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 (~30°C) favoured the threonine-serine resolution on the acids/neutals column while high temperature (~60°C) 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 chromophore 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 femtomole 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 ribonuclease 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.
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 (~200ºC) 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**

![Image of physiological fluid chromatogram]

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

**Post-Column Derivatization is the Industry Standard for Superior Quantitation, Reproducibility and Sensitivity of Amino Acids Analysis**

For 25 years Pickering Laboratories has manufactured instruments, columns and reagents specifically designed for postcolumn derivatization analysis. Over 1,000 laboratories around the world depend on the reliability and quality of these systems and chemistry. These businesses know they can extend their HPLC investments by adding the post-column derivatization system to provide additional high value analysis services to their customers.

For amino acid analysis no other techniques have been shown to match the reproducibility and sensitivity of the post-column methodology. Advantages of this method, such as absence of matrix interferences, are especially important in analysis of native samples. Amino acids can be detected using either a visible or a fluorescent detection method. Pickering’s patented TRIONE® Ninhydrin reagent gives the convenience of ready-made reagent and simultaneous detection of both primary and secondary amino acids. Pickering’s Chromatographic™ Grade o-phthalaldehyde provides excellent sensitivity for primary amines. By using ion-exchange analysis with post-column derivatization sample preparation is greatly simplified over methods using pre-column analysis with reverse-phase liquid chromatography or gas chromatography.

While dedicated amino acid analyzers combine HPLC components with post-column technology into an integrated package they lack the flexibility to perform analysis of any other methods. In today’s business environment flexibility is the key.

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- Cancer Drugs
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- Protein and peptides
- Carbohydrates
- Herbicides and pesticides in the environment
Argininosuccinic aciduria (ASA) is a deficiency of argininosuccinase (argininosuccinate lyase (AL)) that results in markedly elevated plasma and urine levels of argininosuccinic acid.

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.


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