

Spectral Intra-Lot and Inter-Lot Variability in Methacholine

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RAPID COMMUNICATION

Abstract

Methacholine chloride is a parasympathomimetic (cholinergic) bronchoconstrictor agent. It is administered exclusively in solution via inhalation for diagnostic purposes, strictly indicated for diagnosing bronchial airway hyperreactivity in adults and pediatric patients aged five years and older who lack clinically apparent asthma. Spectrometric evaluation of a single manufacturing batch, lot P3055R, revealed distinct anomalies, most notably a broad absorbance peak at 5131 cm⁻¹ that was present in certain vials but entirely absent in others. Principal component analysis orthogonally quantified this intralot variance, demonstrating that Vial 1 deviated by 7.3 standard deviations (SDs) from the cluster center, while a subsequent rotation identified Vial 4 as an outlier positioned 4.3 SDs away. Expanding the evaluation to a 39-vial library across three distinct lots (P3055R, P3048R, and P4009S) demonstrated significant interlot structural variance. This complexity required nine full principal components to model 99.97% of the

cumulative variation. Furthermore, the application of the nonparametric FSOB (Fast Son of BEST) subcluster analysis algorithm inside the spectral clusters confirmed the geometric separation of the data into statistically significant sub-groups. Specifically, vials 8 through 18 naturally separated from the remaining library (vials 1 through 7 and 19 through 39). This geometric separation spanned an extensive correlation distance of 58.8 SDs, defined by the statistical metrics $r_{tn}=0.9944$, $r_{ts}=0.8903$, and $\sigma=0.0019$, with probability-density perturbations achieving a significance level of $p=0.02$. While these quantified deviations do not definitively prove the presence of adulteration or an excess level of impurities, they strongly indicate that the manufacturer's production pipeline may have drifted outside of a state of strict process control, warranting further investigation.

Introduction

The University of Kentucky's (UK) Drug Quality Task Force (DQTF) was established in August of 2019 to engage in consumer-level quality assurance screening for drugs used within UK HealthCare's pharmacies ([Yoon, 2025a](#)). The DQTF currently screens medications using Fourier transform near-infrared spectrometry (FTNIR) and Raman spectrometry for potential quality defects indicated by variability in absorbance peak intensities and locations. Through years of continuous monitoring, DQTF has assembled a spectral library containing medications typically used in a health system setting. Statistical analyses using the DQTF spectral library are performed to identify potential intra-lot and inter-lot variability in medications under review. Using Medwatch and publications in the scientific literature, the DQTF reports its findings in an effort to hold manufacturers accountable for GMP requirements and to improve patient outcomes by providing information on quality to augment the information on price that is already available. The increasing transparency is designed to improve the pharmaceutical supply chain.

Drug Product

The package insert describes methacholine chloride (Provocholine, manufactured by Methapharm, Inc.) as "a parasympathomimetic (cholinergic) bronchoconstrictor agent designed to be administered in solution only, by inhalation, for diagnostic purposes. Each 20 mL vial contains 100 mg and each 50 mL vial contains 1,600 mg of methacholine chloride powder which is to be reconstituted with 0.9% sodium chloride injection or 0.9% sodium chloride injection containing 0.4% phenol (pH 7.0)" ([FDA Methacholine package insert, 2025](#)).

Methacholine chloride is strictly indicated for the diagnosis of bronchial airway hyperreactivity in adults and pediatric patients five years of age and older who do not have clinically apparent asthma. The drug is administered via oral inhalation during a bronchoprovocation procedure known as the methacholine challenge test. It is primarily used to evaluate patients who exhibit atypical asthma symptoms (such as an unexplained, persistent cough) but present with normal baseline pulmonary function tests and spirometry results. Methacholine chloride is a potent

cholinergic agonist (parasympathomimetic) that binds directly to muscarinic receptors in the airway smooth muscle, inducing bronchoconstriction. Patients with asthma or underlying airway hyperresponsiveness are markedly more sensitive to this drug-induced constriction than healthy individuals. The drug has no therapeutic applications.

[Figure 1](#) is a photograph of the methacholine drug product (Provocholine, Methapharm). The lot number for intralot testing was P3055R.



Figure 1. Photo of the methacholine drug product. The lot number for intralot testing was P3055R.

The lot number for intralot testing was P3055R. The lot numbers making up the spectral library were P3055R, P3048R, and P4009S.

Background

Recent studies

Methacholine chloride is a synthetic choline ester and a nonselective muscarinic receptor agonist. As an acetylcholine derivative, it acts directly on receptors in the airway smooth muscle to induce contraction and narrowing. From a formulation standpoint, methacholine chloride (marketed under names like Provocholine) was developed to resolve the deliquescence issues associated with conventional methacholine, providing much greater physical stability and ensuring consistent, reliable drug concentrations during aerosolized delivery.

Recent clinical trials and retrospective studies have focused on comparing exact chemical formulations, optimizing safety profiles for pediatric cohorts, and refining the metrics used to interpret the test. Overall, methacholine chloride continues to serve its purpose exceptionally well, with high efficacy and a strong safety profile.

Efficacy of Chloride Formulation vs. Conventional Methacholine: A large-scale retrospective study published in February 2026 evaluated over 17,300 participants to compare the clinical performance of the newly available inhaled methacholine chloride against conventional methacholine ([Chen, 2026](#)). Methacholine chloride was found to be significantly more sensitive, yielding a higher rate of positive diagnostic tests (36.7% vs. 30.8%) and requiring a lower median dose (PD20-FEV1) to trigger a response. The chloride formulation was deemed highly safe; no serious adverse events were reported, and the most common mild reactions were transient chest tightness (30.8%) and cough (27.2%).

Safety of Abbreviated Pediatric Protocols: A 2025 study explored modifying the MCT dosing escalation strategy to shorten the procedure for children. By escalating doses more aggressively (until FEV1 declined by 10%), the abbreviated protocol reduced the average test duration by over 18 minutes. The researchers concluded that this abbreviated method successfully served its diagnostic purpose with no significant clinical adverse events, making it a highly efficient alternative for pediatric pulmonology ([Hevroni, 2025](#)).

Recalibrating Reference Values: A 2024 aerosol medicine study utilized breathing simulators to test whether current adult-derived MCT reference values are appropriate for children. Because pediatric breathing patterns result in a greater predicted pulmonary dose of methacholine on a µg/kg of body weight basis compared to adults, the standard negative threshold (>400 µg for a 20% FEV1 decrease) might over-diagnose BHR in children. The study suggested that while the drug performs its constrictive purpose well, the reference thresholds for children need recalibration to ensure diagnostic accuracy ([Coates, 2024](#)).

Refining the Diagnostic Benchmark: A 2025 analysis investigated whether the traditional 20% fall in baseline FEV1 (PD20) should be replaced. The study found that measuring a 15% fall in the patient's *predicted* FEV1 (PD15%) is significantly less influenced by the patient's existing

baseline airflow obstruction, offering a more standardized and mathematically sound way of interpreting the methacholine chloride response ([Dean, 2025](#)).

Recalls

While there have been no recent widespread recalls of the primary commercial formulation of methacholine chloride (Provocholine), there is a notable compounding recall on record, and the drug carries a highly restrictive, prominent safety warning due to its pharmacodynamic profile.

The most significant recent regulatory action did not occur in Provocholine, but instead occurred in December 2021, when Edge Pharma, LLC, an FDA-registered 503B outsourcing facility, issued a voluntary nationwide recall of all its compounded sterile drug products ([FDA, 2021](#)).

This recall affected multiple concentrations of compounded Methacholine Chloride (PF) pre-filled syringes (ranging from 0.0625 mg/mL to 16 mg/mL) used in 5-syringe challenge test kits. The recall was initiated due to a systemic lack of sterility assurance at the manufacturing facility, which posed a risk of severe site-specific or systemic infections. The recall was not due to an inherent chemical defect with the methacholine active pharmaceutical ingredient (API) itself.

Shortages

As of late February 2026, there is no reported national shortage of methacholine chloride (brand name Provocholine) in the United States.

A review of the primary drug shortage tracking systems confirms its availability:

- [FDA Drug Shortages Database](#): Methacholine chloride is not currently listed as "Currently in Shortage" or experiencing supply interruptions by the U.S. Food and Drug Administration.
- [ASHP Drug Shortages List](#): The American Society of Health-System Pharmacists, which often tracks supply chain strains earlier and more broadly than the FDA, also does not list methacholine chloride on its current shortages list.

Supply Chain Context

While the primary commercial supply is stable, the distribution landscape has seen historical adjustments to prevent shortages. In the past (such as the late 2021 nationwide recall by Edge Pharma), localized availability issues arose for clinics that relied heavily on specific 503B outsourcing facilities for pre-mixed, pre-filled syringes. This was due to manufacturing compliance issues at the compounding facility, not a shortage of the active methacholine pharmaceutical ingredient itself.

To stabilize the supply chain for bronchoprovocation testing facilities, Methapharm (the primary manufacturer of Provocholine) partnered with Nephron Pharmaceuticals in 2020 to reliably produce stable, ready-to-use solution kits. This collaboration has largely insulated the diagnostic testing market from the supply shocks seen with other respiratory therapeutics ([Nephron Pharmaceuticals Corporation, 2020](#)).

FDA Medwatch

An FDA Form 3500 Medwatch describing the findings of this Rapid Communication was filed.

Methods

FTNIR (Fourier Transform Near-Infrared) Spectrometry

Using nondestructive analytical techniques, FTNIR spectra were collected from inventory as part of routine medication quality screening. A representative sample of individual vials were selected for screening and noted to be stored under the conditions required by the manufacturer in their original packaging. FTNIR spectra were collected noninvasively and nondestructively through the bottom of the vials using a Thermo Scientific Antaris II FTNIR Analyzer (Waltham, MA, USA)([Yoon, 2025b](#)).

Smoothing

Data smoothing is a technique used to remove noise from data. This can be done by fitting a smooth curve to the data, such as a cubic spline. Cubic splines are piecewise cubic polynomials that are continuous and have continuous first and second derivatives. This makes them very smooth and resistant to noise. Cubic splines can be easily fitted to data using least squares ([Matlab, 2024](#))([Pollock, 1998](#)).

Multiplicative Scatter Correction (MSC)

Multiplicative scatter correction (MSC) is a widely used spectrometric normalization technique. Its purpose is to correct spectra in such a way that they are as close as possible to a reference spectrum, generally the mean of the data set, by changing the scale and the offset of the spectra ([Isaksson, 1988](#)).

BEST (Bootstrap Error-Adjusted Single-sample Technique)

The BEST calculates distances in multidimensional, asymmetric, nonparametric central 68% confidence intervals in spectral hyperspace (roughly equivalent to standard deviations)([Dempsey, 1996](#)). The BEST metric can be thought of as a "rubber yardstick" with a

nail at the center (the mean). The stretch of the yardstick in one direction is therefore independent of the stretch in the other direction. This independence enables the BEST metric to describe odd shapes in spectral hyperspace (spectral point clusters that are not multivariate normal, such as the calibration spectra of many biological systems). BEST distances can be correlated to sample composition to produce a quantitative calibration, or simply used to identify similar regions in a spectral image. The BEST automatically detects samples and situations unlike any encountered in the original calibration, making it more accurate in chemical investigation than typical regression approaches to near-IR analysis. The BEST produces accurate distances even when the number of calibration samples is less than the number of wavelengths used in calibration, in contrast to other metrics that require matrix factorization. The BEST is much faster to calculate as well ($O(n)$ instead of the $O(n^3)$ required by matrix factorization.)

Principal Components (PCs)

Principal component analysis is the process of computing the principal components of a dataset and using them to execute a change of basis (change of coordinate system) on the data, usually employing only the first few principal components and disregarding the rest ([Jolliffe, 2016](#)). PCA is used in exploratory data analysis and in constructing predictive models. PCA is commonly utilized for dimensionality reduction by projecting each data point onto only the first few principal components to obtain lower-dimensional data while preserving as much of the original variation in the data as possible. The first principal component is the direction that maximizes the variance of the projected data. The second principal component is the direction of the largest variance orthogonal to the first principal component. Decomposition of the variance typically continues orthogonally in this manner until some residual variance criterion is met. Plots of PC scores help reveal underlying structure in data.

Subcluster Detection

In typical near-infrared multivariate statistical analyses, samples with similar spectra produce points that cluster in a certain region of spectral hyperspace. These clusters can vary significantly in shape and size due to variation in sample packings, particle-size distributions, component concentrations, and drift with time. These factors, when combined with discriminant analysis using simple distance metrics, produce a test in which a result that places a particular point inside a particular cluster does not necessarily mean that the point is actually a member of the cluster. Instead, the point may be a member of a new, slightly different cluster that overlaps the first. A new cluster can be created by factors like low-level contamination, moisture uptake, or instrumental drift. An extension added to part of the BEST, called FSOB (Fast Son of BEST) can be used to set nonparametric probability-density contours inside spectral clusters as well as outside ([Isaacs, 2023c](#))([Lodder, 1988](#)), and when multiple points begin to appear in a certain region of cluster-hyperspace the perturbation of these density contours can be detected at an assigned significance level using r values, and visualized using quantile-quantile (QQ) plots. The detection of unusual samples both within and beyond 3 SDs of the center of the training set

is possible with this method. Within the ordinary 3 SD limit, however, multiple instances are needed to detect unusual samples with statistical significance.

