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**PC1: Detection and
Quantification of Process-
related impurities: Host cell
protein detection in
biological products**

**Bailey, M.J.A.
Hills, A.E.
Jones, C.
O'Connor, G.
Quaglia, M.**

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Detection and Quantification of process-related impurities: Host cell protein detection in biological products

Anna Hills
Quality of Life Division

ABSTRACT

The report contains a review of current industry practice for host cell protein quantification in biopharmaceuticals and identifies alternative technologies for the measurement of these process-related impurities.

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National Physical Laboratory
Hampton Road, Teddington, Middlesex, TW11 0LW

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Approved on behalf of the Managing Director, NPL
by Martyn Sené, Director, *Quality of Life Division*

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1 Introduction

The project “Detection and Quantification of process-related impurities: Host cell protein and DNA detection in biological products” was led and coordinated by the National Physical Laboratory on behalf of a consortium consisting of the UK Pharmaceutical Analytical Sciences Group (PASG), LGC Ltd and NIBSC. It seeks to support the strategy of the National Measurement System Measurements for Biotechnology Programme, to improve the comparability of biological measurements and to remove barriers to the exploitation of biotechnology by UK industry.

The involvement of the Biopharmaceutical Working Party of PASG provides strong industrial collaboration and is a reflection of the commitment that the UK biopharmaceutical industry has for developing methods for the quantification of trace amounts of process-related impurities, which are compatible with regulatory requirements.

1.1 *Aims of the project*

- Build on the PASG position paper to generate a thorough review of current industry practice for host cell protein determination and identify new technologies for the measurement of HCP and HCD in biopharmaceuticals.
- Develop model systems that reproduce the contamination profiles found in biopharmaceuticals that are characteristic of common expression systems.
- Experimentally evaluate selected proteomics-based separation and protein quantitation technologies to determine their fitness for purpose for HCP separation and quantitation, and identify the advantages and disadvantages of these techniques to current industrial practise.
- To undertake case studies with the HCP techniques to ensure their fitness for purpose.

- Proactively work with the PASG BWP and international industry to engage with regulators to ensure the project influences (and gains input from) the ongoing international debate on HCP measurements.

1.2 Deliverables

The project proposal identified a set of work packages to address the aims listed above, and focussed separately on host cell protein (HCP) and host cell DNA (HCD). The host cell DNA work packages are the subject of a separate report (1).

The following deliverables focus on the host cell protein work packages. It was identified that there was a diverse range of end-users who would wish to gain access to the outcomes; those interested in the regulatory debate around HCP analysis, those who wish to learn and apply current industry practise, and analytical scientists who may want to replicate the experimental protocols. As a result, an overall review document was produced that was supported by technical reports (2) (3).

- A model system was demonstrated, that mimicked HCP profile in biopharmaceutical products that can be freely distributed, analysed, and the results published without infringing pre-existing intellectual property.
- A review of current regulatory advice and guidance on HCP quantification was generated, in the project summary report.
- Experimentally evaluated protocols for the application of particular proteomics techniques for the consistent quantification of host cell protein impurities.
- Case studies of new techniques applied to formulated biopharmaceutical products where an HCP profile is available (depending on the availability of commercial materials, and the IP holders' consent).

1.3 End Users

- The consortium members
- The members of the Biopharmaceutical Working Party of the Pharmaceutical Analytical Sciences Group (UK biopharmaceutical manufacturing industry)
- The international biopharmaceutical manufacturing industry
- The biopharmaceutical regulators such as the FDA, EMEA, MHRA
- Industrial and regulatory focus groups such as the WCBP CMC Strategy Forum
- Industrial associations and support activities such as the Biotechnology Industry Association and bioProcess UK.

2 Background

2.1 What are host cell proteins and DNA?

Biopharmaceutical products such as peptides and proteins, viral vaccines and gene therapy products are produced using recombinant DNA technology that directs a host cell to produce the molecule of interest. The most common expression systems used by the biopharmaceutical industry employ bacterial, yeast or mammalian cells. These are cultured *in vitro* and secrete the desired product in to the culture medium or into a compartment of the host cell. Recovery of the product from complex culture media is challenging. Proteins or DNA from the host system may carry through or co-purify with the molecule of interest during the down-stream purification process, and be incorporated into the final administered product.

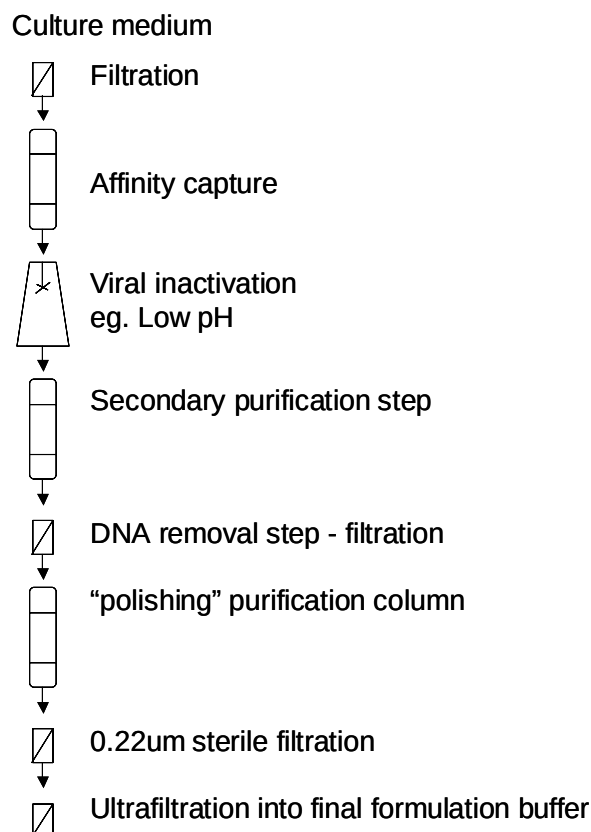


Figure 1. A typical down-stream purification process for a monoclonal antibody produced in a mammalian expression system.

Host cell proteins (HCP) and host cell DNA (HCD) are distinguished by their origin from other process-related impurities which derive from the reagents used in the fermentation process, such as single chemical entities including gene selection agents or media raw materials, or from the purification process (e.g. immobilised staphylococcal protein A used to affinity purify monoclonal antibodies, which may leach and contaminate the final product). Product-related impurities, which usually derive from chemical or biochemical degradation or modification of the product and are largely identical to it, are not classed as process-related impurities in this project.

Regulatory authorities request information on the level of all impurities present in a drug substance, including HCP and HCD, typically stated as parts per million of mass of the drug product. The acceptable level of contamination depends on the nature of the product and its intended therapeutic indication, which will be discussed in later

sections of the report. The current regulation does not require the identification of the HCP or HCD sequences.

The biopharmaceutical industry dedicates much resource to monitor the HCP and HCD during development of a manufacturing process. The diligence in manufacturing process design, taken together with the dosage approach developed and safety data from the clinical trials, should ensure that any biopharmaceutical product that gains regulatory approval will not contain impurities that represent a risk to the patient.

2.2 Why measure HCPs and DNA in biopharmaceutical products?

Host cell proteins are contaminants which may have unwelcome biological effects, and therefore, like any contaminant, their levels are subject to regulation through the application of maximum limits (specifications). These specifications will normally be product-specific; acceptable levels of HCPs should be expected to be different for products administered repeatedly for chronic conditions (eg. insulin, somatropin) compared to products used for rare, one-off life saving interventions (such as tissue plasminogen activator) (4). HCP contaminants have the potential to be themselves immunogenic. They may have adjuvant activity to stimulate an immune response against the active pharmaceutical ingredient (API), may be enzymatically active, have inflammatory or other functional properties, or may lead to disease.

2.3 Effects of process-related impurities on product efficacy

Initial studies with the “follow-on” somatropin product OmnitropeTM suggested that host cell proteins may be associated with the development of antibodies against the active material. To quote, “...the European Public Assessment Report for OmnitropeTM reports that initial high levels of host cell proteins were associated with high levels of anti-somatropin antibodies, which was resolved when the purity was improved” (5). Anti-API antibodies which neutralize biological activity will impact on product efficacy and, in some cases could conceivably lead to more serious long term effects.

2.4 Immunogenicity of HCPs

Host cell proteins also can give rise to antibodies which recognise the HCPs in the drug product (but not the active ingredient). Such antibodies do not normally lead to neutralisation of product potency as measured, for example, in cell-based bioassays of cytokines. Two publications report that administration of GM-CSF to patients resulted in antibody responses against the product, these antibodies reacted with a cell lysate of the (*E. coli*) production system, and blotting of one-dimensional gels showed that they recognised two *E. coli* proteins of molecular mass 20 kDa and 30 kDa.(6,7). Recombinant cytokine products may have adjuvant properties which may enhance the immunogenicity of trace contaminants.

In another study reported (8), anti-yeast antibodies were detected in patients treated with a recombinant *S. cerevisiae*-derived somatropin, but the validity of this data and the clinical significance were unclear. Similarly, antibodies against *E. coli* have been reported in patients treated with OmnitropeTM, an *E. coli*-derived somatropin (9), but these were considered to be of no clinical significance. Antibody responses against a CHO host cell protein present in a recombinant α -L-iduronidase have also been reported (10).

