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METHOD

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High-throughput excipient screening using 384-well plates and a pipetting robot: assessing protein stability after freeze-drying to pre-select viable formulations

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ABSTRACT

Introduction: Formulation research benefits from high-throughput excipient screening methods, considering the ever-growing excipient space. We investigate the use of 384-well plates as freeze-drying containers and for subsequent analyses, to screen the effect of excipients on the stability of proteins during freeze-drying. **Methods:** For both the preparation and analysis methods of a range of β-galactosidase formulations, an 8-tip pipetting robot was used. Formulations were lyophilized in 384-well plates, which were also used for subsequent enzymatic activity assessment, serving as an indication of protein stability. **Results:** Excipient screening revealed that threonine, histidine, arginine, sucrose, and trehalose enhance the recovery of the enzymatic activity of β-galactosidase compared to the protein freeze-dried in buffer without other excipients. Moreover, pullulan only showed a stabilizing effect when it was combined with low-molecular-weight excipients that by themselves were poor stabilizers, which was especially the case for serine and to some extent for valine. **Discussion:** There were no significant differences in enzymatic activity when comparing the automated 384-well plate freeze-drying method with a common in-vial method, while offering the added sustainability benefits of increased throughput, reduced workload, and lower protein and reagent usage. This approach might be suitable for the pre-selection of viable formulations.

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Introduction

In 2023, 15 out of the 25 highest revenue drugs were biopharmaceuticals [1], and this trend is likely to continue. Notwithstanding, process and storage stability remain a major obstacle during development of new products. Some common strategies to improve protein stability include, amongst others, modifying the solution's pH, the concentration and choice of buffer components, and using surfactants to prevent interfacial adsorption [2], as well as stabilizers such as amino acids, sugars or polymers to increase conservation of the protein's native state [3,4]. Alternatively, protein stability can be improved by bringing active pharmaceutical ingredients (API) in the solid state, resulting in reduced degradation reaction kinetics due to reduced molecular mobility. To this end, freeze-drying is often used as a relatively gentle drying method. Nevertheless, for freeze-drying, excipients are also often necessary to improve protein stability. As such, over the past few decades, many excipients have been introduced to the market, resulting in virtually limitless combinations when developing a biopharmaceutical formulation. To illustrate, as of June 2020 there were 211 biopharmaceuticals approved by the FDA, represented by 665 different formulations, out of which 397 formulations used a unique combination of excipients [5]. Besides the marketed excipients, there is also active research into new excipients and repurposing existing excipients for new roles, such as the use of amino acids as lyoprotectants [6]. Due to the virtually limitless excipient combinations, sample sizes quickly become hard to manage through classical freeze-drying screening methods.

To minimize costs, time, and efforts spent during the development phase, high-throughput screening of

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excipients can be used as a strategy to narrow down to a list of viable freeze-drying pre-formulations for subsequent thorough characterization. Firstly, the freeze-drying process can be time-consuming and any method to shorten it increases screening throughput and scalability. Several studies have been published on freeze-drying sample volumes in the microliter range, using 96-well plates as sample containers, which could also be conveniently used during subsequent analyses [7–11]. To take this a step further, Coussot and colleagues [12] were, to the best of our knowledge, the first to freeze-dry using 384-well plates, although their work was focused on preparing freeze-dried well plate assays to detect antigens. To build on these works, in this study, we show that 384-well plates can also be used to screen the effect of excipients on protein stability during freeze-drying, namely the stability of β-galactosidase. To this end, we evaluated the stabilizing capacity of a range of excipient combinations comprising a polysaccharide and low-molecular-weight excipients in various ratios. Such combinations have been shown in prior studies to exhibit strong stabilizing potential [13,14]. To assess β-galactosidase stability, we used its enzymatic activity, before and after freeze-drying, as stability indicating readout, using a straightforward colorimetric assay. In addition to using 384-well plates, we employed a relatively short freeze-drying cycle and an 8-tip pipetting robot to handle all solution processing, as well as preparing and performing the enzymatic activity assays, to further increase throughput. Finally, the results of this proof-of-concept 384-well plate-based screening method were compared to those of a conventional in-vial method, to demonstrate its suitability for a preliminary selection of viable formulations.

