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Cell Culture Media Analysis using Rapid Spectroscopic Methods.

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Abstract: Cell culture media (CCM) used in industrial biotechnology are complex mixtures of molecules and elements that are inherently difficult to analyze comprehensively. CCM quality analysis is of utmost importance for efficient production of protein-based therapeutics with the correct Critical Quality Attributes (CQAs). Here we discuss the use of rapid spectroscopic methods for routine screening of CCM molecular variance which include electronic (UV-visible absorption and fluorescence) and vibrational (Raman, FT-IR, and Near-Infra-red) spectroscopies. CCM analysis needs to provide: identity testing, compositional variance analysis (i.e. lot-to-lot variation), validation of media preparation protocols, and correlations with Critical Process Parameters (CPPs) or product CQAs. Rapid spectroscopic methods can fulfil some of these requirements but only with correct sample handling and preparation. Accurate analysis requires the use of chemometrics combined with a detailed knowledge of sample behavior such as water absorption and chemical stability.

Keywords: Cell culture media (CCM), Analysis, Spectroscopy, Raman, Fluorescence, chemometrics.

Abbreviations:

ASTM American Society for the Testing of Materials
ATR Attenuated Total Reflectance
BSA Bovine Serum Albumin
CCM Cell Culture Media
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CPP</td>
<td>Critical Process Parameter</td>
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<tr>
<td>CQA</td>
<td>Critical Quality Attributes</td>
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<td>CRS</td>
<td>Conventional Raman Spectroscopy</td>
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<tr>
<td>EEM</td>
<td>Excitation Emission Matrix</td>
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<td>FIA</td>
<td>Flow Injection Analysis</td>
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<tr>
<td>FT-IR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
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<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
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<tr>
<td>MIR</td>
<td>Mid Infrared</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>NIR</td>
<td>Near Infrared</td>
</tr>
<tr>
<td>PAT</td>
<td>Process Analytical Technology</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface Enhanced Raman Scattering</td>
</tr>
<tr>
<td>SORS</td>
<td>Spatially Offset Raman Spectroscopy</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to Noise Ratio.</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>TSFS</td>
<td>Total Synchronous Fluorescence Scan.</td>
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</table>
Introduction:

Cell culture media (CCM) are a critical element in biopharmaceutical manufacturing which directly affect process yield and product quality. Therefore, the analysis of media variance and prediction of performance is of high importance. The essential purpose of CCM is to produce and maintain an optimum physiological environment to allow large-scale culturing of cells where they remain healthy for an appropriate amount of time and express the right product with the correct Critical Quality Attributes (CQAs). For protein-based therapeutics the quality attributes include factors such as the glycosylation pattern, stability, aggregation, impurity profiles etc. Many CQAs are directly affected by CCM [1] and unknown changes in composition can have very serious adverse consequences for the process. CCM are carefully designed and optimized for each process (and often for different stages in the process) and cell line, which results in a very wide range of different CCM formulations. The resulting media are highly complex, molecular and elemental mixtures, containing amino acids, carbohydrates, vitamins, growth factors, trace and bulk minerals. The most complex of the raw material components used in media manufacture are the various hydrolysates of protein sources (like yeast or soy). These hydrolysates are a complex mixture of carbohydrates, amino acids, peptides, and a host of other often unknown molecules and minerals [2-5]. These contain many more components than chemically defined media and often display much greater molecular and elemental variance but are often critical for antibody manufacturing processes [6]. This variance can arise from changes in source material, manufacturing issues, or improper handling/storage. For these materials, both molecular [7] and elemental variance are important [8] in the context of their impact on cell culture. This molecular and elemental variance impacts on the CCM quality in terms of its intended purpose, namely sustaining a specific bioprocess. Therefore, when we use the term media quality it refers to a specific media and specific process. It should be noted that the same media could be used in two different processes and for each process, media quality attributes (e.g. concentrations of specific components) may be different.

Another major issue is the fact that components are present in widely varying concentrations, from trace (ppm) levels for some elements to >5% w/w for carbohydrates. In liquid media water is the major component (>90% w/w) diluting components (milli-, micro- and nanomolar concentrations) and making analyte detection more challenging for spectroscopic methods. Because of these compositional and concentration factors, media
analysis using traditional analytical methods, is challenging, very time consuming, requiring multiple methods and techniques. This makes comprehensive analysis too expensive for routine use. As the industrial cell culture production of therapeutic proteins continues to grow so too does the need for rapid, inexpensive, reliable, robust and non-destructive analytical methods that can be integrated into process control to improve end product yield and quality. A recent review [9] provides a more in-depth assessment of these raw materials issues. Whereas here we are focusing on the use of spectroscopic measurements on the media and being critical look into the quality of the data being generated. The quality of the spectral data that these rapid methods can provide is very sensitive to sample handling and failure to recognize this can lead to the generation of erroneous data and ultimately misleading conclusions.

