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RESEARCH ARTICLE

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Characterization of soy protein hydrolysates and influence of its iron content on monoclonal antibody production by a murine hybridoma cell line

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Abstract

A challenging aspect with the use of protein hydrolysates in commercial manufacturing processes of recombinant therapeutic proteins is their impacts on the protein production due to a lack of understanding of batch-to-batch variability. Soy hydrolysates variability and its impact on fed-batch production of a recombinant monoclonal antibody (mAb) expressed in Sp2/0 cells were studied using 37 batches from the same vendor. The batch-to-batch variability of soy hydrolysates impacted cell growth, titer and product quality. Physicochemical characterization of batches confirmed that soy hydrolysates are mainly a source of amino acids and peptides containing lower amounts of other components such as carbohydrates and chemical elements in cell culture media. Soy hydrolysates composition of different batches was consistent except for trace elements. Statistical analyses identified iron as a potential marker of a poor process performance. To verify this correlation, two forms of iron, ferric ammonium citrate and ferrous sulfate, were added to a batch of soy hydrolysates associated to a low level of iron during cell culture. Both forms of iron reduced significantly cell growth, mAb titer and increased level of the acidic charge variants of the mAb. Consequently, trace element composition of soy hydrolysates or of all incoming raw materials might lead to significant impacts on process performance and product quality and therefore need to be tightly controlled.

KEYWORDS
antibody titer, batch variability, characterization, iron, soy hydrolysates

1 | INTRODUCTION

Plant hydrolysates, such as soy hydrolysates have been used in cell culture media or feed supplements to enhance the cell culture performance for large-scale production of recombinant therapeutic proteins, including monoclonal antibodies, as a substitution to animal-derived components. Soy hydrolysates are usually produced by controlled enzymatic hydrolysis of soy grits. They are mainly composed of peptides or amino acids, carbohydrates and minerals. Other components were reported in the literature in lower amounts such as saponins, isoflavones, phospholipids, fatty acids and Maillard reaction products.[1,2] The amounts of these components are subject to batch-to-batch variations that may affect the upstream process output.[1,3,4]

Observed variabilities of recombinant protein yield associated with different batches of soy hydrolysates can be attributed to several factors affecting soybean cultivation (geographic location, harvest and storage conditions) and the manufacturing process.[5,6] As a result, cell culture performance may vary significantly. Several researches have been conducted by the scientific community to better identify these parameters. Certain components present in protein hydrolysates (amino acids, lipids, vitamins, salts, etc.) could promote growth and cause the observed batch-to-batch variation.[1,7-10] Some causes of the

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batch-to-batch variability in performance were investigated and several publications pointed to the potential impacts of seasonal effect during the soybean cultivation or during the production process of soy hydrolysates.[5,6,11,12] The manufacturing process includes several steps from protein hydrolysis, filtrations steps, pasteurization and drying. Hartshorn et al. were able to correlate cell culture performance with the level of some components and changes in seven parameters of the hydrolysates manufacturing process over eight process steps.[5] Likewise, Lau et al identified process parameters which can influence the levels of 20 markers among amino acids, lipids, carbohydrates, nucleotides, peptides and xenobiotics that needed to be closely controlled to get a favorable hydrolysates composition.[11] One key manufacturing step (not disclosed) was found to significantly influence half of the identified shared performance indicators.

Following the observation of a significant variability in product titer due to changing the batch of soy hydrolysates at manufacturing scale of a commercial fed batch process, a systematical assessment of the performance of a given batch of soy hydrolysates before use at manufacturing scale was implemented, in parallel to a physico-chemical characterization of the batches tested. This approach allowed for a better understanding on the composition of soy hydrolysates, and finally the identification of potential composition change(s) in batches over the time. All those elements are presented in this article.

2 | MATERIALS AND METHODS

2.1 | Soy hydrolysates and experimental design

Thirty-seven batches of the same reference product of soy protein hydrolysates manufactured by the same vendor in a 6-year period were used in this study. The soy hydrolysates were stored at room temperature. Information obtained from certificate of analysis of 41 batches of soy hydrolysates were gathered.

2.2 | Media preparation for batch-to-batch comparison

For each experiment, the powder of the basal medium and other required supplements were dissolved in water at 37°C with stirring, except for soy protein hydrolysates powder. The same batch of raw materials was used during media preparations. The cell culture medium was then divided into several aliquots for testing up to 8 batches of soy protein hydrolysates including each time the same reference batch. The reference corresponds to the batch being used at manufacturing scale at the time of this study. Each cell culture medium aliquot was supplemented with batch of soy hydrolysates to a final concentration of 1.25 g/L. The fed-batch process includes a concentrated feeding solution of soy hydrolysates at 250 g/L dissolved at 37°C and stirred for 1 hr. Media and feed solution were 0.22 μm filtered aseptically and stored protected from light at 2 to 8°C. A few hours before their use, cell culture media were incubated at 37°C.

2.3 | Cell line and cell culture

The recombinant cell line derived from the murine hybridoma cell line Sp2/0 was used to produce the therapeutic monoclonal antibody. The cell cultures were maintained in an incubator at 37 °C in a humidified atmosphere with 10% CO_2 and an agitation of 130 rpm. Shake-flasks were fitted with an airy cap for gas exchange. Studies were performed in shake-flasks in triplicate with 40 mL working volume. Cell culture took place in two phases. During the cell expansion phase, the cell line was adapted to its culture medium in two passages with an interval of 2–3 days between passages. The production phase lasted 11 days. It corresponds to a phase of cell growth and production of the monoclonal antibody. Initial seeding density was targeted at 2.8×10^5 viable cells/mL. Samples were taken throughout the culture in order to monitor both cell growth and metabolism. During the production stage, cell culture was carried out in fed-batch mode with the sequential addition feed solutions. On the fourth day of culture, the culture medium was supplemented with a concentrated solution of soy hydrolysates at 10 mL per liter of cell culture medium. The additions of feed solutions were carried out after monitoring growth and metabolism on the second and fourth day of culture.

2.4 | Analytical methods for cell culture

The viable cell density and the cell viability were measured using a Vi-Cell analyzer. During the production phase, after cell counting, the rest of the sample was filtered at 0.22 μm to remove cells. Metabolites, such as glutamate, glutamine, lactate and glucose, and ammonium ions, were determined with the Nova Bioprofile 100+. At the end of cell culture, part of the supernatant was removed, filtered and stored at −80°C prior to determining the concentration of monoclonal antibody. Titer quantifications of the mAb produced were performed using a PA-HPLC (Protein A high performance liquid chromatography) method. Normalized total volumetric productivities calculated at the end of the cell culture were determined. Normalized volumetric productivity was expressed as a percentage and corresponds to the average of the total volumetric productivity compared to the chosen batch reference. Eventually, product quality was assessed after a first chromatography capture step on protein A, after the triplicate shake flasks content were pooled, centrifuged, and filtered. Analysis of the acidic charge variants was performed by CEX-HPLC (Cation exchange high performance liquid chromatography). The relative amount of acidic forms was determined as the sum of the peaks area eluted before the main peaks over the sum of all peaks area and were normalized.

