RESOLUTION OIV-OENO 457-2014

METHOD OF DETERMINATION OF BIOGENIC AMINES IN WINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION

THE GENERAL ASSEMBLY

IN VIEW of article 2, paragraph 2 iv of the agreement establishing the International Organization of Vine and Wine,

On the proposal of the Methods of Analysis Sub-commission

DECIDES to adopt and introduce into the Compendium of International Methods of Analysis of Wines and Must, the following type IV method:

Method of determination of biogenic amines in wine by High-performance liquid chromatography with photodiode array detection

Type IV method

1. Scope

This method is applicable to the analysis of biogenic amines in wines:

Amines	Scope
Histamine	0.500 to 20 mg/L
Methylamine	0.250 to 20 mg/L
Ethylamine	0.450 to 20 mg/L
Tyramine	0.235 to 20 mg/L
Putrescine	0.098 to 20 mg/L
Cadaverine	0.480 to 20 mg/L

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Phenethylamine (or Phenylethylamine)	0.096 to 20 mg/L
Isoamylamine	0.020 to 20 mg/L

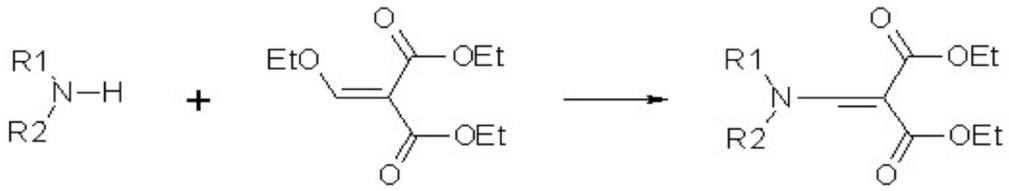
2. Definition

The word biogenic means "created by life". The term "biogenic amines" is therefore given to all the amines produced by the metabolism of living, animal, plant, or microbial cells. Biogenic amines in wine are mainly of microbial origin. The main ones are histamine, putrescine, cadaverine, and tyramine.

3. Principle

The biogenic amines studied here are primary, secondary, tertiary, aliphatic, or aromatic amines. However, only aromatic amines absorb UV. This is because the detection of molecules by UV requires the presence of a chromophore in the molecule, usually a sequence of conjugated double bonds.

In order to use the HPLC/DAD, it is necessary to couple a chromophore to the biogenic amines. To do so, diethyl 2-(ethoxymethylene)malonate (DEEMM) is used which, by alkylation, also known as derivatisation, enables biogenic amines to be obtained that are visible by diode array detector [1].



Derivatisation reaction

NB: The yield of the derivatisation is calculated by adding an internal standard (2,4,6-Trimethylphenethylamine hydrochloride or 2,4,6 TPA). Each of the biogenic amines is quantified against a standard range.

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4. Reagents and Products

4.1. List of reagents

Product references:

	Product	CAS	Purity
4.1.1	Histamine	51–45–6	≥99%
4.1.2	Methylamine	74–89–5	>99.5%
4.1.3	Ethylamine	557–66–4	97%
4.1.4	Tyramine	60–19–5	≥98%
4.1.5	Putrescine (diaminobutane)	333–93–7	≥98%
4.1.6	Cadaverine (diaminopentane)	1476–39–7	≥99%
4.1.7	Phenethylamine	64–04–0	≥99%
4.1.8	Isoamylamine	107-85-7	99%
4.1.9	Boric acid	10043-35-3	≥98.5%
4.1.10	Sodium hydroxide	1310-73-2	≥98%
4.1.11	Sodium azide	26628-22-8	≥99.5%
4.1.12	2,4,6-Trimethylphenethylamine hydrochloride	3167-10-0	97%
4.1.13	DEEMM (Diethyl 2-(ethoxymethylene)malonate)	87-13-8	97%
4.1.14	Glacial acetic acid	64-19-7	≥99.7%
4.1.15	Methanol HPLC	67-56-1	≥99.9%

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	Product	CAS	Purity
4.1.16	Acetonitrile HPLC	75-05-8	≥99.93%
4.1.17	Hydrochloric acid	7646-01-0	≥37%
4.1.18	Ultrapure water (18 MΩ)		

5. Internal standard solution

Preparation of a 2 g/L solution:

Weigh 20 mg of 2, 4, 6-Trimethylphenethylamine hydrochloride (4.1.12)

Dissolve in 10 mL of 0.1 M HCl (5.1)

Storage

The solution is kept at room temperature.

5.1. 0.1 M HCL solution

Preparation of a 0.1 M HCl solution:

Take a sample of approximately 900 ml of ultrapure water (4.1.18) using a graduated cylinder (6.13)

Pour approximately 500 mL of ultrapure water (4.1.18) into a 1 L volumetric flask (6.9)

Take a 100 mL sample of 1M HCl (prepared from the commercial product 4.1.17) using a graduated cylinder (6.11)

Pour the 100 mL of 1 M HCl (4.1.17) into the volumetric flask (6.9)

Top up to 1 L with the remaining ultrapure water

Storage

The solution is kept at room temperature.

