HORIBA Scientific

Application Note

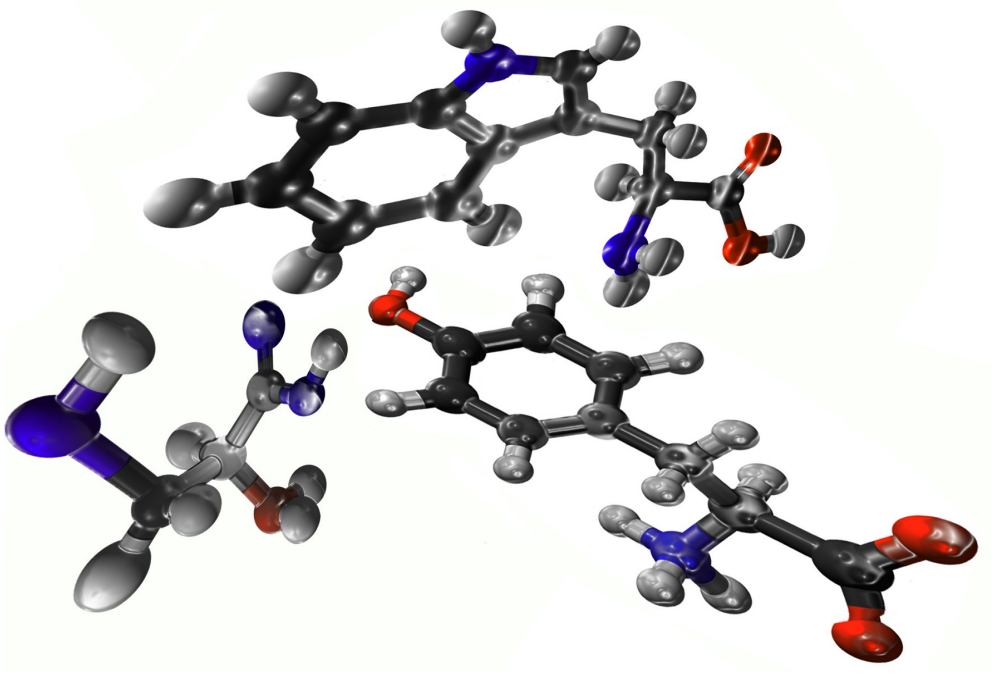
Life Sciences

Capillary Electrophoresis (CE)

