Enzymatic Assay of NITRIC OXIDE SYNTHETASE
(EC 1.14.13.39)

PRINCIPLE:

$$\text{L-Arginine} + \text{ß-NADPH} + O_2 \xrightarrow[Ca^{2+},\ \text{Tetrahydrobiopterin, Calmodulin}]{\text{Nitric Oxide Synthetase}} \text{Citrulline} + NO + \text{ß-NADP}$$

Abbreviations used:
ß-NADPH = ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form
ß-NADP = ß-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form

CONDITIONS: T = 37°C, pH = 7.3

METHOD: HPLC Analysis of Products

REAGENTS:

A. 80 mM HEPES Buffer with 1.0 mM Ethylenediaminetetraacetic Acid and 1.5 mM Calcium Chloride, pH 7.3 at 37°C
(Prepare 100 ml in deionized water using HEPES, Free Acid, Sigma Prod. No. H-3375, Ethylenediaminetetraacetic Acid, Tetrasodium Salt, Hydrate, Sigma Stock No. ED4SS and Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881. Adjust to pH 7.3 at 37°C with 1 M NaOH.)

B. Calmodulin Solution (CaM)
(Immediately before use, prepare a solution containing 5000 units/ml of Phosphodiesterase 3':5'-Cyclic Nucleotide Activator (Calmodulin), Sigma Prod. No. P-2277, in cold Reagent A.)

C. 20 mM DL-Dithiothreitol Solution (DTT)
(Prepare 5 ml in Reagent A using DL-Dithiothreitol, Sigma Prod. No. D-0632.)

D. 1.0 mM Tetrahydrobiopterin Solution (THB)
(Immediately before use, prepare 10 ml in Reagent A using (6R)-5,6,7,8-Tetrahydrobiopterin, Dihydrochloride, Sigma Prod. No. T-4425. **Prepare**

Fresh, Keep on Ice.)

REAGENTS: (continued)

E. 10 µM Citrulline Standard Solution (Cit Std)
(Prepare 10 ml in Reagent A using L-Citrulline, Sigma Prod. No. C-7629.)

F. 1.0 mM Flavin Adenine Dinucleotide Solution (FAD)
(Prepare 10 ml in Reagent A using Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625.)

G. 3.4 mM L-Arginine Solution (L-Arg)
(Prepare 10 ml in Reagent A using L-Arginine, Hydrochloride, Sigma Prod. No. A-5949.)

H. 3.0 mM Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form Solution (NADPH)
(Prepare 10 ml in Reagent A using Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form, Tetrasodium Salt, Sigma Prod. No. N-1630.)

I. 100% (v/v) Methanol (MeOH)
(Use Methanol, Absolute, Sigma Stock No. 17-5.)

J. 10 mM Sodium Phosphate and 10 mM Triethylamine Buffer, pH 5.0 at 37°C (Buffer A)
(Prepare 1 L in deionized water using Sodium Phosphate, Dibasic, Anhydrous, Sigma Prod. No. S-0876, and Triethylamine, Sigma Prod. No. T-0886. Adjust to pH 5.0 at 37°C with 1 M HCl or 1 M NaOH. Before using, filter through a 0.22 µm filter.)

K. Acetonitrile (Buffer B)
(Acetonitrile, Sigma Stock No. 27,071-7.)

L. o-Phthaldialdehyde Reagent Solution (OPA)
(Use o-Phthaldialdehyde Reagent Solution Complete, Sigma Prod. No. P-0532.)

M. Nitric Oxide Synthetase Enzyme Solution
(Immediately before use, prepare a solution containing 40 - 50 units/ml of Nitric Oxide Synthetase in cold Reagent A.)

