CCR No CC-25-0510 Rev 0 Due Date 01/18/25

SECTION 1 – Change Proposal (Initiator)				
Type of Change [1]	Description	Document No.	Revision No.	Initiated By/Date
SOP	Apoaequorin Determination by HPLC using UV-VIS Spectroscopy	D-770	7	AJS 12/19/25

Change Summary

Implemented standard recovery requirement

Rationale

Per CAPA-25-0074

[1] (i.e. Standard Operating Procedure, Forms, Standard Test Methods, Specifications, Product Profiles, Protocols, Reports, Master Batch Records, Materials, Packaging Components, Labeling, Equipment, Suppliers)

Change Request Approved By/Date: JLK 12/18/25

1. Initial Changes made by/Date: AJS 12/19/25
2. Additional Changes made by/Date: N/A MTJ
3. Additional Changes made by/Date: 01-09-26

SECTION 2 – Change Control Review and Impact Assessment/Requirements for Closure (DC/Reviewer/Approver)				
No.	Activity	Responsible	Assigned By/Date	Complete
1.			MTJ	☐
2.		N/A		☐
3.	01-09-26			☐

	Change Control Request Form		
	Form: C-403-F1	CCR No. CC-22-0477	Revision: 8

CCR No CC-25-0510 Rev 0 Due Date 01/18/25

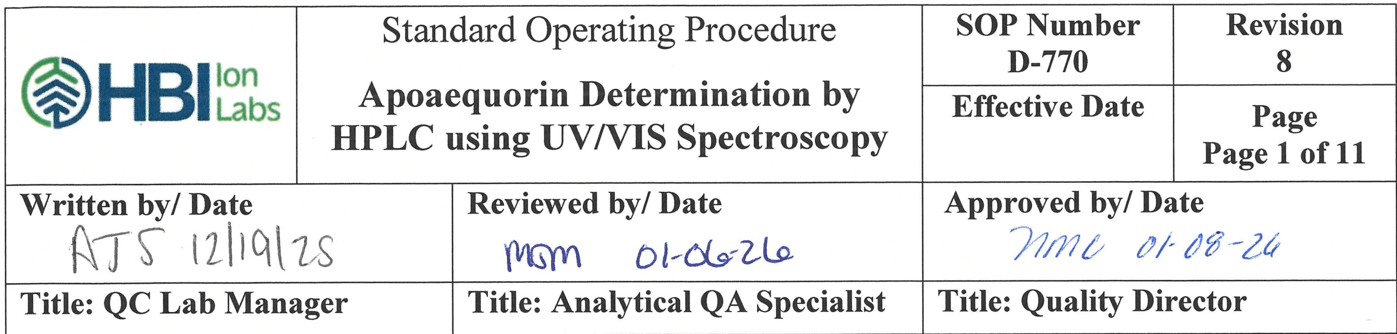
Customer Notification Required: ☐ Yes ☑ No Date of Notification: N/A (attach evidence)

SECTION 3 – Approvals [Reviewed/Approved (Y) Yes or (N) No-Initials/Date. If No, provide explanation							
Department	Print Name	First Routing			Second Routing		
		Y / N	Initials	Date	Y / N	Initials	Date
QA	Melisa Maples	Y	MSM	01-06-26	N/A MTS 01-09-26		
QA	Nicole Motley	Y	NML	01-08-26			
N/A MTS 01-09-26							

SECTION 4 – Change Control Implementation (DC)					
Document Effective Date	01-09-26	By	MTS	Date	01-09-26

SECTION 5 – Change Control Closure (DC)				
☑ All requirements included in the change control verified as complete and closed	By	MTS	Date	01-09-26

Comments N/A MTS 01-09-26



HBI Ion Labs	Standard Operating Procedure **Apoaequorin Determination by HPLC using UV/VIS Spectroscopy**	**SOP Number** **D-770**	**Revision** **8**
		Effective Date	**Page** **Page 1 of 11**

Written by/ Date	**Reviewed by/ Date**	**Approved by/ Date**
AJS 12/19/25	MGM 01-06-26	MMC 01-08-26
Title: QC Lab Manager	**Title: Analytical QA Specialist**	**Title: Quality Director**

1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of Apoaequorin 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 Apoaequorin in raw materials and finished products in the QC laboratory at Ion Labs. Apoaequorin is a good chromophore and was measured at 215 nm.

