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RESEARCH ARTICLE

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# Characterization of Chinese hamster ovary cell culture feed media precipitate

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## Abstract

Process intensification of monoclonal antibody production is leading to more concentrated feed media causing issues with precipitation of solids from the media solution. This results in processing problems since components in the precipitate are no longer in solution, changing the media composition and leading to variability in cell culture performance. The goal of this work is to characterize the feed media precipitate, and in particular to identify the precipitated components so that mitigation strategies can be developed. From the conducted analysis, the precipitate was predominately found to be organic and was analyzed with liquid chromatography-mass spectrometry and inductively coupled plasma-optical emission spectroscopy (ICP-OES) to identify the constituent components. Up to ten amino acids were identified with tyrosine (approximately 77 wt.%) and phenylalanine (approximately 4 wt.%) being the most prevalent amino acids. Elemental analysis with ICP-OES revealed that inorganic components were accounted for less than one weight percentage of the solid precipitate with metal sulfates being the predominant inorganic components.

**KEYWORDS**
cell culture media, LC-MS characterization, organic and inorganic analysis, process development, solid precipitation

## 1 | INTRODUCTION

The manufacturing of biologics via mammalian cell culture is a resource intensive endeavor. This is exacerbated by the fact that most molecules do not make it to market, meaning years of work and experiments for cell line and process development do not result in commercialized product.[1] When a molecule is commercializable, it makes it all the more important to optimize that manufacturing process. Part of that optimization is media design and formulation to increase the productivity and viability of the cell culture process. For most of its history, mammalian cell culture was cultivated using serum-based media. However, there are problems with serum-based media, such as regulatory concerns over viral infections, and composition variability, as well as ethical concerns.[2,3] To avoid these issues, chemically defined media is used for modern biomanufacturing processes, but this introduces new problems.

One of the strategies in the biopharmaceutical industry for maximizing resource use is dubbed "process intensification" where the general principle is to make more product per unit volume. Applied to cell culture media, this means using higher concentration media in smaller volume vessels to increase nutrient uptake and productivity with smaller capital and operating costs.[4] An unintended consequence of such intensification is precipitation in the media due to the increased concentration pushing solubility limits of some of the media components. Precipitation of media components changes the composition of the media and may remove critical components from solution leading to detrimental effects to cell health.

Precipitation in cell culture media is a common problem encountered by industry that is under-reported in academic literature for a

  

variety of reasons. Vendors manufacturing media for commercial use tend to only sell media formulations without precipitation issues and so commercial-media users are unlikely to encounter the problem. Vendors are also unlikely to report their issues with precipitation and approaches for dealing with it due to commercial reasons. Biomanufacturing companies often develop their own media formulations in-house and then contract vendors to manufacture the media for the biomanufacturing process. Similarly, any precipitation issues encountered during formulation development and manufacturing are under-reported for the same commercial reasons.

In recent years, different metabolomics techniques have been adopted to characterize and profiling key metabolites generated by cells throughout the course of a bioprocess. Of these, chromatographic based separation techniques coupled with tandem mass spectrometry served among the most powerful set of tools for identification and quantification of biochemicals due to their capability of analyzing potentially thousands of different constituents from a single sample. As such, gas chromatography-mass spectrometry (GC-MS)[5] and especially liquid chromatography-mass spectrometry (LC-MS)[6,7] have been incorporated into various metabolomic studies to discover critical markers in CHO extracellular environment capable of improving growth and output titer productivity and further provides quantitative assessments of medium additives on cellular metabolism. On the other hand, different elemental analysis techniques have also been used in various studies of cellular physiology, including inductively coupled plasma-optical emission spectroscopy (ICP-OES)[8,9] for quantitative analysis of calcium, phosphorous and silicon to evaluate the level of ion release, and inductively coupled plasma-mass spectroscopy (ICP-MS)[10] for unraveling the effect of iron addition on both antibody productivity and oxidative stress in CHO culture. Though being extensively incorporated in various cell line engineering and cell culture studies along with other transcriptomic and proteomic tools, to the best of our knowledge, application of these metabolomics techniques to evaluate cell culture media currently remains understudied.

The Advanced Mammalian Biomanufacturing Innovation Center[1] (AMBIC) was formed to address a common problem in biomanufacturing research. Namely, that research outcomes are highly dependent on cell line and media composition and those are often proprietary, making it difficult for people across the research, business, and regulatory community to interpret published research. AMBIC uses common cell lines and media formulations accessible to all members to perform biomanufacturing research, thereby addressing the problem around publishing research based on proprietary information.

This work aims to characterize media precipitate formed in an early version of AMBIC's feed media, and by doing so, summarize the analytical techniques available for characterization. The characterization steps are critical for understanding the problem and developing solutions. Ultimately, by understanding the precipitation process in cell culture media, preventive steps can be taken leading to a more consistent process as the uncertainty around media composition due to precipitation is removed. Furthermore, this allows better process logistics where media preparation and process scheduling can be made such that precipitation is avoided.

## 2 | MATERIALS AND METHODS

### 2.1 | Overview of strategy

In this paper, numerous analyses were carried out to identify and characterize the components of solid media precipitate. The analytical techniques used were grouped into qualitative and quantitative categories. The quantitative techniques were further categorized by techniques that target organic or inorganic analytes. A summary of all analytical analysis conducted in the study and their rationale was tabulated in Table 1.

### 2.2 | Precipitate collection

Prior to the analysis, solid precipitate was isolated from the media using a two-step method: (i) collection via gravitational force and (ii) drying via centrifugal force. Since the precipitate was collected directly from the media bottle, excess liquid retained by the precipitate remains a challenge when using gravitational filtration and centrifugal filtration introduces a volume handling problem (media bottles were either 500 ml or 1000 ml). Therefore, two-step filtering was used where gravity separated most of the volume and a centrifuge separated the residual to minimize any further precipitation due to evaporation.

The gravitational filter system consisted of a filter disk (Buchner Funnel Filter Paper, Thomas Scientific, Swedesboro, NJ), glass funnel and 250 ml Erlenmeyer collecting flask. The centrifugal filter system consisted of membrane-inserted spin column (F-spin column, Epigentek, East Farmingdale, NY) inserted on top of a 1.5 ml flex microcentrifuge tube (Eppendorf, Hamburg, Germany). Ultrapure water produced by MilliQ was used for all analytical analysis in this work. Solid precipitate was transferred to the centrifuge tube with the membrane inserted, after which the tube was spun down at 21,300 RCF (Relative Centrifugal Force) in a refrigerated centrifuge (Eppendorf Centrifuge 5424R) for 5 min at 4°C (note that a refrigerated centrifuge is not necessary). For the purpose of further analysis, it is assumed that any mass loss during precipitate collection is negligible, that is, the mass of the final dry precipitate is taken as being the same as the original material that precipitated in the media bottle.

