ION LABS	Standard Operating Procedure **Rosmarinic Acid Determination by HPLC using UV/VIS Spectroscopy**	**SOP Number** **D-756**	**Revision** **1**
		Effective Date 04/25/22	**Page** **Page 1 of 8**

Written by/ Date	**Reviewed by/ Date**	**Approved by/ Date**
SAS 04/11/22	04/12/22	04/12/22
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 rosmarinic acid in raw materials and finished product dietary supplements using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of rosmarinic acid in raw materials and finished products. Rosmarinic acid is a good chromophore and was measured at 327, however to minimize any interference, other wavelengths can be used.

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 **Chlorogenic Acid** – 3CQA; 3-O-Caffeoylquinic acid

5.0 References

5.1 TN-1134, Chlorogenic Acids from Green Coffee by HPLC, Z. Aqeel, D. Truong, J. Preston, S. Lazzaro, and S. Baugh. Joint Phenomenex and Chromadex

5.2 D-741, SOP, Chlorogenic Acid Determination by HPLC using UV/Vis Spectroscopy

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 H_2O

6.1.2 ACN

6.1.3 Formic acid

6.1.4 Methanol

6.1.5 Chlorogenic acid reference standard

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, 0.2µm

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, Dissolution Buffer, Samples, and Standards

7.1 Mobile Phase A – 0.1% Formic acid in 25% ACN (aq)

Prepared by mixing 250ml ACN, 750ml H_2O and 1ml of formic acid together

7.2 Mobile Phase B – 0.1% Formic acid in 10% ACN (aq)

Prepared by mixing 100ml ACN, 900ml H_2O and 1ml of formic acid together

7.3 Dissolution Buffer–60% Methanol (aq)

Prepared by mixing 600ml Methanol and 400ml of H_2O together

7.4 Standard Preparation

7.4.1 The linear range of the method is 0.01 mg/mL – 0.10 mg/mL. All 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 Rosmarinic Acid 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 dissolution buffer then sonicating for 10 minutes.

7.4.4 Dilutions are prepared using dissolution buffer. 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.

7.5 Sample Preparation

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

7.5.2 Based on the fill weight, tablet weight, or raw material potency, transfer an amount that is no less than the minimum weight of the analytical balance into a volumetric flask that is no less than 50 mL to generate an analyte concentration within the linear range of the method. If necessary, a sample stock solution can be prepared at higher concentration and diluted using volumetric glassware or pipets.

7.5.3 The sample is suspended in the final volume and put in the sonicator bath for 10 minutes.

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 the filtrate before collecting a portion in an HPLC vial for analysis.

7.5.4.1 Alternatively, samples and standards can also be centrifuged at 5000 RPM for 3 minutes 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	Gradient Type
0.00	100	0	0
9.00	100	0	0
9.10	0	100	0
15.00	0	100	0

8.2 Column – Phenomenex Luna, C18 (2), 5µm, 100Å, LC column, 150mm x 4.6mm, or equivalent

8.3 Flow Rate – 1.0mL/min

8.4 UV Detection – 327 nm

8.5 3D Spectral Range – 220 nm – 400 nm

8.6 Injection Volume - 20µL

8.7 Column Temperature – 30°C

8.8 Retention Time – about 6.5 min

8.9 Recommended Sequence

8.9.1 Make at least 2 injections of the diluent.

8.9.2 Make five (5) injections of Standard Solution.

8.9.3 Make a single injection of each Sample Preparation.

8.9.4 Make a single injection of the Standard Solution after every six (6) samples and at the end of the run.

8.10 System Suitability Requirements

8.10.1 The %RSD of five (5) injections of the Working Standard is NMT 5.0%.

8.10.2 The %RSD of all injections of the Working Standard is NMT 5%.

8.10.3 Spectral match over the range 220 nm – 400 nm is NLT 900.

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

8.11 Example calculations for determining finished product % label or raw material % purity

8.11.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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)

