REV.ION

RevION, a new way to quantify Oxalate in CGXII media Samples.

Over the course of nearly six months, we have been developing a new (semi-) analytical assay for the detection and quantification of oxalate in CGXII cell medium samples. Although the assay is not yet fully finalized and further refinements are possible, significant progress has been achieved, enabling reliable measurement of oxalate in CGXII medium samples obtained from our reactor runs.

The CGXII samples represent the extracellular fraction of oxalate produced by our modified Corynebacterium glutamicum strains at defined time points during cultivation. Accurate quantification of oxalic acid and oxalate in these samples is essential for assessing the potential of our genetically modified strains to serve as efficient bio factories for our metabolite.

The following summary provides insights into the development, optimization, and current performance of the assay.

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What was the motivation to develop this assay?

Our research team at the Institute of Bioprocess Engineering at University of Stuttgart required a practical assay to quantify oxalate in CGXII cell medium samples. Access to the institute´s HPLC System was limited due to long run times and a waiting list. In addition, the student´s expertise in the field of HPLC was limited and the financial resources from sponsors and donations were insufficient to cover the costs of processing all our samples.

As a second option, we considered an enzymatic assay from Sigma-Aldrich Merck, which was designed to analyze up to 100 samples. However, each sample is required duplicates, a blank, and an

internal standard. This reduced the effective input capacity to a maximum of 25 samples - far too few, considering we had six reactors with approximately 16 Samples per reactor. In total, this would have allowed for only about four samples per reactor at a cost of 25,92€ each, which was insufficient for generating reliable and representative data sets.

Consequently, we decided to develop our own analytical method, specifically tailored to our CGXII medium, and aligned with our financial constraints.

The primary objective of the assay was thereby to establish a laboratory protocol and mathematical model that is inexpensive, user-friendly, and capable of fulfilling as many assay criteria as possible, thereby ensuring suitability for both quantitative and semi-quantitative analysis.

What is the final main protocol structure of our assay?

Our final version of the RevION assay is based on the indirect quantification of oxalate in our CGXII medium samples via the detection and measurement of calcium. The procedure starts with an initial precipitation step (after cell removal) of the oxalate present in our cell culture samples through the addition of calcium chloride in a defined excess, thus providing the necessary calcium ions for the formation of calcium oxalate complexes. Followed by a centrifugation step, the supernatant, containing the unbound excess of calcium ions, is transferred, and subjected to further analysis. After a series of dilution steps, the supernatant samples, along with their corresponding blanks (which did not undergo the precipitation step), are loaded in triplicates onto a standard polystyrene 96-well plate.

Subsequently, a defined amount of our o-Cresolphthalein Complexone (oCPC, also known as Methalphthalein) is added. oCPC forms stable violet-red complexes with the present calcium, whose absorbance intensity is directly proportional to the calcium concentration in the analyzed well. Measurements are then performed via TECAN Spark plate reader absorption analysis in a range from 500-580nm, with its peak absorption at 572nm, thus providing high-precision absorbance data output. By subtracting the measured and blanked excess calcium concentration from the initially added calcium concentration through the precipitation, the effective oxalate content of the sample can be recalculated.

During final testing in CGXII medium, it was observed that calcium not only complexed with oxalate but also with phosphates present in the CGXII mediums composition. This interference required the development of a strategy to correct this phosphate-induced absorbance and to isolate only oxalate-specific signals.

What Problems were encountered throughout the Development Process and what Solutions did we find?

Throughout the optimization process, several challenges emerged that we initially did not anticipate. In the following, the major problems, and our approaches toward solving them are summarized:

Direct Quantification of the analyte

Our initial approach focused on the direct quantification of oxalate in our samples. The oxalate was isolated via calcium oxalate precipitation. After a centrifugation step, a pellet formed at the bottom of the test tube containing the calcium oxalate. The procedure involved removing the excess supernatant, washing the pellet with 80% ethanol to reduce matrix interferences in the measurement process, resuspending the calcium oxalate pellet in ammonium hydroxide or HCl, and subsequently measuring absorbance in the deep-UV range (200–300 nm, typically peaking at 210–216 nm) using the TECAN Spark reader.

