YOUNG RESEARCHER
ISSN: 2277-7911Impact Factor: 5.077 (SJIF 2021)
*Peer-Reviewed, Refereed, Indexed Journal*
*Volume - 12, Issue - II, Apr-May-June 2023*

# GREEN ANALYTICAL CHEMISTRY (GAC) FOR SIMULTANEOUS HPLC METHOD DEVELOPMENT AND VALIDATION OF PIPERINE AND CURCUMIN IN POLYHERBAL FORMULATIONS

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**DOI - 10.5281/zenodo.10039321**

***ABSTRACT:***

*Phytomedicines are flooding developed nations, and customers worldwide prefer natural formulations. Quantifying the main active ingredient makes sense. Modern technologies and procedures that examine substances in a combination may help reach the ideal concentration of therapeutically active components. Thus, this study evaluated Polyherbal formulations for quantitative estimate of therapeutically active phytoconstituents to demonstrate ethno-medicinal value. Piperine and Curcumin in herbal syrup were detected using a gradient RP-HPLC technique. Gradient elution was used on an Agilent eclipse C18 column (250 mm × 4.6 mm i. d., 5m) using mobile phase A (MP A) containing 0.1 percent formic acid in distilled water and MP B of 100% acetonitrile at 1.5 ml/min. Piperine and Curcumin were UV-detected at 342 and 420 nm, respectively. Validation showed that the test was accurate, selective, precise, and robust, confirming the assay parameters. Piperine and Curcumin calibration curves were linear from 100 to 300 µg/mL. Curcumin was linear at 10-50 µg/mL. Toluene: Ethyl acetate: Methanol: Glacial acetic acid (4: 4: 1: 0.5 v/v) was used as the mobile phase to establish a simple, fast, and accurate HPTLC technique for simultaneous measurement of Piperine and Curcumin at 342 and 420 nm. The optimum mobile phase for Curcumin detection was Toluene: Ethyl acetate: Formic acid (6.5: 2: 0.5 percent v/v/v). The calibration plot showed a linear relationship for Piperine at 300-1100 ng/band and Curcumin at 100-500 ng/band with retention values of 0.72 and 0.56. HPLC and HPTLC failed to detect formulation curcumin. The Paired t-test compared techniques statistically.*

***Keywords: Phytoconstituents, HPLC, HPTLC, Validation, Poly Herbal formulation.***

## INTRODUCTION:

India is a nation that is rich in historical knowledge and is home to a diverse collection of flora and fauna. Although it has been practised for close to four thousand years, the efficiency of the Ayurvedic medical system in India has not diminished with time. The use of traditional medicine or herbal remedies is still practised by a sizeable portion of

the population. Studies of stability are carried out to demonstrate that a product has, during the course of its existence, retained all of its original attributes, such as its name, its potency, its integrity, and its quality. The findings of stability studies may also provide information on the effects of long-term changes in factors such as pH (acidic, basic, or neutral), temperature, humidity, and light exposure on the medication. An investigation into the stability of a substance may reveal how numerous medications interact with one another when taken at the same time and whether or not they are suitable for usage in varied environmental conditions (1-4).

It is also feasible to do research on the interactions between drugs and their ingredients. The stability of a drug is defined as the length of time and the conditions that must be met for all of the potentially relevant features of the medicine to remain within the acceptable range. If stability programmes are to be considered acceptable, methodical, repeatable, and compliant, then they need to be carried out in a manner that is in accordance with a number of different standards. The recommendations established by the International Conference on Harmonisation (ICH) are extensively followed. A guideline that was released by the ICH and given the title "Stability Testing of New Drug Substances and Products". Encoders are necessary in order to evaluate the efficacy, safety, and quality of medications under a variety of conditions. For each and every stability test, a method that uses a confirmed stability indicator methodology should be used. The spice Curcuma longa was found to have a terpenoid that was given the name curcumin. It shines brightly and has a high polyphenol content (5, 6).

