Megazyme

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D-LACTIC ACID (D-LACTATE) *(Rapid)* and L-LACTIC ACID (L-LACTATE)

ASSAY PROCEDURES

K-DLATE 08/18

(*50 Assays of each per Kit)

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

Megazyme

INTRODUCTION:

D- and L-lactic acid are found in many foods and beverages. Produced naturally by lactic acid bacteria, D- and L-lactic acid are found in many fermented milk products such as yogurt and cheese, and also in pickled vegetables, and cured meats and fish. L-Lactic acid is supplemented into foods and beverages (E270) where a tart flavour is desired, and is widely used as a non-volatile acidulant. In the egg industry, L-lactic acid is a quality indicator, where levels above 200 mg/kg indicate spoilage by contamination or incubation. Similarly, the quality of milk and fruit juice can be established by measurement of the D- and L-lactic acid content. In the wine industry, the course of malolactic fermentation is monitored by following the falling level of L-malic acid, and the increasing level of L-lactic acid. The production of D-lactic acid can indicate wine spoilage. In the chemical industry, both D- and L-lactic acid are raw materials in the production of compounds such as polylactides and biologically degradable polymers, and applications also exist for these acids in cosmetics and pharmaceuticals.

PRINCIPLE:

The quantification of D-lactic acid requires two enzyme reactions. In the first reaction catalysed by D-lactate dehydrogenase (D-LDH), D-lactic acid (D-lactate) is oxidised to pyruvate in the presence of nicotinamide-adenine dinucleotide (NAD^+) (1).

(1) D-Lactate + NAD^+ $\xrightleftharpoons{\text{(D-LDH)}}$ pyruvate + NADH + H^+

However, since the equilibrium of reaction (1) lies firmly in the favour of D-lactic acid and NAD^+, a further reaction is required to "trap" the pyruvate product. This is achieved by the conversion of pyruvate to D-alanine and 2-oxoglutarate, with the enzyme D-glutamate-pyruvate transaminase (D-GPT) in the presence of a large excess of D-glutamate (2).

(2) Pyruvate + D-glutamate $\xrightarrow{\text{(D-GPT)}}$ D-alanine + 2-oxoglutarate

The amount of NADH formed in the above coupled reaction is stoichiometric with the amount of D-lactic acid. It is the NADH which is measured by the increase in absorbance at 340 nm.

In a similar set of reactions, L-lactic acid (L-lactate) is oxidised to pyruvate by L-lactate dehydrogenase (L-LDH) (3) in the presence of nicotinamide-adenine dinucleotide (NAD^+).

(3) L-Lactate + NAD^+ $\xrightarrow{\text{(L-LDH)}}$ pyruvate + NADH + H^+

Pyruvate is again "trapped" using D-GPT in the presence of a large excess of D-glutamate (2). While the assays for D-lactic acid and L-lactic acid can be performed sequentially, in the standard format they are performed separately as this allows the incubations to be performed concurrently and thus gives a significant reduction in total reaction time.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assays are specific for either D-lactic acid or L-lactic acid. In the assay of lithium D-lactate (MW = 96.0) results of approx. 96% (w/w) can be expected, while a value of 98% (w/w) should be obtained with lithium L-lactate.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.107 mg/L of sample solution at the maximum sample volume of 1.50 mL (or to 1.60 mg/L with a sample volume of 0.1 mL). The detection limit is 0.214 mg/L, which is derived from an absorbance difference of 0.010 and the maximum sample volume of 1.50 mL.

The assay is linear over the range of 0.5 to 30 µg of D- or L-lactic 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 1.50 mL, this corresponds to a D-/L-lactic acid concentration of approx. 0.107 to 0.214 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 D- and L-lactic acid has been completed within the times specified in the assay (D-lactic acid, approx. 5 min; L-lactic acid, approx. 10 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding a D-/L-lactic acid mixture (15 µg of each in 0.1 mL) to the cuvette on completion of the reaction. A significant increase 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 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 50 concurrent determinations of D- and L-lactic acid are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: (x2) Buffer (25 mL, pH 10.0) plus D-glutamate and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+.
Stable for > 5 years below -10°C.