The potential for enzymatic activity of HCP is highlighted in an anonymised report of a case where a host cell protease from a mammalian expression system that co-purified with a monoclonal antibody, leading to degradation of the product (cited at an FDA-led workshop; Follow-on Biologics December 12-14, 2005) (11). Variable amounts of HCP will lead to variable product stability and variable clinical efficacy. Whilst catalytic activity of HCPs which degrade the product are likely to be relatively easy to detect, HCP enzymes which are active in the patient can conceivably lead to clinical consequences.

In general, the specifications for HCP contamination of vaccine products tend to be looser than those for recombinant biotherapeutics. This probably reflects that (i) these products are given only a limited number of times in the life of an individual (ii) vaccine doses tend to be lower than that used for therapeutics, so that the total amount of HCP administered with a vaccine remains small, and (iii) many vaccines are

complex products for which purification is difficult. The key issue with vaccines appears to be allergenicity. Whilst measurement of HCP levels in vaccine products is unusual, there is a general contra-indication against administering vaccines produced in eggs (eg. influenza and MMR vaccines) to infants or adults with an allergy against eggs (See: Contra-indication for egg allergy for vaccine treatment). It has been suggested that contaminating myelin basic protein (MBP) in mumps-measles-rubella (MMR) vaccines may lead to autism in infants. However a detailed immunological study failed to find this particular HCP in products (12).

Whilst not strictly host cell proteins in the sense defined in this report, there are numerous reports of cytokine contamination of clotting factors purified from whole human blood.

2.5 Profiles of HCP in recombinant products

It is known that certain host cell derived biological molecules do have toxic activity in humans. For example, if a biopharmaceutical is manufactured using gene expression systems based on the bacterium *E. coli*, then the manufacturing process will be designed to remove all traces of endotoxin - a proteolipid from the surface of the bacteria that can induce anaphylactic shock. The composition of common process-related impurities – particularly proteins, polysaccharides, nucleic acids, glycolipids and phospholipids from microbes, can be immunogenic to T cells and illicit a cell mediated immune response. Manufacturers rigorously check a drug substance preparation at each stage of the manufacturing process development to confirm that known toxic components are removed, and that non-toxic HCP are reduced to the required safe level.

Factors affecting the immunogenicity of a host cell protein range from the “foreignness” of the molecule to the immune system, its size, and its chemical composition in terms of its primary sequence as well as its conformational determinants - secondary, tertiary and quaternary structure. The HCP physical form also affects its immunogenicity; for example if they are in solution or as insoluble particulates. The effect of host cell proteins in the patient may also depend on the ability of antigen presenting cells to degrade the proteins and present antigenic

peptides to the immune system, and this may be influenced by the genetics of an individual's immune system (responders versus non-responders). The immune systems of certain individuals may also be compromised due to illness, therapy or age.

HCPs may range from a small number of co-purifying proteins at relatively high concentration, to a broad range of proteins that vary in concentration, molecular weight, pI, and solubility. Each of these scenarios presents very different analytical challenges. Immunological detection is successful for detecting a pool of multiple proteins but does not provide information on number, individual levels or identity.

3 Regulatory advice and guidance on HCPs

Regulatory concerns about host cell protein in products date back to the early days of recombinant protein therapeutics. Specifications for protein contamination existed, for example, for bacterial polysaccharides used in vaccine manufacture. In general, a case-by-case approach has been taken for different products (or product classes). Acceptable levels for bacterial and yeast derived proteins (which are considered to have adjuvant activity) may be different from those for products from mammalian cell production systems, such as CHO cells. Similarly, since HCP levels are cited as parts per million in the product, higher levels may be acceptable for a highly active product where a small dose is administered, compared to a product which requires a much higher dose. However, early international discussions on methods to demonstrate recombinant protein quality (13,14) did not consider HCPs.

3.1 WHO recommendations

The early WHO Guidelines for recombinant therapeutic products considered both host cell DNA and host cell protein (4,15), and appeared to consider host cell DNA as the more serious problem. These Guidelines formalised the concept that acceptable limits for “contaminating antigens” should depend on the expected use of the product (eg. with a focus on products which are administered repeatedly or in large quantities).

3.2 ICH guidelines

ICH Guideline S6 “Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals” cautions that “(t)here are potential risks associated with host cell contaminants derived from bacteria, yeast, insect, plants, and mammalian cells. The presence of cellular host contaminants can result in allergic reactions and other immunopathological effects.”

ICH Guidelines Q6B and Q5E also apply. ICH Q6B says that “For host cell proteins, a sensitive assay, e.g. immunoassay, capable of detecting a wide range of protein impurities is generally utilised.” The polyclonal antibody used in such an assay is usually raised by immunisation with a partially purified preparation from a production cell line lacking the product-coding gene. “Clearance studies may sometimes be used to eliminate the need for establishing acceptance criteria for these impurities”. ICH Q5E states that in the event of a process change which may give rise to a different impurity profile, “.... Manufacturers should confirm that the test used to quantify these impurities is still suitable for its intended purpose”. ICH Guideline Q2(R1) “Validation of Analytical Methodology” also applies, the regulatory requirements for the validation of the analytical methodology are the same as those for any other analytical method. The user should demonstrate fitness-for-purpose, precision, robustness and accuracy.

3.3 Pharmacopoeial limits

Measurement of host cell protein levels is covered in the following European Pharmacopoeial monographs.

01/2005:0784	Recombinant DNA Technology, Products of
01/2005:1110	corrected Interferon alfa 2 concentrated solution.
01/2005:1440	corrected Interferon gamma 1b concentrated solution
01/2005:1635	Glucagon, human
01/2005:1641	Molgramostim concentrated solution
07/2005:2031	Monoclonal antibodies for human use.
01/2006:0951	Somatropin
01/2006:0952	Somatropin for injection

01/2006:1316	Erythropoietin concentrated solution
07/2006:2149	Influenza vaccine (surface antigen, inactivated, prepared in cell cultures)
07/2006:2308	Influenza vaccine (whole virion, inactivated, prepared in cell cultures)
01/2007:51400	Gene transfer medicinal products for human use

Validation and reliance on historical data trends are considered appropriate strategies for determination of levels of HCPs, so that testing of every batch prior to release is not necessarily required. The numerical limit for any therapeutic product is agreed individually with the competent authority, rather than a blanket value being cited in the Pharmacopoeia, whilst generic values are cited for some complex vaccine products.

The Pharmacopoeia of the United States of America (USP), 29th Edition discusses briefly methods for HCP quantification in the general chapter, called <1045>, on Biotechnology products, and mentions SDS-PAGE and immunoassay as approaches for *E. coli* and CHO cell proteins, recommending sandwich ELISA techniques. The chapter comments that absolute accuracy and the ability to detect every antigen/HCP cannot be guaranteed. The USP states a limit of 10 ppm for HCPs in recombinant human insulin. No other monographs appear to have general specifications. This value is that used for other contaminant process-related impurities, such as residual Protein A in monoclonal antibodies, and pro-insulin in insulin preparations. The published summary of the USP Biologics and Biotechnology Proteins and Polysaccharides Expert Committee meeting on 15th June 2005 stated that development of a specific chapter on host cell protein and host cell DNA measurement was considered. There is no published information on whether this project is currently being pursued.

3.4 Information and guidance from the FDA

It has been reported that “most biotechnology products reviewed by the FDA contain ELISA-based host cell protein levels of 1-100 ppm” (16). It is not clear over what

time scale this data was collected, or whether there is, for example, a trend towards lower values.

To the best of our knowledge, the FDA Centers have not published, in the form of Notes for Guidance or otherwise, explicit guidance about the risks associated with host protein, or what represents acceptable levels or appropriate methodology. Reading of a number of documents from FDA does seem to suggest that they are happier with validation approaches than are the European agency. An FDA “Points to Consider” document on monoclonal antibodies for human use mentions host cell proteins, but provides no details, and appears to suggest process validation through analysis of three consistency batches, although the phraseology is not clear.

The acknowledged adjuvant activity of some bacterial and yeast HCPs influences the debate on licensing of “follow-on” biological products. Whilst two products may have the same total amount of HCP present, due to different manufacturing and purification processes, the HCP may be different proteins with different biological function. The HCP-stimulated immunogenicity of recombinant human growth hormone (rhHGH) has been cited quite frequently (17,18). Two aspects arise from this debate – firstly, that only innovator companies (and their collaborators) will have access to the unique reagents used to generate the historical data against which a “follow-on” product will be compared, and, secondly, and inconsistently, that even though the identities of the host cell proteins can influence product efficacy, manufacturers seek specifications which relate only to total HCP content.

3.5 EMEA Notes for Guidance and discussions

The EMEA published in 1997 a Guidance Note “Position statement on DNA and host cell protein (HCP) impurities; routine testing versus validation studies” (CPMP/BWP/382/97) which highlights the different regulatory approaches adopted for host cell DNA and host cell proteins. This paper also points to the need to harmonise between the major regions. The European approach for host cell proteins is that the assay should be carried out on all purified bulk materials (“drug substance”) to ensure consistency and compliance with specifications, and that “validation” approaches based on proof of removal of spiked material in test samples will require

special assessment. The paper acknowledges (i) that it is difficult to standardise these assays, since the material present will differ considerably, product by product, and (ii) that the final result depends on the process, and factors such as column use and sanitisation, column storage conditions, and age.