Curcumin, which has been used for a very long time as a dietary supplement, colouring agent, and for the treatment of a variety of diseases, has been shown to have therapeutic effects, including enhancing bioavailability, anti-inflammatory, anti-fungal, anti-bacterial, anti-cancer, anti-fertility, anti-diabetic, antioxidant, antiamoebic, anti-HIV, anti-spasmodic, and nematicidal properties. It has been shown that an intake of 8 grammes per day is not harmful (7-9). The characteristics of curcumin are shown in Figure 1. This compound has the chemical formula $C_{21}H_{20}O_6$, a molecular weight of 368.37g/mol, and a melting point that ranges between 182-184 degrees Celsius. It is referred to as 1, 7-bis-(4-hydroxy-3-methoxyphenyl) in the chemistry.

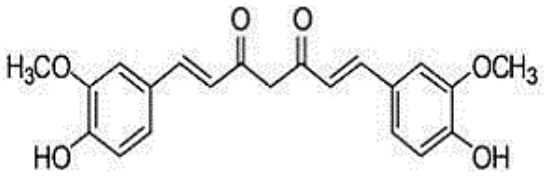



Figure 1: Curcumin

Curcumin is a naturally occurring luminous yellow chemical that may be dissolved in ethanol, acetone,

Dimethylsulphoxide, and oil but is almost insoluble in water. It emits a brilliant yellow light. Piperine, sometimes known as black pepper, is an aromatized alkaloid that may be discovered in the Piper longum and Piper nigrum plants. It is often used in the capacity of a seasoning. Piperine that has been extracted from plants may have a purity level of up to 98%. The therapeutic benefits of piperine include, but are not limited to: CNS depression, antipyretic, anti-inflammatory, antioxidant, cytoprotective, antiulcer, anticonvulsant, insecticide, and bioavailability enhancer. The IUPAC nomenclature for this chemical is piperine, which may also be written as 1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2,4-pentadienyl] piperidine. Its chemical formula is $C_{17}H_{19}NO_3$, and its melting point is somewhere in the range of 282 to 283 degrees Celsius. Piperine's solubility is improved by a number of solvents, including alcohol, ether, and chloroform (10, 11). Piperine's three-dimensional structure is seen in Figure 2.

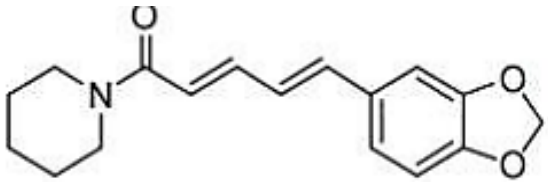



Figure 2: Piperine

Methods for the simultaneous detection of curcumin and piperine have been found. These methods include high-performance thin-layer chromatography, reversed-phase high-performance liquid chromatography with poly (dioxane anhydride), and reversed-phase high-performance liquid chromatography with fast temperature-cooling. Both HPLC-UV and HPLC with silymarin, both of which have been described as analytical methods, have been used in separate research projects to investigate the stability of curcumin. Piperine's stress degradation has been studied utilising a variety of different methodologies, including LC/Q-TOF-dual ESI MS by itself, as well as RP-HPLC technology in conjunction with either rifampicin or Aconitine. Curcumin and piperine, whether taken by alone or in combination with other medications, are the substances that are most often the focus of these detection approaches. Due to the fact that there is currently no method that simultaneously demonstrates a stability-indicating study of curcumin and piperine, we decided to conduct this research in order to develop a unique, validated RP-HPLC method for the simultaneous estimation of curcumin in line with the criteria that were proposed by the ICH (12-14).

## MATERIALS AND METHODS:

### Materials: Chemical Used:

Table 1: List of solvents used in present study

| Sr. No. | Chemical | Grade | Supplier |
|---|---|---|---|
| 1. | Toluene | AR | Merck specialities Pvt. Ltd. |
| 2. | Ethyl acetate | AR | Merck specialities Pvt. Ltd |
| 3. | Formic acid | AR | Merck specialities Pvt. Ltd |
| 4. | Methanol | AR | Merck specialities Pvt. Ltd |
| 5. | Methanol | HPLC | Merck specialities Pvt. Ltd |
| 6. | n-propanol | AR | S D Fine Chem. Ltd. |
| 7. | Acetonitrile | HPLC | Merck specialities Pvt. Ltd. |

