UNITECH SCIENTIFIC CALIFORNIA, USA

UNITAB™ REAGENT

AMMONIA / UREA / L-ARGININE

UV-Method

Product # URAA-60 (30 Tests)
URAA-150 (75 Tests) URAA-500 (250 Tests)

INTENDED USE

Unitech Scientific URAA Reagent is intended for the sequential end-point determination of ammonia, urea, and L-arginine in wine and other liquid samples.

METHODOLOGY & CHEMICAL PRINCIPLES

This method is based on the Ammonia method of Talke and Schubert[1] and the L-Arginine method of Mira de Orduña.[2] The reaction sequence is as follows:

$$NH_3 + \text{ak-G} + NADH + H^+ \xrightarrow{GDH} \text{Glutamate} + NAD^+ + H_20$$

Initially, glutamate dehydrogenase (GDH) catalyzes the condensation of ammonia and alpha ketoglutarate (ak-G) with the concomitant oxidation of reduced nicotinamide adenine dinucleotide (NADH), proportional to ammonia in the reaction.

$$\text{L-Arginine} \xrightarrow{\text{Arginase}} \text{Ornithine} + \text{Urea}$$

$$\text{Urea} \xrightarrow{\text{Urease}} 2\,NH_3 + CO_2$$

Subsequently, L-arginase catalyzes the conversion of L-arginine to ornithine and urea; urease catalyzes the conversion of each mole of urea into two moles of ammonia plus carbon dioxide. Oxidation of NADH causes a decrease in absorbance at 340 nm, proportional to the L-Arginine in the reaction.

REAGENTS

Active Ingredients are:	Concentration as Formulated	30T	75T	250T
AMM Reagent Tablets		12	30	100
Alpha Ketoglurate	1.87 mmol/L			
Adenosine Diphosphate	1.60 mmol/L			
NADH (yeast)	0.35 mmol/L			
YAN Buffer		60mL	150mL	500mL
Triethanolamine, pH 8.6	0.2 M			
GDH Enzyme Trigger		1.3mL	3.3mL	11mL
Glutamate Dehydrogenase	15 kU/L			
Urease Enzyme Trigger		1.3mL	3.3mL	11mL
Urease	1 kU/L			
U/A Enzyme Trigger		1.3mL	3.3mL	11mL
Urease	1 kU/L			
L-Arginase	600 U/L			
	Analyte Conc. (Nitrogen mg/L)			
Standard, Ammonia	110 mg/L (90.6)	1mL	1 mL	5 mL
Standard, L-Arginine	250 mg/L (20.1)	1mL	1 mL	5 mL

(Quantity/Kit: 30T, 75T, 250T)

STORAGE & REAGENT PREPARATION

Components are stable until the labeled expiration date when stored in original container at 2 - 8°C; store tablets tightly sealed with desiccant pack provided. Enzyme, Buffer and Standard solutions are ready to use; gently mix by inversion prior to use. Prepare sufficient WRgt for all samples and standards in the assay using clean glassware; mix by gentle inversion. Examples are provided in the table below.

MANUAL TESTING	5 Tests	10 Tests	20 Tests
Reagent Tablets	2	4	8
YAN Buffer	10mL	20mL	40mL

Note 1: If Ammonia values are not required, add 20uL/mL GDH Enzyme to the working reagent and incubate 20 minutes before reading initial ABS. Omit GDH from the following Procedure.

Note 2: If neither Ammonia nor Urea values are required, add 20uL/mL each of GDH and UREASE Enzymes to the working reagent and incubate 20 minutes before reading initial ABS. Omit these enzyme solutions from the following Procedure.

Bring Working Reagent (WRgt) to room temperature. WRgt is stable 2 days at 2-8°C, discard if it becomes turbid or has an absorbance less than 1.20 at 340 nm read against DI water blank.

PROCEDURE

System requirements: Wavelength 340 nm, absorbance range 0-2A, pathlength 1.0 cm.

