II

(Information)

INFORMATION FROM EUROPEAN UNION INSTITUTIONS, BODIES, OFFICES AND AGENCIES

EUROPEAN COMMISSION

List and description of methods of analysis referred to in the first paragraph of Article 120g of Council Regulation (EC) No 1234/2007 [1]

***(published in accordance with Article 15(2) of Commission Regulation (EC) No 606/2009 of 10 July 2009* [2])**

(2010/C 43/01)

The table below lists the methods of analysis for monitoring the limits and requirements laid down in the Community rules concerning the production of wine products. For each parameter concerned, the third column contains the reference of the corresponding method of analysis described in the latest edition (2009) of the 'Compendium of International Methods of Analysis of Wine and Must' of the International Organisation of Vine and Wine (OIV) available on the date of this publication. For each parameter, only the reference methods ('Category I' or 'Category II' in the 'OIV classification') are described, with the exception of parameters for which there is currently no Category I or II method validated. The description of the methods appears in the Annex to the notice.

NB:

Section of Annex AI of the Compendium of the OIV defines the different categories of methods of analysis, and in particular Category I (Criterion reference method), Category II (Reference method) and Category IV (Auxiliary method).

The methods of analysis for lead and cadmium are now described in Commission Regulation (EC) No 333/2007 of 28 March 2007 (Annex C-3) [3]. Furthermore, Annex II.4 of Regulation (EC) No 401/2006 of 23 February 2006 [4] establishes general criteria for the methods of analysis for ochratoxin A , and it is therefore not necessary to describe a specific method for this substance in relation to wine products.

LIST OF METHODS OF ANALYSIS

No	Parameter	Method in the Compendium of the OIV	Category
1	Specific gravity/Density	AS-2-01-MASVOL	I
2	Refractive index	AS-2-02-SUCREF	I
3	Total dry matter	AS-2-03-EXTSEC	I
4	Isotope ratio $^{18}O/^{16}O$ of water in wine	AS-2-09-MOUO18	II

[1] OJ L 299, 16.11.2007.
[2] OJ L 193, 24.7.2009, p. 1.
[3] OJ L 88, 29.3.2007, p. 29.
[4] OJ L 70, 9.3.2006, p. 12.

No	Parameter	Method in the Compendium of the OIV	Category
5	Folin index	AS-2-10-INDFOL	IV
6	Sugar content (= glucose+fructose)	AS-311-02-GLUFRU	II
7	Sucrose content (HPLC measurement)	AS-311-03-SUCRES	II
8	Nuclear Magnetic Resonance - Deuterium of wine ethanol	AS-311-O5-ENRRMN *(being revised)*	I
9	Alcoholic strength by volume as % (AVS)	AS-312-01-TALVOL	I
10	Isotope ratio $^{13}C/^{12}C$ of ethanol in wine	AS-312-06-ETHANO	II
11	Total acidity	AS-313-01-ACITOT	I
12	Volatile acidity	AS-313-02-ACIVOL	I
13	Citric acid	AS-313-09-ACIENZ	II
14	Sorbic acid	AS-313-14-ACISOR	IV
15	Must pH	AS-313-15-PH	I
16	Ascorbic acid	AS- 313-22 ACASCO	II
17	CO_2 in g/l	AS-314-01-DIOCAR	II
18	CO_2 in g/l (manometry)	AS-314-04-CO2MAN	II
19	CO_2 overpressure	AS-314-02-SURPRES	I
20	Lysozyme	AS-315-14-LYSOZY	IV
21	Potassium sulphate	AS-321-05-SULFAT	II
22	Iron	AS-322-05-FER	IV
23	Copper	AS-322-06-CUIVRE	IV
24	Total Sulphites (SO_2) or Sulphur dioxide	AS-323-04-DIOSU	II

The description of some methods of analysis is being updated by the OIV. These descriptions will be published in the next Commission notice as soon as an updated text is published by the OIV in the 2010 edition of the Compendium of International Methods of Analysis of the OIV.

ANNEX

TABLE OF CONTENTS

1 DENSITY AT 20 °C AND SPECIFIC GRAVITY AT 20 °C (OIV-AS2-01-MASVOL) — CATEGORY I METHOD

1. DEFINITIONS

Density is the mass per unit volume of wine or must at 20 °C. It is expressed in grams per millilitre and denoted by the symbol $\rho_{20\ °C}$.

Specific gravity at 20 °C (or 20 °C/20 °C relative density) is the ratio, expressed as a decimal number, of the density of a given volume of wine or must at 20 °C to the density of the same volume of water at the same temperature, and is denoted by the symbol $d^{20\ °C}_{20\ °C}$

2. PRINCIPLE OF THE METHODS

The density and specific gravity at 20 °C are measured on a test sample:

either by pycnometry: reference method,

or by densimetry using a hydrostatic balance or by electronic densimetry.

Note

For very accurate measurement, the density must be corrected for the effect of sulphur dioxide.

$\rho_{20\ °C} = \rho'_{20\ °C} - 0{,}0006 \times S$

$\rho_{20\ °C}$ = corrected density

$\rho'_{20\ °C}$ = observed density

S = total sulphur dioxide in g/l

3. PRELIMINARY TREATMENT OF SAMPLE

If the wine or must contains appreciable quantities of carbon dioxide, remove most of it by stirring 250 ml of the wine in a 1-litre flask or by filtration under reduced pressure through 2 g of cotton wool placed in an extension tube.

4. REFERENCE METHOD

4.1. **Apparatus**

Normal laboratory equipment, and in particular:

4.1.1. A Pyrex glass pycnometer [1], of approximately 100 ml capacity, with a removable ground-glass jointed thermometer calibrated in tenths of a degree from 10 to 30 °C (Figure 1). The thermometer must be standardised.

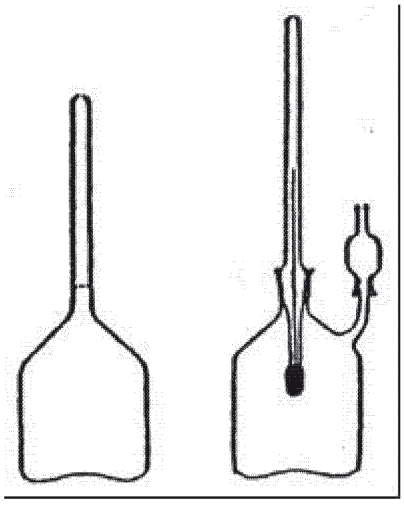

Figure 1

Pycnometer with tare flask

The pycnometer has a side tube 25 mm in length and 1 mm (maximum) in internal diameter, ending in a conical ground joint. This side tube may be capped by a 'reservoir stopper' consisting of a conically tapered ground-glass joint. This stopper serves as an expansion chamber.

[1] Any pycnometer with equivalent properties may be used.

The two ground joints of the apparatus should be prepared with very great care.

4.1.2. A tare flask, consisting of a vessel with the same outside volume (to within less than 1 ml) as the pycnometer and with a mass equal to the mass of the pycnometer filled with a liquid of specific gravity 1,01 (sodium chloride solution 2,0 % m/v).

A thermally insulated container, exactly fitting the body of the pycnometer.

4.1.3. A twin-pan balance with a range of at least 300 g and a sensitivity of 0,1 mg,

or,

a single-pan balance with a range of at least 200 g and a sensitivity of 0,1 mg.

4.2. **Calibration of pycnometer**

Calibration of the pycnometer involves a determination of the following quantities:

— tare of the empty pycnometer,

— volume of the pycnometer at 20 °C,

— mass of the water-filled pycnometer at 20 °C.

4.2.1. *Method using a twin-pan balance*

Place the tare flask on the left-hand pan of the balance and the clean, dry pycnometer, fitted with its 'reservoir stopper', on the right-hand pan. Add weights to the pan holding the pycnometer and record the weight required to establish equilibrium: let it be p grams.

Carefully fill the pycnometer with distilled water at the ambient temperature and fit the thermometer; carefully wipe the pycnometer dry and place it in the thermally insulated container. Mix it by inverting the container until the temperature reading on the thermometer is constant. Accurately adjust the level to the upper rim of the side tube. Wipe the side tube dry and put the reservoir stopper on; read the temperature t °C carefully, correcting it for the inaccuracy in the temperature scale if necessary. Weigh the water-filled pycnometer against the tare and record the weight p′ in grams required to establish equilibrium.

Calculation

Taring of empty pycnometer:

tare of empty pycnometer = p + m

m = mass of air contained in the pycnometer

m = 0,0012 (p − p′).

Volume at 20 °C:

$V_{20\ °C} = (p + m - p') \times F_t$

where F_t = factor taken from Table I for the temperature t °C

$V_{20\ °C}$ should be calculated to within ± 0,001 ml.

Mass of water at 20 °C:

$M_{20\ °C} = V_{20\ °C} \times 0{,}998203$

0,998203 = the density of water at 20 °C.

4.2.2. *Method using a single-pan balance*

Determine:

— the mass of the clean and dry pycnometer: let this be P,

— the mass of the pycnometer filled with water at t °C: following the procedure described in 4.2.1 above: let this be P_1

— the mass of the tare: T_0

Calculation

Taring of empty pycnometer:

tare of empty pycnometer = P − m,

where m = mass of air contained in the pycnometer,

$m = 0{,}0012\ (P_1 - P)$

Volume at 20 °C:

$V_{20\ °C} = [P_1 - (P - m)] \times F_t$

where F_t = factor taken from Table I for the temperature t °C.

The volume at 20 °C should be known to within ± 0,001 ml.

Mass of water at 20 °C:

$M_{20\ °C} = V_{20\ °C} \times 0{,}998203$

0,998203 = the density of water at 20 °C.

4.3. **Measurement method**

4.3.1. *Method using a twin-pan balance*

Fill the pycnometer with the prepared test sample (point 3) and weigh, following the procedure described in 4.2.1 above.

Let p″ be the weight in grams required to establish equilibrium at t °C.

Mass of liquid contained in the pycnometer = p + m − p″

Apparent density at t °C:

$\rho_{t\ °C} = (p + m - p'')/(V_{20\ °C})$

Calculate the density at 20 °C using one of the correction tables given later, in accordance with the nature of the liquid being measured: dry wine (Table II), natural or concentrated must (Table III), sweet wine (Table IV).

The 20 °C/20 °C specific gravity of the wine is calculated by dividing its density at 20 °C by 0,998203.

4.3.2. *Method using a single-pan balance*

Weigh the tare flask and let its mass be T_1.

Calculate $dT = T_1 - T_o$

Mass of the empty pycnometer at the time of measurement = P − m + dT

Fill the pycnometer with the prepared test sample (point 3) and weigh, following the procedure described in 4.2.1 above. Let its mass at t °C be P_2.

Mass of liquid contained in pycnometer at t °C = $P_2 - (P - m + dT)$

Apparent density at t °C:

$\rho_{t\ °C} = (P_2 - (P - m + dT)/(V_{20\ °C})$

Calculate the density at 20 °C of the liquid under test: (dry wine, natural or concentrated must, or sweet wine) as indicated in 4.3.1 above.

The 20 °C/20 °C specific gravity is calculated by dividing the density at 20 °C by 0,998203.

4.3.3. The repeatability of the density measurements

for dry and semi-sweet wines: r = 0,00010

and for sweet wines: r = 0,00018

4.3.4. The repeatability of the density measurements:

for dry and semi-sweet wines: R = 0,00037

and for sweet wines: R = 0,00045

TABLE I

F Factors

by which the mass of water contained in the Pyrex pycnometer at t °C has to be multiplied in order to calculate the pycnometer volume at 20 °C

[Please refer to Table I of Annex II of the AS2 - 01 method described in the International Compendium of methods of analysis of the OIV]

TABLE II

Temperature corrections c to the density of alcohol-free dry wines measured with a Pyrex glass pycnometer at t °C to relate the result to 20 °C

[Please refer to Table II of Annex II of the AS2 - 01 method described in the International Compendium of methods of analysis of the OIV]

$\rho_{20} = \rho_t \pm (c)/(1\ 000)$ – if t °C is less than 20 °C
+ if t °C is more than 20 °C

TABLE III

Temperature corrections c to the density of natural musts and of concentrated musts measured with a Pyrex glass pycnometer at t °C to relate the result to 20 °C

[Please refer to Table III of Annex II of the AS2 - 01 method described in the International Compendium of methods of analysis of the OIV]

$\rho_{20} = \rho_t \pm (c)/(1\ 000)$ – if t °C is less than 20 °C
+ if t °C is more than 20 °C

TABLE IV

Temperature corrections c to the density of wines of 13 % vol and above containing residual sugar measured with a Pyrex glass pycnometer at t °C to relate the result to 20 °C

[Please refer to Table IV of Annex II of the AS2 - 01 method described in the International Compendium of methods of analysis of the OIV]

$\rho_{20} = \rho_t \pm (c)/(1\ 000)$ – if t °C is less than 20 °C
+ if t °C is more than 20 °C

2 EVALUATION BY REFRACTOMETRY OF THE SUGAR CONCENTRATION IN GRAPE MUSTS, CONCENTRATED GRAPE MUSTS AND RECTIFIED CONCENTRATED GRAPE MUSTS (OIV-AS2-02-SUCREF) — CATEGORY I METHOD

1. PRINCIPLE OF THE METHOD

The refractive index at 20 °C, expressed either as an absolute value or as a percentage by mass of sucrose, is given in the appropriate table to provide a means of obtaining the sugar concentration in grams per litre and in grams per kilogram for grape musts, concentrated grape musts and rectified concentrated grape musts.

2. APPARATUS

2.1. **Abbé refractometer**

The refractometer used must be fitted with a scale giving:

— either percentage by mass of sucrose to 0,1 %,

— or refractive indices to four decimal places.

The refractometer must be equipped with a thermometer having a scale extending at least from + 15 °C to + 25 °C and with an arrangement for circulating water enabling measurements to be made at a temperature of 20 °C ± 5 °C.

The operating instructions for this instrument must be strictly adhered to, particularly with regard to calibration and the light source.

3. PREPARATION OF THE SAMPLE

3.1. **Must and concentrated must**

Pass the must, if necessary, through a dry gauze folded into four and, after discarding the first drops of the filtrate, carry out the determination on the filtered product.

3.2. **Rectified concentrated must**

Depending on the concentration, use either the rectified concentrated must itself or a solution obtained by making up 200 g of rectified concentrated must to 500 g with water, all weighings being carried out accurately.

4. PROCEDURE

Bring the sample to a temperature close to 20 °C. Place a small test sample on the lower prism of the refractometer, taking care (because the prisms are pressed firmly against each other) that this test sample covers the glass surface uniformly. Carry out the measurement in accordance with the operating instructions of the instrument used.

Read off the percentage by mass of sucrose to within 0,1 % or read the refractive index to four decimal places.

Carry out at least two determinations on the same prepared sample. Note the temperature t °C.

5. CALCULATION

5.1. **Temperature correction**

5.1.1. Instruments graduated in percentage by mass of sucrose: use Table I to obtain the temperature correction.

5.1.2. Instruments graduated in refractive index: find the index measured at t °C in Table II to obtain (column 1) the corresponding value of the percentage by mass of sucrose at t °C. This value is corrected for temperature and expressed as a concentration at 20 °C by means of Table I.

5.2. **Sugar concentration in must and concentrated must**

Find the percentage by mass of sucrose at 20 °C in Table II and read from the same row the sugar concentration in grams per litre and grams per kilogram. The sugar concentration is expressed in terms of invert sugar to one decimal place.

5.3. **Sugar concentration in rectified concentrated must**

Find the percentage by mass of sucrose at 20 °C in Table III and read from the same row the sugar concentration in grams per litre and grams per kilogram. The sugar concentration is expressed in terms of invert sugar to one decimal place.

