Megazyme

www.megazyme.com

PYRUVIC ACID (PYRUVATE)

ASSAY PROCEDURE

K-PYRUV 04/14

(*100 Assays per Kit) or
(1000 Auto-Analyser Assays per Kit) or
(1000 Microplate Assays per Kit)

* *The number of tests per kit can be doubled if all volumes are halved*

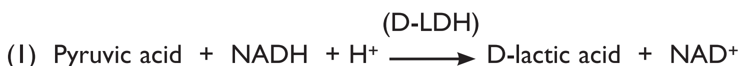
© Megazyme International Ireland 2014

INTRODUCTION:

Pyruvic acid (pyruvate) is an alpha-keto acid that plays a central role in various biochemical pathways. As a product of fermentation, pyruvic acid can be found in large quantities, especially in dark beers. This acid is also found in wine, fruits (e.g. apple) and cheese. The salt form of this acid is the active ingredient in various dietary supplements implicated in the control of body weight. The concentration of pyruvic acid in blood and urine is a useful clinical marker for certain medical conditions.

PRINCIPLE:

D-Lactate dehydrogenase (D-LDH) in the presence of reduced nicotinamide-adenine dinucleotide (NADH) converts pyruvic acid (pyruvate) into D-lactic acid and NAD^+ (1).



$$\text{(1) Pyruvic acid} + \text{NADH} + H^+ \xrightarrow{\text{(D-LDH)}} \text{D-lactic acid} + NAD^+$$

The amount of NAD^+ formed in the above reaction is stoichiometric with the amount of pyruvic acid. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for pyruvic acid.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.0985 mg of pyruvic acid/L of sample solution at the maximum sample volume of 2.00 mL (or to 1.97 mg/L with a sample volume of 0.1 mL). The detection limit is 0.394 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 0.3 to 40 µg of pyruvic acid per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.0 mL, this corresponds to a pyruvic acid concentration of approx. 0.0986 to 0.197 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of pyruvic acid has been completed within the time specified in the assay (approx. 3 min), it can generally be concluded that no interference has occurred. However, this can be further checked by adding pyruvic acid (20 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding pyruvic acid to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 100 assays in manual format (or 1000 assays in auto-analyser format or 1000 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (25 mL, pH 7.4) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: Tablets (21) containing NADH.
Stable for > 2 years at -20°C.

Bottle 3: D-Lactate dehydrogenase suspension (2.2 mL).
Stable for > 2 years at 4°C.

Bottle 4: Pyruvic acid standard solution (5 mL, 0.20 mg pyruvate/mL).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
   Stable for > 2 years at 4°C.

2. Dissolve one tablet from bottle 2 in 1.1 mL of buffer (bottle 1) for every 5 assays planned, and allow to dissolve over 1-2 min. Aid dissolution by shaking or stirring the tube contents. **This is Solution 2.** Once dissolved, the NADH absorbance will

drop slightly over time, but the reagent is suitable for use for approx. 5 days when stored at 4°C, or for approx. 4 weeks when stored at -20°C, though immediate use is recommended.

> **NOTE:** Warm the tablet bottle to room temperature before removing the tablet(s). Opening the tablet bottle while it is still cold will lead to absorption of moisture by the tablets which in turn will reduce the stability of the tablet components.

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. **Swirl the bottle to mix contents before use.** Stable for > 2 years at 4°C.

4. Use the contents of bottle 4 as supplied.
Stable for > 2 years at 4°C.

> **NOTE:** The pyruvic acid standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used, or where it is suspected that inhibition is being caused by substances in the sample. The concentration of pyruvic acid is determined directly from the extinction coefficient of NADH (see page 5).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.2 mL aliquots of NADH (solution 2)].
   - with 25 mL Combitip® (to dispense 2.5 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) and GF/A glass fibre filter papers.

MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.82 mL
Sample solution: 0.3-40.0 µg of pyruvic acid per cuvette (in 0.10-2.0 mL sample volume)
Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C) sample solution 2 (NADH buffer)	2.60 mL - 0.20 mL	2.50 mL 0.10 mL 0.20 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:		
suspension 3 (D-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 3 min).		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATION:

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{pyruvic\ acid}$.