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 spectrum

4.2 **Tris –** Tris(hydroxymethyl)aminomethane base

4.3 **H_3PO_4 –** Phosphoric Acid

4.4 **EDTA –** Ethylenediaminetetraacetic acid disodium salt dihydrate

4.5 **NaCl –** Sodium chloride

4.6 **TFA** – Triflouroacetic acid

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4.7 **ACN** – Acetonitrile

4.8 **CofA** – Certificate of analysis

4.9 **H_2O** – Water (≥ 18.2 MΩ·cm)

4.10 **Apoaequorin** – Fluorescent protein isolated from jellyfish

5.0 References

5.1 MV-LAB-18-167, Protocol, Apoaequorin Determination using HPLC with UV/VIS Spectroscopy

5.2 PRTCL-24-0033, Protocol, Supplemental Validation of D-770 for Determination of Apoaequorin in Chewable Gels by HPLC-UV

6.0 Supplies

6.1 Chemicals: All reagents are ACS grade or better.

6.1.1 H_2O

6.1.2 ACN

6.1.3 TFA

6.1.4 Tris

6.1.5 Apoaequorin reference standard

6.1.6 NaCl

6.1.7 H_3PO_4

6.1.8 EDTA

6.2 Glassware

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

6.2.2 HPLC vial inserts

6.2.3 Erlenmeyer Flasks

6.2.4 Mobile Phase Containers

6.2.5 Volumetric Flasks

6.2.6 Volumetric Pipets

6.3 Disposables

6.3.1 Pipette Tips

6.3.2 1.5mL microfuge tubes

6.3.3 Razor Blades

6.3.4 Disposable Plastic Luer Lock Syringe

6.3.5 0.45µm PVDF low-binding protein filters or equivalent or PTFE

6.3.6 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 Analytical Balance

6.4.4 Stir Plate

6.4.5 Wrist-action Shaker

6.4.6 Microfuge

6.4.7 Adjustable Pipettes

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

7.1 Mobile Phase Preparation

7.1.1 All mobile phases should be prepared in glass only.

7.1.2 Mobile Phase A – 30/70 ACN/H_2O + 0.1% TFA

7.1.2.1 Transfer 300mL ACN to a 1000-mL glass bottle.

7.1.2.2 Add 700mL H_2O.

7.1.2.3 Carefully add 1.0mL TFA, and mix well.

7.1.3 Mobile Phase B – ACN + 0.1% TFA

7.1.3.1 Transfer 1000mL ACN to a 1000-mL glass bottle.

7.1.3.2 Carefully add 1.0mL TFA, and mix well.

7.1.4 Diluent – 60/40 ACN/H_2O

7.1.4.1 Transfer 600mL ACN to a 1000-mL glass bottle.

7.1.4.2 Add 400mL H_2O, and mix well.

7.1.5 Resuspension Buffer – 50mM TRIS, 25mM NaCl, 1mM EDTA, pH8.5

7.1.5.1 Transfer 1.46 g of NaCl to a 1000-mL glass bottle.

7.1.5.2 Add 0.372 g of EDTA.

7.1.5.3 Add 6.06 g of TRIS.

7.1.5.4 Add 1000mL of H_2O, and mix until dissolved.

7.1.5.5 Adjust to pH 8.5 using H_3PO_4.

7.1.6 Standard Preparation

7.1.6.1 The linear range of the method is 0.0068 – 0.8 mg/mL. All final standard and sample preparations must be within this range. Use the actual purity from the CofA or the standard certification for apoaequorin reference material for calculations.

7.1.6.2 All standards are prepared in duplicate (Std A and Std B).

7.1.6.3 Mix solutions gently, being sure to not allow foam to form. Do NOT vortex. Do NOT re-freeze the reference standard after use. Allow to warm to room temperature before use.

7.1.6.3.1 Examine the 8.3mg/ml stock standard after thawing from frozen state. Mix Standard solution gently before making dilution for the working standard.

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Note: The Standard Material can precipitate out of solution causing the standard concentration to be lower than the expected 8.3mg/ml. The precipitation of the standard from the solution causes high results for assay testing. It is essential to verify standard is fully dissolved before proceeding with dilutions.

Examine the 8.3mg/ml stock standard after thawing from frozen state. Mix Standard solution gently before making dilution for the working standard.

7.1.6.4 The standard is prepared by using glass volumetric pipets and glass volumetric flasks.

7.1.6.5 The target concentration of the working standard is generally 0.5 mg/mL, however, see 7.1.6.4.1 if analyzing gummies. The working standard is prepared in Diluent from a pre-prepared stock. Choose aliquot and final volumes to result in a final concentration of about 0.5 mg/mL.

Example: Prepare a 0.5mg/mL working standard from an 8.3mg/mL stock. Using an aliquot volume of 1.0mL, first calculate the final volume.

$M_1*V_1=M_2*V_2$

8.3 mg/mL (stock) * 1mL = 0.5mg/mL * V_2 mL

V_2=16.6mL (most convenient final volume is 16mL)

Using a glass volumetric pipet, add 1.0mL of 8.3mg/mL stock to a 25-mL glass volumetric flask. Using a glass volumetric pipet, add 15.0mL of Diluent to the same 25-mL volumetric flask. Mix gently. Do not dilute to volume.

Std Conc = 8.3mg/mL * 1mL / 16mL = 0.51875 mg/mL

7.1.6.5.1 If analyzing gummies, perform an additional 1:25 dilution in diluent using volumetric glassware for a working standard concentration of ~20µg/ml.

7.1.6.6 Pass standard through 0.45µm PVDF low-protein-binding filter (or equivalent), being sure to discard the first few milliliters to waste.

