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Review

On-line preconcentration strategies for trace analysis of metabolites by capillary electrophoresis

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Abstract

Analysis of low concentrations of metabolites is required for new fields of biological research, such as metabolomics. In this review, recent work in our laboratory aimed at developing improved strategies for on-line sample preconcentration of metabolites by capillary electrophoresis (CE) is presented. Dynamic pH junction, sweeping and dynamic pH junction-sweeping represent three complementary methods for electrokinetic focusing of large volumes of sample directly on-capillary. Focusing selectivity and focusing efficiency are two factors that can be used to assess the suitability of each method for different classes of metabolites. Buffer properties can be selected to enhance the focusing of specific types of metabolites based on knowledge of the analyte physicochemical properties. The application of on-line preconcentration CE for trace analysis of metabolites in real samples of interest, such as biological fluids and cellular extracts, is also demonstrated. Under optimum conditions, up to three orders of magnitude increase in concentration sensitivity can be realized for several classes of metabolites, including catecholamines, purines, nucleosides, nucleotides, amino acids, steroids and coenzymes. Recent work on hyphenating on-line preconcentration with multiplexed CE is highlighted as a promising platform for sensitive and high-throughput analyses of metabolites.

Keywords: Reviews; Metabolites; On-line preconcentration; Dynamic pH junction-sweeping; Sweeping

Contents

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1. Introduction

Since the completion of the human genome project, there is increasing interest in understanding the functional relation of gene products to higher levels of expression in an organism such as phenotype [1,2]. This has given impetus to emerging fields of post-genomic research that include the transcriptome (RNA), proteome (protein) and metabolome (metabolites). The metabolome represents hundreds of low-molecular-mass molecules that play key roles in metabolic processes in the cell, such as amino acids, nucleotides, carboxylic acids, steroids, carbohydrates, lipids, vitamins, inorganic ions and coenzymes. Recently, two related fields of metabolite research have been initiated; metabolomics [3] and metabonomics [4] refer to the comprehensive analysis of metabolites within cells and biological fluids, respectively. In contrast to the more established fields of genome and proteome, there currently exists no single method to analyze the full complement of intracellular metabolites. Metabolites are characterized by a large number of chemically diverse analytes that vary over a wide concentration range, which presents unique challenges for future separation and detection methods, such as liquid chromatography using NMR [1,4] or mass spectrometry [2,3].

We are currently interested in applying capillary electrophoresis (CE) as an alternative technique for the rapid and highly efficient separation of intracellular metabolites [5,6]. Commercial instruments for multiplexed CE using UV absorbance detection have recently been developed [7,8], which can be applied to the high-throughput analysis of a large number of UV-active metabolites. However, CE suffers from poor concentration sensitivity when using UV detection because of the small injection volumes (typically $<$1% capillary length) and narrow optical path length. This presents a significant obstacle for routine analyses of sub-μM levels of metabolites in real samples by CE. Alternative detector formats such as laser-induced fluorescence and electrochemical detection offer greater sensitivity than UV absorbance, but are applicable to fewer types of native analytes unless modified by chemical derivatization. Off-line preconcentration by solid-phase extraction [9] can also be used to enrich analytes, as well as offering a way for sample cleanup prior to CE analysis. However, automation of solid-phase extraction directly on-line to CE is problematic because of the deleterious effect of band broadening after sample elution with an organic solvent.

On-line sample preconcentration represents an effective and versatile way to enhance concentration sensitivity in CE. The high electric field and tunable mobility of analytes can be used to induce electrokinetic focusing within large sample volumes directly on-capillary prior to detection. On-line focusing is normally performed by selecting different buffer properties to modify analyte velocity in two or more electrolyte sections in the capillary, such as sample and background electrolyte (BGE). To date, four major on-line preconcentration techniques have been reported in CE: sample stacking (based on field enhancement) [10–15], transient isotachophoresis (t-ITP) [16–19], sweeping [20–23] and dynamic pH junction [24–27]. Each method relies on a distinct focusing mechanism based on different electrolyte properties between sample and BGE zones, such as conductivity (ionic strength), electrolyte co-ion mobility, additive concentration (analyte–additive interactions) and buffer pH, respectively. Accordingly, such methods are suitable for certain types of analytes based on their specific physicochemical properties in the buffer, such as charge, mobility, equilibria (with additives) and pK_a. Our group has been interested in applying sweeping and dynamic pH junction methods for the on-line preconcentration of metabolites because of their applicability to high-salt samples, unlike conventional stacking [6]. In addition, both sweeping and dynamic pH junction represent complementary methods for the on-line focusing of different classes of metabolites (i.e., weakly ionic and ionic/hydrophobic species) using simple buffer junctions and inexpensive additives, such as SDS surfactants. Recently, we have introduced a new hyphenated on-line focusing method referred to as dynamic pH junction-sweeping for focusing of flavin metabolites, resulting in over 1200-fold improvement in sensitivity relative to conventional injections [28,29]. In this report, a review of our recent work on developing improved on-line preconcentration techniques based on sweeping, dynamic pH junction and dynamic pH junction-sweeping for several classes of metabolites is pre-

sented. High-throughput analysis of metabolites by multiplexed CE with on-line preconcentration is also introduced as a new platform for rapid, sensitive and high-throughput analyses using UV detection [30].

2. On-line preconcentration strategies for metabolites

2.1. Focusing selectivity and focusing efficiency applicable to biological samples

Focusing selectivity and focusing efficiency are two factors to consider when selecting an appropriate on-line preconcentration method for sensitive analysis of sub-μM levels of metabolites. Future CE separations for metabolomics require on-line preconcentration techniques applicable to various classes of metabolites ranging from anionic (carboxylic acids, nucleotides), zwitter-ionic (catecholamines, amino acids), cationic (amines) and neutral (carbohydrates, steroids) species [5]. Most on-line preconcentration methods in CE have limited selectivity for certain types of analytes under restricted buffer and sample conditions. For instance, focusing selectivity in t-ITP is limited to ionic analytes whose mobility is intermediate to selected leading and background electrolytes [19], whereas sweeping with micelles is optimum for hydrophobic or ionic analytes that possess a high retention factor [20]. Thus, multiple on-line focusing strategies are envisioned to be necessary to carry out on-line sample preconcentration for different classes of metabolites by CE. In some cases, on-line focusing methods with high selectivity for specific metabolites (minor components) in complex sample matrixes are desirable for metabolic profiling of key species present at nanomolar levels, such as hormones or coenzymes. Focusing efficiency is also vital for generating sufficient sensitivity enhancement factors in CE (notably when using UV detection), while maintaining high-resolution separations. In general, there exists a compromise between the sensitivity enhancement achievable using large sample injections and the resolution required for complex analyte mixtures. Sensitivity enhancement factors are generally estimated by comparing the peak height using a ‘‘conventional’’ injection relative to a large-volume injection using on-line preconcentration [20,25]. Although greater than a 100 000-fold increase in detector sensitivity has been reported with techniques such as selective cation exhaustive injection-sweeping [31], biological samples that contain high concentrations of salt require extensive desalting prior to analysis. This precludes on-line preconcentration techniques based on conventional sample stacking [10,32] or field-enhanced sample injection [11,33], which require low salt matrixes for analyte focusing. Desalting (e.g., dialysis) of samples containing low-molecular-mass metabolites is particularly difficult since metabolites diffuse with salts across porous membrane filters. Field-enhanced sample injection methods also suffer from poor reproducibility (sample matrix effects) and injection bias for different types of analytes. Thus, on-line focusing techniques using hydrodynamic injection, that are applicable to biological samples (e.g., urine, plasma or cell extracts) with minimal sample pretreatment, would be advantageous for rapid, sensitive and reproducible metabolite analyses by CE. We have previously proposed using the detector-to-injection bandwidth ratio (*DIBR*) as a quantitative measure for comparing analyte focusing that is independent of the choice of conventional injection length and capillary [25]. Analyte focusing is indicated when the original injection plug length is narrower than the analyte bandwidth at the detector or when *DIBR* values are less than 1. Over a 40-fold reduction in original injection bandwidth can be realized under optimum focusing conditions, reflected by a *DIBR* of less than 0.020 [28]. Analyte peaks generated by the on-line focusing of large injection volumes in CE are often sharper than peaks undergoing normal broadening processes, which is important for generating separations of high efficiency and peak capacity.

