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Preconcentration and frontal electroelution of amino acids for in-line solid-phase extraction–capillary electrophoresis

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Abstract

A new frontal electroelution approach that can be used for the preconcentration of amino acids in in-line solid-phase extraction–capillary electrophoresis (SPE–CE) has been developed. A single capillary was employed featuring a short monolithic SPE column created inside the capillary via photo-initiated, free-radical polymerisation of 3-sulfopropyl methacrylate and butyl methacrylate monomers. A weak electrolyte of dilute H_2SO_4, pH 2.9, was found to promote adsorption of the amino acids onto the SPE column. Elution of the amino acids was achieved using a dual solvation/ion-exchange transient boundary mobilised via EOF by using a strong electrolyte containing 62.5 mM ethylenediamine, pH 2.9 with H_2SO_4 and 40% (v/v) acetonitrile. Using these two electrolytes, tryptophan was adsorbed onto the SPE column in weak electrolyte and eluted via a frontal electroelution mechanism in the strong electrolyte. Injections up to 20 min, corresponding to over 14 column volumes (or 1400% of the capillary volume) of sample provided quantitative extraction of tryptophan from the weak electrolyte and were eluted without any loss in efficiency. This represents a practical increase of approximately 300-fold when compared to a typical hydrodynamic injection occupying 5% of the capillary volume.

Keywords: Solid-phase extraction; Capillary electrophoresis; In-line SPE–CE; Frontal electroelution; Focusing

1. Introduction

Over the last decade, capillary electrophoresis (CE) has established itself as a viable alternative to high performance liquid chromatography (HPLC), and can be used for almost any chemical analysis ranging from the analysis of small inorganic ions to large polymers and particles. While its flexibility and versatility are perhaps its greatest strength, the concentration detection limits are generally much lower than those obtained by HPLC and is one of the most significant limitations of this technique.

Various approaches have been developed to enhance the sensitivity of all forms of CE, with the most common being those based on stacking. Stacking is the term used to indicate accumulation, or focusing, of analytes on a boundary via electrophoretic means. The boundary is usually created by the relationship of the sample (and matrix) to the electrolyte, and in its simplest form arises from the sample having at least a ten times lower conductance than the separation electrolyte [1]. pH boundaries can be used to stack ionisable and zwitterionic analytes [2–4], while isotachophoretic boundaries can be exploited for samples that have a naturally high concentration of a particular ion [5,6]. Pseudo-stationary phases used in electrokinetic chromatography can also be used for stacking of neutral and charged analytes via stacking and sweeping [7–9]. While these approaches are impressive in their performance and simplicity, particularly the combination of multiple staking mechanisms, they are all limited by the capillary volume. This limitation can be partly overcome by using electrokinetic injection, but this approach generally suffers from poor reproducibility and it discriminates on the basis of mobility.

The integration of solid-phase extraction (SPE) with CE can potentially overcome these limitations as preconcentration is achieved via adsorption of the analytes onto a stationary phase. Sample can therefore be pumped through the SPE device via pressure allowing multiple capillary volumes of sample to be injected without the use of electrokinetic injection. There are three ways in which SPE can be integrated with CE. Off-line SPE–CE has no integration and involves manual transfer of the sample from the SPE column to the CE. On-line SPE–CE

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involves automatic transfer of the analytes from the SPE column to the CE but both components are housed in separate instruments, and interfacing is commonly achieved by a flow-injection approach. In-line SPE–CE has the SPE column directly coupled to the CE capillary. While each approach has its advantages and disadvantages, in-line SPE provides the simplest and potentially most powerful system.

In-line SPE–CE was first demonstrated by Guzman et al. who used a small packed bed for the preconcentration of methamphetamine [9]. Subsequently, the groups of Landers [10–13] and Tomlinson et al. [14,15], continued the development of in-line SPE–CE, however there were several issues with the use of packed beds. Firstly, it was difficult to make a suitable SPE column. Secondly, continued use of the device resulted in compression of the column bed leading to irreproducible flow rates and sample volumes. Finally, elution of the adsorbed analytes from the SPE material was inefficient and led to peak broadening. The first two issues can be resolved by the use of open-tubular columns, and while these are simple to prepare and offer excellent flow rates, the capacity of these columns is often too small to be practically useful. Continuous monoliths are new generation chromatographic media that are highly permeable and have high surface areas and as such overcome the limitations of packed and open-tubular columns. They are prepared via polymerisation of a precursor mixture inside the capillary thus allowing simple preparation and precise control over the chemical and physical properties of the material. To date, they have been used in capillaries and microchips for a number of SPE applications including proteins[16], DNA [17], drugs [18] and inorganic ions [19].

