OIV-MA-AS315-07A Examination of artificial sweeteners

Type IV method

1. Principle of the method

Examination of saccharine (benzoic sulfimide), Dulcin (*p*-ethoxyphenylurea), cyclamate (cyclohexylsulfamate) and P□4000 (5-nitro-2-propoxyaniline or 1□propoxy-2-amino-4-nitrobenzene).

After concentration of the wine, the saccharine, Dulcin and P□4000 are extracted in an acid medium with benzene; the cyclamate is extracted from the wine after the benzene extraction using ethyl acetate (the order of extraction is important). The residues after solvent evaporation are submitted to thin layer chromatography.

Saccharine and cyclamate are identified by chromatography on cellulose plates (solvent: acetone-ethyl acetate-ammonium hydroxide), the first the benzene extract, the second in the extract by the ethyl acetate after purification by washing with ether.

These sweeteners are developed by spraying with a solution of benzidine; aniline; cupric acetate, and have the following Rf: 0.29 for cyclamate, 0.46 for saccharine.

The P□4000 and Dulcin from the benzene extract are separated by chromatography on polyamide plates, (solvent: toluene; methanol; glacial acetic acid). These sweeteners are developed by spraying a solution of *p*-dimethylaminobenzaldehyde, and have the following Rf: 0.60 for Dulcin, 0.80 for P□4000.

2. Method

Examination of saccharine, cyclamate, Dulcin and the P□4000.

2.1. Apparatus

2.1.1. Chromatography tank

2.1.2. Micrometry syringes or micropipettes

2.1.3. Separator tube 15 mm in diameter and 180 mm long, with a stopcock

2.1.4. Water bath at 100°C

2.1.5. Regulatable oven, able to reach 125°C

2.2. Reagents

2.2.1. Extraction solvent:

- benzene

- ethyl acetate

2. aChromatography solvents:

Mixture No.1:

Acetone	60 parts
Ethyl acetate	30 parts
Ammonium hydroxide (ρ_{20}= 0.92 g/mL)	10 parts

Mixture No 2.:

Toluene	90 parts
Methanol	10 parts
Glacial acetic acid (ρ_{20}= 1.05 g/mL)	10 parts

2.2.3. Chromatography plates (20 x 20 cm):

- with layer of cellulose powder (for ex., Whatman CC 41 or Macherey-Nagel MN300)
- with layer of polyamide powder (for ex., Merck)

4. Indicating reagent for saccharine and cyclamate

Prepare:

- alcoholic solution of benzidine at 250 mg in 100 mL ethanol
- saturated solution of cupric acetate, $Cu(C_2H_3O_2)_2.H_2O$
- freshly distilled aniline

Mix: 15 mL of benzidine solution, 1 mL of aniline and 0.75 mL saturated cupric acetate solution.

This solution must be freshly prepared. It corresponds to the volume required for development of a 20 x 20 cm plate.

2.2.5. Hydrochloric acid 50% (*v/v*),

2.2.6. Nitric acid solution, 25% (*v/v*),

2.2.7. Indicator reagent for the P□4000 and Dulcin: dissolve 1 g of 1,4-paradimethylaminobenzaldehyde in 50 mL methanol; add 10 mL 25% nitric acid; bring to 100 mL with methanol. Use 15 mL of this reagent for the development of a 20 x 20 cm plate.

2.2.8. Cyclo-hexylsulfamic acid in water-ethanol solution, 0.10 g/100 mL

Dissolve 100 mg of the sodium or calcium salt of cyclo-hexylsulfamic acid in 100 mL of an equal part mixture of water and ethanol.

2.2.9. Saccharine aqueous solution, 0.05 g/100 mL

2.2.10. Dulcin, 0.05 g/100 mL of methanol.

2.2.11. P□4000, 0.05 g/100 mL of methanol.

2.3. Procedure

2.3.1. Extraction

100 mL of wine, placed in a beaker, are rapidly evaporated by boiling until the volume is reduced to 30 mL, while directing a current of cold air to the surface of the flask. Allow to cool. Acidify with 3 mL 50% hydrochloric acid (v/v). Transfer to a 500 mL conical flask with a ground stopper, add 40 mL of benzene and stir with a mechanical stirrer for 30 min. Transfer to a separating funnel to separate the organic phase. If an emulsion is formed, it must be separated by centrifugation. Place the organic phase in a conical flask with a ground glass stopper.

Decant the wine previously extracted with benzene, which corresponds to the lower layer in the separating funnel, into a 500 mL conical flask with a ground stopper containing 40 mL of ethyl acetate. Agitate for 30 minutes and separate the organic phase as before taking care to recover only the organic fraction and not the wine.

On a 100°C water bath, evaporate each extraction solvent in 50□60 mm diameter evaporation dishes, in small amounts while directing a stream of cold air on the surface of the dishes. Continue the evaporation until the residue has a syrupy consistency, stopping before the evaporation is complete.

Re-dissolve the benzene extract residue in the evaporation dish with 0.5 mL ethanol-water (1:1) solution (it is advisable to re-dissolve the residue once with 0.25 mL ethanol-water solution and then to rinse the dish with another portion of 0.25 mL of the same solution). Place the ethanol-water extract into a small tube with a ground stopper (extract B).

The residue of the dish in which the ethyl acetate (containing the cyclamate) has been evaporated, is dissolved with 0.5 mL of water and is poured into a small separator tube. Wash the dish with 10 mL ether and add the ether to the contents of the separator tube. Mix vigorously for 2 minutes and separate the lower layer into a small

test tube that contains 0.5 mL ethanol. This comprises a total of 1 mL of ethanol-water solution that contains the possible cyclamate (extract A).

2.3.2. Chromatography

2.3.2.1. Saccharine and cyclamate

For examination of the saccharine and cyclamate, use a cellulose plate, with half of the plate for the identification of cyclamate and the other half for saccharine.

To do this, spot 5 to 10 µL of extract A and 5 µL of the standard cyclamate solution. On the second part of the plate spot 5 to 10 µL of extract B and 5 µL of the standard saccharine solution. Place the prepared plate in the chromatography bath containing solvent No.1 (acetone; ethyl acetate; ammonium hydroxide); allow to migrate until the solvent front reaches 10 to 12 cm. Remove the plate from the bath and dry with warm air. Spray the plate

evenly and gently with the benzidine reagent (17□18 mL for each plate). Dry the plate with cold air. Place the plate in an oven maintained at 120-125°C for 3 minutes. The spots appear dark gray on a light chestnut background; they turn brownish with time.

2.3.2.2. *P-4000 and Dulcin*

Deposit 5 µL of extract B and 5 µL of the standard solutions of Dulcin and P-4000 on a polyamide plate. Place the prepared plate in the chromatography tank containing solvent No. 2 (toluene; methanol; acetic acid). Let the solvent front reach a height of 10 to 12cm.

Remove the plate from the tank; dry in cold air. Spray with 15 mL of the *p*-dimethylaminobenzaldehyde reagent, then dry with cold air until the orange-yellow colored spots appear which correspond to Dulcin and P-4000.

2.3.2.3. *Sensitivity*

The benzidine reagent allows detection of spots corresponding to 2 µg of saccharine and 5 µg of cyclamate. The *p*-dimethylaminobenzaldehyde reagent reveals 0.3 µg of Dulcin and 0.5 µg of P□4000.

This method allows determination of (depending upon the efficiency of the extractions):

Saccharine	2-3 mg/L
Cyclamate	40-50 mg/L
DULCIN	1 mg/L
P-4000	1-1.5 mg/L

Bibliography

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