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Original Article

Effect of Different Buffer Components on IgG4 Stability

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ABSTRACT

This study investigated the impact of four buffer systems (sodium citrate, citrate phosphate, histidine, and acetate) and various excipients on the stability of an IgG4 monoclonal antibody, employing Design of Experiments methodologies and advanced analytical techniques (SEC, IEX, HIC, DSF). While sodium citrate buffer showed superior performance compared to citrate phosphate, an additional peak observed in both systems necessitated further optimization. Histidine buffer formulations with alternative stabilizers (trehalose, sorbitol) demonstrated stability comparable to the approved IgG4 formulation. Acetate buffer emerged as a promising alternative, outperforming citrate-based buffers in some aspects. The study revealed complex buffer-pH-excipient interactions, with pH critically influencing protein stability, particularly in the acetate system. This comprehensive research provides a foundation for rational IgG4 formulation design, highlighting the potential of acetate and histidine buffers, and emphasizing the need for long-term stability and forced degradation studies to fully understand excipient behaviors.

Keywords: Monoclonal antibodies, IgG4, biosimilar, formulation, stability, buffer systems, excipients, Design of Experiments (DOE).

INTRODUCTION

The globalization of monoclonal antibodies (mAbs) market has been on a swift upward trend as sales are forecast to reach $300 billion mark in the year 2025 [1]. Monoclonal antibodies (mAbs) have revolutionized the treatment of many complex diseases such as cancers, autoimmune diseases and diseases of inflammatory nature by their high efficiency and specificity [2]. These biopharmaceuticals have been quite effective in targeting specific molecular pathways and improved patient welfare with fewer side effects as compared to the use of traditional small molecule drugs. However, monoclonal antibodies (mAbs) are complex due to their large size and numbers of functional groups [3]. Consequently, for any given mAb drug product, it is challenging to identify all the potential modifications associated with real storage conditions during its drug product development period. Therefore, formulation development primarily focuses on identifying key modifications such as aggregation, fragmentation, oxidation, and deamidation since all those types of degradation reduce biological efficacy [4].

The optimization of formulation involves a multifaceted approach, incorporating various analytical techniques and stress studies to evaluate the impact of different formulation components on the antibody's stability and functionality. Key considerations include the selection of an appropriate buffer system, pH optimization, and the incorporation of stabilizing excipients such as sugars, amino acids, and surfactants. Changes in the pH of the solution influence the protein charge and could lead to unstable protein formulations. Hence, protein formulations rely on buffers such as histidine, acetate, citrate, aspartate, phosphate, or tris to maintain the solution pH [3,5,6,7,8]. Generally, mAbs with a pI around 8–9 are formulated in mildly acidic buffer, avoiding for example deamidation and aggregation sometimes occurring in mildly alkaline buffer. These conditions, however, are not necessarily the best for the optimal conformational stability. Sugars and polyols are often used to stabilize many proteins and inhibit their aggregation [9,10,11,12]. Among sugars, sucrose and trehalose have been the most frequently used. A variety of amino acids have been used in protein stabilization such as glycine, basic amino acids, acidic

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amino acids and methionine as an anti-oxidizing agent [13]. Recent advancements in high-throughput screening techniques, predictive modeling, and analytical methodologies have significantly enhanced our ability to efficiently optimize biosimilar formulations. These tools allow for the rapid evaluation of multiple formulation parameters and their interactions, enabling a more rational and systematic approach to formulation development [14]. The application of Design of Experiments (DoE) methodologies facilitates the exploration of a wide formulation space while minimizing the number of experiments required. Furthermore, the integration of artificial intelligence and machine learning algorithms in formulation development has shown promise in predicting protein stability and optimizing formulation conditions [15,16,17].

This work was aimed at investigating the degradation behaviors of an IgG4 subtype therapeutic monoclonal antibody associated with excipients, pH and buffer species. The thermal stability and protein-protein interaction of IgG4 in various formulation matrices were evaluated by size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEX). A Design of Experiments (DOE) methodology was utilized to systematically investigate the multidimensional formulation space and optimize the experimental approach. By using a DOE-based approach, we were able to reduce the number of tests needed while effectively examining the relationships between essential formulation parameters and how they affect IgG4 stability. Usually, any given drug product does not show degradation if it is stored at refrigerated temperature such as 2 to 8°C within the duration for formulation development. Therefore, temperature-induced stress study is widely used to obtain adequate degradation which can be captured by characterization methods. The knowledge obtained from the temperature induced study is assumed to be relevant with degradation tendency in a real storage condition. In this study, the most promising formulation candidates were selected on the basis of SEC and IEX results, with the DOE approach providing a robust statistical framework for identifying optimal formulation conditions and predicting stability outcomes.

MATERIALS AND METHODS

Materials

The IgG4 monoclonal antibody used in this study was produced at Research and Development (R&D) laboratories of Abdi İbrahim Biologics manufacturing facility (AbdiBIO, Abdi İbrahim, Türkiye). Sucrose and Trehalose dihydrate were obtained from Merck (USA). Sorbitol, Poloxamer 188, L-Histidine, L-Arginine, Polysorbate 80, L-Methionine and Na-Acetate were purchased from Sigma-Aldrich (USA). L-Histidine HCl monohydrate was also purchased from Merck (USA). Hydrochloric Acid was obtained from Sigma-Aldrich (USA). All chemicals used were of analytical grade and used as supplied without further purification.

Methods

Stability Analysis

The physicochemical stability of the candidate formulations was evaluated under thermal stress conditions. For short (1 month)- and long-term (3 months) thermal stress stability studies, the formulations were incubated at 40 Degrees Celsius (°C) with 75% Relative Humidity (RH) in stability chambers for three months. The formulations were subjected to three freeze-thaw cycles at -80°C to assess freeze-thaw stability. Each cycle consisted of 24 hours of freezing followed by 24 hours of thawing.

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC) was used to evaluate the formation of HMWs of the IgG4 protein. Isocratic elution of samples (50 mM sodium phosphate, 300 mM potassium chloride, pH 5.5) was performed on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an ultraviolet (UV) detector and a Phenomenex, Biozen dSEC-2 column (300 mm x 4.6 mm, pore size 200 Å, particle size 3 µm; Phenomenex, CA, USA) at 280 nm wavelength. The column was loaded with 50 µg of sample per injection and an isocratic method was used for the analysis over 20 minutes.

Ion Exchange-High-Performance Liquid Chromatography (IEX-HPLC)

Cation exchange chromatography (CEX) was employed to separate the charge variants of the IgG4 protein. The CEX separation was performed using a Shimadzu HPLC system (Shimadzu Corporation) equipped with a ProPac WCX-10 cation exchange analytical column (4 mm x 250 mm, 10 µm; Thermo Fisher Scientific Inc., Waltham, MA, USA). The

column temperature was set at 45 °C and the autosampler temperature at 4 °C throughout this study. 45 µg of sample was used in each injection. The flow rate was set to 1.0 mL/min and separation was performed by detection at a UV wavelength of 280 nm. Mobile Phase A was prepared as 20 mM MES buffer at pH 6.4 and Mobile Phase B was prepared as 20 mM MES buffer containing 0.5 M NaCl at the same pH. Mobile Phase B was increased linearly from 0% to 10% for 21 minutes to separate charge variants.

