# DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR QUANTIFYING ISOFLAVONES

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## ABSTRACT

Many plant compounds that function as phytoestrogens in mammals are isoflavones, derivatives of the naturally occurring isoflavone. Beans and other members of the Fabaceae family are the only plants that naturally produce isoflavones (Leguminosae). There is little evidence for the safety of long-term supplementation with isoflavones or closely related phytoestrogens, despite the fact that these compounds are sold as dietary supplements and may have health benefits. There may be dangers associated with taking in too many isoflavones, according to some research. For example, there is a lingering fear that breast cancer may be more common in women with a family history of the disease, but good clinical studies have not confirmed this. The study's overarching goal was to establish a protocol for the validation of Isoflavones analysis, with a focus on High Performance Liquid Chromatography (HPLC). Separation was achieved using reversed-phase high-performance liquid chromatography (RP-HPLC) with an endcapped Waters symmetry C18 column (250 4.6 mm, 5 µm). Acetonitrile and 0.1% acetic acid made up the binary mobile phase (55:45). The injection volume was 25µl, the run time was 25 minutes, and the flow rate was 1.0 ml/min in an isocratic programme. At 254 nm, analytes were detected with a Photo-diode array (PDA). System suitability and selectivity parameters were shown to be within acceptable ranges, validating the developed method.

**Keywords:** Isoflavones, HPLC, Method development, Method Validation.

## INTRODUCTION

Isoflavone is a type of flavone that has a phenyl group added to the 2-position of the chromone. The phenyl group in isoflavone is in the 4th spot. Substituted isoflavone derivatives are made by replacing two or three hydrogen atoms with hydroxyl groups on the parent isoflavone[1].

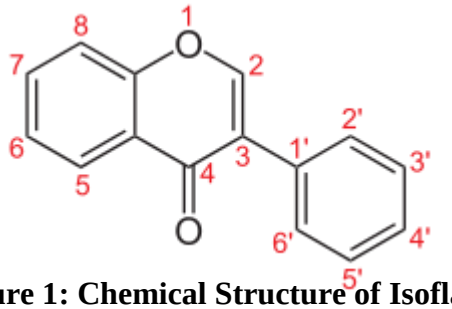


**Figure 1: Chemical Structure of Isoflavone**

"Isoflavone, numbering. Genistein (5-OH, 7-OH, 4'-OH) or daidzein (7-OH, 4'-OH) are e. g. members of the isoflavone family. Isoflavone differs from flavone (2-phenyl-4H-1-benzopyr-4-one) in location of the phenyl group".[1]

Genistein and daidzein[2] are the main isoflavones in soy. The amino acid phenylalanine starts the phenylpropanoid pathway. The intermediate naringenin is turned into the isoflavone genistein by two enzymes that are only found in legumes: an isoflavone synthase and a dehydratase. In the same way, the enzymes chalcone reductase[3], type II chalcone isomerase, and isoflavone synthase work on the intermediate naringenin chalcone to turn it into the isoflavone daidzein.

Isoflavones and related chemicals serve as phytoalexins, which plants use to guard against harmful fungus and other microorganisms that cause disease[4]. Moreover, soybeans use isoflavones to encourage nitrogen-fixing root nodules to grow in the soil bacterium rhizobium[5]. The isoflavone pathway is a subset of the larger phenyl propanoid system responsible for the biosynthesis of flavonoid chemicals in higher plants. For humans, isoflavones are typically obtained through soybeans.[6]

The objective of the study is to develop and validate RP-HPLC analytical method for quantifying Isoflavones. In these current study validation parameters like System suitability, Specificity and Linearity were performed[7].

## MATERIALS & METHOD

"Materials The standard chemicals of Isoflavones were purchased from Sigma (Aldrich, USA). The HPLC grade solvents, water for chromatography (LC-MS grade), methanol and acetonitrile chromatography grade were obtained from E. Merck KgaA (Darmstadt, Germany), acetic acid were also bought from Merck KgaA (Darmstadt, Germany)".

### Preparation of Standard solution

Quantitative analysis of Isoflavones standard was performed. Weighed and transfer each of 25 mg of Isofalvones working standard into a 50 mL volumetric flak, added premix diluent of 80:20(V/V)DMSO:Water diluent, Sonicated for 5minutes to dissolve and dilute up to mark with diluent.

