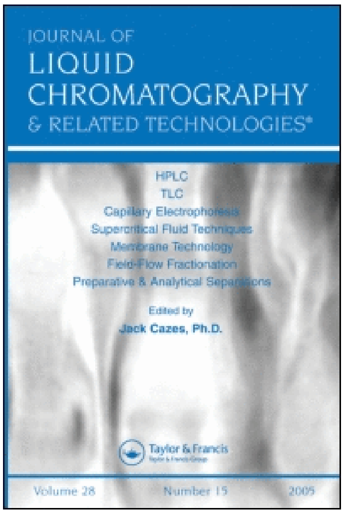

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R. M. Gadzała-Kopciuch[a]; T. Buszewska[a]; B. Buszewski[a]
[a] Department of Environmental Chemistry and Ecoanalytics, Faculty of Chemistry, Nicolaus Copernicus University, PL-87 100 Toruñ, Poland

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ISOLATION AND DETERMINATION OF DENATONIUM BENZOATE IN ANTIFREEZING AGENTS BY SOLID PHASE EXTRACTION AND HPLC

R. M. Gadzała-Kopciuch,[1] T. Buszewska,[2] B. Buszewski[1,]*

[1] Department of Environmental Chemistry and Ecoanalytics
[2] Laboratory of Chemical Education
Faculty of Chemistry
Nicolaus Copernicus University
7 Gagarin St
PL-87 100 Toruñ, Poland

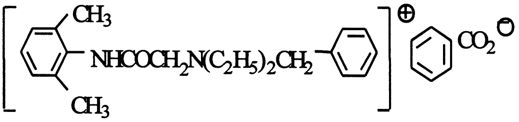
ABSTRACT

The aim of the current work is to discuss a quick method for the isolation and enrichment of denatonium benzoate from low-solidification fluids applied for cooling of internal-combustion engines (liquids based on ethylene and propylene glycol). This method, i.e. solid phase extraction (SPE), can be coupled with other techniques, mostly high performance liquid chromatography in reversed phase mode (RP HPLC).

The optimization of extraction process is based on SPE column and consideration type of packing (different of functional groups); whether there are any joining of beds (sandwich type). Thanks to such solutions, the optimized methodology of preparation of sample permitted the attainment of high recoveries (about 90 %) and reproducibility of quantitative and qualitative results of determined analyte concentration (RSD = ± 1 %).

INTRODUCTION

Denatonium benzoate (benzyldiethyl - N - 2,6 - xylyl-carbamoyl-methyl-ammonium benzoate) (commercial name: Bitrex) is a quaternary amine whose structure, presented below, is much more complex than one could suppose.



With regard to odourless and intensive bitter taste, this substance can be used as an effective denaturant, e.g. in pharmaceutics, cosmetics, and grocery products (vegetable oil, methyl, and ethyl alcohol),[1-5] and in antifreezing agents (*"Petrygo," "Borygo"* - made in Poland). As a result of the addition of Bitrex to these materials (even in a very little quantity), they became unfit for consumption, as such preventing the possible damage to human health. To make sure that the admissible concentrations of the denaturants are not exceeded, selective and precise separation and determination methods are required.[1-4]

Methods available for determination of Bitrex involve colorimetry,[1] thin-layer chromatography,[2] and liquid-liquid extraction,[3,4] but they are either non-specific or time-consuming, requiring large sample volume and many suffer errors inherent to the extraction process.

In this work, a new method for the isolation and quantitative determination of Bitrex has been described. It is based on utilization of a solid phase extraction (a simple and effective procedure) coupled with reversed-phase high performance liquid chromatography (RP HPLC).

EXPERIMENTAL

Apparatus

Chromatographic measurements were made using an HP-1050 liquid chromatograph system (Hewlett Packard, Waldbronn, Germany) equipped with gradient pump and a UV-Vis detector with measuring cell of the volume of 8 μL, and the Vectra QS/HP computer with ChemStation-2 for data collection and the control of the process. Solutes were injected using a Rheodyne (Berkeley, CA, USA) Model 7125 sampling valve with a 20 μL sample loop.

In all chromatographic investigations, the flow rate was 1 mL/min. Separations were performed on columns with different stationary phases (octatedyl, alkylamide, and cyano).

Solid phase extraction of denatonium benzoate prepared from natural samples was performed using a commercial vacuum set of the type of SPE-12 G (J.T. Baker, Gross-Gerau, Germany). The procedure in detail is described according to the scheme presented in Figure 1. Bakerbond SPE columns: octadecyl (C-18), cyano (CN), silica (SiOH) [1000 mg, 6 ml], were purchased from J.T. Baker (S.Witko, Łódż, Poland).

