Analytical Method Validation and Application for Natural Products Analysis

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What is the primary purpose of validating a method of analysis ?

To show that the method is fit for its intended purpose.

- Determine how much of a valuable, necessary, or characteristic ingredient is present in a product.
- Determine if a product meets specifications.
- Determine if a product meets regulatory requirements.
- Survey an environment to determine the presence and amount of a component, contaminant, or a nutrient.
- Identify a product and/or its components

What is this product?

How much of an analyte in the product (matrix)?

METHOD VALIDATION GUIDELINE

- ICH Guideline Q2(R1), **Q2(R2)**
- **ASEAN Guideline for Validation of Analytical Procedures**
- AOAC
  - **Appendix F Guidelines for Standard Method Performance Requirements**
  - Appendix K Guidelines for Dietary Supplements and Botanicals
- U.S. FDA Guidance
- Guideline on Bioanalytical Method Validation (ICH M10)

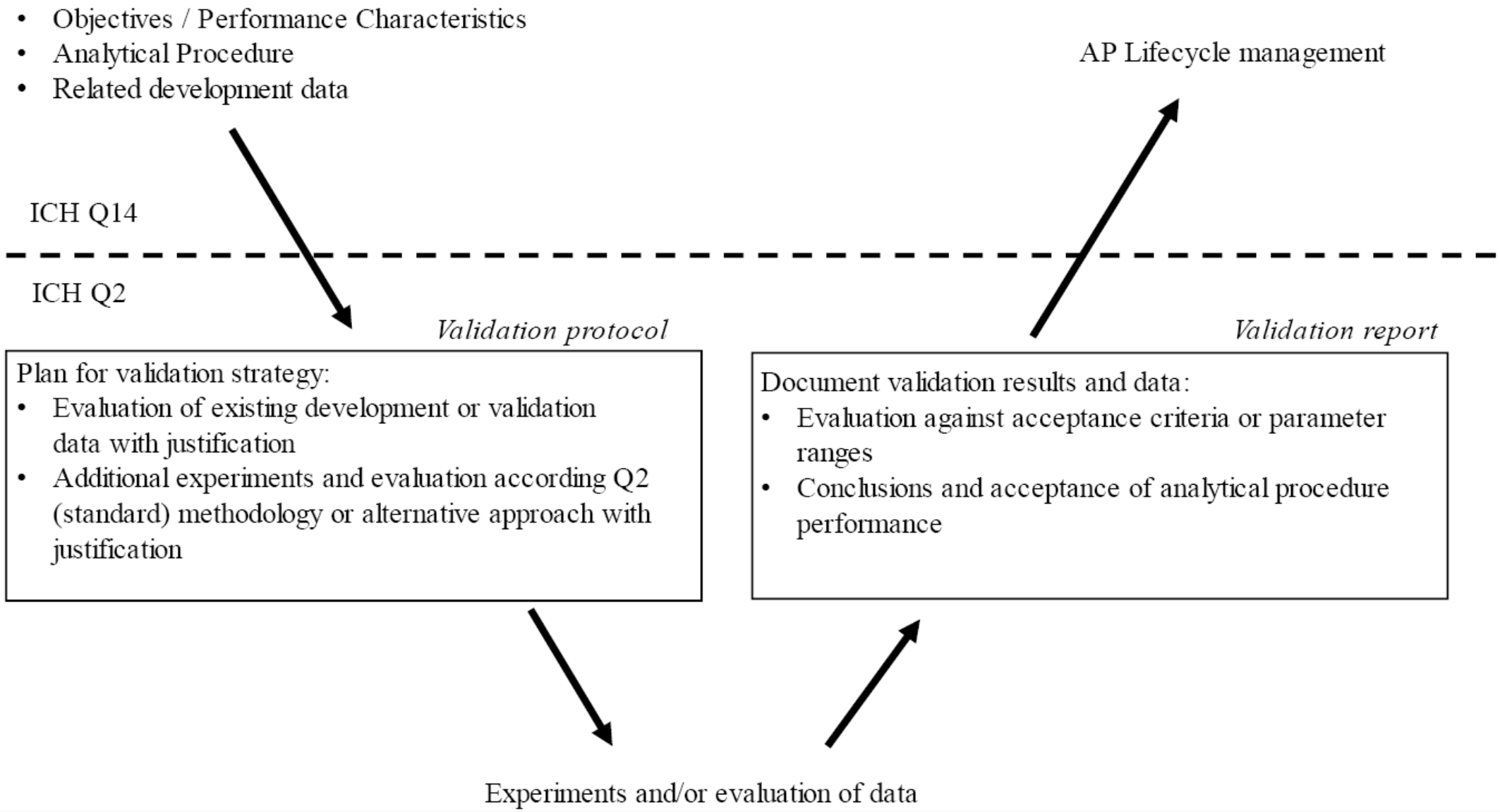
Validation study is
designed to provide sufficient evidence that the analytical procedure meets its objectives.

These objectives are described with a suitable set of performance characteristics and related performance criteria, which can vary depending on the intended use of the analytical procedure and the specific technology selected

ICH guideline Q2 (R2) : Validation of analytical procedures

Type of measured product attribute / Analytical Procedure Performance Characteristics to be demonstrated (2)	Identity	Impurity Other quantitative measurement (1)		Assay content/potency Other quantitative measurement (1)
		Quantitative	Limit	
Specificity (3)				
Specificity Test	+	+	+	+
Working Range				
Suitability of Calibration model	-	+	-	+
Lower Range Limit verification	-	QL (DL)	DL	-
Accuracy (4)				
Accuracy Test	-	+	-	+
Precision (4)				
Repeatability Test	-	+	-	+
Intermediate Precision Test	-	+ (5)	-	+ (5)

Validation Study Design and Evaluation



Validation during the lifecycle of an analytical procedure

Changes

Science and risk-based principles

Revalidation

Co-validation ➡ different laboratory

Cross-validation ➡ different analytical procedures

REPORTABLE RANGE

- The reportable range is typically derived from the product specifications and depends on the intended use of the procedure.
- The reportable range is confirmed by demonstrating that the analytical procedure provides results with acceptable accuracy, precision and specificity.
- The reportable range should be inclusive of the upper and lower specification or reporting limits, as applicable.

Reportable ranges for common uses of analytical procedures

Use of analytical procedure	Low end of reportable range	High end of reportable range
Assay of a drug substance or a finished (drug) product	80% of lower specification limit	120% of upper specification limit
Potency	Lowest specification acceptance criterion -20%	Highest specification acceptance criterion +20%
Content uniformity	70% of declared content	130% of declared content
Dissolution testing	Q-45% (immediate release) of the dosage form strength first measurement timepoint or QL (modified release)	130% of declared content of the dosage form
Impurity testing	Reporting threshold	120% of specification limit
Purity testing (as area %)	80% of specification limit	100% of specification limit

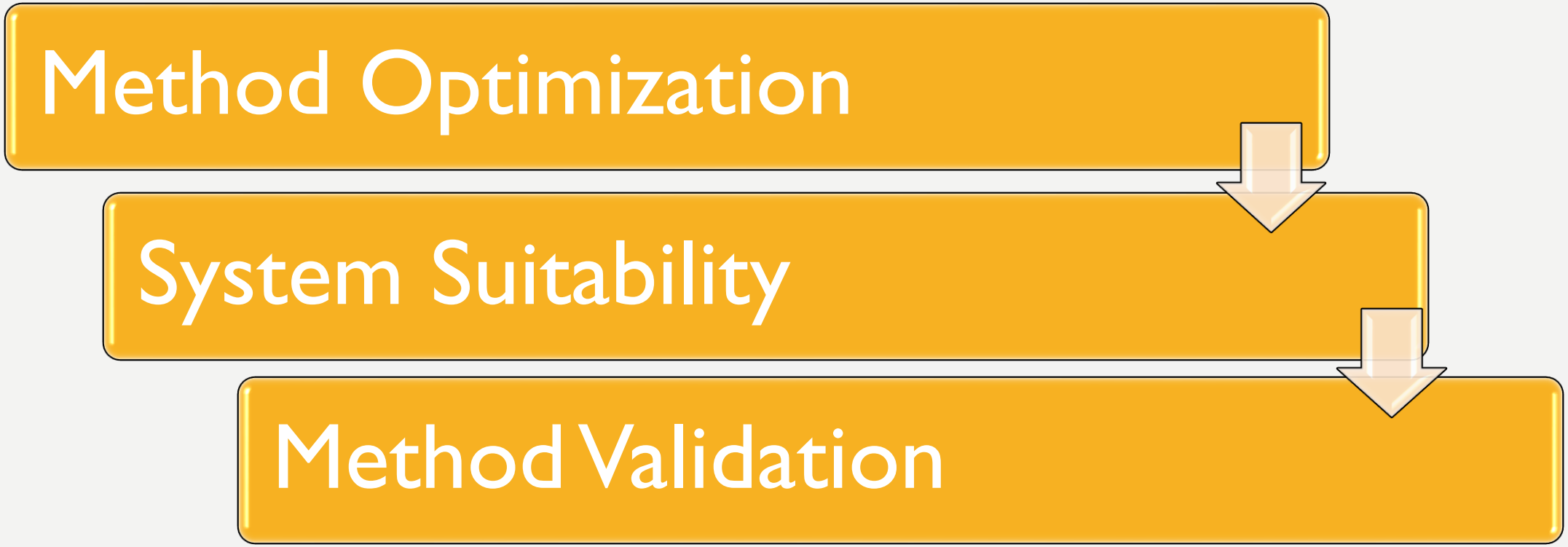
Demonstration of stability indicating properties

procedure that can detect changes in relevant quality attributes of a drug substance or drug product during storage

To demonstrate specificity/selectivity of a stability-indicating test

List	
samples spiked with target analytes and all known interferences	
Samples exposed to stress conditions (Forced degradation)	Example condition
• Higher temperature and/or humidity	Water, reflux at 80 °C
• Acid hydrolysis	0.1 N HCl reflux at 80 °C
• Basis hydrolysis	0.1 N NaOH reflux at 80 °C
• Oxidation	6% v/v H_2O_2
• Photodegradation	White light and/or UV light (6 hrs.)

METHOD VALIDATION PROCEDURE



METHOD VALIDATION PROCEDURE

Method Optimization

Optimization of a separation is principally directed by the following goals:

- to separate better (higher resolution)
- to separate faster (shorter retention time)
- to see more (lower detection limit)
- to separate at lower cost (economic effort)
- to separate more (higher throughput).

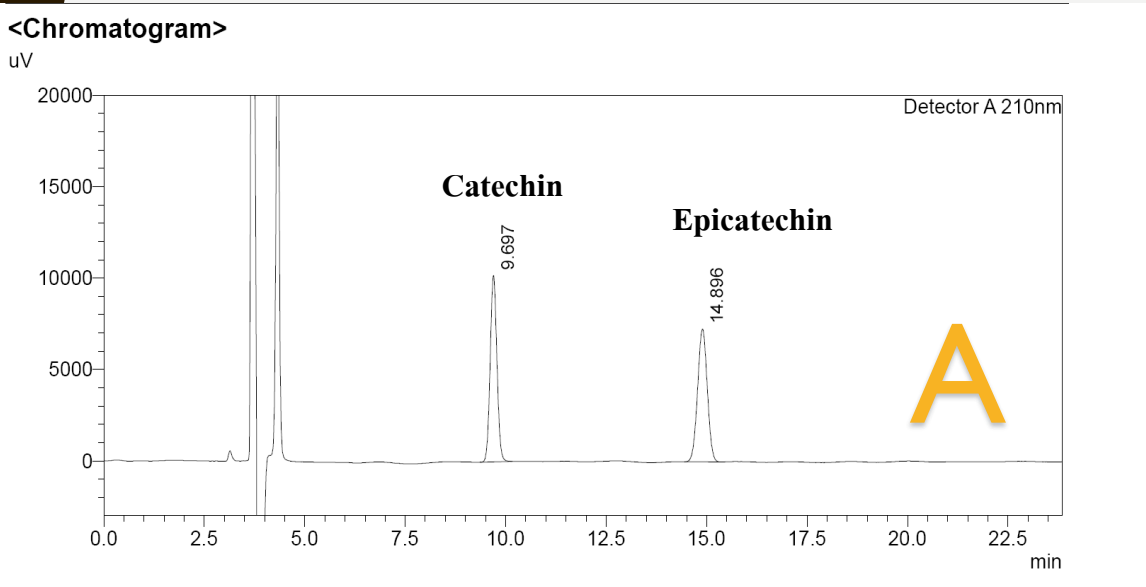
Method Optimization

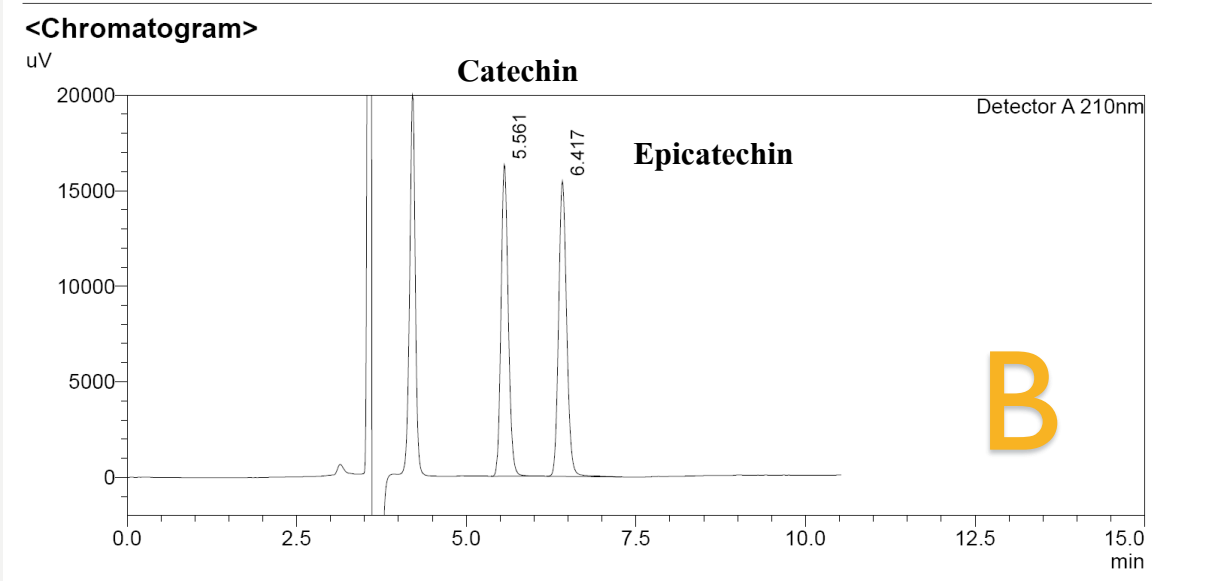
- Condition Analysis : Mobile phase, pH, Temperature
- Concentration Range
- Sample Preparation

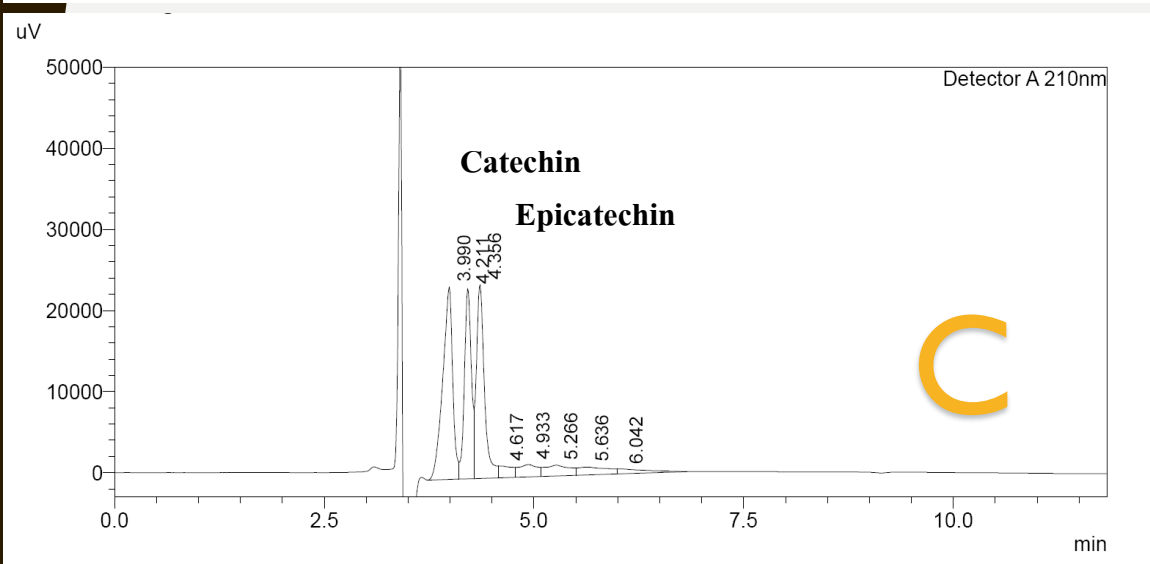
- System suitability parameters
- Standard back calculation