Transfer accurately 2 mL of each standard stock solution into a 25 mL volumetric flask, and dilute to volume with diluent

### HPLC Instrumentation

"All solutions of samples were subjected to RP-HPLC measurement using the condition as follows Waters symmetry C18 column (250 × 4.6 mm, 5µm) was used for the separation. The binary mobile phase consisted of Acetonitrile and 0.1% acetic acid (55:45). An isocratic program was used with a flow rate at 1.0 ml/min, Run time 25mins and the injection volume was 25µl. The analytes were detected by using Photo-diode array (PDA) at 254 nm".

**Validation of the analytical method**

The established procedure was validated utilising ICH guideline-recommended parameters. Specificity, System Suitability, and Linearity were all checked and found to be within acceptable ranges before the procedure was approved.

**Specificity**[7]

Method specificity refers to an analytical technique's propensity to reliably and accurately measure the target analyte despite the presence of confounding factors in a given sample. Separation from, or resolution (Rs) from, other compounds is used to identify the desired ones.

**Linearity**[7]

Daidzin, Glycitin, Genistin, Daidzein, Glycitein, Genistein (500 µg/ml) were used to create a standard stock solution for the linearity test. The HPLC apparatus was fed six different concentrations. For each, we obtained the linear regression equation and the corresponding correlation coefficients. Analytical linearity refers to the straight-line relationship between the response and the analyte concentrations on the calibration curve.

**System suitability criteria**[7]

Isoflavones standard solutions were injected at a concentration of 40 µg/ml, with six time replicates, to determine the system's suitability.

## RESULTS AND DISCUSSION

**HPLC Condition Optimization**

HPLC with a PDA detector was used to analyse isoflavones quantitatively. The PDA detector can generate multiple chromatograms at various wavelengths all at once. UV spectra were recorded online at 200–400 nm using a PDA detector for peak identification. The injection volume was 25 µl, and the PDA detection was carried out at 254 nm. Retention time (Rt) and resolution were affected by the composition of the mobile phase and the flow rate used in the analysis. We first determined the optimum concentration of isoflavones in a standard solution (40µg/ml) and then analysed the chromatographic parameters. Varying amounts of acetonitrile or methanol were mixed with the aqueous solvents to calculate the resolution, tailing factor, and N plate. It was determined what would happen if different concentrations of these solvents were used.

**System Suitability & Specificity:**

The intended compounds are determined by calculating their separation or resolution (Rs) from other compounds.

**Table 1: System Suitability & Specificity Results**

| Isoflavone | Retention Time | Resolution | % RSD | USP Tailing | USP Plate count |
|---|---|---|---|---|---|
| Daidzin | 4.56 | NA | 0.37 | 1.16 | 3791 |
| Glycitin | 6.52 | 1.75 | 0.80 | 1.19 | 6198 |
| Genistin | 7.81 | 2.68 | 0.95 | 1.32 | 6524 |

| | | | | | |
|---|---|---|---|---|---|
| Daidzein | 9.79 | 5.41 | 0.66 | 1.15 | 6258 |
| Glycitein | 15.43 | 6.54 | 1.12 | 1.22 | 7198 |
| Genistein | 19.52 | 7.69 | 1.25 | 1.14 | 7518 |