Based on these constraints, our group has been interested in applying three complementary on-line preconcentration techniques in CE to focus various classes of metabolites under selected buffer conditions. Buffer pH and additive concentration (e.g., micelle, cyclodextrin, ion-pairing reagent, etc.) represent two of the most important properties used to optimize selectivity in CE separations, which are also vital when designing on-line preconcentration strategies. Fig. 1 depicts three formats used for electrokinetic focusing of low concentrations of

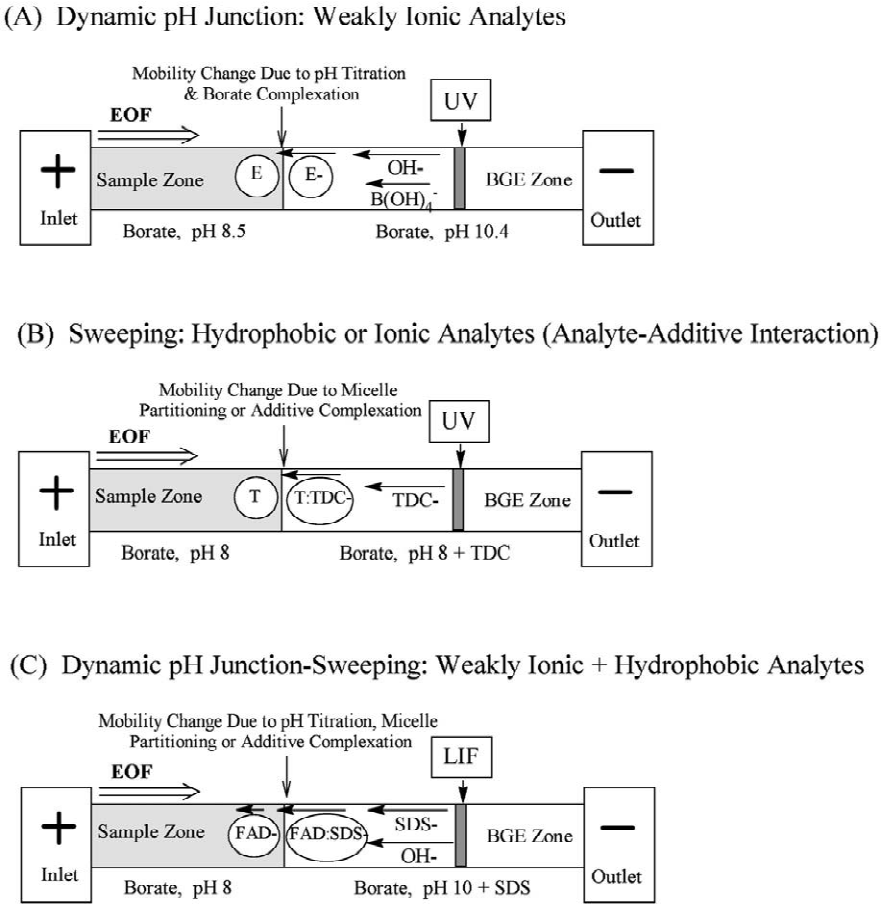

Fig. 1. Schematic diagram comparing three complementary on-line sample preconcentration techniques for the focusing of large injection volumes of metabolites by CE: (A) dynamic pH junction, (B) sweeping and (C) dynamic pH junction-sweeping. Focusing of weakly ionic metabolites (E, epinephrine) by dynamic pH junction is based on changes in analyte mobility (pK_a, vicinal diols) as a result of pH and/or borate complexation. Band narrowing of neutral hydrophobic metabolites (T, testosterone) by sweeping relies on strong partitioning (retention factor, k') to charged (migrating) micelles, such as TDC (taurodeoxycholate). Enhanced focusing performance of weakly ionic and hydrophobic metabolites (FAD, flavin adenine dinucleotide) may be realized by dynamic pH junction-sweeping, which is dependent on analyte velocity differences caused by pH, borate complexation and SDS partitioning.

metabolites in large sample injection volumes: dynamic pH junction, sweeping and dynamic pH junction-sweeping. Under optimum conditions, over two orders in magnitude enhancement in concentration sensitivity has been reported by each method for several classes of metabolites [20,25,29]. Each technique relies on a multi-section electrolyte system involving the hydrodynamic injection of a large sample plug (dissolved in different buffer pH and/or devoid of additive) into a capillary that is previously filled with BGE. The inlet buffer reservoir is then replaced with BGE prior to application of voltage. Analyte focusing occurs under non-stacking conditions since the sample normally contains high concentrations of salt or buffer. On-line focusing of specific classes of metabolites is based on changes in analyte mobility caused by pH titration, micellar partitioning or additive complexation in the sample and BGE. More than one focusing mechanism may be operative under certain conditions, such as using borate as both alkaline buffer and selective complexation agent [25]. Analyte focusing and sub-

sequent zone separation results in extremely narrow and separated bands at the detector window. Knowledge of the physicochemical properties of specific metabolites (e.g., pK_a, charge, size, functional group, equilibria to additives), as well as the properties of the bulk sample matrix (e.g., salt content, pH, chemical interferences), can be used to design optimal on-line preconcentration conditions to enhance detector sensitivity in CE.

2.2. Dynamic pH junction: single buffer type for weakly ionic metabolites

Since a large number of metabolites are weakly ionic, buffer pH plays an essential role in controlling the selectivity, as well as analyte zone electrodispersion during separation. This is exemplified by capillary isoelectric focusing [34], which uses steady-state pH gradients generated by mixtures of ampholytes in the capillary to concentrate ampholyte protein zones based on their isoelectric points. However, the concept of using pH gradients to focus analyte bands is not limited to peptide or protein samples [35]. Moreover, conventional buffers can be used to focus analyte zones instead of synthetic ampholytes solutions. On-line preconcentration by dynamic pH junction represents an alternative technique that uses inexpensive buffer junctions to generate a transient (i.e., non-steady-state) pH gradient within the BGE upon application of voltage [25]. This results in a simple method to focus and separate weakly ionic analytes in a single step without sample mobilization procedures as used in conventional isoelectric focusing. Dynamic pH junction is defined when two or more sections of buffer that possess a different pH are loaded into the capillary to form a discrete step pH junction at the interface of the sample (containing analyte) and BGE zones. The sample may consist of the same buffer or different electrolyte type as BGE to optimize the pH junction range for the focusing of weakly acidic, basic or zwitter-ionic analytes (mobility is pH dependent) based on their pK_a. The principle of dynamic pH junction was first reported by our group when developing a specific assay for epinephrine (adrenaline) in dental anesthetic solutions [24]. Under optimum separation conditions, direct injection of large volumes of anesthetic solution resulted in selective focusing of epinephrine from an over 4000-fold excess of local neutral anesthetic (lidocaine, co-migrates with EOF), as depicted in Fig. 2A. Despite the large volume injected (5.7% of capillary length), high salt content and presence of high concentrations of other additives in the sample, epinephrine migrates as an

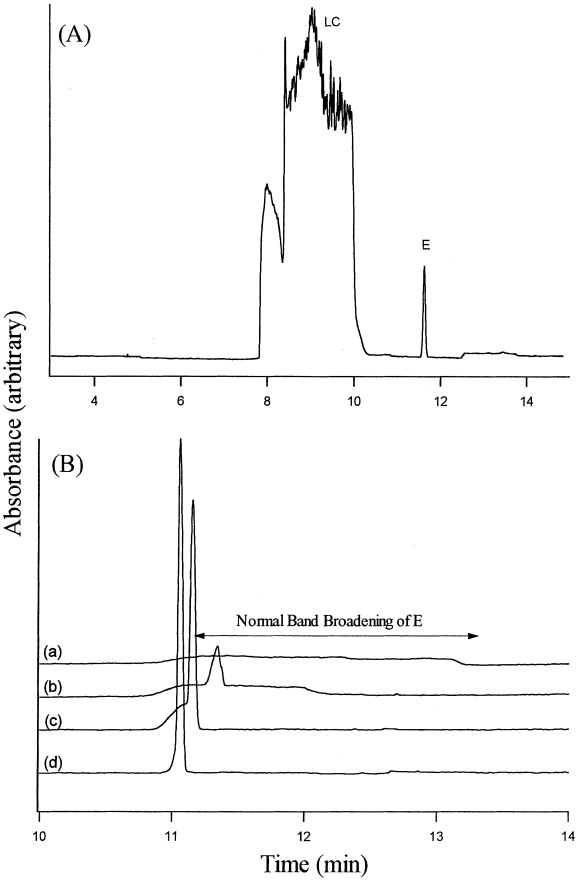

Fig. 2. (A) Electropherogram showing the separation and focusing of 5 μg/mL epinephrine (E) from major amounts of lidocaine (LC) present in multi-use Xylocaine dental anesthetic solutions. Large sample volumes of dental anesthetic solutions were directly injected into the capillary (5.7% of the total capillary length). The BGE used was 160 m*M* borate, 1 m*M* EDTA, pH 10.1. (B) A study of the sample pH dependence of epinephrine focusing (standard solutions) using a dynamic pH junction is depicted as a series of electropherograms. The BGE used is 160 m*M* borate, 1 m*M* EDTA at pH 10.2. All sample solutions contained 27 μ*M* epinephrine, 160 m*M* borate, 155 m*M* NaCl, 3 m*M* sodium disulphite and 1 m*M* EDTA. The pH of the sample is varied from (a) 10.2, (b) 9.5, (c) 9.0 and (d) 8.5. Note that sample overloading of epinephrine due to large sample injections occurs when the sample and BGE have the same buffer pH (a).

extremely sharp band with a detector bandwidth of less than 0.80 cm. It was determined that buffer pH was the most important factor influencing epinephrine focusing since dental anesthetic solutions were weakly acidic [36]. In order to investigate the influence of sample pH on epinephrine focusing, Fig. 2B depicts band narrowing of large volumes of epinephrine (standard solutions), using a dynamic pH junction, where the sample pH is varied from pH 10.2 to 8.5, while BGE is fixed using borate pH 10.2 [25]. When both the sample and BGE have the same pH 10.2, epinephrine migrates as an extremely broad zone with a detector bandwidth of over 10 cm because of sample overloading. However, as the sample borate pH is gradually reduced to 8.5, significant band narrowing of the sample results in sharp epinephrine zones. A pH difference of less than 1.5 units between the sample and BGE was sufficient to sharpen the epinephrine zones. It was observed that epinephrine focusing using dynamic pH junction also occurred with the addition of 155 m*M* sodium chloride to the sample matrix with minimal loss of peak bandwidth. Optimum focusing of epinephrine (23% of total capillary length) was realized using a dynamic pH junction consisting of borate pH 8.5 and 10.4 in the sample and BGE, respectively, which resulted in about a 250-fold enhancement in concentration sensitivity [25]. Since epinephrine is zwitter-ionic with a basic secondary amine ($pK_{a2} \approx 9.9$) and an acidic catechol functionality ($pK_{a1} \approx 8.9$) [37], borate buffer can modify the mobility of the catecholamine by pH titration (hydroxide) and selective complexation with vicinal diols. The high mobility of the hydroxide ion [38] in the BGE and the strong EOF under alkaline conditions results in rapid focusing of catecholamines in the front edge of the sample boundary because of its reduced velocity (i.e., larger negative electrophoretic mobility). For instance, the electrophoretic mobility of epinephrine in borate BGE, pH 8.5, is extremely low at $-2.12 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}, whereas it has a significantly larger negative mobility of $-1.41 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 10.4. This pH is sufficiently greater than the pK_{a2} of epinephrine, thereby reducing the positive charge contributed by the amine and increasing its overall mobility. As hydroxide ions in the BGE continue to titrate the sample zone, the formation of a transient pH gradient is hypothesized to provide an electrokinetic mechanism to focus epinephrine across the entire sample zone. The process continues until a steady state is reached when the sample zone is completely titrated and epinephrine migrates with a constant velocity undergoing normal band broadening.

