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# Validated RP-HPLC Method for the Quantitative Analysis of Fisetin in Pure Form and Mucoadhesive Microemulsion Formulations

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**Abstract**

**A robust and validated reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for the quantitative analysis of fisetin in both its pure form and when encapsulated in mucoadhesive microemulsion (MME) formulations. Chromatographic separation was achieved using a Phenomenex C18 column with a mobile phase comprising methanol and 0.1% orthophosphoric acid in a 55:45 (v/v) ratio. The isocratic method operated at a flow rate of 1.0 mL/min with detection at 362 nm. The retention time of standard fisetin was 7.48 minutes, and encapsulated fisetin eluted at 7.35 minutes, confirming its stability post-formulation. The method was validated according to ICH Q2(R1) guidelines and demonstrated excellent linearity ($R^2$ = 0.993) over the concentration range of 2–12 µg/mL. Precision studies yielded %RSD values below 2%, confirming reproducibility. The method also exhibited good accuracy (96–98% recovery), robustness, and system suitability, with low %RSD values for all tested parameters. The limit of detection and quantification were 0.425 µg/mL and 1.287 µg/mL, respectively. This validated method reliably quantified fisetin in the optimized MME formulation, making it suitable for routine analysis and formulation development.**

**Keywords: Fisetin, Reverse-Phase High-Performance Liquid Chromatography, Mucoadhesive Microemulsion, Analytical Method Validation, International Committee for Harmonization**

## 1. Introduction

Fisetin (3,3′,4′,7-tetrahydroxyflavone), a naturally occurring flavonoid, is abundantly distributed across a range of fruits and vegetables, with the highest concentrations reported in strawberries (160 µg/g), followed by apples (26.9 µg/g) and persimmons (10.5 µg/g). This bioactive molecule has attracted considerable scientific interest due to its multifaceted therapeutic potential. Extensive preclinical investigations have elucidated its neuroprotective and therapeutic efficacy in various models of neurodegenerative disorders, including Alzheimer's disease, vascular dementia, schizophrenia, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, stroke, depression, diabetic neuropathy, and traumatic brain injury. These findings underscore the potential of fisetin as a versatile molecule for addressing the pathophysiological complexity of neurological diseases**(Figure 1).**[1]

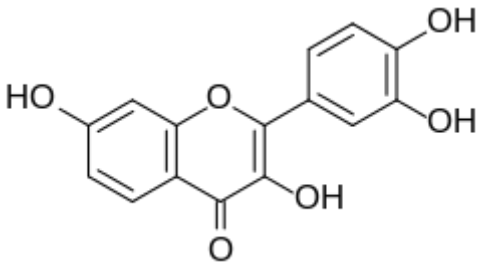


**Figure 1. Structure of Fisetin**

Fisetin, recognized as one of the most potent flavonoids, has demonstrated a broad spectrum of neuroprotective properties within the central nervous system. Notably, extensive studies have revealed its ability to facilitate hippocampal long-term potentiation (LTP), a key mechanism underlying synaptic plasticity, and to improve cognitive performance in both healthy rodents and animal models of cognitive dysfunction.[2]

Schizophrenia is a chronic and severe psychiatric disorder that profoundly impacts an individual's cognition, emotions, and behaviour. It is characterized by positive symptoms, including hallucinations and delusions; negative symptoms, such as diminished emotional expression and reduced participation in daily activities; and cognitive impairments affecting executive function and decision-making.[3] Globally, approximately 21–24 million individuals are affected by schizophrenia, with a higher prevalence among males (12 million) compared to females (9 million), as reported by the World Health Organization (WHO).[4] While schizophrenia is a treatable condition, therapeutic interventions are notably more effective during its early stages. Despite advancements in treatment strategies, over 50% of individuals with schizophrenia do not receive adequate care, highlighting a significant gap in the global management of this disorder.[5]
Schizophrenia is a neuropsychiatric disorder characterized by a disruption in thought processes and impaired emotional regulation. The blood-brain barrier (BBB), a highly specialized structure formed by brain endothelial cells connected via tight junctions (zonula occludens), serves as a critical defence mechanism, preventing the entry of potentially neurotoxic substances into the brain parenchyma. This stringent permeability barrier poses a significant challenge in the development of therapeutic strategies targeting the central nervous system (CNS), as it restricts the delivery of many pharmacological agents to the brain. Consequently, the BBB is a pivotal factor in advancing CNS-targeted drug delivery systems for effective treatment of disorders like schizophrenia.[6,7,8]