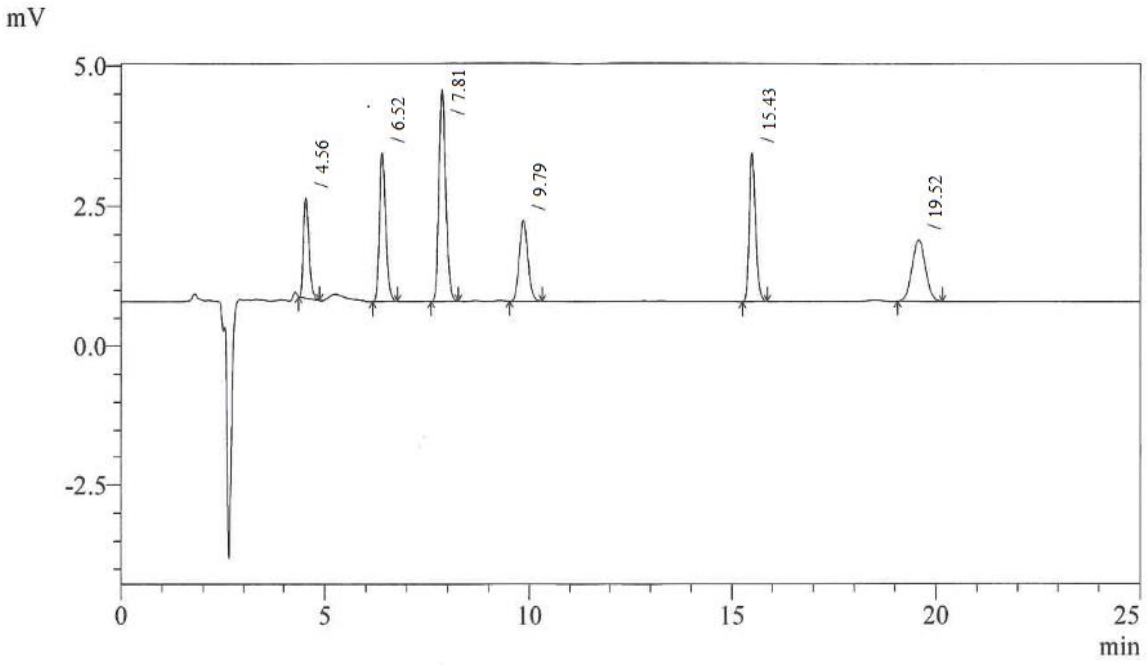


**Figure 2: System suitability & Specificity for Isoflavones**

**Conclusion: "**The results obtained showed that the conditions used for the determination of the levels of Isoflavones standard had good system suitability based on retention time, peak area and peak height requirements, % RSD ≤ 2%"

**Linearity**

Analytical linearity refers to the straight-line relationship between the response and the analyte concentrations on the calibration curve. The Correlation coefficient should be not less than 0.99. The above results reveal that the method is Linear showed in Table 2-7 & Figure 3-8.

**Table 2: Daidzin Linearity**

| | | **Daidzin Linearity** | | | | | |
|---|---|---|---|---|---|---|---|
| S.NO | %Level | **Standard Stock(mg)** | **Dil** | **Vol Taken** | **final Volume** | **Final Conc.** | **Area** |
| 1 | 25 | 25 | 50 | 2 | 100 | 10 | 195562 |
| 2 | 50 | 25 | 50 | 4 | 100 | 20 | 355543 |
| 3 | 75 | 25 | 50 | 3 | 50 | 30 | 563879 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4 | 100 | 25 | 50 | 2 | 25 | 40 | 745852 |
| 5 | 125 | 25 | 50 | 5 | 50 | 50 | 912880 |
| 6 | 150 | 25 | 50 | 3 | 25 | 60 | 1109426 |
| | | | | | | Intercept | 4859.93 |
| | | | | | | Slope | 18352.30 |
| | | | | | | Correlation | 1.000 |
| | | | | | | %Y-Intercept | 0.532 |

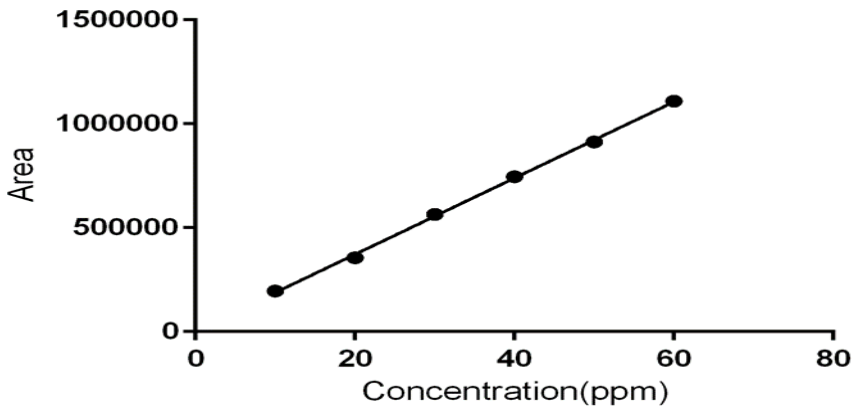


**Figure 3: Linearity for Daidzin**

**Table 3: Glycitin Linearity**

| | | **Glycitin Linearity** | | | | | |
|---|---|---|---|---|---|---|---|
| S.NO | %Level | **Standard Stock (mg)** | **Dil** | **Vol Taken** | **final Volume** | **Final Conc** | **Area** |
| 1 | 25 | 100 | 50 | 2 | 100 | 40 | 1891002 |
| 2 | 50 | 100 | 50 | 4 | 100 | 80 | 3826522 |
| 3 | 75 | 100 | 50 | 3 | 50 | 120 | 5541192 |
| 4 | 100 | 100 | 50 | 4 | 50 | 160 | 7543025 |
| 5 | 125 | 100 | 50 | 5 | 50 | 200 | 9199241 |
| 6 | 150 | 100 | 50 | 3 | 25 | 240 | 11265022 |
| | | | | | | Intercept | 45325.00 |
| | | | | | | Slope | 46421.49 |
| | | | | | | Correlation | 1.000 |
| | | | | | | %Y-Intercept | 0.493 |

