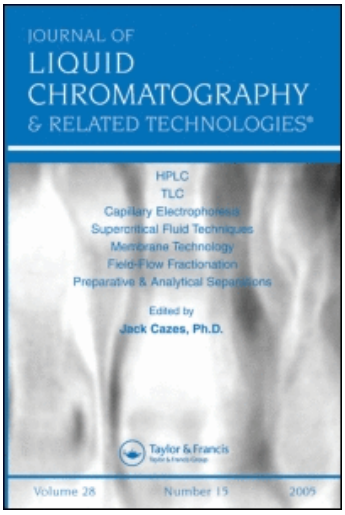

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Accurate HPLC Determination of Piperazine Residues in the Presence of other Secondary and Primary Amines

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Accurate HPLC Determination of Piperazine Residues in the Presence of other Secondary and Primary Amines

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Abstract: A reversed and normal phase HPLC separation technique with diode array detection was used for the investigation of interactions of diethylenediamine (piperazine) and its DNS derivative with different adsorbents, used as HPLC packings. The chromatography in reversed phase mode with s deactivated C_{18} column (BakerBond C_{18} BDC, SUPELCOSIL™ LC-C18-DB) did not give positive results. Piperazine was not retained on the column and eluted at death time. On the other hand, the product of coupling reaction (DNS-diethylenediamine) was strongly retained on the packing, in spite of the addition to the mobile phase of 1-heptanesulfonic acid, to diminish ionic forces between DNS product and column packing, and increase the interactions with octadecyl chains. These results confirmed the published observation, that for the analysis of this compound the use of normal phase system is obligatory. This leads to the elimination of ionic bonding between DNS-diethylenediamine and column packing. For that propose several columns were tested, e.g., BakerBond C_{18} BDC, SUPELCOSIL™ LC-C18-DB, SUPELCOSIL™ LC-CN, and home-made SG-MIX phase. On the basis of these results, the SG-MIX column was selected for further studies comprising the optimization of mobile phase, allowing the obtaining of peaks with high symmetry and good resolution, which is indispensable for the analysis of products containing trace amounts of this compound.

Keywords: Piperazine, Derivatization, HPLC, Secondary and primary amines

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INTRODUCTION

Diethylenediamine is commonly applied in pharmacy, both as a native compound known under the common name piperazine, and as the starting substance for the synthesis of methyl and hydroxyl derivatives, used for the production of drugs such as estropin, clozapine, or cinarazine. Thus, it should be assayed in many cases, during industrial synthesis as an intermediate product, and as technological impurity of the final products, as well as in the pharmacological and environmental analyses. The mode of analytical procedure is determined by the fact that both =NH groups of diethylenediamine easily bind to protons (attach protons) and assume the form $=N^+H_2$, which results in its strongly alkaline (basic) properties. The value pKa = 4.2 is the same as for carboxylic acids, which practically excludes the possibility of using carboxylic sorbents for selective sample enrichment and/or chromatographic separation. Diethylenediamine does not possess chromophores. It absorbs UV light only at a wavelength of 205 nm, and its specific absorption coefficient is very low (<0.01). Therefore, the determination of diethylenediamine at a level below 1 mg/kg is possible only after its transformation into a derivative with sufficiently high optical density, or emitting induced light. Various primary and secondary aliphatic amines are present in considerable amounts in biological and environmental samples. Their selective removal is theoretically possible, but time- and labor-consuming. The chromatographic properties of coupling products of aliphatic diamines with several carbon atoms are affected, to a great degree, by the substituent, whose molecular weight is several times higher and which usually possesses unsaturated bonds and an additional heteroatom. Hydrophobic interactions of derivatives of primary and secondary amines do not differ significantly. Additionally, in contrast to secondary amines, derivatives of primary amines may form hydrogen bonds with relevant groups of stationary phases or solid support.