Artificial Intelligence Tools

Artificial intelligence (AI) tools, principally used for background information, include [Gemini](#) (Google LLC) and [GPT-5](#) (OpenAI). AI can be used in a variety of ways, including to brainstorm, organize thoughts, develop arguments, and edit.

Results and Discussion

Intralot analysis

Graphs of Spectra

[Figure 2](#) displays the near-infrared spectra from individual vials of methacholine belonging to a single lot, P3055R. When zooming in on the spectral region between 4000 and 6200 cm^{-1} , the data reveal a distinct, broad absorbance peak at 5131 cm^{-1} in certain vials. Crucially, this 5131 cm^{-1} peak is entirely absent in the spectra of other vials from the exact same lot.

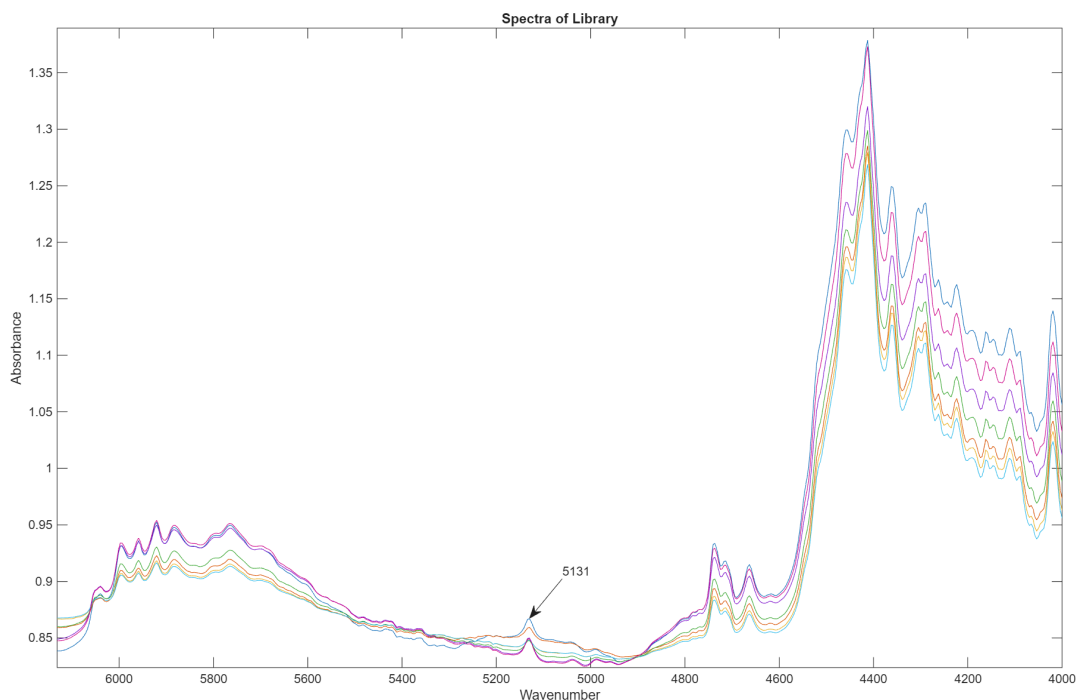


Figure 2. A plot of the spectra from lot P3055R used for intralot testing. Some vials have a very broad peak at 5131 cm^{-1} that does not appear in the spectra of the other vials. This figure zooms in on the spectral region from 4000 to 6200 cm^{-1} .

By circling back to the inconsistent parts (the missing or present peaks) and interpreting them through the lens of the whole (the expectation of lot uniformity), a practical conclusion emerges:

The presence of this intra-lot variability suggests that the manufacturing process for this batch may have been operating outside of a state of strict process control. While the differing spectra clearly show variability, these specific findings do not definitively prove that the drug is adulterated or contains an excess level of impurities. Because the process appears to have deviated from standard control parameters, further investigation into the manufacturing anomalies is required. Ultimately, the individual spectral variations only gain their full diagnostic meaning when viewed against the industry standard of absolute uniformity, highlighting a potential flaw in the production pipeline rather than an immediate chemical danger.

PC Plots

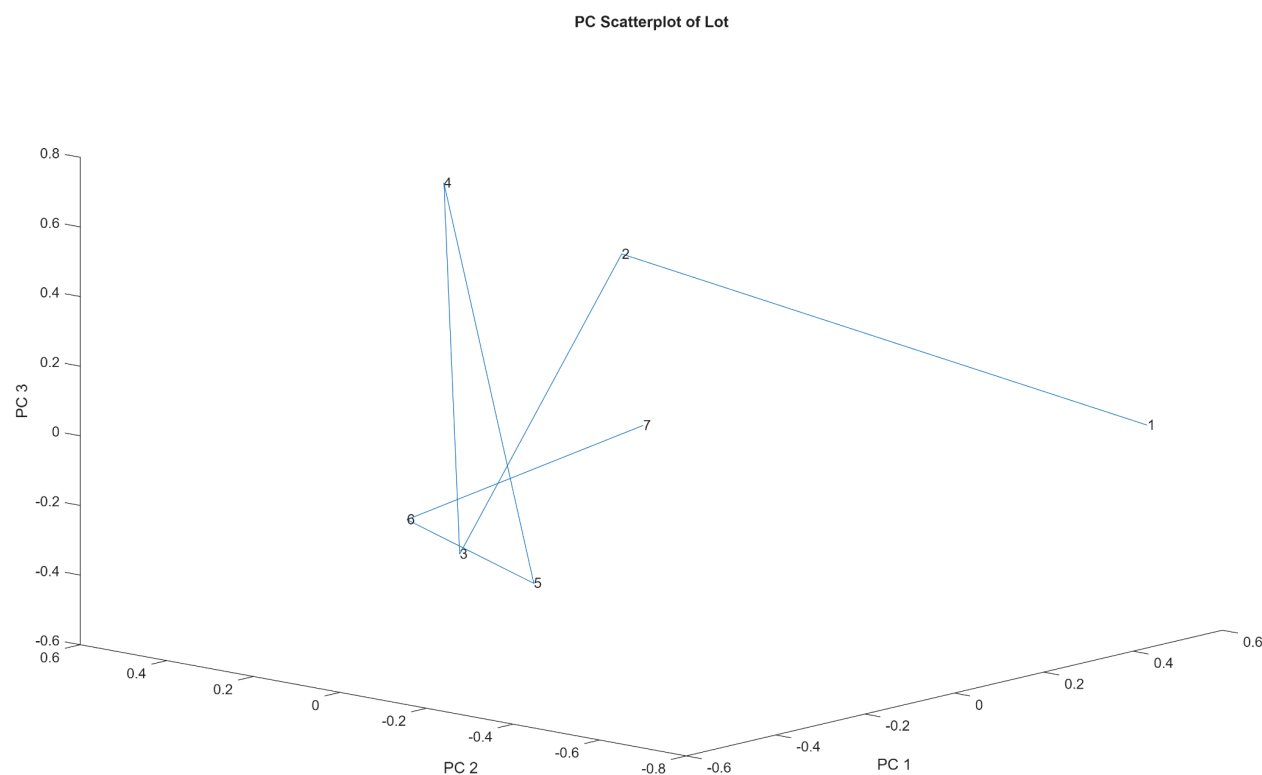


Figure 3. PC scatterplot of the spectra shown in [Figure 2](#). Vial 1, which appears on the right side of the plot, is 7.3 SDs from the center of the other vials.

[Figure 3](#) provides a three-dimensional principal component scatterplot representing the spectral data from the methacholine lot P3055R. It takes the raw near-infrared spectra initially displayed

in [Figure 2](#) and plots them along the first three principal components: PC 1, PC 2, and PC 3. The most prominent feature of the plot is the position of Vial 1, which appears distinctly separated on the right side of the graph. Specifically, the scatterplot demonstrates that Vial 1 is situated 7.3 standard deviations (SDs) away from the center of the other vials within that same lot.

This figure is an essential analytical step because it translates visual spectral anomalies into quantified statistical evidence of intralot variability. Principal component analysis functions by reducing the dimensionality of complex data sets while preserving as much of the original variance as possible. This mathematical change of basis, when plotted as PC scores, exposes the underlying structure and clustering of the data.

While the raw spectra in [Figure 2](#) visually indicated inconsistencies, such as the broad absorbance peak at 5131 cm^{-1} in certain vials, [Figure 3](#) confirms this variance quantitatively. Measuring a distance of 7.3 SDs from the cluster center mathematically confirms Vial 1 as a significant outlier. Identifying a vial that deviates so drastically from the rest of its specific batch (intra-lot analysis) directly supports the broader conclusion that the manufacturer's procedures may have been operating outside of a state of strict process control.

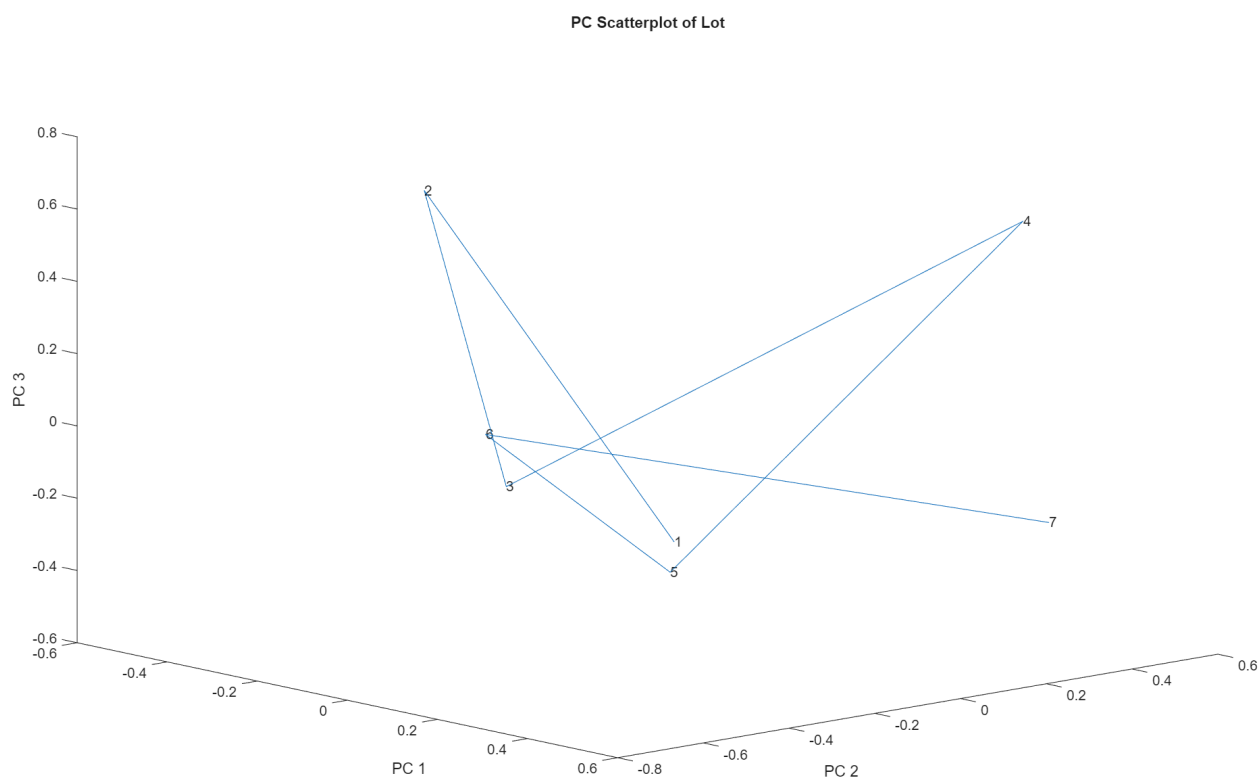


Figure 4. Another rotation of the PC scatterplot shown in [Figure 3](#). This rotation shows that vial 4 is also an outlier. Vial 4, shown on the right side of the plot, is 4.3 SDs from the center of the other vials.

[Figure 4](#) presents another rotation of the principal component (PC) scatterplot initially displayed in [Figure 3](#). By altering the viewing angle of the data plotted against PC 1, PC 2, and PC 3, this figure reveals that Vial 4 is also an outlier within the lot. Specifically, it quantifies this variance by showing Vial 4 situated on the right side of the plot at a distance of 4.3 standard deviations (SDs) from the center of the other vials.

While [Figure 3](#) effectively highlighted Vial 1 as an extreme outlier, it did not clearly illustrate the deviation of Vial 4. [Figure 4](#) demonstrates the necessity of multiple views to capture all significant deviations. Different rotations reveal different perspectives on the points.