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

Bottle 4: L-Lactate dehydrogenase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 5: D-Lactate dehydrogenase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 6: D-/L-Lactic acid standard solution (5 mL, 0.15 mg/mL of each) in 0.02% (w/v) sodium azide.
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

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

NOTE: In order to obtain the fast reaction velocities experienced with this kit, a near saturating level of D-glutamate is employed. On prolonged storage, it is possible that a small amount of D-glutamate may crystallise at the bottom of the bottle. This does not affect the assay and can either be ignored, or resolubilised by incubation (with occasional swirling) in warm water until the solution is clear.

2. Dissolve the contents of one of bottle 2 in 5.5 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes). **Do not** dissolve the contents of the second bottle until required.

3, 4, 5 Use the contents of bottles 3, 4 and 5 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.

6. Use the contents of bottle 6 as supplied.
Stable for > 2 years at 4°C.

NOTE: The D-/L-lactic 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 concentrations of D-lactic acid and L-lactic acid are determined directly from the extinction coefficient of NADH (see page 6).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL and 100 mL).
2. Disposable plastic or glass cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette® - with 25 mL Combitip® (to dispense 0.5 mL aliquots of Buffer 1 and 0.1 mL aliquots of NAD^+ solution).
5. Analytical balance.
6. Stop clock.
7. Spectrophotometer set at 340 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Whatman No. 1 (9 cm) filter papers.

MANUAL ASSAY PROCEDURE *(for D-lactic acid)*:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.24 mL
Sample solution: 0.5-30 µg of D-lactic acid per cuvette (in 0.1-1.5 mL sample volume)

Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (~ 25°C)	1.60 mL	1.50 mL
sample	-	0.10 mL
solution 1 (buffer)	0.50 mL	0.50 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL
suspension 3 (D-GPT)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 5 (D-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 1 min intervals until the absorbances either remain the same, or increase constantly over 1 min** (see Figure 1, page 8).		

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

** if this "creep" rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of addition of suspension 5.

CALCULATION *(for D-lactic acid):*

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

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

The concentration of D-lactic acid can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of D-lactic 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 D-lactic acid:

$$c = \frac{2.24 \times 90.1}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-lactic acid}} \quad [g/L]$$

$$= 0.3204 \times \Delta A_{D\text{-lactic acid}} \quad [g/L]$$

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 D-lactic acid:

$$= \frac{c_{D\text{-lactic acid}}\ [g/L \text{ sample solution}]}{weight_{sample}\ [g/L \text{ sample solution}]} \quad [g/100\ g]$$

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).

MANUAL ASSAY PROCEDURE *(for L-lactic acid)*:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.24 mL
Sample solution: 0.5-30 µg of L-lactic acid per cuvette (in 0.1-1.5 mL sample volume)
Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (~ 25°C)	1.60 mL	1.50 mL
sample	-	0.10 mL
solution 1 (buffer)	0.50 mL	0.50 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL
suspension 3 (D-GPT)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 4 (L-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 5 min intervals until the absorbances either remain the same, or increase constantly over 5 min** (see Figure 2, page 8).		

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

** if this "creep" rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of addition of suspension 4.

CALCULATION *(for L-lactic acid)*:

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

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

The concentration of L-lactic acid is then calculated exactly as described for D-lactic acid on page 6.

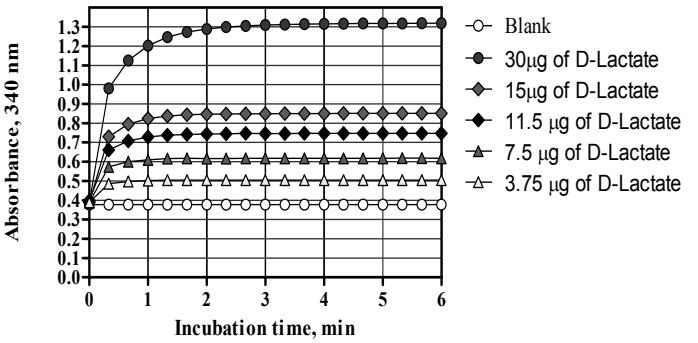

Figure 1. Increase in absorbance at 340 nm on incubation of 3.75-30 µg of D-lactate with D-lactate dehydrogenase plus D-glutamate-pyruvate transaminase in the presence of NAD^+.

