HORIBA Scientific

Application Note

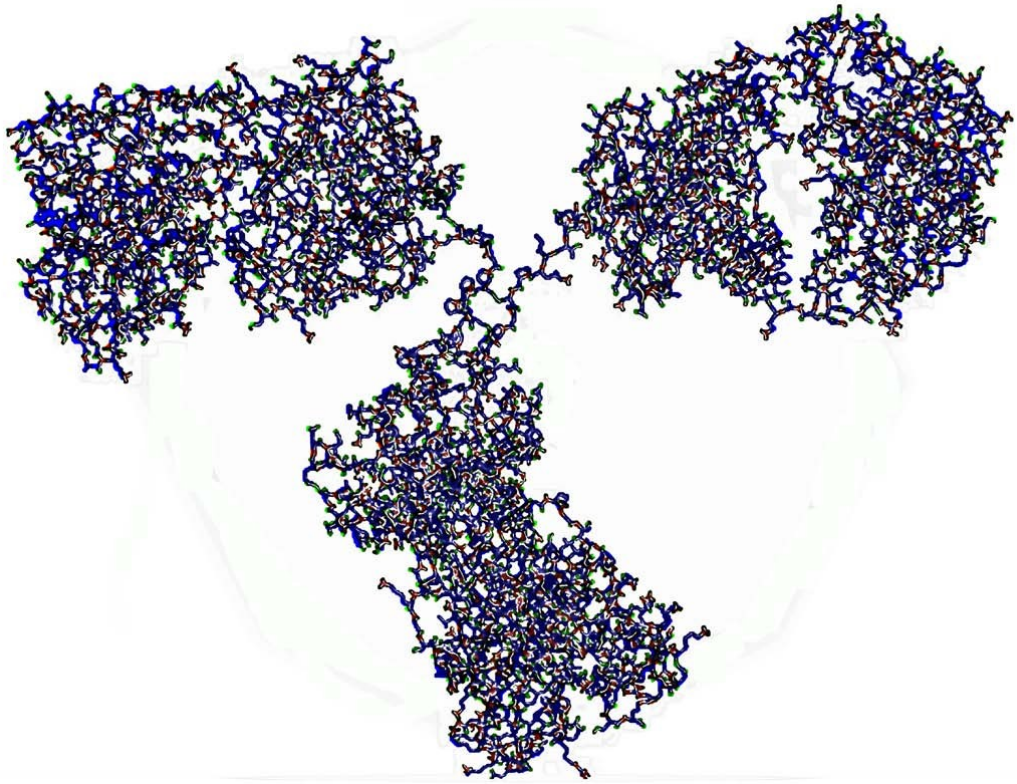
Life Sciences

Capillary Electrophoresis (CE)

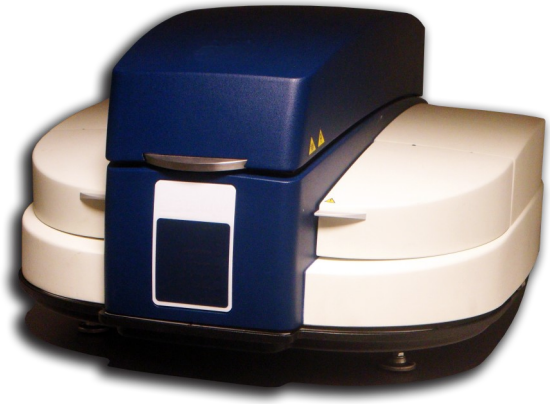
CEAN01

# ANTIBODY ANALYSIS

## By High Performance Capillary Electrophoresis



## Analysis of Immunoglobulin G



Label free analysis using the HPCE-512

## ABSTRACT

Analysis was carried out on several mammalian serum IgGs and commercially available Control Standard IgG using the HPCE-512 instrument to assess IgG stability based on fragmentation, purity and heterogeneity.

Fragmentation pattern and base-line resolved non-glycosylated heavy chain peaks were observed, with excellent reproducibility data obtained (peak time RSD less than 0.03%; quantitation RSD <1%). Accurate quantitation of the non-glycosylated species was also achieved, which came to ~9.6% of total heavy chain.

This application note demonstrates how high performance capillary electrophoresis, using a 512 pixel detector, can be used to assess the fragmentation pattern and post-translational modifications of IgG. The same strategy may be applied in assessing the stability and efficacy of biopharmaceutical mAbs.

