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Effective HPLC method development

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Abstract

High Pressure Liquid Chromatography (HPLC) method development plays an important role in the discovery, development and manufacture of pharmaceutical products and day to day quality work in the laboratory. This article focuses on the effective method development of HPLC. It highlights pertinent conditions and other important perspectives during method development. A sequence of events required for effective method development is described. The steps involved in developing a stability-indicating HPLC method influences the analysis of degradation products/impurities in stability study and its validation demonstrate the suitability for its intended purpose.
Keywords: Method development, High Pressure Liquid Chromatography (HPLC), analytes.

1. **Introduction**

Optimization of high pressure liquid chromatography (HPLC) method development has been discussed extensively in many standard textbooks and journals. However, most of the discussions have been focused on the optimization of HPLC conditions. This article will look at this topic from other perspectives. All critical steps in method development will be summarized and sequence of events that required developing the method efficiently will be proposed. The steps will be discussed in the same order as they would be investigated during the method development process. The rationale will be illustrated by focusing on developing a stability-indicating HPLC-UV method for related substances (impurities). The principles, however, will be applicable to most other HPLC methods (Swartz 2002, Synder 1997) .

In order to have an efficient method development process, the following three questions must be answered:

1.1. What are the critical components for a HPLC method?

The 3 critical components for a HPLC method are: sample preparation, HPLC analysis and standardization (calculations). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a

chance to critically evaluate the method performance in each component and streamline the final method optimization.

1.2. What should be the percentage of time spent on different steps of the method development?

The rest of the article will discuss the recommended sequence of events, and the development timeline. One common mistake is that most scientists and researchers focus too much on the HPLC chromatographic conditions and neglect the other 2 components of the method (i.e., sample preparation, standardization). The recommended timeline would help scientists investigate different aspects of the method development and allocate appropriate time in all steps.

1.3. How should a method development experiment be designed?

A properly designed method development experiment should consider the following important questions:

What sample should be used at each stage?

What should the scientists look for in these experiments?

What are the acceptance criteria?

The authors will answer these questions in the following discussions.

2. Method Development Timeline

The following is a suggested method development timeline for a typical HPLC-UV related substance method. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample preparation, HPLC analysis and standardization) will first be investigated individually. Each of these steps will be discussed in more detail in the following paragraphs.

Step 1: Define method objectives and understand the chemistry (10%)

Determine the goals for method development (e.g., what is the intended use of the method?), and to understand the chemistry of the analytes and the drug product.

Step 2: Initial HPLC condition (20%)

Develop preliminary HPLC conditions to achieve minimally acceptable separations. These HPLC conditions will be used for all subsequent method development experiments.

Step 3: Sample preparation procedure (10%)

Develop a suitable sample preparation scheme for the drug product

Step 4: Standardization (10%)

Determine the appropriate standardization method and the use of relative response factors in calculations.

Step 5: Final method optimization/robustness (20%)

Identify the "weaknesses" of the method and optimize the method through experimental design. Understand the method performance with different conditions, different instrument set ups and different samples.

Step 6: Method validation (30%)
Complete method validation according to ICH guidelines

3. Define Method Objectives

There is no absolute end to the method development process. The question is "what is the 'acceptable' method performance"? The acceptable method performance is determined by the objectives set in this step. This is one of the most important considerations often overlooked by scientists. In this section, the different end points (i.e., expectations) will be discussed in descending order of significance.

3.1 Analytes:

For a related substance method, determining the "significant and relevant" related substances is very critical. With limited experience with the drug product, a good way to determine the significant related substances is to look at the degradation products observed during stress testing. Significant degradation products observed during stress testing should be investigated in the method development.

Based on the current ICH guideline on specifications, the related substances method for a active pharmaceutical ingredients (API) should focus on both products should focus only on the synthetic impurities, while the same method for drug products should focus only on the degradation products. In general practice, unless there are any special toxicology concerns, related substances below the limit of qualification (LOQ) should not be reported and therefore should not be investigated.