**Fast spectroscopic methods:** There are many potential spectroscopic methods that can be applied to media analysis, however, here we limit ourselves to commercially available, tried and tested methods. Relatively recent reviews describe the needs for Process Analytical Technologies (PAT) in biopharmaceutical manufacturing [10-13] and provide more background detail about how rapid spectroscopic techniques could be incorporated into manufacturing processes and are directly relevant to CCM analysis. The most important methods are the electronic spectroscopies (UV-visible absorption and fluorescence) and vibrational spectroscopies (MIR and NIR absorption and Raman scattering). The correct application of all methods requires an in-depth appreciation of the practical difficulties of dealing with these very complex mixtures. Sample handling therefore plays a critical role in the collection of accurate, correct, and reproducible data which is required for the development of robust analytical methods.

**Sample Handling:** A critical issue with CCM analysis relates to sample handling and stability both in powdered and solution forms (Table 1). CCM have complex physicochemical properties which mean that the samples are neither chemically nor physically stable for varying time periods of minutes to hours depending on the type. It is very important to recognize here that stability in the context of measurements is different from stability associated with CCM performance in a bioprocess. For example, powdered CCM are usually hygroscopic, and will quickly absorb atmospheric water rapidly (within a couple of minutes) and are thus unsuitable for vibrational spectroscopy analysis of molecular variance (as opposed to just measuring the effects of water absorption) unless properly dried.
Whereas small amounts of absorbed water are unlikely to have any impact on cell culture performance. Water adsorption from the atmosphere induces variations in baselines and erratic signals which are not always clearly evident to the analyst. One answer is to use specialist powder cells [14] to prevent water adsorption. Another approach is to use Spatially Offset Raman Spectroscopy (SORS) to analyse CCM powder in sealed packages and thus avoid the water adsorption problem [15], while this works well with simple media components it has not been proven with CCM powder, where fluorescence and the large number of components with varying concentrations are the major issues. MIR and EEM fluorescence have been used to measure media aging effects [16] and the authors here concluded that NIR was better. However, the sample handling controls described would suggest that what was observed and measured were the effects of water absorption.

For solution analysis, one needs to be aware that the chemical and physical properties are sensitive to microbial growth, chemical reactions in solution, and environmental conditions (temperature, light, etc.). CCM if not sterilized will change very quickly due to microbial growth. The increased bacterial load will make solutions more turbid and thus increase light scatter leading to increased baselines and noise in spectra which may invalidate the measurements. Even when sterilized and stored at low temperature (2-4°C) chemical changes will occur that can have an effect on the spectral measurements and adversely affect the interpretation of those results. These subtle chemical changes in the liquid solution have been detected and monitored using Surface Enhanced Raman Scattering (SERS) spectroscopy, with most of the changes being associated with cysteine/cystine concentration changes [17].

A similar situation exists with environmental water samples where changes in EEM signal [18] were measured during storage at low temperature (2-4°C). These authors investigated the use of preservatives to minimize microbial growth on their non-sterilized samples and found, as one might expect, that these adversely affected the fluorescence measurements. One approach to minimize and control all these issues which yields very reproducible results is to store all biogenic samples (media or broths) at –70°C [19-22] and then test after defrosting. Most media contain riboflavin and are thus photochemically unstable in solution and this degradation can easily be measured and monitored using fluorescence spectroscopy [23, 24]. High temperature treatment (e.g. pasteurization) of CCM also causes compositional
changes [25] and this also needs to be accounted for during sample handling and chemometric modelling.

Another aspect which is important are spurious signals can be incorporated from the container used to hold solutions. In particular for Raman analysis the use of plastic multi-well plates or glass holders are not recommended because they contribute signals to the already weak and complex sample spectra. The use of electro-polished stainless-steel multi-well plates produced better quality, more reproducible spectra and these holders were also impervious to sample induced corrosion in cases of acidic media components [19, 26]. The most common spectroscopic measurement artefacts in CCM analysis are listed in Table 2.