On-line focusing of the two other natural catecholamines, norepinephrine (noradrenaline) and dopamine, was also investigated by dynamic pH junction [25]. It was determined that optimal focusing of each catecholamine was realized under slightly different pH junction conditions. Fig. 3A depicts optimization of dynamic pH junction by measuring the peak efficiency (i.e., peak sharpness) of catecholamines versus the pH of the BGE while the sample injection length (6.6 cm) and sample pH (borate, pH 8.5) are fixed. The optimal pH required to focus epinephrine, norepinephrine and dopamine in this system was 10.4, 10.0 and 10.6, respectively, as reflected by the maxima of the plots in Fig. 3A. It is important to note that optimization of the focusing conditions for catecholamines is also dependent on the specific length of the sample zone injected. Fig. 3B shows optimal focusing and resolution of about 2 μ*M* of three catecholamines using borate pH 10.6 as BGE. Each catecholamine focuses to a different extent, highlighted by their detector bandwidths of 0.77, 0.91 and 0.19 cm for epinephrine, norepinephrine and dopamine, respectively. In the case of dopamine, analyte focusing by dynamic pH junction results in extremely sharp peaks, reflected by an apparent efficiency (*N*) of about $3.3 \cdot 10^6$ or a *DIBR* of 0.029 (35-fold narrowing of injection plug). Selective catecholamine focusing by a dynamic pH junction reflects the specific chemical properties of the analytes, such as pK_a. For instance, dopamine has a $pK_{a2} \approx 10.6$ [37], thus optimum focusing of dopamine occurs at the highest buffer pH conditions in comparison to epinephrine and norepinephrine. Recently, dynamic pH junction focusing of riboflavin, flavin mononucleotide and flavin adenine dinucleotide was also demonstrated since these metabolites have weakly acidic lactam and vicinal diol groups [28]. Further work is needed to better understand the nature of the pH changes generated within multisection electrolytes, as well as the bell-shape focusing curves depicted in Fig. 3A, where band narrowing gradually deteriorates at higher pH conditions past optimum values.

On-line focusing by dynamic pH junction can also

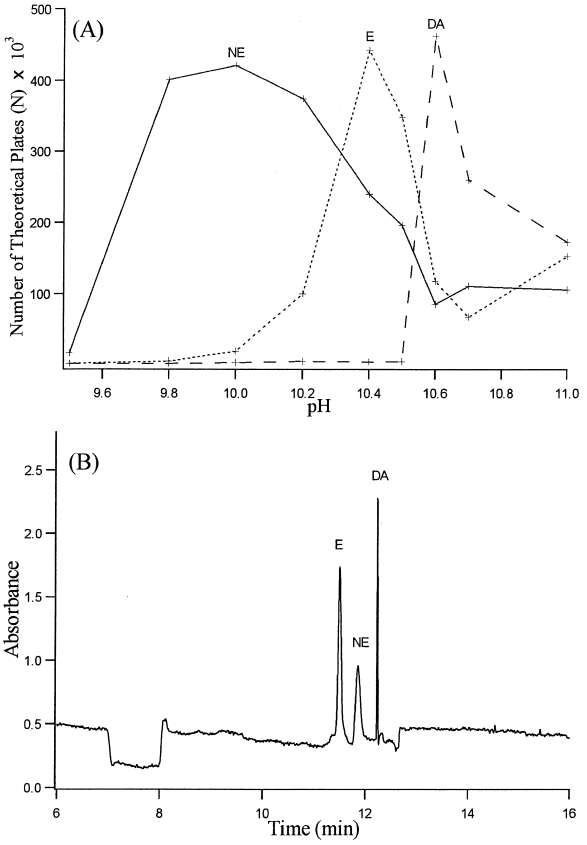

Fig. 3. (A) Optimization of analyte focusing by dynamic pH junction by plotting the apparent plate number (N) versus pH of the BGE for three different catecholamines: epinephrine (E), norepinephrine (NE) and dopamine (DA). All samples contained 10 μ*M* catecholamine in 160 m*M* borate, 155 m*M* NaCl, 3 m*M* sodium metabisulphite and 1 m*M* EDTA, pH 8.5. The BGE is aqueous 160 m*M* borate, 1 m*M* EDTA, and the pH is varied from 8.5. to 11.0. (B) Electropherogram demonstrating optimum separation and focusing of catecholamines by dynamic pH junction based on plot (A). The sample contained about 2.0 μ*M* of each catecholamine in 160 m*M* borate, 155 m*M* NaCl, 3 m*M* sodium disulphite and 1 m*M* EDTA, pH 8.5, whereas the BGE was 160 m*M* borate, 1 m*M* EDTA, pH 10.6. The injection plug corresponds to about 12% of capillary length.

be applied to several classes of weakly acidic metabolites (non-diol), including deoxynucleosides, purines and estrogens. Deoxynucleoside focusing is dependent on the type of purine or pyrimidine base [26]. In the case of deoxyguanosine (dG), which has a weakly acidic lactam purine base ($pK_a \approx 9.4$) [39], optimum focusing by dynamic pH junction was observed to occur at pH 9.7 (sample pH 8, dG is neutral), with a gradual loss of focusing at pH values greater than 10. Deoxynucleosides having weakly acidic bases G, T and U focus into sharp zones with extremely high efficiency ($N \approx 7 \cdot 10^5$), while maintaining good baseline resolution. Fig. 4A depicts optimum focusing and a resolution of 0.2 μ*M* deoxynucleosides using dynamic pH junction, including the acyclic guanosine analogue pencyclovir (PCV), which is used as an antiviral drug for the treatment of the human herpes simplex virus. Neutral deoxynucleosides (e.g., dA and dC) that do not possess weakly acidic groups were not amenable to this technique, since their mobility is independent of

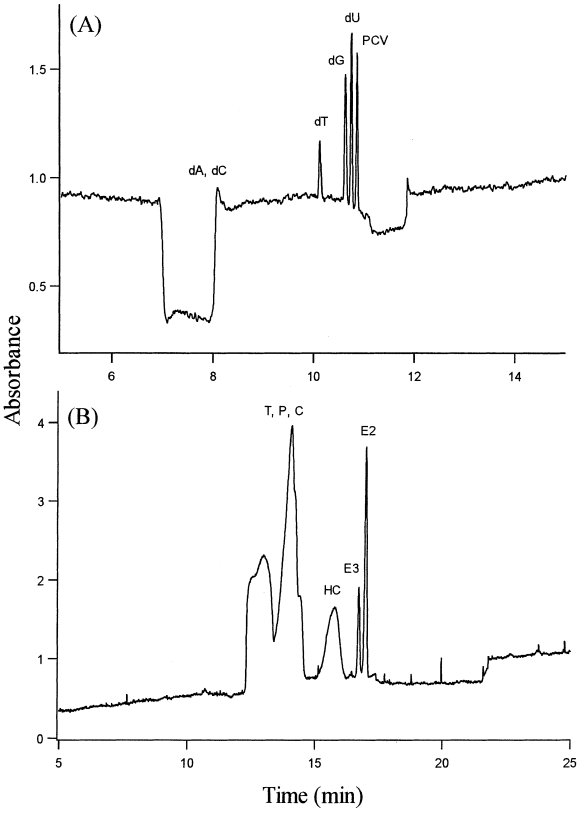

Fig. 4. On-line focusing of weakly acidic (non-diol) metabolites using dynamic pH junction with a single buffer type by CE. (A) Electropherogram showing the separation and focusing of 0.2 μ*M* deoxyribonucleosides (injection length 6.6 cm) with a BGE of 160 m*M* borate, pH 9.7. All sample solutions contained 160 m*M* borate, 150 m*M* NaCl, pH 8.0. (B) Electropherogram depicting selective focusing of 20 μ*M* estrogens by dynamic pH junction in a sample containing a mixture of steroids using a BGE of 160 m*M* borate, 0.5 m*M* γ-CD, pH 11.0. Sample solutions contained 20 μ*M* testosterone (T), cortisone (C) hydrocortisone (HC), prednisone (P), estradiol (E2) and estriol (E3) in 160 m*M* borate, pH 8.5.