The use of monolithic SPE columns addresses the first two limitations with in-line SPE–CE, however the final issue of a loss of efficiency is more serious and is due to the method of analyte elution. Early reports used a small volume of solvent that was flushed through the SPE column under pressure to elute the analytes [9,12–14,20]. The solvent plug was stopped just after the SPE column and when the voltage was applied, various stacking approaches were employed to focus the analytes into a sharp band. This approach was somewhat successful, however the volume of solvent required to elute the analytes was often too large for efficient stacking resulting in broad peaks. The work of Breadmore et al. and Li et al. have shown that if the SPE–CE conditions are selected appropriately, it is possible to simultaneously elute and focus analytes into an exceptionally sharp band. This approach, termed frontal electroelution, allows transferral of the analytes into the CE section of the capillary in a highly efficient manner. The key to this approach is the generation of an appropriate transient boundary and the behaviour of the analytes on either side of this boundary. In a series of papers, Breadmore et al. [21–23] developed the concept and theory of a transient isotachophoretic boundary for the frontal electroelution of inorganic anions preconcentrated via an ion-exchange SPE column, and was subsequently used for the determination of trace ions in water samples [19]. Li et al. [24] have used a transient pH boundary for the frontal electroelution of amino acids from an anion-exchange phase and subsequently separated as cationic compounds in a low pH electrolyte. In this case, electroelution was achieved by a change in ionisation state of the amino acids. While this is a new and interesting approach, the electroelution mechanism is limited in that it can only be applied to low conductivity samples due to the reliance on electric field strength to focus the analytes.

The aim of this work is to develop a new approach for the frontal electroelution of amino acids based on a dual solvation/ion-exchange transient boundary for in-line SPE–CE. A SPE monolith was created via photoinitation of methacrylate monomers to form a mixed-mode ion-exchange reverse-phase material for in-line SPE–CE. Attention is focused on the first part of the process, namely the adsorption and elution of the adsorbed analytes and on understanding the conditions required for frontal electroelution of amino acids that does not rely on a change in ionisation state of the analytes. This new approach is demonstrated by the efficient electroelution of amino acids from multiple column volumes of sample.

2. Experimental

Electrophoretic separations were performed using a HP^{3D}CE (Agilent Technologies, Waldbron, Germany). Separations were carried out using UV-transparent fused silica capillary (Phoenix, AZ, USA) of 50 μm i.d. with a length of 34.5 cm, 8.5 cm to detector, unless otherwise noted. External pressure using in-house compressed air at a pressure of 6 bar was used for on-line flushing of the monolith and for sample injection.

For the preparation of the SPE monolith, an OAI deep UV illumination system series (model LS30/5, San Jose, CA, USA) fitted with a 500 W HgXe lamp was used for UV exposure at a constant intensity of 20.0 mW/cm^2 for 10 min (for calibration, the irradiation power was adjusted to 20.0 mW/cm^2 using an OAI model 306 intensity monitor with a 260 nm probe head).

2.1. Chemicals

3-Sulfopropyl methacrylate (SPMA, 98%), butyl methacrylate (BuMA, 98%), ethylene dimethacrylate (EDMA, 98%), 1-propanol (99.5%), 1,4-butanediol (99%), 2,2-dimethoxy-2-phenylacetophenone (DMAP, 99%), ethylenediamine (technical grade) and NaOH (99%) were purchased from Aldrich (Milwauke, WI, USA). 3-(Trimethoxysilyl)propyl methacrylate (98%), alanine (Ala, 98%), tyrosine (Tyr. 98%), phenylalanine (Phe, 98%) and tryptophan (Trp, 98%) were from Sigma (Sigma, St Louis, MO, USA). Acetone (AR grade), ethanol (HPLC grade), CH_3COOH (99%) and acetonitrile (HPLC grade) were from BDH (Kilsyth, Vic., Australia) and H_2SO_4 (98%) and HCl (99%) were from Ajax Chemicals (Sydney, NSW, Australia). Ethylenediamine was purified by distillation, while BuMA and EDMA were passed through a bed of basic alumina (50–55%, Aldrich) to remove inhibitors and distilled under reduced pressure.