Hydrophobic Interaction Chromatography

The hydrophobic variants and structural changes of IgG4 protein were monitored during development using Hydrophobic Interaction Chromatography (HIC). For this separation, a Shimadzu UPLC system (Shimadzu Corporation) equipped with a UV detector was employed. A YMC-BioPro HIC HT column (100 mm x 4.6 mm, particle size 2.3 µm; YMC, Kyoto, Japan) was used. The column temperature was set at 30 °C and the autosampler temperature at 6 °C throughout this study. The sample injection volume was 5 µL. The flow rate was set to 0.6 mL/min, and separation was performed by detection at a UV wavelength of 280 nm. Mobile Phase B was prepared as a 100 mM sodium phosphate buffer, and Mobile Phase A was prepared as a 100 mM sodium phosphate buffer containing 2 M ammonium sulfate both are in pH 7.0. Mobile Phase C was prepared %20 PEG and performed %35 during gradient. Mobile Phase A decreased linearly from 60% to 26% over 15 minutes to achieve the desired separation.

Differential scanning Fluorimetry (DSF)

The nano Differential Scanning Fluorimetry (nanoDSF) is based on intrinsic fluorescence. nanoDSF measure fluorescence intensity ratio with not needed of fluorescent dye. Proteins containing aromatic amino acid residues (tryptophan, tyrosine, and phenylalanine) show intrinsic fluorescence. When a molecule unfolds, the locations of the aromatic amino acid residues change and cause changes in the fluorescence spectra. The fluorescence spectra of the tryptophan residues, which are buried in the hydrophobic core of a protein, can have a 10–20 nm shift compared to those tryptophans on the surface of the protein [18]. nanoDSF measures the changes in intrinsic fluorescence intensity ratio (350:330 nm) as a function of temperature. During a nanoDSF scan, the intrinsic fluorescence intensity ratio (350:330 nm) is continuously measured and recorded. Plotting the intrinsic fluorescence intensity ratio (or the first derivative of the ratio) as a function of temperature yields a nanoDSF thermogram. The thermal transition (unfolding) temperature (T_m) is obtained in the post-run data analysis. The Tm values can be used to assess the thermal stability of the domains of a protein [19].

The thermal stability of formulated IgG4 protein samples were analyzed using a Prometheus NT.48 system (NanoTemper, PR001), which is a nano differential scanning fluorimeter (nanoDSF). The sample was prepared by filling standard capillaries (NanoTemper, PR-C006) with 10 µL of the solution at a concentration of 25 mg/mL. The thermal unfolding process was carried out by heating the sample from 20 to 95 °C at a rate of 2 °C/min, while monitoring the extinction power at 10. The fluorescence was recorded at two wavelengths, 330 and 350 nm, during the heating process. The data obtained from the experiment were analyzed using the NanoTemper PR Control software.

Design of Experiments (DOE)

We followed two Design of Experiments (DOE) principles: One Factor at A Time (OFAT) and Latin Hypercube statistical design throughout the development of a robust formulation for the biosimilar product. These methodologies allowed us to systematically investigate the impact of different formulation components on the stability and effectiveness of the biosimilar. The development process was advanced according to the number of independent variables within the formulation using these two powerful statistical approaches. When there was only one independent variable, an OFAT design was used to understand the effect of independent variables on IgG4 protein stability, and the results were analyzed using *t*-test slope statistics for comparison of regression lines assuming they have equal variances. On the other hand, significant factors were screened from a large number of factors affecting the process with Latin Hypercube design for more independent variables to understand the effects of independent variables on critical quality attributes (CQA). Variable levels were selected based on previous experiments and existing scientific knowledge. Seven independent variables were examined at two levels (categorical or continuous) with a single repetition at 95% confidence level. A total of 30 formulations were prepared and analyzed based on the Latin Hypercube desing. Statistical

evaluation of the quadratic polynomial equation was performed using ANOVA, including the coefficient of determination (R^2) and Fisher's test (F-test) to assess statistical significance. The goodness of fit of the model was determined by R^2. Response contour plots and pareto plots were analyzed and revealed strong interactions between important factors.

Minitab 19 software [20] was used to create DOE, randomize the design matrix and perform statistical analyses during whole development stages. Microsoft Excel [21] was also used for t-test and slope comparison in OFAT design studies.

RESULTS AND DISCUSSION

Effect of Sodium citrate and Citrate phosphate buffers on IgG4 stability

The selection of appropriate buffer systems is important for maintaining the stability and functionality of therapeutic proteins, particularly monoclonal antibodies such as IgG4 [22]. Recent studies have further emphasized the critical nature of this selection process in protein formulation [23]. In this study, we focused on sodium citrate and citrate phosphate buffers, known for their efficacy in maintaining pH stability over a range of 5.0-7.0 [4]. These buffers were chosen not only for their buffering capacity but also for their potential to enhance protein stability through specific interactions.

Sodium citrate has been shown to have a stabilizing effect on proteins through preferential exclusion mechanisms, where it is preferentially excluded from the protein surface, leading to preferential hydration of the protein [24]. On other hand, citrate phosphate buffer, a mixture of citric acid and sodium phosphate, offers a broader buffering range (pH 2.6 to 7.0) compared to sodium citrate buffer which makes more versatile formulation. This versatility aligns with current trends in biopharmaceutical development, as highlighted by recent comparative studies of citrate-based buffers [23]. The combination of citrate and phosphate ions in this buffer system provides unique properties that may affect protein stability differently than sodium citrate buffer.

To further enhance IgG4 stability in these buffer systems, we incorporated three amino acid excipients: L-Methionine, L-Arginine, and L-Histidine. Each of these amino acids was selected based on their well-documented stabilizing effects on protein formulations. Recent research has further elucidated the multifunctional roles of these amino acids in protein formulations [7,25]. L-Methionine acts as an antioxidant [26,27], L-Arginine suppresses protein aggregation [28,29], and L-Histidine contributes to stability by protecting the hydrophobic regions of the protein exposed to solvent and by its antioxidant properties [30,31].

Building on this theoretical foundation, we designed a comprehensive study to evaluate the effects of these buffer systems on IgG4 stability. Our experimental approach involved a short-term thermal stress analysis, utilizing a well-characterized IgG4 formulation as a control. This control formulation consisted of 10 mM histidine buffer (pH 5.5), 70 mg/mL sucrose, and 0.2 mg/mL polysorbate 80. The IgG4 protein used in the study was obtained using an in-house developed monoclonal antibody, ensuring uniformity in our experimental design and minimizing variability.

To systematically explore the parameter space, we employed a 2-fractional factorial design of experiments (DOE) [32]. This approach allowed us to investigate the effects of various formulation parameters, including buffer concentration (15 mM and 20 mM), Arginine (0 mM and 50 mM), Methionine (0 mM and 15 mM), Histidine (0 mM and 15 mM), and Sucrose (55 mg/mL and 85 mg/mL). The results of our 1-month thermal stress stability study, involving 32 different formulations (DOE) incubated at 40°C for 4 weeks, revealed complex interactions between buffer systems, excipients, and protein stability. Our analysis showed that sodium citrate buffer consistently outperformed citrate phosphate buffer in minimizing acidic variant formation. The Δacidity range for sodium citrate formulations (15.24-20.99) was notably lower than that of citrate phosphate formulations (18.35-24.04). These results were clearly illustrated in Figures 1A and 2A, with further detail provided in the scatter plots of Figures 1B and 2B. Buffer molarity increase (15 mM to 20 mM) showed a slight elevation in acidic variant formation in sodium citrate formulations, while this effect was less pronounced in citrate phosphate buffer. Arginine supplementation (50 mM) generally decreased acidic variant formation in both systems, with a more consistent effect in sodium citrate formulations. The combined use of methionine (15 mM) and histidine (15 mM) exhibited variable effects, reducing Δacidity in sodium citrate formulations but showing minimal or slightly

increased Δacidity in citrate phosphate formulations. Sucrose concentration (55 mg/mL vs 85 mg/mL) had a variable effect on acidity but consistently reduced aggregation in both buffer systems, as shown in Figures 1C and 2C. Notably, sodium citrate formulations displayed significantly lower aggregation propensity (Δaggregation: 0.35-0.68) compared to citrate phosphate formulations (Δaggregation: 0.78-1.37). The lack of clear correlation between Δacidity and Δaggregation suggests independent degradation pathways. Chromatographic analyses by IEX-HPLC and SE-HPLC (Figures 3A, 3B, 3C, and 3D) confirmed the superior stability of selected sodium citrate formulations. Formulations SC-11, SC-19, SC-27, and SC-31 exhibited lower acidic variant formation, while formulations SC-23 and SC29 demonstrated reduced aggregation. These sodium citrate formulations outperformed the top citrate phosphate formulations in both aspects (CP-19, CP-20, CP-27 and CP-31 for acidity; CP-23 and CP-29 for aggregation).

