ION Nutritional Labs	Standard Operating Procedure **Sennosides A and B Determination by HPLC using UV/VIS Spectroscopy**	**SOP Number** **D-762**	**Revision** **3**
		Effective Date 02/04/24	**Page** **Page 1 of 9**

Written by/ Date	**Reviewed by/ Date**	**Approved by/ Date**
SAS 01/31/24	02-01-24	02/05/24
Title: Analytical Development Scientist	**Title: Analytical Development Scientist**	**Title: Quality Control Director**

1.0 Purpose

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

2.0 Scope

This procedure applies to the quantification and identification of the individual or total values of sennosides A and B in raw materials and finished products. Sennosides A and B are moderate chromophores and are measured at 360nm. Other wavelengths should not be used without justification.

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 Management and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

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

4.2 **$Na_2HPO_4 \cdot 12H_2O$** – Dibasic Sodium Phosphate dodecahydrate

4.3 **$NaH_2PO_4 \cdot 2H_2O$** – Monobasic Sodium Phosphate dihydrate

4.4 **BDSAC** – Benzyldimethylstearylammonium chloride

4.5 **H_2O** – Deionized water

4.6 **ACN** – Acetonitrile

4.7 **QC** – Quality Control

5.0 References

5.1 MV-LAB-18-068, Protocol, Sennosides A and B Determination using HPLC with UV/VIS Spectroscopy

5.2 USP 41N36 Monograph for Sennosides

5.3 D-793, SOP, Cryogenic Grinding of Chewable Gels

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 H_2O ($\geq$ 18.2 MΩ·cm)

6.1.2 Sodium Acetate

6.1.3 ACN

6.1.4 $Na_2HPO_4 \cdot 12H_2O$

6.1.5 $NaH_2PO_4 \cdot 2H_2O$

6.1.6 BDSAC

6.1.7 Sennoside A and Sennoside B reference standards

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 Mobile phase containers

6.2.4 Volumetric glassware as required by sample and standard preparations

6.3 Disposables

6.3.1 Tips for adjustable pipettes

6.3.2 Micro-centrifuge tubes

6.3.3 Disposable plastic Luer lock Syringe – 3mL, 6mL, or 10mL

6.3.4 Nylon syringe filters, 0.45µm

6.3.5 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 Micro balance

6.4.4 Ultrasonic bath

6.4.5 Vortex

6.4.6 Stir Plate

6.4.7 Eppendorf centrifuge

6.4.8 Adjustable pipettes

7.0 Preparation of Mobile Phase, Dissolution Buffer, Samples, and Standards

7.1 Mobile Phase A – 200mM Phosphate Buffer pH5.0:ACN:BDSAC (500:500:5)

7.1.1 Transfer 0.062 g of $Na_2HPO_4 \cdot 12H_2O$ to a 1000-mL mobile phase bottle.

7.1.2 Add 1.53 g of $NaH_2PO_4 \cdot 2H_2O$.

7.1.3 Add 5 g of BDSAC.

7.1.4 Add 500 mL of H2O.

7.1.5 Add 500 mL of ACN, and mix to dissolve.

7.1.6 Filter through a 0.45 µm nylon membrane.

7.2 Mobile Phase B – 100% ACN

7.3 Diluent – 1% Sodium Acetate (aq)

7.3.1 Transfer 10 g sodium acetate to a 1000-mL mobile phase bottle.

7.3.2 Add 1000 mL H_2O, and mix to dissolve.

7.4 Stock Standard Preparation

7.4.1 Use a micro-balance with a minimum weight of no less than 5 mg to prepare the Stock Standards.

7.4.2 Prepare individual stock solutions of Sennosides A and B.

7.4.3 Accurately weigh and transfer about 5 mg of each reference standard into separate 50-mL volumetric flasks.

7.4.4 Dilute to volume using Diluent, and sonicate for 10 minutes or until the reference standard is completely dissolved.

7.4.5 Equilibrate to room temperature before performing further dilutions.

7.5 Working Standard Preparation

7.5.1 Transfer 5.0 mL of Sennoside A Stock Standard into a 10-mL volumetric flask.

7.5.2 Dilute to volume with Sennoside B Stock Standard, and mix well.

7.6 Sample Preparation

7.6.1 Specific sample testing details are provided in each products profile. If a specific testing details section is not available, then follow preparation procedure as described below, maintaining concentration within the linear range listed below.

7.6.2 The linear range of the method is 0.01 – 0.1 mg/mL. The analyte concentrations in all standard and sample preparations must be within the linear range of the method.

7.6.3 For raw materials: weigh no less than 20 mg into a suitably sized volumetric flask of no less than 25 mL volume to generate an analyte concentration that is within

the validated linearity range. Dilute to volume with Diluent, and sonicate for 10 min.

7.6.4 For solid and liquid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and fill weight (capsules), serving size (powders and liquids) or tablet weight per dose, weigh no less than 50 mg of the pooled dosages into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linear range. Dilute to volume with Diluent, and sonicate for 10 min.

