentration, termed sweeping, was introduced by Quirino and Terabe [21–28]. The underlying phenomenon, initially observed by Gilges [29], is defined as the picking and accumulating of analyte molecules by a pseudostationary phase (PSP) that enters and fills the sample zone upon application of a voltage. This results in the stacked PSP carrying neutral or positive charged analytes at the interface between sample and running buffer zones. Concentration enhancement for non-ionic hydrophobic solutes of up to 5000-fold have been observed using sodium dodecyl sulfate (SDS) micelles [21].

In this communication, we report the analysis of ADH using CE and LIF detection. Large volume sample stacking, head column field amplified sample stacking, and sweeping are further assessed to reduce concentration limits of detection.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical reagent grade and ethanol and acetonitrile were HPLC grade. All solvents were tested for extraneous fluorescence prior to use. Alcohol dehydrogenase, from baker's yeast (ADH, MW 141 kDa) was obtained from Sigma-Aldrich (Gillingham, Dorset, UK). All aqueous solutions were made up using high-resistivity (18 MΩ) de-ionised water (Elga, Buckinghamshire, UK) and orthophosphoric acid, DMF, sodium hydroxide, acetonitrile, methanol, and ethanol (Merck Chemicals, Dorset, UK) were used as received. BODIPY succinimidyl ester (Molecular Probes Europe BV, The Netherlands) was made up to 10 mg/mL and stored at −40°C.

2.2 Protein labelling

The procedure for the labelling of protein with BODIPY succinimidyl esters and FITC followed a protocol described elsewhere [30]. For standard derivatisation, 50–100 μL of labelling dye (10 mg/mL in DMF) is added to 1 mL of protein (10 mg/mL in 0.05 M phosphate buffer, pH 9.0) and mixed for 4 to 24 hours at 4°C. The resulting mixture is then filtered using a gel filtration column (Sephadex® G-25, Amersham Biosciences UK Limited, Buckinghamshire) and diluted in the desired solvent for CE analysis.

2.3 Preparation of sample solution and running buffer

For studies assessing sweeping, BODIPY succinimidyl ester labelled ADH is prepared in 0.05 M phosphate buffer (pH 2.8). For FASS and combined sweeping/HCFASS studies, BODIPY succinimidyl ester labelled ADH is prepared in water, HCl (0.75 μM) and H_3PO_4 (0.75 μM). Phosphate running buffer was prepared by mixing appropriate aliquots of 0.5 M H_3PO_4, 1 M NaOH, and 1 M SDS to the desired concentration and pH.

2.4 Capillary electrophoresis

All experiments were performed on an P/ACE 2050 capillary electrophoresis system (Beckman Coulter, Buckinghamshire, UK) equipped with an argon ion laser operating at 488 nm. A notch filter (488 nm) was employed at the detection window to eliminate transmission of excitation radiation. All equipment control and data recording was performed using P/ACE Station software (Beckman Coulter, Buckinghamshire, UK). Separations were performed in un-coated fused-silica capillaries with an internal diameter of 50 μm (Supelco, Dorset, UK) and an effective (total) length of 20 (27) cm. A constant voltage of 16 kV (600 V/cm, current, 93–104 μA) was used for all analyses with the positive electrode located on the sampling side. Sample injection was effected by applying a positive voltage of 8 kV for 40 s and pre-injecting a water plug (by immersion in a water vial for 5 s). The temperature of the capillary was maintained at 25°C during the course of all experiments. Prior to use, new capillary columns were preconditioned by rinsing with 0.1 M NaOH for 30 min, followed by water for 10 min and running buffer for 5 min using positive pressure. In addition, capillaries were rinsed with NaOH for 5 min followed by running buffer for 2 min between separations. To prevent capillary blockage, buffers and sample solutions were filtered through 0.2-μm filters (Millisart, Fisher Scientific, Loughborough, UK) prior to entry. Running buffer and analyte concentrations, sample matrix composition and concentrations were as stated in each experiment.

