GRADIENT ELUTION MOVING BOUNDARY ELECTROPHORESIS

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ABSTRACT

A novel method for performing electrophoretic separation and/or purification of compounds in a mixture is described. The method utilizes the electrophoretic migration of chemical species in solution in combination with variable hydrodynamic bulk flow of the solution through a separation capillary or microfluidic channel. The method can be used with the bulk solution flow either in the same direction as the electrophoretic migration or in the opposite direction. Continuous sample introduction eliminates the need for a sample injection mechanism, allowing for implementation in much reduced areas. The method has been designated as gradient elution moving boundary electrophoresis (GEMBE).

With the bulk flow and electrophoretic migration in opposite directions, the method is useful for providing improved separation resolution, particularly in very short separation channels. In this case, only analytes with electrophoretic velocities greater than the bulk fluid velocity can enter the separation column and be detected as boundary zones. As the hydrodynamic flow is swept from high to low, analytes of lower mobilities are introduced, allowing for separation of compounds based upon electrophoretic mobility variations. Slower sweeping rates allow for greater separation resolution.

With the bulk flow and electrophoretic migration in the same direction, the species to be analyzed pass through the separation channel more rapidly, so that analysis times are reduced. The variation of the bulk flow velocity over time insures that the optimal bulk flow velocity is used for each portion of the separation.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates in general to electrophoretic separations, both in capillaries and in microfluidic channels, in order to separate and/or purify ionic compounds from a mixture, and, more particularly, to the combination of electrophoresis in the presence of a variable hydrodynamic flow with continuous introduction of sample. It is expected that the primary application will be in a microfluidic embodiment in order to reduce the total footprint of such microfluidic devices.

1. Brief Description of Related Art

Electrophoresis is a well known analytical method for separation of ionic compounds through use of an applied voltage. The separation mechanism is based mainly on differences in analytes' size and charge, although other factors can be exploited such as shape, density, affinity, *etc*. Early electrophoresis work utilized continuous sample introduction into a relatively large glass U-tube across which a voltage was applied; analytes were detected in free solution as discrete boundaries throughout the tube [1]. Electrophoresis was then applied to hydrated gel supports (e.g., polyacrylamide gel

electrophoresis, PAGE) and later to micron diameter capillary tubes (*i.e.*., capillary electrophoresis or CE). Typically, CE utilizes a discrete sample injection into the capillary in the presence of buffer electrolytes. A fixed detector is placed near the capillary outlet, past which analytes of highest mobility migrate first followed by species of slower mobility; individual analytes are typically detected as bands or peaks. For example, with a negative voltage applied to the capillary inlet, positively charged analytes will be detected first, followed by neutral species, and finally species exhibiting a negative charge. The detector response can then be correlated to analyte migration speed and, through the use of standard mixtures, be used to identify and/or quantify analytes.

The two primary electromotive forces in electrophoresis in a confined separation medium (*i.e.*, tubular, capillary, or microfluidic channel) are analyte electrophoretic mobility and electroosmotic flow (EOF). EOF arises when a surface charge is present along the separation column; in glass-based separations with solutions above pH of approximately 2 the surface silanol groups exhibit net negative charges. Positively charged ions in the solution media (typically a buffered electrolyte solution) are drawn towards the negative surface forming an electrical double layer which can be mobilized axially under an applied voltage along the length the separation media. The mobile cations additionally cause movement of the bulk solution through viscous drag, referred to as EOF [2].

There are two primary disadvantages to the common method of performing CE. First, the method requires an injection to load the mixture to be analyzed into the capillary as a minimally wide plug. Second, long channels are required to produce high-resolution separations. Both of these disadvantages increase the space required to implement CE in a microfluidic chip format. Although long separation channels can be fit into a small area on a microfluidic chip using a serpentine pattern, every turn in the path of the separation channel degrades the separation resolution. Consequently, microchip CE still generally gives lower resolution separations than can be achieved with conventional capillary-based CE.

Two sample loading techniques are in general use for CE: electrokinetic and hydrodynamic injections. The former relies upon electrophoresis to introduce the sample. The inlet is placed in the mixture media to be analyzed, a voltage is applied for a finite time, and the inlet is then placed in a blank solution containing only the background electrolytes. This method introduces preferential injection bias, as those analytes of highest mobility are injected to a greater extent than those of lower mobility. For example, when a negative voltage is applied to the inlet, positive species are injected both by electrophoresis and EOF, while neutral compounds are mobilized solely by EOF and negative species are pulled away from the inlet by electrophoresis while still being injected by EOF.