7.1.6.7 Add standard to a 2mL HPLC vial. (A low-volume insert can be used to extend working life of the 0.5mg/mL working standard.)

7.1.6.8 The 0.5mg/mL working standard can be stored for up to 8 weeks at 2-8°C. (Refrigerated storage does not apply to the 20µg/ml working standard, this standard must be prepared and used fresh.)

7.2 Sample Preparation

7.2.1 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 above.

7.2.2 The target concentration of the final sample solution is generally 0.5 mg/mL. Gummies are analyzed at ~20µg/mL. Sample preparation examples are provided below in 7.2.6. The final sample concentration must be within the linear range of the method.

7.2.3 For raw materials, consult the potency listed on the vendor COA for calculation of the required sample weight.

7.2.4 For finished products, a composite of no less than 10 dosage units is generally used as the sample for analysis.

7.2.5 The sample stock solution should be prepared in Resuspension Buffer. Carefully and gently mix to ensure powder is fully suspended. Then, stir or mix on a wrist action shaker **at low speed** for at least 1 hour being careful to not allow foam to form. For stirring, dilute to volume first then add a stir bar prior to stirring for at least 1 hour. For shaking, dilute to 2/3 volume, shake for at least 1 hour, then dilute to the final volume and mix gently.

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7.2.6 A 10x dilution of the sample stock solution should be performed with Diluent to dilute the Resuspension Buffer salts. Filter through a 0.45µm PVDF low-binding protein filter (or equivalent) being sure to discard the first few milliliters before collecting a portion in a HPLC vial for analysis.

Example: Prevagen capsule (label claim = 20 mg, dosage unit = 467 mg)

Prepare 10mL of a 0.5mg/mL solution

Combine the fill material from 20 capsules. Prepare a sample stock solution by transferring 5.8g of the combined fill material to a 50mL volumetric flask, and gently dissolving in Resuspension Buffer. Transfer 1.0mL of the stock solution to a 10-mL volumetric flask, and dilute to volume using Diluent.

20mg / 0.467g * 5.8 g / 50mL * 1mL / 10mL = 0.4968 mg/mL

Example: Apoaequorin raw material (given 100% potency)

Prepare 10mL of a 0.5mg/mL solution

Prepare a sample stock solution by transferring 250mg of the combined fill material to a 50mL volumetric flask, and gently dissolving in Resuspension Buffer. Transfer 1.0mL of the stock solution to a 10-mL volumetric flask, and dilute to volume using Diluent.

250mg / 50mL * 1mL / 10mL = 0.5 mg/mL

Example: Prevagen gummy (label claim = 5 mg, dosage unit = 1 gummy)

Prepare 100mL of a 0.2mg/mL solution

Quarter four gummies with a razor and prepare a sample stock solution by transferring to a 125mL Erlenmeyer flask and diluting to ~80mL with Resuspension Buffer. Parafilm and stir gently on a stir plate for 1 hour. Ensure gummies are completely dissolved. Quantitatively transfer to a 100mL volumetric flask, QS to volume and gently mix.

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Transfer 5.0mL of the stock solution to a 50-mL volumetric flask, and dilute to volume using Diluent.

20mg / 100mL * 5mL / 50mL = 20 µg/mL

8.0 Test Conditions

8.1 Gradient

Time	%A	%B
0.00	90	10
0.3	90	10
2.7	50	50
2.73	10	90
3.57	10	90
3.6	90	10
6.0	90	10

8.2 Column – Phenomenex Jupiter, C4, 5um, 300Å, LC column, 150mm x 4.6mm, or equivalent.

8.3 Flow Rate – 2.0mL/min

8.4 UV Detection – 215nm

8.5 Recommended 3D Spectral Range – 200nm – 300nm

8.6 Injection Volume – 15.5µL

8.7 Column Temperature – 40°C

8.8 Retention Time – about 2.7 minutes

8.9 Recommended Sequence

8.9.1 Make at least two injections of the diluent.

8.9.2 Make five injections of Standard Solution A.

8.9.3 Make two injections of Standard Solution B.

8.9.4 Make a single injection of each Sample Preparation.

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

8.10 System Suitability Requirements

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

8.10.2 The % recovery of Working Standard A, using Working Standard B is 98-102%

8.10.3 The %RSD of all Standard Solution A injections is NMT 5%.

8.11 Column Wash and Storage

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

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

9.0 Calculations

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

R_u Sample peak area

R_s Mean standard peak area

C_{std} Concentration of pre-prepared Stock Standard in mg/mL

Aq_{std} Aliquot of Stock Standard used to prepare Working Standard in mL

V_{std} Final Volume of the Working Standard in mL

SA Sample amount in mg (solids) or mL (liquids), or 4 for gummies

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 and gummies.

