
**Cheese and processed cheese
products — Determination of citric acid
content — Enzymatic method**

*Fromages et fromages fondus — Détermination de la teneur en acide
citrique — Méthode enzymatique*

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Reference numbers
ISO/TS 2963:2006(E)
IDF/RM 34:2006(E)

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Published in Switzerland

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
- an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 2963|IDF/RM 34 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

This edition of ISO/TS 2963|IDF/RM 34 cancels and replaces ISO 2963:1997, which has been technically revised.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a Standing Committee may decide to publish an other type of normative document which is called by IDF: *Reviewed method*. Such a method represents an agreement between the members of a Standing Committee and is accepted for publication if it is approved by at least 50 % of the committee members casting a vote. A *Reviewed method* is equal to an ISO/PAS or ISO/TS and will, therefore, also be published jointly under ISO conditions.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO/TS 2963|IDF/RM 34 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Lactose and lactate determination*, of the Standing Committee on *Main components of milk*, under the aegis of its project leader, Mr C. Hughes (NZ).

This edition of ISO/TS 2963|IDF/RM 34 cancels and replaces IDF 34C:1992, which has been technically revised.

Introduction

The method described in ISO 2963:1997 and IDF 34C:1992 did not fulfil the requirements for a fully validated International Standard. No new interlaboratory tests could be organized with the method according to ISO 5725-1 and ISO 5725-2 due to a lack of participants, hence the publication of this revision as a Technical Specification/Reviewed method.

Reliable results with enzymatic methods will only be obtained if the good laboratory practice (GLP) rules for such analyses are applied strictly. These GLP rules are given in Annex A.

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Cheese and processed cheese products — Determination of citric acid content — Enzymatic method

1 Scope

This Technical Specification specifies an enzymatic method for the determination of the citric acid content of cheese and processed cheese products.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1

citric acid content

mass fraction of substances determined by the procedure described in this Technical Specification

NOTE The citric acid content is expressed as mass fraction in percent.

3 Principle

An extract of the sample is treated with the following enzymes and biochemical substances:

- a) citrate lyase (CL) to convert citric acid to oxalacetate and acetate;
- b) malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), in the presence of reduced nicotinamide adenine dinucleotide (NADH), to catalyse the reduction of oxalacetate and its decarboxylation product pyruvate to L-malate and L-lactate, respectively, with the subsequent conversion of NADH to its oxidized form (NAD⁺).

The decrease in concentration of NADH is determined by measurement of the absorbance of the test solution at 340 nm. The citric acid content is proportional to the decrease in NADH concentration.

4 Reagents

Use only reagents of recognized analytical grade and distilled water or water of at least equivalent purity, unless otherwise specified. Take note of the production and expiry dates given by the manufacturer of the reagents.

4.1 Enzymes

If an enzyme suspension is applied with other than the prescribed activity, the volume of the suspension stated in the pipetting scheme (8.5.1) shall be increased or decreased proportionally.

The reagents described in 4.7 to 4.10 inclusive may be obtained commercially as a test combination.

4.2 Trichloroacetic acid solution (CCl₃COOH).

Dissolve 200,0 g of trichloroacetic acid in water. Dilute to 1 000 ml with water and mix.

4.3 Sodium hydroxide solution I, $c(\text{NaOH}) = 5,0 \text{ mol/l}$.

Dissolve 200,0 g of sodium hydroxide in water. Dilute to 1 000 ml with water and mix.

4.4 Sodium hydroxide solution II, $c(\text{NaOH}) = 1,0 \text{ mol/l}$.

Dissolve 40,0 g of sodium hydroxide in water. Dilute to 1 000 ml with water and mix.

4.5 Sodium hydroxide solution III, $c(\text{NaOH}) = 0,1 \text{ mol/l}$.

Dissolve 4,0 g of sodium hydroxide in water. Dilute to 1 000 ml with water and mix.