5.2. 1M borate buffer

For 100 mL of solution:

Weigh 6.183 g of boric acid (4.1.9)

Dissolve in a beaker (6.2) by adding 80 mL of ultrapure water (measured using the graduated cylinder) (6.11)

Adjust the pH to 9 with a 4N NaOH solution (prepared from the commercial product

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4.1.10)

Adjust to 100 mL in a volumetric flask (6.7)

Note: To obtain good dissolution, the crystals of boric acid should dissolve at a pH as low as possible. To do so, NaOH should be added in small doses (by 10 drops from a Pasteur pipette) (6.30) over a period of 3 hours.

Storage

The solution is kept at room temperature.

5.3. HPLC mobile phase

Mobile phase A: 25 mM acetate buffer + 0.02% of sodium azide pH 5.8:

Take a 1.8 L sample of ultrapure water in a 2 L beaker (6.3)

Add 2.86 mL of glacial acetic acid (4.1.14) (thoroughly rinse the tip in the beaker)

Then 0.4 g of sodium azide (4.1.11)

Stir with a magnetic stirrer (6.24)

Adjust the pH to 5.80 with the 4M NaOH using a Pasteur pipette (6.30) (about 6.5 mL)

Adjust to 2000 mL in a 2000 mL volumetric flask (6.10)

Mobile phase B: Acetonitrile/Methanol (80/20):

For 2 L of mobile phase

Take a 400 mL sample of methanol (4.1.15) using a graduated cylinder (6.12) and pour it into a 2 L cap bottles (6.20) and add in the same cap bottles 1600 mL sample of acetonitrile (4.1.16) measured using a graduated cylinder (6.14).

Storage

The solutions are kept at room temperature.

5.4. Biogenic amine standard range

Preparation of solutions A :

Stock solution A at 500 mg/L

Weigh about 50 mg (accurately known weight) of histamine (4.1.1), methylamine (4.1.2), ethylamine (4.1.3), tyramine (4.1.4) and putrescine (4.1.5) and dissolve them in the same 100 mL flask (6.7) with 0.1 M HCl (5.1)

Surrogate solution A at 50 mg/L

Take a 25 mL sample of solution A at 500 mg/L and pour into a 250 mL flask (6.8)

Top up to 250 mL with 0.1 M HCl (5.1)

Surrogate solution A at 40 mg/L

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Take a 50 mL sample of 0.1 M HCl (5.1) and pour into a 250 mL flask (6.8)

Top up to 250 mL with the surrogate solution A at 50 mg/L

Preparation of solutions B

Stock solution B at 500 mg/L

Weigh about 50 mg (accurately known weight) of cadaverine (4.1.6), phenethylamine (4.1.7) and isoamylamine (4.1.8) and dissolve them in the same 100 mL flask (6.7) with 0.1 M HCl (5.1)

Surrogate solution B at 50 mg/L

Take a 25 mL sample of solution B to 500 mg/L and pour into a 250 mL flask (6.8)

Top up to 250 mL with 0.1 M HCl (5.1)

Surrogate solution B at 10 mg/L

Take a 50 mL sample of surrogate solution B at 50 mg/L and pour into a 250 mL flask (6.8)

Top up to 250 mL with 0.1 M HCl (5.1)

Combination of solutions A and B - Standard range

In a 100 mL flask (6.7) add 50 mL of solution A at 40 mg/L using a 50 mL volumetric flask (6.6)

Top up to 100 mL with the solution B at 10 mg/L: you obtain the solution at 20 (A) / 5 (B) mg/L

The next table explains how to prepare concentration points for the calibration curve:

Concentration of the initial solution (mg/L)	Volume of initial solution sampled (mL)	Adjusted to 100 mL with a 0.1 M HCl solution (mL)	Concentration of the final solution (mg/L)
20(A) / 5 (B)	50	50	10 (A) / 2.5 (B)
10(A) / 2.5 (B)	50	50	5 (A) / 1.25 (B)
5(A) / 1.25 (B)	20	80	1 (A) / 0.25 (B)

In this way, four concentrations of biogenic amines are contained in solution A (20, 10, 5 and 1 mg/L), and four concentrations of biogenic amines are contained in solution B (5, 2.5, 1.25 and 0.25 mg/L).

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Storage The solutions are kept at –20°C.