Central nervous system (CNS)-related disorders present substantial challenges for the development of effective drug delivery systems, primarily due to the restrictive nature of the blood-brain barrier (BBB). This highly selective barrier prevents the majority of therapeutic agents from accessing the brain, with approximately 98% of small molecules being unable to traverse the BBB owing to unfavourable molecular or physicochemical properties.[9,10]

To address the challenges associated with drug transport across the blood-brain barrier (BBB), various alternative strategies have been explored, including the utilization of non-conventional administration routes such as intranasal and olfactory delivery. Additionally, the development of advanced drug delivery systems, such as nanocarriers, has emerged as a promising approach to enhance the permeability and targeted delivery of therapeutic agents to the central nervous system (CNS).[11]

Microemulsions are thermodynamically stable, isotropic colloidal dispersions consisting of an oil phase, aqueous phase, surfactant, and co-surfactant in appropriate ratios, characterized by droplet sizes typically below 0.15 μm.[12] Due to their lipophilic nature and small globule size, microemulsions have been extensively investigated as a promising drug delivery system to enhance the permeation of therapeutic agents across the nasal mucosa. Furthermore, the incorporation of mucoadhesive agents, such as polyelectrolyte polymers, significantly improves the retention time of the formulation on the nasal mucosa, thereby facilitating prolonged drug absorption and enhanced therapeutic efficacy.[13]

The incorporation of mucoadhesive polymers into microemulsions extends the contact time of the formulation with the nasal mucosa, thereby enhancing the absorption and bioavailability of the active pharmaceutical ingredient. Consequently, mucoadhesive microemulsions (MMEs) administered via the nasal route represent a promising strategy for brain-targeted drug delivery, offering the potential to improve therapeutic efficacy while minimizing dose-dependent side effects.[14]

The development of a robust high-performance liquid chromatography (HPLC) method for fisetin poses challenges due to its limited aqueous solubility, necessitating the incorporation of organic modifiers into the mobile phase. Achieving effective separation of fisetin from other flavonoids and plant-derived matrix components requires careful selection of stationary and mobile phase conditions to ensure specificity. Additionally, optimizing method sensitivity through adjustments in injection volume and detector selection is critical, particularly when employing UV detection, to achieve the desired detection limits.

This study employed a Quality by Design (QbD) approach to systematically optimize the chromatographic method for fisetin, ensuring regulatory compliance and adaptability to evolving industry demands. Using design of experiment (DoE) principles, key chromatographic variables, including 0.1% orthophosphoric acid (OPA), methanol concentration, flow rate, and column oven temperature, were evaluated. Critical method attributes such as retention time, peak resolution, and theoretical plates were optimized using statistical tools, including three-dimensional response surface plots and overlay plots.