3 Results and discussion

3.1 Large volume sample stacking of ADH

Large Volume Sample Stacking (LVSS) with ADH (20 nM) using HCl (0.75 mM) as the low conductivity sample matrix was investigated without the removal of sample matrix by polarity switching. A typical electrophoregram of

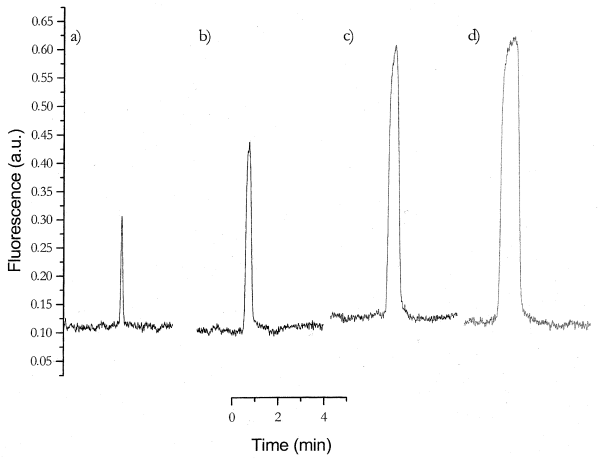

Figure 1. Analysis of ADH (20 nM) by CE with LVSS. (a) No LVSS. LVSS with HCl (0.75 mM) with (b) 30 s, (c) 60 s, (c) 90 s (hydrodynamic) injection time. Separation conditions: uncoated capillary 20 (27) cm × 50 μm ID; 0.05 M phosphate, pH 2.8 running buffer; separation voltage, 16 kV; temperature, 25°C.

ADH is shown in **Figure 1.a**. The concentration efficiency is expressed as the ratio of the solute concentration in the sample zone and its concentration in the stacking area [19]. As injection times are increased (**Figure 1.b–d**) the peak signal intensity increases and then levels off due to column overloading. The achieved concentration efficiency using LVSS is two. Conventionally, the sample matrix removal step is complicated, involving polarity switching and precise current monitoring in order to avoid losing any analyte. A better option for online sample preconcentration is (HCFASS).

3.2 Head-column field amplified sample stacking of ADH

ADH was pre-concentrated using HCFASS (**Figure 2.a**). The BODIPY labelled ADH (0.5 nM), dissolved in water (**Figure 2.aii**) and phosphoric acid (750 μM) (**Figure 2.aiii**), were injected electrokinetically (8 kV) and separated (16 kV) in phosphate buffer (50 mM, pH 2.8). Both conditions show considerable enhancement in signal intensity. A concentration factor of up to 2 orders of magnitude was achieved. The long injection time (90 s) allows a large number of ADH molecules to enter the capillary at high velocity and stack at the interface between the high and low conductivity zones. No apparent band broadening and loss in efficiency were observed.

3.3 Sweeping in a homogeneous electric field

Sweeping of ADH under a homogeneous electric field was investigated using SDS. An electrophoregram showing the normal separation ADH (0.5 nM) is shown in **Figure 2.bi**. The solute was dissolved in a phosphate buffer (50 mM, pH 2.8) and injected electrokinetically (8 kV) into a phosphate running buffer (50 mM, pH 2.8) containing SDS. The conductivity of the sample matrix was adjusted to match the separation buffer, so no HCFASS occurs under these conditions. Hence, the pre-concentration mechanism was purely sweeping. When a voltage is applied, ADH molecules enter the capillary under the influence of EOF. Due to the negative charge on the SDS, it travels towards the positive electrode (the inlet) and intrudes into the sample zone. This results in ADH molecules being trapped and accumulated at the interface between the running buffer and sample matrix after enter-