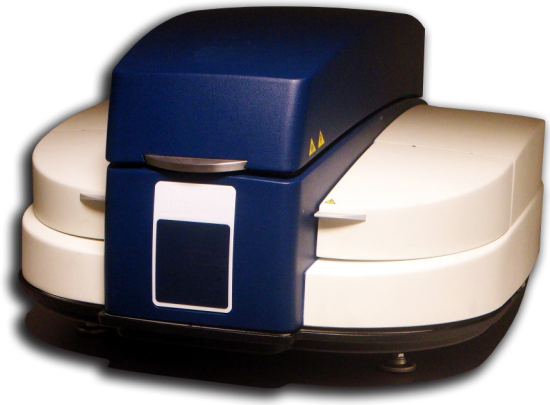
CEAN03

AMINO ACID ANALYSIS

By High Performance Capillary Electrophoresis



Analysis of Amino Acid Standards



Label free analysis using the HPCE-512

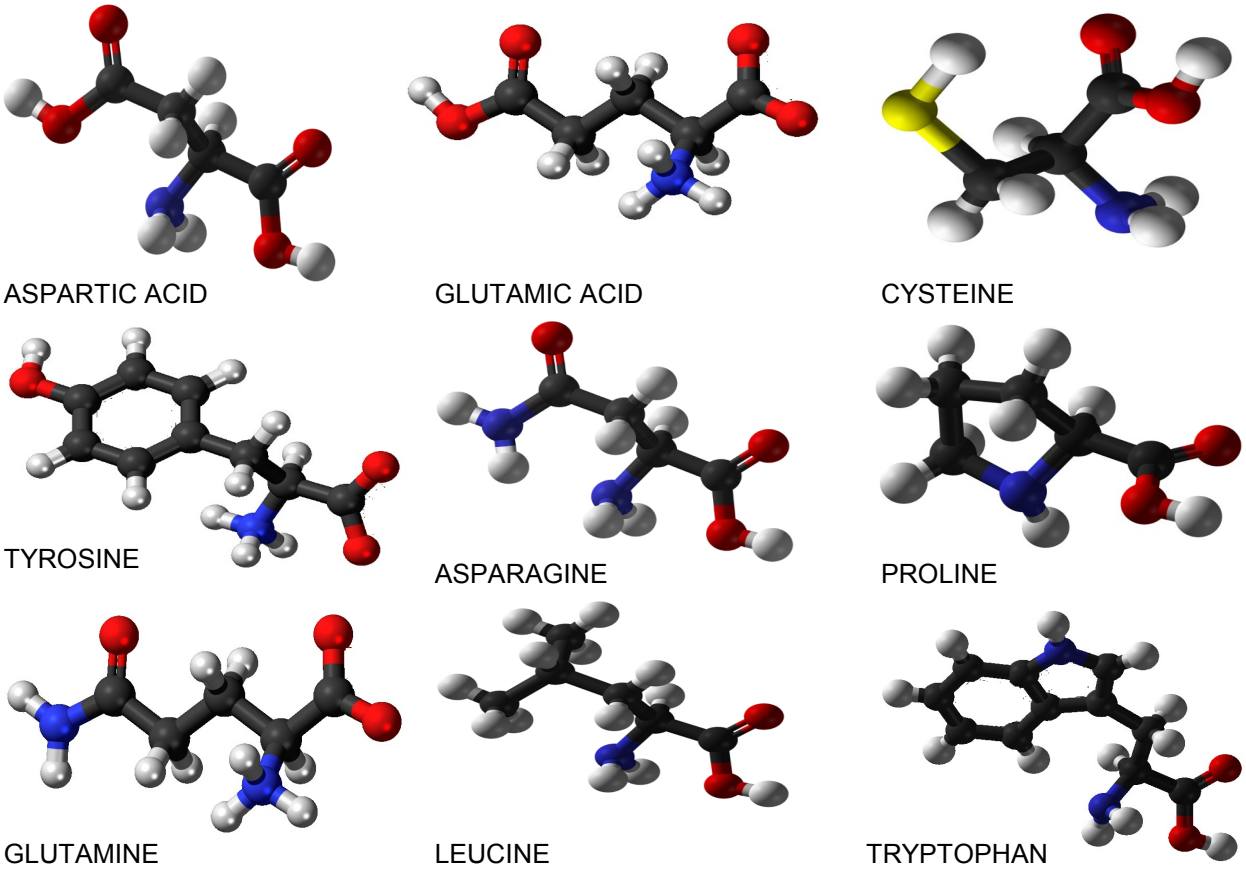
ABSTRACT
Capillary electrophoresis using indirect UV detection was applied for the analysis of 9 amino acids. This is a fast and convenient method for the analysis of amino acids, compared to HPLC. A reversed polarity mode was employed, with the sample being injected at the cathode and electrophoresed towards the detector at the anode.

INTRODUCTION
The label-free HPCE-512 was used with a UV-absorbing buffer. This allows the transmission of light as the non- UV-absorbing analytes pass the detector window. Indirect UV can be used in the analysis of substances inaccessible to the normal methodology. These include carbohydrates, lipids and sugars, which typically absorb little in the 200-300 nm spectral range favoured by the HPCE-512 technology.
In this instance a mixture of amino acids was investigated and analysed using a high pH run buffer in order to fully ionize the amino acid residues. A quaternary ammonium salt was added to the electrolyte in order to reverse the direction of the EOF, allowing anion migration in the same direction, towards the anode.

Indirect UV detection was employed in order to visualise the 9 amino acids (Figure 1). The UV absorbing amino acids of Tyrosine and Tryptophan were analysed within the mixture demonstrating that both chromophore-containing and non-chromophore-containing amino acids can be detected simultaneously.

When applying indirect UV detection, the background electrolyte (BGE) or probe added for UV absorbance is critical. Not only are the pH and wavelength of the chromophore present important, but also the mobility of the probe relative to that of the analytes of interest, as well as the concentration that is added to the run buffer.
Due to the addition of a UV absorbing probe to the running buffer, high currents can be generated, and therefore 50 µM internal diameter capillary was employed. Full preconditioning of the capillary was essential to ensure a stable coating and generation of a reversed EOF.

Figure 1: *Structures for the given 9 amino acids.*



MATERIALS AND METHODS

The data presented in this report were collected on a HPCE-512. All detection was performed at 254nm. Separation of the 9 amino acid residues was carried out in bare fused silica capillaries of 50µm internal diameter, with an effective separation length of 69.9 cm. The total capillary length was 82.1cm. The Peltier cooling system within the instrument was set to 18°C. Amino acids were prepared by dilution in distilled water from their powder forms, to a concentration of 150 µg/ml, for method development and reproducibility studies.