The impact of mixing the solid precipitate sample was also tested. This is important since a representative solid sample cannot be taken for granted as is often the case for liquid sampling. The mixing process was performed by taking dry solid which was previously extracted from liquid media and carefully grinding and stirring with a mortar and pestle. For clarity, throughout the paper samples subjected to the grinding and mixing process will be referred to as "pretreated". It should be noted that all quantitative analyses were performed on pretreated samples. Some qualitative analyses were done on samples

**TABLE 1** Summary of all analytical analysis and their respective rationale

| Analytical technique | Rationale |
|---|---|
| Qualitative – Thermogravimetric analysis (TGA) | Preliminary analysis that can determine relative amount of organic and inorganic components based on the general principle that organic components are volatile at much lower temperature than inorganic components. These results can inform which subsequent quantitative analyses are appropriate. |
| Qualitative – Differential scanning calorimetry (DSC) | Preliminary analysis that can identify the presence of crystalline and/or amorphous material in the precipitate. These results can inform if X-ray diffraction is appropriate since it can only be performed on crystalline samples. |
| Qualitative – X-ray diffraction (XRD) | Directly identifies phases present in the sample by matching diffraction patterns in the sample to a database. XRD is nondestructive and does not require sample preparation removing the potential for chemical transformation affecting the results. XRD can potentially be quantitative but with sources of uncertainty that must be carefully considered. |
| Qualitative – Scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDS) | Directly identifies elements at a specific location of a particle under the microscope. The elemental breakdown of a specific particle/crystal can give clues about its identity but cannot be considered representative without analyzing a large number of particles. |
| Quantitative – Inductively coupled plasma-optical emission spectroscopy (ICP-OES) | Flexible and accurate method for elemental analysis. Due the atomization of the sample during analysis, ICP-OES can only provide the total elemental composition and cannot be used to trace which elements came from which molecules in the sample. |
| Quantitative – Inductively coupled plasma-mass spectroscopy (ICP-MS) | Similar analysis to ICP-OES, provides greater resolution and accuracy but at the cost of more involved method development. If trace elements are critical to the cell culture process the extra effort may be justified. |
| Quantitative – Nuclear magnetic resonance spectroscopy ($^1$H NMR) | Inherently quantitative method that can be used for all organic molecules. In complex sample matrices, the overlap of peaks can make data analysis difficult. |
| Quantitative – Liquid chromatography-mass spectrometry (LC-MS) | The use of a LC column can make analysis highly specific, removing the major limitation with $^1$H NMR, that is, peak overlap. The major drawback is the effort involved in assay development for specific molecules. |

that were not pretreated but this should not affect any conclusion from those results.

## 2.3 | General analysis

### 2.3.1 | Thermogravimetric analysis

Preliminary thermogravimetric analysis (TGA) of the precipitate sample was performed using a TA Instruments TGA5500 thermogravimetric analyzer. Incorporation of TGA into the study allows qualitative assessment of the organic composition of the solid precipitate since organic compounds usually decompose at lower temperature than inorganic compounds.[11] The observed weight loss due to heating can be attributed to the decomposition of organic compounds allowing a rough weight percentage (wt. %) of organic versus inorganic to be measured.[12] As there has not been any previously published work on the characterization of solid precipitation, the experiment was carried out in the presence of nitrogen (inert) and in the presence of air (reactive). For both analyses, high-temperature aluminum pans (pan volume 10 μl) were used. Samples were heated from 25°C to 1000°C and the flow rate of nitrogen and air was set to be constant at 50 ml·min$^{-1}$. Data obtained from the TGA experiment were used to estimate percentage of organic/inorganic of the samples which could then inform decisions regarding further quantitative analysis.

### 2.3.2 | Scanning electron microscopy-energy dispersive X-ray spectroscopy

While TGA can give an estimate of the organic and inorganic content, scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDS) can provide elemental composition information. SEM-EDS was conducted on a JEOL JSM 7401F instrument with a chamber-mounted Everhart-Thornley type secondary electron detector. X-ray microanalysis using EDS for elemental composition analysis was conducted on an EDAX Genesis XM2 Imaging System composed of a 10 mm$^2$ Si (Li) detector with SUTW window for detection of all elements. While SEM-EDS can give quantitative results, it is only specific to the spot at which the X-ray beam is directed. This makes it a powerful tool for investigating the composition of individual crystals in the precipitate, but it is difficult to get quantitative results that are representative of a collected sample. Therefore, it is used here as a qualitative method to support and better inform other analyses.

## 2.4 | Phase identification

### 2.4.1 | Differential scanning calorimeter

Crystallinity of the sample was studied using a TA instrument differential scanning calorimeter (DSC2500) DSC coupled with a RCS90 cooling system. It was unclear how the kinetics of any crystallization

event would affect the heat flow. Therefore, two heating rates (fast and slow) were implemented to ensure any endothermic or exothermic event was not missed due to their kinetics. The fast-ramping experiment ramped up to 400°C at 20°C·min$^{-1}$, followed by a cooling cycle at 10°C·min$^{-1}$ down to 0°C, then a second ramping up to 400°C at 10°C·min$^{-1}$. The slow ramping experiment ramped up to 400°C at 10°C·min$^{-1}$, followed by a cooling ramp of 5°C·min$^{-1}$ down to −90°C, then ramping up to 400°C at 5°C·min$^{-1}$. In the slow ramping case, a minimum temperature of −90°C was used to provide an extreme driving force for crystallization to address the potential of a metastable zone preventing crystallization.

#### 2.4.2 | X-ray diffraction

XRD was a primarily qualitative method to identify the solid phases in the precipitate. The appeal of XRD is that it is a nondestructive way of directly analyzing the solid without sample preparation, save for mounting the sample. This removes some of the uncertainty involved when using methods that analyze liquid samples, since once the sample is dissolved, the identity of any metal salts that precipitated will be lost. Furthermore, the potential for further reactions is introduced which may change the identity of other components. The analysis was performed using a Rigaku SmartLab X-ray diffractometer at the Massachusetts Institute of Technology Materials Research Laboratory.* Data was collected in both the Bragg-Brentano configuration and using a capillary stage to avoid any potential preferred orientation issues in the sample. The scan range was 5–80° and the scan time ranged from 15 min to 2 h.

### 2.5 | Organic analysis

#### 2.5.1 | Nuclear magnetic resonance spectroscopy

Identification of different organic phases was carried out via $^{1}$H NMR analysis of the sample using a JEOL ECZ 400 (400 MHz, 5 mm broad band probe). For the analysis, 6 mg of sample was diluted in 0.1 N HCl solution. Sodium trimethylsilylpropanesulfonate (DSS) at 0.5 mM and difluorotrimethylsilanylphosphonic acid (DFTMP) at 2 mM were incorporated as internal standard (IS) and pH indicator in the final solution, respectively. All data was processed through JEOL Delta v5.3 software. The processing parameter was set to proton mode with autogain enabled. The processing solvent was selected to be deuterated water. In this analysis, $^{1}$H NMR was used as a first attempt for quantification of the organic components since there is less method development associated with this analytical method. The complex nature of cell culture media meant that a more specific method incorporating chromatography might also be needed.

#### 2.5.2 | Liquid chromatography-mass spectrometry

LC-MS came into consideration as an analytical method due to the incorporation of chromatography allowing compounds to be separated prior to analysis for better peak resolution. Furthermore, LC-MS has been used for amino acid quantification in cell culture samples meaning existing methodologies could be directly applied minimizing the need for method development.[13] Amino acid standards (Sigma Aldrich, St. Louis, MO) were used for construction of calibration curves where appropriate. LC-MS grade chemical solvents and reagents (Thermofisher Scientific, Waltham, MA) were used. Individual amino acid standard was injected into an Intrada Amino acid column at 40°C (3 μm, 100 × 3 mm, IMTAKT, Japan). The detailed analytical conditions are shown in Table 2.[13]

#### 2.5.3 | Internal standards

Initial LC-MS analysis of the solid precipitate sample was conducted using existing amino acid calibration curves from previous work.[8] It was found that the curves for phenylalanine and tyrosine were not adequate for accurately quantifying the precipitate. Thus, IS were incorporated into the quantification assay for tyrosine and phenylalanine to account for any variability due to sample preparation and thus.