If the measurement was made on diluted rectified concentrated must, multiply the result by the dilution factor.

5.4. **Refractive index of must, concentrated must and rectified concentrated must**

Find the percentage by mass of sucrose at 20 °C in Table II and read from the same row the refractive index at 20 °C. This index is expressed to four decimal places.

Note: The alcoholic strength by volume of must, concentrated must and rectified concentrated must can be obtained by using the corresponding table shown in Annex I to Commission Regulation (EC) No 1623/2000 of 25 July 2000 (OJEC L 194 of 31 July 2000)

TABLE I

Correction to be made when the percentage by mass of sucrose has been determined at a temperature different from 20 °C.

[Please refer to Table I of the Annex of the AS2 - 02 method described in the International Compendium of methods of analysis of the OIV]

TABLE II

Table giving the sugar concentration (4) in must and concentrated must in grams per litre and grams per kilogram, determined by means of a refractometer graduated either in percentage by mass of sucrose at 20 °C or in refractive index at 20 °C. The density at 20 °C is also given.

[Please refer to Table II of the Annex of the AS2 - 02 method described in the International Compendium of methods of analysis of the OIV]

TABLE III

Table giving the sugar (5) concentration in rectified concentrated must in grams per litre and grams per kilogram, determined by means of a refractometer graduated either in percentage by mass of sucrose at 20 °C or in refractive index at 20 °C. The density at 20 °C is also given.

[Please refer to Table III of the Annex of the AS2 - 02 method described in the International Compendium of methods of analysis of the OIV]

3 TOTAL DRY MATTER (OIV-AS-2-03-EXTSEC) TOTAL DRY MATTER — CATEGORY I METHOD

1. DEFINITION

The total dry matter includes all matter which is non-volatile under specified physical conditions. These physical conditions must be such that the substances forming this matter undergo as little alteration as possible while the test is being carried out.

The sugar-free extract is the difference between the total dry matter and the total sugars.

The reduced matter is the difference between the total dry matter and the total sugars in excess of 1 g/l, potassium sulphate in excess of 1 g/l, any mannitol present and any other chemical substances which may have been added to the wine.

The residual matter is the sugar-free extract less the fixed acidity expressed as tartaric acid.

The matter is expressed in grams per litre and it should be determined to within the nearest 0,5 g.

2. PRINCIPLE OF THE METHOD

[The description of this method of analysis is being updated by the OIV. It will be published in the next Commission notice as soon as an updated text is published by the International Organisation of Vine and Wine in the 2010 edition of the Compendium of International Methods of Analysis of the OIV. By way of illustration, while waiting for this publication, reference may be made to Chapter 4 of the Annex to Commission Regulation (EEC) No 2676/90.]

4 DETERMINATION OF THE ISOTOPIC RATIO $^{18}O/^{16}O$ OF THE WATER IN WINE (OIV-AS-2-09-MOUO18) — CATEGORY II METHOD

(For the record)

[The description of this method of analysis is being updated by the OIV. It will be published in the next Commission notice as soon as an updated text is published by the OIV in the 2010 edition of the Compendium of International Methods of Analysis of the OIV. By way of illustration, while waiting for this publication, reference may be made to Chapter 43 of the Annex to Commission Regulation (EEC) No 2676/90.]

5 FOLIN-CIOCALTEU INDEX (OIV-AS-2-10-INDFOL) — CATEGORY IV METHOD

1. DEFINITION

The Folin-Ciocalteu index is the result obtained using the method described below.

2. PRINCIPLE OF THE METHOD

All the phenolic compounds contained in the wine are oxidised by the Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) which, after oxidation of the phenols, is reduced to a mixture of the blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}).

The blue coloration produced has a maximum absorption in the region of 750 nm, and it is proportional to the total quantity of phenolic compounds originally present.

3. REAGENTS

These must be of analytical reagent quality. The water used must be distilled or water of equivalent purity.

3.1. **Folin-Ciocalteu reagent**

This reagent is available commercially in a form ready for use. It may be prepared as follows: dissolve 100 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) and 25 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) in 700 ml of distilled water. Add 50 ml of 85 % phosphoric acid (ρ_{20} = 1,71 g/ml) and 100 ml of concentrated hydrochloric acid (ρ_{20} = 1,19 g/ml). Bring to the boil and boil for 10 hours under reflux conditions. Then add 150 g of lithium sulphate ($Li_2SO_4 \cdot H_2O$) and a few drops of bromine and boil once more for 15 minutes. Allow to cool and make up to one litre with distilled water.

3.2. Anhydrous sodium carbonate, Na_2CO_3, made up into a 20 % m/v solution.

4. APPARATUS

Normal laboratory apparatus, particularly:

4.1. 100 ml volumetric flasks.

4.2. Spectrophotometer capable of operating at 750 nm.

5. PROCEDURE

5.1. **Red wine**

Introduce the following into a 100 ml volumetric flask (4.1) strictly in the order given:

1 ml of the wine, previously diluted 1:5

50 ml of distilled water

5 ml of Folin-Ciocalteu reagent (3.1)

20 ml of sodium carbonate solution (3.2)

Make up to 100 ml with distilled water.

Stir to homogenise. Wait 30 minutes for the reaction to stabilise. Determine the absorbance at 750 nm through a path length of 1 cm with respect to a blank prepared with distilled water in place of the wine.

If the absorbance is not around 0,3 an appropriate dilution should be made.

5.2. **White wine**

Carry out the same procedure with 1 ml of undiluted wine.

5.3. **Rectified concentrated must**

5.3.1. *Preparation of sample*

Use the solution with a sugar concentration of 25 % (m/m) (25° Brix) prepared as described in the Chapter 'pH', section 4.1.2.

5.3.2. *Measurement*

Proceed as described for the case of red wine (5.1) using a 5 ml sample prepared as described in 5.3.1 and measuring the absorbance with respect to a control prepared with 5 ml of a 25 % (m/m) invert sugar solution.

6. EXPRESSION OF RESULTS

6.1. **Method of calculation**

The result is expressed in the form of an index obtained by multiplying the absorbance by 100 for red wines diluted 1:5 (or by the corresponding factor for other dilutions) and by 20 for white wines. For rectified concentrated musts, multiply by 16.

6.2. **Repeatability**

The difference between the results of two determinations carried out simultaneously or very quickly one after the other by the same analyst must not be greater than 1.

Good repeatability of results is achieved by using scrupulously clean apparatus (volumetric flasks and spectrophotometer cells).

6 GLUCOSE AND FRUCTOSE (OIV-AS-311-02-GLUFRU) — CATEGORY II METHOD

1. DEFINITION

Glucose and fructose may be determined individually by an enzymatic method, with the sole aim of calculating the glucose/fructose ratio.

2. PRINCIPLE OF THE METHOD

Glucose and fructose are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK), and produce glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P):

glucose + ATP → G6P + ADP

fructose + ATP → F6P + ADP

The glucose 6-phosphate is first oxidised to gluconate 6-phosphate by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose 6-phosphate dehydrogenase (G6PDH). The quantity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced corresponds to that of glucose 6-phosphate and thus to that of glucose.

G6P + $NADP^+$ → gluconate 6-phosphate + NADPH + H^+

The reduced nicotinamide adenine dinucleotide phosphate is determined from its absorption at 340 nm.

At the end of this reaction, the fructose 6-phosphate is converted into glucose 6-phosphate by the action of phosphoglucose isomerase (PGI):

F6P → G6P

The glucose 6-phosphate again reacts with the nicotinamide adenine dinucleotide phosphate to give gluconate 6-phosphate and reduced nicotinamide adenine dinucleotide phosphate, and the latter is then determined.

3. APPARATUS

— A spectrophotometer enabling measurements to be made at 340 nm, the wavelength at which absorption by NADPH is at a maximum. Absolute measurements are involved (i.e. calibration plots are not used but standardization is made using the extinction coefficient of NADPH), so that the wavelength scales of and absorbance values obtained from the apparatus must be checked.

  If not available, a spectrophotometer using a source with a discontinuous spectrum which enables measurements to be made at 334 nm or at 365 nm may be used.

— Glass cells with optical path lengths of 1 cm or single-use cells.

— Pipettes for use with enzymatic test solutions, 0,02 — 0,05 — 0,1 — 0,2 ml.

4. REAGENTS

4.1. **Solution 1:** buffer solution (0,3 M triethanolamine, pH 7,6, 4×10^{-3} M in Mg^{2+}): dissolve 11,2 g triethanolamine hydrochloride ($(C_2H_5)_3N$, HCl) and 0,2 g Mg SO_4, $7H_2O$ in 150 ml of doubly distilled water, add about 4 ml of 5 M sodium hydroxide (NaOH) solution to obtain a pH value of 7,6 and make up to 200 ml.

This buffer solution may be kept for four weeks at +4 °C.

4.2. **Solution 2:** nicotinamide adenine dinucleotide phosphate solution (about $11,5 \times 10^{-3}$ M): dissolve 50 mg disodium nicotinamide adenine dinucleotide phosphate in 5 ml of doubly distilled water.

This solution may be kept for four weeks at +4 °C.

4.3. **Solution 3:** adenosine 5′-triphosphate solution (about 81×10^{-3} M): dissolve 250 mg disodium adenosine 5′-triphosphate and 250 mg sodium hydrogen carbonate ($NaHCO_3$) in 5 ml of doubly distilled water.

This solution may be kept for four weeks at + 4 °C.

4.4. **Solution 4:** hexokinase/glucose 6-phosphate dehydrogenase: mix 0,5 ml hexokinase (2 mg of protein/ml or 280 U/ml) with 0,5 ml glucose 6-phosphate dehydrogenase (1 mg of protein/ml).

This solution may be kept for a year at + 4 °C.

4.5. **Solution 5:** phosphoglucose isomerase (2 mg of protein/ml or 700 U/ml). The suspension is used undiluted.

The solution may be kept for a year at + 4 °C.

Note:

All solutions used above are available commercially.

5. PROCEDURE

5.1. **Preparation of sample**

Depending on the estimated amount of glucose + fructose per litre, dilute the sample as follows:

Measurement at 340 and 334 nm	365 nm	Dilution with water	Dilution factor F
Up to 0,4 g/l	0,8 g/l	—	—
Up to 4,0 g/l	8,0 g/l	1 + 9	10
Up to 10,0 g/l	20,0 g/l	1 + 24	25
Up to 20,0 g/l	40,0 /l	1 + 49	50
Up to 40,0 g/l	80,0 g/l	1 + 99	100
Above 40,0 g/l	80,0 g/l	1 + 999	1 000

5.2. **Determination**

With the spectrophotometer adjusted to the 340 nm wavelength, make measurements using air (no cell in the optical path) or water as reference.

Temperature between 20 and 25 °C.

Into two cells with 1 cm optical paths, place the following:

	Reference cell	Determination
Solution 1 (4.1) (taken to 20 °C)	2,50 ml	2,50 ml
Solution 2 (4.2)	0,10 ml	0,10 ml
Solution 3 (4.3)	0,10 ml	0,10 ml
Sample to be measured		0,20 ml
Doubly distilled water	0,20 ml	

Mix, and after about three minutes read off the absorbence of the solutions (A1). Start the reaction by adding:

Solution 4 (4.4)	0,02 ml	0,02 ml

Mix; wait 15 minutes; read off the absorbence and check that the reaction has stopped after a further two minutes (A2).

Add immediately:

Solution 5 (4.5)	0,02 ml	0,02 ml

Mix; read off the absorbence after 10 minutes and check that the reaction has stopped after a further two minutes (A3).

Calculate the differences in the absorbences:

A2 — A1 corresponding to glucose

A3 — A2 corresponding to fructose

for the reference and sample cells.

Calculate the differences in absorbence for the reference cell (ΔA_T) and the sample cell (ΔA_D) and then obtain:

for glucose: $\Delta A_G = \Delta A_D - \Delta A_T$

for fructose: $\Delta A_F = \Delta A_D - \Delta A_T$

Note:

The time needed for the completion of enzyme activity may vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

5.3. **Expression of results**

5.3.1. *Calculation*

The general formula for calculating the concentrations is:

$C = (V \times PM)/(\varepsilon \times d \times v \times 1\,000) \times \Delta A$ (g/l) where

V = volume of the test solution (ml)

v = volume of the sample (ml)

PM = molecular mass of the substance to be determined

d = optical path in the cell (cm)

ε = absorption coefficient of NADPH at 340 nm = 6,3 $mmole^{-1} \times l \times cm^{-1}$)

and V = 2,92 ml for the determination of glucose

V = 2,94 ml for the determination of fructose

v = 0,20 ml

PM = 180

d = 1.

so that:

For glucose: $Cg/l = 0{,}417 \times \Delta A_G$

For fructose: $Cg/l = 0{,}420 \times \Delta A_F$

If the sample was diluted during its preparation, multiply the result by the dilution factor F.

Note:

If the measurements are made at 334 or 365 nm, then the following expressions are obtained:

— measurement at 334 nm: $\varepsilon = 6{,}2$ ($mmole^{-1} \times l \times cm^{-1}$)

For glucose: $Cg/l = 0{,}425 \times \Delta A_G$

For fructose: $Cg/l = 0{,}428 \times \Delta A_F$

— measurement at 365 nm: $\varepsilon = 3{,}4$ ($mmole^{-1} \times l \times cm^{-1}$)

For glucose: $Cg/l = 0{,}773 \times \Delta A_G$

For fructose: $Cg/l = 0{,}778 \times \Delta A_F$

5.3.2. *Repeatability (r)*

$r = 0{,}056\ x_i$

5.3.3. *Reproducibility (R)*

$R = 0{,}12 + 0{,}076\ x_i$

x_i = concentration of glucose or fructose in g/l.

7 **DETERMINING SUGARS USING HPLC (SUCROSE) (OIV-AS-311-03-SUCRES) — CATEGORY II METHOD**

(p.m.)

[The description of this method of analysis is being updated by the OIV. It will be published in the next Commission notice as soon as an updated text is published by the OIV in the 2010 edition of the Compendium of International Methods of Analysis of the OIV. By way of illustration, while waiting for this publication, reference may be made to Chapter 6 of the Annex to Commission Regulation (EEC) No 2676/90.]

8 **DETECTING ENRICHMENT OF GRAPE MUSTS, CONCENTRATED GRAPE MUSTS, RECTIFIED CONCENTRATED GRAPE MUSTS AND WINES BY APPLICATION OF NUCLEAR MAGNETIC RESONANCE OF DEUTERIUM (OIV-AS-311-05-ENRRMN) — CATEGORY I METHOD**

(p.m.)

[The description of this method of analysis is being updated by the scientific departments of the OIV. It will be published in a Communication notice as soon as a definitive text has been adopted by the General Assembly of the OIV. By way of illustration, while waiting for this publication, reference may be made to Chapter 8 of the Annex to Commission Regulation (EEC) No 2676/90.]

9 **ALCOHOLIC STRENGTH BY VOLUME (OIV-AS-312-01-TALVOL) — CATEGORY I METHODS**

(p.m.)

[The description of these methods of analysis are being updated by the OIV. They will be published in the next Commission notice as soon as an updated text is published by the OIV in the 2010 edition of the Compendium of International Methods of Analysis of the OIV. By way of illustration, while waiting for this publication, reference may be made to Chapter 3 of the Annex to Commission Regulation (EEC) No 2676/90.]

10 DETERMINATION OF THE ISOTOPE RATIO $^{13}C/^{12}C$ BY MEANS OF SPECTOMETRY OF THE ISOTOPIC MASS OF THE ETHANOL IN WINE OR THE ETHANOL OBTAINED BY MEANS OF THE FERMENTATION OF MUSTS, CONCENTRATED MUSTS OR RECTIFIED CONCENTRATED MUSTS (OIV-AS-312-06-ETHANO) — CATEGORY II METHOD

1. FIELD OF APPLICATION

The method permits the measurement of the isotope ratio $^{13}C/^{12}C$ of ethanol in wine and that of ethanol obtained after fermentation of products derived from the vine (must, concentrated must, rectified concentrated must).