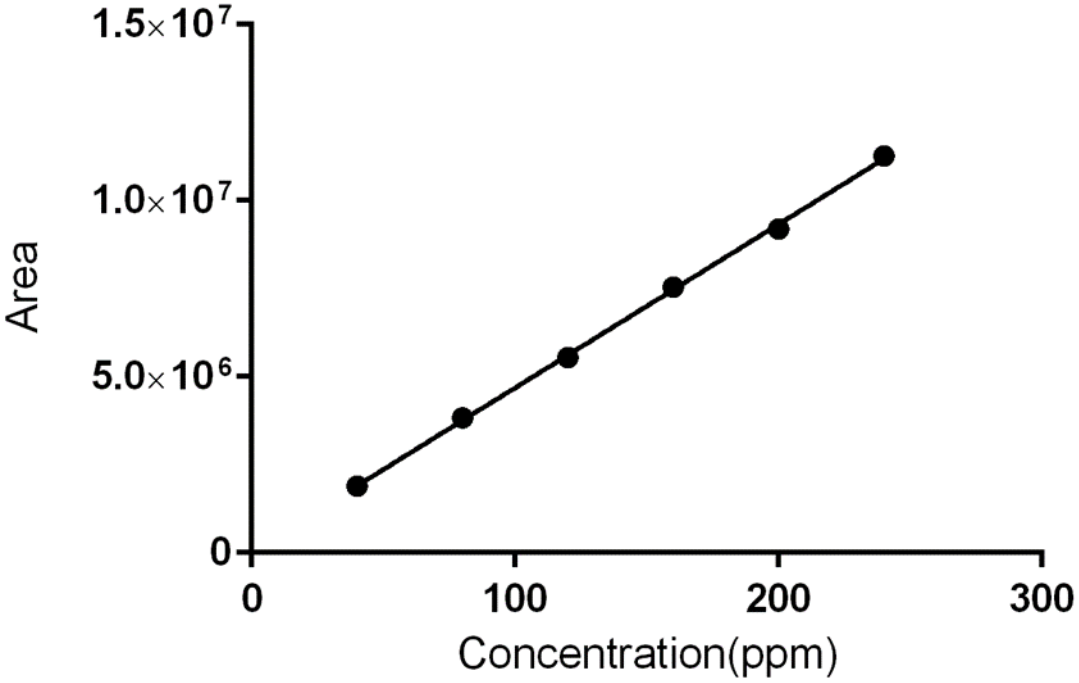


**Figure 4: Linearity for Glycitin**

**Table 4: Genistin Linearity**

| | | Genistin Linearity | | | | | |
|---|---|---|---|---|---|---|---|
| S.NO | %Level | **Standard Stock (mg)** | **Dil** | **Vol Taken** | **final Volume** | **Final Conc** | **Area** |
| 1 | 25 | 100 | 50 | 2 | 100 | 40 | 199100 |
| 2 | 50 | 100 | 50 | 4 | 100 | 80 | 375652 |
| 3 | 75 | 100 | 50 | 3 | 50 | 120 | 564119 |
| 4 | 100 | 100 | 50 | 4 | 50 | 160 | 764802 |
| 5 | 125 | 100 | 50 | 5 | 50 | 200 | 958924 |
| 6 | 150 | 100 | 50 | 3 | 25 | 240 | 1216502 |
| | | | | | | Intercept | -23901.07 |
| | | | | | | Slope | 5026.79 |
| | | | | | | Correlation | 0.998 |
| | | | | | | %Y-Intercept | -2.492 |