The methods for diethylenediamine determination developed up to now do not comply with the standards concerning analysis of xenobiotic compounds in monitoring programs. Fletcher et al.[1] proposed a gas chromatographic method for diethylenediamine determination in urine. However, its detection limit is 50 mg/L, so it cannot be applied to trace analyses. Wellons and Carey[2] developed a method where the coupling product of diethylenediamine and m-toluoyl chloride was assayed by HPLC with ODS column. This method requires the use of a reagent unavailable commercially, and labor-consuming pyridine removal. It enables determination of 5 ng diethylenediamine in a 35 mL sample, which corresponds to a determination limit of 0.14 mg/kg. This method was utilized for commercial product analyses, so it does not include a description of a procedure enabling the determination of this analyte in the presence of other amines from a biological matrix. Sheng, Zhou, and Jin[3] performed a comparative study on separation of substituted piperazine by CZE and HPLC. Again, due to the specific nature of their research, this method cannot be applied to materials containing

a biological matrix. The method proposed by Lau-Cam and Roos[4] seems to be best suited for routine assays. These authors subjected diethylenediamine to dansylation and then assayed in a normal phase system with UV detection. This method has been developed for pharmaceuticals, which do not contain potential co-eluents. Furthermore, the results of chromatographic separation may also be irreproducible, because the cyanopropyl column used in the studies shows very low reproducibility due to its instability during synthesis.

Biological, environmental, and some pharmaceutical samples usually contain low weight aliphatic diamines such as 1,4-diaminobutane, 1,5-diaminopentane, cysteamine, and propanoloamine, which may form with DNS-Cl mono- and di-substituted derivatives. The objective of the present study was the selection of HPLC adsorbents, enabling good separation of DNS-diethylenediamine and potential co-eluents. The research was carried out for two reasons. First, separation of diethylenediamine from other aliphatic amines at the stage of sample preparation requires considerable labor expenditures, and secondly, their determination in the sample may be indispensable in some cases.

EXPERIMENTAL

Materials and Reagents

The following solvents were used for chromatographic analysis (HPLC) and solid phase extraction: methanol (MeOH), acetonitrile (ACN), acetone (ACE), phosphoric acid, heptane sulphonic acid (HPLC-grade, J.T. Baker, Deventer, Holland), alkaline acetonitrile, 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride-DNS-Cl), piperazine (Fluka Chemie AG, Switzerland), and water for HPLC. The aqueous mobile phase was prepared using water purified by means of Mili-Q apparatus (Milipore, El Passo, TX, USA).

Solid phase extraction of piperazine solutions and field samples was performed using a commercial SPE-12 G (J.T. Baker, Gross-Gerau, Germany) vacuum set. BakerBond SPE columns: octadecyl (ODS) and quaternary amine (N^+), were purchased from J.T. Baker. Poultry liver extract, deproteinized, and free from non-polar substances, sugars, and organic acids was chosen as the test sample containing potential co-eluents.

Apparatus

Chromatographic measurements were taken using HP-1050 liquid chromatograph system (Hewlett Packard, Waldbronn, Germany) equipped with a quaternary gradient pump, an autosampler (injection volume 20 μL) and a diode detector with a measuring cell with a volume of 8 μL, and ChemStation for

data collection and control over the process. The flow rate of mobile phase was always 1 mL/min. Separations were performed on different columns, Bakerbond C_{18} BDC (250 × 4.6 mm I.D.; $d_p = 5\,\mu m$ particle size), home-made SG-MIX (125 × 4.6 mm I.D.; $d_p = 5\,\mu m$ particle size and 250 × 4.6 mm I.D.; $d_p = 5\,\mu m$ particle size),[5] SUPELCOSIL™ LC-CN and SUPELCOSIL™ LC-C18-DB (250 × 4.6 mm I.D.; $d_p = 5\,\mu m$ particle size), that characteristics are listed in Table 1.

RESULTS AND DISCUSSION

Preliminary Analyses

Due to a very disadvantageous wavelength, 205 nm, a direct spectrophotometric evaluation of the degree of diethylenediamine extraction from all materials is burdened with a gross error. For that reason, the possibility of recovery of diethylenediamine bonded to an octadecyl sorbent was estimated on the basis of a direct chromatographic analysis at a wavelength of 210 nm, 218, and 230 nm. The degree of elution from this sorbent was evaluated by comparison of results obtained by means of HPLC separation of standards, blank eluates, and eluates of standards, tested at concentrations of 100 and 10 mg/L (Figure 1).

