# Derivatization of Vinyl Aldehydes with Anthrone Prior to High-Performance Liquid Chromatography with Fluorometric Detection

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**Precolumn high-performance liquid chromatography derivatization of several vinyl aldehydes, specifically acrolein, crotonaldehyde, and methacrolein, has been studied by use of the reagent anthrone. After selective condensation of these $\alpha$- or $\beta$-unsaturated aldehydes to form fluorescent benzanthrone derivatives, separation of these compounds was facile on a 10-cm C-18 column. Although ultraviolet detection was possible, fluorescent detection proved more versatile and sensitive. Four liquor samples were subsequently assayed for acrolein and crotonaldehyde. Linearity for these compounds in alcoholic solutions ranged from 0.02 to at least 14 ppm with detection limits reaching down to 0.005 ppm.**

Short-chain vinyl aldehydes are of common concern in air and water pollution studies. These compounds, such as acrolein (1-propenal) and crotonaldehyde (1-butenal), have been found to be produced by automobile engines and photochemical reactions of hydrocarbons in air and are present in rainwater. In addition, unsaturated aldehydes have characteristically given wines and liquors a bitter taste.

Selective gas chromatography (GC) methods for the determination of unsaturated aldehydes such as acrolein, crotonaldehyde, and methacrolein have been reported. These include bromination (*1*) or methyloxylamination prior to bromination (*2*) of the aldehydes to facilitate electron capture detection. However, both derivatization techniques require extensive reagent additions and an extraction step prior to GC analysis of rainwater samples. For the detection of aldehydes in aqueous systems, derivatization prior to high-performance liquid chromatography (HPLC) has been the accepted approach to improve the chromatographic separation capabilities and to increase detectability. 2,4-Dinitrophenylhydrazine (DNP) has been the standard reagent for these methods. Both UV (*3–11*) and electrochemical detection (*12, 13*) of the hydrazone products are possible. Derivatization with 2-(diphenylacetyl)-1,3-indandione-1-hydrazone (*14*) will permit fluorometric detection of the analytes after HPLC. One inherent difficulty with either of these reactions was the ease with which all similar carbonyl compounds, including saturated and unsaturated molecules, produced very similar products. Despite the use of gradient elution, overlap or poor resolution of peak pairs such as acrolein–propanal/acetone or crotonaldehyde–butanal derivatives in chromatograms of some studies was unavoidable. Separation of isomeric compounds such as methacrolein and crotonaldehyde would also be expected to be a problem. Other derivatizing agents employed for HPLC assays of aldehydes in aqueous systems include 1,3-cyclohexanedione (*15*) and bisulfite addition (*16*). However, a selective derivatization reaction for $\alpha,\beta$-unsaturated aldehydes would simplify the chromatography and give positive identification of these analytes.

Two reagents, anthrone (*17, 18*) and *m*-aminophenol (*19*) have been used previously to produce fluorometric derivatives of vinyl aldehydes. Selectivity and detection limits were comparable for both methods. However, the reaction time required was only 10 min at room temperature for the anthrone method as opposed to 30 min at 98 °C for the *m*-aminophenol system. In this work, the anthrone reagent has been utilized as a selective reagent for derivatizing the $\alpha,\beta$-unsaturated aldehydes acrolein, crotonaldehyde, and methacrolein. In an acidic medium, acrolein condenses with anthrone to produce benzanthrone, while the isomers crotonaldehyde and methacrolein produce 1-methylbenzanthrone and the 2-methyl analogue, respectively (*20*). The reaction is shown in Figure 1. For separation by reversed-phase HPLC, the subsequent products were directly injected onto an octadecylsilane column, and a comparison of UV and fluorescent detection was made. Application of this method to commercial liquors was possible.

