VALIDATION AND UNCERTAINTY ESTIMATION OF HPLC METHOD COMBINED WITH ULTRASOUND-ASSISTED EXTRACTION PROCEDURE FOR QUANTITATIVE DETERMINATION OF HESPERIDIN OBTAINED FROM CITRUS PEEL

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Quality Assurance Challenges of Measurements from Field to Laboratory with a Focus on ISO/IEC 17025:2017 Requirements

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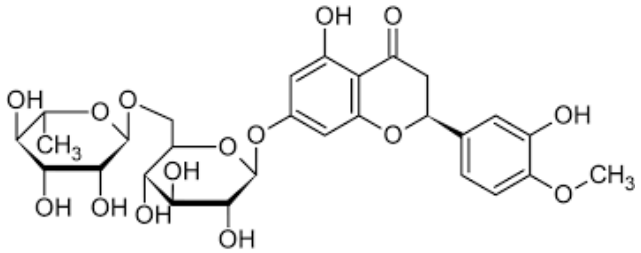
INTRODUCTION

Research Goal

- To validate a new method obtained with a combination of
  - ✓ *alternative, effective, selective, reproducible, low cost and high-yield sequential two-step extraction procedure using ultrasound-assisted technique for obtaining hesperidin from citrus peel;*
  - ✓ *specific, selective and simple analytical HPLC procedure for quantitative determination of hesperidin in the obtained dry extracted product and citrus peel.*
- To evaluate measurement uncertainty of the combined method based on the validation study.

INTRODUCTION

Hesperidin



Hesperidin *- $C_{28}H_{34}O_{15}$ MW=610.56 (3,5,7-trihydroxyflavanone-7-rhamnoglucoside) [CAS #520-26-3] is a flavanone glycoside (flavonoid) found abundantly in citrus fruits.*

Biological and Pharmacological Effects:

- ✓ *Anti-oxidant, anti-inflammatory, anti-carcinogenic, antimicrobial, antihyperlipidemic, cardioprotective, antihypertensive, antidiabetic, UV protective, skin lightening;*
- ✓ *Stimulating of epidermal proliferation, enhancing epidermal permeability barrier homeostasis; wound healing.*

Citrus Agro-Industrial Waste Material – Citrus Peel

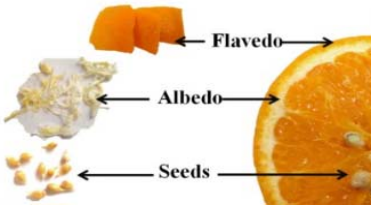
Tangerine (Citrus Unshiu)

Juice (30-40 %)

Tangerine waste

Rag and Pulp (10-20 %)

Tangerine Peel (30-40 %)

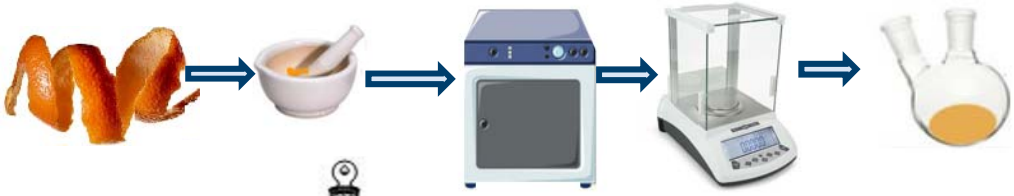


Tangerine processing waste – >70 % citrus peel (Flavedo – cellulosic material, carotenes, chlorophylls, flavonoids, essential oil, vitamins, triterpenoids and paraffin waxes and **Albedo –** pectin, flavonoids)