Hydrodynamic injections can be accomplished similarly to electrokinetic methods by using a pressure differential across the separation column, as opposed to an electrical differential. Most commonly, this is employed using either a sample vial elevated relative to the column outlet (gravity fed) or by using a pump. While eliminating electrical injection bias, hydrodynamic injections suffer from possible sample injection plug broadening due to the laminar flow profile induced by the pressure differential, ultimately

leading to analyte zone broadening and a loss in separation efficiency. Additionally, hydrodynamic injections are much more difficult to implement in a microfluidic format, either necessitating off-chip injection equipment or complicated fabrication procedures, as the gravity fed method of injection is not readily available.

There are many examples in the prior art of the use of bulk flow in conjunction with electrophoresis for enhanced resolution or reduced analysis times. The most common example is the use of the native, unmodified EOF of the silica capillary to induce a bulk flow in the direction opposite to the direction of migration of anionic analytes. The time required for the migration of analytes through the length of the separation capillary and the resolution of the separation are thus increased. In addition, there are many examples of the use of coatings [3;4] or external electric fields [5;6] to vary the EOF of silica capillaries so that the bulk flow is set to the value desired for a given separation problem.

In addition to controlling bulk flow through modifications of the EOF, pressure-driven flow has also been used in conjunction with EOF as a means of better regulating bulk flow during electrophoresis. In this manner, the bulk flow rate can be decoupled from the electrical characteristics of the system and employed in a way to generate a constant, bulk flow for CE using discrete injections [7-l0]. U.S. Pat. Nos. 5,429,728 and 5,482,608 are illustrative of the method.

Generally, methods to control the bulk flow are used to either increase analyte residence time in the electric field in order to enhance the separation or to decrease the residence time in order to reduce analysis times. Use of a bulk counter flow and continuous introduction of the sample can also allow for the purification of species [11-13], provided that the mobility of the species of interest is either the greatest or least of all mixture components.

SUMMARY AND DESCRIPTION OF THE INVENTION

The present invention is a method for the separation of the constituents of a sample mixture. The present invention incorporates a process for coupling continuous sample introduction with a variable hydrodynamic bulk flow for electrophoretic separations in a capillary, microfluidic channel, or other separation column. By changing the bulk flow velocity over time during the separation, analytes are sequentially eluted onto the separation column from the sample vessel where they can individually be detected as zone boundaries.

The method can be applied to a capillary or micro fluidic system. A typical apparatus includes a sample reservoir, a separation column, an outlet reservoir, and a detector for detecting the presence of analyte at some point along the length of the column. Both the inlet and outlet ends of the separation column are connected electrically to a voltage control device. Also included in the apparatus is a means for controlling the variable bulk flow velocity. For example, a regulated pressure control device could be connected to either the inlet reservoir or outlet reservoir. Schematic representations are shown in Figure 1. The pressure differential across the inlet and outlet of the separation column (and, hence, the bulk flow rate) can be made to vary with time, either linearly, or non-linearly (*e.g.*, exponentially or step-wise) throughout the electrophoretic separation.

The primary advantage of this invention over prior art methods of electrophoresis is that it requires much shorter separation lengths and a much smaller total area or footprint on a microfluidic chip. The use of continuous sample introduction and variable bulk flow has the major advantage over conventional CE methods of not requiring any injection mechanism, eliminating the associated costs and footprints of such mechanisms. Consequently, much smaller chips can be used, reducing the cost of fabrication. Additionally, reduction of the area required for electrophoretic separation allows for the integration of more analysis elements (sample preparation, chemical reaction, *etc*.) into a fixed chip area.

Prior art electrophoresis methods have generally employed a constant bulk flow during the separation, either arising solely from native EOF or through the use of controlling mechanisms. A modified constant bulk flow, such as a pressure induced flow counter to EOF, can be used to provide increased resolution for a separation of two closely migrating analytes. However, it is of limited utility for separations involving more than two analytes. For example, if three analytes of interest are present, a constant counter bulk flow can be set to provide enhanced resolution of the first two analytes; however the third analyte will then likely move through the column in the opposite direction, away from the detector. In a defined injection electrophoretic method as is commonly practiced, the third analyte peak would likely migrate out of the column and never be detected. On the other hand, if the constant bulk flow is chosen so that the third analyte does migrate in the correct direction towards the detector, the desired resolution may not be achieved between the first two analytes.

In the method of the current invention, the bulk flow is varied over time during the separation in a systematic way to provide the desired resolution between all analyte zones. In the three-analyte example, the bulk flow would be varied so that early in the separation, it would provide the desired resolution between the first two analytes, and then later in the separation it would provide the desired resolution between the second and third analytes. Furthermore, the fact that the third analyte moves away from the detector early in the separation is used to advantage in that the third analyte does not enter the column until after the first two analytes have been resolved and detected. In the commonly used defined injection methods, should the analyte zone migrate out of the inlet, the analyte zone would be diluted within the blank electrolyte reservoir to which the inlet is connected, likely making it wholly undetectable. This is in contrast to the method described herein, which utilizes a continuous injection, whereby during the portion of the separation that the third analyte migrates away from the detector, it remains in the sample reservoir at an effectively constant concentration, available to be eluted into the column later in the separation.

