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REVIEW

Examining the sources of variability in cell culture media used for biopharmaceutical production

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Abstract Raw materials, in particular cell culture media, represent a significant source of variability to biopharmaceutical manufacturing processes that can detrimentally affect cellular growth, viability and specific productivity or alter the quality profile of the expressed therapeutic protein. The continual expansion of the biopharmaceutical industry is creating an increasing demand on the production and supply chain consistency for cell culture media, especially as companies embrace intensive continuous processing. Here, we provide a historical perspective regarding the transition from serum containing to serum-free media, the development of chemically-defined cell culture media for biopharmaceutical production using industrial scale bioprocesses and review production mechanisms for liquid and powder culture media. An overview and critique of analytical approaches used for the characterisation of cell culture media and the identification of root causes of variability are also provided, including in-depth liquid phase separations, mass spectrometry and spectroscopic methods.

Keywords Biopharmaceuticals · Cell culture · Characterisation · Media · Raw material · Variability

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Media history and the problem with serum

Media history

Mammalian cells have become the dominant system for the production of recombinant therapeutic proteins (Wurm 2004), and concentrated media are used as a cost effective and proficient way of using cultures to improve cell growth and viability (McCoy et al. 2015; Decaria et al. 2009). While some cells can be maintained in a basal medium with no supplementation, the majority of cells require the addition of up to 100 components such as hormones, growth factors, vitamins and amino acids and hydrolysates to maintain, proliferate and/or differentiate (van der Valk et al. 2010). For this reason, medium supplementation with serum, most commonly foetal bovine serum (FBS) was primarily used in early days of recombinant protein production (Zang et al. 2011) as it provided a source of vital nutrients for cell growth including vitamins, fatty acids, proteins and peptides and also performed as a chelator for water-insoluble nutrients,

protecting grown cells against shear damage (Lobo-Alfonso et al. 2010).

It soon became clear however, that there were a number of risks posed with the use of animal sera; predominantly its undefined nature, demonstration of batch-to-batch variability leading to differences in cell growth and productivity (Richardson et al. 2015). There was also risk of introduction of contaminants into the final product during antibody production (Zhang and Robinson 2005). In addition, it became clear that serum addition might be problematic for certain cell culture applications (Jayme and Gruber 1998); serum factors were shown to promote fibroblast overgrowth in mixed cell populations and often failed to provide adequate essential growth factors to promote epithelial cell growth. Progenitor cells proved difficult to maintain in serum-supplemented media without undergoing spontaneous differentiation or apoptosis (Jayme 2007). The use of FBS in particular became restricted due to the risk of transmissible diseases such as bovine spongiform encephalopathy (Gupta et al. 2013). Furthermore, there were ethical issues to consider, as it has been reported that as much as 20–50% of commercial FBS is virus-positive (Wessman and Levings 1999). The challenge faced, therefore, was to produce a media with no animal derived products in a cost-effective manner, with increased productivity and relatively easy purification.

Development of serum-free (SFM) and chemically-defined media (CDM)

Although attempts at devising a CDM have been in place since 1907, when Harrison utilized blood plasma as a growth medium for nerve-fibre cells, there was a lack of real understanding of the nutritional requirements of mammalian cells (Taub 1990). The first partially CDM was developed by Eagle (1995) (Eagle's Modified Essential Medium) and contained a mixture of nutrients such as a carbohydrate source (usually glucose and galactose, sometimes maltose or fructose), amino acids, vitamins, fatty acids, lipids as well as trace elements such as zinc and copper (Jayme 2007; Jeffs 2007), in addition to a small amount of serum, which was the most efficient additive for cell growth (Eagle 1955). Efforts to replace serum with its defined components were moderately effective (Pazos et al. 2004; Taub 1990), but did not take into account serum properties including bulk protein functions and specific biological activities (Jayme et al. 1997).

Typically, serum-free media are composed of a number of cell growth factors including hydrolysates, amino acids, vitamins and inorganic salts, allowing the minimisation of risks associated with the undefined character and potential introduction of contaminants in serum-containing media (McElearney et al. 2015). Serum-free media may also however contain undefined animal-derived products such as serum albumin (purified from blood), hormones, carrier proteins and attachment factors, which contain complex contaminants.

Serum-free media proved particularly successful for anchorage-independent cells, as attachment factors are not a requirement. However, there were a number of drawbacks related to the use of SFM, including the variability of any animal-derived factors present, their complexity (which makes analytical characterisation difficult) and viral contamination which can be problematic. Another serum-free alternative is protein-free media (PFM), which do not contain high-molecular-weight proteins, are used for isolation of monoclonal antibodies and the downstream processing of recombinant proteins (van der Valk et al. 2010). However, PFM are not chemically defined due to the possible presence of protein hydrolysates (Jayme 2007). Chemically-defined media, in contrast, require that all of the components are identified with exact concentrations known and are therefore totally free of animal-derived components such as bovine serum albumin, human serum albumin or foetal bovine serum. Chemically-defined media are regularly supplemented with recombinant versions of albumin and growth factors (Huang et al. 2007; Rodrigues et al. 2012; Zhang et al. 2013). The first step towards the development of an effective CDM was made by Hayashi and Sato, who replaced serum with selected hormones (Hayashi and Sato 1976). Following this study, extensive work was carried out using SFM with supplementation of hormones (Hodge 2005).