## INTRODUCTION

In order to assess the bioactivity and safety of mAbs (recombinant monoclonal antibodies) as biopharmaceutical products, it is important to characterise the molecule and any impurities present. Not only is the information crucial for the assessment of the suitability of a potential biopharmaceutical product, it is also important for optimisation and QA/QC of the manufacturing process, storage conditions and life-time of the drug.

Out of five defined classes of antibody, all currently licensed mAbs are of the immunoglobulin G (IgG) class with applications in the both therapeutic and immunodiagnostic sectors. Recently, many research groups have reported both qualitative and quantitative mAbs analysis by SDS-CGE (SDS-Capillary Gel Electrophoresis) systems. Some have reported the detection of fragments from monoclonal antibody samples, which correspond to H2L, H2, HL, H and L portions of the antibodies. The fragmentation was believed to be caused by incomplete assembly, lack of intermolecular disulfide bridges and/or proteolysis due to microbial contamination. The level of fragmentation has been used as a measure of antibody stability. The effects of pH, temperature and buffer system on fragmentation have also been studied using SDS-CGE.

## MATERIALS AND METHODS

Mammalian serum IgGs were prepared in reducing and non-reducing conditions. Reduced samples were prepared with a proprietary methodology and incubated for 10 minutes at 95 °C. Non-denaturing samples were fragmented by heating for 10 minutes at 95°C. The reduced control standard IgG was prepared with the same proprietary methodology as above and heated for 5 minutes at 95°C.

Separation by capillary electrophoresis was performed in a commercial run buffer in bare fused silica capillaries of 50 µm internal diameter and a separation length of 20 cm. Total length of capillary was 32 cm. All serum IgG samples were injected electrokinetically at 5 kV for 10s and separated at 18 kV for 25 minutes. Control standard IgG samples were pressure injected at 4 psi for 10 seconds and separated at 18 kV for 25 minutes. The capillary was held at 22 °C during the separation. All detection was at 214 nm. At the beginning of the experiment and between the runs, the capillary was conditioned using a proprietary methodology. Between runs the capillary was rinsed with 1M NaOH for 5 minutes followed by 0.1M NaOH, 0.1M HCl and run buffer for 5 minutes each. After conditioning, a buffer conductivity check was performed.

Analysis of the separations was performed using the proprietary 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.