In this stage, relevant related substances should be separated into 2 groups:

3.1.1. Significant related substances: Linearity, accuracy and response factors should be established for the significant related substances during the method validation. To limit the workload during method development, usually 3 or less significant related substances should be selected in a method.

3.1.2. Other related substances: These are potential degradation products that are not significant in amount. The developed HPLC conditions only need to provide good resolution for these related substances to show that they do not exist in significant levels.

3.2 Resolution (Rs)

A stability indicating method must resolve all significant degradation products from each other. Typically the minimum requirement for baseline resolution is 1.5. This limit is valid only for 2 Gaussian-shape peaks of equal size. In actual method development, Rs = 2.0 should be used as a minimum to account for day to day variability, non-ideal peak shapes and difference in peak sizes.

3.3 Limit of Quantitation (LOQ)

The desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1%, the method LOQ should be 0.05% or less to ensure the results are accurate up to one decimal place.

However, it is of little value to develop a method with an LOQ much below this level in standard practice because when the method is too sensitive, method precision and accuracy are compromised.

3.4 Precision, Accuracy

Expectations for precision and accuracy should be determined on a case by case basis. For a typical related substance method, the RSD of 6 replicates should be less than 10%. Accuracy should be within 70% to 130% of theory at the LOQ level.

3.5 Analysis time

A run time of about 5-10 minutes per injection is sufficient in most routine related substance analyses. Unless the method is intended to support a high-volume assay, shortening the run time further is not recommended as it may compromise the method performance other aspects (e.g., specificity, precision and accuracy.)

3.6 Adaptability for Automation

For methods that are likely to be used in a high sample volume application, it is very important for the method to be "automatable". The manual sample preparation procedure should be easy to perform. This will ensure the sample preparation can be automated in common sample preparation workstations.

4. Understand the Chemistry

Most sample preparations involve the use of organic-aqueous and acid-base extraction techniques. Therefore it is very helpful to understand the solubility and pKa of the analytes. Solubility in different organic or aqueous solvents determines the best composition of the sample solvent. pKa determines the pH in which the analyte will exist as a neutral or ionic species. This information will facilitate an efficient sample extraction scheme and determine the optimum pH in mobile phase to achieve good seperations.

4.1 Chemical Properties

Most sample preparation involves the use of organic-aqueous and acid-base extraction techniques. Therefore it is very helpful to understand the solubility and pKa of the analytes. Solubility in different organic or aqueous solvents determines the best composition of the sample solvent. pKa determines the pH in which the analyte will exist as a neutral or ionic species. This information will facilitate an efficient sample extraction scheme and determine the optimum pH in mobile phase to achieve good separations.

4.2 Potential Degradation Products

Subjecting the API or drug product to common stress conditions provides insight into the stability of the analytes under different conditions. The common stress conditions include acidic pH, basic pH, neutral pH, different temperature and humility conditions, oxidation, reduction and photo-degradation. These studies help to determine the significant related substances to be used in method development, and to determine the sample solvent that gives the best sample solution stability.

In addition, the structures of the analytes will indicate the potential active sites for degradation. Knowledge from basic organic chemistry will help to predict the reactivity of the functional groups. For example, some excipients are known to contain trace level of peroxide impurities. If the analyte is susceptible to oxidation, these peroxide impurities could possibly produce significant degradation products (Fig 1).

4.3 Sample Matrix

Physical (e.g., solubility) and chemical (e.g., UV activity, stability, pH effect) properties of the sample matrix will help to design an appropriate sample preparation scheme. For example, Hydroxypropy1 Methylcellulose (HPMC) is known to absorb water to form a very viscous solution, therefore it is essential to use mostly organic solvents in sample preparation.

5. Initial Method Conditions

The objective at this stage is to quickly develop HPLC conditions for subsequent method development experiments. A common mistake is that scientists spend too much time at this stage trying to get a perfect separation.

