NOVEL LCMS TECHNIQUE FOR THE MEASUREMENT OF PHYTOESTROGENS IN SERUM AND URINE OF NEONATES IN MALAYSIA

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DECLARATION OF INTEREST

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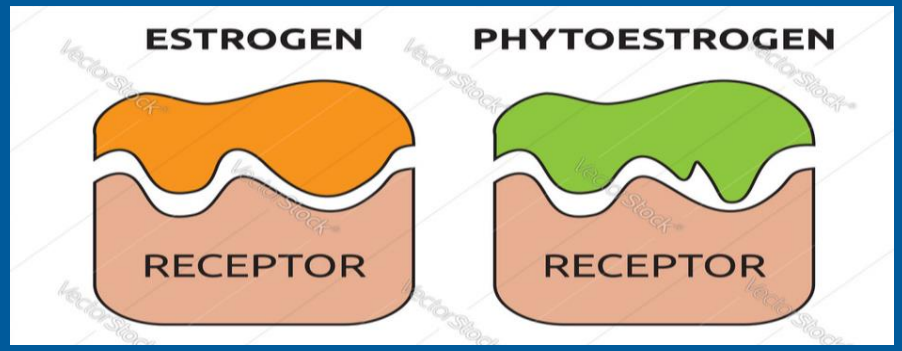
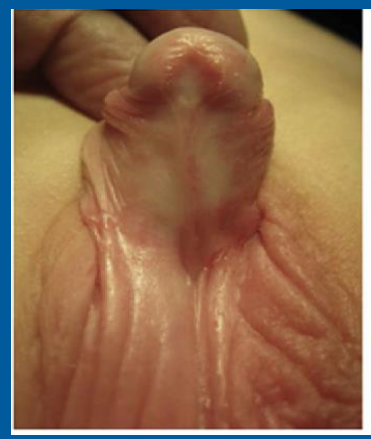
- This study received a **grant** from Ministry of Education, Malaysia
- FRGS/1/2017/SKK08/UKM/01/1

BACKGROUND





STUDY AIMS

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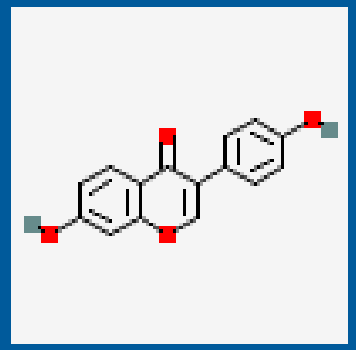
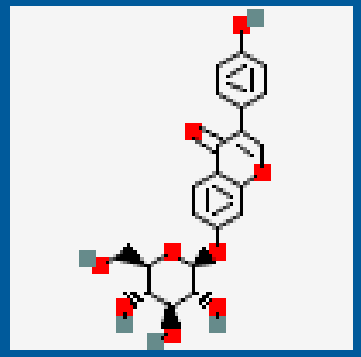
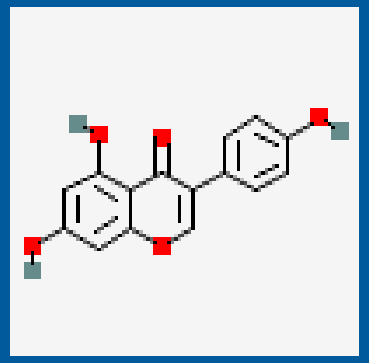
- To develop a **sensitive assay** for **phytoestrogen metabolites** in neonatal serum and urine.



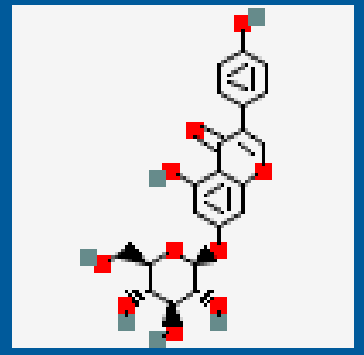
Daidzein



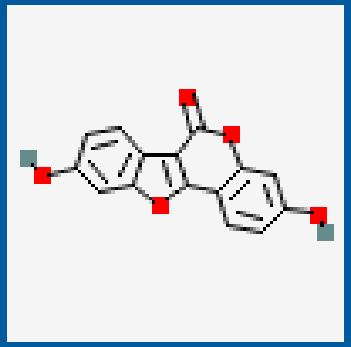
Daidzin



Genistein



Daidzin



Coumesterol

EQUIPMENT & CHEMICALS

- Phytoestrogen metabolites measured using Q-Exactive Quadropole Orbitrap LC-MS interphased with Dionex Ultimate 3000 liquid chromatograph
- Thermo Scientific Vacuum Mannifold