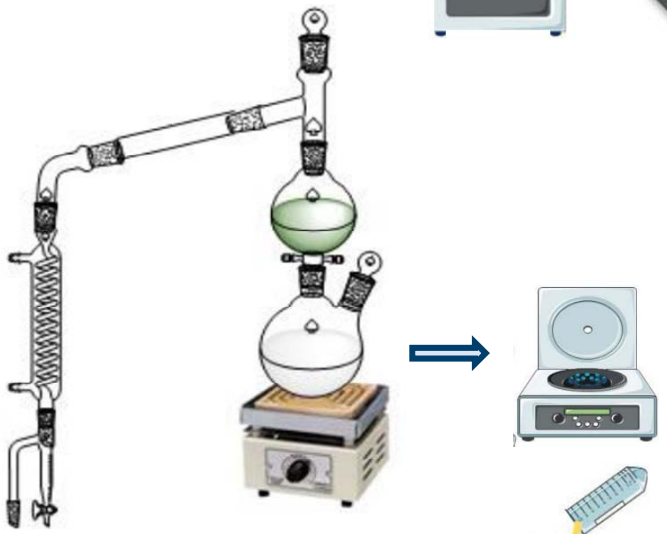
MATERIALS AND METHODS

Sequence of Extraction and Analytical Combined Process

Drying, grinding to powder and weighting of the dried citrus peel

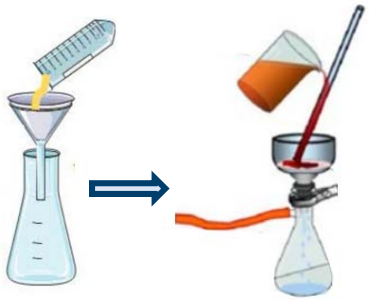


Extraction Stage I – steam distillation – obtaining essential oil and pectin



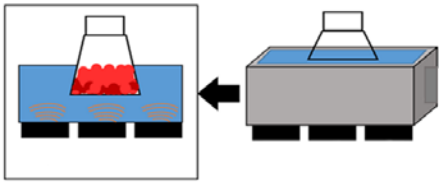
Further procedure for essential oil

Washing of the remained residue with hot acidified water and filtration



Further procedure for pectin

Extraction Stage II – UAE extraction – obtaining hesperidin and carotenes



Dual-frequency ultrasonic bath DW-5200 DTS

MATERIALS AND METHODS

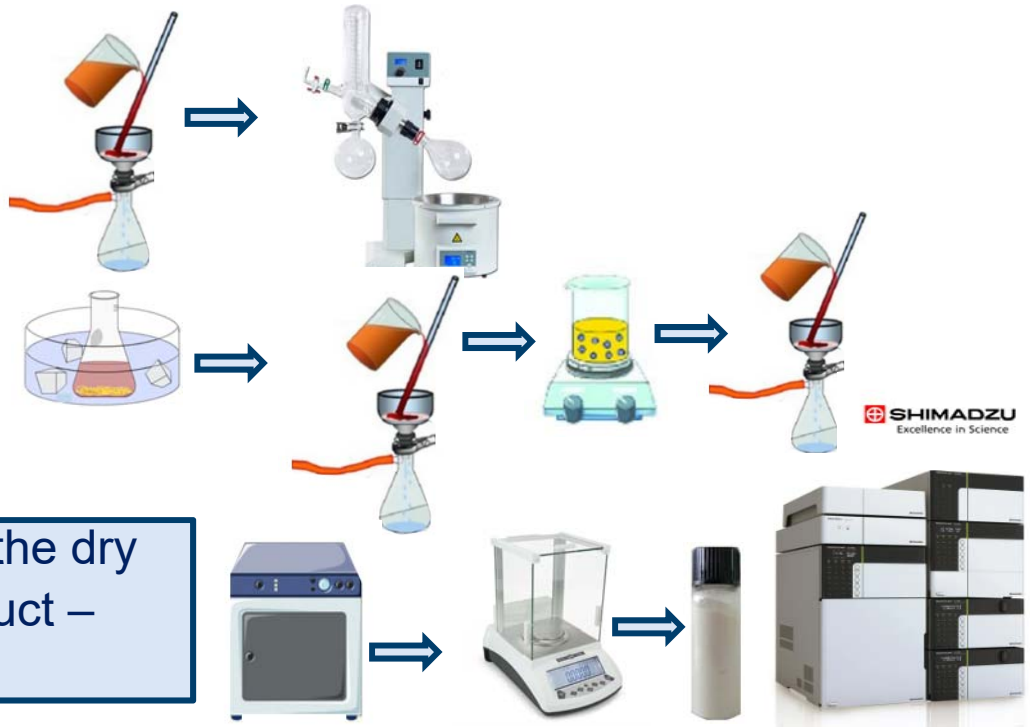
Sequence of Extraction and Analytical Combined Process *(continued)*

Filtration and solvent vaporization to obtain syrup

Dilution, cooling to crystalize hesperidin and filtration

Further procedure for beta-carotene

Drying to obtain the dry extracted product – hesperidin



LC-20AD Prominence HPLC System

Standard and sample preparation - weighing of the dry extracted product and dilution

HPLC analysis of hesperidin

ISO 17034
ANAB Cert# AR-1470

ISO/IEC 17025
ANAB Cert# AT-1467

15 mg→50 mL
(DMSO + Methanol 1:9)

SIGMA-ALDRICH

HESPERIDIN

CERTIFIED REFERENCE MATERIAL

Column - Agilent SB-C18 4.6×250 mm, 5 µm; Standard solution/test solution – 0.25 mg/mL. External standard method for quantification.

METHOD VALIDATION

Method validation and measurement uncertainty according to *ICH, USP, EDQM, Eurachem guidelines*

Validation Parameters:

- ✓ **Robustness** – *standard solution stability/ filter compatibility test/study of critical factors effect*
- ✓ **Specificity -** *Forced degradation*
- ✓ **Linearity-Range**
- ✓ **Accuracy**
- ✓ **Sensitivity - Limit of Detection (LOD); Limit of Quantitation (LOQ)**
- ✓ **Precision** – *repeatability (intraday) and intermediate precision (inter day) (n=6)*
- ✓ **System Suitability Test (SST)**

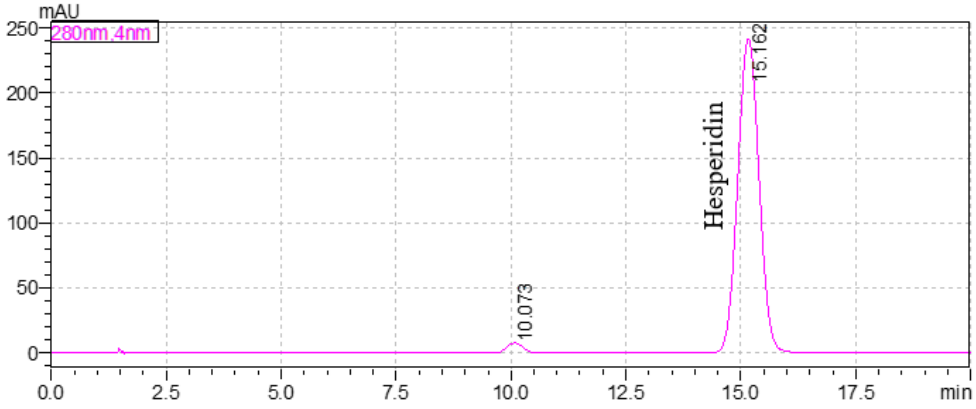
METHOD VALIDATION

- **System Suitability Test (SST) –** standard solution with 6 replicate injections (n=6) at 0.25 mg/mL

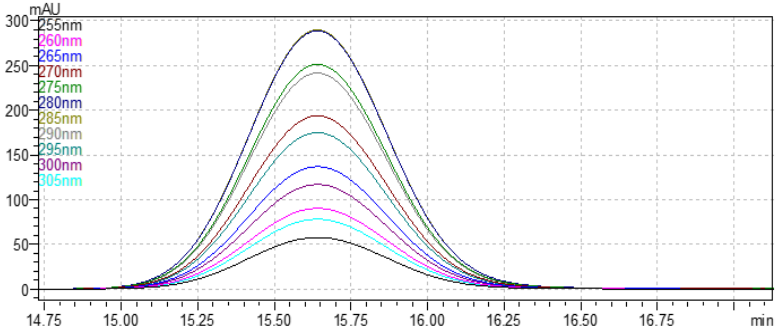
SST Parameter	Hesperidin	Acceptance criteria
Column efficiency - N	>4953	≥2000
RSD_A (n=6)	0.70 %	≤2 %
RSD_{RT} (n=6)	0.15 %	≤1 %
Tailing factor - S	1.03	0.8-1.5

Chromatographic system is suitable and has a good performance.