Even for separations of just two analytes, the method of the current invention has advantages over prior art electrophoretic methods employing constant bulk flow. With a constant bulk flow, in order to achieve a desired resolution between two closely migrating analytes requires accurate and precise control of the value of the bulk flow velocity. In many situations (because of variation of the electroosmotic flow, for example), it is difficult or impossible to control a fixed bulk flow velocity to the required accuracy and precision; this difficulty is exacerbated when using surface modifications to

control EOF, which can be irreproducible in production and can degrade over time within a given column. With the current invention, the desired resolution is set by the choice of the rate of change of the bulk flow velocity, or the bulk flow acceleration. Because column-to-column or run-to-run variations in the electroosmotic mobility do not effect the bulk flow acceleration, it is easier to set the acceleration with the necessary level of precision.

The method of the invention is based upon balancing of electrophoretic velocities and bulk fluid velocity in order to discriminate different constituents within a mixture. Typically, the analyte electrophoretic velocities and the bulk velocity are in opposite directions in the separation column in order to increase species' resolutions. The magnitude of the bulk flow velocity is initially greater than the magnitude of the electrophoretic velocity of any of the analyte species, and analyte is therefore excluded from entering the column. The bulk flow is then decreased while electrophoresis remains constant. When the magnitude of the bulk flow velocity becomes less than the magnitude of the electrophoretic velocity of an analyte, that analyte will enter the column and migrate towards the detector. As the analyte moves past the detector it will be detected as a step or moving boundary. Analytes with different electrophoretic mobilities will be resolved because they will enter the column at different times (as the bulk velocity is decreased) and because, once in the column, they will migrate towards the detector at different speeds. Because different analytes are made to enter the capillary at different times, very short separation columns can be used to produce high-resolution separations. The method has been designated as gradient elution moving boundary electrophoresis (GEMBE).

The resolution of the separation can be increased by reducing the rate at which the bulk velocity is decreased (the bulk flow acceleration) in order to increase the time between the points at which each analyte enters the capillary. As an example, two similar fluorescent dyes, fluorescein and carboxyfluorescein were separated in a GEMBE apparatus at different rates of bulk flow acceleration. The apparatus used was similar to that depicted in Figure 1a. The separation column was a 3 cm length capillary with an inner diameter of 30 μ m and an outer diameter of360 μm. The inlet end of the capillary was connected via a 360 μm i.d. hole to a 150 μL volume sample reservoir. The outlet end of the capillary was connected via a silicone septum to a sealed, pressure-controlled waste reservoir. A sample solution containing 1 μmol/L each of fluorescein and carboxyfluorescein in 0.5 mol/L Tris borate buffer was placed in the sample reservoir and the waste reservoir and capilary were filled with blank 0.5 mol/L Tris borate buffer. A voltage of -3000 V was applied to the sample reservoir, while the waste reservoir was electrically grounded. During the separation, the bulk flow was comprised of a combination of electroosmotic flow resulting from the applied voltage and pressure-driven flow resulting from the applied pressure at the waste reservoir. The bulk flow velocity was varied by changing the pressure applied to the waste reservoir. Detection of the analytes was performed at a distance of 1 cm from the sample inlet using a fluorescence microscope and color CCD camera. The applied pressure at the start of the separation was made great enough to exclude both analytes from entering the separation capillary, typically on the order of 1000 Pa. The pressure was then swept at a fixed rate (producing a fixed bulk flow acceleration) to sequentially introduce the analytes,

typically on the order of 1 Pa/s. The detector response produced a stepwise pattern, similar to Figure 2a. The derivative of the detector response with respect to time shows a pattern of peaks similar to a conventional electropherogram, as in Figure 2b. Figure 3a demonstrates the effect of different pressure sweep rates (or bulk flow acceleration rates) on the resolution between the analytes in a GEMBE separation while Figure 3b shows the relationship between resolution and total analysis time. The separations were performed with the detector only 1 cm from the capillary inlet.

As an alternative to increasing resolution between species, the analysis time ofa separation can be shortened in instances when more than sufficient resolution is obtained with conventional electrophoresis, leading to faster analysis times and higher throughput. An example of decreasing analysis time is shown in Figure 4, wherein five amino acids are separated by GEMBE. In this example, five fluorescent dansyl-labeled amino acids (tryptophan, phenylalanine, serine, glycine, and aspartic acid) in 0.5 mol/L Tris borate buffer were placed in the sample reservoir at equal concentrations of 1 mmol/L. Other experimental conditions were the same as the example of Figure 3, above. Figure 4a shows the results of a linear pressure sweep (constant bulk flow acceleration), while Figure 4b shows the results of a two-step gradient to elute the four fastest migrating amino acids at one sweep rate, followed by a faster rate to elute the slowest amino acid, aspartic acid.