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

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9.2. % Difference from CofA

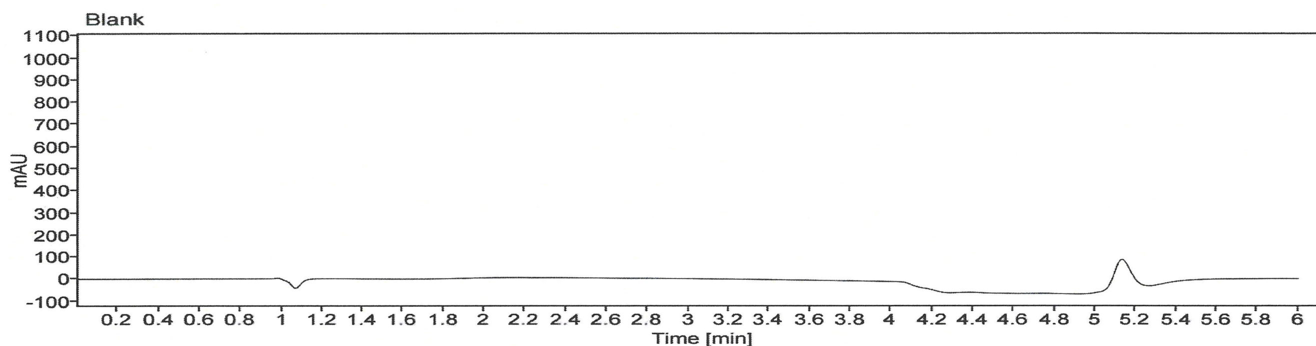
$$\% \text{ Difference from CofA} = \frac{|A_D - A_{CofA}|}{A_{CofA}} \times 100$$

A_D Assay value determined

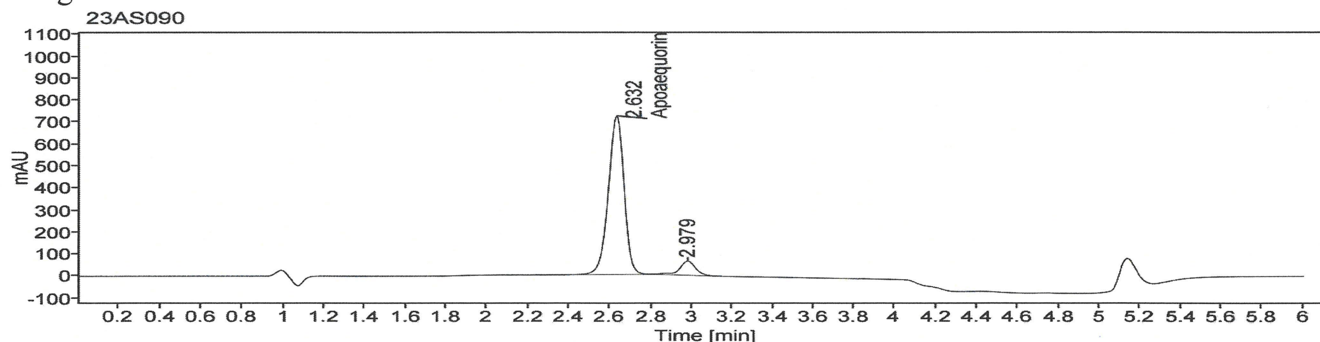
A_{CofA} Assay value reported on manufacturer's Certificate of Analysis