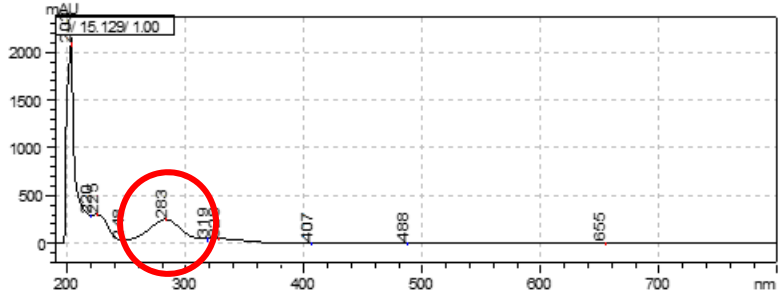
- **Specificity –** standard solution, test solution and the background control - blank (diluent) solution.



The chromatogram of standard solution detected at 280 nm



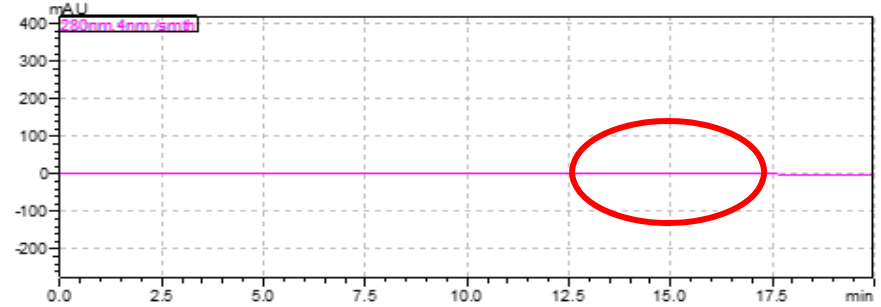
The UV-Vis absorption spectra of hesperidin peak obtained with standard solution measured at different wavelengths



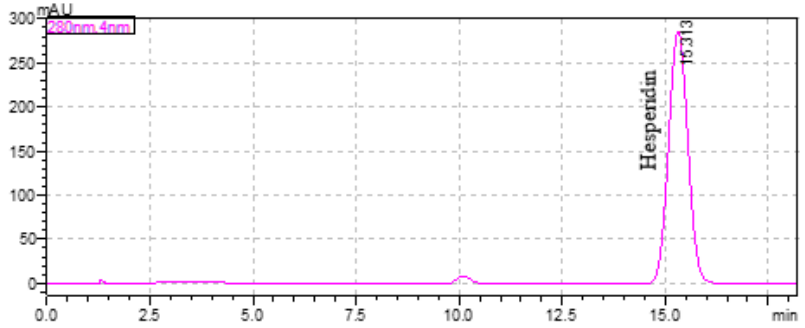
The UV-Vis absorption spectra obtained with standard solution scanned at 200-800 nm

METHOD VALIDATION

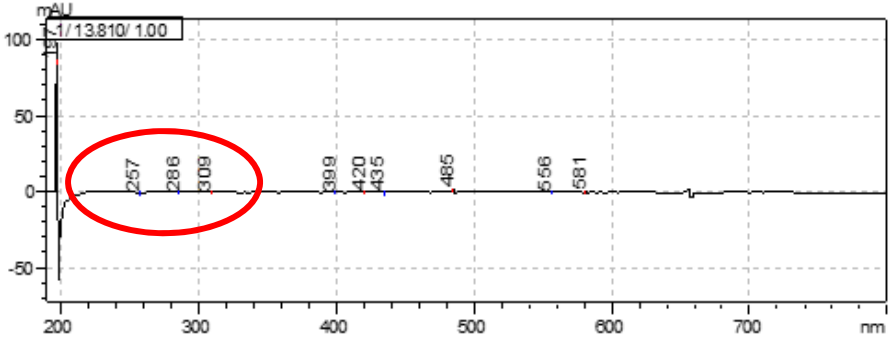
❑ Specificity



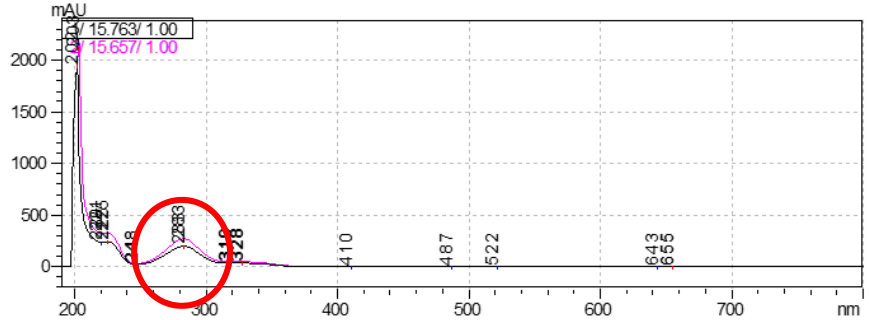
The chromatogram of blank solution detected at 280 nm



The chromatogram of test solution detected at 280 nm

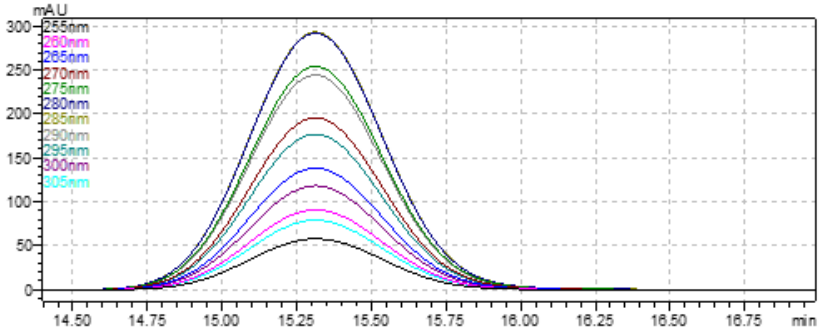


The UV-Vis absorption spectra obtained with blank solution scanned at 200-800 nm



The overlay UV-Vis absorption spectra obtained with standard and test solutions scanned at 200-800 nm

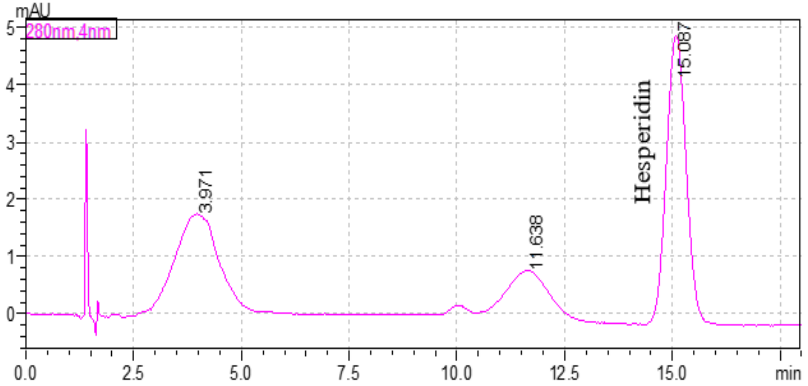
No interference from blank and hesperidin peak is pure (>99 %). Diff, % between retention times <1%.



