Written by/ Date	Reviewed by/ Date	Approved by/ Date
SAS 09/07/22	CJP 09-07-22	09/13/22
Title: Analytical Development Scientist	Title: Analytical Development Scientist	Title: QC Director

1.0 Purpose

The purpose of this procedure is to describe a method for the quantitative analysis and identification of creatine in finished products and raw materials using HPLC coupled with UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to creatine quantification and identification. Some excipients and dietary ingredients used in the finished products can interfere with the analysis of creatine.

3.0 Responsibility

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

3.2 It is the responsibility of QC Laboratory Management to implement this procedure and to ensure that the 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 **ACN** – Acetonitrile

4.3 **H_3PO_4** – Phosphoric Acid

4.4 **H_2O** – Deionized Water ($\geq$ 18.2 MΩ·cm)

4.5 **CofA** – Certificate of Analysis

4.6 **RT** – Room Temperature

4.7 **QC** – Quality Control

4.8 **HPLC** – High Performance Liquid Chromatography

5.0 References

5.1 MV-LAB-12-007, Protocol, Creatine Determination by HPLC

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Reagents: All reagents are HPLC grade or better

6.1.1 H_2O

6.1.2 ACN

6.1.3 H_3PO_4

6.1.4 Creatine monohydrate traceable standard

6.2 Supplies and Glassware

6.2.1 HPLC vials, 12mm X 32mm with screw cap enclosures w/ septa

6.2.2 1L mobile phase container

6.2.3 10mL, 50mL, 100mL, 500mL, and 1L volumetric flasks

6.2.4 200µL, 1mL, and 10mL pipette tips

6.2.5 10mL Plastic luer-lock syringes

6.2.6 0.2µM or 0.45µM 25mm Nylon syringe filters

6.2.7 22mL screw cap vials

6.2.8 1.5mL and 2.0mL micro centrifuge tubes

6.2.9 Weigh paper and weigh boats

6.3 Equipment

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

6.3.2 Analytical Balance

6.3.3 Stir Plate

6.3.4 Wrist Action Shaker

6.3.5 Vortex

6.3.6 Centrifuge

6.3.7 Sonicator Bath

6.3.8 200µL, 1mL, and 10mL Pipettes- adjustable

7.0 Procedure

7.1 7.1 Mobile Phase Preparation

7.1.1 Mobile Phase A - 0.1% H_3PO_4 in H_2O

7.1.1.1 Transfer 1000 mL H_2O to a mobile phase bottle.

7.1.1.2 Add 1.0 mL H_3PO_4, and mix well.

7.1.2 Mobile Phase B - 0.1% H_3PO_4 in ACN

7.1.2.1 Transfer 1000 mL ACN to a mobile phase bottle.

7.1.2.2 Add 1.0 mL H_3PO_4, and mix well.

7.1.3 Diluent – H_2O

7.2 Standard Preparation

7.2.1 Use the actual purity from the CofA for the reference standard in your calculations.

7.2.2 All standards are prepared by weighing no less than the minimum weight of the analytical balance. Dissolve and bring to final volume using H_2O.

7.2.3 Dilutions are prepared using H_2O. Dilutions can be made using volumetric flasks or using 10mL, 1mL and 200µL variable pipettes. Working 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. Final dilutions may be prepared directly in HPLC vials.

7.3 Sample Preparation

7.3.1 The linear range of the method is 0.005 mg/mL – 0.05 mg/mL. Sample preparations must be within the linear range of the method.

7.3.2 10 or more dosage units can be pooled and ground by mortar and pestle as necessary.

7.3.3 Based on the fill weight, tablet weight per dose, or raw material potency, weigh a portion of the pooled dosages to generate an analyte concentration that is within the validated linear range for the analyte being tested.

7.3.4 Dilute the sample to the calculated volume with H_2O, cap, and sonicate for 10 minutes to facilitate dissolution. Alternatively, samples can be shaken on a wrist action shaker for 20 minutes at RT in two thirds their initial volume then brought up to final volume.

7.3.5 Remove the sample from the sonicator and allow it to cool to RT.

7.3.6 Samples can be dissolved in diluent at any volume starting from 10 mL. To manage large volumes the sample can be initially dissolved in a smaller volume that is within the solubility range and a portion further diluted to bring the analyte concentration into the linear range of measurement. The final diluted sample must be filtered or centrifuged before analyzing by HPLC.

7.3.7 For filtration, using the final large scale diluted sample withdraw up to 10 mL using a 10 mL plastic syringe then filter and discard at least 0.5 mL of sample before collecting a portion for analysis.

7.3.8 For centrifugation using the final large scale diluted sample, fill an even number of 1.5 or 2.0 mL micro-centrifuge tubes and pellet insoluble matter for 3 minutes at 6000 rpm.

7.3.9 For finished products or raw materials being analyzed for the first time using this method an in process validation is required to demonstrate spectral purity, baseline separation of peaks and extraction efficiency as a part of system suitability before the formulation can be analyzed.