2. STANDARDIZING REFERENCES

ISO:	5725:1994 'Accuracy (trueness and precision) of measurement methods and results Determination of repeatability and reproducibility of a standard measurement method by means of a collaborative interlaboratory experiment'.
V-PDB:	Vienna-Pee-Dee Belemnite (R_{PDB} = 0,0112372).
OIV AS-311-O5-ENRRMN method	'Detecting the enrichment of musts, concentrated musts, rectified concentrated musts and wines by application of nuclear magnetic resonance of deuterium (RMN-FINS)'.

3. TERMS AND DEFINITIONS

$^{13}C/^{12}C$:	Ratio of carbon isotopes 13 (^{13}C) and of carbon 12 (^{12}C) for a given sample.
$\delta^{13}C$ C:	carbon content 13 (^{13}C) expressed in parts per thousand (‰).
RMN-FINS:	Specific natural isotope splitting studied by means of nuclear magnetic resonance.
V-PDB:	Vienna-Pee-Dee Belemnite. The PDB, the primary reference for the measurement of natural variations in isotope carbon 13 content, was a calcium carbonate stemming from a belemnite rostrum of the Cretacious of the Pee-Dee formation of South Carolina (United States of America). Its isotope ratio $^{13}C/^{12}C$ or R_{PDB} is R_{PDB} = 0,0112372. The PDB was exhausted long ago, but has remained the primary reference for expressing the natural variations in isotope carbon 13 contents, against which the available reference materials are calibrated, available to the International Atomic Energy Agency (IAEA) in Vienna (Austria). The isotope determinations of natural carbon 13 ratios are therefore expressed, by convention, in relation to V-PDB.
m/z:	Mass-to-charge-ratio.

4. PRINCIPLE

During photosynthesis, the assimilation of carbon dioxide by plants takes place in accordance with two principle types of metabolism which are the metabolisms C_3 (Calvin cycle) and C_4 (Hatch and Slack). These two mechanisms of photosynthesis present different isotope fractionation. Thus, products from C_4 plants, such as sugars and alcohol derived from fermentation, present carbon 13 contents higher than those of their homologues from C_3 plants. The majority of plants such as the vine and the beet belong to group C_3. Sugar cane and maize belong to group C_4. Measurement of the carbon 13 content thus permits the detection and evaluation of C_4 sugar (cane sugar or maize isoglucose) added to products derived from the grape (grape musts, wines, etc.). The combined information of the carbon 13 content with that obtained by RMN-FINS also permits the quantification and addition of sugar mixtures or alcohols of C_3 and C_4 plant origin.

The carbon 13 content is determined by the carbon dioxide resulting from the complete combustion of the sample. The ratios of the isotopomeric principles 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$) and 46 ($^{12}C^{16}O^{18}O$), resulting from possible different combinations of ^{18}O, ^{17}O, ^{16}O, ^{13}C and ^{12}C isotopes, are determined on the basis of ionic currents measured on three different collectors of an isotopic mass spectrometer. The contributions of the isotopomers $^{13}C^{17}O^{16}O$ et $^{12}C^{17}O_2$ may be disregarded on account of their

weak ratio. The ionic current for m/z = 45 is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is calculated as a function of the intensity of the current measured for m/z = 46 taking into consideration the relative ratios of ^{18}O and ^{17}O (Craig correction). Comparison with a reference calibrated against the international reference V-PDB permits the calculation of the carbon 13 content on the $\delta^{13}C$ relative scale.

5. REAGENTS

The materials and consumables depend on the apparatus (6) used by the laboratory. The systems generally used are those based on the elementary analyser. This may be equipped to introduce samples placed in grouted metallic capsules, or to inject liquid samples via a septum using a syringe.

Depending on the type of instruments used, the following reference materials, reagents and consumables can be used:

— reference materials

— available from the IAEA:

Name	Material	δ^{13} C versus V-PDB (9)
— IAEA-CH-6	saccharose	– 10,4 ‰
— IAEA-CH-7	polyethylene	– 31,8 ‰
— NBS22	oil	– 29,7 ‰
— USGS24	graphite	– 16,1 ‰

— available from the Institute for Reference Materials and Measurements (IRMM) in Geel (BE):

Name	Material	δ^{13}C versus V-PDB (9)
— CRM/BCR 656	wine alcohol	– 26,93 ‰
— CRM/BCR 657	glucose	– 10,75 ‰
— CRM/BCR 660	Hydro-alcoholic solution (AVS 12 %)	– 26,72 ‰

— Standard work sample with a known ratio $^{13}C/^{12}C$ calibrated against international reference materials.

— The indicative list of consumables below is drawn up for continuous-flow systems:

  — helium for analysis (CAS 07440-59-7),

  — oxygen for analysis (CAS 07782-44-7),

  — carbon dioxide for analysis, used as a secondary reference gas for the carbon 13 content (CAS 00124-38-9),

  — oxidation reagent for the oven of the combustion system, for example, copper oxide (II) for elementary analysis (CAS 1317-38-0),

  — desiccant to eliminate water produced by combustion, for example, anhydrone for elementary analysis (magnesium perchlorate) (CAS 10034-81-8) (not necessary for apparatus equipped with a water elimination system using cryotrapping or a selectively permeable capillary).

6. APPARATUS AND MATERIAL

6.1. **Mass spectrometer for determining isotope ratios (SMRI)**

Mass spectrometer for determining isotope ratios (SMRI), permitting determination of the relative ^{13}C content of CO_2 gas in natural ratio with an internal accuracy of 0,05 ‰ or better expressed as a relative value (point 9). The internal accuracy is here defined as the difference between two measurements of the same sample of CO_2. The mass spectrometer, intended to measure isotope ratios, is generally equipped with a triple collector to simultaneously measure the intensities for m/z = 44, 45 and 46. The mass spectrometer for determining isotope ratios must either be equipped with a double introduction system, to measure alternately the known sample and a reference sample, or use an integrated system which performs quantitative combustion of samples and separates the carbon dioxide from other combustion products prior to measurement in the mass spectrometer.

6.2. **Combustion apparatus**

Combustion apparatus capable of quantitatively converting ethanol into carbon dioxide and of eliminating all other products of combustion including water without any isotope fractionation. The apparatus may be either a continuous-flow system integrated into the instrumentation of mass spectrometry (point 6.2.1), or an autonomous combustion system (point 6.2.2). The apparatus must permit an accuracy at least equivalent to that indicated in point 11 to be obtained.

6.2.1. *Continuous-flow systems*

These comprise either an elementary analyser or a gas chromatograph equipped with an in-line combustion system.

For systems equipped for the introduction of samples contained in metal capsules, the following laboratory material is used:

— microsyringe or volumetric micropipette with appropriate cones,

— balance graded to 1 µg or better,

— clip for encapsulation,

— tin capsules for liquid samples,

— tin capsules for solid samples,

Note:

to limit the risks of evaporation of samples of ethanol, it is possible to place an absorbent material in the capsules (for example, *chromosorb W 45-60 mesh*) which has been examined previously by means of a plain measurement to check that it does not contain a significant quantity of carbon likely to alter the measurements.

Where use is made of an elementary analyser equipped with an injector for liquids or in the case of a chromatography combustion preparation system, the following laboratory material is used:

— syringe for liquids,

— bottles with a waterproof closing system and inert septa.

The laboratory materials indicated in the lists above are examples and are liable to be replaced by other materials with an equivalent performance according to the type of combustion apparatus and mass spectrometry used by the laboratory.

6.2.2. *Autonomous preparation systems*

In this case, the carbon dioxide samples resulting from the combustion of analysis and reference samples are collected in ampoules which are then installed in the double entrance system of the spectrometer to perform the isotope analysis. Several types of combustion apparatus described in the literature can be used:

— closed combustion system filled with circulating oxygen gas,

— elementary analyser with helium and oxygen flux,

— sealed glass ampoule filled with copper oxide (II) as an oxidation agent.

7. PREPARATION OF TEST SAMPLES

Ethanol must be extracted from the wine before isotope determination. This extraction is performed by distilling the wine as described in point 3.1 of the RMN-FINS (OIV - MA-E-AS311-05-ENRRMN) method.

In the case of grape must, concentrated grape must and rectified concentrated grape must, the sugars must be fermented in ethanol first as described in point 3.2 of the RMN-FINS (OIV - MA-E-AS311-05-ENRRMN) method.

8. PROCEDURE

All the preparatory stages must be performed without any significant loss of ethanol through evaporation which would change the isotopic composition of the sample.

The following description refers to procedures generally used for the combustion of samples of ethanol by means of automated commercial combustion systems. Any other method, ensuring that the ethanol sample is quantitatively converted into carbon dioxide without any loss of ethanol through evaporation may be used for the preparation of carbon dioxide for isotopic analysis.

Experimental procedure based on the use of an elementary analyser:

(a) encapsulation of samples:

— use clean capsules, a clean grip and a clean preparation dish,

— take one capsule of a size appropriate to the grip,

— introduce the necessary volume of liquid into the capsule with the aid of the micropipette,

— *Note:*

3,84 mg of absolute ethanol or 4,17 mg of distillate with an alcoholic strength of 92 % m/m are necessary in order to obtain 2 mg of carbon. The appropriate quantity of distillate must be calculated in the same way according to the quantity of carbon necessary in relation to the sensitivity of the mass spectrometry instrumentation.

— close the capsule again with the aid of the grip,

— each capsule must be completely airtight when closed. Otherwise, it must be rejected and a new capsule must be prepared,

— for each sample, prepare two capsules,

— place the capsules in the appropriate place on the turntable of the automatic sample changer of the elementary analyser. Each capsule must be carefully identified by a serial number,

— place the capsules containing working references at the beginning and end of the series of samples.

— regularly insert control samples into the series of samples;

(b) control and adjustment of the elementary analysis and mass spectrometry instrumentation

— adjust the temperature of the elementary analyser ovens and the flux of helium and oxygen gas for optimum combustion of the sample,

— verify that there is no leakage in the elementary analysis and mass spectrometry system (for example, by checking the ionic current for m/z = 28 corresponding to N_2),

— adjust the mass spectrometer to measure the intensities of the ionic currents for m/z = 44, 45 and 46,

— verify the support system for known control samples before starting to measure the samples;

(c) sequence of a series of measurements

The samples placed on the automatic sample changer of the elementary analyser (or of the chromatograph) are successively introduced. The carbon dioxide of each sample combustion is eluted towards the mass spectrometer which measures the ionic currents. The computer interfaced with the instrumentation registers the intensities of the ionic currents and calculates the δ values for each sample (point 9).

9. CALCULATION

The aim of the method is to measure the isotopic ratio $^{13}C/^{12}C$ of the ethanol extracted from the wine or from products derived from grapes after fermentation. The isotopic ratio $^{13}C/^{12}C$ can be expressed by its variation in relation to a working reference. The isotopic carbon 13 variation ($\delta\ ^{13}C$) is thus calculated on a delta scale for a thousand (δ/1 000) by comparing the results obtained for the sample to be measured against those of the working reference previously calibrated in relation to the primary international reference (V-PDB). The $\delta^{13}C$ values are expressed in relation to the working reference in accordance with:

$$\delta^{13}C_{ech/ref}\ ‰ = 1\ 000 \times (R_{ech}-R_{ref})/R_{ref}$$

where R_{ech} and R_{ref} are respectively the isotopic ratios $^{13}C/^{12}C$ of the sample and those of the carbon dioxide used as a reference gas.

The $\delta^{13}C$ values are therefore expressed in relation to V-PDB in accordance with:

$$\delta^{13}C_{ech/V\text{-}PDB}\ ‰ = \delta^{13}C_{ech/ref} + \delta^{13}C_{ref/V\text{-}PDB} + (\delta^{13}C_{ech/ref} \times \delta^{13}C_{ref/V\text{-}PDB})/1\ 000$$

where $\delta^{13}C_{ref/V\text{-}PDB}$ is the isotopic variation previously determined for the working reference against the V-PDB.

During on-line measurement, small derivates due to the variation in instrumental conditions can be observed. In this case, the $\delta\ ^{13}C$ of the samples must be corrected in relation to the difference between the $\delta^{13}C$ value measured for the working reference and its real value, previously calibrated against the V-PDB by comparison with one of the international reference materials. Between two measurements of the working reference, the derivate, and thus the correction to apply to the sample results may be assumed to be linear. The working reference must be measured at the beginning and end of every series of samples. A correction may then be calculated for each sample by means of a linear interpolation.

10. QUALITY ASSURANCE AND CONTROL

Check that the ^{13}C value for the working reference does not differ by more than 0,5 ‰ from the admitted value. Otherwise, the settings of the spectrometer instrumentation must be checked and readjusted if necessary.

For each sample, verify that the difference in the result between the two capsules measured successively is less than 0,3 ‰. The final result for a given sample is therefore the mean value of the two capsules. If the variation is greater than 0,3 ‰, the measurement must be repeated.

An inspection of the correct functioning of the measurement may be based on the intensity of the ionic current for m/z = 44 which is proportional to the quantity of carbon injected into the elementary analyser. In type conditions, the intensity of this ionic current should be practically constant for analysis samples. A significant variation must give rise to the suspicion of ethanol evaporation (for example, an improperly sealed capsule) or instability in the elementary analyser or mass spectrometer.

11. METHOD PERFORMANCE CHARACTERISTICS (PRECISION)

An initial collaborative study (point 11.1) has been conducted on distillates containing alcohol of vinous origin, and alcohols from sugar cane and beet sugar as well as different mixtures of these three origins. As this study has not taken into consideration the distillation phase, supplementary information from other inter-laboratory tests performed on wines (point 11.2), and in particular, on aptitude test circuits (point 11.3), for isotopic measurements, have also been taken into account. The results show that the different distillation systems used in satisfactory conditions, and in particular, those applicable to the RMN-FINS measurements, do not result in significant variability for $\delta^{13}C$ determinations of ethanol in wine. The parameters of reliability observed for wines are almost identical to those obtained during the collaborative study (point 11.1) on distillates.

11.1. **Collaborative study on distillates**

Year of interlaboratory test: 1996

Number of laboratories: 20

Number of samples: 6 double-blind samples

Analyte: $\delta^{13}C$ of ethanol

Sample code	Alcohol of vinous origin	Alcohol from beet sugar	Alcohol from cane sugar
A & G	80 %	10 %	10 %
B & C	90 %	10 %	0 %
D & F	0 %	100 %	0 %
E & I	90 %	0 %	10 %
H & K	100 %	0 %	0 %
J & L	0 %	0 %	100 %

Samples	A/G	B/C	D/F	E/I	H/K	J/L
Number of laboratories retained after elimination of abnormal results	19	18	17	19	19	19
Number of results accepted	38	36	34	38	38	38
Mean value ($\delta^{13}C$) ‰	– 25,32	– 26,75	– 27,79	– 25,26	– 26,63	– 12,54
S_r^2	0,0064	0,0077	0,0031	0,0127	0,0069	0,0041
Deviation type of repeatability (S_r) ‰	0,08	0,09	0,06	0,11	0,08	0,06
Limit of repeatability r (2,8 × S_r) ‰	0,22	0,25	0,16	0,32	0,23	0,18
S_R^2	0,0389	0,0309	0,0382	0,0459	0,0316	0,0584
Deviation type of reproducibility (S_R) ‰	0,20	0,18	0,20	0,21	0,18	0,24
Limit of reproducibility R (2,8 × S_R) ‰	0,55	0,49	0,55	0,60	0,50	0,68

11.2. **Interlaboratory study on two wines and one alcohol**

Year of interlaboratory test: 1996

Number of laboratories: 14 for the distillation of wines of which 7 also for the $\delta^{13}C$ measurement of ethanol in wines, 8 for the $\delta^{13}C$ measurement of the sample of alcohol

Number of samples: 3 (white wine AVS 9,3 % vol., white wine AVS 9,6 % vol. and alcohol with a strength of 93 % m/m)

Analyte: $\delta^{13}C$ of ethanol

Samples	Red wine	White wine	Alcohol
Number of laboratories	7	7	8
Number of results accepted	7	7	8
Mean value ($\delta^{13}C$) ‰	– 26,20	– 26,20	– 25,08
Reproducibility variance S_R^2	0,0525	0,0740	0,0962
Deviation type of reproducibility (S_R) ‰	0,23	0,27	0,31
Limit of reproducibility R (2,8 × S_R) ‰	0,64	0,76	0,87

Different distillation systems have been used by the participating laboratories. The $\delta^{13}C$ isotopic determinations performed in a single laboratory on all the distillates returned by the participants reveal neither abnormal values nor values significantly distinct from the mean values. The variance in the results (S^2 = 0,0059) is comparable with the S_r^2 repeatability variances of the collaborative study on distillates (point 11.1).