### Standard Used:

Table 2: List of standard markers used in present study

| Sr. No. | Name of extract | Name of Standards | Potency (%) | Supplier |
|---|---|---|---|---|
| 1 | *Curcuma longa* | Curcumin | 97% | Sigma |
| 2 | *Piper longum* | Piperidine | 99% | Sigma |

### Instruments Used:

Table 3: List of Instruments used in present study

| Sr. No | Equipment | Manufacture | Model |
|---|---|---|---|
| 1 | Electronic balance-1 | Mettle Toledo | Classic plus<br>D value -0.1 mg |
| 2 | HPLC pump Uv visible detector Column $C_{18}$ [150 mm × 4.6 mm, 5µ] | waters<br>Phenomenex | E 26952489<br>Phens |
| 3 | Sonicator | sonorex | Sonorex dig 10 p |
| 4 | pH meter | Elico | |
| 5 | Moisture analyser | Metter Toledo | HR84 halogen |

### Methodology:

### Identification of Phytoconstituents:

Since ancient times, India's medical practitioners have successfully treated a wide variety of conditions utilising various plants. It's possible that a plant's leaves, bark, roots, stem, flowers, seeds, and fruits all contain substances that have therapeutic properties and might be used to treat a variety of ailments. Phytochemicals, also known as phytoconstituents, are chemical components of plants that have been found to have therapeutic benefits. Extensive research has been conducted on the phytoconstituents' pharmacological properties, such as their antibacterial, antimicrobial, and antiprotozoal actions (15). When a plant is attacked by a bacterium, an insect, a disease, or a predator, phytoconstituents spring into action to

protect the plant from the many threats. Some are in charge of organoleptic characteristics such as colour and aroma, for example (16).

**Melting Point Determination:**

The measurement of a material's melting point is the kind of thermal analysis that is performed most often for the purpose of characterising solid crystalline substances. It is used in a variety of sectors for quality control and research and development reasons to establish the identification of solid crystalline compounds and evaluate the purity of those chemicals. The melting points of piperine and curcumin were determined with the use of an equipment that measures melting points (17).

**Identification by IR Spectroscopy:**

Infrared spectroscopy is the term given to the scientific study of how different substances either absorb, emit, or reflect infrared light. Infrared spectroscopy may also be referred to by its other term, vibrational spectroscopy. Utilising this technique, chemical compounds or functional groups may be examined and identified regardless of whether they are in the solid, liquid, or gaseous state. Infrared (IR) spectroscopy is a typical technique of absorption that may be used for qualitative and quantitative assessments (18).

**Solubility Study:**

Compiling research may be done in a number of different methods, and the one you choose will depend entirely on the requirements of the current project. The active pharmaceutical ingredient (API) is generally added to a solvent or solvent combination on an individual basis at regular intervals until the required concentration for the pharmaceutical product is achieved. The degree to which piperine and curcumin are soluble in acetonitrile, methanol, and water is studied (19).

**Estimation of Curcumin in Poly Herbal Formulation by HPLC Method:**

The plant known as Curcuma longa Linn produces a chemical known as curcumin (CMN), which is yellow in colour. Antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer are just some of the therapeutic roles that CMN plays in the body. CMN is an effective medicine for the treatment of a wide variety of illnesses, including diabetes, arthritis, Alzheimer's disease, and allergies.

**Estimation of Piperine in Poly Herbal Formulation by HPLC Method:**

Because it contains the primary active component piperine, black pepper, also known as Piper nigrum, is considered to be one of the most valuable components that can be derived from a plant. Piperine is the primary compound responsible for the biological action of black pepper. The Scoville heat index ranges from 100,000 to 20,000,000 for this substance. Piperine is a carboxamide alkaloid that is generated from Piperic acid and piperidine. Piperine is also known as piperidine alkaloid. In recent years, the

piperine market has received more interest from the pharmaceutical sector. Antibacterial, anti-inflammatory, and anti-arthritic are just a few of the numerous beneficial effects that this substance has. It is especially remarkable because piperine enhances the absorption of a broad range of medicines and phytochemicals. Piperine is found in black pepper (20).