However, several issues arose: the ethanol wash proved inefficient due to the strong insolubility of the calcium oxalate complexes, as the pellet remained intact even after several vortexing steps. Attempts to resuspend and redissolve the pellet with ammonium hydroxide or HCl were only partially successful, and a complete solubilization of the calcium oxalate complexes remained nearly unattainable. Consequently, reliable semi-analytical measurement of oxalate was not possible with this direct quantification method.

Following extensive literature research and experimental trials, we transitioned to the current method based on the indirect oxalate quantification via oCPC-mediated calcium detection, which significantly improved the robustness and reproducibility of our RevION Assay.

Scalability from mM to µM

In the final stages of our research on the RevION´s development, an enzymatic assay revealed that the oxalate concentrations in several of our reactor samples were not within the anticipated millimolar range but instead in the lower micromolar range. Consequently, our assay had to be rescaled to achieve accurate quantification within this lower concentration range.

Over a period of four months, a mathematical system was developed in Microsoft Excel to autonomously calculate all key experimental parameters, including reagent volumes, stock concentrations, and dilution factors, based on the users defined calibration range. This

approach not only ensured time saving but also the accommodation of both the lowest and highest expected oxalate concentrations in our samples and therefore reliability on the calibration output.

In the final calibration experiment of one of the last assay designs, a mean absolute error (MAE) of 1.2 mM was achieved, corresponding to a match of about 93,18% between original and measured values. Additionally, the calibration curve exhibited an R^2 value of 0,9692, representing a strong linear correlation of 96,92% across all calibration standards.

Our primary concern during the transition to quantification of lower concentrations was the potential amplification of variances and absolute errors, which could negatively impact the measurements precision. However, after adjusting the scaling parameters within the mathematical model and conducting laboratory validation with minor protocol refinements, we observed only a minimal increase in the MAE that did not significantly affect the overall accuracy or linearity of the final calibration curve.

Matrix Effects Through Unknown Substances

One of the primary challenges in bio-analytics is addressing all matrix-induced interferences and developing strategies to minimize or eliminate them. During the early stages of RevIONs development, calibration experiments were conducted exclusively in deionized water to optimize the chemical parameters and establish a stable reaction environment. Once reproducible results were achieved, we switched the matrix to CGXII medium to better simulate real sample analysis conditions.

Unexpectedly, when observing the blank calibration standard—which contained no oxalate and therefore was not expected to show any precipitation—a distinct precipitate formed at the bottom of the test tube. Further investigation in the matter revealed that the calcium ions were also complexing with phosphates present in the CGXII medium. This presented a critical issue, since calcium could bind both oxalate and phosphate, it became impossible to distinguish between calcium-oxalate and calcium-phosphate complexes within our samples. To address this problem, two potential approaches were developed:

a. Phosphate Precipitation Prior to Analysis

The first approach involved performing phosphate precipitation using a protocol routinely applied at our institute for HPLC related sample preparation. The idea was to selectively remove phosphate ions before oxalate quantification, thereby obtaining a

clarified supernatant only containing all other CGXII components and the oxalate of interest.

However, this procedure introduces magnesium ions into the sample, which also possess the ability to form complexes with the oCPC during the final colorimetric reaction step, potentially interfering with the measurement. Theoretically, this interference can be corrected by blanking the samples´ raw absorption results with a non-precipitated reference sample. This blank would allow quantification of the native magnesium and calcium ion fractions already present in the CGXII medium—as well as those introduced during phosphate precipitation—so that their contribution to oCPC complex formation can be mathematically subtracted from the measured signal.

This method is currently still under experimental validation but shows promising potential for integration into the final RevION assay design.

b. Phosphate Quantification via External Assay

The second approach focuses on quantifying the phosphate concentrations directly through an external phosphate assay. This strategy involves measuring phosphate levels in at least two reactor samples, ideally one from the mid-point of the reactor run and one from the final sampling time point. Combined with the known initial phosphate concentration in the CGXII medium (at t = 0), these data points can be used to construct an exponential model that describes the theoretical phosphate consumption by our engineered Corynebacterium strain during specific reactor run times. The accuracy of this model improves with the number of phosphate concentration data points collected.