IS for amino acid assays are typically very expensive due to the use of labeled monoisotopes. However, the media formulation investigated here contained no alanine and the amino acid assay can detect alanine without any additional method development. This presents alanine as an ideal candidate as a low-cost alternative to typical monoisotope IS. Another advantage of incorporating alanine as an IS is that the methyl side chain of alanine is widely known as inactive and, hence, is a stable compound in solution that generally can avoid the risk of being methylated into a different compound.[14] However, we appreciate that this method cannot be applied to samples from cell culture (since the cells may produce or consume the alanine changing the concentration) or media formulations that may contain alanine as an unknown.

The IS calibration curves were constructed by dissolving the amino acid standard compound in 0.1 N hydrochloric acid (HCl) solution to obtain individual stock solutions of 180 mM phenylalanine, 32 mM tyrosine, and110 mM alanine. The standards were vortexed at room temperature to allow dissolution. Tyrosine calibration standard solutions were prepared at concentration ratio of tyrosine/alanine ranging from 3 to 5 with 0.5 increments. Each tyrosine standard solution was spiked with alanine so that the final concentration of alanine in each standard is 3 mM. Phenylalanine calibration standard solutions were prepared at concentration ratio of phenylalanine/alanine (0.1, 0.15, 0.25, 0.5, 0.75, 1). Each standard solution was spiked with alanine so that the final concentration of alanine in each standard solution is 10 mM. For analysis, 6 mg of solid sample was dissolved with 110 mM alanine stock solution and 50:50 (%v/v) mixture of 0.1 N HCl and MilliQ water to 600 μl total volume of 3 mM alanine.

It should be noted that the raw LC-MS results are for the concentration of the amino acid in the prepared sample that was injected. When reporting results here, it is more convenient to use the weight percentage of the analyte in the solid sample since that is ultimately the composition of interest in this work. The equation used to perform this conversion is below (Equation 1).

**TABLE 2** LC-MS operating condition and input parameters

| Instrument | Shimadzu LC-MS/MS 8040 Triple Quad |
|---|---|
| Column | Intrada amino acid (3 μm, 100 × 3 mm, IMTAKT) |
| Software | Labsolutions (v 5.72, Shimadzu, Japan) |
| LC specifications | |
| Mobile phase A[b] | Acetonitrile/100 mM Ammonium formate = 20/80 (% v/v) |
| Mobile phase B[d] | Acetonitrile/Formic acid = 100/0.3 (% v/v) |
| Gradient program | B 20% (0 min)–B 20% (4 min)–B 100% (14 min)–B 100% (16 min)–B 20% (16.1 min)–B 20% (18 min) |
| Flow rate | 0.6 ml·min$^{-1}$ (5 Mpa) |
| Column temperature | 40°C |
| Injection volume | 10 μl (1000 nmol·ml$^{-1}$ [= 1 mM], 0.1 N HCl) |
| MS specifications | |
| Ionization mode | ESI (negative and positive) MRM and SIM |
| Nebulizer gas flow | 3 L·min$^{-1}$ |
| Drying gas flow | 15 L·min$^{-1}$ |
| DL temperature | 300°C |
| Heat block temperature | 500°C |

Abbreviation: LC-MS, liquid chromatography-mass spectrometry.

$$wt.\%_{analyte} = \frac{m_{analyte,sample}}{m_{solid\ sample}} \times 100 = \frac{C_{analyte,sample} V_{prepared\ solution} MW_{analyte}}{m_{solid\ sample}} \times 100, \quad (1)$$

where $wt.\%_{analyte}$ is the weight percentage of the analyte in the solid precipitate [dimensionless], $m_{analyte,sample}$ is the mass of the analyte in the solid sample taken from the precipitate [mg], $m_{solid\ sample}$ is the mass of the solid sample taken from the precipitate (mg), $C_{analyte,sample}$ is the concentration of the analyte in the prepared solution that is injected to the LCMS (mM), $V_{prepared\ solution}$ is the volume of the prepared solution that is injected (L), and $MW_{analyte}$ is the molecular weight of the analyte (mg·mmol$^{-1}$). Note that this would assume 100% of the analyte in the starting solid sample is present in the final prepared solution.

## 2.6 | Inorganic analysis

### 2.6.1 | Inductively coupled plasma-optical emission spectroscopy and inductively coupled plasma-mass spectroscopy

Identification and quantitation of the inorganic components of the solid precipitate was carried out with ICP-OES and ICP-MS. The inorganic analysis was initially developed based on ICP-OES. Later in the study, ICP-MS was also incorporated into the study as an alternative to ICP-OES due to the lack of fundamental source of continuum background in ICP-MS that allows detection of analyte at lower concentration range.[15,16] In this study, ICP-MS analysis was conducted based on a protocol previously developed.[10] ICP-MS Agilent 5110 ICP-OES (Agilent Technologies) and Agilent 7900 ICP-MS with SPS4 Autosampler was used for single element detection. Calibration standards were purchased from Sigma Aldrich at a stock concentration of 1000 mg·ml$^{-1}$. All calibration standards are TraceCERT™ graded, and suitable for use with ICP. A total of 11 different calibration standards were prepared with concentration gradient as illustrated in Table 3. Each ICP-OES and ICP-MS measurement was done in triplicate measurement.

**TABLE 3** ICP element standards and calibration range

| Standards | Concentration range (μg·ml$^{-1}$) |
|---|---|
| Ca, Co, Cu, Fe, K, Mg, Mn, Na, P, S, and Zn | 0 |
| | 0.01 |
| | 0.1 |
| | 1 |
| | 10 |
| | 50 |

Abbreviation: ICP, inductively coupled plasma.

The solid samples were completely dissolved with 70% trace-metal graded nitric acid. Nitric acid was selected due to its ability to form stable and soluble salts. For solid analysis, samples were digested with 1 ml of concentrated 70% $HNO_3$ in a heating block at 70°C, then diluted to 10 ml total volume with water. For ICP-MS and ICP-OES, the calculated mass of a chemical compound in a sample can also be obtained similar to Equation 1 as shown below (Equation 2):

$$m_{analyte,sample} = C_{analyte,sample} V_{prepared\ solution}, \quad (2)$$

where $m_{analyte,sample,ICP}$ is the mass of the analyte in the solid sample taken from the precipitate [mg], $m_{solid\ sample,ICP}$ is the mass of the solid

sample taken from the precipitate (mg), $C_{analyte}$ is the concentration of the analyte in the prepared solution that is injected and measured through ICP (mg·ml$^{-1}$), $V_{prepared\ solution}$ is the volume of the prepared solution that is injected for ICP-OES or ICP-MS (ml).

## 2.7 | Mass loss calculation

Prior to quantitative analysis, all solid precipitate from one bottle was extracted and treated as described previously to ensure content uniformity and representative sampling. The total precipitate weight collected from a given bottle is measured. The mass of each analyte lost from solution due to precipitation can then be calculated from the LC-MS, ICP-OES, and ICP-MS results assuming the analyzed samples are representative of the whole:

$$m_{analyte,bottle} = \frac{wt.\%_{analyte}}{100} \times m_{total\ precipitate}, \quad (3)$$

where $m_{total\ precipitate}$ is the mass of the total collected precipitate from a given bottle of media from which the samples were taken for subsequent analysis (mg). The mass concentration of each analyte per bottle ($C_{analyte,bottle}$ [mg·ml$^{-1}$]) can be determined:

$$C_{analyte,bottle} = \frac{m_{analyte,bottle}}{V_{bottle}}, \quad (4)$$

Finally, the percentage weight loss of each analyte due to precipitation can be calculated:

$$wt\ loss\%_{analyte} = \frac{C_{analyte,bottle}}{C_{analyte,formulation}}, \quad (5)$$

Here, the concentration of each analyte from the formulation ($C_{analyte,formulation}$ [mg·ml$^{-1}$]) in Equation 3 was given by the manufacturer.