All samples were injected hydrodynamically at 1.0 psi for 5 seconds, and run in reversed polarity mode. Electrophoresis was performed in a proprietary buffer system (pH 12.0). New capillaries were conditioned by flushing with run buffer for 15 minutes at a pressure of 30 psi.

RESULTS AND DISCUSSION

A HPCE-512 system was used for the separation of 9 amino acids residues. Analysis of the separations was performed using the Equiphase Vertexing Algorithm (EVA) and Generalised Separation Transform (GST) algorithm. GST is a method of combining the data from the 512 pixels in a natural way which preserves the peak shape information of the electropherograms while at the same time maximising the signal-to-noise ratio. Typically a 10-fold increase in signal-to-noise using GST is observed compared to single electropherograms. EVA is an advanced pattern-recognition tool which maximizes the system resolution. In EVA the electropherograms are first analyzed to find local peaks. These are used first to perform vertexing (determine the point of origin of the bands) and then to produce a signal output.

EVA is an advanced pattern-recognition tool which maximizes the system resolution, and converts quantitative information to a peak height rather than area using vertexing to determine the point of origin of the bands and produce a signal output. The results quoted in this report are based upon the GST and EVA analyzed traces.

All 9 amino acid residues were resolved and identified with baseline resolution using GST processed data. **Figures 2** and **3** demonstrate representative GST and EVA traces respectively. The EVA processing software was modified to depict negative inflection peaks within the GST processed traces as positive peaks.

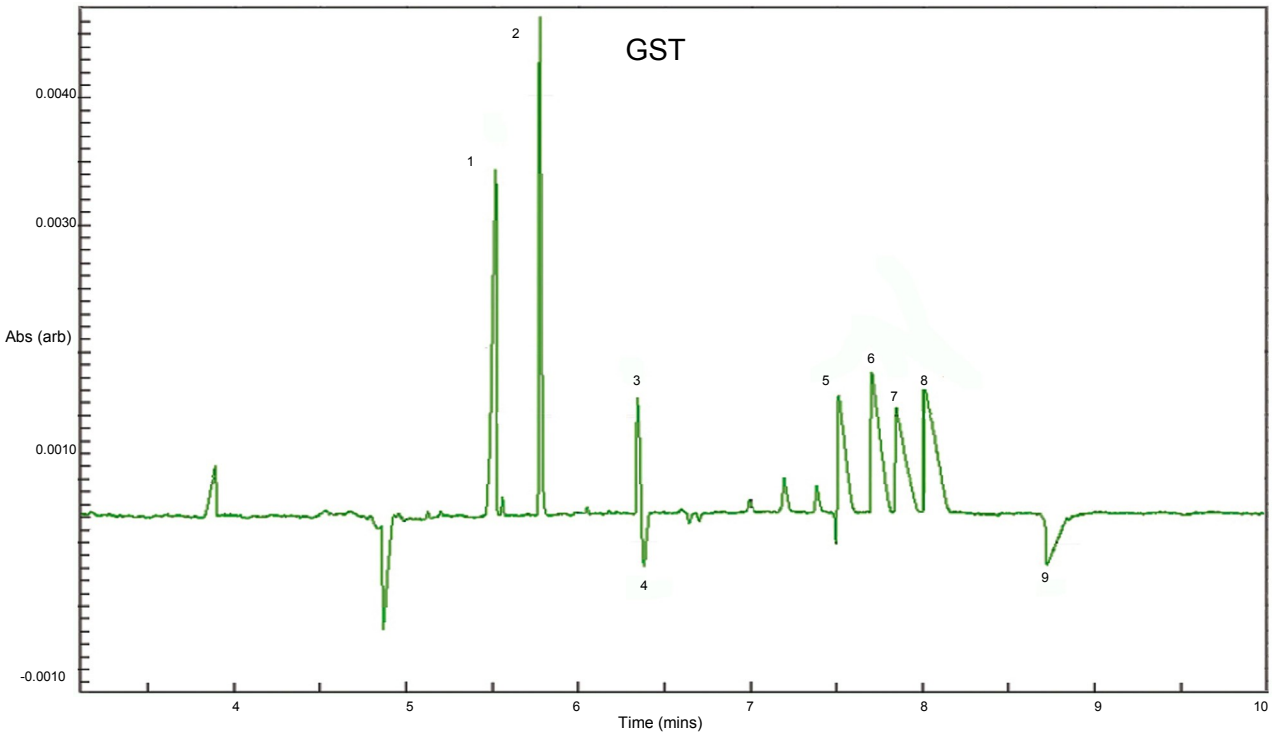


Figure 2: *GST separation of all 9 amino acids. The analytes are (1) Aspartic acid, (2) Glutamic acid, (3) Cysteine, (4) Tyrosine, (5) Asparagine, (6) Proline, (7) Glutamine, (8) Leucine, (9) Tryptophan (4 and 9 both contain a chromophore).*