Method Optimization

Catechin and Epicatechin Determination in Fermented Tea

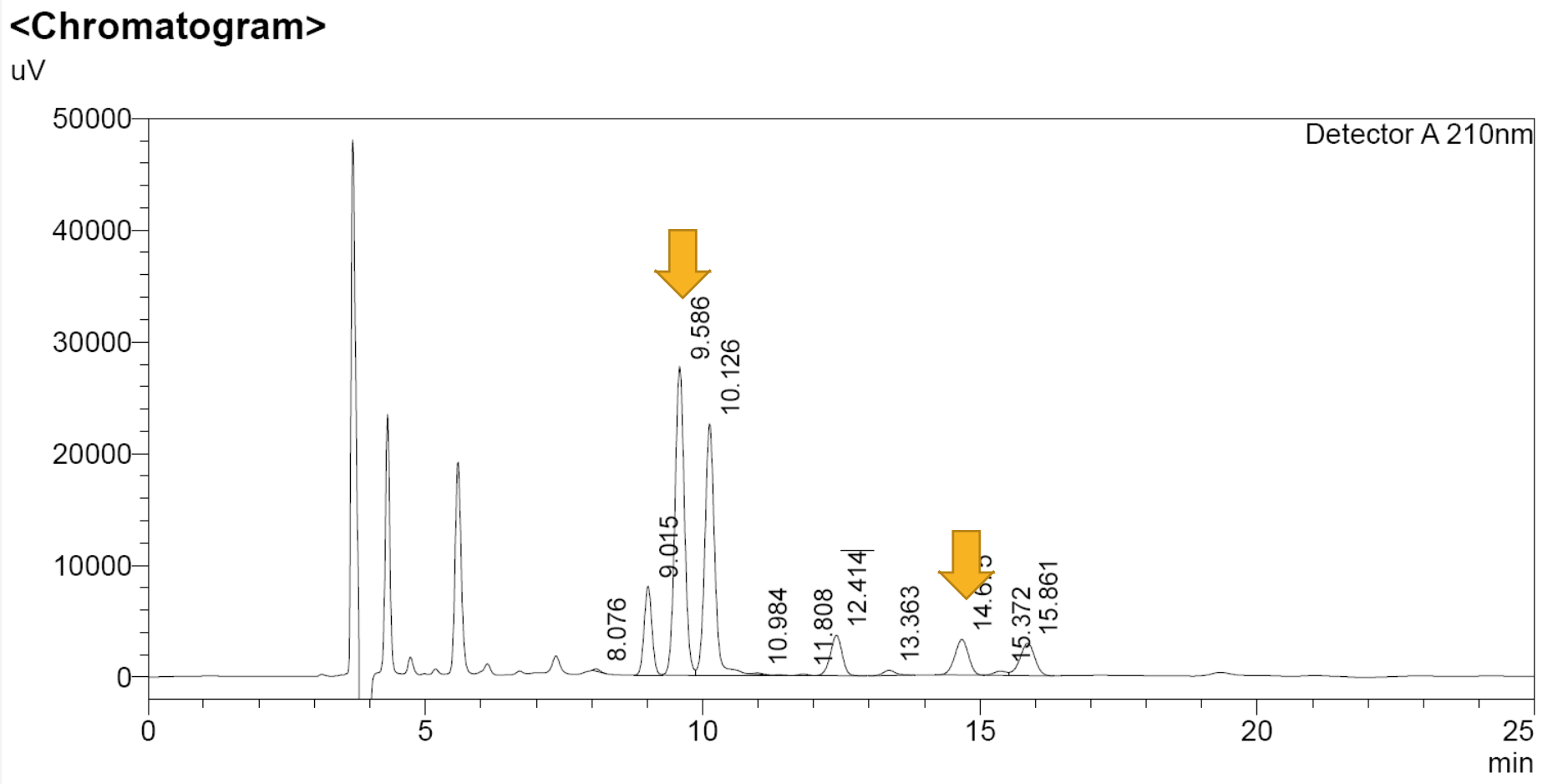




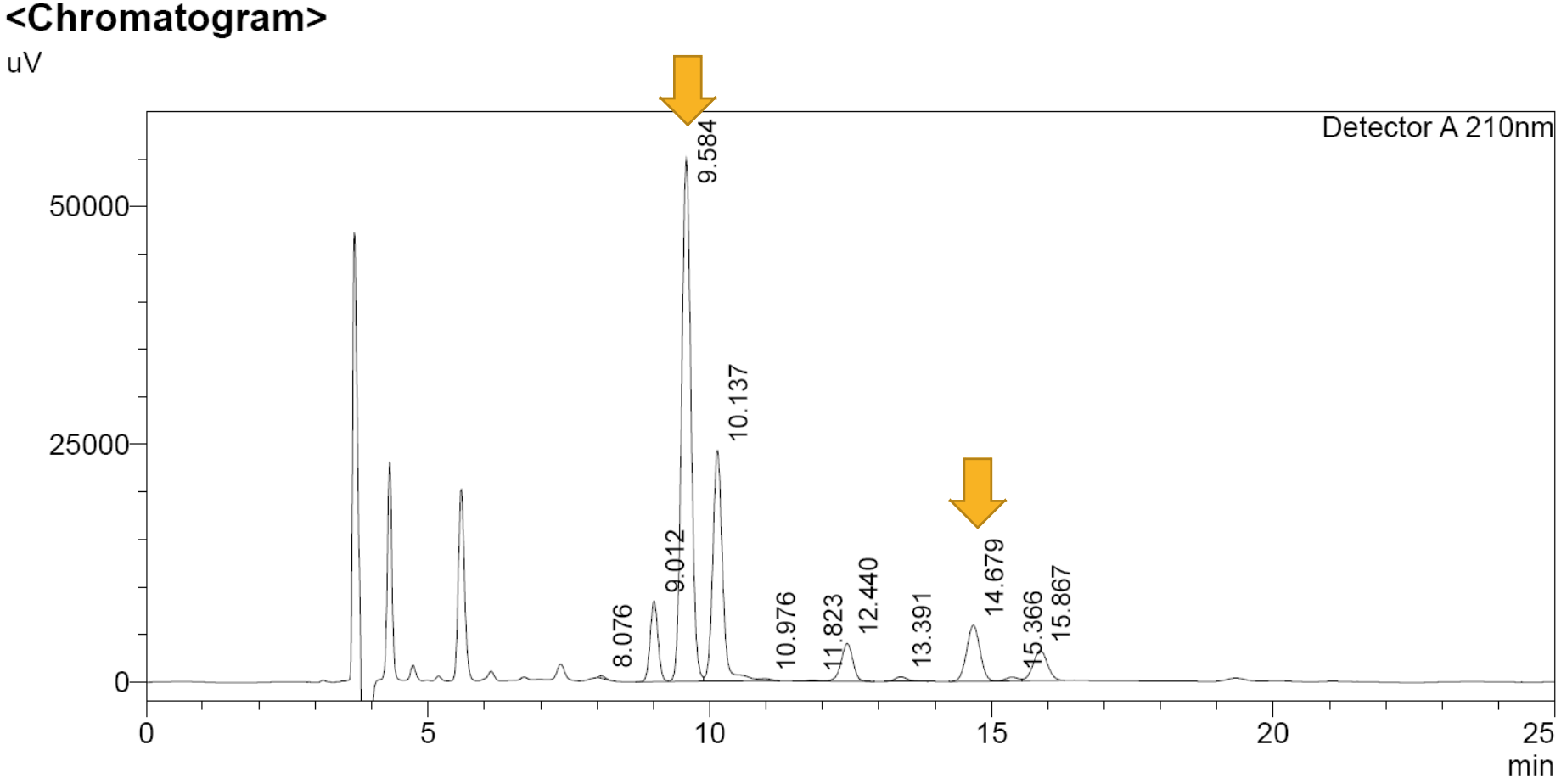


- Condition : mobile phase, pH, temp
- Concentration of standard
- Concentration of sample

Sample

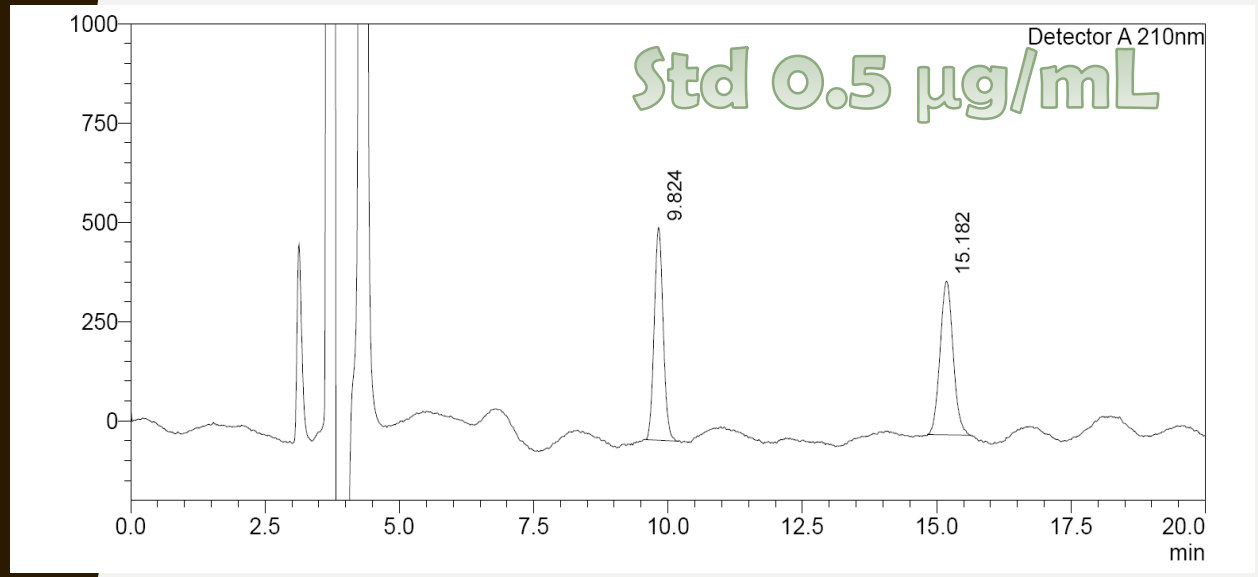

<Chromatogram>
uV
50000
40000
30000
20000
10000
0
Detector A 210nm
8.076
9.015
9.586
10.126
10.984
11.808
12.414
13.363
14.6
15.372
15.861
0
5
10
15
20
25
min

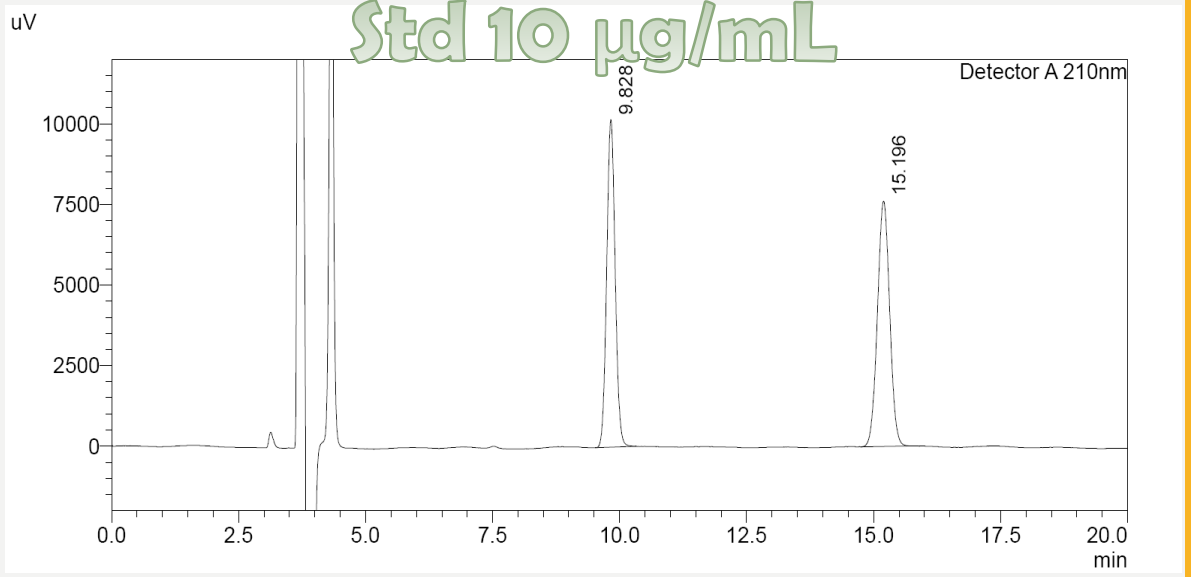
Sample (spike standard)