Reaching the Linear Zone in the Plate reader and avoiding overreads

Another recurring challenge during the RevION assay´s optimization was maintaining measurements within the linear detection range of the TECAN Spark® plate reader. Accurate quantification results strongly depend on ensuring that the absorbance values of all samples remain within this linear range, as over- or under-saturation of the detector can lead to significant deviations and unreliable results.

To address this, one of our early approaches involved adjusting the dilution of each sample individually, so that all samples would ideally yield similar absorbance values within

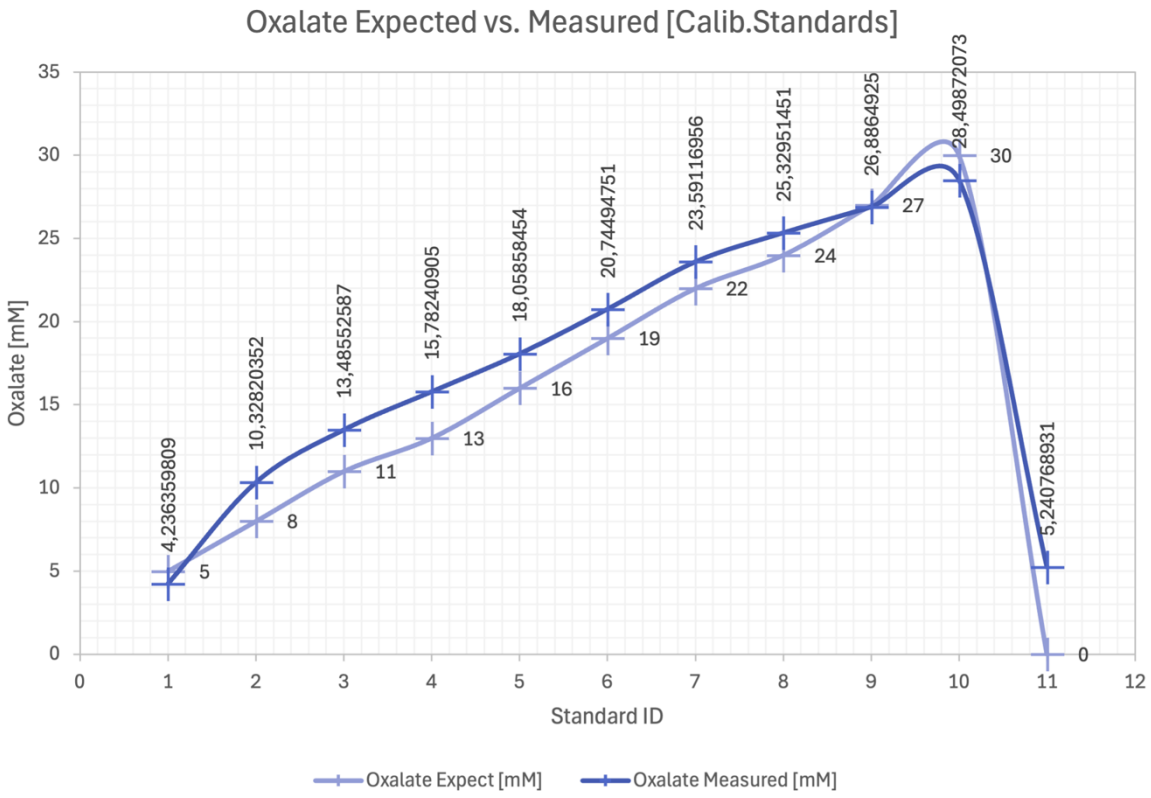
the plate reader's optimal range. In theory, this would result in a perfectly horizontal output line, from which the original concentrations could be recalculated using their respective dilution factors.

While this approach functioned conceptually, it introduced additional variance into the dataset. The use of multiple dilution factors increased the cumulative error propagation, and due to the very small pipetting volumes, often in the sub-microliter range, minor inaccuracies or calibration deviations of the pipettes had a disproportionately large effect on the final results.