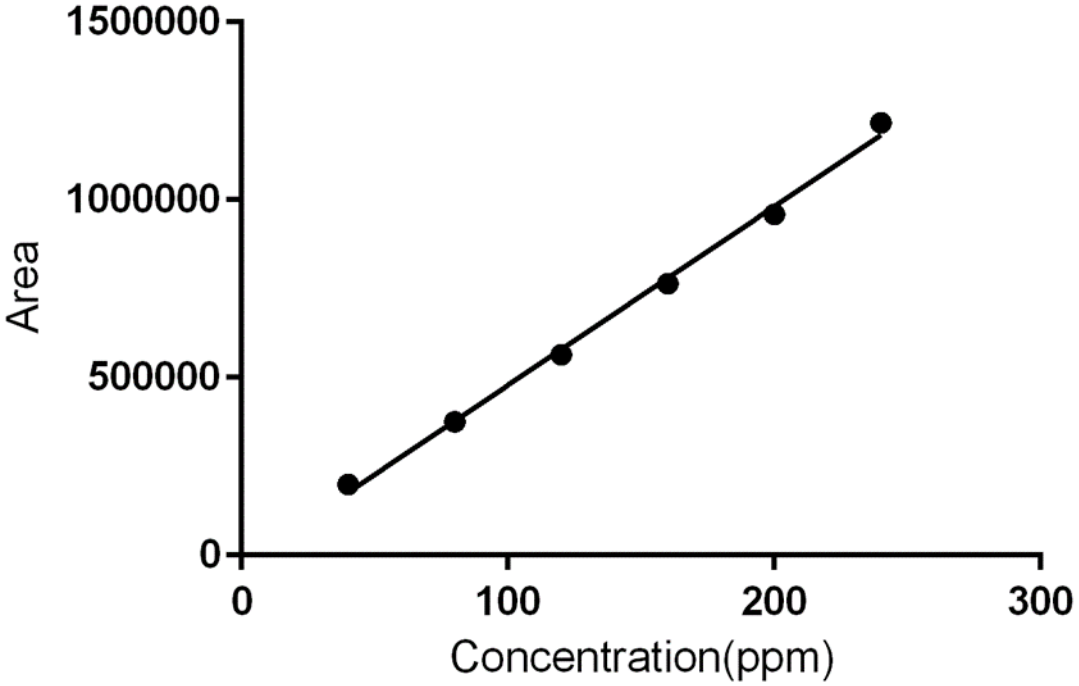


**Figure 5: Linearity for Genistin**

**Table 5: Daidzein Linearity**

| | | Daidzein Linearity | | | | | |
|---|---|---|---|---|---|---|---|
| S.NO | %Level | **Standard Stock (mg)** | **Dil** | **Vol Taken** | **final Volume** | **Final Conc** | **Area** |
| 1 | 25 | 100 | 50 | 2 | 100 | 40 | 189101 |
| 2 | 50 | 100 | 50 | 4 | 100 | 80 | 376652 |
| 3 | 75 | 100 | 50 | 3 | 50 | 120 | 564119 |
| 4 | 100 | 100 | 50 | 4 | 50 | 160 | 758551 |
| 5 | 125 | 100 | 50 | 5 | 50 | 200 | 988924 |
| 6 | 150 | 100 | 50 | 3 | 25 | 240 | 1226502 |
| | | | | | | Intercept | -37850.47 |
| | | | | | | Slope | 5155.90 |
| | | | | | | Correlation | 0.998 |
| | | | | | | %Y-Intercept | -3.827 |