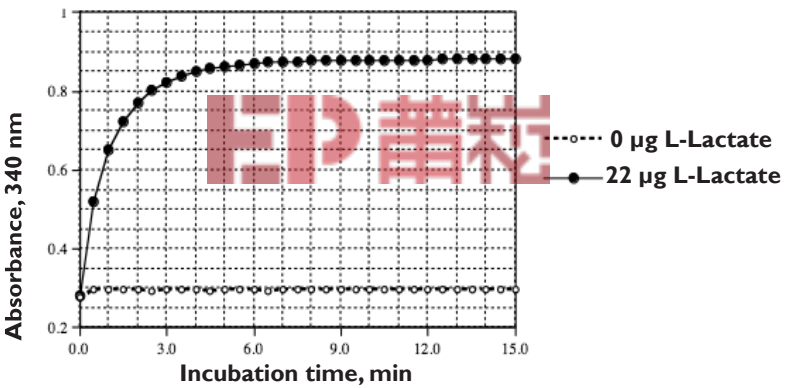

Figure 2. Increase in absorbance at 340 nm on incubation of 22 µg of L-lactate with L-lactate dehydrogenase plus D-glutamate-pyruvate transaminase in the presence of NAD^+.

MANUAL ASSAY PROCEDURE *(for the sequential assay of D-lactic acid and L-lactic acid)*:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.24 mL (D-lactic acid)
2.26 mL (L-lactic acid)
Sample solution: 0.5-30 µg of total lactic acid per cuvette
(in 0.1-1.5 mL sample volume)
Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (~ 25°C)	1.60 mL	1.50 mL
sample	-	0.10 mL
solution 1 (buffer)	0.50 mL	0.50 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL
suspension 3 (D-GPT)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 5 (D-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 1 min intervals until the absorbances either remain the same, or increase constantly over 1 min** (see Figure 3, page 13).		
suspension 4 (L-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_3) at the end of the reaction (approx. 10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 5 min intervals until the absorbances either remain the same, or increase constantly over 5 min** (see Figure 3, page 13).		

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

** if this "creep" rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of addition of suspension 4 or 5.

CALCULATION *(for sequential assay):*

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

Determine the absorbance difference (A_3-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{L\text{-lactic acid}}$.

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

The concentration of D-and L-lactic acid can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of lactic 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 D-lactic acid:

$$c = \frac{2.24 \times 90.1}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-lactic acid}} \quad [g/L]$$

$$= 0.3204 \times \Delta A_{D\text{-lactic acid}} \quad [g/L]$$

It follows for L-lactic acid:

$$c = \frac{2.26 \times 90.1}{6300 \times 1.0 \times 0.1} \times \Delta A_{L\text{-lactic acid}} \quad [g/L]$$

$$= 0.3232 \times \Delta A_{L\text{-lactic acid}} \quad [g/L]$$

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 D-lactic or L-lactic acid:

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

MANUAL ASSAY PROCEDURE *(for total lactic acid)*:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.26 mL
Sample solution: 0.5-30 µg of total lactic acid per cuvette (in 0.1-1.5 mL sample volume)

Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (~ 25°C)	1.60 mL	1.50 mL
sample	-	0.10 mL
solution 1 (buffer)	0.50 mL	0.50 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL
suspension 3 (D-GPT)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 5 (D-LDH)	0.02 mL	0.02 mL
suspension 4 (L-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 5 min intervals until the absorbances either remain the same, or increase constantly over 5 min** (see Figure 4, page 13).		

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

** if this "creep" rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of addition of suspensions 4 and 5.

CALCULATION *(for total lactic acid):*

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

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

The concentration of lactic acid can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of lactic 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 lactic acid:

$$c = \frac{2.26 \times 90.1}{6300 \times 1.0 \times 0.1} \times \Delta A_{lactic\ acid} \quad [g/L]$$

$$= 0.3232 \times \Delta A_{lactic\ acid} \quad [g/L]$$

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 lactic acid:

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

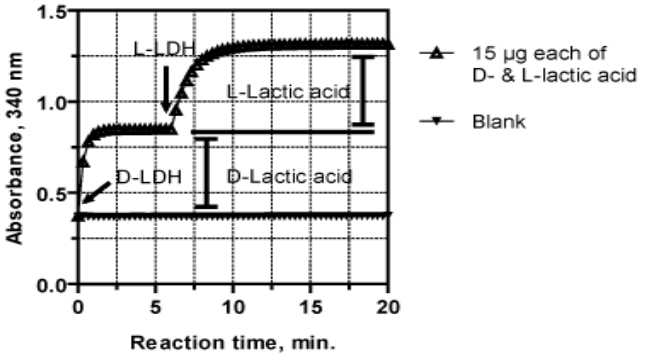

Figure 3. Sequential assay. Increase in absorbance at 340 nm on incubation of 15 µg each of D- and L-lactic acid (30 µg total lactic acid) in sequential reactions with D-LDH followed by L-LDH, plus D-glutamate-pyruvate transaminase in the presence of NAD^+ at 25°C using 1 cm path-length cuvettes.