2.5 | Iron supplementation assays

Cell culture medium and feed solution containing a batch of soy hydrolysates associated to a low level of iron (60 μg/g of soy hydrolysates or 60 ppm for batch 10) were spiked with ammonium ferric citrate (Merck Millipore) or with ferrous sulfate heptahydrate

(Sigma–Aldrich®) to a final concentration of iron of 80, 100, 130, 175, 250, 500 ppm. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder. These conditions were tested in shake flasks in triplicates as previously.

2.6 | Characterization of soy hydrolysates

Total amino acids in soy hydrolysates samples were quantified as descripted in the European pharmacopeia, method 2.2.56. Free amino acids in soy hydrolysates were measured by AccQ-tag ultra-high-performance liquid chromatography—diode array detector—tandem mass spectrometry. Molecular weight distribution was determined by size exclusion chromatography (SEC). Vitamins were quantified using a hydrophilic interaction chromatography column (HILIC) based approach on a UPLC-QDa instrument. Maillard reaction products such as carboxymethyllysine (CML), carboxyethyllysine (CEL) and furosine (Fur), and the cross-link amino acid lysinoalanine were qualitatively analyzed on Xevo®-QTof instrument. Monosaccharides and disaccharides analyses of soy hydrolysate samples (fructose, galactose, glucose, inositol, sucrose, lactose, maltose, raffinose and stachyose) were performed using HILIC and LC–MS instrument. Fatty acid analyses were performed on a GC–MS (Gas chromatography–mass spectrometry) instrument. For the screening of trace elements in soy hydrolysates, two different ICP-MS (Inductively Coupled Plasma Mass Spectrometry) devices and methods were used: a semi-quantitative overview analysis (Totalquant®) using quadrupole ICP-MS and a quantitative re-measurement using high resolution ICP-MS. Pesticides and mycotoxines were quantified using methods gas chromatography with conventional detectors ECD (electron capture detector) and FPD (flame photometric detector). Sulfuric acid was quantified by using optimized Monier-Williams method.

2.7 | Data analysis

Data were first assembled in Excel 2003 (Microsoft, Redmond, WA). Minitab® 18 statistical software (Minitab Inc., 2018, Pennsylvania) was used for univariate data analysis. The comparison of batches of soy hydrolysates samples was performed with one-way ANOVA (analysis of variance) and linear regressions.

3 | RESULTS

3.1 | Impacts of batch-to-batch variability of soy hydrolysates on cell culture, on mAb production and product quality

To study the impact of soy hydrolysates batch-to-batch variability on cell growth, metabolism and on mAb production, SP2/0 cells stably expressing a monoclonal antibody were grown in shake-flasks using medium and feed solution prepared with a given batch of soy hydrolysates.

3.2 | Cell growth and cell metabolism

We investigated cell growth using 37 batches of soy hydrolysates. The batch-to-batch variability of soy hydrolysates was found not to impact the evolution of cell viability during the cell culture (Figure 1). However, the batch of soy hydrolysates used had an impact on the viable cell density beyond the second day of the production phase. The peak cell density ranges from 2.5×10^6 cells/mL for batch 22 to 4.0×10^6 cells/mL for batch 3 (Figure 1). The batch-to-batch variability of soy hydrolysates did not impact significantly the production of glutamate, lactate and ammonium measured in the cell culture supernatant during the production phase. However, beyond the fourth day of culture after soy hydrolysate addition, the concentrations of the substrates in the cell culture supernatant, glucose and glutamine, were affected. For example, lower levels of glucose and glutamine were measured in the supernatant for batch 3, 19, the reference, 21, 20, and 22, respectively (Figure 1). The observed higher consumption of glutamine and glucose present in the culture medium could be related in part to the effect of a batch of soy hydrolysates on cell density.

3.3 | Volumetric productivity

Figure 2 shows the average volumetric productivity observed for 36 batches of soy hydrolysates relative to the chosen reference batch. The reference corresponds to the batch being used at manufacturing scale at the time of this study. The productivity varied from 75% to 111% compared to the reference batch. Therefore, the batch-to-batch variability of soy hydrolysates can trigger productivity variations up to 36%. Overall, more recent batches of soy hydrolysates tend to be associated to lower volumetric productivity compared to the older batches of soy hydrolysates.

3.4 | Acidic charge variants upon antibody production

The comparison of the acidic charge variants of produced antibody of 10 batches of soy hydrolysates are represented in Figure 3. These results highlighted that a change of soy hydrolysates batch was also associated to a variation of the product acidic charge variants.

Depending on the batch of soy hydrolysates used, cell growth was impacted from the second day of cell culture and concentration of metabolites from the fourth day of the cell culture. These results pointed to a batch-to-batch variability in the composition of soy hydrolysates. Some batches might be composed of elements promoting or inhibiting cell growth and therefore impacting volumetric productivity and product quality. Consequently, physico-chemical characterization of batches of soy hydrolysates was conducted to evaluate if any variation in process outputs was correlated with a variation in quality of soy hydrolysates.