10.0 Example Chromatography

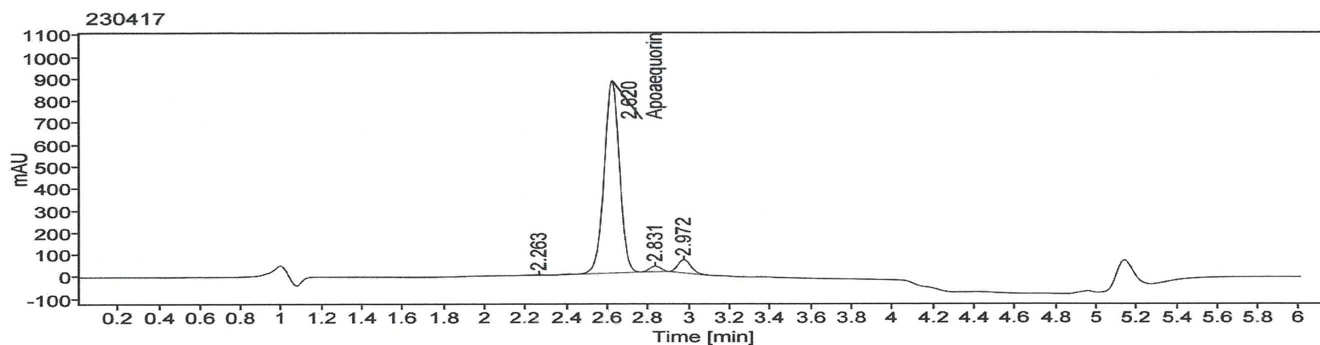
10.1 Blank



rking Standard



10.2 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 details for prep of suspension buffer, add recommended sequence, add system suitability requirements, add column wash and storage, add example chromatography.	CC-22-0177	S. Sassman
2	11/16/22	Clarified spectral range. Remove retention time requirement.	CC-22-0441	J. Sassman
3	02/23/23	Add instruction to check the product profile for test details, remove language requiring in-process validation for new products, remove spectral match as system suitability requirement.	CC-23-0095	S. Sassman
4	12/09/23	Added more representative chromatography.	CC-23-0592	J. Sassman
5	04/19/24	Revise method to include expanded linear range and new finished product matrix.	CC-24-0165	C. Perry
6	11/27/24	Added calculation for determination of % difference from value on manufacturer's Certificate of Analysis	CC-24-0538	K. Bittler
7	04/30/25	Added standard solution examination after thawing	CC-25-0196	K. Bittler
8	12/18/25	Implemented standard recovery requirement.	CC-25-0510	A. Shannon

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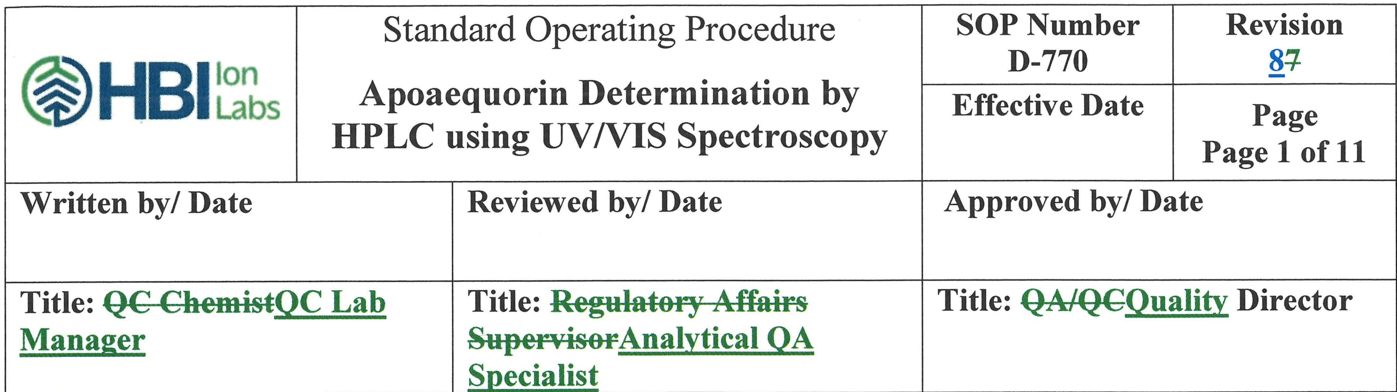

HBI Ion Labs	Standard Operating Procedure **Apoaequorin Determination by HPLC using UV/VIS Spectroscopy**	**SOP Number** **D-770**	**Revision** **8~~7~~**
		Effective Date	**Page** **Page 1 of 11**
Written by/ Date	**Reviewed by/ Date**	**Approved by/ Date**	
Title: ~~QC Chemist~~QC Lab Manager	**Title: ~~Regulatory Affairs Supervisor~~Analytical QA Specialist**	**Title: ~~QA/QC~~Quality Director**	

Redline

1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of Apoaequorin 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 Apoaequorin in raw materials and finished products in the QC laboratory at Ion Labs. Apoaequorin is a good chromophore and was measured at 215 nm.

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 spectrum

4.2 **Tris –** Tris(hydroxymethyl)aminomethane base

4.3 **H_3PO_4 –** Phosphoric Acid

4.4 **EDTA –** Ethylenediaminetetraacetic acid disodium salt dihydrate

4.5 **NaCl –** Sodium chloride

4.6 **TFA** – Triflouroacetic acid

4.7 **ACN** – Acetonitrile

4.8 **CofA** – Certificate of analysis

4.9 **H_2O** – Water (≥ 18.2 MΩ·cm)

4.10 **Apoaequorin** – Fluorescent protein isolated from jellyfish

5.0 References

5.1 MV-LAB-18-167, Protocol, Apoaequorin Determination using HPLC with UV/VIS Spectroscopy

5.2 PRTCL-24-0033, Protocol, Supplemental Validation of D-770 for Determination of Apoaequorin in Chewable Gels by HPLC-UV