The value of $\Delta A_{pyruvic\ acid}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of pyruvic acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{pyruvic\ acid} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of pyruvic acid [g/mol]

ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for pyruvic acid:

$$c = \frac{2.82 \times 88.06}{6300 \times 1.0 \times 0.1} \times \Delta A_{pyruvic\ acid} \quad [g/L]$$

$$= 0.3942 \times \Delta A_{pyruvic\ acid} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of pyruvic acid

$$= \frac{c_{pyruvic\ acid}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

> **NOTE:** These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for pyruvic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of pyruvic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
bottle 1 (buffer) bottle 2 (tablets) distilled water	7 tablets 7.7 mL 77 mL
Total volume	84.7 mL

Preparation of R2:

Component	Volume
bottle 4 (D-LDH) distilled water	0.7 mL 7.7 mL
Total volume	8.4 mL

EXAMPLE METHOD:

R1: 0.250 mL
Sample: ~ 0.01 mL
R2: 0.025 mL

Reaction time: ~ 3 min at 37°C
Wavelength: 340 nm
Prepared reagent stability: > 2 days when refrigerated
Calculation: endpoint
Reaction direction: decrease
Linearity: up to 0.4 g/L of pyruvic acid using 0.01 mL sample volume

MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for pyruvic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of pyruvic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.282 mL
Linearity: 0.1-4 µg of pyruvic acid per well (in 0.01-0.2 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water sample solution standard solution solution 2 (NADH/buffer)	0.260 mL - - 0.020 mL	0.250 mL 0.010 mL - 0.020 mL	0.250 mL - 0.010 mL 0.020 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
suspension 3 (D-LDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 3 min).			

* for example using microplate shaker, shake function on a microplate reader, or repeated aspiration (e.g. using a pipettor set at 50 - 100 µL volume).

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of pyruvic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.3 and 40 µg. The sample solution must therefore be diluted sufficiently to yield a pyruvic acid concentration between 0.003 and 0.40 g/L.

Dilution Table

Estimated concentration of pyruvic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.40	No dilution required	1
0.40-4.0	1 + 9	10
4.0-40	1 + 99	100
> 40	1 + 999	1000

If the value of $\Delta A_{pyruvic\ acid}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.0 mL with the volume of distilled water adjusted accordingly so that the sum volume of the sample and distilled water components are not altered. Use the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P-9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z-4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: for clear, slightly coloured liquid samples, adjust the pH to approx. 7.4 and use directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 7.4 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 7.4 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no D-LDH, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min to allow the fat to separate and then filter. Discard the first few mL of filtrate, and use the clear supernatant (which may be slightly opalescent) for assay.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of pyruvic acid in wine.

For white wine, use 0.20 mL in the assay. Volumes up to 2.0 mL can be used for samples containing low acid content. For red wine containing approx. 0.2 g of pyruvic acid/L, use 0.10 mL of sample without decolourising in the assay. For red wine containing less than 0.1 g of pyruvic acid/L, decolourise by adding 0.2 g of PVPP per 10 mL of sample and stir for 5 min. Filter an aliquot through Whatman No. 1 filter paper, and adjust the pH to approx. 7.4. Adjust the volume to twice that of the original volume of sample taken. Use up to 2.0 mL of sample in the assay, and allow for the dilution and sample volume in the calculations. *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory for white wine, and 0.1 mL for red wine.*

(b) Determination of pyruvic acid in fruit juices (e.g. apple).
With fruit juices containing a high level of pyruvic acid (approx. 0.5 g/L), dilute an aliquot of the sample with an equal volume of water and use 0.1 mL for assay. If a large volume of sample is required, adjust the pH of the solution to approx. 7.4 before analysis. Coloured juices should be decolourised as described in "General considerations (e)" on page 9. Use 0.10 to 2.00 mL of sample for the assay (adjust to pH 7.4 if larger volumes are required). *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(c) Determination of pyruvic acid in beer.
Degass the beer by filtration or by stirring for 5 min with a glass rod. Analyse the sample without dilution. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(d) Determination of pyruvic acid in cheese.
Accurately weigh approx. 2 g of ground cheese into a 100 mL volumetric flask and add 60 mL of distilled water. Incubate the flask at approx. 60°C for 20 min, with intermittent shaking. Cool the flask to 20-25°C and fill to the mark with distilled water. Store the flask at 4°C for 30-60 min and then filter an aliquot of the solution through Whatman GF/A glass fibre filter paper. Use the clear filtrate in the assay. *Typically, no dilution is required and a sample volume of 0.5 mL is satisfactory.*