**UV-visible absorption:** The method is quick and can produce data which can enable the identification of gross changes in media quality such as relatively large concentration changes or microbial contamination. For specific media component quantification there are many colorimetric assays based on UV-visible measurements available which could be applied once interferents and matrix effects have been considered. For example, the use of a hexokinase reagent was recently demonstrated for glucose quantification in media [27]. Unfortunately, this approach is too time-consuming and impractical for the analysis of more than a few analytes. However, it may be suitable for some analytes which have a validated high impact on the process of product CQAs. Overall, UV-visible spectroscopy lacks the sensitivity and specificity required to measure molecular variance to a level that is required for routine CCM analysis.

**Fluorescence:** Most CCM contain multiple fluorophores (Trp, Tyr, Riboflavin, Pyridoxine, etc.,) and other photophysically active molecules which either emit or affect fluorescence emission. This means that there are multiple processes such as energy transfer, static quenching, and dynamic quenching which affect the wavelength and intensity of the emission [28]. The complete fluorescence emission space can be recorded with either an Excitation Emission Matrix (EEM) or Total Synchronous Scan Fluorescence (TSFS) measurement and these represent a unique media ‘molecular fingerprint’. EEM is a very effective and useful tool for the qualitative characterization of nearly all the different solutions that are encountered in biopharmaceutical manufacturing: CCM [20, 21, 23, 29], biogenic raw materials [22], bioprocess broths [30-32], and proteins [33]. To achieve this, once needs to correctly use multivariate analysis combined with chemical knowledge, “chemometrics”, to
best interpret, understand, and use EEM. A recent IUPAC technical document [34] provides more information and guidance about the correct implementation of these methods and the chemometric data analysis procedures.

One of the key goals in media analysis is to establish correlations between the measured variance and Critical Process Parameters (CPPs) such as product yield [20] or quality. In one case, EEM fluorescence proved to be much better than Raman spectroscopy, which indicated that the critical components related to process performance were present at too low a concentration to be detected by Raman [19, 20, 26, 35]. A more recent study [36] suggested that EEM fluorescence measurements could be correlated with antioxidant behavior for a soy-based hydrolysate and also looked at the filtration process. More recently, the combination of anisotropy with the multidimensional EEM measurement has been used to quantify protein content in a model cell culture media [33] and had similar accuracy to the Bradford assay.

**Vibrational spectroscopy:** One driver for the use of vibrational spectroscopy is the provision of much more informative molecular fingerprints for each component in a mixture. However, analyte SNR, large variety of different analytes, and concentration variations severely limit the application of vibrational spectroscopy techniques. A recent review has discussed in detail the use of Raman spectroscopy in biopharma [37] and covered most of the important issues involved in CCM analysis many of which are also relevant to MIR and NIR spectroscopic analysis.

In 2010, Li et al. showed that Raman spectroscopy could characterize variance and discriminate different CCM components in solution from a commercial recombinant-protein manufacturing process [35]. However, this study and others [38] highlighted some of the difficulties associated with Raman spectroscopy of media solutions: the water signal is stronger than the weak analyte spectra, thus only relatively large composition changes are measurable. SERS [39] could be used to increase sensitivity, however, the compositional complexity of media means that it is difficult to implement. In 2011, Wen et al. suggested SERS as a potential quality-control method for certain cell-culture-media components. They looked at melamine adulteration of several media raw materials and in a commercially-available CCM [40]. They showed reasonable results for simple components (with minimal SERS response) but that for media with multiple components it was not possible. In 2012, SERS was evaluated as a method for assessing variance in yeastolate, a complex hydrolysate...
[22], which could not be analyzed by Raman because of its intense fluorescence. SERS could detect small variations in composition (mainly associated with adenine) that were associated with different manufacture/source and could be used as a rapid screening tool for lot-to-lot consistency [22]. Unfortunately, a variety of issues including competing surface adsorption processes, the efforts required to development robust measurement protocols, and high numbers of replicate measurements required make it an impractical method for routine CCM analysis [17, 22].