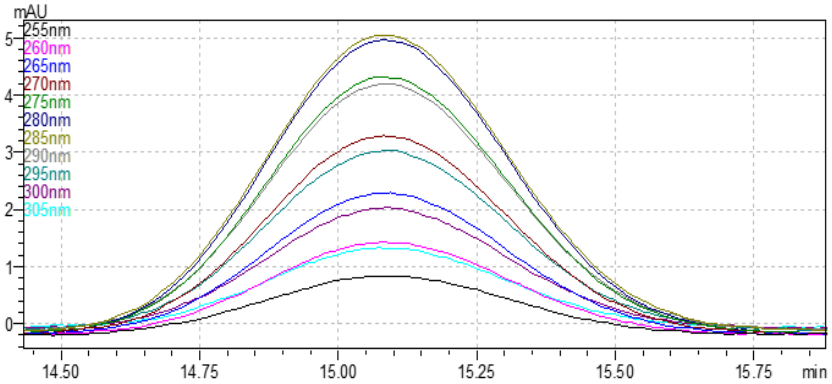
The intensity of hesperidin peak obtained with test solution measured at different wavelengths

METHOD VALIDATION

- **Specificity – forced degradation -** samples of the dry extracted product treated under stress conditions before sample preparation.

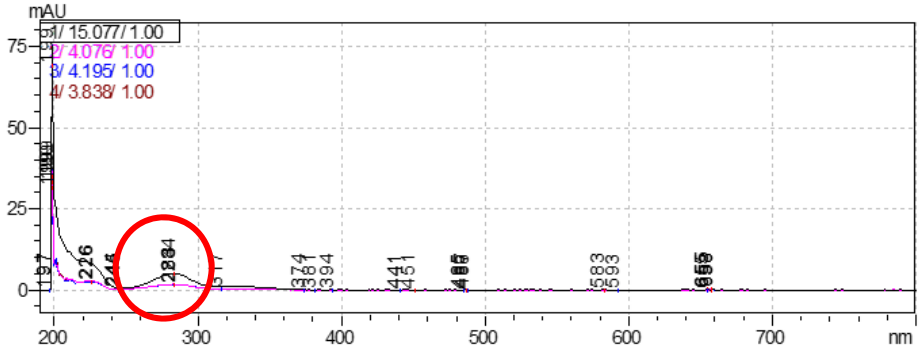


The chromatogram obtained with the test (oxidative degradation) detected at 280 nm



The intensity of hesperidin peak measured at different wavelength and obtained on the test solution (oxidative degradation) – worst condition

Condition	Concentration of hesperidin, mg/mL	Degradation, %
Acid degradation - 5 mL of 1M HCl for 60 mins and then neutralized	0.216	18.49
Alkali degradation - 5 mL of 1M NaOH for 60 mins and then neutralized	0.222	16.23
Oxidative degradation - 0.5 mL of 30 % H_2O_2	0.029	89.06
Thermal degradation - 80°C for 24 h	0.125	15.54
UV degradation – 254 nm for 60 mins	0.198	44.15
Normal condition	0.265	-



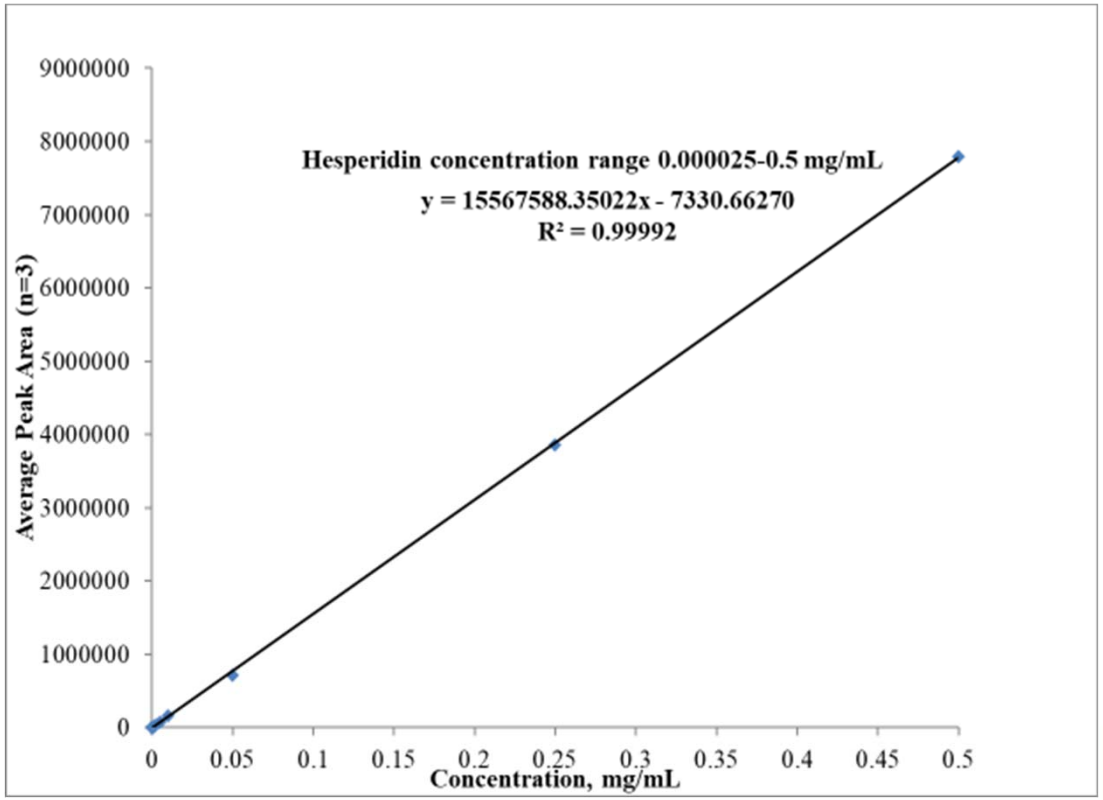
The UV-Vis absorption spectra obtained with the test solution (oxidative degradation) scanned at the range 200-800 nm

Hesperidin peak is pure (>99 %); Absence of any other peak in the same retention time; peak purity passed in worst conditions.

