



## THE ROLE OF OXIDANT STRESS AND GENDER IN THE ERYTHROCYTES ARGININE METABOLISM AND AMMONIA MANAGEMENT IN PATIENTS WITH TYPE 2 DIABETES 👄

PLOS One

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Works for me dx.doi.org/10.17504/protocols.io.q5qdy5w



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#### ABSTRACT

The erythrocytes are not simply gas transporting cells, but they have a very important role in the regulation and transport of metabolites related to nitrogen metabolism both in healthy subjects and in patients with type 2 DM. The different levels of metabolites are associated with gender differences.

We have two particular objectives: 1) To study the differences in the levels of nitrogen metabolites, such ammonium and nitric oxide, and the correlations that exist between them in RBC and serum, in healthy subjects and patients with type 2 DM. And 2) to determine the differences in the levels of nitrogen metabolites, such ammonium and nitric oxide, and the correlations that exist between them in relation to gender in healthy subjects and patients with type 2 DM.

This cross-sectional study included 80 patients diagnosed with type 2 DM (40 female and 40 male patients) and their corresponding controls paired by gender (n = 80). We separated serum and red blood cells (RBC) and determined metabolites mainly through colorimetric and spectrophotometric assays. We evaluated changes in the levels and correlations of the main catabolic by-products of blood nitrogen metabolism, as ammonium, nitric oxide (NO), malondialdehyde (MDA), among others.

Healthy female and male controls showed a differential blood metabolites distribution involved in arginine metabolism, as ornithine, ammonium, urea and . NO. Patients with DM had increased ammonia, citrulline, urea, uric acid, and ornithine, mainly in the RBC, while the level of arginine was significantly lower in men with type 2 DM. These findings were associated with hyperglycemia, glycosylated hemoglobin (Hb A<sub>1C</sub>), as well as with levels of RBC's MDA. Most of the DM-induced alterations in nitrogen-related metabolites seem to be associated with a different RBC capacity for the release of these metabolites and resulting in an abrogation of the gender-related differential management of nitrogen metabolites in healthy subjects.

This study provides the first documented findings giving strong evidence of a putative role of RBC as an extra-hepatic mechanism for controlling serum levels of nitrogen-related metabolites, which differs according to gender in healthy subjects. Type 2 DM promotes higher ammonia, citrulline, and MDA blood levels, which culminate in a loss of the differential management of nitrogen-related metabolites seen in healthy women and men and a characteristic pattern of metabolic disturbances that culminates in a loss of the differential management of nitrogen-related metabolites seen in healthy women and men. Therefore, it is not unlikelythat these characteristic patterns of blood metabolites elicited by type 2 DM might be involved in the specific physiopathology of this disease, and changes in the oxidative status, i.e., increased lipid peroxidation in RBC membranes from diabetic patients, are playing a role in the metabolic alterations found in these blood cells

**EXTERNAL LINK** 

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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MATERIALS

**VENDOR** NAME CATALOG #

NAME ~	CATALOG #	VENDOR V
Citrulline	C7629	Sigma – Aldrich
Arginine	A5006	Sigma - Aldrich
Urea	U5378	Sigma - Aldrich
HEPES	RDD002	Sigma - Aldrich
Uric	U2625	Sigma - Aldrich
Glucose	158968	Sigma - Aldrich
Potassium dihydrogen phosphate	NIST200B	Sigma - Aldrich
Monobasic potassium phosphate	PHR1330	Sigma - Aldrich
Sodium bicarbonate	S6014	Sigma - Aldrich
Sodium carbonate	223484	Sigma - Aldrich
Sodium barbiturate	11715-100G	Sigma - Aldrich
Sodium chloride	746398	Sigma - Aldrich
Perchloric acid	244252	Sigma - Aldrich
Potassium nitrite	310484	Sigma - Aldrich
Potassium nitrate	1548349	Sigma - Aldrich
Tris base	T1503	Sigma - Aldrich
Hydrochloric acid	320331-500ML	Sigma - Aldrich
Ninhydrin	151173	Sigma - Aldrich
Sulfuric acid	258105	Sigma - Aldrich
Acetic acid	320099-6X2.5L	Sigma - Aldrich
alpha-Ketoglutaric acid sodium salt	K1875	Sigma - Aldrich
Adenosine 5-diphosphate sodium salt	A2754	Sigma - Aldrich
Tetraethyl ammonium chloride monohydrate	86605	Sigma - Aldrich
Beta-nicotinamide adenine dinuclotide, reduced disodium salt hydrate	N8129	Sigma – Aldrich
Glutamate dehydrogenase (Gldh)	10197734001	Sigma - Aldrich
Trichloroacetic acid (TCA)	T6399	Sigma - Aldrich
Potassium bicarbonate	237205	Sigma - Aldrich
Succinic acid	398055	Sigma - Aldrich
Urease from Canavalia ensiformis (Jack bean)	U7752	Sigma - Aldrich
Ninhydrin	N4876	Sigma - Aldrich
Sodium hydroxide	S8045	Sigma – Aldrich
Phenazone	P0800000	Sigma – Aldrich
2,3-Butanedione monoxine	31550	Sigma – Aldrich
N-(1-Naphthyl)ethylenediamine dihydrochloride	222488	Sigma – Aldrich

