ION LABS	Standard Operating Procedure **Salicin Determination by HPLC using UV/VIS Spectroscopy**	**SOP Number** **D-767**	**Revision** **1**
		Effective Date 05/03/22	**Page** **Page 1 of 7**

Written by/ Date	**Reviewed by/ Date**	**Approved by/ Date**
SAS 04/15/22	04/19/22	04/27/22
Title: Analytical Development Scientist	**Title: Analytical Development Manager**	**Title: QC Laboratory Director**

1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of Salicin in raw materials and finished products using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of salicin in raw materials and finished products in the QC laboratory at Ion Labs. Salicin is a decent chromophore and was measured at 268, other wavelengths can be used to maximize signal to noise.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to ensure that this procedure is being followed.

3.3 It is the responsibility of QC Laboratory and Analytical Development Management to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **HPLC** – High Performance Liquid Chromatography

4.2 **UV/VIS** – Ultraviolet and Visible Electromagnetic Spectrums

4.3 **ACN** – Acetonitrile

4.4 **CofA** – Certificate of Analysis

4.5 **H_2O** – Water (≥ 18.2 MΩ·cm)

4.6 **Salicin** – (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(Hydroxymethyl)-6-[2-(hydroxymethyl)phenoxy]oxane-3,4,5-triol

5.0 References

5.1 MV-LAB-18-168, Protocol, Salicin Determination by HPLC using UV/Vis Spectroscopy

5.2 0069-Willow Bark by HPLC: Chromadex application note

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 Water ($\geq$ 18.2 MΩ·cm)

6.1.2 ACN

6.1.3 Salicin reference standard

6.1.4 Methanol

6.1.5 Glacial acetic acid

6.2 Glassware

6.2.1 HPLC vials, 12mm x 32mm with screw cap enclosures with septa

6.2.2 Scintillation Vials

6.2.3 1L Mobile Phase Container

6.2.4 50mL Volumetric Flask

6.2.5 100mL Volumetric Flask

6.3 Disposables

6.3.1 10mL Pipette Tips

6.3.2 1mL Pipette Tips

6.3.3 200µL Pipette Tips

6.3.4 1.5mL microfuge tubes

6.3.5 16mL Test Tubes

6.3.6 Disposable Plastic Luer Lock Syringe – 3mL, 6mL, or 10mL

6.3.7 Nylon Syringe Filters, 0.45µm

6.3.8 Weigh paper

6.4 Equipment

6.4.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV detector with a chromatographic data handling system

6.4.2 Analytical Balance

6.4.3 Ultrasonic bath

6.4.4 Vortex

6.4.5 Stir Plate

6.4.6 Eppendorf Centrifuge

6.4.7 10mL Pipette

6.4.8 1mL Pipette

6.4.9 200µL Pipette

7.0 Preparation of Mobile Phase, Diluent, Samples, and Standards

7.1 Mobile Phase A – 1% Acetic acid in H_2O

Prepared by mixing 10mL glacial acetic acid with 990mL H_2O (scale as necessary).

7.2 Mobile Phase B – Acetonitrile

7.3 Diluent– 20% Methanol (aq)

Prepared by mixing 200mL methanol and 800mL H_2O (scale as necessary).

7.4 Standard Preparation

7.4.1 The linear range of the method is 0.4 mg/mL – 6.0 mg/mL. The final standard and sample preparations must be within the linear range.

7.4.2 Use the actual purity from the CofA or the standard certification for salicin reference material for calculations. The stock standard preparation reflects 100% content for the analyte assayed.

7.4.3 The standard is prepared by weighing no less than the minimum weight of the analytical balance, then bringing up to the final volume in an appropriate volumetric flask using Diluent then sonicating for 5 minutes.

7.4.4 Dilutions are prepared using Diluent. Dilutions can be made using volumetric flasks or using 10mL, 1mL, and 200µL variable pipettes. Specific standard concentrations will approximate the concentration expected to be found in the product being tested based on the sample dilution and calculated from the label. Dilutions can be prepared in HPLC vials.

7.5 Sample Preparation

7.5.1 For finished products, 10 or more dosage units can be pooled and ground by mortar and pestle as necessary.

7.5.2 Samples can be dissolved in Diluent at any volume starting from 50mL and any weight greater than the minimum weight of the analytical balance.

7.5.3 The sample is suspended in the final volume and put in the sonicator bath for at least 10 minutes with occasional shaking to ensure large chunks are dispersed.

7.5.4 Before injection, insoluble matter should be removed via filtration using a 0.45µm nylon syringe filter. Discard at least 0.5mL of filtrate before collecting a portion for analysis. Dilute filtrate as needed then add 1mL of the final dilution to an HPLC vial for analysis.

7.5.4.1 Alternatively, samples and standards can also be centrifuged at 6,000 RPM for at least 200 seconds in an Eppendorf centrifuge to pellet insoluble matter.

7.5.5 For raw materials or finished products being analyzed for the first time using this method, in-process verification is required to demonstrate spectral purity and extraction efficiency before the method can be implemented.