After transitioning to the micromolar concentration range, this issue was effectively resolved. The lower concentration range allows the use of uniform dilution factors and practical pipetting volumes, minimizing variance and ensuring consistent and reproducible measurements. Consequently, the assay now maintains excellent linearity while avoiding overreads and detector saturation in the TECAN Spark®, thereby improving both precision and reproducibility.

Proof of concept

Throughout the course of our research and assay development, several major breakthroughs were achieved. One of the most significant milestones was the first successful detection and quantification of oxalate within the millimolar concentration range. The graph displayed below illustrates a representative test using standards with known oxalate concentrations created in CGXII medium as their matrix. It compares both the expected values with those measured by our RevION assay.



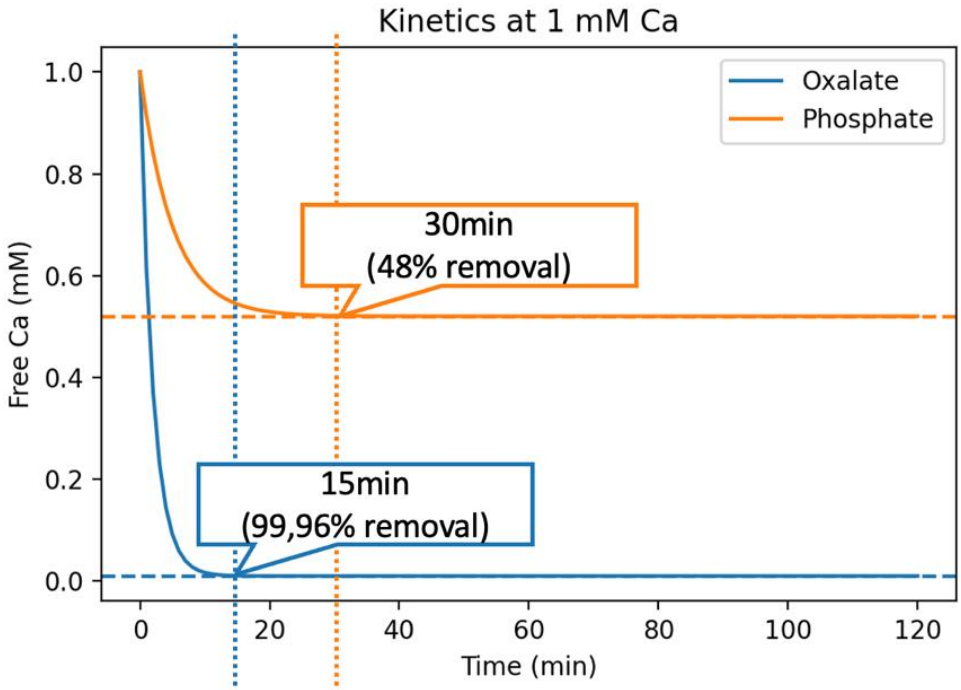
The results clearly demonstrate that the assay´s core system and workflow functions effectively and is fully operational. We are now in the process of further optimizing the RevION method to enhance its precision and fully exploit its analytical potential. As noted earlier, the mean absolute error (MAE) of this calibration set was approximately 6,82%, which is an excellent outcome for the first successful validation. Moreover, the calibration equation achieved an R^2 value of 0,9692, indicating that the model describes oxalate concentrations with a precision of roughly 97% relative to the measured absorbance data.

Interestingly, this calibration also provided a crucial insight that significantly advanced our understanding of the system's chemistry. In calibration standard 11 (designed as a blank containing no oxalate) a measurable signal was detected, revealing that not only oxalate but also phosphate species present in the CGXII medium were forming complexes with calcium. This unexpected observation marked a turning point, leading to the ongoing resolution of phosphate-induced interferences within the system. This standard blank was expected to show a concentration of

approximately 13.09 mM, corresponding to the effective phosphate concentration in the CGXII medium. However, the measured value was only 5.24 mM, indicating that the calcium–phosphate complexation efficiency is relatively low. This finding highlights the poor complexing rate of phosphate with calcium compared to oxalate and provides key evidence for the instability of calcium phosphate complexes under the given assay conditions, an aspect that will be discussed in greater detail in the following section.