The finalized method was rigorously validated in accordance with ICH Q2(R1) guidelines, with assessments of robustness, accuracy, precision, limit of detection (LoD), and limit of quantitation (LoQ) confirming its reliability and consistency. The method demonstrated exceptional sensitivity, enabling the detection of trace levels of fisetin, and high selectivity, ensuring precise differentiation from other components. Its rapidity and cost-effectiveness further enhance its utility for routine pharmaceutical applications, particularly in the analysis of fisetin formulations such as Microemulsion. This comprehensive validation underscores the method's suitability for high-quality pharmaceutical analysis and its potential for widespread adoption in industry settings.[15]

## 2. Materials and Methods

Fisetin (3,3′,4′,7-tetrahydroxyflavone) was procured from Sigma-Aldrich (Mumbai, India). Various oils including Capmul MCM and Transcutol HP, were received as gift samples from Gattefosse Company (Mumbai, India). Tween 80 was purchased from Merck Pvt. Ltd. (Mumbai, India). All solvents and reagents used in high-performance liquid chromatography (HPLC) analysis were of HPLC grade and procured from Merck Pvt. Ltd. (Mumbai, India). Milli-Q water, employed throughout the experimental

procedures, was supplied by KLE College of Pharmacy (Belagavi, India). Unless otherwise specified, all other chemical and reagents used were of pharmaceutical or analytical grade standards.

**Chromatographic Instrumentation and Conditions**

The chromatographic analysis was conducted using a Shimadzu Agilent 1220 Infinity II LC system (LC-20AD, Japan), equipped with a quaternary pump with degasser (G7111A), an auto-injector (G7129A), and a photodiode array (PDA) detector (G7115A). Data acquisition and processing were performed using OpenLab CDS software. Separation and analysis were achieved using a Phenomenex Luna C18(2) analytical column (150 mm × 4.6 mm, 5 µm particle size), manufactured by Phenomenex Inc., USA, which is known for its precision and reliability in chromatographic applications[16].

The optimized mobile phase consisted of methanol and 0.1% orthophosphoric acid (OPA) in a ratio of 55:45 (v/v), delivered at a constant flow rate of 1.0 mL/min. The mobile phase was degassed prior to use using a 0.45 µm polyvinylidene fluoride (PVDF) filter membrane (Millex HV, Millipore, USA) and ultrasonic techniques to eliminate any entrapped gases. Samples were injected in a fixed volume of 10 µL, and chromatographic detection was performed at a wavelength of 362 nm, selected to ensure optimal sensitivity for fisetin. The column oven was maintained at 35°C to ensure consistent retention and peak resolution.This instrumental setup provided high specificity and precision, enabling the accurate quantification and reliable identification of fisetin in the analyzed samples.

**Preparation of Stock and Working Standard Solutions**

A primary stock solution of fisetin was prepared at a concentration of 1 mg/mL using methanol as the solvent. Working standard solutions were subsequently prepared by serial dilution of the stock solution with the mobile phase to achieve concentrations ranging from 0.05 to 0.4 µg/mL. All prepared solutions were stored in tightly sealed volumetric flasks at room temperature to maintain stability prior to high-performance liquid chromatography (HPLC) analysis.

**Preliminary Method Development Studies**

Fisetin's low solubility in aqueous media necessitated the use of organic solvents, with methanol identified as the optimal solvent for complete dissolution. Methanol was initially selected as the primary component of the mobile phase for chromatographic analysis. However, early attempts using methanol-water combinations as diluents failed to produce discernible peaks within a 30-minute chromatographic run.

Subsequently, water was replaced with 0.1% orthophosphoric acid (OPA) to enhance peak resolution and retention. Various methanol:0.1% OPA ratios, including 48:52, 50:50, 52:48, and 55:45 (v/v), were evaluated, resulting in observable peaks with improved chromatographic performance. To optimize separation and peak characteristics, a systematic Quality by Design (QbD) approach was employed, incorporating design of experiments (DoE) methodologies. This robust optimization process ensured reliable and reproducible analytical results, facilitating the development of a validated method for fisetin analysis.

**Preparation of Microemulsions**

Microemulsions were prepared using the water titration method. An accurately weighed quantity of the drug was first dissolved in precise amounts of oil phase and surfactant-co-surfactant mixture (Smix) under magnetic stirring. To form a transparent microemulsion, distilled water was added dropwise to the oil-Smix-drug mixture. The resulting system was then subjected to ultrasonic treatment for 10 minutes at a frequency of 20 kHz using an ultrasonic bath to ensure uniform dispersion and the formation of a stable microemulsion[17].