Chemically-defined media can be developed by firstly selecting an established chemically-defined basal formulation and supplementing it with specific additives for the targeted cell (Zhang and Robinson 2005). Van der Valk et al. (2010) have described a "media pyramid", describing a typical approach for the development of serum-free or chemically-defined media where there is usually a weaning approach used

where serum is gradually reduced over several passages until the media is serum-free, which can prove time consuming and costly. Furthermore, there has been some confusion with the classification of SFM and CDM, with both being used interchangeably in an attempt of manufacturers to attract the commercial market, suggesting a need for a common terminology regarding GMP compliance and regulation (Gottipamula et al. 2013). Jayme and Smith (2000) have described and defined the most commonly used types of cell culture media.

The addition of hydrolysates

Serum-free media remained the most commonly employed commercial media despite the associated drawbacks, due to the lack of a viable replacement with the ability to support the desired range of cell growth. As a result, poorly defined components, such as peptones and hydrolysates, were added to simulate the growth achieved with serum-containing media (Saad et al. 1993). Hydrolysates are complex mixtures that contain oligopeptides, amino acids, iron salts, lipids, vitamins and other low molar mass substances in trace elements (Kim and Lee 2009), which have shown to be a suitable replacement for recombinant insulin and serum components in media (Ling et al. 2015). Considerable work has been carried out using media containing plant-derived hydrolysates (Franek et al. 2000; Heidemann et al. 2000; Nyberg et al. 1999), which were typically chosen over animal tissue hydrolysates due to safety concerns. Soy protein hydrolysates significantly enhance cell growth and recombinant protein production in cell cultures (Gupta et al. 2014), and have been widely used as additives for serum-free cell culture medium for approx. 40 years (Luo and Chen 2007a, b). There can be significant variability with hydrolysates, however, due a number of qualitative and quantitative factors, such as the heterogeneous nature of the raw material used, in addition to geographic factors that may affect the crop, e.g. location, harvest and storage conditions patterns (Luo and Chen 2007a, b; Milo et al. 1976). The addition of peptones results in higher cell growth and productivity (Heidemann et al. 2000); however, variations in the seed composition and seed/meal processing in soy hydrolysates results in batch-to-batch variations in the enhancement in cell growth and IgG production. These variations can also be attributed to the abundance of different classes of compounds and their quality, or the presence of specific compounds.

Early work often employed Primatone or peptones derived from bovine milk; however, since the elimination of animal and human-derived proteins is a primary goal for the production of protein therapeutics, several new hydrolysates have been tested as protein free medium for recombinant therapeutic production, including peptones of soy, rice, wheat and gluten (Heidemann et al. 2000). In recent work, media supplemented with wheat gluten hydrolysates resulted in growth similar to serum supplemented media, where yeast and soy hydrolysates demonstrated growth inhibition (Radosevic et al. 2016). More recently, a shift towards chemically-defined formulations was observed whereby only fully characterised low-molecular-weight compounds, such as plant or bacterial recombinant proteins, were used to provide nutritional supplementation for cell growth (Decaria et al. 2009; Gilbert et al. 2013; Huang et al. 2010). Ho et al. (2016) evaluated soy, yeast and wheat hydrosylates, in addition to a CD supplement in a commercially available media formulation used to culture mAb producing cell lines.

The origin of the problem

Media production demands

In recent years there has been a demand for increased efficiency in media production operations and subsequently a large number of demands made on media, which had previously been selected due to its ability to support large-scale culture of cells (Decaria et al. 2009; Hudson 2013; Jerums and Yang 2005). Complete characterisation of animal serum remains challenging due to the large number of proteins and metabolites present (Anderson and Anderson 2002; Anderson et al. 2004; Psychogios et al. 2011). Original formulations faced considerable challenges in guaranteeing consistent production lines due to variations among different lots of materials, caused in particular by the presence of undefined animal-derived components which ultimately affected the reproducibility of cell growth processes (McElearney et al. 2015; Sinacore et al. 2000; van der Valk et al. 2010). This variation is the largest source of both process variation and project rejection, resulting in quality control

failure and huge financial losses to biopharmaceutical companies.

In order to be used commercially for large-scale biopharmaceutical manufacturing, media production must also have the capability to address industrial scale demand, must support higher growth rates and culture densities and allow both the promotion of high product yield and the reduction of process related impurities (Decaria et al. 2009; Jayme et al. 1997; Jerums and Yang 2005). Further concerns include the cost, supply and biochemical stability of critical raw materials and the compatibility of all ingredients and components with regulatory directives. In addition, medium must be stable under all storage conditions for the desired storage duration.

The commercial distribution of serum is also a reason of concern for biopharmaceutical companies based in the US or EU, since its availability is strictly correlated to beef consumption in countries such as South America, South Africa and New Zealand where environmental/governmental policies can restrict exportation, potentially causing supply chain disruptions (Gstraunthaler et al. 2013). Furthermore, regulatory guidelines regarding the import of animal sera were previously undetailed and often led to the distribution of impure products. Due to the potential contamination by adventitious agents, for example those that cause BSE, regulations have become more rigid regarding the biopharmaceutical production process (Zhang and Robinson 2005). These restrictions have shifted global interest towards the development of formulations which are free from animal-components (Rios 2010).

The removal of serum and animal-derived products from media has also significantly simplified downstream processing, as it reduces purification difficulties and process-related impurities. In turn, purification performance is more consistent, and there is better control over physiological responsiveness when unknowns are removed (Carlini et al. 2007; Decaria et al. 2009).