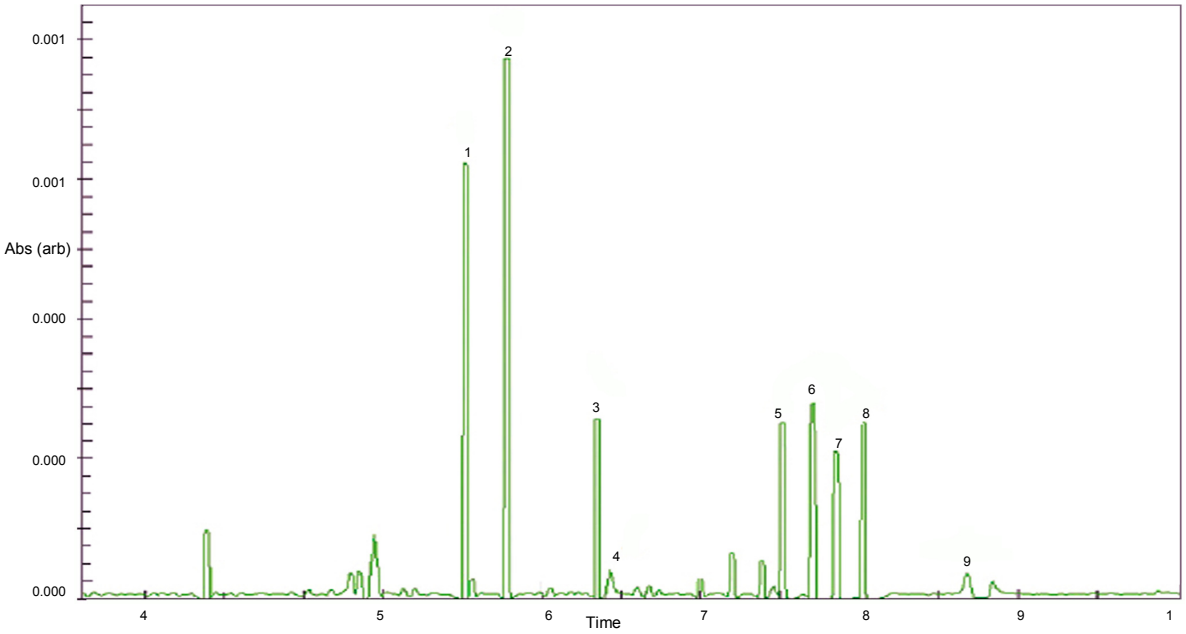


Figure 3: *EVA separation of all 9 amino acids. The analytes are (1) Aspartic acid, (2) Glutamic acid, (3) Cysteine, (4) Tyrosine, (5) Asparagine, (6) Proline, (7) Glutamine, (8) Leucine, (9) Tryptophan.*

Repeatability studies were performed using pressure injections of the mixture of 9 amino acids, at a concentration of 150 µg/ml. The repeatability data for the peaks arising from aspartic acid, glutamic acid and leucine are presented for both GST and EVA data in the **Tables 1** and **2.**

GENERALISED SEPARATION TRANSFORM	Peak Migration Repoducibility			Peak Area Reproducibility		
	Mean	Std dev	% RSD	Mean	Std Dev	% RSD
Aspartic Acid	5.514	0.012	0.212	0.043	0.001	1.973
Glutamic Acid	5.781	0.014	0.238	0.035	0.001	1.896
Leucine	8.035	0.022	0.271	0.045	0.001	1.372

Table 1: *GST repeatabilities for 3 amino acids.*

EQUIPHASE VERTEXING ALGORITHM	Peak Migration Repoducibility			Peak Area Reproducibility		
	Mean	Std dev	% RSD	Mean	Std Dev	% RSD
Aspartic Acid	5.521	0.012	0.212	0.006	0.0002	4.142
Glutamic Acid	5.782	0.012	0.224	0.007	0.0001	1.597
Leucine	8.031	0.019	0.234	0.002	2.47E-05	1.054