When viewing a three-dimensional representation of multidimensional data, points that are vastly separated along one axis might appear clustered together if that axis is pointing directly toward or away from the viewer. Rotating the axes ensures that critical distances (such as the 4.3 SD separation of Vial 4) are not obscured by the specific angle of the projection.

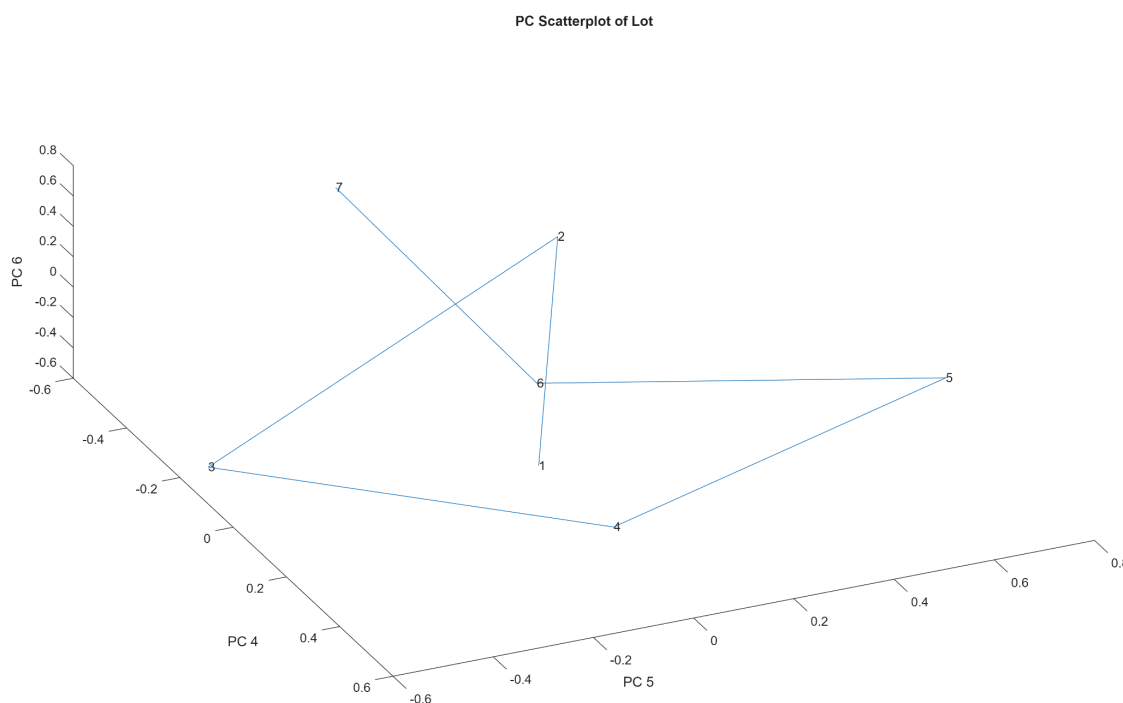


Figure 5. Another projection of the data shown in [Figure 4](#). PC scatterplot of PCs 4, 5, and 6. These points are from the spectra shown in [Figure 2](#). In this rotation none of the spectra appear to be outliers, which is not unusual. Different rotations reveal different perspectives on the points.

[Figure 5](#) displays another projection of the intralot spectral data, projecting the spectra onto principal components 4, 5, and 6. In contrast to the earlier plots, this specific dimensional view shows that none of the individual vials appear to be outliers. [Figure 5](#) demonstrates that multidimensional data structures are highly dependent on the coordinate system used for observation. The fact that no outliers are visible in this specific rotation is not unusual. It visually reinforces that different rotations reveal distinct perspectives on the data points, highlighting the necessity of examining multiple dimensional combinations to ensure no geometric angle obscures an anomalous sample.

Principal component analysis orthogonally decomposes dataset variance, a process that typically continues until a specific residual variance criterion is met. According to Table 1, PCs 1 through 3 account for 99.18% of the cumulative variance, while PCs 4, 5, and 6 account for much smaller fractions (0.0049, 0.0019, and 0.0013, respectively). Despite this minimal overall variance, examining higher-order PCs is a critical and necessary analytical step. The highest-variation components almost exclusively capture dominant macroscopic or physical effects. For instance, the loadings of PC 1 mostly reflect residual baseline shifts after multiplicative scatter correction (MSC), and massive variances can also arise from amber vial color baseline distortions or particle-size distributions.

By projecting data orthogonally, PCA partials out these dominant physical effects. The residual variance remaining in higher PCs is frequently where subtle, yet critical, chemical variations emerge. Factors like low-level contamination, moisture uptake, or instrumental drift often govern these lower-variance dimensions.

In spectral hyperspace, new and slightly different clusters can easily overlap the primary sample cluster. A vial might be perfectly aligned with the main cluster along the axes of maximum variance (PCs 1-3) but separate distinctly along a minor variance axis. Discarding lower-variance PCs prematurely risks collapsing the specific dimension where a unique defect is quantified, effectively masking a chemically distinct subcluster.

Table 1: Variation accounted for by each of the principal components of the spectra of one lot

PC Number	Variation in this PC	Cumulative PC Variation
1	0.8476	0.8476
2	0.1072	0.9548
3	0.0371	0.9918
4	0.0049	0.9968
5	0.0019	0.9987
6	0.0013	1.0000

[Table 1](#) displays the proportion of statistical variance accounted for by the principal components (PCs) derived from the near-infrared spectra of a single lot of methacholine. It specifically lists the PC number, the fraction of variation captured by that individual component, and the cumulative variation captured as successive components are added. The table demonstrates that PC 1 accounts for the vast majority of the variance at 0.8476 (84.76%), while PCs 1 through 3 cumulatively account for 0.9918 (99.18%) of the total variation. By the sixth principal component, a cumulative variation of 1.0000 (100%) is reached. The fact that the first PC captures less than 90% of the total variation suggests that the manufacturing process might not be in complete control.

[Table 1](#) is important because it mathematically justifies the exploratory data analysis and dimensionality reduction used to evaluate the drug's quality. The goal of PCA is to project complex data points onto a lower-dimensional space while preserving as much of the original variation in the data as possible. [Table 1](#) quantifies exactly how much information is retained or lost when disregarding higher-order components. By establishing that over 99% of the variation is contained within the first three components, it confirms that the 3D PC scatterplots (such as [Figure 3](#)) provide a highly accurate and representative geometric mapping of the spectral data.

The difference in the number of principal components between [Table 1](#) and [Table 2](#) stems from the scope and complexity of the datasets they analyze. [Table 1](#) represents the *intra*lot analysis, meaning it only evaluates the spectra from a single batch (lot P3055R). [Table 2](#) represents the *inter*lot analysis, which evaluates the entire spectral library compiled from three different lots (P3055R, P3048R, and P4009S). The interlot library encompasses a much larger dataset of 39 individual vials, reflecting a broader array of manufacturing conditions and physical variances across different batches. PCA continues to orthogonally decompose variance until some residual variance criterion is met. Because the intralot dataset ([Table 1](#)) is smaller and simpler, it accounts for 100% of its variation within just 6 PCs. The interlot dataset ([Table 2](#)) contains more complex structural variance, requiring 9 PCs to meet the residual variance threshold and reach a cumulative variation of 0.9997. And of course, it is mathematically impossible to create more PCs than the number of vials unless there are more than 1557 vials. The possible number of PCs is limited by the total number of vials or the total number of wavenumbers in the spectra, whichever is less.

Interlot analysis

Spectra graphs of library

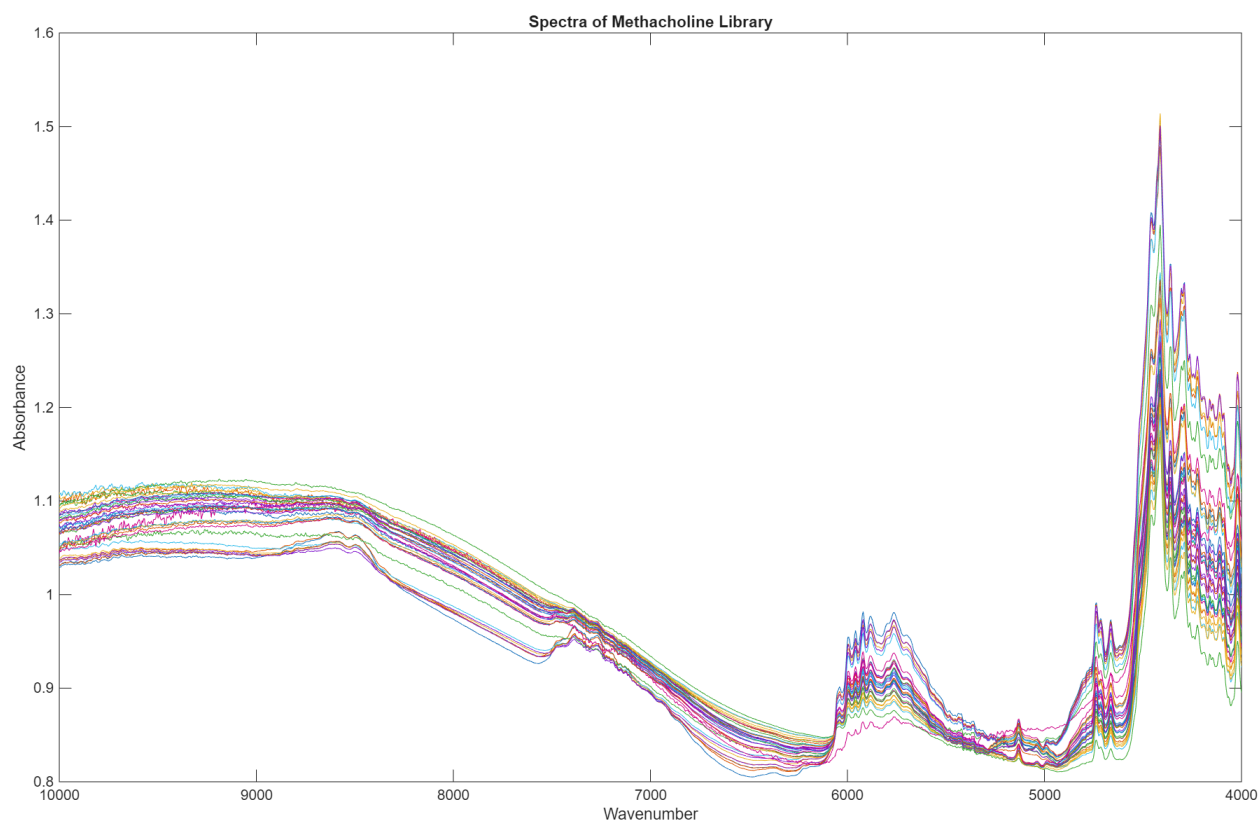


Figure 6. Full spectra of all the vials in all the lots forming the spectra library. These spectra cover the near-IR range from 4000 to 10000 cm^{-1} . The low, broad peak centered at 5131 cm^{-1} appears in this figure in some vials like it does in [Figure 2](#). However it is harder to see because of the number of vials plotted and the zoom factor. These spectra also show the typical baseline distortions from 6500 to 10000 cm^{-1} caused by the amber vial color.

[Figure 6](#) provides a comprehensive visual overview of the full near-infrared spectra for every vial across all the lots that make up the complete spectral library. The plotted data cover the near-IR wavenumber range spanning from 4000 to 10000 cm^{-1} .

Some of the spectra contain the low, broad peak centered at 5131 cm^{-1} that was previously identified in [Figure 2](#) for certain vials. However, this peak is much more difficult to visually isolate here due to the zoomed-out scale and the large number of overlaid spectra. The graph explicitly displays typical baseline distortions occurring between 6500 and 10000 cm^{-1} , which are introduced physically by the amber color of the storage vials.

[Figure 6](#) represents a shift from evaluating a single batch to evaluating the manufacturer's broader production landscape. While earlier figures focused on an intralot analysis of a single batch, [Figure 6](#) establishes the interlot baseline by representing a larger dataset of 39 individual vials compiled from the three available different lots of methacholine (P3055R, P3048R, and P4009S). By visualizing the macroscopic baseline distortions caused by the amber vials, the figure highlights the dominant physical effects present in the raw data. This is important because the statistical models must mathematically separate these massive physical variances from the subtle chemical variations to find defects.

The fact that the 5131 cm^{-1} anomaly is harder to see in this aggregated view practically demonstrates why simply looking at raw spectra is insufficient for quality control across multiple lots. It visually justifies the necessity of applying Principal Component Analysis (PCA) to project this complex, overlapping data into a lower-dimensional space where chemical outliers can be mathematically distinguished.