# 3 | RESULTS AND DISCUSSION

## 3.1 | Phase identification

In preparation for XRD analysis for phase identification, DSC was performed to ascertain the sample crystallinity and therefore its suitability for XRD.

### 3.1.1 | Differential scanning calorimeter

Preliminary DSC results for three replicate runs of heterogeneous samples – replicate 1 (2.1 ± 0.1 mg), replicate 2 (2.5 ± 0.1 mg) and replicate 3 (2.5 ± 0.1 mg) are shown in Figure 1. All replicates (1, 2 and 3) showed a distinct melting event around 300°C. In addition, replicate 1 showed some endothermic activities around 150°C which was attributed to residual moisture in the sample. To address this, the other samples were left in an oven at a gentle temperature of 37°C for at least 24 h before analysis. Replicate 2 and 3 did not exhibit the same endothermic behavior at 150°C suggesting the hypothesis of residual moisture in the sample was correct.

Furthermore, there was no obvious sign of a glass transition (usually indicated by an inverse sigmoid shape). This observation coupled with the clearly observable melting events suggest the sample is crystalline enough for XRD analysis to yield usable data for phase identification. The absence of any exothermic event which serves to indicate recrystallization is an unexpected result, and the smaller minima next to the minimum at 300°C is also unexplained. One hypothesis is that the heat allowed a chemical transformation to take place changing the material properties so that crystallization was not possible within the experimental parameters. However, the main purpose of this analysis was to determine the material's crystallinity, and further analysis to address these unexpected observations were considered out of scope for this work.

## 3.2 | General analysis

### 3.2.1 | Thermogravimetric analysis

The TGA analysis results are reported in Figure 2. For both environmental conditions, the first major weight loss happened at approximately 150°C. There was less degradation observed when the sample was heated under nitrogen (12.1% remaining) vs. air (2.8% remaining). It is speculated that first observed mass loss between 100°C and 200°C corresponds to bound water in the sample (i.e., not residual moisture), as previously demonstrated in comparable work.[17,18] Additionally, the consistent onset temperature (around 150°C) of the weight loss suggests it is not affected by the environment. This further supports the hypothesis that the first observed mass loss was due to the loss of bound water molecules. The other mass loss events, indicated by changes in the gradient, are clearly affected by the environment. The approximate breakdown of bound water, organic compounds, and inorganic compounds in the precipitate is presented in Table 4.

The main purpose of TGA was to provide a rough estimation of the organic and inorganic content of the precipitate. Therefore, the root causes of the differences between both mass loss profiles, and the identification of compounds which could be attributed to specific mass loss events were not interrogated further. Coupling TGA with a mass spectrometer or Fourier transform infrared spectroscopy can be a powerful technique if the TGA analysis was to be taken further.[19]

### 3.2.2 | Scanning electron microscopy-energy dispersive X-ray spectroscopy

The results obtained from the SEM analysis are shown in Figure 3. Surface morphology reveals the crystalline structure of much of the

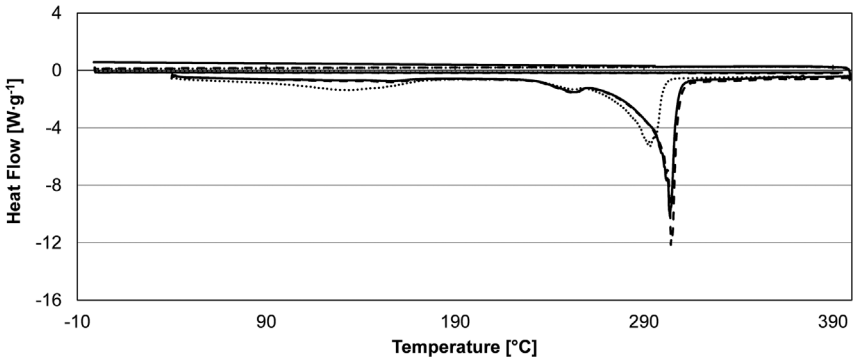


**FIGURE 1** Differential scanning calorimeter (DSC) thermogram of the precipitate. Here, the solid precipitate sample was exposed to under different heating rates: (i) Replicate 1: 27–400°C at 20°C·min$^{-1}$, 400–0°C at 10°C·min$^{-1}$, 0–400°C at 10°C·min$^{-1}$, (ii) Replicate 2 and 3: 27–400°C at 10°C·min$^{-1}$, 400−−90°C at 5°C·min$^{-1}$, −90–400°C at 5°C·min$^{-1}$

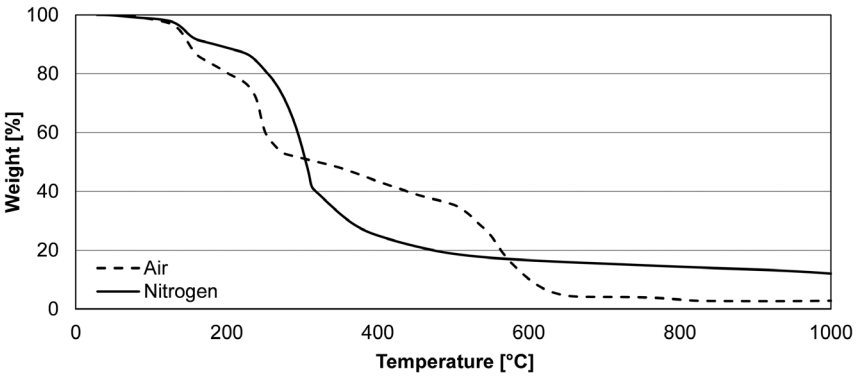


**FIGURE 2** Thermogravimetric analysis (TGA) mass loss plot of the precipitate. The solid precipitate sample was heated from 25 to 1000°C under air and nitrogen at constant flow rate of 50 ml·min$^{-1}$

media precipitation. The crystal morphology in Figure 3(a)–(d) shows long aspect ratio crystals throughout the sample. Elemental composition at specific points on individual crystals was revealed through EDS and the results are shown in Figure 3(e). EDS analysis showed the analyzed crystals to be made up of mostly carbon, nitrogen, and oxygen with trace amounts of magnesium, phosphate, sodium, and chlorine. This result supports the TGA analysis which suggested the precipitate is largely organic. Note that while EDS yields a quantitative elemental analysis for specific individual crystals, that cannot be extended to the sample as a whole without analyzing a restrictively large number of crystals. Therefore, the technique was applied here only for qualitative insights into the precipitate properties.

### 3.2.3 | X-ray diffraction

The data generated from the Rigaku SmartLab for nonpretreated samples from two different manufacturers of the media are shown in Figure 4. The crystallinity suggested by DSC and SEM was confirmed by the clear observable peaks in Figure 4. Furthermore, the scans of both samples are almost identical. This is not a surprising result since the formulation is the same for both manufacturers. The results suggest that are no major phases present in one sample that is not present in the other.

**TABLE 4** Approximate organic and inorganic content of the precipitate

| Components | Composition (%) |
|---|---|
| Bound water | 8%–13% |
| Volatile content (assumed organic content) | 80%–85% |
| Nonvolatile content (assumed inorganic content) | Less than 13% |

Phase identification via XRD takes place by comparing the sample scan to a database of scans for known phases using phase identification software. This analysis took place with Highscore Plus and using the commercial ICDD Organics PDF4+ 2020 database for organic components and ICDD PDF4+ 2020 database for inorganic components. While opensource databases are available (Crystallography Open Database), the quality of the scans in the database is not verified making commercial databases a better option if available. When performing the phase identification, it was clear that the best match between the sample and the database was for tyrosine. In fact, when performing a whole pattern matching analysis using the Pawley method,[20] the tyrosine database scan was a near identical fit as seen in Figure 5. This is not to suggest that the sample is 100% tyrosine, just that there were no major peaks in the sample could not be accounted for by tyrosine.