6. Equipment and apparatus

6.1. 25 mL Beakers
6.2. 250 mL Beakers
6.3. 2000 mL Beakers
6.4. 10 mL volumetric flasks
6.5. 25 mL volumetric flasks
6.6. 50 mL volumetric flasks
6.7. 100 mL volumetric flasks
6.8. 250 mL volumetric flasks
6.9. 1000 mL volumetric flasks
6.10. 2000 mL volumetric flasks
6.11. 100 mL graduated cylinder
6.12. 500 mL graduated cylinder
6.13. 1000 mL graduated cylinder
6.14. 2000 mL graduated cylinder
6.15. 200 µL automatic pipette
6.16. 1 mL automatic pipette
6.17. 5 mL automatic pipette
6.18. 10 mL automatic pipette
6.19. Tips for 1 mL, 5 mL and 10 mL automatic pipette
6.20. 2-litre cap bottles
6.21. Pyrex 10 mL hydrolysis tubes with screw top
6.22. 2 mL screw cap bottles adapted to the auto-sampler
6.23. Scales for weighing from 0 to 205 g
6.24. Magnetic stirrer
6.25. High-performance liquid chromatography (HPLC)
6.26. Data acquisition software
6.27. DAD detector (diode array)
6.28. Octadecyl-type column (for example HP® C18 - HL, 250 mm x 4.6 mm, 5 µm).
6.29. Dry bath at 70 ° C
6.30. Pasteur pipette

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6.31. Ultrasonic bath

7. Sampling (sample preparation)

This method does not require special sampling in that 1 mL of wine to be analysed is collected and deposited directly into a Pyrex 10 mL hydrolysis tube with a screw cap (6.21) (see procedure).

However, it is recommended to carry out the derivatisation reaction with DEEMM on receipt of the sample because the histamine concentration in wine may reduce over time.

8. Procedure

8.1. Test sample

The manipulation must be done under a fume hood because of the toxicity of certain of the reagents.

If the buffer contains borate crystals, heat it to 50°C while stirring (lower initially, until the solution has heated up).

To avoid any risk of adsorption on the tips of the automatic pipettes, it is advisable to use the micropipette as follows:

- Pre-wet the cone once with the solution to be sampled
- Add the solution to the recipient without rinsing the tip with the contents of the recipient unless otherwise specified

Shake the solutions well before use (especially the frozen wine)

In a Pyrex 10 mL hydrolysis tube with a screw cap (6.21), introduce using suitable micropipettes:

- 1.75 mL of borate buffer (5.2)
- 750 µL of methanol (4.1.15)
- 1 mL of the sample to be derivatised (1 mL automatic pipette) (6.16)
- 40 µL of the internal standard (2,4,6 TPA to 2 g/L) (5.1)
- 30 µL of DEEMM (4.1.13)

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Close the tube (fully tighten to avoid any evaporation) and shake manually.

Turn on the dry bath (6.29) to 70°C.

Place the tube in the ultrasonic bath (6.31) for 30 minutes (2 times 15 minutes, stirring every 5 minutes). Always use a plastic rack suitable for the water bath because the derivatisation is unsatisfactory when a metal rack is used.

Heat the reaction mixture to 70°C for 1h in the dry bath (6.29) to degrade the surplus DEEMM.

Turn off the dry bath

After the reaction mixture has returned to room temperature, fill the 2 mL bottles using Pasteur pipettes (6.30) (change Pasteur pipette with each tube). Shake the tubes manually before sampling.

8.2. Operating conditions

The operating conditions below are given as an example.

Mobile phase:

- A: 25 mM acetate buffer + 0.02% of sodium azide pH 5.8 :
- B: Acetonitrile/Methanol (80/20):

Gradient elution as follows, with a flow rate of 0.9 mL/min:

Time (min)	% A	% B
0	90	10
5	90	10
10	83	17
35	60	40
43	28	72
48	18	82
52	0	100

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57	0	100

Column temperature: 15°C
Detection wavelength: 280 nm
Flow rate: 0.9 ml/min
Volume injected: 50 µL
Analysis time: 57 minutes
Identification of biogenic amines:
The biogenic amines are identified by their retention time. To do so, each biogenic amine was analysed individually in order to determine its retention time (Tr).

Amines		Average Tr (min)
Histamine	HI	25.46
Methylamine	ME	33.11
Ethylamine	ET	39.00
Tyramine	TY	41.50
Putrescine	PU	46.00
Cadaverine	CA	48.00
Phenethylamine	PH	48.75
Isoamylamine	IS	50.25
Internal standard	2,4,6-TPA	54.75

9. Calculations (Results)

Bias caused by the uncertainty on the derivatisation yield and the injection volume can be corrected using the internal standard.

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Once the value of the peak has been corrected, the concentration of biogenic amine is calculated based on the slope value of the standard range of the corresponding biogenic amine. To do so, for each series of analyses a standard range is also derivatised and injected.

The results are expressed in mg/L to one figure after the decimal point.