11.3. **Results of the aptitude circuit exercises in isotopic tests**

Since December 1994, international aptitude exercises for isotopic determinations on wines and alcohols (distillates with an AVS of 96 % vol) have been organised regularly. The results permit participating laboratories to control the quality of their analyses. Statistical use of the results permits an assessment of the variability of determinations in conditions of reproducibility and thus an evaluation of the parameters of variance and limit of reproducibility. The results obtained for the $\delta^{13}C$ determinations of ethanol in wines and distillates are summarised in the following table:

Date	Wines				Distillates			
	N	S_R	S_R^2	R	N	S_R	S_R^2	R
December 1994	6	0,210	0,044	0,59	6	0,151	0,023	0,42
June 1995	8	0,133	0,018	0,37	8	0,147	0,021	0,41
December 1995	7	0,075	0,006	0,21	8	0,115	0,013	0,32
March 1996	9	0,249	0,062	0,70	11	0,278	0,077	0,78
June 1996	8	0,127	0,016	0,36	8	0,189	0,036	0,53
September 1996	10	0,147	0,022	0,41	11	0,224	0,050	0,63
December 1996	10	0,330	0,109	0,92	9	0,057	0,003	0,16
March 1997	10	0,069	0,005	0,19	8	0,059	0,003	0,16
June 1997	11	0,280	0,079	0,78	11	0,175	0,031	0,49
September 1997	12	0,237	0,056	0,66	11	0,203	0,041	0,57
December 1997	11	0,127	0,016	0,36	12	0,156	0,024	0,44
March 1998	12	0,285	0,081	0,80	13	0,245	0,060	0,69
June 1998	12	0,182	0,033	0,51	12	0,263	0,069	0,74
September 1998	11	0,264	0,070	0,74	12	0,327	0,107	0,91
Weighted mean		0,215	0,046	0,60		0,209	0,044	0,59

N: number of participating laboratories.

11.4. **Limits of repeatability and reproducibility**

The data from different interlaboratory exercises presented in the preceding tables thus permit the establishment for this method, also including the distillation stage, of the following repeatability and reproducibility limits:

Limit of repeatability r: 0,24

Limit of reproducibility R: 0,6.

11 TOTAL ACIDITY (OIV-AS-313-01-ACITOT) — CATEGORY I METHOD

1. DEFINITION

The total acidity of the wine is the sum of its titratable acidities when it is titrated to pH 7 against a standard alkaline solution.

Carbon dioxide is not included in the total acidity.

2. PRINCIPLE OF THE METHOD

Potentiometric titration or titration with bromothymol blue as an indicator and comparison with an end-point colour standard.

3. REAGENTS

3.1. Buffer solution pH 7,0:

Monopotassium phosphate KH_2PO_4: 107,3 g

1 M sodium hydroxide (NaOH) solution: 500 ml

Water to 1 000 ml.

Alternatively, ready made buffer solutions are available commercially.

3.2. 0,1 M sodium hydroxide (NaOH) solution.

3.3. 4 g/l bromothymol blue indicator solution.

Bromothymol blue ($C_{27}H_{28}Br_2O_5S$)	4 g
Neutral ethanol 96 % vol.	200 ml

Dissolve and add:

Water free of CO_2	200 ml

1 M sodium hydroxide solution

sufficient to produce blue-green colour (pH 7)	7,5 ml
Water to	1 000 ml

4. APPARATUS

4.1. Water vacuum pump.

4.2. 500 ml vacuum flask.

4.3. Potentiometer with scale graduated in pH values, and electrodes. The glass electrode must be kept in distilled water. The calomel/saturated potassium chloride electrode must be kept in a saturated potassium chloride solution. A combined electrode is most frequently used: it should be kept in distilled water.

4.4. Measuring cylinders 50 ml (wine), 100 ml (rectified concentrated must).

5. PROCEDURE

5.1. **Preparation of sample**

5.1.1. *Wines*

Elimination of carbon dioxide. Place about 50 ml of wine in a vacuum flask; apply vacuum to the flask with the water pump for one to two minutes, whilst shaking continuously.

5.1.2. *Rectified concentrated must*

Introduce 200 g of accurately weighed rectified concentrated must. Make up to the mark with 500 ml water. Homogenise.

5.2. **Potentiometric titration**

5.2.1. *Calibration of pH meter*

The pH meter is now calibrated for use at 20 °C, according to the manufacturer's instructions, with the pH 7,00 buffer solution at 20 °C.

5.2.2. *Method of measurement*

Into a measuring cylinder (4.4), introduce a volume of the sample, prepared as described in 5.1, equal to 10 ml in the case of wine and 50 ml in the case of rectified concentrated must. Add about 10 ml of distilled water and then add the 0,1 M sodium hydroxide solution (3.2) from the burette until the pH is equal to 7 at 20 °C. The sodium hydroxide must be added slowly and the solution stirred continuously. Let n ml be the volume of 0,1 M NaOH added.

5.3. **Titration with indicator (bromothymol blue)**

5.3.1. *Preliminary test: end-point colour determination*

Into a measuring cylinder (4.4) place 25 ml of boiled distilled water, 1 ml of bromothymol blue solution (3.3) and a volume prepared as in (5.1) equal to 10 ml in the case of wine and 50 ml in the case of rectified concentrated must. Add the 0,1 M sodium hydroxide solution (3.2) until the colour changes to blue-green. Then add 5 ml of the pH 7 buffer solution (3.1).

5.3.2. *Determination*

Into a measuring cylinder (4.4) place 30 ml of boiled distilled water, 1 ml of bromothymol blue solution (3.3) and a volume of the sample, prepared as described in (5.1), equal to 10 ml in the case of wine and 50 ml in the case of rectified concentrated must. Add 0,1 M sodium hydroxide solution (3.2) until the same colour is obtained as in the preliminary test above (5.3.1). Let n ml be the volume of 0,1 M sodium hydroxide added.

6. EXPRESSION OF RESULTS

6.1. **Method of calculation**

6.1.1. *Wines*

The total acidity expressed in milliequivalents per litre is given by:

$A = 10\ n$

It is recorded to one decimal place.

The total acidity expressed in grams of tartaric acid per litre is given by:

$A' = 0{,}075 \times A$

It is recorded to one decimal place.

6.1.2. *Rectified concentrated musts*

— The total acidity expressed in milliequivalents per kilogram of rectified concentrated must is given by $a = 5 \times n$

— The total acidity expressed in milliequivalents per kilogram of total sugars is given by:

$A = (500 \times n)/(P)$

P = % concentration (m/m) of total sugars.

It is recorded to one decimal place.

6.2. **Repeatability (r) for titration with the indicator**

r = 0,9 me/l

r = 0,07 g of tartaric acid/litre

for white, rosé and red wines.

6.3. **Repeatability (r) for titration with the indicator (5.3)**

For white and rosé wines:

R = 3,6 me/l

R = 0,3 g of tartaric acid/litre

For red wines:

R = 5,1 me/l

R = 0,4 g of tartaric acid/litre

12 VOLATILE ACIDITY (OIV-AS-313-02-ACIVOL) — CATEGORY I METHOD

1. DEFINITION

The volatile acidity is formed from the acids of the acetic series present in wine in the free state and combined as a salt.

2. PRINCIPLE OF THE METHOD

Titration of the volatile acids separated from the wine by steam distillation and titration of the distillate.

Carbon dioxide is first removed from the wine.

The acidity of free and combined sulphur dioxide distilled under these conditions should be deducted from the acidity of the distillate.

The acidity of any sorbic acid which may have been added to the wine must also be deducted.

Note:

Part of the salicylic acid used in some countries to stabilise the wines before analysis is present in the distillate. This must be determined and deducted from the acidity. The method of determination is given in section 7 of this chapter.

3. REAGENTS

3.1. Crystalline tartaric acid ($C_4H_6O_6$)

3.2. 0,1 M sodium hydroxide (NaOH) solution

3.3. 1 % phenolphthalein solution in 96 % vol neutral alcohol.

3.4. Hydrochloric acid (ρ_{20} = 1,18 à 1,19 g/ml) diluted 1/4 (v/v)

3.5. 0,005 M iodine (I_2) solution

3.6. Crystalline potassium iodide (Kl)

3.7. 5 g/l starch solution.

Mix 5 g of starch with about 500 ml of water. Bring to the boil, stirring continuously and boil for 10 minutes. Add 200 g sodium chloride. When cool, make up to one litre.

3.8. Saturated solution of sodium borate ($Na_2B_4O_7$, 10 H_2O), i.e. about 55 g/l at 20 °C.

4. APPARATUS

4.1. Steam distillation apparatus consisting of:

1) a steam generator; the steam must be free of carbon dioxide;

2) a flask with steam pipe;

3) a distillation column;

4) a condenser.

This equipment must pass the following three tests:

(a) Place 20 ml of boiled water in the flask. Collect 250 ml of the distillate and add to it 0,1 ml of 0,1 M sodium hydroxide solution (3.2) and two drops of the phenolphthalein solution (3.3). The pink colouration must be stable for at least 10 seconds (i.e. steam to be free of carbon dioxide).

(b) Place 20 ml of a 0,1 M acetic acid solution in the flask. Collect 250 ml of the distillate. Titrate with the 0,1 M sodium hydroxide solution (3.2): the volume of this used must be at least 19,9 ml. (i.e. at least 99,5 % of the acetic acid entrained with the steam).

(c) Place 20 ml of a 1 M lactic acid solution in the flask. Collect 250 ml of the distillate and titrate the acid with the 0,1 M sodium hydroxide solution (3.2).

The volume of sodium hydroxide solution added must be less than or equal to 1,0 ml (i.e. not more than 0,5 % of lactic acid is distilled).

Any apparatus or procedure which passes these tests satisfactorily fulfils the requirements of official international apparatus or procedures.

4.2. Water vacuum pump.

4.3. Vacuum flask.

5. PROCEDURE

5.1. **Preparation of sample: elimination of carbon dioxide.**

Place about 50 ml of wine in a vacuum flask; apply vacuum to the flask with the water pump for one to two minutes, whilst shaking continuously.

5.2. **Steam distillation.**

Place 20 ml of wine, freed from carbon dioxide as in 5.1, in the flask. Add about 0,5 g of tartaric acid (3.1). Collect at least 250 ml of the distillate.

5.3. **Titration**

Titrate with the 0,1 M sodium hydroxide solution (3.2) using two drops of phenolphthalein (3.3) as indicator. Let n ml be the volume of sodium hydroxide used.

Add four drops of 1/4 dilute hydrochloric acid (3.4), 2 ml starch solution (3.7) and a few crystals of potassium iodide (3.6). Titrate the free sulphur dioxide with the 0,005 M iodine solution (3.5). Let n′ ml be the volume used.

Add the saturated sodium borate solution (3.8) until the pink coloration reappears. Titrate the free sulphur dioxide with the 0,005 M iodine solution (3.5). Let n″ ml be the volume used.

6. EXPRESSION OF RESULTS

6.1. **Method of calculation**

The volatile acidity, expressed in milliequivalents per litre to 1 decimal place, is given by:

$A = 5\ (n - 0{,}1\ n' - 0{,}05\ n'')$

The volatile acidity expressed in grams of tartaric acid per litre to two decimal places is given by:

$0{,}300\ (n - 0{,}1\ n' - 0{,}05\ n'')$

6.2. **Repeatability (r)**

r = 0,7 me/l

r = 0,04 g of acetic acid/litre

6.3. **Reproducibility (R)**

R = 1,3 me/l

R = 0,08 g of acetic acid/litre

6.4. **Wine with sorbic acid present**

Since 96 % of sorbic acid is steam distilled with a distillate volume of 250 ml, its acidity must be deducted from the volatile acidity, knowing that 100 mg of sorbic acid corresponds to an acidity of 0,89 milliequivalents or 0,053 g of acetic acid and knowing the concentration of sorbic acid in mg/l as determined by other methods.

7. DETERMINATION OF SALICYLIC ACID ENTRAINED IN THE DISTILLATE FROM THE VOLATILE ACIDITY

7.1. **Principle**

After the determination of the volatile acidity and the correction for sulphur dioxide, the presence of salicylic acid is indicated, after acidification, by the violet colouration that appears when an iron III salt is added.

The determination of the salicylic acid entrained in the distillate with the volatile acidity is carried out on a second distillate having the same volume as that on which the determination of volatile acidity was carried out. In this distillate, the salicylic acid is determined by a comparative colorimetric method. It is deducted from the acidity of the volatile acidity distillate.

7.2. **Reagents**

7.2.1. Hydrochloric acid (HCl) (ρ_{20} = 1,18 to 1,19 g/ml).

7.2.2. Sodium thiosulphate ($Na_2 S_2 O_3 \times 5 H_2O$) in a 0,1 M solution.

7.2.3. 10 % (m/v) solution of iron III ammonium sulphate [$Fe_2 (SO_4)_3 (NH_4)_2 SO_4$ 24 H_2O].

7.2.4. 0,01 M solution of sodium salicylate. Solution containing 1,60 g/l of sodium salicylate ($Na C_7 H_5 O_3$).

7.3. **Procedure**

7.3.1. *Identification of salicylic acid in the volatile acidity distillate.*

Immediately after the determination of the volatile acidity and the correction for free and combined sulphur dioxide, introduce into a conical flask 0,5 ml hydrochloric acid (7.2.1), 3 ml of the 0,1 M sodium thiosulphate solution (7.2.2) and 1 ml of the iron (III) ammonium sulphate solution (7.2.3).

If salicylic acid is present, a violet coloration appears.

7.3.2. *Determination of salicylic acid*

On the above conical flask, indicate the volume of the distillate by a reference mark. Empty and rinse the flask.

Subject a new test sample of 20 ml wine to steam distillation and collect the distillate in the conical flask up to the reference mark. Add 0,3 ml pure hydrochloric acid (7.2.1), and 1 ml of the iron III ammonium sulphate solution (7.2.3). The contents of the conical flask turn violet.

Into a conical flask identical to that carrying the reference mark, introduce distilled water up to the same level as that of the distillate. Add 0,3 ml pure hydrochloric acid (7.2.1), and 1 ml of the iron III ammonium sulphate solution (7.2.3). From the burette run in the 0,01 M sodium salicylate solution (7.2.4) until the violet coloration obtained has the same intensity as that of the conical flask containing the wine distillate.

Let n‴ ml be the volume of solution added from the burette.

7.4. **Correction to the volatile acidity**

Subtract the volume 0,1 × n‴ ml from the volume n ml of 0,1 M sodium hydroxide solution used to titrate the acidity of the distillate during the determination of volatile acidity.