Materials and Reagents

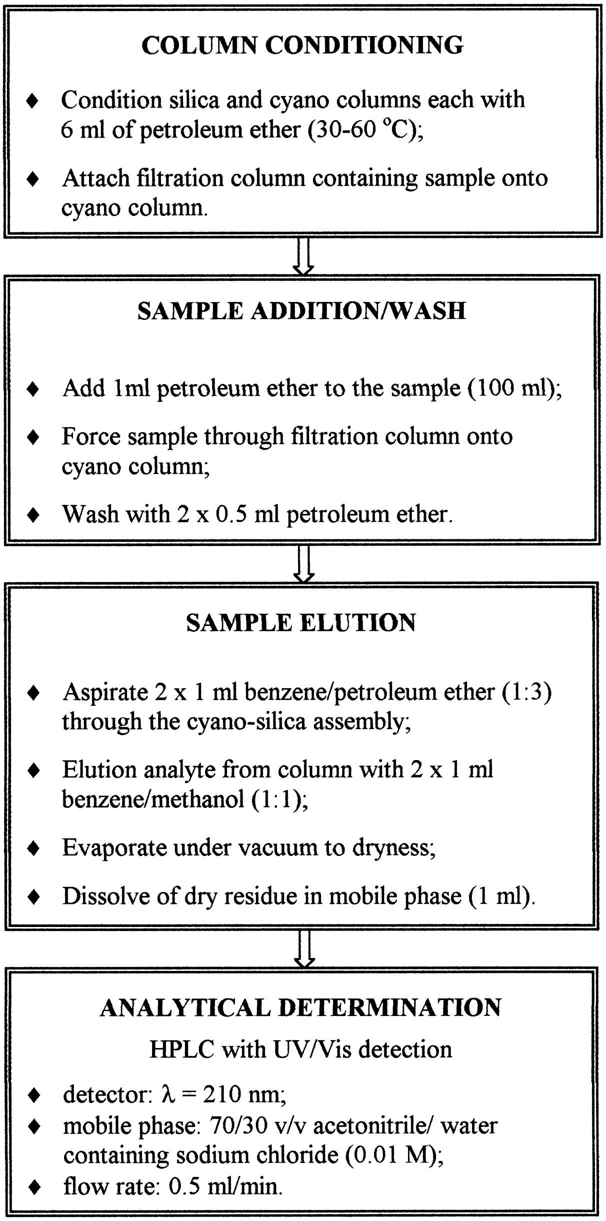
All organic solvents and chemicals used for sample preparation and chromatographic analysis were of analytical grade (J. T. Baker, Deventer, The Netherlands). The deionized water was freshly prepared in our laboratory by the Milli-Q system (Millipore Corp., Bedford, USA). The denatonium benzoate (Aldrich Chemie, Steinheim, Germany) was used for the preparation of standard solutions.

The phases prepared (SG-AP[7,8] and SG-CN[9]) were packed into 150 mm x 4.6 mm I.D. stainess-steel tubes purchased from Bischoff (AG Leonberg, Germany). All columns were packed using a DT 122 packing pump (Haskel Ltd., Burbank, CA) under a pressure of 50 MPa according to procedure described in Ref. [9]. Also, the separation of denatonium benzoate was accomplished using a 5 μm spherical particle silica gel with C-18 stationary phases (Alltech Association, Cartforth, England).

RESULTS AND DISCUSSION

For the accomplishment of the proposal, the optimization of the extraction process was tested with a series of SPE columns (Table 1). The packings of this SPE column differed in the under-surface structure of chemically bonded phases. The extraction of denatonium benzoate from samples fortified with standard solutions of concentrations 5 and 15 ppm is based on the procedure presented in Figure 1. The choice of values of the concentrations was made based on the range of concentration of denatonium benzoate in samples of low-solidification fluid.[10]

Presented in Table 1 is the recovery values (R) and standard deviations (SD) for standard solutions of denatonium benzoate (5 and 10 ppm). Recoveries obtained from individual SPE columns, i.e., C-18, SiOH, and CN are below ($R \approx 67\%$), and with high values of standard deviation - testifying to the lack of reproducibility. These were discarded on the basis of their considerable low R values, which are situated in the range of $32.5\% < R < 66.4\%$ (Table 1).



COLUMN CONDITIONING

- Condition silica and cyano columns each with 6 ml of petroleum ether (30-60 °C);
- Attach filtration column containing sample onto cyano column.

SAMPLE ADDITION/WASH

- Add 1ml petroleum ether to the sample (100 ml);
- Force sample through filtration column onto cyano column;
- Wash with 2 x 0.5 ml petroleum ether.

SAMPLE ELUTION

- Aspirate 2 x 1 ml benzene/petroleum ether (1:3) through the cyano-silica assembly;
- Elution analyte from column with 2 x 1 ml benzene/methanol (1:1);
- Evaporate under vacuum to dryness;
- Dissolve of dry residue in mobile phase (1 ml).