buffer pH [26]. Corresponding purine bases, guanine and adenine, were also readily focused by CE using dynamic pH junction [27]. Steroids represent a functionally complex class of metabolite that serve important functions as hormones for intercellular signaling. Fig. 4B demonstrates the selective resolution and focusing of two estrogen metabolites, estradiol (E2) and estriol (E3), due to their weakly acidic phenolic functionality ($pK_a \approx 10.4$) among a mixture of neutral androgen and corticosteroids [40]. Optimum conditions for dynamic pH junction focusing of estrogens required a strongly alkaline pH of 11.0 in the BGE, whereas the sample pH was 8.5 (estrogen is neutral). On-line preconcentration of neutral steroid metabolites can be performed using sweeping or dynamic pH junction-sweeping methods. It is important to note that borate is not essential as the BGE to focus weakly acidic analytes that do not possess vicinal diol groups, such as deoxynucleoside, purines or estrogen metabolites, since analyte velocity is mediated by buffer pH differences alone. Thus, other types of alkaline buffers, including phosphate or carbonate, can also be used as the BGE. In addition, dynamic pH junction under acidic buffer conditions (sample is neutral or alkaline) can also be used to focus metabolites, such as zwitter-ionic peptides [41] or proteins [42]. Recently, a mechanism based on a transient moving chemical reaction boundary method was proposed to describe the on-line focusing of phenylalanine and tryptophan in high salt solutions using a dynamic pH junction format with formic acid (BGE is acidic/sample is alkaline) [43]. The application of pH gradients in CE also has important implications towards selectivity enhancement for complex mixtures of weakly ionic analytes [44], similar to the use of solvent gradients for elution in HPLC separations. Recently, a novel on-line focusing method was reported for generating pH gradients by electrolysis of water (using a dilute buffer in BGE) after inserting a short piece of platinum wire inside the capillary [45]. Further work is needed to better characterize dynamic pH junction in terms of the pH changes occurring within the sample, as well as modeling of analyte band narrowing based on fundamental physicochemical properties of analytes and the electrolyte, such as pK_a, electrophoretic mobility (pH dependence), buffer type, pH, ionic strength and conductivity.

2.3. Dynamic pH junction: mixed buffer type for weakly ionic metabolites

Different buffer types or electrolytes can also be used in the sample matrix to further lower the pH range when using an alkaline BGE in order to focus relatively stronger acids (e.g., $pK_a < 8$), such as some purines, nucleosides and nucleotides. Optimum focusing of nucleosides with ribose sugar moieties was performed using an acidic acetate buffer pH 4.0 (nucleosides are neutral) in the sample matrix and borate pH 9.7 as BGE [26], since nucleosides have a significant negative mobility in borate pH 8.0. In this case, analyte focusing is dependent on pH and buffer type (borate–ribose complexation), where borate as the BGE plays the dual role of alkaline buffer and selective complexation agent. However, the use of strongly acidic sample conditions for analyte focusing by dynamic pH junction is not applicable to certain types of metabolites that are labile in acid, such as nucleotides. Fig. 5A depicts optimum focusing and resolution of 0.2 μM levels of 24 different deoxyribonucleotide and ribonucleotide standards using 150 mM sodium chloride in the sample matrix [26]. The concentration of salt is vital to adjust the sample conductivity to a similar level as the BGE, thereby maintaining a uniform electric field throughout the capillary, in contrast to techniques based on sample stacking. Excellent concentration sensitivity (LOD $\approx 4.0 \cdot 10^{-8}$ M for ATP) and good separation performance was achieved even with the injection of large volumes of sample. Over a 50-fold enhancement in concentration sensitivity was realized with sufficient peak retention to resolve complex mixtures of nucleotides. This method was also applicable to the direct analysis of nucleotides in cell extracts derived from mouse lymphoma cell lines as a way to monitor in vitro genotoxic effects of drug therapy. A study of the influence of buffer pH, type of electrolyte co-ion and ionic strength was recently reported for on-line focusing of purine metabolites using a dynamic pH junction [27]. The type and specific mobility of electrolyte co-ion in the sample matrix relative to BGE may also contribute to analyte focusing when using mixed buffer systems, notably for strongly acidic purines such as uric acid, whose mobility is less affected by pH. In contrast, guanine and adenine can be selectively focused by

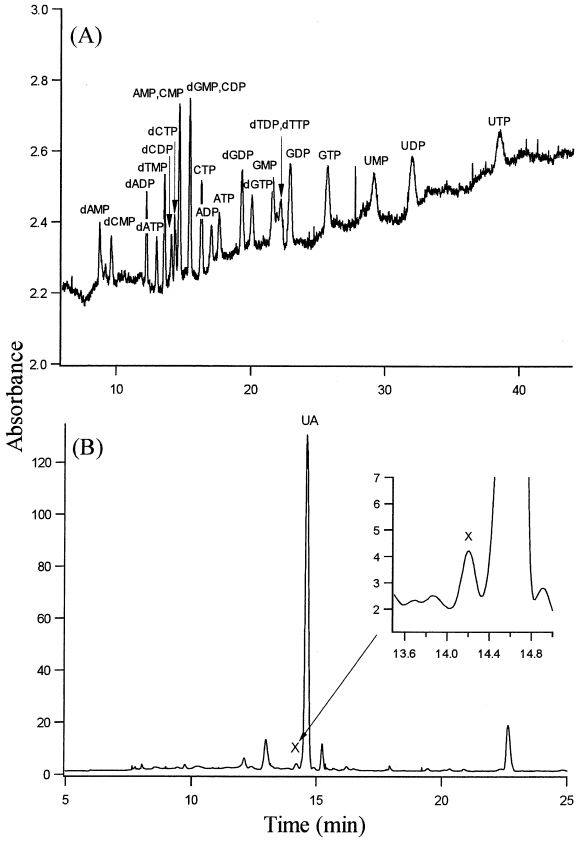

Fig. 5. On-line focusing of weakly acidic metabolites using dynamic pH junction with a mixed buffer type by CE. (A) Electropherogram showing focusing and separation of 24 different nucleotides by dynamic pH junction using an injection of 12% of capillary length. Samples contained 0.2 μ*M* of each nucleotide in 150 m*M* NaCl. (B) Selective analysis of xanthine (X) from uric acid (UA) in human urine using a dynamic pH junction by CE. Pooled urine was diluted 20-fold in 75 m*M* phosphate, pH 6.0, prior to analysis. The BGE was 160 m*M* borate buffer, pH 9.5, using a large volume injection of 18% of capillary length. Other peaks in (B) correspond to unidentified urinary metabolites.

dynamic pH junction (borate pH 8/9.7 in sample/BGE) using a single buffer type because of their higher pK_a [27]. Alternative mechanisms have been proposed to describe theophylline [46] and hypoxanthine [47] focusing in borate buffer systems based on t-ITP, where borate may act as terminating ion and phosphate (or chloride) as the leading ion. This mechanism may also play a role in the focusing of selected catechols and sugars using borate as BGE and salt or phosphate in the sample, which was previously described by sweeping via borate complexation [48]. Our previous study demonstrated poor band narrowing and resolution of purine metabolites when using phosphate in a sample that had the same pH as the borate BGE (see Fig. 9d), which emphasizes the requirement for a dynamic pH junction for optimum analyte focusing [30]. A dual focusing mechanism by dynamic pH and t-ITP modes may occur when using mixed buffer pH junctions based on different types of electrolyte co-ions. Enhanced focusing performance can result from the additive band narrowing effects of dynamic pH junction and t-ITP for selected metabolites. For instance, about a two-fold enhancement in xanthine (p$K_a \approx 7.4$) band narrowing was obtained using weakly acidic phosphate buffer in the sample compared to (non-buffered) salt solution [27]. Fig. 5B depicts the focusing and resolution of μ*M* levels of xanthine (from high levels of uric acid) in pooled human urine using on-line focusing of large sample volumes (18% of capillary length) by CE with dynamic pH junction [27]. The limit of detection (LOD, $S/N = 3$) for xanthine determination by the CE method was about $4 \cdot 10^{-8}$ *M*. The reproducibility of xanthine analysis (triplicate injection of urine sample) gave a RSD of 1.0 and 2.9% for migration time and peak area, respectively. Further work is needed to investigate the use of dynamic pH junctions based on mixed buffer types and its relationship to conventional ITP, as a novel method for improved focusing of weakly ionic (e.g., phenolates, carboxylates), as well as strongly ionic (e.g., phosphates, sulfates), metabolites.

2.4. Sweeping: ionic and hydrophobic metabolites

Different types of additives can be added to the run buffer in CE to improve the selectivity for chiral and neutral analytes based on differential equilibria, such as the use of micelles in MEKC-based separations [49]. Sweeping represents a versatile on-line preconcentration method that uses additives in the BGE (sample is devoid of additive) for electrokinetic focusing of large injection volumes of analyte that possess a strong affinity for specific types of additives, including ionic and non-ionic micelles [20,23,50], cyclodextrins [51–53] and polyethylene oxide [54]. Single or multiple additives can be

employed, provided that the velocity of analytes in the sample zone is modified upon interaction with the additive, resulting in analytes being ''swept'' or focused into a narrow band. Previous reports of sweeping have generally used anionic SDS micelles to focus neutral or cationic analytes, where the length of the focused analyte zone is dependent on the magnitude of the retention factor [21]. Analytes that partition with a strong affinity due to hydrophobic and/or electrostatic attraction can be focused into extremely sharp zones. In most cases, sweeping functions optimally when the conductivity of the sample is similar to the BGE, although higher sample conductivities can also be used provided that the sample injection length is adjusted [22,55]. Over three orders of magnitude improvement in concentration sensitivity has been reported by sweeping when operating under acidic conditions to suppress EOF [20], however this condition can restrict the type of buffer and pH required for resolution of complex sample mixtures. The lower sensitivity enhancement performance of sweeping under alkaline conditions has been attributed to additional band broadening caused by mismatches in EOF in sample and BGE zones [56]. A recent review of the focusing mechanism and application of sweeping has been reported [57].