2.2. Monolith SPE column fabrication

Teflon-coated fused silica capillaries were rinsed using a Harvard syringe pump (Harvard Apparatus, Holliston, MA, USA)

and a 250 μL gas-tight syringe (Hamilton Company, Reno, NE, USA) with water, acetone, 0.2 mol/L NaOH for 30 min, water, 0.2 mol/L HCl for 30 min, water, and ethanol. A 20% (w/w) solution of 3-(trimethoxysilyl)propyl methacrylate in 95% ethanol adjusted to pH 5 using acetic acid was pumped through the capillary at a flow rate of 30 μL/Hr for 1 h. The capillary was then washed with ethanol and dried with a stream of air overnight. Short monolith columns (~10 cm in length) were made by filling the capillary via capillary action with a deaerated polymerisation mixture consisting of 26.5% (w/w) BMA, 3.5% (w/w) SPMA, 20% (w/w) EDMA, 6.4% (w/w) H_2O, 28% (w/w) 1-propanol, 16% (w/w) 1,4-butanediol and 1% (w/w) DMAP (with respect to monomers) and irradiated with UV light for 10 min at a distance of 30 cm. Unreacted monomers were removed by flushing the column with methanol for 4 h before being conditioned with water and electrolyte, both for 1 h, at a flow rate of 100 μL/Hr. Capillaries were subsequently mounted in the Agilent HP^{3D}CE cassette, cut to length and conditioned with a combination of pressure (6 bar) and incremental increases of voltage (every 5 kV up to 20 kV) until a stable baseline and current were obtained.

2.3. In-line SPE–CE

Stock solutions containing 1 mM of each amino acid were prepared in 10 mL of H_2O acidified with 1 drop of HCl and diluted in H_2O as required. Weak electrolytes were prepared by dilution of H_2SO_4 with water to the appropriate pH. Strong electrolytes were prepared by titration of ethylenediamine with H_2SO_4 to pH 2.9 and diluted with H_2O and acetonitrile to the appropriate composition. All electrolytes were degassed using vacuum sonication for 2 min and filtered through a 0.45 μm filter.

3. Results and discussion

3.1. Conditions for frontal electroelution

Frontal electroelution has been developed specifically for in-line SPE–CE and it exploits fundamental characteristics of both HPLC and CE. It is based on the generation of an elution boundary, mobilisation of this boundary via electrophoretic means, and elution of the adsorbed analytes via the moving elution boundary. When the experimental conditions are selected appropriately, it is possible to elute and focus the analytes into an extremely narrow band. In order for this simultaneous elution and focusing to occur, the following three conditions must be met. First, the electrolyte in front of the boundary, called the weak electrolyte, must promote strong adsorption of the analytes to the SPE phase. Second, analytes in the electrolyte on the rear of the boundary, called the strong electrolyte, must have no interaction with the SPE phase. Third, the analytes must have a velocity in the strong electrolyte that is quicker than that of the boundary. When these three requirements are met, analytes adsorbed onto the SPE phase are eluted by the strong electrolyte on the rear of the boundary, and then because their velocity is quicker than that of the boundary they move back into the weak electrolyte where they are re-adsorbed onto the SPE phase. The analytes remain adsorbed on the phase until the boundary catches up and they are eluted by the strong electrolyte again. As the boundary moves through the SPE column these processes continue and the analytes become focused into a very sharp band. At the end of the SPE column, the boundary pushes the focused band of analytes into the CE separation capillary and the analytes separate according to normal electrophoretic principles. A schematic diagram representing these processes for in-line SPE–CE is shown in Fig. 1. This approach has been shown to provide no loss in separation performance due to the introduction of the SPE phase, and eliminates the problem of band broadening associated with early studies of in-line SPE–CE. However, for any new system, it is necessary to optimise the composition of the weak and strong electrolytes to ensure that analytes are eluted and focused from the SPE phase via this mechanism. This work focuses on the development of appropriate weak and strong electrolyte combinations for the frontal electroelution of amino acids from the SPE column, and as such all of the electropherograms shown were obtained with detection immediately after the SPE column.