6.0 Supplies

6.1 Chemicals: All reagents are ACS grade or better.

6.1.1 H_2O

6.1.2 ACN

6.1.3 TFA

6.1.4 Tris

6.1.5 Apoaequorin reference standard

6.1.6 NaCl

6.1.7 H_3PO_4

6.1.8 EDTA

6.2 Glassware

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

6.2.2 HPLC vial inserts

6.2.3 Erlenmeyer Flasks

6.2.4 Mobile Phase Containers

6.2.5 Volumetric Flasks

6.2.6 Volumetric Pipets

6.3 Disposables

6.3.1 Pipette Tips

6.3.2 1.5mL microfuge tubes

6.3.3 Razor Blades

6.3.4 Disposable Plastic Luer Lock Syringe

6.3.5 0.45µm PVDF low-binding protein filters or equivalent or PTFE

6.3.6 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 Analytical Balance

6.4.4 Stir Plate

6.4.5 Wrist-action Shaker

6.4.6 Microfuge

6.4.7 Adjustable Pipettes

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

7.1 Mobile Phase Preparation

7.1.1 All mobile phases should be prepared in glass only.

7.1.2 Mobile Phase A – 30/70 ACN/H_2O + 0.1% TFA

7.1.2.1 Transfer 300mL ACN to a 1000-mL glass bottle.

7.1.2.2 Add 700mL H_2O.

7.1.2.3 Carefully add 1.0mL TFA, and mix well.

7.1.3 Mobile Phase B – ACN + 0.1% TFA

7.1.3.1 Transfer 1000mL ACN to a 1000-mL glass bottle.

7.1.3.2 Carefully add 1.0mL TFA, and mix well.

7.1.4 Diluent – 60/40 ACN/H_2O

7.1.4.1 Transfer 600mL ACN to a 1000-mL glass bottle.

7.1.4.2 Add 400mL H_2O, and mix well.

7.1.5 Resuspension Buffer – 50mM TRIS, 25mM NaCl, 1mM EDTA, pH8.5

7.1.5.1 Transfer 1.46 g of NaCl to a 1000-mL glass bottle.

7.1.5.2 Add 0.372 g of EDTA.

7.1.5.3 Add 6.06 g of TRIS.

7.1.5.4 Add 1000mL of H_2O, and mix until dissolved.

7.1.5.5 Adjust to pH 8.5 using H_3PO_4.

7.1.6 Standard Preparation

7.1.6.1 The linear range of the method is 0.0068 – 0.8 mg/mL. All final standard and sample preparations must be within this range. Use the actual purity from the CofA or the standard certification for apoaequorin reference material for calculations.

~~7.1.6.1~~7.1.6.2 All standards are prepared in duplicate (Std A and Std B).

~~7.1.6.2~~7.1.6.3 Mix solutions gently, being sure to not allow foam to form. Do NOT vortex. Do NOT re-freeze the reference standard after use. Allow to warm to room temperature before use.

~~7.1.6.2.1~~7.1.6.3.1 Examine the 8.3mg/ml stock standard after thawing from frozen state. Mix Standard solution gently before making dilution for the working standard.

Note: The Standard Material can precipitate out of solution causing the standard concentration to be lower than the expected 8.3mg/ml. The precipitation of the standard from the solution causes high results for assay testing. It is essential to verify standard is fully dissolved before proceeding with dilutions.

Examine the 8.3mg/ml stock standard after thawing from frozen state. Mix Standard solution gently before making dilution for the working standard.

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~~7.1.6.4~~7.1.6.5 The target concentration of the working standard is generally 0.5 mg/mL, however, see 7.1.6.4.1 if analyzing gummies. The working standard is prepared in Diluent from a pre-prepared stock. Choose aliquot and final volumes to result in a final concentration of about 0.5 mg/mL.

Example: Prepare a 0.5mg/mL working standard from an 8.3mg/mL stock. Using an aliquot volume of 1.0mL, first calculate the final volume.

$M_1*V_1=M_2*V_2$

8.3 mg/mL (stock) * 1mL = 0.5mg/mL * V_2 mL

V_2=16.6mL (most convenient final volume is 16mL)

Using a glass volumetric pipet, add 1.0mL of 8.3mg/mL stock to a 25-mL glass volumetric flask. Using a glass volumetric pipet, add 15.0mL of Diluent to the same 25-mL volumetric flask. Mix gently. Do not dilute to volume.

Std Conc = 8.3mg/mL * 1mL / 16mL = 0.51875 mg/mL

~~7.1.6.4.1~~7.1.6.5.1 If analyzing gummies, perform an additional 1:25 dilution in diluent using volumetric glassware for a working standard concentration of ~20µg/ml.

~~7.1.6.5~~7.1.6.6 Pass standard through 0.45µm PVDF low-protein-binding filter (or equivalent), being sure to discard the first few milliliters to waste.

~~7.1.6.6~~7.1.6.7 Add standard to a 2mL HPLC vial. (A low-volume insert can be used to extend working life of the 0.5mg/mL working standard.)

~~7.1.6.7~~7.1.6.8 The 0.5mg/mL working standard can be stored for up to 8 weeks at 2-8°C. (Refrigerated storage does not apply to the 20µg/ml working standard, this standard must be prepared and used fresh.)

7.2 Sample Preparation

7.2.1 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 above.

7.2.2 The target concentration of the final sample solution is generally 0.5 mg/mL. Gummies are analyzed at ~20µg/mL. Sample preparation examples are provided below in 7.2.6. The final sample concentration must be within the linear range of the method.