To date, there have been few applications of sweeping for the on-line focusing of metabolites present in biological samples, since many metabolites are anionic (hydrophilic) and thus have a low retention factor with common additives, such as SDS micelles. However, specific classes of metabolites may be well suited to on-line preconcentration by sweeping, such as selected types of steroids, vitamins and aromatic carboxylic acids or amines. Fig. 6A demonstrates the application of sweeping under alkaline conditions using 100 m*M* SDS to selectively focus vitamin B2 (riboflavin, RF) by CE using laser-induced native fluorescence detection [28]. Since the retention factor of RF is only $k' \approx 0.6$ under these buffer conditions, only a four-fold reduction in injection bandwidth was realized, reflected by a *DIBR* of 0.26. However, phosphorylated flavin coenzymes, FMN and FAD, migrate as extremely broad peaks due to a very weak interaction with the micelle. In this case, improved sweeping performance under acidic conditions is not applicable since

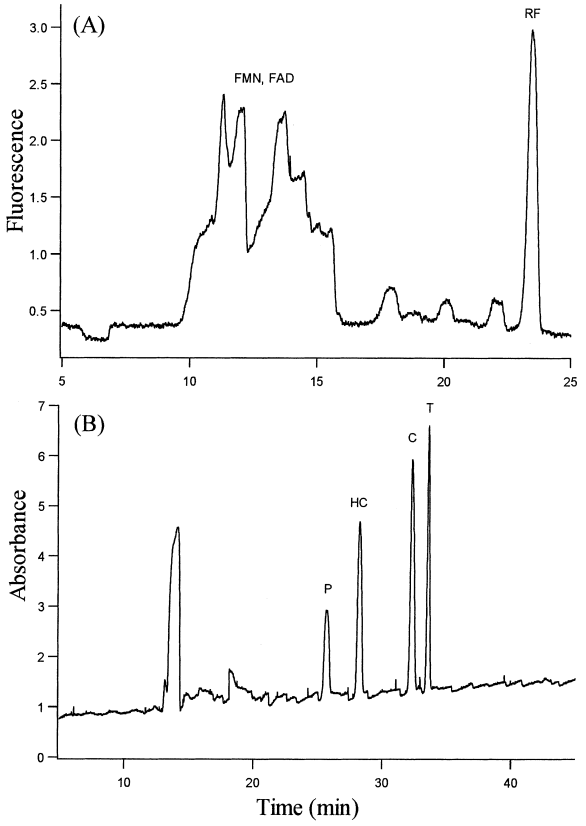

Fig. 6. On-line focusing of hydrophobic metabolites in CE using sweeping with charged micelles. (A) Electropherogram showing selective focusing and separation of riboflavin (RF) from hydrophilic flavin coenzymes, FMN and FAD, by sweeping using 140 m*M* borate, 100 m*M* SDS, pH 8.5. Sample solutions contained 0.2 μ*M* flavins dissolved in 140 m*M* borate, pH 8.5, which was injected to about 17% of the capillary length. (B) Electropherogram depicting optimum focusing and resolution of hydrophobic steroids by sweeping under alkaline conditions using 160 m*M* borate, 80 m*M* sodium taurodeoxycholate (bile salt), 10 m*M* γ-CD, pH 8.0. Sample solutions contained 20 μ*M* steroids: prednisone (P), hydrocortisone (HC), cortisone (C) and testosterone (T) dissolved in 140 m*M* borate, pH 8.0. Focusing efficiency (i.e., detector bandwidth) is dependent on the magnitude of the retention factor to the micelle.

several classes of phosphorylated metabolites, including nucleotides, sugar pyrophosphate derivatives, flavin and nicotinamide coenzymes, are acid-labile [58]. The use of different types of micelles, such as cationic [56] or non-ionic micelles [50], has been reported to improve the sweeping of some anionic analytes under alkaline conditions. However, in the

case of cationic micelles, the capillary must be coated to suppress EOF.

Sweeping represents a particularly useful method for on-line preconcentration of steroids because of their high retention factor for micelles. In addition, sweeping using charged micelles is essential for the selectivity in CE since most steroids are neutral. Fig. 6B depicts the separation and on-line focusing of 20 μ*M* of steroids using a combination of an anionic bile salt micelle (sodium taurodeoxycholate) and neutral γ-CD to optimize resolution [40]. In general, the w_{det} of the apolar steroids testosterone and corticosterone are narrower than the more polar steroid hydrocortisone and the synthetic cortisone analogue prednisone, because of their larger retention factor. Over a 1000-fold enhancement in concentration sensitivity has been realized for apolar testosterone and progesterone steroids using sweeping with SDS micelles under acidic conditions [20]. Sodium cholate micelles and anionic cyclodextrin derivatives may also be used for sweeping to enhance the detectability of corticosteroids [22]. Estrogens are an important class of metabolite which have an important clinical value as biomarkers in the early diagnosis of cancer [59], as well as environmental significance as being potential hormone disruptors in exposed fish and other organisms [60]. Recently, Harino et al. [61] reported about a 10 000-fold enhancement in sensitivity for estrogens present in water using a combination of sweeping with SDS micelles and off-line preconcentration by solid-phase extraction. Sweeping of complex biological samples may require more than one buffer condition for optimum resolution, focusing and analysis time for different classes of metabolites. The selectivity (retention factor) in MEKC can be easily modified by changing the buffer pH, organic solvent content or cyclodextrin concentration in the BGE.

The derivatization of specific types of metabolites with hydrophobic fluorescent or UV probes may be advantageous to enhance sweeping focusing performance, as well as overall detector sensitivity. For example, amino acids are relatively small hydrophilic metabolites that have weak chromophores (excluding aromatic moieties), resulting in their poor concentration sensitivity when using CE with UV detection. Fig. 7A depicts a conventional small volume injection of a mixture of 21 different PTH-labeled amino acids (5 ppm) by CE using SDS micelles under acidic conditions [62]. A low pressure towards the detector is applied before the elution of tyrosine in order to reduce the total analysis time,

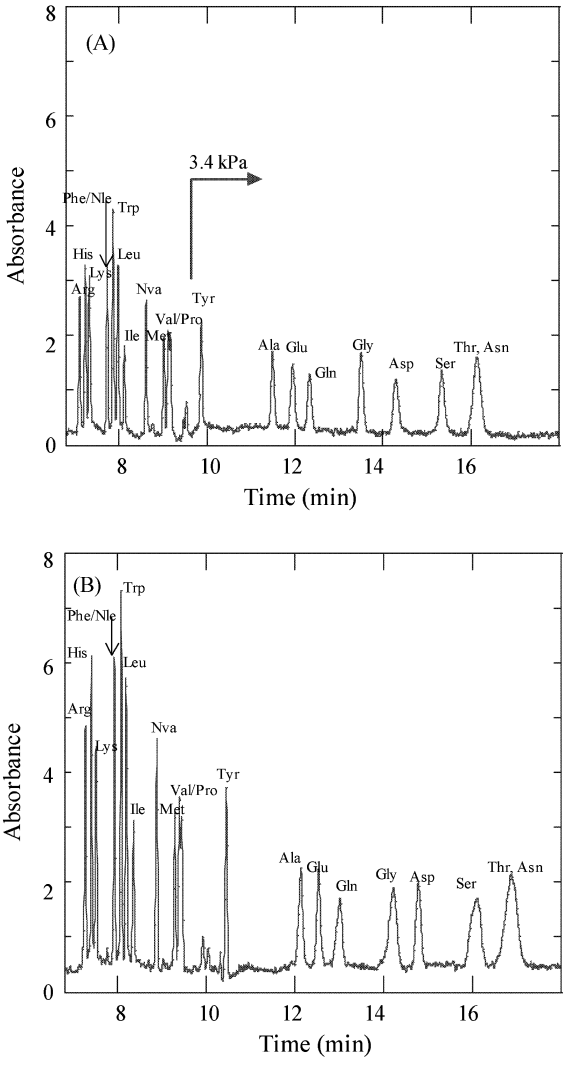

Fig. 7. On-line preconcentration of 21 PTH–amino acid derivatives (5 ppm) for improved detectability and sweeping performance of hydrophilic metabolites (weak chromophores) using 10 m*M* phosphate, 50 m*M* SDS, 20% methanol, pH 2.2. A positive pressure (3.4 kPa) was applied before the migration of Tyr in order to shorten the analysis time for late-migrating peaks. Electropherograms depict the use of (A) conventional injection (0.2 cm) and (B) large volume injection (1.8 cm), resulting in up to a 10-fold improvement in concentration while retaining the separation resolution.

while retaining the resolution of earlier-migrating PTH–amino acids. Fig. 7B shows up to a 10-fold improvement in concentration sensitivity with sweeping while retaining a similar resolution obtained in Fig. 7A. Higher sensitivity enhancement factors can be realized with longer injection plugs, however resolution is compromised for complex sample mixtures. Sweeping of amino acid derivatives that possess larger hydrophobic labels may permit improved sensitivity enhancement using UV absorbance or LIF detection. Further work is needed to explore the applicability of sweeping for the on-line preconcentration of native and chemically labeled metabolites in biological samples.