## EXPERIMENTAL SECTION

**Solutions.** All chemicals were reagent grade. Anthrone was obtained from MCB (Norwood, OH), and sulfuric acid was purchased from Fisher Scientific (Springfield, NJ). Gold Label acrolein, crotonaldehyde, and methacrolein were obtained from Aldrich (Milwaukee, WI) and were kept refrigerated. Aldehyde stock solutions were prepared by weighing the cold aldehyde liquid previously pipetted into a flask on an analytical balance. To keep down evaporative losses, approximately 10 mL of solvent was already contained in the tared flask. The solvents used were either deionized, doubly distilled water or 50/50 water/absolute ethanol. Because of the volatility and polymerizing capabilities of these unsaturated aldehydes, the stock solutions were kept refrigerated in the dark and used for no more than a week for analytical work. All analytical aldehyde standard solutions were pipetted fresh daily from the stock solutions. The 0.04% anthrone solution (w/v) was prepared weekly with concentrated $H_2SO_4$. Only HPLC grade solvents were used for the chromatography. Four actual liquor samples were obtained at a local liquor store.

**Equipment.** The HPLC system was composed of an Altex (Beckman Instruments, Berkeley, CA) Model 110A pump, a Rheodyne (Berkeley, CA) 7010 injector with a 130-$\mu$L loop, a Waters Associates (Milford, MA) radial compression module equipped with a Nova-PAK C-18 Radial-PAK cartridge (100 × 8 mm) and a $\mu$Bondapak C-18 Guard-PAK precolumn insert, and an AMINCO (SLM Instruments, Urbana, IL) scanning spectrofluorometer SPF-125 fitted with an HPLC flow cell. Both excitation and emission slit widths of the detector were set at 2 mm (22 nm band-pass). The excitation and emission wavelengths were set at 405 and 480 nm, respectively. For comparison work, a Beckman Instruments (Berkeley, CA) Model 153 UV detector was used with the 254-nm filter. The detector signal was recorded by a series 5000 Fisher Recordall (Houston Instruments, Houston, TX) chart recorder. Chromatographic data were also collected on an Apple II Plus computer (Apple Computers, Inc., Cupertino, CA), and peak area integration was carried out with Autovideogration software (Heydon & Sons, Philadelphia, PA).

**Procedure.** The reaction conditions were essentially the same as those used by Kwon and Watts (*17*). A 3-mL volume of 0.04%

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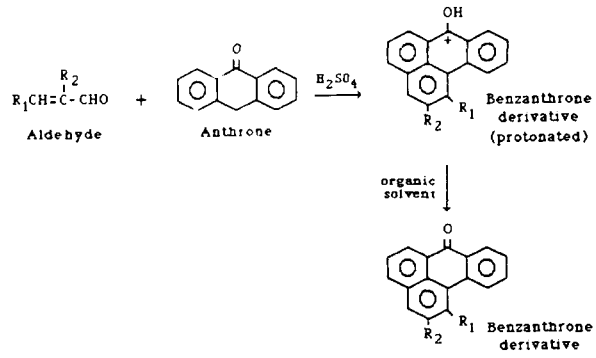


**Figure 1.** Condensation reaction of unsaturated aldehydes with anthrone: acrolein ($R_1 = R_2 = H$), crotonaldehyde ($R_1 = CH_3$, $R_2 = H$), and methacrolein ($R_1 = H$, $R_2 = CH_3$).

anthrone was added to 2 mL of an aqueous or alcoholic sample in a 10-mL volumetric flask and mixed by inversion. The solution was allowed to cool 10 min, and then acetonitrile was added with constant mixing to the mark. Thorough mixing upon addition of acetonitrile was important to ensure that the volume was reproducible. The flask was allowed to cool another 10 min before injection of the reacted sample. Aqueous sample work performed in the early part of this investigation omitted the addition step of acetonitrile to the reaction mixture. However, the high viscosity of these solutions induced a presure pulse with each sample injection, and the acidity limited the guard cartridge lifetime. Thus, acetonitrile was added to the reaction mixture to reduce its viscosity and acidity. Periodic replacement of the guard column after 50–70 injections was still carried out.

## RESULTS AND DISCUSSION

**Optimization of Reaction and Chromatography Conditions.** Very few changes from the original work by Kwon and Watts (*17*) were necessary in the derivatization of unsaturated aldehydes. A 1:1 ratio of anthrone reagent to sample caused precipitation of the benzanthrone products. Thus, the 3:2 ratio originally reported was maintained. Due to the nature of the fluorescent detector, the anthrone reagent concentration was dropped from 0.2% to 0.04% so that the photomultiplier tube would not be overexposed on sensitive scales. This drop in reagent concentration caused no significant loss of derivatized product within the linear range of the method. The original work (*17*) explained the red color produced by the anthrone reaction with aldehydes as being a dimer of benzanthrone. However, when the solution was diluted with an organic solvent, a yellow color was apparent. The dimerization of benzanthrone in acid is doubtful, but it can, however, occur in base (*20*). Thus, protonation of the benzanthrone in the acidic medium (Figure 1) was believed to cause the reddish color.