To investigate this further, we performed an additional test in the micromolar range, including two calibration sets, one consisting only of CGXII medium and ddH_2O, to specifically assess the calcium phosphate complex formation. The results yielded a precise calibration equation for different phosphate concentrations in the CGXII medium. Although a direct comparison graph (as shown in the millimolar example) could not be constructed due to issues with the oxalate calibration, the data confirmed that oxalate and phosphate can be independently calibrated. Moreover, it demonstrated that scaling the system from the millimolar to the micromolar range does not significantly alter the variance between expected and measured oxalate concentrations.

This can be attributed to the high stability and precision of calcium–oxalate complexation, which reaches up to 99.96% complexing rates at 1mM Ca concentration, compared to phosphate complexation, which peaks at roughly 48% and remains highly unstable particularly at lower concentrations as shown in the graphic below. Consequently, as both analyte and phosphate levels decrease, the nucleation rate of phosphate diminishes, reducing the standard error and the phosphate-induced deviations, and thus enabling more reliable analytical outputs.



Overall, the assay performs with remarkable consistency in the millimolar range, achieving a mean match rate of about 93.18%. When scaled into the micromolar range using samples with low concentrations and a maximum spiking concentration of 5 mM theoretically, the reduced phosphate nucleation rate is expected to further enhance the RevIONs precision beyond this benchmark. Having demonstrated both the chemical and mathematical reliability of the RevION system, it becomes evident that the accuracy of the final analysis predominantly depends on procedural precision. Even minor pipetting inaccuracies, decalibrated instruments, or slight deviations in pH or reagent concentrations can accumulate and visibly impact the assays output which will be reflected in the quality control (QC) results.

With continued refinement of pipetting accuracy, blanking procedures, and reagent handling, the RevION assay is expected to shift further into the optimal analytical zone, unlocking the system's complete potential and setting a new standard for accessible analytical biochemistry, where precision and simplicity converge.

How Does the Mathematical Workflow Operate?

The final mathematical framework, modeled in Microsoft Excel, is composed of four main submodules that together enable the precise and automated calibration of the assay.

1. Calibration Standard Design

The first submodule defines the calibration standard set. Here, the concentrations, dilution factors, and corresponding reagent volumes are systematically calculated and optimized to achieve a consistent and accurate set of calibration results across the entire target range.

2. Sample Spiking and Precipitation Parameters

The second submodule incorporates all parameters related to sample spiking and the calcium chloride ($CaCl_2$) precipitation step. It ensures that the correct stoichiometric excess of calcium ions is provided for efficient oxalate complex formation and subsequent quantification.

