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Determination of Triclosan as Its Pentafluorobenzoyl Ester in Human Plasma and Milk Using Electron Capture Negative Ionization Mass Spectrometry

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A sensitive method for the determination of triclosan in plasma and milk is presented. Following hydrolysis of possible conjugates, triclosan is extracted with *n*-hexane/acetone, partitioned into alcoholic potassium hydroxide, and converted into its pentafluorobenzoyl ester. After sulfuric acid cleanup, sample extracts are analyzed by gas chromatography/electron capture negative ionization mass spectrometry. The limit of quantification was 0.009 ng/g for a 5-g plasma sample and 0.018 ng/g for a 3-g milk sample. The coefficient of variation for the method was 6%. The method was tested on more than 70 human plasma and milk samples, of which all plasma samples and more than half of the milk samples were above the limit of quantification. The presented method has lowered the limit of quantification for triclosan in human matrixes significantly as compared to previous methods and makes possible the analysis of triclosan in humans under normal exposure conditions.

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether, CAS 3380-34-5) (Figure 1) is a lipophilic and phenolic compound (log K_{OW} = 4.76; pK_a = 7.9).[1,2] Due to its antibacterial properties, triclosan has found widespread use in a variety of consumer products, including toothpastes, deodorants, soaps, polymers, and fibers.[3−5] Following its discovery in the bile of fish living downstream of sewage treatment plants,[5] triclosan has attracted increasing interest as a potentially toxic environmental contaminant. The acute toxicity of triclosan to mammals is low, but in vitro studies on rat and human material have shown that low concentrations of triclosan may disturb metabolic systems and hormone homeostasis.[6−9] Triclosan is absorbed by humans and has been found in human milk and plasma.[5,10,11] In humans, triclosan is metabolized to and excreted as glucuronide and sulfate conjugates.[8,10] Considering the widespread use of triclosan and the potential adverse effects from long-term exposure, there is a need to further study triclosan concentrations in human body fluids.

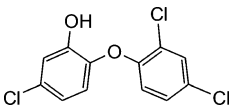

Figure 1. Molecular structure of triclosan.

Few methods for analyzing triclosan in human matrixes are described in the literature. However, Hoar and Sissons[12] presented a method for analyzing triclosan in plasma, urine, fish tissue and bile with a reporting limit of 10 ng/mL. More recently, Lin[13] analyzed triclosan in human plasma with a reported limit of detection (LOD) of 10 ng/mL; Bagley and Lin[14] reported a limit of quantification (LOQ) of 10 ng/mL. Ye et al.[15] reported an LC/MS/MS method for the analysis of triclosan in urine with a LOD of 2 ng/mL.

To study triclosan in humans under normal exposure conditions, the high reporting limits of previous methods are insufficient. As a result, we have worked during the last several years at improving these methods. Recently, our group analyzed triclosan in milk and plasma at reporting limits of 0.3 ng/g

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(personal communication for fresh weight basis) and 0.1 ng/g, respectively.[5,10] In this paper, we give a detailed description of the analytical methodology, which includes more recent improvements that have further lowered the LOQ.

EXPERIMENTAL SECTION

Chemicals. Solvents and chemicals used in the extraction and cleanup of the samples: *n*-hexane (LiChrosolv) (Merck, Darmstadt, Germany) was of liquid chromatography grade; acetone (Suprasolv) (Merck) was of gas chromatography grade; hydrochloric acid (HCl, 37%, w/w), sodium chloride (NaCl) (Scharlau, Barcelona, Spain), sulfuric acid (H_2SO_4, 98%) (Fisher Scientific, Loughborough, UK), and potassium hydroxide (KOH) (Eka Chemicals, Bohus, Sweden) were of pro analysis quality; ethanol (99.5%) (Kemetyl, Haninge, Sweden); pentafluorobenzoyl chloride (PFBCl, ≥ 98.5%) (Fluka, UK). Water was purified employing a Milli-Q Reagent Water System (Millipore Corporation, Billerica, MA). Universal indicator (Merck) was used for pH measurement. The gases used were helium (99.997%), nitrogen (99.998%), argon−methane (90:10, 99.9997%/99.995%) (Air Liquid, Kungsängen, Sweden), and ammonia (99.998%) (AGA Gas AB, Sundbyberg, Sweden). $^{13}C_{12}$-labeled triclosan was purchased from Cambridge Isotope Laboratories (Andover, MA). Triclosan (Irgasan DP300) (calibration standard) was a gift from Ciba-Geigy. CB 106 (2,3,3′,4,5-pentachlorobiphenyl) was synthesized by Peter Haglund (now at Umeå University).

Samples. The analytical method described in the following was applied to 72 plasma and 71 milk samples from 36 Swedish women.[16] The study was approved by the Regional Ethical Review Board, Huddinge University Hospital, Sweden (dnr: 395/03). Informed consent by the mothers was compulsory for their participation in the study.

Hydrolysis, Extraction, and Cleanup. Test tubes were machine-washed and heated to 420 °C for 4 h before use. During analysis, the caps of the test tubes were lined with a double layer of aluminum. The test tubes were centrifuged for 5 min at 2000 rpm (900 g) after each mixing of aqueous and organic phases to accelerate phase separation.

The sample (3 g milk, 5 g plasma, or 3 mL H_2O as the blank) was transferred to a 15-mL screw capped test tube and fortified with ^{13}C-labeled triclosan. To cleave any metabolic conjugates of triclosan, the sample was hydrolyzed in H_2SO_4 (9 M when in sample) on a heating unit (0.5 h, 60 °C). The sample was cooled to room temperature and extracted twice with *n*-hexane/acetone (9:1, v/v, 6 + 4 mL) by inverting the tube 60 times by hand. Triclosan was partitioned into an aqueous KOH solution (0.5 M, 50% ethanol, 2 mL), which was mixed with the extract on a vortex mixer, followed by inverting the test tube for 5 min on a rotary mixer. The organic phase containing neutral compounds was discarded after centrifugation. The aqueous KOH solution with the deprotonated triclosan was acidified with HCl (2 M, 1 mL), and triclosan was extracted twice with *n*-hexane/acetone (9:1, v/v, 4 + 2 mL) by mixing on a vortex mixer. The extract was cleaned up by adding H_2SO_4 (13.7 M, 4 mL) and inverting the test tube for 5 min on a rotary mixer. The extract was transferred to a second test tube, and the H_2SO_4 layer was reextracted with *n*-hexane/acetone (9:1, v/v, 2 mL). The combined organic phases were adjusted to 3 mL before derivatization.

Derivatization. All samples and calibration standards were treated as follows: Triclosan was converted to its pentafluorobenzoyl ester by adding H_2O (Milli-Q, 2 mL), KOH (2 M, 50 μL), pentafluorobenzoyl chloride (10% in toluene, 10 μL) and NaCl (0.3 g; more NaCl was added if emulsion formed upon mixing) to the extract and shaking the test tube vigorously for 2 min. The extract was transferred to another test tube, and the aqueous phase was reextracted with *n*-hexane (2 mL). H_2SO_4 (98%, 3 mL) was added to the extract, and the tube was inverted 60 times. The extract was transferred to another test tube, and the H_2SO_4 layer was reextracted with *n*-hexane (2 mL). The final extract was reduced to 2 mL under a gentle flow of nitrogen gas at room temperature after addition of volumetric standard (CB 106). Approximately 0.5 mL of the extract was transferred to a GC/MS vial and analyzed as described below.

Triclosan was also converted to its methyl ether and acetate ester for comparison purposes. The methyl ether was formed by the reaction of triclosan in *n*-hexane with diazomethane for 3 h at 4 °C in darkness. Excess reagent and ether were removed under a gentle flow of nitrogen gas at 30 °C. The acetate ester was formed by reaction of triclosan in *n*-hexane with acetic anhydride/pyridine (1:1) for 30 min at 60 °C. Excess reagent was washed out from the organic phase with H_2O (5 mL).