While both buffer systems showed higher degradation compared to the control, sodium citrate formulations demonstrated better overall stability, indicating promising directions for further optimization. Although there are promising candidates for acidic and aggregation levels, an extra peak indicated by red arrows in Figures 3A, 3B, 3C and 3D was observed in all formulation candidates. Therefore, more comprehensive screening is necessary in the development process using these buffers.

In conclusion, our study demonstrates the superior performance of sodium citrate buffer in maintaining IgG4 stability compared to citrate phosphate buffer. The incorporation of amino acid excipients, particularly arginine, showed potential in further enhancing stability. These findings provide valuable insights for the development of stable IgG4 formulations and highlight the importance of buffer selection in biopharmaceutical development.

A

S. ID	Buffer (mM)	Arginine (mM)	Methionine (mM)	Histidine (mM)	Sucrose (mg/mL)	ΔAggregation	ΔAcidity
Control						0.65	14.51
SC-1	15	0	0	0	55	0.64	20.26
SC-3	15	50	0	0	55	0.57	16.6
SC-5	15	0	15	0	55	0.54	19.71
SC-7	15	50	15	0	55	0.45	16.22
SC-9	15	0	0	15	55	0.54	18.26
SC-11	15	50	0	15	55	0.48	15.39
SC-13	15	0	15	15	55	0.38	18.3
SC-15	15	50	15	15	55	0.5	16.15
SC-17	15	0	0	0	85	0.63	19.89
SC-19	15	50	0	0	85	0.41	15.4
SC-21	15	0	15	0	85	0.45	19.87
SC-23	15	50	15	0	85	0.38	16.75
SC-25	15	0	0	15	85	0.41	17.86
SC-27	15	50	0	15	85	0.44	15.24
SC-29	15	0	15	15	85	0.35	18.09
SC-31	15	50	15	15	85	0.4	15.49
SC-2	20	0	0	0	55	0.66	20.99
SC-4	20	50	0	0	55	0.62	17.4
SC-6	20	0	15	0	55	0.54	20.17
SC-8	20	50	15	0	55	0.58	17.63
SC-10	20	0	0	15	55	0.55	18.96
SC-12	20	50	0	15	55	0.56	16.49
SC-14	20	0	15	15	55	0.5	18.8
SC-16	20	50	15	15	55	0.54	16.6
SC-18	20	0	0	0	85	0.68	20.56
SC-20	20	50	0	0	85	0.54	16.89
SC-22	20	0	15	0	85	0.52	20.25
SC-24	20	50	15	0	85	0.49	16.95
SC-26	20	0	0	15	85	0.49	18.5
SC-28	20	50	0	15	85	0.53	16.36
SC-30	20	0	15	15	85	0.44	19.08
SC-32	20	50	15	15	85	0.47	16.44

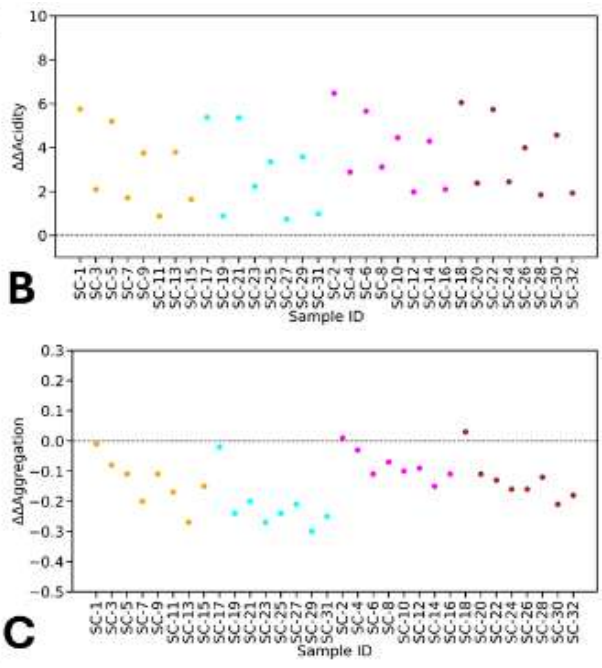


Figure 1. The figure shows detailed comparison of data derived from DOE for sodium citrate formulation buffer optimization, along with critical quality attributes (CQAs). (A) The table presents aggregation and acidic variant increases for 32 experiments obtained from the DOE. (B) The scatter plot illustrates the distribution of acidic changes across these 32 formulations compared to the original formulation. (C) Another scatter plot shows the aggregation changes relative to the original formulation. The generated formulation designs are colored based on similarities in their buffer or stabilizer molarities.

A

S. ID	Buffer (mM)	Arginine (mM)	Methionine (mM)	Histidine (mM)	Sucrose (mg/mL)	ΔAggregation	ΔAcidity
Control						0.65	14.51
CP-1	15	0	0	0	55	1.16	23.78
CP-3	15	50	0	0	55	1.15	19.52
CP 5	15	0	15	0	55	0.9	22.87
CP-7	15	50	15	0	55	1.06	19.14
CP-9	15	0	0	15	55	1.08	21.13
CP-11	15	50	0	15	55	1.23	19.38
CP-13	15	0	15	15	55	1.07	22.23
CP-15	15	50	15	15	55	1.23	19.97
CP-17	15	0	0	0	85	1.05	24.04
CP-19	15	50	0	0	85	0.96	18.62
CP-21	15	0	15	0	85	0.89	23.44
CP-23	15	50	15	0	85	0.79	20.39
CP-25	15	0	0	15	85	0.96	21.86
CP-27	15	50	0	15	85	0.85	18.53
CP-29	15	0	15	15	85	0.78	20.38
CP-31	15	50	15	15	85	0.85	18.35
CP-2	20	0	0	0	55	1.08	23.92
CP-4	20	50	0	0	55	1.15	19.26
CP-6	20	0	15	0	55	0.99	23.64
CP-8	20	50	15	0	55	1.06	19.14
CP-10	20	0	0	15	55	1.19	22.14
CP-12	20	50	0	15	55	1.37	19.49
CP-14	20	0	15	15	55	1.14	22.35
CP-16	20	50	15	15	55	1.25	20.72
CP18	20	0	0	0	85	1.02	23.98
CP-20	20	50	0	0	85	1.1	18.35
CP-22	20	0	15	0	85	0.89	23.99
CP-24	20	50	15	0	85	0.94	20.33
CP-26	20	0	0	15	85	0.97	21.08
CP-28	20	50	0	15	85	1.02	19.16
CP-30	20	0	15	15	85	0.83	21.99
CP-32	20	50	15	15	85	0.85	18.78