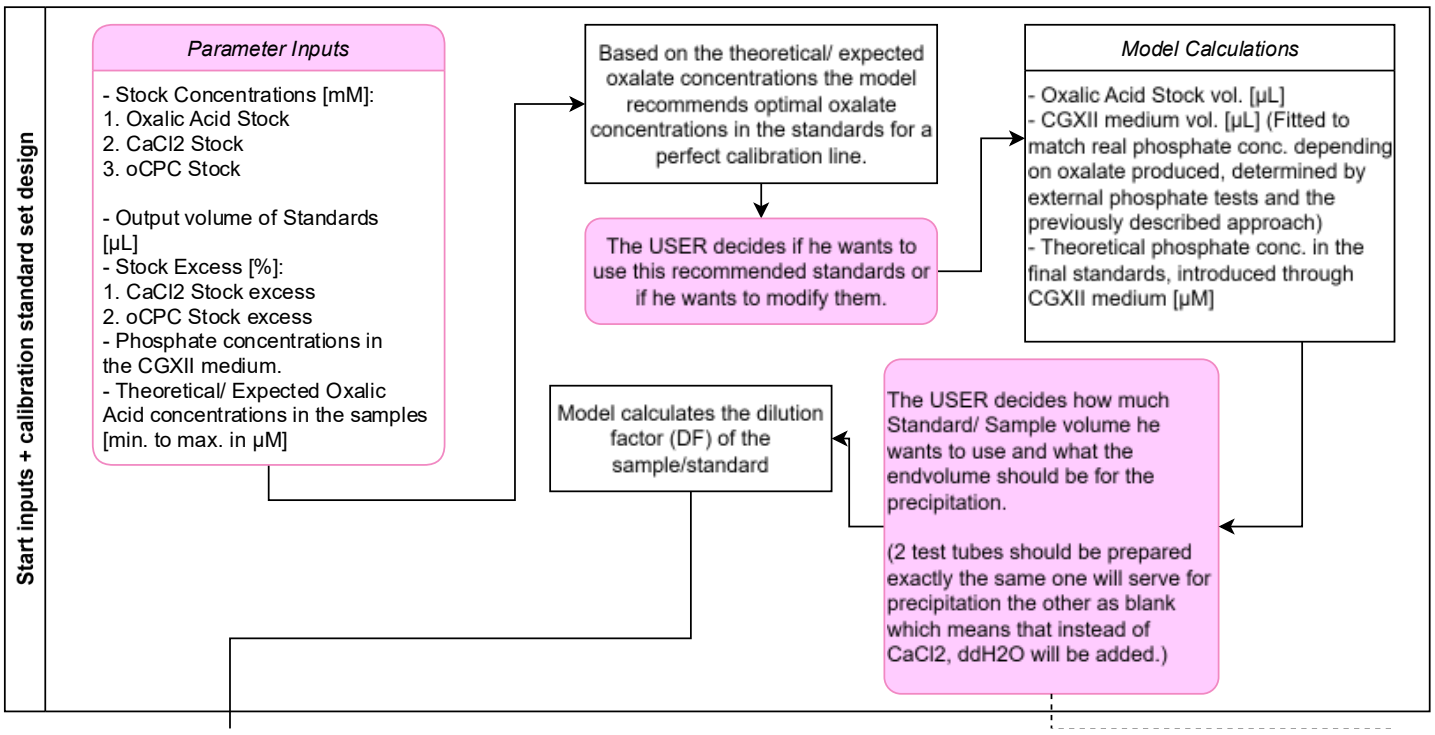
3. oCPC Complexation and Plate Configuration

