	Standard Operating Procedure Determination of Isoflavones in Raw Materials and Finished Products by HPLC-UV	SOP Number D-1001	Revision 0
		Effective Date <i>01/17/22</i>	Page Page 1 of 8
Written by/ Date <i>C. P. 11-08-21</i>	Reviewed by/ Date <i>Jm 11/08/21</i>	Approved by/ Date <i>SS 11/08/21</i>	
Title: Analytical Development Scientist	Title: Analytical Development Manager	Title: QC Laboratory Director	

1.0 Purpose

This document describes the analytical procedure for the determination of Isoflavones in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of Isoflavones in raw materials and finished products. Total Isoflavones is calculated as the sum of Daidzein, Genistein, Formononetin and Biochanin A. This method was validated under protocol PRTCL-21-0049.

3.0 Responsibility

- 3.1 It is the responsibility of QC and Analytical chemists who have verified their ability to execute this procedure to follow this procedure.
- 3.2 It is the responsibility of the QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.
- 3.3 It is the responsibility of the QC Laboratory Management and AD Personnel to keep this procedure current with the associated monographs and laboratory practices.

4.0 Definitions

- 4.1 QC – Quality Control
- 4.2 AD – Analytical Development
- 4.3 ACN – Acetonitrile
- 4.4 DMSO – Dimethylsulfoxide
- 4.5 MeOH – Methanol
- 4.6 TFA – Trifluoroacetic Acid
- 4.7 ACS – American Chemical Society
- 4.8 HPLC – High Performance Liquid Chromatography
- 4.9 UV – Ultraviolet (Detection)

Standard Operating Procedure Determination of Isoflavones in Raw Materials and Finished Products by HPLC-UV	SOP No D-1001	Rev 0	Page 2 of 8
--	--------------------------	------------------	--------------------

5.0 References

- 5.1 PRTCL-21-0049, Protocol, Validation of an Analytical Method for the Determination of Isoflavones in Raw Materials & Finished Products by HPLC-UV
- 5.2 D-793, SOP, Cryogenic Grinding of Chewable Gels

6.0 Supplies

- 6.1 Chemicals – All reagents are ACS grade or better.
 - 6.1.1 Milli-Q Water
 - 6.1.2 ACN
 - 6.1.3 MeOH
 - 6.1.4 DMSO
 - 6.1.5 TFA
 - 6.1.6 Daidzein Reference Standard
 - 6.1.7 Genistein Reference Standard
 - 6.1.8 Formononetin Reference Standard
 - 6.1.9 Biochanin A Reference Standard
- 6.2 Supplies and Glassware
 - 6.2.1 HPLC vials, 12mm X 32mm with screw cap enclosures w/ septa
 - 6.2.2 Volumetric glassware and/or adjustable pipettes and tips
 - 6.2.3 Weigh paper and/or funnels
 - 6.2.4 Syringes with 0.45 μ Nylon Syringe Filters
- 6.3 Equipment
 - 6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV detector with a chromatographic data handling system
 - 6.3.2 Analytical and/or Top Loading Balance
 - 6.3.3 Analytical Micro Balance
 - 6.3.4 Sonicator Bath

7.0 Procedure

- 7.1 Mobile Phase & Diluent Preparation
 - 7.1.1 Mobile Phase

Standard Operating Procedure Determination of Isoflavones in Raw Materials and Finished Products by HPLC-UV	SOP No D-1001	Rev 0	Page 3 of 8
--	--------------------------------	------------------------	--------------------

7.1.1.1 Mobile Phase A: Combine 250 mL ACN + 750 mL Water + 500 µL of TFA and mix well.

7.1.1.2 Mobile Phase B: Combine 1000 mL ACN + 500 µL of TFA and mix well.

7.1.2 Extraction Solvent / Diluent

7.1.2.1 Combine equal volumes of MeOH and DMSO and mix well. Allow the resulting solution to equilibrate to room temperature prior to use.