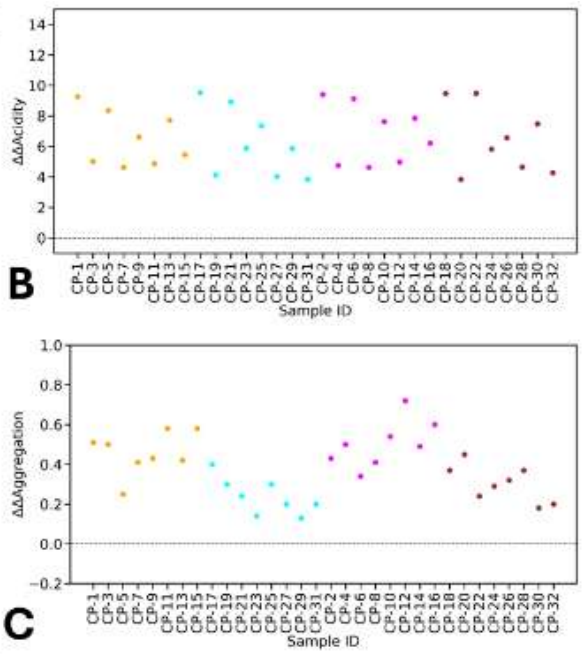


Figure 2. The figure shows detailed comparison of data derived from DOE for citrate-phosphate formulation buffer optimization, highlighting the critical quality attributes (CQAs). (A) The table presents aggregation and acidic variant increases for 32 experiments obtained from the DOE. (B) The scatter plot illustrates the distribution of acidic changes across these 32 formulations compared to the original formulation. (C) Another scatter plot shows the aggregation changes relative to the original formulation. The generated formulation designs are colored based on similarities in their buffer or stabilizer molarities.

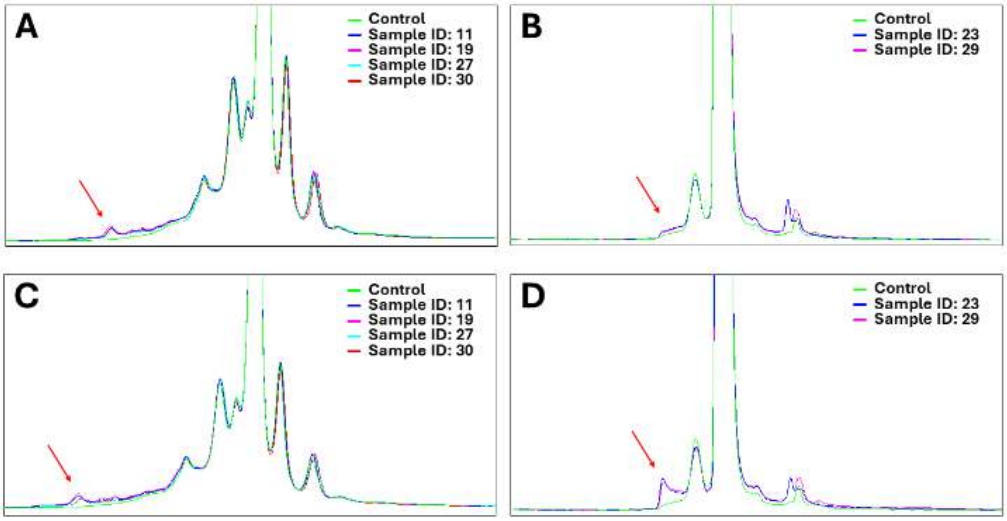


Figure 3. Comparative chromatographic analyses of sodium citrate and citrate phosphate buffer formulations. All chromatograms are normalized and presented alongside the control for peak-to-peak comparison. (A-D) IEX-HPLC and SE-HPLC chromatograms of formulations showing the lowest acidic variant formation and least aggregation increase for both buffer systems. Sodium citrate formulations 11, 19, 27, and 31 (IEX-HPLC) and 23, 29 (SE-HPLC) are shown in A and B, while citrate phosphate formulations 19, 20, 27, 31 (IEX-HPLC) and 23, 29 (SE-HPLC) are presented in C and D. Red arrows indicate an additional peak observed in all formulations, not present in the control.

Effect of Histidine buffer on IgG4 stability

Histidine buffer was selected as a crucial component in our formulation buffer development for IgG4 stability due to its unique properties and growing popularity in biopharmaceutical formulations. Histidine offers buffering capacity in the pH range of 5.5-7.5, which is often optimal for maintaining conformational stability and minimizing chemical

degradation of monoclonal antibodies, including IgG4 [23]. Unlike traditional buffers such as phosphate or citrate, histidine demonstrates minimal ionic strength contribution, which can be advantageous in reducing protein-protein interactions that may lead to aggregation [14]. Moreover, histidine's indirect metal-chelating properties can help mitigate oxidative damage catalyzed by trace metal ions, a common concern in protein formulations [33]. Recent studies have also suggested that histidine may interact directly with antibody molecules, potentially providing additional stabilizing effects through mechanisms such as preferential hydration and surface charge modulation [31,34].

Previously, the formulation buffer contained histidine, sucrose and polysorbate 80 has been shown used for IgG4 formulation [35]. Therefore, six different formulations were designed with alternative excipients and surfactants as described in table 1. Instead of sucrose, trehalose and sorbitol were used as these were proved as alternative stabilizers for monoclonal antibodies formulations. Trehalose has demonstrated superior protein-stabilizing properties, particularly during lyophilization and long-term storage at elevated temperatures, due to its higher glass transition temperature and ability to form stronger hydrogen bonds with proteins compared to sucrose [36]. On the other hand, sorbitol has shown promise in liquid formulations due to its ability to preferentially hydrate proteins and its compatibility with high-concentration antibody formulations [37]. It also has been reported in recent studies that both trehalose and sorbitol have demonstrated synergistic effects with histidine buffer, potentially offering enhanced protection against various stress conditions such as freeze-thaw cycles, mechanical stress, and elevated temperatures [38]. Moreover, the combination of histidine with either trehalose or sorbitol has shown potential in reducing the viscosity of antibody formulations, which is a critical factor in the development of formulations [39,40]. Poloxamer 188, a non-ionic triblock copolymer surfactant, has emerged as a promising alternative due to its superior chemical stability and lower propensity to form harmful degradation products [41]. Grapentin et al. [42] evaluated poloxamer 188 as an alternative to polysorbate 80 for IgG4 formulation and obtained promising results in terms of both sub-visible particle count and aggregation rate. Additionally, recent studies have demonstrated that poloxamer 188 can effectively prevent protein aggregation and particle formation in antibody formulations, particularly under conditions of mechanical stress, freeze-thaw cycles and also shown potential in reducing the immunogenicity of therapeutic proteins [43,44,45]. The combination of poloxamer 188 with histidine buffer has also demonstrated synergistic effects in stabilizing monoclonal antibodies against various stress conditions, making it an attractive option for our IgG4 formulation development [46]. Therefore, the effect of poloxamer 188 on IgG4 stability in combination with other excipients was checked.

The formulation buffer development study for the monoclonal antibody involved an OFAT DOE approach, evaluating different stabilizers and surfactants while maintaining a constant 10 mM Histidine buffer and pH 5.5 (Table 1).