PC Plots

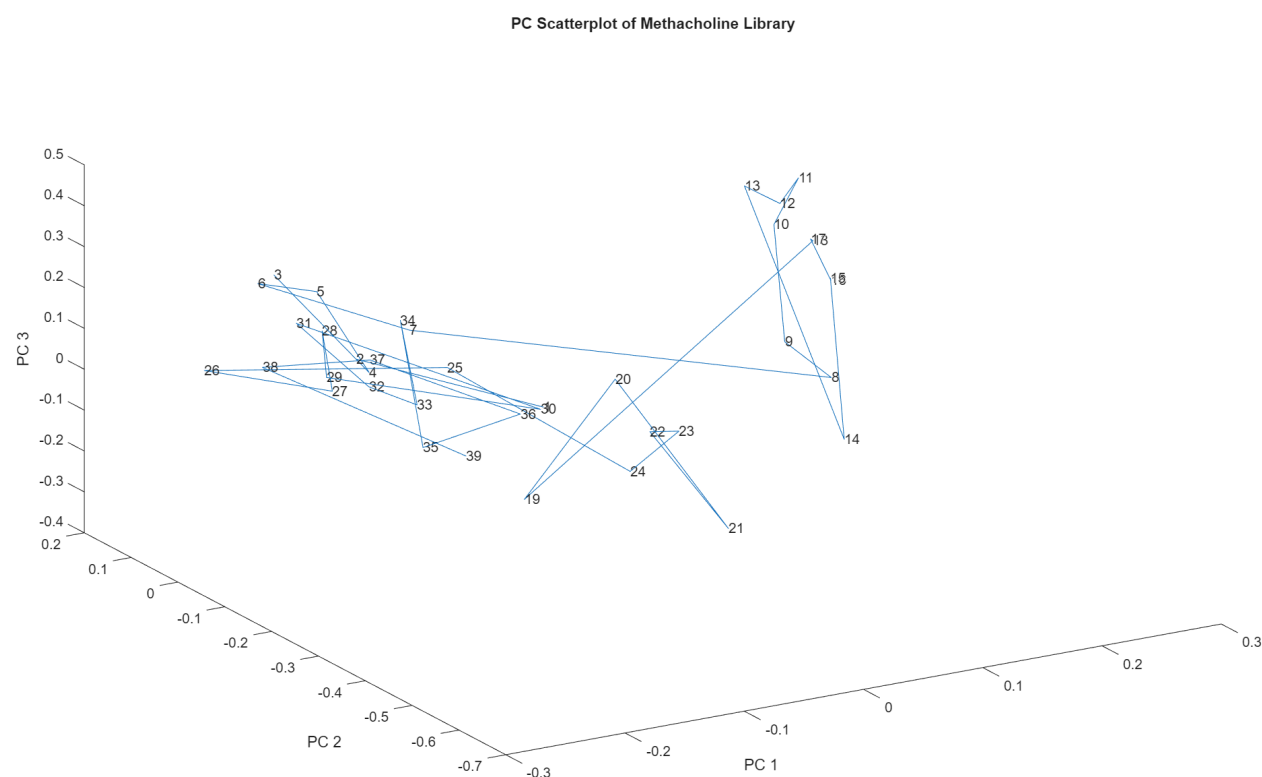


Figure 7. PC scatterplot of spectra in [Figure 6](#). PCs 1 through 3 are plotted.. It appears there are two groups in the spectral data. Vials 8-18 form one group, and vials 1-7 and 19-39 form the other group ($p=0.02$). The two groups are 58.8 SDs apart using the subscluster analysis test ($r_{in}=0.9944$, $r_{is}=0.8903$, $\sigma=0.0019$)

[Figure 7](#) presents a three-dimensional principal component (PC) scatterplot derived from the methacholine spectral library that was previously displayed in [Figure 6](#). It plots the spectral data along the first three principal components: PC 1, PC 2, and PC 3. The scatterplot reveals that the spectral data naturally separate into two distinct groups. Specifically, vials 8 through 18 constitute one group, while vials 1 through 7 and 19 through 39 constitute the other group.

The importance of [Figure 7](#) lies in its ability to mathematically quantify interlot variability across the tested samples, providing detailed statistical evidence of structural differences within the spectral hyperspace. The division of the vials into these two distinct groups is statistically significant, at a p-value of 0.02. By applying the subcluster analysis test, the analysis demonstrates that these two groups are separated by a massive distance of 58.8 SDs. The figure provides precise test parameters for this geometric separation, specifically identifying $r_{tn}=0.9944$, $r_{ts}=0.8903$, $\sigma=0.0019$. Because the analyzed library consists of vials from three separate lots (P3055R, P3048R, and P4009S), identifying two completely distinct clusters separated by 58.8 SDs provides concrete evidence of substantial physical or chemical variance across the manufacturer's broader production output.

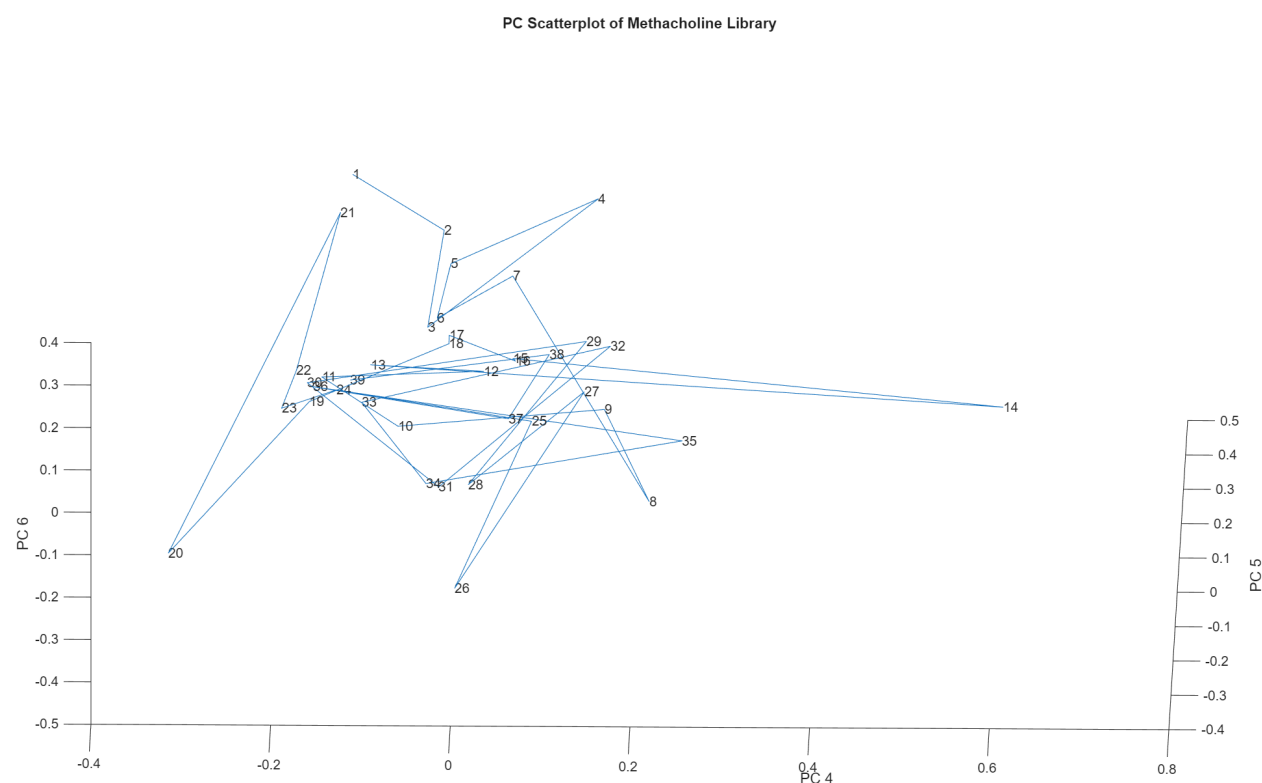


Figure 8. PC scatterplot of spectra in [Figure 6](#) and [Figure 7](#). PCs 4 through 6 are plotted. This rotation suggests vial 14 might be an outlier.

[Figure 8](#) presents another three-dimensional principal component (PC) scatterplot of the complete methacholine spectral library, initially introduced in [Figure 6](#) and [Figure 7](#). Instead of plotting the largest sources of variance, it projects the spectral data onto the next three principal components: PC 4, PC 5, and PC 6. From this specific rotational perspective, the plot visually separates Vial 14 from the rest of the data points, suggesting it might be an outlier.

It is useful to look at this isolated figure within the broader framework of dimensionality reduction and quality control. As established earlier, the highest-variation components (PCs 1-3) primarily capture dominant physical effects. The residual variance remaining in higher PCs is frequently where subtle, yet critical, chemical variations emerge. Vial 14's emergence in PCs 4-6 suggests its variance is likely chemical or systemic rather than a gross physical difference.

In spectral hyperspace, a vial might be perfectly aligned with the main cluster along the axes of maximum variance but separate distinctly along a smaller, minor variance axis. [Figure 8](#) perfectly illustrates this principle: if the analysis had stopped at [Figure 7](#) (PCs 1-3), Vial 14 would have remained hidden within the larger group.

Discarding lower-variance PCs prematurely risks collapsing the specific dimension where a unique defect is quantified. By finding an outlier in PCs 4-6, [Figure 8](#) validates the practice of thoroughly examining different PCs an anomalous sample.

[Figure 9](#) displays a three-dimensional principal component scatterplot of the methacholine spectral library, specifically projecting the data onto principal components 7, 8, and 9. From this specific mathematical perspective, the visualization suggests that vial 2, and potentially vial 19, separate from the main data cluster and might be orthogonal outliers.

Applying a hermeneutic circle to understand this figure requires us to situate these isolated data points (the parts) within the overarching framework of pharmaceutical quality assurance and dimensionality reduction (the whole). According to the interlot analysis, PCs 7, 8, and 9 account for a minuscule fraction of the total spectral variation—specifically 0.0005, 0.0001, and 0.0001, respectively. Because the highest-variation components almost exclusively capture dominant physical effects like baseline shifts and amber vial color distortions, the residual variance remaining in these much higher-order PCs is frequently where critical, subtle chemical variations like low-level contamination, moisture uptake, or instrumental drift emerge. [Figure 9](#) is therefore highly important because it practically demonstrates that a vial might perfectly align with the main sample cluster along axes of maximum variance while remaining distinctly separated along a minor variance axis. By exposing vials 2 and 19 as anomalies only at this deep level of statistical decomposition, the figure shows that safely validating a manufacturing process's state of control requires examining even the smallest mathematical variances to ensure no geometric angle obscures a chemically distinct sample.

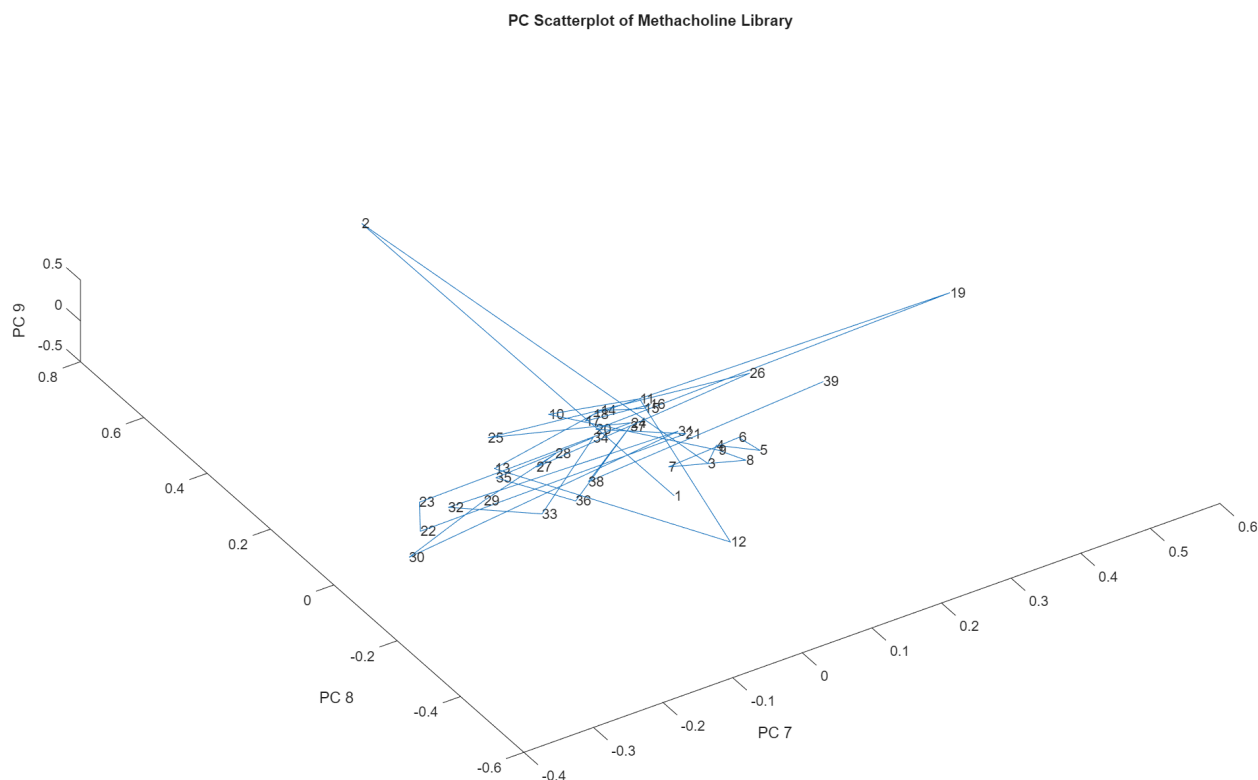


Figure 9. PC scatterplot of spectra in [Figure 6](#), [Figure 7](#), and [Figure 8](#). PCs 7 through 9 are plotted. This rotation suggests vial 2 and perhaps vial 19 might be outliers.