- **CHEMICALS**
  - Methanol, Acetic Acid, Dimethyl sulfoxide (DMSO), Formic acid, Acetonitrile, Ammonium Acetate, LCMS water
  - ***Analytical standards***: Genistein, Genistin, Daidzein, Daidzin, Coumesterol
  - ***Internal Standards***: Deuterated Genistein , Deuterated Daidzein
  - ***Deconjugating Agents:*** Beta Glucuronidase, 4 methylumbelliferone glucuronide, 4 methylumbeliferone sulphate

STEP 1:COMPOUND DETECTION

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- 1ml of 7 in 1 Working Solution containing 7 phytoestrogens
  - Genistein
  - Genistin
  - Genistein-D4 –deuterated sample
  - Daidzin
  - Daidzein
  - Daidzein-D4
  - Coumesterol

STEP 1:COMPOUND DETECTION

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COMPOUND	MASS	RETENTION TIME 1	RETENTION TIME 2	RANGE
Genistein	271.061	5.31 mins	5.51 mins	5-6
Genistin	433.1129	4.11mins	4.25 mins	3.75- 4.75
Genistein-D4	275.0852	5.30 mins	5.53 mins	5-6
Daidzin	417.1180	3.68 mins	3.80 mins	3.3-4.3
Daidzein	255.0652	4.76 mins	4.92 mins	4.4 – 5.4
Daidzein-D4	259.0903	4.75mins	4.94 mins	4.4- 5.4
Coumesterol	269.0444	5.40 mins	5.63 mins	5.1- 6.1

STEP 2: RANGE DETECTION

- **Working stock solutions diluted to 8 different concentrations to obtain a calibration curve**

1ppb	5ppb	10ppb	50ppb	100ppb	500ppb	1000ppb	5000ppb

- **Calibration curve obtained for each phytoestrogen component**
- **Need in order to obtain limit of quantification (LOQ and LOD)**

STEP 3: OPTIMAL SOLID PHASE EXTRACTION TECHNIQUE

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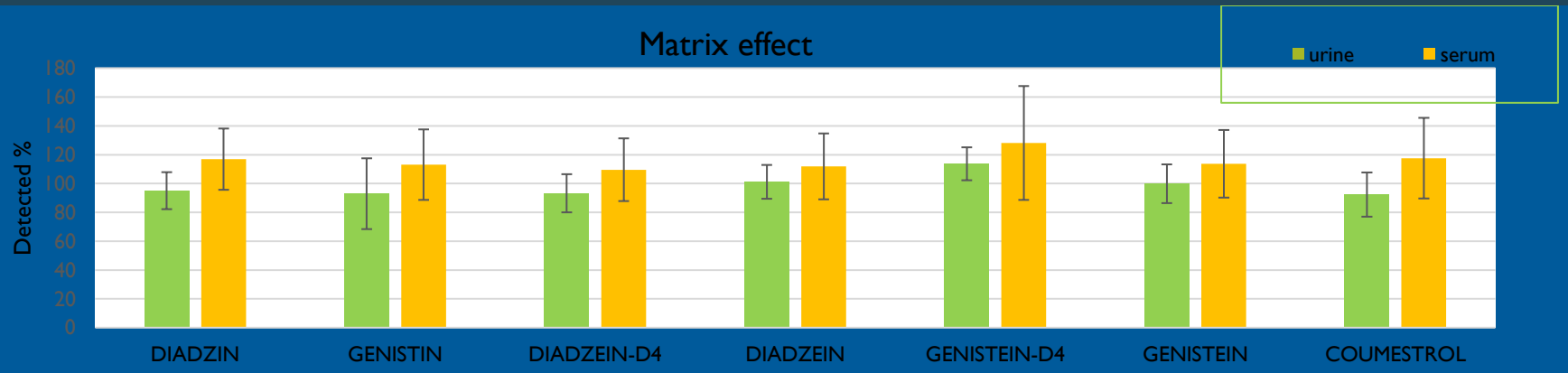
- **SERUM**
- 1ml serum + 1ml [water +2% Formic Acid]
- **A**: SPE Column conditioned with:
  - 2ml MeOH
  - 2ml LCMS Water
- **B**: SPE **loaded** with sample
- **C**: SPE column **washed** with 2ml 5% MeOH
- **D**: SPE column **elution** with 2ml 5% MeOH
- **E**: Eluent **dried** in speed vac at 45C
- **F**: **Reconstitution** 1ml of MeOH
- **G**: **Filter** reconstituted sample
- **H**: **Process** with LCMS

- **URINE**
- 2ml of urine used instead of mixture
- Similar steps **A to H** repeated.