3.6 Product Licences

Product licenses for individual products contain the specifications which that product is expected to meet, and as such, provide the most comprehensive data set which, potentially could be analysed by product type, host cell line, submission date etc. However, these documents are confidential and such analyses have, to the best of our knowledge, never been reported.

3.6.1 WCBP CMC Strategy Forum

Whilst not a regulatory body, the CMC Strategy Forum issues discussion documents derived from workshops attended both by industry and regulatory bodies. They combine aspects of current and proposed best practice. In 2005, CMC published four documents on “Defining your product profile and maintaining control over it”. One of these documents covered host cell protein determination (16). This considered the advantages of ELISA-based methods compared to SDS-PAGE methods, and distinguishes between product-specific, manufacturer-specific multi-product and generic approaches, and highlighted the advantages and disadvantages of each. The article suggests that manufacturer-specific multi-product approaches are the most generally applicable.

4 Existing Methodology for HCP determination

Current methods for host cell protein detection rely on immunodetection principles, where polyclonal anti-HCP antibodies are raised in mammals such as rabbits or goats for use in enzyme-linked immunosorbent assays (ELISA) or Western blotting. These approaches require development and validation of the reagents involved, particularly in production of the antiserum. This is often an expensive and time consuming exercise which is beyond the resources of some laboratories. As a result,

“generic” kits have become commercially available which supply antibodies and standards for a wide range of commonly used expression systems in the biopharmaceutical industry. The benefits and disadvantages of using this approach versus a process-specific one are discussed in the following sections.

Some organisations with legitimate interests in measuring HCP in commercial products, such as laboratory facilities answering to national regulatory authorities, do not have access to the innovators antiserum or the reference preparations, and so cannot use the identical tests to those used by the manufacturer. In addition, since they may have to test a wide variety of products from different manufacturers, they would need very extensive collections of the antiserum - reference compound combinations, which would be very difficult to achieve. Such organisations will benefit from access to generic methodology which they can apply to multiple products and which are independent of specific antisera.

4.1 ELISA Methodology

4.1.1 How to prepare antiserum

Section 6.2.1(a) of Q6B) recommends that assays to detect host cell proteins should include “...a sensitive assay, e.g. immunoassay, capable of detecting a wide range of protein impurities...” and gives further guidance on producing the necessary antibodies for an immunoassay test. In general, a representative collection of host cell proteins are produced by a “sham” production and purification from a production cell line containing the vector but not the product gene. The HCP are then “purified” through the normal down stream purification procedure to a point at which the product would be 95-99 % pure. This preparation is then used as immunogen, the resulting antisera are pooled and/or purified and used in western blot or ELISA techniques. Although this procedure is well established, there are some fundamental flaws in overall concept as well as technical issues with both western and ELISA methodology. An assumption is made that HCPs in the sham production and purification will be the same as in the full production situation and that they will behave the same way in the sham purification as in the presence of the product. This may not be the case, and the higher the product yield, in terms of the amount of

product as a proportion of total host cell proteins, the more likely that co-purifying host cell proteins will behave and interact differently with the purification resins and filters. There are also likely to be scale-up issues involved – the sham process is unlikely to be done at the same scale as the full-scale production process. Special consideration must be given to processes which purify monoclonal antibodies in a sham purification – typically the first purification step for monoclonal antibodies is a protein A affinity column which clears >99% of all other proteins. The product is likely to have a very reduced host cell protein profile compared to the sham situation, therefore it is not representative of real values in product.

A position paper from the Biopharmaceutical Working party of the Pharmaceutical Analytical Sciences Group (PASG) in the UK was written in Autumn 2002 to outline the concerns above (see Appendix 1). The paper points out that quantitative or semi-quantitative assays using antisera may not produce results that accurately reflect the true levels of HCPs because of biases introduced by the differing immunogenicities and abundances of the HCPs in the immunogen preparation. This may be mitigated to some extent if one accepts that one of the major reasons for wanting to exclude HCPs from the final product is that they may be immunogenic in the patient, and therefore an assay biased towards the detection of such potential contaminants is desirable.

4.1.2 Advantages and limitations of immunoassays; process specific vs generic antiserum

Immunoassays are only as sensitive and specific as the antibodies that are used, non-immunogenic proteins will not be represented in the assay. There are a number of immunoassay kits and antisera for detecting host cell proteins that are commercially available, and are termed “generic” assays. The antisera in these instances are often raised using a lysate of the most common cell lines used as biopharmaceutical expression systems, and are therefore broadly reactive to as many HCPs as possible, the majority of proteins are highly conserved from clone to clone in the same species (>95%, (19)). Generic assays are often used in process development and for product used for early stage safety testing. In addition, many biopharmaceutical production processes are not fully defined until a very late stage of the drug development process (Phase III), particularly if processes are required to transfer between manufacturing

sites. Therefore the effort and expense of developing a process specific assay before this point may be wasted, as minor changes to the fermentation conditions and purification process may have an effect on the quantity and number of HCPs present. For example, a production run that has fewer viable cells by the time of harvest (due to changes in media, feed rates, harvest method) may have a considerably higher load of HCPs due to cell lysis and release of their cytoplasmic contents into the medium. Changes to chromatographic resin or filter chemistry may affect the loading capacity or “break-through” of HCPs. When a new or atypical HCP occurs in the final product, a process specific-antisera may not detect it. However, generic antisera in kits should still be validated for the individual circumstances.

As described in the previous section, a process specific assay measures those HCPs that co-purify with the product, often mimicked by sham production runs using a relevant cell line. Sham runs make the critical assumption that absence of the product from the growth and purification procedure will not alter the number or recovery of HCPs. Both methods for preparing antisera have advantages and limitations that should be considered depending on the stage of development of the product. Process specific assays may be more appropriate for well-defined, large scale manufacturing processes where little variation or a few discrete proteins are known to co-purify. Depending on the resources of the manufacturer, there may be antisera available from each stage of the development or purification process.

When preparing the antisera for use in these two approaches, affinity purification can improve both specificity and sensitivity. Specificity is enhanced by the removal of pre-existing irrelevant antibodies and other proteins from the polyclonal serum that could result in artefacts or high background values in the immunoassay approach. Purification of HCP-reactive antibody species increases the concentration of HCP reactive antibodies and improves assay linearity and analytical range (see section 4.1.5 for discussion). Care should be taken to compare Western Blots of unpurified antiserum with those obtained from affinity-purified antibody, to indicate whether significant antibody populations have been lost in the purification process. The antisera may distinguish non-HCP bands from non-specific binding or other artefacts. Affinity-purified antibody can yield additional bands over the raw antiserum because of the selective enrichment and sensitivity improvement, but a general guide for

assessing the suitability of affinity-purified antibodies is that fewer than 5% of the raw antiserum HCP bands are missing afterward. Alternatively, the use of two-dimensional gel electrophoresis can be an even higher resolution method for determining the reactivity of an antibody preparation (19).

4.1.3 Immunoassay calibration

HCP immunoassays measure a pool of antigens, which are unidentified. The assay may detect a few discreet co-purifying proteins or hundreds, but report one value for the total amount present, typically as part per million (ppm) or ng/mg. Absolute determination of HCPs is extremely challenging as the identity and molecular weight of multiple proteins would have to be determined. As a result, determining the accuracy of the immunoassay is complicated, and requires a relevant HCP standard. Inaccuracies can arise when HCPs are solubilised from the relevant cell line and a protein concentration assay (for example biuret or bicinchoninic acid dye binding assays) are performed. Detergents used for solubilisation of the cell line, as well as lipids from the cell wall can interfere with the colorimetric detection and over-estimate the amount of protein present in the standard. In addition, not all HCPs are immunogenic and so will not be detected in the immunoassay. Both of these problems can lead to the immunoassay appearing insufficiently sensitive. A different approach to making HCP standards is to immobilise antibodies generated from an HCP whole cell lysate immunogen onto an affinity support resin. Solubilised HCPs are then passed over the resin, and non-immunoreactive HCPs and excess antigens from very high concentration HCPs are not retained. The bound immunoreactive material is eluted and that protein quantitated by a protein assay for use as the HCP assay standard (19).

4.1.4 Choice of immunised animal species

The choice of animal used to generate the antiserum depends on the ability to raise large enough quantities of antibodies, and how likely they are to represent an immune response in humans. Often larger animals such as rabbit or goats are used as their muscle mass can receive large enough doses to receive a volume equivalent to a full human dose, and can produce large enough volumes of antiserum. A successful immunoassay validation strategy should identify critical reagents that will be required

over the biopharmaceutical product development lifetime, and ensure continuity of supply for lot release in manufacturing regimes, a period of time that can last ten to twenty years or longer. If the stock of antiserum runs out, then comparability and re-validation activities studies should be conducted to ensure that the antiserum will detect the same range of HCPs with equivalent sensitivity.