With GEMBE, the analyst is afforded all the experimental parameters available to traditional capillary electrophoresis separations, including electric field strength, separation column dimensions, temperature, background electrolyte composition, etc., as well as the new parameters of bulk flow velocity and acceleration. All these parameters can be adjusted to realize a separation of analytes. For a given separation column and background electrolyte, the voltage range can be determined through the use of Ohm's Law plots, which present the relationship between observed current and applied voltage. Positive deviations from linearity at higher voltages indicate Joule heating of the capillary, which can degrade the separation, and establish an upper voltage limit (along with physical limitations of available voltage sources). Common field strengths are between 100 and 1000 V cm. As with conventional CE, temperature control of the separation column can be utilized to extend the working voltage range, especially in the cases of highly conductive solutions. Either polarity of applied voltage can be used, so that the direction of electrophoretic migration of the analytes can be from inlet to outlet or from outlet to inlet. The magnitude and direction of bulk flow would then be set accordingly so that during the separation the analytes would move from the inlet to the outlet.

Separation column cross sectional areas are typically less than 0.1 mm^2 in order to allow rapid heat transfer and prevent Joule heating of the capillary. The lower limit of cross sectional area is dictated both by availability of substrate and, when using pressure to control the bulk fluid flow, the limitation of the pressure control device to affect bulk flow against high back pressures. Commonly 100 to 20000 µm2 cross sectional areas can be used with good effect. As one major benefit of GEMBE is the use of short separation columns, separation lengths are typically limited by geometrical constraints including electrical and/or hydrodynamic connections to the apparatus, as well as having available

room for detection instruments. Using a standard fluorescent microscope as a detector, column lengths on the order of 1 cm can be utilized without great difficulty.

Background electrolyte compositions are typically dependent upon the analytes of interest, and are most commonly conductive aqueous buffered ionic solutions. The background electrolyte pH and ionic strength are two primary factors in modifying electroosmotic flow and realizing separation of two distinct analytes. Additionally, additives, such as organic modifiers or polymers, can be used to enhance selectivity. A buffer usually will have a pK_a at or near the desired working pH and usually wil be less than 100 mmol/L in order to avoid excessive conductivity.

When using an applied pressure to control the bulk flow rate, the initial pressure is typically chosen to exclude entrance of any detectable analytes onto the separation column. In a standard linear method (constant bulk flow acceleration), the pressure is decreased in a linear fashion until all analytes have entered the column for detection. The pressure sweep rate (acceleration) can be reduced in order to increase the resolution of analytes or increased in order to decrease analysis time. Commonly 0.1 Pa/s to 10 Pa/s rates can be utilized on a standard pressure control system (whether gravity, gas, or piston driven) with the other separation parameters similar to that described above. Alternatively, the pressure can be held constant, stepped, or varied in other non-linear manners throughout the separation.

It should be noted that this method can be used for the separation of both anionic and cationic species in the same separation with analysis times greatly reduced as compared to conventional methods. For example, if a negative voltage is applied to the inlet end of the separation column, anions will electrophoretically migrate from the inlet to the outlet, and cations will electrophoretically migrate from the outlet to the inlet. The bulk flow velocity will initially be set in the direction from outlet to inlet and at a magnitude that is greater than the electrophoretic migration speed of the anionic analytes. As the bulk flow velocity is reduced each of the anionic analytes will be eluted into the column and detected as above. The bulk flow velocity would then be varied further until it changes direction (from inlet to outlet). The magnitude of the bulk velocity would then increase with time, and each cationic analyte would be pulled into the column when its electrophoretic velocity is overcome by the increasing bulk flow velocity.

As in prior art electrophoretic methods, it is also possible to use this method in conjunction with additives to modify the electrophoretic mobility of analytes and provide different separation selectivity. Examples of additives include cyclodextrins or other chiral selectors for chiral separations, micelles, microemulsions, liposomes, dendrimers or other additives that act as a pseudo stationary phase to provide selectivity based upon the strength of interaction between the additives and the analytes. Note that with appropriate additives, this method can be used to separate neutral molecules.

The steps required to implement the method of the present invention are:

providing a reservoir of solvent or solution in fluid contact with the outlet end of the separation column;

filling the separation column with the solvent or solution;

introducing a sample solution to the inlet end of the separation column;

producing a bulk flow of the solvent or solution through the separation column;

applying a voltage along the length of the separation column to drive the electrophoretic motion of analyte(s) through the column;

changing the bulk flow velocity over time, so that each of the analytes of interest will sequentially be eluted from the sample solution into the column.

A possible additional step is: 7) detecting the analytes as they move through the column. A plot of detector signal vs. time will have steps similar to the result of prior art moving boundary electrophoresis methods. Each step will correspond to an analyte, and the height of each step will correspond to the concentration of that analyte in the sample. Any detector usable with prior art electrophoresis methods could be used.