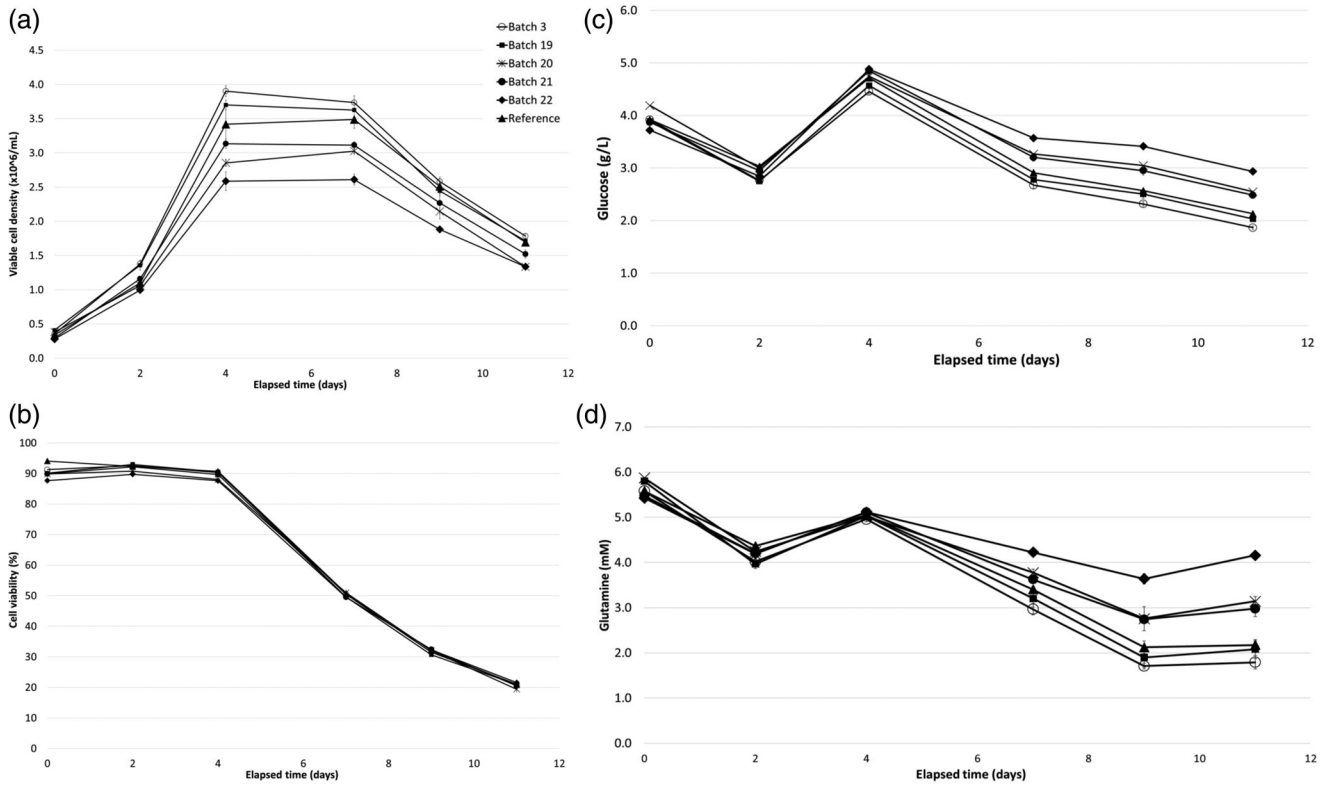


FIGURE 1 Impact of soy hydrolysate batches in cell culture media and feed solution on cell density (a), cell viability (b), glucose (c) and glutamine (d). Each batch of soy hydrolysates was tested in triplicate in shake-flasks except for batch 22 which was tested in duplicate only. On the fourth day of culture, the culture medium was supplemented with a concentrated solution of the same batch of soy hydrolysates. Bars are one standard error from the mean

3.5 | Soy hydrolysates characterization

In addition to information available in certificates of analyses of soy hydrolysates batches, batches were subjected to a panel of analyses.

3.6 | Certificate of analyses of soy hydrolysates

Certificate of analyses allows to ensure that the product complies with a predefined quality. The panel of tests and the acceptance criteria are based on standards or guidelines required by the buyers. The information of 41 certificates of analysis were gathered and compared. All batches met the acceptance criteria. Batches were manufactured from 2013 to 2019. Test results were consistent, except for clarity (Table 1). Clarity results were not correlated to the volumetric productivity but were often associated with lowest filterability of the feed solutions containing soy hydrolysates (data not shown).

3.7 | Amino acids and molecular weight distribution

Soy hydrolysates contain on average about 56 ± 2% (w/w) of peptides/amino acids. The proportion of each amino acid is represented in Table 2. The three most abundant free amino acids are arginine, leucine and lysine (1.1%, 1.0%, and 0.7%, respectively), while the two most abundant amino acids present in peptides are glutamic acid and aspartic acid (12.4% and 7.2%, respectively). Molecular weight distribution was obtained from 23 batches of soy hydrolysates (Table 3). Most peptides had a molecular weight below 500 Da (73%). These peptides were therefore composed of less than 5 amino acids.

3.8 | Carbohydrates

Soy hydrolysates also contain about 16.2% (w/w) of carbohydrates. Sucrose and stachyose were the main sugars at 7.1% and 7.0%, respectively. Raffinose and fructose were detected in lower proportion (1.4% and 0.7% respectively). Low levels of glucose were reported for some batches. Lactose, maltose, galactose and inositol were not detected (Table 4).

3.9 | Chemical elements

Chemicals elements represents about 7% (w/w) of the total composition of soy hydrolysates. High levels (>200 μg/g of soy hydrolysates)

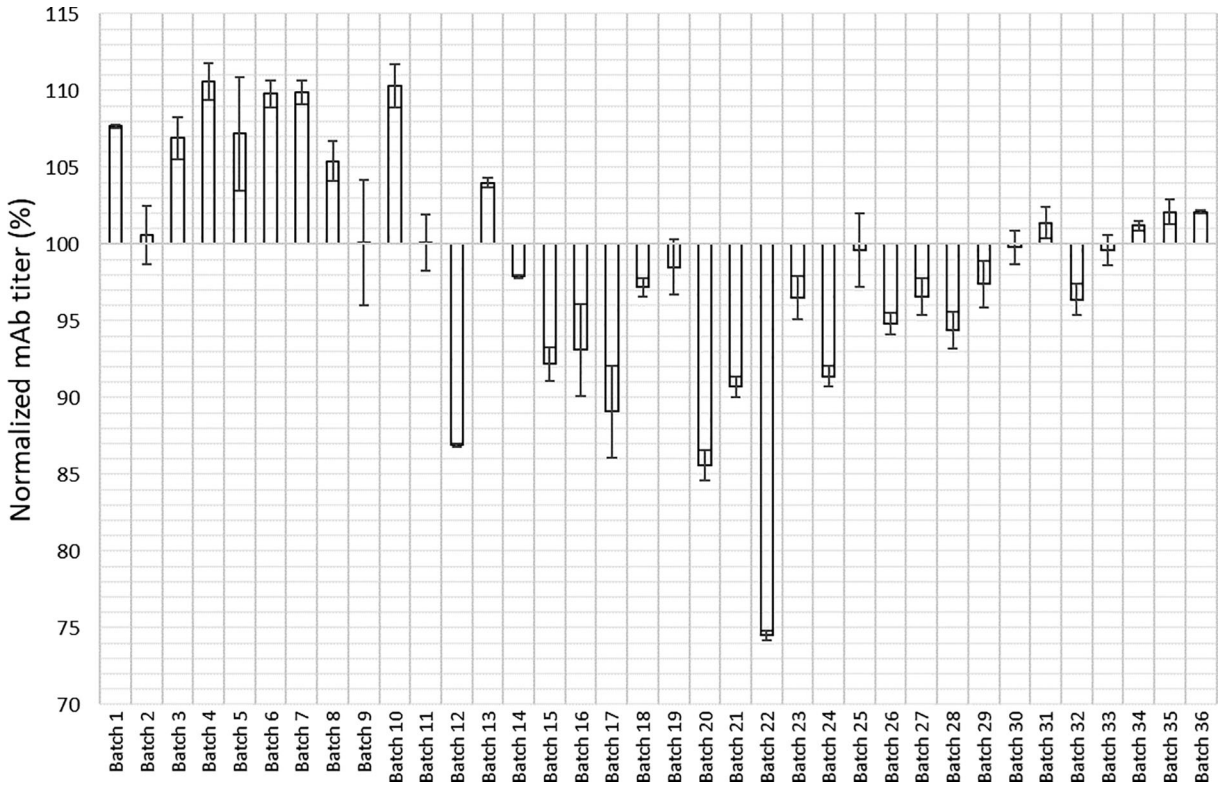
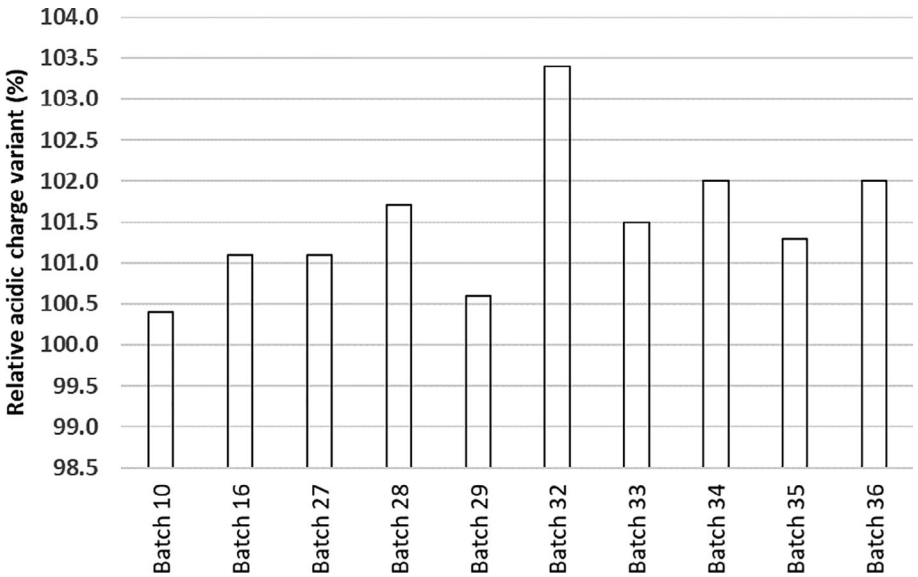