Table 1. Candidate formulations generated with 10 mM Histidine buffer at pH 5.5. The table presents various buffer compositions, including the control formulation (HB01) and five alternative formulations (HB02-HB06). Each formulation candidate was combined with a different stabilizer (Sucrose, Sorbitol or Trehalose) and surfactant (Polysorbate 80 or Poloxamer 188).

Candidate Buffer	Buffer (mM)	Stabilizer	Surfactant	pH
HB01 (Originator)	10 mM Histidine	70 mg/mL Sucrose	0.02 (v/v%) Polysorbate 80	5.5
HB02		50 mg/mL Sorbitol	0.02 (v/v%) Polysorbate 80	
HB03		70 mg/mL Trehalose	0.02 (v/v%) Polysorbate 80	
HB04		70 mg/mL Sucrose	0.02 (v/v%) Poloxamer 188	
HB05		50 mg/mL Sorbitol	0.02 (v/v%) Poloxamer 188	
HB06		70 mg/mL Trehalose	0.02 (v/v%) Poloxamer 188	

The study assessed the impact of these formulations on protein stability under thermal stress conditions (40°C for 3 months), analyzing size variants by SE-HPLC, charge variants by IEX-HPLC, and hydrophobic variants by HIC-UPLC. The chromatograms showing changes in charge, size, and hydrophobic variants of histidine buffer formulations (compared with the control) after 3 months at 40°C

are presented in Figure 4. Initially, all formulations showed comparable stability profiles to the control (HB01), with minimal differences in size, charge, and hydrophobic variants. After 3 months of thermal stress, all formulations, including HB01, exhibited similar degradation pathways, indicating consistent behavior across different excipient combinations. The progression of acidic variants and aggregation over the 3-month period is illustrated in Figure 5A and 5B, respectively, showing a linear increase in both degradation markers for all formulations. In addition, initial and 3-month stability data results are given in Table 2 (1st and 2nd month data was not given). Charge variant analysis by IEX-HPLC demonstrated a comparable increase in acidic species across all formulations, from about 15% initially to 57-60% after stress. Size exclusion chromatography revealed an increase in high and low molecular weight species (HMW+LMW) from approximately 0.4% to 2.5-3.4%. Hydrophobic interaction chromatography showed a consistent increase in pre-peak species, from approximately 10.5% to 27-33%.

A *t*-test analysis was conducted on the slopes (β) of regression lines to compare the stability profiles of different formulations. This test was used to evaluate the rate of change in critical quality attributes (CQAs) over time, including acidic variant formation and aggregation. The null hypothesis (H_0: $\beta_1=\beta_2$) suggested that the slopes were equal, indicating no difference in the rate of change of CQAs over time, while the alternative hypothesis (H_1: $\beta_1\neq\beta_2$) suggested a difference in slopes. The *t*-test accounted for data variability and standard errors of the slope estimates, with the resulting *p*-value indicating the statistical significance of any difference between the slopes [47]. Results were evaluated against a significance level of $\alpha=0.05$, with a *p*-value less than 0.05 indicating a statistically significant difference. The *t*-test slope comparison results, shown in Figure 5C, revealed no significant differences ($p > 0.05$) in degradation rates between formulations HB02-HB05 and the control (HB01) for both acidic variant formation and aggregation. However, HB06 formulation showed a statistically significant difference ($p = 0.04$) in aggregation rate compared to HB01, while maintaining a similar acidic variant formation rate ($p = 0.54$). These results suggest that while all formulations, including the originator, underwent similar changes under thermal stress, the HB06 formulation (Trehalose/Poloxamer 188) may offer a slight improvement in aggregation stability. The consistent behavior observed across all formulations indicates that the excipient combinations tested maintain the general stability profile of the original formulation.

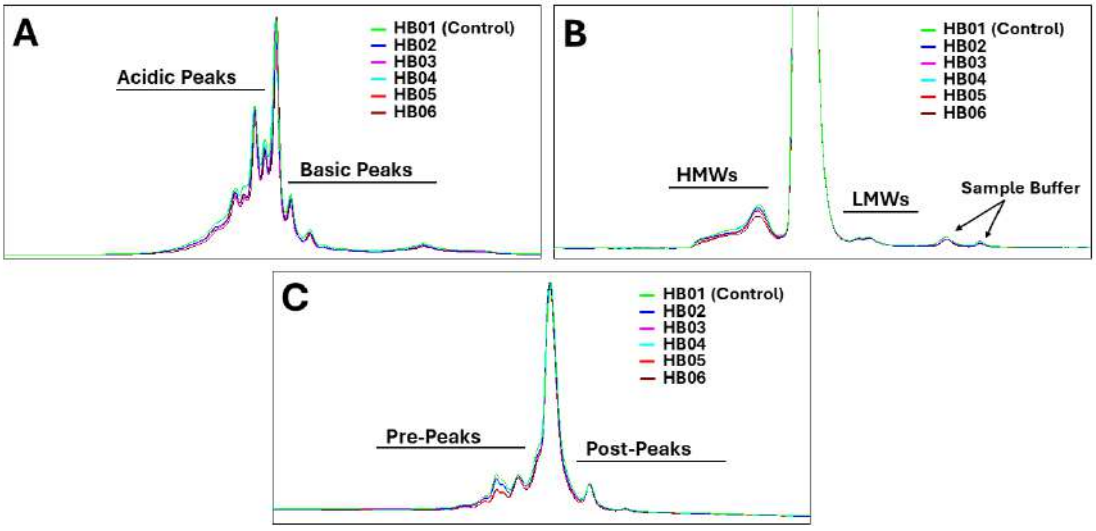
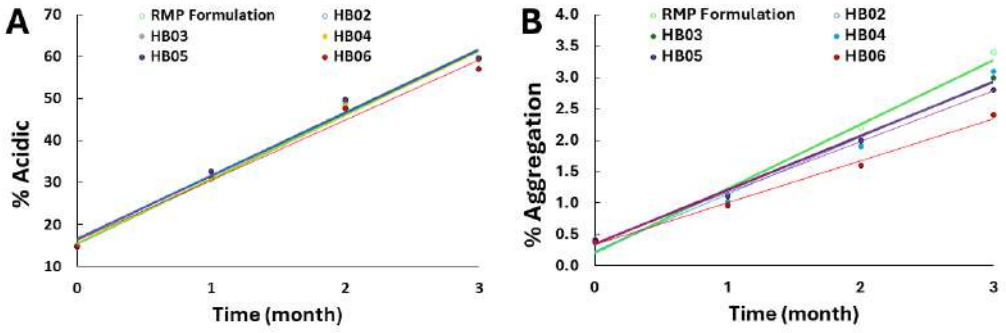


Figure 4. Chromatographic profiles of Histidine buffer formulations after thermal stress. (A) IEX-HPLC chromatograms showing charge variant distribution. (B) SE-HPLC chromatograms displaying size variant profiles. (C) HIC-UPLC chromatograms illustrating hydrophobic variant changes. All formulations (HB02-HB06) and the control (HB01) are shown after 3 months of incubation at 40°C. Peaks are labeled to indicate main species and relevant variants.



C

p-value Comparison

Sample ID	Acidic	HMWs+LMWs
HB02	0.87	0.24
HB03	0.84	0.31
HB04	0.90	0.46
HB05	0.90	0.13
HB06	0.54	0.04

Figure 5. Illustrates (A) monthly increase in acidic variants and (B) Monthly aggregation increase over 3-month period. (C) Statistical evaluation of slope comparisons between the originator formulation and candidate formulations, with p-values resulting from the t-test comparing the slopes (β1 and β2) of the regression lines. A p-value less than 0.05 indicated a statistically significant difference between the slopes.