METHOD VALIDATION

- **Linearity-Range** - working standard solutions (n=3) at 11 different concentration levels. **AC:** Square of corr. coefficient – $R^2 \geq 0.998$.

Linearity curve over the range - 0.000025-0.5 mg/mL is linear and R^2=0.99992 is highly significant; Peak area is directly proportional to the concentration of hesperidin. The method has a good linearity.



Calibration (linearity) curve over the concentration range - 0.000025-0.5 mg/mL for hesperidin

- **Sensitivity - LOQ and LOD** - a number of series of stepwise diluted working standard solutions (n=3)

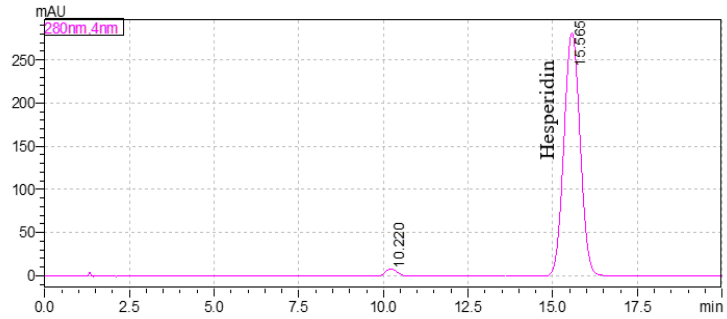
Parameter	Value
LOQ, mg /mL	0.000025
LOD, mg /mL	0.00001
RSD_A, % for LOQ (n=3)	8.199
Acceptance criteria	≥10
RSD_{RT}, % for LOQ (n=3)	0.452
Acceptance criteria	≥1
s/N for LOQ (n=3)	45.50
Acceptance criteria	≥10
s/N for LOD (n=3)	6.52
Acceptance criteria	≥3

The method is highly sensitive.

METHOD VALIDATION

- **Precision - repeatability (intra-day precision)** and time dependent **intermediate precision (inter-day precision)** - standard solution with 6 replicate injections (n=6) and 6 individual determinations of hesperidin in test solutions (100 %).

Standard solution Inj. №	Repeatability (intra-day)		Intermediate precision (inter-day)	
	Peak Area	Retention Time	Peak Area	Retention Time
1	3716668	15.162	3534531	14.288
2	3757935	15.127	3529578	14.227
3	3784866	15.162	3528953	14.262
4	3791810	15.141	3513652	14.241
5	3766331	15.169	3523335	14.269
6	3766198	15.113	3537583	14.213
Average	**3763968.00**	**15.15**	**3527938.67**	**14.25**
RSD, %	**0.70**	**0.15**	**0.24**	**0.20**
Acceptance Criteria	**≤2**	**≤1**	**≤2**	**≤1**



The chromatogram obtained with the test solution detected at 280 nm

Test solution №	Concentration, mg/mL	
	Repeatability (intra-day)	Intermediate Precision (inter-day)
1	0.281	0.238
2	0.275	0.245
3	0.243	0.224
4	0.274	0.223
5	0.249	0.217
6	0.269	0.231
Average (n=6)	**0.265**	**0.230**
RSD, % (n=6)	**5.84**	**4.60**
Acceptance Criteria	**≤6**	
Average (n=12)	**0.247**	
RSD, % (n=12)	**9.11**	
Acceptance Criteria	**≤10**	
F-test	**2.14**	
Acceptance Criteria	**5.05**	
Percentage Difference, %	**12.28**	
Acceptance Criteria	**≤15**	

The method gives the repeatable and reproducible results. The method has a good precision.

METHOD VALIDATION

❑ Accuracy

- ✓ 2 standard solutions and 3 spiked test solutions prepared using standard addition method by spiking the known amounts at 100 % concentration level with 3 individual determinations with 3 replicate injections (n=3).
- ✓ The **recovery – Rec, %** of the method including extraction procedure:

$$Rec, \% = \frac{W_d \times 100}{W_a}$$

- ✓ The **similarity factor (Sf):** $$Sf = \frac{W_{st1} \times A_{st2} \times 100}{W_{st2} \times A_{st1}}$$

- ✓ **AC:** Rec, % - 90.0 –110.0%; the RSD of Rec, % (n=3×3=9) ≤10.0%; The similarity factor (Sf) between 2 standard solutions 98.0 %-102.0 %

Name of solution	Percentage Recovery - Rec, %			The Average Recovery, %	SD, % (n=9)	RSD, % (n=9)	The Mean Recovery - R, %
	Rec_1, %	Rec_2, %	Rec_3, %				
Spiked test solution I	91.65	92.15	90.36	**91.39**	3.74	**4.09**	**91.48**
Spiked test solution II	86.86	87.23	87.45	**87.18**			
Spiked test solution III	96.51	95.99	95.12	**95.87**			
Similarity factor between two standard solutions							**101**

The method gives the accurate results and has a good recovery.

METHOD VALIDATION

❑ **Robustness – study of critical factors effect** - small changes in the critical parameters as critical factors affected on the results of analysis.