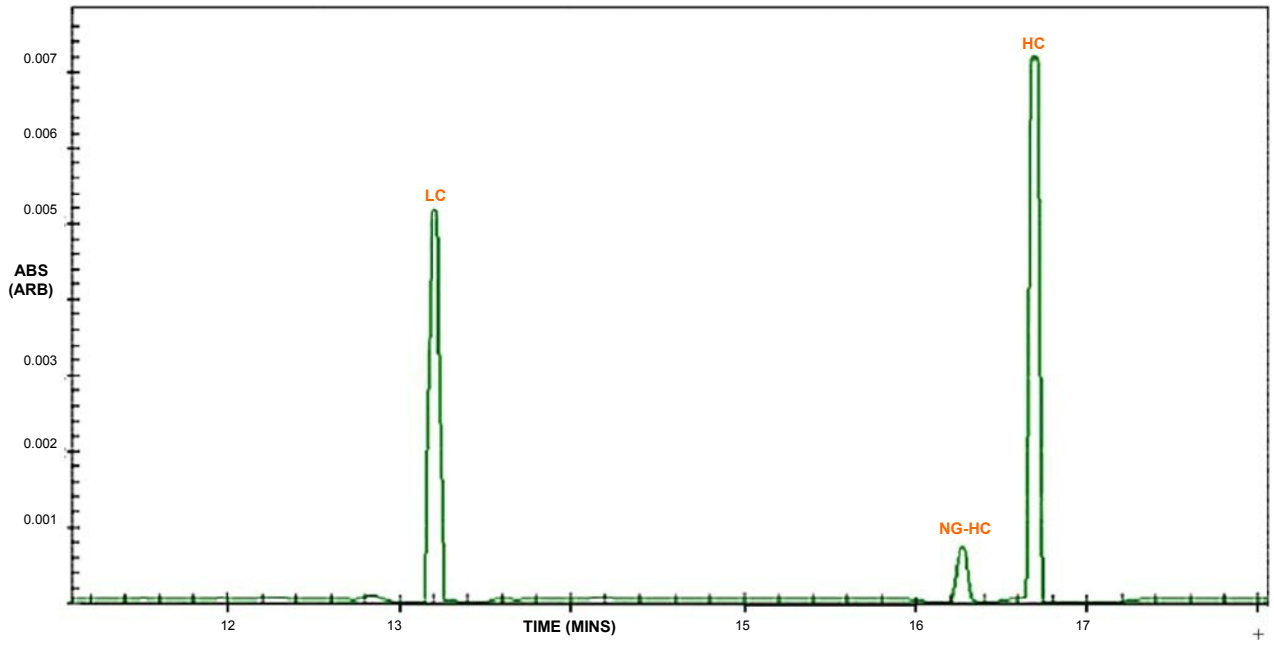



***Figure 2***: *Commercial control standard under denaturing conditions, EVA processed data, LC Light Chain, HC Heavy Chain, NG-HC Non-Glycosylated Heavy Chain.*

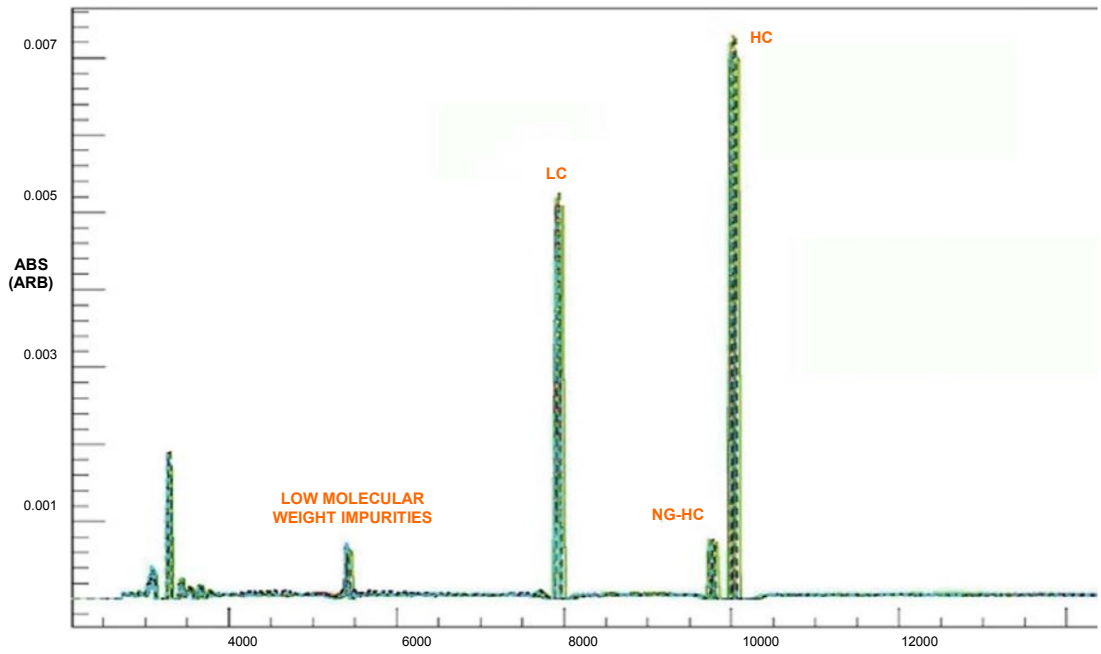



***Figure 3:*** *An overlay of EVA-processed data of 8 consecutive runs of Commercial control standard IgG In reducing conditions.*

| Antibody fragment | | Mean | Standard Deviation | RSD |
|---|---|---|---|---|
| Light Chain | Peak time | 13.21 | 0.0409 | 0.3096 |
| | Peak area | 0.02 | 0.0006 | 2.9335 |
| | %LC | 39.44 | 0.0986 | 0.2500 |
| Non-glycosylated Heavy Chain | Peak time | 16.27 | 0.0476 | 0.2927 |
| | Peak area | 0.003 | 0.0001 | 3.3228 |
| | % NG-HC | 5.76 | 0.0470 | 0.8124 |
| Heavy Chain | Peak time | 16.69 | 0.0496 | 0.2974 |
| | Peak area | 0.029 | 0.0009 | 3.10107 |
| | %HC | 54.78 | 0.0762 | 0.1390 |

***Table 1:***

*Mean, Standard Deviation, Relative Standard Deviation of peak time and relative quantitation for Light Chain, Non-Glycosylated Heavy Chain and Heavy Chain of Control Standard IgG separated using denaturing conditions*

## RESULTS AND DISCUSSION

Three mammalian serum IgGs (bovine, goat and rabbit) were separated under reducing and non-reducing conditions. In reducing conditions, the SDS-protein complex was heated for 10 minutes at 95°C in the presence of a reducing agent. This treatment breaks the covalent disulphide bond between the heavy and light chain (HC and LC respectively), reducing the IgG structures to two species with distinct molecular weights. In non-reducing conditions, the SDS-protein complex was heated at 100°C for 10 minutes in the absence of a reducing agent to facilitate the fragmentation process of IgG structure.

