Customs Laboratories
European Network

ILIADe 280:2024 | CLEN Method

Determination of Denatonium Benzoate in Alcoholic Products by HPLC-UV

Version 13 February 2024

This table shows the most important changes that have been made compared with the latest former version	
Date of the latest former version: 25 July 2023	
Section	**Changes**
8. Precision	Enrichment of the precision data with minimum requirements for LOD and LOQ

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1. Scope

The purpose of this method is verification of fulfilment of the legislative requirements on denatured alcohol, particularly the Regulation (EC) 3199/93 of 22 November 1993 and its amendments, concerning the mutual recognition procedures for the complete denaturing of alcohol (CDA) for the purposes of exemption from excise duty. The common denaturing procedure for completely denatured alcohol defines the amount of denaturing agents in litre (or gram) per hectolitre of absolute ethanol. According to the Implementing Regulation (EU) 2017/2236 the amount of denatonium benzoate (DB) added to 100 L (1 hL) of absolute ethanol is 1 gram.
This method is suitable for the determination of Denatonium Benzoate in denatured alcohol and alcohol containing solutions such as burning alcohol and screen-wash.

2. Principle

This document describes a standard method for the determination of denatonium benzoate (DB) in CDA (completely denatured alcohol) formulations using HPLC with UV detection at 210 nm. The chemical structure of denatonium benzoate is shown in Fig.1. The samples are directly injected into the HPLC system after membrane filtration, if necessary. The working range for quantitative determination of DB is 0.5 to 20.0 mg/L.

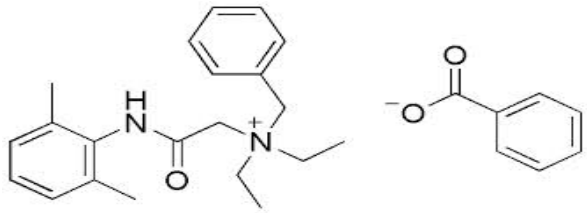

Fig.1. Denatonium benzoate (DB) chemical structure

3. Reagents and materials

3.1 Reagents

- ✓ Denatonium benzoate (CAS: 3734-33-6), purity ≥ 99 %. Handle it with gloves.
- ✓ Ethanol 96 % vol.
- ✓ Sodium chloride, extrapure
- ✓ Acetonitrile, HPLC grade
- ✓ Water, HPLC grade
- ✓ 0.2 % sodium chloride solution
  Weigh 0.4 g of sodium chloride in a weighing bottle and dissolve it in a beaker with 200 ml of water HPLC grade.
- ✓ Mobile phase
  Add in a 1000 ml volumetric flask, 200 ml of 0.2 % sodium chloride solution and 800 ml of acetonitrile HPLC grade.

3.2 Materials

- ✓ Analytical balance with a precision of 0.1 mg
- ✓ Technical balance with a precision of 0.01 g
- ✓ 0.5, 2, 5, 10 and 20 ml pipettes
- ✓ 100 and 1000 ml volumetric flasks
- ✓ Weighing bottle
- ✓ Syringes
- ✓ 0.45 µm cellulose membrane filters
- ✓ 250 ml beaker
- ✓ 1000 ml graduated cylinder

4. Apparatus

- ✓ HPLC system equipped with:
  - Analytical column, for example: LiChrospher® 100 CN (5 µm) in LiChroCART® 250-4 guard column
  - Thermostated column compartment
  - Diode array detector (DAD) or UV detector
- ✓ Electronic densimeter: used only to measure the density of the denatonium benzoate calibration solutions and not to measure the ethanol content of an unknown sample. The ethanol content of an unknown sample must be determined using the method of ethanol content in CDA.

Alternative HPLC columns (C_{18} / C_8), buffers and chromatographic parameters may be used provided that good peak shape is obtained for denatonium benzoate and good separation of denatonium benzoate from potential interferences can be achieved. A list of possible alternative HPLC columns and experimental conditions are provided in Annex.