✓ *Both quantitative and qualitative critical parameters of the method were assessed and selected using risk assessment approach;*

✓ ***Risk parameters:***
- o *Risk severity (S)*
- o *Risk probability (P)*
- o *Risk detectability (D)*

✓ ***Risk level:***
RL=(S)+(P)+(D)

✓ ***Risk category:***
- o ***Critical***
- o ***Significant***
- o ***Negligible***

Method Parameter	Procedure	Character	S	P	D	RL	Risk Category	Critical Parameter (Risk Factor) – Xi
			L - 1	L - 1	L - 1	3-4	Negligible	
			M - 2	M - 2	M - 2	5-6	Significant	
			H - 3	H - 3	H - 3	7-9	Critical	
Sample size	UAE	Experimental	3	3	3	9	Critical	X1
Volume of ethanol	UAE	Experimental	3	3	3	9	Critical	X2
Ultrasound power	UAE	Controlled automatically	3	3	1	8	Critical	X3
Ultrasonication time	UAE	Controlled automatically	3	3	1	7	Critical	X4
Extraction temperature	UAE	Controlled automatically	2	2	1	5	Significant	-
Centrifugation	Separation	Controlled automatically	2	2	1	5	Significant	-
Membrane filtration	Separation	Experimental	3	3	3	9	Critical	Checked within filter compatibility test
Rotary vaporization	Concentration	Controlled automatically	3	3	3	9	Negligible	-
Solvent before crystallization	Clean-up	Experimental	3	3	3	9	Critical	X5
Delay time for crystallization	Clean-up	Controlled manually	2	3	1	6	Significant	-
Solvent I	Clean-up	Experimental	3	3	2	8	Critical	X6
Solvent II	Clean-up	Experimental	2	2	2	6	Significant	-
Volume of solvent II	Clean-up	Experimental	3	3	2	8	Critical	X7
Heating temperature	Clean-up	Controlled manually	3	3	2	8	Critical	X8
Heating time	Clean-up	Controlled manually	2	2	2	6	Significant	-
Ratio of MP components	HPLC	Experimental	3	3	1	7	Critical	X9
Membrane filtration	HPLC	Experimental	3	3	3	9	Critical	Checked within filter compatibility test
Flow rate of MP	HPLC	Controlled automatically	3	3	1	7	Critical	X10
Stationary phase of column	HPLC	Experimental	3	3	1	7	Critical	Checked within intermediate precision
Column temperature	HPLC	Controlled automatically	2	2	2	6	Significant	-
Wavelength of detector	HPLC	Controlled automatically	3	3	1	7	Critical	X11
Injected volume	HPLC	Controlled automatically	2	2	1	5	Significant	-

METHOD VALIDATION

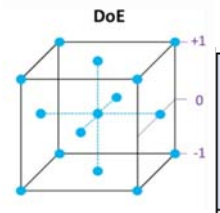
❑ Robustness – study of critical factors effect

✓ Based on the risk assessment 11 critical parameters or factors (Xi) with small variations (low "-" and high "+" levels) of nominal "0" level

№	Critical parameter - Xi	Unit	Level		
			Low level (-)	Nominal level (0)	High level (+)
1	Sample size – X1	g	15	20	25
2	Volume of ethanol – X2	mL	175	200	225
3	Ultrasound power – X3	kHz	-	25	40
4	Ultrasonication time – X4	min	25	30	35
5	Solvent before crystallization – X5	-	4 % acetic acid	6 % acetic acid	8 % acetic acid
6	Solvent I for clean-up – X6	-	Methanol	Isopropanol	-
7	Volume of solvent II – X7	mL	45 (3×15)	60 (3×20)	75 (3×25)
8	Heating temperature – X8	ºC	65	70	75
9	Ratio of MP components – X9	v/v	5:15:25:55	5:10:30:55	5:5:35:55
10	Flow rate of MP – X10	mL/min	1.3	1.5	1.7
11	Wavelength of detector – X11	nm	278	280	282

✓ 12-run experiments with 11 critical factors according to the DoE by Placket-Burman approach.

METHOD VALIDATION



The results of 12-run experiments for the robustness parameter

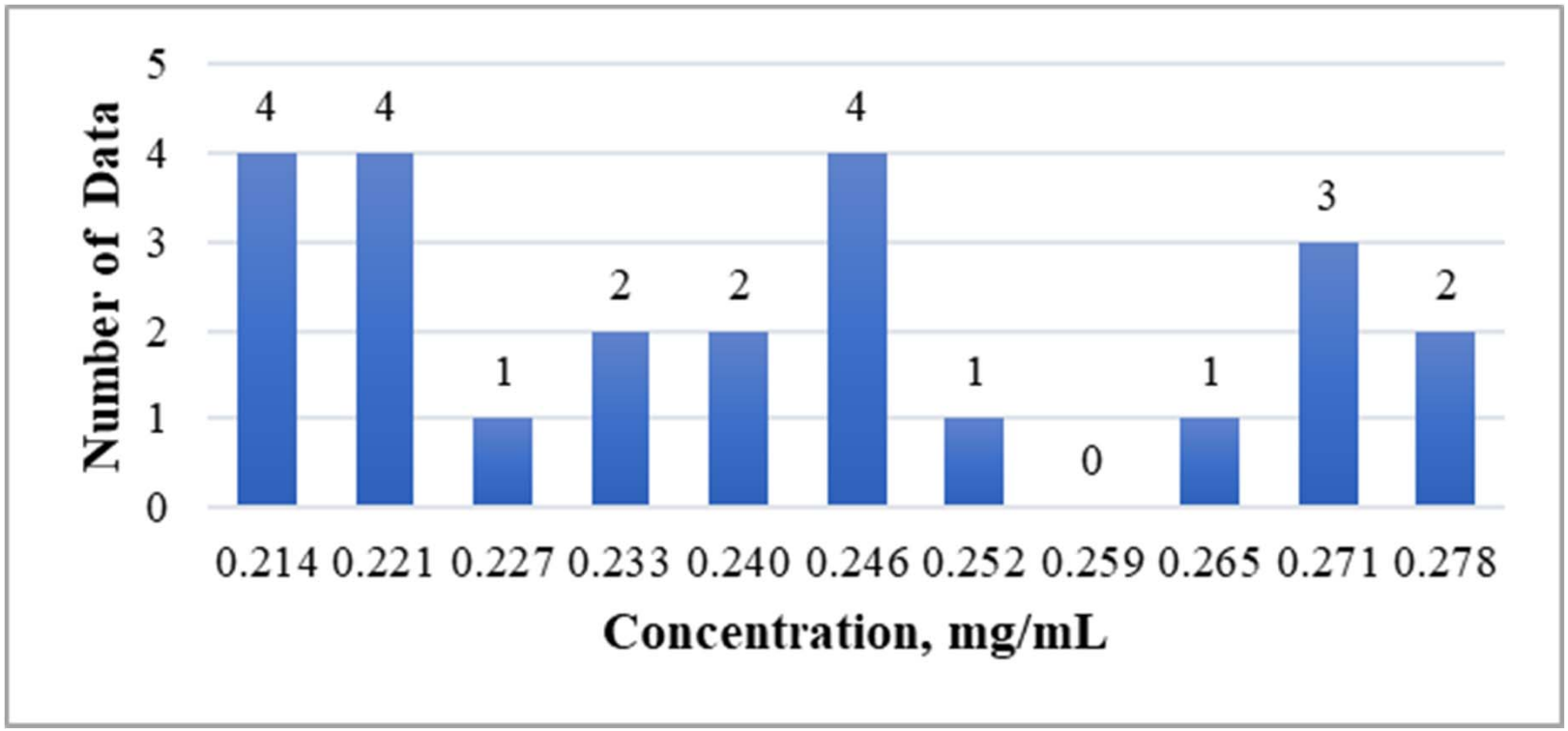
12-run experiments of 11 critical factors with two "+" and "-" levels