4.6 Zinc chloride solution, $c(\text{ZnCl}_2) = 800 \text{ mg/l}$.

Dissolve 800 mg of zinc chloride in water. Dilute to 1 000 ml with water and mix.

4.7 Buffer solution, pH 7,8.

Dissolve 71,3 g of glycylglycine in about 700 ml of water. Adjust to pH 7,8 with sodium hydroxide solution I (4.3). Add 100 ml of zinc chloride solution (4.6). Dilute to 1 000 ml with water and mix.

The buffer solution may be kept for 4 weeks if stored in a refrigerator at between 0 °C and +5 °C.

4.8 Reduced nicotinamide adenine dinucleotide solution

Dissolve 50 mg of reduced nicotinamide adenine dinucleotide disodium salt ($\text{C}_{21}\text{H}_{27}\text{N}_7\text{O}_{14}\text{P}_2\text{Na}_2$) and 100 mg of sodium hydrogen carbonate (NaHCO_3) in 10 ml of water.

The reduced nicotinamide adenine dinucleotide solution may be kept for 4 weeks if stored in a refrigerator at between 0 °C and +5 °C.

4.9 Malate dehydrogenase/lactate dehydrogenase suspension

Mix suitable amounts of malate dehydrogenase (MDH from pig heart; suspension in ammonium sulfate solution, 3,2 mol/l, at pH $6,0 \pm 0,2$; EC 1.1.1.37)¹⁾ and lactate dehydrogenase (LDH from rabbit muscle; suspension in ammonium sulfate solution, 3,2 mol/l, at pH $7 \pm 0,2$; EC 1.1.1.27). Dilute with ammonium sulfate solution (3,2 mol/l) so as to obtain a final suspension containing about 600 units²⁾ of MDH/ml and 1 400 units²⁾ of LDH/ml.

The malate dehydrogenase/lactate dehydrogenase suspension may be kept for 1 year if stored in a refrigerator at between 0 °C and +5 °C.

4.10 Citrate lyase solution

Dissolve a suitable amount of citrate lyase [lyophilisate (CL) from *Aerobacter aerogenes*; EC 4.1.3.6] in ice-cold water so as to obtain a solution containing 40 units/ml²⁾.

The citrate lyase solution may be kept for 1 week if stored at between 0 °C and +5 °C, and for 4 weeks if stored at -20 °C.

4.11 Citric acid standard solution.

Dissolve 1,600 g of citric acid monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) in water. Dilute to 1 000 ml with water and mix.

1) The EC number refers to the Enzyme Classification number as given in Reference [4].

2) This unit (often called International or Standard Unit) is defined as the amount of enzyme which will catalyse the transformation of 1 μmol of substrate per minute under standard conditions.

5 Apparatus

Usual laboratory equipment and, in particular, the following:

- 5.1 **Analytical balance**, capable of weighing to the nearest 1 mg, with a readability of 0,1 mg.
- 5.2 **pH meter**.
- 5.3 **Glass beakers**, of capacity 50 ml.
- 5.4 **Macerator**, equipped with a suitable beaker.
- 5.5 **One-mark volumetric flasks**, of capacity 100 ml.
- 5.6 **Pipettes**, capable of delivering 0,02 ml, 1 ml, 2 ml, 5 ml, 25 ml and 40 ml, respectively.
- 5.7 **Graduated pipettes**, capable of delivering 10 ml, graduated in 0,1 ml divisions.
- 5.8 **Measuring cylinder**, of capacity 50 ml.
- 5.9 **Filter funnel**, of diameter approximately 7 cm.
- 5.10 **Filter paper**, medium grade, of diameter approximately 15 cm.
- 5.11 **Spectrometer**, suitable for measuring at a wavelength of 340 nm, equipped with cells of optical path length 1 cm.
- 5.12 **Plastic paddles**, suitable for mixing the sample-enzyme mixture in the spectrometer cell.
- 5.13 **Water bath**, capable of maintaining a temperature of 20 °C to 25 °C, with rack suitable for holding the spectrometer cell (5.11) during the incubation period (optional; see 8.5.1).