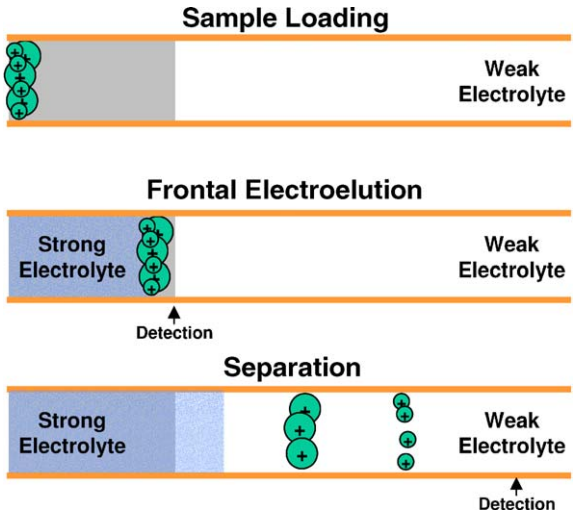

Fig. 1. Schematic representation of the processes involved in in-line SPE–CE. Sample is loaded under pressure/voltage with the analytes in weak electrolyte. The components are then eluted from the SPE column with voltage via a frontal electroelution mechanism based on electrophoretic movement of the strong electrolyte. After exiting the SPE column, analytes migrate ahead of the strong electrolyte front and separate according to their electrophoretic mobility. This work focuses on the first two steps, namely adsorption and electroelution.

3.2. Optimisation of the weak electrolyte composition

The monolith used as the SPE column in this work contains both cation-exchange sites, from the SPMA monomer, and reverse-phase sites, from the BuMA monomer. The use of a SPE material with both ion-exchange and reverse-phase properties means that a suitable weak electrolyte will ideally promote adsorption of the analytes onto the SPE phase by both of these mechanisms. Conditions for promoting reverse-phase adsorption can be achieved by the use of a purely aqueous solvent,

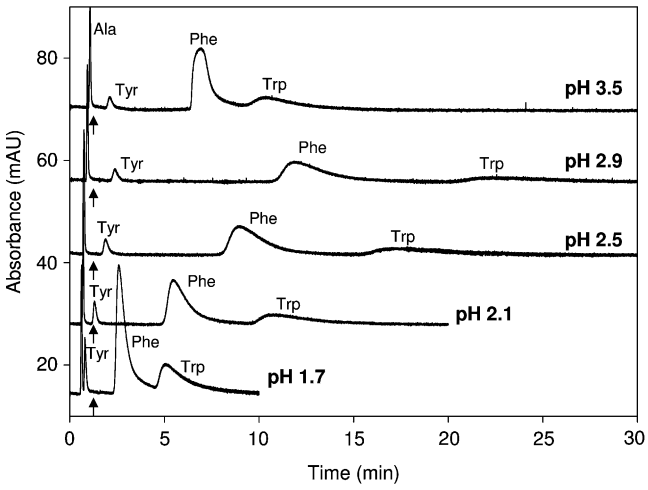

Fig. 2. Optimisation of the weak electrolyte pH to maximise interaction of the amino acids with the SPE monolith. Column: 34.5 cm × 50 μm id (8.5 cm effective length), with an 8.5 cm SPMA monolith, −25 kV, 25 °C, 214.10 nm. Sample 0.1 mM of each amino acid injected 6 bar for 6 s. The position of the EOF at each pH is indicated by the position of the arrow.