Manufacturing processes

The high transportation costs and limited shelf life have always restricted the applicability of liquid media formulations. In an attempt to overcome these issues, highly concentrated pre-solubilised liquid nutrient formulas have also been developed with partial success (Jayme et al. 1992, 1993). These were either reconstituted in batch mode (Jayme et al. 1998) or used in conjunction with in-line mixing devices to deliver up to 30,000 l of diluted media to bulk containers (Jayme et al. 1996). In the 1960s, a new type of media in powder format (dry powder media, DPM), manufactured by a mixing-milling process, was also introduced (Young et al. 1966); however, initial difficulties were observed for generating a homogeneous powdered-form product with favourable solubility, similar potency and performance of ready-to-use liquid solutions (Fike et al. 2001). Ball milling, where a number of ceramic balls located inside a cylinder, crush the raw materials present, resulting in the formation of micron sized particles, was the traditionally accepted method for the manufacturing of DPM (Young et al. 1966). Difficulties were observed however, in relation to the production of a pure and homogeneous product since components present in microgram quantities were required to mix with kilograms of a particular media. Further issues were also encountered in relation to the stability of heat-labile components and the generation of large quantities of dust, which required considerable cleaning efforts to prevent cross-batch contamination. Human intervention was also required to replace the ceramic balls due to wearing over time. These limitations have led towards the implementation of other high-speed milling manufacturing equipment such as 'FitzMills', where materials are displaced rapidly within the grinding chamber and are pulverised under the action of rotating blades, or hammer mills, which are characterised by continuously rotating stainless steel hammers that grind the introduced raw materials against a screen. Powders are held until the desired particle size is achieved and an evenly distributed powdered medium is obtained (Jayme and Smith 2000). Recently developed serum-free and protein-free media formulations were not found to be compatible with attrition-based manufacturing methods due to the generation of heat which can potentially damage key media components present. Hence, alternative types of high-speed-impact mills such as pin mills and air classifier mills were developed. Pin mills consist of a feed inlet, a set of two disks with concentric rings of pins and a product outlet. The raw materials are fed continuously through the inlet and when they reach the centre of the disk, are centrifugally propelled outward and reduced in size by the

rotating intermeshing pins. The powder is fluidised and transported to a collection bin without being by screens hence preventing the generation of heat while being cooled under a closed-loop circulation of N_2 gas (Rios 2010). Similarly, as described for pin mills, air classifier mills (Ray and Caple 2001) are used in continuous processes which are used to avoid typical over-grinding that occurs with ball mills, achieving a narrower particle size distribution. The product is ground until it reaches the desired size where it is moved under airflow to a vertical ribbon blender while large particles are returned to the grinding chamber until they reach the desired size.

The introduction of Advanced Granulation Technology (Fike et al. 2001; Jayme et al. 2001, 2002), where fluid-bed technology was utilised for producing granules containing trace elements and labile materials, was a major breakthrough for the manufacturing of cell culture media as it eliminated the health hazards associated with the generation of aerosol powders present with traditional milling manufacturing methods. Dry powders, originally milled to a uniform particle size, are suspended in a column of conditioned air and sprayed with an aqueous solution containing trace elements which are distributed homogeneously, leading to the formation of porous granules which demonstrated improved solubility relative to the powder alternatives. The particle size reduction and granulation process are performed in stainless steel containers hence facilitating sanitization and clean-up. The medium formulation is also complete and ready for use by the end-user upon hydration since no further components need to be added meaning that no pH/osmolality adjustments are required offering considerable cost-saving benefits relative to DPM (Fig. 1).

Powder properties and effect on media performance

The physical properties of powdered media are generally described in terms of particle size distribution, flow properties and solubility. To achieve the desired particle size, which would allow for favourable solubility properties, each milling process must be finely tuned for a particular medium formulation. For example, the milling speed can be increased to yield smaller particles, but care should be taken to prevent over-heating of powders which could affect the quality of the product. If the powder formed is too coarse, dissolution will be slow and hence, undesirable. If the particles are too fine, a larger surface area is available for the liquid solvent to act on, allowing for faster dissolution rates. However there is a threshold, since particles that are too fine can also agglomerate leading to further solubility complications (Sundgren 2010). Basic particle characterisation techniques such as filtering through mesh screens can provide approximate information of particle sizes present in a sample blend. However, ultra-fine particles can often agglomerate, leading to inaccurate results. Laser diffraction analysers are preferred for accurately measuring particles from the nanometer to millimeter dimensions (Sundgren 2010). Another important property of powders is their ability to flow under desired conditions. This important characteristic is often determined by the Hausner ratio, which is the ratio of tapped density over bulk density (USP 2012). As a general rule, bulk density decreases correlating to a decrease in particle size due to the presence of air present between finer particles, leading to larger Hausner values which would indicate a poorly flowing product. It is considered beneficial to keep the particle size distribution as high as possible without going above the solubility threshold.