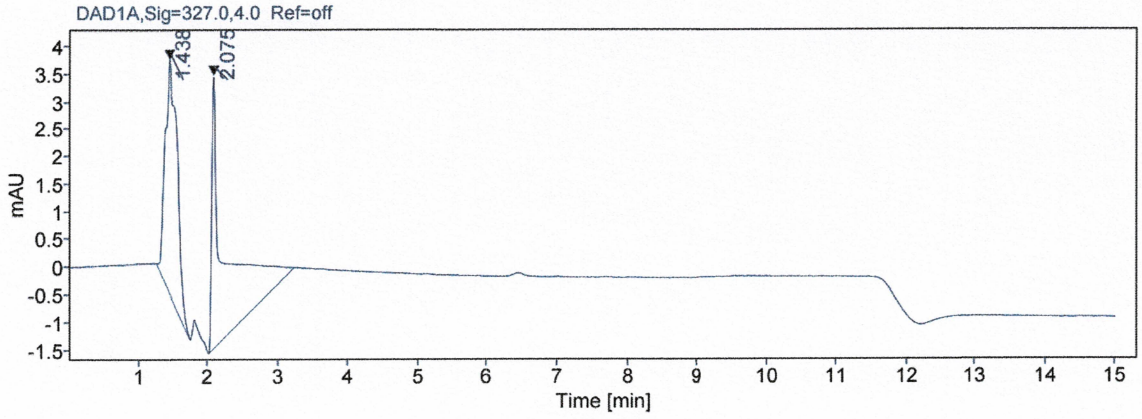
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

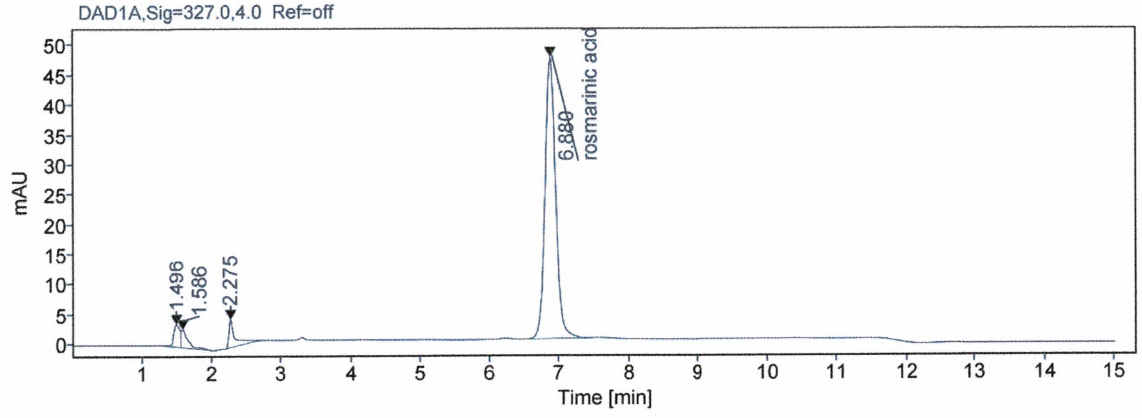
LA Label amount in mg per dose or 1 for raw materials

8.12 Example Chromatography

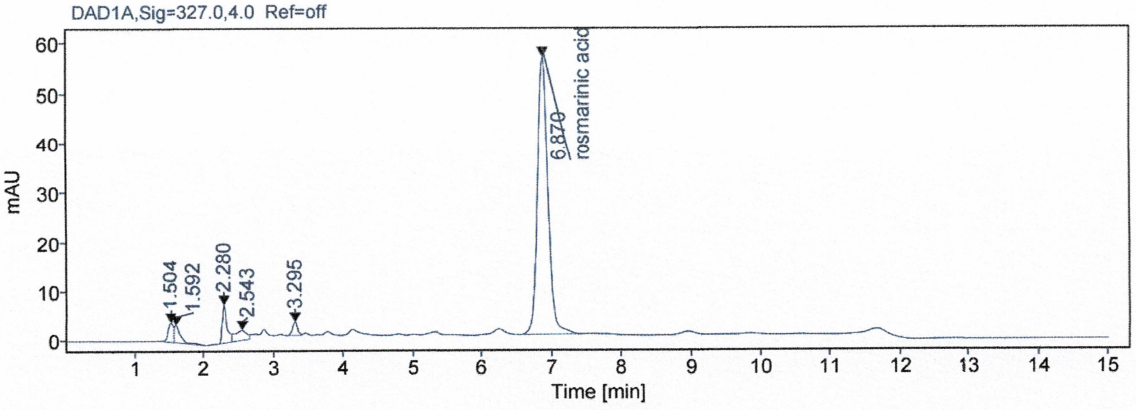
8.12.1 Blank



8.12.2 Working Standard



8.12.3 Sample



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	08/09/18	New procedure.	N/A	J. Maignan
1	04/11/22	Update for consistency with current methods and lab practices, add recommended sequence, add system suitability section, add example chromatography, remove extraneous information.	CC-22-0173	S. Sassman