Applying a hermeneutic circle to [Figure 10](#) involves examining this specific statistical graph (the part) through the overarching requirement of detecting subtle pharmaceutical manufacturing flaws (the whole). [Figure 10](#) displays a quantile-quantile (QQ) plot generated by the FSOB subcluster analysis algorithm applied to the complete methacholine spectral library. It visualizes the perturbation of probability-density contours by plotting the empirical cumulative distribution function against the theoretical cumulative distribution function, yielding specific statistical metrics of $r_{tn}=0.9904$, $r_{ts}=0.8903$, and a significance level of $p=0.02$. This figure is important because standard discriminant analysis using simple distance metrics can mistakenly categorize a point as a normal member of a cluster when it actually belongs to a new, overlapping cluster created by factors like low-level contamination, moisture uptake, or instrumental drift. [Figure 10](#) overcomes this blind spot by mapping nonparametric probability-density contours directly inside the spectral clusters. By successfully detecting unusual samples that hide within the ordinary 3 standard deviation limit, this plot mathematically confirms the presence of statistically significant subclusters ($p=0.02$) across the different lots. In practical terms, this deep level of statistical

scrutiny bridges the gap between individual spectral variations and the broader conclusion that the methacholine manufacturing process may have drifted outside of strict process control parameters.

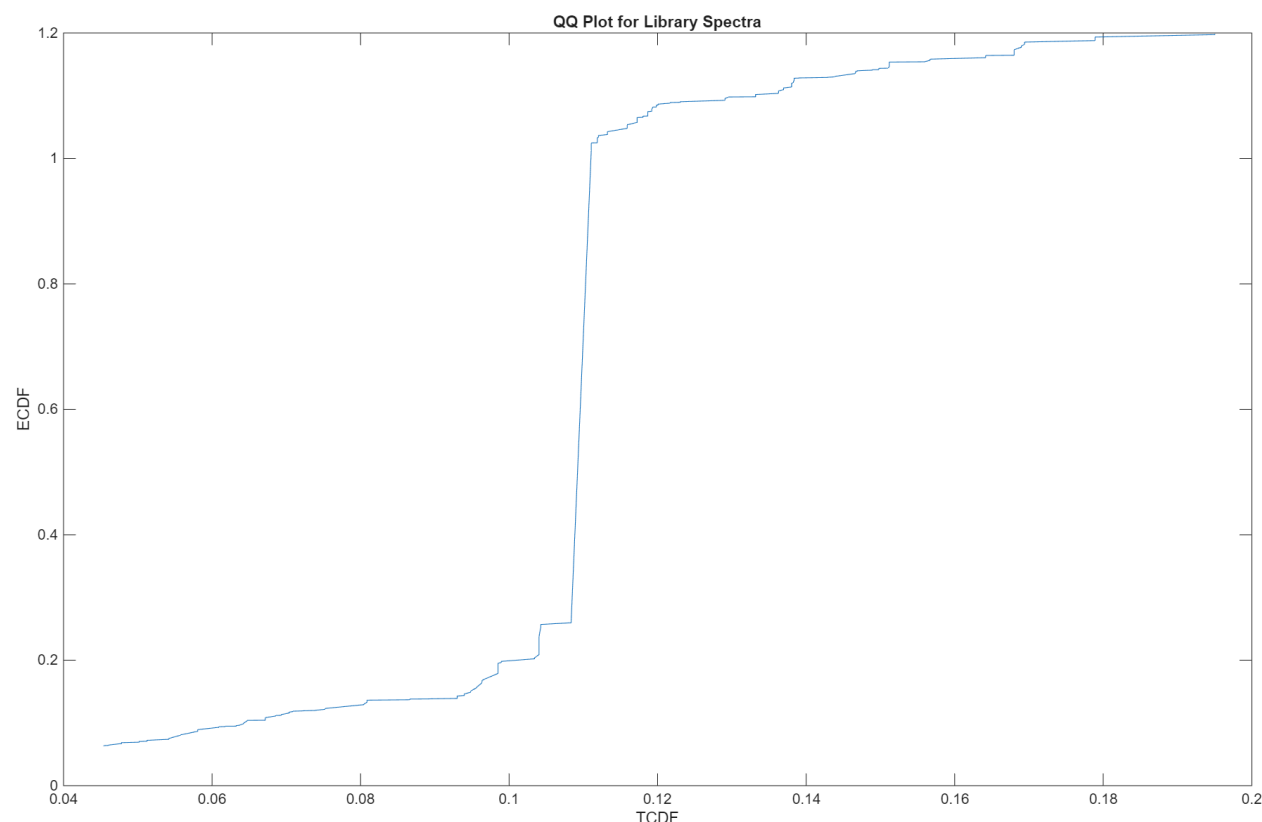


Figure 10. QQ plot from the subcluster analysis algorithm applied to the spectral library. $r_{tn}=0.9904$, $r_{ts}=0.8903$, $p=0.02$

Applying the hermeneutic circle to [Table 2](#) requires users to examine its specific mathematical breakdown of variance (the parts) through the broader lens of multi-batch pharmaceutical manufacturing complexity (the whole). [Table 2](#) displays the proportion of statistical variation accounted for by each of the nine principal components (PCs) derived from the entire interlot spectral library. It explicitly shows that while the first principal component captures 91.17% of the variance, it takes nine full components to reach a cumulative variation of 99.97%. This table is practically important because it mathematically quantifies the increased structural complexity of the interlot dataset compared to the analysis of a single batch. Because this library encompasses a much larger dataset of 39 individual vials compiled from three different lots (P3055R, P3048R, and P4009S), it inherently reflects a broader array of manufacturing conditions and physical variances across different batches. [Table 2](#) fundamentally justifies exactly why nine PCs are required to meet the residual variance threshold for this specific dataset. By thoroughly documenting this dimensionality reduction, the table ensures that

higher-order components—which contain the subtle residual variances necessary to detect chemical anomalies across different batches—are rigorously tracked, ultimately allowing users to evaluate whether the manufacturer's overall production pipeline is operating under a state of strict control.

[Table 1](#) reflects an intralot analysis of a single batch, where the first principal component (PC 1) accounts for 84.76% of the variance. In the context of evaluating a single, supposedly uniform batch, the fact that PC 1 captures less than 90% of the total variance suggests that the manufacturing process might not be in complete control. This lower percentage implies that an unusually high proportion of the variance is spilling into higher-order components, which typically represent subtle chemical inconsistencies or localized anomalies rather than overarching physical traits. Conversely, [Table 2](#) evaluates an interlot library compiled from three different lots encompassing 39 individual vials, which inherently reflects a much broader array of manufacturing conditions and physical variances across different batches. Principal component analysis is designed such that the highest-variation components almost exclusively capture dominant macroscopic or physical effects, such as residual baseline shifts after multiplicative scatter correction, particle-size distributions, or baseline distortions caused by the amber vial color. When pulling back to observe the whole production landscape across multiple batches, the gross physical and systemic differences between the distinct manufacturing runs become the overwhelmingly dominant source of variation. Therefore, these massive interlot physical differences statistically overshadow the smaller intralot chemical inconsistencies, driving the variance captured by PC 1 in [Table 2](#) up to 91.17%.

Table 2: Variation accounted for by each of the principal components of the spectra in the library

PC Number	Variation in this PC	Cumulative PC Variation
1	0.9117	0.9117
2	0.0515	0.9632
3	0.0189	0.9821
4	0.0127	0.9948
5	0.0026	0.9973
6	0.0017	0.9990
7	0.0005	0.9996
8	0.0001	0.9997
9	0.0001	0.9997

PC Loadings Plots

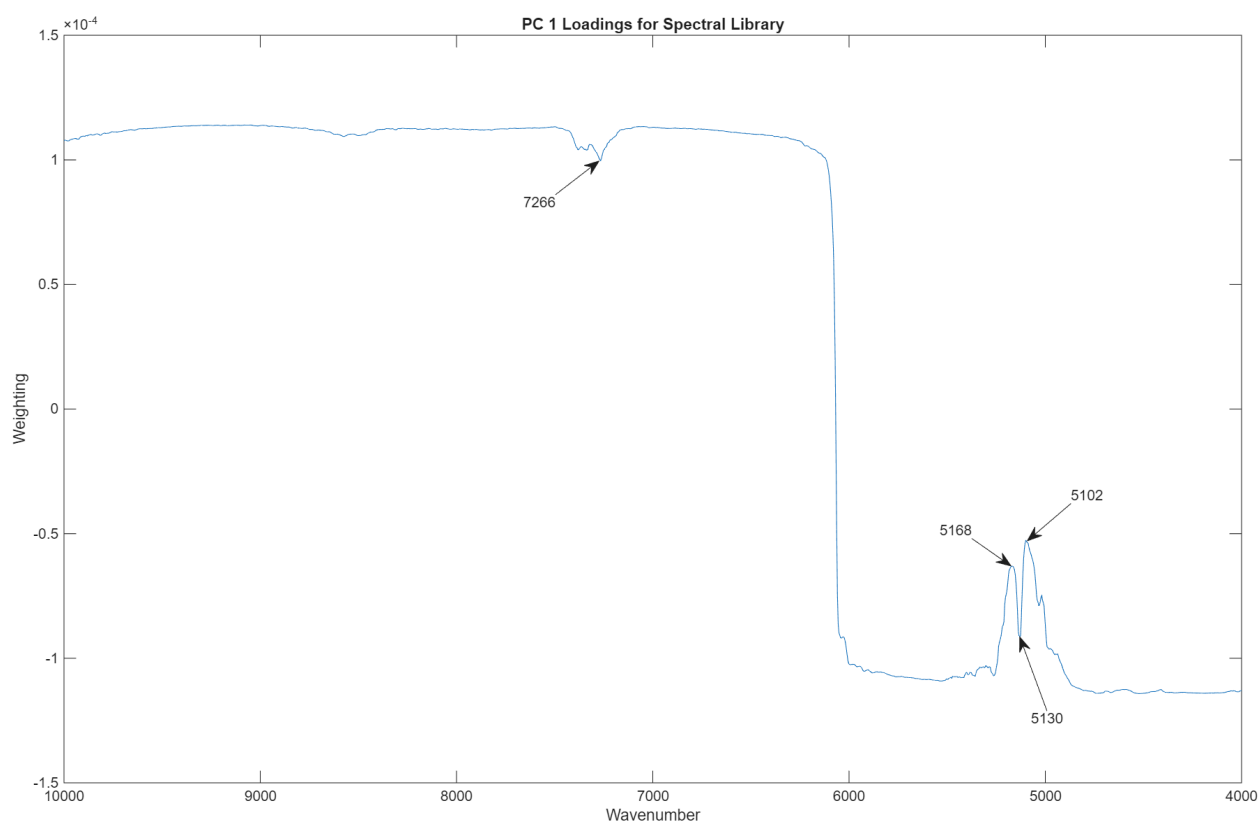


Figure 11. The loadings of the first principal component mostly show the effects of the multiplicative scatter correction on the baseline of the library spectra.

[Figure 11](#) displays the loadings plot for the first principal component (PC 1) of the interlot methacholine spectral library. The plot graphs the statistical weighting against the wavenumber, highlighting features at specific points like 7266, 5168, 5130, and 5102 cm^{-1} . Crucially, the figure demonstrates that these PC 1 loadings primarily reflect the residual baseline shifts resulting from the multiplicative scatter correction (MSC) applied to the library spectra. This is practically important because it confirms how the statistical model mathematically isolates macroscopic physical variance. The highest-variation components almost exclusively capture dominant physical effects like these residual MSC baseline shifts, particle-size distributions, or baseline distortions from the amber vials. By showing that the massive 91.17% variance captured by PC 1 is overwhelmingly driven by these physical baseline adjustments, [Figure 11](#) illustrates how the model effectively partials out dominant physical effects. This orthogonal projection is the necessary first step to ensure that the subtle, critical chemical variations indicating manufacturing anomalies are cleanly isolated in the residual variance of the higher-order principal components.