STEP 4:MATRIX EFFECT



- acceptable matrix effect = < ± 15%
- Urine: all within acceptable range except genistein-D4 (114 %)
- Serum: only Genistein-D4 outside acceptable range, others:
  - Diadzin (116%)
  - Genistin (113%)
  - Diadzin (112%)
  - **Genistein-D4** (128 %)
  - Genistein (114%)
  - Coumestrol (118%)

STEP 5: CONSTRUCTION OF STANDARD CURVE

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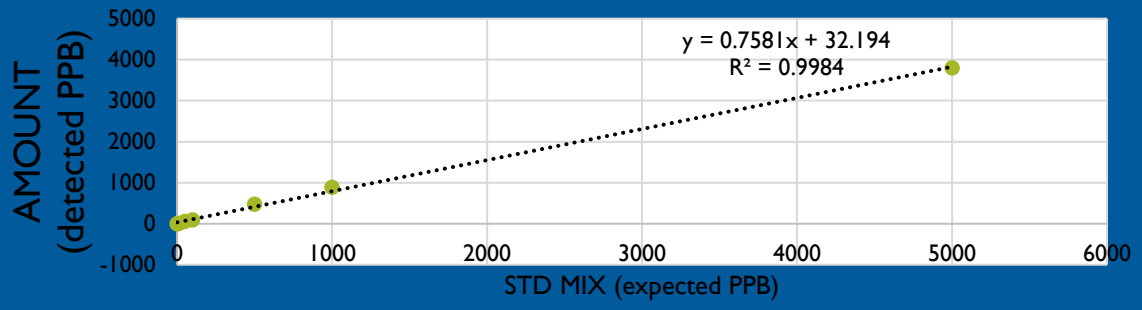
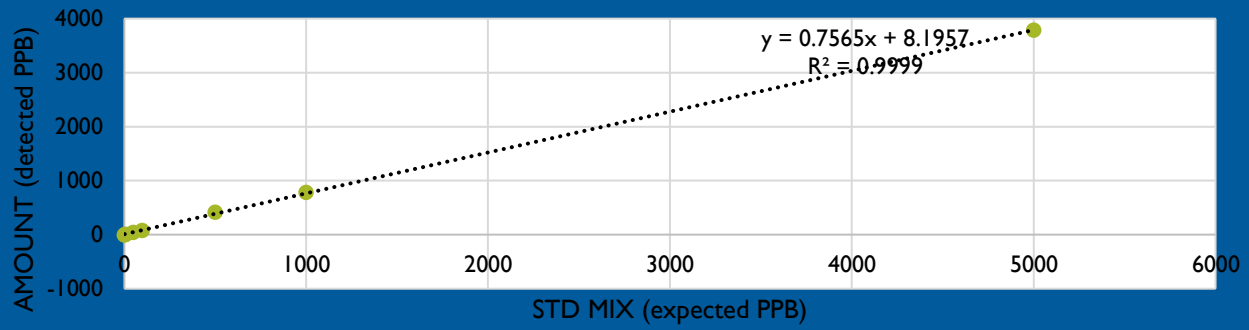
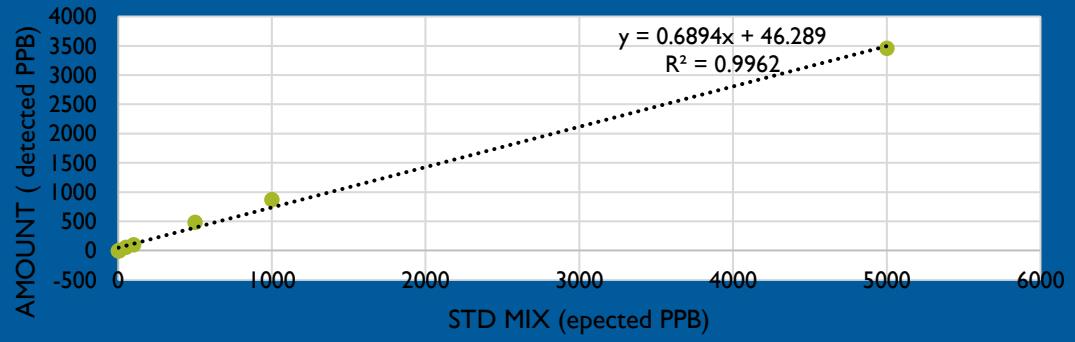
DIADZIN