**Figure 1** shows the EVA processed data of heat-treated, non-reduced bovine serum IgG. A profile of 8 species with distinct migration times was observed, where the LC and HC could be easily identified based on migration time information derived from the GST profile of the reduced IgG (data not shown). With the latest migrating peak assigned as the whole IgG molecule, the rest of the peaks were identified as 2HC+1LC, 2HC, 1HC+1LC and low-level impurities present in the sample.

The same fragmentation pattern was observed in both non-reduced goat and rabbit serum IgGs – with entire IgG, 2HC +1LC, 2HC, 1HC+1LC, HC/LC portions and low-level impurities (data not shown). The migration time of each portion varies across the three mammalian IgGs examined.

Given SDS-CGE's superior resolving power over traditional SDS-PAGE, it is possible to examine the heterogeneity of post-translational modifications in IgG through mass-based separation. One important aspect of post-translational modification is glycosylation. HPCE resolving power in the SDS-CGE system with HPCE technology can be demonstrated by the base-line resolved separation of the non-glycosylated (NG HC) and glycosylated (HC) heavy chain molecules. The sample used was a commercial control standard IgG where a known quantity of non-glycosylated heavy chain is present. **Figure 2** shows the EVA processed data for the commercial control standard IgG, where the non-glycosylated heavy chain is baseline-resolved from the glycosylated heavy chain.

**Figure 3** shows the overlay of EVA-processed data of 8 consecutive runs of commercial control standard IgG in denaturing conditions. The analysis results of those 8 runs are summarized in **Table 1**. The relative standard deviations (%RSD) for migration of all three species (light chain, non-glycosylated heavy chain and glycosylated heavy chain) are all <0.03% while the quantitations of each species (%LC, %NG-HC, and %HC) are all <1% RSD. Through the reproducibility study, the precision of the HPCE instrument in both resolution and quantitation is clearly demonstrated. Based on quantitation analysis, the percentage of non -glycosylated heavy chain present in the sample was calculated, which came to 9.55% ± 0.007 of the total heavy chain, compared to the supplier's figure of 9.5%.