**MIR & NIR absorption:** MIR and NIR because they are absorption based and operate under different selection rules are much more efficient processes than Raman spectroscopy, but they are also much more sensitive to the presence of polar compounds and water in particular. Furthermore, the wavelengths of light used in each technique have significant impacts on the material light interaction which has practical consequences for media analysis. For example, infra-red light (approx. 2.5–50 µm) results in very short, sample path lengths in samples (~µm) which can lead to sub-sampling problems. NIR utilizes shorter-wavelength light (800–2,500 nm) which provides for many practical benefits in terms of sampling and instrument design. As NIR spectra comprise combinations and overtones of molecular vibrations and this produces spectra (for solids and liquids) with broad, overlapping bands. This means that NIR does not generate the same level of molecular specificity compared to MIR or Raman spectroscopy. However, the light wavelengths involved enable the use of simple lamp-based light sources and relatively inexpensive glass-based optical components, fibers, and sampling systems. Secondly, the lower molar absorptivity in the NIR allows for longer sample path-lengths/more homogeneous sampling and facilitate sampling through relatively thick container walls. NIR has been applied to media [41] and hydrolysate powder analysis [42] and to assess the quality of complex media such as corn steep liquor used in bioprocesses [43]. However, experimental details for the sample handling and/or measurement processes were insufficiently detailed to enable one to determine the quality/reliability of the NIR data. Some authors have mentioned that the materials are hygroscopic [42], however, no control measures were implemented. An interesting application of FT-IR microscopy and elemental composition analysis to the study of filter fouling by media was demonstrated by Cao et al., [44]. FT-IR microscopy was used to show
that most fouling arose from cellular debris arising from microbial contamination rather than
the precipitation of specific inorganic components of the media.

**Combined Analysis:** The reliance on a single analytical technique for CCM characterization
is limited because cell culture performance is affected by both molecular and elemental
components. Thus, one needs a combination of techniques to properly identify and
characterize relevant media variance. NIR, fluorescence, X-ray fluorescence, and Raman
spectroscopy were used to investigate hydrolysate compositional variability with a goal of
predicting CPPs such as integrated viable cell density (IVCD) and product yield from spectral
measurements [45]. Techniques were compared individually and then in combination
however, details on sample handling and replicate measurements of some techniques (to
assess measurement variability) were not provided. Furthermore, the Raman solution spectra
presented showed sharp peaks associated with the immersion probe sapphire window. The
authors concluded that this data-fusion approach offers significant benefits in assessing media
variance. A similar study [38] compared several methods to evaluate wheat hydrolysate
variability. Unfortunately, sample preparation and handling details were vague and thus one
would be unsure as to the quality/reliability of the solid-state measurements. Likewise, the
solution measurements were only single measurements for the fluorescence. Furthermore,
the Raman spectra were recorded from solution which does not provide very good quality
data because of several factors [19].

**Conclusions**
Rapid spectroscopic methods if correctly applied can be used for the rapid and effective
screening of cell culture media to identify molecular variance and potential issues with media
manufacture. However, the effective application of these methods is very dependent on
correct sample handing which in turn is based on an in-depth appreciation of the physical and
chemical stability of these complex materials. In many publications, critical information
about sample storage, handling, preparation, measurement variance, and robustness was
missing which makes an objective assessment of the data quality impossible. Furthermore, it
makes repeating these studies difficult. Some of this is due to publisher page/word limits and
Chemometrics can deal with some measurement error, however, for CCM one cannot often easily identify measurement and sample induced errors. The quality (robustness, reliability, and repeatability) of the output chemometric models is highly dependent on input spectra quality. For each spectroscopic technique different sample factors (stability, hygroscopicity, etc.) will have greater or lesser impact on the measurements and each should be risk assessed and controlled appropriately (Table 1 and 2). Once data has been collected it needs to be validated prior to analysis/interpretation and the utilization of the results for process control.

Media analysis requires a set of recognized standard methods and technical guidance documents for spectroscopic measurements similar, for example to those produced by the ASTM [46] and by IUPAC [47].

