**August 22, 2007**

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# On the Persistence of Cation-exchange Chromatography for Analysis of Free Amino Acids

*a report by*
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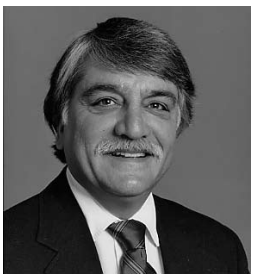


Dr. Michael Pickering is President of Pickering Laboratories Incorporated (Mountain View, California), founded in 1980. Pickering Laboratories was established to supply the liquid chromatography (LC) market with application-specific chemistry and hardware, with an emphasis on post-column technology. He has held research and development positions with Durrum Instruments (now Dionex Corp., Sunnyvale, California) and Spectra Physics (San Jose, California). In 1974 he earned a PhD in chemistry from the Oregon Center for Science and Technology, now the Oregon Graduate Institute. His research area was synthetic and medicinal chemistry. He received an MS in chemistry from San Francisco State University in 1970, and a BS in Chemistry from St Mary's College of California in 1964.

## Introduction

Although it was not called a liquid chromatograph, the first such instrument was introduced in late 1959 by Beckman Instruments and was designated the M.S. Amino Acid Analyzer in honour of Moore and Stein.[1] Inject valves had yet to be invented, the buffer switching valves were glass stopcocks and the column temperature was controlled by circulating water baths. Two positive displacement pumps eluted two glass columns (acids and neutrals basics). The eluants were a set of four buffers plus a fifth solution of the appropriate hydroxide called the column regenerant. Low temperature (~30ºC) favoured the threonine/serine resolution on the acids/neutrals column while high temperature (~60ºC) favoured the tyrosine phenylalanine on the basics column. Amino acids were detected with a spectrophotometer after derivatisation with ninhydrin reagent. Analysis time for physiological fluids was 72 hours. Approximately 10 years later, Waters Corporation introduced the first instrument called a 'liquid chromatograph'. The three most significant differences between this liquid chromatograph and the amino acid analysers of the time were:

- its ability to form continuous gradients between two eluants;
- a single column temperature; and
- silica- or alumina-based stationary phases.

As instrument and column technology improved, liquid chromatography (LC) became increasingly popular as a method of separating and detecting a wide variety of organic compounds. In the early 1980s, Pickering Laboratories introduced the first isothermal, two-eluant gradient amino acid analysis based on the then available high-performance liquid chromatography (HPLC) technology of ternary gradient pumps and single temperature elution. Adding a specially designed post-column derivatisation system made it possible to analyse amino acids using contemporary HPLC equipment. Total analysis time was shortened. A little later, most HPLC manufacturers offered one of several pre-column derivatisation methods for amino acid analysis by reversed-phase chromatography. The need for this pre-column derivatisation step derives from the facts that too many of the amino acids are poorly retained on such phases and that they do not possess any useful chromphore for optical detection These pre-column methods did not significantly impact the popularity of ion-exchange analysis with ninhydrin post-column visualisation in spite of the advertised short analysis times and femptomole sensitivity. The reasons were straightforward. Preanalysis derivatisation means performing chemical synthesis in a very complex medium: the native sample. Matrix complexity produces both competition and inhibition in chemical synthesis. This phenomenon results in variance in area reproducibility. In this regard, even the most common of these precolumn methods (o-phthalaldehyde/2-mercaptoethanol), produces an unstable derivative.[2] Thus, the poorly reproducible synthesis and the well-known vagaries of matrix influences on reversed-phase elution time reproducibility resulted in double-digit relative standard deviations for both area and retention times.

Due to the fact that the reversed-phase HPLC methods were not successful in displacing the hoary dedicated amino acid analyser, the artificial isolation of the two instrument technologies persists today. Dedicated amino acid analysers still employ series isocratic elution with buffers and temperature gradients, and HPLC systems use gradient elution with two to four eluants and constant temperature.

Dedicated amino acid analysers employ buffers from low to close to neutral pH and intermediate cation concentration. Using such buffers as eluting solutions requires column temperature adjustments to achieve acceptable resolution and help to manage analysis time. Although buffers provide good stability for retention times, using isocratic elution makes it difficult to significantly improve resolution. Usually a new buffer needs to be developed in order to 'open' select areas of the chromatogram. Buffer substitution, however, affects the rest of the chromatogram. Thus, the cost of resolving one co-elution may be gaining another later in the chromatogram.