5.1 Preliminary HPLC Conditions

In order to develop preliminary HPLC conditions in a timely fashion, scientists should use artificial mixtures of active pharmaceutical ingredients and related substances at relatively high concentrations (e.g., 1-2% of related substance relative to API) to develop the preliminary HPLC conditions. This concentration ratio between API and the related substances should be maintained to ensure the chromatography represents that of a real sample. Alternatively, a highly stressed sample (e.g., 5% degradation) can also be used at this stage. With the known composition and high levels of degradation products in the sample, one can evaluate the chromatography to determine whether there are adequate separations for all analytes. The high concentrations of related substances are used to ensure all peaks will be detected.

Computer assisted method development can be very helpful in developing the preliminary HPLC conditions quickly. Since the objective at this stage is to quickly develop HPLC conditions for subsequent method development experiments, scientists should focus on the separation of the significant related substances (section 3.1.1) instead of trying to achieve good resolution for all related substances. These significant related substances should be baseline resolved from each other with Rs > 2.0. After the preliminary method development, the HPLC conditions can be further specificity for the other related substances.

5.2 Aged HPLC Column

An aged HPLC column should be used to develop the initial HPLC conditions. Usually it is more difficult to achieve the required resolution with an aged column (e.g., column with about 200 injections). This will reflect the worst case scenario likely to be encountered n actual method uses, and help the long-term method robustness.

In general, develop all methods with HPLC columns from the same vendor. The preferred brand of HPLC column should be selected primarily based on the long term stability and lot to lot reproducibility.

6. Sample Preparation

6.1. Selection of Sample Solvent

This stage focuses on the selection of the sample solvent (for extraction) and the proper sample preparation procedures. Investigate the effect of sample solvents of different % organic, pH, extraction volume and extraction procedure on accuracy, precision, sensitivity (LOQ) and the changes in the chromatography (e.g., peak shape, resolution). Whenever possible use the mobile phase in the sample preparation. This will ensure that there will not be any compatibility issues between the sample solution and the HPLC conditions.

6.1.1 Accuracy: To investigate the accuracy in sample preparation (i.e., extraction efficiency), prepare a spiked solution by adding known amounts of related substances into a sample matrix. Compare responses of the spike solutions and the neat standard solutions to assess the recovery from the sample preparation. In this stage, since only one particular step is being investigated (i.e., sample preparation), close to theoretical recovery should be observed at this point (e.g., 90-110%). (Fig 2).

6.1.2 Precision: Use the stressed sample to represent the worst case scenario and perform replicate sample preparations from the same composite. Investigate the consistency of the related substance profile (i.e., any missing peaks?) and the repeatability results from these preparations.

6.2 Another objective is to determine the sample concentration that gives an acceptable LOQ (Signal to Noise ratio, S/N) in low level spike concentrations. The sample concentration should be low enough to maintain linearity and precision, but high enough to achieve the desired LOQ. For example, if the ICH reporting limit for this drug product is 0.1%, the LOQ of the method should be less than 0.05% (i.e., desired LOQ, in %). By using spike sample solutions of very diluted concentrations for the significant related substances, estimate the concentrations that give a S/N of about 10 for significant related substances. This estimated concentration is the appropriate LOQ concentration (i.e., estimated LOQ concentration, in µg/mL).

The following equation can be used to estimate the target sample concentration for the method:

Target sample concentration =

estimated LOQ concentration (µg/mL) x 1/desired LOQ (%) x 100%

7. Standardization

7.1 Area % method

If the response of the active pharmaceutical ingredient is linear from LOQ to the nominal sample concentration, use the % area approach where the related substance is reported as % area. This is the most straightforward approach, and doesn't require the preparation of standard solutions. It also has the highest precision since preparation to preparation variation will not affect the results. However, in order to ensure the concentration is linear within this range, the sample concentration is usually limited and this will reduce

the method sensitively (i.e., increase LOQ). In general, use this approach as long as the desired LOQ can be achieved.

7.2 External Standard method

Use the external standard method if the response of the active pharmaceutical ingredient is not linear throughout the whole range, or the desired LOQ cannot be achieved by the area % method. The concentration of standard solution should be high enough to ensure the standard solution can be prepared accurately and precisely on a routine basis, it should be low enough to approximate the concentration of related substance in the sample solution. In general, the standard concentration should correspond to about 5% of related substances.

