Qualitative and quantitative analysis/method validation in metabolomics

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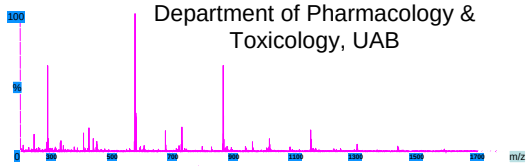
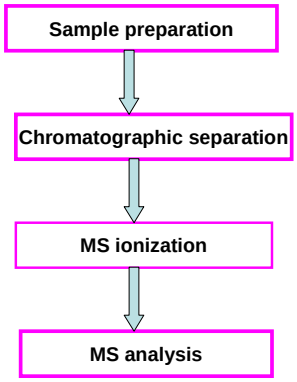


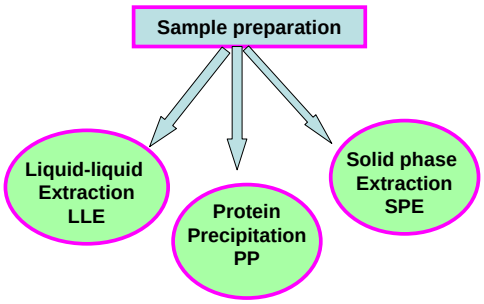
Class Overview

- **Introduction to bioanalysis**
- **Quantitative analysis of puerarin, EGCG and isoflavones in biological samples by LC-MS/MS**

Bioanalysis Flow Chart



Sample preparation is a crucial step in removing the interfering compounds from biological matrix



The method of choice will be determined by the sample matrix and the concentration of compounds In samples

Properties of Good Internal Standards

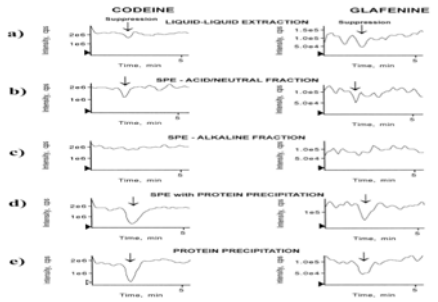
- **Is not found in the original sample**
- **The structure of the internal standard needs to be similar to the analyte.**
- **For isoflavone conjugates:**
- **a. Hydrolysis of 4-Methylumbelliferyl sulfate**
- **b. Hydrolysis of Phenolphthalein glucuronide**
- **c. Extraction Efficiency (Apigenin)**

Problems encountered in LC-MS analysis

Ion suppression?

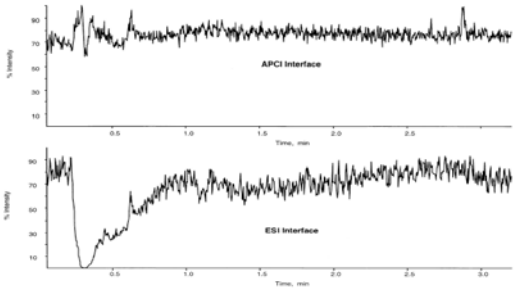
- the presence of endogenous substances from matrix, i.e. organic or inorganic molecules present in the sample and that are retrieved in the final extract
- exogenous substances, i.e. molecules not present in the sample but coming from various external sources during the sample preparation

Severe ion suppression effect for codein and glafenin was observed with PPT and SPE-PPT



Muller *et al. J. Chrom B (2002)*

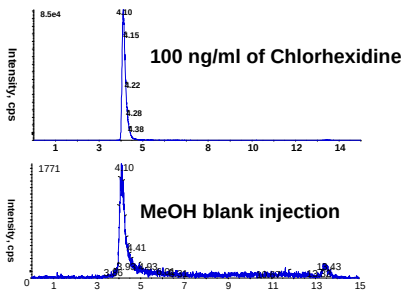
APCI is less prone to than ESI to the effects of ion suppression



King et al. J. Am Soc Mass Spectrom 2000

Carry over a big problem?

Previously injected sample when appears upon subsequent analyses due to physico-chemical property of the sample, analysis system or both.



Why quantification of drug/drug metabolites in plasma/tissues PK studies is so important?

- An accurate and fast analytical method for measuring the concentrations of a compound in plasma or tissue is the first step in order to yield the PK of a compound
- Established assay for human sample analyses (plasma, serum or urine matrix) needs to be more rugged, robust and be able to withstand the test of time during this the longest phase of clinical development. The requirements and adherence to specificity, selectivity and stability will become very important

G (good) L (laboratory) P (practices)

- Standard operating procedures (SOP)
- Installation, operational, and performance qualification of facilities, instrumentation, and software
- Personnel selection, staffing, and training
- Quality control (QC) procedures and staffing
- Quality assurance
- Procedures and staffing
- Documentation and archival process

Analytical method validation

- Should demonstrate specificity, linearity, recovery, accuracy, precision
- Lower limit of quantification
- Stability (freeze/thaw)
- Robustness

Method validation..