4.1.5 Published recommendations on immunoassay validation

The ICH guidelines Q2 (R1) provide information for laboratories needing to validate analytical procedures. Quantitative tests for measurement of impurities are one of the most common tests requiring validation for product release. ICH Q6B guidelines (Specifications: Test Procedures and Acceptance Criteria for Biotechnological /Biological Products) highlight the use of immunoassays to detect host cell proteins in biological products:

“For host cell proteins, a sensitive assay, e.g. immunoassay, capable of detecting a wide range of protein impurities is generally utilized. In the case of an immunoassay, a polyclonal antibody used in the test is generated by immunization with a preparation of a production cell minus the product-coding gene, fusion partners, or other appropriate cell lines. Clearance studies, which could include spiking experiments at the laboratory scale, to demonstrate the removal of cell substrate-derived impurities such as nucleic acids and host cell proteins may sometimes be used to eliminate the need for establishing acceptance criteria”.

The guidelines outline the assay characteristics that must be examined in order to demonstrate that it is suitable for its intended purpose. These have been related to the HCP immunoassay in the paragraphs below.

Specificity is the ability to measure the antigen/s (multiple HCPs) in the presence of components that may be expected to be present. In the case of a host cell protein immunoassay, these might include process buffers at different pH, detergents and high salt concentrations as well as large concentrations of the product and its degradants.

The **accuracy** of an analytical procedure expresses the agreement between the value reported in a control sample and the value which is accepted either as a conventional true value or an accepted reference value. This is often achieved by demonstrating that the assay can report HCP from a reference or standard spiked at known levels in to the sample matrix (in this case the biopharmaceutical product).

The **precision** of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same sample under the prescribed conditions. Precision may be considered at three levels: repeatability (over a short period of time under the same operating conditions), intermediate precision (on different days, with different analysts or equipment) and reproducibility (precision between laboratories, for example in collaborative studies). It is normally expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

The **detection limit** of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The **quantitation limit** of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. It is important to remember that HCP immunoassays are multi-antigen assays.

The **linearity** of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. For HCP immunoassays, this relies on a standard preparation of HCPs provided from an in-house process, or a blend of HCPs from common cell lines used for expressing biopharmaceuticals

The **range** of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision,

accuracy and linearity. The **Linear Dynamic range** is often reported as the ratio between the smallest and largest values in the linear range.

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage. For HCP immunoassays, this could test the washing, incubation and substrate development steps. The critical reagent is the antiserum, which should be tested for its stability during long term storage. Robustness of the assay itself should not be confused with the ability of the assay to detect changes in the downstream process.

In addition to the ELISA validation protocol mentioned above, the specificity and sensitivity of any antibody-based assay used for the detection of HCP is related directly to the quality of the antibodies themselves. Is the polyclonal antibody preparation specific for intended application? Western blots are often used to characterize the antibodies used in immunoassays as they are a one-off experiment to demonstrate that the majority of protein bands separated by gel electrophoresis (either one or two- dimensional) will have corresponding antibody reactive western blot bands. However an ELISA is a more sensitive and quantitative procedure than Western blot yielding detection limits more than 100 fold lower. ELISA is typically the only method with adequate sensitivity to detect multiple contaminants in downstream samples and final product. ELISA in general can be less prone to false positive, non-specific reactions that are encountered in many Western blot procedures.

5 What HCP values do manufacturers actually deliver?

Information in the public domain is scarce as data submitted to the regulators is confidential. However the FDA comment that most licensed products have HCP values of between 1 and 100 ppm. The problem of host cell contamination was discussed at the Recovery XII meeting in Arizona in April 2006. Informal discussions with a senior bioprocessing staff member from Genentech indicated that they expected to deliver monoclonal antibody products with approximately 5 ppm of CHO HCP as standard, whilst a “good” product would have levels of approximately 1 ppm.

These values seemed consistent with those reported verbally by other attendees at the conference. The general guidance from FDA CBER has been to have a limit of 1% (20) (21).

6 Why develop novel methodology for HCP determination?

6.1 Increasingly rigorous regulatory standards

As analytical methods for product characterisation progress, regulatory requirements for biopharmaceutical products become ever higher, and it is a reasonable assumption that at some stage in the near future regulators will seek information about the identity of HCPs and well as total quantity.

6.2 Application to biosimilars and “follow-on” products

As an increasing number of licensed products come to the end of their patent protection, biosimilars or “follow-on” biologics will appear. Manufacturers of such products will have access to some of the information used in the original licensing process, and are likely to be asked to meet similar product specifications. HCP specifications relate to total material present, and it is theoretically possible that differences in HCP profile, but not total amount, can affect product efficacy and safety. This suggests that there will be increasing demand to determine both HCP amount and HCP identity. This is not feasible by current immunological approaches, and requires, almost certainly, some combination of separation, mass spectrometric identification and quantitation. It is arguable whether a biosimilar product with a different HCP profile is indeed essentially identical to the originator product.

6.3 Tracking counterfeit products

The methodology under development aims to quantify trace protein components of biopharmaceutical products. Similar methods are applicable to defining the original manufacturer of products - i.e. to distinguish genuine from counterfeit products.

NIBSC has been asked to provide evidence of authenticity in a number of cases in the past few years, and the availability of appropriate methodology will support this work.

7 Project approaches to HCP determination - proteomics

There has been much interest from biopharmaceutical manufacturers in proteomic techniques such as LC-MS and gel electrophoresis for the determination of HCPs. Advances in the sensitivity and accuracy of analytical instrumentation have provided feasible methods for the detection of trace amounts of HCPs. The advantages in using such instrumentation is that methods have the potential to be applicable to a wide range of products and do not rely on antisera raised against a specific expression system. The proliferation of databases for protein sequences during the last twenty years has enabled identification of proteins from a wide range of organisms, some of which are used for creating cell lines and clones in the biopharmaceutical industry. The information available is likely to increase due to genome and proteome sequencing initiatives.

There is significant debate amongst the biopharmaceutical manufacturing community over the advantages and disadvantages of replacing the existing immunodetection methods for HCP detection. There are different analytical and regulatory burdens involved in producing a global figure of HCP, compared to those for analytical techniques that quantify individual components. Immunoassays are very successful in detecting multiple proteins which may collectively report a total value of 1 ppm (or 1 ng/mL), despite possibly having different sensitivities for different contaminants. For proteomic approaches the analytical challenges of quantifying hundreds of proteins in trace amounts is very different to that of quantifying two or three proteins at 1 or 10 ppm.

The early development of proteomic methods were conducted by John Yates et al. and applied to the proteome of *Saccharomyces cerevisiae*. The authors commented that the ratio between the most abundant protein identified and the least abundant protein was believed to be 10,000:1 (ie. 100 ppm of the least abundant material) (22). Since

this study involved deconvolution of extremely complex chromatograms, a greater dynamic range may be achieved with purified materials, where peptides from minor components are well resolved from intense peaks contributed by major components. These experiments used multiple chromatographic separations, in which peptides were initially trapped on cation exchange chromatography resin at the front of the column. Some of this material was eluted using a step salt gradient, to be trapped by reversed phase resin packed in the same column. A chromatographic separation was carried out on the reversed phase using an increasing gradient of organic solvent (typically acetonitrile), with the column eluent diverted into the mass spectrometer. The gradient elution of the reversed phase column did not affect peptides still bound on the cation exchange resin, and a second portion of material was then eluted using a higher concentration of salt. This process could be repeated with up to fifteen different salt elution steps.

Liquid chromatography coupled with mass spectrometry (LC/MS) has demonstrated significant potential for the separation, identification and quantification of complex protein mixtures. Furthermore the development of new chromatographic and mass spectrometry instrumentation, designed specifically for proteomic studies, certainly expatiated the use of LC/MS methodologies for the analysis of biological fluids, cell lysates and digested proteins. These types of complex mixtures had previously been limited to separation/analysis by gel electrophoresis methodologies.

Liquid chromatography techniques (e.g. ion exchange, size exclusion, affinity and reverse phase), as well as electrophoretic separation in liquid phase (capillary isoelectrofocusing, capillary zone electrophoresis), are established methods for protein separation. The retention mechanisms of these methods can be modulated by the choice of the mobile phase, and gradients (changes in mobile phase concentration or composition) can be applied in order to optimize the separation. The most common chromatographic approach coupled with mass spectrometry is reverse phase, as this generally uses volatile buffers and MS compatible mobile phase components. Modern reverse phase high performance liquid chromatography (HPLC) columns utilize a wide range of packing materials to separate proteins and peptides. Pore size, surface area and stationary phase chemistry (polymeric or silica based) play a decisive role in the separation and retention of proteins and peptides.

Chromatographic methods for the analysis of HCP have to separate and quantify all the proteins contained in the product. This is best achieved without the application of sample clean-up, which could cause the loss of some of the host cell proteins. Due to the large differences in concentration of the biopharmaceutical product, when compared to the HCP and the low levels of these proteins, high column “loadability” and low detection limits are fundamental to the successful application of LC/MS. It is known that the loadability of the column is dependant on the superficial area of the material packed into the column and, particularly for protein separation, from the inner diameter of the columns. Columns characterised by inner diameters of 4.6mm offer good loadability, however the best performance, is obtained at flow rates not compatible with mass spectrometers. A successful combination between column loadability, column dimensions, flow rate and mass spectrometer conditions has therefore to be considered.