Incubation of the cells in the water bath is only necessary if the room temperature is below 20 °C.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this Technical Specification. A recommended sampling method is given in ISO 707/IDF 50.

7 Preparation of test sample

Prepare a homogeneous sample taking care to avoid loss of moisture, using the following procedure.

- a) In the case of cheese, remove the rind or mouldy surface layer of the cheese in such a way as to provide a sample representative of the cheese as it is usually consumed.

Grind or grate the sample by using an appropriate device, mix the ground or grated mass quickly and, if possible, grind or grate a second time and again mix thoroughly by intensive stirring and kneading.

- b) In the case of processed cheese, remove a sample representative of the product. Mix the sample mass quickly and grind if necessary. Mix thoroughly by intensive stirring and kneading.

- c) In the case of processed cheese containing pieces of other foods (e.g. ham, fruit, nuts, herbs), determine whether the objective of the analysis is to determine the citric acid content of the processed cheese proper or of the entire product. In the former case, separate the pieces of other food and then proceed as for processed cheese.

Transfer the test sample into a container provided with an airtight lid, for storage prior to analysis. Close the container immediately. Analysis should be carried out as soon as possible after preparation of the test sample.

8 Procedure

8.1 Check tests

8.1.1 Carry out the test in 8.1.2 to 8.1.4 to check the recovery of citric acid whenever

- a new batch of reagents (4.7 to 4.10 inclusive) is brought into use,
- such reagents have been kept in a refrigerator without being used for more than 2 weeks,
- restarting analytical work after a period of analytical inactivity, or
- when conditions justify such a test.

8.1.2 Pipette 5,0 ml and 10,0 ml of citric acid standard solution (4.11) into each of two 100 ml one-mark volumetric flasks (5.5).

Add 10 ml of trichloroacetic acid solution (4.2) to each flask. Dilute the contents of each flask to the 100 ml mark with water and mix.

Determine the citric acid content of both solutions as described in 8.4.3 to 8.5.3 inclusive.

8.1.3 Calculate the citric acid monohydrate content of the citric acid standard solution (4.11) according to Equation (2) in 9.1, but using the following values.

- V_5 is the volume, in millilitres, of the citric acid standard solution (4.11) ($V_5 = 1\ 000$ ml);
- V_6 is the volume, in millilitres, of the citric acid standard solution (8.1.2) ($V_6 = 5$ ml and 10 ml respectively);
- V_7 is the total volume, in millilitres, of the diluted citric acid standard solution (8.1.2) ($V_7 = 100$ ml).

8.1.4 Taking into account the purity of the citric acid monohydrate, the recovery obtained for both dilutions (8.1.2) shall be within the range $100\% \pm 5\%$. If the recoveries are not within this range, the reagents, the operating technique, the accuracy of the pipettes and the condition of the spectrometer shall be checked and the required action shall be taken to obtain the appropriate results. The test shall be repeated until satisfactory results are obtained.

8.2 Test portion

Weigh, to the nearest 0,1 mg, approximately 1 g of the prepared test sample (Clause 7) in a beaker (5.3). Suspend the test portion in about 50 ml of water preheated to between 40 °C and 50 °C using the macerator (5.4). Transfer the contents of the beaker quantitatively to a 100 ml one-mark volumetric flask (5.5). Cool the contents of the flask to about 20 °C.

8.3 Reagent blank test

Carry out a blank test in duplicate. Proceed as specified in 8.4 and 8.5, using all reagents but omitting the test portion.

8.4 Deproteination

8.4.1 Add 10 ml of trichloroacetic acid (4.2) to the suspension (8.2) in the 100 ml one-mark volumetric flask. Dilute to the mark with water and mix thoroughly.