Instrumentation. For gas chromatography/electron capture (GC/ECD) determinations, a Varian Star (Varian, Walnut Creek, CA) equipped with a spilt/splitless injector and a DB5-MS capillary column (15 m; i.d 0.25 mm; 0.10-μm film thickness; J&W, USA) coupled to a ^{63}Ni-ECD detector was used. The temperature of the split/splitless injector was 275 °C, and the detector temperature was 320 °C. The column temperature was held at 90 °C for 1 min, ramped 20 °C min^{-1} to 300 °C, and held for 10 min. Helium was used as the carrier gas (1 mL/min), and argon-methane (20 mL/min), as the makeup gas.

For gas chromatography/mass spectrometry/electron capture negative ionization (GC/ECNI/MS), a HP5890A gas chromatograph connected to a Finnigan SSQ 7000 quadropole mass spectrometer was used. The extract (1 μL) was injected in splitless mode on a DB5-MS capillary column (15 m; i.d 0.25 mm; 0.10-μm film thickness; J&W, USA). The temperature of the split/splitless injector was 280 °C, and the transfer line temperature was 300 °C. The column temperature was held at 90 °C for 2 min, ramped 20 °C min^{-1} to 315 °C, and held for 10 min. The ion source temperature was 180 °C. The electron energy was 70 eV. Helium was used as the carrier gas (1.4 mL/min), and ammonia, as the reagent gas (7000 mTorr). The ions monitored for quantification and identification purposes were *m/z* 287, 289, and 482 for the triclosan pentafluorobenzoyl ester and its fragments; *m/z* 299, 301, and 494 for the ^{13}C-labeled triclosan pentafluorobenzoyl ester and its fragments; and *m/z* 326 and 328 for CB 106. The surrogate standard was used to correct the measured concentrations.

RESULTS AND DISCUSSION

In the following, triclosan concentrations, if not from spiking, refer to the sum of triclosan in either unchanged or conjugated form present in plasma or milk.

Hydrolysis and Extraction. In humans, triclosan is metabolized to and excreted as glucuronide and sulfate conjugates.[8,10]

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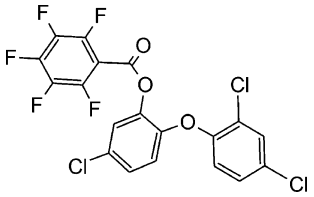

Figure 2. Molecular structure of the triclosan pentafluorobenzoyl derivative.

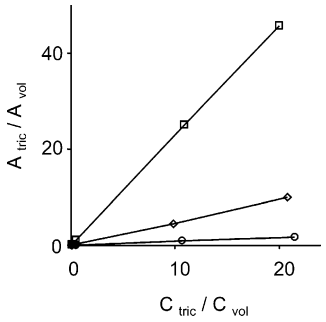

Figure 3. Relative response for the different triclosan derivatives from the GC/ECNI/MS analysis. Square = pentafluorobenzoyl triclosan (relative response factor, $k = 2.3$), diamond = acetyl triclosan ($k = 0.5$), circle = methyl triclosan ($k = 0.08$). C_{tric}/C_{vol} and A_{tric}/A_{vol} = concentration and area ratio between triclosan and the volumetric standard.

Hence, both unchanged and conjugated triclosan may be present simultaneously in the body.[10] To determine the total amount of triclosan in milk and plasma, hydrolysis was conducted to cleave triclosan from any conjugates that might be present. The hot sulfuric acid hydrolysis method for triclosan conjugates used in this work was previously described by Hoar and Sissons.[12] Initially a vortex mixer was used for the first extraction step for both milk and plasma. However, in some cases, problematic emulsions were formed, especially in plasma. This was avoided by instead inverting the test tube 60 times by hand.