NAME Y	CATALOG #	VENDOR V
Hemoglobin human	H7379	Sigma – Aldrich
Potassium ferricyanide (III)	702587	Sigma - Aldrich
Potassium phosohate dibasic	60356	Sigma - Aldrich
Phosphoric acid	7664-38-2	Meyer
Acetic Acid, Glacial	0040	Meyer
Potassium cyanide	207810	Sigma - Aldrich
1,1,3,3-Tetraethoxypropane	9889	Sigma - Aldrich
Copper (II) sulfate pentahydrate	209198	Sigma - Aldrich
Folin & Ciocalteus phenol	F9253	Sigma - Aldrich
Sulfanilamide	S9251	Sigma - Aldrich
Sodium nitrite	237213	Sigma - Aldrich
Potassium sodium tartrate tetrahydrate	217255	Sigma - Aldrich
Ethanol absolute	13225483	JT Baker
Bovine serum albumin	A7906	Sigma - Aldrich
Manganese (II) chloride tetrahydrate	221279	Sigma - Aldrich
L-Ornithine dihydrochloride	75440	Sigma - Aldrich
Pyridine	360570	Sigma - Aldrich
Butyl alcohol	W217808	Sigma - Aldrich
Potassium Chloride	746436	Sigma - Aldrich
Potassium hydroxide	P1767	Sigma - Aldrich
2-Thiobarbituric acid		Sigma - Aldrich
Ammonium chloride	254134	Sigma - Aldrich
Triethanolamine	90279	Sigma - Aldrich
L-Ornithine monohydrochloride	02375	Sigma - Aldrich
Iron (III) sulfate hydrate	307718	Sigma - Aldrich
Sodium nitrate	S5506	Sigma - Aldrich
STEPS MATERIALS		
NAME ×	CATALOG #	VENDOR ~
Perchloric acid	244252	Sigma – Aldrich
Sodium hydroxide	SB0617.SIZE.500G	Bio Basic Inc.
Potassium hydroxide	P1767	Sigma - Aldrich
2-Thiobarbituric acid		Sigma – Aldrich
Perchloric acid	244252	Sigma – Aldrich
Acetic Acid, Glacial	0040	Meyer

NAME ~	CATALOG #	VENDOR ~
Potassium hydroxide	P1767	Sigma – Aldrich
2-Thiobarbituric acid		Sigma - Aldrich
1,1,3,3-Tetraethoxypropane	9889	Sigma - Aldrich
Manganese (II) chloride tetrahydrate	221279	Sigma - Aldrich
Sodium carbonate	222321	Sigma - Aldrich
Acetic Acid, Glacial	0040	Meyer
Ninhydrin	N4876	Sigma - Aldrich
Acetic Acid, Glacial	0040	Meyer
Phosphoric acid	7664-38-2	Meyer
Manganese (II) chloride tetrahydrate	221279	Sigma - Aldrich
Sodium carbonate	223484	Sigma - Aldrich
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Sodium carbonate	223484	Sigma - Aldrich
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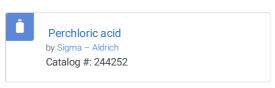
NAME Y	CATALOG #	VENDOR ~
Urease from Canavalia ensiformis (Jack bean)	U7752	Sigma - Aldrich
Ammonium chloride	254134	Sigma – Aldrich
Acetic Acid, Glacial	0040	Meyer
Ninhydrin	N4876	Sigma – Aldrich
Phosphoric acid	7664-38-2	Meyer
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2,3-Butanedione monoxine	31550	Sigma – Aldrich
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N-(1-Naphthyl)ethylenediamine dihydrochloride	222488	Sigma - Aldrich
Sulfanilamide	S9251	Sigma – Aldrich
Phosphoric acid	7664-38-2	Meyer
Sodium nitrate	S5506	Sigma - Aldrich