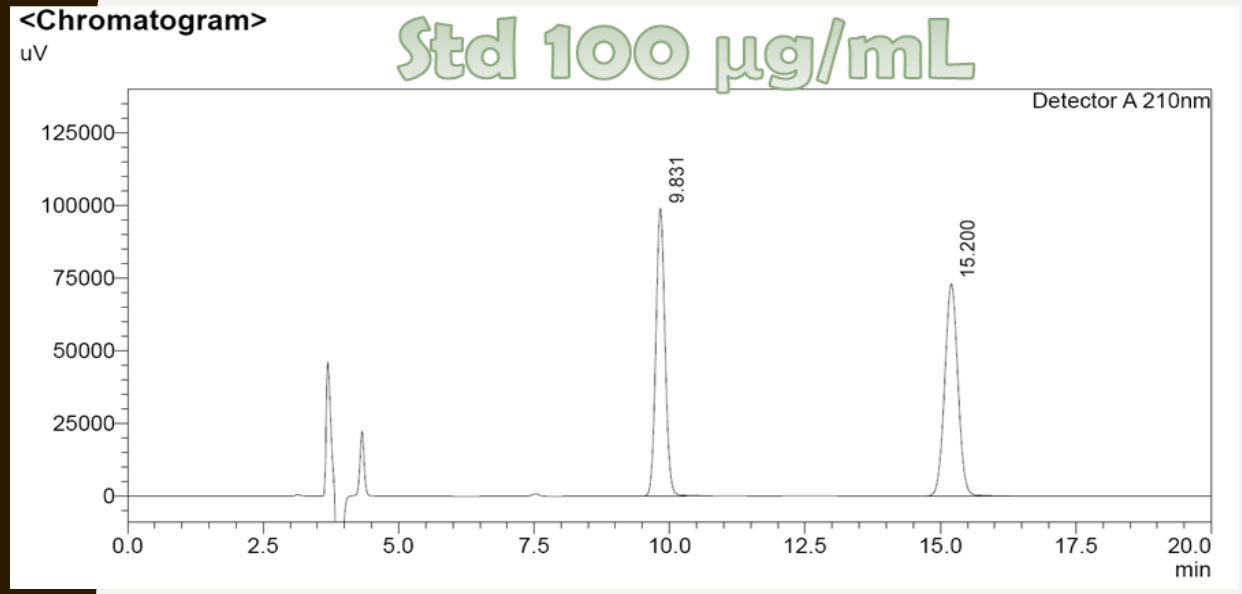


Method Optimization

Catechin and Epicatechin Determination in Fermented Tea



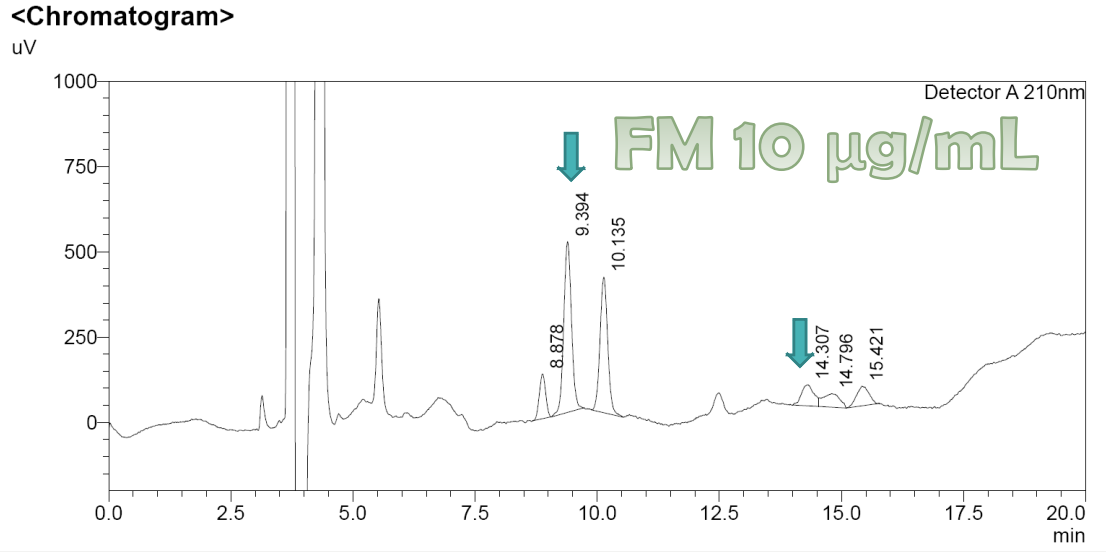


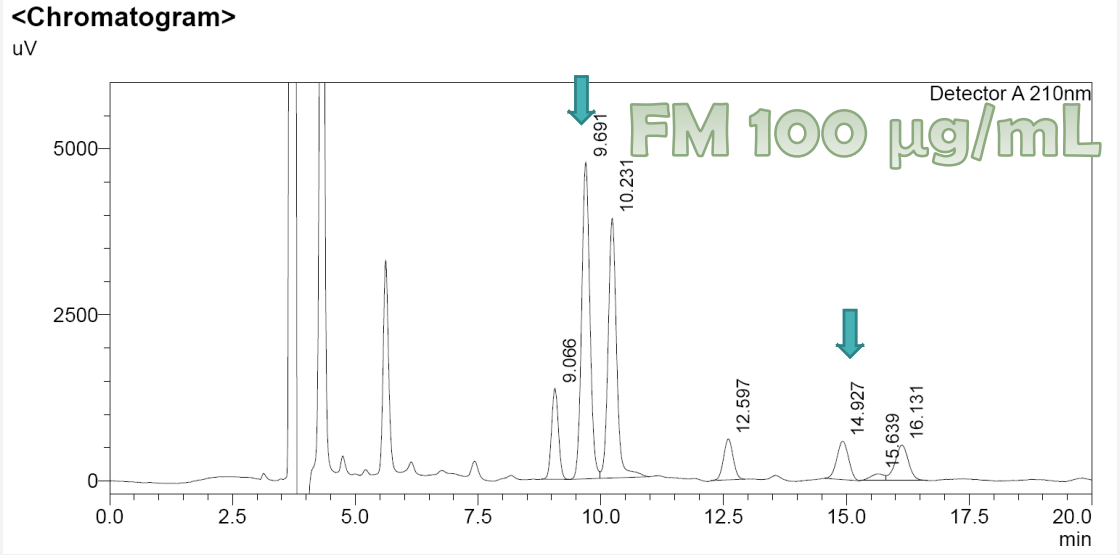


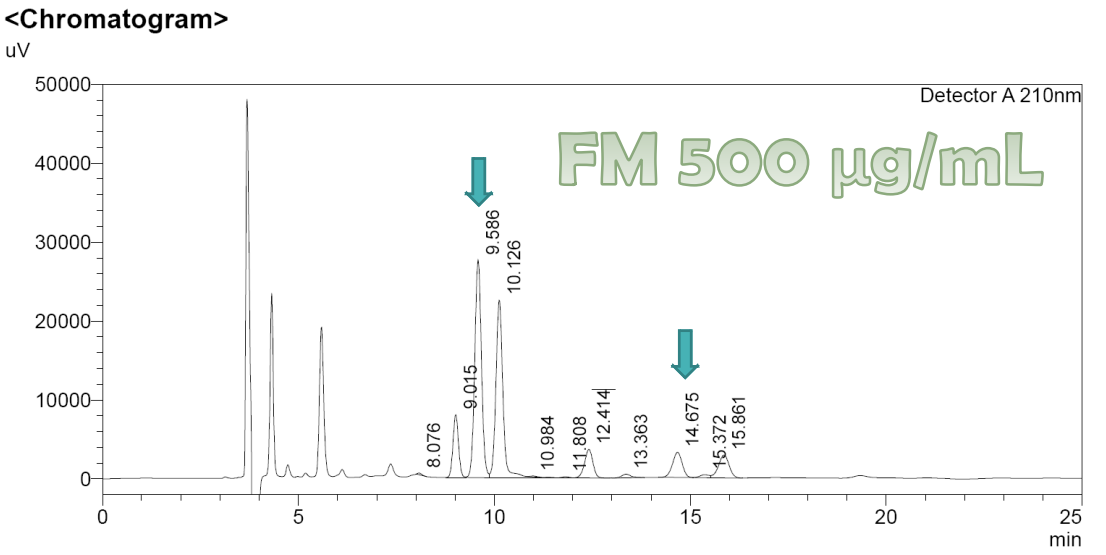
- Condition : mobile phase, pH, temp
- Concentration of standard
- Concentration of sample

Method Optimization

Catechin and Epicatechin Determination in Fermented Tea





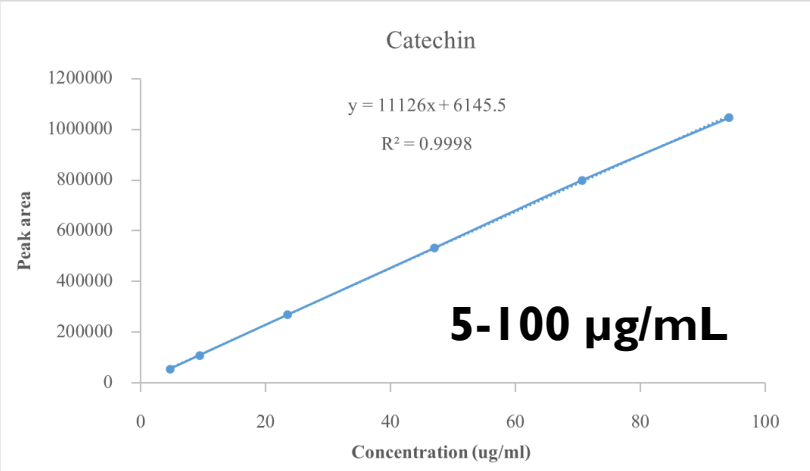


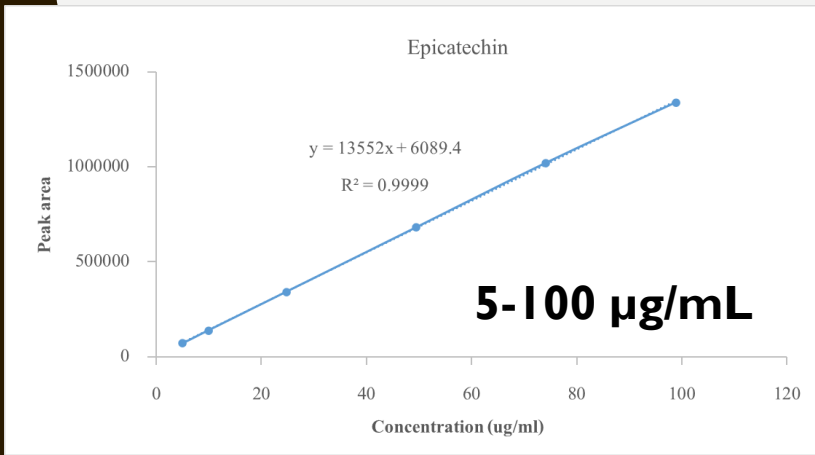
- Condition : mobile phase, pH, temp
- Concentration of standard
- Concentration of sample

Method Optimization

Catechin and Epicatechin Determination in Fermented Tea

The objective : stability indicating => LOQ – XX %





Find out the concentration of API in the test substance = The test level of 100%

Test substance concentration 500 µg/mL had Catechin: **~30 µg/mL (FM Tea)**

The test level of 100% = Catechin 30 µg/mL
80% = 24 µg/mL & 120% = 36 µg/mL

METHOD VALIDATION PROCEDURE

System Suitability

- Confirmatory test(s) procedures and parameters to ensure that the system (equipment, electronics, and analytical operations and controls to be analyzed) will function correctly as an integrated system at the time of use. The system suitability acceptance criteria applied to standards controls and samples, such as **peak tailing, precision, capacity factor, S/N, Theoretical plate and resolution acceptance criteria**, may be required as applicable. For system suitability of chromatographic systems, refer to the FDA guidance for industry on *Validation of Chromatographic Methods* and **USP General Chapter <621> *Chromatography***.

METHOD VALIDATION PROCEDURE

Method Validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose.

- Identification
- Quantitative tests for impurities' content
- Limit tests for the control of impurities
- Quantitative tests of the active moiety in samples

SPECIFICITY / SELECTIVITY

ICH Q2 R2

- **Specificity** is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, related substances, matrix, or other components.

- **Absence of interference :** identification and/or quantitation of an analyte is not impacted by the presence of other substances
- **Orthogonal procedure comparison :** measured result of an analyte is comparable to the measured result of orthogonal procedure
- **Technology inherent justification :** specificity of the analytical technology can be ensured and predicted by technical parameters (e.g., resolution of isotopes in mass spectrometry, chemical shifts of NMR signals), no experimental study may be required.

SPECIFICITY / SELECTIVITY

Identification Method

- Reference material
- Sample containing the analyte (positive result)
- Sample do not contain the analyte (negative result)
- Material structurally similar or closely related to the analyte (negative result)

SPECIFICITY / SELECTIVITY

Assay, purity- and impurity test(s)

The specificity/selectivity of an analytical procedure should be demonstrated to fulfil the accuracy requirements

- Specificity can be demonstrated by the resolution of the two components which elute closest to each other.

- Reference material
- Sample (unspiked)
- Spiked sample (with impurities and/or excipients)
- Blank matrix (placebo)
- Dilution solvent
- Impurities (stress condition) => Stability indicating method

SPECIFICITY / SELECTIVITY

AOAC (2023)

Selectivity is the degree to which the method can quantify the target analyte in the presence of other analytes, matrices, or other potentially interfering materials.

- Selective solvent extraction
- Chromatographic
- Phase separations

Resolution, Rs, is expressed as a function of both the absolute separation distance expressed as retention times (minutes) of the two peaks

- At least 1.5 is usually sought
- At least 2 for active drug dosage forms, including hydrolytic, photolytic, and oxidative degradation products (USFDA suggest)

SPECIFICITY / SELECTIVITY

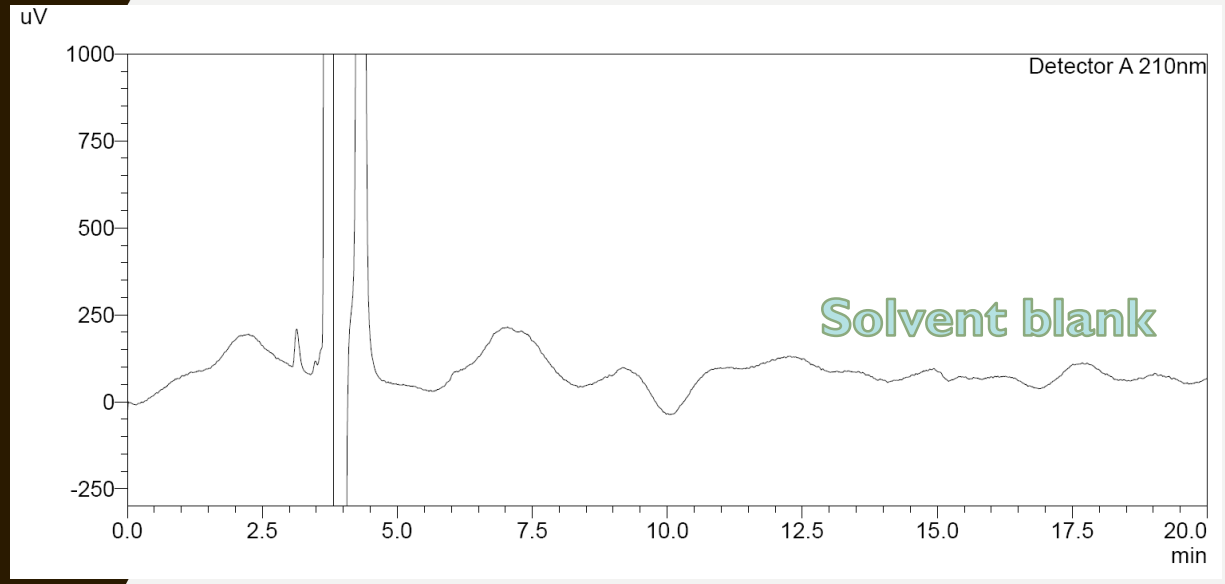
AOAC (2023)

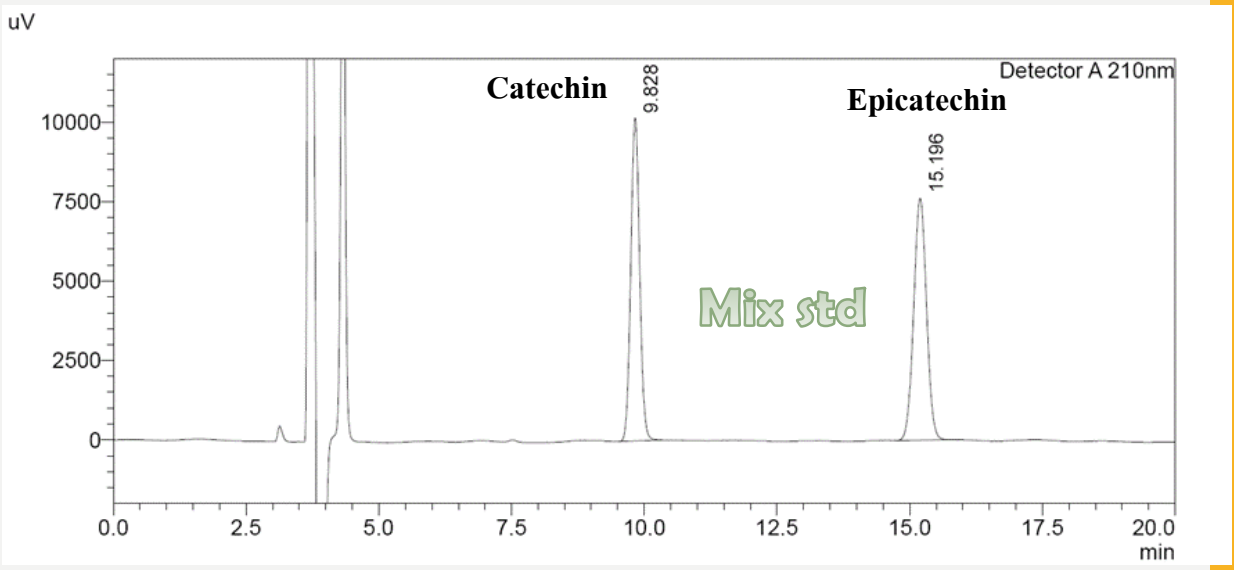
Selectivity is the degree to which the method can quantify the target analyte in the presence of other analytes, matrices, or other potentially interfering materials.

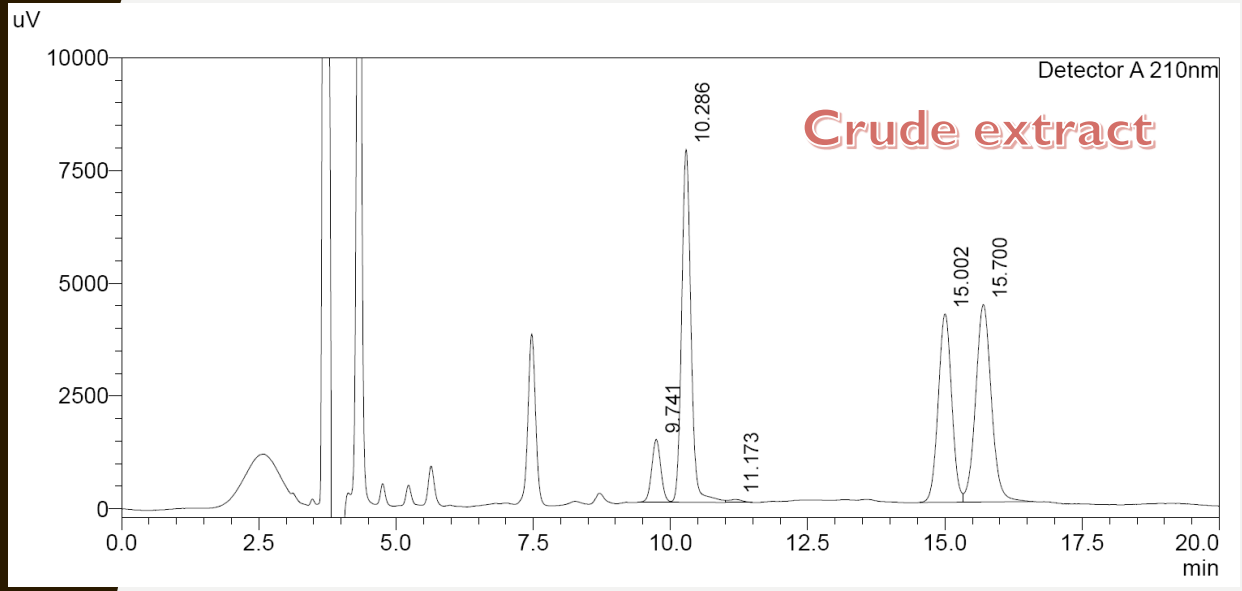
- Reference material
- Sample
- Spiked sample
- Blank matrix (placebo, formulation)
- Dilution solvent
- Impurities (stress condition) => Stability indicating method

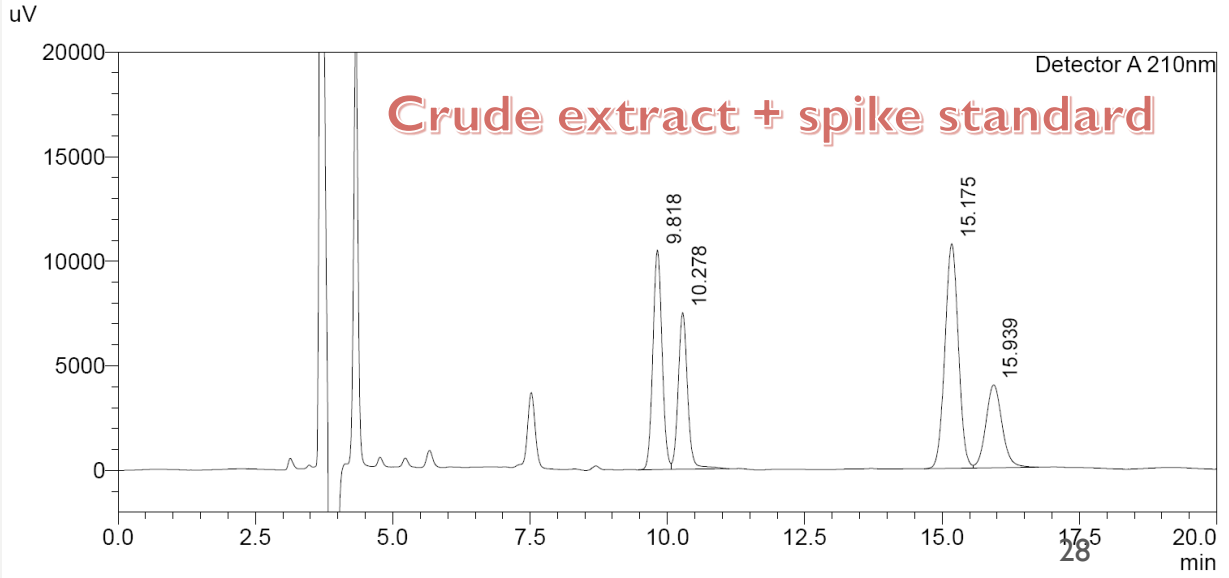
SPECIFICITY / SELECTIVITY

Catechin and Epicatechin Determination in Fermented Tea









WORKING RANGE

ICH Q2 R2

Depending on the sample preparation (e.g., dilutions) and the analytical procedure selected, the reportable range will lead to a specific working range

Linear Response

The response can be demonstrated directly on the drug substance (e.g., by dilution of a standard stock solution) or separate weightings of synthetic mixtures of the drug product components, using the proposed procedure.