**Method validation**

According to the criteria of the ICH standards, the method validation was carried-out[17, 18].

**Linearity**

To assess the method's linearity, Fisetin solutions were prepared at concentrations ranging from 0.05 to 0.4% µg/mL. Each concentration was prepared in triplicate. The calibration curve was constructed by plotting the peak area against the respective concentrations for each compound. Linearity testing was conducted over a period of three days, with results consistently obtainedacross the defined concentration ranges, confirming the method’s reliability for accurate quantification.

**Sensitivity**

The limits of detection (LOD) and quantification (LOQ) were determined based on the calibration curve, following standard procedures. LOD was calculated as the concentration corresponding to a signal-to-noise ratio of 3:1, while LOQ was defined as the concentration yielding a signal-to-noise ratio of 10:1. These values were derived to assess the method’s sensitivity and its ability to reliably detect and quantify trace amounts of the analytes.

**Precision**

Precision was evaluated in two categories: intra-day (repeatability) and inter-day (intermediate precision).

**Intra-day (Repeatability):** The standard solutions of both phytoconstituents were analyzed in triplicate at concentrations of 0.05 µg/mL, 0.2 µg/mL, and 0.4 µg/mL for fisetin, with measurements performed three times within the same day.

**Inter-day (Intermediate Precision):** For inter-day precision, three replicates of each phytoconstituent standard solution were analyzed over three consecutive days at the same concentrations (0.05 µg/mL, 0.2 µg/mL, and 0.4 µg/mL) for fisetin.

Both intra-day and inter-day precision were assessed to ensure the method’s consistency and reliability across different time periods.

**System Suitability**

System suitability was assessed by evaluating the peak area for fisetin, based on six independent measurements taken during the chromatographic analysis. The repeatability of the peak area across these

readings was used to verify the suitability and performance of the chromatographic system, ensuring that it met the required criteria for accuracy, precision, and reliability in quantitative analysis.

**Robustness**

The robustness of the method was evaluated by introducing small deliberate variations in key chromatographic parameters. For fisetin analysis, the following adjustments were tested: wavelength (360 nm and 364 nm), flow rate (0.9 mL/min and 1.1 mL/min), and mobile phase ratio (43:57 and 47:53, methanol:0.1% OPA). These variations were designed to assess the method's reliability and performance under slight changes in conditions, ensuring consistent results and confirming its robustness for routine application.

**Accuracy**

Accuracy was assessed through recovery experiments, where the mean percentage recovery of fisetin was determined at three distinct concentrations: 50%, 100%, and 150% of the target concentration. The samples were analyzed in triplicate, and the percentage recoveries were calculated to evaluate the method's accuracy and its ability to consistently quantify fisetin in the presence of potential interferences. The results were reported as the mean recovery values, providing a measure of the method's reliability in real-world conditions.

## 3. Results and Discussion

**HPLC Analysis**

A validated reverse-phase HPLC method was used to quantitatively analyze fisetin in both its pure form and when encapsulated in MMEs (Mixed Micelle Emulsions). Chromatographic separation was performed using a Phenomenex C18 column (250 × 4.6 mm, 5 µm particle size). The mobile phase consisted of methanol and 0.1% orthophosphoric acid in water in a 55:45 volume ratio, delivered isocratically at a flow rate of 1.0 mL/min. Detection was carried out at an optimized wavelength of 362 nm. The retention time (Rt) for standard fisetin was consistently found to be 7.48 minutes. Chromatograms of fisetin-loaded MME formulations showed clearly defined peaks at approximately the same retention time (7.35 minutes), indicating that fisetin remained chemically stable after encapsulation**(Figure 2)**. The close match in retention times between the standard and encapsulated samples demonstrates both the specificity of the method and the chemical integrity of fisetin within the formulation. To ensure the method’s reliability for routine use, validation was performed according to the International Council for Harmonisation (ICH) Q2(R1) guidelines. The method was assessed for linearity, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ). All parameters met the ICH acceptance criteria, confirming the method's suitability for the accurate and consistent quantification of fisetin in MME formulations.