8.4.2 Let the mixture stand for 30 min. Do not remix the contents of the volumetric flask prior to filtration.

8.4.3 Filter the supernatant liquid through a filter paper (5.10), discarding the first fraction of filtrate.

8.4.4 Pipette 25 ml of the filtrate into a glass beaker (5.3). Adjust the pH to approximately 4 by adding sodium hydroxide solution II (4.4) and, subsequently, to approximately pH 8 by adding sodium hydroxide solution III (4.5) while checking using the pH meter (5.2).

Transfer the contents of the beaker quantitatively to a 100 ml one-mark volumetric flask (5.5). Dilute to the mark with water and mix.

8.4.5 Filter through a filter paper (5.10), discarding the first fraction of filtrate.

8.5 Determination

8.5.1 Scheme for procedure

Carry out the determination according to the scheme in Table 1, taking care to bring the buffer solution (4.7) and the water to be used to room temperature just before use.

Table 1 — Determination scheme

Pipette into the spectrometer cells	Test portion or check test	Blank test
Buffer solution (4.7)	1,00 ml	1,00 ml
NADH solution (4.8)	0,10 ml	0,10 ml
MDH/LDH suspension (4.9)	0,02 ml	0,02 ml
Test or check test filtrate	2,00 ml	—
Blank test filtrate	—	2,00 ml
Mix the contents of the cells, using the plastic paddles (5.12) and incubate at a temperature above 20 °C for 5 min (see 5.13). Measure the absorbance, A_0 , of the solution in each cell against air at a wavelength of 340 nm. Then add to the spectrometer cells:		
Citrate lyase solution (4.10)	0,02 ml	0,02 ml
Mix the contents of the cells and incubate at a temperature above 20 °C for 10 min (see also 5.13). Measure the absorbance A_{10} of the solution in each cell, against air.		

8.5.2 Calculation of absorbance

Calculate the absorbance, A , of each cell content to be used for the calculation of citric acid content (9.1) using the following equation:

$$A = A_0 - A_{10} \quad (1)$$

where

A_0 is the numerical value of the absorbance measured before the addition of the citrate lyase solution;

A_{10} is the numerical value of the absorbance measured after the addition of citrate lyase solution and an incubation time of 10 min.

8.5.3 Verification

If the absorbance, A , exceeds 0,800, repeat the procedure specified in 8.5.1 and 8.5.2, using an appropriate aqueous dilution of the filtrate from both the test portion (8.2) and the blank test (8.3).

9 Calculation and expression of results

9.1 Calculation

Calculate the citric acid content, w , expressed as a mass fraction in percent of anhydrous citric acid, by using the following equation:

$$w = \frac{(A_s - A_r) \times M_r}{K \times l \times m} \times \frac{V_1 \times V_3 \times V_5 \times V_7}{V_2 \times V_4 \times V_6} \times 100 \% \quad (2)$$

where

A_s is the numerical value of the measured absorbance for the test portion or the check test;

A_r is the numerical average value of the measured absorbance for the blank test;

M_r is the relative molecular mass of citric acid (for anhydrous citric acid, $M_r = 192,1$);

K is the molar absorption coefficient of NADH at 340 nm (i.e. $K = 6,3 \times 10^6 \text{ cm}^2/\text{mol}$);

l is the numerical value of the optical path length, in centimetres, of the spectrometer cells ($l = 1 \text{ cm}$);

m is the mass, in grams, of the test portion (8.2);

V_1 is the total volume, in millilitres, of liquid in the spectrometer cell ($V_1 = 3,14 \text{ ml}$);

V_2 is the volume, in millilitres, of the filtrate (8.4.5) in the spectrometer cell ($V_2 = 2,00 \text{ ml}$);

V_3 is the volume, in millilitres, to which the deproteinated, filtered solution (8.4.3), after adjusting the pH to 8, was diluted (8.4.4) ($V_3 = 100 \text{ ml}$);

V_4 is the volume, in millilitres, of the deproteinated, filtered solution (8.4.3) taken for adjusting the pH to 8 (8.4.4) ($V_4 = 25 \text{ ml}$);

V_5 is the volume, in millilitres, of the solution in 8.1.2 ($V_5 = 100 \text{ ml}$);

V_6 is the volume, in millilitres, of the filtrate (8.4.5) taken for dilution (8.5.3), if appropriate; if not $V_6 = 1$;

V_7 is the volume, in millilitres, to which the filtrate taken for dilution (8.5.3) was diluted, if appropriate; if not $V_7 = 1$;

9.2 Expression of results

Report the test result to three significant figures.