**Estimation of Piperine and Curcumin in Water extract of formulation and crude drugs by developed HPLC Method:**

For the purpose of demonstrating the viability of this notion, a combination of these phytoconstituents and traditional medications was constructed. After that, the solutions were injected into the HPLC column. Comparisons were made between the retention periods of the chromatographic peaks of piperine and curcumin standards found in methanolic extracts. A regression equation was used in order to determine the amount of piperine that was present in both the unprocessed drugs and the final product. A crude methanolic extract of Curcuma longa was analysed, and the quantity of curcumin that present was measured (21).

**Analytical Method Validation:**

The RP-HPLC approach was utilised to identify curcumin in a combination with other botanicals. To evaluate the accuracy of the technique, the following parameters were taken into consideration. The process of conducting tests in a laboratory as part of analytical method validation ensures that the method's performance characteristics are up to par for the applications for which they are intended. Method validation, also known as "the process of providing documented evidence that the method performs as intended," ensures that the method will continue to be reliable even after extensive usage. When an analytical method is validated, it demonstrates not only that it is suitable for the application for which it was designed but also that it is accurate, specific, and precise throughout the spectrum of analyses that are to be analysed. Techniques of Analyses Validation of both the new and the old analytical procedures is necessary if there is a change in either the method itself, the composition of the medicinal product, or the synthesis of the active pharmaceutical components. Specificity Because there were no unexpected contaminants discovered, the findings of the study may be relied upon. Method Validation is an essential analytical method that should always be used since it ensures that analytical procedures are accurate and precise. With the use of this procedure, one may identify the limits of detection and quantification for calculating the components of a drug. In addition to the techniques of validation, we also evaluate the suitability of the system. Statistical approaches are also used in

the process of communicating the analytic outcomes of the validation features. Validating analytical methods requires both the statistical processing of analytical data and the execution of distinguishing parameters. Validation cannot take place without both of these components. By carrying out these procedures, we validate the reliability of the analytical results (20-21).

**Specificity:**

The capacity to accurately and selectively identify an analyte in the presence of other elements that may be present in the sample matrixsuch as contaminants, degradation products, and matrix components, is what is meant by the term "specificity." It is necessary to provide evidence that the analytical process is unaffected by the presence of materials that have been tampered with. The approach must have the capacity to discriminate between chemicals that share structural connections and are likely to be present in identification tests.

**Precision:**

Determine the accuracy of the analytical approach by using the two different methodologies.

**A) Repeatability:** Calculating the assay for six unique sample preparations derived from the same batch is one way to evaluate the repeatability of an analytical method. Determine the % RSD for each of the six different example solutions, and then report your findings.

**B) Intermediate precision (Ruggedness):** You may figure out how reliable the analytical method is by calculating the assay for six distinct sample preparations taken from the same batch, each of which was performed on a different day by a different analyst using a different HPLC machine with a different amount of column. This will allow you to compare the results. After you have determined the proportion of each of the six sample preparations, compute the associated % RSD.

**Linearity & Range:**

You may demonstrate that the linearity of your assay analytical technique can be shown by injecting into the chromatograph five distinct volumes of the standard preparation varying in concentration from 80% to 120%. A graph depicting the greatest reactivity to curcumin vs its concentrations measured in ppm was created. For a scatter plot depicting peak response vs curcumin concentration, the slope, intercept, and regression coefficient were provided for analysis (22).

**Accuracy:**

The analytical technique's precision was determined using the following method. Recuperation efforts: Performed recovery studies by combining known quantities of Curcumin working standard with known quantities of placebo (diazen Tablets excipient mixture) in the range of 80% - 120% of the test concentration specified in the method of analysis. Reported were the percentage of

recovery in the presence of placebo and relative standard deviations for all values of percent recovery.

**System Suitability:**

The method of analysis was used to determine the suitability of the chromatographic system by establishing system suitability test parameters such as relative retention times, resolution, tailing factor, number of theoretical plates for system suitability preparation, and % relative standard deviation of Curcumin standard preparation. This parameter must be examined daily (21, 22).

**RESULT AND DISCUSSION:**

**Identification of Phytoconstituents:**

**Melting Point Determination:**

Piperine and Curcumin's melting points were determined using Melting Point apparatus.