5. Procedure

5.1 Standard solutions

5.1.1 Preparation of the stock solution (100 mg DB / L)

Weigh, recording the exact weight, 0.1 g of denatonium benzoate in a weighing bottle and dissolve it in a 1000 ml volumetric flask with ethanol 96 % vol. Mix gently.

Measure the mass of this solution with a technical balance and the density at 20ºC with an electronic densimeter.

Calculate the concentration of the stock solution as follows:

$$\left[DB, \frac{mg}{L}\right]_{stock} = \frac{DB\ mass\ (g)\ x\ 10^6}{\frac{stock\ solution\ mass\ (g)}{stock\ solution\ density\ \left(\frac{g}{cc}\right)}}$$

5.1.2 Preparation of the working calibration solutions

Add, in 100 ml volumetric flasks, 20 ml of ethanol 96 % vol. (to minimize weighing errors), then 0.5, 2, 5, 10 or 20 ml (weighing) of the stock solution and top up to the filling mark with ethanol 96 % vol. Mix gently.

Measure the mass of each working solution with an analytical balance and the density at 20ºC with an electronic densimeter.

Calculate the concentration of the working solutions as follows:

$$\left[DB, \frac{mg}{L}\right]_{working\ i} = \left[DB, \frac{mg}{L}\right]_{stock} \cdot f_i \qquad f_i = \frac{\frac{stock\ solution\ mass\ i\ (g)}{stock\ solution\ density\ \left(\frac{g}{cc}\right)}}{\frac{working\ solution\ mass\ i\ (g)}{working\ solution\ density\ i\ \left(\frac{g}{cc}\right)}}$$

Calibration solutions should be stored in a refrigerator.

5.2 Chromatographic and calibration parameters

When using LiChrospher® 100 CN (5 µm) column, chromatographic and calibration parameters recommended are:

- ✓ Column flow: 1.2 ml / min
- ✓ Stoptime: 14 min
- ✓ Detector: signal 210 nm (bandwidth 8 nm), reference 360 nm (bandwidth 100 nm)
- ✓ Mobile phase: acetonitrile 80:20 0.2 % sodium chloride solution
- ✓ Injection volume: 20 µl
- ✓ Column oven temperature: 27ºC
- ✓ Calibration: external standard
- ✓ Signal: peak area
- ✓ Curve type: linear
- ✓ Origin: included (origin is used as one of the calibration point)
- ✓ Weight: equal (all calibration points have the same importance)

5.3 Calibration

Working solutions containing the following concentrations of denatonium benzoate 0.5, 2, 5, 10 and 20 mg/l are analysed by injecting one replicate of each working solution. Peak areas corresponding to denatonium benzoate are plotted according to the respective concentrations in order to obtain a linear regression. The correlation coefficient must be > 0.999. Otherwise, the system must be checked to improve the linear regression if possible, or the working solutions must be discarded and a new set of calibration solutions should be prepared.

5.4 Quality control

Daily, before the first measurement one of the calibration solutions, or a separately prepared reference solution or a reference material, is injected for performing QC verification. If the results are within ± 5 % of their theoretical values, analysis may proceed. If not, an investigation should be made to find the cause of the inaccuracy and remedial action taken as appropriate (i.e. new calibration curve).

5.5 Sample preparation and analysis

The samples are directly injected into the HPLC system after 0.45 µm cellulose membrane filtration, if necessary.

6. Calculation

The analytical results obtained in mg DB / L are converted in g DB / 100 L of absolute ethanol (g / hL absolute ethanol) using the ethanol content of the sample as measured by GC-FID.

$$\left[DB, \frac{g}{hL\ AA}\right] = \frac{[DB, mg/L]\ x\ 10}{Alcoholic\ strength\ (\%\ vol.)}$$

7. Expression of results

Results and uncertainty are expressed with maximum 3 significant figures and maximum 2 decimal places (example 1.05 ±0.72)

8. Precision

Precision data (limits of r and R) obtained from the CLEN 1st and 2nd proficiency test on Completely Denatured Alcohol, performed in 2019 (by 41 laboratories on 3 samples; final report issued 4 September 2019) and in 2021 (by 49 laboratories on 4 samples; final report issued 14 April 2022):