- **Specificity is established by the lack of interference peaks at the retention time for the internal standard and the analyte.**
- **Accuracy is determined by comparing the calculated concentration using calibration curves to known concentration. The LLQ is defined as the smallest amount of the analyte that could be measured in a sample with sufficient precision (%CV) and accuracy (within 20% for both parameters) and is chosen as the lowest concentration on the calibration curve.**

LC/MS/MS Method for Puerarin

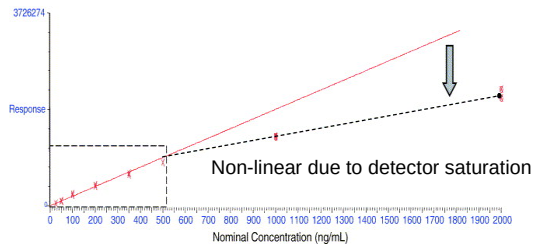
Column:	**Waters X-Terra C18 with guard, 2.1 x 100 mm, 3.5 micron**
Mobile Phase A:	**10% MeCN + 10 mM NH4OAc**
Mobile Phase B:	**70% MeCN + 10mM NH4OAc**
Gradient:	**0 minutes = 100% A** **6 minutes = 100% B** **7 minutes = 100% A** **10 minutes = Stop**
Injection Volume:	**20 ul**
Flow Rate:	**0.2 ml/min split flow**
Mass Spectrometer:	**Negative Electrospray**
Mass Transitions:	**415/267 (Puerarin)** **415/295 (Puerarin)** **269/149 (apigenin, IS)**

Table 1.
Summary of calibration curves (n =5)

Concentration (ng/ml)	Mean ± S.D.	CV (%)	Accuracy (%)
2.0	2.21 ± 0.16	7.00	110.7
5.0	5.22 ± 0.28	5.30	104.48
50	45.32 ± 2.53	5.60	90.64
500	473.60 ± 26.57	5.60	94.72
1000	1021.20 ± 71.53	7.00	102.12
5000	5340 ± 420.18	7.90	106.80

Mean r = 0.996

Standard curve non-linearity is possible due to Detector saturation, dimer/multimer formation, and or ESI droplet saturation at higher concentration

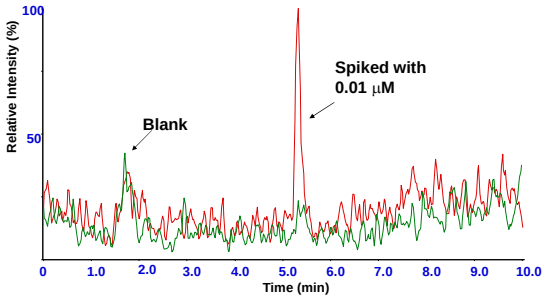


Source: Bakhtiar & Majumdar.
Journal of Pharmacological and Toxicological methods, 2007

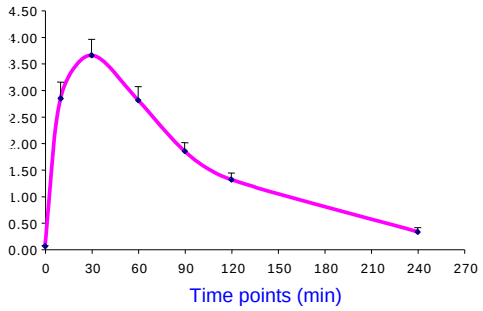
Table 2.
Assay validation characteristics of the method for the determination of puerarin in rat serum (n =5)

Concentration (ng/ml)	Mean ± S.D.	CV (%)	Accuracy (%)
2.0	2.21 ± 0.16	7.00	110.7
4.0	3.96 ± 0.30	7.90	99.20
8.32	7.32 ± 1.00	14.40	113.30
20	19.20 ± 1.20	6.30	96.00
200	203.20 ± 19.41	9.60	101.60
832	821.18 ± 55.86	6.80	101.31
2000	2240 ± 96.70	4.30	112.00

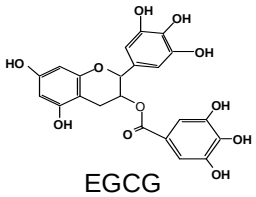
Ion chromatograms of a rat serum spiked sample (0.01 μM of puerarin) vs. blank serum



Average serum concentration of puerarin versus time after Oral administration of 50 mg/kg puerarin

