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Molecular Spectrometry

A rapid fluorescence based method for the quantitative analysis of cell culture media photo-degradation

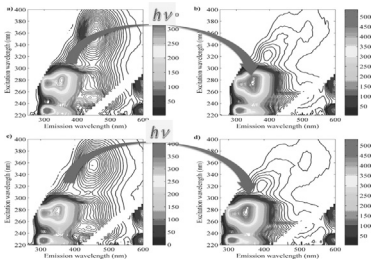
Amandine Calvet, Boyan Li, Alan G. Ryder*

Nanoscale BioPhotonics Laboratory, School of Chemistry, National University of Ireland, Galway, Galway, Ireland

HIGHLIGHTS

- Quantitative method for monitoring the photo-degradation of cell culture media.
- Chemically defined media degrades under visible light illumination.
- Analysis uses fluorescence spectroscopy and robust chemometrics.
- Monitor photodegradation using PARAFAC or MCR.

GRAPHICAL ABSTRACT



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ABSTRACT

Cell culture media are very complex chemical mixtures that are one of the most important aspects in biopharmaceutical manufacturing. The complex composition of many media leads to materials that are inherently unstable and of particular concern, is media photo-damage which can adversely affect cell culture performance. This can be significant particularly with small scale transparent bioreactors and media containers are used for process development or research. Chromatographic and/or mass spectrometry based analyses are often time-consuming and expensive for routine high-throughput media analysis particularly during scale up or development processes.

Fluorescence excitation–emission matrix (EEM) spectroscopy combined with multi-way chemometrics is a robust methodology applicable for the analysis of raw materials, media, and bioprocess broths. Here we demonstrate how EEM spectroscopy was used for the rapid, quantitative analysis of media degradation caused by ambient visible light exposure. The primary degradation pathways involve riboflavin (leading to the formation of lumichrome, LmC) which also causes photo-sensitised degradation of tryptophan, which was validated using high pressure liquid chromatography (HPLC) measurements. The use of PARallel FACtor analysis (PARAFAC), multivariate curve resolution (MCR), and *N*-way partial least squares (NPLS) enabled the rapid and easy monitoring of the compositional changes in tryptophan (Trp), tyrosine (Tyr), and riboflavin (Rf) concentration caused by ambient light exposure. Excellent agreement between HPLC and EEM methods was found for the change in Trp, Rf, and LmC concentrations.

1. Introduction

Chemically defined media (CD-media) are widely used in industrial mammalian cell culture and comprise an integral element of the manufacturing process. These CD-media are usually highly complex mixtures, containing amino acids, carbohydrates, vitamins, and other materials [1–4]. An enriched basal RDF (eRDF) media is one such example, which has relatively high concentrations of amino acids and glucose to sustain high density growth [5]. eRDF can vary in composition but typically comprises in excess of 30 different chemical species [6,7]. It has been previously noted that these media are not chemically stable [8], but can undergo some slow rate chemical reactions when stored in the dark between 2 and 8 °C, the industry standard temperature for

* Corresponding author. Tel.: +353 91 492943.
E-mail address: alan.ryder@nuigalway.ie (A.G. Ryder).

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storing large volumes of cell culture media. It is also well-known that cell culture media, and particularly those containing riboflavin are very sensitive to photo-chemically induced changes which can adversely affect cell growth [9–13]. Thus monitoring media stability is critical at a variety of stages in the development and manufacture of biological APIs. One generally develops and refines biologics manufacture on a small scale, paying particular attention to the generation of the best possible cell culture media that optimizes yield yet retains the other critical quality attributes required (*e.g.* glycosylation). Often this development work is undertaken with the use of transparent bioreactors, media storage vessels, or single-use disposable bioreactors [14], which can be transparent to wavelengths of light that can induce photo-damage.

Ideally for detailed analysis of media changes one would utilize chromatographic methods for the identification of specific component changes. For example a recently reported study on retinoic acid stability in cell culture media used a HPLC method which required a ~40 min runtime per sample after a very complex and multi-step sample handling procedure [15]. Another time-consuming approach is to use metabolomics based methods to generate a high resolution picture of the composition [16]. These types of methods are often not practical from a cost/time consideration particularly the media quality for multiple small scale bioreactors has to be analyzed over days or weeks. What is needed is a rapid, inexpensive method capable of first detecting the onset of photo-damage and second quantifying the degree of change. We suggest using a combination of excitation–emission matrix (EEM) spectroscopy with multi-way chemometrics methods for rapid monitoring of media change [17]. The efficacy of EEM combined with chemometric methods for the identification, quality analysis, and quantification of various constituents of cell culture media has been established [8,17–19]. EEM-chemometric methods are an ideal process analytical technology for the assessment of critical quality and performance attributes of the complex materials used to prepare cell culture media [20–24]. Raman spectroscopy can also be used for the identification and quality monitoring of CD-media [25,26]. However, conventional Raman spectroscopy does not have sufficient sensitivity for measuring the small changes in the photophysically active analytes which are only present in low concentrations for many media. Here we demonstrate methods for using EEM methods to rapidly identify, monitor, and quantify both photo-chemically and chemically induced changes in cell culture media. Furthermore, EEM measurements can provide quantitative analysis [19] of some specific photo-active species present in media such as riboflavin (Rf), tryptophan (Trp), and tyrosine (Tyr). The ability to rapidly identify and quantify variances in the concentrations of these fluorophores in the complex cell culture media is of interest from both quality control and quality assurance points of view. These rapid spectroscopic methods provide a relatively inexpensive and rapid approach for media monitoring over extended timeframes, which should be of particular use during process development, scale up and general operations in biotechnology.