Table 2: *EVA repeatabilities for 3 amino acids.*

LIMIT OF DETECTION AND QUANTIFICATION

1. Pressure injection - For pressure injection of 1 psi for 5 seconds, a serial dilution between 10 to 0.039 mM was created for each of the analyte listed in the **Table 3**. LoD and LoQ were calculated on five points ranging from 1.25 mM to 0.039 mM for each of the analytes (with the exception of lysine) The LoDs ranged from 10µM to to 450µM and LoQs ranged from 45µM to 1380 µM. Lysine was injected at 7 psi for 3 seconds using a different UV absorbing buffer the LoD was calculated to be 0.06 µM and LoQ was calculated to be 0.2 µM

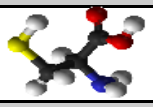
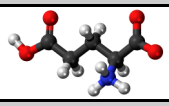
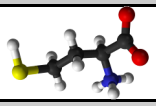
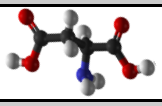
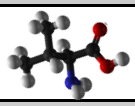
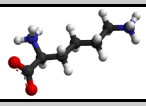
	Cysteine	Glutamic Acid (N=6)	Homocysteine (N=4)	Aspartic Acid (N=1)	Valine (N=1)	Lysine (N=1)
Structure						
MW	240.30	147.13	268.35	133.1	117.15	182.65
LoQ	10µM	150µM	1380µM	250µM	400µM	0.2µM
LoD	45µM	37µM	450µM	80µM	130µM	0.06µM
R2	0.9968	0.999	0.996	0.9816	0.9816	0.9995

Table 3

2. Electrokinetic Stacking injection -Stacking is a process which is generally used when an analyte present at low concentrations in a long injected sample zone, is concentrated into a short zone (stack). The stacked analytes are then separated and individual zones are detected. Thus stacking can provide better separation efficiency and detection sensitivity.

The graph in **Figure 4** demonstrates a linear relationship between injection voltage and time when the analyte is made in water. The greatest peak area was observed when analyte made in water was injected at 10 kV for 60 seconds. Samples made in 0.1 M and 0.02M HCl had large effect on peak area at the two different injection voltage conditions at the four different time points. To determine LoD and LoQ, a serial dilution of Aspartic acid and Glutamic acid made in water were injected at 5 kV for 15 seconds.

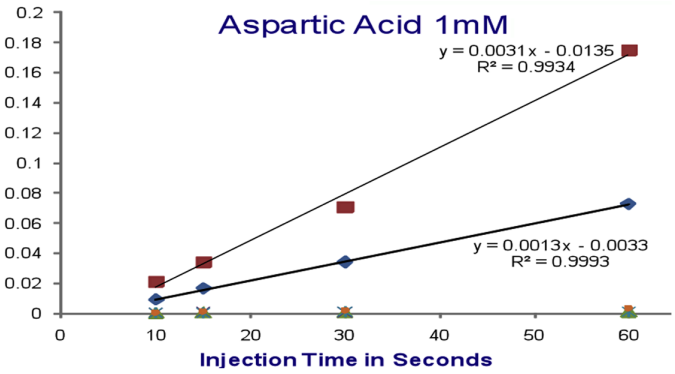


Figure 4: *The effect of four injection time in seconds (10,15, 30 and 60 seconds) using two different injection voltage conditions (5 kV and 10 kV) for aspartic acid made in three different stack buffer.*

LoD and LoQ for aspartic acid is 0.1 and 0.3 µM respectively and for Glutamic acid 0.085 and 0.250 µM - **Table 4**).

CONCLUSION

The HPCE-512 system using indirect UV detection was successfully applied for the analysis of 9 amino acid residues, demonstrating both rapid and efficient separation throughout the concentration range.

	Glutamic Acid (N=6)	Aspartic Acid (N=1)
LoQ	0.250µM	0.300µM
LoD	0.085µM	0.100µM
R_2	0.9995	0.9952

Table 4.

Excellent resolution has been demonstrated between all 9 amino acid residues, with high reproducibility for migration time (<0.5%) and peak area (<2%). Using the appropriate UV absorbing probe added to the run buffer at high pH, it is possible to simultaneously identify a variety of non- chromophore and chromophore containing analytes. It is interesting to note the negative deflection of the UV absorbing amino acids. The GST data shows an impressive signal to noise increase, and excellent resolution between amino acid residues, while the EVA processed data shows dramatically increased resolution of all 9 peaks, by applying the vertexing.

A minor modification to the EVA software algorithms allows us to continue to employ this powerful processing technique, allowing the amino acids to be highly resolved and clearly identified.

Analysis of these 9 amino acids demonstrates the powerful capability of the HPCE-512 indirect UV analysis technique, and using a universal buffer this technique can allow the analysis of many analytes such as carbohydrates, lipids, anions and cations, all in the same system.