- a plot of the data
- the correlation coefficient (r) or coefficient of determination (r^2)
- y-intercept and slope of the regression line
- the residuals plot (non-random pattern)

WORKING RANGE

Linear Response

ICH Q2R2

For the establishment of linearity, a minimum of five concentrations appropriately distributed across the range is recommended; however, additional concentrations may be required for more complex models.

AOAC (2023)

Calibration : Six to 8 points, approximately equally spaced over the concentration range of interest, performed in duplicate.

- the correlation coefficient (r) > 0.99
- the residuals plot (non-random pattern)

WORKING RANGE

Use of analytical procedure	Low end of reportable range
Assay	80% of lower specification limit
Potency	±20% of specification
Content uniformity	70-130% of declared content
Dissolution testing	Q-45% to 130% (immediate release) QL to 130% (modified release) ± 20% of specified range (USP)
Impurity testing	Reporting threshold to 120% 50-120% acceptance criterion (USP)
Purity testing (as area %)	80-100% of specification limit

WORKING RANGE

ICH Q2 R2

Depending on the sample preparation (e.g., dilutions) and the analytical procedure selected, the reportable range will lead to a specific working range

Sample Preparation (aspirin tablet)

ASSAY

- **PROCEDURE**

Mobile phase: 2 g/L of sodium 1-heptanesulfonate in a mixture of acetonitrile and water (15:85). Adjust with glacial acetic acid to a pH of 3.4.

Diluent: Acetonitrile and formic acid (99:1)

Standard solution: 0.5 mg/mL of USP Aspirin RS in *Diluent*

Sample stock solution: Nominally 5 mg/mL of aspirin prepared as follows. Transfer a quantity, equivalent to about 100 mg of aspirin from NLT 20 finely powdered Tablets, to a suitable container. Add 20.0 mL of *Diluent* and 10 glass beads. Shake vigorously for about 10 min, and centrifuge.

Sample solution: Nominally 0.5 mg/mL of aspirin in *Diluent* from *Sample stock solution*

→ **5 mg/mL** →

0.4 mg/mL

↑

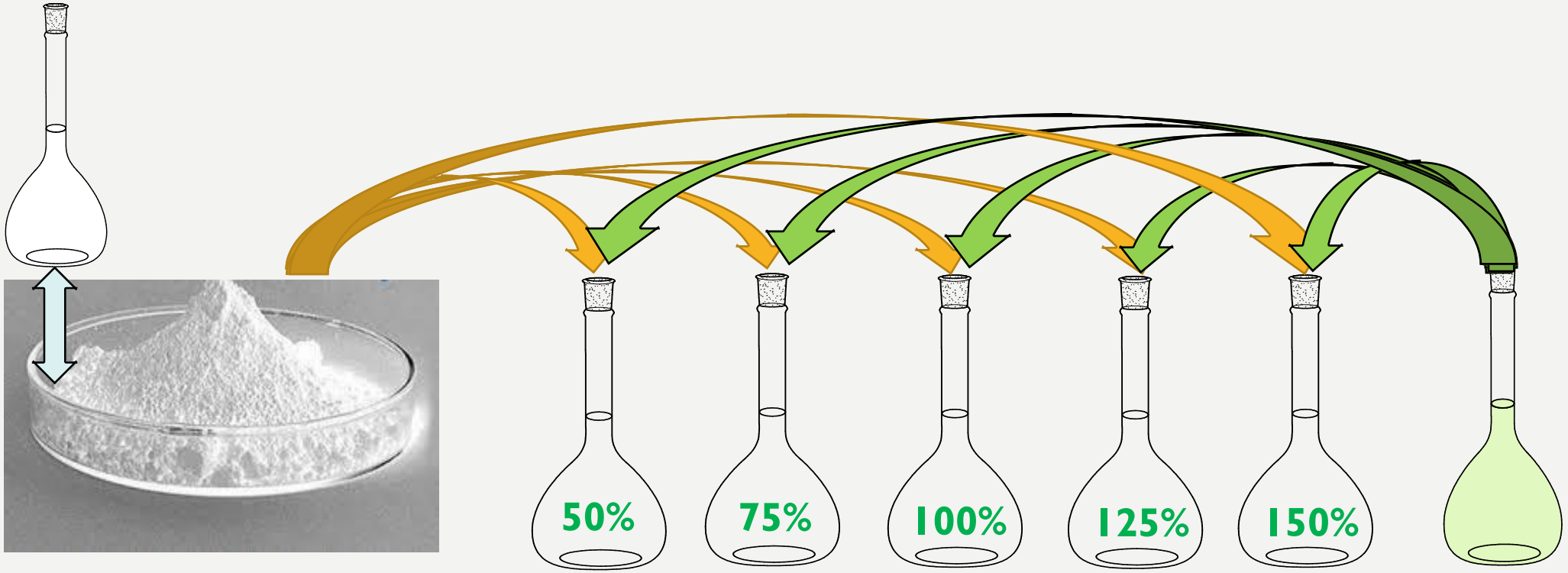
0.5 mg/mL

↓

0.6 mg/mL

WORKING RANGE

Drug product (aspirin tablet)

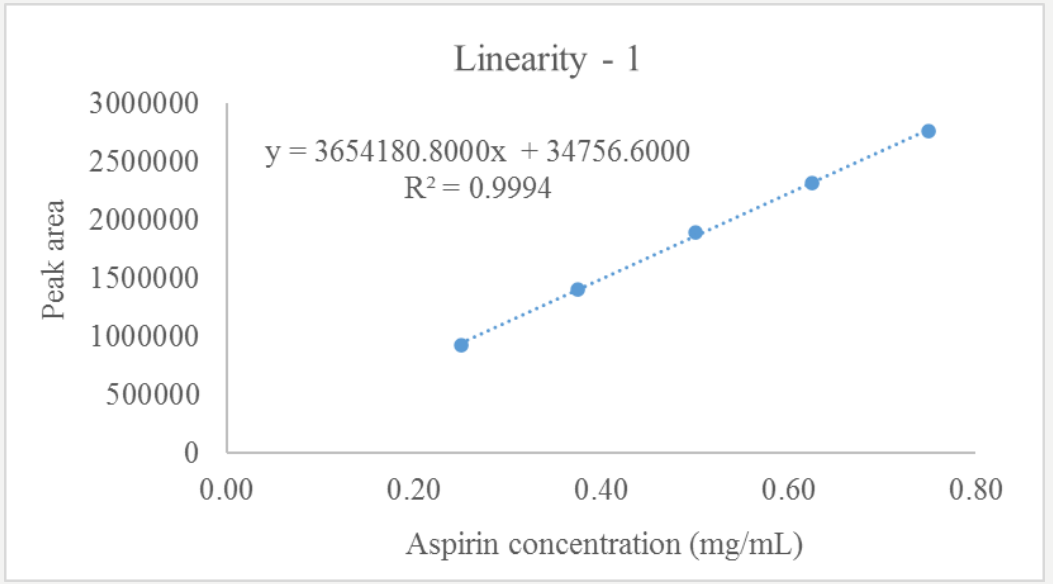


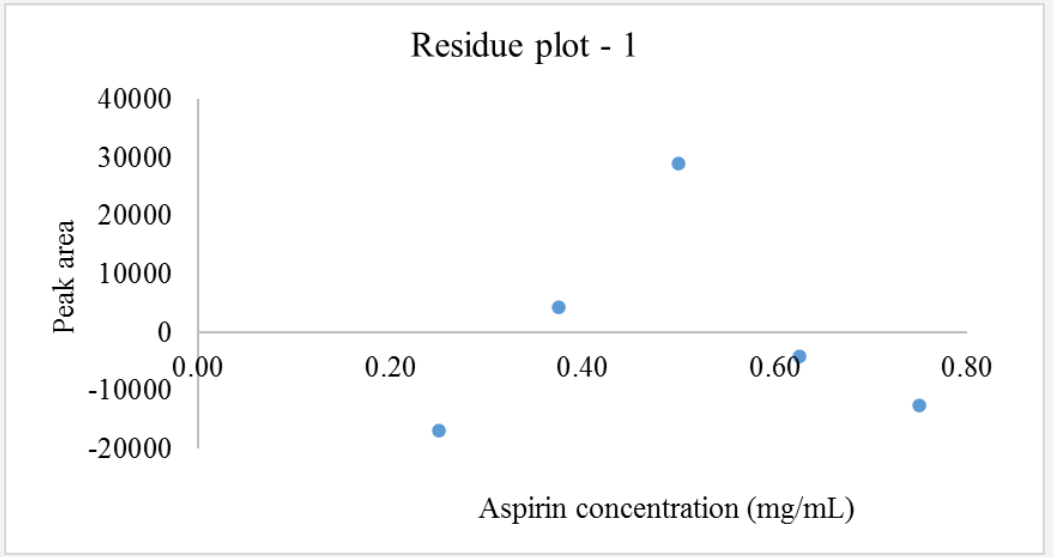
Sample matrix (placebo)

5 points x Triplicate

Std stock 5 mg/mL

Standard curve solutions spike matrix				
Standard aspirin concentraion (mg/mL)	Actual peak area			
	Linear 1	Linear 2	Linear 3	Average
0.2500	931521.0	929511.0	912307.0	924446.3
0.3750	1409467.0	1376515.0	1391157.0	1392379.7
0.5000	1890856.0	1851009.0	1838093.0	1859986.0
0.6250	2314547.0	2421425.0	2350228.0	2362066.7
0.7500	2762844.0	2806480.0	2806480.0	2791934.7
r	**0.9997**	**0.9986**	**0.9998**	**0.9998**
r^2	**0.9994**	**0.9973**	**0.9997**	**0.9996**
Slope	**3654180.8**	**3839078.4**	**3797933.6**	**3763730.9**
Intercept	**34756.6**	**-42551.2**	**-39313.8**	**-15702.8**
% y-intercept	**1.84**	**-2.30**	**-2.14**	**-0.84**
Predicted Area				
Standard aspirin concentraion (mg/mL)	Linear 1	Linear 2	Linear 3	Average
0.2500	948301.8	917218.4	910169.6	925229.9
0.3750	1405074.4	1397103.2	1384911.3	1395696.3
0.5000	1861847.0	1876988.0	1859653.0	1866162.7
0.6250	2318619.6	2356872.8	2334394.7	2336629.0
0.7500	2775392.2	2836757.6	2809136.4	2807095.4
Residue				
Standard aspirin concentraion (mg/mL)	Linear 1	Linear 2	Linear 3	Average
0.2500	-16780.8	12292.6	2137.4	-783.6
0.3750	4392.6	-20588.2	6245.7	-3316.6
0.5000	29009.0	-25979.0	-21560.0	-6176.7
0.6250	-4072.6	64552.2	15833.3	25437.6
0.7500	-12548.2	-30277.6	-2656.4	-15160.7

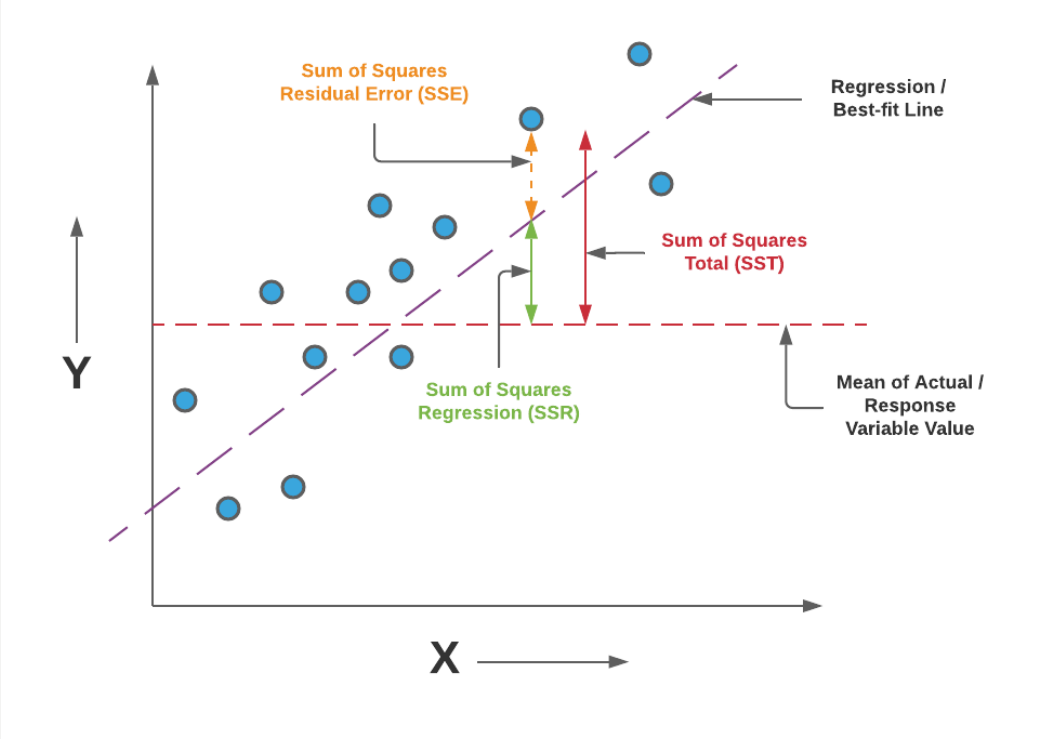




Residuals = Area Observed – Area Predicted

RESIDUAL PLOT

Residuals = Area Observed – Area Predicted



Residual values are randomly spaced around the horizontal axis and no specific trend of data are observed

WORKING RANGE

Drug product (aspirin tablet)

Standard curve solutions spike matrix				
Standard aspirin concentraion (mg/mL)	Actual peak area			
	Linear 1	Linear 2	Linear 3	Average
0.2500	931521.0	929511.0	912307.0	924446.3
0.3750	1409467.0	1376515.0	1391157.0	1392379.7
0.5000	1890856.0	1851009.0	1838093.0	1859986.0
0.6250	2314547.0	2421425.0	2350228.0	2362066.7
0.7500	2762844.0	2806480.0	2806480.0	2791934.7
r	0.9997	0.9986	0.9998	0.9998
r^2	0.9994	0.9973	0.9997	0.9996
Slope	3654180.8	3839078.4	3797933.6	3763730.9
Intercept	34756.6	-42551.2	-39313.8	-15702.8
% y-intercept	1.84	-2.30	-2.14	-0.84

%y-intercept

- assay ±2%, impurity ±5%
- multiple point calibration
- single point calibration

% Intercept = (y-intercept x 100) / response at 100%
= -15702.8 x 100 / 1859986.0
= -0.8442

WORKING RANGE

Sample Preparation (Herbal)

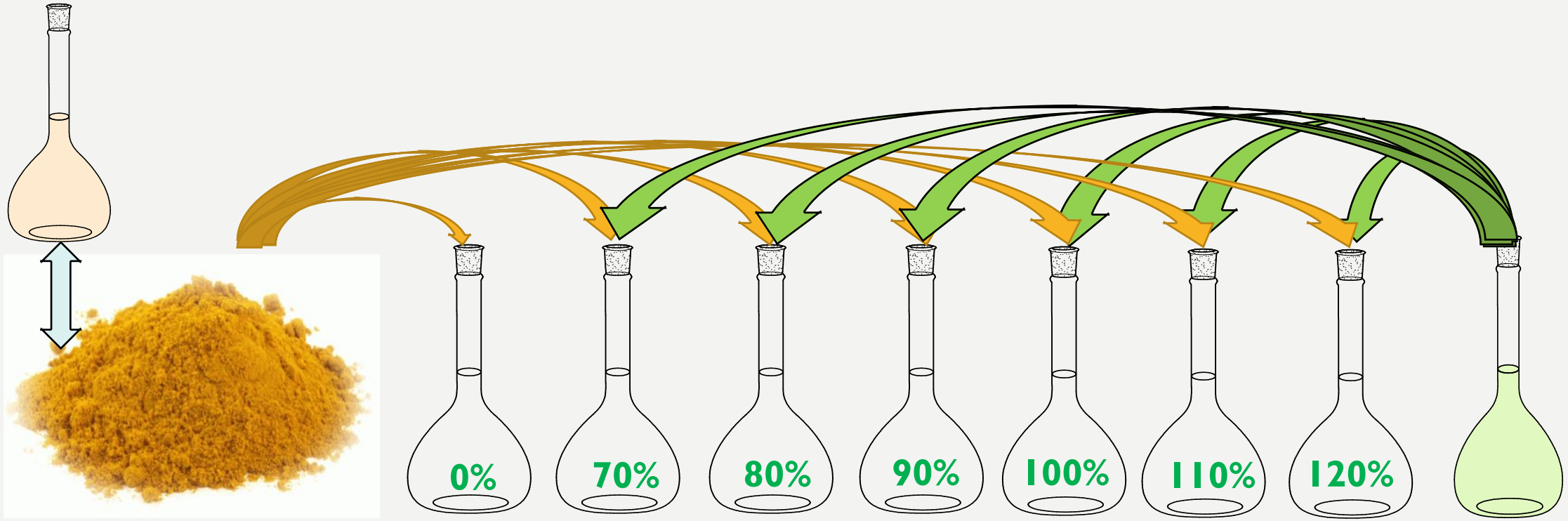
2.4. Preparation of standard and sample solutions

Stock solutions of curcumin, DEMC and BDEMC (about 1 mg/ml) were prepared in 95 ml/100 ml ethanol in water and stored at around 4 °C condition. The stock solution was then diluted with 95 ml/100 ml ethanol in water to the appropriate concentration range to establish calibration curves.

