CORESTA RECOMMENDED METHOD N° 33

DETERMINATION OF ACETATE IN CIGARETTE PAPER

(January 1993)

1 - FIELD OF APPLICATION

The method is applicable to all kinds of cigarette paper.

2 - DEFINITIONS

Acetate in cigarette paper influences the burning speed of the cigarette paper and therefore the puff number of the cigarette. Acetate usually is added to the cigarette paper as sodium or as potassium salt.

3 - REFERENCES

BERGMEYER H.U. (Hrsgb.) (1974) Methoden der enzymatischen Analyse. 3. Aufl. Bd. 1, 119 - 125. Verlag Chemie, Weinheim, FRG.

BERGMEYER H.U. (Hrsgb.) (1974) Methoden der enzymatischen Analyse, 3. Aufl. Bd. 2, 1566 - 1574. Verlag Chemie, Weinheim, FRG.

ISO 187: 1990
Paper, board and pulps - Standard atmosphere for conditioning and testing and procedure for monitoring the atmosphere and conditioning of samples.

ISO 287: 1985
Paper and board - Determination of moisture content - Oven drying method.

4 - PRINCIPLE

Acetate (acetic acid) is converted in the presence of the enzyme acetyl-CoA synthetase (ACS) with adenosin-5'-triphosphate (ATP) and coenzyme A (CoA) to acetyl-CoA (1).

$$\text{(1)}\quad \text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{acetyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

Acetyl-CoA reacts with oxaloacetate to citrate in the presence of citrate synthase (CS) (2).

$$\text{(2)}\quad \text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \xrightarrow{\text{CS}} \text{citrate} + \text{CoA}$$

The oxaloacetate required for reaction (2) is formed from malate and nicotinamide-adenine dinucleotide (NAD) in the presence of malate dehydrogenase (MDH) (3). In this reaction NAD is reduced to NADH.

(3) Malate + NAD^+ $\xleftarrow{\text{MDH}}$ oxaloacetate + H^+ + NADH

The determination is based on the formation of NADH measured by the increase in absorbance at 340 nm. Since a preceding indicator reaction is used, the amount of NADH formed is not linearly proportional to the acetic acid concentration (for calculations, see below).

5 - MATERIALS AND EQUIPMENT

It is recommended to use test kits for the enzymatic acetate determination. Such test kits are available from various suppliers (*e.g.* BOEHRINGER, Mannheim).

A test kit contains:

- a bottle with approximately 32 cm^3 of solution, consisting of: triethanolamine buffer, pH 8.4; L-malic acid, 134 mg; magnesium chloride, 67 mg; stabilizers (bottle 1).
- a bottle with approximately 280 mg lyophilisate, consisting of: ATP, 175 mg; CoA, 18 mg; NAD, 86 mg; stabilizers (bottle 2).
- a bottle with approximately 0.4 cm^3 of enzyme suspension, consisting of: malate dehydrogenase, 1100 U; citrate synthase, 270 U (bottle 3).
- a bottle with lyophilisate acetyl-CoA-synthetase, 5 U (bottle 4).

Sodium acetate trihydrate, p.A.
Distilled water
ERLENMEYER flasks, 250 cm^3
Calibrated flasks, 1000 cm^3, 100 cm^3
Filtration funnel, 8 cm diameter
Folded filters, 125 mm diameter
Pipettes, 10.0 cm^3, 5.0 cm^3, 2.50 cm^3, 1.00 cm^3
0.200 cm^3 Micropipette
UV Spectrophotometer, double beam
Glass cuvettes, volume 5 cm^3, 10 mm light path
Ultrasonic bath
Analytical balance

6 - STANDARD SOLUTIONS

For the calibration of the method standard solutions containing 300, 100 and 50 ppm sodium acetate trihydrate in distilled water are used.

7 - PROCEDURE

a) *Preparation of the reagent solutions.*

- The solution of bottle 1 is used undiluted (solution 1).
- Dissolve the content of bottle 2 in 7 cm^3 of distilled water (solution 2).
- Use the suspension of bottle 3 undiluted (solution 3).
- Dissolve the content of bottle 4 with 0,25 cm^3 of distilled water (solution 4).