13 CITRIC ACID (OIV-AS-313-09-ACIENZ) — CATEGORY II METHOD

1. PRINCIPLE OF THE METHOD

Citric acid is converted into oxaloacetate and acetate in a reaction catalysed by citrate-lyase (CL):

citrate ⟷oxaloacetate + acetate

In the presence of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), the oxaloacetate and its decarboxylation derivative, pyruvate, are reduced to L-malate and L-lactate by reduced nicotinamide adenine dinucleotide (NADH):

oxaloacetate + NADH + H^+ ⟷L-malate + NAD^+

pyruvate + NADH + H^+ ⟷L-lactate + NAD^+

The amount of NADH oxidised to NAD^+ in these reactions is proportional to the amount of citrate present. The oxidation of NADH is measured by the resultant decrease in absorbence at a wavelength of 340 nm.

2. REAGENTS

2.1. Buffer solution pH 7,8

(glycylglycine 0,51 M; pH = 7,8; Zn^{2+}: 0,6 × 10^{-3}M)

Dissolve 7,13 g of glycylglycine in approximately 70 ml of doubly distilled water.

Adjust the pH to 7,8 with approximately 13 ml of 5 M sodium hydroxide solution, add 10 ml of zinc chloride ($ZnCl_2$ 80 mg in 100 ml H_2O) solution and make up to 100 ml with doubly distilled water.

The solution remains stable for at least a 4 weeks at 4 °C.

2.2. Reduced nicotinamide adenine dinucleotide (NADH) solution (approximately 6 × 10^{-3} M). Dissolve 30 mg NADH and 60 mg $NaHCO_3$ in 6 ml of doubly distilled water.

2.3. Malate dehydrogenase/lactate dehydrogenase solution (MDH/LDH, 0,5 mg MDH/ml, 2,5 mg LDH/ml).

Mix together 0,1 ml MDH (5 mg MDH/ ml), 0,4 ml ammonium sulphate solution (3,2 M and 0,5 ml LDH (5 mg/ml). This suspension remains stable for at least a year at 4 °C.

2.4. Citrate-lyase (CL, 5 mg protein/ml). Dissolve 168 mg lyophilisate in 1 ml ice-cold water. This solution remains stable for at least a week at 4 °C and for at least four weeks if frozen.

It is recommended that, prior to the determination, the enzyme activity should be checked.

2.5. Polyvinyl polypyrolidone (PVPP)

Note: All the reagents above are available commercially.

3. APPARATUS

3.1. A spectrophotometer enabling measurements to be made at 340 nm, the wavelength at which absorption by NADH is at a maximum.

Failing that, a spectrophotometer, with a discontinuous spectrum source permitting measurements to be made at 334 nm or 365 nm, may be used. Since absolute absorbence measurements are involved (i.e. calibration curves are not used but standardization is made by consideration of the extinction coefficient of NADH), the wavelength scales and spectral absorbence of the apparatus must be checked.

3.2. Glass cells with optical path lengths of 1 cm or single-use cells.

3.3. Micropipettes for pipetting volumes in the range 0,02 to 2 ml.

4. PREPARATION OF THE SAMPLE

Citrate determination is normally carried out directly on the wine, without preliminary removal of pigmentation (colouration) and without dilution provided that the citric acid content is less than 400 mg/l. If this is not so, dilute the wine until the citrate concentration lies between 20 and 400 mg/l (i.e. between 5 and 80 µg of citrate in the test sample).

With red wines that are rich in phenolic compounds, preliminary treatment with PVPP is recommended:

Form a suspension of about 0,2 g of PVPP in water and allow to stand for 15 minutes. Filter using a fluted filter.

Place 10 ml of wine in a 50 ml conical flask, add the moist PVPP removed from the filter with a spatula. Shake for two to three minutes. Filter.

5. PROCEDURE

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbence using the 1 cm cells, using air as the zero absorbence (reference) standard (no cell in the optical path). Place the following in the 1 cm cells:

	Reference cell	Determination
Solution 2.1	1,00 ml	1,00 ml
Solution 2.2	0,10 ml	0,10 ml
Sample	—	0,20 ml
Doubly distilled water	2,00 ml	1,80 ml
Solution 2.3	0,02 ml	0,02 ml

Mix, and after about five minutes read the absorbence of the solutions in the reference and sample cells (A_1).

Add:

Solution 2.4	0,02 ml	0,02 ml

Mix; wait until the reaction is completed (about five minutes) and read the absorbences of the solutions in the reference and sample cells (A_2).

Calculate the absorbence difference (A_1-A_2) for the reference and sample cells.

Finally, calculate the difference between those differences:

$\Delta A = \Delta A_D - \Delta A_T$

Note: The time needed for the completion of enzyme activity may vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

6. EXPRESSION OF RESULTS

Citric acid concentration is given in milligrams per litre (mg/l) to the nearest whole number.

6.1. **Method of calculation**

The general formula for calculating the concentration in mg/l is:

$C = ((V \times PM)/(\varepsilon \times d \times v)) \times \Delta A$

V = volume of test solution in ml (here 3,14 ml)

v = volume of the sample in ml (here 0,2 ml)

P.M = molecular mass of the substance to be determined (here, for anhydrous citric acid, M = 192,1)

d = optical path in the cell in cm (here, 1 cm)

ε = absorption coefficient of NADH (at 340 nm,

ε = 6,3 $mmole^{-1} \times l \times cm^{-1}$.

so that:

$C = 479 \times \Delta A$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

Note: At 334 nm: $C = 488 \times \Delta A$ ($\varepsilon = 6{,}2\ mmole^{-1} \times l \times cm^{-1}$)

At 365 nm: $C = 887 \times \Delta A$ ($\varepsilon = 3{,}4\ mmole^{-1} \times l \times cm^{-1}$)

6.2. **Repeatability (r)**

Citric acid concentration less than 400 mg/l: r = 14 mg/l.

Citric acid concentration greater than 400 mg/l: r = 28 mg/l.

6.3. **Reproducibility (R)**

Citric acid concentration less than 400 mg/l: R = 39 mg/l.

Citric acid concentration greater than 400 mg/l: R = 65 mg/l.

14 SORBIC ACID (OIV-AS-313-14-ACISOR) — CATEGORY IV METHOD

1. PRINCIPLE OF METHODS

1.1. **Determination by ultraviolet absorption spectrophotometry**

Sorbic acid (trans, trans, 2.4-hexadienoic acid) extracted by steam distillation is determined in the wine distillate by ultraviolet absorption spectrophotometry. Substances that interfere in the ultraviolet are removed by evaporation to dryness using a lightly alkali, calcium hydroxide. Thin layer chromatography is used for confirmation of levels (1 mg/l) less than 20 mg/l.

1.2. **Determination by gas chromatography**

Sorbic acid extracted in ethyl ether is determined by gas chromatography with an internal standard.

1.3. **Identification of traces by thin-layer chromatography**

Sorbic acid extracted in ethyl ether is separated by thin layer chromatography and its concentration is evaluated semi-quantitatively.

2. DETERMINATION BY ULTRAVIOLET ABSORPTION SPECTOPHOTOMETRY

2.1. **Reagents**

2.1.1. Crystalline tartaric acid ($C_4H_6O_6$).

2.1.2. Calcium hydroxide, Ca $(OH)_2$ solution, approximately 0,02 M.

2.1.3. Reference sorbic acid solution, 20 mg/l.

Dissolve 20 mg of sorbic acid, $C_6H_8O_2$, in approximately 2 ml of 0,1 M sodium hydroxide solution. Pour into a 1 000 ml volumetric flask, and make up to the mark with water. It is also possible to dissolve 26,8 mg of potassium sorbate, $C_6H_7KO_2$, in water and make up to 1 000 ml with water.

2.2. **Apparatus**

2.2.1. Steam distillation apparatus (see Chapter *'Volatile acidity'*).

2.2.2. Water bath at 100 °C.

2.2.3. Spectrophotometer enabling absorbence measurements to be made at a wavelength of 256 nm and having a quartz cell with a 1 cm optical path.

2.3. **Procedure**

2.3.1. *Distillation*

Place in the flask of the steam distillation apparatus 10 ml of wine and add 1 to 2 g tartaric acid (2.1.1). Collect 250 ml of the distillate.

2.3.2. *Preparation of the calibration curve*

Prepare, by dilution of the reference solution (2.1.3), four dilute reference solutions with 0,5, 1,0, 2,5 and 5 mg of sorbic acid per litre. Measure their absorbences with the spectrophotometer at 256 nm using that of distilled water as a blank. Plot a curve showing the variation of absorbence as a function of concentration. The variation is linear.

2.3.3. *Determination*

Place 5 ml of the distillate in an evaporating dish of 55 mm diameter, add 1 ml of calcium hydroxide solution (2.1.2). Evaporate to dryness on a water bath.

Dissolve the residue in several ml of distilled water, transfer completely to a 20 ml volumetric flask and make up to the mark with rinsing water. Measure the absorbence at 256 nm using the spectrophotometer against a blank consisting of a solution obtained by diluting 1 ml of calcium hydroxide solution (2.1.2) to 20 ml with water.

Plot the value of the measured absorbence on the calibration curve and from this find the concentration C of sorbic acid in the solution.

Note: In this method the loss due to evaporation can be neglected and the absorbence measured on the treated distillate diluted ¼ with distilled water.

2.4. **Expression of results**

2.4.1. *Method of calculation*

The sorbic acid concentration in the wine expressed in mg per litre is given by

100 × C

where C = concentration of sorbic acid in the solution analysed by spectrophotometry expressed in mg per litre.

3. DETERMINATION BY GAS CHROMATOGRAPHY

3.1. **Reagents**

3.1.1. Ethyl ether, $(C_2H_5)_2O$, distilled just before use.

3.1.2. Internal reference solution: solution of undecanoic acid, $C_{11}H_{22}O_2$, in 95 % vol ethanol at a strength of 1 g/l.

3.1.3. Aqueous solution of sulphuric acid, H_2SO_4 (ρ_{20} = 1,84 g/ml) diluted 1:3 (v/v).

3.2. **Apparatus**

3.2.1. Gas chromatograph fitted with a flame ionization detector and a stainless steel column (4 m × 1/8 inch) previously treated with dimethyl dichlorosilane and packed with a stationary phase consisting of a mixture of diethylene glycol succinate (5 %) and phosphoric acid (1 %) (DEGS – H_3PO_4) or of a mixture of diethylene glycol adipate (7 %) and phosphoric acid (1 %) (DEGA – H_3PO_4) bonded on Gaschrom Q 80 – 100 mesh.

Treatment of column with DMDCS – pass through the column a solution containing 2 to 3 g of DMDCS in toluene. Immediately wash with methanol, followed by nitrogen and then wash with hexane followed by more nitrogen. It is now ready to be packed.

Operating conditions:

Oven temperature: 175 °C.

Temperature of the injector and detector: 230 °C.

Carrier gas: nitrogen (flow rate = 20 ml/min.).

3.2.2. Microsyringe, 10 µl capacity graduated in 0,1 µl.

Note: Other types of columns that give a good separation can be used, particularly capillary columns (e.g. FFAP).

The working method described is given as an example.

3.3. **Procedure**

3.3.1. *Preparation of sample to be analysed*

Into a glass test tube of approximately 40 ml capacity and fitted with a ground glass stopper, introduce 20 ml of wine, add 2 ml of the internal reference solution (3.1.2) and 1 ml of dilute sulphuric acid (3.1.3).

After mixing the solution by repeatedly turning the tube over, add to its contents 10 ml of ethyl ether (3.1.1). Extract the sorbic acid in the organic phase by shaking the tube for five minutes. Leave to settle.

3.3.2. *Preparation of the reference solution*

Select a wine for which the chromatogram of the ether extract shows no peak corresponding to the elution of sorbic acid. Overload this wine with sorbic acid at a concentration of 100 mg per litre. Treat 20 ml of the sample prepared in this way according to the procedure described in 3.3.1.

3.3.3. *Chromatography*

Using a microsyringe, inject into the chromatograph in turn 2 µl of the ether-extract phase obtained in 3.3.2 and 2 µl of the ether-extracted phase obtained in 3.3.1.

Record the respective chromatograms: check the identity of the respective retention times of the sorbic acid and the internal standard. Measure the height (or area) of each of the recorded peaks.

3.4. **Expression of results**

3.4.1. *Method of calculation*

The concentration of sorbic acid in the analysed wine, expressed in mg per litre, is given by

$100 \times (h/H) \times (l/i)$

where

H = height of the sorbic acid peak in the reference solution

h = height of the sorbic acid peak in the sample for analysis

l = height of the internal standard peak in the reference solution

i = height of the internal standard peak in the sample for analysis

Note: The sorbic acid concentration may be determined in the same way from measurements of the areas under the respective peaks.

15 **PH (OIV-AS-313-15-PH) — CATEGORY I METHOD**

1. PRINCIPLE

The difference in potential between two electrodes immersed in the liquid under test is measured. One of these two electrodes has a potential which is a function of the pH of the liquid, while the other has a fixed and known potential and constitutes the reference electrode.

2. APPARATUS

2.1. **pH meter with a scale calibrated in pH units and enabling measurements to be made to at least ± 0,05 pH unit.**

2.2. **Electrodes:**

2.2.1. Glass electrode, kept in distilled water.

2.2.2. Calomel-saturated potassium chloride reference electrode, kept in a saturated solution of potassium chloride.

2.2.3. Or a combined electrode, kept in distilled water.

3. REAGENTS

3.1. **Buffer solutions**

3.1.1. Saturated solution of potassium hydrogen tartrate, containing at least 5,7 g of potassium hydrogen tartrate per litre ($C_4H_5KO_6$) at 20 °C. This solution may be kept for up to two months by adding 0,1 g of thymol per 200 ml.

pH
- 3,57 to 20 °C
- 3,56 to 25 °C
- 3,55 to 30 °C

3.1.2. Solution of potassium hydrogen phthalate, 0,05 M, containing 10,211 g of potassium hydrogen phthalate ($C_8H_5KO_4$) per litre at 20 °C. (Maximum keeping period, two months).

pH
- 3,999 to 15 °C
- 4,003 to 20 °C
- 4,008 to 25 °C
- 4,015 to 30 °C

3.1.3. Solution containing:

Monopotassium phosphate, KH_2PO_4	3,402 g
Dipotassium phosphate, K_2HPO_4	4,354 g
Water to	1 l

(Maximum keeping period, two months).

pH
- 6,90 to 15 °C
- 6,88 to 20 °C
- 6,86 to 25 °C
- 6,85 to 30 °C

Note: Alternatively, ready made buffer solutions are available commercially.

4. PROCEDURE

4.1. **Preparation of sample to be analysed**

4.1.1. *For must and wine:*

use the must or wine directly.

4.1.2. *For rectified concentrated must:*

Dilute the rectified concentrated must with water to produce a concentration of 25 ± 0,5 % (m/m) of total sugars (25 º Brix.).

If P is the percentage concentration (m/m) of total sugars in the rectified concentrated must, weigh a mass of 2 500/P,

and make up to 100 g with water. The water used must have a conductivity below 2 microsiemens per cm.

4.2. **Zeroing of the apparatus**

Zeroing is carried out before any measurement is made, according to the instructions provided with the apparatus used.

4.3. **Calibration of pH meter**

Calibrate the pH meter at 20 °C using buffer solutions of pH 6,88 and 3,57 at 20 °C.

Use the buffer solution of pH 4,00 at 20 °C to check the calibration of the scale.

4.4. **Determination**

Dip the electrode into the sample to be analysed, the temperature of which should be between 20 and 25 °C and as close as possible to 20 °C. Read the pH value directly off the scale.

Carry out at least two determinations on the same sample.