0.4 ml of water-soluble or oil-soluble turmeric colour principals and about 0.0800 g of solid powder of turmeric colour principals were accurately dissolved in 10 mL 95 ml/100 ml ethanol in water, respectively. Curry powder and rhizoma curcumae longae (about 1.0000 g) were accurately weighed and extracted by ultrasonication at 40 °C for 20 min with 20 mL 95 ml/100 ml ethanol in water. The solution of four times extractions was combined and adjusted to the 100 mL. The primary filtrate was discarded. The subsequent filtrate was used for HPLC analysis. Then each solution was filtered through a 0.45 μm nylon 66 membrane prior to analysis and injected into HPLC system. The injection volume was 10 μL.

Optimization data

WORKING RANGE



Sample

6 to 8 points x duplicate

Std stock
1 mg/mL

WORKING RANGE (LOWER RANGE LIMITS)

Detection limit (DL) and Quantitation limit (QL)

ICH Q2R2

1. **Based on visual evaluation**
2. **Based on Signal-to-Noise (DL = 3:1, QL = 10:1)**
3. **Based on the Standard Deviation of a Linear Response and a Slope**

$$DL = \frac{3.3\,\sigma}{S}$$

$$QL = \frac{10\,\sigma}{S}$$

σ = the standard deviation of the response
S = the slope of the calibration curve

WORKING RANGE (LOWER RANGE LIMITS)

Detection limit (DL)

ICH Q2R2

- Based on visual evaluation
- Based on Signal-to-Noise (DL = 3:1)
- By calculation or extrapolation : this estimate can subsequently be validated by the analysis of a suitable number of samples known to be near or at the DL

Quantitation limit (QL)

- If the QL was estimated, the limit should be subsequently validated by the analysis of a suitable number of samples known to be near or at the QL
- For impurity tests, the quantitation limit for the analytical procedure should be equal to or below the reporting threshold.

Based on the Standard Deviation of the Response and the Slope

std curve	Intercept	Slope	r^2
1	6145.51	11125.55	0.9998
2	-4368.61	12848.38	1.0000
3	4217.89	13877.75	0.9999
4	6145.50	10480.00	0.9998
5	4883.37	14772.40	0.9997
6	16204.59	15355.30	0.9996
Ave	5538.04	13076.56	0.9998
SD	5983.68	1794.02	0.0001

LOD = 3.3 x 5983.68/1307.56

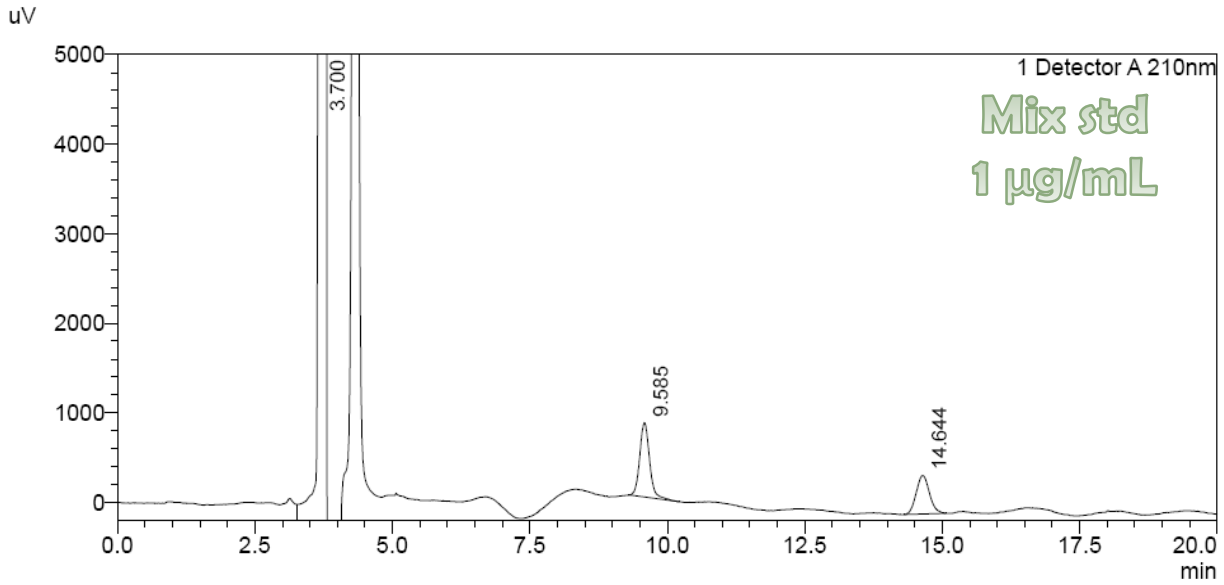
= 1.51 µg/mL

LOQ = 10 x 5983.68/1307.56

= 4.58 µg/mL

Based on Signal-to-Noise

<Chromatogram>

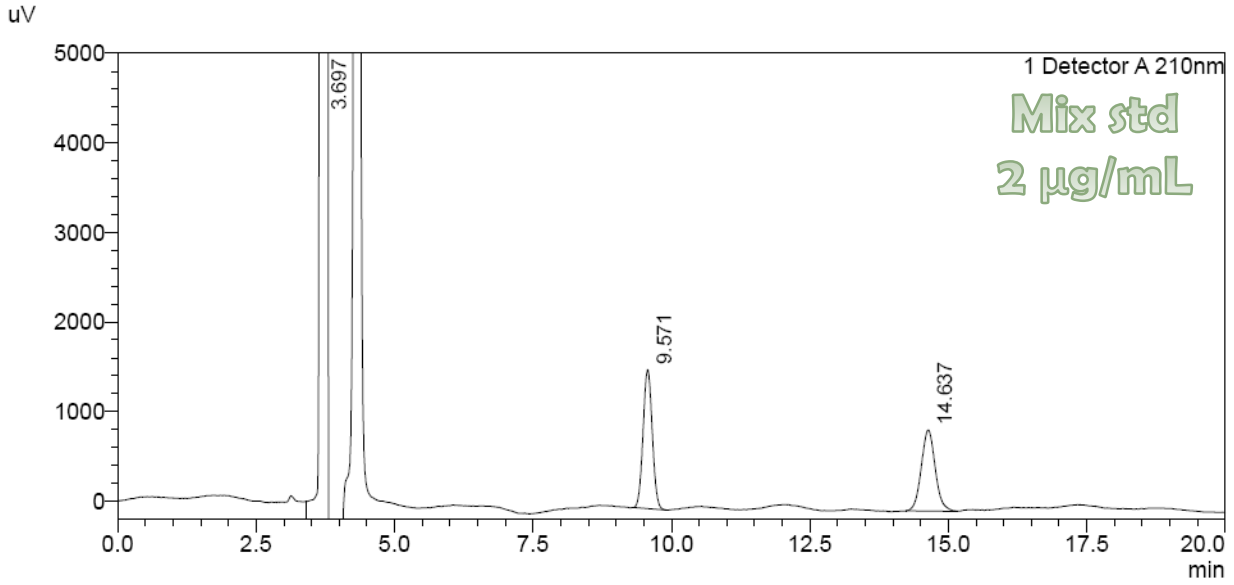


<Peak Table>

Detector A 210nm

Peak#	Ret. Time	Area	Tailing Factor	Resolution(USP)	Capacity Factor(k')	S/N	Noise
1	3.700	1499745	0.700	--	--	55.88	1890.66
2	9.585	10232	1.134	19.772	1.591	0.44	1890.66
3	14.644	7182	1.133	13.208	2.958	0.23	1890.66
Total		1517160					

<Chromatogram>



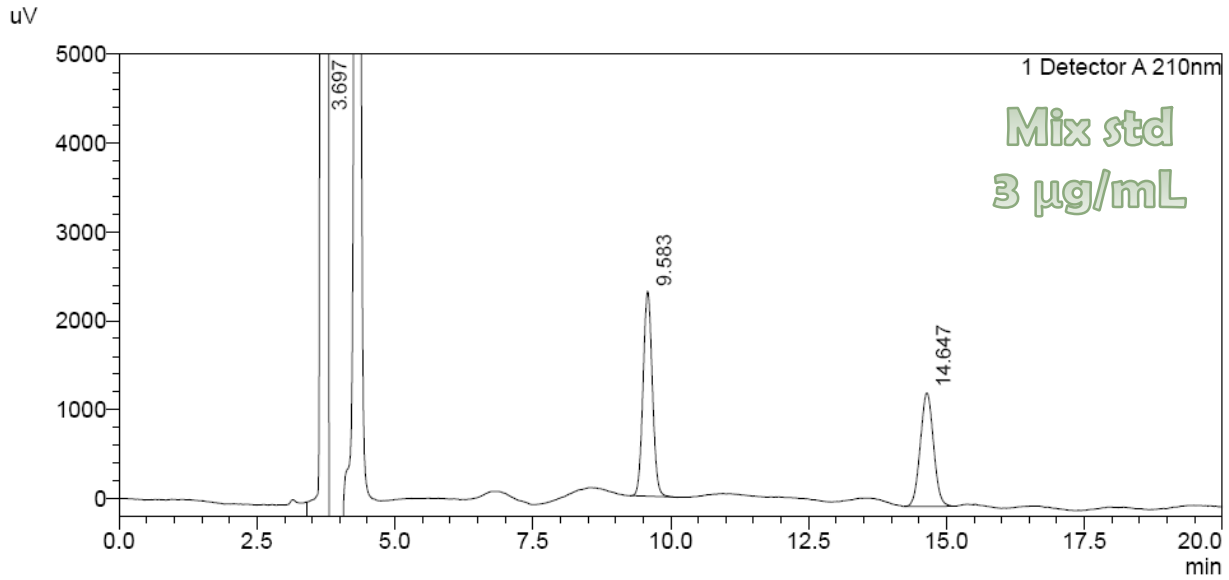
<Peak Table>

Detector A 210nm

Peak#	Ret. Time	Area	Tailing Factor	Resolution(USP)	Capacity Factor(k')	S/N	Noise
1	3.697	1164169	0.811	--	--	52.96	1867.69
2	9.571	17824	1.048	20.533	1.589	0.83	1867.69
3	14.637	15833	1.086	13.162	2.959	0.48	1867.69
Total		1197826					

Based on Signal-to-Noise

<Chromatogram>

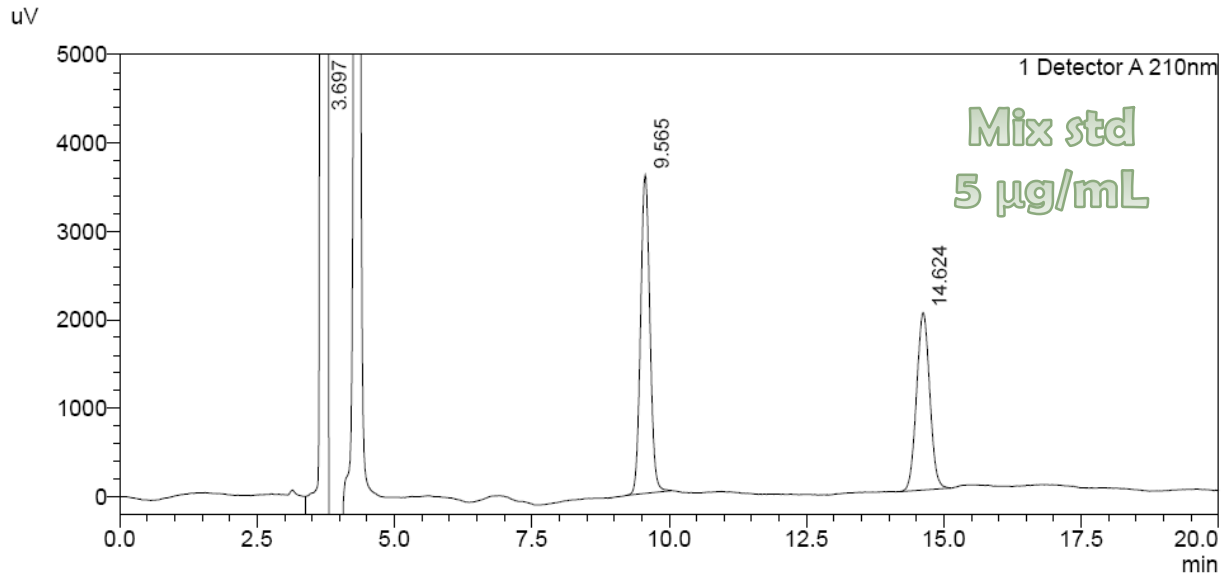


<Peak Table>

Detector A 210nm

Peak#	Ret. Time	Area	Tailing Factor	Resolution(USP)	Capacity Factor(k')	S/N	Noise
1	3.697	1168213	0.801	--	--	53.16	1849.54
2	9.583	26922	1.065	20.420	1.592	1.25	1849.54
3	14.647	21337	1.040	13.327	2.962	0.69	1849.54
Total		1216472					

<Chromatogram>



<Peak Table>

Detector A 210nm

Peak#	Ret. Time	Area	Tailing Factor	Resolution(USP)	Capacity Factor(k')	S/N	Noise
1	3.697	1181741	0.777	--	--	54.46	1770.16
2	9.565	42169	1.066	20.314	1.588	2.03	1770.16
3	14.624	33461	1.040	13.332	2.956	1.13	1770.16
Total		1257371					

DETECTION & DETERMINATION LIMIT

AOAC 2023

- limit of detection and limit of determination is based upon the variability of the blank. The blank value, x_{Bl}, plus 3 times the standard deviation of the blank ($x_{Bl} + 3s_{Bl}$) is taken as the detection limit and the blank value plus 10 times the standard deviation of the blank ($x_{Bl} + 10s_{Bl}$) is taken as the determination limit.