FIGURE 2 Impact of batch-to-batch variability of soy hydrolysates on monoclonal antibody titer. Thirty-six batches of soy hydrolysates from the same vendor were compared to a given batch for which the mAb titer was fixed at 100%. Tests were performed in shake-flaks in triplicate. Results are expressed as the percentage of the titer obtained with a given soy hydrolysate batch at the end of the culture compared to the titer obtained with the reference batch. Bars are one standard error from the mean

FIGURE 3 Impacts of batch-to-batch variability of soy hydrolysates on mAb acidic charge variants. Ten batches of soy hydrolysates from the same vendor were compared. Results represent the increase in the mAb acidic charge variants level obtained with a given soy hydrolysates batch at the end of the culture compared to the mAb acidic charge variants obtained with the reference batch for which the level was fixed at 100%



of potassium, sodium, magnesium, and calcium were detected at 3.5%, 3%, 0.3%, and 0.1%, respectively and they are the major chemical elements present in soy hydrolysates. Medium levels (20–200 μg/g of soy hydrolysates) of boron, aluminum and iron were measured. Finally, very low levels of vanadium, manganese, nickel, zinc, rubidium and molybdenum were detected (Table 5). Considering levels of these trace elements in the basal medium, these levels are considered as not significant. Soy hydrolysates represent a significant source of iron, boron and aluminum compared to the other raw materials present in cell culture medium and feed solutions.

TABLE 1 Summary of information from certificates of analysis of 41 batches of soy hydrolysates

Analysis	Mean ± SD
Residue on ignition (%)	9.5 ± 1.7
Degree of digestion (%)	23.6 ± 1.2
Amino nitrogen (%)	2.2 ± 0.1
pH	7.1 ± 0.1
Total nitrogen (%)	9.2 ± 0.2
Clarity (NTU)	0.43 ± 0.49
Color	0.55 ± 0.07

TABLE 2 Free and total amino acid composition of soy hydrolysates. The free amino acid composition of 33 batches were analyzed as well as the total amino acid composition of 24 batches of soy hydrolysates. Free and total amino acid compositions are expressed in gram of a given amino acid per 100 g of soy hydrolysates (%)

	Free amino acid (%, w/w)	Total amino acid (%, w/w)
Alanine	0.28 ± 0.04	2.64 ± 0.09
Arginine	1.07 ± 0.13	3.76 ± 0.16
Asparagine	0.30 ± 0.03	ND
Aspartic acid	0.22 ± 0.03	7.17 ± 0.28
Cysteine	ND	ND
Cystine	ND	0.30 ± 0.03
Glutamic acid	0.48 ± 0.05	12.43 ± 0.49
Glutamine	ND	ND
Glycine	0.30 ± 0.04	2.56 ± 0.09
Histidine	0.16 ± 0.02	1.42 ± 0.06
Isoleucine	0.05 ± 0.02	2.26 ± 0.10
Leucine	1.03 ± 0.13	4.08 ± 0.14
Lysine	0.66 ± 0.08	3.57 ± 0.15
Methionine	0.16 ± 0.02	0.64 ± 0.05
Phenylalanine	0.33 ± 0.06	2.44 ± 0.10
Proline	0.02 ± 0.01	2.71 ± 0.13
Serine	0.54 ± 0.08	3.26 ± 0.11
Threonine	0.29 ± 0.04	2.49 ± 0.09
Tryptophan	0.13 ± 0.02	ND
Tyrosine	0.23 ± 0.04	1.69 ± 0.08
Valine	0.10 ± 0.03	2.51 ± 012
Total Free amino acid	6.35 ± 0.77	55.93 ± 1.91

Abbreviations: ND, not detected.

3.10 | Fatty acids

The fatty acid composition of 6 batches of soy hydrolysates were measured. Levels of fatty acids were below 0.002% in soy hydrolysates. Levels of fatty acids were much lower than the overall concentration of fatty acids in the cell culture medium and feeds (Table 6).

TABLE 3 Molecular weight distribution of batches of soy hydrolysates. Twenty-three samples of different soy hydrolysates batches from the same vendor were analyzed

Molecular weight	Proportion (%, w/w)
<0.5 kDa	72.8 ± 2.6
0.5–1 kDa	26.5 ± 2.4
1–2 kDa	0.8 ± 0.3

TABLE 4 Carbohydrates content in soy hydrolysates. Thirty-two samples of different soy hydrolysates batches from the same vendor were analyzed

Carbohydrate composition	Proportion (%, w/w)
Sucrose	7.1 ± 1.16
Stachyose	7.0 ± 1.02
Raffinose	1.4 ± 0.36
Fructose	0.7 ± 0.25
Galactose	ND
Glucose	ND
Inositol	ND
Lactose	ND
Maltose	ND
Total	16.2 ± 1.89

Abbreviations: ND, not detected.

TABLE 5 Chemical elements content in soy hydrolysates. Chemical elements of 35 samples of different soy hydrolysates batches from the same vendor were analyzed

Chemical elements	Quantity (μg/g of soy hydrolysates)
K	>36′000
Na	>27′000
Mg	>2′600
Ca	>1′100
Fe	95 ± 25
Al	65 ± 35
B	48 ± 11
Rb	17 ± 4
Ni	10 ± 1
V	9 ± 4
Zn	6 ± 1
Mn	5 ± 1
Mo	5 ± 2

TABLE 6 Fatty acids content. Contents in fatty acids of six different batches of soy hydrolysates from the same vendor were analyzed

Fatty acids	Average (μg/g of soy hydrolysates)
Myristic *14:0*	3,8 ± 0.8
Palmitic *16:0*	7,3 ± 1.5
Palmitoleic *16:1w7*	1,8 ± 0.4
Stearic *18:0*	2.0 ± 0.3
Oleic *18:1w9*	0,7 ± 0.2
Linoleic *18:2w6*	0,2 ± 0.1
Arachidic *20:0*	0,2 ± 0.1
11-Eicoenoic *20:1w9*	0,2 ± 0.3
Total	16.1 ± 2.0

TABLE 7 Maillard reaction products and cross-link amino acid lysinoalanine for 33 batches of soy hydrolysates

	Mean (area under curve)
Carboxymethyllysine, CML	6773 ± 360
Carboxylethyllysine, CEL	6246 ± 541
Furosine, Fur	ND
Cross-link lysinoalanine, LAL	7229 ± 525

Abbreviations: ND, not detected.