Solution 1 is stable for 1 year at +4°C. Solution 1 has to be brought up to 20-25°C before use. Solution 2 is stable for 4 weeks at +4°C. Solution 3 is stable for 1 year at +4°C. Solution 4 is stable for 5 days at +4°C.

The overall activities of the enzyme systems must be 100 ± 5% .

b) *Sample preparation.*

1.000 g of sliced cigarette paper (conditioned according to ISO 187) is extracted in 100 cm^3 of redistilled water in a 250 cm^3 ERLENMEYER flask for 30 minutes by the aid of an ultrasonic bath. The paper extract is filtered through a folded filter. Reaction solutions with distilled water (blank), with the standard solutions and with the paper extract are prepared in the following manner:

Pipette into cuvettes	Blank	Sample and standard
Solution 1	1.00 cm^3	1.00 cm^3
Solution 2	0.20 cm^3	0.20 cm^3
Dist. Water	2.00 cm^3	1.90 cm^3
Paper extract or standard solution	-	0.10 cm^3-
Mix and read absorbances of the solutions (A_0) Addition of:		
Solution 3	0.01 cm^3	0.01 cm^3
Mix and read absorabnaces of the solutions (A_1) after approximately 3 minutes. Start the reaction by additon of :		
Solution 4	0.02 cm^3	0.02 cm^3
Mix, wait until the reaction has stopped (approximately 10 - 15 minutes) and read the absorbances of the solutions (A_2). If the reaction has not stopped after 15 minutes, continue to read the absorbances at 2 minutes intervals until the absorbance increases constantly for 2 minutes. If the absorbance A_2 increases constantly, extrapolate the absorbance to the time of the addition of solution 4.		

Extreme care must be taken by pipetting sample extracts.

Parameters of the UV spectrophotometer:

Wavelength:	340 nm
Cuvettes:	Glass, 5 cm^3 volume, 10 mm light path
Temperature:	20 - 25°C
Final volume:	3.23 cm^3

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

Determine the absorbance differences ($A_1 - A_0$) and ($A_2 - A_0$) for the blank and the sample. With preceding indicator reactions, there is no linear proportionality between the measured absorbance difference and the acetic acid concentration.

The following formula, which should generally be used for preceding indicator reactions, serves to calculate the $\delta A_{\text{Acetic acid}}$.

$$\delta A_{\text{Acetic acid}} = \left[(A_2 - A_0)_{\text{sample}} \frac{(A_1 - A_0)^2_{\text{sample}}}{(A_2 - A_0)_{\text{sample}}} \right]$$

$$- \left[(A_2 - A_0)_{\text{blank}} - \frac{(A_1 - A_0)^2_{\text{blank}}}{(A_2 - A_0)_{\text{blank}}} \right]$$

The absorbance differences measured should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results. The amount of acetic acid present in the cuvette should range between 1 µg and 15 µg (measured at 340 nm). The sample solution must therefore be diluted sufficiently to yield an acetic acid concentration between 0.01 and 0.15 g/l.

The absorbance difference is recommended to be between 0.2 to 0.4.

8. CALCULATION

According to the general equation for calculating the concentration:

$$\mathbf{C} = \frac{\mathbf{V} \times \mathbf{MW}}{\in \times \mathbf{d} \times \mathbf{v} \times \mathbf{1000}} \times \delta \mathbf{A} [\mathbf{g/l}], \text{ where}$$

V = final volume [cm^3]
v = sample volume [cm^3]
MW = molecular weight of the substance to be assayed [g x mol^{-1}]
d = light path [cm]
$\in$ = absorption coefficient of NADH at 340 nm = 6.3 [l x $mmol^{-1}$ x cm^{-1}]

It follows for acetic acid (calculated as anhydrous acetic acid):

$$\mathbf{C} = \frac{\mathbf{3.23} \times \mathbf{60.05}}{\mathbf{6.3} \times \mathbf{0.1} \times \mathbf{1000}} \times \delta \mathbf{A} = \frac{\mathbf{1.940}}{\mathbf{6.3}} \delta \mathbf{A}$$

C = [g anhydrous acetic acid / l sample solution]

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

If 1.000 g paper is extracted in 100 cm^3 distilled water, the reading of C corresponds directly to % anhydrous acetic acid in the paper at the equilibrium humidity.