2.5. Dynamic pH junction-sweeping: weakly ionic and hydrophobic analytes

The design of new focusing strategies in CE based on several distinct focusing mechanisms (e.g., micelle partitioning, borate complexation, dynamic pH junction, t-ITP, etc.) may result in enhanced band narrowing and selectivity performance for different classes of metabolites than conventional on-line preconcentration techniques. For instance, optimum analyte focusing by sweeping is favored for species that possess a strong interaction with additives (e.g., retention factor to micelle) in the buffer, whereas the dynamic pH junction is limited to weakly ionic analytes (e.g., analytes with pK_a) whose mobility changes as a function buffer pH. Alkaline buffer conditions are generally required for adequate selectivity of complex mixtures of weakly acidic metabolites, but hydrophilic species also have lower retention factors to micelles. Our group has recently introduced a new hyphenated on-line preconcentration technique referred to as dynamic pH junction-sweeping for improved focusing performance of flavin metabolites in biological samples by CE using LIF detection [6,28]. Flavin coenzymes, FMN and FAD, serve as essential redox-active coenzymes associated with flavoenzymes which catalyze a variety of biochemical reactions involving carbohydrate, protein or fat metabolism [63]. Dynamic pH junction-sweeping is defined when the sample is devoid of SDS (sweeping condition) and has a different buffer pH (dynamic pH junction condition) relative to the BGE. Over a 1200-fold improvement in concentration sensitivity was realized compared to conventional injections, resulting in a limit of detection ($S/N = 3$) of about 4.0 pM for the flavin coenzymes FAD and FMN. In our study, dynamic pH junction-sweeping resulted in over a four-fold enhancement in band narrowing compared to either sweeping or dynamic pH junction techniques alone, when using sample injection plugs of 12 cm or about 22% of capillary length. Enhanced analyte band narrowing resulted from the additive focusing effects of buffer pH, borate complexation and SDS partitioning, since flavins possess a weakly acidic lactam, vicinal diols and hydrophobic isoalloxazine tricyclic ring system [28]. Fig. 8A depicts the analysis of nanomolar levels of free flavins (RF, FMN and FAD) in pooled human plasma by dynamic pH junction-sweeping CE [29]. Plasma was diluted four-fold in phosphate buffer prior to heat deproteinization to release tightly bound flavins with excellent recovery. Triplicate measurements of flavins in pooled plasma resulted in concentrations of 10.2 ± 0.7, 8.4 ± 0.2, and 44.0 ± 3.0 nM for RF, FMN and FAD, respectively [29]. This method was also applied to quantitate sub-μM levels of flavin coenzymes in cell extracts from *B. Subtilus*, the flavin content in selected flavoenzymes (e.g., nitric oxide synthase), as well as nanomolar levels of riboflavin present in human urine [29]. Since few metabolites are natively fluorescent when using Ar-ion laser emission (excitation at 488 nm), this method is extremely selective for flavins in complex biological matrixes with few chemical interferences.

Separation in CE under alkaline conditions may be advantageous for certain classes of metabolites for increased selectivity, as well as detectability in terms of a higher absorptivity coefficient or fluorescent quantum yield. Dynamic pH junction-sweeping represents a promising method to improve the selectivity of complex mixtures of analytes, as well as the focusing performance since it is applicable to both weakly ionic and neutral (hydrophobic) metabolites. When operating under alkaline buffer conditions in CE, the retention factor (i.e., micelles) of weakly acidic metabolites can be enhanced by preparing the sample in a neutral or acidic buffer (provided the analytes are stable) using a multi-section electrolyte

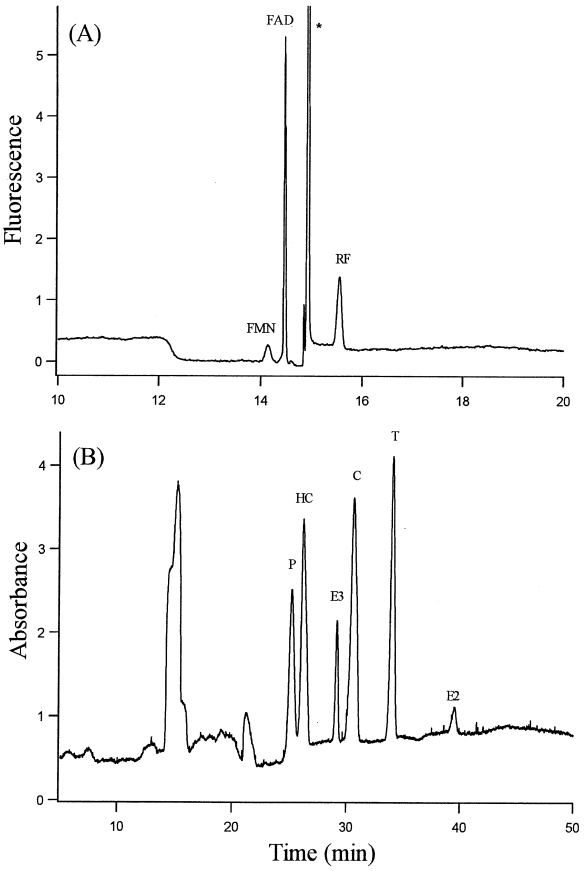

Fig. 8. On-line focusing of weakly ionic and hydrophobic metabolites by dynamic pH junction-sweeping. (A) Electropherogram depicting analysis of nanomolar levels of flavins in pooled human plasma by CE with LIF detection using an injection length of 8.2 cm. Plasma samples were diluted five-fold in 75 m*M* phosphate, pH 6.0, heat denatured to release bound flavins from enzyme (deproteinization) and centrifuged prior to injection. The BGE used was 140 m*M* borate, 100 m*M* SDS, 5 m*M* β-CD, pH 8.5. (B) Electropherogram depicting the optimum focusing and resolution of a mixture of steroids (20 μ*M*) by dynamic pH junction-sweeping CE. The BGE used was 160 m*M* borate, 32 m*M* sodium taurodeoxycholate, 5 m*M* γ-CD, pH 11.0, whereas the sample was dissolved in 160 m*M* borate, pH 8.0.

system. For example, there was over an eight-fold increase in the retention factor of riboflavin for SDS when the sample was prepared in phosphate, pH 6.0, relative to borate, pH 8.5, which was used as the BGE [28]. Fig. 8B demonstrates optimum resolution and focusing of different classes of steroids (i.e., androgens, estrogens and corticosteroids) when using dynamic pH junction-sweeping under alkaline conditions with anionic bile salt micelles and neutral cyclodextrin [40]. In comparison to Fig. 6B, which shows conventional sweeping at lower alkaline conditions (borate buffer, pH 8), baseline resolution for all six types of steroids, including hydrophobic estrogens, is achieved with minimal loss of focusing efficiency. In addition, conventional sweeping under the same BGE conditions (i.e., borate, pH 11) resulted in poorer band narrowing and resolution of polar steroids (i.e., cortisol, prednisone, estriol) [40]. Thus, dynamic pH junction-sweeping represents a complementary format to optimize the focusing and resolution of diverse classes of metabolites in cases when conventional sweeping or dynamic pH junction are less effective.

2.6. High-throughput and sensitive separation formats for metabolomics

New separation platforms for high-throughput analyses based on multiplexed CE (capillary array format) promise rapid and highly efficient separations, as highlighted by its important role in DNA sequencing used in the human genome project [64]. Our group is currently interested in developing sensitive and high-throughput methods for metabolomics using on-line preconcentration techniques in conjunction with multiplexed CE with UV detection [30]. Because of the large number of UV-active metabolites (e.g., nucleotides, coenzymes, vitamins, aromatic carboxylic acids and amino acids, etc.), multiplexed CE with UV detection represents a versatile format for the rapid screening of the hundreds of different metabolites present in a cell. This would be particularly attractive in a clinical setting for the high-throughput screening of metabolites in biological samples for diagnosis of chronic diseases. Since many buffer properties can be changed to alter the on-line focusing performance, multiplexed CE can be used for the rapid determination of the optimum focusing conditions using up to 96 different sample matrixes in a single run [30]. Fig. 9 depicts a series of 12 electropherograms (capillary #49 to #60 in the array) derived from multiplexed CE experiments, which were used to optimize purine focusing by systematically changing