A peak with retention time of 2.0 min. was recorded in the chromatogram of a standard diethylenediamine solution. This was column dead time, but taking into account the detector response to a solution of 100 ppm, constituting 90.7% of the 10 ppm solution, i.e., remaining within the linearity range, and the fact that this peak was visible only at 210 nm, it may be assumed that it was a diethylenediamine peak. DL for the standard solution, measured by a noise level at 210 nm, was 1.043 mA, and QL–2.009 mA, which corresponds to 0.07 and 0.14 mg/L, respectively. This shows that detection sensitivity was sufficient for testing the behavior of standard solutions on extraction columns.

Table 1. Surface characteristics of chemically bonded phases

Type of phase	Types of functional groups	Pore size (Å)	Carbon P_C (%)
SUPELCOSIL™ LC-C18-DB	C_{18}	120	11
SUPELCOSIL™ LC-CN	CN	120	4
BakerBond C18 BDC	C_{18}	120	12
SG-MIX	C_{18}, C_8, Ph, CN, NH_2	110	15
SG-MIX	C_{18}, C_8, Ph, CN, NH_2	300	11

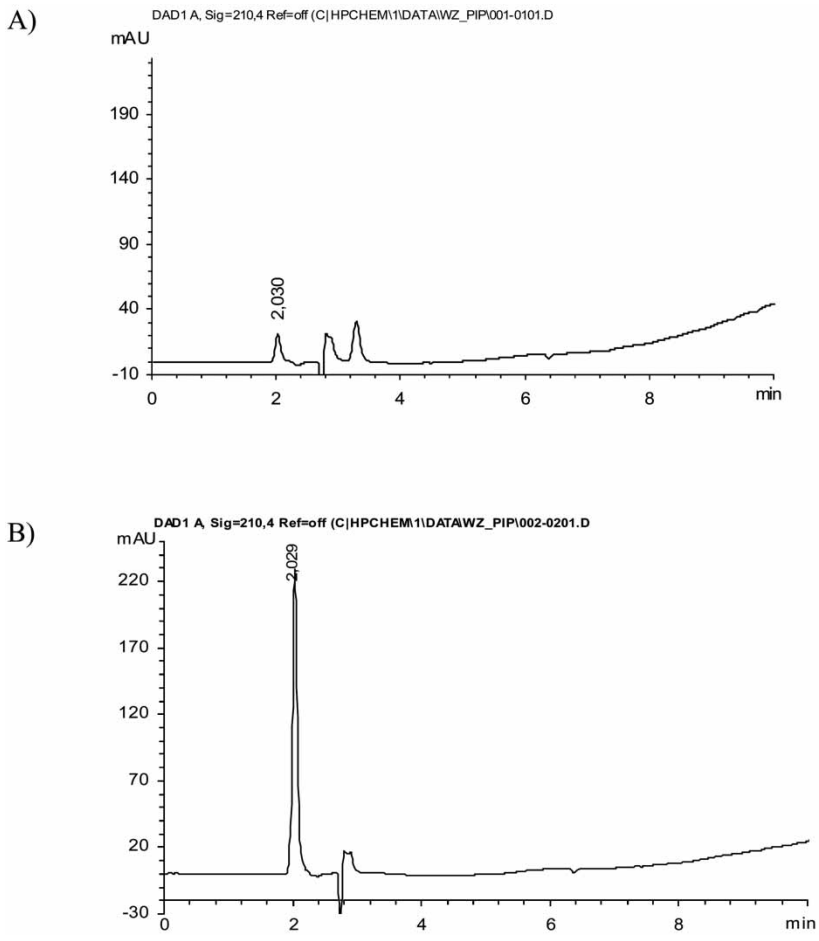

Figure 1. Chromatograms of piperazine standard solutions at concentrations 10 mg/L (A) and 100 mg/L (B). Chromatographic conditions: BakerBond C18 BDC column (250 × 4.6 mm; particle size 5 μm), mobile phase: phase A (30:70% v/v methanol:water + 0.15 mL phosphoric acid + 5 mM heptane sulfonic acid); phase B (90:10% v/v methanol:water + 0.15 mL phosphoric acid + 5 mM heptane sulfonic acid); gradient elution 1 min 100% phase A, 15 min 100% phase B, 30 min 100% phase B, 35 min 100% phase A, 50 min 10% phase A; mobile phase flow rate 1 mL/min.