The final result is taken to be the arithmetic mean of the determinations.

5. EXPRESSION OF RESULTS

The pH of the must, the wine or the 25 % (m/m) (25 º Brix) solution of rectified concentrated must is quoted to two decimal places.

16 SIMULTANEOUS DETERMINATION OF L-ASCORBIC ACID AND D-ISOASCORBIC ACID BY MEANS OF HPLC AND UV DETECTION (OIV-AS-313-22-ACASCO) — CATEGORY II METHOD

1. INTRODUCTION

Ascorbic acid is an antioxidant naturally present in a whole series of foods. The normal amount of ascorbic acid in the grape diminishes during the preparation of musts and in the course of wine making. It can be added to musts and to wines within certain limits.

The method described has been validated within the framework of interlaboratory tests, by means of analyses of samples of wine with added amounts of L-ascorbic acid and D-isoascorbic acid respectively of 30 mg/L to 150 mg/l and of 10 mg/l to 100 mg/l.

2. FIELD OF APPLICATION

This method is suitable for the simultaneous determination of L-ascorbic acid and D-isoascorbic acid (erythorbic acid) in the wine by means of high-performance liquid chromatography and UV detection in a range of 3 to 150 mg/l.

For contents greater than 150 mg/l, the sample must be diluted.

3. PRINCIPLE

The samples are injected directly into the HLPC system after membrane filtration. The analytes are separated in a reverse-phase column and are subjected to UV detection at 266 nm. The quantification of L-ascorbic acid and D-isoascorbic acid is performed in relation to an external standard.

Note: The columns and the operating conditions are given by way of example. Other types of columns can also ensure good separation.

4. REAGENTS AND PRODUCTS

4.1. **Reagents**

4.1.1. n-octylamine, purity ≥ 99,0 %

4.1.2. Sodium acetate × 3 H_2O, purity ≥ 99,0 %

4.1.3. Pure acetic acid, 100 %

4.1.4. Phosphoric acid, approx. 25 %

4.1.5. Oxalic acid, purity ≥ 99,0 %

4.1.6. Ascorbate oxidase

4.1.7. L-ascorbic acid, ultra ≥ 99,5 %

4.1.8. D-isoascorbic acid, purity ≥ 99,0 %

4.1.9. Doubly distilled water

4.1.10. Methanol, p.A. 99,8 %

4.2. **Preparation of the mobile phase**

4.2.1. Solutions for the mobile phase

Prepare the following solutions for the mobile phase:

4.2.1.1. 12,93 g of n-octylamine in 100 mlL of methanol

4.2.1.2. 68,05 g of sodium acetate × 3 H_2O in 500 ml of doubly distilled water

4.2.1.3. 12,01 g of pure acetic acid in 200 ml of doubly distilled water

4.2.1.4. Buffer solution (pH 5,4): 430 mol of sodium acetate solution (0) and 70 ml of acetic acid solution (0)

4.2.2. *Preparation of the mobile phase*

Add 5 ml of n-octylamine solution (4.2.1.1) to approximately 400 ml of doubly distilled water in a beaker. Adjust this solution to a pH of 5,4 to 5,6, by adding 25 % phosphoric acid drop by drop (4.1.4). Add 50 ml of the buffer solution (4.2.1.4) and transfer the compound into a 1 000 ml volumetric flask, then make up with doubly distilled water. Before use, the mobile phase must be filtered with the aid of a membrane (0,2 µm regenerated cellulose) and if possible degased with helium (for approximately 10 minutes) according to the requirements of the HLPC system used.

4.3. **Preparation of the standard solution**

Note:

All the standard solutions (stock solution 4.3.1. and working solutions 4.3.2) must be prepared each day and preferably stocked in a refrigerator before injection.

4.3.1. Preparation of the stock solution (1 mg/ml)

Prepare an aqueous solution of 2 % oxalic acid and remove the dissolved oxygen by bubbling nitrogen.

Weigh precisely 100 mg of L-ascorbic acid and 100 mg of D-isoascorbic acid in a 100 mlL volumetric flask and make up with aqueous solution of 2 % oxalic acid.

4.3.2. Preparation of working solutions

For working solutions, dilute the stock solution (4.3.1) to the concentrations desired with the 2 % oxalic acid solution. Concentrations between 10 mg/l and 120 mg/lL are recommended. For example, 100 µl, 200 µl, 400 µl, 800 µl, 1 200 µl to 10 ml, corresponding to 10, 20, 40, 80 and 120 mg/l.

5. APPARATUS

Current laboratory material, in particular the following equipment:

5.1. **HLPC pump**

5.2. **20 µl loop injector**

5.3. **UV detector**

6. SAMPLING

Samples of wine are filtered through a membrane with a pore diameter of 0,2 µm before injection.

For contents greater than 150 mg/l, the sample must be diluted.

7. PROCEDURE

7.1. **Conditions of use of the HLPC system**

Inject 20 µl of the membrane-filtered sample into the chromatographic apparatus.

Guard column:	for example Nucleosil 120 C18 (4 cm × 4 mm × 7 µm)
Column:	for example Nucleosil 120 C18 (25 cm × 4 mm × 7 µm)
Injection volume:	20 µl
Mobile phase:	see 4.2.2, isocratic
Flow rate:	1 ml/min
UV detection:	266 nm
Rinsing cycle:	at least 30 mlL of doubly distilled water followed by 30 ml of methanol and 30 ml of acetonitrile

7.2. **Identification/Confirmation**

Peaks are identified by comparing the retention times of the standards and the samples. With the chromatographic system described as an example, the retention times are 7,7 min for L-ascorbic acid and 8,3 min for D-isoascorbic acid (see figure 1, chromatogram A) respectively.

To confirm positive results, these samples must be treated with a spatula of ascorbate oxidase and measured again (see figure 1, chromatogram B).

Because of the degradation of the L-ascorbic acid and the D-isoascorbic acid provoked by the ascorbate oxidase, no signal should be found at the time at which the L-ascorbic acid and the D-isoascorbic acid are retained. In the event of the detection of parasite peaks, their surface must be taken into account in the calculation of the concentration of analytes.

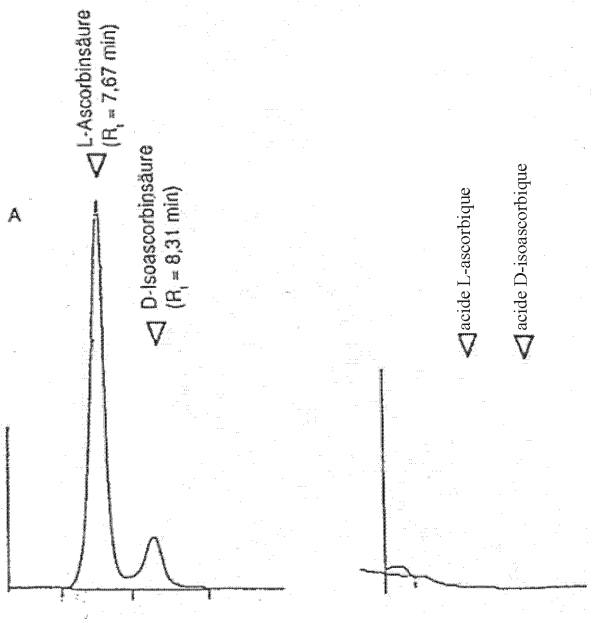

Figure 1

Example of chromatogram of a white wine: A before ascorbate oxidase treatment; B after treatment

Note: It is recommended that the samples treated with ascorbate oxidase be analysed at the end of a sequence followed by a rinsing cycle to eliminate ascorbate oxidase residue in the column, otherwise L-ascorbic acid and D-isoascorbic acid could be converted by the ascorbate oxidase residue during HPLC measurement and the result could be affected.

8. CALCULATIONS

Prepare a calibration curve based on working solutions (4.3.2). According to the external standard method, L-ascorbic acid and D-isoascorbic acid are quantified by measuring the surfaces of the peaks and comparing them to the corresponding concentration on the calibration curve.

Expression of results

The results are expressed to one decimal in mg/l of L-ascorbic acid and D-isoascorbic acid respectively (for example, 51,3 mg/l).

For contents greater than 150 mg/l, take the dilution into account.

9. RELIABILITY

The method has been tested within the framework of an interlaboratory test organised in 1994 by the former Federal Board of Health (Bundesgesundheitsamt, Germany), in which 27 laboratories participated. The programme of the interlaboratory test followed section 35 of the German Act on Foodstuffs, which has been accepted by the OIV until the introduction of the new protocol (OENO 6/2000).

The study focused on four different samples of wine – two white wines and two red wines – with five repetitions of each sample requested. Seeing that it was not possible to prepare samples with sufficiently stable analytes (different speeds of degradation), a decision was taken to send participants defined quantities of pure standard substances as well as samples of wine. The laboratories were instructed to transfer the standards quantitatively in the samples of wine and to analyse them immediately. Volumes of 30 to 150 mg/L for L-ascorbic acid and of 10 to 100 mg/L for D-isoascorbic acid were analysed. The detailed results of the study are presented in the ANNEX published by the OIV. The evaluation was performed in accordance with DIN/ISO 5725 (1988 version).

The deviation types of repeatability (s_r) and reproducibility (s_R) were in accordance with the concentrations of L-ascorbic acid and D-isoascorbic acid. The actual precision parameter can be calculated with the aid of the following equations:

L-ascorbic acid

$s_r = 0{,}011\ x + 0{,}31$

$s_R = 0{,}064\ x + 1{,}39$

x: concentration of L-ascorbic acid (mg/L)

D-isoascorbic acid

$s_r = 0{,}014\ x + 0{,}31$

$s_R = 0{,}079\ x + 1{,}29$

x: concentration of D-isoascorbic acid (mg/ll)

Example:

50 mg/L of D-isoascorbic acid s_r = 1,0 mg/l

s_R = 5,2 mg/L

10. OTHER CHARACTERISTICS OF THE ANALYSIS

10.1. **Detection limit**

The detection limit of this method has been estimated at 3 mg/l for L-ascorbic acid and D-isoascorbic acid.

10.2. **Accuracy**

The average recovery calculated on the basis of the interlaboratory test conducted on four samples (see the ANNEX published in the Compendium of the OIV) was:

— 100,6 % for L-ascorbic acid

— 103,3 % for D-isoascorbic acid

17 CARBON DIOXIDE (OIV-AS-314-01-DIOCAR) — CATEGORY II METHOD

1. PRINCIPLE OF THE METHOD

1.1. **Still wines (overpressure of CO_2 ≤ 0,5 × 10^5 Pa)** ([1])

The volume of wine taken from the sample is cooled to around 0 °C and mixed with a sufficient quantity of sodium hydroxide to give a pH of 10 to 11. Titration is carried out with an acid solution in the presence of carbonic anhydrase. The carbon dioxide content is calculated from the volume of acid needed to change the pH from 8,6 (bicarbonate form) to 4,0 (carbonic acid). A blank titration is carried out in the same conditions on decarbonated wine in order to take account of the volume of sodium hydroxide solution taken up by the wine acids.

1.2. **Sparkling and semi-sparkling wines**

The sample of wine to be analysed is cooled near to freezing point. After removal of a quantity to be used as a blank after decarbonising, the remainder of the bottle is made alkaline to fix all the carbon dioxide in the form of Na_2CO_3. Titration is carried out with an acid solution in the presence of carbonic anhydrase. The carbon dioxide content is calculated from the volume of acid solution needed to change the pH from 8,6 (bicarbonate form) to 4,0 (carbonic acid). A blank titration is carried out in the same conditions in decarbonated wine in order to take account of the volume of sodium hydroxide solution taken up by the wine acids.

2. DESCRIPTION OF THE METHOD

2.1. **Still wines (overpressure of carbon dioxide ≤ 0,5 × 10^5 Pa).**

2.1.1. *Apparatus*

2.1.1.1. Magnetic stirrer.

2.1.1.2. pH meter.

2.1.2. *Reagents*

2.1.2.1. Sodium hydroxide (NaOH) solution, 0,1 M.

2.1.2.2. Sulphuric acid solution, (H_2SO_4), 0,05 M.

2.1.2.3. Carbonic anhydrase solution, 1 g/l.

2.1.3. *Procedure*

Cool the wine sample to approximately 0 °C together with the 10 ml pipette used for sampling.

Place 25 ml of sodium hydroxide solution (2.1.2.1) in a 100 ml beaker; add two drops of aqueous solution of carbonic anhydrase (2.1.2.3). Introduce 10 ml of wine using the pipette cooled to 0 °C.

Place the beaker on the magnetic stirrer, set up the pH electrode and stir moderately.

When the liquid has reached room temperature, titrate slowly with the sulphuric acid solution (2.1.2.2) until the pH reaches 8,6.

Continue titrating with the sulphuric acid (2.1.2.2) until the pH reaches 4,0. Let nml be the volume used between pH 8,6 and 4,0.

Remove CO_2 from approximately 50 ml of the wine sample by agitation under vacuum for three minutes, the flask being heated in a water bath to about 25 °C.

Carry out the above procedure on 10 ml of the decarbonated wine. Let n′ ml be the volume used.

2.1.4. *Expression of results*

1 ml of the titrated 0,1 M sulphuric acid solution corresponds to 4,4 mg of CO_2.

The quantity of CO_2 in grams per litre of wine is given by the formula:

$0{,}44\ (n - n')$

It is quoted to two decimal places.

Note: Where wines contain little CO_2 (CO_2 < 1 g/l), the addition of carbonic anhydrase to catalyse the hydration of CO_2 is unnecessary.

([1]) 10^5 pascal (Pa) = 1 bar.

2.2. **Sparkling and semi-sparkling wines**

2.2.1. *Apparatus*

2.2.1.1. Magnetic stirrer.

2.2.1.2. pH meter.

2.2.2. *Reagents*

2.2.2.1. Sodium hydroxide, NaOH, 50 % (m/m).

2.2.2.2. Sulphuric acid solution, (H_2SO_4), 0,05 M.

2.2.2.3. Carbonic anhydrase solution, 1 g/l.

2.2.3. *Procedure*

On the bottle of wine for analysis, mark the level to which it has been filled and then cool until it begins to freeze.

Allow the bottle to warm up slightly, while shaking, until ice crystals disappear.

Remove the stopper rapidly and place 45 to 50 ml of wine in a measuring cylinder for blank titration. The exact volume removed, v ml, is determined by reading on the cylinder after it has returned to room temperature.

Immediately after the blank sample has been removed, add 20 ml of the sodium hydroxide solution (2.2.2.1) to the bottle with a capacity of 750 ml.

Wait until the wine has reached room temperature.

Place 30 ml of boiled distilled water and two drops of the carbonic anhydrase solution (2.2.2.3) into a 100 ml cylindrical bulb. Add 10 ml of alkalinised wine.

Place the beaker on the magnetic stirrer, set up the pH electrode and magnetic rod and stir moderately.

When the liquid has reached room temperature, titrate slowly with the sulphuric acid solution (2.2.2.2) until the pH reaches 8,6.

Continue titrating with the sulphuric acid (2.2.2.2) until the pH reaches 4,0. Let n ml be the volume used between pH 8,6 and 4,0.

Remove CO_2 from approximately v ml of the wine, by agitation under vacuum for three minutes, the flask being heated in a water bath to about 25 °C. Remove 10 ml of decarbonated wine and add to 30 ml of boiled distilled water, add two to three drops of sodium hydroxide solution (2.2.2.1) to bring the pH to 10 to 11. Then follow the above procedure. Let n′ ml be the volume of 0,5 M sulphursulphuric acid added.

2.2.4. *Expression of results*

1 ml of 0,05 M sulphuric acid corresponds to 4,4 mg of CO_2.

Empty the bottle of wine which has been made alkaline and determine to within 1 ml the initial volume of wine by making up to the mark with water, say V ml.