Denatonium benzoate (in mg/L)	**Matrices**			
	Completely Denatured Alcohol (CDA)	Burning Alcohol	Screen Wash	Disinfectant
Denatonium benzoate (robust mean), mg/L	9.66-9.97	7.64-10.6	1.29-12.7	6.42
Repeatability, *mg/L*	0.18	0.20	0.16	0.08
Reproducibility *mg/L*	0.73	1.47	0.87	0.57

Denatonium benzoate (expressed in g/hL of absolute ethanol)	**Matrices**			
	Completely Denatured Alcohol (CDA)	Burning Alcohol	Screen Wash	Disinfectant
Denatonium benzoate (robust mean), g/hL EtOH	1.02-1.12	0.85-1.18	0.55-3.73	1.05
Repeatability, *g/hL EtOH*	0.02	0.02	0.05	0.01
Reproducibility, *g/hL EtOH*	0.08	0.15	0.28	0.13

For Denatonium benzoate, the minimum achievement

- for the **limit of detection**, LOD, is 0.2 mg/L
- and for the **limit of quantification**, LOQ is 0.5 mg/L.

Annex

(annex to section 4.)

possible alternative HPLC columns and experimental conditions:

Zorbax Eclipse XDB-CN 4.6 x 250 mm (5µm) Flow rate: 1.0 ml/min ; 20 µl injection Column temperature 28°C, DAD at 210 nm Eluent: identical to SOP	Pursuit XRS 5µ Diphenyl 4.6 x 150 mm (5 µm) Flow rate : 1.0 ml/min ; 40 µl injection Eluent 900 ml MeOH + 100 ml H_2O + 1 ml formic acid
Hichrom Lichrospher CN-5 (5 µm) 25 cm x 4.6 mm i.d. Flow rate: 1.2 ml/min, 20 µl injection Eluent: 800 ml Acetonitrile, 200 ml 0.2% NaCl solution	Phenomenex Sy nergy 4u Polar-RP 80A (4.6 x 250 mm) Flow rate: 1.2 ml/min ; 30 µl injection Ethanol:Formic Acid (0.15% in water) = (85;15; v.v)
Phenomenex Prod'gy 5u ODS3 100A (250 x 4.6 mm x 5 µm) Flow rate: 1.5 ml/min Water:Acetonitrile (80:20; v.v)	Labiospher PSI C8 4.6 x 150 mm (5 µm) Flow rate: 1.5 ml/min ; 20 µl injection Acetonitrile:NH_4COOH (2mM) 75:25 (v.v)
Zorbax SBC18 4.6 x 150 mm (5µm) Flow rate: 0.9 ml/min ; 7 to 50 µl injection A (45%): H_2O + CH_3COOH (1000:1) B (55%): CH_3OH + $(C_2H_5)_3N$ (1000:2)	Eclipse XDB C18 4.6 x 250 mm Flow rate: 1.2 ml/min ; 20 µl injection Acetonitrile:Buffer (50:50 ; v.v) Buffer: NaH_2PO_3 0.01M + 25nM natrium lauryl sulphate
Agilent Zoabax Eclipse XD B-C 18 4.6 x 150 mm (5µm) Flow rate: 1.5 ml/min ; 20µl injection	Sprerisob S3-CN 4.6 x 150 mm Flow rate: 1.2 ml/min ; 25 µl injection
Kinetex (core shell) Phenyl hexyl 3 x 150 mm, 2,6 micron Flow rate: 0,35 ml/min Inj. Vol: from 10 to 20µl Eluent: 3,5 ± 0,1 g of SDS + 400 ml of acetonitrile +50 ml buffer NaH2PO3 0,1 M pH 3, brought to 1 liter with water T = 40°C	Column Macherey-Nagel 3 x 250 mm particle size 3 µm Flow rate: 1 ml/min ; 10 µl injection Acetonitrile:Buffer (50:50 ; v.v) Buffer: NaH_2PO_3 0.01M + 25nM natrium dodecyl sulphate