Run (N) №	Critical parameters - Xi											Results (Response Variables) of Robustness		Results of Precision (At nominal level "0")
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Conc. of hesperidin, mg/mL	SST parameters (N, S, RSD_A, RSD_{RT})	Conc. of hesperidin, mg/mL
1	+	–	+	+	+	–	–	–	+	–	–	0.269	N>3545; S=0.96; RSD_A=1.12 %; RSD_{RT}=0.72 %	0.281
2	+	–	+	+	+	–	–	–	+	–	+	0.211	N>3342; S=0.91; RSD_A=0.89 %; RSD_{RT}=0.46 %	0.275
3	–	+	+	+	–	–	–	+	–	+	+	0.217	N>3133; S=0.97; RSD_A=1.33 %; RSD_{RT}=0.12 %	0.243
4	+	+	+	–	–	–	+	–	+	+	–	0.254	N>2345; S=0.88; RSD_A=1.32 %; RSD_{RT}=0.45 %	0.274
5	+	+	–	–	–	+	–	+	+	–	+	0.263	N>3145; S=0.96; RSD_A=0.49 %; RSD_{RT}=0.33 %	0.249
6	+	–	–	–	+	–	+	+	–	+	+	0.221	N>3212; S=0.97; RSD_A=0.98 %; RSD_{RT}=0.61 %	0.269
7	–	–	–	+	–	+	+	–	+	+	+	0.233	N>3041; S=0.92; RSD_A=1.37 %; RSD_{RT}=0.88%	0.238
8	–	–	+	–	+	+	–	+	+	+	–	0.211	N>2984; S=0.93; RSD_A=1.32 %; RSD_{RT}=0.54 %	0.245
9	–	+	–	+	+	–	+	+	+	–	–	0.219	N>3977; S=1.03; RSD_A=0.95 %; RSD_{RT}=0.74 %	0.224
10	+	–	+	+	–	+	+	+	–	–	–	0.239	N>4977; S=1.00; RSD_A=0.47 %; RSD_{RT}=0.12 %	0.223
11	–	+	+	–	+	+	+	–	–	–	+	0.243	N>4132; S=1.02; RSD_A=0.41 %; RSD_{RT}=0.21 %	0.217
12	–	–	–	–	–	–	–	–	–	–	–	0.222	N>4912; S=0.99; RSD_A=0.43 %; RSD_{RT}=0.46 %	0.231
Average, mg/mL												0.234	Acceptance Criteria	0.247
Minimal Value, mg/mL												0.211	N>200; S=0.8-1.5;	0.217
Maximal Value, mg/mL												0.269	RSD_A≤2 %;	0.281
Abs. Diff. between the maximal and minimal values, mg/mL												0.058	RSD_{RT}≤1 %	0.064
Diff., %													5.73	

METHOD VALIDATION

❑ **Robustness – study critical factors effect**

- ✓ The histogram plotted based on the analytical data obtained the precision and robustness parameters (N=24). There is a multi-modal data distribution;
- ✓ The analytical data spread is from 0.211 mg/mL to 0.281 mg/mL;
- ✓ Abs. Diff.=0.058 mg/mL of the robustness is very close to Abs. Diff.=0.064 mg/mL of the precision; Diff.,%= 5.73 % between the precision (n=12) and robustness (N=12) average results ≤15 % (Precision AC).



METHOD VALIDATION

❑ Robustness

✓ Standard solution stability

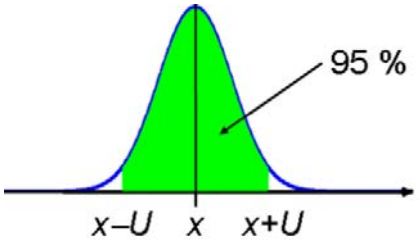
Standard solution stored under refrigeration is stable within 7 day – Diff, % between peak areas obtained with two standard solutions, one stored under refrigeration for 7 days and another prepared freshly - 1.75 %<3 % (AC);

✓ Filter compatibility test

- o Both type membrane filters – 0.45 µm membrane PVDF filter and MCE membrane filter were evaluated;
- o The Diff, % between peak areas of filtered and non-filtered standard solutions (0.25 mg/mL) - 0.59 % and 0.89 %, respectively (AC≤2 %);
- o No adsorption of each analyte on the filter and no affect on the result of analysis.

The method has a good robustness.

MEASUREMENT UNCERTAINTY



Step I – Identification of the measurand

Step II – Identification of uncertainty contributors and sources by Ishikawa diagram

Step III – Quantification of measurement uncertainty by a combination of bottom-up and top-down approaches