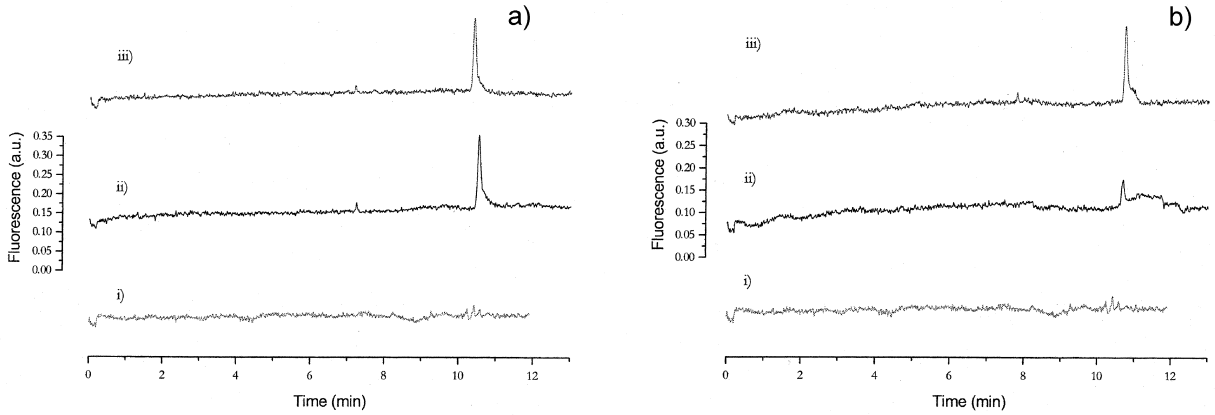

Figure 2. a) Analysis of ADH (0.5 nM) by CE with HCFASS. (i) No HCFASS. HCFASS with (ii) water and (iii) phosphoric acid (0.75 mM) as the sample matrix. Separation conditions: uncoated capillary 20 (27) cm × 50 μm ID; 0.05 M phosphate, pH 2.8 running buffer; electrokinetic injection, 8 kV, 90 s; separation voltage, 16 kV; temperature, 25°C. b) Analysis of ADH (0.5 nM) by CE with Sweeping. (i) No sweeping, (ii) Sweeping with 20 mM SDS and (iii) Sweeping with 200 mM SDS in the running buffer. Separation conditions: uncoated capillary 20 (27) cm × 50 μm ID; sample matrix, 0.05 M phosphate, pH 2.8; 0.05 M phosphate, pH 2.8 running buffer with (b) 20 mM SDS and (C) 200 mM SDS; electrokinetic injection, 8 kV, 90 s; separation voltage 16 kV; temperature, 25°C.

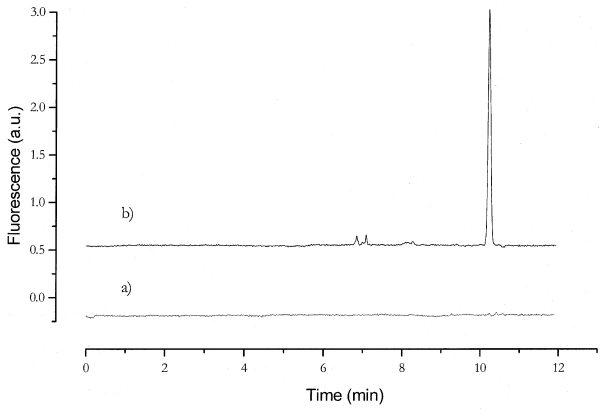

Figure 3. Analysis of ADH (0.5 nM) by CE with HCFASS and sweeping. (a) No sweeping or HCFASS. (b) Sweeping and HCFASS. Separation conditions: uncoated capillary 20 (27) cm × 50 μm ID; sample matrix, water; 0.05 M phosphate, pH 2.8 running buffer with 200 mM SDS; electrokinetic injection, 8 kV, 90 s; separation voltage 16 kV; temperature, 25°C.

ing the capillary. The signal intensity increases significantly with an increase in SDS concentration and maximises at 200 mM (**Figure 2.biii**) yielding a concentration factor of up to two orders of magnitude.

3.4 Incorporation of HCFASS and sweeping with ADH

To further increase sample pre-concentration a combination of both HCFASS and sweeping was used in the analysis of ADH. ADH (0.5 nM) was dissolved in water. Electrokinetic injection (8 kV) was carried out prior to separation (16 kV) in a phosphate buffer (50 mM, pH 2.8) with SDS (200 mM). On application of a drive voltage, the ADH molecules enter the capillary at high velocity. Once analyte molecules have entered the capillary, they slow down and stack at the interface between the high and low conductivity zones. At the same time, SDS in the running buffer is driven towards the positive electrode (near the inlet) and therefore intrudes the ADH molecules. This yields additional concentration of the analyte by sweeping. The combination of both effects produces a concentration factor of approximately 4 orders of magnitude. **Figure 3** illustrates analysis of ADH (0.5 nM) using this combined method. The limit of detection for the current system, based on a minimum signal to noise ratio of three, was found to be 5×10^{-13} M for ADH. Reproducibility data were determined by performing five consecutive analyses of ADH (0.5 nM). The mean standard deviation and RSD (%) for migration time and peak height were 10.41 minutes, 1.077% and 2.682, 2.189% respectively. Furthermore, it is noted that whilst the migration time of ADH is identical when using LVSS or HCFASS, it is slightly increased when sweeping is used for sample pre-concentration. This effect is primarily due to changes in the electrophoretic mobility of ADH caused by association with SDS molecules.