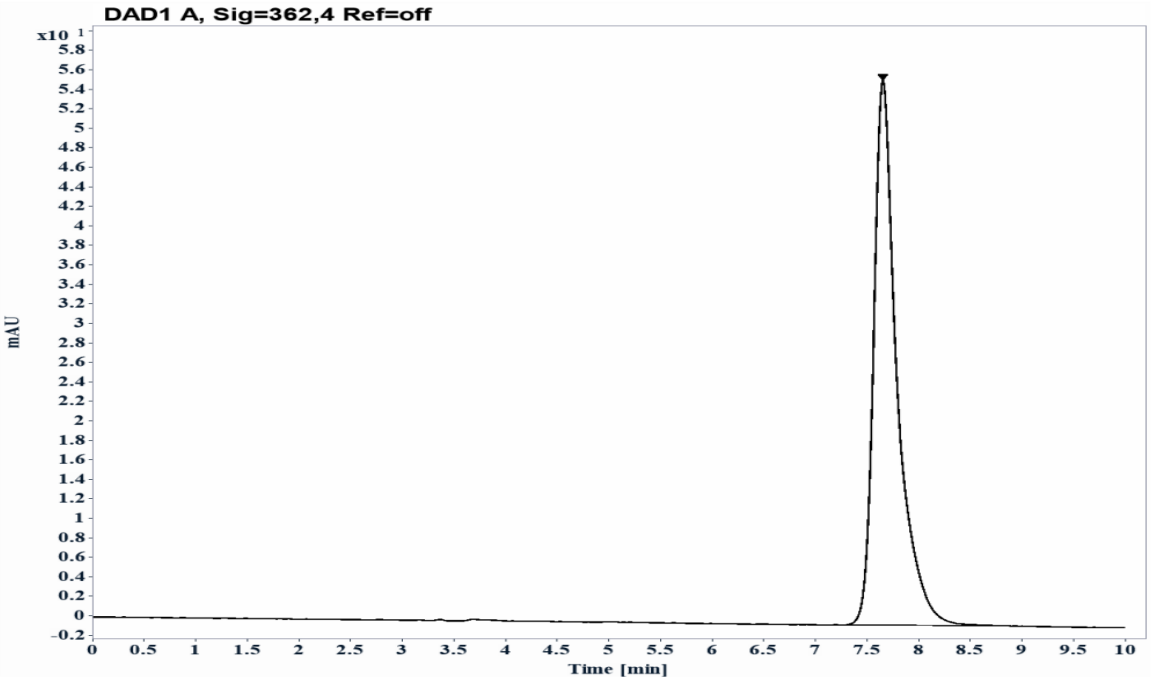


**Figure 2. HPLC optimized chromatograms of Fisetin pure drug**

**Method validation**

**Linearity**

At concentrations between 2 µg/mL and 12 µg/mL, the peak areas ranged from 335.06 to 2236.27. The resulting calibration equation was y = 187.99x - 33.748, with a correlation coefficient ($R^2$) of 0.993, as shown in the calibration curve. These results indicate that peak areas increase with concentration, suggesting a strong correlation between the two**(Table 1 and Figure 3).**

| **Concentration(µg/ml)** | **Area** |
|---|---|
| 0 | 0 |
| 2 | 335.06 |
| 4 | 681.83 |
| 6 | 1091.8 |
| 8 | 1458.06 |
| 10 | 1856.13 |
| 12 | 2236.27 |