**Conflicts of Interest**
The author declares no conflict of interest.

**Acknowledgements**
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<th>Powders</th>
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<th>Solutions</th>
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<tbody>
<tr>
<td></td>
<td>Hydrolysates</td>
<td>Chemically Defined</td>
<td>Hydrolysates</td>
<td>Chemically Defined</td>
</tr>
<tr>
<td><strong>Hygroscopic</strong></td>
<td>Yes, very [42].</td>
<td>Yes, very [16].</td>
<td>n/a, unless very concentrated</td>
<td>n/a, unless very concentrated</td>
</tr>
<tr>
<td><strong>Drying required before NIR/IR/Raman measurements</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Light sensitivity</strong></td>
<td>Some</td>
<td>Some</td>
<td>Yes.</td>
<td>Yes [23].</td>
</tr>
<tr>
<td><strong>Protection measures</strong></td>
<td>Avoid intense or prolonged light exposure</td>
<td>Avoid intense or prolonged light exposure</td>
<td>Store in dark containers.</td>
<td>Store in dark containers.</td>
</tr>
<tr>
<td><strong>Temperature sensitivity</strong></td>
<td>Some</td>
<td>Some</td>
<td>Yes.</td>
<td>Yes [17].</td>
</tr>
<tr>
<td><strong>Long-term storage conditions</strong></td>
<td>−20°C, dry. Need to minimize potential microbial growth as these materials are usually not sterilized.</td>
<td>−20°C, dry. Need to minimize potential microbial growth as these materials are usually not sterilized.</td>
<td>−70°C [20].</td>
<td>−70°C.</td>
</tr>
<tr>
<td><strong>Microbial growth</strong></td>
<td>Yes, if sufficient water absorbed.</td>
<td>Yes, if sufficient water absorbed.</td>
<td>Yes, very sensitive [22].</td>
<td>Yes, very sensitive.</td>
</tr>
<tr>
<td><strong>Protection measures</strong></td>
<td>Store dry.</td>
<td>Store dry.</td>
<td>Aseptic sample preparation and 0.2 μm filtering recommended [16].</td>
<td>Aseptic sample preparation and 0.2 μm filtering recommended [16, 23].</td>
</tr>
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</table>

**Table 1:** Some general sample handling and storage requirements for the spectroscopic methods used for cell culture media analysis.
<table>
<thead>
<tr>
<th>Method</th>
<th>Key strengths</th>
<th>Weakness</th>
<th>Common measurement issues</th>
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<tbody>
<tr>
<td><strong>Raman spectroscopy</strong></td>
<td>Best for powder samples. Minimal sample handling required.</td>
<td>Liquid formulations, less useful: Good only for &gt; millimolar concentrations, not always possible at that level for complex mixtures [37]. Can not be used with strongly fluorescent samples.</td>
<td>Weak analyte signals [19, 26, 38]. Signals from fiber optic probe (usually sapphire) [45]. Signals from sample containers (e.g. glass slides or plastic multi-well plates) [26]. Fluorescence from powder and liquid hydrolysates [22, 38, 45].</td>
</tr>
<tr>
<td><strong>FT-IR</strong></td>
<td>Best for powder samples. High molecular specificity for component identification. Widely used technology in small molecule API manufacture. Immersion probes available for in-situ measurements.</td>
<td>Less useful for liquid media because of very strong water signal.</td>
<td>Water contamination in powder samples not always obvious. Sub-sampling an issue with small sample areas available with ATR systems used for FT-IR.</td>
</tr>
<tr>
<td><strong>NIR</strong></td>
<td>Best for powder samples. Minimal sample handling required.</td>
<td>Less useful for liquid media because of very strong water signal. Less good for component identification due to broad and overlapping spectral bands.</td>
<td>Water contaminated signals, change baselines &amp; bands in solid media [42].</td>
</tr>
<tr>
<td><strong>Fluorescence (2 or 3D)</strong></td>
<td>Milli- and micromolar detection of some analytes like Trp, Tyr, Riboflavin, Pyridoxine possible which are intrinsically fluorescent [29]. Immersion probes available for in-situ measurements.</td>
<td>Liquid samples only, not suitable for powdered media.</td>
<td>Increased scatter from suspended particles and/or microbes [28, 36]. Unknown measurement variance because of the reporting of single or unknown replicate measurements [36, 38, 48].</td>
</tr>
</tbody>
</table>

Table 2: Summary of the key strengths, weaknesses, and common measurement issues encountered during spectroscopic analysis of cell culture media. In some published cases, the effect has been mentioned but not explicitly dealt with during measurement.
References and recommended reading:

Papers of particular interest, published within the annual period of the review, have been highlighted as:

- of special interest
- of outstanding interest

Recommendations:

- A good overview of many of the critical issues involved with the raw materials and cell culture media used in biopharmaceutical manufacturing.

- A good example of some of the issues involved in the measurement of complex samples via fluorescence spectroscopy. This case is for water analysis and is also relevant for media analysis. However, the use of preservatives is not recommended.

- A recent report which covers all of the major issues involved in the use of multidimensional fluorescence spectroscopy measurements of complex samples such as CCM. Provides a series of recommendations that should facilitate the collection of more reproducible data and correct implementation of chemometric methods of analysis.

- Recent overview of the application of Raman spectroscopy to applications in the biopharma sector. Includes assessments of the major issues with a variety of different samples included solid and solution media.

- A nice example of the application of FT-IR spectroscopy to the analysis of filter membrane clogging. Experimental details are comprehensive and the integration with EDAX results for elemental screening is very useful.
References: