Method Optimization for Purity Analysis of IgG Isotypes and F-Fusion Proteins by Intact Reversed Phase

Laurence Brill, Ivan Lebedev, and Brian Rivera
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Overview

A primary analytical technique for purity for recombinant proteins is reversed phase LC (RPLC). This simple, robust methodology has a relatively short analysis time, as well being high resolution, capable of separation of hydrophobic variants such as oxidation, glycoforms, and clipping. As such, it is a common analytical approach for monoclonal antibodies (mAbs), especially considering it can be implemented at both intact and subunit levels without extensive method optimization.

However, different recombinant proteins present other challenges, requiring deviation from a platform method that was developed on a humanized, IgG1 mAb. For example, different isotypes of IgG, including IgG2 and IgG4s, have disulfide variants and Fab arm exchange variants, which must be characterized. Fc-fusion proteins are another, well established recombinant protein modality. These present many analytical challenges, as their glycosylation can vary considerably, Consequently, this makes the analysis of Fc-Fusions at the intact level somewhat challenging.

Methods for intact reversed phase often employ shallow gradient slopes (less than <1% B per column volume) over short periods of time (<15 minutes). For example, a gradient program used might be 30-40% B in 5 minutes, with gradient slope being 0.4% B per column volume. This shallow gradient within a tight window of organic might work reasonably well for mAbs of the same isotype, e.g. humanized IgG1. However, deviation in isotype or working with Fc-fusions may not work under these conditions.

Figure 1 shows Etanercept, a recombinant protein composed of a humanized Fc conjugated to the Tumor Necrosis Factor (TNF) receptor. Using an intact reversed phase LC method developed for mAbs, the analyte elutes very early in the gradient, with a k' of <2. Reducing the sample causes the protein to almost elute in the void. Figure 2 shows Panitumumab, an IgG2, that is too hydrophobic for the 40% B gradient, thus elutes in gradient program wash.

Consequently, if one is working with proteins of different physicochemical properties, it might be more prudent to then increase the gradient slope slightly and extend the organic window to facilitate separation of analytes of varying hydrophobicities. Figure 3 implements method where the gradient program starts earlier and elutes later than the aforementioned IgG1 method; 20-45% B. To ensure that gradient slope is relatively shallow, the gradient time is extended to 10 minutes.

The resulting method uses a 0.6% B per column volume gradient slope. Although this is steeper than the previously run method, when working with multiple protein formats, one can use this method as a platform for screening, then optimize further depending on the method needs. As shown in Figure 3, a range of analytes, from bispecifics, to different isotypes and fusion proteins, all elute within an acceptable portion of the gradient program. Purity can be assessed accordingly and again, as method requirements become more stringent, a shallower gradient might be implemented.

In summary, purity analysis by reversed phase LC is a primary method for the analysis of recombinant proteins. Fc-fusions and other monoclonal antibody isotypes may behave quite differently chromatographically. However, by simply adjusting gradient programs to optimize capacity factor or retention time, one can develop a streamlined, "platform" method for purity analysis that may be optimized depending on method requirements.

HPLC Conditions

Column:	bioZen 2.6 µm WidePore C4
Dimension:	100 x 2.1 mm
Part No.:	00D-4786-AN
Mobile Phase A:	0.1 % TFA in Water
Mobile Phase B:	0.1 % TFA in Acetonitrile
Gradient Program:	30-40% B in 5 minutes (Figure 1,2), 0.4 % B/CV 20-45% B in 10 minutes (Figure 3), 0.6 % B/CV
Flow-rate:	0.8 mL/min
Temperature:	80° C
Detection:	UV-Vis @ 280 nm
Injection:	2 µL
Samples:	As indicated (0.5 mg/mL)

Figure 1. Etanercept Using Standard IgG1 Gradient

Overlay of etanercept, intact and non-reduced. Because it is heavily glycosylated and thus more hydrophilic, it elutes early in the gradient program.