Market trends

Current market trends reveal that over 90% of media supplied at large scale is in powder format (Langer and Rader 2014) since it provides biopharmaceutical companies an element of flexibility in terms of scaling up or down production processes depending on requirements without large investments for the storage of large quantities of unused liquids. The recent technological developments delivered by granulation methods are likely to enforce the current market scenario as most of the issues associated with powdered-format media, such as poor solubility upon hydration and batch-to-batch variations, have been addressed (Jayme et al. 2002). This concept emphasized by recent investments of companies like Thermo Fisher Scientific (2016), who invested £14 million in a new AGT manufacturing facility in Scotland, and Lonza (2012). Despite the popularity of a powdered-form media, ready-to-use liquid solutions still maintain market share since they are likely to be employed by smaller-sized companies who do not wish to invest large capitals in infrastructure investments. The

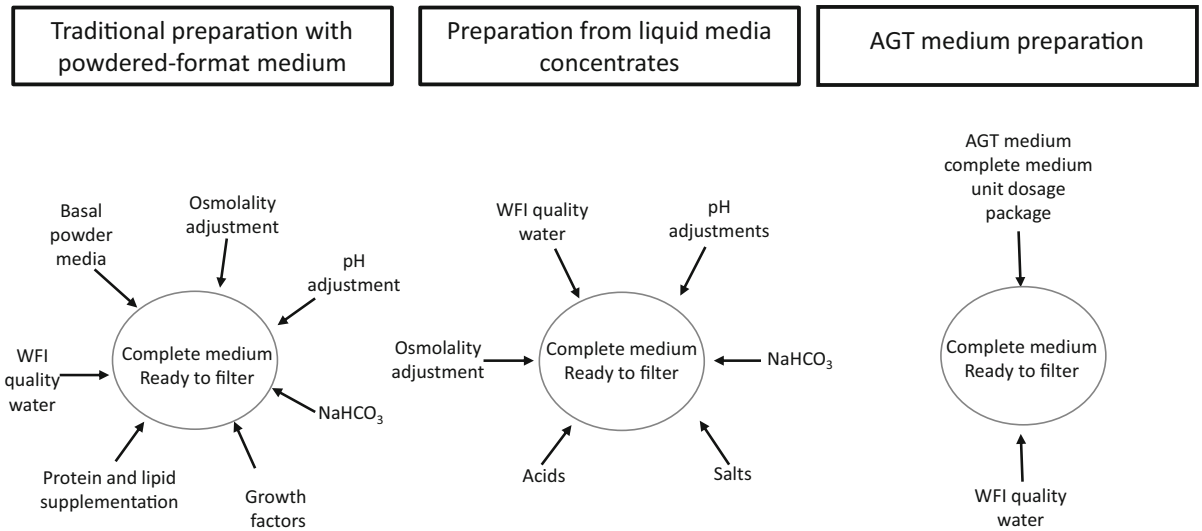

Fig. 1 Advantages of AGT relative to traditional powder media formulations and liquid concentrates

facilities required to handle liquid formulations are less complex than the powder-oriented media and mostly require large storage/refrigeration areas while higher equipment investment costs are necessary for facilities that handle powdered-format formulations, in addition to factoring in higher staff costs, since a larger number of personnel must be trained to allow for correct product handling in order to facilitate a contamination-free manufacturing process (Table 1).

Analytical methods for raw material characterisation

Separation methods

Cell culture performance can be significantly affected by subtle variations in media composition, hence analytical techniques have been often applied as screening tools to determine both qualitatively and quantitatively specific components that can be correlated to productivity. Among the various constituents of media, hydrolysates are particularly difficult to characterise due to variability in the parameters chosen during their production and hydrolysis processes which results in lot-to-lot variability. Initial efforts aimed at the identification of components which had a significant impact on cell growth patterns involved the separation by size-exclusion principles of peptide-containing sub-fractions from the remaining soy components, revealing a 41% improvement in cell growth performance relative to original soy samples (Franek et al. 2000). To obtain further information regarding the composition of these peptide fractions, adsorption-partition based hyphenated techniques have been implemented whereby peptide 'maps' were developed to characterise wheat gluten hydrolysate samples thereby creating 'profiles' which could be applied as rapid screening tools for media characterisation (Schwartz et al. 2002).

Essential components such as glucose and glutamine (which have been found to affect the rate of glycosylation) (Nyberg et al. 1999) have also been detected using chromatographic methods coupled with spectroscopic detection methods. Depending on pH conditions, weak acids such as carbohydrates can be ionised and separated by high performance anion exchange with pulsed amperometric detection (PAD) (Hanko and Rohrer 2000). However, sample interferences can severely affect sensitivity due to the high concentration of undefined components present. Alternative reversed-phase methods coupled with fluorescence detection have shown to be promising (Alwael et al. 2011), with up to 10 monosaccharaides in femtomole concentrations separated within 20 min. These methods are particularly labour intensive since initial pre-concentration extraction steps are required followed by pre-column derivatisation of the monosaccharides which is required due to the lack of

Table 1 Overview of media formulations

Media formulations	Year introduced	Advantages	Disadvantages
Bulk liquid, 1× strength	1955	Low upfront cost	Cost/litre
		Ready to use, QC certification performed by supplier	Non-economical for large production scale of biological therapeutics
		Supplied sterile	Large refrigerating storage space required
		Most suitable for small-scale system such as disposable bioreactors	Restricted stability (several weeks)
			Limited batch size
Milled powders	1963	Cost/litre	Labour intensive and time consuming
		Improved stability over liquids (3 years Merck)	Higher risk of contamination relative to bulk liquids
		Larger batch sizes relative to bulk liquids	Upfront capital investment required
		Most suitable for large-scale systems	Component solubility issues in particular with regards to serum-free media
			Humidity must be controlled to prevent cake
			Heating is required but can damage media components
			Dust can arise after user manipulation due to pouring in containers
Liquid concentrates (LCM), 50× strength	1992	Improved solubility of complex media components	Supplementation needs to be performed after dilution
		Increased nutrient shelf life (up to 1 year stability)	pH/osmolality adjustments need to be performed
		Can be used with in-line dilution to increase medium lot size	Lower precision than bulk liquids
		Less labour intensive relative to DPM	Specialised personnel required for production of large volume batches
Granular format	2001	Superior homogeneity to ball-milled formulations	N/A
		Minimal dust generation	
		Scalable	
		Lot-to-lot consistency	
		Complete medium package	
		Comparable cell growth to liquid media	
		Longer shelf life (5 years) relative to DPM	

suitable chromophores or fluorophores present in these compounds.