8.0 Test Conditions

8.1 Gradient

Time	%A	%B

0.00	97	3
8.00	97	3
10.00	0	100
15.00	0	100
17.00	97	3
22.00	97	3

8.2 Column – Phenomenex Kinetex, C18, 5um, 100Å, 150mm x 4.6mm, or equivalent.

8.3 Flow Rate – 1.0mL/min

8.4 UV Detection – 268 nm

8.5 3D Spectral Range – 220 nm – 320 nm

8.6 Injection Volume - 10µL

8.7 Column Temperature – Not controlled

8.8 Recommended Sequence

8.8.1 Make at least two injections of the diluent.

8.8.2 Make five injections of Standard Solution.

8.8.3 Make a single injection of each Sample Preparation.

8.8.4 Make a single injection of the Standard Solution after every six samples and at the end of the run.

8.9 System Suitability Requirements

8.9.1 The %RSD of the first five standard injections is NMT 5.0%.

8.9.2 The %RSD of all standard injections is NMT 5%.

8.9.3 Spectral match over the range 220 nm – 320 nm is NLT 900.

8.9.4 The retention time of the sample is within 0.3 min of the standard.

8.10 Column Wash and Storage

8.10.1 Wash the column with H_2O:ACN (50:50) at 1 mL/min for at least 15 min.

8.10.2 Store the column with H_2O:ACN (50:50).

9.0 Calculations

9.1 $$\% \text{ assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of reference standard in mg

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount in mg (solids) or mL (liquids)

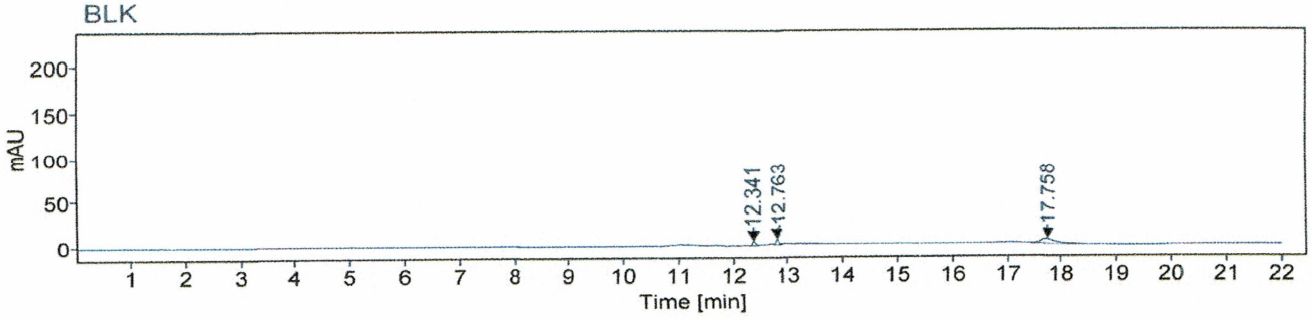
V_{spl} Volume of the sample preparation accounting for dilutions in mL

SS Serving size: Weight of a single dosage unit in mg for tablets and capsules, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

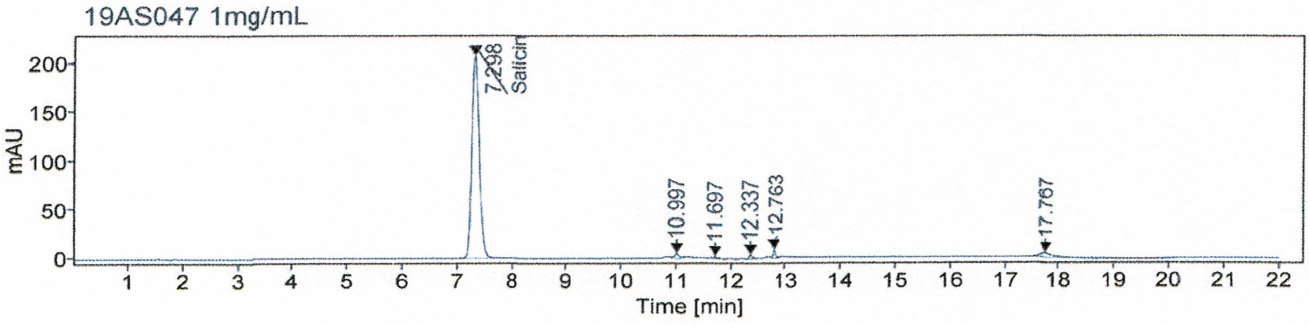
LA Label amount in mg per dose or 1 for raw materials

10.0 Example Chromatography

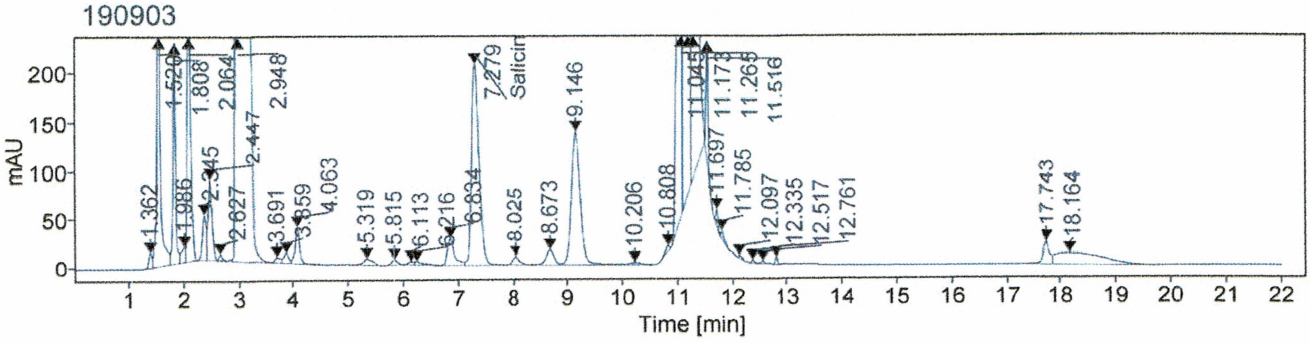
10.1 Blank



10.2 Working Standard



10.3 Sample



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/02/19	New	N/A	J. Maignan
1	04/15/22	Update to reflect current practices and for clarity, add reference to the validation, add linear range, add recommended sequence, add system suitability requirements, add column wash and storage, add example chromatography.	CC-22-0178	S. Sassman