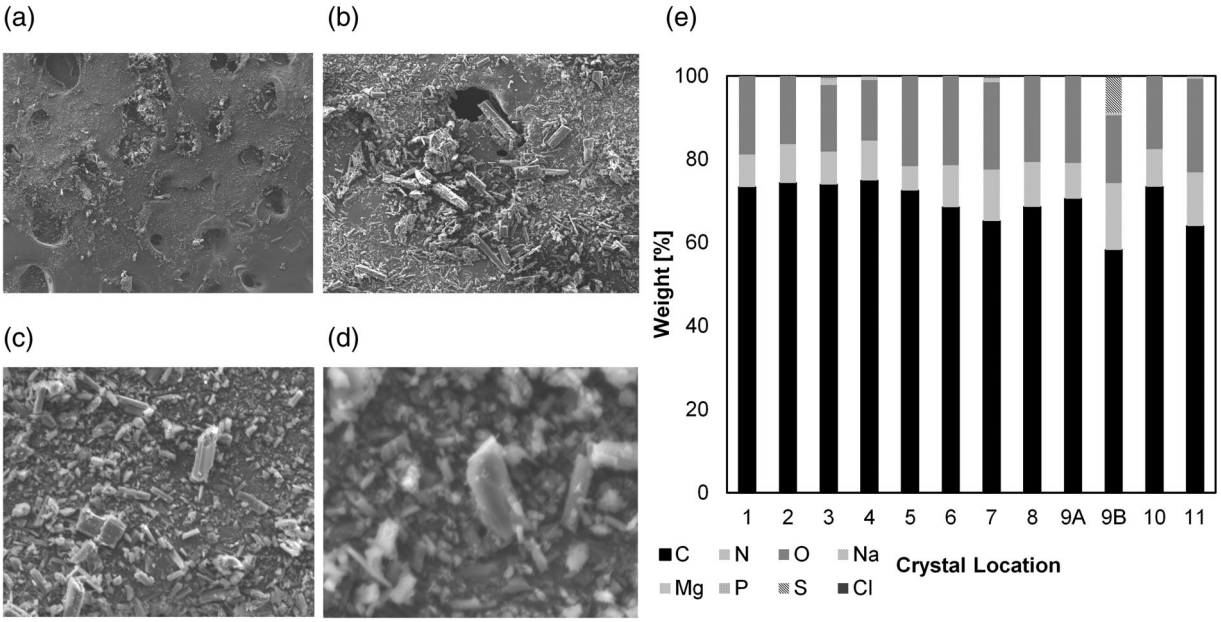


**FIGURE 3** Inorganic analysis of the precipitate. (a)–(d) Scanning electron microscopy (SEM) surface morphology. (e) Energy dispersive X-ray spectroscopy (EDS) elemental composition. As shown here, crystal morphology of the solid precipitate observed under SEM at different zoom levels: (a) 100×, (b) 400×, (c) 1500×, and (d) 5000×. Elemental composition at 12 locations on the surface of 11 crystals, and the weight percentage of elements each location is shown (e)

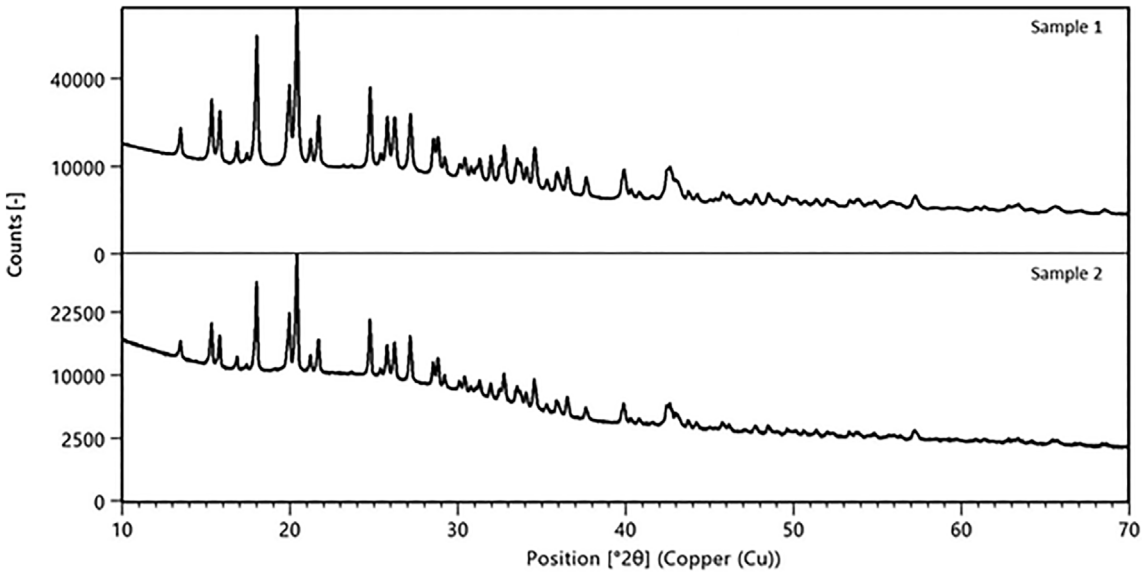


**FIGURE 4** X-ray diffraction (XRD) diffraction patterns of the precipitate. The analysis was conducted on samples obtained from Vendor A (sample 1) and Vendor B (sample 2) using a capillary stage in transmission XRD mode for a 21-minute scan time

The results show that XRD can be a powerful qualitative method for phase identification, but only for major components. For minor, or trace components, a more sensitive analytical method is needed. It should also be noted that minor components in the precipitate do not necessarily mean they will have a negligible impact on cell culture. For example, some critical components are added to cell culture media at ppm and ppb levels, so any loss of these components due to precipitation could have significant consequences for cell culture.

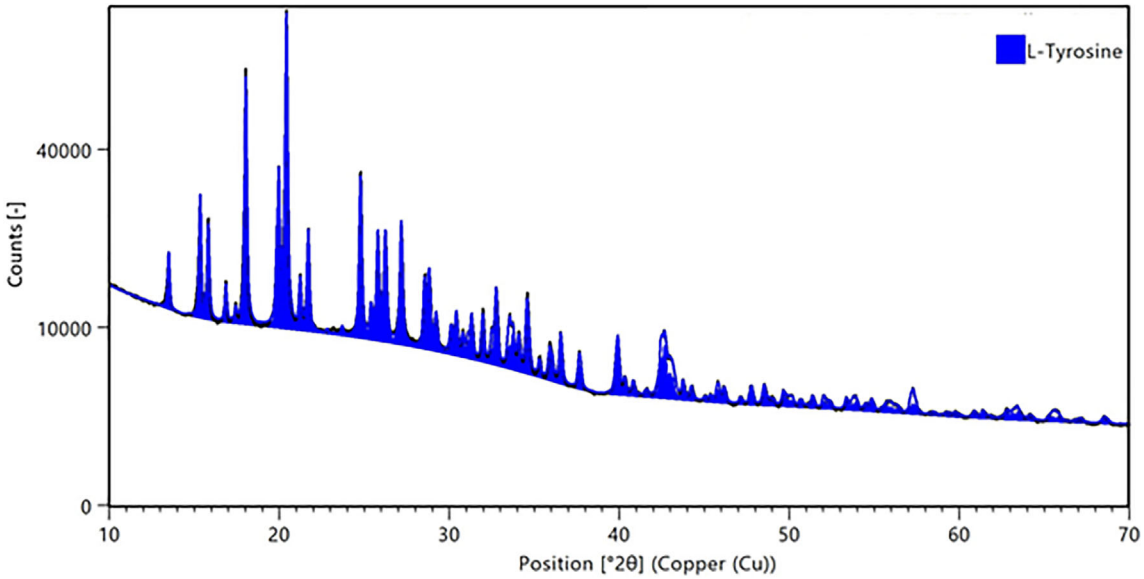


**FIGURE 5** X-ray Diffraction (XRD) whole pattern matching of the precipitate. Here, the analysis was conducted on a sample collected from bottle commercialized by Vendor A using the Pawley method[20]