7.2.3 For raw materials, consult the potency listed on the vendor COA for calculation of the required sample weight.

7.2.4 For finished products, a composite of no less than 10 dosage units is generally used as the sample for analysis.

7.2.5 The sample stock solution should be prepared in Resuspension Buffer. Carefully and gently mix to ensure powder is fully suspended. Then, stir or mix on a wrist action shaker **at low speed** for at least 1 hour being careful to not allow foam to form. For stirring, dilute to volume first then add a stir bar prior to stirring for at least 1 hour. For shaking, dilute to 2/3 volume, shake for at least 1 hour, then dilute to the final volume and mix gently.

7.2.6 A 10x dilution of the sample stock solution should be performed with Diluent to dilute the Resuspension Buffer salts. Filter through a 0.45µm PVDF low-binding protein filter (or equivalent) being sure to discard the first few milliliters before collecting a portion in a HPLC vial for analysis.

Example: Prevagen capsule (label claim = 20 mg, dosage unit = 467 mg)

Prepare 10mL of a 0.5mg/mL solution

Combine the fill material from 20 capsules. Prepare a sample stock solution by transferring 5.8g of the combined fill material to a 50mL volumetric flask, and gently dissolving in Resuspension Buffer. Transfer 1.0mL of the stock solution to a 10-mL volumetric flask, and dilute to volume using Diluent.

20mg / 0.467g * 5.8 g / 50mL * 1mL / 10mL = 0.4968 mg/mL

Example: Apoaequorin raw material (given 100% potency)

Prepare 10mL of a 0.5mg/mL solution

Prepare a sample stock solution by transferring 250mg of the combined fill material to a 50mL volumetric flask, and gently dissolving in Resuspension Buffer. Transfer 1.0mL of the stock solution to a 10-mL volumetric flask, and dilute to volume using Diluent.

250mg / 50mL * 1mL / 10mL = 0.5 mg/mL

Example: Prevagen gummy (label claim = 5 mg, dosage unit = 1 gummy)

Prepare 100mL of a 0.2mg/mL solution

Quarter four gummies with a razor and prepare a sample stock solution by transferring to a 125mL Erlenmeyer flask and diluting to ~80mL with Resuspension Buffer. Parafilm and stir gently on a stir plate for 1 hour. Ensure gummies are completely dissolved. Quantitatively transfer to a 100mL volumetric flask, QS to volume and gently mix.

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Transfer 5.0mL of the stock solution to a 50-mL volumetric flask, and dilute to volume using Diluent.

20mg / 100mL * 5mL / 50mL = 20 μg/mL

8.0 Test Conditions

8.1 Gradient

Time	%A	%B
0.00	90	10
0.3	90	10
2.7	50	50
2.73	10	90
3.57	10	90
3.6	90	10
6.0	90	10

8.2 Column – Phenomenex Jupiter, C4, 5um, 300Å, LC column, 150mm x 4.6mm, or equivalent.

8.3 Flow Rate – 2.0mL/min

8.4 UV Detection – 215nm

8.5 Recommended 3D Spectral Range – 200nm – 300nm

8.6 Injection Volume – 15.5μL

8.7 Column Temperature – 40°C

8.8 Retention Time – about 2.7 minutes

8.9 Recommended Sequence

8.9.1 Make at least two injections of the diluent.

8.9.2 Make five injections of Standard Solution A.

8.9.28.9.3 Make two injections of Standard Solution B.

8.9.38.9.4 Make a single injection of each Sample Preparation.

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

8.10 System Suitability Requirements

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

8.10.18.10.2 The % recovery of Working Standard A, using Working Standard B is 98-102%

8.10.28.10.3 The %RSD of all SStandard Solution A injections is NMT 5%.

8.11 Column Wash and Storage

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

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

9.0 Calculations

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

R_u Sample peak area

R_s Mean standard peak area

C_{std} Concentration of pre-prepared Stock Standard in mg/mL

Aq_{std} Aliquot of Stock Standard used to prepare Working Standard in mL

V_{std} Final Volume of the Working Standard in mL

SA Sample amount in mg (solids) or mL (liquids), or 4 for gummies

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 and gummies.

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

9.2. % Difference from CofA

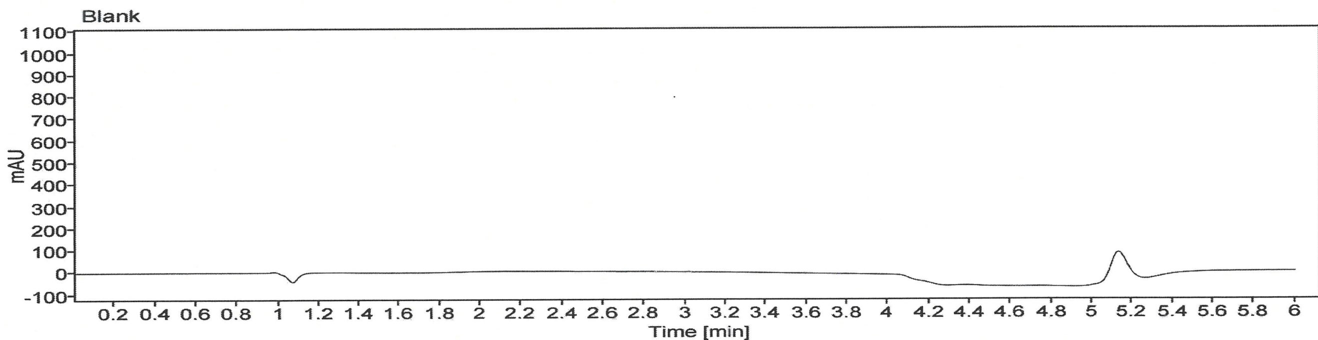
$$\% \text{ Difference from CofA} = \frac{|A_D - A_{CofA}|}{A_{CofA}} \times 100$$

