Current Standard Operating Protocols (SOP), NCBS-CCAMP MS-Facility Metabolomics –

Quantification of Isoflavones

Purpose: To provide general guidelines for conducting the quantification of isoflavones using tandem triple quadrupole mass spectrometry.

Reagents: All solvents and reagents used are of LC-MS quality.

Protocol:

A. Preparation of Standards:

- Prepare the individual stock solutions (STDs) of each isoflavone(~1mg/mL) in methanol (Stock A).Next prepare 100µg/mL stocks in methanol (Stock B) by taking the required amounts (~100µl) from Stock A.

- Prepare mixed stock of all thirteen STDsin methanol (Stock C):
  - 5µg/mL (Galangin, Quercetin dihydrate, Isoquercetin, Astragalin and Glabridin),by taking ~50µl from respective Stock B solutions
  - 2.5µg/mL (Liquiritin, Genistin, Naringenin and Isovitexin),by taking ~25µl from respective Stock B solutions
  - 0.5µg/mL (Formononetin, Ononin, Licochalcone A), by taking ~5µl from respective Stock B solutions, and
  - 25µg/mL (Kaempferol), by taking ~250µl from respective Stock B solution.

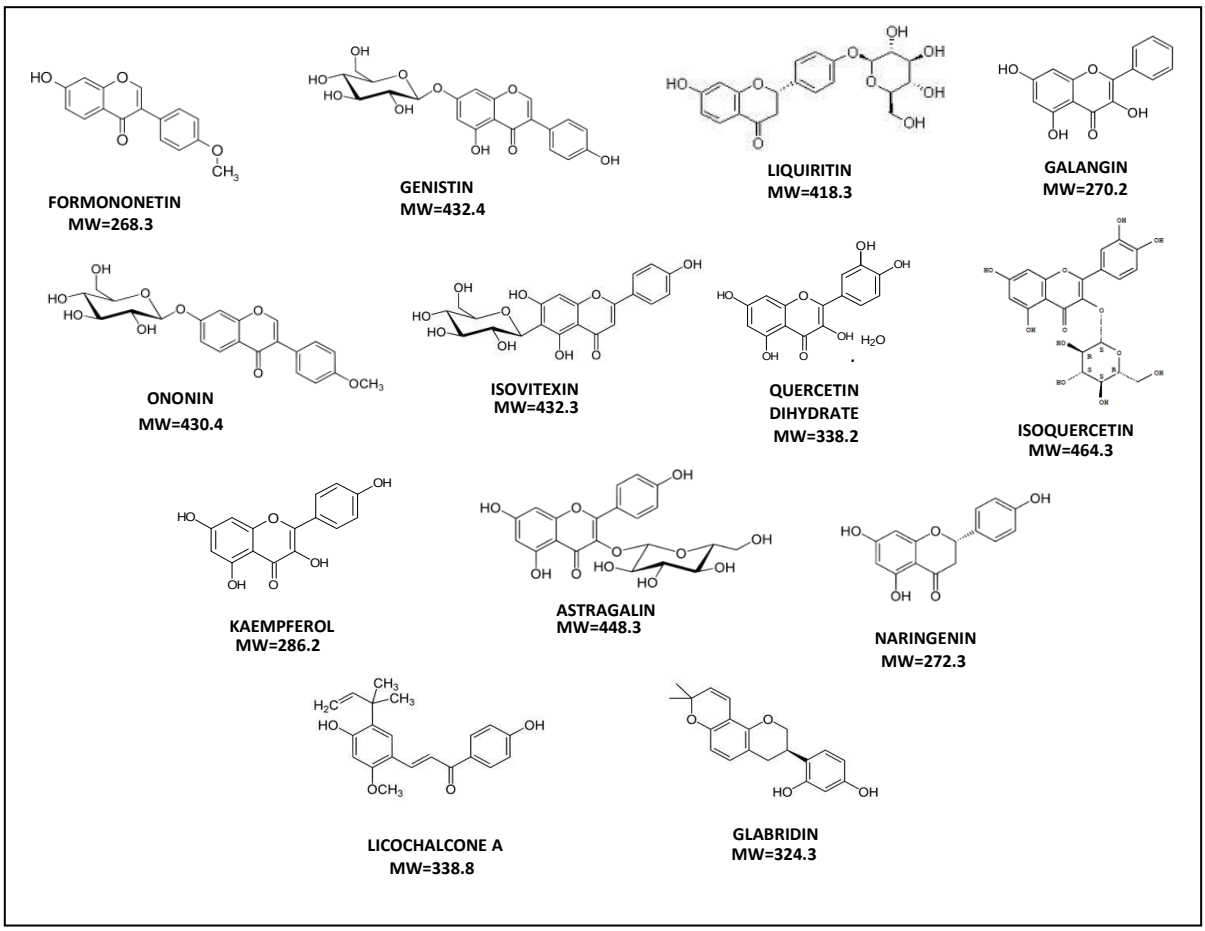

Figure 1: List of thirteen isoflavones

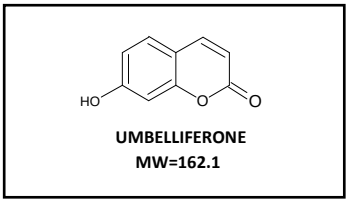

Figure 2: Internal standard- Umbelliferone

- Prepare individual stock solution of ISTD, Umbelliferone (~1mg/mL) in methanol (Stock D).
- Prepare 5µg/mL stock (Stock E) of ISTD in methanol by taking ~5µl from Stock D.
- Take 10µL each from Stock C and Stock E and dilute it into 50µL of 50% MeOH and transfer this into an HPLC vial and place it in the auto sampler for analysis.