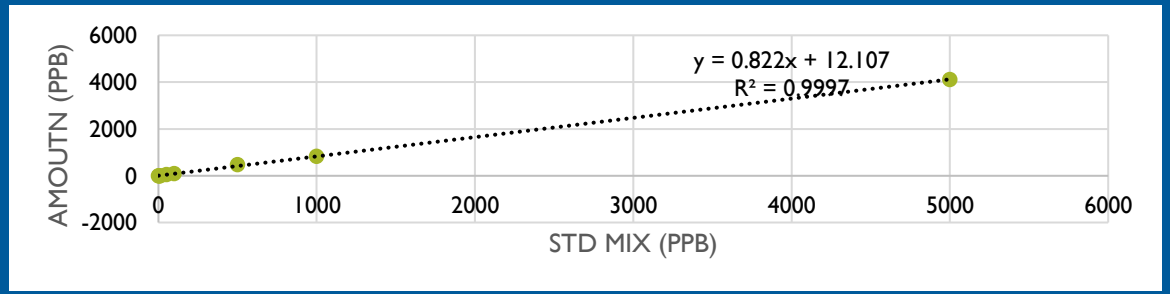
GENISTIN



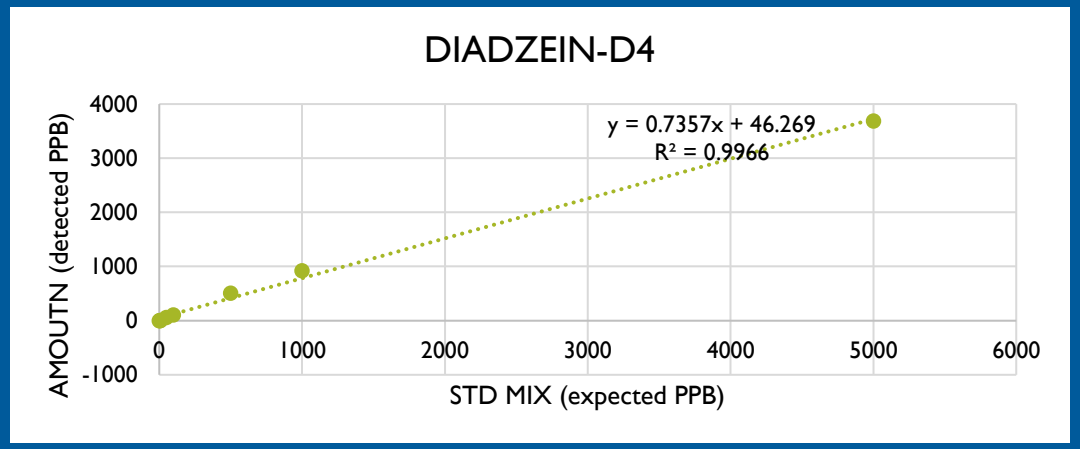
DIADZEIN

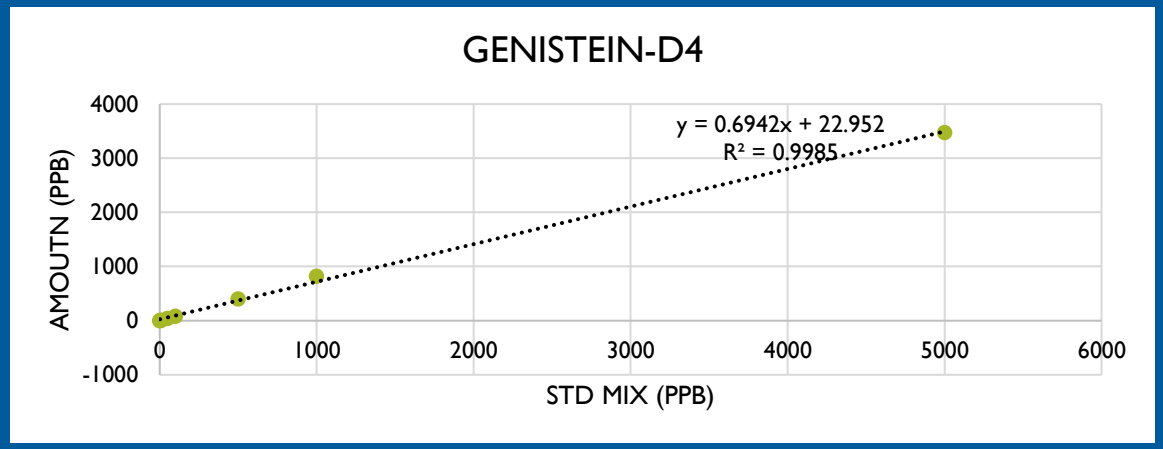


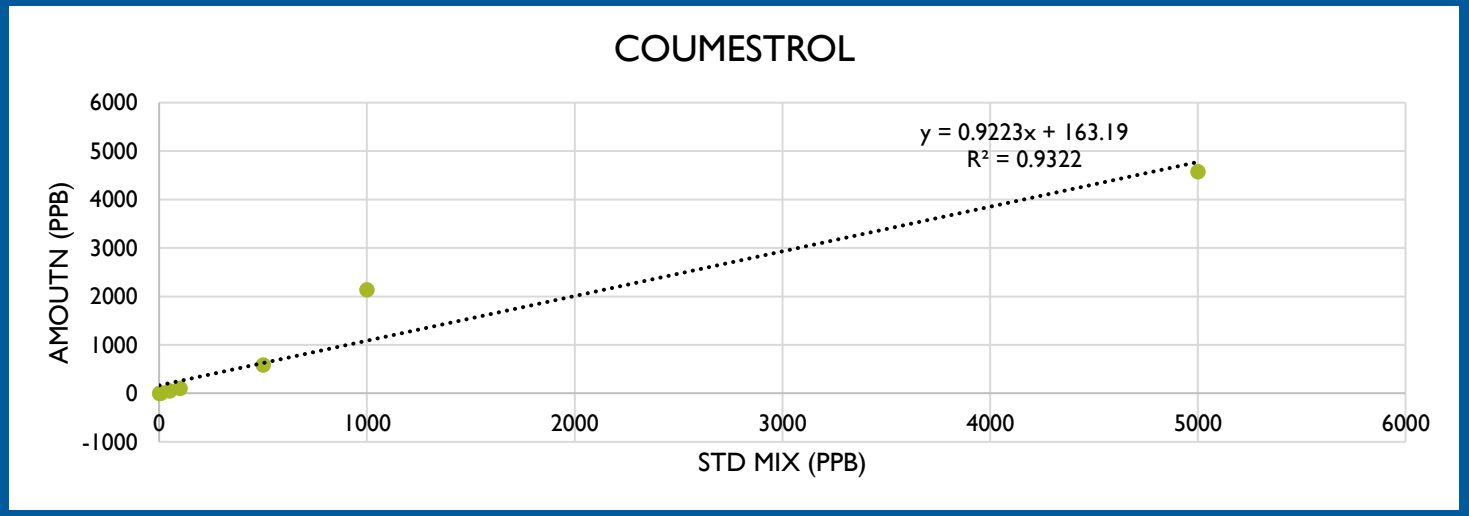
GENISTEIN



STEP 5: CONSTRUCTION OF STANDARD CURVE







STEP 5: CONSTRUCTION OF STANDARD CURVE

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	Daidzin	Genistin	Daidzein- D4	Daidzein	Genistein-d4	Genistein	Coumesterol
SD	0.016681	0.015588	0.078147	0.030707	0.081949	0.067686	0.038423
slope	0.7581	0.7565	0.7357	0.6894	0.6942	0.822	0.9223
LOQ	0.06601	0.061818	0.318663	0.133625	0.354143	0.247028	0.12498
LOD	0.220035	0.20606	1.06221	0.445416	1.180476	0.823325	0.416601

*LOD= 3XSD/SLOPE OF CURVE
*LOQ= 10XSD/SLOPE OF CURVE

- acceptable r2 value (all > 0.99);
- Except **coumestrol,** r2 = 0.9322

- LOQ (limit of quantitation) in between 0.066-0.354
- **Genistein-d4** has the highest LOQ (> 0.354 ppb)

Phytoestrogen	R2 value
Daidzin	0.99843
Genistin	0.99987
Daidzein- D4	0.99658
Daidzein	0.99621
Genistein- D4	0.99853
Genistein	0.99972
Coumesterol	0.93223

STEP 5: ENZYME DECONJUGATION AND OPTIMISATION

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- Enzyme deconjugation to release conjugated phytoestrogen to free serum phytoestrogen
- Optimal timing for enzyme activity is 16 hours for serum and 24 hours for urine
- As no degradation in rate of phytoestrogen after 16 hours in serum for ease of process we chose 24hours for enzyme incubation.
- SPE recovery noted to be above 80% for all phytoestrogens in serum
- Urine: Genistin 70%
- Daidzein 122%
  - Genistein 76%
  - Coumesterol 72%

CONCLUSION

- This method is feasible for analysis of phytoestrogen in serum and urine

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THANK YOU