Table 2. Includes data obtained from SE-HPLC, WCX-HPLC and HIC-UPLC techniques at the initial time point and 3 months after the thermal stress stability study initiated for histidine buffer formulation.

Analysis		SE-HPLC		WCX-HPLC			HIC-UPLC		
Time	Candidate Buffer	%HMW+LMW	%Main	%Acidic	%Main	%Basic	%Pre-peaks	%Main	%Post-peaks
İnitial	HB01 (Originator)	0.40	99.6	14.8	69.3	15.9	10.5	71.6	17.9
	HB02	0.42	99.6	14.9	69.0	16.1	10.4	71.5	18.1
	HB03	0.40	99.6	14.7	69.3	16.0	10.5	71.6	17.9
	HB04	0.37	99.6	14.8	69.2	16.0	10.5	72.1	17.4
	HB05	0.40	99.6	14.9	69.1	16.0	10.4	71.4	18.2
	HB06	0.39	99.6	14.9	69.0	16.1	10.5	71.9	17.6
After 3 Months	HB01 (Originator)	3.40	96.6	59.9	28.6	11.5	33.3	59.0	7.7
	HB02	3.10	96.9	59.4	29.5	11.1	31.6	60.6	7.8
	HB03	3.10	96.9	59.1	30.0	11.0	32.2	60.0	7.9
	HB04	3.20	96.8	59.4	28.2	12.4	30.8	61.4	7.8
	HB05	2.80	97.2	59.5	29.4	11.1	28.3	64.2	7.5
	HB06	2.50	97.5	57.1	30.1	12.8	27.7	64.9	7.4

Effect of Acetate buffer on IgG4 stability

Acetate buffer was selected as a key component in our formulation buffer development for IgG4 stability. This selection was based on its well-established use in biopharmaceutical formulations and its ability to maintain pH stability in the range critical for IgG4 stability (typically pH 4.8-6.0). The critical role of pH in protein stabilization is well-documented, with pH control being of paramount importance considering the charge effect of each formulation component on the protein [35,48]. In our study, special attention was paid to the buffering capacity when choosing a buffer, especially for biotherapeutic formulations with multiple ingredient changes. The choice of acetate buffer is supported by its excellent buffering capacity within the pH range of 3.8-5.8 [49], which is crucial for minimizing chemical degradation and maintaining the conformational stability of biotherapeutics [23]. This range overlaps significantly with the optimal pH range for IgG4 stability, making acetate buffer an ideal candidate. Furthermore, acetate buffer has demonstrated minimal interactions with protein

molecules, potentially reducing the risk of buffer-induced aggregation or other instabilities that can occur with some buffer species [50,51]. To ensure compatibility with the control buffer, we compared the buffering capacity of acetate buffer to that of the control formulation. The control formulation using 10 mM Histidine has a buffering pH range of 5.0-6.0 [5], while 10 mM Acetate buffer covers the range of 3.8-5.8 [49]. Based on these considerations, we selected a pH value of 5.0 for the acetate buffer formulation. This choice ensures that the buffering capacity is not too close to its upper limit, providing optimal stability for the IgG4 molecule. On the other hand, Kim et al. [52] have shown that acetate buffer can contribute to the reduction of charge variants and aggregation in monoclonal antibodies, which is a critical quality attribute in biopharmaceutical development. Interestingly, a study by Mieczkowski [53] revealed a trend towards lower pH in mAb formulations released in the market between 1986 and 2023. The authors suggest that this trend is driven by efforts to increase protein stability and reduce protein-protein interactions. Our choice of acetate buffer aligns well with these criteria, supporting its selection for IgG4 formulation development. Moreover, a comprehensive review by Strickley and Lambert [35] found that 14 out of 126 commercialized mAb formulations utilized acetate buffer, ranking it among the top buffer choices alongside histidine, citrate, and phosphate. This prevalence in commercial formulations further validates our selection of acetate buffer for IgG4 stability optimization.

Having established the suitability of acetate buffer, we subsequently focused on selecting complementary excipients to further enhance the formulation's stability. The selection of L-Arginine, L-Methionine, and L-Histidine as excipients in our acetate buffer-based formulation for IgG4 protein was informed by an extensive literature review and their frequent use in mAb formulations [25,30,54]. These amino acids were chosen for their complementary stabilizing effects and potential synergistic interactions with the buffer system. Literature evidence suggests that L-Methionine serves as an antioxidant and scavenger of reactive oxygen species, protecting against oxidative stress. It has been reported to inhibit protein aggregation by weakening non-covalent interactions and suppress the formation of high molecular weight species during long-term storage [55,56,57]. These properties made L-Methionine an attractive candidate for inclusion in our formulation. L-Arginine was selected based on its reported ability to suppress protein aggregation and increase solubility in concentrated antibody formulations. Previous studies have shown that L-Arginine can enhance thermal stability by increasing the melting temperature of antibodies [29,58]. These characteristics aligned well with our formulation goals for IgG4 mAb stability.

The inclusion of L-Histidine in our formulation was motivated by its multifaceted properties reported in the literature. Beyond its buffering capacity, L-Histidine has been shown to offer metal-chelating properties and act as a pH-dependent stabilizer. Studies have indicated that it can improve thermal stability and reduce aggregation during long-term storage [59,60]. These literature-based findings supported our decision to incorporate L-Histidine into the formulation. The combination of L-Arginine, L-Methionine, and L-Histidine with acetate buffer was strategically chosen based on their potential to modulate various degradation pathways in monoclonal antibodies. Previous studies have demonstrated the effectiveness of these amino acids in reducing oxidation, deamidation, and aggregation [60,61]. In our formulation development process, we carefully optimized the molarities of these excipients to maximize their stabilizing effects while minimizing potential negative interactions or over-stabilization that could impact the biological activity of the IgG4 mAb. To validate our theoretical considerations and optimize the formulation, we conducted a series of experiments using nanoDSF. We performed conformational stability monitoring with 10 mM acetate buffer to understand how different excipients react to pH changes in acetate buffer and to find the ideal pH range. To study this, the effect of 20 mM fixed concentrations of Histidine, Methionine, and Arginine on protein melting points in acetate buffer at different pH levels was examined. The pH-dependent changes in thermal stability parameters are illustrated in Figure 6, where the initial denaturation temperature ($T_{initial}$) and the melting temperature of the second transition (Tm2) are depicted across the examined pH range in (A) and (B), respectively. The pH range indicated by the black dash lines in Figure 6 is the optimal range for the DOE design as it deviates less when compared with the control formulation. In addition, the pH range of 4.8-5.4 was considered

suitable for the DOE study to keep the DOE operating range slightly wider.

To confirm our theoretical considerations and optimize the formulation, we conducted a series of experiments using nano Differential Scanning Fluorimetry (nanoDSF). This technique allowed us to monitor conformational stability with 10 mM acetate buffer and understand how different excipients respond to pH changes in the buffer system. Our primary goal was to identify the ideal pH range for optimal stability. In these experiments, we examined the effect of 20 mM fixed concentrations of Histidine, Methionine, and Arginine on protein melting points in acetate buffer across different pH levels. The pH-dependent changes in thermal stability parameters are illustrated in Figure 6. Specifically, Figure 6A depicts the initial denaturation temperature (Tinitial), while Figure 6B shows the melting temperature of the second transition (Tm2) across the examined pH range.