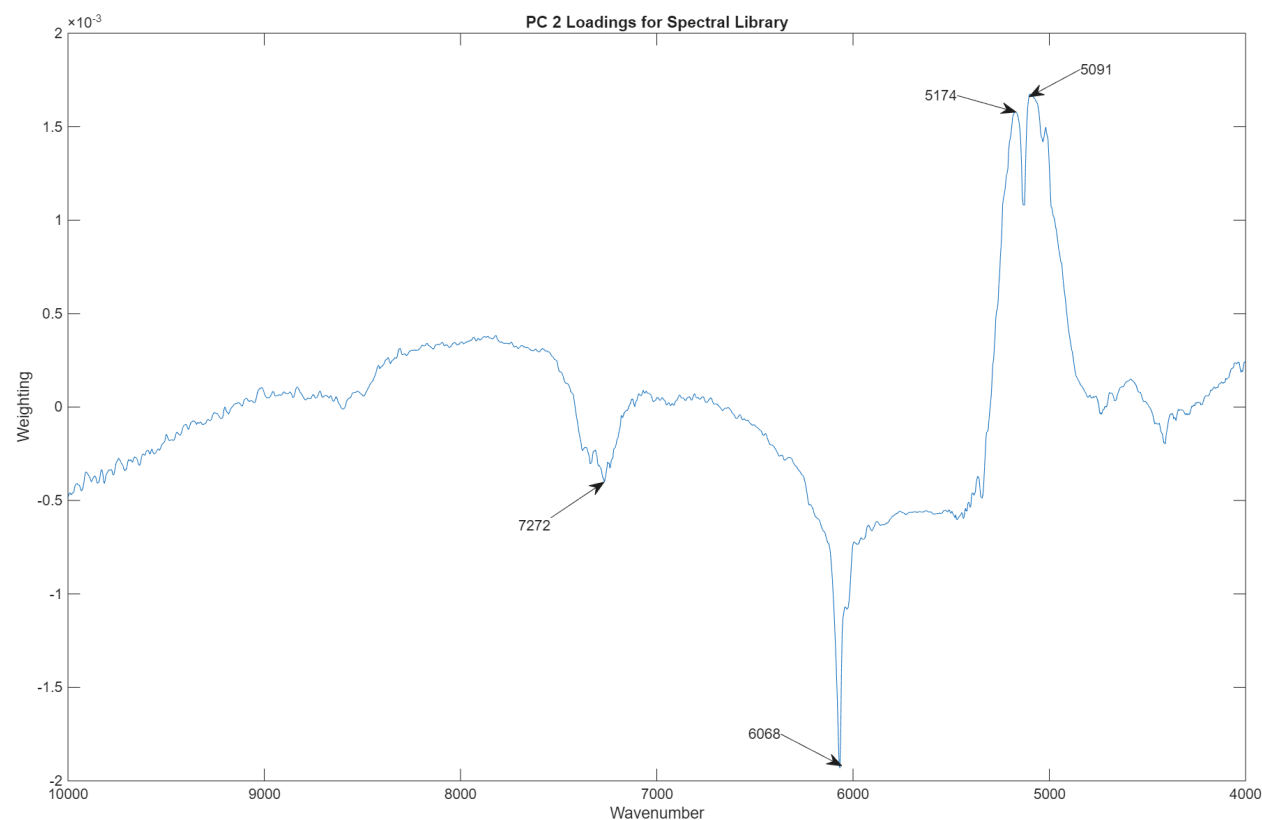


Figure 12. The loadings of the second PC reveal chemical information in the spectral library through peaks at 5091, 5174, 6068, and 7272 cm^{-1} .

[Figure 12](#) displays the spectral loadings for the second principal component (PC 2) of the interlot methacholine library, specifically revealing peaks at 5091, 5174, 6068, and 7272 cm^{-1} . This figure is important because it marks the critical analytical transition from observing physical artifacts to observing actual chemical variance. As established in the broader context of the analysis, the first principal component almost exclusively captures dominant macroscopic or physical effects, such as residual baseline shifts remaining after multiplicative scatter correction. By projecting the data orthogonally into the second principal component, the statistical model effectively partials out those overwhelming physical effects. This exposes the residual variance, which is frequently where subtle, yet critical, chemical variations emerge. Therefore, the specific wavenumber peaks shown in [Figure 12](#) are essential because they provide the first direct mathematical evidence of underlying chemical differences across the tested vials, confirming that the variations detected between the different manufacturing lots involve the actual chemical composition of the drug product rather than just physical packaging or particle-size distortions.

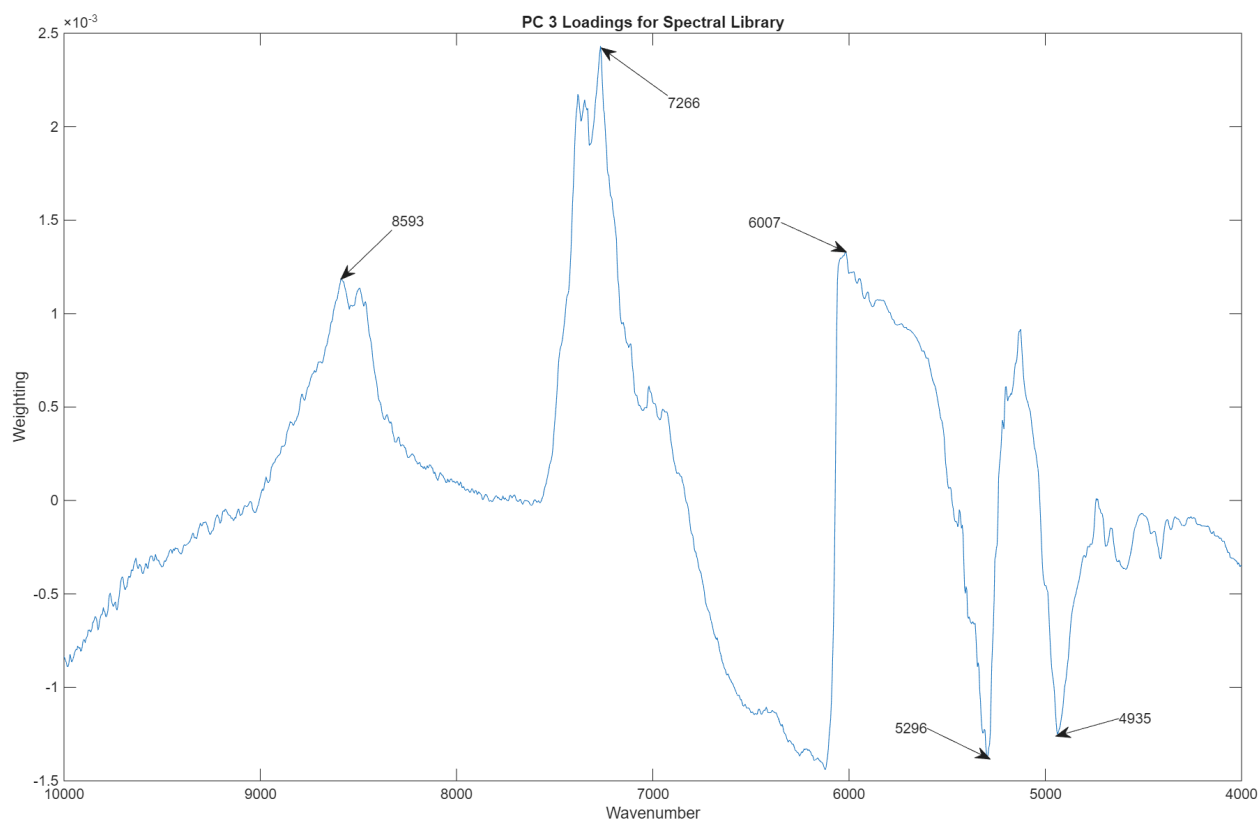


Figure 13. The loadings for PC 3 show peaks at 4935, 5296, 6007, 7266 and 8593 cm^{-1} .

[Figure 13](#) presents the spectral loadings for the third principal component (PC 3) of the interlot methacholine library, pinpointing specific absorption peaks at 4935, 5296, 6007, 7266, and 8593 cm^{-1} . This visualization is important because it represents the continued extraction of hidden chemical variance from the dataset. In the broader context of principal component analysis, the algorithm continuously partials out dominant physical and macroscopic effects, such as the residual baseline shifts captured by PC 1, by projecting the data orthogonally. While PC 2 introduced the initial chemical variance, PC 3 drills down further into the residual variance to expose additional subtle structural differences across the lots. By mathematically isolating these specific peaks, [Figure 13](#) ensures that the unique dimensional defects contributing to the variation captured by this component are meticulously quantified, preventing critical indicators of manufacturing drift from being masked by overlapping spectral clusters.

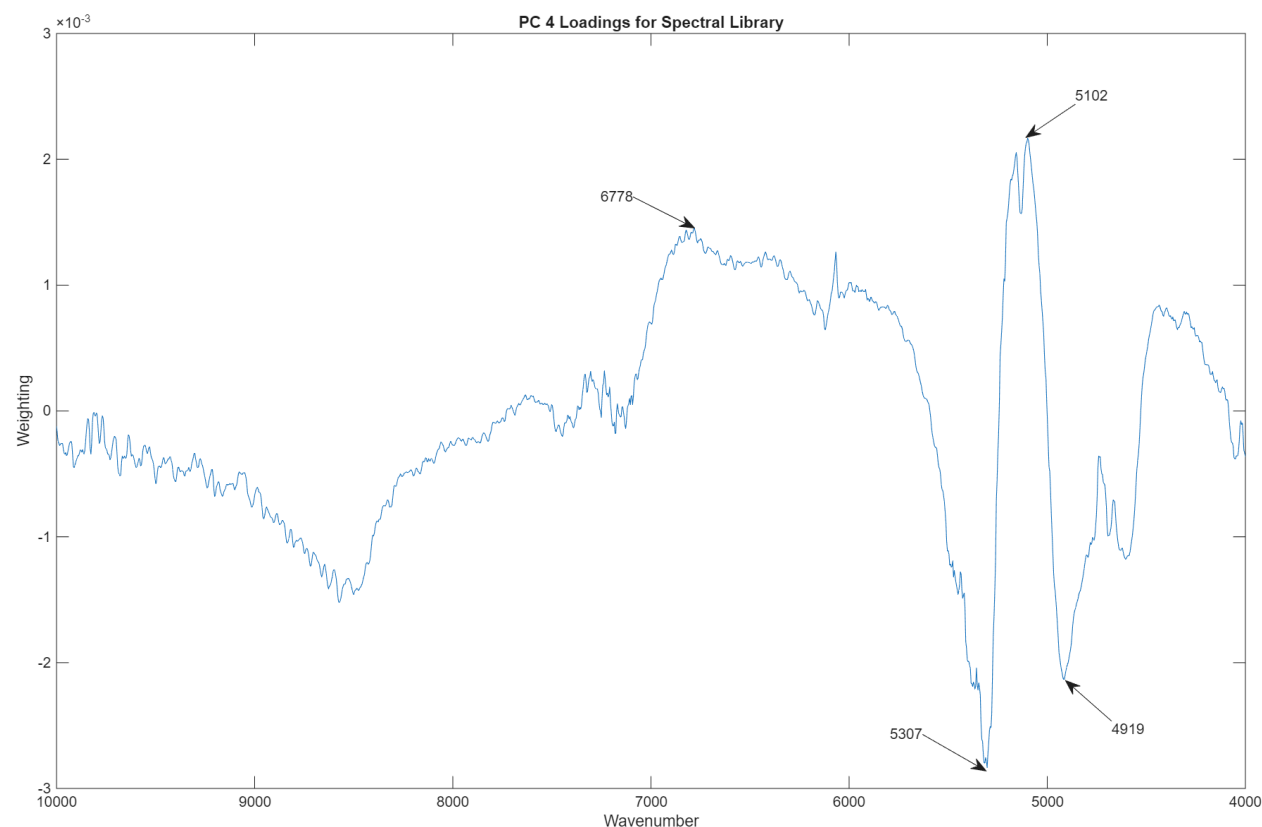


Figure 14. The loadings for PC 4 show peaks at 4919, 5102, 5307, and 6778 cm^{-1} . Noise is beginning to appear in the high wavenumber region of the spectrum.

[Figure 14](#) displays the spectral loadings for the fourth principal component (PC 4) of the methacholine library, isolating specific residual peaks at 4919, 5102, 5307, and 6778 cm^{-1} . Crucially, this specific rotation also reveals that noise is beginning to prominently appear in the high-wavenumber region of the projected spectrum. In near-infrared (NIR) spectrometry, this detector noise arises from several fundamental physical and electronic sources that inherently limit measurement precision. Physically, ambient thermal energy causes the spontaneous generation of electron-hole pairs within the semiconductor lattice of the detector, creating a continuous "dark current" that introduces random Poisson noise even in the absence of external light. Furthermore, the thermodynamic agitation of charge carriers inside the resistive elements and electrical interconnects produces constant Johnson-Nyquist (thermal) noise. From an electronics standpoint, the readout integrated circuits and multiplexers used to quantify the signal introduce electronic "read noise," while structural defects and electron traps within the semiconductor material produce low-frequency $1/f$ (flicker) noise. [Figure 14](#) is profoundly important because it visually establishes the mathematical boundary line of the quality control analysis; it shows the exact dimensional threshold where the subtle, trace chemical variations distinguishing the drug subclusters degrade to the point of competing directly with the random

quantum and thermal fluctuations of the spectrometer's hardware. By exposing this noise floor, the figure validates that the preceding components (PCs 1 through 3) successfully stripped away the dominant, measurable physical and chemical variances across the lots, while also demonstrating that any structures discovered in subsequent, lower-variance components must be heavily scrutinized to ensure they represent true chemical anomalies rather than mere instrumental artifacts.