ACCURACY

ICH Q2 R2

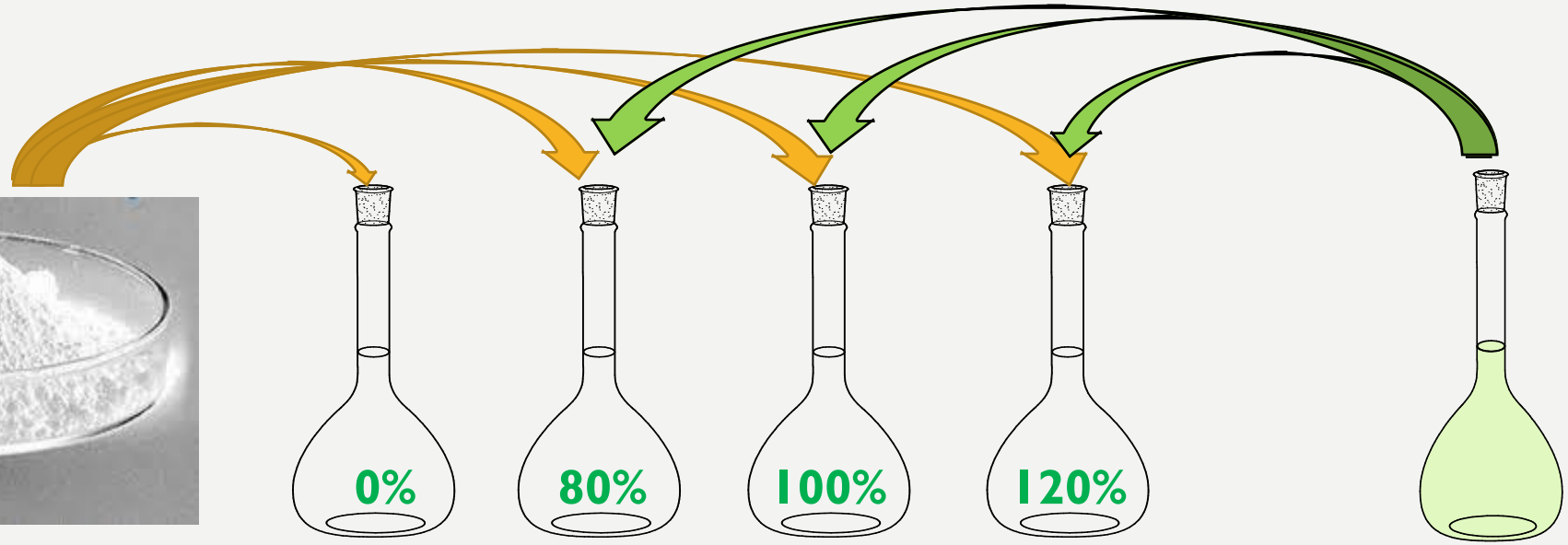
- Accuracy should be established across the reportable range of an analytical procedure
- Accuracy should be demonstrated under regular test conditions of the analytical procedure (in the presence of sample matrix)

- Reference material comparison (drug substance)
- Spiking Study
- Orthogonal Procedure comparison

ACCURACY

At least 3 conc x 3 replicates

Sample matrix (placebo)



x 3 replicates

Std stock

ACCURACY

ICH Q2 R2

Accuracy should be reported as the mean percent recovery by the assay of a known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

$$\% \text{ Recovery} = \frac{\text{Conc. Found}}{\text{Conc. Added}} \times 100$$

$$\text{The 95\% Confidence interval} = \text{mean} \pm (t_{(0.05,n-1)} \times SD)/ \sqrt{n}$$

Sample	No.	Peak area	Observed concentration (mg/mL)	Recovery (%)
Accuracy solutions (0.25 mg/mL)	1	931521	0.2457	98.30
	2	929511	0.2452	98.08
	3	958150	0.2529	101.15
Accuracy solutions (0.50 mg/mL)	1	1890856	0.5026	100.52
	2	1851009	0.4919	98.39
	3	1904496	0.5063	101.25
Accuracy solutions (0.75 mg/mL)	1	2762844	0.7361	98.14
	2	2806480	0.7478	99.70
	3	2825335	0.7528	100.38
Mean (n=9)				**99.55**
SD (n=9)				**1.33**
%RSD (n=9)				**1.34**
95% confidence interval				**1.02**

ACCURACY

- Acceptance criteria: 98-102%

The 95% Confidence interval = mean $\pm$ ($t_{(0.05,n-1)}$ x SD)/ $\sqrt{n}$

t Table

cum. prob	$t_{.50}$	$t_{.75}$	$t_{.80}$	$t_{.85}$	$t_{.90}$	$t_{.95}$	$t_{.975}$	$t_{.99}$	$t_{.995}$	$t_{.999}$	$t_{.9995}$
one-tail	0.50	0.25	0.20	0.15	0.10	0.05	0.025	0.01	0.005	0.001	0.0005
two-tails	1.00	0.50	0.40	0.30	0.20	0.10	0.05	0.02	0.01	0.002	0.001
df											
1	0.000	1.000	1.376	1.963	3.078	6.314	12.71	31.82	63.66	318.31	636.62
2	0.000	0.816	1.061	1.386	1.886	2.920	4.303	6.965	9.925	22.327	31.599
3	0.000	0.765	0.978	1.250	1.638	2.353	3.182	4.541	5.841	10.215	12.924
4	0.000	0.741	0.941	1.190	1.533	2.132	2.776	3.747	4		
5	0.000	0.727	0.920	1.156	1.476	2.015	2.571	3.365	4		
6	0.000	0.718	0.906	1.134	1.440	1.943	2.447	3.143	3		
7	0.000	0.711	0.896	1.119	1.415	1.895	2.365	2.998	3		
8	0.000	0.706	0.889	1.108	1.397	1.860	2.306	2.896	3		
9	0.000	0.703	0.883	1.100	1.383	1.833	2.262	2.821	3		
10	0.000	0.700	0.879	1.093	1.372	1.812	2.228	2.764	3		
11	0.000	0.697	0.876	1.088	1.363	1.796	2.201	2.718	3		
12	0.000	0.695	0.873	1.083	1.356	1.782	2.179	2.681	3		
13	0.000	0.694	0.870	1.079	1.350	1.771	2.160	2.650	3.012	3.852	4.221
14	0.000	0.692	0.868	1.076	1.345	1.761	2.145	2.624	2.977	3.787	4.140
15	0.000	0.691	0.866	1.074	1.341	1.753	2.131	2.602	2.947	3.733	4.073
16	0.000	0.690	0.865	1.071	1.337	1.746	2.120	2.583	2.921	3.686	4.015
17	0.000	0.689	0.863	1.069	1.333	1.740	2.110	2.567	2.898	3.646	3.965

= 99.55 $\pm$ (2.306 x 1.33)/ $\sqrt{9}$

= 99.55 $\pm$ 1.02

= 98.53 – 100.57 %

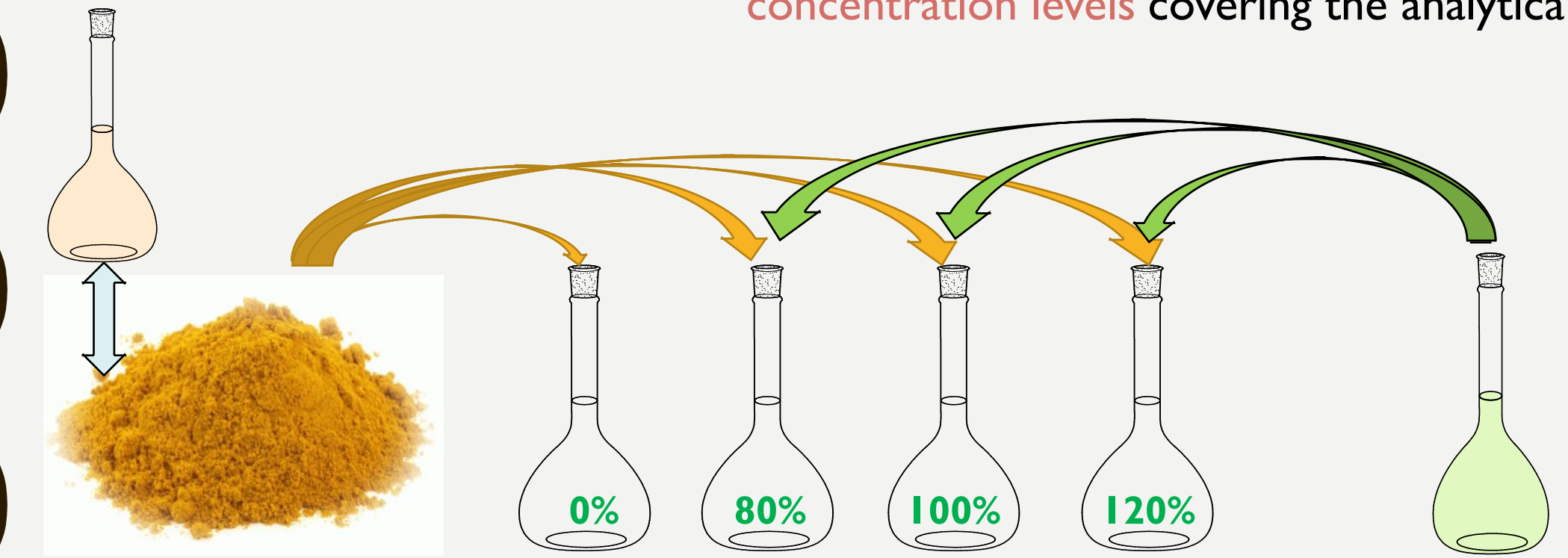
ACCURACY

AOAC 2023

- The available certified or commercial analyte standard, diluted if necessary, is added to typical analyte-free matrices at levels about 1x or 2x the expected concentration.
- Analyte-free matrices for residues are obtained from growers who certify that the chemical is not used in their cultivation, growth, or feeding and verified analytically. They may also be obtained from the residues of previously extracted materials or from test samples shown to be negative for the analyte.
- If an analyte-free matrix is not available, the analyte standard is added to separate test portions and the recovery is calculated from the base determined by the method of addition.

ACCURACY

Determined from spiked blanks or samples with at least seven independent analyses per concentration level at a minimum of three concentration levels covering the analytical range



Sample or (analyte-free matrices)

3 conc x 7 replicates

Std stock 1 mg/mL

ACCURACY

AOAC 2023

- Marginal % recovery = $(C_f - C_u) \times 100 / C_A$
- Total % recovery = $100(C_f) / (C_u + C_A)$

where

C_f = concentration of fortified samples,

C_u = concentration of unfortified samples

C_A = concentration of analyte added to the test sample

ACCURACY

Expected recovery as a function of analyte concentration

AOAC 2023

Analyte, %	Mass fraction (C)	Unit	Mean recovery, %
100	1	100%	98-102
10	10^{-1}	10%	
1	10^{-2}	1%	97-103
0.1	10^{-3}	0.1%	95-105
0.01	10^{-4}	100 ppm (mg/kg)	90-107
0.001	10^{-5}	10 ppm (mg/kg)	80-110
0.0001	10^{-6}	1 ppm (mg/kg)	
0.00001	10^{-7}	100 ppb (µg/kg)	
0.000001	10^{-8}	10 ppb (µg/kg)	60-115
0.0000001	10^{-9}	1 ppb (µg/kg)	40-120

ACCURACY (EXAMPLE)

Accuracy of the three curcuminoids in turmeric pigment.

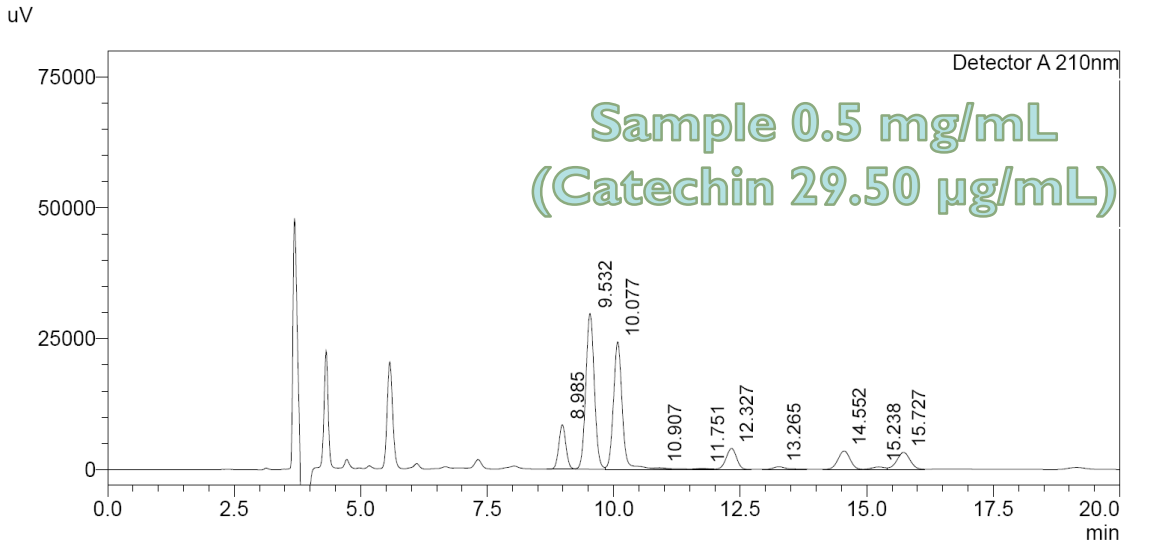
Analytes	Compound	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)[a]	RSD (%)[b]
Water-soluble	Curcumin	189.2	95.8	284.3	99.3	2.18
			191.7	384.3	104.0	2.14
			268.4	454.2	98.8	1.72
	DEMC	55.8	35.5	92.2	102.6	2.46
			53.2	111.3	100.1	2.00
			74.5	131.5	101.6	3.11
	BDEMC	154.1	85.7	238.5	98.5	
			171.4	325.0	101.3	
			239.9	392.7	99.4	1.82
Oil-soluble	Curcumin	60.7	31.6	92.6	100.8	3.09
			79.1	14		
			102.8	16		
	DEMC	33.1	14.8	4		
			37.1	70.9	102.0	2.62
			48.2	80.7	98.8	2.90
	BDEMC	70.4	35.9	107.1	101.9	2.94
			89.9	162.8	102.7	3.56
			116.9	187.1	99.8	2.82
Solid powder	Curcumin	336.8	160.0	493.4	97.8	2.32
			319.9	664.7	102.4	2.99
			415.9	767.5	103.5	2.97
	DEMC	66.8	48.8	114.8	98.2	3.08
			63.5	132.7	103.7	2.21
			82.5	147.4	97.7	1.46
	BDEMC	33.6	24.5	58.4	101.2	2.80
			31.9	66.4	103.0	3.36
			41.4	76.6	103.9	2.54

Marginal % recovery = $(C_f - C_u) \times 100 / C_A$

Recovery (%) = 100 x (measured amount - original amount) / spiked amount

ACCURACY

Catechin and Epicatechin Determination in Fermented Tea

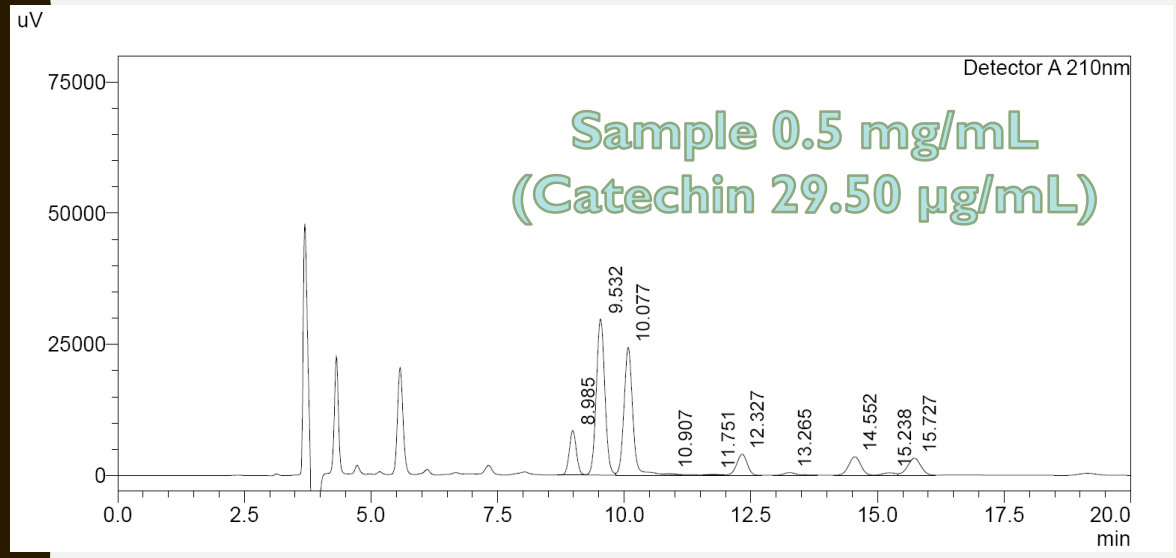


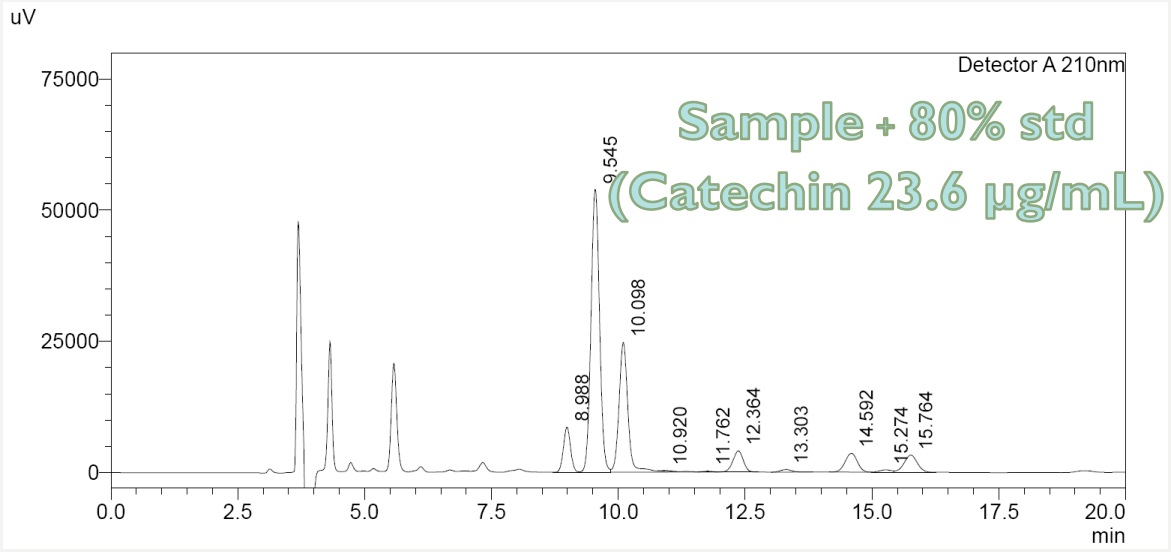
- At least **3 conc x 7 replicates**
  - 80%, 100%, 120%

