OIV-MA-AS323-11 Method for the determination of potassium polyaspartate in wine by high-performance liquid chromatography coupled with a fluorescence detector

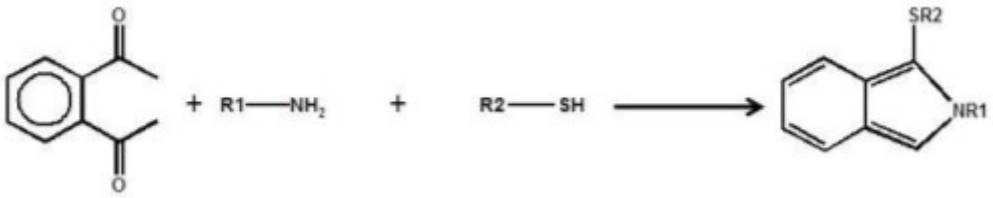
Type IV method

1. Scope of application

This method is applicable to the analysis of potassium polyaspartate (KPA) in wines at concentrations higher than 40 mg/L.

2. Principle

The procedure consists of carrying out the determination of aspartic acid in wine before and after acid hydrolysis, by derivatisation with ortho-phthalaldehyde (OPA) followed by chromatographic analysis coupled with a fluorescence detector. The difference in the aspartic acid content between the hydrolysed sample and non-hydrolysed sample will indicate the level of addition of polyaspartate.



Ortho-Ortho-phthalaldehyde (OPA) Derivatised AA
R1-NH_2: aspartic acid
R2-SH: mercaptoethanol

3. Reagents and products

Use ultra-pure water (EN ISO 3696 Grade 3 or double-distilled water)

For acid hydrolysis:

3.1. 10 g/L Sodium metabisulphite ($Na_2S_2O_5$, CAS No.: 7681-57-4) solution: weigh 5 grams of sodium metabisulphite into a 500-mL Class A flask and make up to the mark with ultra-pure water.

3.2. 6 M Hydrochloric acid (HCl, CAS No.: 7647-01-0)

3.3. 5 M Sodium hydroxide (NaOH, CAS No.: 1310-73-2)

Standard solutions:

3.4. Aspartic acid (DL-aspartic acid $C_4H_7NO_4$, purity $\geq$ 99%, CAS No.: 617-45-8)

3.4.1. Stock solution 1: solution of 8000 mg/L aspartic acid in ultra-pure H_2O

3.4.2. Stock solution 2: solution of 200 mg/L aspartic acid in ultra-pure H_2O

3.5. Aminocaproic acid ($C_6H_{13}NO_2$, purity ≥ 99%, CAS No.: 60-32-2)

3.5.1. Stock solution of aminocaproic acid at 1000 mg/L in ultra-pure H_2O (internal standard)

Calibration solutions prepared through dilution of stock solutions 1 and 2 in double-distilled H_2O. The reference values are as follows:

- 2 mg/L STD1: take 0.200 mL of stock solution 2 (3.4.2.) and make up to the mark in a 20-mL flask with ultra-pure H_2O
- 10 mg/L STD2: take 1.000 mL of stock solution 2 (3.4.2.) and make up to the mark in a 20-mL flask with ultra-pure H_2O
- 50 mg/L STD3: take 5.000 mL of stock solution 2 (3.4.2.) and make up to the mark in a 20-mL flask with ultra-pure H_2O
- 100 mg/L STD4: take 0.250 mL of stock solution 1 (3.4.1.) and make up to the mark in a 20-mL flask with ultra-pure H_2O
- 250 mg/L STD5: take 0.625 mL of stock solution 1 (3.4.1.) and make up to the mark in a 20-mL flask with ultra-pure H_2O
- 500 mg/L STD6: take 1.250 mL of stock solution 1 (3.4.1.) and make up to the mark in a 20-mL flask with ultra-pure H_2O

Derivatising solution:

3.6. Sodium tetraborate decahydrate (solid, purity > 99%, CAS No. 1303-96-4)

3.6.1. 0.1 M Sodium tetraborate decahydrate buffer solution with a pH of 10.5: dissolve 19.1 g sodium tetraborate and make up to the mark in a 500-mL flask with ultra-pure water. Check the pH value.

3.7. Ortho-phthalaldehyde (OPA) ($C_8H_6O_2$, purity ≥ 99%, CAS No.: 643-79-8)

3.8. Mercaptoethanol (C_2H_6OS, purity ≥ 99%, CAS No.: 60-24-2)

3.9. Derivatising solution: add 100 mg OPA, 200 µL mercaptoethanol and 1 mL methanol to a 10 mL-flask and make up to the mark with the 0.1 M sodium tetraborate decahydrate buffer solution with a pH of 10.5. The solution should be prepared just before use.

Mobile phases for HPLC:

3.10. HPLC-grade methanol (liquid)

3.11. HPLC-grade tetrahydrofuran (liquid)

3.12. Anhydrous sodium acetate (CAS No.: 127-09-3)

3.12.1. 0.05 M Sodium acetate buffer solution: dissolve 2.05 g anhydrous sodium acetate and make up to the mark in a calibrated 500-mL flask with ultra-pure water.