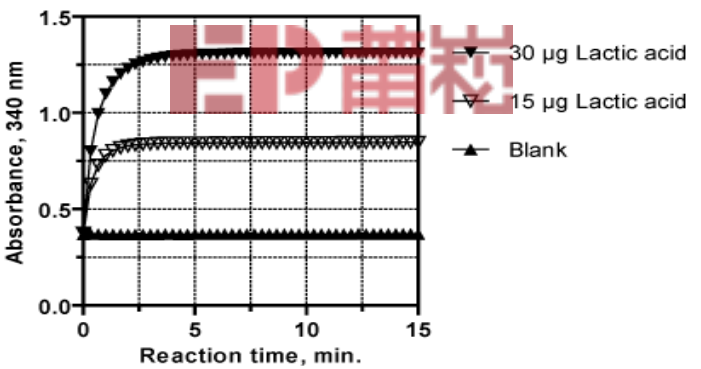

Figure 4. Total lactic acid assay. Increase in absorbance at 340 nm on incubation of 15 µg of each of D- and L-lactic acid (30 µg total lactic acid) with D-LDH and L-LDH together plus D-glutamate-pyruvate transaminase in the presence of NAD^+ at 25°C using 1 cm path-length cuvettes.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of D-lactic acid or L-lactic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.5 and 30 µg. The sample solution must therefore be diluted sufficiently to yield a D- or L-lactic acid concentration between 0.005 and 0.30 g/L.

Estimated concentration of D- or L-lactic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.30	No dilution required	1
0.30-3.0	1 + 9	10
3.0-30	1 + 99	100
> 30	1 + 999	1000

If the value of $\Delta A_{D\text{-}}$ or $_{L\text{-lactic}}$ 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 1.50 mL, making sure that the sum of the sample and distilled water components in the reaction is 1.60 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) $\{K_4[Fe(CN)_6].3H_2O\}$ (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: clear, slightly coloured and approximately neutral, liquid samples can be used 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 sample should be increased to approx. 10.0 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. 10.0 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 (or L-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) per 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 at 60°C. Allow to cool 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. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively use Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of free D- and L-lactic acid in wine.

The free D-/L-lactic acid concentration [F] of white and red wine can generally be determined without any sample treatment (except dilution according to the dilution table). *Typically, a dilution of 1:10 and sample volume of 0.1 mL are satisfactory.*

(b) Determination of free and esterified D- and L-lactic acid in wine.

The concentration of both free and esterified D- or L-lactic acid [F + E] in white and red wine can be determined as follows: add

2 mL of 2 M NaOH to 20 mL of wine and heat under reflux for 15 min with stirring. After cooling, carefully adjust the pH of the solution to 10.0 with 1 M H_2SO_4 and adjust the volume to 100 mL with distilled water. Then analyse the sample according to the general procedure, with dilution where necessary. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

The concentration obtained is the sum of the free and esterified D- or L-lactic acid [F + E], and thus the esterified D- or L-lactic acid concentration alone [E] can be calculated as follows:

$$[E] = [F + E] - [F] \qquad [g/L]$$

(c) Determination of D- and L-lactic acid in beer.
The D- or L-lactic acid concentration of beer can generally be determined without any sample treatment, except removal of carbon dioxide by stirring for approx. 1 min with a glass rod. *Typically, no dilution is required, and a sample volume of 0.2 mL is satisfactory.*

(d) Determination of D- and L-lactic acid in yogurt and milk.
Accurately weigh approx. 1 g of homogenised yogurt or 10 g of milk into a 100 mL volumetric flask containing 60 mL of distilled water. Add the following solutions and mix after each addition: 2 mL of Carrez I solution, 2 mL of Carrez II solution and 4 mL of NaOH solution (100 mM). Fill up to the mark with distilled water, mix and filter. *Typically, no further dilution is required and sample volumes of 0.1 mL (for yogurt) and 1.0 mL (for milk) are satisfactory.*