ANALYTICAL DETERMINATION

HPLC with UV/Vis detection

- detector: $\lambda = 210$ nm;
- mobile phase: 70/30 v/v acetonitrile/ water containing sodium chloride (0.01 M);
- flow rate: 0.5 ml/min.

Figure 1. Solid phase extraction of denatonium benzoate from antifreezing agents.

Table 1

Comparison of Denatonium Benzoate Recovery Obtained on Studied SPE Columns

Type of SPE Columns	**Denatonium Benzoate**			
	5 ppm Added		**15 ppm Added**	
	Recovery (%)	**RSD (%)***	**Recovery (%)**	**RSD (%)***
Octadecyl C-18	64.1	8.1	66.4	8.8
Silica gel SiOH	32.5	6.7	36.5	6.4
Cyano CN	56.3	4.7	58.2	4.5
SiOH + CN	89.2	0.9	91.3	1.1

* Standard deviations for three independent samples.

For these reasons, and taking into consideration the obtained results and the choice for construction of column type used, an extraction was done that is a combination SPE columns packed with silica gel (SiOH) and cyano (CN) phases. Use of an arrangement of the sandwich type gave considerable improvement of recovery value (R > 90 % ± 1.1, Table 1). Surely, this is caused by the selective sorption of analytes on this of kind joint fillings (ionic interactions). High recovery value and good reproducibility of the process (a low value of SD being situated in the range of 0.9 ÷ 1.1%) assure effective sorption and good elution of the analyte.

The selection of chromatographic columns for the determination of analyte in eluate testing columns is different for different kinds of packing (with octadecyl, alkylamide, and cyano phase). Addition of sodium chloride to the mobile phase provides a symmetrical band broadening of denatonium benzoate (10 ppm) for SG-AP ($f_{AS} = 0.987$) and SG-CN columns ($f_{AS} = 1.173$) (Figure 2, Table 2).

With regards to repeatability of surface area ($s = 618.42 \pm 1.58\%$) and a short retention time ($t_R = 2.754$ min) of matter analysed, the column SG-AP was chosen for further analysis. The results given in Table 2 provide evidence that SG-AP surface area contains an alkylamide group, together with the mobile phase used, containing sodium chloride, making possible quick and effective identification of denatonium benzoate in samples analysed.

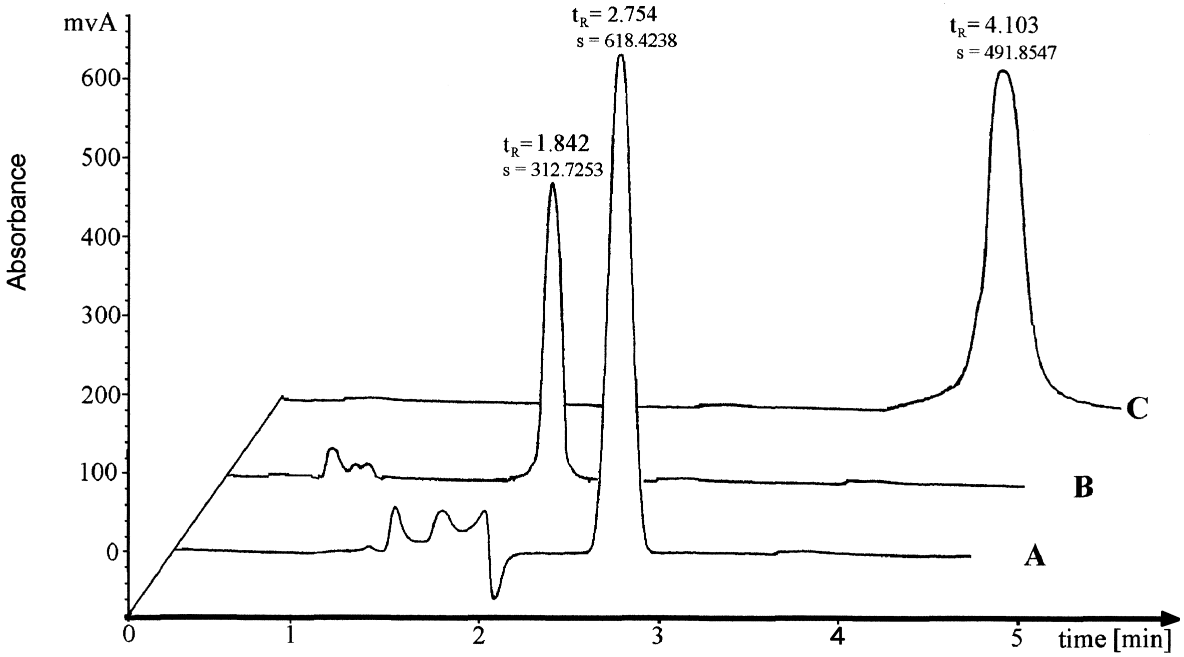


Figure 2. Normalized chromatograms for standards of denatonium benzoate (10 ppm) obtained on home-made SG-AP (A), SG-CN (C) columns and commercial Alltech C-18 (B) column.