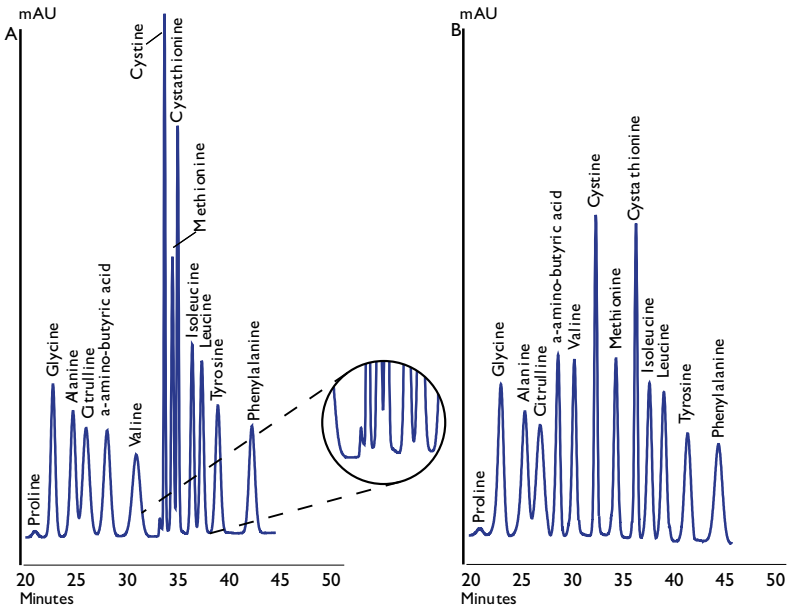
*1. Stanford Moore, William H Stein and Christian B Anfinsen received the 1972 Nobel Prize in chemistry for their work concerning amino acids and the ribonucelease molecule. The work was the result of findings made due to their invention of amino acid analysis based on ion-exchange chromatography with Ninhydrin detection.*

*2. Frister H, Meisel H, Schlimme E, "OPA method modified by use of N,N-dimethyl-2-mercaptoethylammonium chloride as thiol component", Fresenius Z Anal Chem (1988);330: pp. 631–633.*

Isothermal amino acid analysis gradient systems use as eluants a low pH and ionic-strength buffer and high-ionic-strength but low-buffering solution. These eluants allow for linearly increasing cation normality and slowly increasing pH. Although such systems provide good resolution and analysis times, they may be insufficient to resolve certain coelutions. This is especially true for amino acids eluting at high ionic strength.

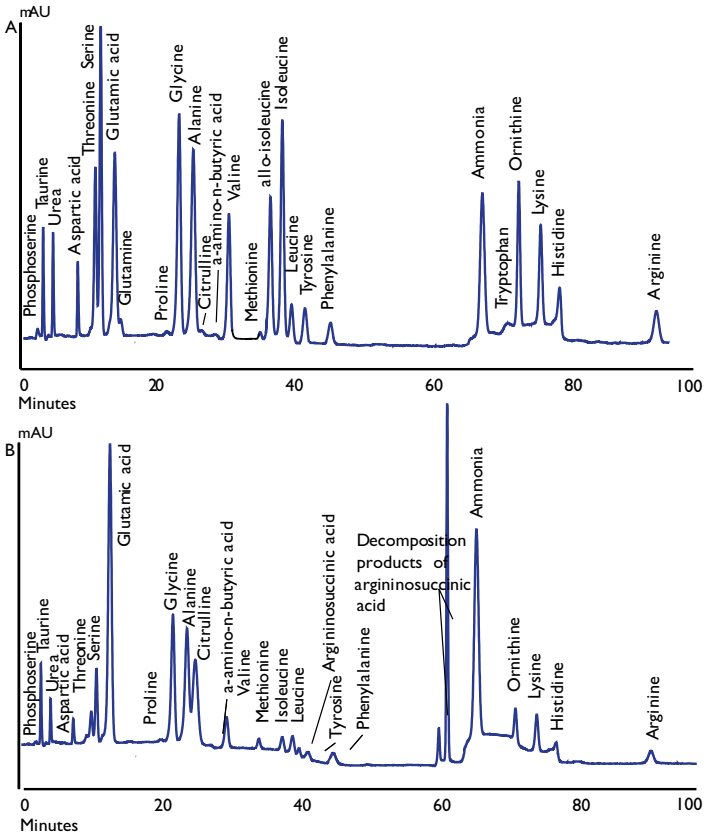
Over the years, HPLC has exponentially multiplied resolving power and detection level with new and improved stationary phases, the number of eluants possible, higher operating pressures and more sophisticated detectors. Temperature gradients and high-temperature (~200°C) chromatography are very recent innovations. With the advent of the Pinnacle PCX, the first post-column derivatisation system equipped with a column oven capable of temperature programming, it is possible to benefit from the engineering improvements of the modern HPLC (i.e. sophisticated quaternary pumps, low refractive index sensitivity detectors, etc.) coupled with the temperature gradients associated with the dedicated analysers.