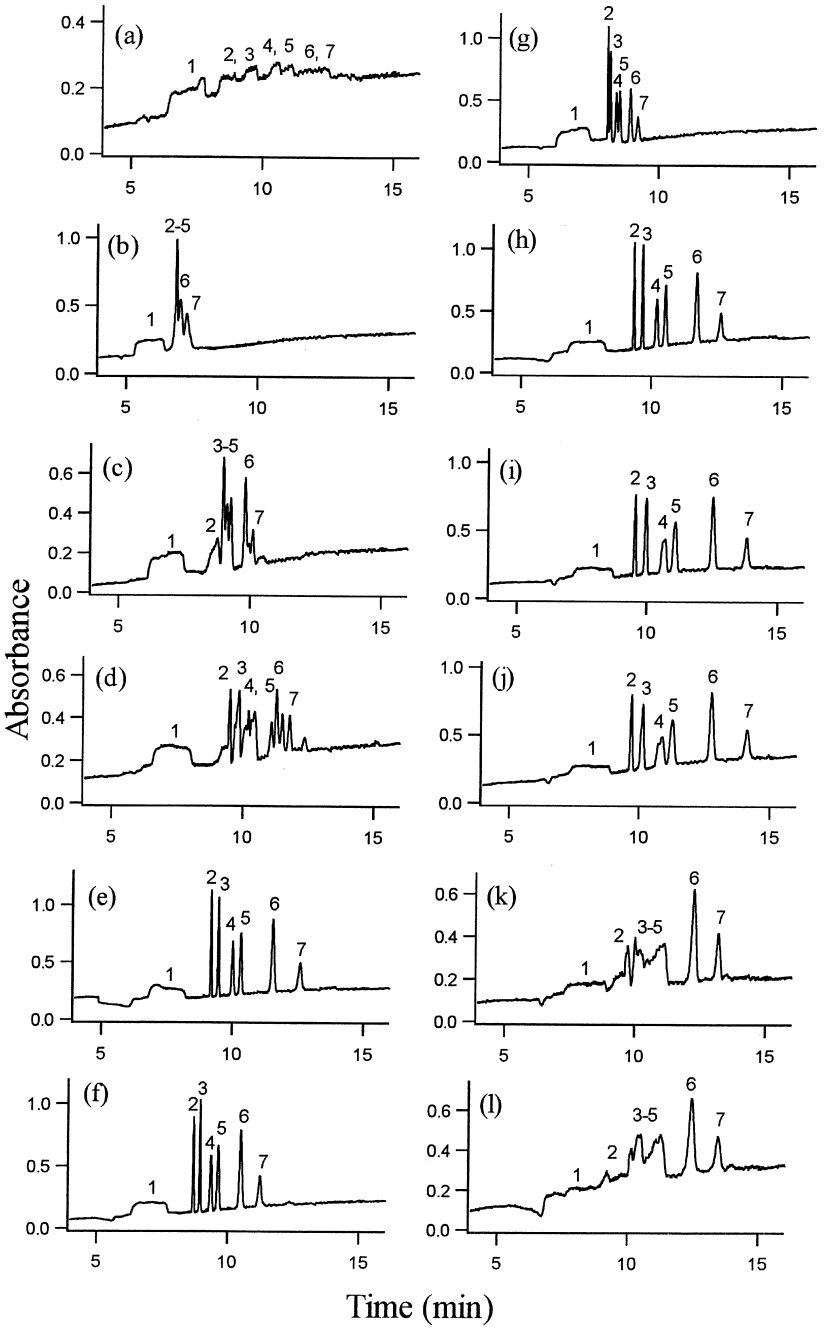

Fig. 9. Series of 12 electropherograms showing rapid optimization of purine focusing by multiplexed CE with UV detection. The sample matrix conditions are changed in terms of the buffer type, pH and ionic strength. The BGE used in all cases was 70 m*M* borate, pH 9.5, and the injection length used was 13.1 cm. All sample solutions contained 40 μ*M* purine in a sample matrix consisting of: (a) 70 m*M* borate, pH 9.5, (b) 7 m*M* borate, pH 9.5, (c) 20 m*M* carbonate, pH 9.5, (d) 20 m*M* phosphate, pH 9.5, (e) 20 m*M* phosphate, pH 3.0, (f) 20 m*M* phosphate, pH 6.0 (optimum conditions), (g) 10 m*M* phosphate, pH 6.0, (h) 25 m*M* phosphate, pH 6.0, (i) 40 m*M* phosphate, pH 6.0, (j) 50 m*M* phosphate, pH 6.0, (k) 75 m*M* phosphate, pH 6.0 and (l) 100 m*M* phosphate, pH 6.0. Analyte peak numbering corresponds to: 1 = deoxyadenosine (EOF marker), 2 = adenine, 3 = guanine, 4 = theobromine, 5 = allopurinol, 6 = hypoxanthine, and 7 = xanthine.

the sample matrix conditions (i.e., buffer co-ion, pH, ionic strength). In all cases, the separation buffer was 70 m*M* borate, pH 9.5, and a large sample volume of 80 s (7.2 cm or 13.1% of capillary length to detector) was injected. Six different purine test analytes (40 μM) were analyzed, including the xanthine oxidase inhibitor allopurinol, which is a drug used for the treatment of hyperuricemia [65]. Fig. 9a depicts the effects of sample overloading when purines are prepared in the same buffer as the BGE, 70 m*M* borate, pH 9.5. Conventional sample stacking using a 10-fold diluted sample (7 m*M* borate, pH 9.5) results in an extremely poor resolution of purines peaks because of the large voltage drop associated with the low conductivity sample zone as shown in Fig. 9b. Figs. 9c and 9d demonstrate the effect of changing the buffer co-ion type (the pH is the same as the BGE) in the sample matrix using 20 m*M* carbonate and phosphate, respectively, based on t-ITP using borate as the BGE [46,47]. Partial focusing of the purine peaks was observed, however significant peak splitting resulted in poor resolution [30]. In contrast, when the sample pH of phosphate is lowered from 9.5 to 3.0 and 6.0, as shown in Figs. 9e and 9f, respectively, a significant improvement in the peak focusing and resolution is realized for all purine metabolites. The conductivity of the solutions was measured to be 3.66 and $2.05 \cdot 10^{-3}$ Ω^{-1} cm^{-1} for 70 m*M* borate, pH 9.5, and 20 m*M* phosphate, pH 6.0, respectively, which indicates that focusing occurs mainly under non-stacking conditions. Figs. 9g–l demonstrate the dependence of sample ionic strength (conductivity) on purine focusing, where phosphate is varied from 10 to 100 m*M*. Significant band broadening (destacking) of purine peaks was observed to occur when the phosphate ionic strength in the sample was >75 m*M*. Thus, the effect of buffer type, pH and ionic strength in the sample matrix can be rapidly assessed by multiplexed CE in order to determine the optimum conditions for focusing and resolution of purine metabolites. Capillary arrays normally suffer from poor inter-capillary reproducibility because of the variations in EOF (capillary surface), temperature (Joule heating), and the optical alignment of each capillary window to the detector. However, precision can be improved by adequate pre-separation rinsing, operation of low currents to ensure minimal Joule heating, as well as the use of internal standards to normalize the analyte migration times and peak areas [8]. The inter-capillary precision of dynamic pH junction multiplexed CE was determined to be excellent, as reflected by the average relative standard deviation (%RSD) of 1.8 and 2.4 for the normalized purine migration times and peak areas, respectively [30]. Over a 50-fold improvement in the concentration sensitivity was realized with a limit of detection ($S/N = 3$) of less than $8.0 \cdot 10^{-8}$ M under optimum conditions when using UV absorbance (214 nm). Further work will be carried out on applying on-line preconcentration formats for other classes of metabolites using multiplexed CE with UV detection.

2.7. Future prospects for on-line preconcentration of metabolites by CE

Although zwitter-ionic amino acids such as tyrosine can be focused by dynamic pH junction [25], most non-aromatic amino acids have low absorption coefficients, resulting in their poor concentration sensitivity when using UV detection. On-line sample preconcentration in conjunction with on-line chemical derivatization (UV or fluorescent probe) represents a promising method to enhance the detectability of weakly absorbing amino acids using UV or LIF detection. Recently, sample stacking with on-line derivatization of amino acids with 1,2-naphthoquinone-4-sulphonate was reported to improve sensitivity by over 1000-fold with respect to conventional pre-capillary derivatization using CE with UV detection [66]. On-line preconcentration techniques for sensitive analyses by CE are also critical to new types of detectors such as NMR, which provide a high information content for the qualitative identification of unknown metabolites, but suffer from very poor detection sensitivity [4]. Recent interest in the adaptation of microchip electrophoresis systems for rapid separations also benefit from on-line focusing methods because of the poor concentration sensitivity and low column efficiency even when using LIF detection [67]. Electrokinetic focusing strategies that can be applied to new modes of separation in CE, such as capillary electrochromatography using monolithic silica columns [68], can also be useful for enhancing detector sensitivity.

The validation of on-line sample preconcentration

strategies for real biological samples by CE merits further investigation. In most cases, off-line sample pretreatment is still required to either cleanup, preconcentrate or modify the sample matrix prior to injection. For example, plasma or protein samples require deproteinization by acid or high temperature, followed by centrifugation [29]. Because of the high concentration of salts and particulates in urine, centrifugation and subsequent dilution of urine samples are performed to ensure adequate reproducibility and prevent capillary blockages [27]. Analysis of cell extracts often involves neutralization (acid extract) or evaporation (methanol) of the sample in order render it compatible (e.g., adjusting buffer pH, conductivity, etc.) with on-line preconcentration techniques in CE [29]. In some cases, direct injection of large volumes of pharmaceutical solutions is possible without sample pretreatment [24]. The combination of off-line solid-phase extraction with on-line sample preconcentration CE represents a powerful method to achieve extremely high enrichment applicable to real samples [61]. With proper capillary rinsing and adequate sample pretreatment, sensitive, yet reproducible, analyses of several classes of metabolites in biological samples can be realized by CE using dynamic pH junction, sweeping and dynamic pH junction-sweeping formats of on-line sample preconcentration [24,26,27,29,30].