2. Materials and methods

2.1. Materials

eRDF was obtained from Kyokuto Pharmaceuticals Industrial (Japan). NaOH (97+%), $NaHCO_3$ (99.7+%), L-tyrosine (≥98%), L-tryptophan (≥98%), pyridoxine, (−)-riboflavin, lumichrome, methanol, and folic acid dihydrate (97%) were obtained from Sigma-Aldrich and used without further purification. Potassium phosphate monobasic (≥99%) was obtained from Acros. An aliquot of sterilized high purity water was used to dissolve eRDF (4.4248 g), to which was added $NaHCO_3$ (0.2832 g) before making the solution up to a final volume of 250 mL (17.7 g L^{-1} working concentration, eRDF stock). The solution was immediately sterilized by membrane (0.22 μm) filtration and then dispensed as 1.25 mL aliquots into sterile containers (2 mL translucent polypropylene eppendorf tubes) before being placed in one of the four storage conditions: (1) RT–L: room temperature in the light; (2) RT–D: room temperature in the dark; (3) C–L: cold (fridge) with light, and (4) C–D: cold (Fridge) in the dark. Control samples were stored in the dark at −70 °C.

The dark stored samples were placed in a cardboard box covered with tin foil at all times. For the light exposed samples, a similar light source was used inside and outside of the fridge: Phillips warm white 827 Genie stick energy saving bulb (420 lumen) on for 24 h a day (Fig. S1, Supplemental information). The temperatures inside and outside the fridge were recorded throughout the experiment and the room temperature (r.t.) samples were kept at 16.4 ± 3.0 °C whereas the fridge temperature 6.0 ± 1.5 °C. The temperature variation in the fridge is explained by the necessity to have the power inlet for the lamp hindering perfect closure. The four boxes containing the samples were rotated regularly in order to prevent inequalities between sample *vis-à-vis* temperature and light exposure conditions. Fluorescence EEM spectral data were collected over 32 days (Day 0, 7, 11, 14, 18, 21, 25, 28, and 32). At every sampling point three samples were removed from each of the four storage conditions and immediately placed in the dark at 4 °C to limit any further change in the samples during the 3–4 h time period required to collect the EEM from the 12 samples in random order.

2.2. Sample preparation for calibration

For the quantification of Tyr, Trp, Rf, folic acid (FA), and pyridoxine (Py) in the photo-degraded eRDF we used a NPLS quantitative method in which the calibration samples were generated from spiked eRDF solutions.[19] The method was extended in this study to quantify of Py, Rf, and FA. In this method a *Test* (reference) 17.7 g L^{-1} eRDF solution was spiked 10 times in order to triple the initial analyte concentration (c_0). This lead to a total of 31 samples comprising a *Test* sample and 10 spiked samples for each analyte. The EEM of these samples were collected in triplicate and then used for calibration (dataset **quant_cal**). A second, independently prepared dataset (**quant_pred**) was used for prediction (see supplemental information for details).

2.3. Fluorescence instrumentation and data collection

EEM were measured over various excitation and emission spectral ranges with a data interval of 5 nm using a Cary Eclipse (Varian) fluorescence spectrometer with semi-micro quartz cuvettes[1] [8]. The EEM matrices varied according to the specific target analytes being analyzed *e.g.* for amino acids, dataset **AFaa**: λ_{ex} = 220–400 nm/λ_{em} = 250–600 nm/*slit widths* = 5 nm; for the vitamins, dataset **AFv**: λ_{ex} = 315–540 nm/λ_{em} = 330–600 nm/*slit widths* = 5 nm (ex.)/10 nm (em.).

2.4. Chemometric methods and data analysis

All calculations were performed using PLS_Toolbox 4.0®, supplemented by in-house-written MATLAB® (ver. 7.4) code. The NPLS quantification methods used modified standard addition method (MSAM),[27,28] and unfolded principal component analysis (UPCA) which have been described in detail elsewhere [19]. Calibration

[1] In this set up a 4 mm excitation pathlength and 10 mm emission pathlength were used.