7.3 Wavelength Selection and Relative Response Factor

Generate the linearity plot of API and related substances at different wavelengths. At this point, Photodiode Array Detector can be used to investigate the linearity of the active pharmaceutical ingredient and related substances in the proposed concentration range. By comparing the linearity slopes of the active pharmaceutical ingredient and the related substances, one can estimate the relative response factors of the related substances at different wavelengths.

Disregard of whether Area % or External Standard approach is used, if the relative response factors of some significant related substances are far from unity, a response factor correction must be applied.

The optimum wavelength of detection is the wavelength that gives the highest sensitivity (λmax) for significant related substances and minimize the difference in response factors between those of the active pharmaceutical ingredient and the related substances.

After the optimum wavelength is determined, use a highly stress sample (e.g., 5% degradation) to verify that the selected wavelength will give the highest % related substances results.

7.4 Overall accuracy

A final check of the method performance is to determine the overall accuracy of the method. Unlike the accuracy from sample preparation (section 6.1.1), which simply compares the response of the analyte with and without spiking with matrix, the overall accuracy compares the % related substances calculated from an accuracy solution with that of the theoretical value. The accuracy solutions are the solutions spiked with known concentrations of related substances and matrix. Since the extraction efficiency, choice of wavelength and the bias in standardization influence the calculated related substance result, this is the best way to investigate the accuracy of the method. Overall accuracy reflects the true accuracy of the method.

8. Method Optimization/Robustness

After the individual components of the method are optimized, perform the final optimization of the method to improve the accuracy, precision and LOQ. Use an experimental design approach to determine the experimental factors that have significant impact on the method. This is very important in determining what factors need to be investigated in the robustness testing during the method validation (see section 9). To

streamline the method optimization process, use Plackett Burmann Design (or similar approach to simultaneously determine the main effects of many experimental factors (Debebe et al).

Some of the typical experimental factors that need to be investigated are:

HPLC conditions: % organic, pH, flow rate, temperature, wavelength, column age.

Sample preparation: % organic, pH, shaking/sonication, sample size, sample age.

Calculation/standardization: integration, wavelength, standard concentration, response factor correction.

Typical responses that need to be investigated are:

Results: precision (%RSD), % related substance of significant related substances, total related substances.

Chromatography: resolution, tailing factor, separation of all related substances (section 3.1.1 and 3.1.2).

9. Method validation

9.1 Robustness

Method validation should be treated as a "final verification" of the method performance and should not be used as part of the method development. Some of the typical method validation parameters should be studied thoroughly in the previous steps (Korany 2012). In some cases, robustness can be completed in the final method optimization before method validation. At this point, the robustness experiments should be limited only to the most significant factors (usually less than 4 factors). In addition, unlike the final method optimization (see section 8), the experimental factors should be varied within a narrow range to reflect normal day to day variation. During the method validation, the purpose is to demonstrate that the method performance will not be significantly impacted by slight variations of the method conditions.

9.2 Linearity, Accuracy, Response Factor

Linearity, accuracy and response factors should be established for the significant related substances (section 3.1.1) during the method validation. In order to limit the workload of the method development, usually less than 3 significant related substances should be selected in a method. Therefore, the other related substances (section 3.1.2) should not be included in the experiments.

9.3 System suitability criteria

It is advisable to run system suitability tests in these robustness experiments. During the robustness testing of the method validation, critical method parameter such as mobile phase composition and column temperature are varied to mimic the day-to-day variability. Therefore, the system suitability results from these robustness experiments should reflect the expected range. Consequently, the limits for system suitability tests can be estimated from these experiments.

10. Conclusion

All of the critical steps in method development have been summarized and prioritized. The steps for method development are discussed in the same order as they would be investigated in the actual method development process. These steps will ensure all critical method parameters are optimized before the method validation.

In order to develop a HPLC method effectively, most of the effort should be spent in method development and optimization as this will improve the final method performance. The method validation, however, should be treated as an exercise to summarize or document the overall method performance for it intended purpose.