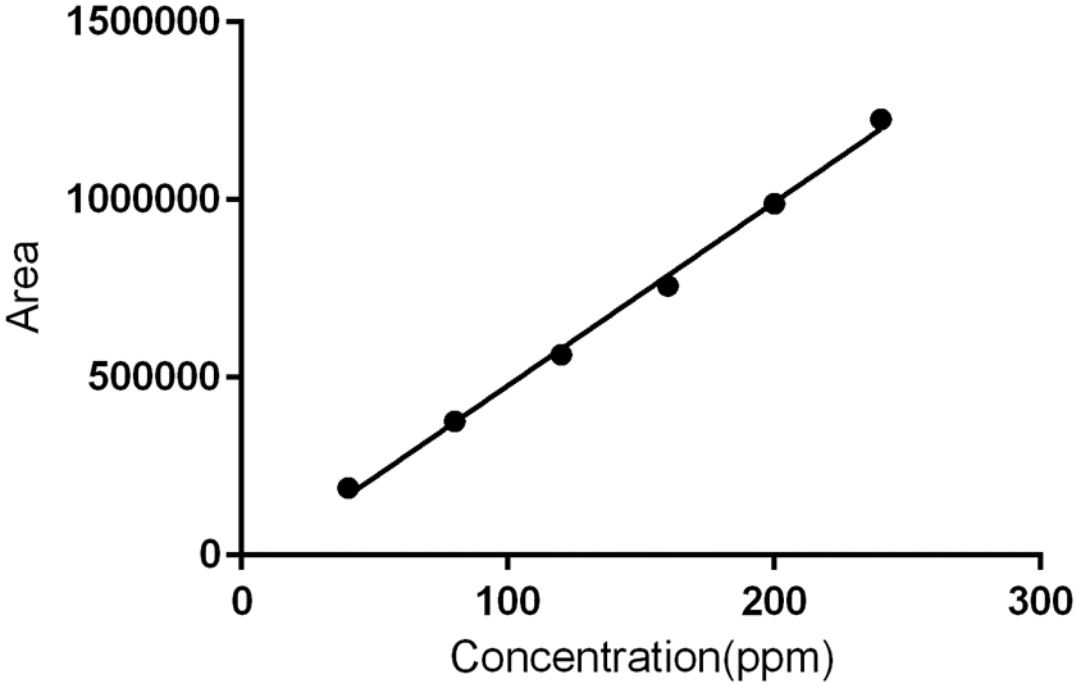


**Figure 6: Linearity for Daidzein**

**Table 6: Genisten Linearity**

| | | **Genisten Linearity** | | | | | |
|---|---|---|---|---|---|---|---|
| S.NO | %Level | **Standard Stock (mg)** | **Dil** | **Vol Taken** | **final Volume** | **Final Conc** | **Area** |
| 1 | 25 | 100 | 50 | 2 | 100 | 40 | 179101 |
| 2 | 50 | 100 | 50 | 4 | 100 | 80 | 358453 |
| 3 | 75 | 100 | 50 | 3 | 50 | 120 | 554518 |
| 4 | 100 | 100 | 50 | 4 | 50 | 160 | 815351 |
| 5 | 125 | 100 | 50 | 5 | 50 | 200 | 1008924 |
| 6 | 150 | 100 | 50 | 3 | 25 | 240 | 1286242 |
| | | | | | | Intercept | -74363.60 |
| | | | | | | Slope | 5534.25 |
| | | | | | | Correlation | 0.998 |
| | | | | | | %Y-Intercept | -7.371 |