7.1.3 Preparations may be scaled as necessary

7.2 Standard Prep

7.2.1 Solution A: Accurately weigh and transfer about 5 mg of Daidzein reference standard into a 100 mL volumetric flask. QS to volume with Diluent and sonicate until dissolved.

7.2.2 Solution B: Accurately weigh and transfer about 5 mg of Genistein reference standard into a 100 mL volumetric flask. QS to volume with Diluent and sonicate until dissolved.

7.2.3 Working Standard: Accurately weigh and transfer about 10 mg of Formononetin reference standard into a 50 mL volumetric flask with the aid of ~15 mL Diluent. Accurately weigh and transfer about 10 mg of Biochanin A reference standard into the same 50 mL volumetric flask with the aid of ~15 mL Diluent. Using glass volumetric pipets, transfer 5 mL of Solution A and 5 mL of Solution B into the 50 mL volumetric flask containing the Formononetin and Biochanin A. QS to volume with Diluent and sonicate until dissolved.

7.3 Sample Preparation

7.3.1 The validated linear range for the analytical method is 0.96034 – 4.80168 µg/mL Daidzein, 0.98529 – 4.92646 µg/mL Genistein, 41.81429 – 209.07144 µg/mL Formononetin and 42.46386 – 212.31930 µg/mL Biochanin A.

7.3.2 Extract sufficient sample (based on the raw material manufacturer assay value or finished product label claim) with Extraction Solvent in order to generate a concentration that is within the validated linear range.

7.3.3 When analyzing powders, fill the flask to 80% of the chosen volume with Extraction Solvent and sonicate for 10 minutes. Cool to ambient temperature then QS to volume. Filter a 5ml aliquot for analysis, discarding the first 3-4ml of filtrate.

7.3.4 When analyzing gummies, prepare the samples as per D-793 utilizing a 20 minute beaker stir time. QS to volume in the volumetric flask and add a 10 minute sonication. Filter a 5ml aliquot for analysis, discarding the first 3-4ml of filtrate.

7.3.5 For materials being analyzed for the first time using this method, an in process validation is required to demonstrate spectral purity and extraction efficiency as a part of system suitability before data can be reported using this method.

7.4 HPLC Parameters

7.4.1 Column: Supelco Ascentis Express 90Å C₈, 4.6 x 100mm, 2.7µm SPP (Or Equivalent)

7.4.2 Column Temperature: 45°C

7.4.3 Flow rate: 0.5 mL/min

7.4.4 Mobile Phase: Gradient

7.4.4.1	<u>Time, min</u>	<u>%B</u>
	0.00	0
	2.00	0
	20.00	60
	20.10	0
	25.00	0

7.4.5 Wavelength: 254 nm

7.4.6 Injection Volume: 5 µL

7.4.7 Run Time: 25 minutes

7.4.8 3-D Spectral Range (for Identification): 210nm - 350nm

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the Diluent.

7.5.2 Make at least five (5) injections of Working Standard.

7.5.3 Make a single injection of each Sample Preparation.

7.6 System Suitability Requirements

7.6.1 The %RSD of five (5) consecutive standard injections is NMT 2.0%

7.6.2 If present, any interference in the diluent should be subtracted out of the sample and standard peak areas.

7.7 Example calculations for determining % assay / label claim:

Standard Operating Procedure Determination of Isoflavones in Raw Materials and Finished Products by HPLC-UV	SOP No D-1001	Rev 0	Page 5 of 8
--	--------------------------------	------------------------	--------------------

$$7.7.1 \quad \% = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{SS}{SA} \times \frac{V_{spl}}{LA} \times 100$$

R_u Sample peak area

R_s Mean (n=5) standard peak area

Wt_{std} Weight of the reference standard

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount

SS Serving size: Average weight of ten dosage units or theoretical weight in mg for tablets / gummies, theoretical fill weight for capsules, theoretical mass of a single serving in mg for powders, volume of a single serving from the theoretical formula in ml for liquids, or use 1 for raw materials.