Materials and methods

Materials

Bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), magnesium dichloride, arginine hydrochloride (denoted as arginine), L-histidine, L-serine, L-threonine, L-valine, and ortho-nitrophenyl-β-galactoside (ONPG) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dihydrate were obtained from Merck (Rahway, NJ, United States). β-Galactosidase was obtained from Sorachim (Lausanne, Switzerland). Sucrose and trehalose dihydrate were gifts from DFE Pharma (Goch, Germany). Pullulan (average molecular weight 200–300 kDa, <10% mono-, di- and oligosaccharides) was a generous gift from Hayashibara (Okayama, Japan).

Formulation preparation and freeze-drying

Stock solutions of arginine, histidine, serine, threonine, valine, sucrose, trehalose, and pullulan were prepared in 2 mM HEPES buffer of pH 7.4±0.1 at a concentration of 1 mg/mL. Solutions of β-galactosidase of 1 mg/mL were prepared fresh in 2 mM HEPES buffer of pH 7.4±0.1. To assess reproducibility and whether freeze-drying in outer wells resulted in significantly different enzymatic activity from inner wells, so-called edge effects, a β-galactosidase-trehalose formulation with an excipient to protein weight ratio of 99 to 1 was prepared manually. The formulation had a final β-galactosidase concentration of 10 µg/mL. Subsequently, the entire 384-well plate except row A, which was used for a calibration curve, was filled with 10 µL per well and then freeze-dried.

For subsequent screening experiments, a JANUS Expanded AJL8001 Automated Liquid Handler (Perkin Elmer, Waltham, MA, United States) was used to make 30 unique protein formulations, by mixing stabilizer and protein stock solutions in a 99 to 1 volume ratio in a 96-deep-well plate, including the control formulation of β-galactosidase in buffer. Subsequently, 10 µL of each of these formulations were transferred from the 96-deep-well plate in triplicate into a 384-well plate. To freeze-dry the samples, the 384-well plate was centrifuged and placed on a custom-made aluminum platform, which was pre-cooled to −55 °C in a Martin Christ Epsilon 2–4 LSC plus freeze-dryer (Salm & Kipp, Breukelen, The Netherlands). The aluminum platform was used to facilitate heat transfer from the well bottoms to the cooling plate of the freeze-dryer. Primary drying was then performed for 10 h at a shelf temperature of −35 °C and 0.220 mbar, after which the pressure was reduced to 0.055 mbar and the temperature was raised to −10 °C during 30 min. Thereafter, the temperature was increased with a rate of 0.33 °C/min, until a temperature of 20 °C was reached. Then, secondary drying was performed for 5 h, at a temperature of 20 °C and a pressure of 0.055 mbar. After drying for 17 h in total, the well plates were transferred to a nitrogen flushed climate box, sealed, and then stored at −20 °C, until used for further analysis. In total, three full replicates of screening experiments were performed. Each of these replicates involved the fresh preparation of the aforementioned 30 formulations, which were then pipetted in triplicate into a 384-well plate, freeze-dried, and analyzed.

To compare the results of the experiments using 384-well plates to a common in-vial approach, a

selection of formulations was freeze-dried in common rubber-stoppered glass vials. Each vial contained 100 µL of formulation with the same composition as was used for freeze-drying in the 384-well plates. Subsequently, three vials per formulation were freeze-dried using the same program used for the 384-well plates. After the freeze-drying cycle was complete, samples were closed under vacuum and stored at −20 °C, until used for further analysis.

Enzymatic activity assessment

Enzymatic activity of β-galactosidase was assessed by modifying a previously described method [13], based on conversion of the colorless ONPG to the yellow *ortho*-nitrophenol. In short, samples that were freeze-dried in 384-well plates were diluted to 0.83 µg/mL protein in the wells, using 120 µL 0.05 M phosphate buffer at pH 7.5, containing 0.1% BSA and 1.43 mM $MgCl_2$. Then, the 384-well plates were incubated at room temperature for 10 min. Subsequently, from each individual well, 40 µL of dissolved sample was transferred to an empty well. Subsequently, 80 µL of substrate solution comprising 1 mM $MgCl_2$ and 2 mM ONPG in 0.1 M phosphate buffer at pH 7.5 was added to the 40 µL. Thereafter, absorption at 405 nm was determined every 56 s for 10 min at room temperature. The rate of increase in absorption over time, due to substrate conversion, was compared to the rates for known concentrations of freshly prepared β-galactosidase solutions, to determine the residual enzymatic activity. The enzymatic activity after traditional vial-based freeze-drying was assessed as previously described [13].