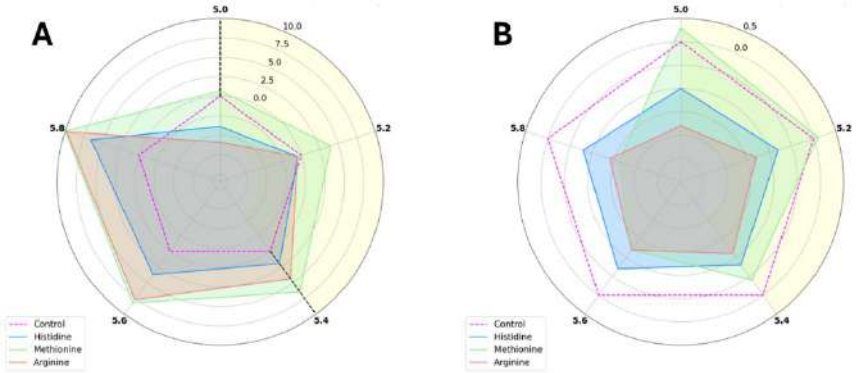


Figure 6. The effect of each excipient on the melting points of the IgG4 protein and the subsequent selection of the appropriate pH based on these melting point changes. Deviations in T_i (A) and T_m2 (B) values of the screened excipients for each pH level compared to the reference formulation (control) are shown.

The results revealed a pH range, indicated by black dashed lines in Figure 6, that showed minimal deviation compared to the control formulation. This range was identified as optimal for the Design of Experiments (DOE) study. To ensure a comprehensive analysis, we slightly expanded this range and found the pH range of 4.8-5.4 to be suitable for the DOE study. Following our initial experiments, we conducted an extensive literature review to determine the appropriate buffer molarity. Analysis of commercial antibodies reveals that products using acetate buffer have concentrations ranging from 10 mM to 80 mM (80 mM was observed in only one product), with most using concentrations of 20 mM or 25 mM [35]. Furthermore, Desai et al. [62] recommend buffer concentrations below 50 mM in biologics formulations, considering patient comfort (pain generation) during administration. Consequently, acetate buffer was considered as a categorical factor at 10, 20 and 30 mM levels in the DOE.

In addition to optimizing the buffer system, we explored trehalose as an alternative stabilizer to sucrose in our acetate buffer-based formulation. This decision was based on several factors supported by recent research. Trehalose has demonstrated superior protein-stabilizing properties in various stress conditions, particularly during lyophilization and long-term storage at elevated temperatures. The exceptional stabilizing effect of trehalose is attributed to its higher glass transition temperature and its ability to form stronger hydrogen bonds with proteins compared to sucrose, leading to better preservation of the native protein structure [36]. Recent studies have shown that trehalose, when used in combination with acetate buffer, can provide enhanced protection against freeze-thaw stress and mechanical stress, which are critical considerations in the manufacturing and handling of biopharmaceuticals [49,63]. Furthermore, trehalose has demonstrated a synergistic effect with acetate buffer in reducing sub-visible particle formation, a key indicator of protein stability [64]. Recent studies utilizing differential scanning calorimetry (DSC) have compared the stabilizing effects of trehalose and sucrose on protein formulations. While both excipients demonstrated protein stabilization capabilities, trehalose showed slightly superior performance in terms of thermal stability for some proteins [65].

Additionally, trehalose has shown a lower tendency to crystallize during freeze-thaw cycles and storage, a

critical factor in maintaining the stability of liquid and lyophilized formulations. This improved resistance to crystallization can lead to better long-term stability profiles, potentially extending the shelf life of the IgG4 formulation [66,67]. These advantages, combined with the observed synergistic effects between trehalose and the chosen amino acid excipients in our acetate buffer system, suggest that trehalose could serve as a viable and potentially superior alternative to sucrose in our IgG4 formulation.

Initially, we utilized a Latin Hypercube design, a statistical methodology that ensures comprehensive coverage of the parameter space for even sampling. This approach allows for efficient analysis of multiple factors with limited experiments, which is particularly valuable in the early stages of formulation development when the effects of various excipients and their interactions are still poorly understood [17,68]. The DOE investigated a wide range of parameters: acetate buffer concentration (10-30 mM), arginine (0-100 mM), methionine (0-40 mM), histidine (0-40 mM), trehalose (2-10% w/v), polysorbate 80 (0.02-0.04% v/v), and pH (4.8-5.4). The results were analyzed using Lasso regression, a powerful statistical method that performs variable selection and regularization simultaneously [69]. This approach allowed for the identification of the most relevant parameters affecting protein stability while minimizing the risk of overfitting the model. Analysis of the results revealed several key observations. Notably, pH emerged as a critical factor in the stability of proteins, with lower pH (4.8) strongly correlated with increased aggregation propensity. This finding is consistent with previous studies that have highlighted the critical role of pH in maintaining the conformational stability of proteins and in minimizing intermolecular interactions leading to aggregation [23,70,71].

Altering the concentration of acetate buffer between 10 and 30 mM did not reveal a definitive pattern in enhancing either aggregation or the formation of acidic variants, although higher concentrations of the buffer resulted in a slight increase in aggregation. This observation implies that reduced buffer concentrations may be adequate for preserving stability, thereby possibly minimizing the likelihood of buffer-induced degradation as indicated by Zbacnik et al. [23] and further investigated by Thorat et al. [72]. Arginine exhibited a protective effect against aggregation, mainly at high concentration (>80 mM) and high pH. Such a synergistic effect concerning arginine and pH was previously reported in various protein formulations. This is generally believed to emanate from the ability of arginine to suppress protein-protein interaction while possibly binding to protein areas prone to aggregation [28,73].

Contrary to early expectations, high trehalose concentrations (10% w/v) only slightly increased aggregation compared to lower concentrations (2% w/v), which might reflect an optimum concentration of trehalose between those two values. This indicates a complex process regarding excipient-mediated protein stabilization and points out the importance of very specific concentration optimization [35,74]. The DOE did not elucidate the effects of methionine and histidine, revealing only minimal or inconsistent influences on both aggregation and the formation of acidic variants. This observed variability in the effects of amino acids has been documented in existing literature and underscores the protein-specific characteristics inherent to stabilization strategies [59,75]. Therefore, to better understand these two excipients, they should be studied in long-term stress studies or forced degradation studies during further optimizations. There was no clear difference at examined concentrations (0.02-0.04% v/v) that gave Polysorbate 80 a distinct advantage about stability enhancement; this is not surprising because the effectiveness of surfactants can vary with the particular protein and under specific formulation conditions [76,77]. A significant observation was the absence of a definitive relationship between aggregation (Δaggregation) and the formation of acidic variants (Δacidity), indicating that these two degradation processes may operate independently. This result aligns with the prevailing knowledge regarding protein degradation mechanisms and underscores the necessity for diverse stabilization strategies [7]. This result showed similarity with our previous studies using sodium citrate and citrate phosphate. To facilitate comparison and provide a clear visual representation of these results, the data were normalized relative to the control formulation, as illustrated in Figure 7.