It was calculated that an ODS column retains approx. 75% diethylenediamine from 20% methanol, and approx. 30% from 40% methanol. Diethylenediamine bounded on this sorbent could be eluted with 40% acetonitrile, which indicates that it is not permanently retained on an ODS column. It follows that this column should also enable analysis of the DNS-derivative. N^+ sorbent trapped diethylenediamine from 5% acetonitrile. The analyte peak was

present in the eluate containing 10% acetonitrile, where it accounted for 35% of the standard, and in 20% ACN eluate, where its surface area constituted 65% of the standard dosed to the extraction column.

These results suggest that diethylenediamine binding by bonded phases is mostly associated with hydrophobic interactions, and its separation from other diamines with similar molecular weights is possible only on a carboxylic sorbent, or more effectively, on a sulfone sorbent, following very careful pH adjustment. A positive effect of using a quaternary amine (N^+) column at the stage of sample preparation, is obtaining eluate in 20% acetonitrile with neutral pH. This allows passing to the coupling stage.

Effects of Acetonitrile and Acetone on Piperazine Derivatization

The surface area of peaks of the reagent sample for 5 mg DNS-Cl in ACN was 70% of the value of the surface area of peaks of the reagent sample calculated on the basis of 2 mg DNS-Cl in ACN. The surface area of peaks of the reagent sample for 5 mg DNS-Cl in ACN was 20% of the value of the surface area of peaks of the reagent sample for 5 mg DNS-Cl in ACE. The surface area of DNS-diethylenediamine obtained for the solution DNS-Cl 5 mg/mL was 172.6% of the surface area for 2 mg/mL.

The surface area of the peak of piperazine coupled with DNS-Cl at concentration of 5 mg/mL acetone was 99.9% of the surface area of the peak obtained for the solution DNS-Cl 2 mg/mL acetonitrile. The height of the peak of piperazine derivative obtained by incubation with a 5 mg/mL acetonitrile solution was twofold higher, compared with the height of the peak of the derivate obtained by incubation with an acetone solution of DNS-Cl at the same concentration.

These results suggest that the maximum concentration of acetonitrile-DNS-Cl solutions may be 3.5 mg/mL. The solubility of DNS-Cl in acetone is fivefold higher than its solubility in acetonitrile. Despite this fact, an acetonitrile medium ensures twofold higher detection sensitivity. Therefore, further analyses were performed with an acetonitrile solution of DNS-Cl. DNS-Cl (1.6 mg) was sufficient for coupling 0.2 mg diethylenediamine. This means that the weight ratio of DNS-Cl to diethylenediamine was 8:1, corresponding to a 4:1 ratio for one amine group. At a molar ratio of 269.75:86.14 $\geqslant$ 3:1, a 2.5 excess of the coupling reagent should be used.

Coupling Procedure

The following procedure was adopted on the basis of the above results: 0.8 mL of solution containing 3.5 mg dansyl chloride in 1 mL acetonitrile was added to 2 mL of diethylenediamine solution in alkaline acetonitrile, immersed in an ultrasound bath for 10 min., and stored in a darkroom for 30 min. Then 2.25 mL water was added and a dansyl derivative was extracted with 2 mL

chloroform for one minute. The phases were separated by centrifugation (5 min, 3500 rpm), the chloroform layer was removed and passed through quantitative filter paper (5.5 cm in diameter) containing 500 mg of anhydrous sodium sulfate. The filter paper was then washed with 1 mL of chloroform. The filtrate was concentrated to 1 mL and stored in an ice bath in a closed test tube.

Chromatographic Analyses

Three types of columns were used for the chromatographic analysis of the dansyl derivative, i.e., a Bakerbond C18 BDC and SUPELCOSIL LC-C18-DB RP column, and three columns for a normal phase system, i.e., a cyanopropyl SUPELCOSIL LC-CN 250 × 4.6 mm column and our own SG-MIX (125 × 4.6 mm; 250 × 4,6 mm) columns, prepared according to the procedure described in Ref. [5]. The mobile phase in a reversed phase system was a methanol-water mixture modified with o-phosphoric acid (0.15%) and heptane sulfonic acid (5 mmol/L), in the following gradient: phase A (MeOH:water—30:70% v/v), phase B (MeOH:water—90:10% v/v). The mobile phase in a normal phase system was a hexane-2-propanol mixture in the gradient 90:10–70:30% v/v. Mobile phase flow rate was 1.0 mL/min in both cases. To ensure chloroform solubility in the water-methanol mobile phase, 20 μL sample was transferred to all columns. Identification was performed using a UV detector at a wavelength of $\lambda = 335$ nm and $\lambda = 365$ nm.