The quantity of CO_2 in grams per litre of wine is given by the formula:

$0{,}44(n - n') \times (V - v + 20)/(V - v)$

It is quoted to two decimal places.

2.3. **Calculation of theoretical overpressure**

Overpressure at 20 °C, $Paph_{20}$, expressed in pascals, is given by the formula:

$Paph_{20} = (Q)/(1{,}951 \times 10^{-5}(0{,}86 - 0{,}01\ A)(1 - 0{,}00144\ S)) - Patm$

with:

Q: Content in grams of CO_2 per litre of wine

A: Alcoholic strength of wine at 20 °C

S: Sugar content of wine in grams per litre

Patm: Atmospheric pressure, expressed in pascals

18 **DETERMINATION OF CARBON DIOXIDE IN THE WINE USING MANOMETRIC METHOD (OIV–AS-314-04-CO2MAN) — CATEGORY II METHOD**

(p.m.)

[The description of this method of analysis is being updated by the OIV. It will be published in the next Commission notice as soon as an updated text is published by the OIV in the 2010 edition of the Compendium of International Methods of Analysis of the OIV]

19 MEASUREMENT OF THE EXCESS PRESSURE IN SPARKLING AND SEMI-SPARKLING WINES (OIV-AS-314-02-SURPRES) — CATEGORY I METHOD

1. PRINCIPLE

After thermal stabilisation and shaking of the bottle, the excess pressure is measured using an aphrometer (pressure gauge). It is expressed in Pascals (Pa) (Category I method). The method is also applicable to sparkling and semi-sparkling wines.

2. APPARATUS

The apparatus enabling the measurement of excess pressure in bottles of sparkling and semi-sparkling wine is called an aphrometer. It takes a different form according to the way in which the bottle is stoppered (metallic top, cap, cork or plastic stopper).

2.1. **For bottles with a top**

It comprises three parts (figure 1):

— the upper part (or needle carrier) is composed of the manometer, a manual tightening ring, an endless screw which slides through the intermediate part, and a needle which goes through the top. The needle has a lateral hole which conveys the pressure to the manometer. A seal ensures that the whole bottle top is airtight.

— the intermediate part (or nut) serves to centre the upper part. It screws into the lower part in such a way as to keep the assembly tightly on the bottle

— the lower part (or clamp) has a pin which slides on the ring of the bottle, in such a way as to retain the assembly. There are rings adapted to each type of bottle.

2.2. **For bottles with a stopper**

It comprises two parts (figure 2):

— The upper part is identical to the previous apparatus; however, the needle is longer. The needle is a long hollow tube at the end of which there is a point which helps to go through the stopper. This point is detachable, it falls into the wine once the stopper has been pierced

— the lower part is formed by the screw and a base resting on the stopper. This has four fastening screws serving to keep the assembly on the stopper.

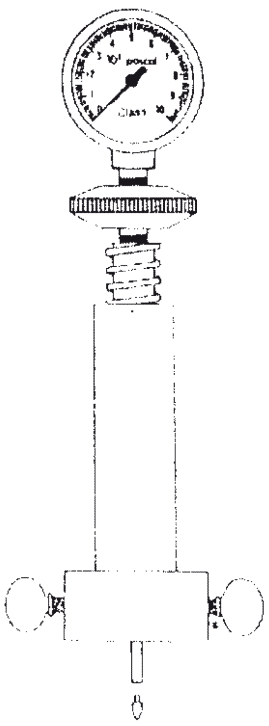

Figure 2: Aphrometer for stoppers

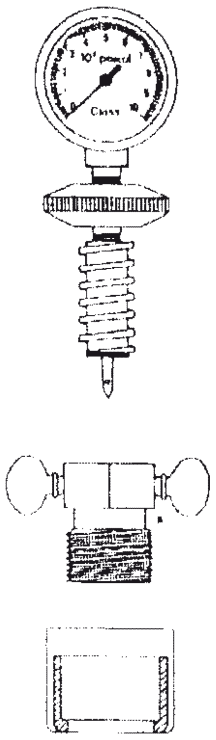

Figure 1: Aphrometer for tops

Notes regarding the manometers on these two types of apparatus:

— They can be either mechanical with a Bourdon tube or numerical with a piezoelectric sensor. In the former case, the Bourdon tube must be made of stainless steel.

— They are calibrated in Pascals (abbreviation Pa). For sparkling wines, it is more practical to use 10^5 pascals (10^5 Pa) or the kilopascal (kPa) as a unit.

— They fall into different classes. The class of a manometer is the precision of a reading in relation to a full scale reading expressed as a percentage (e.g. a 1 000 kPa manometer class I means that the maximum pressure of 1 000 kPa can be read to within ±10 kPa). A class I instrument is recommended for accurate measurement.

3. PROCEDURE

Measurements must be carried out on bottles whose temperature has been stabilised for at least 24 hours. After having pierced the cap, the cork or the stopper, the bottle must then be shaken vigorously until the pressure is constant in order to take the reading.

3.1. **Aphrometer for bottles with caps**

Slip the clamp over the spur binders under the ring of the bottle. Tighten the nut until the entire apparatus is tight on the bottle. The top part is screwed onto the nut. To avoid any gas escaping, the capsule should be pierced as quickly as possible to bring the joint into contact with the capsule. The bottle must be shaken vigorously until it reached a constant pressure, when a reading can be taken.

3.2. **Aphrometer for bottles with corks and stoppers**

Put a point in place at the end of the needle. Place the whole assembly on the stopper. Fasten the four screws on the stopper. Screw down the upper part (the needle then goes through the stopper). The point must fall into the bottle to enable the pressure to be conveyed to the manometer. The bottle must then be shaken vigorously until the pressure is constant in order to take the reading. Recover the point after taking the reading.

4. EXPRESSION OF RESULTS

The excess pressure at 20 °C ($Paph_{20}$) is expressed in pascals (Pa) or in kilopascals (kPa). It must be quoted in a form consistent with the precision of the manometer (e.g. $6{,}3 \times 10^5$ Pa or 630 kPa and not $6{,}33 \times 10^5$ Pa or 633 kPa for a class I manometer with a full scale reading of 1 000 kPa).

If the temperature at which the measurement is carried out is different from 20 °C, a correction should be made by multiplying the measured pressure by the coefficient given in Table I.

Table 1

Relationship of the excess pressure $Paph_{20}$ of a sparkling or semi-sparkling wine at 20 °C to the overpressure $Paph_t$ at a temperature t

°C		°C	
0	1,85	13	1,24
1	1,80	14	1,20
2	1,74	15	1,16
3	1,68	16	1,13
4	1,64	17	1,09
5	1,59	18	1,06
6	1,54	19	1,03
7	1,50	20	1,00
8	1,45	21	0,97
9	1,40	22	0,95
10	1,36	23	0,93
11	1,32	24	0,91
12	1,28	25	0,88

5. MONITORING OF RESULTS

Method of direct determination of physical parameters (Category I criterion method)

Checking of aphrometers

Aphrometers must be checked regularly (at least once a year).

Checking is performed using a calibration bench. It permits comparison of the manometer for testing with a reference manometer of a higher class, adjusted to national standards, mounted in parallel. Checking is used to compare the values indicated by the two pieces of equipment for increasing and decreasing pressures. If there is a difference between the two, a fastening screw permits the necessary corrections to be made.

Laboratories and authorised bodies all have such calibration benches; they are also available from manometer manufacturers.

20 DETERMINATION OF LYSOZYME IN WINE USING HPLC (OIV-AS-315-14) — CATEGORY IV METHOD

1. INTRODUCTION

It is preferable to use an analytical method not based on enzymatic activity for lysozyme.

2. FIELD OF APPLICATION

This method permits the quantification of lysozyme (mg of protein/l) present in white and red wines independently of enzymatic activity (which might be compromised by partial denaturating or by complexing and co-precipitation phenomena) of the matrix.

3. DEFINITION

High-performance liquid chromatography (HPLC) offers an analytical approach based on steric, polar or absorption interaction between the stationary phase and the analyte and, as a result, not associated with the actual enzymatic activity of the protein.

4. PRINCIPLE

The analysis is performed by high-performance liquid chromatography (HPLC) associating a spectrophotometric detector and a spectrofluorimetric detector. The unknown content of the wine sample is calculated in relation to the surface of the chromatographic peak using the external calibration method.

5. REAGENTS

5.1. **Solvents and solutions**

Acetonitrile (CH_3CN) for HPLC analysis

Pure trifluoroacetic acid (TFA)

De-ionised water for HPLC analysis

Standard solution: tartaric acid 1 g/L, ethyl alcohol 10 % v/v adjusted to pH 3,2 with neutral potassium tartrate

5.2. **Eluents**

A: CH_3CN 1 %, TFA 0,2 %, H_2O= 98,8 %

B: CH_3CN 70 %, TFA 0,2 %, H_2O= 29,8 %

5.3. **Reference solutions**

From 1 to 250 mg/L of standard lysozyme dissolved in the model solution by continuous shaking for a minimum of 12 hours.

6. MATERIAL

6.1. HPLC apparatus with vacuum system provided to obtain an elution gradient.

6.2. Housing for thermostated column (oven)

6.3. Spectrophotometric detector associated with a spectrofluorimetric detector

6.4. Injection loop, 20 µL

6.5. Inverse-phase polymer column with phenyl functional groups (pore diameter = 1 000 Å, exclusion limit = 1 000 000 Da), Tosoh Bioscience TSK-gel Phenyl 5PW RP 7,5 cm × 4,6 mm ID, as an example

6.6. Guard column in the same material as the column, Tosoh Bioscience TSK-gel Phenyl 5 PW RP Guardgel 1,5 cm × 3,2 mm ID, as an example

7. PREPARATION OF SAMPLE

The wine samples are acidified with HCl (10 M) diluted to 1/10th and filtered with a polyamide filter with pores of 0,22 µm in diameter, 5 minutes after addition. The chromatographic analysis is performed immediately after filtration.

8. OPERATING CONDITIONS

8.1. Flow rate of eluent: 1 ml/min

8.2. Temperature of the column: 30 °C

8.3. Spectrophotometric detection: 280 nm

8.4. Spectrofluorimetric detection: λ ex = 276 nm; λ em = 345 nm; Gain = 10

8.5. Elution gradient programme

Temps (min)	Sol A %	Sol B %	gradient
0	100	0	
			isocratic
3	100	0	
			linear
10	65	35	
			isocratic
15	65	35	
			linear
27	40,5	59,5	
			linear
29	0	100	
			isocratic
34	0	100	
			linear
36	100	0	
			isocratic
40	100	0	

8.6. Average retention time of lysozyme: 25,50 minutes

9. CALCULATION

Reference solutions containing the following concentrations of lysozyme are analysed in triplicate: 1; 5; 10; 50; 100; 200; 250 mg/l. On each chromatogram, the areas of the peak corresponding to the lysozyme are reported in a diagram in relation to their respective concentrations in order to obtain the lines of linear regression expressed by the formula Y = ax+b. The coefficient of determination r^2 must be > 0,999.

10. CHARACTERISTICS OF THE METHOD

With the aim of assessing the suitability of the method for the objective formulated, an assessment study was performed taking into account the linearity, the detection and quantification limits, and the precision of the method. This last parameter was determined by defining the level of precision and accuracy of the method.

	Range of linearity (mg/L)	Gradient of the straight line	Coefficient of determination (r^2)	LD (mg/L)	LQ (mg/L)	Repeatability (n=5) RSD %			Reproducibility (n=5) RSD %
						Std[1]	R.W.[2]	W.W.[3]	Std[1]
UV	5-250	3,786	0,9993	1,86	6,20	4,67	5,54	0,62	1,93
FLD	1-250	52,037	0,9990	0,18	0,59	2,61	2,37	0,68	2,30

Table 1: Data relative to the characteristics of the method: Std[1] standard solution; R.W.[2] red wine; W.W.[3] white wine

10.1. **Linearity of the method**

Based on the results obtained thanks to the linear regression analysis, the method has proved to be linear for the ranges indicated in Table 1.

10.2. **Detection and quantification limit**

The detection limit (LD) and quantification limit (LQ) have been calculated as the signal equivalent to 3 times and 10 times respectively the chromatographic background noise in working conditions on an actual matrix (Table 1).

10.3. **Accuracy of the method**

The parameters taken into consideration are repeatability and reproducibility. Table 1 shows the values of these parameters (expressed as a standard deviation percentage of measurements repeated with different concentrations) based on the standard solution, on white wine and on red wine.

10.4. **Accuracy of the method**

The recovery percentage has been calculated on the basis of standard solutions containing 5 and 50 mg/L of lysozyme, with a specified added amount of lysozyme, as shown in the following table.

	[C] initial nominal (mg/L)	Addition (mg/L)	[C] theoretical (mg/L)	[C] found (mg/L)	Standard deviation	Recovery %
UV 280 nm	50	13,1	63,1	62,3	3,86	99
FD	50	13,1	63,1	64,5	5,36	102
UV 280 nm	5	14,4	19,4	17,9	1,49	92,1
FD	5	14,4	19,4	19,0	1,61	97,7

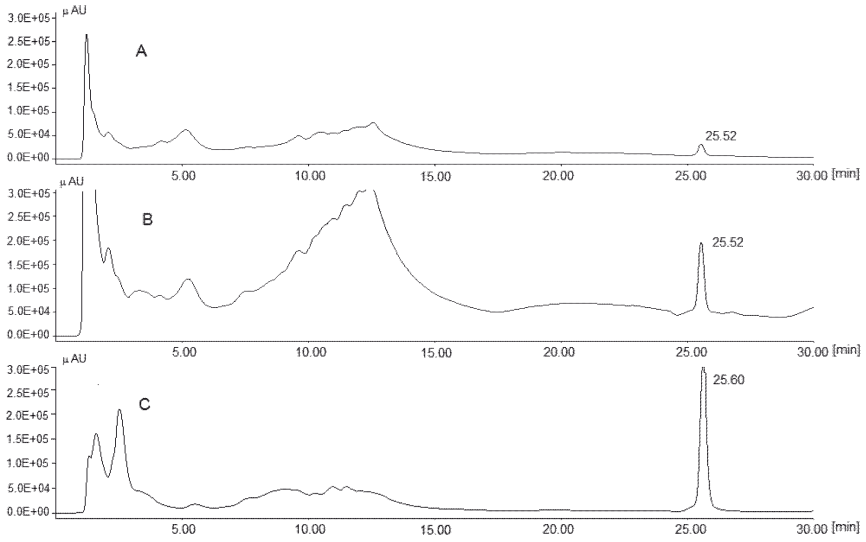

Fig.1: Chromatogram of red wine containing pure Lysozyme (a standard solution containing 1 000 mg/l of Lysozyme has been added to the wine to obtain a final concentration of 125 mg/l of Lysozyme). A: 280 nm UV detector; B: 225 nm UV detector; C: FLD detector (λ ex 276 nm; λ em 345 nm).

21 SULPHATES (OIV-AS-321-05-SULPHATE) — CATEGORY II METHOD

1. PRINCIPLE OF METHODS

1.1. **Reference method**

Precipitation of barium sulphate and weighing. The barium phosphate precipitated in the same conditions is eliminated by washing the precipitate in hydrochloric acid.

In the case of musts or wine rich in sulphur dioxide, prior de-sulphiting by boiling in an airtight vessel is recommended.

1.2. **Quick test method**

Wines are classified into several categories using the so-called limits method, based on the precipitation of barium sulphate using a barium ion titrant.

2. REFERENCE METHOD

2.1. **Reagents**

2.1.1. 2 M solution of hydrochloric acid.