7.4 Test Conditions

7.4.1 Gradient - Isocratic

7.4.1.1 Time	%A	%B
0.00	98	2
5.00	98	2

7.4.2 Column - Luna C5, 5µm, 150 mm X 4.6 mm or equivalent

7.4.3 Flow Rate - 1.0 mL/min

7.4.4 UV Detection – 200 nm

7.4.5 Injection Vol – 10 µL

7.4.6 Temperature – 45 °C

7.5 Recommended Sequence

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

7.5.2 Make five (5) injections of the Working Standard.

7.5.3 Make a single injection of each Sample Solution.

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

7.6 System Suitability Requirements

7.6.1 %RSD of five consecutive injections of Working Standard is NMT 5.0%.

7.6.2 %RSD of all injections of Working Standard is NMT 5%.

7.7 Column Rinse and Storage

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

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

7.7.3 Store the column with H_2O/ACN (10/90).

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

7.8.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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)

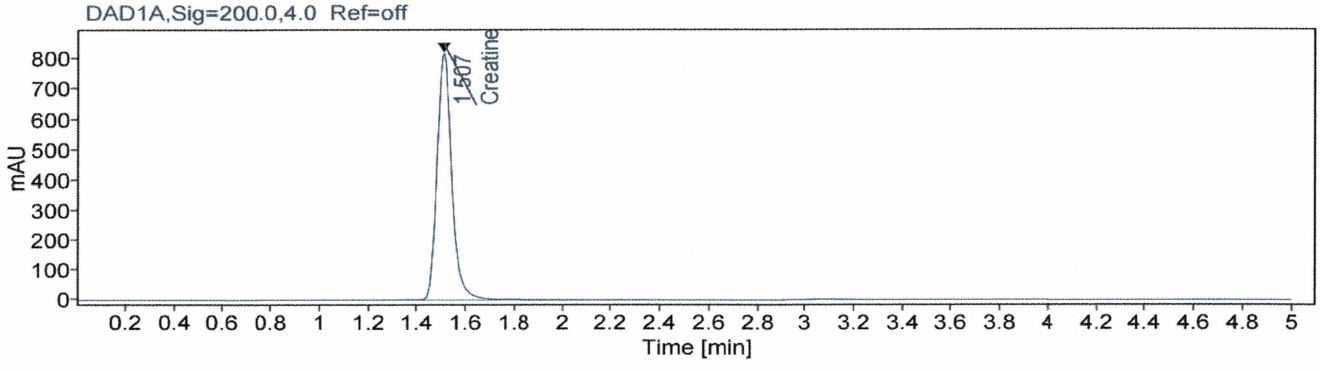
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

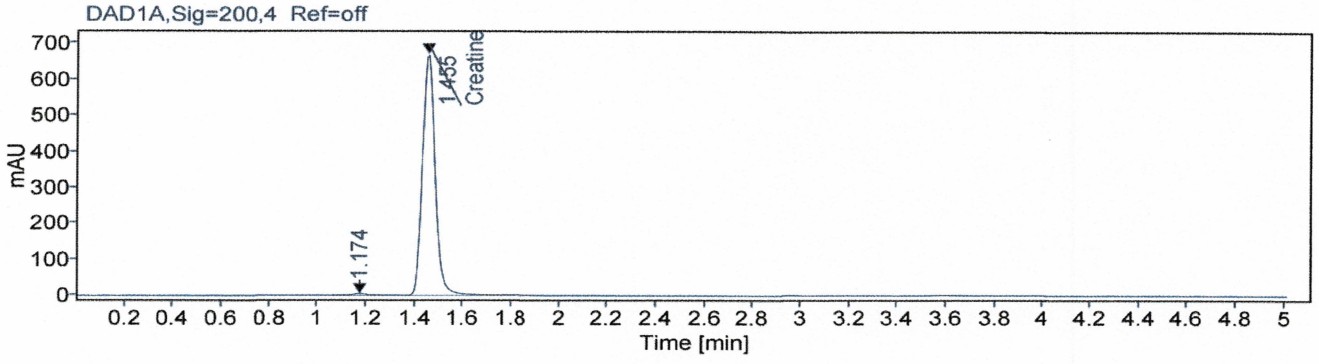
LA Label amount in mg per dose or 1 for raw materials

7.9 Example Chromatography

7.9.1 Working Standard



7.9.2 Working Sample



8.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	12/10/12	New	-	-
2	03/28/13	Added % label calculations, added Attachment 1, expanded on sample and standard preparation.	13-0243	B. Johns
3	09/08/15	Scheduled review: updated SOP Format. Update HPLC method format.	15-0602	B. Johns
4	01/02/19	Scheduled review: updated SOP format, stability requirement, weight requirement, and number of pooled tablets.	19-0011	J. Maignan
5	09/07/22	Update for consistency with current methods, simplify standard preparation, add recommended sequence, add system suitability section, add column rinse and storage section, add example chromatography.	CC-22-0362	S. Sassman