7.6.5 For chewable gels (gummies), homogenize at least 10 dosage units according to the procedure outlined in D-793 Cryogenic Grinding of Chewable Gels. Quickly weigh a portion of the pooled and homogenized dosages into a beaker. Use several small portions of Diluent to completely transfer the sample into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Dilute to volume, and sonicate for 10 min.

7.6.6 To manage large volumes, the standard can be initially prepared at a higher concentration and further diluted into the linear range using Diluent. **Equilibrate to room temperature prior to performing further dilution.** Dilutions can be made using volumetric glassware and/or adjustable pipettes. Dilutions can be prepared in HPLC vials

7.6.7 If particulates remain in the final sample preparation, a portion may be centrifuged at 10,000 rpm for 5 min prior to HPLC analysis. Alternatively, the sample may be filtered through a 0.45 µm membrane discarding the first 3 – 4 mL.

8.0 Test Conditions

8.1 Gradient

Time	%A	%B
0.00	100	0
35.00	100	0

40.00	30	70
50.00	30	70
55.00	100	0
60.00	100	0

8.2 Column – Kinetex XB-C18, 5µm, 100Å, LC column, 250mm x 4.6mm, or equivalent

8.3 Flow Rate – 1.0mL/min

8.4 UV Detection – 360nm

8.5 Injection Volume - 10µL

8.6 Column Temperature – 40°C

8.7 Recommended 3-D Spectral Range – 200 to 400 nm

8.8 Retention Times

8.8.1 Sennoside A is about 43 min

8.8.2 Sennoside B is about 25 min

8.9 Recommended Sequence

8.9.1 Make at least 2 injections of a Blank (Diluent).

8.9.2 Make a single injection of the Sennoside A Stock Standard.

8.9.3 Make a single injection of the Sennoside B Stock Standard.

8.9.4 Make five injections of the Working Standard.

8.9.5 Make a single injection of each Sample Preparation.

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

8.10 System Suitability

8.10.1 The %RSD of five consecutive injections of Working Standard is NMT 5.0%.

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

8.11 Column Wash and Storage

8.11.1 Rinse the column with H_2O / ACN (90/10) at 1 mL/min for at least 15 min.

8.11.2 Rinse the column with H_2O / ACN (50/50) at 1 mL/min for at least 10 min.

8.11.3 Store the column with H_2O / ACN (50/50).

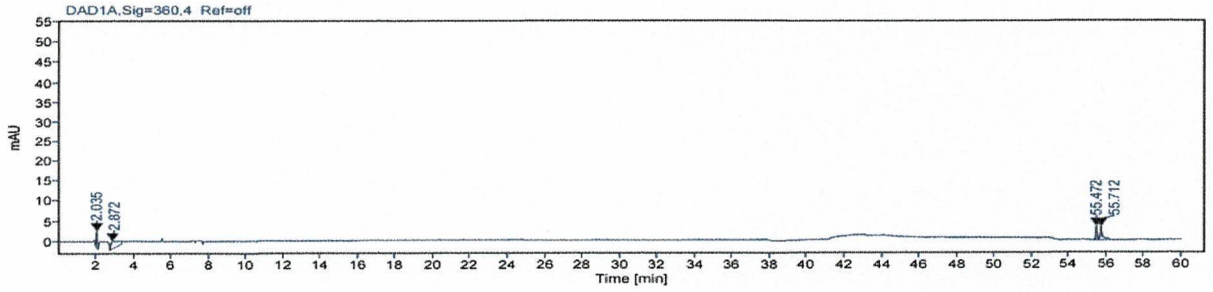
9.0 Example Calculation

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

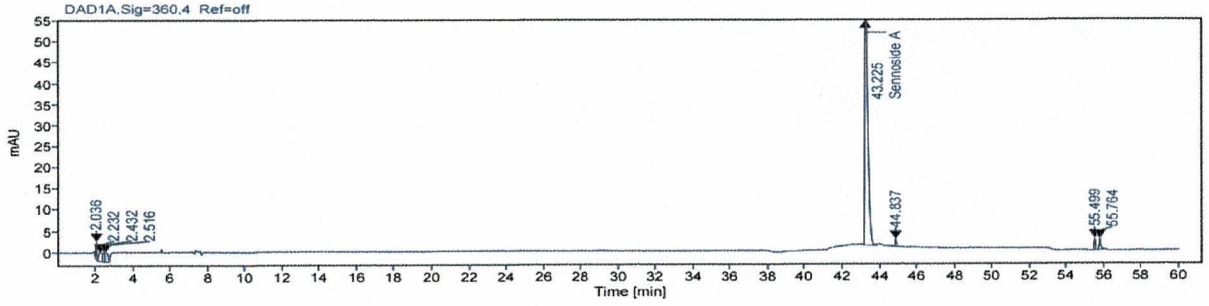
R_u	Sample peak area
R_s	Mean standard peak area
Wt_{std}	Weight of reference standard in mg
V_{std}	Volume of the standard preparation accounting for dilutions in mL
P	Purity of the reference standard in decimal format
SA	Sample amount in mg
V_{spl}	Volume of the sample preparation accounting for dilutions in mL
SS	Serving size: Weight of a single dosage unit in mg or 1 for raw materials.
LA	Label amount in mg per dose or 1 for raw materials