### **Biochemical Measurements**

- 1 a) Whole blood was poured directly into ice-cold perchloric acid (8% w/v, final concentration). After centrifugation, we obtained perchloric acid-extracts of serum and RBC.
  - b) Whole blood was centrifugated to separate RBC and serum samples  $\,$

## [M] 8 Mass/Volume Percent







#### § 4 °C on ice

The perchloric acid-extracts obtained in the previous step were neutralized: we added sodium hydroxide until we reach a pH of 7.

These preparations were used to determine the following metabolites: ammonia, urea, arginine, citrulline, ornithine, nitrites, malondialdehyde (MDA), uric acid.



3 For the determination of glucose, insulin, HbA1C, triglycerides, cholesterol, alanina aminotransferasa (ALT), and aspartato aminotransferasa (AST), we only used serum samples (1b).

#### Glucose determination

4 Glucose was determined in serum samples with the glucose oxidase reaction using the Glucosa-LQ GOD-POD kit (SPINREACT). The color developed was read at 450 nm using Biotek instruments.



#### Insulin determination

5 Insulin was determined in serum samples with the Human Insulin ELISA kit (ELH-Insulin-2 RayBio, USA). The color developed was read at 450 nm using Biotek instruments.



#### HbA1C determination

6 HbA1C was determined in serum samples with the glycosylated HbA1c kit (BoiSys-Kovalent, Brazil; 4190045K R1 2). The color developed was read at 450 nm using Biotek instruments.



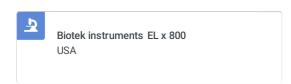
#### Quantitative determination of triglycerides

7 Triglycerides were determined in serum samples with the GPO-POD enzimatic colorimetric kit (Spinreact, Spain). The color developed was read at 450 nm using Biotek instruments.



#### Uric acid determination

8 Uric acid was determined in serum samples with the Uricase-POD Enzimatic colorimetric kit (Spinreact, Spain). The color developed was read at 450 nm using Biotek instruments.



#### Aspartate aminotransfefase (AST) determination

9 AST was determined in serum samples with the NADH.Cinetic UV.IFCC kit (Spinreact, Spain). The color developed was read at 450 nm using Biotek instruments.



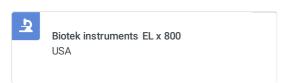
#### Alanine aminotransferase (ALT) determination

10 ALT was determined in serum samples with the NADH.Cinetic UV.IFCC kit (Spinreact, Spain). The color developed was read at 450 nm using Biotek instruments.



## Cholesterol determination

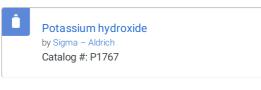
11 Cholesterol was determined in serum samples with the CHOD-POD lit (Spinreact, Spain).
The color developed was read at 450 nm using Biotek instruments.



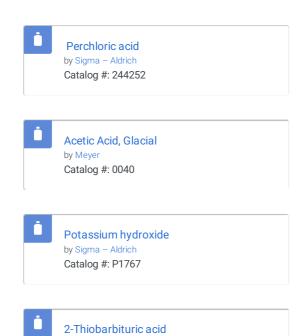
Malondialdehyde (MDA) determination (Hernandez-Munoz et al. (1984); Biochem Pharmacol 33(16); pp 2599-2604)

MDA was quantified by the thiobarbituric acid method using acid extracts from RBC and serum (6% perchloric acid w/v). Samples of these extracts (0.5ml) were mixed with 1.5 ml of 20% acetic acid (adjusted to pH 2.5 with KOH) and with 1.5 ml of 8% thiobarbituric acid.

## [M]8 Mass/Volume Percent 8% 2-thiobarbituric acid







[M]6 Mass/Volume Percent 6% perchloric acid w/v

[M]20 Volume Percent 20% acetic acid

Malondialdehyde (MDA) determination

by Sigma - Aldrich

13 The samples were kept for 45 minutes a boiling water bath and 1 ml of 2% KCL was added at the end of the incubation to each sample.

© 00:45:00 boiling water bath

[M]2 Mass/Volume Percent 2% KCL

■1 ml of 2% KCL

The color complex formed at the end of the incubation (in the last step) was extracted with the mix butanol-pyridin (15:1, v/v) and detected at 532 nm in a spectrophotometer (Beckman Coulter).