**Figure 1: The Methionine Region of Physiological Fluid Chromatogram**




*A) Series isocratic elution. B) Linear gradient elution. The inset shows how the cystine area reproducibility is compromised. mAU = milli absorbance unit.*

**Figure 2: Analysis of Amino Acids**




*A) Serum of a maple syrup urine disease (MSUD) patient. B) Serum of a patient with argininosuccinic aciduria (ASA). mAU = milli absorbance unit.*

Ion exchange stationary phases embody an array of challenges and opportunities. These gel-type resins are soft and easily compressed and they change size based on interactions with the eluants. The first property makes them difficult to pack. The osmotic effect makes getting the correct quantity to pack a challenge and alters the size during elution. Yet, in spite of these vexations, the overall benefits prevail, because the dominant retention mechanism is nearly perfectly matrix-insensitive. The same conditions used to analyse human serum will suffice for urine, wine, orange juice, etc. Furthermore, it will be possible to see how the myriad sub-dominant retention mechanisms, including partitioning, absorption, exclusion, etc., between the stationary phase and the various amino acids provide very keen selectivity. It is the plurality of retention mechanisms characteristic of the stationary phases used in ionexchange amino acid analysis that provides the opportunity to resolve co-elutions. In the more prevalent reversed phases, co-elutions represent the commonality of a single property (hydrohobicity). Thus, changes in eluant strength or temperature (below 100ºC) will tend to affect both peaks similarly and so stymie resolution. In contrast, ion-exchange resins exhibit many retention mechanisms; this confusion results in incidental co-elutions. As the elution parameters include pH, cation concentration, temperature and organic modifiers, the competition of slopes provide resolution.

There are several advantages of using a linear gradient over series isocratic elution. One of them is illustrated in *Figure 1*. The step change of the buffer always creates a front that can be seen on the chromatogram and could interfere with separation and/or integration of the peaks close to it. The front is a refractive index change caused by an osmotic shrinking of the resin due to the sudden change in normality. Gradient elution does not have such refractive index anomalies. Combining eluant and temperature gradients gives greater control of the peak position and allows full advantage to be taken of the stationary phase's complex behaviour. As all the elution parameters affect each retention process differently, they all have significant influences on selectivity. Now, with the same set of eluants, it is possible to carefully adapt the method to achieve resolution of the peaks of interest even in 'busy' parts of the chromatogram.

The latter is illustrated in *Figure 2* by separation of such metabolic markers as allo-isoleucine (maple syrup urine disease (MSUD))[3] and argininosuccinic aciduria (ASA)[4]. Under isothermal conditions, these amino acids co-elute with cystathionine and isoleucine, respectively, but are each resolved using a localised temperature gradient. Such nuancing of the chromatogram does not compromise resolution of subsequently eluting amino acids, nor does it extend analysis time. The flexibility of the separation also saves analysis time without sacrificing resolution. Another advantage of postcolumn derivatisation with ninhydrin is that each amino acid shows a signal at 440nm and 570nm. The ratio of these areas is a constant for each amino acid and thus provides confirmation of peak purity. Picomole sensitivity is easily accomplished with modern detectors.

Cation-exchange chromatography justifiably remains the method of choice for amino acid analysis. It is also clear that the oldest analytical method greatly benefits from the newest hardware. Current analysis time for physiological fluids is 111 minutes, inject to inject.

*3. Schadewaldt P, Bodner-Leidecker A, Hammen H-W, Wendel U, "Significance of L-alloisoleucine in plasma for diagnosis of maple syrup urine disease", Clin Chem (1999);45: pp. 1,734–1,740.*

*4. Argininosuccinic aciduria (ASA) is a deficiency of argininosuccinase (argininosuccinate lyase (AL)) that results in markedly elevated plasma and urine levels of argininosuccinic acid.*