A_D Assay value determined

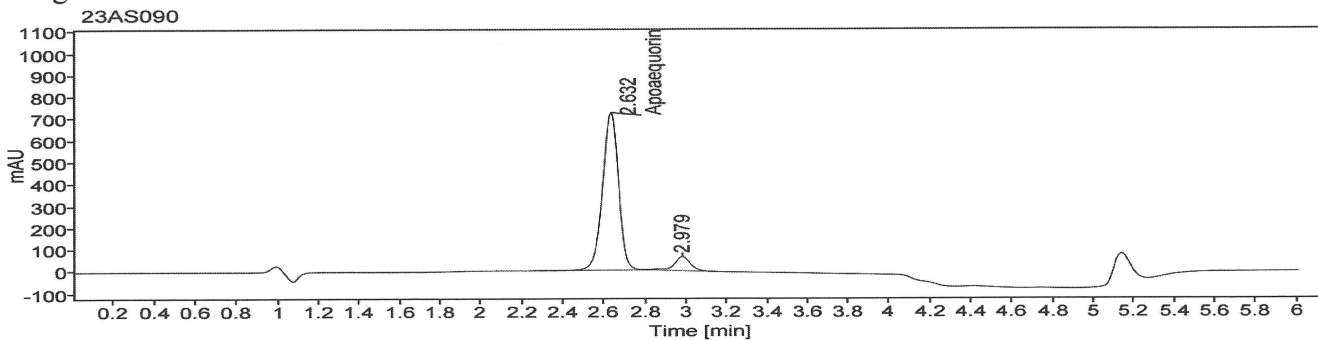
A_{CofA} Assay value reported on manufacturer's Certificate of Analysis

10.0 Example Chromatography

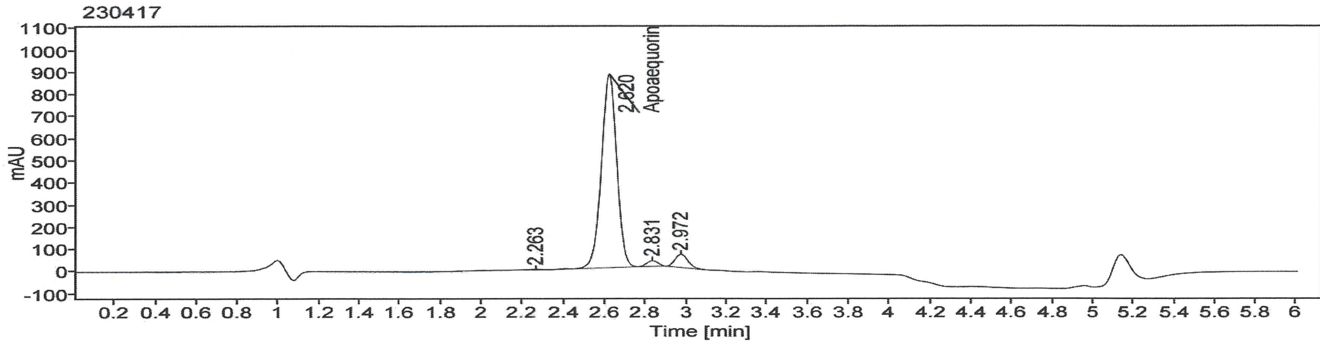
10.1 Blank



rking Standard



10.2 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 details for prep of suspension buffer, add recommended sequence, add system suitability requirements, add column wash and storage, add example chromatography.	CC-22-0177	S. Sassman
2	11/16/22	Clarified spectral range. Remove retention time requirement.	CC-22-0441	J. Sassman
3	02/23/23	Add instruction to check the product profile for test details, remove language requiring in-process validation for new products, remove spectral match as system suitability requirement.	CC-23-0095	S. Sassman
4	12/09/23	Added more representative chromatography.	CC-23-0592	J. Sassman
5	04/19/24	Revise method to include expanded linear range and new finished product matrix.	CC-24-0165	C. Perry
6	11/27/24	Added calculation for determination of % difference from value on manufacturer's Certificate of Analysis	CC-24-0538	K. Bittler
7	04/30/25	Added standard solution examination after thawing	CC-25-0196	K. Bittler
8	12/18/25	Implemented standard recovery requirement.	CC-25-0510	A. Shannon