The solvent or solution used in step 1 can be any solvent or solution useable for electrophoretic separations: aqueous or non-aqueous buffer, with or without a pseudostationary phase or other additives.

Examples of appropriate separation columns would be capillaries or microchannels, made from glass or polymer or any other electrically insulating material. The cross-sectional size of the inside of the column would typically be less than 0.1 mm^2, but could be larger as in prior art of tubular free zone electrophoresis [14]. The column could also contain a stationary phase, present either as a packed bed or wall coating or any other form used for capillary electrochromatography [15; 16].

The sample solution of step 3 can be prepared in a solvent or buffer that is similar or dissimilar to the solvent or buffer of step 1. The types of analytes that can be separated include any analytes that can be separated by conventional CE methods (small molecules, large molecules, particles, amino acids, nucleic acids, carbohydrates, proteins, peptides, pesticides, pharmaceuticals, cells, viruses, bacteria, etc.)

The bulk flow of step 4 can be driven either by electroosmosis (using the same applied voltage as step 5) or by applied pressure gradients or both. The direction of the bulk flow can be in either direction through the column, from inlet to outlet or from outlet to inlet. The terms 'inlet' and 'outlet' refer to the net direction of motion of the analyte molecules. During a separation, analytes move from the sample solution through the column inlet, traverse the column length, and exit the column through the column outlet.

The sign of the applied voltage depends on the direction of bulk flow and the sign of the charge of the analyte molecules. For example, if the analyte molecules are negatively charged in solution (or if they partition into a negatively charged pseudostationary phase) and the bulk flow is from outlet to inlet, then the voltage applied to the inlet will be negative relative to the voltage applied to the outlet. In most cases, when the desired result is high resolution in a short column, the bulk flow of the solvent and the electrophoretic motion of the analytes should be in opposite directions. However, in cases

where the resolution is more than adequate, the analysis time can be reduced with this method by using a bulk flow that is in the same direction as the electrophoretic motion.

The key step to this process (and the one that makes it distinguishable from the prior atr) is step 6. The bulk flow is initially set so that all or nearly all of the analytes of interest do not enter the column. During the separation, the bulk flow is changed over time so that each of the analytes of interest will sequentially move into the column.

There are different ways that the bulk flow rate could be varied over time: In the reduction to practice described above, the bulk flow is driven by a combination of electroosmosis and externally applied pressure difference between the column inlet and outlet. The applied pressure is varied over time to vary the bulk flow rate.

The bulk flow could also be driven entirely by electroosmosis and the electroosmotic mobility could be varied over time to vary the bulk flow rate. One example of this would be the use of a voltage applied across the walls of the separation column as described in ref. [5;6]

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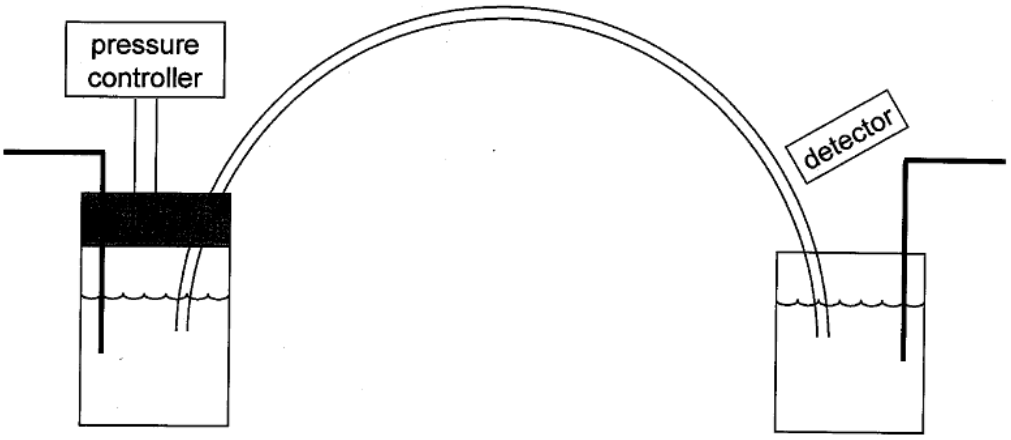
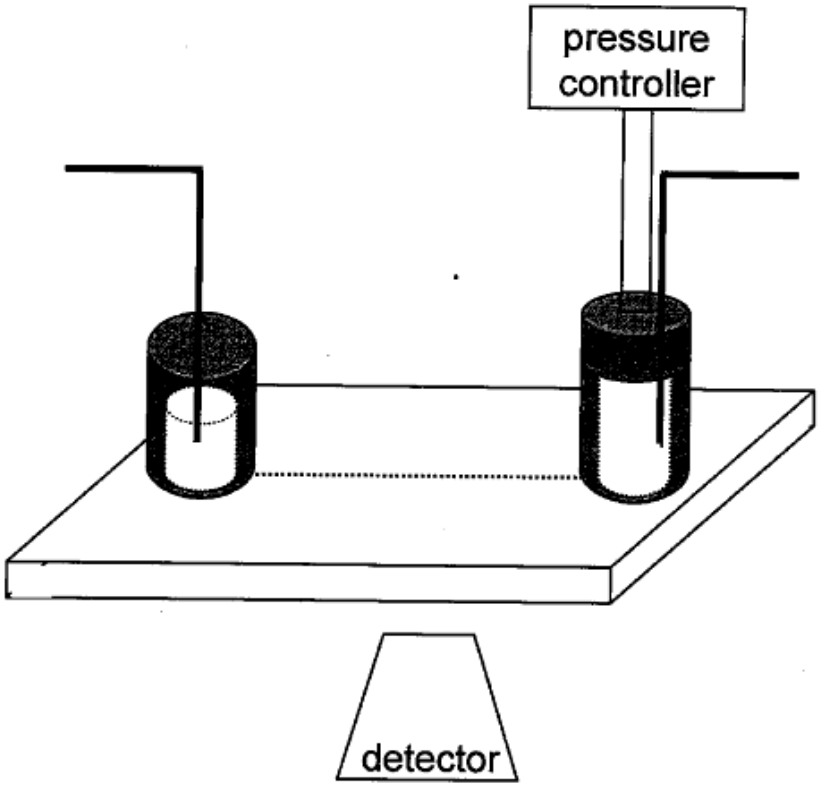