LA Label amount in mg of analyte (use 1 for raw materials)

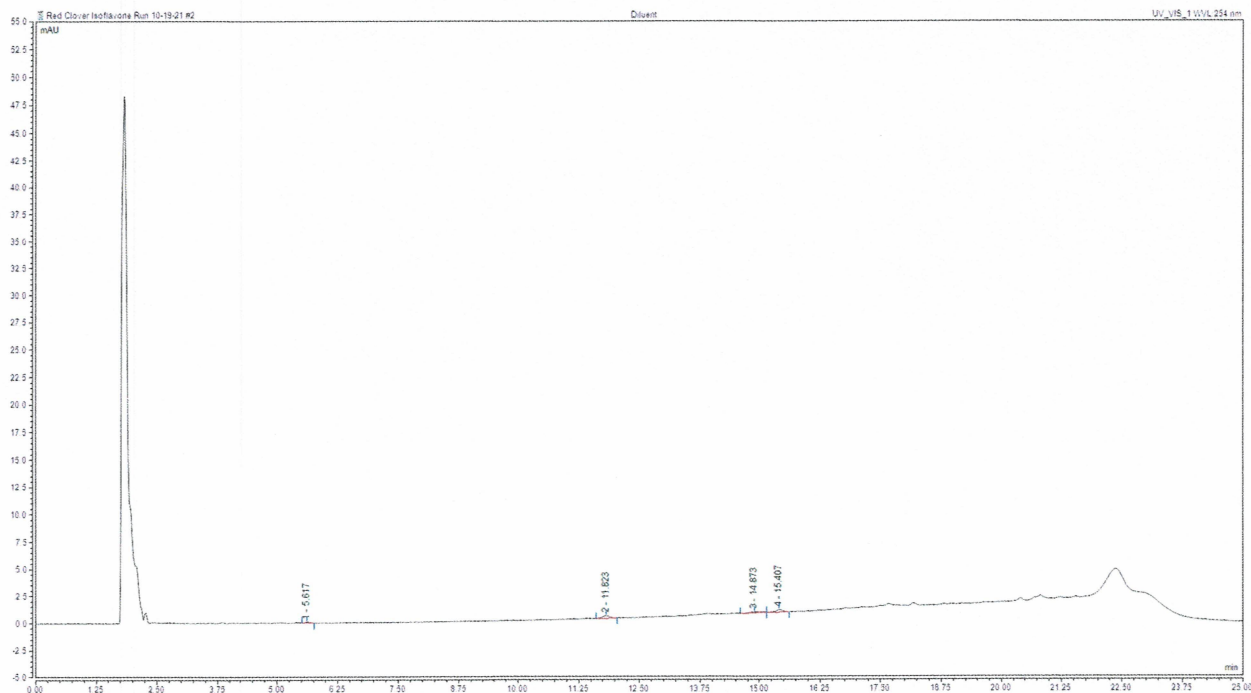
V_{spl} Volume of the sample preparation accounting for dilutions in mL