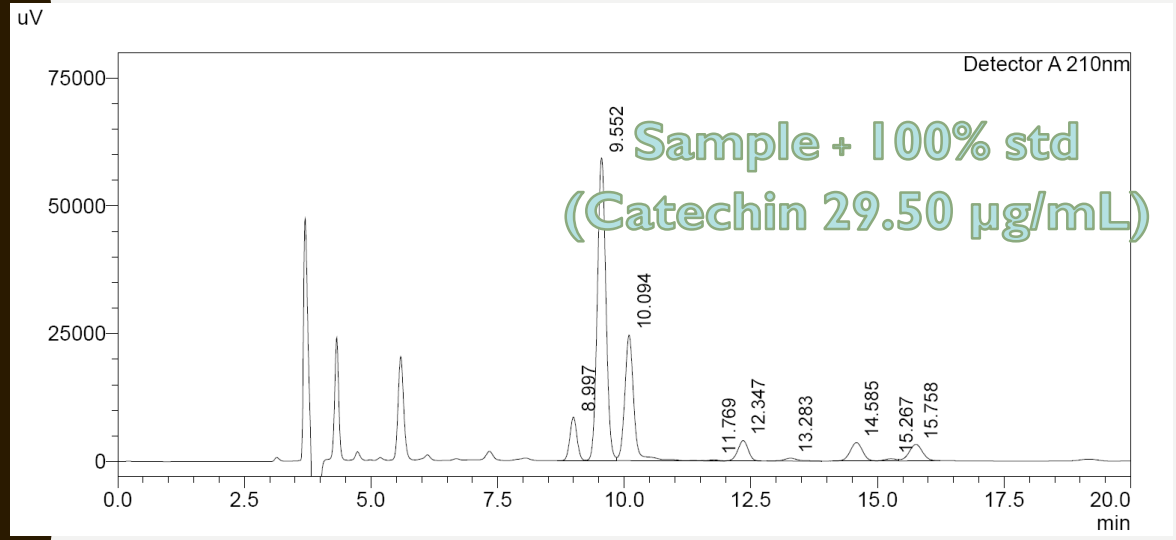
$$\% \text{ Recovery} = \frac{\text{Conc. Found}}{\text{Conc. Added}} \times 100$$

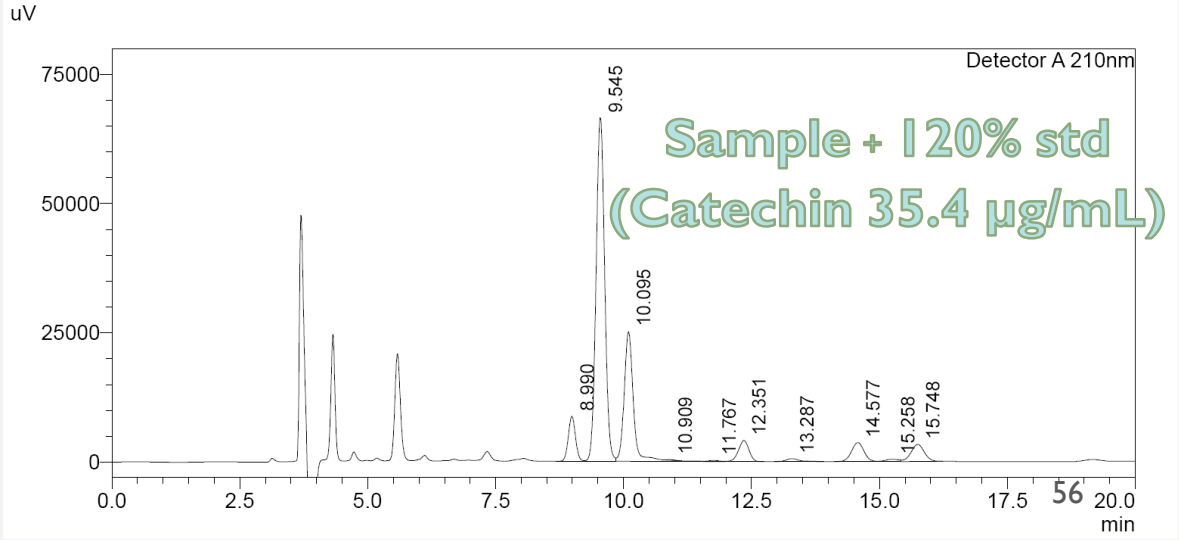
ACCURACY

- At least 3 conc x 7 replicates
- 80%, 100%, 120%









sample	%std	added conc.(ug/ml)	added conc * purity/100	PA	average PA	(sp PA+std) - sp PA	found conc.(ug/ml)	Accuracy (% recovery)	Precision (%RSD)
ซาหมัก	0			328539	325311		29.82		
				328468			29.81		
				324748					
				325342					
				323096			29.32		
				323097			29.32		
				323889			29.39		
	80	23.75	22.37	590462	593428	265151	24.00	107.26	1.48
				590941		265630	24.04	107.46	
				596206		270895	24.52	109.62	
				595107		269796	24.42	109.17	
				595105		269794	24.42	109.17	
				598729		273418	24.75	110.66	
				587448		262137	23.72	106.03	
	100	29.67	27.95	629877	633278	304566	27.62	98.81	2.38
				635291		309980	28.12	100.59	
				632419		307108	27.85	99.65	
				632420		307109	27.85	99.65	
				647288		321977	29.22	104.54	
				623405		298094	27.02	96.68	
				631387		306076	27.76	99.31	
	120	35.58	33.52	706195	703627	380884	34.63	103.32	2.07
				693527		368216	33.47	99.84	
				693530		368219	33.47	99.85	
				705425		380114	34.56	103.11	
				699899		374588	34.05	101.59	
				701285		375974	34.18	101.97	
				715433		390122	35.48	105.85	
							Mean	**103.53**	**1.98**
							SD	**4.20**	
							%RSD	**4.06**	

$$\text{Marginal \% recovery} = (C_f - C_u) \times 100 / C_A$$

ACCURACY

The 95% Confidence interval = mean $\pm$ ($t_{(0.05,n-1)}$ x SD)/ $\sqrt{n}$

t Table

cum. prob	$t_{.50}$	$t_{.75}$	$t_{.80}$	$t_{.85}$	$t_{.90}$	$t_{.95}$	$t_{.975}$	$t_{.99}$	$t_{.995}$	$t_{.999}$	$t_{.9995}$
one-tail	0.50	0.25	0.20	0.15	0.10	0.05	0.025	0.01	0.005	0.001	0.0005
two-tails	1.00	0.50	0.40	0.30	0.20	0.10	0.05	0.02	0.01	0.002	0.001
df											
1	0.000	1.000	1.376	1.963	3.078	6.314	12.71	31.82	63.66	318.31	636.62
2	0.000	0.816	1.061	1.386	1.886	2.920	4.303	6.965	9.925	22.327	31.599
3	0.000	0.765	0.978	1.250	1.638	2.353	3.182	4.541	5.841	10.215	12.924
4	0.000	0.741	0.941	1.190	1.533	2.132	2.776	3.747	4.604	7.173	8.610
5	0.000	0.727	0.920	1.156	1.476	2.015	2.571	3.365	4.032	5.893	6.869
6	0.000	0.718	0.906	1.134	1.440	1.943	2.447	3.143	3.707	5.208	5.959
7	0.000	0.711	0.896	1.119	1.415	1.895	2.365	2.998	3.499	4.785	5.408
8	0.000	0.706	0.889	1.108	1.397	1.860	2.306	2.896	3.355	4.501	5.041
9	0.000	0.703	0.883	1.100	1.383	1.833	2.262	2.821	3.250	4.297	4.781
10	0.000	0.700	0.879	1.093	1.372	1.812	2.228	2.764	3.169	4.144	4.587
11	0.000	0.697	0.876	1.088	1.363	1.796	2.201	2.718	3.106	4.025	4.437
12	0.000	0.695	0.873	1.083	1.356	1.782	2.179	2.681	3.055	3.930	4.318
13	0.000	0.694	0.870	1.079	1.350	1.771	2.160	2.650	3.012	3.852	4.221
14	0.000	0.692	0.868	1.076	1.345	1.761	2.145	2.624	2.977	3.787	4.140
15	0.000	0.691	0.866	1.074	1.341	1.753	2.131	2.602	2.947	3.733	4.073
16	0.000	0.690	0.865	1.071	1.337	1.746	2.120	2.583	2.921	3.686	4.015
17	0.000	0.689	0.863	1.069	1.333	1.740	2.110	2.567	2.898	3.646	3.965
18	0.000	0.688	0.862	1.067	1.330	1.734	2.101	2.552	2.878	3.610	3.922
19	0.000	0.688	0.861	1.066	1.328	1.729	2.093	2.539	2.861	3.579	3.883
20	0.000	0.687	0.860	1.064	1.325	1.725	2.086	2.528	2.845	3.552	3.850
21	0.000	0.686	0.859	1.063	1.323	1.721	2.080	2.518	2.831	3.527	3.819

= 103.53 $\pm$ (2.086 x 4.20)/ $\sqrt{21}$

= 103.53 $\pm$ 1.91

= 101.62 – 105.44 %

Test concentration : 23-35 (ug/ml)

Acceptance criteria:

100 ppm => recover 90-107%

PRECISION

% RSD ≤ 2.0%

ICH Q2 R2

Precision should be investigated using homogeneous, authentic samples or artificially prepared samples (e.g., matrix mixtures spiked with relevant amounts of the analyte in question).

- **Repeatability**

1. a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each);

2. a minimum of 6 determinations at 100% of the test concentration

PRECISION

% RSD ≤ 2.0%

ICH Q2 R2

- **Intermediate Precision**

Typical variations to be studied include different days, environmental conditions, analysts and equipment, as relevant. Ideally, the variations tested should be based on and justified by using analytical procedure

- **Reproducibility**

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias.

PRECISION

artificially prepared samples

- 9 determinations
- At least 3 conc x 3 replicates
- 80%, 100%, 120%

Sample matrix (placebo)

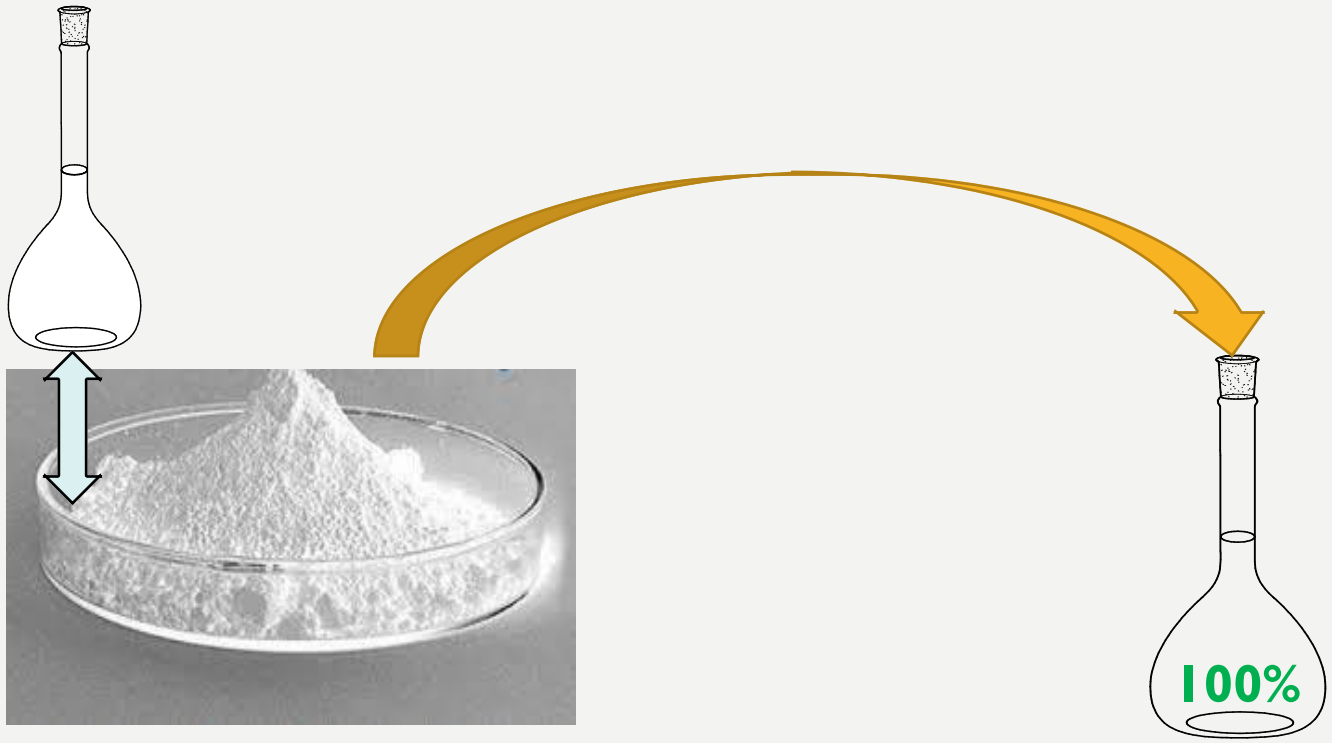
x 3 replicates

Std stock 1 mg/mL

PRECISION

authentic samples

- 6 determinations
- At 1 conc x 6 replicates
- Conc 100%



Sample

x 6 replicates

PRECISION

$$\% \text{ RSD} = \frac{SD}{\bar{x}} \times 100$$

$$\% \text{ RSD} \leq 2.0\%$$

Standard aspirin (mg/mL)		Actual peak area (day 1)		Actual peak area (day 2)	
0.2500		948329.0		947542	
0.3750		1384826.0		1385555	
0.5000		1891255.0		1884253	
0.6250		2412344.0		2412344.0	
0.7500		2768835.0		2768830	
r^2		0.9967		0.9968	
Slope		3734824.0		3735492.0	
Intercept		13705.80		11958.80	
Sample	**No.**	**Intraday precision**		**Interday precision**	
		Area	**% Recovery**	**Area**	**% Recovery**
Accuracy solutions (0.25 mg/mL)	1	931521	98.3	935540	98.90
	2	929511	98.08	937758	99.14
	3	958150	101.15	959000	101.41
Accuracy solutions (0.50 mg/mL)	1	1890856	100.52	1892741	100.70
	2	1851009	98.39	1854592	98.66
	3	1904496	101.25	1903785	101.29
Accuracy solutions (0.75 mg/mL)	1	2762844	98.14	2798854	99.47
	2	2806480	99.7	2823345	100.35
	3	2825335	100.38	2835597	100.79
Average (n=9)			**99.55**		**100.08**
% RSD (n=9)			**1.34**		**1.05**
Average (n=18)					**99.81**
% RSD (n=18)					**1.20**

PRECISION

$$\% \text{ RSD} = \frac{SD}{\bar{x}} \times 100$$

% RSD ≤ 2.0%

Standard aspirin concentration (mg/mL)		Actual peak area (day 1)		Actual peak area (day 2)	
0.5000		1851009.0		1855428.0	
		1890856.0		1894862.0	
		1891255.0		1842578.0	
		1838093.0		1897253.0	
		1859986.0		1852463.0	
average		1866239.8		1868516.8	
Sample	**No.**	**Intraday precision**		**Interday precision**	
		Area	**%Assay**	**Area**	**%Assay**
Sample solutions (equivalent aspirin 0.50 mg/mL)	1	1890856	101.32	1892741	101.30
	2	1851009	99.18	1854592	99.25
	3	1904496	102.05	1903785	101.89
	4	1878900	100.68	1877452	100.48
	5	1904582	102.05	1885259	100.90
	6	1887582	101.14	1900054	101.69
Average (n=9)			**101.07**		**100.92**
% RSD (n=9)			**1.06**		**0.95**
Average (n=18)					**100.99**
% RSD (n=18)					**0.96**

PRECISION

AOAC (2023)

- **Repeatability**

Prepare and homogenize three unknown samples at different concentrations to represent the full, claimed range of the method. Analyze each unknown sample by the candidate method seven times, beginning each analysis from weighing out the test portion through to final result with no additional replication

- **Intermediate Precision (within-laboratory deviation)**

PRECISION

AOAC (2023)

- **Reproducibility**

Quantitative methods: Recruit 10– 12 collaborators; must have eight valid data sets; two blind duplicate replicates at five concentrations for each analyte/ matrix combination to each collaborator.

Qualitative methods: Recruit 12– 15 collaborators; must have 10 valid data sets; six replicates at five concentrations for each analyte/ matrix combination to each collaborator.