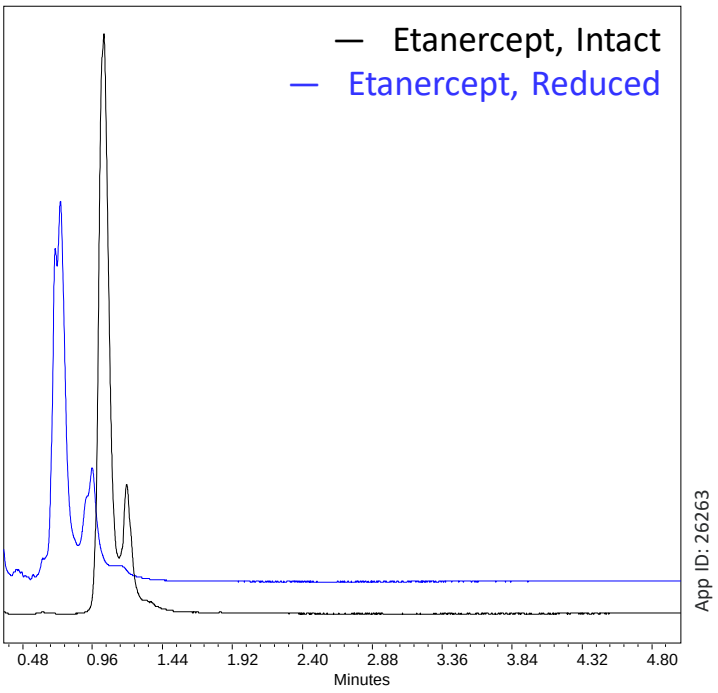


Figure 2. IgG2 Using Standard IgG1 Gradient

Chromatogram of panitumumab, a hydrophobic IgG2 which elutes at the end of the gradient program, thus eluting almost completely in the wash.

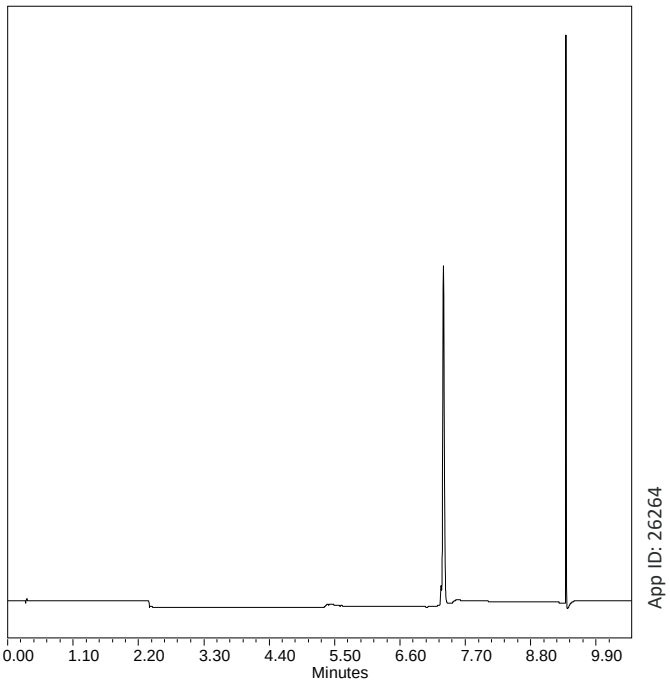
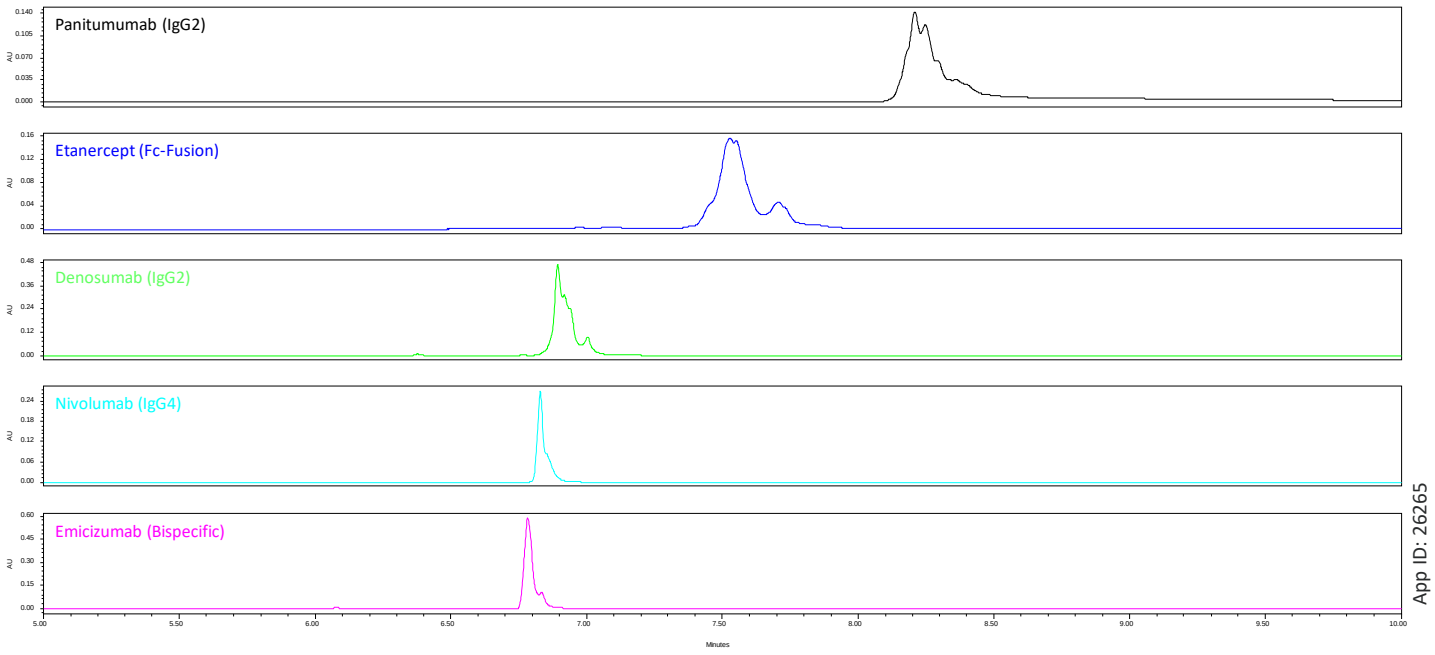