9 - SPECIFITY AND QUALITY OF THE METHOD

The method is specific for acetic acid. In collaborative tests, carried out by the CORESTA Smoke Study Group Task Force: "Analytical Methods for Cigarette Papers", a standard deviation of 0.0087 % and a coefficient of variation of 1.46 % (0.59 % anhydrous acetic acid in the cigarette paper) were determined.

10 - ANALYTICAL REPORT

The analytical report must contain:

- Brand name of the paper
- Name of the manufacturer or supplier of the paper
- Details of sampling procedure
- Details of conditioning
- Date of test
- Room temperature and relative humidity in the test room
- Humidity of the paper (ISO 287)
- % anhydrous acetic acid in the paper at the equilibrium humidity.

When paper samples are taken from cigarettes the results might be influenced by external parameters (*e.g.* additives of the tobacco blend).

CORESTA RECOMMENDED METHOD N° 34

DETERMINATION OF CITRATE IN CIGARETTE PAPER

(January 1993)

1 - FIELD OF APPLICATION

The method is applicable to all kinds of cigarette paper.

2 - DEFINITIONS

Citrate in cigarette paper influences the burning speed of the cigarette paper and therefore the puff number of the cigarette. Citrate usually is added to the cigarette paper as trisodium salt, as tripotassium salt or as a mixture of trisodium and tripotassium salts.

3 - REFERENCES

BERGMEYER H.U. (Hrsgb.) (1974) Methoden der enzymatischen Analyse. 3. Aufl. Br. 2, pp 1609; 1613 - 1615. Verlag Chemie, Weinheim, FRG.

BOEHRINGER Mannheim. (1989) Methoden der enzymatischen Lebensmittel-analytik. Arbeitsanleitungen zur Analyse. (Internal Company document).

ISO 187: 1990
Paper, board and pulps - Standard atmosphere for conditioning and testing and procedure for monitoring the atmosphere and conditioning of samples.

ISO 287: 1985
Paper and board - Determination of moisture content - Oven drying method.

4 - PRINCIPLE

Citric acid (citrate) is converted to oxaloacetate and acetate in the reaction catalyzed by the enzyme citrate lyase (CL) (1).

$$\text{(1) Citrate} \xrightarrow{\text{CL}} \text{oxaloacetate + acetate}$$

In the presence of the enzymes malate dehydrogenase (MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by reduced nicotinamide-adenine dinucleotide (NADH) (2) (3).

$$\text{(2) Oxaloacetate + NADH + } H^+ \xrightarrow{\text{MDH}} \text{L-malate + } NAD^+$$

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

The amount NADH oxidized in reactions (2) and (3) is stoichiometric with the amount of citrate. NADH is determined by means of its absorbance at 340 nm.

5 - MATERIALS AND EQUIPMENT

It is recommended to use test kits for the enzymatic citrate determination. Such test kits are available from various suppliers (e.g. BOEHRINGER, Mannheim).

A test kit contains:

- a bottle containing 1.4 g lyophilisate, consisting of: glycilglycine buffer, pH 7.8; malate dehydrogenase, 136 U; L-lactate dehydrogenase, 280 U; NADH, 6 mg; stabilizers (bottle 1).
- a bottle with 50 mg lyophilisate citrate lyase, 12 U (bottle 2).