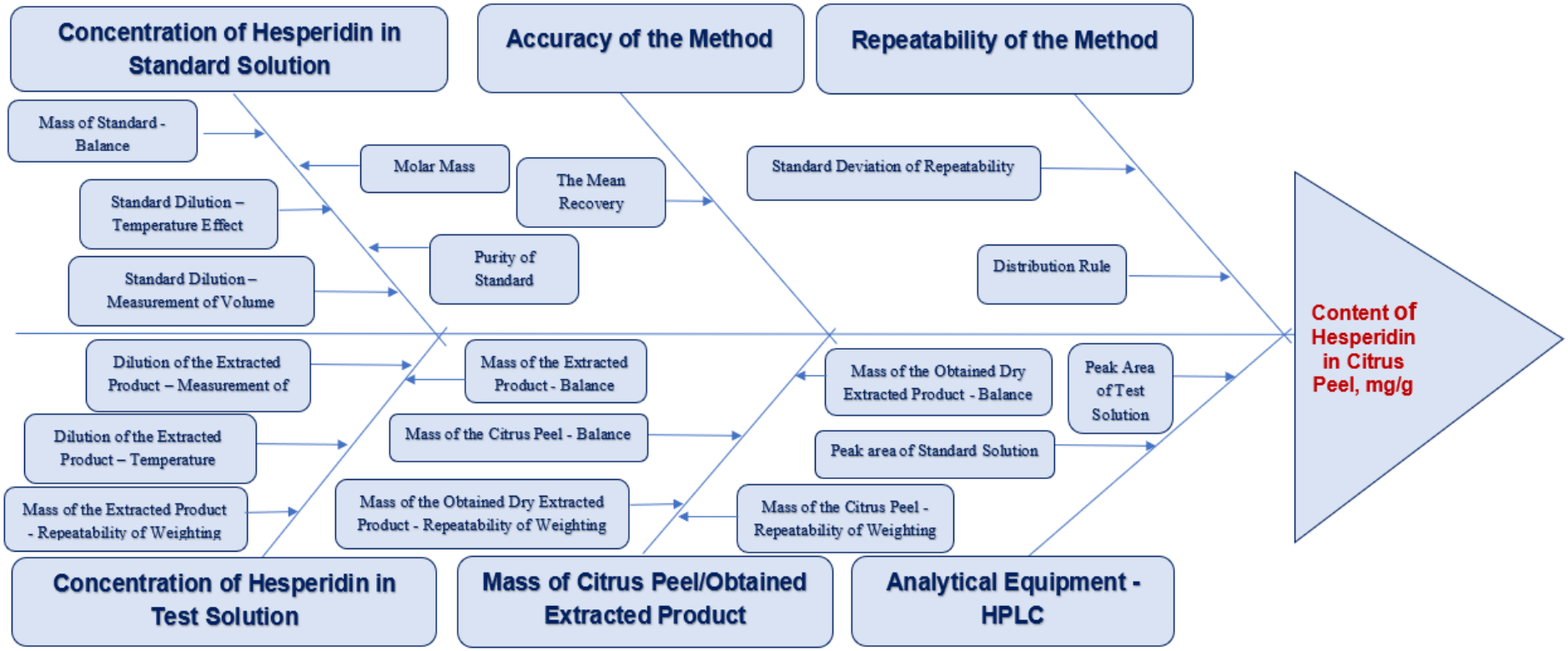
Standard uncertainty arising from each individual source (B Type)

Standard uncertainty by replicate measurements (A Type)

Step IV – Calculation of combined standard uncertainty and expanded uncertainty

MEASUREMENT UNCERTAINTY

Ishikawa Diagram for Identification of Uncertainty Contributors



Measurand - The content of hesperidin – X, mg per 1 g of the dry sample of citrus peel was calculated by the formula:

$$X = \frac{A_s \times W_{st} \times V_S \times W_d \times P}{A_{st} \times W_s \times W \times V_{St} \times 100}$$

where, A_S – The peak area of hesperidin obtained with the test solution; A_{St} – The peak area of hesperidin obtained with the standard solution; W_{St} – The weight of the standard, mg; V_{St} – The dilution of the standard, mL; P – The purity of the standard, %; W - The weight of the dry sample of citrus peel, g; W_S - The weight of the extracted product sample taken for test solution, mg; VS – The dilution of the extracted product sample, mL; W_d - The weight of the obtained extracted product after extraction, mg.

MEASUREMENT UNCERTAINTY

Measurement Uncertainty of the Method

Combined Standard Uncertainty, mg/g		
$u = \sqrt{u_A{}^2 + \left(X \times \sqrt{(c_1 \times \frac{u_{St}}{X})^2 + (c_2 \times \frac{u_S}{X})^2 + (c_3 \times \frac{u(m)}{W})^2 + (c_4 \times \frac{u(m_d)}{W_d})^2 + (c_5 \times u_R)^2 + (c_6 \times \frac{u(E)}{A})^2} \right)^2}$		0.32
Coverage Factor		1.96
Expanded Uncertainty, mg/g	$U = u \times k$	0.62
Expanded Uncertainty, %	$U, \% = \frac{U \times 100}{X}$	1.76

QUANTITATIVE ESTIMATION OF HESPERIDIN

- The content of hesperidin in mg per 1 g of tangerine peel varies from 34.13 to 36.32 mg/g (from 3.41 % to 3.63 %); The average content of hesperidin is 35.36 mg ± 0.62 mg (U; k=2 (1.98), approximately 95% level of confidence) per 1 g tangerine peel;
- The purity of the extracted product is high and varies from 85.17 % to 92.62 %;
- The average value of the total content of hesperidin equals to 90.13 % ± 1.76 % (U; k=2, approximately 95% level of confidence) .

The content of hesperidin in tangerine peel and the extracted product.

Sample №	Content of hesperidin in the tangerine peel, mg/g		Percentage content of hesperidin in the extracted product, %	
	Repeatability	Intermediate Precision	Repeatability	Intermediate Precision
1	34.4767	36.2097	89.928	90.699
2	36.1451	34.7775	91.483	86.869
3	34.1271	35.6890	85.170	89.762
4	35.2015	35.1494	93.919	90.388
5	35.4447	35.9559	88.063	91.793
6	34.8331	36.3241	90.871	92.622
Average (n=6)	**35.0380**	**35.6843**	**89.91**	**90.699**
Average (n=12)	**35.36**		**90.13**	

CONCLUSION

- The developed method obtained with a combination two-stage sequential extraction and analytical HPLC procedures of hesperidin is a simple, effective, eco-friendly, reproducible, low cost, selective, sensitive, specific and full validated with measurement uncertainty.
- The proposed method can be successfully used to apply for determination the content and the purity of hesperidin in the dry extracted product and citrus waste material in routine and stability study analyses;

- The method fully supports the developed laboratory technologies for utilization of citrus agro-industrial waste materials to obtain simultaneously four bioactive valuable compounds – essential oil, pectin, hesperidin and beta-carotene from one citrus waste material in the same process which can easily be adapted to industrial conditions and to design a manufacturing technological process.

Thank You

For Your Attention!

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