Table 2

Parameters Characterising HPLC Columns used for Determination of the Denatonium Benzoate*

Type of Prepared Columns for HPLC	Producer	Functional Group	t_R (min)	f_{AS}	S (mAU·sec)
C=18	Alltech	$-C_{18}$, ≡Si-OH	4.103	1.225	491.8547
SG-CN	UMK Toruń	≡Si-OH, -CN	1.842	1.173	312.7253
SG-AP	UMK Toruń	≡Si-OH, NH_2, -NH-CO-C_{15}	2.754	0.987	618.4238

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* Where: t_R = the retention time, f_{AS} = the peak asymmetry, S = the peak surface area.

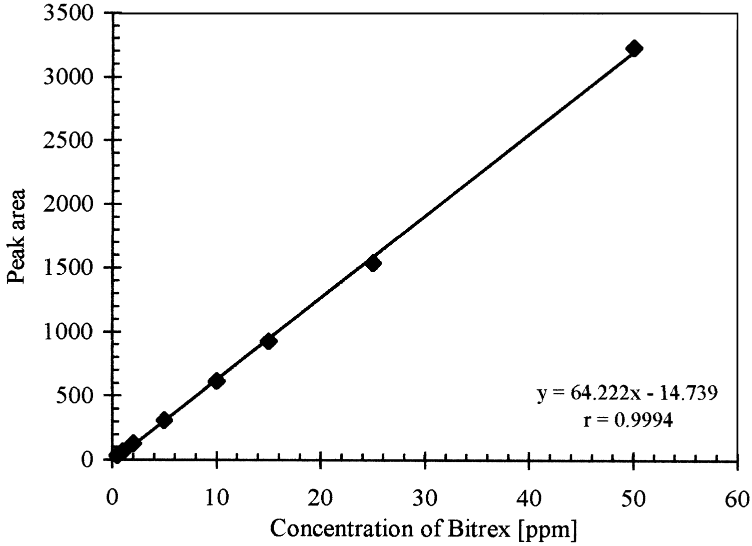

Figure 3. Typical calibration curve of denatonium benzoate (standards prepared in methanol).

Table 3

Concentrations of Denatonium Benzoate in Antifreezing Agents

Type of Antifreezing Agents	Concentration of Bitrex (ppm)*
Petrygo	11.75 ± 1.4
Borygo	10.58 ± 0.95

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* Mean values ± standard deviations calculated for six independent samples.

Surely, the chloride ion in this system serves to block spaces which were found unblocked in residual silanols during modification, and usually interacted with the particles of separated substance (denatonium benzoate).

In Figure 3 is presented the typical calibration curve obtained of a standard solution of denatonium benzoate prepared in methanol. The linearity of the responses in the concentration limits (0.5 to 50 ppm) of the calibrations solutions calculated by linear regression and expressed as correlation coefficients (r = 0.9994), is satisfactory.

The results presented in Table 1, are obtained from joint SiOH+CN columns (like a sandwich) in solid-phase extraction, used in isolation of denatonium benzoate contained in low-solidification fluid used in coolants of engines ("Petrygo," "Borygo"). Samples of fluids of about volume of 100 mL were filtered before bringing in deposit sorbents, and then isolated and enriched in accordance with the procedure outlined on Figure 1.

Extracts obtained were analysed by HPLC in reversed phase mode using a column with SG-AP phase, and obtained results are presented in Table 3. Enumerated values of concentrations for both liquids "Petrygo" (11.75 ± 1.4 ppm) and "Borygo" (10.58 ± 0.95 ppm) do not exceed the permissible concentration of Polish regulations,[10] that is, exceeding 15 ppm.

CONCLUSIONS

Optimization of the isolation process of denatonium benzoate from antifreezing agents (*Borygo* and *Petrygo*) showed that a type of the stationary phase, as well as undersurface properties of packings, have significant influence on recovery values;

Reproducible and best results (RSD < 0.9 ÷1.2) were obtained for extraction on BakerBond SPE-columns {silica (SiOH) combination with cyano (CN)};

Determination of the denatonium benzoate on the SG-AP column give a possibility of obtaining better efficiency, i.e. smaller band broadening and very good surface area, with an insignificant increase of retention, compared to Alltech C-18 and SG-CN phases;

Combination of the SPE and HPLC method presented here is accurate, efficient, economic, and sensitive. It is less hazardous than liquid-liquid extraction methods, easier to perform, and less time consuming than other existing methods.

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