The application of reversed phase chromatography and a deactivated C_{18} column brought quite unexpected results. The native substance was eluted from the column at dead time, whereas the coupling product (DNS-diethylenediamine) was very strongly retained on the packing. Too high width and asymmetry of peaks made it impossible to use this column for trace analyses.

The results obtained showed that a normal phase system should be applied, since it enables the elimination of ion interactions between DNS-diethylenediamine and column packing. Therefore, we decided to test the column with cyano packing, recommended by Lau-Cam and Ross,[4] and our own column with SG-MIX phase, which contains octadecyl and octyl chains, as well as amine, cyanic, and phenyl units. This column permits various analyte-sorbent interactions, so it can be used in both reversed phase and normal phase systems.

Peaks were very wide on the cyanopropyl column. This suggests that on the CN column, DNS-diethylenediamine interacted strongly with the stationary phase despite an anhydrous normal phase system, such as in the case of deactivated ODS phase. This confirms the supposition that despite major technological advances and recent developments in the preparation of chemically bounded phases, the CN group in cyanic columns is often decomposed to an amide or even a carboxylic group. The poor quality of chromatograms, inconsistent with relevant standards, could also result from inadequate silica

coverage density. Since it was impossible to obtain reproducible peaks with good characteristics with the cyanopropyl column, it was eliminated from further studies, similarly as ODS and BDC ones.

As expected, the SG-MIX column obtained a sharp main peak with two additional, not completely separated, ones. These peaks were very well separated from those representing the product of hydrolysis of the derivatizing reagent, i.e., DNS-OH. The application of the optimum mobile phase gradient

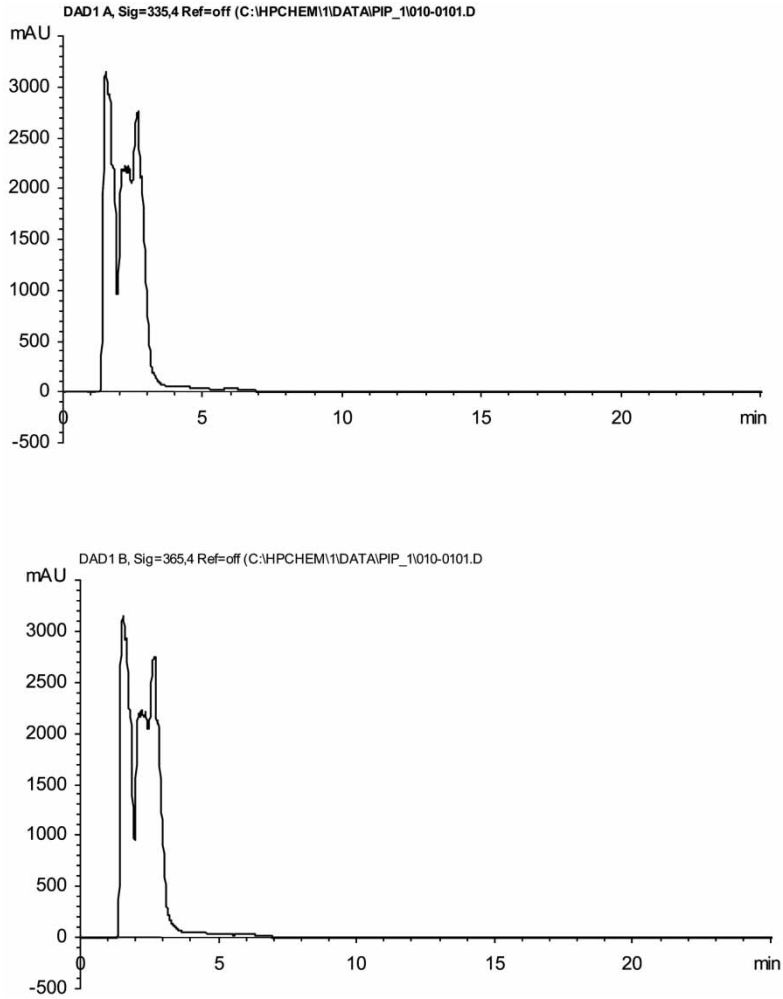

Figure 2. Chromatogram of the reagent sample. Chromatographic conditions: SG-MIX column (125 × 4.6 mm; particle size 5 μm), mobile phase: gradient 0 min to 90/10% hexane/2-propanol; 10 min–80/20% hexane/2-propanol; 20 min 80/20% hexane/2-propanol; 25 min 90/10% hexane/2-propanol; 30 min 90/10% hexane/2-propanol; mobile phase flow rate 1 mL/min.

resulted in almost total band separation and obtained high-symmetry peaks of the product determined (Figures 2–4).