Initial chromatographic studies were performed with UV detection at 254 nm because of the common use of fixed wavelength detectors. Figure 2 shows chromatograms of acrolein (A) and crotonaldehyde (B) derivatized aqueous samples with the respective derivative peaks indicated as D. The three major peaks besides the derivative peaks correlate in order to (1) sulfuric acid, (2) anthrone reagent, and (3) a reaction product from the anthrone reagent in acid. From a review of the literature, the third blank peak was expected to be dianthrone (bianthronyl) produced from the acidic photodehydrodimerization of anthrone (*21*). Dianthrone can exist in fresh reagent solutions and will increase in concentration with aging. This compound, synthesized in-house, was used to substantiate the existence of dianthrone within the anthrone reagent. An injected dianthrone standard dissolved in acetonitrile had the same retention time as the third peak in the blank chromatogram. Benzanthrone recrystallized from 1-propanol was used to prove chromatographically that benzanthrone was actually produced from the derivatization of

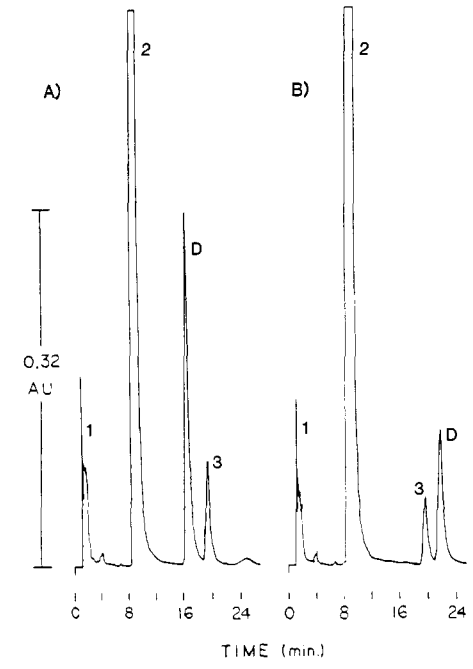


**Figure 2.** UV detection of anthrone derivatized (A) 22.2 ppm acrolein and (B) 21.5 ppm crotonaldehyde in water with respective products benzanthrone and 1-methylbenzanthrone labeled D. Other major peaks are as follows: (1) sulfuric acid, (2) anthrone, and (3) dianthrone. The mobile phase of 52/48 acetonitrile–water was pumped at 2.0 mL/min.

acrolein. To achieve a separation between dianthrone and the crotonaldehyde derivative (1-methylbenzanthrone), a mobile phase of 52/48 acetonitrile–water was considered the best compromise. At a lower organic content in the mobile phase, the 1-methylbenzanthrone peak merged with that of dianthrone; at percentages higher than 54% acetonitrile, retention of benzanthrone and dianthrone became similar. Because of the close overlap of the 1-methylbenzanthrone and dianthrone peaks, poor resolution occurred when crotonaldehyde concentrations were 1 ppm or lower.

Because of the likelihood of improving detection limits for the aldehyde derivatives, interest switched to chromatographic studies with fluorescent detection. In the acidic reaction mixture, the optimum wavelengths as found by Sawicki et al. (*18*) for excitation and emission were 480 and 560 nm, respectively. When the mixture was introduced into the organic/aqueous mobile phase, the wavelengths necessary for optimum response moved to shorter wavelengths. Initial fluorescent maxima were set by scanning the reacted mixture after a 10-fold dilution with the mobile phase. The excitation and emission were determined to be 410 and 555 nm, respectively. Unfortunately, any small amount of acid present can perturb these wavelength maxima. Once the benzanthrone products have chromatographically been separated from the acid in the reaction mixture, the excitation and emission maxima will change again, as determined by scanning of the benzanthrone standard prepared in acetonitrile. A 405-nm excitation wavelength and a 480-nm emission wavelength were approximately the maxima for all three aldehyde products in the chromatographic system. The chromatogram in Figure 3 shows a separation of the acrolein, crotonaldehyde, and methacrolein derivatized aldehydes. These three products could be base-line resolved in approximately 10 min. Only two minor blank peaks, one due to the anthrone reagent (A), are seen in Figure 3. Noticeably missing is the dianthrone peak. Dianthrone can undergo fluorescence at these wavelength maxima (22), but it would be expected to be weak because of its low concentration and the likelihood of quenching through free rotation of the bond connecting the methylene carbons of each anthrone substituent.