3. Conclusion

Metabolomics represents a new field of biological science that requires highly efficient and sensitive separation techniques to analyze the hundreds of diverse classes of metabolites present in the cell or biological fluids. Dynamic pH junction, sweeping and dynamic pH junction-sweeping represent three complementary on-line preconcentration methods in CE that can be used to improve concentration sensitivity by over three orders of magnitude, while generating extremely sharp detector bandwidths for high resolution separations. The injection of large sample plugs ($>$20% capillary length) and electrokinetic focusing of analyte zones (non-stacking conditions) prior to detection using multi-section electrolytes can be designed based on the physicochemical properties of the metabolites. Weakly acidic, basic or zwitter-ionic metabolites can be optimally focused by CE using dynamic pH junction based on the pK_a and/or the presence of specific functional groups, such as vicinal diols. In general, the sample is prepared in a buffer where the mobility of the analyte is zero (about 1 pH unit $<$pK_a), and the BGE is selected such that the analyte has a large mobility (about 0.5 pH unit $>$pK_a). Focusing is hypothesized to be caused by the formation of a transient pH gradient (pH titration) within the sample zone, which results in rapid focusing of metabolites that undergo velocity changes in the pH range selected even under strong EOF conditions. Single or mixed buffer types can be used to generate an appropriate dynamic pH junction for different metabolites of interest, such as catecholamines, deoxynucleosides, estrogens, amino acids, nucleotides and purines. Additional focusing mechanisms (other than pH) may occur when using dynamic pH junction under certain conditions, such as borate complexation when using borate buffers or t-ITP when applying mixed buffers that contain suitable terminating and leading co-ions. On-line preconcentration by sweeping using charged micelles is optimal for hydrophobic (neutral) metabolites that possess a high retention factor ($k' > 2$) under suppressed EOF conditions. Steroids (i.e., estrogens, androgens, corticosteroids) are particularly well suited for sweeping since these metabolites have strong affinity for many micelles and are acid-stable, so that sweeping can be performed under acidic conditions to suppress EOF using uncoated capillaries. Chemical derivatization of metabolites (hydrophilic) with a hydrophobic UV or fluorescent probe can be an effective way to enhance sweeping performance and detectability using UV and LIF detection. Dynamic pH junction-sweeping represents a hyphenated on-line preconcentration technique that is suitable for both weakly ionic and hydrophobic (neutral) metabolites. Enhanced focusing performance may be realized for certain metabolites, since focusing is based on multiple (additive) mechanisms, such as pH, borate complexation and micelle partitioning, compared to conventional techniques. Dynamic pH junction-sweeping may also offer unique advantages in terms of selectivity and resolution for the separation of complex mixtures. Analysis of

sub-μM of metabolites by on-line preconcentration CE has been demonstrated for several biological samples of interest such as cell extracts, plasma, urine and protein. Improved understanding of the mechanism of on-line preconcentration techniques and their application to different classes of metabolites in biological samples is required for future metabolomic studies by CE.

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References

[1] L.M. Raamsdonk, B. Teusink, D. Broadhurst, N. Zhang, A. Hayes, M.C. Walsh, J.A. Berden, K.M. Brindle, D.B. Kell, J.J. Rowland, H.V. Westerhoff, K. van Dam, S.G. Oliver, Nat. Biotechnol. 19 (2001) 45.
[2] D. Delneri, F.L. Brancia, S.G. Oliver, Curr. Opin. Biotechnol. 12 (2001) 87.
[3] W. Weckwerth, O. Fiehn, Curr. Opin. Biotechnol. 13 (2002) 156.
[4] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Nature Rev. 1 (2002) 153.
[5] S. Terabe, M.J. Markuszewski, N. Inoue, K. Otsuka, T. Nishioka, Pure Appl. Chem. 73 (2001) 1563.
[6] P. Britz-McKibbin, S. Terabe, Chem. Rec. 2 (2002) 397.
[7] X. Gong, E.S. Yeung, Anal. Chem. 71 (1999) 4989.
[8] X. Gong, H.M. Pang, E.S. Yeung, Anal. Chem. 71 (1999) 2642.
[9] D. Figeys, A. Ducret, R.J. Aebersold, J. Chromatogr. A 763 (1997) 295.
[10] D.S. Burgi, R.L. Chien, Anal. Biochem. 202 (1992) 306.
[11] R. Chien, D.S. Burgi, J. Chromatogr. 559 (1991) 141.
[12] C.X. Zhang, W. Thormann, Anal. Chem. 68 (1996) 2523.
[13] J.P. Quirino, S. Terabe, Anal. Chem. 70 (1998) 149.
[14] Z.K. Shihabi, J. Capillary Electrophor. 6 (1995) 267.
[15] Y. Xiong, S. Park, H. Swerdlow, Anal. Chem. 70 (1998) 3605.
[16] J.L. Beckers, J. Chromatogr. 641 (1993) 363.
[17] L. Krivankova, A. Vrana, P. Gebauer, P. Bocek, J. Chromatogr. A 772 (1997) 283.
[18] P. Gebauer, W. Thormann, P. Bocek, J. Chromatogr. 608 (1992) 47.
[19] P. Gebauer, W. Thormann, P. Bocek, Electrophoresis 16 (1995) 2039.
[20] J.P. Quirino, S. Terabe, Science 282 (1998) 465.
[21] J.P. Quirino, S. Terabe, Anal. Chem. 71 (1999) 1638.
[22] J. Palmer, N.J. Munro, J.P. Landers, Anal. Chem. 71 (1999) 1679.
[23] J.B. Kim, J.P. Quirino, K. Otsuka, S. Terabe, J. Chromatogr. A 916 (2001) 123.
[24] P. Britz-McKibbin, A.R. Kranack, A. Paprica, D.D.Y. Chen, Analyst 123 (1998) 1461.
[25] P. Britz-McKibbin, D.D.Y. Chen, Anal. Chem. 72 (2000) 1242.
[26] P. Britz-McKibbin, D.D.Y. Chen, Anal. Chem. 72 (2000) 1729.
[27] P. Britz-McKibbin, D.D.Y. Chen, Chromatographia 57 (2003) 87.
[28] P. Britz-McKibbin, K. Otsuka, S. Terabe, Anal. Chem. 74 (2002) 3736.
[29] P. Britz-McKibbin, M.J. Markuszewski, T. Iyanagi, K. Matsuda, T. Nishioka, S. Terabe, Anal. Biochem. 313 (2003) 89.
[30] P. Britz-McKibbin, T. Nishioka, S. Terabe, Anal. Sci. 19 (2003) 99.
[31] J.P. Quirino, S. Terabe, Anal. Chem. 72 (2000) 1023.
[32] G. McGrath, W.F. Smyth, J. Chromatogr. B 681 (1996) 125.
[33] J.P. Quirino, S. Terabe, Anal. Chem. 70 (1998) 1893.
[34] S. Hjerten, M.J. Zhu, J. Chromatogr. 346 (1985) 265.
[35] P.G. Righetti, A. Bossi, Anal. Chim. Acta 372 (1998) 1.
[36] P. Britz-McKibbin, J. Wong, D.D.Y. Chen, J. Chromatogr. A 853 (1999) 535.
[37] Data for Biochemical Research, 2nd ed., Oxford University Press, New York, 1969.
[38] A.B. Duso, D.D.Y. Chen, Anal. Chem. 74 (2002) 2938.
[39] Handbook of Biochemistry: Selected Data for Molecular Biology, CRC Press, Cleveland, 1970.
[40] P. Britz-McKibbin, T. Ichihashi, K. Tsubota, D.D.Y. Chen, S. Terabe, J. Chromatogr. A (2003) in press.
[41] R. Aebersold, H.D. Morrison, J. Chromatogr. 516 (1990) 79.
[42] S.J. Wang, W.L. Teng, Y.W. Lin, H.T. Chang, J. Chromatogr. A 979 (2002) 261.

[43] C.X. Cao, Y.Z. He, M. Li, Y.T. Qian, M.F. Gao, L.H. Ge, S.L. Zhou, L. Yang, Q.S. Qu, Anal. Chem. 74 (2002) 4167.
[44] W.E. Rae, J.E. Wong, C.A. Lucy, J. Chromatogr. A 781 (1997) 3.
[45] W. Wei, G. Xue, E.S. Yeung, Anal. Chem. 74 (2002) 934.
[46] T. Adam, J. Sevcik, Z. Svagera, L.D. Fairbanks, P. Bartak, Electrophoresis 20 (1999) 564.
[47] Z.K. Shihabi, Electrophoresis 23 (2002) 1612.
[48] J.P. Quirino, S. Terabe, Chromatographia 53 (2001) 285.
[49] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[50] M.R.N. Monton, J.P. Quirino, K. Otsuka, S. Terabe, J. Chromatogr. 939 (2001) 99.
[51] J.P. Quirino, S. Terabe, K. Otsuka, J.B. Vincent, G. Vigh, J. Chromatogr. A 838 (1999) 3.
[52] N.J. Munro, J. Palmer, A.M. Stalcup, J.P. Landers, J. Chromatogr. B 731 (1999) 369.
[53] K. Otsuka, M. Matsumura, J.B. Kim, S. Terabe, J. Chromatogr. A 979 (2002) 425.
[54] M.M. Hsieh, W.L. Tseng, H.T. Chang, Electrophoresis 21 (2000) 2904.
[55] J.P. Quirino, S. Terabe, P. Bocek, Anal. Chem. 72 (2000) 1934.
[56] J.B. Kim, K. Otsuka, S. Terabe, J. Chromatogr. A 912 (2001) 343.
[57] J.P. Quirino, J.B. Kim, S. Terabe, J. Chromatogr. A 965 (2002) 357.
[58] R.P. Maharjan, T. Ferenci, Anal. Biochem. 313 (2003) 145.
[59] Z. Chen, W. Zheng, L.M. Dunning, K.G. Anderson, R.S. Parrish, J.L. Holtzman, Steroids 64 (1999) 856.
[60] P. Matthiessen, J.P. Sumpter, Effects of Estrogenic Substances in the Aquatic Environment, Birkhauser, Basel, 1998.
[61] H. Harino, S. Tsunoi, T. Sato, M. Tanaka, Fresenius J. Anal. Chem. 369 (2001) 546.
[62] C. Saeki, K. Otsuka, S. Terabe, unpublished results.
[63] Modern Chromatographic Analysis of Vitamins, Marcel Dekker, New York, 2000.
[64] E. Zubritsky, Anal. Chem. 74 (2002) 22A.
[65] T. Wessel, C. Lanvers, S. Frends, G. Hempel, J. Chromatogr. A 894 (2000) 154.
[66] R.M. Latorre, J. Saurina, S. Hernandez-Cassou, Electrophoresis 22 (2001) 4355.
[67] Y. Sera, N. Matsubara, K. Otsuka, S. Terabe, Electrophoresis 22 (2001) 3509.
[68] H. Kobayashi, C. Smith, K. Hosoya, T. Ikegami, N. Tanaka, Anal. Sci. 18 (2002) 89.