Pipet into Cuvettes	Reagent Blank Cuvette	Sample or Standard Cuvettes
Sample		25 µL
DI water	25 µL	
Working Reagent	2 mL	2 mL
Mix cuvettes and wait 3 minutes. Zero spectrophotometer (water). Read A_0 (Initial absorbance).		
GDH Enzyme Soln.	35 uL (1 drop)	35 uL (1 drop)
Mix and wait 10-20 min. Read A_1		
Urease Enzyme Soln.	35 uL (1 drop)	35 uL (1 drop)
Mix and wait 15-25 min. Read A_2 (Final absorbance)		
U/A Enzyme Soln.	35 uL (1 drop)	35 uL (1 drop)
Mix and wait 15-25 min. Read A_2 (Final absorbance)		

1. Label one cuvette for each sample, standard and blank.
2. Refer to Sample Dilution Section. Pipette standard, samples, and water into cuvettes as shown in the Table above using calibrated micropipettes.
3. Dispense WRgt into each cuvette, mix and wait 3 minutes. Zero spectrophotometer with deionized water. Read initial absorbance A_0 values.
4. Gently mix the GDH Enzyme reagent by inversion and dispense, as shown above. Mix each cuvette, wait 10-20 minutes and read absorbance A_1.
5. Gently mix the UREASE Enzyme reagent by inversion and dispense, as shown above. Mix each cuvette, wait 10-20 minutes and read absorbance A_2.
6. Gently mix the U/A Enzyme reagent by inversion and dispense, as shown above. Mix each cuvette, wait 15-25 minutes and read absorbance A_3.

CALCULATIONS

A. AMMONIA Net Absorbance Change

1. Calculate delta $A_{Amm} = A_0 - A_1$ for each cuvette.

2. Calculate the sample & std. Net A change by subtracting delta A_{Blank} ($A_0 - A_1$ of Reagent Blank): Net A_{Amm} = delta A_{Amm} - delta A_{Blank}.

B. UREA Net Absorbance Change

1. Calculate delta $A_{Urea} = A_1 - A_2$ for each cuvette.
2. Calculate the Net A values as in Step I.#2:

Net A_{Urea} = delta A_{Urea} - delta A_{Blank}

C. L-ARGININE Net Absorbance Change

1. Calculate delta $A_{Arg} = A_2 - A_3$ for each cuvette.
2. Calculate the Net A values as in Step #2:

Net A_{Arg} = delta A_{Arg} - delta A_{Blank}

D. CALCULATION METHODS

1. Extinction Coefficient

$$\text{Analyte, mg/L} = \frac{\text{Net A} \times \text{MW} \times \text{TV}}{(\varepsilon)\,(P)\,(SV)\,(R)}$$

Ammonia, mg/L = 225.2 x Net A_{Amm}

Urea , mg/L = 404.6 x Net A_{Arg}

L-Arginine, mg/L = 1193 x Net A_{Arg}

Where:	Amm.	Urea	L-Arginine
MW molecular weight	17	60.06	174.2 g/mole
TV total reaction vol.	2.060	2.095	2.130 mL
SV sample volume	0.025	0.025	0.025 mL
ε absorptivity, 340 nm	6.22	6.22	6.22
P light path, cm	1	1	1
R molar ratio, NH_3 formed	1	2	2

2. Single-point Standard

Analyte mg/L = Conc. Std x Net A_{SAMPLE}/Net A_{STD}

Ammonia mg/L = 110* x Net A_{SAMPLE}/Net $A_{STD-AMM}$

Urea mg/L = 55* x Net A_{SAMPLE}/Net $A_{STD-AMM}$

L-Arginine mg/L = 250* x Net A_{SAMPLE}/Net $A_{STD-ARG}$

3. Multi-point Standards (e.g. Unitech 5-Point Standards) Sample concentrations are calculated from the best-fit standard curve.

* Note: To estimate Primary Amino Nitrogen, substitute the labeled Nitrogen Concentration on the respective Standard vial and multiply the result by 3.3. Refer to the following discussion.

DEFINITIONS & SIGNIFICANCE OF MEASUREMENT

Ammonia and L-Arginine are abundant nitrogen sources in grape juice.[2] Yeast Assimilable Nitrogen,YAN is made up of Ammonia Nitrogen and PAN. Ammonia Nitrogen content is 82.4% of the Ammonia mg/L. L-Arginine comprises 30 – 50% of the total PAN value. Therefore, total PAN may be estimated by (3.3)(L-Arginine mg/L). L-Arginine determination is critically important as a potential cause of EC formation, as described below.