B. LC-SRM Analysis:

- Equilibrate the Shim-pack ODSIII column (Phenomenex 2µ, 2 X 150 mm) with 10%acetonitrile.
- Use the mobile phase solvents A: water (10 mM ammonium acetate, 0.1 % FA), B: Acetonitrile (0.1% FA),and a flow rate of 200 µL/min for analysis.
- Set the following gradient (0 to 3 min-10 % B, 3 to 10 min- 10 to 50% B, 10 to 17 min-50-95% B, 17 to 20 min- 95%, 20 to 20.1 min-95 to 10% B, and 20.1 to 25 min- 10%) in the LC system.
- In the MS set the source parameters like spray voltage, (+ve): 4000 V and (-ve): 2800V; source temperature, 300º C; ion transfer capillary temperature, 270 °C; collision gas argon, S-lens voltage- as per table 1; sheath gas-20 and auxiliary gas-10; and scan time-50 milli sec for each transition. Set the following injector settings: 0-2 min: waste, 2-22 min: load, 22-25 min: waste.
- Select the most intense product ion and the corresponding collision energy and S-lens voltage of each transition for the LC-SRM analysis as shown in table 1.
- Inject 10 µL of the sample (1-50ng on column) for the actual analysis.
- The expected result is shown in figure 3

Table 1: SRM Table for isoflavones analyzed in the method

S.No.	Name	Polarity	Precursor ion (m/z)	Product ion (m/z)	Collision Energy	S-lens Voltage
1	Isovitexin	+ve	433.1	283.01	26	86
2	Isoquercetin	-ve	463.0	299.96	29	113
3	Liquiritin	-ve	417.0	254.99	22	75
4	Genistin	+ve	433.2	271.02	19	87
5	Astragalin	-ve	447.0	254.90	41	110
6	Ononin	+ve	431.1	269.04	19	84
7	Quercetin dihydrate	-ve	301.0	150.93	23	81
8	Naringenin	-ve	271.0	151.06	20	75