Expected precision as a function of analyte concentration

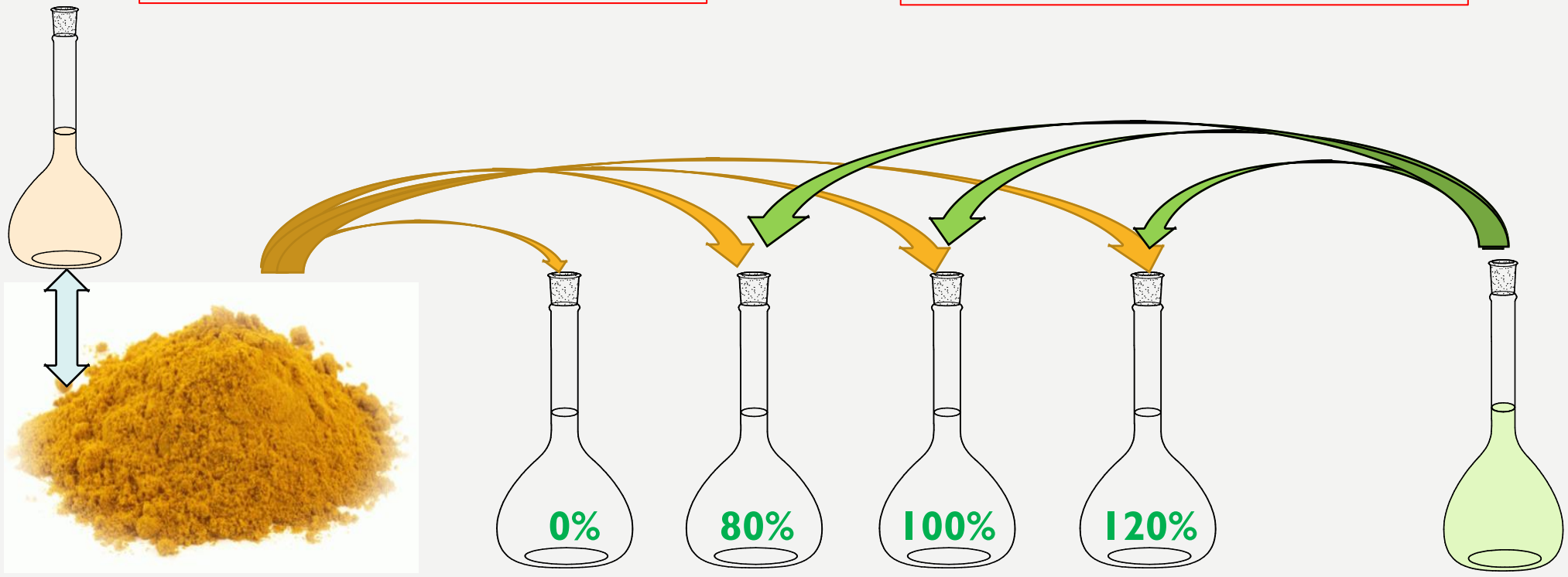
AOAC 2023

Analyte, %	Mass fraction (C)	Unit	RSD, %	
			Repeatability	Reproducibility
100	1	100%	1.3	2
10	10^{-1}	10%	1.9	3
1	10^{-2}	1%	2.7	4
0.1	10^{-3}	0.1%	3.7	6
0.01	10^{-4}	100 ppm (mg/kg)	5.3	8
0.001	10^{-5}	10 ppm (mg/kg)	7.3	11
0.0001	10^{-6}	1 ppm (mg/kg)	11	16
0.00001	10^{-7}	100 ppb (µg/kg)	15	22
0.000001	10^{-8}	10 ppb (µg/kg)	21	32
0.0000001	10^{-9}	1 ppb (µg/kg)	30	45

PRECISION

Accuracy
- At least 3 conc x 7 replicates
- 80%, 100%, 120%

Precision
- At least 3 conc x 7 replicates
- 80%, 100%, 120%



Sample

x 7 replicates

Std stock 1 mg/mL

sample	%std	added conc.(ug/ml)	added conc * purity/100	PA	average PA	(sp PA+std) - sp PA	found conc.(ug/ml)	Accuracy (% recovery)	Precision (%RSD)
ซาหมัก	0			328539	325311		29.82		
				328468			29.81		
				324748					
				325342					
				323096			29.32		
				323097			29.32		
				323889			29.39		
	80	23.75	22.37	590462	593428	265151	24.00	107.26	1.48
				590941		265630	24.04	107.46	
				596206		270895	24.52	109.62	
				595107		269796	24.42	109.17	
				595105		269794	24.42	109.17	
				598729		273418	24.75	110.66	
				587448		262137	23.72	106.03	
	100	29.67	27.95	629877	633278	304566	27.62	98.81	2.38
				635291		309980	28.12	100.59	
				632419		307108	27.85	99.65	
				632420		307109	27.85	99.65	
				647288		321977	29.22	104.54	
				623405		298094	27.02	96.68	
				631387		306076	27.76	99.31	
	120	35.58	33.52	706195	703627	380884	34.63	103.32	2.07
				693527		368216	33.47	99.84	
				693530		368219	33.47	99.85	
				705425		380114	34.56	103.11	
				699899		374588	34.05	101.59	
				701285		375974	34.18	101.97	
				715433		390122	35.48	105.85	
							Mean	**103.53**	**1.98**
							SD	**4.20**	
							%RSD	**4.06**	

$$\text{Marginal \% recovery} = (C_f - C_u) \times 100 / C_A$$

Expected precision as a function of analyte concentration

AOAC 2023

Analyte, %	Mass fraction (C)	Unit	RSD, %	
			Repeatability	Reproducibility
100	1	100%	1.3	2
10	10^{-1}	10%	1.9	3
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EXAMPLE: CURCUMIN QUANTITATION AND STABILITY STUDY

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journal homepage: www.elsevier.com/locate/lwt

Research note

Isolation of three curcuminoids for stability and simultaneous determination of only using one single standard substance in turmeric colour principles by HPLC with ternary gradient system

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WHAT IS THE DATA FROM LITERATURE?

- Analytical procedure (condition)
- Sample preparation
- Working range

respectively. Curry powder and rhizoma curcumae longae (about 1.0000 g) were accurately weighed and extracted by ultrasonication at 40 °C for 20 min with 20 mL 95 ml/100 ml ethanol in water. The solution of four times extractions was combined and adjusted to the 100 mL. The primary filtrate was discarded. The subsequent filtrate was used for HPLC analysis. Then each solution was filtered through a 0.45 μm nylon 66 membrane prior to analysis and injected into HPLC system. The injection volume was 10 μL.

Table 1
Calibration curve, linear range, LOQ, LOD and relative response factors of the investigated compounds.

Compounds	Linear regression data			LOQ (μg/ml)	LOD (μg/ml)
	Regression equation	r^2	Linear range (μg/ml)		
Curcumin	$y = 76.811x + 15.291$	0.9996	9.50–152.00	0.84	0.27
DEMC	$y = 109.09x + 64.093$	0.9996	8.83–141.33	0.55	0.18
BDEMC	$y = 79.592x + 10.68$	0.9994	10.33–165.33	0.75	0.23

LOQ, limits of quantification, S/N = 10; LOD, limits of detection, S/N = 3.

Optimized condition

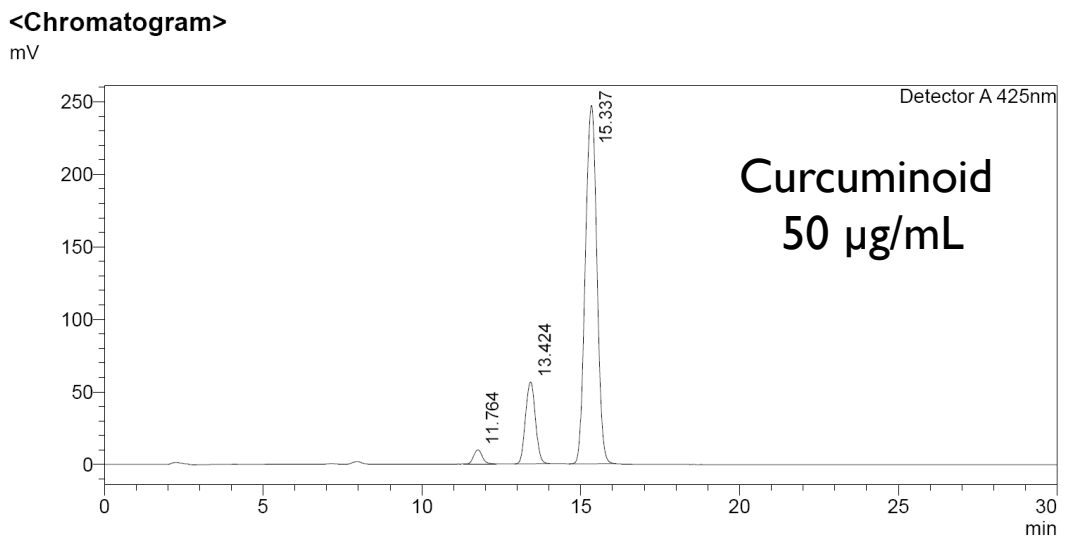
Condition
Stress testing
Working range
Sample preparation

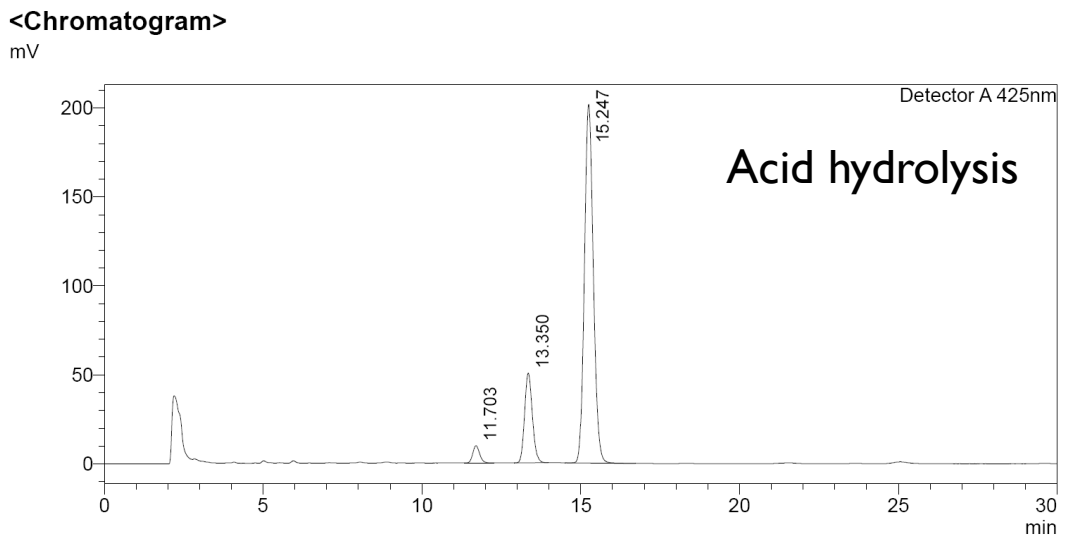
Working range selection ← Sample preliminary determination

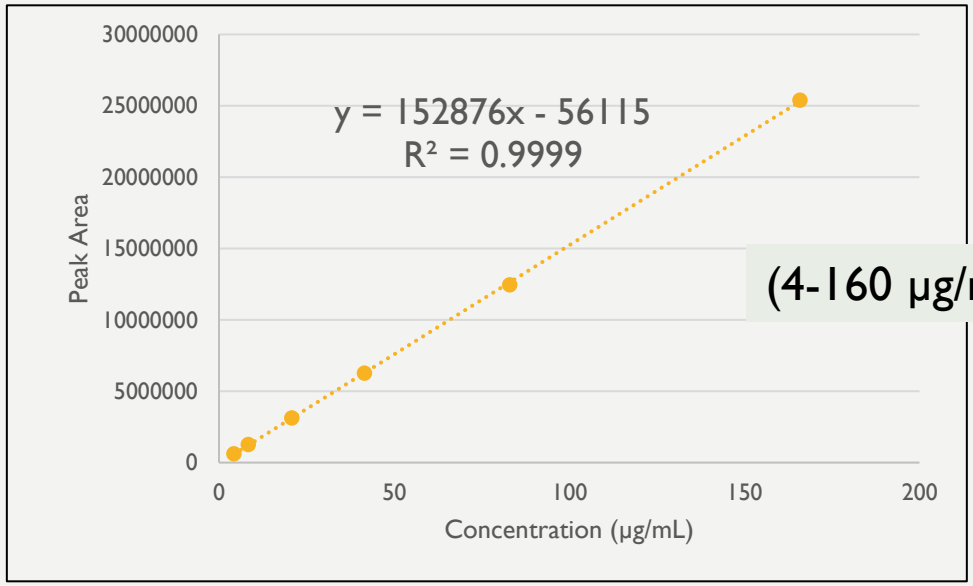
LOQ-XX

Designed method validation protocol

Optimized condition







(4-160 µg/mL)

Sample 200 mg/25 mL

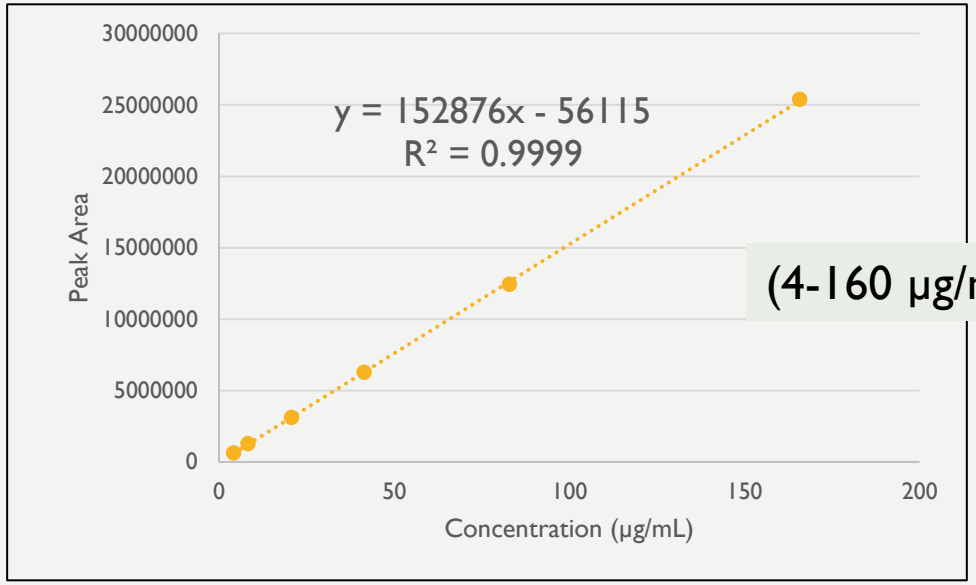
Dilute 1:10

Sample 800 µg/mL

Curcumin 30 µg/mL → 15-45 µg/mL

(10-50 µg/mL)

Working range selection



(4-160 µg/mL)

Sample 200 mg/25 mL

↓ Dilute 1:10

Sample 800 µg/mL

↓

Curcumin 30 µg/mL → 15-45 µg/mL → 5 concentration

(10-50 µg/mL)

(10, 20, 30, 40, 50 µg/mL)

↓

5, 10, 20, 30, 40, 50 µg/mL

Designed method validation protocol

System suitability	Standard curcuminoid at concentration of curcumin 30 µg/mL (5-6 injection)
Specificity	Standard curcumin (curcuminoid) at concentration of curcumin 30 µg/mL, Sample 800 µg/mL, Sample (stress condition), Mixtures, Etc.
linearity	Standard curcumin 5,10, 20, 30, 40, 50 µg/mL spike with sample 800 µg/mL (triplicate)
Accuracy	Standard curcumin 5,10, 20, 30, 50 µg/mL spike with sample 800 µg/mL (triplicate)
Precision	Sample 800 µg/mL (6 replicates)
Quantitation limit	Standard curcumin 5,10 µg/mL spike with sample 800 µg/mL (triplicate)

SEQUENTIAL DESIGNED

Name	(n x inj)	min	Name	(n x inj)	min
System suitability	1 x 5	150	Calibration curve		
Specificity			- Std cur 5 µg/mL	1 x 1	30
- Standard curcumin 30 µg/mL	1 x 1	30	- Std cur 10 µg/mL	1 x 1	30
- Standard curcumin	1 x 1	30	- Std cur 20 µg/mL	1 x 1	30
- Sample 800 µg/mL	1 x 1	30	- Std cur 30 µg/mL	1 x 1	30
- Stress sample 800 µg/mL	1 x 1	30	- Std cur 40 µg/mL	1 x 1	30
- Solvent	1 x 1	30	- Std cur 50 µg/mL	1 x 1	30
- Etc	1 x 1	30	blank		30
linearity			accuracy		
- Std cur 0 µg/mL + sample	3 x 1	90	- Std cur 0 µg/mL + sample	3 x 1	90
- Std cur 5 µg/mL + sample	3 x 1	90	- Std cur 5 µg/mL + sample	3 x 1	90
- Std cur 10 µg/mL + sample	3 x 1	90	- Std cur 10 µg/mL + sample	3 x 1	90
- Std cur 20 µg/mL + sample	3 x 1	90	- Std cur 20 µg/mL + sample	3 x 1	90
- Std cur 30 µg/mL + sample	3 x 1	90	- Std cur 30 µg/mL + sample	3 x 1	90
- Std cur 40 µg/mL + sample	3 x 1	90	- Std cur 40 µg/mL + sample	3 x 1	90
- Std cur 50 µg/mL + sample	3 x 1	90	- Std cur 50 µg/mL + sample	3 x 1	90
blank		30	blank		30
			Precision (sample 800 µg/mL)	6 x1	180
Summary		16.5 h			16 h

SEQUENTIAL DESIGNED

Name	(n x inj)	min
System suitability	1 x 5	150
Calibration curve		
- Std cur 5 µg/mL - Std cur 10 µg/mL - Std cur 20 µg/mL - Std cur 30 µg/mL - Std cur 40 µg/mL - Std cur 50 µg/mL	30 30 30 30 30 30	30 30 30 30 30 30
blank	30	30
Precision (sample 800 µg/mL)	6 x1	180
Sample determination	3 x1	90
Summary		7.35 h

THANK YOU
ANY QUESTION?