Method Validation

The analyses of standard solutions show that:

1. in order to obtain good characteristics of peaks, the volume of a chloroform sample introduced into the column cannot be higher than 50 μL;

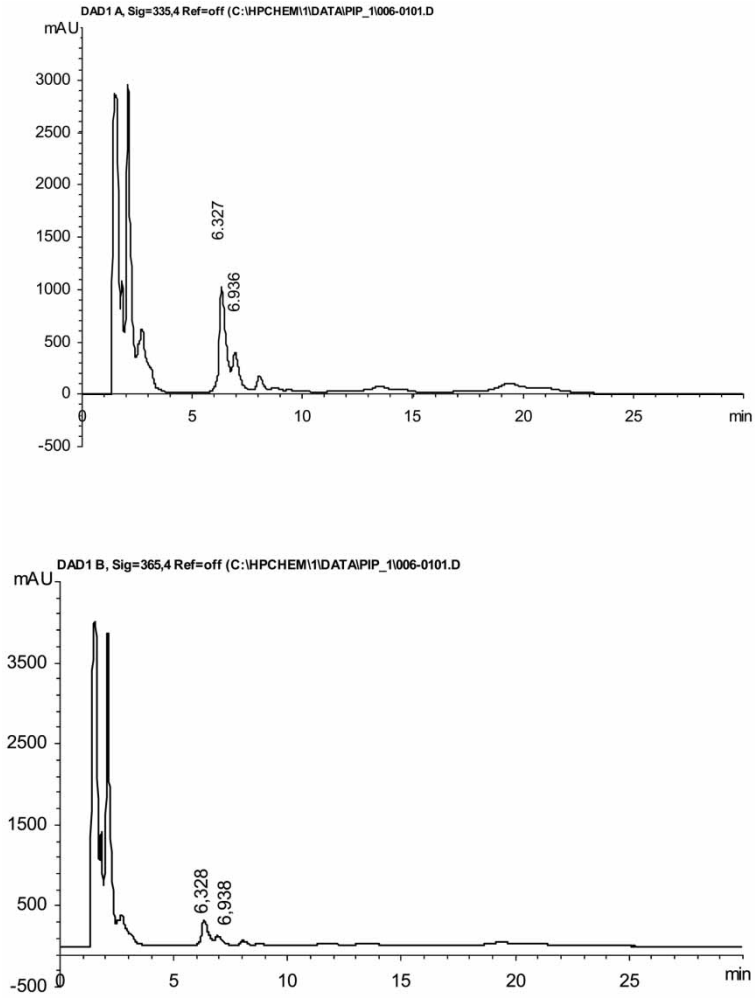

Figure 3. Chromatogram of the standard sample (chromatographic conditions as in Figure 2).