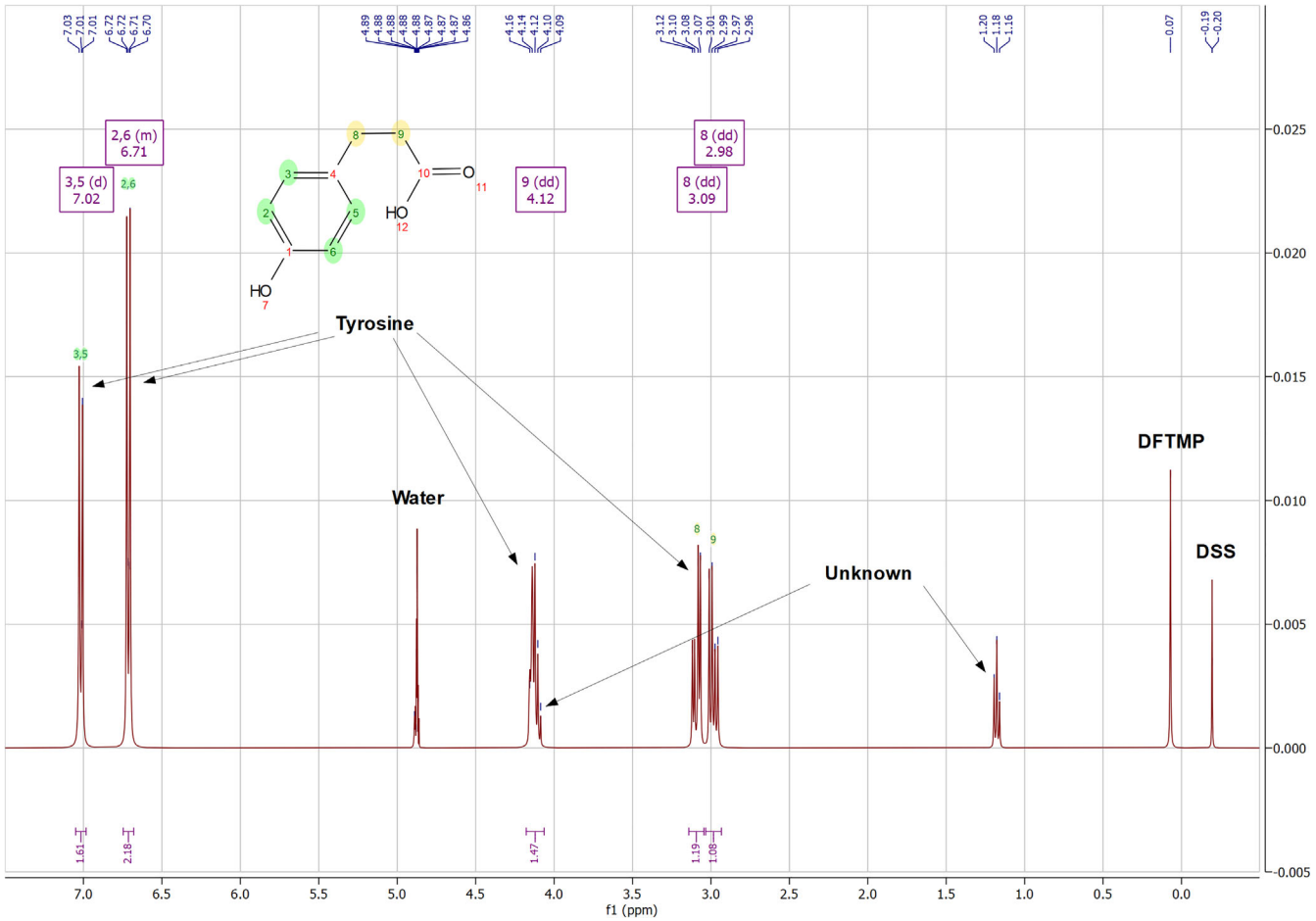


**FIGURE 6** $^{1}$H NMR chromatogram of the precipitate. Here, sodium trimethylsilylpropanesulfonate (DSS) (0.5 mM) and difluorotrimethylsilanylphosphonic (DFTMP) (2 mM) were incorporated into the analysis as IS and pH indicator, respectively

## 3.3 | Organic analysis

### 3.3.1 | Nuclear magnetic resonance spectroscopy

$^1$H NMR spectra of the solid precipitate is shown in Figure 6. Readers interested in the background and further information on interpretation of amino acid NMR results are referred to Kellenbach et al., (2008) for a detailed review.[21] Processed NMR spectra reveals the presence of an aromatic compound indicated by the presence of two multiplets downfield (6.632–6.653 ppm and 6.935–6.956 ppm). Additionally, three different doublets of doublets can also be found in the NMR spectra (4.051–4.032 ppm, 3.011–2.996 ppm, and 2.940–2.992 ppm). Since a similar splitting pattern from tyrosine was previously reported in the HMDB (Human Metabolome Database),[22] and thus the presence of tyrosine is structurally confirmed through $^1$H NMR.

It is important to note that the intensity of the aromatic multiplets from tyrosine is almost three time as much as the intensity of the DSS IS (−0.266 ppm). This would suggest the abundant presence of tyrosine in the precipitate sample as also suggested in XRD. However, due to the possible contribution of peaks from different compounds in the solid precipitate that could not be separated this cannot be treated as an accurate quantification method. For example, although it was confirmed that the splitting pattern between 3.011–2.996 ppm was due to the predominant presence of tyrosine in the solid precipitate, cysteine which is another component of liquid media also expresses a similar multiplet splitting pattern in this region.[22] Thus, it is difficult to deconvolute the pattern for accurate quantification. Software packages can be used to perform this deconvolution through the use of reference databases (e.g., CHENOMX) but this was not pursued as part of this work.

**TABLE 5** List of amino acids analyzed in this study and their corresponding method of quantification

| Amino acid | Data quality | Concentration detected in sample (mM) |
|---|---|---|
| Tyrosine | High | $3.6 \times 10^{1} \pm 3\%$ |
| Phenylalanine | High | $2.1 \times 10^{0} \pm 6\%$ |
| Arginine | Medium | $2.4 \times 10^{-1} \pm 3\%$ |
| Glutamine | Low | $2.2 \times 10^{-2} \pm 1\%$ |
| Histidine | Low | $1.0 \times 10^{-1} \pm 2\%$ |
| Lysine | Low | $4.0 \times 10^{-2} \pm 2\%$ |
| Methionine | Low | $2.7 \times 10^{-2} \pm 3\%$ |
| Threonine | Low | $1.0 \times 10^{-1} \pm 2\%$ |
| Tryptophan | Low | $7.2 \times 10^{-2} \pm 1\%$ |
| Valine | Not detected | N/A |
| Asparagine | Not detected | N/A |
| Cystine | Not detected | N/A |
| Proline | Not detected | N/A |
| Aspartic acid | Not detected | N/A |
| Threonine | Not detected | N/A |
| Serine | Not detected | N/A |
| Glutamic acid | Not detected | N/A |

*Note:* High quality data involves calibration curves and samples with internal standards. Medium quality data involves interpolating from existing calibration curves without an internal standard. Low quality data involves extrapolating existing calibration curves without an internal standard.