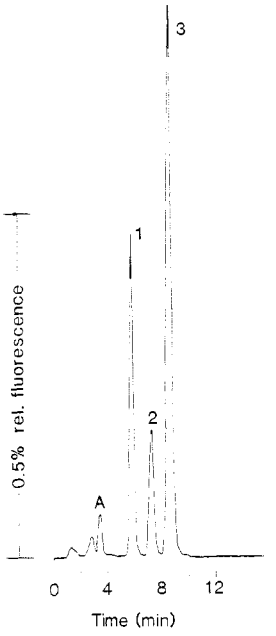


**Figure 3.** Fluorescent detection of an anthrone derivatized mixture of three vinyl aldehydes acrolein, crotonaldehyde, and methacrolein, all at approximately 9 ppm in 50% ethanol. The respective products are (1) benzanthrone, (2) 1-methylbenzanthrone, and (3) 2-methylbenzanthrone. The anthrone reagent peak is indicated by A. The mobile phase of 60/40 acetonitrile–water was pumped at 2.5 mL/min.

**Table I. Comparison of Peak Areas and Their Standard Deviations (SD) for Acrolein Standard Solutions with Either UV or Fluorescent Detection**

| acroelin, ppm | UV detection peak area[a] ($\times 10^4$) ± SD | acrolein ppm | fluorescence Peak area[a] ($\times 10^4$) ± SD |
|---|---|---|---|
| 33.4 | 344 ± 2.7 | 37.0 | 114 ± 5.4 |
| 11.1 | 118 ± 1.8 | 14.8 | 49.6 ± 2.1 |
| 5.56 | 57.5 ± 0.9 | 3.7 | 13.7 ± 0.4 |
| 1.11 | 10.4 ± 0.1 | 1.18 | 4.6 ± 0.2 |
| 0.445 | 3.3 ± 0.1 | 0.370 | 1.4 ± 0.04 |
| 0.111 | 0.6 ± 0.03 | 0.071 | 0.4 ± 0.007 |
| 0.067 | 0.3 ± 0.03 | 0.022 | 0.2 ± 0.01 |

[a] At least four trials for each concentration from two different aliquots.

**Calibration Data.** Table I compares the peak area output for a range of acrolein standard solutions with UV and fluorescent detection. Direct comparison of peak areas for the two systems is not, however, appropriate since the area is related to data acquisition time which was set differently for the two systems. In all cases, at least four trials were run for each standard concentration, and in the fluorescent detection system, the four trials consisted of two different reacted aliquots which were each run at least twice. The seven-point calibration curve for the aqueous acrolein standards with UV detection had a slope of $(1.0 \times 10^5) \pm (3.0 \times 10^2)$, a $y$ intercept of $(-3.0 \times 10^3) \pm (3.9 \times 10^3)$, and a correlation coefficient of 0.9999. The range of linearity was from 0.067 to at least 33 ppm with an average relative standard deviation (RSD) of 3.9% for all data points. Crotonaldehyde had a similar range of linearity but with a slope of approximately half that of acrolein. Methacrolein was not studied with UV detection, but no expected difficulties are foreseen.