(e) Determination of D- and L-lactic acid in cheese.
Accurately weigh approx. 1 g of grated cheese into a 100 mL volumetric flask containing approx. 70 mL of distilled water and heat at 60°C with occasional shaking for 20 min, or until fully dispersed. Fill up to the mark with distilled water, place in a refrigerator (or ice-water) for approx. 20 min to allow separation of the fat, and then filter. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(f) Determination of D- and L-lactic acid in vinegar and vinegar-containing liquids.
The D- or L-lactic acid concentration of vinegar or other pickling liquids can generally be determined without any sample treatment (except filtration where necessary and dilution according to the dilution table). *Typically, no dilution is required, and a sample volume of 0.1 mL is satisfactory.*

(g) Determination of D- and L-lactic acid in sauerkraut juice.
The D- or L-lactic acid concentration of sauerkraut juice can generally be determined without any sample treatment (except filtration where

necessary and dilution according to the dilution table). *Typically, a dilution of 1:100 and sample volume of 0.1 mL are satisfactory.*

(h) Determination of D- and L-lactic acid in meat products.
Accurately weigh approx. 5 g of homogenised sample into a beaker containing 20 mL of 1 M perchloric acid and homogenise with an Ultra-turrax® (or equivalent) for 5 min. Add approx. 40 mL of distilled water and adjust the pH to approx. 10.0 with 2 M KOH, using pH test strips. Transfer the contents quantitatively to a 100 mL volumetric flask and fill to the mark with distilled water (if a fat layer develops, make sure this is above the mark, and the aqueous layer is at the mark). Place in a refrigerator (or ice-water) for approx. 20 min to allow separation of fat and precipitation of potassium perchlorate. Filter, discarding the first few mL of filtrate and use the clear, possibly slightly turbid, solution diluted, if necessary, for the assay. *Typically, a dilution of 1:2 and sample volume of 0.1 mL are satisfactory.*

(i) Determination of D- and L-lactic acid in liquid whole egg.
Homogenise the whole egg sample by vigorous stirring and accurately weigh approx. 10 g into a 50 mL volumetric flask containing approx. 20 mL of distilled water. Add 2 drops of *n*-octanol and heat at approx. 100°C with occasional shaking for 15 min and then allow to cool. With mixing after each addition, add 2 mL of **concentrated** Carrez I solution {15 g of $K_4[Fe(CN)_6].3H_2O$ in 100 mL of distilled water} and 2 mL of **concentrated** Carrez II solution (30 g of $ZnSO_4.7H_2O$ in 100 mL of distilled water). Fill up to the mark with 0.1 M NaOH, thoroughly mix and filter. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory for contaminated or incubated eggs, or 0.5 mL for fresh eggs.*

The L-lactic acid content of whole egg powder is determined by accurately weighing approx. 2 g of whole egg powder into a volumetric flask containing 25 mL of distilled water. After mixing, add 2 drops of *n*-octanol and proceed as described above, except after the addition of concentrated Carrez II solution, the pH should be adjusted to 9.0 using 1 M NaOH. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory for poor quality egg powder, or 0.5 mL for good quality egg powder.*

(j) Determination of D- and L-lactic acid in vegetable juices, fruit juices and similar beverages.
Dilute the sample to yield a D- or L-lactic acid concentration of less than 0.35 g/L (see dilution table). Clear, neutral solutions can generally be determined without any sample treatment. Turbid liquids generally only require filtering before the dilution step.

If coloured vegetable juice (such as tomato juice) requires decolourising, proceed as follows: accurately weigh approx. 5 g of homogenised sample into a 100 mL volumetric flask containing 60 mL of distilled water. Add the following solutions and mix after each addition: 2 mL of Carrez I solution, 2 mL of Carrez II solution and 4 mL of 0.1 M NaOH solution. Fill up to the mark with distilled water, mix and filter. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

If coloured fruit juice (such as orange juice) requires decolourising, proceed as follows: adjust 25 mL of filtered sample to a pH of approx. 10.0 using 2 M NaOH. Quantitatively transfer the solution to a 50 mL volumetric flask and adjust to volume with distilled water. Transfer to a beaker and add 1 g of PVPP. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 (9 cm) filter paper. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

(k) Determination of D- and L-lactic 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.

(l) Determination of D- and L-lactic 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 an Ultra-turrax® 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.

(m) Determination of D- and L-lactic 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.

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