### 3.3.2 | Liquid chromatography-mass spectrometry

LC-MS can provide more specificity by using chromatography to separate molecules prior to analysis, thereby removing the potential for peak overlap when analyzing a complex mixture. As mentioned in the methods section, new calibration curves with IS were used for tyrosine and phenylalanine whereas existing curves were used for the others.[13] For most of the amino acids, their concentrations were below the minimum value of the existing calibration curves. However, due to their low concentration in comparison to tyrosine and

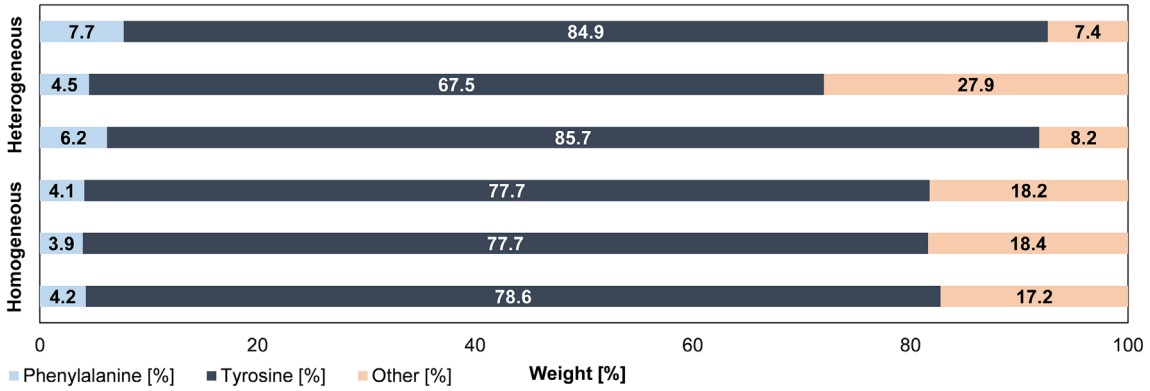


**FIGURE 7** Comparison of solid precipitate composition between pretreated and nonpretreated samples. Here, the analysis was conducted to assess how mixing the solid via grinding in a mortar and pestle affects sampling

phenylalanine, the increase in accuracy from recreating the calibration curves was not deemed significant. Therefore, the results presented for those compounds are extrapolated from the existing calibration curves. In the event expected peaks could not be distinguised from the noise in the mass spectrum, the compound was assessed to be "not detected". Table 5 shows the concentration of amino acids in the analyzed sample of precipitate that was not pretreated by mixing and are reported with the relative standard deviation of technical triplicate measurements.

Representative sampling is critical for any quantitative analysis. In liquid systems this is easily achieved by shaking or agitating the mother solution from which the sample is taken. However, mixing solid samples is not as simple. To test a method for representative sampling, the impact of mixing was assessed by LC-MS. The solid collected from one bottle was split into two lots where one lot was mixed via grinding in a mortar and pestle. Three sample were then taken from each lot and analyzed via LC-MS (see Figure 7), where only phenylalanine and tyrosine were compared due to their high quality of quantification.

In addition, it was clearly shown from Figure 7 that at without pretreatment (grinding via mortar and pestle), representative sampling is not a valid assumption. Therefore, to ensure a valid quantification analysis can be carried out, the samples must be pretreated. This can be seen visually but is also seen when calculating the standard deviation of the pretreated results (0.1 wt.% and 0.4 wt.% for phenylalanine and tyrosine, respectively) and nonpretreated results (1.3 wt.% and 8.4 wt.%, for phenylalanine and tyrosine, respectively). The standard deviation increases 12-fold and 20-fold for the two measured amino acids further supporting the observation that without solid pretreatment, the samples cannot be considered representative.

Quantification of the amino acids must therefore proceed with pretreated samples. The concentration of all amino acids precipitated in a given bottle of the AMBIC feed media was quantified via LC-MS, from which their weight percentage can be calculated (Equation 1). The amino acid composition of the precipitate is presented in Table 6 where the relative standard deviation of technical triplicate measurements is also presented.

The media provided for AMBIC projects is manufactured by two vendors, here referred to Vendor A and Vendor B. A quantitative analysis was performed on the precipitate from the different vendors to provide some insight on the variability that arises from two manufacturers making the same formulation (see Figure 8). Note that all samples analyzed here were pretreated and therefore can be treated as quantitative. It can be seen that the composition of the precipitate is different between the two manufacturers. This is likely due to differences in impurities, the way the media was hydrated, the date on which the media was hydrated, and storage conditions. Ultimately, the thermodynamic conditions under which precipitation took place are likely different enough to produce precipitates with different compositions.

**TABLE 6** Media precipitate composition as measured by LC-MS

| Species | Weight (%) |
|---|---|
| Tyrosine | 78 ± 3% |
| Phenylalanine | 3.9 ± 6% |
| Arginine | 0.5 ± 3% |
| Glutamine | <0.1% |
| Histidine | 0.2 ± 2% |
| Lysine | 0.1 ± 2% |
| Methionine | 0.1 ± 3% |
| Threonine | 0.2 ± 2% |
| Tryptophan | 0.2% ± 1% |

Abbreviation: LC-MS, liquid chromatography-mass spectrometry.

### 3.3.3 | Inorganic analysis

For ICP-OES analysis, calibration standards were prepared to the concentration range as noted in Table 3. Samples for inorganic analysis were taken from the same bottle used for the quantitative LC-MS analysis. The concentration by mass of all elements detected through ICP-OES analysis was calculated and presented in Table 7. Here, sulfur was found to be the most abundant inorganic elements found in the precipitate, followed by sodium and magnesium. It should be noted that sulfur can also be present in organic molecules, however,

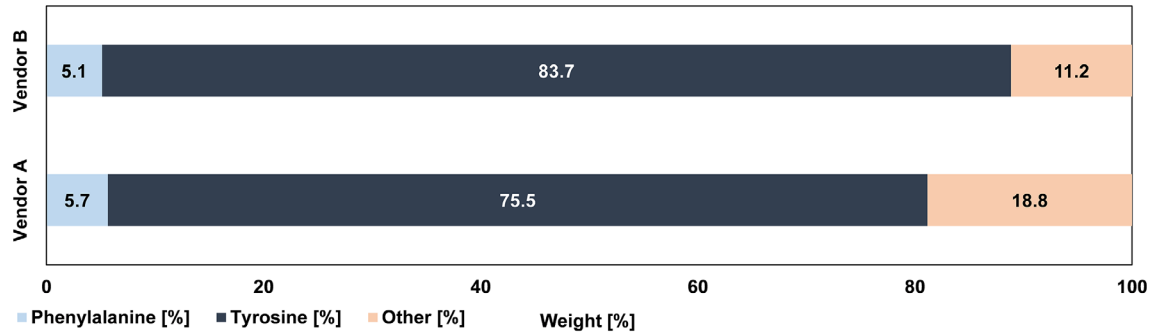


**FIGURE 8** Comparison of solid precipitate composition from different manufacturers. Here, samples were collected from bottles made of the same media formulation, but contracted through different vendors