Citric acid monohydrate, p.A.
Distilled water
ERLENMEYER flasks, 250 cm^3
Calibrated flasks, 1000 cm^3, 100 cm^3
Filtration funnel, 8 cm diameter
Folded filters, 125 mm diameter
Pipettes, 10.0 cm^3, 5.0 cm^3, 2.50 cm^3, 1.00 cm^3
0,200 cm^3 Micropipette
UV Spectrophotometer, double beam
Glass cuvettes, volume 5 cm^3, 10 mm light path
Ultrasonic bath
Analytical balance

6 - STANDARD SOLUTIONS

For the calibration of the method, standard solutions containing 50, 25 and 12.5 ppm citric acid monohydrate p.A. in distilled water are used.

7 - PROCEDURE

Extreme care should be taken by pipetting sample extracts.

a) *Preparation of the reagent solutions (for ten determinations).*

- Dissolve the content of the bottle 1 of the test kit in 12 cm^3 of distilled water (solution 1).
- Dissolve the content of the bottle 2 of the test kit in 0.3 cm^3 of distilled water (solution 2).

Solution 1 is stable for 2 weeks at +4°C or for 4 weeks at -20°C. Solution 1 has to be brought up to 20-25°C before use. Solution 2 is stable for 1 week at +4°C or for 4 weeks at -20°C.

The overall activity of the enzyme systems should be 100 ± 5 %.

b) *Sample preparation.*

1.000 g of sliced cigarette paper (conditioned according to ISO 187) is extracted in 100 cm^3 of distilled water in a 250 cm^3 ERLENMEYER flask for 30 minutes by the aid of an ultrasonic bath. The paper extract is filtered through a folded filter. Reaction solutions with distilled water (blank), with the standard solutions for calibration and with the paper extract are prepared in the following manner:

Pipette into cuvettes	*Blank*	Sample and standard
Solution 1	1.00 cm^3	1.00 cm^3
Dist. Water	2.00 cm^3	1.80 cm^3
Paper extract or standard solution	-	0.20 cm^3
Mix, read absorbance of the solutions (A_1) after approximately 5 minutes, and start the reaction by additon of:		
Solution 2	0.02 cm^3	0.02 cm^3
Mix; on completion of the reaction (approximately 5 minutes), read the absorbance of the solution (A_2).		

Parameters of the UV spectrophotometer:

Wavelength:	340 nm
Cuvettes:	Glass, 5 cm^3 volume, 10 mm light path
Temperature:	20 - 25°C
Final volume:	3.02 cm^3

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

Determine the absorbance differences ($A_1 - A_2$) for both blank and sample. Substract the absorbance difference of the blank from the absorbance difference of the sample.

$$\mathbf{A} = \delta\mathbf{A}_{\mathbf{sample}} - \delta\mathbf{A}_{\mathbf{blank}}$$

Occasionally a negative value with $(A_1 - A_2)_{blank}$ is obtained. This value is then to be added to $(A_1 - A_2)_{sample}$ according to the calculation formula.

The absorbance differences measured should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results. If the absorbance difference of the sample (δA_{sample}) is higher than 0.850 (measured at 340 nm), the concentration of citric acid in the sample solution is too high. The sample solution is to be diluted to get a citric acid concentration in the cuvette below of 80 µg.

The absorbance difference is recommended to be between 0.2 to 0.4.

8 - CALCULATIONS

According to the general equation for calculating the concentration :

$$\mathbf{C} = \frac{\mathbf{V} \times \mathbf{MW}}{\in \times \mathbf{d} \times \mathbf{v} \times \mathbf{1000}} \times \delta\mathbf{A}\,[\mathbf{g/l}], \text{ where}$$

V = final volume [cm^3]
v = sample volume [cm^3]
MW = molecular weight of the substance to be assayed [g x mol^{-1}]
d = light path [cm]
$\in$ = absorption coefficient of NADH at 340 nm = 6.3 [l x mol^{-1} x cm^{-1}]

It follows for citric acid (calculated as the anhydrous acid):

$$\mathbf{C} = \frac{\mathbf{3.02} \times \mathbf{192.1}}{\mathbf{6.3} \times \mathbf{0.2} \times \mathbf{1000}} \times \delta\mathbf{A} = \frac{\mathbf{2.90}}{\mathbf{6.3}} \times \delta\mathbf{A}$$

