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Simultaneous determination of substituted diphenylamine antioxidants and benzotriazole ultra violet stabilizers in blood plasma and fish homogenates by ultra high performance liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

Analytical methods were developed for the determination of eight substituted diphenylamines (SDPAs) and six benzotriazole UV stabilizers (BZT-UVs) in blood plasma and fish homogenate matrices. Liquid-liquid extraction by methyl *tert*-butyl ether and denaturation by KOH following silica gel packed column clean-up was employed for blood plasma preparation. For the fish homogenate samples, ultrasonic assisted solvent extraction combined with automated gel permeation chromatography and silica gel packed column clean-up was used. The target compounds were determined by optimized ultra performance liquid chromatography-tandem mass spectrometry in positive electrospray ionization mode. The method limits of quantification (MLOQs) of the 14 analytes ranged from 0.002 to 1.5 ng g^{-1} and 0.001 to 2.3 ng g^{-1} (wet weight, w.w.) for blood plasma and fish homogenate, respectively. The total recoveries of the target compounds varied from 61% to 100% (mean 77 ± 9%). Eleven targets including monobutyl- (C4), dibutyl- (C4C4), monooctyl- (C8), monobutyl monooctyl- (C4C8), dioctyl-(C8C8), monononyl- (C9), dinonly-(C9C9) and 4,4′-bis(α,α-dimethylbenzyl)-(diAMS) DPAs, as well as 2-(2H-Benzotriazol-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol (UV234), 2,4-di-*tert*-butyl-6-(5-chloro-2H-benzotriazol-2-yl) phenol (UV327) and 2-(2H-Benzotriazol-2-yl)-4,6-di-*tert*-pentylphenol (UV328) were identified in the environmental biota samples, with concentrations in the range of <MLOQ–934 pg g^{-1}, <MLOQ–4.2 × 10^3 pg g^{-1} and <MLOQ–3.9 × 10^3 pg g^{-1} w.w. for bottle nose dolphin (*Tursiops truncatus*) plasma, northern pike (*Esox lucius*) plasma and white sucker (*Catostomus commersonii*) homogenate, respectively. This is the first report of an analytical method development for SDPAs in biotic matrices and BZT-UVs in blood plasma samples.

1. Introduction

Anthropogenic SDPAs are commonly added in lubricants, engine oils, plastics, polyurethane foams and rubbers as antioxidants to prevent degradation of the materials by destroying free radicals [1–3]. BZT-UVs are also anthropogenic additives and are widely used in plastic commodities and industrial products to protect them from sunlight ultraviolet radiation caused yellowing and degradation [4,5]. These contaminants may enter the aquatic environment through direct discharge during application and manufacture processes and wastewater treatment plant effluent, or indirect release from the wastes containing these compounds (e.g., small plastic debris) [6,7]. Once released into the aquatic environment, some of these compounds may be toxic, persistent and bioaccumulative [3–5,8–15]. However, the occurrence and environmental fate processes information of these contaminants in North America is very limited. Thus, SDPAs and BZT-UVs are currently being assessed under the Chemical Management Plan (CMP) of the Canadian Environmental Protection Act (CEPA) for human and environmental risk.

Very limited data is available for the occurrence of SDPAs in environmental samples. For example, the concentrations of C8C8 SDPA were only reported in natural beach sand in the Mediterranean Sea [16]. In contrast, BZT-UVs have been determined in many environmental matrices [17] including surface water [5,18,19], wastewater [5,19–23], sewage sludge [24,25], sediments [4,25], soil [13], indoor

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dust [26,27], mussels [28], marine mammals [29], birds [4] and fish [4,15,30]. However, analytical methods and data are absent for the determination of SDPAs in any biota and BZT-UVs in blood samples. Blood serum and plasma have been widely used to monitor contaminants in wildlife [31,32]. This is because the contaminants levels/patterns in the blood are related to levels/patterns in other tissues and/or in the environment [32]. In addition, the blood samples can be obtained with minimal harm to the animal [31,32]. Moreover, blood is a possible reservoir of BZT-UVs due to the possible binding interactions of these contaminants with circulating proteins [11].

The objective of this study was to develop a suitable multi-residue analytical method to simultaneously determine SDPAs and BZT-UVs in blood serum, plasma and fish homogenate samples. The affecting factors during the sample preparation were optimized. Finally, the developed methods were evaluated and applied to the analysis of the targets in bottle nose dolphin (*Tursiops truncatus*) plasma from Florida (USA), northern pike (*Esox lucius*) plasma from St. Lawrence river (Canada) and homogenized white sucker (*Catostomus commersonii*) samples from an urban creek of Canada.

2. Materials and methods

2.1. Materials

SDPA standards were either purchased from Accustandard (New Haven, CT, USA) wherever possible or provided by industry. The SDPA standards used in this work including (1) benzenamine, N-phenyl- reaction products with 2,4,4-trimethylpentene and isobutylene (CAS# 184378-08-3; containing C4-, C4C4-, C8-, C4C8- and C8C8-DPAs); (2) benzenamine, N-phenyl-, tripropenyl derivatives (CAS# 68608-79-7; containing C9- and C9C9- DPAs) and (3) diAMS (CAS#10081-67-1). The compositions of SDPA mixtures are confidential business information and therefore could not be reported here; however, the composition was determined by mass spectrometry and verified by flame ionization detection and used to correct the concentrations of each individual compound in this study. BZT-UV analytical standards including UV234 (CAS# 70321-86-7), 2-*tert*-butyl-6-(5-chloro-2H-benzotriazol-2-yl)-4-methylphenol (UV326; CAS# 3896-11-5), UV327 (CAS# 3864-99-1), UV328 (CAS# 25973-55-1), 2-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol (UV329; CAS# 3147-75-9), and internal standard 2-allyl-6-benzotriazol-2-yl-4-methylphenol (ABZTMP; CAS# 2170-39-0) were purchased from Sigma-Aldrich (Oakville, ON, Canada). 2-(2H-benzotriazol-2-yl)-4-(*tert*-butyl)-6-(sec-butyl) phenol (UV350; CAS# 36437-37-3) was purchased from BOC Science (Shirley, NY, USA). The structures of the target compounds are shown in Table S1 in Supplementary material.