4 Conclusions

The studies presented herein demonstrate the analysis of ADH extracted from yeast using capillary electrophoresis. ADH molecules were fluorescently labelled with a BODIPY dye and detected by LIF. The fluorescence signal is enhanced by on-capillary pre-concentration using two mechanisms. The cumulative effect of sweeping using SDS was shown to narrow the length of sample zone prepared in a non-micellar aqueous buffer. This enables over 100-fold enhancement in LOD. When the analyte is dissolved in a low conductivity matrix such as water, sample stacking occurs. Consequently, the combination of sweeping and HCFASS has proved to yield a concentration factor of up to 4 orders of magnitude with a LOD of approximately 5×10^{-13} M and no apparent loss in resolution.

Acknowledgement

The authors would like to thank Dr. Catherine M. Halliwell and Dr. Chao-Xuan Zhang for useful discussions. S.A. Leung is grateful to AstraZeneca for her PhD studentship.

References

[1] E. Gassmann, J.E. Kuo, R.N. Zare, *Science* **1985**, *230*, 813–814.

[2] X. Pares, A. Moreno, J.M. Peralba, M. Font, L. Bruseghini, A. Esteras, *Meth. Findings Exper. Clin. Pharmacol.* **1991**, *13*, 37–42.

[3] M. Orellana, E. Valdes, J. Fernandez, R. Rodrigo, *Gen. Pharmacol.* **1998**, *30*, 719–723.

[4] S. Trivic, V. Leskova, G.W. Winston, *Biotechn. Lett.* **1999**, *21*, 231–234.

[5] S. Trivic, V. Leskovac, *J. Serb. Chem. Soc.* **2000**, *65*, 207–227.

[6] H.K. Seitz, U.A. Simanowski, G. Egerer, R. Waldherr, U. Oertl, *Digestion* **1992**, *51*, 80.

[7] S. Vakevainen, J. Tillonen, M. Blom, H. Jousimies-Somer, M. Salaspuro, *Alcoholism-Clin. Exper. Res.* **2001**, *25*, 421–426.

[8] H.Y. Yu, *Nucleic Acids Res.* **1994**, *22*, 3226–3232.

[9] R.B. Mujumdar, *Bioconjugate Chem.* **1993**, *4*, 105–111.

[10] C.M. Halliwell, *unpublished material*.

[11] R.L. Chien, D.S. Burgi, *J. Chromatogr.* **1991**, *559*, 141.

[12] D.S. Burgi, R.L. Chien, *Anal. Chem.* **1991**, *63*, 2042–2047.

[13] N.E. Baryla, C.A. Lucy, *Electrophoresis* **2001**, *22*, 52–58.

[14] G.B. Harland, G. McGrath, S. McClean, W.F. Smyth, *Anal. Commun.* **1997**, *34*, 9–11.

[15] Y. He, H.K. Lee, *Anal. Chem.* **1999**, *71*, 995–1001.

[16] J.P. Quirino, S. Terabe, *Electrophoresis* **2000**, *21*, 355–359.

[17] C.X. Zhang, Y. Aebi, W. Thormann, *Clinical Chem.* **1996**, *42*, 1805–1811.

[18] C.X. Zhang, W. Thormann, *Anal. Chem.* **1996**, *68*, 2523–2532.

[19] C.X. Zhang, W. Thormann, *Anal. Chem.* **1998**, *70*, 540–548.

[20] S.A. Leung, A.J. de Mello, *J. Chromatogr. A* **2002**, *979*, 171–178.

[21] J.P. Quirino, S. Terabe, *Science* **1998**, *282*, 465–468.

[22] J.P. Quirino, S. Terabe, *Chromatographia* **2001**, *53*, 285–289.

[23] J.P. Quirino, S. Terabe, *Anal. Chem.* **2000**, *72*, 1023–1030.

[24] J.P. Quirino, S. Terabe, P. Bocek, *Anal. Chem.* **2000**, *72*, 1934–1940.

[25] J.P. Quirino, Y. Iwai, K. Otsuka, S. Terabe, *Electrophoresis* **2000**, *21*, 2899–2903.

[26] J.P. Quirino, S. Terabe, *Anal. Chem.* **1999**, *71*, 1638–1644.

[27] L.P. Quirino, S. Terabe, K. Otsuka, J.B. Vincent, G. Vigh, *J. Chromatogr. A* **1999**, *838*, 3–10.

[28] J.P. Quirino, S. Terabe, *J. High Resol. Chromatogr.* **1999**, *22*, 367–372.

[29] M. Gilges, *Chromatographia* **1997**, *44*, 191–196.

[30] G.T. Hermanson, *Bioconjugate Techniques*. Academic Press, New York 1996.

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