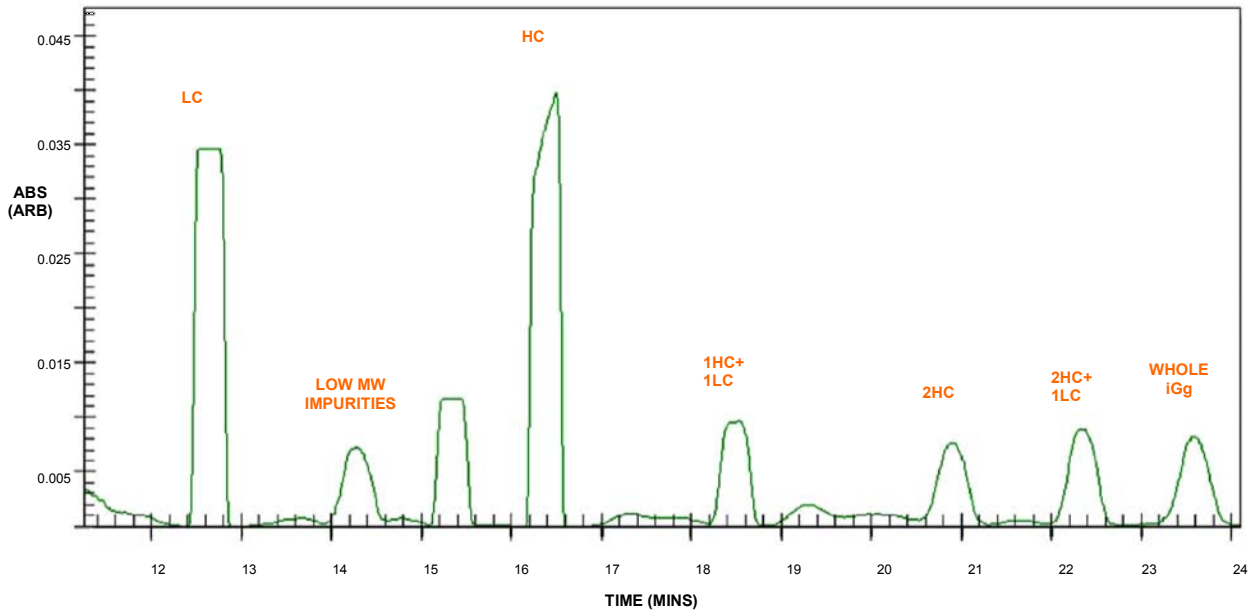



***Figure 1:*** *EVA processed data of SDS-CGE separation of bovine serum IgG using commercial SDS gel buffer under non-reducing conditions*

Light chain glycosylation can also be observed using the proprietary protocols. Due to the presence of a 'shoulder' on the light chain peak of some antibody samples, an alternative CGE method was utilised in an attempt to further resolve any potential glycoforms present in these samples. The separation length of the capillary was increased to 40cm in order to improve resolution between the glycosylated and non-glycosylated light chains of the antibody (**Figure 4**), and some adjustments made to the buffer regime.

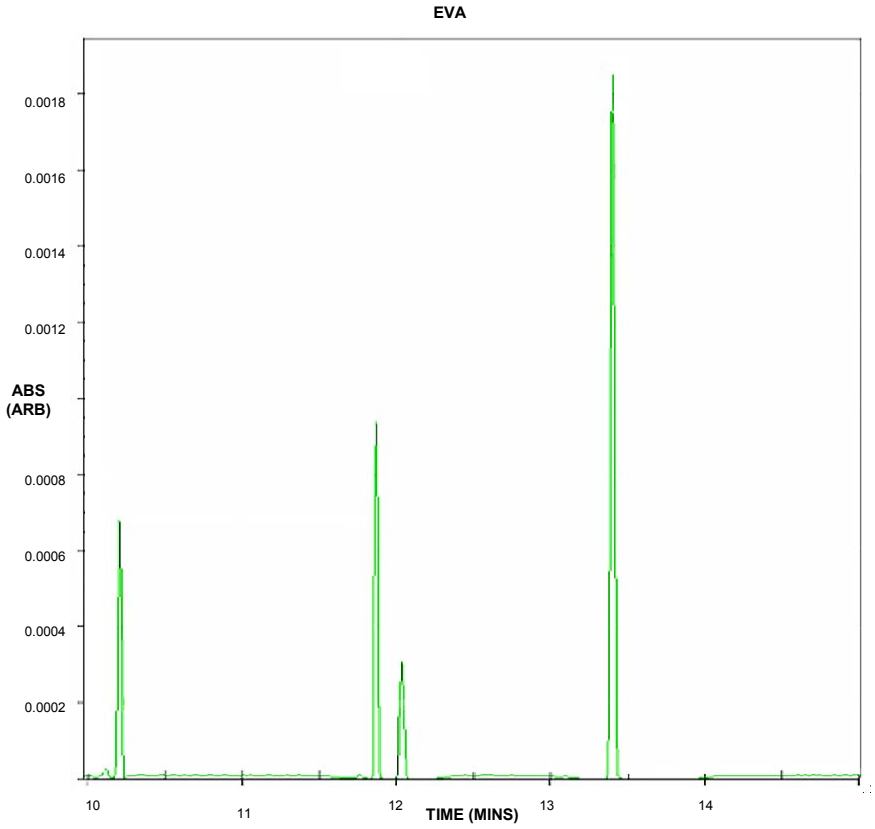



***Figure 4:*** *Glycosylation on the light chain of an antibody by CGE analysis.*

## Conclusion

This application note demonstrates how SDS-CGE on the HPCE-512 system can be used to obtain valuable information on IgG sample stability, purity and heterogeneity in a rapid and reproducible way. Fragmentation patterns and base-line resolved non-glycosylated heavy chain peaks were observed, with excellent reproducibility (peak migration time <0.03%; quantification <1% RSD).

Accurate quantification of the non-glycosylated species was also achieved , which came to 9.55% of total heavy chain. Light chain glycosylation was also observed.

When applied to the production of biopharmaceutical products, the HPCE system can be a powerful tool in the assessment of the bioactivity and safety potential products. This information will be vital in the optimisation and quality control of the manufacturing process, storage conditions and life-time of the biopharmaceutical product.