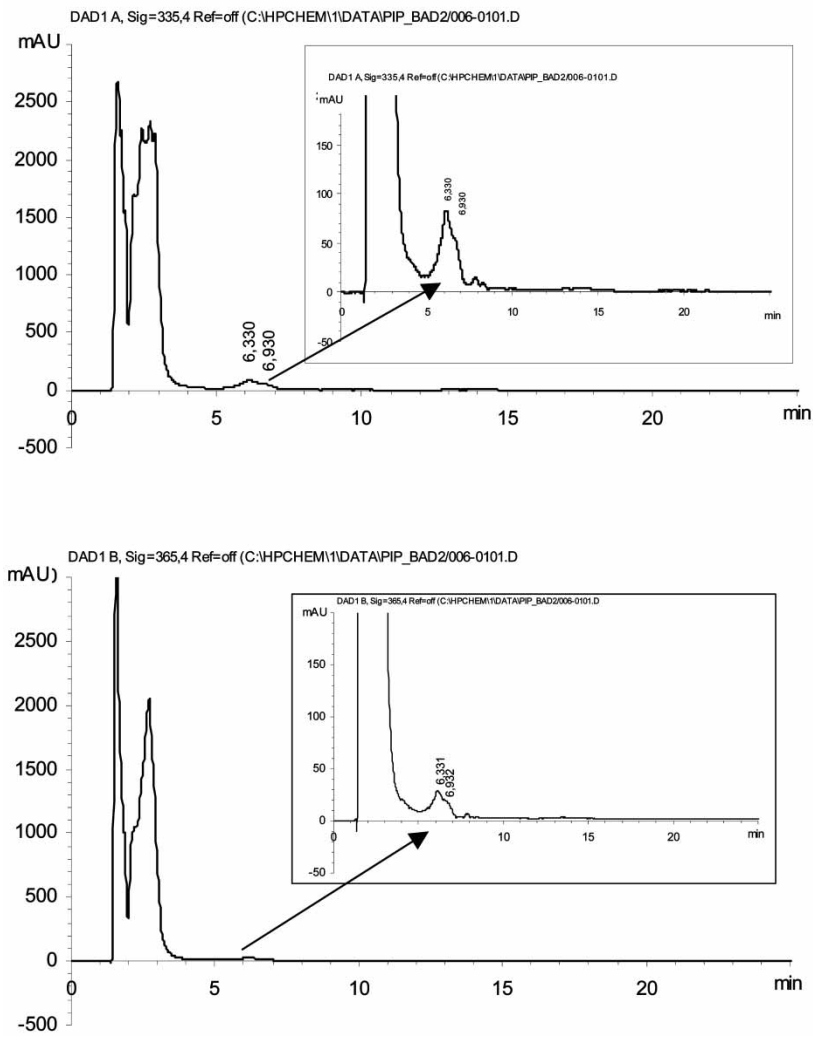

Figure 4. HPLC analysis of the fish extract sample (chromatographic conditions as in Figure 2).

2. diethylenediamine forms two derivatives with DNS-Cl, whose total separation was impossible under the present experimental conditions. These are probably mono- and di-substituted compounds. The basic parameters of the method tested could be evaluated despite incomplete separation of these peaks.

Due to the fact that much better readability was obtained at 335 nm than at 365 nm, the results recorded at 335 nm were subjected to evaluation,

although the base line was more stable at 365 nm. Mean noise surface area was 1.53 ± 2.16 mAU, and the mean surface area of peaks of a sample at concentration 1 mg/L was 181.5 mAU. Reproducibility was assessed by performing 10 replicate injections of a solution containing 0.2 ppm piperazine. It follows that the detection limit for the standard solutions introduced into the column in a volume of 50 μL ($x_{śr} + 3\,s$) was 0.012 ng/μL, and determination limit ($x_{śr} + 10\,s$)−0.020 ng/μL (Table 2). Compared with the data given in Ref. 3, these values are by almost one order of magnitude higher. Following sample enrichment, this enables achieving the limits regulated by law.

The mean variation coefficient of detector response over the whole measuring range was 7.7%, reaching 6.9% at concentration of 0.2 ppm. The coefficient of variation of retention time of the DNS-diethylenediamine peak was 0.32%. High repeatability of retention times was conducive to high reliability of results. Detector response was linear in a concentration range of 0.2 to 25 ppm (Table 2).

Verification of Measurement Result Reliability

Positive results of analytical method validation for standard solutions do not guarantee the reliability of measurements performed on real samples. To find out whether there occurs co-elution with other amines, a comparison was made between chromatograms of standard solutions and extracts of fish tissue–the richest source of amines (Figure 4). It indicated the possibility of co-elution of amines being the products of metabolic changes of amino acids and the xenobiotic examined, i.e., diethylenediamine. This shows that despite very good results obtained with standard solutions, the 125 mm SG-MIX column cannot be used for assays of real samples. To obtain a separation factor enabling accurate measurements, it was necessary to use a 250 mm column. Due to a considerable 2-propanol content, the use of a standard column with 5 μm sorbent with 80–120 μm pores would result in