3.11 | Maillard reaction products and cross-linked lysinoalanine

Carboxymethyllysine, carboxyethyllysine and lysinoalanine were detected but there was no significant variation observed between samples. Furosine was not detected (Table 7).

3.12 | Pesticides, mycotoxins and sulfuric acid

Soy hydrolysates were analyzed to detect the potential presence of pesticides, mycotoxins and sulfuric acid in 8 batches. Among pesticides, only aminomethylphosphonic acid (AMPA) and glyphosate were detected in trace quantity (about 1 μg/g of soy hydrolysates), in addition to the presence of sulfuric acid in larger proportion (<135 μg/g of soy hydrolysates) (data not illustrated).

3.13 | Vitamins

No significant levels of vitamins were detected in samples of soy hydrolysates.

In summary, soy hydrolysates were found to be composed of about 56 ± 2% (w/w) of amino acids/peptides, 16 ± 2% (w/w) of carbohydrates, and 7% (w/w) of chemical elements. Very low levels of fatty acids were detected. Some Maillard reaction products were detected. Glyphosate, aminoethylphosphonic acid (AMPA) and sulfuric acid were detected in trace quantities. Vitamins were not observed. Results from the characterization of batches of soy hydrolysates and the effect on cell growth, cell viability, protein expression and product quality were compared to identify some relationships that might explain the observed impacts on cell growth, productivity and on product quality.

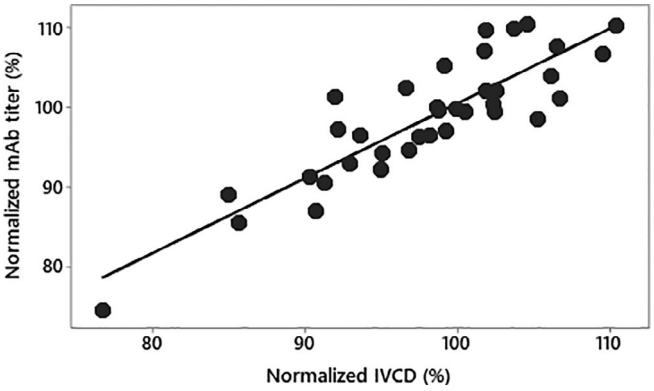


FIGURE 4 Correlation between normalized integral of viable cell density and normalized volumetric productivity. The relationship between productivity and integral of viable cell density (IVCD) is statistically significant ($p < 0.001$). 76% of the variation in productivity is explained by the regression model. The positive correlation ($r = 0.87$) indicates that when IVCD increases, productivity also tends to increase

3.14 | Viable cell density and productivity

The integral viable cell density at the end of the cell culture were calculated for each batch. Normalized results were calculated compared to the reference batch. A linear regression indicated that the relationship between the normalized productivity and integral viable cell density (IVCD) is statistically significant ($p < 0.001$, $R^2 = 76\%$). The positive correlation ($r = 0.87$) indicates that when IVCD increases, productivity also tends to increase (Figure 4).

3.15 | Characterization and productivity

No significant correlation between volumetric productivity and concentrations of amino acids, total amino acids, molecular weight distribution, carbohydrates, Maillard reaction products, pesticides, glyphosate, sulfuric acid, chemical elements was found. However, there was a correlation between the level of iron in soy hydrolysates and productivity (p-value <0.001, $R^2 = 0.48$). The higher is the concentration of iron in a batch of soy hydrolysates, the lower is the volumetric productivity (Figures 5 and 6). A one-way Anova indicates that the mean of iron concentration in good performing batches differs from means of intermediate and bad performing batches at the 0.05 level of significance (p-value = 0.004). It is important to notice that

soy hydrolysates represent a major source of iron in the cell culture medium and in the feed solution in comparison with the basal medium composition for which the iron content is negligible.

Given that the negative correlation between the level of iron in soy hydrolysates and productivity, supplementation assays were carried out to confirm or disprove this hypothesis.

3.16 | Effect of ferric ammonium citrate supplementation

In order to test the hypothesis of iron impact on the cell culture process performance, iron supplements made of ferric ammonium citrate, ranging from 80 to 500 ppm in cell culture medium and in the concentrated soy hydrolysates solution were tested. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder. This complexed form of iron was chosen knowing the intermediate chelating properties of citric acid and the potential prior complexation form of iron in soy hydrolysates illustrated in some publications.[13,14] After the first passage, the cell viability of the condition with the highest level of iron tested (500 ppm) decreased to 15% when compared to the control (91%). The cell viabilities for remaining conditions were consistent except for the condition with 250 ppm of iron that was associated to a slight decrease of the cell viability to 87%. After the second passage, the cell viability of the condition with 250 ppm dropped to 7.2%, while other conditions remained consistent compared to the control. Therefore, it was hypothesized that conditions with iron concentration above 175 ppm reduced drastically the cell growth and induced cell death. It was not possible to continue the cell culture for these conditions. For all remaining conditions, no major impact was observed on cell growth (Figure 7) or cell metabolism (results not shown). We confirmed experimentally an inverse correlation between level of iron and productivity (Figure 8). An elevated level of iron in the cell culture medium and in the soy hydrolysates feed solution resulted in about 7% lower productivity when the equivalent iron concentration was above 80 ppm. An impact on the acidic charge variants distribution was clearly observed. At elevated iron

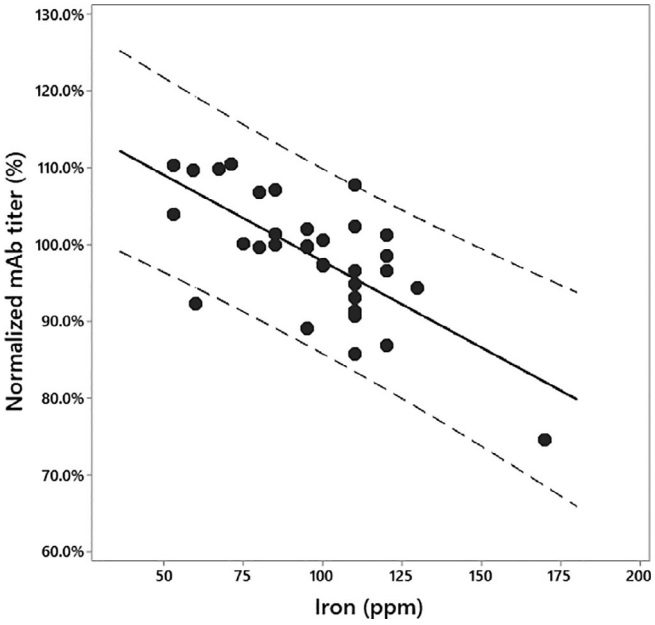


FIGURE 6 Prediction plot for the correlation between productivity and iron concentration in soy hydrolysates. The fitted line is the predicted productivity for any iron concentration in a batch of soy hydrolysates. The dashed lines correspond to the upper and lower 95% prediction intervals. The relationship between productivity and iron is statistically significant ($p < 0.05$). 48.7% of the variation in productivity is explained by the regression model. The negative correlation ($r = -0.70$) indicates that when iron increases, volumetric productivity tends to decrease. Iron content indicated in ppm stands for μg of iron per g of soy hydrolysates