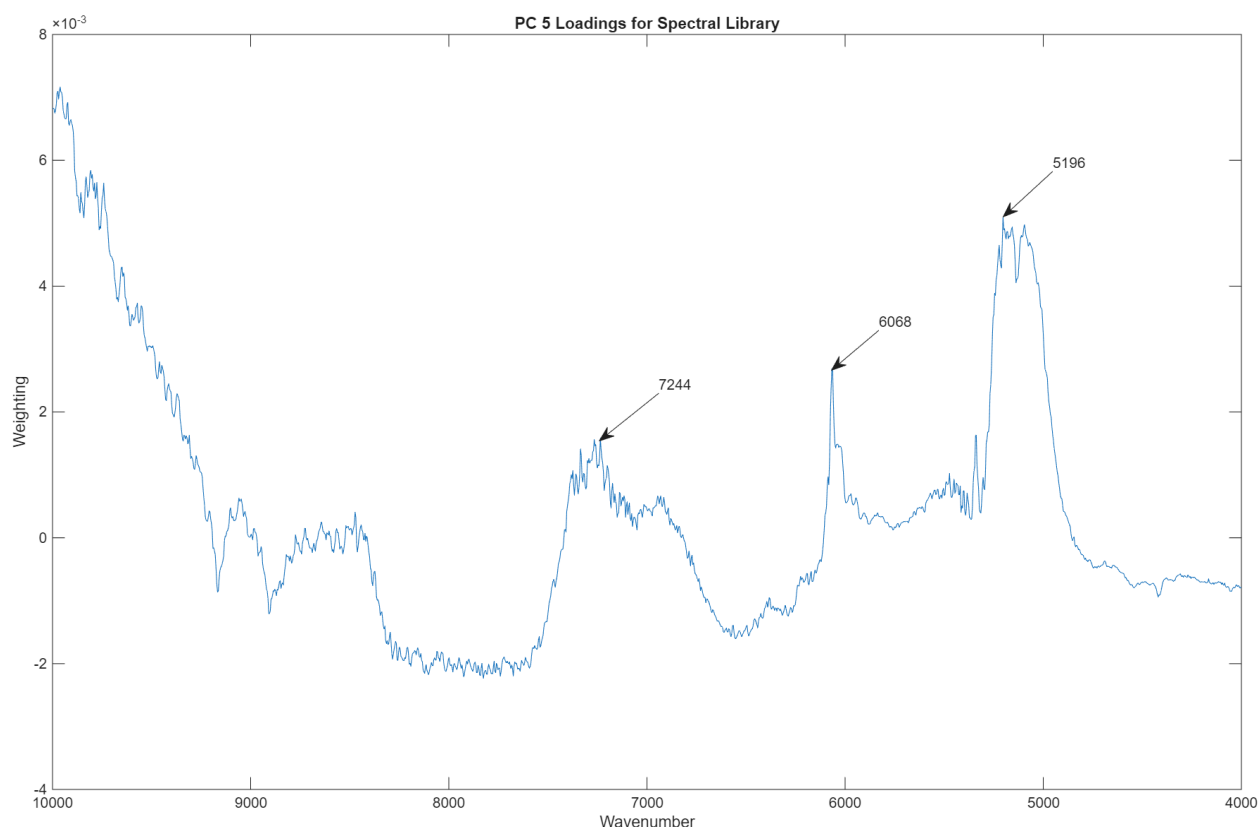


Figure 15. The loadings for PC 5 show peaks at 5196, 6068 and 7244 cm^{-1} .

[Figure 15](#) displays the spectral loadings for the fifth principal component (PC 5) of the interlot methacholine library, pinpointing precise near-infrared absorption peaks at 5196, 6068, and 7244 cm^{-1} . This visualization is of importance because it demonstrates the depths of residual variance that the statistical model must probe to fully evaluate the production pipeline. In the broader context of the interlot analysis, the fifth principal component accounts for an incredibly small fraction of the total dataset variance, specifically 0.0026. Because the previous component (PC 4) already revealed the emergence of high-wavenumber instrumental noise competing with the chemical signal, [Figure 15](#) represents the extraction of exceedingly subtle, trace structural differences that exist right at the edge of the spectrometer's physical detection limits. By mathematically isolating these minute near-infrared features, the figure ensures that

potential localized chemical anomalies, such as trace low-level contamination or moisture uptake, are meticulously quantified rather than prematurely discarded as random noise. Ultimately, this deep level of statistical decomposition confirms that fully understanding the significant structural discrepancies between the different manufacturing lots requires mapping even the faintest residual chemical variances.

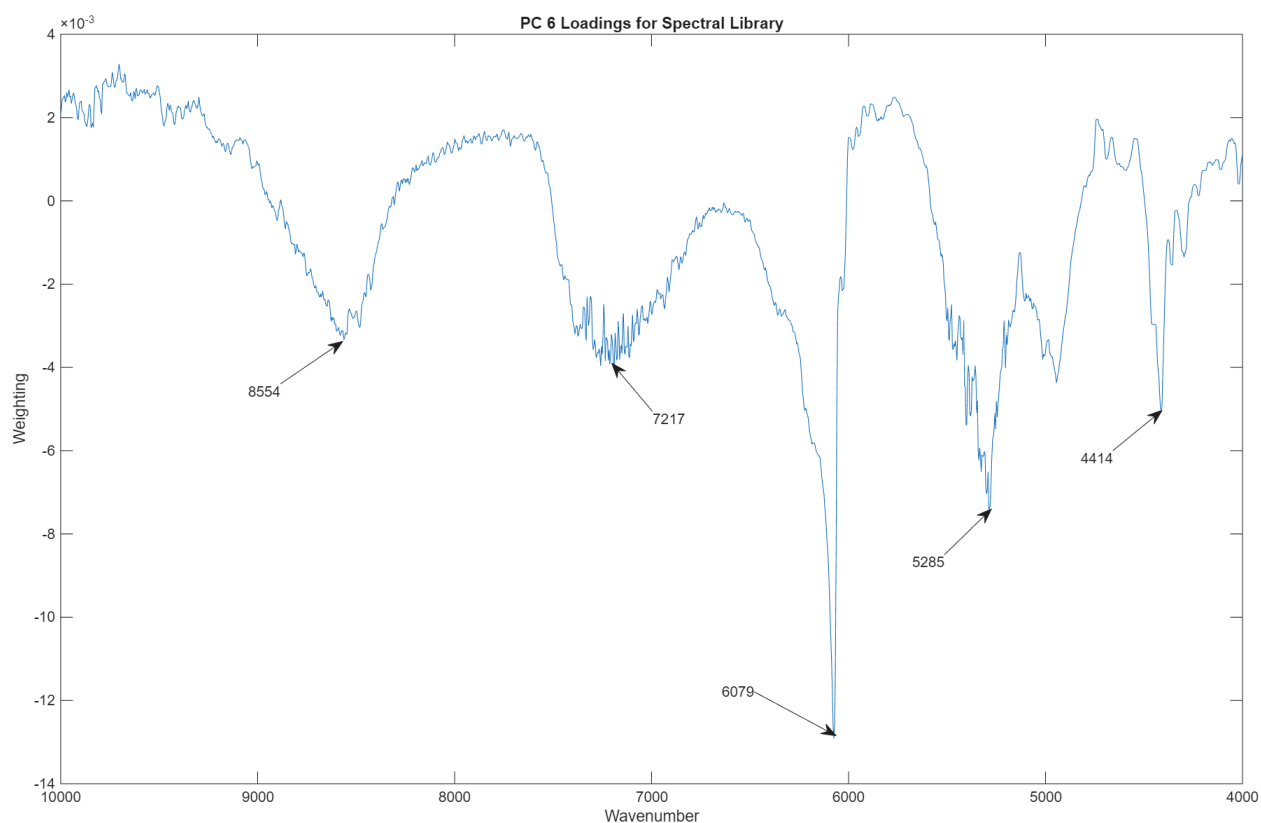


Figure 16. The loadings for PC 6 show peaks at 4414, 5285, 6079, 7217, and 8554 cm^{-1} .

[Figure 16](#) displays the spectral loadings for the sixth principal component (PC 6) of the interlot methacholine library, pinpointing precise near-infrared absorption peaks at 4414, 5285, 6079, 7217, and 8554 cm^{-1} . This visualization is of critical importance because it demonstrates the absolute depths of residual variance that the statistical model must probe to fully evaluate the production pipeline. In the broader context of the interlot analysis, the sixth principal component accounts for an incredibly small fraction of the total dataset variance, specifically capturing only 0.0017 of the cumulative variation. Because the preceding principal components have already orthogonally stripped away the dominant macroscopic physical effects and the most prominent chemical differences, [Figure 16](#) represents the extraction of exceedingly subtle, trace structural differences. By mathematically isolating these minute near-infrared features, the figure ensures that potential localized chemical anomalies are meticulously quantified rather than prematurely

discarded, confirming that fully understanding the structural discrepancies between the different manufacturing lots requires mapping even the faintest residual chemical variances. In a near-infrared spectra-structure correlation table, the isolated peak at 6079 cm^{-1} falls squarely within the broad region spanning from 6250 to 5000 cm^{-1} , which is primarily assigned to first overtones and O-H combination bands. More specifically, the 6079 cm^{-1} wavenumber sits structurally right on the boundary between two defined functional group signatures; it is located slightly below the 6175 to 6095 cm^{-1} range associated with the first overtone of a terminal alkene ($\text{C}=\text{CH}$) stretch, and just above the 5990 to 5915 cm^{-1} range that characterizes the first overtone of an aromatic C-H stretch. Therefore, this subtle signal uncovered in PC 6 most likely represents a minute, isolated variation involving carbon-hydrogen overtone stretching within the tested vials.

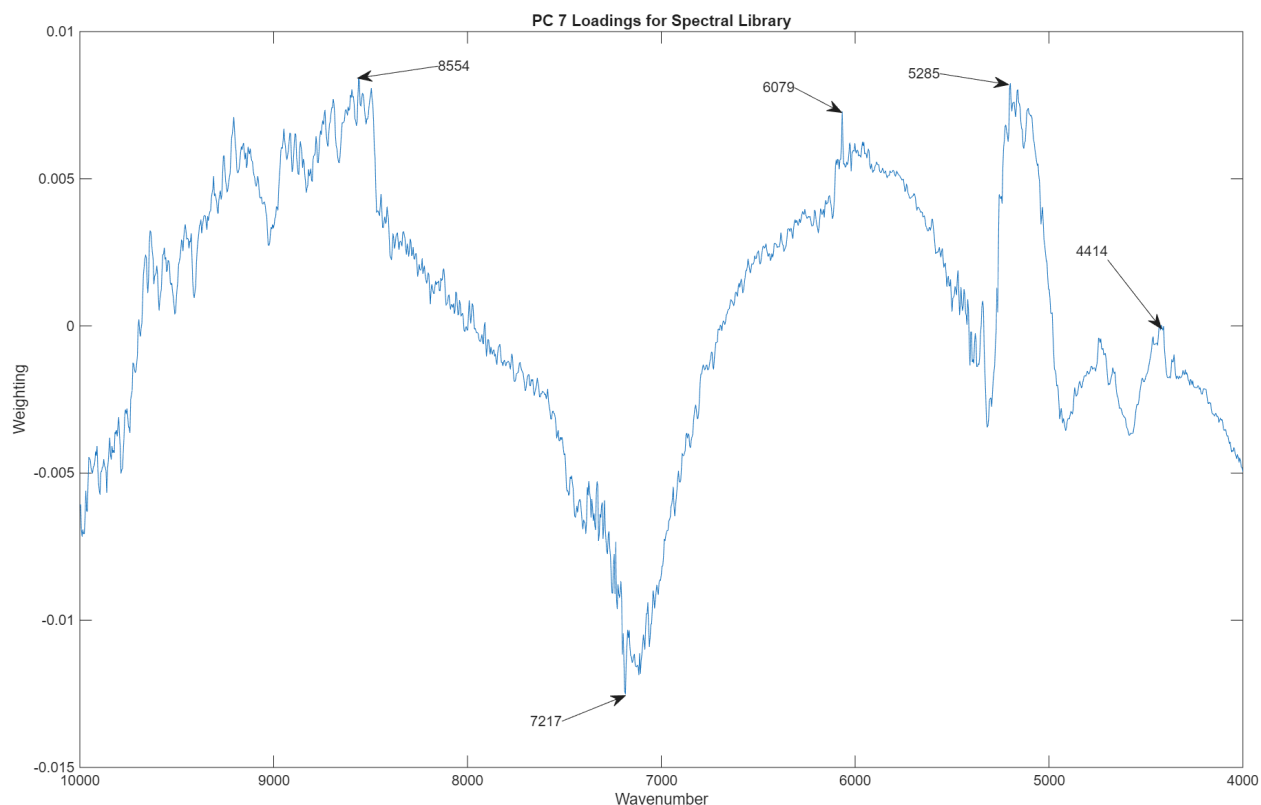


Figure 17. The loadings for PC 7 show peaks at 4414 , 5285 , 6079 , 7217 , and 8554 cm^{-1} .