Statistical testing

To compare rows and columns and assess possible aforementioned edge effects (Figure 1), an ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used. To compare the means of formulations freeze-dried in the 384-well plates (Figure 2) with the control (β-galactosidase with only buffer and no other excipients), an ordinary one-way ANOVA with a post-hoc Dunnett's multiple comparison test was used. To compare the effect of pullulan on the stabilizing effect of low-molecular-weight excipients (Figure 2), an ordinary one-way ANOVA with a post-hoc Tukey's multiple comparison test was used. To compare the effect of the preparation method on enzymatic activity for each formulation when comparing manual vs. automated formulation and sample processing (Figure 3), a paired t-test was used. For all of the above analyses, p-values < 0.05 were considered significant. All statistics were performed using Prism 10.4.1 (532).

Results

Edge effects

During preliminary experiments, so-called "edge effects" were observed in plastic 96-well plates, which showed significantly higher enzymatic activities in the

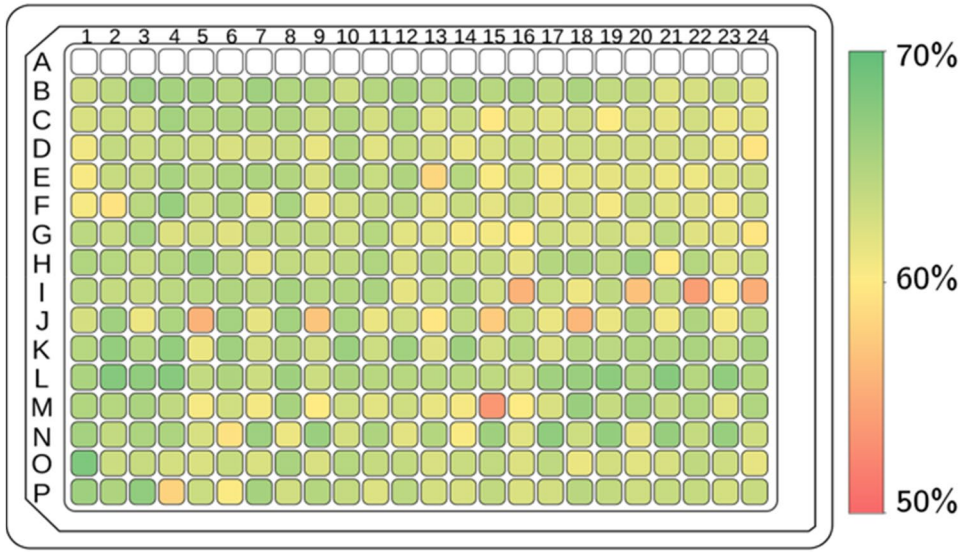

Figure 1. Average enzymatic activity of β-galactosidase – trehalose (1:99 w:w) formulations after freeze-drying. The residual enzymatic activity is represented by a color gradient. Each of the 360 wells in this figure represents the average of the respective well from three independent experiments. The average relative standard deviation (SD) across these 360 wells, calculated from the triplicates for each well was 8.11% and is not shown in the figure. Row A was used for the calibration curve. The variation between the slopes of three calibration curves was < 5%.