3.12.2. HPLC-grade acetonitrile (CH_3CN) (liquid)

3.12.3. Ultra-pure water (e.g. EN ISO 3696 Grade 3 or double-distilled water)

3.12.4. Mobile phase:

[eluent A]: ultra-pure water,

[eluent B]: 0.05 M sodium acetate / tetrahydrofuran (96:4) buffer solution,

[eluent C]: methanol,

[eluent D]: acetonitrile.

4. Equipment and apparatus

Unless otherwise specified, the glassware required to prepare the solutions should be class A.

4.1. Hot plate

4.2. 4-mL Tinted-glass vial with screw cap

4.3. 0.100-1.000-mL Micropipette

4.4. Cellulose-acetate-membrane syringe filter with porosity of 0.20 µm

4.5. Precision balance

4.6. Calibrated flasks

4.7. HPLC system that includes a quaternary pump, automatic sampler, compartment with thermostat for the column and FLD

4.8. Column: polar endcapped C18 (e.g. Syncronis aQ 4.6 x 250 mm; 5 µm)

5. Procedure

The procedure is divided into three phases: hot acid hydrolysis of the wine sample; the process of preparation of the samples (both of the calibration solutions and of the wines before and after hydrolysis), which are analysed by HPLC-FLD to determine the aspartic acid concentration; and HPLC-FLD analysis.

Phase 1: Acid hydrolysis

Pour the following successively into a 4-mL tinted-glass vial with screw cap (4.2.):

- 0.2 mL solution of 10 g/L sodium metabisulphite (3.1.),

- 2 mL wine sample,
- 2 mL 6 M HCl (3.2.);
- heat to 108°C (± 2 °C) on a hot plate for 72 hours;

pour into a 10-mL flask, add 2.5 mL 5 M NaOH (3.3.) and make up to the mark with ultra-pure water.

The verification of the acid hydrolysis process is detailed in paragraph 6.

Phase 2: Preparation for HPLC analysis

The method envisages a derivatisation reaction of aspartic acid with ortho-phthalaldehyde (OPA).

To prepare the samples for analysis by HPLC, proceed as follows:

Calibration solutions and wine samples before hydrolysis:

- take a 1 mL sample of the solution for analysis and micro-filter (0.20 µm filter) it into a 20-mL flask,
- add 0.2 mL internal standard (3.5.1.),
- make up to the mark with ultra-pure water.

Samples after hydrolysis:

- take a 5 mL sample of the solution for analysis and micro-filter (0.20 µm filter) it into a 20-mL flask,
- add 0.2 mL internal standard (3.5.1.),
- make up to the mark with ultra-pure water.

Phase 3: HPLC analysis

The instrumental parameters for analysis by HPLC-FLD, for example, are as follows:

Oven temperature: 40 °C

Injection: 10 µL

FLD Wavelength (□): □ex = 340 nm; □em = 450 nm

The separation is conducted in gradient mode (see the eluents in point 3.15.):

Time	%B	%C	%D	Flow
0.00	100.0	0.0	0.0	1.1

3.00	100.0	0.0	0.0	1.1
15.00	50.0	25.0	25.0	1.1
17.00	84.0	8.0	8.0	1.1
18.00	100.0	0.0	0.0	1.1
Stop time: 21 min + 2 min post time				

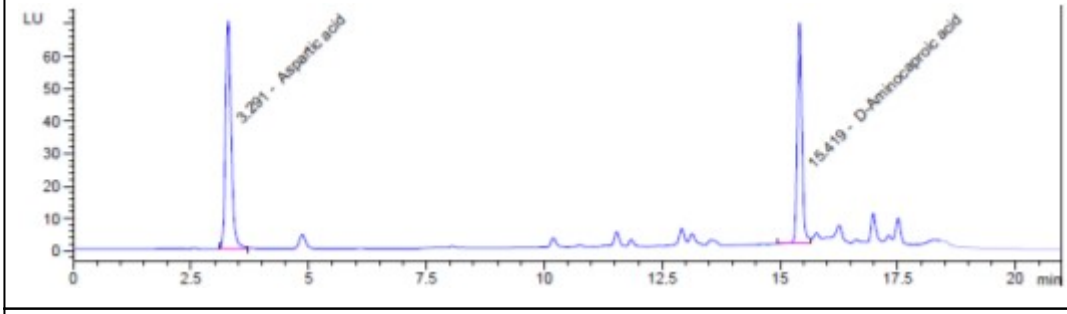
The following is an example of the automated derivatisation mode with an autosampler:

Reagent positions in the autosampler:

- position 1: methanol,
- position 3: OPA,
- position 4: empty vial,
- position 11: ultra-pure water.

Derivatisation phases:
draw 2.0 µL from the air,
draw 20.0 µL from vial 1,
transfer 20.0 µL into vial 4,
draw 5.0 µL from the sample,
transfer 5.0 µL into seat,
draw 0.0 µL from vial 1 (to clean the outside of the needle),
draw 5.0 µL from vial 3,
transfer 5.0 µL into seat,
mix 10.0 µL in seat, 10 times,
wait 0.50 min,
inject.