Derivatives. Derivatization was used for enhancing the chromatographic and spectrometric properties of triclosan. We compared the pentafluorobenzoylated triclosan (Figure 2) with the acetylated and methylated triclosan with respect to its sensitivity on GC/ECD and GC/ECNI/MS. Applying GC/ECD, the relative response factor (relative to the volumetric standard) of the pentafluorobenzoylate was 4 and 7 times higher than the acetylate and the methylate, respectively. On GC/ECNI/MS, the relative response factor of the pentafluorobenzoylate was 5 and 28 times higher than the acetylate and methylate, respectively (Figure 3). This suggests that pentafluorobenzoylation is a suitable method for enhancing the chromatographic and, in particular, the spectrometric properties of triclosan.

The use of concentrated sulfuric acid to destroy matrix components of biological samples is a simple and effective method often employed in environmental analysis. The different derivatives were tested regarding their stability toward H_2SO_4 treatment (>98%, 3 mL, inverting the tube 60 times by hand). The results showed that acetylated triclosan was degraded completely in H_2SO_4, but both the methylate and pentafluorobenzoylate showed satisfactory stability (results not shown).

Stability of the derivatives over time was also compared by storing a set of derivatized extracts in a refrigerator and repeatedly analyzing them on GC/ECD. Over the course of 1 month, the response of the pentafluorobenzoyl and the acetyl derivative relative to the volumetric standard CB 106 dropped by ~50 and 13%, respectively, as compared to the first analysis. The response for the methylated triclosan relative to the volumetric standard remained unchanged. The results suggest that the slow degradation of the pentafluorobenzoyl derivative could reduce the method sensitivity and cause errors in quantification if the derivatized extracts were stored for longer periods prior to instrumental analysis. However, if ^{13}C-labeled triclosan is used as the surrogate standard, the degradation of the native triclosan and the surrogate standard should occur at the same rate, and the determination of triclosan should remain unaffected.

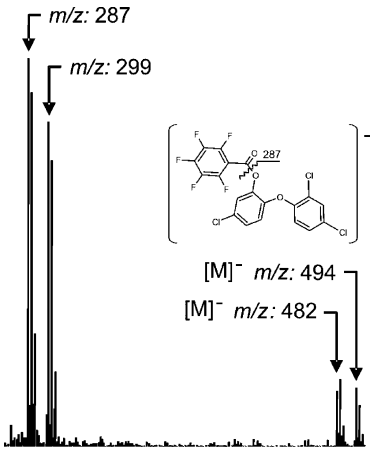

Figure 4. Full scan ECNI mass spectra of triclosan and ^{13}C-labeled triclosan from a milk sample, m/z 270−500. $[M]^-$ = the molecular ions for the derivatives. The molecular ion structure of the derivative is depicted. The zigzag sign in the molecular structure denotes where the major fragmentation occurs.

Pentafluorobenzoylation was selected as the derivatizing method, primarily because of the high sensitivity of the derivative on the instruments tested. In addition, the method is quick and easy, and it allows an effective cleanup with H_2SO_4. The use of hazardous chemicals such as diazomethane is avoided as well.

Method Specificity. The ECNI of the triclosan pentafluorobenzoyl ester resulted in the molecular ion m/z 482 and 484 and a major dissociation to the fragment ions m/z 287 and 289. The corresponding ions for the ^{13}C-labeled triclosan derivative were m/z 494 and 496 and m/z 299 and 301 for the fragments (Figure 4). Figure 4 depicts the full scan mass spectrum (m/z 270−500) for a milk sample. Both the full scan spectra of the triclosan and the ^{13}C-labeled triclosan derivatives show the characteristic molecular ion cluster patterns for trichlorinated compounds. Full scan spectra for standards and samples were used for the identification of triclosan. However, for the quantification of triclosan, the fragment ions m/z 287 and 289 for the triclosan derivative and m/z 299 and 301 for the ^{13}C-labeled triclosan derivative were used. The absolute and relative abundance of these ions was used for identification and quantification of triclosan. Figure 5 depicts the GC/ECNI/MS-SIM chromatograms for triclosan and ^{13}C-triclosan from a triclosan calibration standard and from the milk sample with the lowest concentration above the LOQ (0.018 ng/g) in this study.

Blanks. Blanks were analyzed together with each set of 16 samples. All blanks contained triclosan at levels well above the LOD (see definition in next section). Initially, the levels were

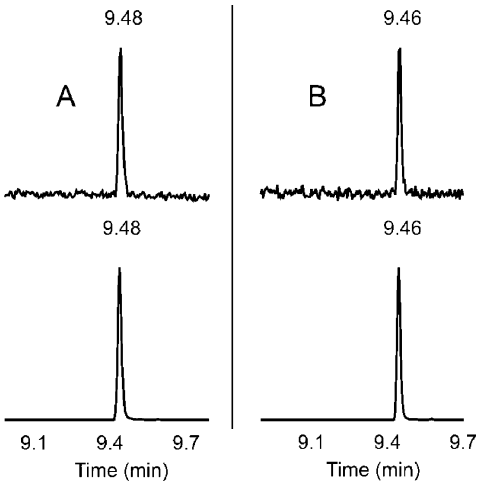

Figure 5. GC/ECNI/MS-SIM chromatograms showing triclosan (upper chromatograms, fragment ions *m*/*z* 287 and 289) and ^{13}C-triclosan (lower chromatograms: fragment ions *m*/*z* 299 and 301) from (A) a triclosan calibration standard and (B) the milk sample with the lowest triclosan concentration above the LOQ in this study.

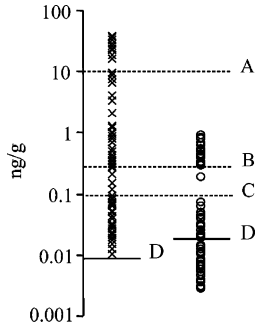

Figure 6. Triclosan concentrations (ng/g of fresh weight) in human blood plasma and milk. Cross = plasma, circle = milk, solid lines = LOQ in plasma and milk, respectively, in the present study; dashed lines = reporting limits for previous studies: (A) = Hoar and Sissons (1976); Lin (2000); Bagley and Lin (2000), (B) = Adolfsson-Erici et al. (2002), (C) = Sandborgh et al. (in press), (D) = present study.

unacceptably high. To identify the source, the Teflon-coated test tube caps used in the procedure were rinsed with solvent, which was then analyzed. Elevated levels of triclosan were found, even though the caps had been machine-washed with alkaline detergent after previous use. The use of aluminum foil to line the caps significantly reduced the method blank. The blanks were also consistent, the coefficient of variation of ($A_{\text{triclosan}}/A_{^{13}\text{C-triclosan}}$) in the blanks amounting to 5% ($n = 5$) and 14% ($n = 5$) in milk and plasma analysis, respectively.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The limit of detection was defined as a signal height of three times the standard deviation of the chromatographic noise. The LOQ was defined by the triclosan content in the blanks. As noted above, the variability in the blanks was low. Consequently, the LOQ was defined as 4 times the blank level. This corresponded to 0.06 ng per sample, or 0.018 ng/g of milk and 0.009 ng/g of plasma for ~3 g of milk and 5 g of plasma.

Method Recovery. The relative recovery of native triclosan compared with the ^{13}C-labeled triclosan was assessed by spiking five aliquots of a milk sample to nominal triclosan concentrations of 0.6 ng/g and an additional five to a concentration of 4.6 ng/g of milk. ^{13}C-labeled triclosan was then added, and the samples were worked up and analyzed as described above. The relative recovery for both levels of triclosan was 95%.

Plasma ($n = 72$) and milk ($n = 71$) samples were fortified with ^{13}C-labeled triclosan and analyzed as described. The mean absolute recovery of the ^{13}C-labeled triclosan was 46% (CV = 23%) in plasma (85% in blanks) and 49% (CV = 18%) in milk (91% in blanks). The main loss of triclosan is believed to occur in the derivatization step, in which varying amounts of precipitate formed in the sample extract, possibly hindering the derivatization of the triclosan. This precipitate was not seen in the blanks, which were treated in the same way as the samples, and for which the recovery was considerably higher. It would probably enhance the recovery if an additional cleanup procedure were introduced before the derivatization step. Variability in recovery of the surrogate standard was also observed in seven replicates analyzed for repeatability (recovery 42% (CV 22%)); however, the repeatability was still very good (see below).

Method Repeatability. The method repeatability test was assessed by repeatedly analyzing triclosan in three different milk samples: one with a high, one with an intermediate, and one with a low triclosan concentration. The replicates were not analyzed together; rather, they were analyzed on different occasions with freezing and thawing of the sample between analyses. The higher concentration was among the highest concentrations in milk in this study, and the lower concentration was near the LOQ for milk. The coefficient of variation was 6% for the high concentration (mean = 0.84 ng/g, $n = 7$), 1% for the intermediate concentration (mean = 0.31 ng/g, $n = 3$), and 5% for the low concentration (mean = 0.020 ng/g, $n = 3$). The instrumental precision was evaluated by analyzing two samples three times distributed over a sequence of 184 injections on the GC/ECNI/MS. In both cases, the instrumental coefficient of variation was 1%.

In addition to the repeated determination of triclosan in milk, 10 g of milk with only trace amounts of triclosan was fortified with triclosan to a nominal concentration of 7.4 ng/g. The measured triclosan concentration in that sample was 7.1 ng/g (mean, $n = 3$). These results show that the repeatability of the method was good and that the reliability was satisfying.

Method Applications. The present work was aimed at developing a method for the determination of triclosan, possible metabolic conjugates in plasma and milk, or both; however, the method or parts from it may also be applicable to other matrixes. Provided that the extract is clean enough, the derivatization of triclosan to its pentafluorobenzoylate should be suitable for any analysis of triclosan when using GC/ECNI/MS or GC/ECD; however, the use of a GC/ECD for determination excludes the possibility of using ^{13}C-labeled triclosan as the surrogate standard.

More than 70 plasma and milk samples were analyzed for triclosan with this method. Figure 6 depicts the results. Triclosan was present in the plasma samples at concentrations ranging over almost 4 orders of magnitude. All of the concentrations were above the LOQ. In milk, triclosan was found in detectable amounts in all samples, but in 42% of them, the concentration was below the LOQ. Overall, the concentrations in milk were lower than in plasma, but also the sample size was smaller for milk (3 g) than it was for plasma (5 g). If a method would be required for quantifying triclosan in milk from the vast majority of women, it

would be necessary to increase the sample size or reduce the method blank.

The reporting limits from published methods are also shown in Figure 6. If the methods reported by other groups[12−14] (line A) had been used to analyze these samples, all of the milk samples and 85% of the plasma samples would have been below the LOQ or LOD. The earlier methods developed in our group[5,10] would have performed better but would still not have allowed for the quantification of triclosan in most of the milk samples and ∼45% of the plasma samples.

The increased sensitivity of the present method was primarily due to the use of pentafluorobenzoylation as a means to enhance the spectrometric properties of triclosan. Furthermore, the pentafluorobenzoylation of triclosan allows an effective final cleanup of the extract with concentrated sulfuric acid, which reduces the interferences in the GC/MS analysis. A significant prerequisite for the method improvement was the reduction of triclosan in the blanks, which, in fact, defined the LOQ.

The development work resulting in the method presented in this paper has lowered the LOQ for triclosan in human body fluids by 3 orders of magnitude. This opens the possibility to study the level of triclosan exposure in the general population and to better explore the sources of that exposure and the behavior of triclosan in the body.

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