Table 4: Melting Point of Piperine and Curcumin

| | |
|---|---|
| Piperine | 128-130°C. |
| Curcumin | 183°C |

**Identification by IR Spectroscopy:**

It is used to analyse and identify solid, liquid, or gaseous chemical substances or functional groups. By utilising an FT-IR Spectrophotometer, the IR spectra of Piperine and Curcumin were acquired.

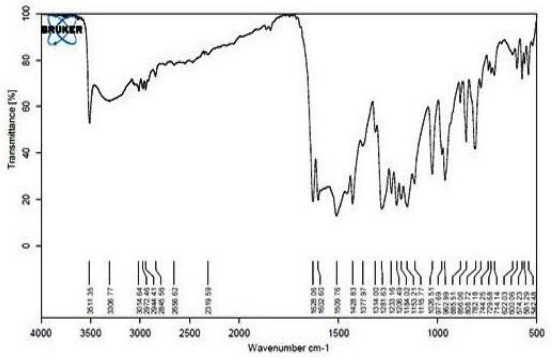



Figure 3: Piperine (Sample spectrum)

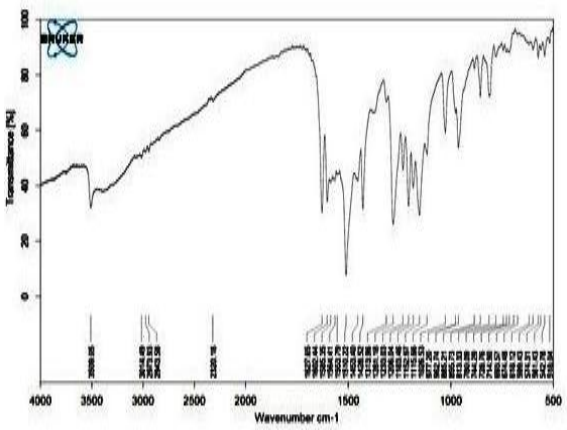



Figure 4: Curcumin (Sample spectrum)

**Solubility Study:**

When establishing the formulation, the first and most important stage is to determine how effectively the active pharmaceutical ingredient (API) dissolves in the topical excipients. Most topical formulations strive for an API that has been dissolved since this both speeds up API migration through the epidermal layers and brings it closer to the site of action, as well as preventing API precipitation while the formulation is being stored. For the length of the product's shelf life, the active pharmaceutical ingredient (API) shall have the same particle size and shape it had when it was first manufactured, assuming the product is a suspension. Piperine and curcumin

were subjected to tests to determine whether or not they were soluble in a number of different solvents, such as water, methanol, and acetonitrile.

Table 5: Solubility data

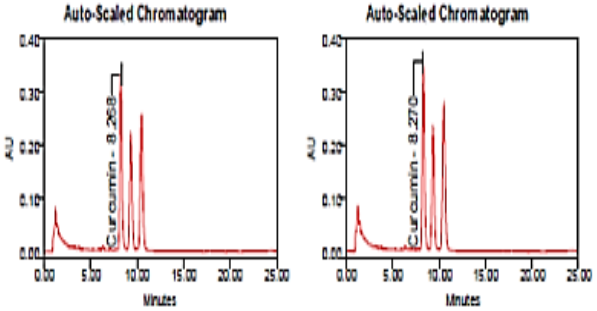
| Solvent | Observation | |
|---|---|---|
| | Piperine | Curcumin |
| Water | Slightly soluble | soluble |
| Methanol | Soluble | Insoluble |
| Acetonitrile | Freely Soluble | Soluble |

**Estimation of Curcumin in Poly Herbal Formulation by HPLC Method:**

We devised and verified a straightforward method that is economical, kind to the environment, and efficient for determining the amount of curcumin in herbal capsules without taking into account the effects of other phytochemicals that make up formulation components. According to the findings and the statistical analysis, this method may prove to be very useful for assessing the amount of curcumin present in Polyherbal medications or turmeric extracts.