7.8 System Wash, Column Wash and Column Storage

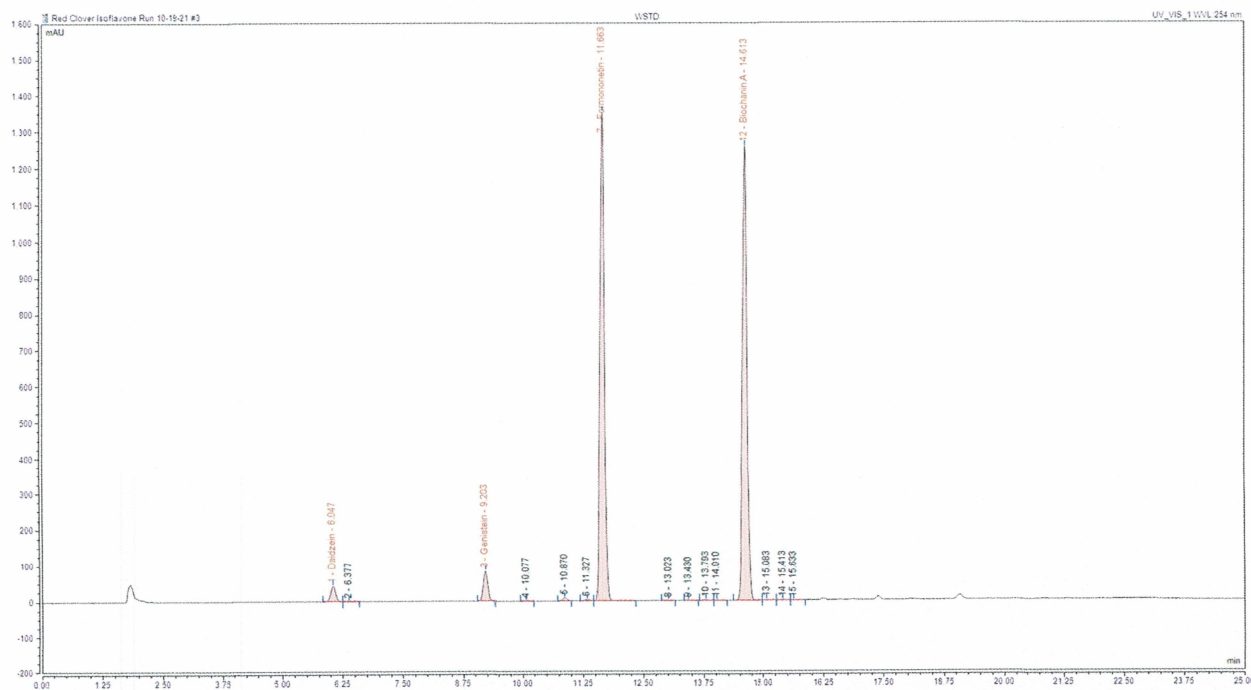
7.8.1 Wash and store the column in 75:25 ACN / Water.