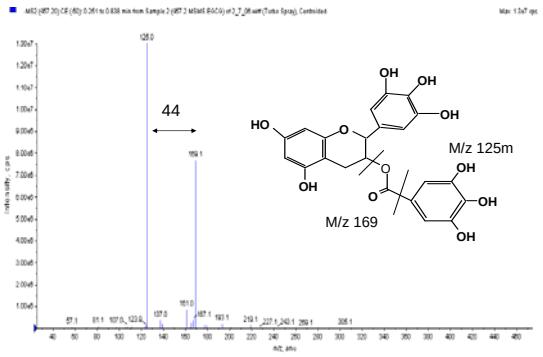


Analysis of tea catechin in biological samples

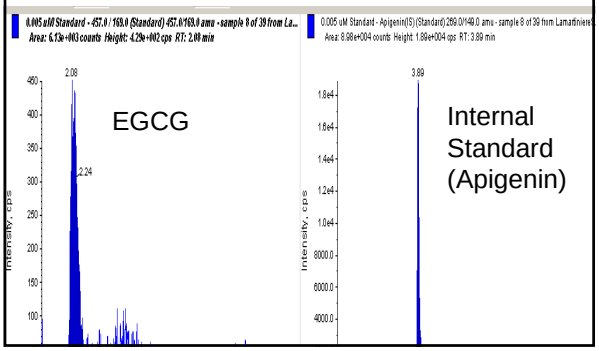


EGCG
[M-H]- m/z 457

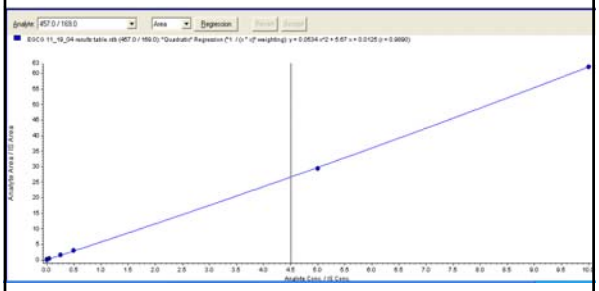
Product ion spectrum of the ion m/z 457



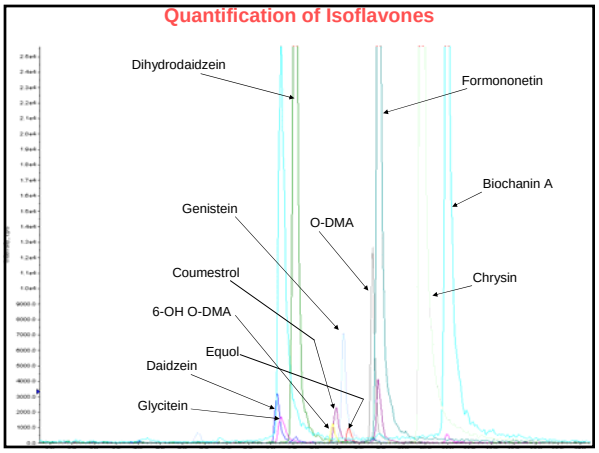
Typical chromatogram of a serum sample spiked with 5 nM after protein precipitation

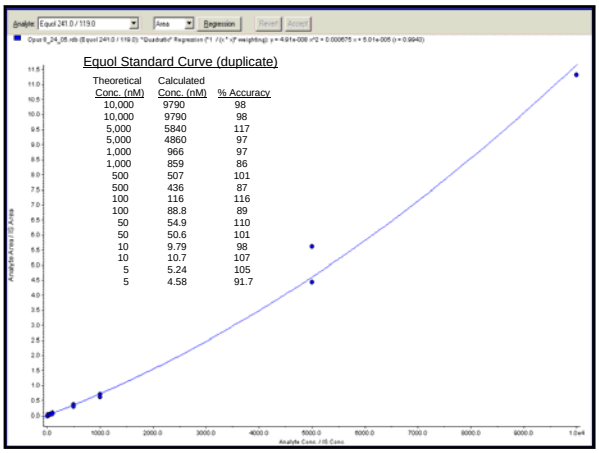


Calibration curve for EGCG (1-10,000 nM) after extracting from rat serum



Quantification of Isoflavones





Equol Standard Curve (duplicate)

Theoretical Conc. (nM)	Calculated Conc. (nM)	% Accuracy
10,000	9790	98
10,000	9790	98
5,000	5840	117
5,000	4860	97
1,000	966	97
1,000	859	86
500	507	101
500	436	87
100	116	116
100	88.8	89
50	54.9	110
50	50.6	101
10	9.79	98
10	10.7	107
5	5.24	105
5	4.58	91.7