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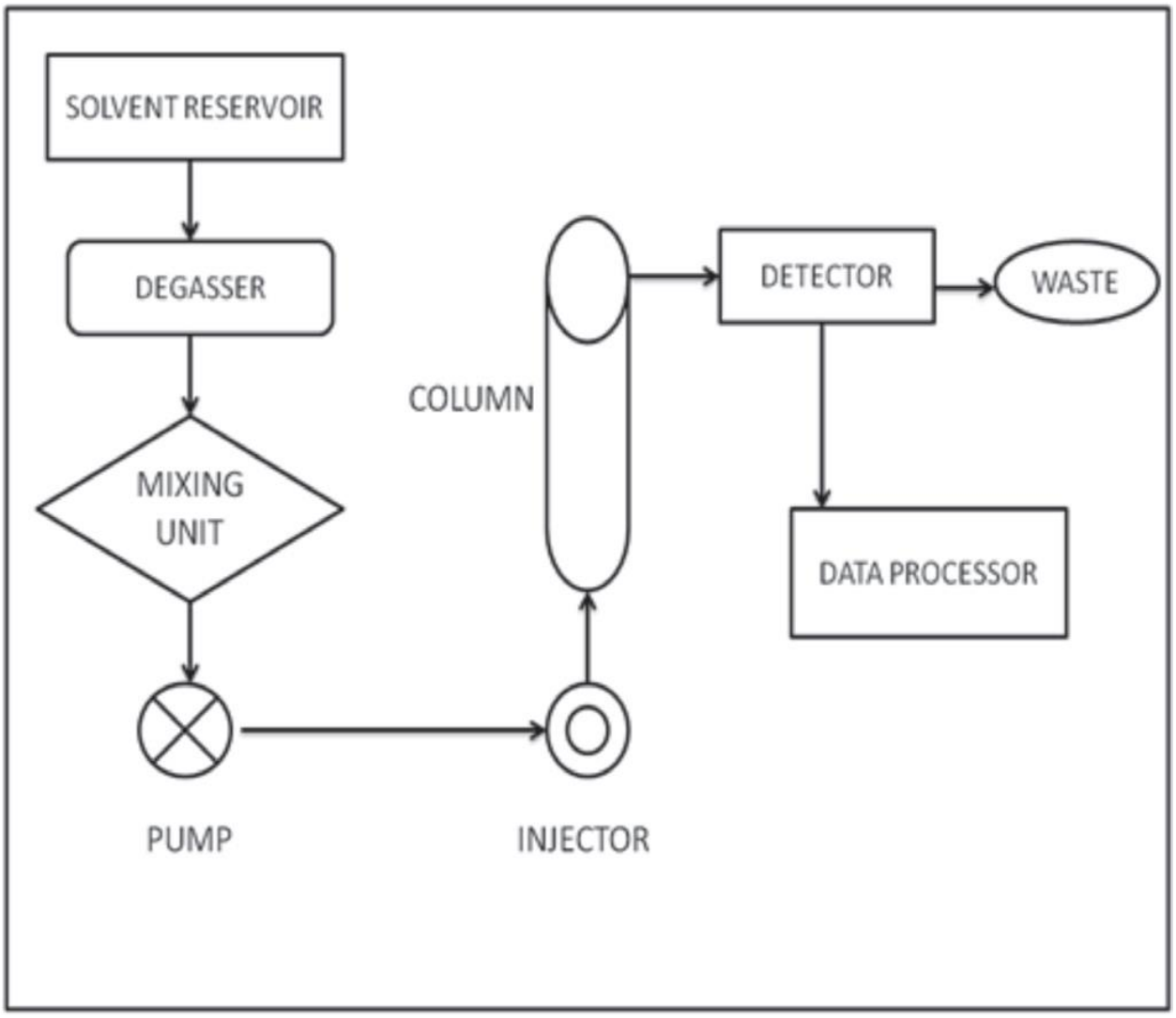