Proteins have been identified and quantified by characterisation of their derived peptides using either electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI). MALDI does not allow direct on-line coupling to HPLC, however LC fractions can be deposited in series on a metal target before automated analysis. Electrospray ionization is generally applied in proteomics when coupled to HPLC. The most common mass spectrometers used in proteomics are based around: quadrupole mass filters, time of flight (TOF) analysers or quadrupole ion traps. Most modern mass spectrometers combine a number of analysers enabling tandem mass spectrometry, such as in quadrupole-TOF mass spectrometry.

The ability to perform accurate mass measurement and resolve ions of similar mass to charge ratio is also important in proteomic applications. Quadrupole mass filters normally operate with nominal mass resolution, whereas TOF instruments, with a resolution of >10000 can easily resolve the ^{13}C isotopomers for multiply charged peptides as well as provide accurate mass information. Sequencing information is obtained by tandem MS, where a particular peptide ion is isolated, energy is imparted by collision with an inert gas and this energy causes the fragmentation of the peptide. A product ion spectrum is therefore generated, with the mass differences between many of the product ions being indicative of the peptide sequence.

Our investigation focussed on three analytical techniques; gel electrophoresis of fluorophore labelled proteins (at NIBSC), chromatographic and mass spectrometric methods for quantification of individual proteins or peptides in the presence of a large amount of protein “product” (NIBSC and LGC), and isotopic labelling approaches followed by LC/MS for protein and peptide quantification (LGC).

The evaluation of each method was aided by the following decision process; which was used to decide whether the approach could deliver sufficient dynamic range to justify further development or replacement of existing methods;

- If the impurities were not detected when they were present at 100 ppm in the model biopharmaceutical product, then this approach was unlikely to be usable in the long term.
- If the impurities were detected when they were present at 100 ppm in the model biopharmaceutical product, but not when present at 10 ppm, then this approach may be usable in the long term but would require considerably more development work.
- If the impurities are detected when they were present at 10 ppm in the model biopharmaceutical product, then this approach is likely to be a workable method.

Two complementary approaches were examined for the determination of HCPs by chromatography and mass spectrometry. Both employed a “model” drug preparation consisting of bovine insulin, “spiked” with a mixture of known protein “contaminants”. In the first approach, contaminant proteins were separated chromatographically and quantified as intact molecules by LC/MS.

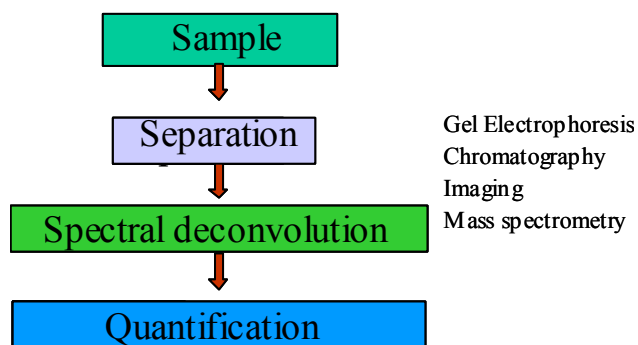


Figure 2. Experimental scheme for chromatographic approaches to isolate individual HCPs from the sample, with quantitation by mass spectrometry.

In the second approach, contaminant proteins were identified and quantified as non-product related peptides following tryptic digestion of the complete “spiked” insulin mixture (Figure 3).

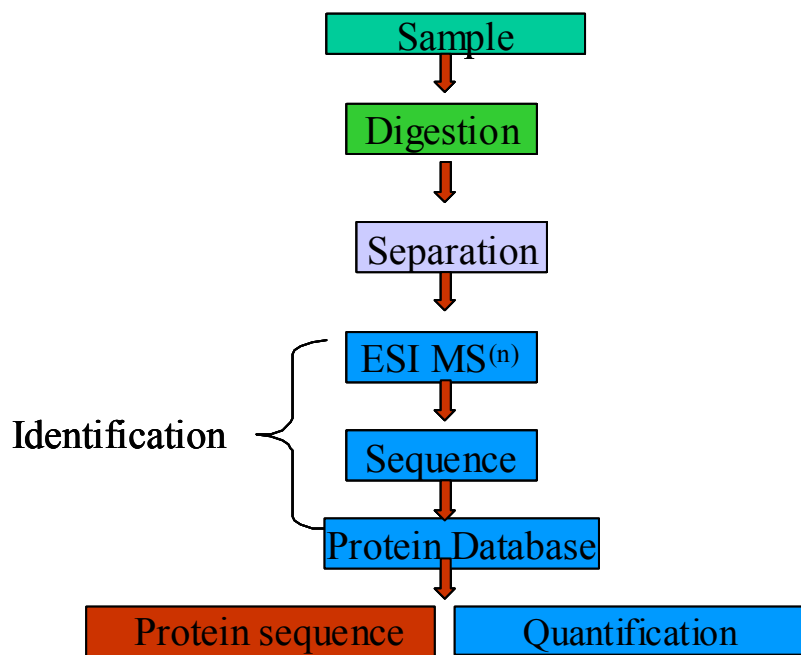


Figure 3. Experimental scheme for digestion of the sample to peptides and identification and quantification of non-product related peptides by mass spectrometry.

Peptides from the product protein degradation products are largely identical to those from the intact material and are not quantified as HCPs. The peptides were quantified directly by chromatographic separation coupled to mass spectrometry, using either label-free or isotopic labelling techniques.

The consensus in a recent WCBP CMC (2006) strategy forum discussion was that the peptide identification approach is the most favoured. HCP contaminants that have co-purified with the product during manufacture are unlikely to be easy to separate as intact proteins prior to quantitation. Proteolytic digestion to peptides generates species which can be separated, due to there being a greater range of physical properties and the higher resolution of HPLC systems for peptides, and therefore offers a workable approach to identifying and quantifying trace impurities. Since tryptic digestion of any realistic potential products, such as monoclonal antibodies or coagulation factors, will result in complex peptide maps of product peptides, two-dimensional HPLC approaches are likely to be necessary to achieve sufficient resolution. The methodology should reflect common practice by, for example, using essentially standard protocols for proteolytic digestion and commonly available HPLC-mass spectrometry systems in order to promote uptake into analytical laboratories and quality control laboratories.

If a “peptide identification” method is implemented, one would be seeking to quantify HCPs at the 1 ppm level, and it was recognised that digestion with, for example, 1 % w/w trypsin (vs product) represented deliberate addition of 10,000 ppm of an “impurity” which could interfere with the process. At that stage development of “low trypsin” digestion protocols could prove valuable. However, the addition of trypsin to the final product will not influence the quantification of single HCP if separation of the single peptides is employed. Therefore, the most important outcome of this study is to assess whether this approach is likely to be able deliver sufficient dynamic range to make further experimental development worthwhile.

7.1 *Spiking rationale*

In order to examine the sensitivity and detection limit for these methods, a model system using a protein “spike” set was used, rather than a proprietary product which would preclude publication of data from this analysis. Another advantage of this approach is that known spiked proteins at quantified levels provide a positive quality control in each experiment and allow platform comparison, and challenges the instrumentation at high and low molecular weight ranges rather than a product

specific range. A representative drug substance was chosen; Insulin from Bovine Pancreas purchased from Sigma Aldrich (I5500).

An HCP profile was simulated by spiking with the following proteins, that represent a range of molecular weights and isoelectric points.

Protein	Mw (kDa)	Supplier/Cat No
Phosphorylase b, rabbit muscle	80	Sigma Aldrich #P6635
Alcohol dehydrogenase, bakers yeast	35	Sigma Aldrich #A7011
Bovine carbonic anhydrase, bovine erythrocytes	31	Fluka #21803
Ribonuclease B, bovine pancreas	14.7	Sigma Aldrich #R7884
Lysozyme, chicken egg white	14.4	Sigma Aldrich #L7651
Insulin b chain, bovine	3.5	Sigma Aldrich # I6383

The assumptions made in the spiking rationale are that the proteins represent the molecular weight range of HCPs present in a typical biopharmaceutical product (given that many are process specific and may not be widely differing in nature) and that the bovine insulin chosen as the “product” is free from contaminating proteins itself, which is unlikely to be the case. Sigma Aldrich does not determine the purity of the insulin preparation used in this project, the product is sold based on the activity (≥ 27 USP units/mg (HPLC), powder).

8 Summary of intact protein analysis - Gel electrophoresis

Proteomic studies have historically focussed on the separating power of two-dimensional gel electrophoresis for the quantitative analysis of protein amounts in complex extracts. However, the limitations of this approach in terms of the dynamic range, throughput and reproducibility of the techniques has precluded their use in quality control or lot release of biopharmaceutical products.

The detection limit and dynamic range of spiked proteins in the presence of “product” was achieved by taking the advantage of dual-fluorophore labelling and separation of the proteins by gel electrophoresis. The labelling method was tested first to ensure consistency between components labelled separately and together.

This is difficult for a real situation of impurity detection where HCP co-purify with the product. Therefore, further experiments were designed to test the detection limit of the spike protein mixture in the presence of the product using single fluorophore labelling.