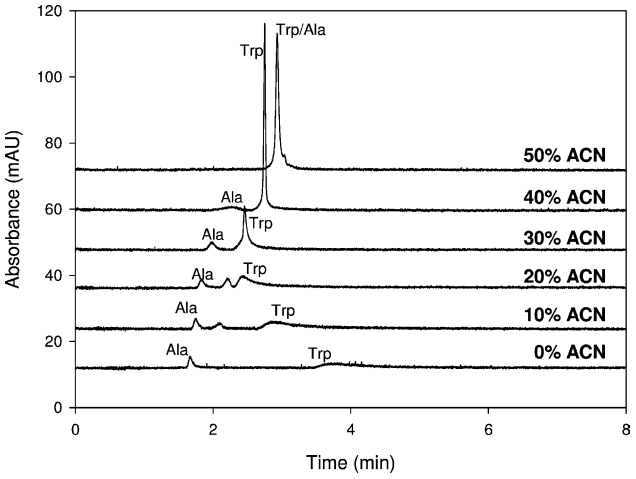

Fig. 3. Optimisation of the strong electrolyte composition for efficient elution of the amino acid Trp from the SPE monolith. Electrolyte: 62.5 mM ethylenediamine adjusted to pH 2.9 with H_2SO_4 with the addition of X% (v/v) acetonitrile. Sample of 0.1 mM of Ala and Trp in water acidified with HCl. All other conditions as in Fig. 2.

however, maximising the cation-exchange adsorption of the amino acids is more complex. The pK_as of the carboxylate group in each of the amino acids is approximately 2.0–2.3 and a low pH should therefore provide the highest net positive charge and provide strong cation-exchange interaction with the SPE column. However, a low pH will provide a high concentration of H^+ and the electrolyte will be a stronger ion-exchange eluent and will reduce the cation-exchange interaction of the analytes with the SPE monolith. Thus a higher pH is more desirable to minimise the ion-exchange elution strength of the electrolyte. The optimal pH will therefore be a compromise between maximising the net positive charge of the amino acids and minimising the elution strength of the electrolyte. The significance of both of these factors can be seen in Fig. 2 which shows the separation of four amino acids in dilute H_2SO_4 with the monolithic SPE column. The position of the EOF is indicated by the arrow at each pH. As expected, an increase in pH from 1.7 to 2.9, results in longer migration times for Tyr, Phe and Trp due to more interaction with the monolith as a consequence of a decrease in the concentration of H^+ (20 mM at pH 1.7 compared to 1.2 mM at pH 2.9). Above a pH of 2.9, the retention decreases as the net positive charge of the amino acids decreases due to deprotonation of the carboxylate group. In all cases, the migration of Ala was almost constant and migrated before the EOF indicating that this amino acid has very little interaction with the monolith under any of the conditions examined. This is in contrast to the other three amino acids which migrate well after the EOF due to considerable interaction with the monolith. Without the monolith, all amino acids migrate well before the EOF due to their net positive charge. Trp showed the greatest interaction with the monolith and shows the most potential for preconcentration in SPE–CE.

A weak electrolyte consisting of aqueous dilute H_2SO_4, pH 2.9 was selected as the optimal weak electrolyte for the preconcentration of the four amino acids examined.

3.3. Optimisation of the strong electrolyte composition

The strong electrolyte must be able to elute the analytes from the SPE monolith by suppression of both the ion-exchange and reverse-phase interactions. Suppression of the ion-exchange component can be achieved by varying the pH (as shown above) however this introduces a pH boundary into the elution mechanism which can cause the analytes to stack via electrophoretic means. An alternative approach is to keep the pH constant and to add a high concentration of a strong competing ion. Ethylenediamine was selected as the competing ion due to its small size and +2 charge, and its compatibility with the acetonitrile required to eliminate residual reverse phase interactions with the SPE monolith. Fig. 3 shows the separation of the least retained amino acid, Ala, and the most retained, Trp, with the SPE monolith using a electrolyte made from 62.5 mM ethylenediamine titrated to pH 2.9 with H_2SO_4. It can be seen that without acetonitrile, Trp migrates after Ala indicating a small amount of interaction with the SPMA monolith via residual reverse-phase interactions. Adding acetonitrile to the electrolyte results in a decrease in migration times due to suppression of the reverse-phase interaction of the analytes with the monolith. With 40% (v/v) acetonitrile added to the electrolyte a single sharp peak is obtained for Trp indicating that there was no interaction with the SPE monolith in this electrolyte suggesting that this electrolyte will be suitable for the efficient elution of the amino acids after adsorption in the weak electrolyte. Interestingly, the peak of Ala becomes slightly slower and broader in 40% ACN and migrates under the Trp peak in 50% ACN. This was attributed to a slight change in the ionisation of the amine group due to the presence of ACN.