Figure 1:HPLC system with its different components

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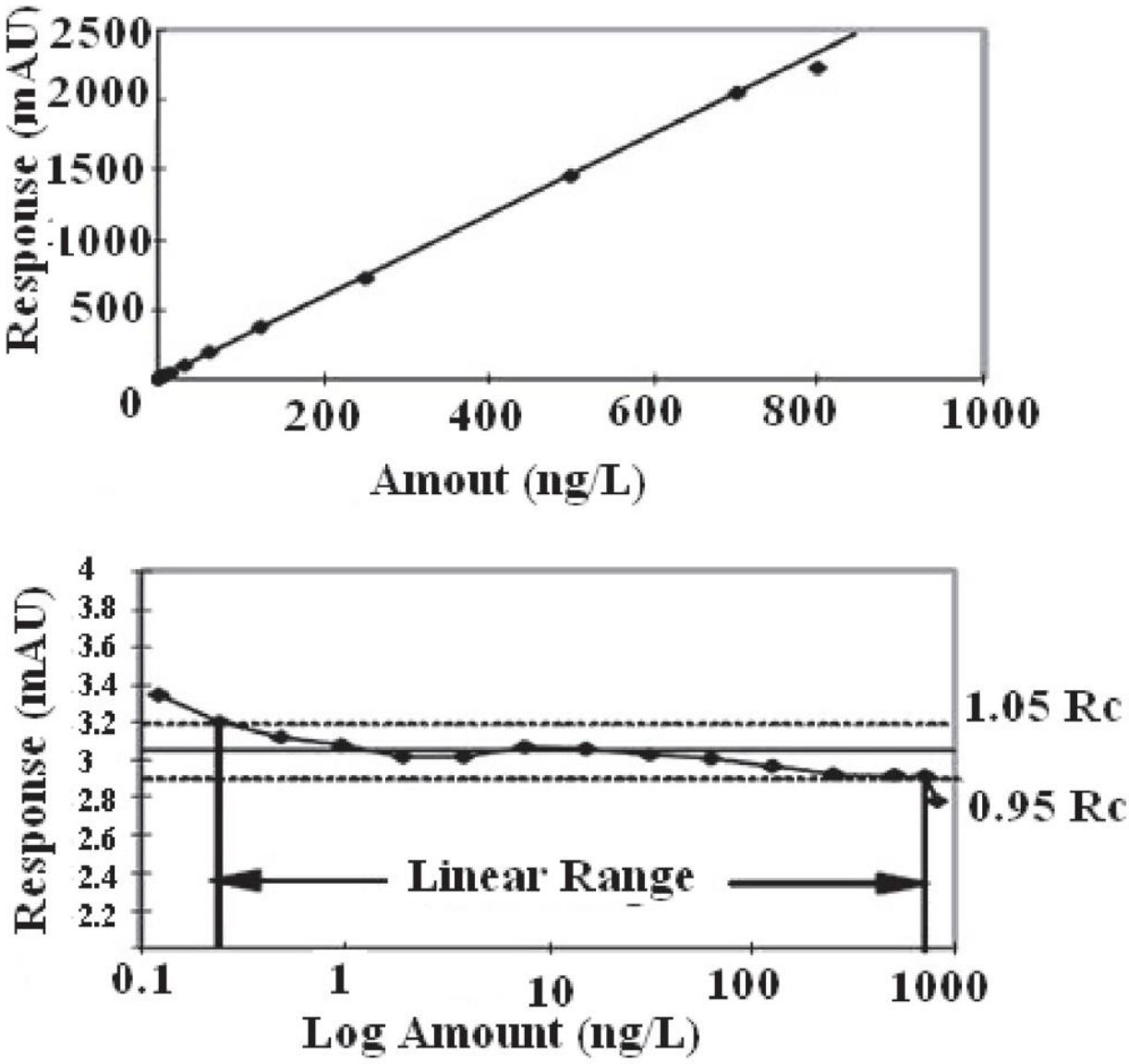

Figure 2: Graphical presentations of linearity plot of caffeine sample using HPLC.

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