2.1.2. Barium chloride solution of 200 g/l of $BaCl_2 \cdot 2H_2O$.

2.2. **Procedure**

2.2.1. *General procedure:*

Measure 40 ml of the analysis sample into a 50 ml centrifuge tube; add 2 ml of 2 M hydrochloric acid and 2 ml of barium chloride solution at 200 g/l. Stir with a glass stirrer; rinse the stirrer with a little distilled water and leave to stand for five minutes. Centrifuge for five minutes, then carefully decant the supernatant liquid.

Next wash the barium sulphate precipitate as follows: add 10 ml of 2 M hydrochloric acid, place the precipitate in suspension and centrifuge for five minutes, then carefully decant the supernatant liquid. Repeat the washing procedure twice in the same conditions using 15 ml distilled water each time.

Quantitatively transfer the precipitate, by rinsing with distilled water, into a tared platinum capsule and place over a water bath at 100 °C until fully evaporated. The dried precipitate is calcined several times briefly over a flame until a white residue is obtained. Leave to cool in a desiccator and weigh.

Let m = the mass in milligrams of barium sulphate obtained.

2.2.2. *Special procedure:* sulphited must and wine with a high sulphur dioxide content.

Beforehand, eliminate the sulphur dioxide.

Measure 25 l of water and 1 ml of pure hydrochloric acid (ρ_{20} = 1,15 — 1,18 g/ml) into a 500 ml conical flask equipped with a dropping funnel and an outlet tube. Boil the solution to remove the air and introduce 100 ml of wine through the dropping funnel. Continue boiling until the volume of liquid in the flask has been reduced to about 75 ml and quantitatively transfer it, after cooling, to a 100 ml volumetric flask. Make up to the mark with water. Determine the sulphates in a 40 ml sample as indicated in 2.2.1.

2.3. **Expression of results**

2.3.1. *Calculations*

The sulphate content, expressed in milligrams per litre of potassium sulphate, K_2SO_4 is:

18,67 × m

The sulphate content in musts or wine is expressed in milligrams per litre of potassium sulphate, with no decimal point.

2.3.2. *Repeatability*

up to 1 000 mg/l: r = 27 mg/l

about 1 500 mg/l: r = 41 mg/l

2.3.3. *Reproducibility*

up to 1 000 mg/l: R = 51 mg/l

about 1 500 mg/l: R = 81 mg/l

22 IRON (OIV-AS-322-05-IRON) — CATEGORY IV METHOD

1. PRINCIPLE OF METHODS

REFERENCE METHOD

After suitable dilution of the wine and removal of alcohol, iron is determined directly by atomic absorption spectrophotometry.

USUAL METHOD

After mineralisation of the wine by the hydrogen peroxide solution, the total iron, now in the Fe(III) state, is reduced to the Fe(II) state and is determined using the red coloration produced by orthophenanthroline.

2. REFERENCE METHOD

2.1. **Reagents**

2.1.1. Concentrated standard iron solution containing 1 g FeIII per litre.

Use a standard commercial solution (1 g/l). This solution may be prepared by dissolving 8,6341 g of ferric ammonium sulphate ($FeNH_4(SO_4)_2 \cdot 12H_2O$) in distilled water slightly acidified with 1 M hydrochloric acid and making up to one litre.

2.1.2. Dilute standard iron solution containing 100 mg iron per litre.

2.2. **Apparatus**

2.2.1. Rotary evaporator with thermostatically controlled water bath.

2.2.2. Atomic absorption spectrophotometer equipped with an air-acetylene burner.

2.2.3. Iron hollow cathode lamp.

2.3. **Procedure**

2.3.1. *Preparation of sample*

Remove the alcohol from the wine by reducing the volume of the sample to half its original volume using a rotary evaporator (50 to 60 °C). Make up to the original volume with distilled water.

If necessary, dilute prior to the determination.

2.3.2. *Calibration*

Place 1, 2, 3, 4 and 5 ml of the solution containing 100 mg iron per litre (2.1.2) respectively into a set of 100 ml volumetric flasks and make up to 100 ml with distilled water. The solutions prepared in this way contain 1, 2, 3, 4 and 5 mg of iron per litre respectively.

These solutions should be kept in polyethylene bottles.

2.3.3. *Determination*

Set the wavelength to 248,3 nm. Zero the absorbence scale using distilled water. Aspirate the diluted sample directly into the burner of the spectrophotometer, followed in succession by the five standard solutions (2.3.2). Read off the absorbences. Repeat each measurement.

2.4. **Expression of results**

2.4.1. *Method of calculation*

Plot a graph giving the variation in absorbence as a function of the iron concentration in the standard solutions. Record the mean value of the absorbence obtained with the diluted wine sample on this graph and determine its iron concentration C.

The iron concentration in milligrams per litre of the wine to one decimal place will be

$C \times F$

where F = the dilution factor.

23 COPPER (OIV-AS-322-06) — CATEGORY IV METHOD

1. PRINCIPLE OF THE METHOD

The method is based on the use of atomic absorption spectrophotometry.

2. APPARATUS

2.1. Platinum dish.

2.2. Atomic absorption spectrophotometer.

2.3. Copper hollow cathode lamp.

2.4. Gas supplies: air-acetylene or nitrous oxide/acetylene.

3. REAGENTS

3.1. Metallic copper.

3.2. Nitric acid, HNO_3, concentrated 65 % (HNO_3, ρ_{20} = 1,38 g/ml).

3.3. Dilute nitric acid, 1:2 (v/v).

3.4. **Solution containing copper at 1 g/l**

Use a standard commercial copper solution at 1 g/l. This solution may be prepared by weighing 1 000 g of metallic copper and transferring it without loss to a 1 000 ml volumetric flask. Add 1:2 (v/v) dilute nitric acid (3.3) in just sufficient quantity to dissolve the metal, add 10 ml of concentrated nitric acid (3.2) and make up to the mark with doubly distilled water.

3.5. **Solution containing copper at 100 mg/l**

Introduce 10 ml of the solution prepared as in 3.4 into a 100 ml volumetric flask and make up to the mark with doubly distilled water.

4. PROCEDURE

4.1. **Preparation of sample and determination of copper**

Place 20 ml of the sample in a 100 ml volumetric flask and bring to the reference mark with doubly distilled water. Modify the dilution if necessary.

Read the absorbence of the diluted sample on the atomic absorption spectrophotometer, at a wavelength of 324,8 nm, after having zeroed the absorption scale with distilled water. If necessary, prepare a suitable dilution with doubly distilled water.

4.2. **Preparation of the calibration curve**

Take 0,5-1-2 ml of solution (3.5) (100 mg of copper per litre), put them in 100 ml volumetric flasks making up the volume with doubly distilled water; the solutions obtained contain 0,5-1-2 mg/l of copper respectively. With the absorbence values of these solutions, measured as described in 4.1., plot the calibration curve.

5. EXPRESSION OF RESULTS

Using the measured absorbence of the samples, read off the concentration C in mg/l from the calibration curve.

If F is the dilution factor, the concentration of the copper present is given in milligrams per litre: F × C.

It is quoted to two decimal places.

Notes:

(a) Select the solutions for establishing the calibration curve and the dilutions of the sample appropriate to the sensitivity of the apparatus to be used and the concentration of the copper present in the sample.

(b) Proceed as follows when very low copper concentrations are expected in the sample to be analysed. Place 100 ml of the sample in a platinum dish and evaporate on a water bath at 100 °C until it becomes syrupy. Add 2,5 ml of concentrated nitric acid (3.2) drop by drop, covering the bottom of the dish completely. Carefully ash the residue on an electric hotplate or over a low flame; then place the dish in a muffle furnace set at 500 ± 25 °C and leave for about one hour. After cooling, moisten the ash with 1 ml of concentrated nitric acid (3.2) while crushing it with a glass rod; allow the mixture to evaporate and ash again as before. Place the dish in the muffle furnace again for 15 minutes; repeat the treatment with nitric acid at least three times. Dissolve the ash by adding 1 ml of concentrated nitric acid (3.2) and 2 ml of doubly distilled water to the dish and transfer to a 10 ml flask. Wash the dish three times using 2 ml of doubly distilled water each time. Finally, make up to the volume with doubly distilled water. Make a determination as shown in 4.1. using 10 ml of solution, bearing in mind the concentration factor for the expression of the results.

24 SULPHUR DIOXIDE (OIV-AS-323-04-DIOSU) — CATEGORY II METHOD

1. DEFINITIONS

Free sulphur dioxide is defined as the sulphur dioxide present in the must or wine in the following forms: H_2SO_3, H SO_3 The equilibrium between these forms is a function of pH and temperature.

$H_2SO_3 \leftrightarrow H^+ + HSO_3^-$

H_2SO_3 represents molecular sulphur dioxide.

Total sulphur dioxide is defined as the total of all the various forms of sulphur dioxide present in the wine, either in the free state or combined with its constituents.

2. FREE AND TOTAL SULPHUR DIOXIDE

2.1. **Principle of the methods**

2.1.1. *Reference method*

2.1.1.1. For wines and musts

The sulphur dioxide is carried over by a current of air or nitrogen; it is fixed and oxidised by being bubbled through a dilute and neutral hydrogen peroxide solution. The sulphuric acid formed is determined by titration with a standard solution of sodium hydroxide. Free sulphur dioxide is purged from the wine by entrainment at low temperature (10 °C).

Total sulphur dioxide is purged from the wine by entrainment at high temperature (approximately 100 °C).

2.1.1.2. Rectified concentrated musts

Total sulphur dioxide is extracted from the previously diluted rectified concentrated must by entrainment at high temperature (approximately 100 °C).

2.1.2. *Rapid method of determination (for wines and musts)*

Free sulphur dioxide is determined by direct iodometric titration.

Combined sulphur dioxide is subsequently determined by iodometric titration after alkaline hydrolysis. When added to the free sulphur dioxide, it gives the total sulphur dioxide.

2.2. **Reference method**

2.2.1. *Apparatus*

2.2.1.1. The apparatus used should conform to the diagram shown below, particularly with regard to the condenser.

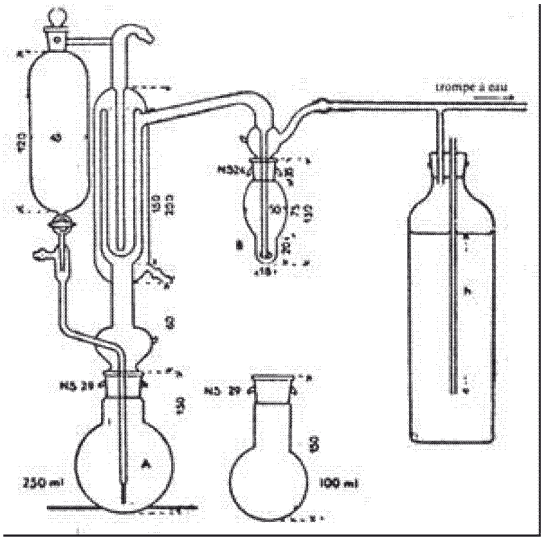

Figure 1

The dimensions given are in millimetres. The internal diameters of the four concentric tubes forming the condenser are 45, 34, 27 and 10 mm

The gas feed tube to the bubbler B ends in a small sphere of 1 cm diameter with 20 0,2 mm diameter holes around its largest horizontal circumference. Alternatively, this tube may end in a frit glass plate which produces a large number of very small bubbles and thus ensures good contact between the liquid and gaseous phases.

The gas flow through the apparatus should be approximately 40 litres per hour. The bottle on the right of the diagram is intended to restrict the pressure reduction produced by the water pump to 20 to 30 cm of water. To regulate the vacuum to its correct value, a flow meter with a semi-capillary tube should be installed between the bubbler and the bottle.

2.2.1.2. A microburette.

2.2.2. *Reagents*

2.2.2.1. Phosphoric acid, 85 % (H_3PO_4) (ρ_{20} = 1,71 g/ml).

2.2.2.2. Hydrogen peroxide solution, 9,1 g H_2O_2/litre (three volumes).

2.2.2.3. Indicator reagent:

Methyl red	100 mg
Methylene blue	50 mg
Alcohol, 50 % vol.	100 ml

2.2.2.4. Sodium hydroxide (NaOH) solution, 0,01 M.

2.2.3. *Determination of free sulphur dioxide*

2.2.3.1. Procedure

The wine must be kept in a full and stoppered bottle at 20 °C for two days before the determination.

— Place 2 to 3 ml of hydrogen peroxide solution (2.2.2.2) and two drops of the indicator reagent in the bubbler B and neutralise the hydrogen peroxide solution with the 0,01 M sodium hydroxide solution (2.2.2.4). Connect the bubbler to the apparatus.

— Introduce 50 ml of the sample and 15 ml of phosphoric acid (2.2.2.1) into flask A of the entrainment apparatus. Connect the flask into the apparatus.

— Bubble air (or nitrogen) through it for 15 minutes. The free sulphur dioxide carried over is oxidised to sulphuric acid. Remove the bubbler from the apparatus and titrate the acid which has formed against the 0,01 M sodium hydroxide solution (2.2.2.4). Let n ml be the volume used.

2.2.3.2. Expression of results

The liberated sulphur dioxide is expressed in mg/l to the nearest whole number.

2.2.3.2.1. Calculation

The free sulphur dioxide in milligrams per litre is 6,4 n.

2.2.4. *Determination of total sulphur dioxide*

2.2.4.1 Procedure

2.2.4.1.1. For rectified concentrated musts, use the solution obtained by diluting the sample to be analysed to 40 % (m/v) as indicated in the Chapter 'Total acidity', section 5.1.2. Introduce 50 ml of this solution and 5 ml of phosphoric acid (2.2.2.1) into the 250 ml flask A of the entrainment apparatus. Connect the flask into the apparatus.

2.2.4.1.2. Wines and musts

If the estimated concentration in the sample is no greater than 50 mg of total SO_2 per litre, introduce 50 ml of the sample and 15 ml of phosphoric acid (2.2.2.1) into flask A of the entrainment apparatus. Connect the flask into the apparatus.

If the estimated concentration in the sample is greater than 50 mg of total SO_2 per litre, introduce 20 ml of the sample and 5 ml of phosphoric acid (2.2.2.1) into flask A of the entrainment apparatus. Connect the flask into the apparatus.

Place 2 to 3 ml of hydrogen peroxide solution (2.2.2.2) in the bubbler B, neutralised as before, and bring the wine in the flask A to the boil using a small flame of 4 to 5 cm height which should directly touch the bottom of the flask. Do not put the flask on a metal plate but on a disc with a hole of approximately 30 mm diameter in it. This is to avoid overheating substances extracted from the wine that are deposited on the walls of the flask.

Maintain boiling while passing a current of air (or nitrogen). Within 15 minutes the total sulphur dioxide has been carried over and oxidised. Determine the sulphuric acid which has formed by titration with the 0,01 M sodium hydroxide solution (2.2.2.4).

Let n ml be the volume used.

2.2.4.2. Expression of results

Total sulphur dioxide is expressed in mg/l and in mg/kg of total sugar to the nearest whole number.

2.2.4.2.1. Calculation

— Wines and musts

Total sulphur dioxide in milligrams per litre:

— samples low in sulphur dioxide (50 ml test sample):

$6,4 \times n$

— other samples (20 ml test sample):

$16 \times n$

— Rectified concentrated musts

Total sulphur dioxide in milligrams per kilogram of total sugars (50 ml prepared test sample) (2.2.4.1.1):

$(1,600 \times n)/(P)$

where P = percentage concentration (m/m) of total sugars

2.2.3.4.2. Repeatability (r)

50 ml test sample < 50 mg/l; r = 1 mg/l

20 ml test sample > 50 mg/l; r = 6 mg/l

2.2.3.4.3. Reproducibility (R)

50 ml test sample < 50 mg/l; R = 9 mg/l

20 ml test sample > 50 mg/l; R = 15 mg/l