Figure 1a - capillary GEMBEF



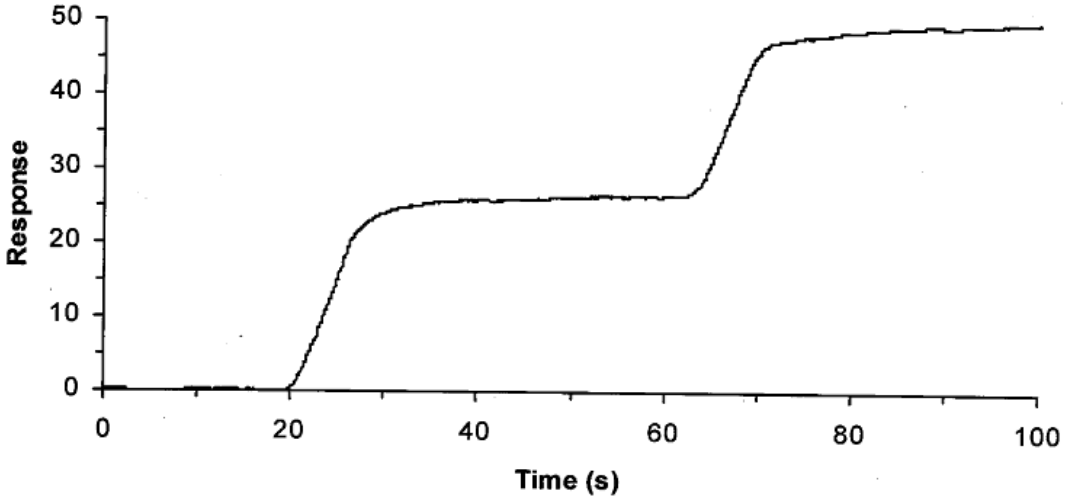


Figure 2a - GEMBE raw data

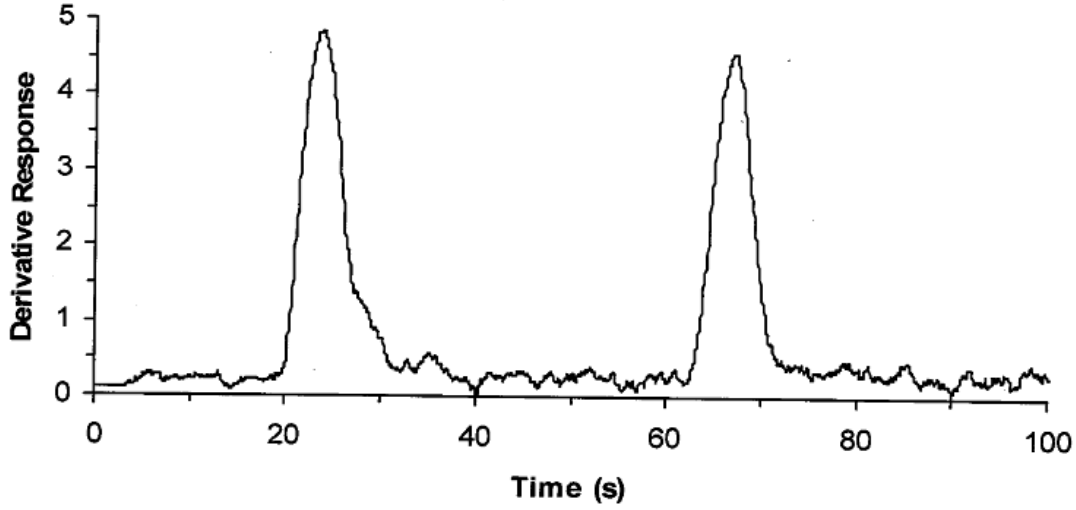


Figure 2b – GEMBE derivative