C = [g citric acid / l sample solution]

It follows for citric acid (calculated as citric acid monohydrate):

$$\mathbf{C} = \frac{\mathbf{3.02} \times \mathbf{210.1}}{\mathbf{6.3} \times \mathbf{0.2} \times \mathbf{1000}} \times \delta\mathbf{A} = \frac{\mathbf{3.17}}{\mathbf{6.3}} \times \delta\mathbf{A}$$

c = [g citric acid monohydrate / l sample solution]

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

If 1.000 g paper is extracted with 100 cm^3 of distilled water, the reading "c" corresponds directly to % citric acid monohydrate or citric acid in the paper at equilibrium humidity.

9 - SPECIFITY AND QUALITY OF THE METHOD

The method is specific for citric acid. In collaborative tests, carried out by the CORESTA Smoke Study Group Task Force: "Analytical Methods for Cigarette Papers", a standard deviation of 0.021% and a coefficient of variation of 5.8% (0.37% citric acid monohydrate in the cigarette paper) and a standard deviation of 0.105% and a coefficient of variation of 4.0% (2.57% citric acid monohydrate in the cigarette paper), respectively, were determined.

10 - ANALYTICAL REPORT

The analytical report must contain:

- Brand name of the paper
- Name of the manufacturer or supplier of the paper
- Details of the sampling procedure
- Details of conditioning
- Date of test
- Room temperature and relative humidity in the test room
- Humidity of the paper (ISO 287)
- % of citric acid monohydrate in the paper at equilibrium humidity.

When paper samples are taken from cigarettes the results might be influenced by external parameters (e.g. additives of the tobacco blend).

CORESTA RECOMMENDED METHOD N° 45

DETERMINATION OF PHOSPHATE IN CIGARETTE PAPER

(January 1998)

1. FIELD OF APPLICATION

The method is applicable to all types of cigarette paper.

2. DEFINITIONS

Phosphates are salts of orthophosphoric acid. Phosphates in cigarette paper influence the ash appearance of a burning cigarette and the burning rate of the cigarette paper and therefore the puff number of the cigarette. Phosphates are usually added to the cigarette paper as disodium hydrogen phosphate or monoammonium dihydrogen phosphate.

3. REFERENCES

ISO 186:1994
Paper and board - Sampling to determine average quality.

ISO 187:1990
Paper, board and pulps - Standard atmosphere for conditioning and testing and procedure for monitoring the atmosphere and conditioning of samples.

ISO 287:1985
Paper and board - Determination of moisture content - Oven-drying method.

ISO 5725-2:1994
Accuracy (trueness and precision) of measurement methods and results -
Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

4. PRINCIPLE

Phosphate ions (PO_4^{3-}) react in an acid solution with ammonium vanadate and ammonium heptamolybdate to form a yellow-coloured molybdenum vanadate phosphoric acid complex which can be measured photometrically.

5. APPARATUS AND MATERIALS

5.1. *Apparatus*

- analytical balance with an accuracy of 0,001 g;
- UV Spectrophotometer, double beam;
- glass or plastic cuvettes, 10 mm light path;
- micro pipettes;
- pipette 50 cm^3;
- calibrated flask 50 cm^3 and 100 cm^3.

5.2. *Materials*

- distilled water;
- 1 mol·L^{-1} hydrochloric acid;
- ashfree filter papers;
- P-reagent.

  Note: reagent will deteriorate with time as a result of temperature and radiation. The response should be checked periodically.

It is recommended that test solutions containing ammonium vanadate and ammonium heptamolybdate hereafter referred to as P-reagent are used. Such test kits are available from various suppliers (*e.g.* E. MERCK, Darmstadt).

6. STANDARD SOLUTIONS

6.1. *Preparing the standard solutions for the calibration curve*

To calibrate the equipment, standard solutions containing 0, 5, 10, 15 and 20 mg PO_4^{3-} per 50 cm^3 of 0,5 mol·L^{-1} hydrochloric acid are used. Various phosphates can be used, *e.g.* disodium phosphate, potassium phosphate, monoammonium phosphate or phosphoric acid, all with a purity of p.a.