Only in recent years, reports highlighting the chemometric evaluation of analytical data derived from metabolite analysis in complex samples by LC (Gupta et al. 2014; Pyke et al. 2015), GC (Koek et al. 2011; Shi et al. 2015) and CE (Ramautar et al. 2009, 2015) coupled with MS detection started to emerge due to the large amount of mass-specific data which can be gathered to facilitate the identification of individual species. An untargeted analysis approach, which is aimed at detecting all possible components in a sample, is often performed at initial profiling stages and correlation analysis is applied to identify the presence of patterns which can be potentially impact the production rate of therapeutic proteins. Relative to similar statistical models derived from the spectroscopic analysis of the same materials, which can only reveal partial sample information due to their limited sensitivity, the models obtained from the MS/MS data

provide accurate information regarding the identity of the majority of components present since ion fragmentation can be performed and mass information for each ion can be matched against a pre-compiled database (Vinaixa et al. 2016). An untargeted metabolomics approach using liquid chromatography in tandem with an Orbitrap mass spectrometer, using either electrospray ionisation (ESI) or atmospheric-pressure chemical ionisation (APCI), was applied for the identification of 125 small molecule metabolites and 4131 unique peptides in 111 different soy samples which were used to supplement CHO cell cultures derived from of DXB-11 cell lines (Richardson et al. 2015). Despite the large amount of data collected, the soy composition was still not fully characterised since the identification process is dependent on the completeness of the reference library used (NIST012 in this case) and instrument performance. Amino acids such as citrulline and ornithine in particular, which are produced by bacterial fermentation during the soy manufacturing process, were identified as productivity enhancers while several nucleosides and short hydrophobic peptides revealed a negative correlation to productivity. In a separate study (Gupta et al. 2014), the analysis of 30 soy samples used as nutritional supplements for the growth of a CHO cell line, CRL-11397, revealed that the levels of compounds such as phenyllactate, lactate, trigonelline and chiro-inositol correlated strongly with productivity. However, these findings were more than likely correlated to the specific type of cell line adopted, since contradictory information is available in the literature regarding the nutritional benefits of lactate (Ozturk et al. 1992).

Despite the complexity of media compositions, LC–MS/MS techniques were also applied for the simultaneous quantification of multiple media components (Qiu et al. 2016). Using a simple dilution step of CHO cells and media samples, 21 amino acids and 9 water-soluble vitamins, which are responsible for controlling several metabolic functions during cell growth, were detected and quantified using ion-pairing liquid chromatography and a triple quadrupole mass spectrometer (QqQ), operated in selected reaction monitoring (SRM) mode (Qiu et al. 2016). A statistical *t* test was performed to assess the variations in the target species levels for two different cell lines which were monitored over a culturing period of 12 days. Despite the low number of analytes observed in this study, powerful analytical methods such as the one described could be potentially applied for larger scale studies using higher-end QqQ detectors which could quantify hundreds of metabolites in relative short run times and facilitate the development of highly efficient media formulations.

Spectroscopic methods

Commonly employed spectroscopic methods for the analysis of culture media and raw materials include near infrared (NIR), mid-infrared (MIR), Raman, fluorescence and nuclear magnetic resonance (NMR) spectroscopy. These methods require minor or no sample preparation and allow for simple, rapid, inexpensive and most importantly non-destructive analysis which can be performed in-/on- or at-line. Spectroscopic methods are "fingerprinting" approaches that result in large datasets of multiple spectra from which results have to be extracted by chemometric tools such as principle components analysis (PCA), soft independent modeling of class analogy (SIMCA), partial least square modelling (PLS) or parallel factor analysis (PARFAC) (Jose et al. 2011; Li et al. 2010; Ryan et al. 2010).

Vibrational spectroscopy

IR and Raman spectroscopy are the most widely used methods for the identification and quality assessment of raw materials. Their ability to analyse samples through plastic or glass packaging materials presents a significant advantage over most other analytical techniques, as this does not require the opening of containers, thus eliminating the risk of cross-contamination and quality risk of potentially hydroscopic materials (Kayat et al. 2012).