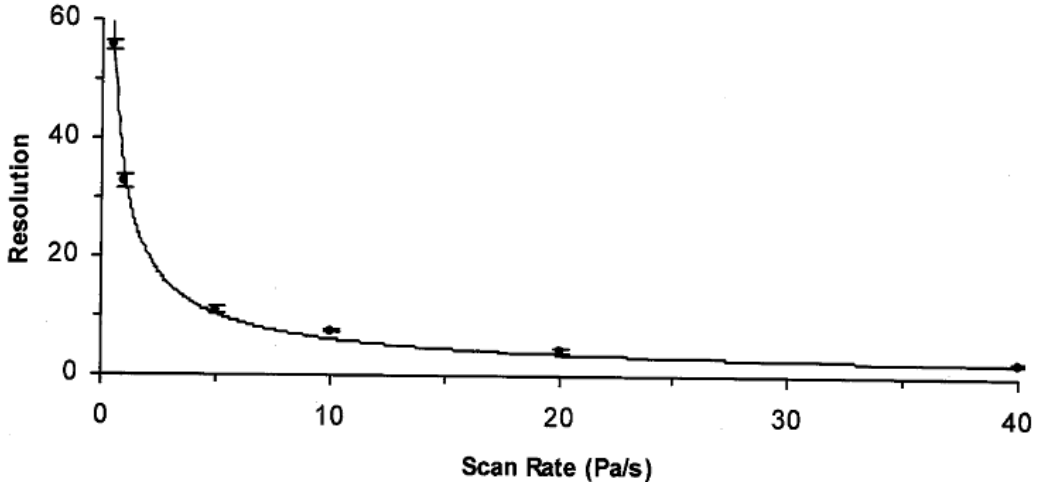


Figure 3a - RS vs Rate

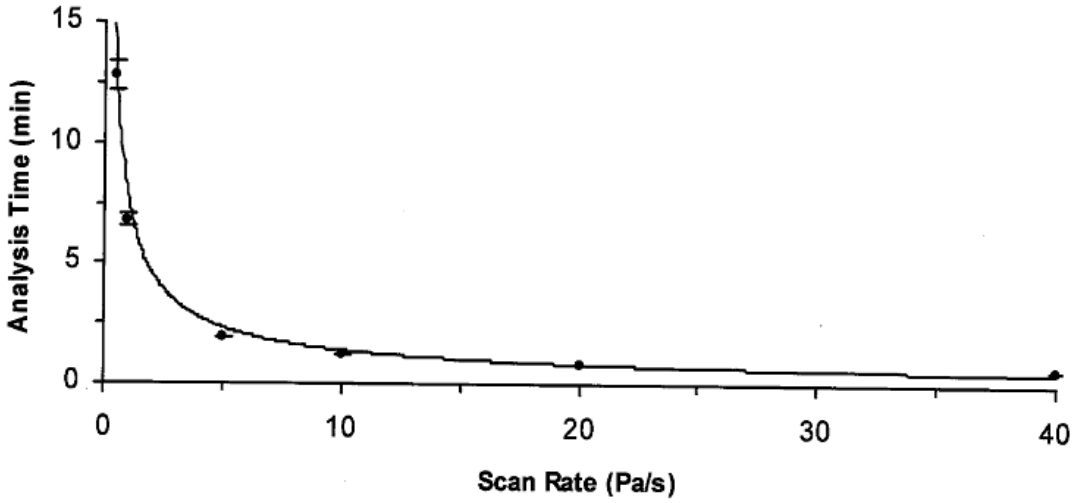


Figure 3b – Time vs Rate

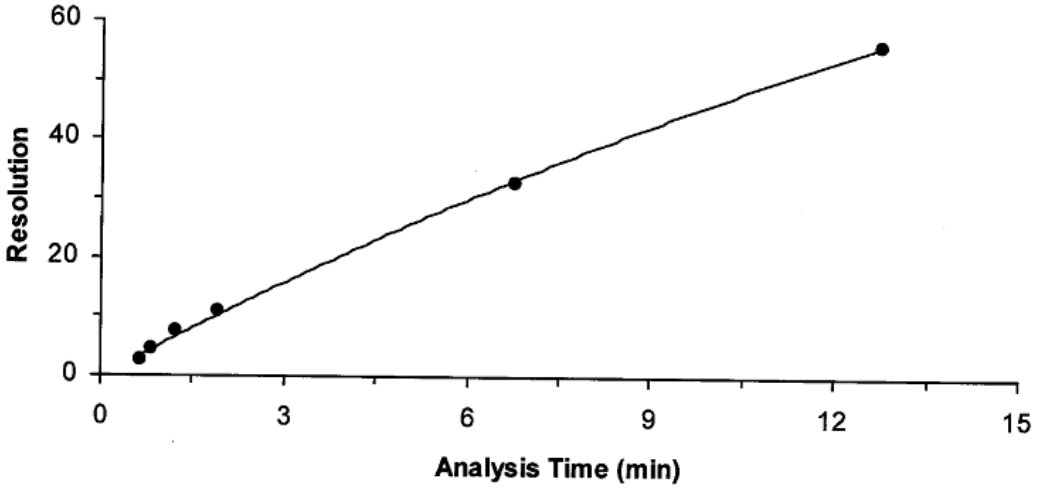