If the results obtained are higher than the calibration curve limit, dilute the sample as appropriate and perform the test again.



Chromatogram derived from a red wine

6. Quality control

For each series of analyses, quality control should be carried out through analysis of a wine sample to which 100 mg/L aspartic acid is added.

The sample, prepared according to point 5, is analysed at the beginning of the series. The results obtained, given in terms of the percentage yield, are recorded on a control chart.

7. Method characteristics: Intra-laboratory validation parameters

Linearity

Non-hydrolysed, non-derivatised calibration solutions of known concentration are used to simulate the entire analytical process. Each sequence, to be acceptable, should contain calibration curves with an $R^2 > 0.990$ (Figure 1).

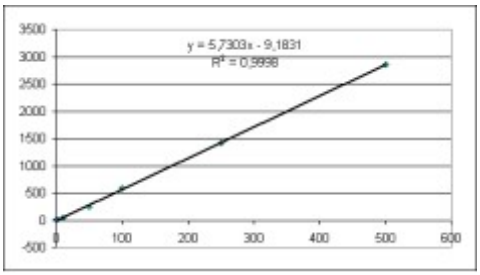


Figure 1

Recovery and matrix effect

Aqueous solutions:

The recovery rate for the processes of acid hydrolysis and derivatisation is verified through comparison of the pre-hydrolysis and post-hydrolysis aqueous stock solutions of aspartic acid. Three solutions of known concentration (25, 100 and

200 mg/L) were prepared; the data obtained is shown in the following table:

	Test 1	Test 2	Test 3
Before hydrolysis (mg/L)	25.4	109	213
After hydrolysis (mg/L)	25.2	108	213
Recovery rate (%)	99.2	99.1	100

Wine:

The method of standard additions to white wine and red wine was applied (50 mg/L and 200 mg/L potassium polyaspartate gradual additions to verify matrix interference in the determination of KPA). For each level, 5 repeatability tests were carried out.

	Red wine w/o addition (mg/L)	Red wine + 50 mg/L	Red wine + 200 mg/L	White wine w/o addition (mg/L)	White wine + 50 mg/L	White wine + 200 mg/L
Repetitions (in Asp. Ac.)	84.0	123.9	258.2	121.9	164.6	294.2
	85.4	127.3	259.4	123.2	163.3	291.5
	83.8	125.1	250.2	121.9	170.3	291.3
	87.7	124.4	253.5	119.5	161.9	284.8
	83.2	126.1	256.9	123.3	160.0	287.4
Mean (in Asp. Ac.)	84.8	125.4	255.6	122.0	164.0	289.8
S_r (in Asp. Ac.)	1.8	1.4	3.8	1.5	3.9	3.7
Mean (in added KPA)	-	46.6	196.3	-	48.3	193.0

S_r (in added KPA)	-	2.8	5.0	-	4.9	3.8
Horrat r	-	0.99	0.52	-	1.71	0.48
Theoretical KPA	-	50	200	-	50	200
Rec KPA %	-	93	98	-	94	96

S_r (in Asp. Ac.): standard deviation of repeatability tests expressed in aspartic acid

S_r (in KPA): standard deviation of repeatability tests expressed in KPA

Rec KPA %: recovery expressed in KPA

The recovery should be within the range of 80-110%.

The repeatability meets the Horrat criterion.

Limits of detection and quantification:

Considering that the wine naturally contains aspartic acid, the decision was made to determine the LoD and LoQ based on the signal-to-noise ratio determined for the wine samples.

CALCULATION OF THE LIMITS OF DETECTION AND QUANTIFICATION CASE N°3: THE SIGNAL-TO-NOISE (S/N) RATIO IS KNOWN FOR A LOW CONCENTRATION		
C	24.74	Value of the wine sample concentration
S/N	122.5	Signal-to-noise ratio
LoD	0.7	Limit of detection
LoQ	2.1	Limit of quantification (3 x LoD)

8. **Calculation and expression of results**

The aspartic acid concentration in milligrams per litre (mg/L) present in the samples is calculated through the acquisition programme's processer.

The quantity of potassium polyaspartate (KPA) added is obtained through the

difference between the sample subjected to hydrolysis and the non-hydrolysed sample without addition:

$$KPA(mg/L) = (AsparticAcid_{hydrolysed_{wine}} - AsparticAcid_{wine_{w/o_addition}}).f_{KPA}$$

where f_{KPA} is the factor of conversion of potassium polyaspartate into aspartic acid, calculated from the ratio of the molecular mass of the potassium polyaspartate monomer to that of aspartic acid, according to the following equation:

$$f_{KPA} = \frac{MM_{KPA_{monomer}}}{MM_{asparti\,c_{acid}}} = 1.15$$

The results are expressed in mg/L to 1 significant figure.

Bibliography

- OIV-MA-AS1-10 (OENO 7/2000)