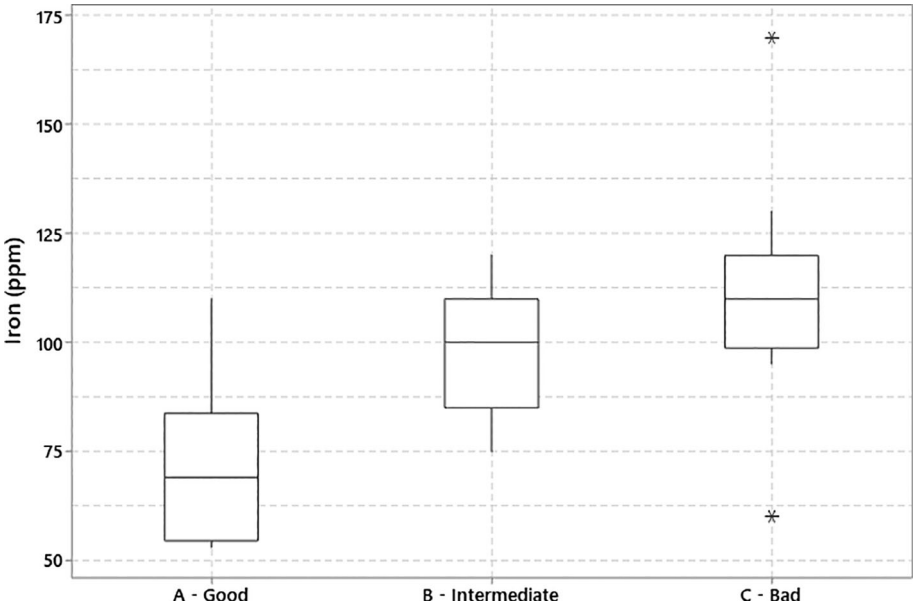


FIGURE 5 Box plot of iron content for batches of soy hydrolysates categorized according to their impact on monoclonal antibody titer. Three categories were defined. Batches with a mAb titer higher than 104% were identified as good batches ($n = 8$). Batches with a mAb titer between 96% and 104% were identified as intermediate batches ($n = 12$). Batches with a mAb titer below 96% were identified as bad batches ($n = 12$). Iron content indicated in ppm stands for μg of iron per g of soy hydrolysates

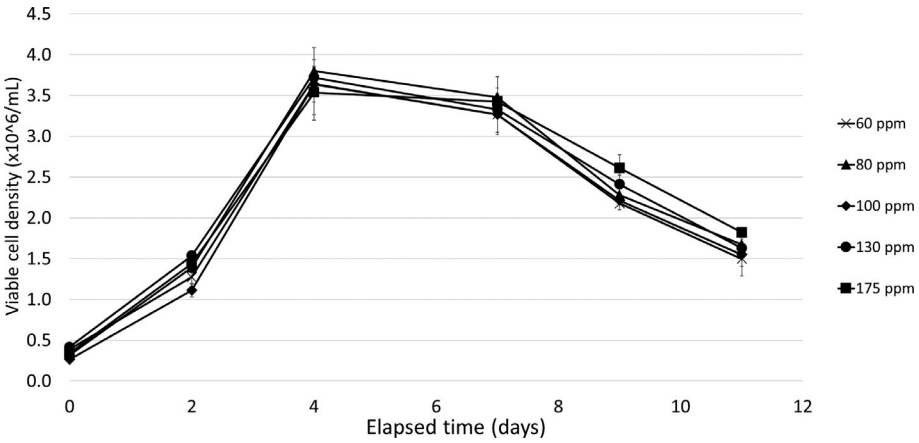


FIGURE 7 Effect of ferric ammonium citrate supplementation on viable cell density. Tests were performed in shake-flaks in triplicate. Ferric ammonium citrate was supplemented in both cell culture medium and soy hydrolysates feed solution added at the fourth day of cell culture. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder. Bars are one standard error from the mean

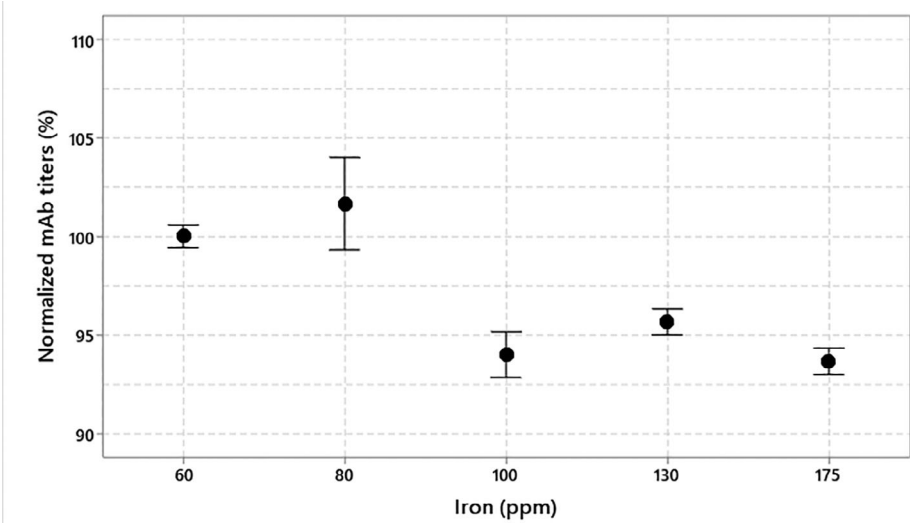


FIGURE 8 Effect of ferric ammonium citrate supplementation on antibody titer. Tests were performed in shake-flaks in triplicate. Ferric ammonium citrate was supplemented in both cell culture medium and soy hydrolysates feed solution added at the fourth day of cell culture. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder. Bars are one standard error from the mean

concentrations, a significantly larger fraction of acidic forms of the monoclonal antibody was observed (p-value = 0.005 and R^2 = 94.8%) (Figure 9). Notably, usage of ferric ammonium citrate, which is already a form of chelated iron, could result in less negative effects on process performance compared to a noncomplexed form of iron.