10 Precision

10.1 Interlaboratory test

The values for repeatability and reproducibility are derived from an interlaboratory test that, however, did not fulfil the complete requirements of ISO 5725³⁾, thus the reliability of its results is doubtful.

NOTE The results of the interlaboratory test will be published in the *Bulletin of the International Dairy Federation*.

10.2 Repeatability

The absolute difference between two single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than 5 % (relative) of the arithmetic mean of the results.

10.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 8 % (relative) of the arithmetic mean of the results.

11 Test report

The test report shall specify:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, with reference to this Technical Specification;
- d) all operational details not specified in this Technical Specification, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- e) the test result(s) obtained and, if the repeatability has been checked, the final quoted result obtained with reference to the comment in 10.1.

3) ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests* (now withdrawn), was used to obtain the precision data.

Annex A (normative)

Good Laboratory Practice (GLP) rules for the performance of enzymatic analyses

A.1 Introduction

Good Laboratory Practice rules for enzymatic analyses are often less well known than those for other chemical analyses.

It is advisable to pay more attention to such rules in order to obtain results with a satisfactory accuracy and precision.

Therefore, before the analyses, read the GLP rules described below conscientiously.

A.2 Reagents

A.2.1 Use only enzymes of the prescribed grade (specific activity, concentration, contaminants with enzymatic activities, solvent).

A.2.2 Use only coenzymes of the prescribed grade (purity grade, salt or acid form, contaminants).

A.2.3 All reagents other than the enzymes and the coenzymes shall be of analytical grade.

A.2.4 The water for the preparation of the enzyme solutions and the other reagents shall be doubly glass-distilled.

A.2.5 The water for the preparation of the sample solutions shall be glass-distilled or de-ionized.

A.2.6 Store the reagents and enzyme suspensions/solutions according to the instructions (usually between 2 °C and 8 °C).

A.2.7 Do not freeze enzyme suspensions.

A.2.8 When the expiry date of a reagent has been exceeded, either discard the reagent or check the efficiency of this reagent by examining standard solutions with varying amounts of analyte. The absorbances obtained shall be proportional to the concentrations.

A.2.9 Buffer solutions taken from the refrigerator shall be warmed up to room temperature before being added to the assay mixture.

A.3 Photometric and spectrometric cells

A.3.1 Use glass or plastic cells with an optical path length of 1 cm.

NOTE Plastic cells have the following advantages over glass cells:

- they are cheap (disposable);
- there is the possibility of greater numbers of analyses;
- within one batch, plastic cells agree very well regarding the absorbance.

A.3.2 Whenever a new batch of cells is put into use, check the optical path length of the cells against that of a precision cell (e.g. quartz cell), as follows.

Fill the precision cell and plastic cells with water and measure the absorbance (A_1) of each cell against air. Fill the cells, after rinsing, with a solution of NADH (approximately 0,15 mg/ml) and again measure the absorbance (A_2) against air.

For both the precision cell and the plastic cells, calculate ($A_2 - A_1$). The differences ($A_2 - A_1$) between the two types of cell shall not deviate significantly. If the differences ($A_2 - A_1$) exceed 0,5 % of the net absorbance measurement for the precision cell, calculate the average percentage difference and take this into account for the path length (1 cm) in the calculation (9.1).

A.3.3 Always use clean and unscratched cells. Dry or clean the optical sides of the cells with a soft tissue only.