10. Quality Control

Quality controls can be carried out with certified reference materials, wines whose characteristics are derived from consensus or wines to which standard additions have been regularly made during the analytical series and in accordance with the accompanying control charts.

11. Characteristics of the method: intralaboratory validation parameters

The validation parameters were determined according to [4].

11.1. Linearity

The approach chosen for the study of linearity is that of comparing the residual standard deviations from a linear regression model and a second-order polynomial regression model.

This study was conducted on two different wines spiked with biogenic amines at concentrations of 0, 1, 5, 10, and 20 mg / L (solution A) and 0, 0.25, 1.25, 2.5, and 5 mg/L (solution B).

Summary of the results for biogenic amines:

biogenic Amine	S_{res} Linear	S'_{res} Order 2	DS 2	PG	F (5%)	Conclusion

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Methylamine	0.766	0.606	3.218	8.757	4.75	Linear
Ethylamine	0.371	0.371	0.140	1.014		Linear
Tyramine	1.065	1.065	1.135	1.000		Linear
Putrescine	0.524	0.523	0.286	1.043		Linear
Cadaverine	0.276	0.267	0.134	1.881		Linear
Phenethylamine	0.251	0.248	0.082	1.328		Linear
Isoamylamine	0.216	0.215	0.055	1.199		Linear
Histamine	0.591	0.589	0.316	1.084		Linear

11.2. Specificity

The principle of specificity measurement consists in examining the regression line r = a + bv and verifying that slope b is equal to 1 ($T_{obs} < T_{critical}$) and that intercept point a is equal to 0 ($T'_{obs} < T_{critical}$). The hypotheses are tested using a t-test associated with the 1% risk of error.

The value of $T_{critical}$, bilateral [p–2, 1%] associated with the 1% risk of error for 3 degrees of freedom is 4.541.

Summary of the results for biogenic amines

biogenic Amine	Wine A		Wine B		Wine C		Wine D	
	T_{obs}	T'_{obs}	T_{obs}	T'_{obs}	T_{obs}	T'_{obs}	T_{obs}	T'_{obs}
Methylamine	4.482	2.321	2.933	0.013	1.563	0.007	5.199	2.864
Ethylamine	0.411	0.002	0.081	0.010	0.546	10.556	0.169	2.537
Tyramine	1.834	0.005	0.636	0.005	2.151	4.485	3.420	37.419
Putrescine	7.605	0.041	0.604	0.000	3.257	0.064	2.135	0.011

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Cadaverine	5.499	0.033	1.719	1.314	10.929	0.049	8.466	0.026
Phenethylamine	3.348	0.016	1.265	0.001	10.238	0.034	5.925	0.009
Isoamylamine	12.980	0.016	2.297	0.004	12.996	0.020	11.121	0.000
Histamine	4.978	0.250	1.222	0.006	3.128	0.014	1.229	0.004

11.3. Repeatability

For this repeatability study, seven different red wines were selected, and three different repetitions were performed on each. Concentrations were from 0.5 mg/L to 15 mg/L depending on the biogenic amine and the wine.

biogenic Amine	S_r (mg/L)	r (mg/L)	Validation range (mg/L)
Methylamine	0.335	0.937	3 - 16
Ethylamine	0.173	0.486	2 - 7
Tyramine	0.276	0.773	2 - 20
Putrescine	0.500	1.400	7 - 26
Cadaverine	0.025	0.069	0.2 - 0.8
Phenethylamine	0.028	0.079	0.3 - 1.1
Isoamylamine	0.017	0.048	0.1 - 0.8
Histamine	0.108	0.303	5 - 16

11.4. Reproducibility

For this reproductibility study, three different red wines were selected, and two repetitions were performed with each.

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biogenic Amine	S_r (mg/L)	R (mg/L)	Validation range (mg/L)
Methylamine	0.533	1.492	3 - 16
Ethylamine	0.884	2.475	2 - 7
Tyramine	0.341	0.955	2 - 20
Putrescine	0.419	1.172	7 - 26
Cadaverine	0.172	0.482	0.2 - 0.8
Phenethylamine	0.053	0.150	0.3 - 1.1
Isoamylamine	0.056	0.157	0.1 - 0.8
Histamine	1.333	3.732	5 - 16

11.5. Limits of detection (LOD) and limits of quantification (LOQ)

According to an intralaboratory study using the method of successive dilutions from a solution to 0.5 mg/L serially diluted to 0.01 mg/L :

Amines		LD (mg/L)	LQ (mg/L)
Histamine	HI	0.167	0.500
Methylamine	ME	0.083	0.250
Ethylamine	ET	0.150	0.450
Tyramine	TY	0.078	0.235
Putrescine	PU	0.033	0.098
Cadaverine	CA	0.160	0.480

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Phenethylamine	PH	0.032	0.096
Isoamylamine	IS	0.007	0.020

12. Bibliography

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