Figure 3. Gradient Program Optimization for Different mAb Formats and Fc Fusions

Chromatogram stack for various recombinant proteins. From top to bottom: Panitumumab (IgG2), Etanercept (Fc-Fusion), Denosumab (IgG2), Nivolumab (IgG4), and Emicizumab (Bispecific). By modifying the gradient slope- widening the elution program and extending the gradient length, acceptable impurity profiles are obtained for all molecules.



Need a different column size or sample preparation format?

No problem! We have a majority of our available dimensions up on www.phenomenex.com, but if you can't find what you need right away, our super helpful Technical Specialists can guide you to the solution via our online chat portal www.phenomenex.com/LiveChat.

Australia
t: +61 (0)2-9428-6444
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
info@phenomenex.com

China
t: +86 400-606-8099
cninfo@phenomenex.com

Czech Republic
t: +420 272 017 077
cz-info@phenomenex.com

Denmark
t: +45 4824 8048
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
anfrage@phenomenex.com

India
t: +91 (0)40-3012 2400
indiainfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
nordicinfo@phenomenex.com

Poland
t: +48 22 104 21 72
pl-info@phenomenex.com

Portugal
t: +351 221 450 488
ptinfo@phenomenex.com

Singapore
t: +65 800-852-3944
sginfo@phenomenex.com

Slovakia
t: +420 272 017 077
sk-info@phenomenex.com

Spain
t: +34 91-413-8613
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
nordicinfo@phenomenex.com

Switzerland
t: +41 (0)61 692 20 20
swissinfo@phenomenex.com

Taiwan
t: +886 (0) 0801-49-1246
twinfo@phenomenex.com

Thailand
t: +66 (0) 2 566 0287
thaiinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
info@phenomenex.com

All other countries/regions
Corporate Office USA
t: +1 (310) 212-0555
info@phenomenex.com

www.phenomenex.com

Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com

TN7784220