**Table 1. Linearity of Fisetin**

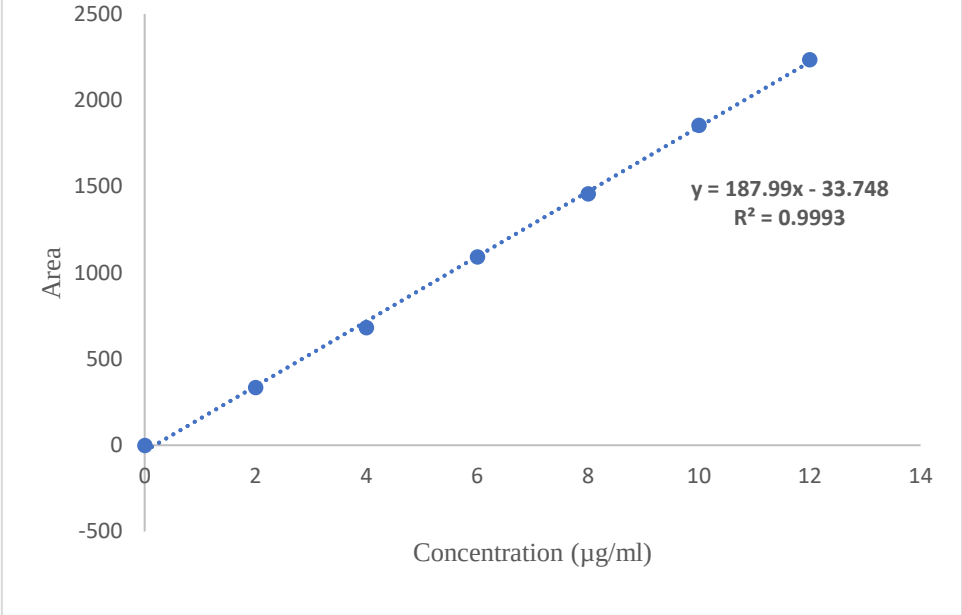


**Figure 3. Standard Calibration curve of Fisetin**

**Precision**

The precision of the HPLC method was thoroughly evaluated through interday and intraday measurements at three different Fisetin concentrations (2 µg/mL, 6 µg/mL, and 12 µg/mL). The %RSD values obtained were all less than 2%, confirming the excellent precision of the developed method**(Table 2).**

| Concentration (µg/mL) | Intraday (%RSD) | Interday(%RSD) |
|---|---|---|
| 2 | 0.47 | 0.95 |
| 6 | 0.38 | 0.97 |
| 12 | 0.41 | 0.92 |

**Table 2. Precision results of Fisetin**

**Robustness**

The robustness of the HPLC method was assessed by examining its performance under slight variations in flow rate, wavelength, and mobile phase ratio at drug concentrations of 6 µg/mL. The results were consistent, with all %RSD values remaining below 2%. This confirms that the method can reliably deliver accurate measurements even when minor operational changes are introduced**(Table 3).**

| Robustness | % RSD |
|---|---|

| | |
|---|---|
| Flowrate (mL/min) | 0.81 |
| Mobilephaseratio (v/v) | 1.06 |
| wavelength(nm) | 0.91 |

**Table 3.Robustness results of Fisetin**

**System Suitability**

System suitability was evaluated using the medium concentration (6 µg/mL) analyzed in six replicate injections. The obtained data showed that the %RSD values were within the acceptable limits**(Table 4).**

| **Validation parameter** | **%RSD** |
|---|---|
| Peak area (%RSD) | 0.22 |
| Retention time (%RSD) | 0.42 |
| Theoretical plates (%RSD) | 0.99 |
| Tailing factor (%RSD) | 1.09 |

**Table 4.Results of System suitability**

**LOD and LOQ**

The LOD and LOQ were found to be 0.425 µg/mL and 1.287 µg/mL respectively, at 362 nm, demonstrating that the developed HPLC method is sensitive for detectingFisetin concentrations.These results confirm the method's ability to accurately analyze samples containing very low levels of Fisetin.