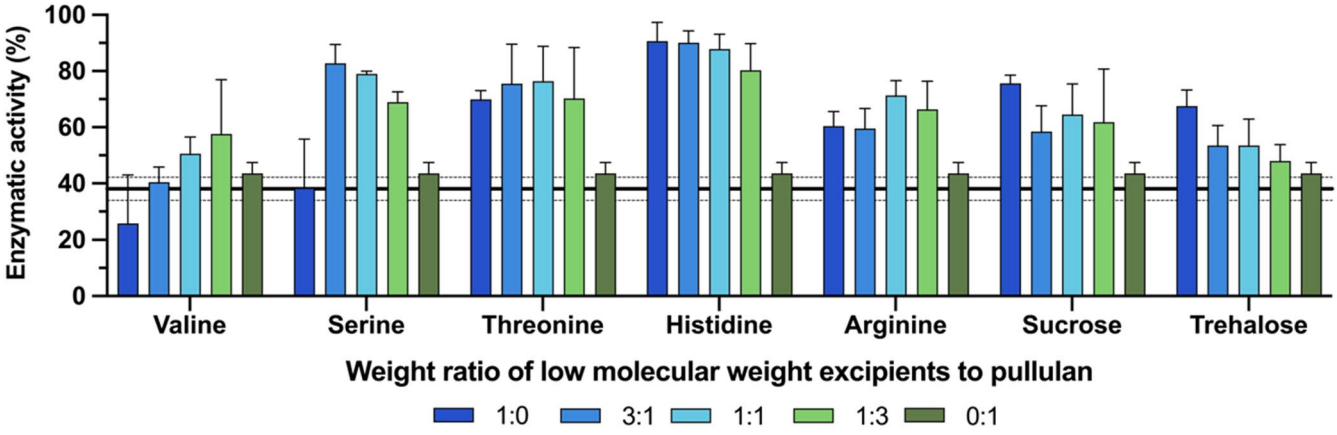

Figure 2. Enzymatic activity of β-galactosidase formulations containing various weight ratios of low molecular weight excipients to pullulan. The black line represents β-galactosidase freeze-dried in buffer without other excipients, with the dotted lines representing the SD; $n = 3$ with SD, where each replicate involved a full experimental cycle: preparation of fresh formulations, which were pipetted in triplicate into a 384-well plate, freeze-dried, and then analyzed.

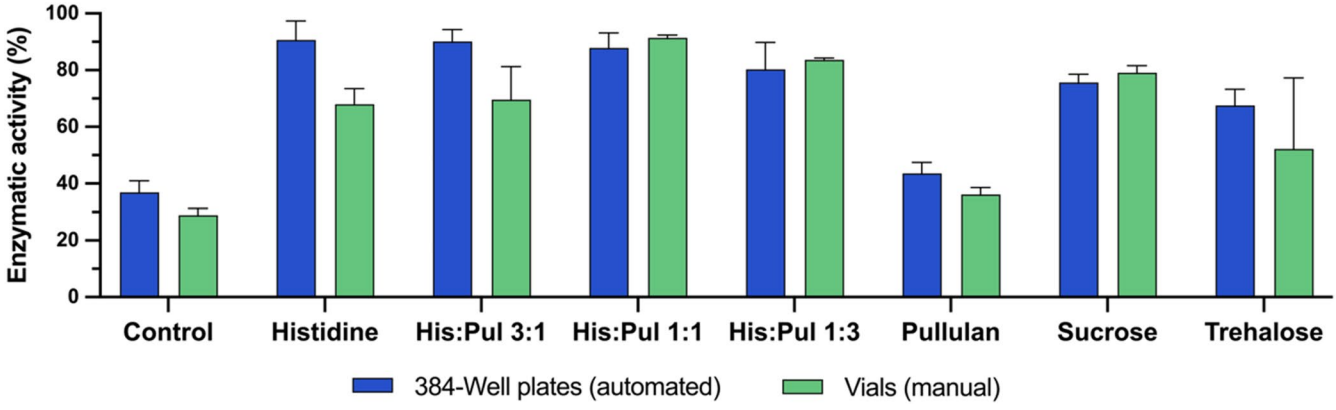

Figure 3. Enzymatic activity of a selection of β-galactosidase formulations to compare the new method to the traditional method ($n = 3$ with SD, of which the well plate data originates from Figure 2 and of which the vial data are of 3 separately prepared samples freeze-dried in the same cycle). The control is β-galactosidase freeze-dried in buffer without other excipients.

outer wells than in the inner wells (data not shown). As visualized by the color gradient in Figure 1, neither clear edge effects nor specific activity patterns were observed when freeze-drying in the 384-well plate in this study. Moreover, there were no significant differences between the columns. When comparing rows, some significant differences were observed. While no clear patterns were observed, statistical analysis revealed that enzymatic activity in rows L and K was significantly higher than in most other rows. Row L showed higher activity than all rows except B, N, and K, while row K's activity was higher than all rows except B, H, L, N, O, and P.

Excipient screening

To exemplify how freeze-drying using 384-well plates might be leveraged, β-galactosidase was freeze-dried in a range of formulations containing various weight ratios of pullulan to low-molecular-weight excipients. Several amino acids with different side-chain characteristics were selected, namely valine (non-polar), serine and threonine (polar), and arginine and histidine (positively charged). In addition, sucrose and trehalose were also included as they are gold standards for protein stabilization. The enzymatic activity of β-galactosidase was determined after preparation and freeze-drying, as shown in Figure 2. The low-molecular-weight excipients threonine, histidine, arginine, sucrose and trehalose acted as stabilizers, resulting in significantly higher ($p < 0.05$) enzymatic activity than β-galactosidase freeze-dried in buffer without other excipients. For formulations containing threonine, histidine, arginine, sucrose, and trehalose, increasing the high-molecular-weight stabilizer pullulan fraction did not significantly impact protein stability. Moreover, by itself, pullulan did not exhibit a significant stabilizing effect, compared to β-galactosidase freeze-dried in buffer. Nevertheless,

pullulan had a significant effect ($p < 0.05$) when combined with low-molecular-weight excipients that by themselves were poor stabilizers, as was the case for the formulations with a serine-to-pullulan weight ratio of 3:1, 1:1, and 1:3. When combined with valine, pullulan showed an increase in average β-galactosidase activity compared to the β-galactosidase freeze-dried in buffer, though the effect was not significant. However, compared to valine alone, valine combined with pullulan at a 1:3 weight ratio was significantly more effective ($p < 0.05$).

Comparison to traditional freeze-drying

To compare the 384-well plate freeze-drying method to a more common freeze-drying screening approach, a selection of formulations was manually prepared in 5 mL freeze-drying glass vials with rubber stoppers. These formulations were prepared with the same concentrations of components and an identical freeze-drying cycle as those used in the well plate experiments. The results of this comparison are shown in Figure 3. While overall the manual method tended to yield lower values than the automated 384-well plate method, there was no significant difference between the manual and automated methods.

Discussion

Edge effects

Edge effects observed during preliminary experiments could be due to increased evaporation rates in the outer wells during the analysis; a phenomenon that has been described before [15]. The same edge-related drying behavior has also been observed when freeze-drying in 96-well plates, resulting in slightly higher drying rates in the outer wells [8]. Nevertheless, no apparent edge effects were observed in the activity data from 384-well plates, possibly explained by low incubation temperatures used during analysis. While the incubation temperature was modified in this study to facilitate sample processing, incubation at room temperature has also been shown to significantly reduce edge effects in both 96 [16,17] and 384-well plates [18], likely due to reduced evaporation.

Excipient screening

The superior stabilizing capacity of sucrose, and trehalose compared to the buffer control can be attributed to their ability to form hydrogen bonds with the protein and to form a stable amorphous matrix [19], which likely is also the case for the amino acids threonine, histidine and arginine (Figure 2). In contrast, the poor stabilizing capacity of valine and serine could be due to valine's inability to form hydrogen bonds with its side-chain [6] and the low (predicted) glass transition temperature (T_g) of 11 and 28 °C for valine and serine, respectively [20]. In these formulations, pullulan increases the system T_g, thereby providing kinetic stability and protecting β-galactosidase. Moreover, in previous studies, it has been shown that combining low molecular weight and high molecular weight excipients can be beneficial under prolonged storage conditions, in particular if formulations are exposed to moisture. Water acts as a plasticizer and depresses the glass transition temperature of the system, potentially leading to crystallization, which is detrimental to protein stability [13]. In such cases, including a polymer in a formulation can increase the glass transition temperature to prevent the glass to rubber transition, and eventually crystallization. As such, the addition of pullulan to formulations containing threonine, histidine, arginine, sucrose, and trehalose makes them significantly more robust under such conditions.

Nevertheless, samples were not subjected to accelerated storage conditions in the current study design. Rather, the stresses of freeze-drying itself were assessed to screen the impact of excipients on process stability. The results show that combinations of trehalose and pullulan followed similar trends in process stabilizing capacity as observed in a previous study [14]. Similarly, arginine alone and in combination with pullulan performed better as a process stabilizer than pure pullulan, consistent with previous findings [13]. When comparing the 384-well plate to a manual method in Figure 3, similar and clear distinctions could be made between poor and good stabilizers, e.g., pullulan vs. sucrose. However, there were slight differences in absolute values between both methods, for example for histidine and trehalose. The tendency for some excipients to result in slightly lower activities in the common in-vial method compared to the 384-well plate method might be attributed to subtle differences in the drying process, such as in the heat transfer and container material. The large surface area-to-volume ratio in the 384-well plates could lead to a more uniform and efficient drying, which may be particularly beneficial for certain excipients. However, when comparing both methods overall, the results were not significantly different, indicating that the 384-well plate method can be a reliable tool for initial screening and pre-selection of formulations.