NIR is based on the absorption of radiation by organic molecules in the near-infrared region of the electromagnetic spectrum (700–2500 nm) (Tamburini et al. 2014). Most NIR applications focus on the analysis of powders, as the use of NIR for liquid applications is limited due to the strong absorption band of water which can mask important spectral details (Li et al. 2010; Tamburini et al. 2014). NIR allows for the identification of batch-to-batch variability as well as vendor-to-vendor differences (Lee et al. 2012). In addition, NIR can be used for the determination of water content, particle size, mixing and dissolution of powdered media which can have a

significant effect on its performance attributes (Hagen 2013; Punzalan et al. 2014; Strother 2008). Indeed, many studies described the use of NIR for the prediction of media components including soy hydrolysates and yeastolates on their performance in cell culture (Kasprow et al. 1998; Kirdar et al. 2010; Lee et al. 2012). Generally, the predictive power of NIR and other spectral methodologies largely depends on the extent of the spectral library used and previous experience from historical data (Hagen 2013). Very low concentrations of the critical media components can negatively impair the prediction of raw material on cell culture performance as observed for soy dosages of $\leq$ 5 g/l by Lee et al. (2012).

Mid-infrared spectroscopy (MIR; 2500–40,000 nm) is less commonly used for the analysis of raw materials, although it exhibits a higher spectral resolution and intensity than NIR. However, due to its spectral characteristics MIR results in a stronger absorbance band for water than NIR, can penetrate less deep into material and probes are generally more fragile and expensive (Teixeira et al. 2009). The potential of MIR for on-line monitoring of mammalian cell culture components including HEPES, potassium phosphate and sodium hydrogen carbonate has been evaluated by Foley et al. (2012).

Raman spectroscopy is a complimentary method to IR and measures the changes in molecular polarizability. Water is a weak Raman scatterer which is particularly advantageous for the analysis of complex aqueous-based media used in biotechnology. This was recently demonstrated by Li et al. (2010), who used Raman spectroscopy for the rapid characterization and quality control of five complex cell culture media solution. As Raman spectroscopy is prone to fluorescent interferences (e.g. from excipients), careful selection and evaluation of an appropriate excitation wavelength is required (Kayat et al. 2012).

Conventional Raman spectroscopy is not suited for analytes with a concentration below 0.5% (w/w) which accounts for a considerable number of components in liquid cell culture media (Calvet et al. 2014). Recent attempts to increase Raman sensitivity include Raman-subsampling techniques which generate a large array of heterogeneous Raman spectra and Surface enhanced Raman Spectroscopy (SERS) (Calvet et al. 2014; Li et al. 2015). SERS is a very sensitive analytical method where molecules are adsorbed onto nanosized noble metal structures thereby enhancing the Raman scattering effect. Calvet et al. (2014) used SERS to monitor the compositional changes in cell culture media upon dark storage. However, SERS is difficult to implement reproducibly and is not yet developed for commercial validation (Kayat et al. 2012). Efforts for SERS experimental optimization are currently ongoing (Fisk et al. 2016).

Due to the limited sensitivity for vibrational spectroscopy, small concentration differences in bioprocessing media components may not be detected by vibrational spectroscopy as was shown for seven culture media with essentially similar formulations which could only be differentiated after targeted monitoring of folic acid and glucose using complementary techniques (Mallya et al. 2016). In addition, Raman and IR spectroscopy are both indirect techniques which depend on the calibration of both the spectral and chemometric methods (Tamburini et al. 2014). Raman and IR spectral properties are highly subjective to variability. Sampling inconsistency, process changes or changes in media pH and temperature can have a significant effect on spectral properties (Foley et al. 2012; Kayat et al. 2012). It is therefore critical to perform profound robustness studies and to integrate physical and chemical spectral variety from multiple batches into spectral calibrations (Kayat et al. 2012).

Optical spectroscopy

Culture media compounds, such as riboflavin, pyridoxine, proteins and free amino acids including tryptophan, tyrosine and phenylalanine, exhibit a characteristic fluorescent emission after excitation by UV (250–500 nm) (Groza et al. 2014; Pons et al. 2004). Multidimensional fluorescence (MDF) such as excitation–emission matrix (EEM) fluorescence is best suited for their analysis in complex media in order to distinguish them from emission overlaps from other media components. Fluorescence spectroscopy is less affected by the turbidity of the media and more sensitive than absorbance methods. Ryan et al. (2010) demonstrated the combined use of EEM and chemometrics for the identification of compositional changes and the correlation of small compositional changes of fed-batch media to their protein product yield with an accuracy of $\pm$ 0.13 g/l. In addition, fluorescence spectroscopy is a useful tool to study the degree of photo-damage during media storage and light

exposure which can be monitored by the decrease of photo-active media components and the increase of photo-degradation products (Calvet et al. 2014; Ryan et al. 2010). Secondary effects including changes in absorption or changes in quenching rates have to be taken into account (Calvet et al. 2014). Fluorescence signal intensity may not always be linearly related to changes in the concentration of the respective photo-active analyte.

Fluorescence anisotropy is an advanced fluorescence technique where the polarization state of the emitted light is characterized and related to the physical size and movement of a fluorophore. It was recently introduced by Groza et al. (2014) for the measurement of low-concentration proteins such as insulin and serum albumin (1–10 mg/ml) in culture media that are difficult to analyse by vibrational spectroscopy due to their low spectral resolution and signal strength.

Data fusion methods

The combination of data from orthogonal spectroscopic methods is a powerful strategy to obtain enhanced predictive potential. Jose et al. (2011) recently studied the predictability of thirteen lots of two different culture media types on their mAb product yields by using NIR, MIR and 2D-fluorescence. Predictive models for the combined approach out-performed the models obtained when using only one spectroscopic method. In another study four different spectroscopic techniques including NIR, Raman, 2D fluorescence and X-ray fluorescence were used for the characterization of 15 different soy hydrolysates on two cell lines and the data were subsequently fused using ensemble partial least squares (EPLS) and multi-block partial least squares (MBPLS) (Lee et al. 2012). For both cell lines, Raman and NIR spectra had the most significant influence in obtaining accurate predictions by EPLS. On the contrary, MBPLS models did not demonstrate superiority over models from single spectroscopic techniques.