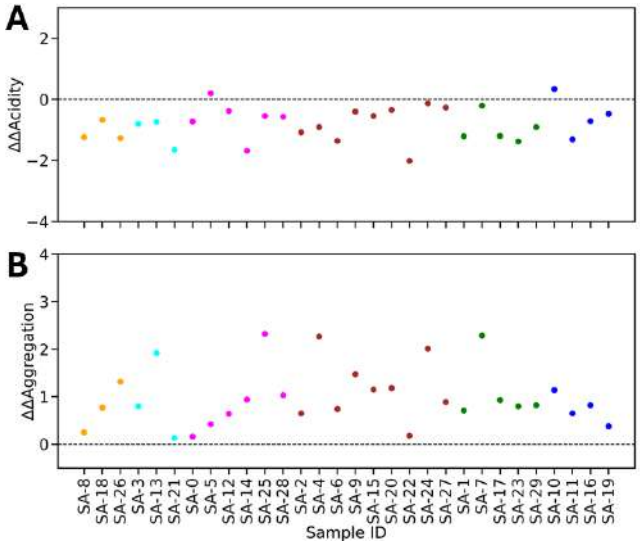


Figure 7. The illustration shows the critical quality attributes (CQA) changes of 30 design experiments for the acetate buffer formulation after 1 month of thermal stress stability testing. The figure represents the comparison of (A) acidic variant, (B) aggregation change with the original formulation. The generated formulation designs are colored based on similarities in their buffer or stabilizer molarities.

When examining the most promising results from the normalized acidity (Figure 7A) and aggregation (Figure 7B) data compared to the control, we selected representative samples for further analysis. Chromatograms of SA-14, SA-21, and SA-22 for acidity, and SA-0, SA-8, SA-21, and SA-22 for aggregation are presented in Figure 8A and 8B, respectively. While the acidity profiles showed improvement compared to the control, the opposite trend was observed for aggregation. Although lower aggregation than the control was not achieved, importantly, no new peaks were observed. These findings highlight the potential outcomes of our DOE results and underscore the need for further optimization studies to balance the trade-offs between improved acidity profiles and aggregation control.

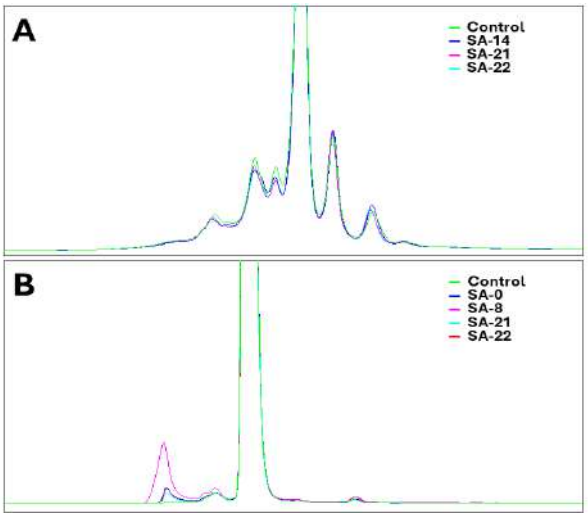


Figure 8. Chromatographic profiles of selected formulations from the DOE studies under thermal stress conditions. (A) Acidity profiles of promising formulations (SA-14, SA-21, and SA-22) from the DOE compared to the control after 1-month thermal stress at 40°C. (B) Aggregation profiles of selected formulations (SA-0, SA-8, SA-21, and SA-22) from the DOE compared to the control after 1-month thermal stress at 40°C.

In conclusion, this systematic approach to formulation development, moving from a broad exploratory DOE using Latin Hypercube sampling to a focused optimization study, allowed us to efficiently identify promising excipient combinations and concentrations for enhancing the overall stability profile of our protein in an acetate buffer system. The selection of acetate buffer for our IgG4 formulation development is well-supported by its excellent buffering capacity, minimal protein interactions, potential for charge variant reduction, and alignment with current trends in mAb formulation pH. The addition of carefully selected amino acid excipients, along with the exploration of trehalose as a stabilizer, further enhances the stability profile of our formulation. These results set the stage for further refinement and potentially the development of a more stable and effective IgG4 formulation.

Table 3.

Sample ID	Buffer (mM)	Arginine (mM)	Methionine (mM)	Histidine (mM)	Trehalose (w/v%)	Polysorbate 80 (v/v%)	pH	Δaggregation	Δacidity
Control								0.64	16.0
SA-8	10	92	30	23	2	0.04	5.4	0.89	14.8
SA-18	10	2	9	21	2	0.02	5.1	1.41	15.3
SA-26	10	28	37	9	2	0.02	4.8	1.96	14.7
SA-3	10	38	33	10	10	0.02	4.8	1.44	15.2
SA-13	10	82	27	37	10	0.04	4.8	2.56	15.3
SA-21	10	85	19	13	10	0.04	5.4	0.77	14.4
SA-0	20	65	23	11	2	0.02	5.4	0.80	15.3

SA-5	20	5	38	25	2	0.04	5.4	1.06	16.2
SA-12	20	22	11	26	2	0.02	5.4	1.28	15.6
SA-14	20	78	31	17	2	0.04	5.1	1.58	14.3
SA-25	20	72	26	5	2	0.02	4.8	2.96	15.5
SA-28	20	12	34	22	2	0.04	5.1	1.67	15.4
SA-2	20	88	39	29	10	0.04	5.1	1.29	14.9
SA-4	20	98	2	35	10	0.04	4.8	2.91	15.1
SA-6	20	62	3	30	10	0.02	5.1	1.38	14.6
SA-9	20	48	14	33	10	0.02	4.8	2.11	15.6
SA-15	20	55	18	2	10	0.02	4.8	1.79	15.5
SA-20	20	35	17	7	10	0.04	4.8	1.82	15.7
SA-22	20	68	29	31	10	0.02	5.4	0.82	14.0
SA-24	20	58	22	38	10	0.02	4.8	2.65	15.9
SA-27	20	18	25	6	10	0.02	4.8	1.53	15.7
SA-1	30	95	15	19	2	0.02	5.1	1.35	14.8
SA-7	30	52	7	14	2	0.02	4.8	2.93	15.8
SA-17	30	42	1	18	2	0.02	5.1	1.57	14.8
SA-23	30	45	35	15	2	0.04	5.1	1.44	14.6
SA-29	30	25	13	3	2	0.02	5.1	1.46	15.1
SA-10	30	8	10	34	10	0.02	4.8	1.78	16.3
SA-11	30	75	5	27	10	0.02	5.1	1.29	14.7
SA-16	30	32	6	39	10	0.04	5.1	1.46	15.3
SA-19	30	15	21	1	10	0.02	5.1	1.02	15.5

CONCLUSION

The present study on IgG4 monoclonal antibody formulation development has elucidated significant insights into the effects of various buffer systems and excipients on protein stability. Through the application of Design of Experiments (DOE) methodologies and advanced analytical techniques, it was demonstrated that sodium citrate and citrate phosphate buffer systems exhibit considerable potential for further optimization. Histidine buffer formulations displayed effectiveness when used in conjunction with alternative stabilizers, while acetate buffer emerged as a promising alternative, showing superior results in certain aspects compared to histidine and citrate-based buffers.

The investigation of sodium citrate, citrate phosphate, and acetate buffer systems at varying concentrations and in combination with different excipients has revealed promising avenues for enhancing IgG4 formulation stability. The exploration of alternative stabilizers such as trehalose and sorbitol, particularly in histidine buffer systems, has opened new possibilities for formulation optimization. However, the study also highlighted the need for further investigation of certain excipients, specifically methionine and arginine, in the acetate buffer system. The behavior of these amino acids was not fully elucidated in the current experimental framework, necessitating long-term stability studies and forced degradation experiments to comprehensively understand their effects and interactions within the acetate buffer environment. This research establishes a robust foundation for the rational design of IgG4 formulations with enhanced stability profiles. The findings provide new perspectives for the development of more stable and effective formulations in the field of biopharmaceutical product development. Furthermore, the identified need for extended studies on specific excipients in buffer systems paves the way for more targeted formulation strategies in future investigations.

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