9	Kaempferol	-ve	285.0	184.99	29	78
10	Formononetin	+ve	269.1	197.07	37	89
11	Galangin	-ve	269.2	169.08	27	75
12	LicochalconeA	+ve	339.0	121.09	27	91
13	Glabridin	-ve	323.0	201.00	25	118
14	Umbelliferone	-ve	161.0	133.14	21	54

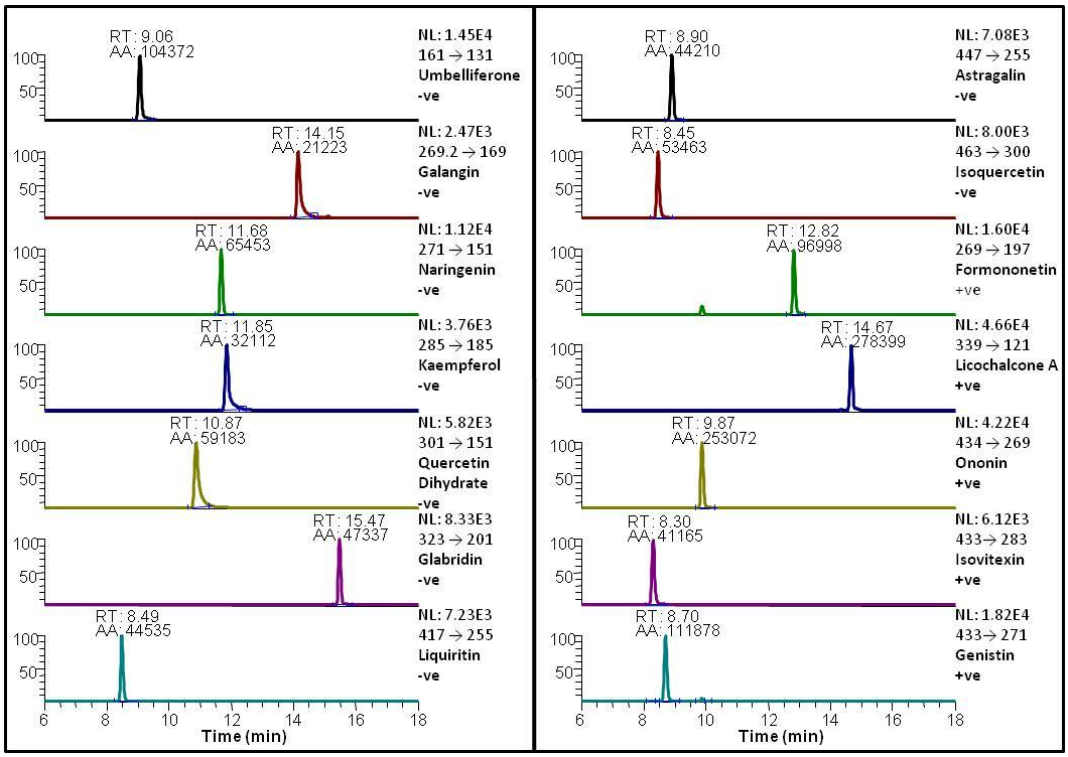

Figure 3: LC-SRM chromatogram of thirteen isoflavones

C. Preparation of samples for analysis:

- Obtain the extract as a lyophilized powder. Solubilize the extract in methanol (approximately 1ml for 2.5mg of extract).
- Vortex the sample for 2mins and then sonicate (bath) for 3min thrice, with intervals of 2mins.
- Centrifuge at 13000rpm for 5mins. Separate the supernatant and spike with ISTD, 10µl from Stock E.
- Dry the spiked supernatants in a speed vac.
- Reconstitute the residue in 50µl of 50%MeOH, vortex for 2min and sonicate (bath) again for 3min.
- Centrifuge at 13000rpm for 2min. Transfer the supernatant to an HPLC vial and place it in the auto sampler for analysis.
- Inject 10µl for actual analysis.