3.17 | Effect of ferrous sulfate supplementation

As iron in soy hydrolysates might be present in an uncomplexed form, supplementation with ferrous sulfate was also tested as described above. Figure 10 illustrates the impact of ferrous sulfate supplementation on viable cell densities after 2 passages. Above 100 ppm, a clear negative impact was observed. It was not possible to maintain the condition at 500 ppm, as the viability reached 10.4% after the first passage. In the same way, for conditions at 175 ppm and 250 ppm, cell densities did not allow one to obtain enough cells after the second passage for the production phase. At 250 ppm, cell viability dropped to 38% while the control (60 ppm) was at 94%. At 175 ppm, only a slight decrease of the cell growth was observed as cell viability was

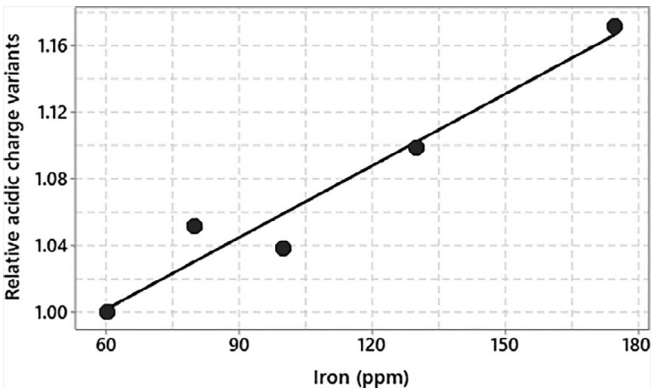


FIGURE 9 Effect of ferric ammonium citrate supplementation on acidic charge variants of the monoclonal antibody. Ferric ammonium citrate was supplemented in both cell culture medium and soy hydrolysates feed solution added at the fourth day of cell culture. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder. The positive correlation indicates that at elevated iron concentrations, a significantly larger fraction of acidic forms of the monoclonal antibody was obtained (p-value = 0.005 and R^2 = 94.8%)

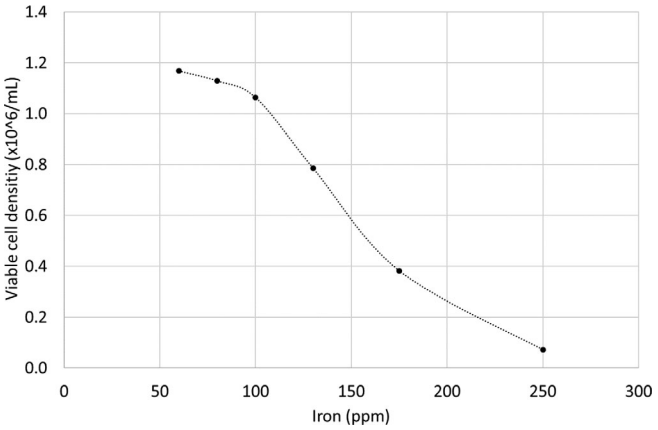


FIGURE 10 Effect of ferrous sulfate supplementation on viable cell density during cell expansion after two passages. Tests were performed in shake-flaks in duplicate. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysate powder. The condition at 500 ppm is not represented given that the viable cell density reached 0.02×10^6 cells/mL after the first passage

kept above 86% after the second passage. Therefore, cells from the control at 60 ppm were used to inoculate 175, 250, and 500 ppm conditions. The effect of ferrous sulfate supplementation in the cell culture medium and in the soy hydrolysates on viable cell densities is shown in Figure 11, on antibody titers in Figure 12 and on acidic charge variants in Figure 13. During the production phase, viable cell density and viability dropped to 0.04×10^6 cells/mL and to 10% respectively at the second day. For all remaining conditions, viabilities were comparable throughout the production phase even at 250 ppm (results not shown). This might be explained by higher cell densities that may reduce the toxicity effect of iron. Nevertheless, inhibition of cell growth was observed for conditions above 100 ppm (Figure 11). This was associated to a significant decrease of titer by 10% at 130 ppm, by 18% at 175 ppm, and by 32% at 250 ppm (Figure 12). On top of that, acidic charge variants tend to be higher for conditions spiked with ferrous sulfate compared to the control (60 ppm) (Figure 13).

In conclusion, the inverse correlation between level of iron in media during cell culture and productivity for both ferric ammonium

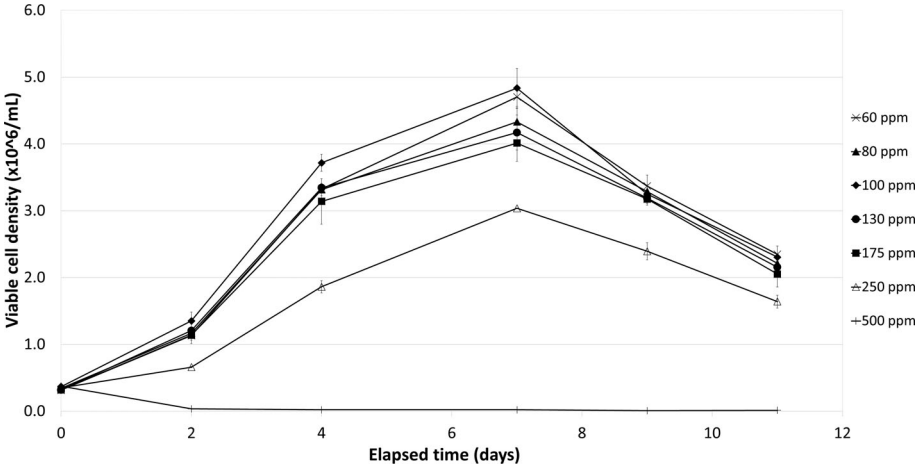


FIGURE 11 Effect of ferrous sulfate supplementation on viable cell density. Tests were performed in shake-flaks in triplicates. Ferrous sulfate was supplemented in both cell culture medium and soy hydrolysates feed solution added at the fourth day of cell culture. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder. Bars are one standard error from the mean

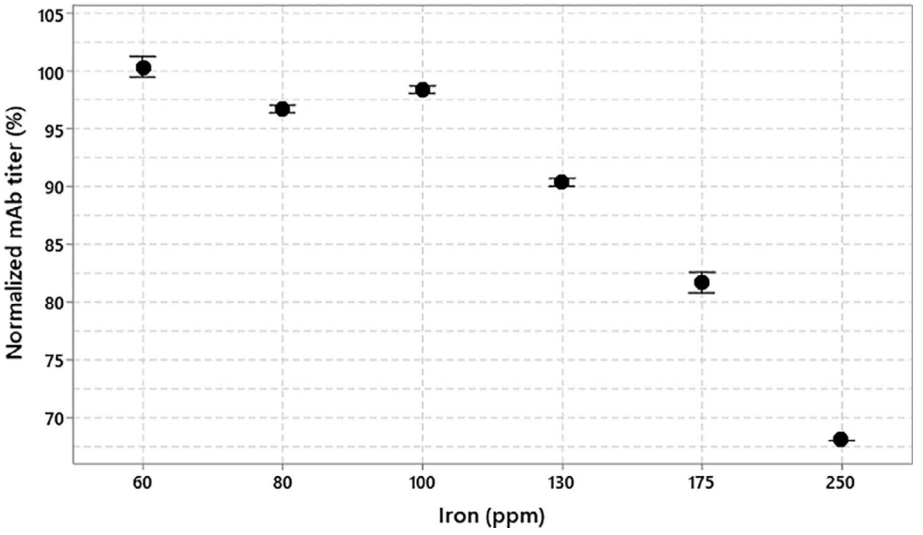


FIGURE 12 Effect of ferrous sulfate supplementation on monoclonal antibody titer. Tests were performed in shake-flaks in triplicate. Ferrous sulfate was supplemented in both cell culture medium and soy hydrolysates feed solution added at the fourth day of cell culture. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder. Bars are one standard error from the mean