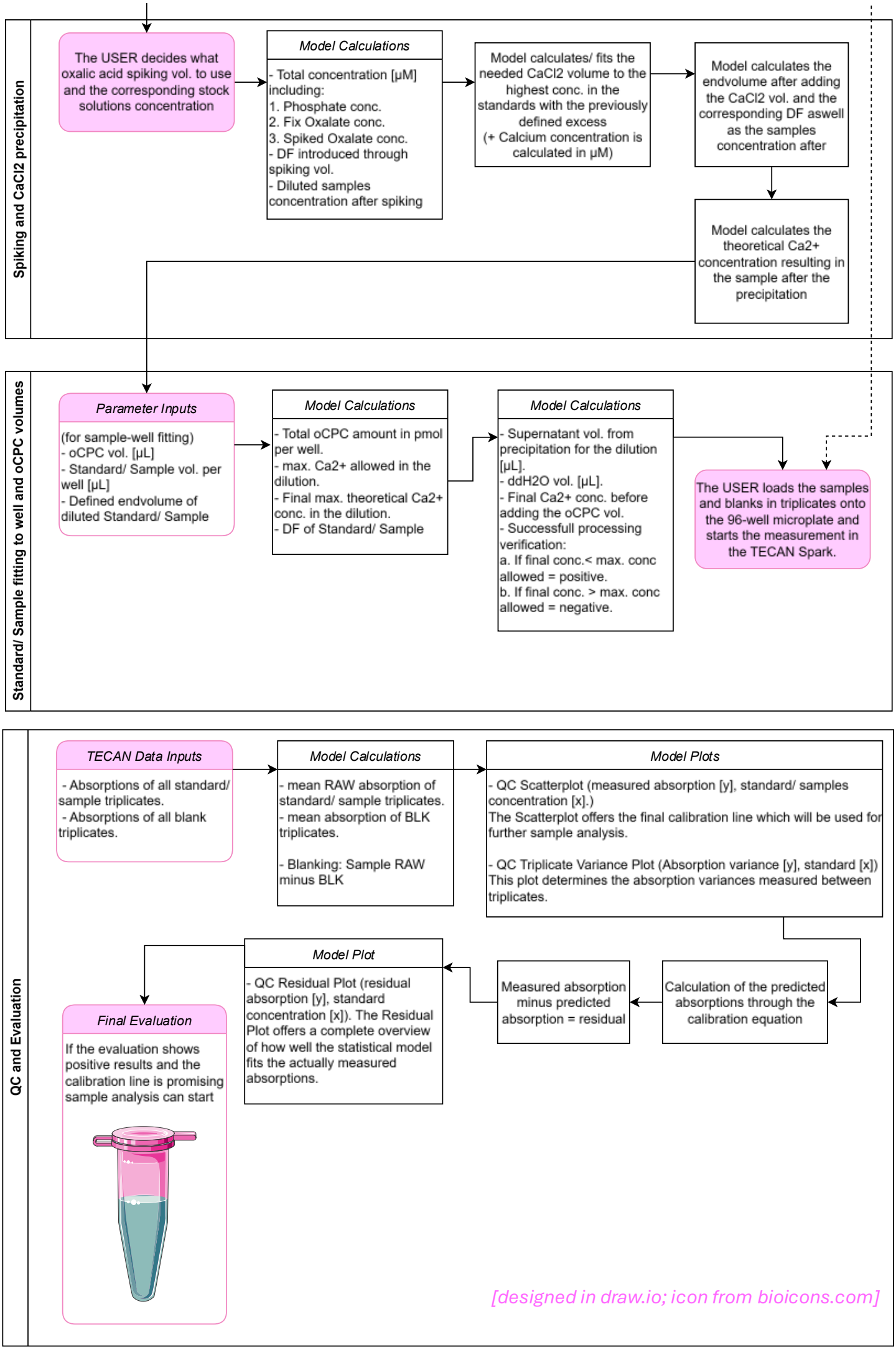
The third submodule models and optimizes the conditions relevant to oCPC complex formation and the 96-well plate setup. This includes calculations for reagent volumes and concentrations, reaction ratios, and absorbance normalization, ensuring compatibility with the TECAN Spark® plate reader's operational and linear range.

4. Quality Control and Evaluation

The fourth and final submodule performs a quality control (QC) and data evaluation prior to sample result analysis. It assesses calibration performance, linearity (R_2), and statistical accuracy metrics such as residuals and variances throughout the protocol and measurement, thereby validating the system before proceeding with actual sample evaluation.

For improved clarity, the schematic below provides a visual overview of the complete mathematical workflow framework modelled in Microsoft Excel.