Figure 3c – RS vs rate

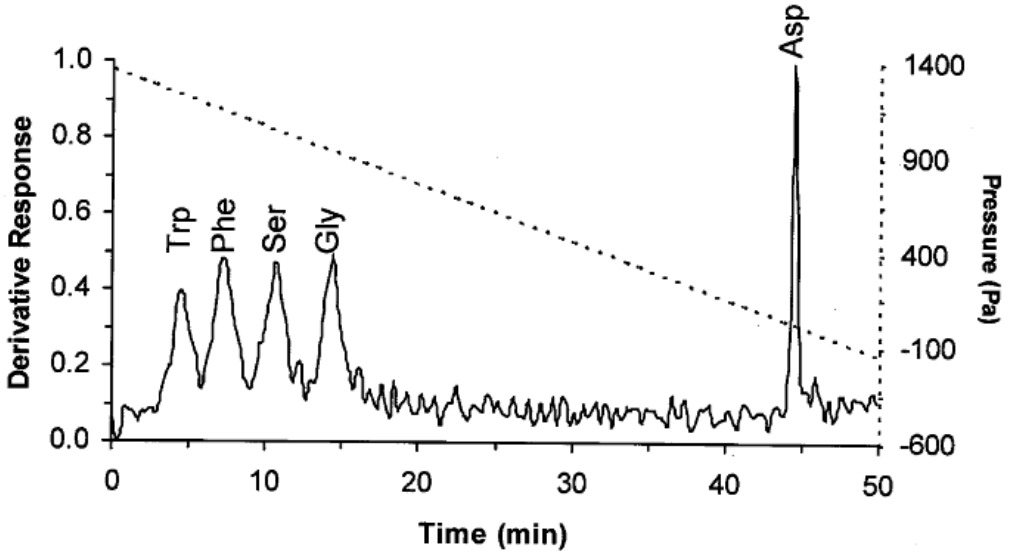


Figure 4a - linear aa

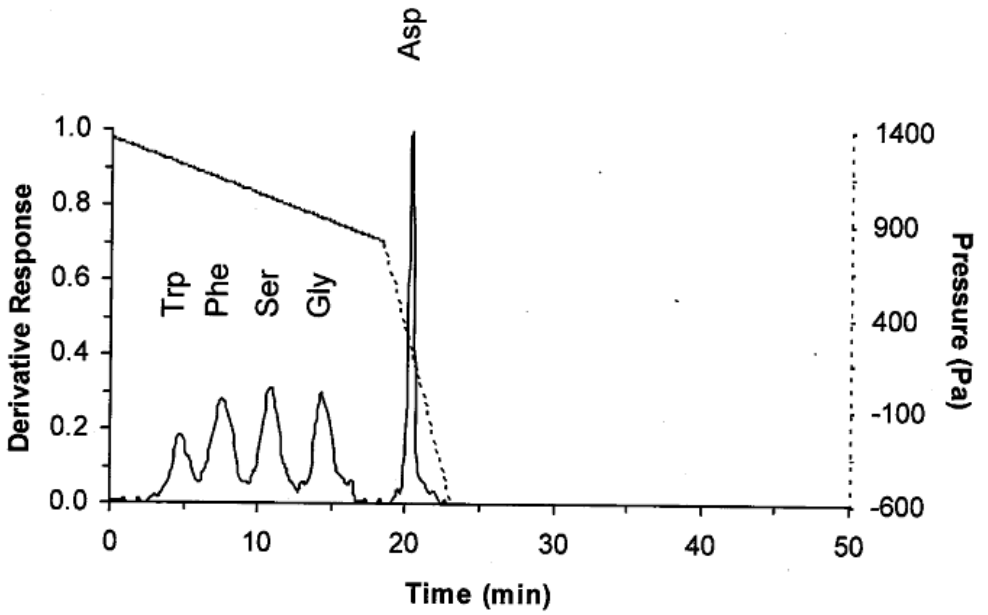


Figure 4b – stepped aa