10.0 Example Chromatography

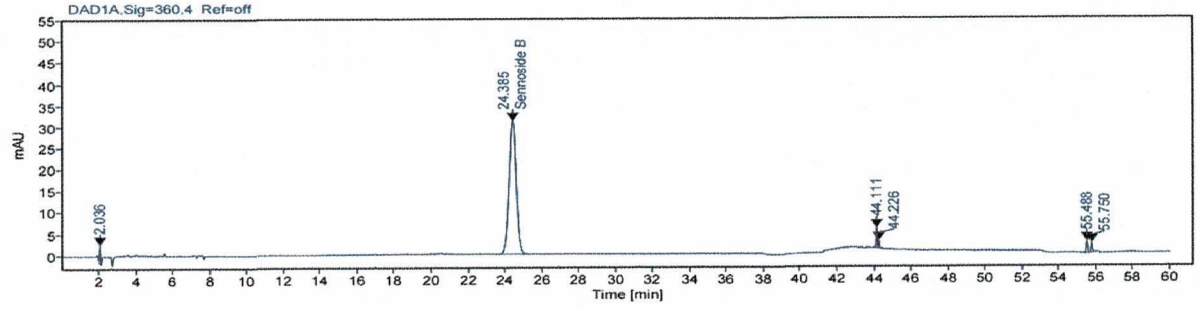
10.1 Blank



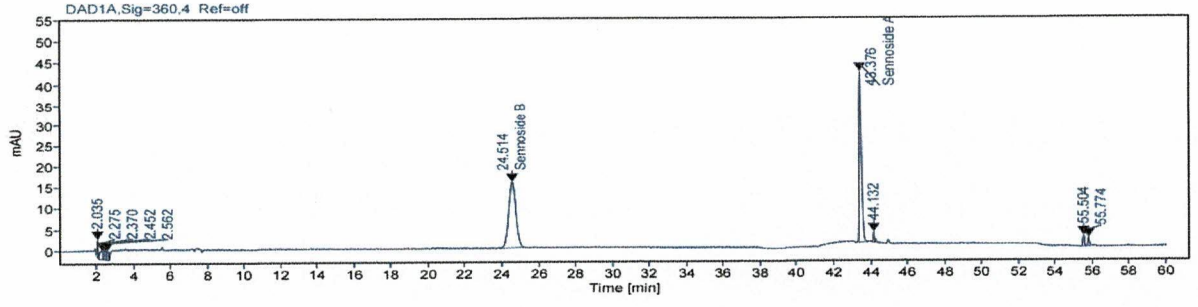
10.2 Sennoside A Stock Standard



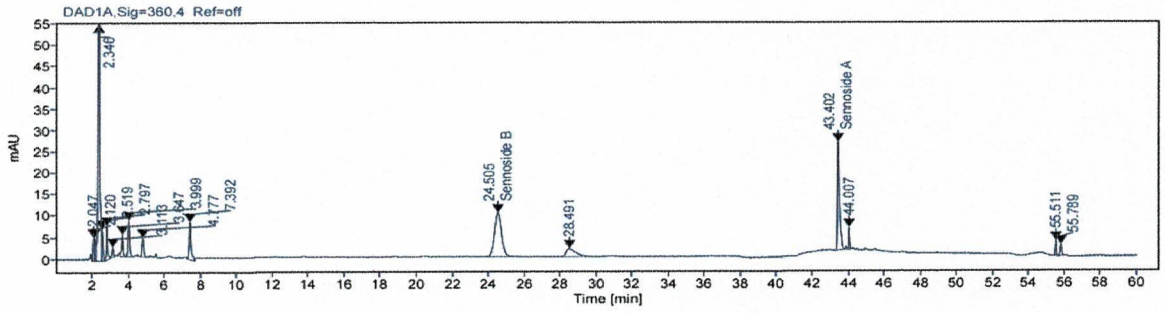
10.3 Sennoside B Stock Standard



10.4 Working Standard



10.5 Finished Product Sample



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/02/19	New procedure.	N/A	J. Maignan
1	06/20/22	Updated for consistency with current methods. Added recommended sequence section. Replaced requirements with system suitability section. Added column wash and storage. Added example chromatography. Updated logo and format.	CC-22-0278	S. Sassman
2	06/06/23	Added instruction to follow test details containing product specific sample preparation. Added specific sample prep instructions for different dosage forms. Adjusted retentions times. Updated example chromatograms. Updated logo and format.	CC-23-0266	S. Sassman
3	01/17/24	Changed NaOAc to sodium acetate since it was not defined. Corrected typo in gradient.	CC-24-0023	S. Sassman