Dual fluorophore labelling followed by SDS-PAGE was used to determine the detection limit and dynamic range of spiked proteins (HCP mimic) in the presence of “product”: CRM197 (cross reacting material) at 61.15 mg/ml, Mw 58,408 Da from NIBSC. The labelling method used was lysine minimal labelling, which labels the proteins via the epsilon amino group of lysine. The ratio of dye to protein is kept low to ensure that the only protein visualized on the gel is that which contains a single dye molecule, which aids quantitation. The gel was imaged by ImageQuant software and the best results are achieved by using low fluorescent glass plates for the gel support.

A Rainbow molecular weight range marker, (GE Healthcare, 10-250 kDa recombinant protein mix) was used to optimise the labelling protocol and detection conditions. Six recombinant proteins were reliably detected in the marker and exhibited a distinctive migration depending on the fluorescent label used.

The detection limit and dynamic range of the Rainbow marker protein that had been spiked into CRM197 product was then determined, by observation of the bands. The approach used was to label the two components with different dyes, then mix the “product” protein and Rainbow markers in different ratios.

Mix of spike protein in the presence of matrix protein Cy3 (300PMT) and
Cy5 (800PMT) 040704

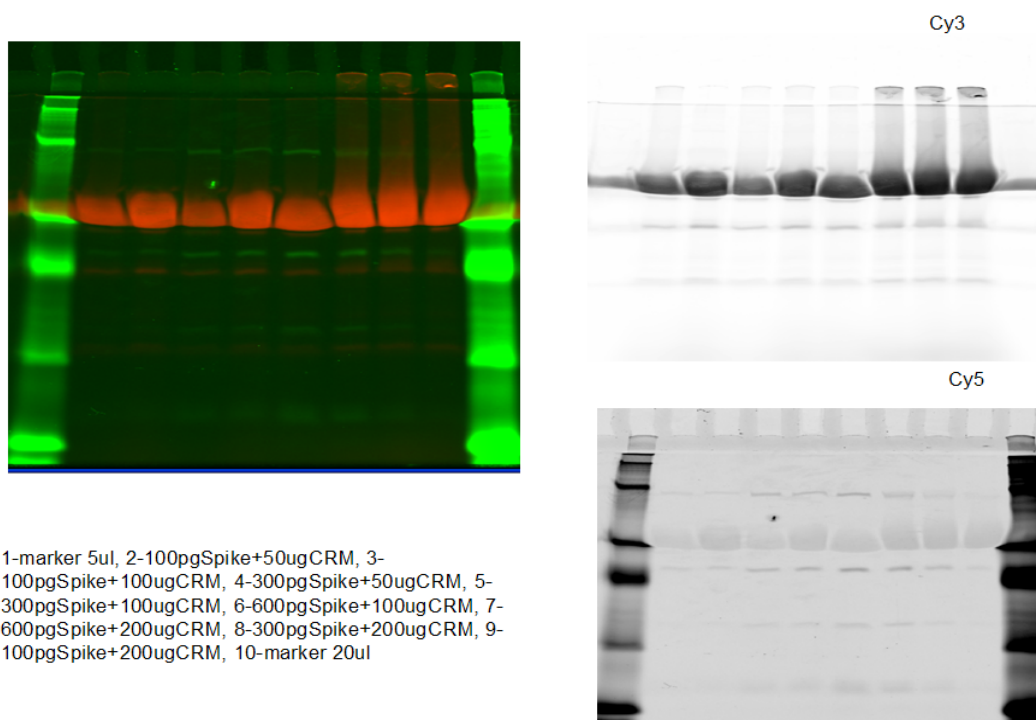


Figure 4. Presence of Rainbow marker protein spiked into CRM197.

The Rainbow spiked proteins were labelled with Cy5 and the CRM product with Cy3 fluorophore. Each spiked protein was visible in the range 3 ppm to 12.5 ppm.

Table 1. Detection limit and dynamic range of Rainbow marker proteins spiked into CRM197

Lane No.	CRM197 (ug)	Total Rainbow marker spike (ug)	Spike protein (approx pg per protein)	Dynamic range
2	50	100	16	3.1 ppm
3	100	100	16	6.3 ppm
4	50	300	50	1.0 ppm
5	100	300	50	2.0 ppm
6	100	600	100	1.0 ppm
7	200	600	100	2.0 ppm
8	200	300	50	4.0 ppm
9	200	100	16	12.5 ppm

In practice, the HCP and the product would be labelled with the same fluorophore. 10 ug of Cy3 labelled CRM197 was used, in this approach, at higher concentrations the CRM197 process and product-related impurities masked the bands from the Rainbow marker spiked proteins.

Spike protein mixture Cy5 labelled-310806

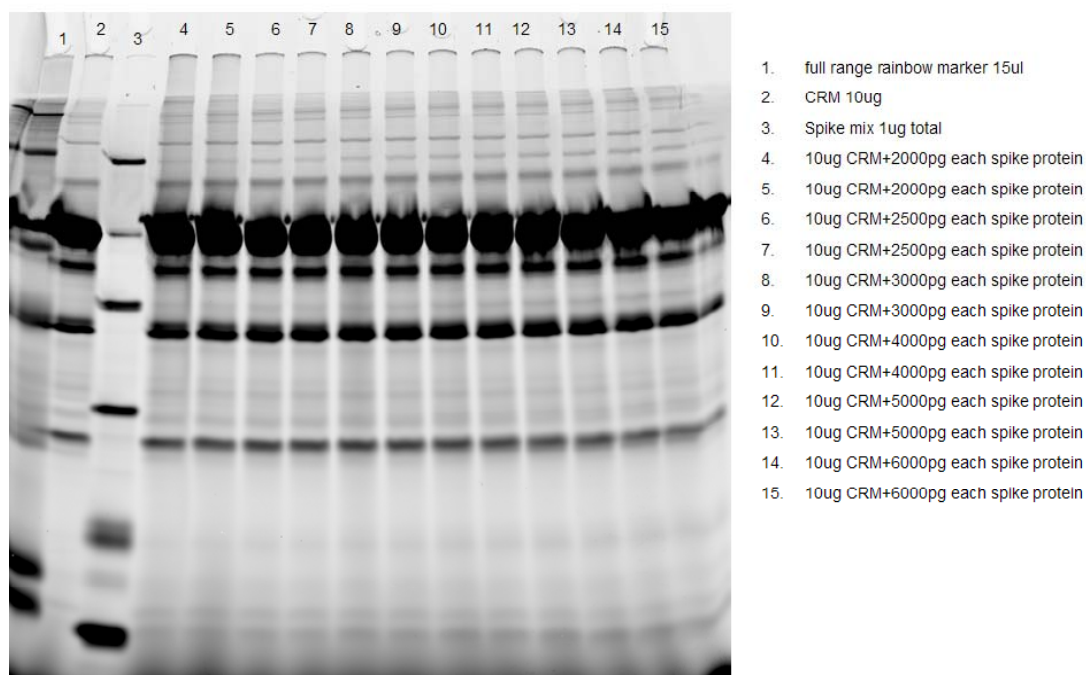


Figure 5. Detection of Rainbow marker proteins spiked into CRM197, using one-colour Cy3 fluorophore labelling

The detection limit for the Rainbow spiked proteins was 2.5 ng per protein, in the presence of 10 ug of CRM197 (PMT=500). This equates to a value of 250 ppm, and a dynamic range of 4×10^4 . This is a much smaller dynamic range compared to the dual-fluorophore detection system, but this is more representative of a real case of HCP detection. It seems unlikely that we can develop the system in such a way as to increase the dynamic range sufficiently.

9 Summary of quantification of process-related impurities as intact proteins

The potential of liquid chromatography mass spectrometry (LC/MS) for the analysis of host cell proteins (HCPs) as intact proteins was investigated. A mixture of proteins characterised by different molecular weights (Mw) and isoelectric points (pI) was used in order to identify the most appropriate LC/MS detection strategy. Bovine insulin spiked with the protein mixture at ppm levels, to mimic a bio-product, was also analysed.

Size exclusion chromatography (SEC) and ion exchange (IX) and reverse phase (RP) chromatography columns were evaluated and peak capacity, speed and loadability of the columns was considered. Ultraviolet (UV) or mass spectrometry (MS) detection was employed and detection limits calculated. The possibility to detect and quantify proteins spiked at ppm levels, corresponding to the HCP levels, was investigated.

Using size exclusion chromatography (SEC):

- A method for the separation of the protein mixture was developed.
- Proteins at 1000ppm were detected by UV.

Using ion exchange chromatography (IXC):

- A method for the separation of the protein mixture was developed.
- Proteins spiked at ppm levels in bovine insulin as “product” eluted with insulin and were not detected by UV.

Using reverse phase (RP) chromatography:

- Three reverse phase chromatography columns were evaluated for the analysis of intact proteins and the most appropriate column used for quantitative studies.
- The complexity of analysing proteins by mass spectrometry increases with the increasing molecular weight of the protein. Separation of proteins characterised by Mw higher than 25 kDa is therefore necessary prior to quantification.
- No significant differences were observed between the three methods considered for quantifying intact proteins by mass spectrometry: the most abundant mass/charge state, the summation of the three most abundant mass/change states, and de-convolution.
- Detection limits (fmols) were calculated and narrow linearity ranges (pmols) were observed.
- No significant differences were noticed by comparing detection limits calculated in the presence or absence of bovine insulin as “product”.
- Proteins spiked at 50-200 ppm were detected by LC/MS.