Fluorescent detection of six acrolein standards dissolved in 50/50 water–ethanol afforded a least-squares fit with a slope of $(3.3 \times 10^5) \pm (2.7 \times 10^2)$, a $y$ intercept of $(5.7 \times 10^3) \pm (1.9 \times 10^3)$, and a correlation coefficient of 0.9988. Linearity ranged from 0.022 to 14.8 ppm, and the average RSD for these

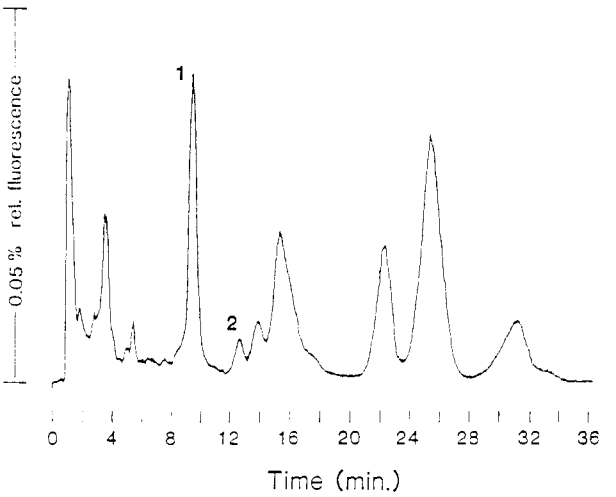


**Figure 4.** Separation of a derivatized Scotch whiskey (brand X) sample, with fluorescent detection. Acrolein and crotonaldehyde peaks are as follows: (1) benzanthrone and (2) 1-methylbenzanthrone. The mobile phase of 45/10/45 acetonitrile–methanol–water was pumped at 2.7 mL/min.

standard solutions was 4.0%. Crotonaldehyde had a similar linear range, but again, the slope of the line was less by about one-third of that for acrolein. There was little difference between aqueous and water–ethanol standard calibration curves for acrolein. However, for the determination of crotonaldehyde in liquor samples, it was important to prepare the standards in a 50/50 ethanol–water mixture.

Detection limits of the original sample solutions were determined at three times the signal to noise ratio. By use of UV detection, acrolein had a detection limit of 0.04 ppm. As stated previously, crotonaldehyde had a high UV detection limit of 0.4 ppm because of peak overlap with dianthrone. Methacrolein was not run in the UV system but would be expected to have a detection limit similar to acrolein. Significantly lower fluorescent detection limits of 0.005 ppm for both acrolein and methacrolein were found. The fluorescent crotonaldehyde detection limit was 0.010 ppm in the 50/50 ethanol–water solvent system. Crotonaldehyde did not react as readily in the alcohol–water solvent mixture as in water; the detection limit could be lowered by about a factor of 2 in the latter solvent. No such detection limit difference between alcohol and water samples of either acrolein or methacrolein was noted. The fluorescent HPLC study carried out by Swarin and Lipari (*14*) on acrolein and crotonaldehyde as well as other aldehydes and ketones reported detection limits for the vinyl aldehydes of 84 pg. This is less than a factor of 2 below that of the anthrone-derivatized acrolein which possessed an absolute detection limit of 130 pg.

**Liquor Samples.** In the study of liquor samples, Puputti et al. have reported two techniques for aldehydes. Direct HPLC separation of acrolein and crotonaldehyde in alcohol was performed after distilling the sample to preconcentrate the aldehydes (*23*). A more complete HPLC method for studying carbonyls in whiskey employed DNP derivatization with diode-array detection (*24*). Sufficient separation of acrolein and other related compounds was not achieved using a gradient elution program until the column temperature was set to 60 °C. Even then, real sample analysis proved questionable for crotonaldehyde because the corresponding UV–vis spectrum for the hydrazone product was not pure.

Four liquor samples were assayed for acrolein and crotonaldehyde by using the anthrone derivatization method. Methacrolein had not been previously reported to be found in such samples. The chromatogram respresenting an anthrone-derivatized Scotch whiskey sample is shown in Figure 4. Injection of the underivatized samples proved that there

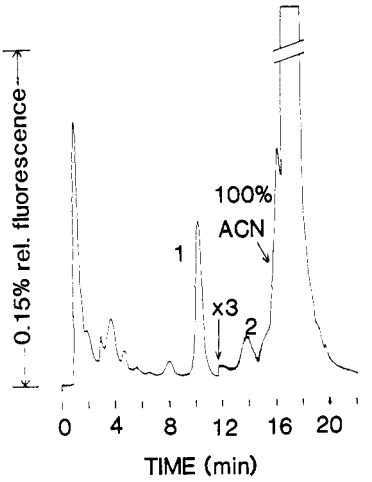


**Figure 5.** Separation of a derivatized bourbon sample, with fluorescent detection. Acrolein and crotonaldehyde peaks are as follows: (1) benzanthrone and (2) 1-methylbenzanthrone. Sensitivity of the fluorometer increased by 3 at 12 min. Mobile phase as in Figure 4 until switched to 100% acetonitrile at 15.8 min.