[Figure 17](#) displays the spectral loadings for the seventh principal component (PC 7) of the interlot methacholine library, pinpointing precise near-infrared absorption peaks at 4414 , 5285 , 6079 , 7217 , and 8554 cm^{-1} . In the broader context of the interlot analysis, this seventh principal component accounts for a microscopic fraction of the total dataset variance—specifically 0.0005 . At this extreme depth of orthogonal decomposition, the extracted chemical signal is visibly breaking down, becoming heavily entangled with the fundamental physical and electronic

noise floor of the spectrometer. However, this figure is important precisely *in spite of* that heavy noise. The fact that specific, quantifiable absorption peaks still manage to emerge as coherent mathematical structures against the backdrop of random thermodynamic and electronic scatter demonstrates that the statistical model is successfully mapping the absolute furthest boundaries of the drug's residual chemical variance. By exposing these trace signals buried within the noise, [Figure 17](#) proves that understanding the complete reality of the manufactured lots requires us to trace the data down to its very limits. It ensures that no subtle manufacturing drift, trace contamination, or molecular anomaly is overlooked merely because it exists at the extreme edge of the analytical instrument's detection capabilities.

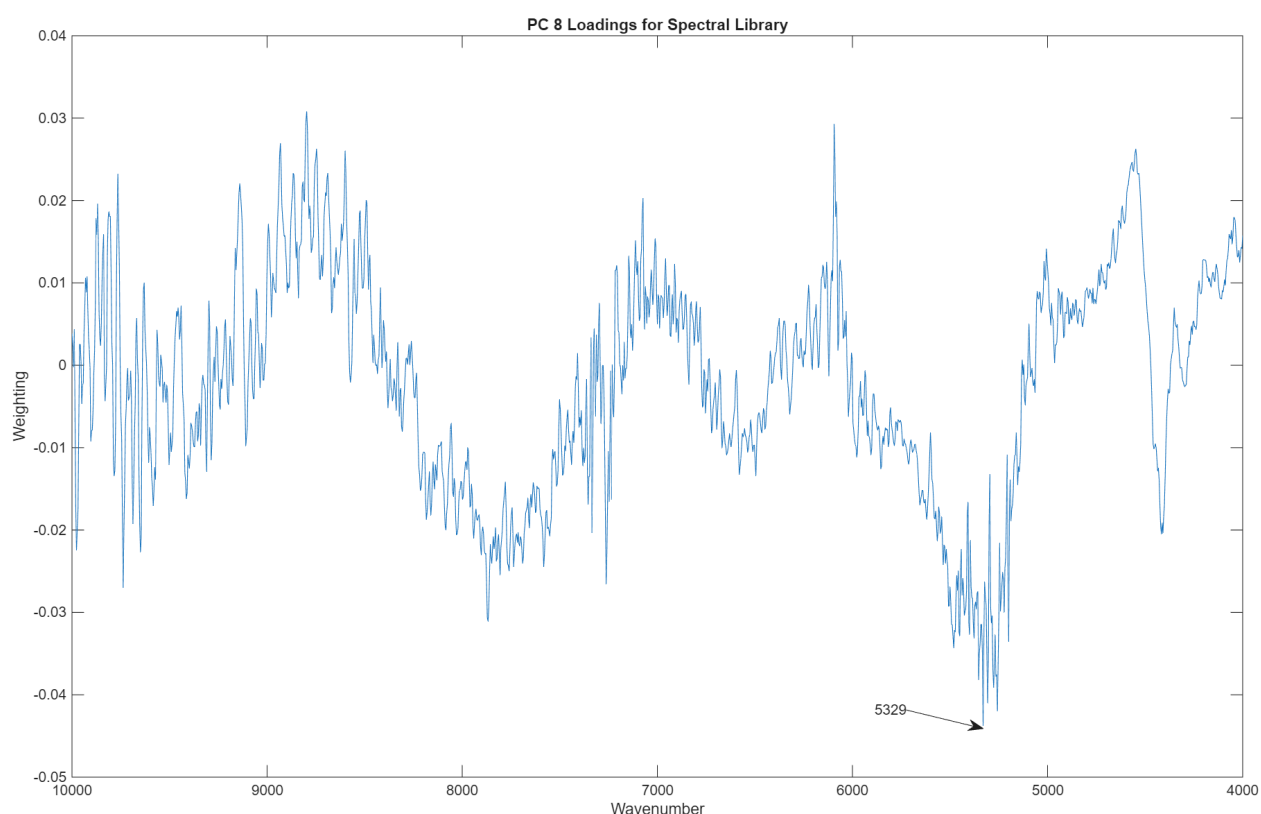


Figure 18. The loadings for PC 8 show one likely peak at 5329 cm^{-1} . The peaks from 4400 to 4600 cm^{-1} may be real as well. The rest of the features are probably noise.

[Figure 18](#) displays the spectral loadings for the eighth principal component (PC 8) of the complete interlot methacholine library, revealing one likely peak at 5329 cm^{-1} and potentially real peaks between 4400 and 4600 cm^{-1} , while the rest of the plotted features are categorized as probable noise. In the broader context of the mathematical variance decomposition, this specific principal component accounts for an infinitesimally small fraction of the total dataset variance, specifically capturing just 0.0001 of the cumulative variation. This visualization is important because it illustrates the ultimate boundary of the quality control analysis, representing the exact

threshold where legitimate chemical signals are almost entirely overtaken by the random thermodynamic and electronic fluctuations of the spectrometer. However, by successfully extracting a solitary, likely real chemical signature at 5329 cm^{-1} from this overwhelming noise floor, the figure mathematically demonstrates that the structural variations between the manufactured lots extend down to the very limits of instrumental detection. It proves that to definitively evaluate whether a pharmaceutical manufacturing process has drifted out of control, analysts must relentlessly interrogate the mathematical data until the chemical signal is completely exhausted, ensuring that no minuscule molecular anomaly is overlooked simply because it borders on the noise floor.

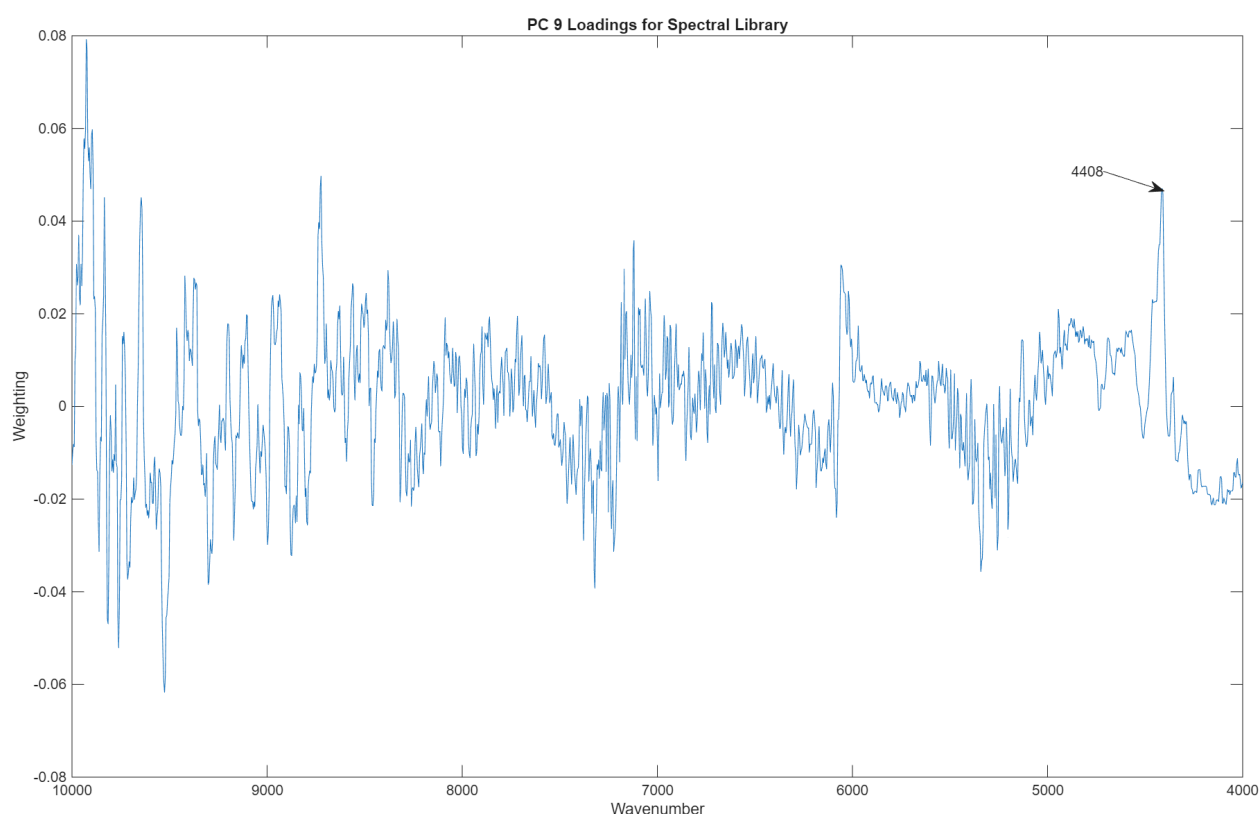


Figure 19. The loadings for PC 9 show a candidate peak at 4408 cm^{-1} .

[Figure 19](#) displays the spectral loadings for the ninth and final principal component (PC 9) of the complete interlot methacholine library, revealing a single candidate peak at 4408 cm^{-1} surrounded by an overwhelming amount of random scatter noise. As established in near-infrared (NIR) spectrometry, the largest source of this degrading noise floor is typically thermal noise (Johnson-Nyquist noise) combined with shot noise originating from the detector's dark current; because the semiconductor materials required to detect low-energy NIR photons must have fundamentally narrow bandgaps, ambient thermal energy spontaneously excites electrons, generating a continuous, random noise floor even in the absence of light. This figure

is fundamentally important because it visually demonstrates the exact mathematical limit of the quality control analysis. According to the interlot variance breakdown, this ninth principal component captures an incredibly minuscule 0.0001 of the total dataset variation, bringing the cumulative modeled variation to 0.9997 and representing the absolute end of the orthogonal decomposition. By isolating a mere "candidate" peak just before the signal is completely swallowed by the thermodynamic and electronic limits of the spectrometer hardware, the figure demonstrates that the analytical model has rigorously stripped away every possible layer of macroscopic physical distortion and subtle chemical variation across the manufactured lots. It confirms that the extraction of residual variance has been exhausted, ensuring that no structural anomaly between the drug batches was left unmapped.

Conclusion

The package insert defines methacholine chloride (Provocholine, manufactured by Methapharm, Inc.) as a parasympathomimetic (cholinergic) bronchoconstrictor agent designed exclusively for diagnostic administration in solution via inhalation, indicated strictly for the diagnosis of bronchial airway hyperreactivity in adults and pediatric patients (five years and older) lacking clinically apparent asthma. Spectrometric evaluation of a single manufacturing batch (lot P3055R) exposed distinct physical anomalies, most notably a broad absorbance peak at 5131 cm^{-1} present in certain vials but entirely absent in others. Principal component analysis orthogonally quantified this intralot variance, revealing that Vial 1 deviated by 7.3 standard deviations (SDs) from the cluster center, while a subsequent rotation identified Vial 4 as an outlier positioned 4.3 SDs away. Expanding the analysis to a 39-vial library across three distinct lots (P3055R, P3048R, and P4009S) demonstrated significant interlot structural variance requiring nine full principal components to model 99.97% of the cumulative variation. While initial components isolated macroscopic physical baseline shifts, higher-order orthogonal projections systematically extracted subtle chemical discrepancies (including isolated carbon-hydrogen overtone stretching signals near 6079 cm^{-1}) tracing variance down to the spectrometer's fundamental thermodynamic and electronic noise floor. Furthermore, application of the nonparametric FSOB subcluster analysis algorithm inside the spectral clusters confirmed the geometric separation of the data into statistically significant sub-groups. Specifically, vials 8 through 18 naturally separated from the remaining library (vials 1–7 and 19–39). This separation spanned a correlation distance of 58.8 SDs, defined by the statistical metrics $r_{\text{tn}}=0.9944$, $r_{\text{ts}}=0.8903$, and $\sigma=0.0019$, with probability-density perturbations achieving a significance level of $p=0.02$. These spectrometric results do not prove an excess level of impurities or adulteration. However, they suggest that the manufacturing process may have been operating outside of a state of process control. Additional investigation is needed.

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