The extinction coefficient of malonal dehyde color complex was  $1.56 \times 105 \, \text{cm}^{-1} \, \text{M}^{-1}$ , as determined using a standard curve with tetraethoxypropane.

[M]15 Volume Percent mix butanol-pyridin (15:1, v/v)





Arginine determination (Colombo, JP and Konarska L.; Arginase in Bergmeyer HU and Bergmeyer J, Grassi M., Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie; 1984)

15 Content of arginine was determined in neutralized perchloric extracts of RBC and serum as arginine converts to ornithine using rat liver cytosol-containing activated arginase.

Liver cytosol enzyme was activated by a 20 min-incubation with 10 mM Manganese chloride (MnCl2) at  $55\,^{\circ}$ C.

© 00:20:00 20 minutes incubation

**\( \text{protocols.io} \)** 8 07/18/2019

#### A 55 °C for incubation

## [M]10 Molarity (m) 10mM Manganese chloride (MnCl2)



#### Arginine determination

16 To a sample of 0.5 ml acid-extract, 0.3 ml of sodium carbonates buffer (pH = 9.5) was added and the reaction started with 10 μg of cytosolic protein.

The mixture was incubated by 40 min at 37 °C, and stopped by addition of 1.5 ml of acetic acid.

#### § 37 °C incubation

#### **© 00:40:00** incubation



■10 μg 10 μg of cytosolic protein.



The color resulting from the reaction was developed through a ninhydrin solution (140 mM dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v) in boiling water during 45 min.

The colored was detected at 515 nm, contrasting with a standard curve for ornithine (30, 50, 100, 200 500, 1000 nmoles in the reaction mixture).







[M]140 Molarity (m) 140 mM dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v

**©00:45:00** 

Arginase was determined in RBC. Five mg of hemoglobin from RBC lysate were activated for arginase activity by a 20 min-incubation with 10 mM MnCl<sub>2</sub>.

#### © 00:20:00 incubation time



Manganese (II) chloride tetrahydrate

by Sigma - Aldrich

Catalog #: 221279

#### **■5** mg hemoglobin from RBC

19 Samples were now incubated in 0.3 ml sodium carbonate buffer (pH = 9.5) by 10 min at 37 °C, and stopped by addition of 1.5 ml of acetic acid.

#### **© 00:10:00** incubation



Sodium carbonate

by Sigma - Aldrich

Catalog #: 223484

#### § 37 °C incubation temperature



Acetic Acid, Glacial

by Meyer

Catalog #: 0040

The color was developed through a ninhydrin solution (140 mM dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v) in boiling water by 45 min.

The colored was detected at 515 nm in a spectrophotometer (Beckman Coulter), contrasting with a standard curve for ornithine (30,50, 100, 200 500, 1000 nmoles in the reaction mixture).

Activity expressed as nmoles·min-1 ·mg of hemoglobin protein.



Ninhydrin

by Sigma - Aldrich

Catalog #: N4876



Acetic Acid, Glacial

by Meyer

Catalog #: 0040



Phosphoric acid

by Meyer

Catalog #: 7664-38-2

© 00:45:00 duration in boiling water

## [M]140 Molarity (m) mM ninhydrin solution dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v [M]6 Volume Percent v/v acetic acid:6 M phosphoric acid



Hb determination (Colombo, JP and Konarska L.; Arginase in Bergmeyer HU and Bergmeyer J, Grassi M., Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie; 1984)

21 Add 1ml of any type of sample (an extract, RBC or serum) 2 ml Drabkin solution.

Drabkin's solution:

Dissolve 200 mg K3[(CN)6], 50 mg KCN, 140 mg KH2PO4. in 1000 ml water. We omitted the addition of antifoam because it is not available (no supply).

The sample was centrifugated when needed. In this case we measured the absorbance of the supernatant at 546 nm. When centrifugation was not needed, the sample was directly measured in the spectrophotometer (Beckman Coulter).

Calculate the concentration of hemoglobin (gr/l) with a hemoglobin cyanide-standard or using the molar absortion coefficient E=44 lnmol<sup>-1</sup> /mm<sup>-1</sup>.

Molecular weight of Hb is 64 458.







- **200 mg** K3[(CN)6]
- **■50 mg KCN**
- **■140 mg KH2P04**
- ■1000 ml water
- **2** ml Drabkin solution



Protein determination (Lowry OH et al. (1951); Protein measurement with the Folin phenol reagent. J of Biol Chem. Nov; 193(1):265-75.)

We prepared the following reagents:

Reagent A, 2 per cent NaCO3 in 0.10 N NaOH

Reagent B, 0.5 per cent CuSO4.5 H20 in 1 per cent potassium sodium tartrate.

Reagent C, alkaline copper solution. Mix 50 ml. of Reagent A with 1 ml. of Reagent B. Discard after 1 day. Reagent D, carbonate-copper solution, is the same as Reagent C except for omission of NaOH.

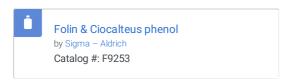
Reagent E, diluted Folin reagent (Sigma-Aldrich) diluted 1:1 in water.

We prepared a solution from bovine serum albumine and used it as a standard curve.











Procedure for Proteins in Solution or Readily Soluble in Dilute Alkali- (Directions are given for a final volume of 1.1 to 1.3 ml., but any multiple or fraction of the volumes given may be employed as desired'.)

To a sample of 5 to 100 ul of protein in 0.2 ml. or less in a 3 to 10 ml. test-tube, add 1 ml. of Reagent C.

Mix well and allow to stand for 10 minutes or longer at room temperature.

#### **© 00:10:00**

24 Add 0.10 ml. of Reagent E very rapidly and mixed within a second or two (see below).

After 30 minutes or longer, the sample is read in a calorimeter or spectrophotometer.

For the range 5 to 25 ul of protein per ml. of final volume, it is desirable to make readings at or near 750 nm, the absorption peak. Calculate from a standard curve.

## **© 00:30:00**



Ammonia determination (Bergmeyer HU, Beutler HO. Ammonia, in: Bergmeyer HU, Bergmeyer J, Grassl M, Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie, 1984:454-461.)

Ammonia reacts with 2-oxoglutarate and NADH to form I-glutamate in the presence of de glutamate dehydrogenase. As a result we obtain oxidized NADH (NAD). The amount of NADH consumed in the reaction is stoichiometric with the amount of ammonia.

We use neutralized perchloric extracts of RBC or serum samples. All operations are made at 0-4°C.

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Pipette successively into the cuvettes:

- -1 ml of reaction buffer (2-oxoglutarate 35 mM; ADP: adenine diphosphate 1.82 mM; TEA: trietanolamine 0.5M. pH 8).
- 0.10 ml NADH (6 mM)
- -0.10 0.5 ml sample

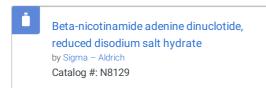
Final volume with water to 3.2 ml.

Mix and monitor change in absorbance until constant. Read absorbance (0).

#### § 4 °C at all time before incubation









Add 0.02 ml GIDH (glutamate dehydrogenase,3,000 U/ml in triethanolamine 150 mM pH 8 buffer; diluted 1:400)
Mix. After, read every 20 min until stability using a spectrophotometer.

Calculate from a standard curve with NH4Cl solution.







Urea determination (Kerscher L, Ziegenhorn J. Urea, in: Bergmeyer HU, Bergmeyer J, Grassl M, Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie, 1984:444-453.)

Urea in the presence of urease is transformed to 2 ammonium molecules, which is determined by the aforementioned method. Pipette successively into the cuvettes:

-1.75 ml of reaction buffer (Tris 150 mM, succinate 60 mM, 2-oxoglutarate 12.5 mM, ADP. Adenosine 5-diphosphate sodium salt 2 mM, pH

8)

- -0.1 ml NADH 5 mM
- 0.66 ml GIDH (glutamate dehydrogenase,3,000 U/ml Tris\_HCl buffer 150 mM pH 8; diluted 1:400)
- 0.05 ml neutralized perchloric extracts of RBC or serum diluted 10-fold

Mix thoroughly alter 10 min read (339 nm) A(0)

## © 00:10:00 mix thoroughly



#### Tris base

by Sigma - Aldrich

Catalog #: T1503



## Succinic acid

by Sigma - Aldrich

Catalog #: 398055



#### alpha-Ketoglutaric acid sodium salt

by Sigma - Aldrich

Catalog #: K1875



#### Adenosine 5-diphosphate sodium salt

by Sigma - Aldrich

Catalog #: A2754



#### Glutamate dehydrogenase (Gldh)

by Sigma - Aldrich

Catalog #: 10197734001

## 28 Add 0.005 ml urease solution (2.5kU/L). Mix.

After 15 min read ( 339 nm) the final absorbance A using a spectrophotometer.