**Table II. Determination of Two Vinyl Aldehydes in Four Different Liquor Samples**

| sample ($n$)[a] | acrolein, ppm | crotonaldehyde, ppm |
|---|---|---|
| Scotch whiskey (4) (brand x) | 0.67 ± 0.04 | 0.03 ± 0.01 |
| Scotch whiskey (4) (brand y) | 11.1 ± 0.42 | 0.21 ± 0.02 |
| Kentucky bourbon (3) | 1.62 ± 0.03 | 0.04 ± 0.002 |
| Vodka[b] (3) | <0.03 | <0.02 |

[a] Total number of assays from two different aliquots. [b] Detected but could only be estimated.

were no detectable fluorescent compounds present that had a retention time longer than 6 min. Therefore the additional peaks in Figure 4 were derived from the reaction step. To improve sample throughput, the mobile phase was switched to 100% acetonitrile to quickly elute off the later peaks (Figure 5). This procedure was used to generate the quantitative data for acrolein and crotonaldehyde in the whisky and bourbon samples presented in Table II. Chromatograms of liquor samples such as vodka were clean with no such extra peaks. In all four samples, these two aldehydes were present, but in the case of vodka, the responses for both aldehydes were below the linear range. In a whiskey sample determined previously by another method (*24*), acrolein was found at a concentration of 9.1 mg/L which correlates closely with the determined amount of acrolein in one of the two Scotch whiskey samples. The RSD for the determination of acrolein in the liquor samples was less than 7.0%. Since only a very low concentration of crotonaldehyde was present in the liquor samples, the RSD values were higher but still under 9.0% for two of the samples. Use of a fluorometer designed for HPLC would have undoubtedly allowed better quantitation of crotonaldehyde.

**Interference Studies.** A variety of compounds including formaldehyde, acetaldehyde, propionaldehyde, acetone, methyl ethyl ketone, acrylic acid, acrylamide, and benzaldehyde at concentrations of approximately 50 ppm in water were tested using this derivatization procedure with subsequent HPLC analysis with both UV and fluorescent detection. Other than a small amount of crotonaldehyde impurity found in the acetaldehyde, no peaks with retention greater than anthrone were observed in the chromatograms for any of these test compounds. Only formaldehyde produced a weakly fluorescent derivative which had a retention time slightly longer than the anthrone reagent peak. Therefore, none of the compounds in this study posed an interference problem with the three $\alpha,\beta$-unsaturated aldehydes.

Glycerol has been reported to dehydrate in sulfuric acid to acrolein which can subsequently condense with the anthrone reagent (*25*). However, reaction conditions for such a derivatization include heating of the reaction mixture to at least 100 °C for 20 min. Considering these conditions, interference from glycerol was not expected.

A possible group of interferents expected in the liquor samples were sugars. Pentoses are known to react rapidly with anthrone; the accepted procedure for reacting hexoses and anthrone has been to heat them in a water bath. It has been postulated that the dehydration of sugars in the sulfuric acid medium to furfural or analogous furfural derivatives is necessary for the reaction with the anthrone reagent to occur (*26*). Therefore, most sugars existing in liquors should give very similar products with the anthrone reagent. The analysis of a mixture of 100 ppm glucose spiked with 1.5 ppm crotonaldehyde using the anthrone derivatization method and HPLC mobile phase conditions described in Figure 4 was carried out. Despite a variety of peaks, only one sugar derivative peak that could possibly interfere was found. This peak was retained over a minute longer than the crotonaldehyde derivative peak and was only half the magnitude. Identity of the sample components causing the extraneous peaks in Figure 4 as sugars was not established.