It is important to note that because both acetonitrile and ethylenediamine are necessary to obtain efficient elution of Trp from the SPE column and the fact that acetonitrile is neutral, movement of the electroelution boundary is accomplished by

EOF. This was experimentally confirmed by monitoring the elution of Trp from the monolith with and without a short injection of thiourea, an EOF marker. The focused peak of Trp was observed to coincide exactly with the position of the thiourea peak indicating that the amino acid was eluted and focused along the monolith with an EOF-mobilised elution front. This indicates that this electrolyte will function suitably as the strong electrolyte in an in-line SPE–CE system.

An electrolyte composition of 62.5 mM ethylenediamine titrated to pH 2.9 with H_2SO_4 and 40% (v/v) acetonitrile was judged to be the best strong electrolyte for these analytes and used for all subsequent separations.

3.4. Demonstration of preconcentration and electroelution of Trp

To demonstrate the potential of the developed weak and strong electrolytes for the preconcentration and frontal electroelution of amino acids for in-line SPE–CE, Trp was selected as the test analyte due to its strong adsorption onto the monolith in the weak electrolyte and efficient elution in the strong electrolyte. Prior to injection of the sample, the SPE monolith was flushed with weak electrolyte to ensure that the column was conditioned appropriately and that the capillary was completely filled with weak electrolyte. The bottom trace in Fig. 4 shows a blank 20 min injection of sample matrix (14.7 capillary columns), namely the dilute H_2SO_4 identified above for promoting maximum amino acid adsorption. The position of the weak electrolyte/strong electrolyte boundary, the elution boundary, can be seen clearly at approximately 1.5 min and the absence of any positive peaks indicates that there is no contamination and that no system peaks occur due to the generation and movement of the boundary. Subsequent traces in Fig. 4 show the effect of decreasing the concentration of Trp by a factor of 10 while increasing the injection time by the same factor. It is important to note that detection in the traces shown in Fig. 4 was performed just after the end of the SPE monolith to allow the efficiency of the electroelution front to be evaluated. Comparison of the three traces show a single sharp peak obtained for Trp superimposed on the electroelution boundary for all three separations. The peak is similar in height, area and width, with recoveries relative to the 0.2 min injection of 99.2% and 98.7% for the 2.0 and 20.0 min injections, respectively.

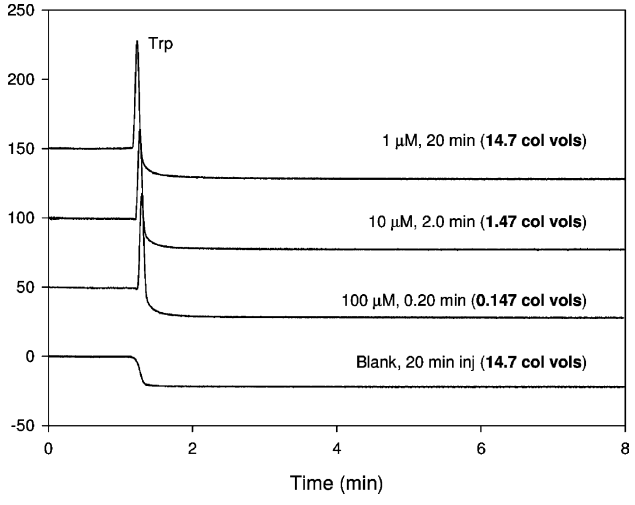

Fig. 4. Preconcentation and frontal electroelution of the amino acid Trp using the optimised weak and strong electrolytes. Weak electrolyte: dilute H_2SO_4, pH 2.9. Strong electrolyte: 62.5 mM ethylenediamine pH 2.9 with H_2SO_4 and 40% (v/v) acetonitrile. Prior to all injections, the SPE monolith was conditioned for 20 min with weak electrolyte. Injection was performed for the appropriate time (0.2–20 min) at a pressure of 6 bar.