Table 2. Parameters of calibration of the diethylenediamine determined by HPLC

Parameter	Value
Concentration range (ng/μL)	0.2–25
Equation of regression line (for peak area)	y = 212,26x
Concentration levels (n)	5
Correlation coefficient	0,9999
Limit of detection (ng/μL)	0.012
Limit of determination (ng/μL)	0.020

too high a resistance, making accurate analysis impossible in the case of full column protection (saturation column, pre-column and filter). Therefore, the new sorbent was synthesized on a porous support (300 μm.). Figure 5 shows the results obtained with this column, which enabled successful separ-

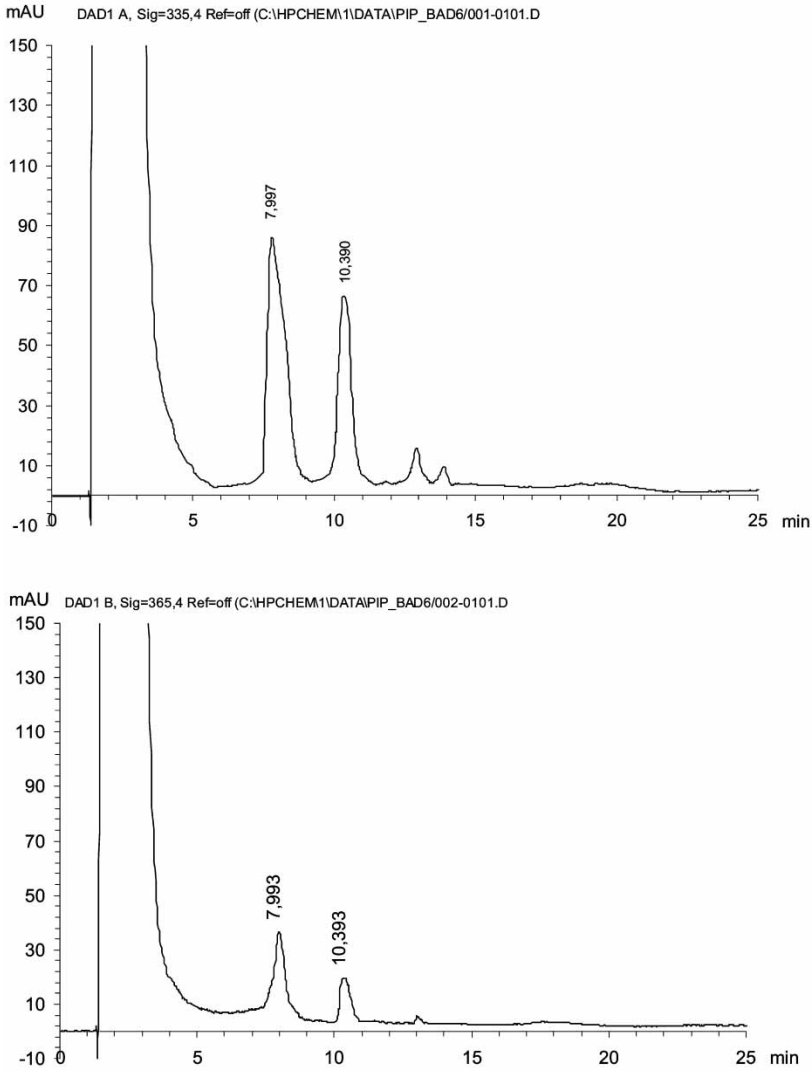

Figure 5. HPLC chromatogram of the fish extract sample. Chromatographic conditions: SG-MIX column (250 × 4.6 mm; particle size 5 μm), mobile phase: gradient 0 min to 90/10% hexane/2-propanol; 10 min-80/20% hexane/2-propanol; 20 min 80/20% hexane/2-propanol; 25 min 90/10% hexane/2-propanol; 30 min 90/10% hexane/2-propanol; mobile phase flow rate 1 mL/min.

Table 3. Quantitative results of the analysis of spiked and naturally fish tissue

Sample no.	Concentration level (ng/μL)	Found concentration (ng/μL)	Recovery (%)	Reproducibility, (RSD %)
1	0.1 (spiked)	0.093	93	1.1 (n = 3)
2	0.4 (spiked)	0.36	90	1.5 (n = 3)
3	1.0 (spiked)	0.89	89	1.9 (n = 3)
4	Naturally contaminated	0.037	n.d.	4.5 (n = 5)

ation of diethylenediamine from fish extract interferents. The method has been successfully applied for determination of residual piperazine in fish (Table 3). The method is reproducible and free from interferences, such as amino compounds.

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