Advantages of the automated approach

When further comparing the automated and traditional methods, several distinct advantages emerge for the automated approach in freeze-drying studies. The

automated 384-well plate method substantially increases throughput while reducing workload and human interference, thereby minimizing the risk of experimental errors. The freeze-dryer used in this study could accommodate approximately 250 freeze-drying 5 mL vials in a traditional setup. However, it could also hold around 9,384-well plates. When accounting for assay calibration curves, these 384-well plates can hold 3,240 samples per freeze-drying cycle. Moreover, only 10 µL per well was used which was spread out over a relatively large surface. Therefore, shorter freeze-drying cycles might be possible than those used for conventional vials, which typically hold up to several milliliters per vial. In this study, a freeze-drying cycle of 17 h was chosen to fit within a one-day timeframe, based on previous research where 100 µL per well was freeze-dried [7,10]. However, since in this study only 10 µL per well was freeze-dried, an even shorter freeze-drying cycle might be worth exploring. Together, the almost 13-fold higher sample capacity and reduced drying times result in a marked decrease in energy consumption by the 384-well plate method compared to common vial-based methods. The small volume per sample also results in very low protein usage. For example, for the screening of the excipients shown in Figure 2, all formulations together amounted to only 13.2 µg of β-galactosidase with a total of 1.32 mL volume of water to be removed. This might be beneficial for newly developed enzymes or therapeutic proteins that are available in limited quantities. The increased throughput, reduced workload, and lower reagent usage are key benefits that contribute to sustainability in drug discovery [21]. Furthermore, the only manual tasks during the 384-well plate experiments were preparing stock solutions and transferring the 384-well plates between the automated liquid handling system, the freeze-dryer and spectrophotometer. Standard manual procedures like mixing stabilizer solutions, dilutions, aliquoting, preparing calibration curves and assay reagent mixtures could be performed by the 8-tip pipetting robot. Additionally, labeling of samples is not required when using well plates. It is worth noting that not all novel proteins are enzymes. Nevertheless, there are several techniques that can in such cases still be used for high-throughput screening, for example fluorescence spectroscopy [7], isothermal denaturation fluorometry [22], differential scanning fluorometry [23], and light scattering [24]. Additionally, during formulation development it is crucial to thoroughly evaluate not only enzymatic activity but also properties of the product. These include protein structure, aggregation propensity, matrix properties, dissolution behavior, and storage stability [19]. Lastly, it is worth noting that this study focused on a single model protein, β-galactosidase. While the well plate method proved effective for this specific case, future research could explore its applicability and limitations for a broader range of proteins with varying structural characteristics to further validate its universal utility. Nevertheless, a screening method like the one described in this study can serve as a first step to select a range of viable formulations for more thorough characterization.

Conclusions

In this work, a range of β-galactosidase formulations was freeze-dried in 384-well plates, which were also used to assess its enzymatic activity. For both the preparation and analysis method, an 8-tip pipetting robot was used. Excipient screening revealed that most low molecular weight excipients helped to preserve the enzymatic activity, while pullulan only showed a stabilizing effect when it was combined with low molecular weight excipients that were ineffective stabilizers on their own. This observation highlights the potential of excipient combinations, and the benefit of high-throughput screening methods to investigate them. The automated 384-well plate freeze-drying method demonstrated enzymatic activity trends similar to those of a common in-vial method, while offering the added benefits of increased throughput, reduced workload, and lower protein and reagent usage. These are important advantages featuring sustainability in drug discovery. Overall, the automated 384-well plate method offers an efficient and high-throughput approach for freeze-drying studies, particularly suitable for the pre-selection of viable formulations.

Author contributions

CRediT: **Daan Zillen**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing; **Noa N. van der Ploeg**: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing; **Ronald van Merkerk**: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing; **Gerrit J. Poelarends**: Funding acquisition, Resources, Supervision, Writing – review & editing; **Henderik W. Frijlink**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing; **Wouter L.J. Hinrichs**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Disclosure statement

The authors declare no conflicts of interest.

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Data availability statement

Data can be made available upon request.

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