In all SPE methods, the capacity of the SPE column governs the amount of sample that can be analysed. In conventional SPE, this manifests as a plateau of peak area and peak height as the injection volume increases due to saturation of the surface area of the column. This is a limitation in the adsorption component of the in-line SPE–CE approach. For methods based on frontal electroelution, the unique focusing and elution mechanism can easily be overloaded. Analytes are continuously focused around the transient boundary and high concentrations of analytes in front of the boundary can exceed the localised column capacity in that region. This leads to a decrease or plateau in peak height (due to localised saturation of the column capacity) while the peak area linearly increases with injection time (the whole column has not yet been saturated). In effect, this results in a decrease in the efficiency of the electroelution mechanism, and a broadening of the focused peak. To determine whether the adsorption or electroelution component of the in-line SPE–CE method was the major limiting factor, the injection time of Trp was increased from 0.2 min (0.147 column volumes of sample) to 80 min (58.8 column volumes of sample). Fig. 5 shows the change in peak height, peak area and peak width of Trp as the injection time is increased over this range. It can be seen that in all three cases, there is a discontinuity around 20 min. The peak area and peak height increase linearly ($r^2 > 0.99$) and the peak width remains constant for injections up to 20 min indicating that Trp is quantitatively extracted up to this point. Above 20 min, the peak area continues to increase, although it is no longer linear, the peak height levels off and the peak width increases. The fact that the peak area continues to increase in a small fashion

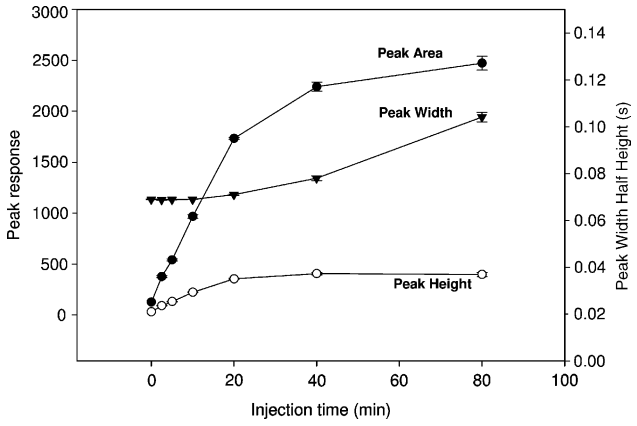

Fig. 5. Variation in peak area and peak height for the preconcentration and fronal electroelution of Trp using the SPE monolith. The sample contained 1 μM of Trp and was injected at 6 bar for 0.2–80 min.

after 20 min indicates that the limiting factor governing the preconcentration and frontal electroelution of Trp is the adsorption component of the in-line SPE–CE mechanism, and not the electroelution component. From the concentration of Trp (1 μM) and the volume of sample injected (14.7 column volumes, 1 column volume = 511 nL), the capacity of the monolith was estimated to be 5.1×10^{-12} equivalents.

The maximum injection time that provided quantitative extraction of Trp examined here was 20 min and represents an injection of 14.7 capillary volumes of sample and is a 100-fold improvement over the lowest injection volume of 0.147 capillary volumes examined experimentally. Practically, this represents approximately 300 times more sample injected than is possible to inject with a standard injection occupying 5% of the capillary length. Of particular note is that there is no loss in elution efficiency as the injection volume is increased, a problem that was frequent in early studies of in-line SPE–CE. However, the practicality of this approach is limited due to the low capacity of the SPE column making the method unsuitable for moderate- and high-ionic strength samples. Optimisation of the monolith properties to increase the capacity of the SPE column and demonstration of this approach for the preconcentration and separation of amino acids will be dealt with in forthcoming publications.

4. Conclusions

A new method for the frontal electroelution of amino acids from a monolithic SPE column in in-line SPE–CE has been developed. The monolith used for in-line SPE–CE was created via photoiniated free radical polymerisation and possessed both ion-exchange and reverse-phase adsorption sites. Optimisation of the weak electrolyte showed that maximum interaction of the amino acids was obtained in dilute aqueous H_2SO_4 at a pH of 2.9. Elution of the amino acids was achieved using a strong electrolyte consisting of 62.5 mM ethylenediamine with the pH adjusted to 2.9 with H_2SO_4 and 40% (v/v) acetonitrile. Using these weak and strong electrolytes, Trp was preconcentrated and focused via a frontal electroelution mechanism with high efficiency from the SPE monolithic column. Injections of 0.147 to 14.7 capillary volumes of Trp showed no loss in elution efficiency and quantitative extraction of the analyte from the sample. This allows a 300-fold increase in sensitivity over a conventional hydrodynamic injection to be obtained.

Acknowledgments

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