

Megazyme

PYRUVIC ACID (PYRUVATE)

ASSAY PROCEDURE

K-PYRUV 03/07

(100 Assays per Kit)

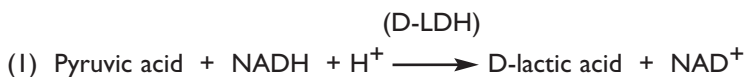


INTRODUCTION:

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).



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 reagents used in the determination of pyruvic acid are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 100 assays are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1:** Tris/HCl (25 mL, 1.0 M, pH 7.4) plus magnesium chloride (30 mM) and 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, 2000 U/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 Tris/HCl 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.

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.

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)	2.60 mL	2.50 mL
sample	-	0.10 mL
solution 2 (NADH/Tris/HCl buffer)	0.20 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

$\Delta A_{\text{pyruvic acid}}$

The value of $\Delta A_{\text{pyruvic acid}}$ 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:

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

where:

V = final volume [mL]

MW = molecular weight of pyruvic acid [g/mol]

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

It follows for pyruvic acid:

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

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

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

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

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).

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 $\Delta A_{\text{pyruvic acid}}$ 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, making sure that the sum of the sample and distilled water components in the reaction is 2.1 mL and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6] \cdot 3H_2O\}$ (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 ($ZnSO_4 \cdot 7H_2O$) (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 polyvinylpyrrolidone (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 decolorising in the assay. For red wine containing less than 0.1 g of pyruvic acid/L, decolorise 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 are 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 6. 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 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 biological samples.

If necessary, heat biological samples at approx. 80°C for 20 min to denature any enzymes present that may interfere with the assay (alternatively, deproteinise using Carrez reagents as described on page 6). After filtering, use the clear supernatant, with dilution if necessary, for the assay. *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory.*

REFERENCE:

I. 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.



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