Spiking and CaCl2 precipitation
The USER decides what oxalic acid spiking vol. to use and the corresponding stock solutions concentration
Model Calculations
- Total concentration [µM] including:
1. Phosphate conc.
2. Fix Oxalate conc.
3. Spiked Oxalate conc.
- DF introduced through spiking vol.
- Diluted samples concentration after spiking
Model calculates/ fits the needed CaCl2 volume to the highest conc. in the standards with the previously defined excess
(+ Calcium concentration is calculated in µM)
Model calculates the endvolume after adding the CaCl2 vol. and the corresponding DF aswell as the samples concentration after
Model calculates the theoretical Ca2+ concentration resulting in the sample after the precipitation
Standard/ Sample fitting to well and oCPC volumes
Parameter Inputs
(for sample-well fitting)
- oCPC vol. [µL]
- Standard/ Sample vol. per well [µL]
- Defined endvolume of diluted Standard/ Sample
Model Calculations
- Total oCPC amount in pmol per well.
- max. Ca2+ allowed in the dilution.
- Final max. theoretical Ca2+ conc. in the dilution.
- DF of Standard/ Sample
Model Calculations
- Supernatant vol. from precipitation for the dilution [µL].
- ddH2O vol. [µL].
- Final Ca2+ conc. before adding the oCPC vol.
- Successfull processing verification:
a. If final conc.< max. conc allowed = positive.
b. If final conc. > max. conc allowed = negative.
The USER loads the samples and blanks in triplicates onto the 96-well microplate and starts the measurement in the TECAN Spark.
QC and Evaluation
TECAN Data Inputs
- Absorptions of all standard/ sample triplicates.
- Absorptions of all blank triplicates.
Model Calculations
- mean RAW absorption of standard/ sample triplicates.
- mean absorption of BLK triplicates.
- Blanking: Sample RAW minus BLK
Model Plots
- QC Scatterplot (measured absorption [y], standard/ samples concentration [x].)
The Scatterplot offers the final calibration line which will be used for further sample analysis.
- QC Triplicate Variance Plot (Absorption variance [y], standard [x])
This plot determines the absorption variances measured between triplicates.
Calculation of the predicted absorptions through the calibration equation
Measured absorption minus predicted absorption = residual
Model Plot
- QC Residual Plot (residual absorption [y], standard concentration [x]). The Residual Plot offers a complete overview of how well the statistical model fits the actually measured absorptions.
Final Evaluation
If the evaluation shows positive results and the calibration line is promising sample analysis can start
[designed in draw.io; icon from bioicons.com]

After careful evaluation and validation of the calibration set output, the analysis continues with the sample measurements. Each sample and its corresponding blank have been parallelly processed following the exact same procedure as the calibration standards to ensure methodological consistency. After blank correction of the raw absorbance values, the adjusted data are inserted into the established calibration equation, which then provides the corresponding oxalic acid concentration for each sample under the conditions the QC analysis provided.

Conclusion

Over the course of several months, the development of our (semi-)analytical RevION assay has evolved from a concept into a versatile analytical tool for the detection and quantification of oxalate in CGXII cell media samples. Despite the challenges encountered during the process, ranging from matrix-induced interferences to achieving measurement linearity and precise scalability, the assay has advanced through systematic experimentation, quantitative modeling, and iterative refinement.

A major breakthrough was achieved through the transition from direct oxalate quantification to an indirect, calcium-based detection method which uses the ability of oCPC to complexate with calcium ions, enabling reliable colorimetric analysis with high reproducibility rates. Critical analytical limitations, such as the phosphate's interference in measurements, were met with innovative solutions, such as the selective phosphate precipitation and model-based absorption correction approaches. Furthermore, the assay's mathematical framework was optimized to ensure scalability across several orders of magnitude, successfully adapting from the millimolar to the micromolar concentration range without significant loss in precision.

Equally important, attention to the plate reader's linear detection zone and optimized dilution strategies minimized instrumental bias and variance, resulting in a stable and reliable analytical workflow. Together, these developments not only demonstrate the assay's analytical strength but also its adaptability to diverse experimental conditions and sample matrices.

Finally, the cost per sample was approximately €0.18, including both the samples and their corresponding blanks which are run in triplicates, as well as the expenses for all needed reagent stock solutions and the 96-well microplate. This low cost per sample highlights the assay's accessibility and suitability for routine, high-throughput applications.

The RevION assay now stands as far more than just another analytical tool, it is the embodiment of persistence, innovation, and the belief that precision science can be made

accessible to all in need of it. With continued refinements, such as automated phosphate correction and standardized calibration routines, it is poised to become a reliable and affordable method for the quantification of oxalate in CGXII medium across diverse biological systems. Designed around simplicity and accuracy, the assay relies only on low-cost, widely available reagents and standard laboratory equipment, supported by an intuitive mathematical framework designed in excel that transforms complexity into clarity.

What once began as a challenge born from limitation has evolved into a system that empowers others who face the same constraints we once did. The RevION assay breaks down barriers between resource-rich and resource-limited laboratories, between theory and application, between uncertainty and understanding. It stands as a symbol of how science, driven by curiosity and determination, can create not only precise and reproducible results, but also open doors for future discoveries.

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