NMR spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is based on the absorption and emission of electromagnetic radiation of nuclei in a magnetic field. Its high specificity and reproducibility, fast throughput, large dynamic range (1 μM–500 mM) and ability to simultaneously analyse up to 100–150 components qualitatively as well as quantitatively make this technique highly attractive for the analysis of culture media (Girard 2013). Compounds can be identified by their characteristic NMR resonances and systematic instrument calibration is not required, facilitating the comparison of results across research sites. Luo and Chen (2007a, b) used a combined approach of NMR and chemometrics for the screening of 25 soy peptone lots in cell culture media which allowed for the distinction of lots resulting in high titres from lots resulting in low titres and for the identification of lactate as the component with most influence on the predictive power. However, NMR for the analysis of culture media is not widely used to date, mainly due to its high capital and operational costs and space requirements (Table 2).

Culture media in the context of quality by design (QbD)

The quality testing of the final product is no longer considered a sufficient quality measure. Instead, regulatory guidelines recommend a "quality by design" approach where quality is designed and built into the process leading to consistent and predefined product quality parameters and a more flexible regulatory approach. The concept of QbD is outlined by the International Conference on Harmonization in ICH Q8(R2) (2009), Q9 (2005) and Q10 (2008) and is based on an improved understanding of the product, its manufacturing process and associated risk factors. Essentially, QbD requires the definition of Critical Quality Attributes (CQAs) which form the basis for the development of a design space. The latter undergoes regulatory assessment and approval followed by continuous monitoring, implementation of appropriate control strategies and continual improvement of the process.

Culture media components are important quality attributes as they can significantly influence the characteristics and yield of the final product. To facilitate their risk assessment, their classification into "critical"—i.e. with significant impact on product quality, "key"—i.e. with impact on the process rather than the product and "non-key" components was

Table 2 Overview of spectroscopic and chromatographic techniques in relation to cell culture media

	Spectroscopic analytical methods			Chromatographic techniques
	(N)IR	Raman	Fluorescence	LC, GC, CE, IC
Advantages	Widely accepted in regulation	Powder and liquids	Real-time	Hyphenated techniques: analysis can be optimised by coupling appropriate detection methods (spectroscopic, PAD, MS) with separation techniques to target specific subsets of media constituents
	NIR penetrates deeper and has less absorbance of water than MIR has 10–100× higher intensity		On-line, in-line, at-line	
	MIR higher spectral resolution but probes more fragile and expensive for MIR		Suitable for low concentrations	Capillary systems can provide increased sensitivity and require minimal initial quantities of sample, allowing the determination of compounds presence at trace levels
				Analysis can be either untargeted, which monitors all detectable media components, or targeted, for the quantification of specific compounds
				Chemometrics can be applied for the determination of key compounds which can be correlated to productivity
	Hand-held			
	Fast and efficient (real-time)			
	Little/no sample prep, no sample dilution			
	Possibility for in situ			
	Holistic approach (sample and matrix)			
	Complementary			
Challenges	Mainly limited to powder applications (water results in predominant peak)	Fluorescence interference (careful choice of wavelength)	Secondary effects: quenching, overlap	Destructive techniques
	Not suited for in-solution BP		Limited to components with fluorescent properties	Larger capital investment required for sourcing of equipment
	Complex chemometrics			Lack of comprehensive mass spectral databases for metabolites
	Secondary methods, require calibration → prediction strength depending on calibration → calibration only valid if process does not change			
	Sensitivity not sufficient for low-concentration components			
	High-energy laser may decompose sensitive samples			
	Representative sampling (probe positioning, opacity change)			

Table 2 continued

	Spectroscopic analytical methods			Chromatographic techniques
	(N)IR	Raman	Fluorescence	LC, GC, CE, IC
Recent developments	N/A	SERS enables analysis of lower concentrations More selective bands than NIR SERS limited to in-solution, stringent reproducibility control required and not yet developed for commercial validation Raman sub-sampling	Fluorescence anisotropy (inclusion of hydrodynamic volume and rotational speed) for protein quantitation	Undefined media constituents, such as hydrolysates, can be screened and peptide maps can be developed for quality-monitoring High-end triple quadrupole and Hi-Res MS instruments can provide unprecedented sensitivity and specificity, thus allowing the determination of molecular weight and structure specific measurement for a given analyte
	Improved portable instruments Improved concentration ranges	Improved probe design Extended libraries		

proposed (Rathore and Low 2010). Whereas "critical" raw materials have to meet defined acceptance criteria and require appropriate analytical testing, "key" raw materials can be monitored on a more risk-based decision (Kirdar et al. 2010). However, the supply chain structure of culture media components is rather complex. In addition, the analysis of raw materials is often outsourced which requires special attention to supply chain management and traceability in order to fulfil the QbD concept (Kirdar et al. 2010; Elliott et al. 2013).