Under the conditions studied, methyl vinyl ketone, also an isomer of crotonaldehyde and methacrolein, was reacted with anthrone. Chromatography of the derivatized product showed a peak with a similar retention time to that of methacrolein. The expected product for methyl vinyl ketone was 3-methylbenzanthrone. No attempt was made to separate this product from 2-methylbenzanthrone since methyl vinyl ketone has not been observed as a fermentation byproduct in liquor analyses. However, we would expect this derivatization method to be applicable to the determination of methyl vinyl ketone in aqueous samples.

## ACKNOWLEDGMENT

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**Registry No.** Acrolein, 107-02-8; crotonaldehyde, 4170-30-3; methacrolein, 78-85-3; benzanthrone deriv. ($R_1 = R_2 = H$), 82-05-3; benzanthrone deriv. ($R_1 = CH_3$, $R_2 = H$), 112533-08-1; benzanthrone deriv. ($R_1 = H$, $R_2 = CH_3$), 82-03-1; anthrone, 90-44-8.

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# Determination of Tri-*n*-butyltin and Di-*n*-butyltin in Fish as Hydride Derivatives by Reaction Gas Chromatography

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**A method is described for the determination of tri-*n*-butyltin (TBT) and di-*n*-butyltin (DBT) by reaction gas chromatography. The method involves formation of hydride derivatives of TBT and DBT in a packed reactor inside the injection port of a gas chromatograph (GC). Following hydride formation, the hydrides are separated on a wide bore capillary column (5% phenylmethylsilicone) and detected with a flame photometric detector (FPD) equipped with a 600-nm filter. The method is sensitive to approximately 100 pg of TBT injected (as Sn) and its application to the determination of TBT and DBT in salmon tissue is described. For this application, organotins are extracted with a methanol/methylene chloride mixture and the extracts are cleaned up on a small silica gel column prior to reaction GC. For 18 salmon samples analyzed, TBT levels ranged from <5 to 188 ppb (as Sn) with an average recovery of 93%. The method may have applications for other organometallic pesticides and environmental pollutants that can undergo hydridization.**

Organotin compounds are widely used as miticides, fungicides, and molluscides and as stabilizers for poly(vinyl chloride) polymers. For the former two applications, trialkyl tin compounds are most commonly utilized with the organo group being butyl, cyclohexyl, or phenyl. Recently, questions have been raised regarding potential adverse environmental impacts of tri-*n*-butyltin (TBT) residues leached from ships and boats treated with antifouling paints containing TBT. TBT is extremely toxic to a wide variety of aquatic organisms (*1*, *2*) and, at sublethal levels, causes shell thickening in oysters (*3*). Its use as an antifouling treatment for net pens used to hold fish at aquaculture facilities has resulted in substantial fish mortalities (*4*).

Based on the concerns of the impact and ultimate fate of organotin compounds in the environment and the possibility that detectable levels of TBT may be present in human foods, a need exists for a rapid and sensitive method to determine the levels of the various organotin compounds in water, sediment, and biological samples, including human foods. Although little is known currently about the toxicological significance of low levels of TBT in foods, a rapid, sensitive analytical method is needed to support any future regulatory guidance.

A variety of analytical techniques have been developed for speciating organic tin compounds. Although HPLC techniques have been developed (*5*, *6*), the most widely utilized methods are based on gas chromatography. Generally, the mono-, di-, and trisubstituted organotin compounds do not chromatograph well and some form of derivatization is usually required. A widely utilized technique is the formation of a tetrasubstituted derivative via a Grignard reaction. Techniques have been described for GC speciation based on forming the ethyl (*7*), butyl (*8*), and pentyl (*9*) derivatives of TBT. In addition to the alkylation reactions, hydridization has been widely utilized to form volatile derivatives prior to gas chromatography (*10*, *11*). Sodium borohydride is an effective hydridizing reagent and is therefore utilized most frequently. In many cases, the volatile hydrides formed are extracted with an organic solvent, but purge and trap techniques for the hydrides have also been described (*12-14*).

A variety of detection techniques have been utilized for alkyl tin compounds. These include atomic absorption spectrometry both with (*8*, *12*) and without (*1*, *12*, *13*) prior chromatographic separation and gas chromatography with electron capture (*10*), mass spectrometric (*10*), and flame photometric detectors (*7*, *9*, *11*). Flame photometric detectors have several advantages in being widely available and sensitive and are quite specific for organotin compounds, with the proper filter selection.