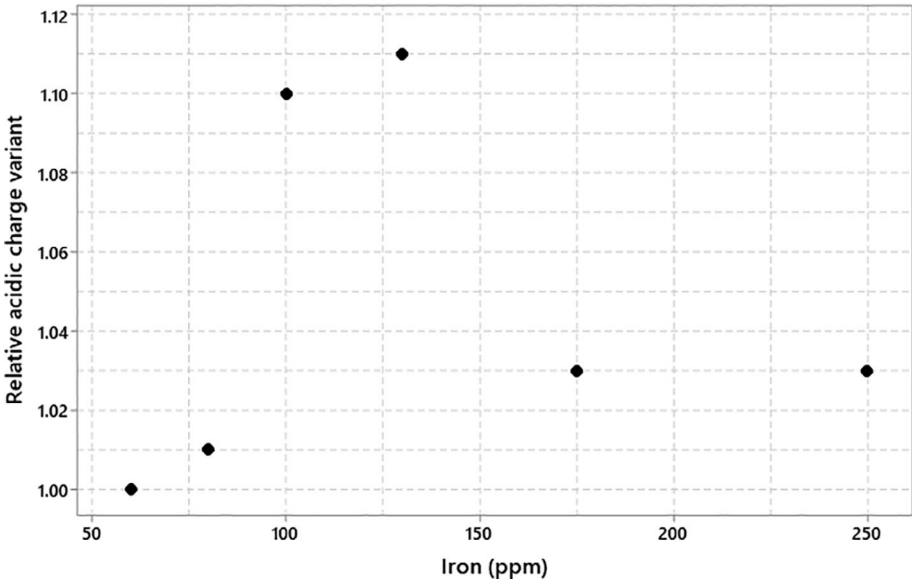


FIGURE 13 Effect of ferrous sulfate supplementation on acidic charge variants of the monoclonal antibody. Ferrous sulfate was supplemented in both cell culture medium and soy hydrolysates feed solution added at the fourth day of cell culture. Levels of iron indicated in ppm mimic quantities of iron present in soy hydrolysates powder

citrate and for ferrous sulfate was experimentally confirmed. An increase of the acidic charge antibody variants was also brought to light.

4 | DISCUSSION

The importance of iron content in cell culture media and its impacts on cell growth, mAb production and acidic charge variants was described in several publications.[15-22] Cellular iron deficiency can be associated with a lower cell growth, while higher level of iron has shown to be potentially cytotoxic due to Fenton reaction.[15,16] Impact on cell growth due to transitional metals variations, here e.g., iron, in cell culture media is a mechanism described by Dixon et al. in 2012 as ferroptosis.[23] Ferroptosis is a non-apoptotic form of cell death that can be triggered by conditions inhibiting glutathione biosynthesis of the glutathione-dependent antioxidant enzyme GPx4. This lethal process is defined by the iron Fe(II)-dependent accumulation of lipid reactive oxygen species and the peroxidation of polyunsaturated fatty acids leading to their depletion and the accumulation of toxic lipid reactive oxygen species (ROS).[23-25] This mechanism might explain in part the cause of batch variability of soy hydrolysates. In addition, it was reported that iron forms ROS that tend to increase acidic mAb species.[17-22]

Consequently, our data indicate that it is advisable to identify the source of iron variation in soy protein hydrolysates. Two main sources can be envisioned: the soy grit itself or the manufacturing process. In fact, soybeans are sources of trace elements, and environmental conditions and agricultural practices may impact the final trace elements composition of soybean crops. Plants need small amounts of essential trace elements for their growth and metabolism. Soybean crops are mainly sensitive to trace elements present in the soil including iron. In response to a deficiency in trace elements, supplementation can be made. This supplementation is made by adding fertilizer, a product containing the trace element in its mineral form or formulated within organic complexes which promote absorption by plants.[26,27] The potential impact of agricultural practices on the final quality of soybean protein hydrolysates need to be assessed. On top of that, the soy hydrolysates manufacturing process can be a source of trace elements. The filters aids made of perlite or diatomaceous earth used during filtration steps of manufacturing processes are known to contains trace elements.[28,29] The metals used to shape stainless steel tanks can also be released under specific physicochemical conditions.[30] It is advisable to establish a close collaboration with the vendor of soy protein hydrolysates to reach a better understanding and management of the observed iron batch-to-batch variability. In this context, final iron content in soy hydrolysates measured by ICP-MS should be added as a specification with an acceptance criteria in certificates of analysis. Therefore, it is also recommended to demand more control by vendor when qualifying them by considering for example the potential impact in mAb manufacturing processes of trace elements batch-to-batch variation.

5 | CONCLUSION

Soy protein hydrolysate is a plant derived raw material, commonly used as a cell culture medium supplement to promote cell growth and mAb production. They are produced by soy grits hydrolysis. The characterization of several batches of soy hydrolysates manufactured by the same vendor in a 6-year period showed that they are the main source of amino acids and peptides, carbohydrates and chemical elements. Some other components were detected in low amount such as fatty acids, some pesticides and sulfuric acids. In addition, Maillard reaction products were detected such as carboxymethillysine, carboxyethyllysine and cross-linked amino acid lysinoalanine. Vitamins and mycotoxins were not detected by our assays.

Although the global composition of soy hydrolysates was consistent, it was demonstrated that a batch-to-batch variability of soy

hydrolysates lead to significant impacts on cell growth, cell metabolism, productivity and on acidic charge variants of the mAb. Indeed, the batch-to-batch variability of soy protein hydrolysates led to an impact on the final yield of monoclonal antibody up to 36%. The use of a small-scale cell culture model before using a given batch of soy hydrolysates at manufacturing scale allowed us to select batches associated with better performance.

Among all components measured in soy hydrolysates, iron correlated negatively with final productivity. Ferric ammonium citrate and ferrous sulfate spiking assays were performed to verify this correlation. Increasing the level of iron up to 175 ppm in cell culture medium and in soy hydrolysates feed solution led to about 7% and 18% lower productivity for ferric ammonium citrate and ferrous sulfate respectively and to a significant increase of acidic charge variants. Highest levels of iron (250 and 500 ppm) were associated in both cases to a drastic drop of the cell viability. Our data corroborate the notion that iron concentrations in cell culture media need to be precisely controlled and limited as they may have an impact on cell growth, productivity and product quality.

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AUTHOR CONTRIBUTIONS

Leïla DJEMAL: Conceptualization; formal analysis; investigation; supervision; visualization; writing-original draft. **Joerg von Hagen:** Supervision; writing-review & editing. **Harald Kolmar:** Supervision; writing-review & editing. **Veronique Deparis:** Conceptualization; supervision; writing-review & editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research data are not shared.

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