Analytical methods

Process Analytical Technologies (PAT) aim to enhance the understanding and real-time control of the manufacturing process, and are therefore enabling components of QbD (Read et al. 2010). Analytical methods used for PAT have to be suitable for in-, on- or at-line process control, and include a broad range of spectroscopic methods for the monitoring of culture media components as outlined in the section "Spectroscopic methods". In order to be used for routine operations analytical methods have to be approved by regulatory authorities. Non-compendial methods can be used if they are appropriately validated and if the instruments are qualified according to GMP (EudraLex 2014). Many pharmaceutical companies have recently implemented NIR for raw material identification, characterization and PAT. However, spectroscopic instrument qualification and method validation according to regulatory guidelines remains challenging. Spectroscopic methods differ significantly from other conventional methods including the need for chemometric data interpretation and their high susceptibility to variability leading to a high risk of false-positive and false-negative results.

Regulatory bodies have therefore initially been slow in adopting NIR techniques (Reich 2005). To minimize regulatory hurdles and to support the development, calibration and validation of NIR methods for qualitative and quantitative analysis and in PAT applications, EMEA released a guideline which includes data requirements for new submissions and variations (EMEA 2012). A QbD approach was adopted for the NIR method lifecycle which should facilitate their continuous adaptation throughout their lifecycle. However, the requirements for calibration and validation of qualitative and quantitative NIR methods remain extensive. They include both the experimental as well as chemometric aspects, and both the method and spectral library require continuous updating. In addition, sample outliers should be re-evaluated by reference analytical methods and sample

spectra should pass a statistical spectral quality test for the respective chemometric model used in order to reduce out-of-specification classifications resulting from false-positive and false-negative results.

Design of experiments (DoE)

Due to the complexity of culture media components used in biotechnological processes, it is not feasible to examine the effect of each component on a product's characteristics or yield individually (Rathore and Low 2010). Instead, statistical design of experiments (DoE) methods are applied to understand possible interactions of multiple parameters on the final process and product which are not observed when changing one factor at a time (Shivhare and McCreath 2010).

Design of Experiments approaches are facilitated by the combination of PAT with high-throughput cell culture scale-down systems such as commercially available from Sartorius or Applikon (e.g. Ambr- or MiniBio-series). Those allow the structured performance of parallel experiments in low-volume ml to liter ranges (Ellert and Vikström 2014). However, as the timeframe for process optimization is relatively short, batch-to-batch, time- and source-dependent changes of culture media may for the first time occur during manufacturing. To include long-term variability within raw materials, the retrospective analysis of historical data by chemometric methods should be considered (Read et al. 2010).

Conclusions and future trends

The advantages of serum-free cell culture media have been detailed in this review, the main benefits namely; the reduction of batch-to-batch variation to improve product safety, the potential optimisation of production processes and improved cost efficiency (Ling et al. 2015). Although most cells still need some supplementation, an industrial push has driven the standard use of SFM or CDM for most cell culture production modes due to their high yields, and their ability to support cell division rates which can yield $> 10^6$ ml^{-1} viable cells in batch culture and $> 20 \times 10^6$ ml^{-1} viable cells in fed-batch cultures (Decaria et al. 2009). As a result, the use of a serum-containing media in the biotherapeutics industry is now rare and typically only used where particularly advantageous, for example normal human cells and embryonal cells grown routinely in SFM to control growth and differentiation status (Draper et al. 2004; Keenan et al. 2006; Yao et al. 2003).

The question has long stood as to whether or not a truly CDM is possible (Summers and Biggers 2003). As each cell type has its own requirements for media due to the different receptors for cell growth, a universal CDM has not yet been developed despite considerable efforts (van der Valk et al. 2010). However, as a result of the development of CDM, the nutritional demands of mammalian cells have evolved, and in turn cell culture media is now regularly studied during process development, with each cell line and process thoroughly optimised. Improvements in both media composition and process control have led to an increase in volumetric productivity of mammalian cells cultivated in bioreactors (gram per litre in some cases), which is significantly higher than reported values in the mid-1980s (Wurm 2004).

There has been a relatively slow adoption within the biotherapeutics industry of continuous bioprocessing in place of the standardly used batch systems. With this gentle but steadily increasing move due to numerous advantages such as reduced equipment size, high volumetric productivity and overall lower cost (Konstantinov and Cooney 2015), there is a definite need for a robust CDM that can support very high cell densities at low volumetric perfusion rates. Chemically-defined media must perform well during both development and demonstrate reproducibility by commercial media vendors (Ling et al. 2015). Cell viabilities are currently maintained at over 5 days at peak cell densities (Decaria et al. 2009) using optimised processes and further advancements may see this duration extended. Product quality is also of the utmost importance during the entire continuous process, and further improvements are needed through new fit-for-purpose operations and processes (rather than connecting existing batch equipment) to avoid mutations and alterations of gene expressions in CDM. Further opportunities in advancements in vector and host cell engineering should also be explored to ensure the constant improvement of production of therapeutics using CDM (Wurm 2004), and the removal of waste products should also be monitored (Chu and Robinson 2001). A major challenge for the development of an internal CDM is the transition from old to

new media (Ling et al. 2015), and thorough knowledge of processes may be required to ensure successful implementation of a new medium. As previously mentioned, it is imperative to maintain critical product quality attributes, especially for the production of larger proteins to reduce variability (Decaria et al. 2009).

In conclusion, although great advances towards the development of a truly CDM have been made since the origin of SFM, further work must be carried out focussing on a scalable SFM that can support cells for longer term processes without altering the cell metabolism. It is also clear that for ethical reasons it is imperative to remove animal derived products from cell culture medium unless absolutely necessary.

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