Adult bovine serum (Sigma-Aldrich; Oakville, ON, Canada) and farmed salmon fish plasma (Meridian Life Science Inc.; Memphis, TN, USA) were used for blood plasma method development. Homogenates of lake trout (*Salvelinus namaycush*) collected from the Great Lakes were used for fish homogenate method development. The spike levels for recovery test were 80 ng g^{-1} and 20 ng g^{-1} of SDPA mixture or individual BZT-UV. OASIS HLB SPE cartridges (200 mg) were purchased from Waters (Mississauga, ON, Canada). Solvents (methanol, isopropanol, acetonitrile, methyl *tert*- butyl ether (MTBE) and water) were HPLC grade and purchased from Fisher Scientific (Ottawa, ON, Canada). Hexane and dichloromethane (Distilled in Glass grade) were purchased from Caledon Laboratory Chemicals (Georgetown, ON, Canada). Formic acid (98%) was purchased from Sigma-Aldrich (Oakvile, ON, Canada). Bio-Beads S-X3 (200–400 mesh) was purchased from Bio-Red Laboratories (Hercules, CA, USA). Potassium hydroxide (KOH), hydrochloric acid (HCl) and anhydrous sodium sulfate (Na_2SO_4) were purchased from VWR (Mississauga, ON, Canada). Na_2SO_4 and neutral alumina (100–200 mesh, Fisher Scientific, NJ, USA) were activated at 450 °C overnight before use. Silica gel (63–200 μm, Caledon Laboratory Chemicals, ON, Canada) was activated at 130 °C overnight before use. Both neutral alumina and silica gel were deactivated by 5% of water prior to use. Instruments used for sample preparation are listed in the Supplementary material.

2.2. Environmental sample collection

Blood plasma samples were collected from live-captured and released bottlenose dolphins from Sarasota Bay at Florida (USA) in 2014 and northern pike from St. Lawrence River in 2011. Blood plasma was obtained by centrifugation of the blood within few hours after collection [33]. White sucker samples were captured from an urban creek in Ontario (Canada) in 2014 using a backpack electrofisher (Halltech HT-2000) and transported live to the on-site laboratory in aerated tank. Fish liver, bile and blood plasma were removed before homogenization of the carcass. Fish homogenization was conducted by a RETSCH GM200 GRINDOMIX homogenizer. All samples were stored at −20 °C until extraction.

2.3. Finalized method for blood serum and plasma extraction

The main procedures of sample preparation methods are summarized in Fig. 1. Blood serum or plasma samples were thawed and weighted before extraction. About 0.1–1 g of blood plasma was used for analysis, depending on the available sample size. Plasma with internal standard (4 ng) was extracted by 3 mL of MTBE, followed by vortex (1 min), sonication (5 min) and centrifugation (5 min). The liquid–liquid extraction (LLE) was repeated for 3 times and all the organic fractions were combined and concentrated to about 1 mL by gentle N_2. The remaining aqueous phase of blood sample was treated by 1 mL of 5 M KOH and followed by 30 s of vortex. The aqueous phase was then extracted by 2 × 5 mL of MTBE. All of the organic extracts were combined and concentrated to dryness and reconstituted in 1 mL of 1/1 dichloromethane/hexane mixture for further cleanup. The cleanup column was packed by 5 g of 5% water deactivated silica gel with 1.5 g of Na_2SO_4 on the top. The column was preconditioned by 50 mL of hexane, and then the extracts were loaded and eluted with 30 mL of dichloromethane/hexane (1/1, *v*/*v*) mixture. The eluents were concentrated to dryness and reconstituted in 1 mL of acetonitrile for instrumental analysis.

2.4. Finalized method for fish homogenate extraction

Wet fish homogenate sample (0.5 g) with internal standard (4 ng) was mixed with 2 g of Na_2SO_4. A mixture solvent of 5 mL dichloromethane/hexane (*v*/*v*, 1/1) was used for fish homogenate extraction. Two minutes of vortex was conducted for the samples, followed by 10 min of mechanical shaking and sonication. The tube was centrifuged for 5 min and the organic phase was transferred to a pre-weighed new glass centrifuge tube. This extraction procedure was repeated three times and the supernatants were combined and then concentrated to dryness using N_2 gas. The lipid content was measured gravimetrically. The extract was then reconstituted in 1 mL of 1:1 dichloromethane/hexane. This extract was further filtrated by 0.45 μm (13 mm i.d.) nylon filter and then cleaned up by automatic gel permeation chromatography (GPC) (Express Performance column, 25cm × 3.5 cm i.d.; packed with 50 g of Bio-Beads S-X3) (J2 scientific; Columbia, MI, USA) using 1:1 dichloromethane/hexane as mobile phase at a flow rate of 5 mL min^{-1}. The fraction corresponding to 7.5–22.5 min during the GPC clean-up was collected and concentrated to about 1 mL using rotary evaporation. This extract was further cleaned by silica gel