A full report on the study, including experimental conditions, is available (2).

10 Summary of quantification of process-related impurities after enzymatic digestion

The possibility to detect and quantify host cell proteins (HCP) after enzymatic digestion by liquid chromatography mass spectrometry (LC/MS) has been evaluated. Synthetic and labelled peptides were employed to optimize the LC/MS method and the sensitivity of the system was evaluated by analysing synthetic peptide and bovine insulin as a surrogate “product” spiked with a standard protein mixture at ppm levels.

The use of isobaric tagging reagents, such as iTRAQ, for cell expression profiling and its potential use for the relative quantification of peptides and proteins in the bio-marker, diagnostic and biopharmaceutical areas is relatively novel. However, multiplexed protein quantification by amine reactive isobaric tagging reagents was considered as the most appropriate approach to quantify host cell proteins after tryptic digestion as it offers the possibility of identifying and quantifying digested proteins in multiple samples simultaneously. Furthermore, the targeting of an amine group results in the labelling of most peptides in a sample with no loss of sequence information. However, a full understanding of the derivatisation reaction and awareness of the variability of the methodology are required before using labelling strategies for host cell protein quantification.

Systematic investigations on the variability of the iTRAQ derivatisation reaction and its implications on the relative quantification of peptides and proteins were carried out. A mixture of five synthetic peptides, from standard proteins, were derivatized with the individual isobaric tagging reagents, and the percentage of derivatisation and the kinetics of the reaction were evaluated. Detection limits (DL) between 0.5-10 fmols on-column were calculated. Digested proteins spiked at 1 and 10 ppm in bovine insulin acting as a surrogate “product” were detected by LC/MS. The labelling reaction required for relative protein quantification by iTRAQ was evaluated. The kinetics of the reaction for the four isobaric tagging reagents was the same, although the percentage of derivatisation was greater than 90 % for only three of the five peptides employed. High percentages of derivatisation are fundamental for appropriate relative quantification of synthetic peptides. Four synthetic peptide mixtures at the same concentration were derivatized with the four isobaric tagging

reagents, pooled together and analysed by LC/MS/MS. A ratio of 1:1:1:1 was expected; a variability of ± 0.2 from the expected ratio was observed. Four protein mixtures at the same concentration were digested, labelled with the four isobaric tagging reagents, pooled together and analysed by LC/MS/MS. A ratio of 1:1:1:1 was expected; a variability of less than 5% from the expected ratio was observed. Quantification of digested protein mixtures, in a range of relative concentrations from 0.1-10 (two orders of magnitude) was possible. However a greater variability (30 %) was observed, mainly due to the low intensity of the reporter ion 114 corresponding to the lower concentration. iTRAQ derivatisation could be successfully applied to the quantification of HCPs by introducing standards at a concentration equal to the maximum concentration allowed. The use of standard protein mixtures is recommended due to the high variability of the ratio of the individual peptides identified. The low intensity of the product ion spectrum, of proteins spiked at the 1 and 10 ppm level, did not allow accurate quantification by iTRAQ. An improvement in the intensity of the product ion spectrum has therefore to be performed in order to identify and quantify proteins spiked at 1 and 10 ppm.

A full report on the study, including experimental conditions, is available (3).

11 Conclusions and Recommendations

FDA Guidelines and EU Position Statements for HCP quantification have not been revised in the last ten years, however down-stream processing separation technology has improved in this time and there is an acknowledged reduction in HCP levels in new products, and there is a lack of any significant clinical problem that has arisen from the presence of HCPs in a product to date. Nonetheless, advances in the sensitivity and accuracy of protein analytical methods have led to interest in new methods for HCP quantification.

Liquid chromatography coupled with mass spectrometry allows the separation and the quantification of individual HCP. Equipment is common in research and quality control laboratories despite the initial outlay for expensive equipment, and can be converted to high throughput formats. Proteins spiked at 1 and 10 ppm were detected,

however an improvement in sensitivity is necessary in order to identify and quantify proteins by using the iTRAQ labelling strategy. This strategy also requires HCP standards to allow relative quantitation of known HCPs. Despite the need to develop methods specific for the individual products, liquid chromatography coupled to mass spectrometry showed potential for the use in the determination of individual host cell proteins. At the time of writing, ELISA for HCP quantitation is still the most applicable method for lot release of biopharmaceutical product for quality control laboratories.

Table 2. Summary of the techniques for HCP quantification reviewed in this project.

Technique	Included in this study?	Sample	Quantification (ppm)
ELISA	N	N/A	1 ppm (global HCP, individual components may be lower)
Separation of intact proteins by PAGE + fluorophore labelling	Y	CRM197 + spike protein set	250 ppm per protein
Separation of intact proteins by RP-LC/MS	Y	Insulin + spike protein set	50 to 200 ppm depending on protein
Detection of tryptic peptides by LC/MS	Y	Insulin + spike protein set	Detected at 1-10 ppm total spike
Isotopic labelling of proteins + detection of tryptic peptides by LC/MS	Y	Insulin + spike protein set	Detected at 1-10ppm total spike

If proteomic approaches are sensitive enough for HCP quantification, it is likely that the ability to identify individual HCPs is in place in research and development laboratories. Currently this information is proprietary which has precluded general dissemination of the case study data. The implications of identifying HCPs has prompted discussion of the testing required for biopharmaceutical regulatory approval and the utility of techniques to examine “biosimilar” or “follow-on” products. It is hoped that these discussions will result in a pragmatic approach to the issues without an increase in the burden of analytical testing quality control.

We are grateful for the support and encouragement from the scientists involved in this study and the helpful comments during dissemination of this work. The process of generating material transfer agreements, intellectual property and consortium agreements was lengthy and the amount of time and resource necessary for these activities was underestimated at the start of the project.

12 Knowledge transfer activity and links with international initiatives

Web dissemination:

- A project page on the MfB website, to provide a focus for dissemination and a location for freely accessible copies of the project reports to be downloaded. At the end of the project, the outputs will be transferred to the PASG and MfB websites for long term maintenance and dissemination. At least 50 paper copies of the best practice guide to be disseminated to interested parties.

Oral Presentations:

- To PASG at their biannual meetings (April and October) for the duration of the project (Bailey M.J.A., Parkes, H.)
- Invited speakers at a major conference for the biopharmaceutical manufacturing industry, WCBP 2007 CMC Strategy Forum (Bailey, M.J.A., Parkes, H.) 23rd January 2006, San Francisco, USA)
- 11th International Symposium on the Separation of Proteins, Peptides and Polyoligonucleotides (ISPPP2006), Innsbruck, Austria (October 2006). Quaglia*, M.; Burkitt, W. and O'Connor, G.
- Data from the study, with acknowledgement of NMS MfB funding, included in: Measurement of Biological Molecules at the European Society for Animal Cell Technology UK meeting (Hills, A., 4-5th January 2007, Wyboston, UK)

- Measurements for Biotechnology Programme dissemination workshop, 9th March 2007. The workshop was attended by over one hundred delegates, which included representatives from the biopharmaceutical industry, regulators, the DTI and academia. The day of presentations and posters on all projects in the 2004 to 2007 programme an oral presentation on this project.
- Invited speaker at the 31st International Symposium on High Performance Liquid Phase Separations and related techniques” (HPLC 2007), Ghent, Belgium (June 2007). Quaglia*, M.; Hall, Z.; O’Connor, G
- Analytical Research Forum, Glasgow, UK (July 2007). Hall, Z.*; Quaglia, M.; O’Connor, G.

Posters:

- Quaglia, M.; Oudgenoeg, G.; Vickers, P.; Griffins, M.; Keer, J.; O’Connor, G.; Parkers, H.; Jones, C.; Bailey, M. Poster “PC1: Detection and Quantification of process related impurities: host cell proteins and host cell DNA in biological products” PASG meeting (April 2006)
- Quaglia, M.; Hall, Z.; O’Connor, G.; Parkes, H. “Evaluation of liquid chromatography-mass spectrometry approaches for the analysis of host cell proteins in bio-pharmaceuticals” MfB workshop (March 2007)
- Detection and Quantification of process-related impurities: Host cell protein and DNA detection in biological products.
WCBP 25-27 January 2006, Washington DC, USA
WCBP 2007 29-31 January 2007, Washington DC, USA
MfB Dissemination workshop 9th March 2007, Teddington, UK

Articles:

- Hall, Z.; O'Connor, G.; Quaglia, M. "Systematic study on relative and absolute quantification of peptides and proteins by using amine-reactive isobaric tagging reagents". In preparation for J. of Proteomics Res.
- Quaglia, M.; O'Connor, G. "Intact protein quantification: practical strategies" in preparation for Anal.Biochem
- Quaglia, M.; Hall, Z., O'Connor, G. "Potential of LCMS for the analysis of HCP in bioproducts" in preparation

Links with international initiatives:

The California Separation Science Society (CaSSS) has organised a Chemistry Manufacturing Control (CMC) strategy forum as part of its Well Characterised Biological Product conference series, now in its 11th year. The forum is steered by a committee of industry and regulators to foster collaborative technical and regulatory interactions on matters of concern to the industry.