**Optimized Chromatographic Condition:**

Column: symmetry C18, 150 × 4.6mm × 5µ
Mobile phase: THF: 1% Citric acid (450:550)
Flow rate: 1 ml/min
Column temp: Ambient
Detection wavelength: 425 nm
Run time: 25 min
PH: 2.8
Injection volume: 20 µl
Elution technique: Isocratic technique




Component Summary Table
Name: Curcumin

| | Sample Name | Name | RT | Area |
|---|---|---|---|---|
| 1 | ASSAY PREPARATION-1 | Curcumin | 8.268 | 5469658 |
| 2 | ASSAY PREPARATION-1 | Curcumin | 8.270 | 5440343 |
| Mean | | | 8.269 | 5455000.5 |
| Std. Dev. | | | 0.001 | 20728.84 |
| % RSD | | | 0.0 | 0.4 |

**Observation:** peaks were well separated and RSD was passed.

**Method Validation:**

The RP-HPLC method was designed and validated to estimate curcumin in a multi-herbal tablet dosage form, meeting ICH requirements for system appropriateness, linearity and range, precision, accuracy, robustness, and specificity. Results from developing and validating analytical techniques are summarised here.

Table 6: Data and Result

| **Parameters** | **Acceptance** | **Results** |
|---|---|---|
| System suitability | % RSD NMT 2%<br>Tailing factor NMT 2<br>Theoretical plates NLT 2000 | 3.171<br>1.47<br>Passes |
| Specificity | No interference with blank and placebo preparation | No interference |

| | | |
|---|---|---|
| Precision (Repeatability) | % RSD NMT 2% | 0.135 |
| Accuracy | 98-102% | 101.73 |
| Linearity | Correlation co-efficient NLT 0.999 | 0.9994 |
| Ruggedness | % RSD NMT 2% | 0.2 |

In terms of the suitability of the system, linearity and range, accuracy (repeatability), intermediate precision, specificity, and durability, the compiled observations and conclusions fall within the acceptable range. In light of the aforementioned information, the method that was proposed was user-friendly, specific, linear, exact, accurate, robust, and generally applicable when it came to estimating the dose of curcumin in multiherbal tablets. Moreover, the method was linear. It has become possible to conduct contemporary chromatography using the reverse-phase high-performance liquid chromatography technology. A chromatogram of the pure substance was performed by using a number of distinct mobile phases, such as acetic acid, tetrahydrofuran, and citric acid at a concentration of 1%. In addition to other columns, the $C_8$ and $C_{18}$ columns were used. The mobile phase was a 45:55 combinations of 1% citric acid in THF. This mixture served as the mobile phase. Because of its tendency to generate a sharp, symmetric peak with 1.51 tailing, Phenolex $C_{18}$ was selected to serve as the analytical column. It was discovered that the calibration graph had a linear relationship between 20 and 30 µg/ml. The five different concentrations of curcumin that were described above were placed into the HPLC in equal amounts. The value of the correlation coefficient, often known as $r^2$, was found to be 0.9994. It was shown that the concentration range and the concentration were correlated with one another. The measurement in percentages revealed that the formulation contained an average of 109.23% of the curcumin that was called for. The relatively low values of standard deviation and coefficient of variation at each stage of the recovery experiment provide evidence that the procedure has a high degree of accuracy.

**Estimation of Piperine in Poly Herbal Formulation by HPLC Method:**

**Optimized Chromatographic Condition:**

The investigation was carried out at a temperature of 25 $^oC$ utilising an isocratic elution mode with a flow rate of 1.5 millilitres per minute. The detection was done at 250 nanometres. The retention period of piperine in the standard was determined to be 12 minutes, and the quantitative analysis of the chosen markers in the formulation was carried out with reference to a linear regression equation.

| Parameter | Condition |
|---|---|
| Stationary Phase | Phenomenex Luna C18 (250 × 4.6mm, 5µm) |
| Mobile Phase | Buffer: ACN (80: 20) |
| Flow Rate | 1.5 ml/min |
| Detection | 250 nm |
| Pump Mode | Isocratic |
| Injection Volume | 20µl |
| Run Time | 12 min |
| Column Temperature | Ambient |
| Retention Time | About 7 to 9 min |
| Needle wash | Water : ACN (50:50) |

High-performance liquid chromatography was used to determine the amount of piperine present. In order to verify the findings of the piperine test performed on herbal pills, this method is carried out.