Used A - A(0) = delta A for calculation.

Calculation The value thus obtained has to be multiplied by the factor of sample pre dilution and using a standard curve with NH4Cl solution.

## **७** 00:15:00 mix



# Urease from Canavalia ensiformis (Jack bean)

by Sigma - Aldrich

Catalog #: U7752



## Ammonium chloride

by Sigma - Aldrich

Catalog #: 254134



Ornithine determination (A spectrophotometric Method for the direct determination of cysteine in the presence of other naturally occurring Amino Acids. M.K. Gaitonde. Biochem J, 1967, 104: 627.)

We added water to 0.3 0.5 ml acid-extracts of serum or RBC to have a final volume of 1 ml. Mix.

Add 0.5 ml acetic acid, glacial. Mix.

Add 0.5 ml ninhydrin \*. Mix.

\*ninhydrin solution: 250 mg ninhydrin in 6 ml glacial acetic, 4ml phosphoric acid 0.6 M.

Put in boiling water bath for 10 min.

Allow cooling to room temperature and add 3 ml EtOH 95%

Read at 515 nm using a spectrophotometer.

Calculation using a standard curve with 5, to 100 nmoles of ornithine.









## **७** 00:10:00 boiling water bath





Citrulline determination (Ceriotti G. Ornithine Carbamoyltransferase, in: Bergmeyer HU, Bergmeyer J, Grassl M, Eds. Methods of Enzymatic Analysis, Vol. IV. Deerfield Beach, Florida, Verlag Chemie, 1984:319-334.)

The citrulline is formed from serum ornithine and carbamyl phosphate by ornithine carbamoyl transferase. This method is based on the

30 citrulline determination by the diacetylmonoxime-phenazone reaction.

For the colorimetric reaction, we add 0.2 - 0.5 ml of acid extracts of serum and RBC to a mixture\* of 1.5 ml of diacetylmonoxime and phenazone, prepared immediately before use.

\*Phenazone: 4 g are dissolved in 1 L of 40% v/v sulfuric acid solution; 50 mg of ferric sulfate are added. Diacetylmonoxime solution: 0.5% in 5% acetic acid.



Sulfuric acid

by Sigma - Aldrich

Catalog #: 258105



Iron (III) sulfate hydrate

by Sigma - Aldrich

Catalog #: 307718



2,3-Butanedione monoxine

by Sigma - Aldrich

Catalog #: 31550



Phenazone

by Sigma - Aldrich

Catalog #: P0800000



Acetic Acid, Glacial

by Meyer

Catalog #: 0040

31 We performed the colorimetric reaction in a 15 min boiling water bath incubation.

The color formed is read at 460 nm using a spectrophotometer against a blank with water treated in the same manner.

The absorbance at 460 nm is divided by 0.038 (the absorbance for 1 pg of citrulline, giving the amount of micrograms of citrulline contained).

#### © 00:15:00 boiling water bath



Beckman Coulter DU 640 spectrophotometer

Nitrites determination (Green, L.C. et al. (1982). Analysis of Nitrtate, nitrite, and [15N]Nitrate in Biological Fluids. Analytical Biochemistry, 126, pp. 131-138)

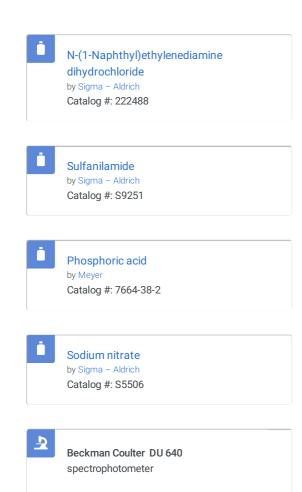
Based on the technique of Green, L.C. et al. (1982). Analysis of Nitrtate, nitrite, and [15N]Nitrate in Biological Fluids. Analytical Biochemistry, 126, pp. 131-138, modified by Dr. Rolando Hernandez Munoz (IFC, UNAM, Mexico).

Content of nitrites was determined in neutralized perchloric extracts of RBC and serum.

A sample of 0.5 ml acid-extract was incubated 20 min at room temperature with the 0.5 ml of Griess reagent (0.1% naphtyl-ethylene diamine dihydrichloride and 1% sulfanilamide in 5% phosphoric acid).

After developing the color, assays were read at 550 nm using a spectrophotometer, contrasting with a standard curve for sodium nitrate in deionized water.

© 00:20:00 incubation time



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