Enzymatic Assay of NITRIC OXIDE SYNTHETASE (EC 1.14.13.39)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers:

	Test	Blank
Reagent A (Buffer)	1.95	1.75
Reagent B (CaM)	0.20	0.20
Reagent C (DTT)	0.20	0.20
Reagent D (THB)	0.20	0.20
Reagent F (FAD)	0.05	0.05

Mix by inversion and equilibrate to 37°C. Then add:

Reagent M (Enz Soln)	0.10	------
Reagent A (Buffer)	------	0.10

Incubate at 37°C for 30 - 60 seconds. Then add:

Reagent G ($_L$-Arg)[1]	0.10	0.10
Reagent H (NADPH)[1]	0.20	0.20

Immediately mix by inversion and incubate for exactly 10 minutes at 37°C.

After incubating for 10 minutes, pipette 0.5 ml of the Test and Blank into a 2 ml Eppendorf tube containing 0.5 ml of ice cold Reagent I (Methanol). **Store on ice**. Prepare Citrulline Standards by pipetting (in milliliters) the following reagents into 2 ml Eppendorf tubes:

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std Blank
Deionized Water	0.49	0.45	0.40	0.30	0.20	----	0.50
Reagent E (Cit Std)	0.01	0.05	0.10	0.20	0.30	0.50	----
Reagent I (MeOH)	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Store on ice. Centrifuge all samples to remove any precipitates.

PROCEDURE: (continued)

Step 2:

HPLC analysis of OPA derivatized samples:

1. Column: Supelcosil LC-18, Supelco Catalog No. 5-8230, 4.6 x 150 mm, C18, 5µm particle size.

   Mobile Phase: Equilibrate the column with 5% Buffer B at 1.0 ml/min. Run a linear gradient of 5 - 15% Buffer B over 10 minutes, followed by 15 - 100% Buffer B over 3 minutes. Hold at 100% Buffer B for 5 minutes. Re-equilibrate the column for 15 minutes with 5% Buffer B. The gradient may need adjusting as the column ages.

   Detection: Use fluorescence detection at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

2. Warm a 10 ml aliquot of Reagent L (OPA) to room temperature (23°C).

3. Set up auto sampler to dispense 0.1 ml Reagent L (OPA) into 0.1 ml of sample in a HPLC vial. If auto sampler is not available then, accurately add 0.1 ml Reagent L (OPA) solution to 0.1 ml of the sample to be assayed. Mix for 1 minute, immediately inject 20 ul of derivatized sample onto the HPLC column. **This reaction is carried out at room temperature.** All of the reaction times need to be consistent with OPA + Sample before each injection. The OPA reagent will react with the primary amino acid(s) and the derivatized product detected by fluorescence. NOTE: Ammonia, Proline and Cysteine are not detected by OPA.

4. Inject blank then standards of Citrulline and samples. A comparison can then be made between standard curve of Citrulline and Citrulline generated from the sample reaction.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\text{nmoles of citrulline})(df)(3)}{(0.1)(10)}$$

df = Dilution factor
3 = Volume (in milliliters) of assay
0.1 = Volume (in milliliter) of enzyme used

10 = Time (in minutes) of assay as per the Unit Definition

Enzymatic Assay of NITRIC OXIDE SYNTHETASE (EC 1.14.13.39)

CALCULATIONS:

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will convert 1.0 nmole of arginine to citrulline per minute at pH 7.3 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 80 mM HEPES, 1 mM ethylenediaminetetraacetic acid, 1.5 mM calcium chloride, 1000 units calmodulin, 1.3 mM DL-dithiothreitol, 0.07 mM tetrahydrobiopterin, 0.02 mM flavin adenine dinucleotide, 0.1 mM arginine, 0.2 mM ß-nicotinamide adenine dinucleotide phosphate, reduced form, and 4 - 5 units nitric oxide synthetase.

REFERENCE:

Ohshima, H., Oguchi, S., Adachi, H., Iida, S., Suzuki, H., Sugimura, T., and Esumi, H. (1992) *Biochemical and Biophysical Research Communications* **183**, 238-244

Bredt, D.S. and Snyder, S.H. (1990) *Proceedings National Academy of Sciences*, USA, **87**, 682-685

NOTES:

1. Reagent G (L-Arg) and Reagent H (NADPH) should be prewarmed to 37°C before adding to the reaction.

2. This assay is based on the cited references.

3. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.