A.3.4 Do not measure the absorbance of the sample test cell against that of the blank test cell, since no information will be obtained about the order of magnitude of the absorbance of the blank test itself. Measure the absorbance of both the sample and the blank test cell against air and calculate the difference.

A.3.5 Do not measure the absorbance of a sample or blank test cell against an empty cell (because of light diffusion).

A.3.6 Mix the contents of a cell with a plastic paddle or by sealing the cell with parafilm and gently swirling.

A.3.7 Remove air bubbles from the walls of the cells using a paddle. Avoid scratching the optical side of the cell.

A.3.8 Always use the same kind of cells for the measurement of the sample test and the blank test.

A.3.9 Always place cells in the same position and orientation in the cell holder. For this purpose, mark one optical side of the cell.

A.4 Photometers and spectrometers

A.4.1 General

Use a spectrometer (bandwidth ≤ 10 nm) provided with an interference filter (bandwidth ≤ 10 nm), or a spectrum line photometer equipped with a mercury vapour lamp. Measurements, carried out using a spectrometer or filter photometer, shall be made at the absorption maximum of NADH or NADPH, i.e. 340 nm; those carried out using a spectral line photometer with a mercury vapour lamp shall be made at 365 nm or 334 nm.

NOTE The molar absorption coefficients of NADH and NADPH measured at 334 nm, 340 nm and 365 nm are as follows.

— NADH and NADPH at 334 nm (Hg):	$6,18 \times 10^6 \text{ cm}^2/\text{mol};$
— NADH and NADPH at 340 nm:	$6,3 \times 10^6 \text{ cm}^2/\text{mol};$
— NADPH at 365 nm (Hg):	$3,5 \times 10^6 \text{ cm}^2/\text{mol};$
— NADH at 365 nm (Hg):	$3,4 \times 10^6 \text{ cm}^2/\text{mol}.$

A.4.2 Linearity check

A linear relationship up to an absorbance of 2,0 shall exist between the absorbance and the concentration of NADH or NADPH. Check this as follows.

- a) Pipette 2,00 ml of distilled water into a cell. Measure the absorbance A_0 against air.
- b) Pipette 0,10 ml of NADH solution (0,5 mg/ml) into the cell and mix. Measure the absorbance A_1 .

Calculate the reduced absorbance, A_{r1} , using the following equation:

$$A_{r1} = (A_1 - A_0) \times \frac{2,1}{3,5}$$

where

A_1 is the numerical value of the absorbance obtained with the measurement of the NADH solution [see b)];

A_0 is the numerical value of the absorbance obtained with the measurement of the water [see a)].

- c) Repeat the procedure described in point b) above 14 times. After each pair of measurements, calculate the reduced absorbance, A_{rn} , with the following equation:

$$A_{rn} = (A_n - A_0) \times \frac{V}{3,5}$$

where

A_n is the numerical value of the absorbance obtained at measurement n ;

V is the volume of the cell contents at measurement n .

- d) For each measurement, plot the volume of NADH solution present in the cell against the corresponding reduced absorbances. A straight line shall be obtained connecting the obtained intersection points.

A.5 Automatic pipettes and other dispensers

A.5.1 Use automatic pipettes and other dispensers in accordance with the manufacturer's instructions.

A.5.2 Use the appropriate tips for each pipette.

A.5.3 Check the specifications of volume and repeatability of automatic pipettes and other dispensers periodically (e.g. monthly) as follows:

- a) weigh a glass beaker with water at time t ;
- b) pipette or dispense one measure of water into the beaker and weigh exactly at $t + 1$ min after the first weighing;
- c) repeat the pipetting or dispensing procedure as described in point b) 9 times;
- d) weigh, without pipetting or dispensing, the beaker at the moments $t + 11$ min, $t + 12$ min, $t + 13$ min, $t + 14$ min and $t + 15$ min; calculate from these weighings the evaporation loss per minute;
- e) calculate the volume and repeatability of the pipette or dispenser, taking into account the loss of water by evaporation.