Weight of phosphate salts for a concentration of 20 mg PO_4^{3-} / 50 cm^3

	mol. weight (g/mol)	weight (mg/50 cm^3)
KH_2PO_4	136,09	28,66
$K_3PO_4 \cdot 3H_2O$	266,32	56,09
$Na_3PO_4 \cdot 12H_2O$	380,12	80,05
Na_2HPO_4	141,96	29,90
$(NH_4)_2HPO_4$	132,06	27,81
PO_4^{3-}	94,97	

Prepare 5 dilutions between 0 and 20 mg PO_4^{3-} in 50 cm^3 in 0,5 mol·L^{-1} hydrochloric acid equivalent to 0-2% (m/m) of phosphate in cigarette paper. Perform six replicate measurements per calibration point.

(PO_4^{3-}) content in solution (%) (m/m)	(PO_4^{3-}) in cigarette paper (%) (m/m)
0	0
0,01	0,5
0,02	1,0
0,03	1,5
0,04	2,0

Preparation of the blank sample:
Pipette 1 cm^3 P-reagent + 3 cm^3 0,5 mol·L^{-1} hydrochloric acid into a cuvette.

6.2. *Measuring the standard solutions*

Pipette 1 cm^3 P-reagent + 2,5 cm^3 0,5 $mol \cdot L^{-1}$ hydrochloric acid + 0,5 cm^3 standard solution into a cuvette. After approx. 5 minutes the absorbance can be measured on the UV spectrophotometer at a wavelength of 430 nm. Perform six replicate measurements for each calibration point.

7. TEST PROCEDURE

Preparation and measurement of the paper sample.

7.1. *Preparation*

- weigh approx. 1 g of shredded cigarette paper conditioned according to ISO 187:1990 in a 100 cm^3 Erlenmeyer-flask and correct absorbancy reading of the UV spectrophotometer to 1,000 g of cigarette paper;
- add 50 cm^3 0,5 $mol \cdot L^{-1}$ hydrochloric acid;
- leave suspension of paper in 0,5 $mol \cdot L^{-1}$ hydrochloric acid for 20 min, at room temperature (leave to stand, stir or swirl);
- filter the suspension after 20 min. through an ashfree filter paper;
- use the filtrate for the photometric determination of PO_4^{3-}.

7.2. *Measurement of the paper sample*

Pipette 1 cm^3 P-reagent and **a** cm^3 0,5 $mol \cdot L^{-1}$ hydrochloric acid and **b** cm^3 of test solution into a cuvette (**a** cm^3 + **b** cm^3 = 3 cm^3) and by using the UV Spectrophotometer measure the absorbance at a wavelength of 430 nm after approx. 5 min.

Choose **a** cm^3 and **b** cm^3 test solution so that the absorbancy is in the upper quartile range of the calibration curve. The PO_4^{3-} content of the cigarette paper can be read from the calibration curve once the absorbancy has been corrected to 0,5 cm^3 test solution.

8. REPEATABILITY AND REPRODUCIBILITY

A collaborative test (8 laboratories / 6 replicates each) covering the range of 0,2-1,0% (m/m) phosphates in cigarette paper gave the following results:

r = repeatability

R = Reproducibility

see ISO 5725-2:1994

Mean PO_4^{3-} (%) (m/m)	1,70	0,68	0,95
r (%)	0,06901	0,04056	0,02703
R (%)	0,196	0,102	0,129

9. TEST REPORT

The test report shall contain:

- date of test;
- material identification (brand name, supplier name, etc.);
- sampling procedure must be stated if different to ISO 186:1994;
- the paper conditioning parameters must be stated if different to ISO 187:1990;
- percent PO_4^{3-} in cigarette paper as mean value and standard deviation and number of replicate measurements;
- origin of paper (bobbin, spill etc,).

Note: when paper samples are taken from cigarettes (spills) the results may be influenced by external parameters (*e.g.* additives in the tobacco blend).