**Method Validation:**

| Parameters | Acceptance | Results |
|---|---|---|
| System suitability | % RSD NMT 2%<br>Tailing factor NMT 2<br>Theoretical plates NLT 2000 | 3.101<br>1.26<br>Passes |
| Specificity | No interference with blank and placebo preparation | No interference |
| Precision (Repeatability) | % RSD NMT 2% | 0.82 |
| Accuracy | 98-102% | 1.009 |
| Linearity | Correlation co-efficient NLT 0.999 | 0.9994 |
| Ruggedness | % RSD NMT 2% | 0.2 |

**Estimation of Piperine and Curcumin in Water extract of formulation and crude drugs by developed HPLC method:**

**Optimized Chromatographic Condition:**

Column: Agilent eclipse C18 column (250 mm × 4.6 mm i. d., 5µm)

Mobile phase A (MP A): 0.1% Formic acid in distilled water Mobile Phase B (MP B): 100% Acetonitrile

Mode of Separation: Gradient method

Diluent: Water

Flow rate: 1.5 mL/min

Injection volume: 20µL

Wavelength: 340 nm (Piperine) and 420 nm (Curcumin)

Run time: 52 min

Temperature: 30°C

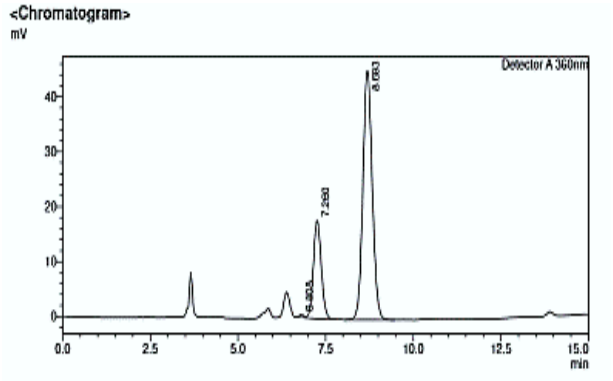



Figure 5 Chromatogram of curcumin and piperine using optimum chromatographic conditions

**Method Validation:**

Method validation parameters are summarized as follows:

| Parameters | Piperine | Curcumin |
|---|---|---|
| Linearity (µg/ml) | 100-300 | 10-50 |
| Accuracy (% Recovery) | 98.6 – 100.4 % | 99.2-100.2 % |
| LOD (µg/ ml) | 11.8 | 0.15 |
| LOQ (µg/ ml) | 35.9 | 0.46 |
| Precision (% RSD) | | |
| Repeatability | 1.35 | 1.95 |
| Intraday (n=3) | 1.24 - 1.74 | 1.11-1.37 |
| Interday (n=3) | 1.83-1.99 | 1.55-1.99 |
| Robustness (% RSD) | | |
| Change in flow rate | 1.34-2.06 | 1.66-1.8 |
| Change in wavelength | 1.50-1.76 | 1.6-1.72 |
| Change in temperature | 1.21-2.01 | 1.2-1.74 |

**SUMMARY AND CONCLUSION:**

7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-Curcumin is referred to by its chemistry name, dione. It is an herbal drug that acts as a hepatoprotective agent, an anti-mutagenic agent, an anti-carcinogenic agent, and an anti-bacterial agent. An easy, sensitive, and cost-effective RP-HPLC method with increased system compatibility factors has been developed specifically for the purpose of determining the amount of curcumin present in various formulations. Estimations of formulations including many herbal combinations may be made with the use of this method. Based on the results and parameters obtained from the experiments, it was concluded that the RP-HPLC technology that was developed offers the opportunities that are listed below:

- There is a reduction in the amount of time spent preparing the standard and the sample.
- The chromatographic method that was developed for curcumin was found to be simple, precise, accurate, and cost-effective.
- Additionally, it was found that this method could be effectively applied for routine analysis in research institutions, quality control departments in industries, approved testing laboratories, bio-pharmaceutics, and bioequivalence standards.

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