8.0 Chromatograms

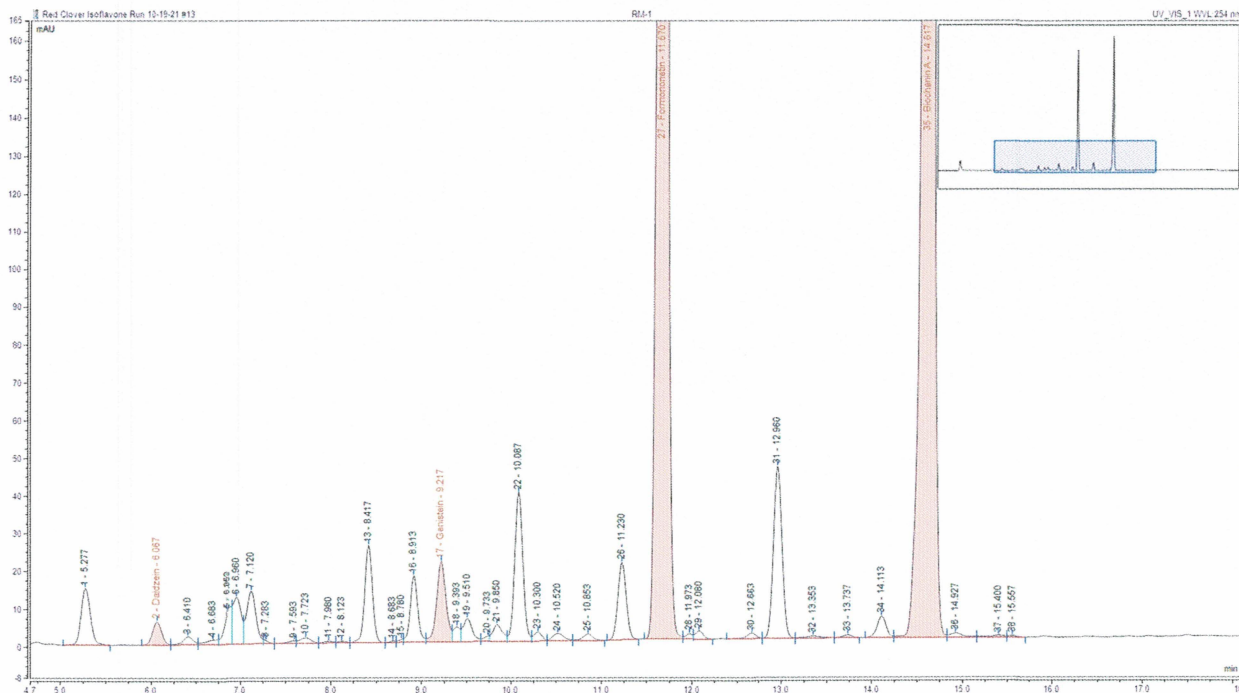
8.1 Typical Diluent Chromatogram



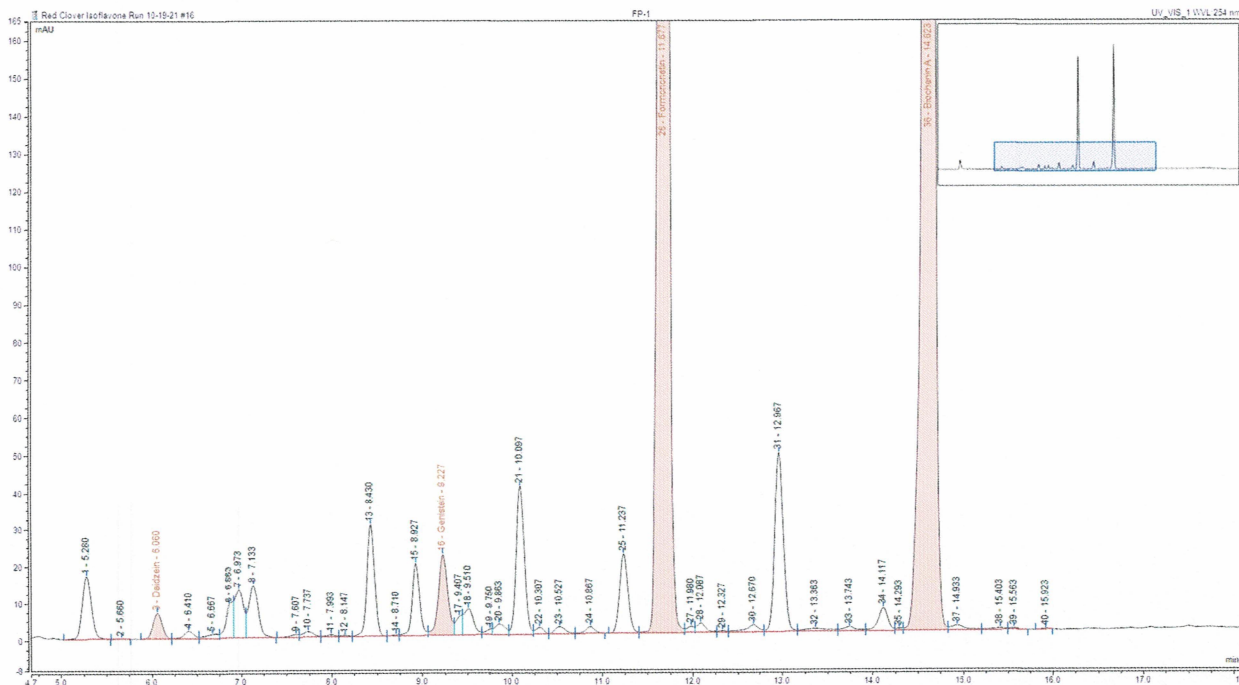
8.2 Typical Standard Chromatogram



8.3 Typical Raw Material Chromatogram



8.4 Typical Finished Product Chromatogram



<p style="text-align: center;">Standard Operating Procedure Determination of Isoflavones in Raw Materials and Finished Products by HPLC-UV</p>	<p style="text-align: center;">SOP No D-1001</p>	<p style="text-align: center;">Rev 0</p>	<p style="text-align: center;">Page 8 of 8</p>
---	--	--	---

9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	11/05/21	New	N/A	C. Perry