Typical total YAN compounds range from 40 – 560, expressed in mg Primary Amino Nitrogen mg/L. Low Arginine and/or Ammonia levels have been associated with sluggish fermentation and sensory imperfections.[9] Lower limits of 35 mg/L L-Arginine and 50 – 70 mg/L Total Nitrogen (YAN) have been recommended. Nitrogen status of must is monitored to: 1) ensure sufficient Nitrogen sources to sustain yeast and lactic bacteria fermentation;[3,4,5,6,7] 2) to maintain L-Arginine below 1000 mg/L[8], thus minimizing the content of ethyl carbamate (EC), a potential carcinogen.[8] Nitrogen content of grape juice may be supplemented, e.g. with diammonium phosphate (DAP.) Neither routine nor excessive nitrogen supplementation is recommended.[8]

SPECIFICITY

The current arginase method is much more specific for L-arginine than previous methods.[2] Nevertheless, polyphenolics present in grape juice may inhibit GDH; citric acid[10] and proline[11] inhibit L-arginase. Endogenous fermentation enzymes in unclarified grape juice can result in creep reactions.[2]

QUALITY CONTROL

It is recommended that known Amm and L-Arg samples (or standards) be included in each set of assays to assist in verifying procedure and reagents. Factors that may affect the performance of this test include instrument performance, temperature, cleanliness of glassware and pipetting accuracy. Linearity standards are available.

SAMPLE PREPARATION

Dilution & Linearity: Measurement of Ammonia is linear to 225 mg/L, Arginine to 500-750 mg/L (40-60 mg/L Nitrogen). Test Ammonia samples neat. For L-Arginine analysis, pre-dilute juice and early fermentation samples 1+4 with DI water (i.e. to 20%) prior to testing; due to potential interferences in L-Arginine recovery, dilute any sample containing > 225 mg/L Ammonia or > 500 mg/L L-Arginine, or any sample with <0.10 Final ABS. Multiply all calculated G/L results by dilution factor. If non-standard TV or SV are used, adjust calculations as appropriate.

Sample Preparation: Clarify samples by centrifugation; if anthocyanin interference is suspected, decolorize using PVPP (add 1 g polyvinylpolypryrrolidone per 10 mL juice, mix for 1 minute, filter) prior to analysis. Decolorize Red juice/wine if SV is larger than 100 µL. Minimize Creep Reactions by: 1) placing fermentation samples into a water bath at 80°C to inactivate fermentation enzymes; 2) Prepare and analyze Sample Blanks [Rgt + Sample] in addition to the Samples [Rgt + Sample + Trigger]; calculate corrected delta A = delta A_{Sample} – delta $A_{Sample\ Blank}$.

NOTES

1. Traditionally, YANC is calculated based on Primary Amino Nitrogen (i.e. NOPA) plus Ammonia determinations. (Yeast assimilable nitrogen compounds = YANC= Ammonia Nitrogen + 1°Amino Nitrogen). This URAA reagent provides L-Arginine, Urea, and Ammonia results; L-Arginine nitrogen constitutes about a third (or one-half) of the total NOPA content. As such, URAA provides an under-estimate of total YANC.

REFERENCES

1. Talke, H., and G.E. Schubert. (1965) Klin.Wchnschr.**43**, 174.
2. Mira de Orduña, R. (2001) J. Agric. Food Chem., **49** (2), 549.
3. Ough, C.S. (1970) Amer. J. Enol. Vit. **21**, 213.
4. Yang, H.Y. (1953) J. Agr. Food Chem. **1**, 331.
5. Tressler, D.K., Celmer, R.F., Beavens, E.A. (1941) J. Ind. Eng. Chem. **33**, 1027.
6. Fuleki, T. (1965) Food Technol. **19**, 1287.
7. Peynaud, E. (1984) Eng. Trans., John Wiley & Sons, p. 37.
8. Butzke, C.E. and L.F. Bisson, USFDA Center for Food Safety and Applied Nutrition, Ethyl Carbomate Prevention Manual: 1997.
9. Austin, KT and CE Butzke, Spectrophotometric Assay for Arginine in Grape Juice and Must, AJEV, Vol 51, 2000, 227
10. Bisson, LF and CE Butzke, Diagnosis and Rectification of Stuck and Sluggish Fermentations, AJEV, 2000, 168-177.
11. Austin, KT and C.E. Butzke, Spectrophotometric Assay for Arginine in Grape Juice and Must, AJEV, 51, 3, 2000, 227 ff.
12. Müller, E.; Peternel, M.; Löhnertz, O. Aktuelle Forschungsergebnisse: Stickstoff und Qualität. Dtsch, Weinbau. 1998, 7, 14-17.
13. Greenberg, DM. Arginase. In The Enzymes; Boyer, PD, Ed. Academic Press: New York, 1960.

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