**TABLE 7** Inorganic composition of solid media precipitate as measured by ICP-OES and ICP-MS

| Elements | wt.% from ICP-OES | wt.% from ICP-MS |
|---|---|---|
| Ca | Not detected | Not detected |
| Co | Not detected | N/A |
| Cu | Not detected | N/A |
| Fe | Not detected | $2.9 \times 10^{-7}$ ± 22% |
| K | Not detected | N/A |
| Mg | $1.3 \times 10^{-3}$ ± 1% | N/A |
| Mn | Not detected | Not detected |
| Na | $3.7 \times 10^{-2}$ ± 19% | N/A |
| P | $2.6 \times 10^{-3}$ ± 9% | N/A |
| S | $2.8 \times 10^{-1}$ ± 12% | N/A |
| Zn | Not detected | $4.3 \times 10^{-7}$ ± 7% |

*Note:* The relative standard deviation of technical triplicate measurements is also presented.
Abbreviations: ICP-OES, inductively coupled plasma-optical emission spectroscopy; ICP-MS, inductively coupled plasma-mass spectroscopy.

**TABLE 8** Percentage loss by mass of all detected compounds through precipitation

| Species | wt.% in precipitate sample | Percentage loss |
|---|---|---|
| Tyrosine[a] | 77.7 ± 3% | 15.8 ± 2% |
| Phenylalanine[a] | 3.9 ± 6% | 1.3 ± 7% |
| Sulfur[b] | $2.7 \times 10^{-1}$ ± 12% | $1.7 \times 10^{-1}$ ± 6% |
| Zinc[b] | $4.3 \times 10^{-3}$ ± 7% | $1.5 \times 10^{-1}$ ± 8% |
| Glutamine[b] | <0.10% | $1.3 \times 10^{-1}$ ± 1% |
| Histidine[c] | $2.1 \times 10^{-1}$ ± 2% | $7.8 \times 10^{-2}$ ± 2% |
| Iron[c] | $2.9 \times 10^{-7}$ ± 22% | $5.1 \times 10^{-2}$ ± 22% |
| Arginine[c] | $4.9 \times 10^{-1}$ ± 3% | $4.4 \times 10^{-2}$ ± 3% |
| Threonine[c] | $2.2 \times 10^{-1}$ ± 2% | $2.3 \times 10^{-2}$ ± 2% |
| Methionine[c] | $5.7 \times 10^{-2}$ ± 3% | $1.9 \times 10^{-2}$ ± 3% |
| Lysine[c] | $8.4 \times 10^{-2}$ ± 2% | $8.2 \times 10^{-3}$ ± 2% |
| Tryptophan[c] | $1.5 \times 10^{-1}$ ± 1% | $5.9 \times 10^{-3}$ ± 1% |
| Sodium[c] | $3.7 \times 10^{-2}$ ± 19% | $5.7 \times 10^{-3}$ ± 6% |
| Magnesium[c] | $1.3 \times 10^{-3}$ ± 1% | $3.0 \times 10^{-3}$ ± 6% |
| Phosphorous[c] | $2.6 \times 10^{-3}$ ± 9% | $6.6 \times 10^{-4}$ ± 6% |
| Residual | 15.57% | — |

[a]Significant losses (more than 1 wt.%).
[b]Intermediate losses (1 wt.% to 0.1 wt.%).
[c]Minor losses (less than 0.1 wt.%).
*Note:* The uncertainty presented is the relative standard deviation of the measurements.

none of the major amino acids detected contain sulfur, and it was therefore assumed that the majority of the sulfur measured here came from metal sulfates.

The ICP-MS results for all the elements detected are also shown in Table 7. Here, only iron and zinc were detected out of four total scanned elements (iron, copper, manganese, and zinc). Interestingly, iron and zinc, which were previously not detected through ICP-OES, were detected with ICP-MS. This suggests that for critical metals in the formulation at low concentrations, ICP-MS may be needed to detect if it precipitated or not. If an element is detected in the supernatant and not the precipitate, it would provide enough evidence that any precipitation was negligible. Overall, elemental composition via ICP-OES in conjunction with ICP-MS allows identification and quantification of inorganic elements.

### 3.4 | Approximate composition and mass loss estimates

All quantitative analyses were performed on pretreated samples from a single bottle of media; therefore, the samples and weight percentages can be treated as representative of the precipitate formed in the bottle. The percentage loss of each component in the precipitate can then be calculated as well as the residual weight percentage (see Table 8). The percentage lost is an important calculation, because while it is useful to know the composition of the precipitate, it is far more relevant to know how much of component was lost from solution and is no longer available to cells during culture. Here, unsurprisingly, tyrosine shows the most amount of lost. Losses of other components were also listed and sorted in decreasing order. Due to the high presence of sulfur detected by ICP-OES, coupled with the fact that the chemical structure of tyrosine being $C_9H_{11}NO_3$ which lacks sulfur, it was reasonable to conclude most of the inorganic losses occurred through metal sulfates.

While the residual mass of the precipitate is quite high, with 15.57% of the mass being unaccounted for, it should be noted that only accurately quantifiable weight percentages are reported in Table 8. Recall from the TGA analysis that approximately 8%–13% of the precipitate is likely made up of bound water. Furthermore, the lighter elements associated with the metal salts were not detected by the ICP-OES or ICP-MS analyses (e.g., oxygen in $SO_4^{2-}$ and $PO_4^{3-}$, and any hydroxides) and would be part of the residual weight percentage. These two sources account for the residual mass within the degree of experimental uncertainty.

## 4 | CONCLUSION

In conclusion, different characterization methodologies were applied to characterize solid precipitate from CHO cell culture feed media. Simple analyses such as TGA, NMR, and XRD are sufficient to provide a general understanding of the precipitate (major component/most abundant phase, etc.), however, these techniques are not sensitive enough for detailed quantitative information. From the conducted analysis, ICP-OES/ICP-MS was adequate as a characterizing tool for analyzing elements that make up inorganic components in the precipitate. However, the identity of the components from which the elements came cannot be known due to the destructive nature of the analysis (the molecules are dissociated and atomized). From our study,

LC-MS was a good analysis technique to analyze amino acids and could potential be used for other organic molecules and/or molecules that do not dissociate upon dissolution. Developing a good LC-MS protocol is an exhaustive process, however, with a good LC and MS parameter condition (i.e., flow rate, buffer composition, elution gradient, etc.), LC-MS can serve as a powerful quantification tool for any media component analysis. In our study of solid precipitate collected from AMBIC feed media bottle, preliminary characterization of the solid precipitate through TGA, DSC and $^{1}$H NMR suggested the precipitate was made up mainly from organic compounds. LC-MS allowed both identification and quantification of up to ten different amino acids found in the solid precipitate, with tyrosine (approx. 77 wt.%) and phenylalanine (approx. 4 wt.%) being the major components. The residual weight percentage not accounted for by quantitative analysis was 15.57 wt.% which is largely bound water based on the TGA results.

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## CONFLICT OF INTEREST

All authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

**Duc Hoang:** Formal analysis; investigation; methodology; writing-original draft; writing-review & editing. **Shaun Galbraith:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; visualization; writing-review & editing. **Bingyu Kuang:** Methodology; resources; writing-review & editing. **Amy Johnson:** Project administration; resources; supervision; writing-review & editing. **Seongkyu Yoon:** Funding acquisition; project administration; resources; supervision; writing-review & editing.

## ENDNOTE

* https://xrd.mit.edu/

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/btpr.3188.

## DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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