**Accuracy**

**Table 5** shows percentage recovery values ranging from 97% to 100%, confirming the method's accuracy in quantifying the analyte.

| **Concentration (µg/mL)** | **Level(%)** | **Recovery(%)** |
|---|---|---|
| 6 | 50 | 96.62±0.26 |
| 6 | 100 | 97.54±0.52 |
| 6 | 150 | 98.37±0.85 |

**Table 5. Accuracy results of Fisetin**

**Quantification of Fisetin in the Optimized Mucoadhesive Microemulsion Formulation**

The amount of fisetin in the optimized mucoadhesive microemulsion (MME) formulation was determined using the developed HPLC method. A sample containing 10 mg of the optimized MME was diluted with 10 mL of methanol and analyzed by HPLC at 362 nm**(Figure 4)**. The resulting chromatogram was compared with that of the standard pure Fisetin. The fisetin in the MME showed a retention time similar to that of the standard pure Fisetin, confirming its presence.Thus, the HPLC analysis provided strong analytical evidence for the presence and identification of the loaded drug.

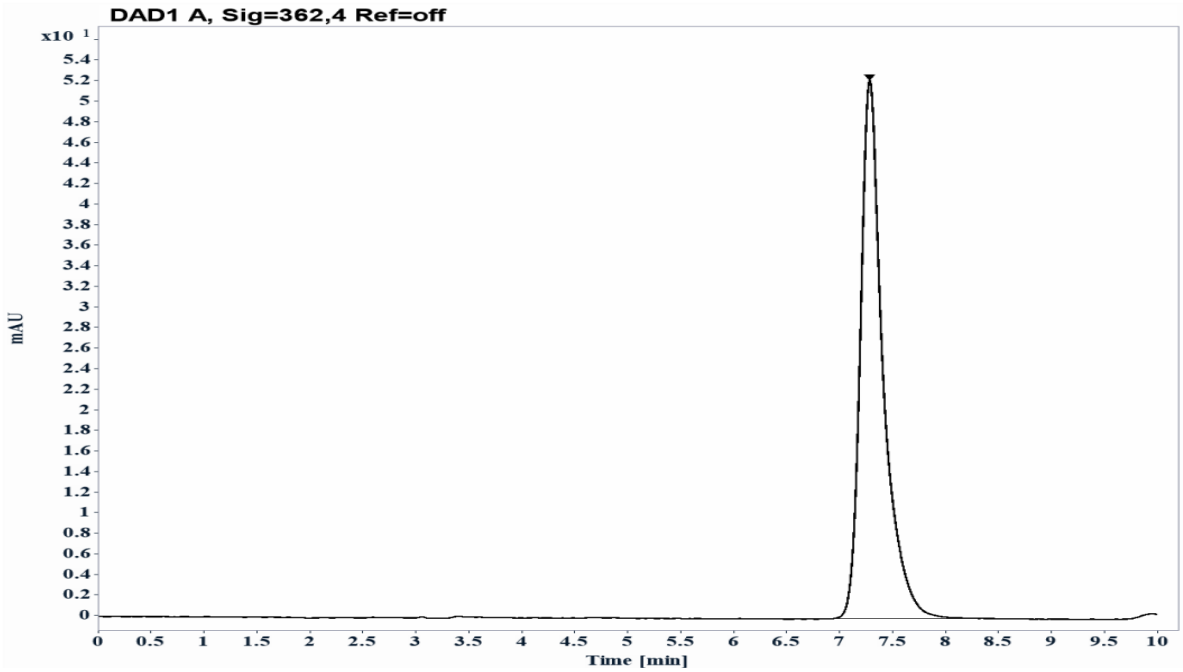


**Figure 4. HPLC optimized chromatograms of Fisetin-loaded Mucoadhesive Microemulsion**

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