Following an invitation from the forum organisers, the work from this project was presented to WCBP on 23rd January 2006 in San Francisco with an update in January 2007. The meeting provided an opportunity to discuss developments in proteomic and genomic methods and practical applications to biopharmaceutical manufacture.

Positive feedback was received from the FDA and members of the US biopharmaceutical industry. Genentech raised the concern that if we can identify all the HCPs then this might become a regulatory requirement. Genentech efforts to identify host cell proteins from a Chinese Hamster Ovary cell expression system was focussing on resolution of the whole proteome by two-dimensional electrophoresis approach rather than identification of co-purifying process related impurities. However there was consensus that the digestion to peptides approach was the most likely approach to achieve success in HCP quantitation. There was strong interest in the project outputs.

12.1 Acknowledgements

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Paula Vickers and Gideon Oudgenoeg; LGC

Chris Jones, Sonia Nisar, Litao Fu and Jun Wheeler; NIBSC

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14 Appendix

PASG Position Paper – Host Cell Protein Determination – Is There Best Practice?

*PASG Biopharm Group Autumn Meeting 2002
Simon Forster, CAT; Richard Branton, Celltech*

14.1 Host Cell Proteins

The assay of host cell proteins (HCP's) in biopharmaceutical products produced by cell culture methods is one of the most controversial areas in the characterisation and analysis of these products – there are both technical and conceptual problems. Current regulatory guidelines are scanty in their coverage of this topic.

Why assay for HCP's? Obviously any contaminant in a biopharmaceutical product is undesirable. The reasons for the undesirability of the presence of HCP's is twofold: firstly they may be toxic; secondly, even if not directly toxic, they may be immunogenic. Therefore there is a regulatory requirement to assay for HCP's. Apart from safety and regulatory reasons, assay of HCP's may also be an aid to process development (although this would only be true for a generic type assay – see below). This position paper arose out of a workshop held by the Biopharmaceutical subgroup of the Pharmaceutical Analytical Sciences Group during the Autumn 2002 meeting of the PASG at Warwick University, during which delegates discussed current techniques for the determination of HCP's and their potential shortcomings, the current regulatory guidance, and tried to suggest ways forward.

14.2 HCP Assays

Two approaches to HCP assay can be distinguished – generic and process-specific. The generic approach attempts to measure all HCP contaminants that could be present i.e. any protein in the host cell apart from the desired product. The process-specific approach measures only those HCP contaminants that co-purify with the product.

Although technically less demanding, this approach suffers from a number of potential problems. By definition, the assay is not available until the process has been defined. Furthermore, it can be “too specific” in the sense that a process failure could introduce into the final product HCP’s that would not normally co-purify and thus would not be picked up by the assay (although it is unlikely that such a failure would not be picked up by other assays as well). A related issue with process-specific assays is that a decision has to be made as to at what point in the development process should the HCP assay be developed; a factor here is that the assay ought to be available to characterise material used for toxicological studies before entry into clinical trials, although this is probably the exception rather than the rule, and in any case the process may change quite substantially between toxicology and late stage clinical trials.

14.3 Current Approaches to HCP Assays

The commonest current approach is an immunochemical one, in which anti-HCP antibodies are used in a western blot or ELISA-type procedure. Insofar as there is any regulatory guidance on these assays, this is referenced by the regulatory bodies: ICH Q6B “suggests” this approach. Other approaches currently used include SDS-PAGE, usually with silver staining, or HPLC, or indeed a combination of two or more of these.

Another potential approach is “validating out” – that is, demonstrating, usually by means of spiking studies during process validation, a high level of confidence in the clearance of HCP during the DSP procedures and that therefore there is no likelihood of a batch being produced with raised levels of HCP. However, if this approach is taken, very careful attention must be given to change control and the need for revalidation following process changes.

14.4 ICH Q6B Approach

This approach (section 6.2.1(a) of Q6B) recommends “...a sensitive assay, e.g. immunoassay, capable of detecting a wide range of protein impurities...” and gives some further guidance on producing the necessary antibodies for such a test. In general, antigen is produced by “sham” production and purification from a null production cell, containing the vector but not the product gene. This is then “purified” by going through the normal DSP procedure to a point at which the product would be 95-99% pure. This preparation is then used as immunogen, the resulting antisera are pooled and/or purified and used in western blot or ELISA techniques. This type of procedure is fairly well established, but there are some fundamental flaws in overall concept as well as technical issues with both western and ELISA methodology.

14.5 ICH Q6B Approach – Conceptual Issues

First of all, the assumption is made that HCP’s in the sham production and purification will be the same as in the true production situation and that they will behave the same way in the sham purification as in the true production situation, in the presence of the product. This is probably not the case, and the higher the product yield, in terms of the amount of product as a proportion of total intracellular or medium proteins, the less likely it is to be the case. There are also likely to be scale-up issues involved – the sham process is unlikely to be done at the same scale as the

full scale production process. There is also a particular problem with monoclonal antibodies, which form a substantial proportion of all biopharmaceuticals currently in development, regarding the sham purification – typically the first purification step for monoclonals is a protein A affinity column which immediately clears >99% of all other proteins.

Secondly, given that a representative immunogen preparation can be produced despite the above, any quantitative or semi-quantitative assay using sera raised in this way is unlikely to produce results which accurately reflect the true levels of HCP's because of biases introduced by the differing immunogenicities and abundances of the HCP's in the immunogen preparation. This may be mitigated to some extent if one accepts that one of the major reasons for wanting to exclude HCP's from the final product is that they may be immunogenic in the patient, and therefore an assay biased towards the detection of such potential contaminants is a good thing.

14.6 ICH Q6B Approach – Technical Issues

In addition to these general issues, there are specific issues with both Western and ELISA methodologies. The western blot method gives some idea of the specific identity of contaminant(s), in terms of patterns of banding and approximate molecular weights obtained from the blot. However it is at best semiquantitative; it is also generally less sensitive than the ELISA method and its sensitivity is limited by sample load. Conversely, the ELISA method is usually more sensitive than the western blot, depending on the exact nature of the antibodies and detection technique used, and is quantitative, although with the proviso that it is not clear exactly what is being quantified. This leads to difficulty with some aspects of validation (limits of detection and quantitation, range, accuracy) due to multiple components being assayed simultaneously. Both methods are clearly only as good as the antibodies used, and the discussion above has highlighted some of the issues around this.

14.7 Points emerging from discussion

Some of the flaws in the Q6B approach could be addressed by a more controlled approach and better characterisation of the detection antibodies, such as developing a cocktail of well characterised monoclonals, and ultimately by the use of reference standard antisera across the biopharmaceutical industry. Only a few cell types are in wide use across the biopharmaceutical industry – for example the vast majority of monoclonal antibodies are produced in either NS0 or CHO cell lines, and there is also a degree of commonality in the purification schemes adopted. It is reasonable to assume, therefore, that despite the variety of different expression systems in use, that it would not be out of the question to develop well-characterised monoclonals against the commonly co-purifying HCP's from these lines; the same argument could apply to E.coli HCP's, although in the case of biopharmaceuticals manufactured using E. coli there is likely to be much less commonality of purification schemes between different products. In addition, commercially available “generic” HCP ELISAs and Western Blotting kits exist which could be employed to support product and process development. However, despite the use of common parental cell lines, clone differences still need to be considered.

A proteomics-based approach was suggested as an alternative way forward, perhaps incorporating techniques such as 2D gels or multi-dimensional chromatographic techniques. In the case of the former, the presence of large amounts of product would present a problem for staining techniques, which might be at least partially solved by

2D westerns (although the presence of large amounts of product is likely to be a potential problem in virtually every type of HCP assay).

For both gel and chromatographic techniques it is likely that there will be a movement, as techniques develop and become more sensitive, towards directly identifying HCP spots on 2D gels or westerns and peaks on chromatograms by mass spectrometry and other proteomics-based approaches.

A further suggestion was the generation of a database of HCPs from different licensed biopharmaceuticals and development samples that have already been given to patients.

14.8 Summary

The current best practice for the measurement of HCPs in biopharmaceutical products remains ELISA or Western Blotting. Despite the theoretical flaws with these approaches that have been described in this paper and their semi-quantitative nature, these methods are highly sensitive and have been proven in practice. These approaches are also recognised and accepted by the regulators.

Generic methodologies, such as commercially available ELISAs, can be employed to support the development of biopharmaceutical products and processes and represent a realistic approach to HCP measurement. Demonstration that these assays are fit for purpose probably has to be demonstrated on a case-by-case basis, as the processes by which the products are made are not generic.

The possibility of generating a database of HCPs from different licensed biopharmaceuticals and development samples that have already been given to patients should be investigated as a means to expedite the development of new biopharmaceuticals.

Quantitative proteomics-based methods such as 2D electrophoresis or multidimensional chromatography, coupled with MS, may provide an opportunity to both better characterise the nature of potential HCP contaminants and provide quantitative measurement. For these reasons, such techniques should be investigated as potential improvements on our current methodologies. These methods need to be thoroughly investigated to demonstrate their suitability to this application, as there are significant challenges to overcome, such as the analysis of very low concentrations of HCP in the presence of high concentrations of product.