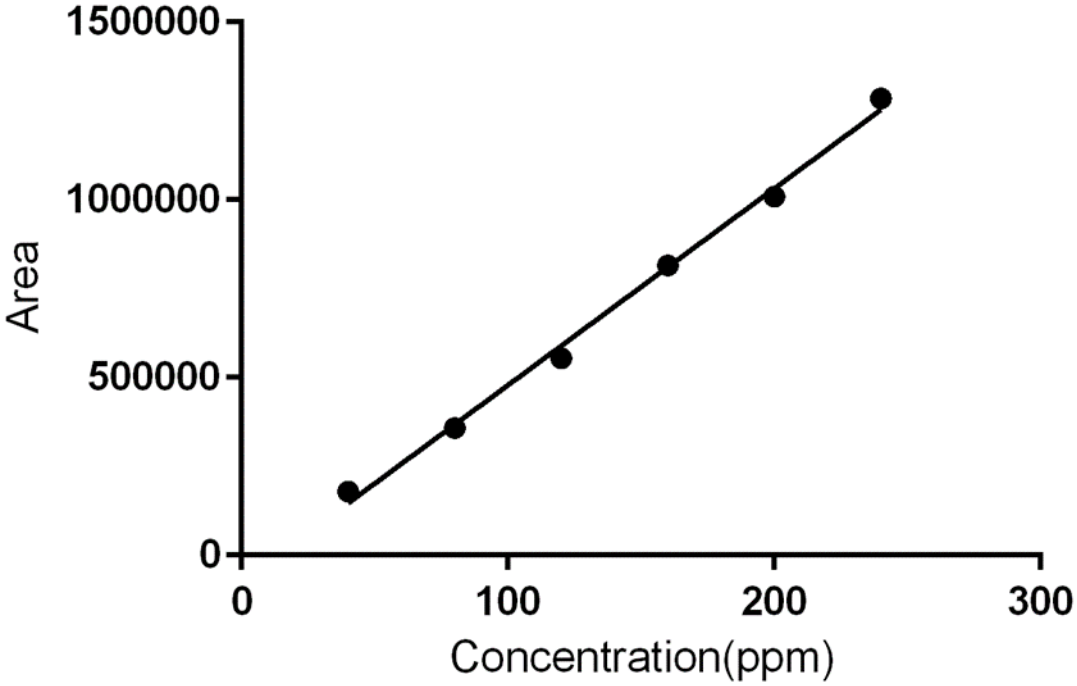


**Figure 7: Linearity for Genisten**

**Table 7: Glycitein Linearity**

| | | Glycitein Linearity | | | | | |
|---|---|---|---|---|---|---|---|
| S.NO | %Level | **Standard Stock (mg)** | **Dil** | **Vol Taken** | **final Volme** | **Final Conc** | **Area** |
| 1 | 25 | 100 | 50 | 2 | 100 | 40 | 209101 |
| 2 | 50 | 100 | 50 | 4 | 100 | 80 | 385652 |
| 3 | 75 | 100 | 50 | 3 | 50 | 120 | 575119 |
| 4 | 100 | 100 | 50 | 4 | 50 | 160 | 778541 |
| 5 | 125 | 100 | 50 | 5 | 50 | 200 | 999924 |
| 6 | 150 | 100 | 50 | 3 | 25 | 240 | 1116502 |
| | | | | | | Intercept | 19148.87 |
| | | | | | | Slope | 4702.32 |
| | | | | | | Correlation | 0.998 |
| | | | | | | %Y-Intercept | 1.915 |

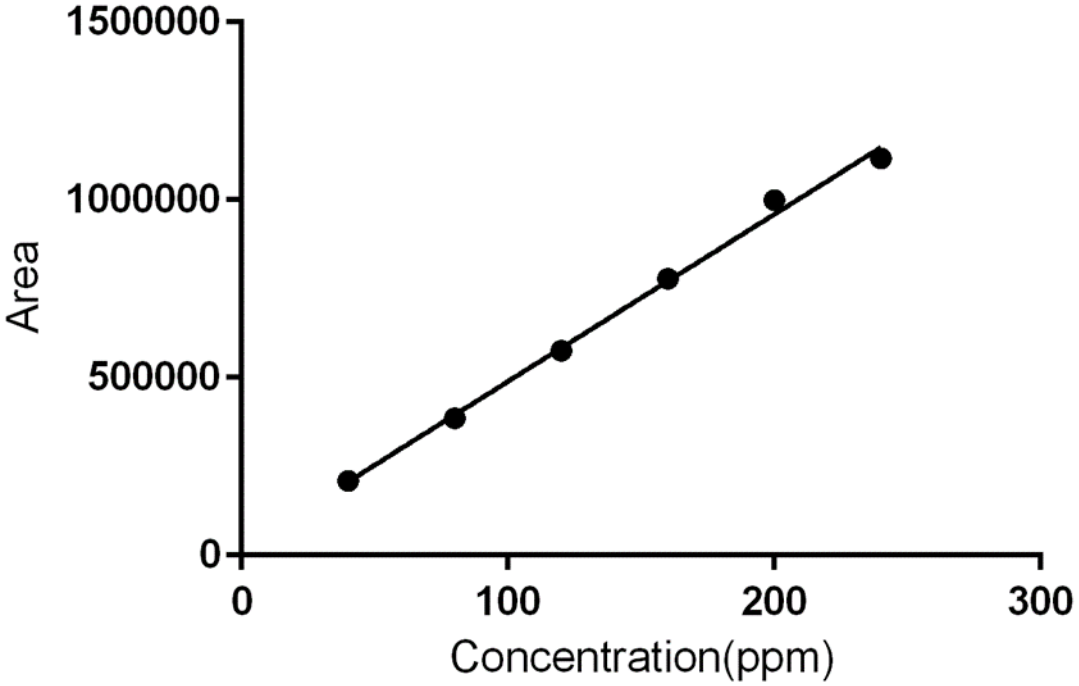


**Figure 8: Linearity for Glycitein**

## CONCLUSION

Although HPLC has found widespread use in the laboratory already. Using a cost-effective technique, we have developed the first HPLC approach for simultaneous measurement of Isoflavones. Although the analytes have different physicochemical properties, careful method development allowed for their measurement using a single method. The HPLC method in reversed phase mode provided a robust and well-validated approach to the determination of isoflavone concentrations. The developed technique is straightforward, and it could be used in regular quality assurance tests. The method is specific and Linearity for multiple drug dosage form.

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