(e) Determination of pyruvic acid in dietary supplements.
In general, the concentration of pyruvic acid in dietary supplements can be determined as follows: accurately weigh approximately 5 g of representative material into a 100 mL volumetric flask. After addition of approx. 60 mL of distilled water, stir the contents until fully dissolved or suspended, and fill up to the mark with distilled water. Mix and if necessary filter through Whatman No. 1 filter paper. Use the clear filtrate, with dilution according to the dilution table if necessary. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(f) Determination of pyruvic acid in whole blood samples.

a. Solutions:

Concentrated Carrez I solution. Dissolve 30 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6].3H_2O$} (Sigma cat. no. P-9387) in 200 mL of distilled water. Store at room temperature.

Concentrated Carrez II solution. Dissolve 60 g of zinc sulphate {$ZnSO_4.7H_2O$} (Sigma cat. no. Z-4750) in 200 mL of distilled water. Store at room temperature.

b. Procedure:

Heat 1 mL of whole blood sample at approx. 80°C for 20 min in a microfuge tube then centrifuge at 13,000 x g for 10 min and recover the supernatant. Add 20 µL Carrez Reagent II and mix thoroughly, then add 20 µL Carrez Reagent I and mix thoroughly. Centrifuge the sample again at 13,000 x g for 10 min and recover the clarified supernatant for use in the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The final volume of the clarified supernatant will be approximately one quarter of the starting volume of the original sample. Therefore adjust the volume of the starting material as required to obtain sufficient volume of clarified sample for the test.

(g) Determination of pyruvic acid in biological tissue samples.

Accurately weigh approx. 5 g of representative biological tissue into a 100 mL Duran® bottle. Add 20 mL of 1 M perchloric acid and homogenise for 2 min using a Ultraturrax® or Polytron® homogeniser (or equivalent). Quantitatively transfer to a 40 mL glass beaker and adjust the pH to approx. 8.0 using 2 M KOH. Quantitatively transfer to a 100 mL volumetric flask and adjust to the mark with distilled water (ensuring the fat containing layer is "above" the mark, and the aqueous layer is "at" the mark). Store on ice for 20 min to precipitate potassium perchlorate and allow separation of the fat (if present). Centrifuge an appropriate volume of the sample at 13,000 x g for 10 min and recover the clarified supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the clear filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The amount of starting material and volumes used can be adjusted accordingly depending on the amount of analyte present in the sample.

(h) Determination of pyruvic acid in biological fluid samples (e.g. urine and serum).

For some biological fluid samples it may be sufficient to test them directly without any sample preparation other than appropriate dilution in distilled water. If this is not adequate then deproteinisation with either perchloric acid or trichloracetic acid may be required.

Deproteinise biological samples by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge an appropriate volume of the sample at 1,500 x g for 10 min and recover the supernatant for use in the assay, alternatively filter through Whatman No. 1 filter

paper, discarding the first 3-5 mL, and use the filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay. Alternatively, use 50% (w/v) trichloroacetic acid instead of perchloric acid.

REFERENCE:

Lamprecht, W. and Heinz, F. (1988). Pyruvate. In *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 3rd ed., Vol. VI, pp. 570-577, VCH Publishers (UK) Ltd., Cambridge, UK.

NOTES:

NOTES:

NOTES:

Megazyme International Ireland,
Bray Business Park, Bray,
Co. Wicklow,
IRELAND.

Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com

WITHOUT GUARANTEE
The information contained in this booklet is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents.