Study Title

READY BIODEGRADATION OF 8-2 TELOMER B ALCOHOL (MODIFIED OECD 301D CLOSED BOTTLE TEST)

Test Guideline

Organization for Economic and Cooperative Development (OECD) Guideline for Testing of Chemicals; Section 3: Ready Biodegradability: 301 D Closed Bottle Test (1992).

Author

Ning Wang, Ph.D.

Study Completion Date

20-March-2003

Test Facilities

E.I. du Pont de Nemours and Company
Central Research & Development
Corporate Center for Engineering Research
Environmental and Microbiological Sciences & Engineering
Glasgow Building 300, P.O. Box 6101
Newark, DE 19714-6101, USA

and

E.I. du Pont de Nemours and Company
Haskell Laboratory for Health and Environmental Sciences
Newark, DE 19714

Submitter

E.I. du Pont de Nemours and Company
DuPont Chemical Solutions Enterprise
Wilmington, DE 19898, USA

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CERTIFICATION OF AUTHENTICITY

READY BIODEGRADATION OF 8-2 TELOMER B ALCOHOL (MODIFIED OECD 301D CLOSED BOTTLE TEST)

We, the undersigned, declare that the work described in this report was performed under our supervision, and that this report provides an accurate record of the procedures and results.

Report by:

Ning Wang, Ph.D.
Senior Research Biologist

03/20/03
Date

Approved by:

John T. Gannon, Ph.D.
Research Manager

03/20/03
Date

Study Initiation Date:
26-December-2002

Date Study Completed:
20-March-2003

Submitter:
E.I. du Pont de Nemours and Company
DuPont Chemical Solutions Enterprise
Wilmington, DE 19898, USA

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READY BIODEGRADATION OF 8-2 TELOMER B ALCOHOL (MODIFIED OECD 301D CLOSED BOTTLE TEST)

Author
Ning Wang, Ph.D.

1.0 SUMMARY

Rationale of the Study

This test generates environmental fate information relevant to assess the potential for environmental persistence of a test substance. When a chemical enters the environment, biodegradation is one of the major routes that determines the environmental fate of the chemical. The "OECD 301 D Closed Bottle Test" is a widely accepted test to assess the "ready" biodegradability of a test chemical. Due to the stringent test conditions, if it passes this test, a given chemical is unlikely to be persistent in the environment. Because activated sludge will be used as the inoculum for the test, the outcome of the test will indicate what may occur if this chemical enters a domestic sewage treatment plant (POTW). The low water solubility, volatility, and strong surface adsorption properties of 8-2 Telomer B Alcohol (8-2 TBA, CAS # 678-39-7) to be used in this test requires a modification of the 301 D test guidelines. Because the duration of the test is relatively short (28 days) and 8-2 TBA is not expected to be ultimately metabolized, this specific study will only provide information on primary biodegradation potential of 8-2 TBA, through measurement of parent loss and formation of fluoride, not the ultimate environmental fate of the test chemical. To assess full biodegradation potential of fluorinated chemicals such as 8-2 TBA, a test system with adapted microorganisms to the test chemical and a favorable growth medium (mineral medium plus additional carbon source) may be needed.

Test System:

The biodegradability and biotransformation potential of the test substance 8-2 Telomer B Alcohol in mineral medium plus activated sludge inoculum (5 mL activated sludge per liter of mineral medium) from a POTW was determined. The test system consisted of individually crimped test vessels (glass serum bottles) and the test was conducted at room temperature (~22°C). The saturated solution of 8-2 Telomer B Alcohol in mineral medium was inoculated with activated sludge and kept in closed bottles in the dark at room temperature. Periodically (days 0, 7, 14, and 28), sample bottles of different experimental treatments were sacrificed for extraction and analysis. Potential degradation of the test chemical and formation of potential metabolites (transformation products) was followed by analysis of the concentration of the test chemical and by analysis for fluoride (F^- ion) during the 28-d study.

Findings:

Under the test conditions, the loss of 8-2 TBA from the test vessels was ~8% more at day 7 and 13% more at day 14 compared with the abiotic control vessels. At day 28, the concentrations of 8-2 TBA were not distinguishable between the test system and the abiotic controls. No defluorination of 8-2 TBA was observed during the test.

Conclusion:

- Under the test conditions, 8-2 TBA is not readily biodegradable.
- Confirmed expectations that abiotic removal mechanisms – volatility and/or adsorption – will be factors that need consideration when designing future studies with objective of achieving mass balance.

2.0 GENERAL STUDY INFORMATION

Study Objectives

- Determine the biotransformation potential of 8-2 TBA by monitoring its concentration during the test
- Determine the degree of defluorination of 8-2 TBA
- Determine if abiotic removal mechanisms – volatility and/or adsorption – will be factors that may affect ability to achieve mass balance.

Test System Justification

The test system is outlined by the OECD 301D guideline and readily accepted.

Study Personnel

E.I. du Pont de Nemours and Company

- Central Research and Development – Corporate Center for Engineering Research - Environmental and Microbiological Sciences & Engineering
- Haskell Laboratory for Health and Environmental Sciences

Management:	John T. Gannon, Ph.D. E.I. du Pont de Nemours and Company Central Research and Development Corporate Center for Engineering Research Environmental and Microbiological Sciences & Engineering Glasgow Building 300, P.O. Box 6101 Newark, DE 19714-6101 USA and S. Mark Kennedy, Ph.D. E.I. du Pont de Nemours and Company Haskell Laboratory for Health and Environmental Sciences Newark, DE 19714 USA
Study Director:	Ning Wang, Ph.D. E.I. du Pont de Nemours and Company Central Research and Development Corporate Center for Engineering Research Environmental and Microbiological Sciences & Engineering Glasgow Building 300, P.O. Box 6101 Newark, DE 19714-6101 USA

Analytical Chemist:	Bogdan Szostek, Ph.D. E.I. du Pont de Nemours and Company Haskell Laboratory for Health and Environmental Sciences Newark, DE 19714, USA
Technical Personnel:	Patrick W. Folsom, DuPont Central Research & Development Keith B. Prickett, DuPont Haskell Laboratory

Study Execution Dates

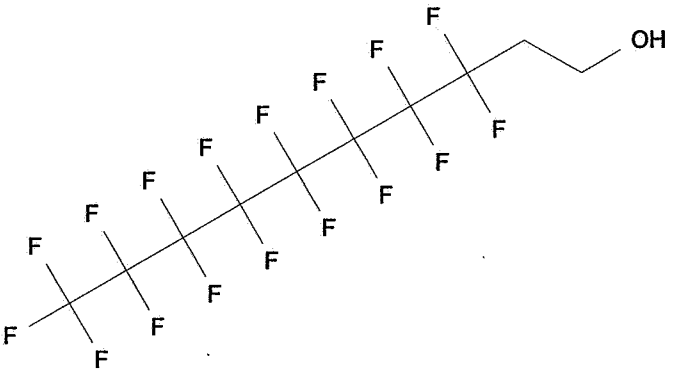
Experimental Start Date:	31-December-2002
Experimental Completion Date:	28-January-2003
Study Completion Date:	20-March-2003

3.0 MATERIALS AND METHODS

3.1 Test System

3.1.1 Test Substance

Name:	8-2 Telomer B Alcohol
Synonym:	(Perfluorooctyl)ethanol, 8-2 TBA
Active substance(s)	8-2 Telomer B Alcohol, 99%
CAS Name:	1-Decanol, 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10- heptadecafluoro-
Molecular weight	464.12 g mole^{-1}
CAS Number(s):	678-39-7
Structure:	



Lot Number:	P.00/001
EMSE Sample Number:	E93386-80
Concentration of a.s., nominal:	99%
Concentration of a.s., analyzed:	99.2%
Major impurity	0.8% as $C_7F_{15}CF{=}CHCH_2OH$

Certificate of Analysis Date:	13-Sept-2001
Date Received:	26-Mar-2002
Solubility at 25°C.:	~140 μg L^{-1}
Vapor pressure:	0.023 mm Hg
Stability:	Stable at ambient room temperature
Appearance/Color:	White solid
Storage Conditions:	Room temperature; keep tightly closed
Safety Precautions:	Wear lab coat, protective gloves, and safety glasses

3.1.2 Reference Substance

None

3.1.3 Preparation of Mineral Medium

One day before the initiation of the test, 2 mL each of mineral stock solutions A, B, C, and D (See Table A-4 for mineral medium stock solution preparation) were added to 2 liters of sterile deionized water and the mineral medium was sterile filtered into two Nalgene 1 liter filter units.

3.1.4 Container Coating with 8-2 TBA Solution

Several polypropylene gallon jugs with lids (Pretium packaging, Case ID# YZ2Q07) were sterilized under UV light overnight in a Biohood. Five days before the initiation of the test, one of the jugs was filled with 4 liters of sterile water from Barnstead E-Pure system (Megohm-cm = 17.5). A 0.4 mL aliquot of 8-2 TBA stock solution (3 mg/mL in ethanol) was added to the jug for a final concentration of 300 μg/L and was stirred for 4 days. After rinsing with sterile water, the jug was capped for later use to make 8-2 TBA saturated mineral medium.

3.1.5 Preparation of Saturated 8-2 TBA Solution

Five days before the initiation of the test, another jug was filled with 3 liters of sterile water from Barnstead E-Pure system (Megohm-cm = 17.5) and was autoclaved. After cooling down, 0.6042 g of 8-2 TBA was added to the jug for a final concentration of 201 mg 8-2 TBA/L and the solution was stirred on a stir plate for 4 days. The solution in the jug was transferred to sterile centrifuge bottles and was centrifuged to remove the particular matter. The supernatant was transferred to the pre-coated jug prepared in *Section 3.1.4* and was referred as saturated 8-2 TBA solution.

3.1.6 Preparation of 8-2 TBA Saturated Mineral Medium

Three milliliter each of Mineral stock solutions A, B, C, and D was added to the saturated 8-2 TBA solution (3 liters) and was mixed by inversely shaking the jug. The mixed solution was referred as 8-2 TBA saturated mineral medium and was used the next day.

3.1.7 Activated Sludge Collection

Approximately 4 liters of activated sludge was collected from the City of Wilmington (DE) Municipal Waste Treatment Facility (POTW) - Aeriation Basin #2 on December 31, 2002. After arriving at the test lab, the sludge was assigned an ID number E93386-105. The sludge was mixed by briefly shaking the jug to suspend the microorganisms. After settling the sludge for approximately 15 min to remove coarse matters, the upper aqueous phase of the

sludge was filtered through a Nylon net with a pore size of 85 μm. This is referred to as the activated sludge filtrate. A 20 mL aliquot of the filtrate was transferred to a glass scintillation vial with a plastic transfer pipette and was autoclaved. The autoclaved sludge filtrate was referred to as killed sludge filtrate.

3.1.8 Test Units

Test vessels were 60 mL borosilicate glass serum bottles with pre-cleaned aluminum-lined crimp caps. The pre-cleaning was done by rinsing the aluminum foil and septa with methanol once and then with sterile deionized water three times.

3.2 Test Conduct

Four types of experimental treatments were conducted with pre-cleaned glassware and septa and aluminum foil. The pre-cleaning was done by rinsing them with methanol once and then with sterile deionized water 3 times.

3.2.1 Treatment 1 – 8-2 TBA Saturated Mineral Medium plus Activated Sludge Filtrate

For a total of 16 glass serum bottles (4 replicates × 4 sampling time points), 34.82 mL of 8-2 TBA saturated mineral medium and 0.175 mL of activated sludge filtrate was added to each of the glass serum bottles with plastic transfer pipettes. The bottles were crimped with pre-cleaned aluminum foil and PTFE/silicone septa.

3.2.2 Treatment 2 – 8-2 TBA Saturated Mineral Medium plus Killed Sludge Filtrate

For a total of 16 glass serum bottles (4 replicates × 4 sampling time points), 34.82 mL of 8-2 TBA saturated mineral medium and 0.175 mL of killed sludge filtrate was added to each of the glass serum bottles with plastic transfer pipettes. The bottles were crimped with pre-cleaned aluminum foil and PTFE/silicone septa. This treatment served as an abiotic control.

3.2.3 Treatment 3 – Mineral Medium plus Activated Sludge Filtrate for 8-2 TBA Spike Recovery

For a total of 16 glass serum bottles (4 replicates × 4 sampling time points), 34.82 mL of the mineral medium and 0.175 mL of activated sludge filtrate was added to each of the glass serum bottles with plastic transfer pipettes. The bottles were crimped with pre-cleaned aluminum foil and PTFE/silicone septa.

At each sampling time points (Days 0, 7, 14, and 28), 4 bottles were spiked (dosed) with 140 μL of 8-2 TBA stock solution (112.5 mg/L in ethanol) for a final concentration of 448 μg/L. The bottles were incubated for approximately 30 min with 200-300 RPM shaking with an orbitory shaker (New Brunswick Scientific Company, Model G-10, Serial number 880511137) before sample collection and sample extraction.

3.2.4 Treatment 4 – Mineral Medium plus Activated Sludge Filtrate

For a total of 8 glass serum bottles (2 replicates × 4 sampling time points), 34.82 mL of mineral medium and 0.175 mL of activated sludge filtrate was added to each of the glass serum bottles with plastic transfer pipettes. The bottles were crimped with pre-cleaned aluminum foil and PTFE/silicone septa. These samples served as sample matrix controls during quantification of 8-2 TBA and its possible transformation products.

3.2.5 *Test Conditions and Sampling*

3.2.5.1 *Sample Incubation*

The crimped glass serum bottles were incubated with 200-300 RPM of shaking with an orbitory shaker (New Brunswick Scientific Company, Model G-10, Serial number 880511137) at room temperature in the dark inside a chemical hood. The temperature of the test system was monitored and recorded throughout the course of the study.

3.2.5.2 *Sampling Interval*

Four crimped serum bottles for *Treatment 1-3* and 2 crimped serum bottles for *Treatment 4* were sampled for extraction of 8-2 TBA, fluoride (F^- ion), and other metabolites at day 0 (31-Dec-2002), day 7 (7-Jan-2003), day 14 (14-Jan-2003), and day 28 (28-Jan-2003).

3.2.5.3 *Sample Storage*

Analytical samples were stored at approximately -20°C.

3.3 ***Sample Extraction and Analysis***

3.3.1 *Sample Collection and Extraction*

3.3.1.1 *Sample Collection*

At days 0, 7, 14, and 28, crimped sample bottles were removed from the shaker and the bottles were turned upside down. A total of 10 mL of the test medium from each of the bottles was withdrawn with a 10-mL polypropylene syringe. Five milliliter was injected into a 30 mL glass serum bottle and covered with pre-cleaned aluminum foil and was stored in a freezer for further analysis. Another 5 mL left in the syringe was injected into a 15 mL polypropylene tube that contained 0.05 mL of 5 N sodium hydroxide for fluoride extraction.

3.3.1.2 *Sample Extraction*

Fluoride extraction: The 15 mL polypropylene tubes containing the 5 mL test medium plus NaOH were incubated at room temperature for 3 - 4 h with 250 - 300 RPM of shaking. Then 0.042 mL of 6N H_2SO_4 was added to each of the tubes to neutralize the test medium. The sample tubes were stored in a –20°C freezer for later fluoride analysis.

8-2 TBA extraction: Before extraction, MTBE-H_2SO_4 solvent system was prepared by adding 5.56 mL of 6N H_2SO_4 in 500 mL of chilled MTBE. A 30 mL aliquot of the chilled MTBE-H_2SO_4 was then injected into each of the crimped sample bottles after the 10 mL of test medium was withdrawn from the bottles. After the MTBE- H_2SO_4 was injected, the sample bottles were incubated at room temperature for approximately 2 h with 250 - 300 RPM shaking. After settling the MTBE phase, the crimped sample bottles were decapped and the MTBE phase from each of the sample bottles was transferred with a glass pipette to 50 mL polypropylene centrifuge tubes and kept in a freezer. A 20 mL aliquot of MTBE was added to the sample bottles after the first MTBE phase was transferred to the polypropylene centrifuge tubes. The sample bottles were recapped with the original aluminum-lined Teflon® septa and were incubated at room temperature for approximately 1 h with 250 – 300 RPM shaking. The MTBE phase was then transferred with a glass pipette to the polypropylene centrifuge tubes that contained the first MTBE phase. The combined MTBE phase was centrifuged at approximately 2000 RPM for 10 min. A 20 mL aliquot of the MTBE phase from each of the centrifuge tubes was transferred with a glass pipette to a 20 mL glass scintillation vial with a foil-lined cap. From each of the glass scintillation vials,

2 mL aliquot of the MTBE phase was transferred to a GC vial and was sealed with pre-cleaned aluminum-lined crimp cap for GC/MS quantification of 8-2 TBA.

3.3.1.3 Temperature Measurements

The temperature of the test system was monitored and recorded throughout the course of the study.

3.3.2 Analytical Methods for Test Substance and Products

3.3.2.1 Analysis of Test Substance

8-2 TBA Analysis:

Analytical standards:

The 1H,1H, 2H, 2H-perfluorodecan-1-ol (8-2 TBA, CAS# 678-39-7, 97.6%, Oakwood Products, West Columbia, SC) was used as the analytical standard. The 1D, 1D, 2D, 2D, 3-^{13}C-heptadecafluoro decanol (D-8-2 TBA, DuPont) was used as the internal standard. Stock solutions (1000 mg/L) of the analytical standard and the internal standard were prepared in methanol and refrigerated. The calibration standards were prepared freshly for each calibration in MTBE by dilution of the freshly made 50-mg/L stocks in methanol. Typically, the calibration standards were made in the range of 25-1000 µg/L 8-2 TBA. Constant level of internal standard was used: ~300 µg/L of D-8-2 TBA. The calibration curves were constructed using the ratio of the peak area for ion m/z 31 (8-2 TBA) and m/z 33 (D-8-2 TBA) and the ratio of the concentrations of 8-2 TBA and the internal standard. An example of a calibration curve was given in Figure A-2 and an example of a chromatograph of sample (E93384EJ-29) separation was given in Figure A-3.

A 0.5 mL aliquot of the MTBE phase from the GC vials (***Section 3.3.1.2***) was placed in a glass GC vial (1.7 mL volume), 3 µL of 50 µg/mL of D-8-2 TBA internal standard was added to the vial using a GC syringe, the vial was capped and subjected to analysis. Each sample was analyzed twice by a GC/MS instrument according to the following conditions:

GC/MS system:		HP 6890 Plus GC (Agilent), HP 5973 Mass Selective Detector (Agilent), MPS2-MultiPurposeSampler (Gerstal)
Column:		DB-5MS, 30 m x 0.25 mm, 1 µm film (Agilent)
Temp. ramp:	Initial:	80°C for 2 min
		20°C/min to 120°C
		50°C/min to 300°C and hold for 3 min
Flow rate:		1.0 mL/min; He; constant flow mode
Split:		5:1
Inlet temp.:		250°C
Injection volume:		2 µL
MSD transfer line temp.:		280°C
Ionization:		EI, 70 eV
SIM ions monitored:		m/z: 31, 33, 95, 98; dwell time: 25 ms for each ion

Retention time:

8-2 Telomer B Alcohol (8-2 TBA):	4.97 min
Internal standard (D-8-2 TBA):	4.95 min

Fluoride Analysis:

Analytical standard:

Certified standard of 100 mg/L of fluoride in water (Thermo Orion) was used for standard calibration. The calibration standards were made in the range of 5 – 100 µg/L by dilution of the 100 mg/L of fluoride standard with TISABII® (total ionic strength adjustment buffer II; Thermo Orion) and deionized water from Barnstead E-Pure system (Megohm-cm = 16 - 18). Half strength TISABII® (one part of TISABII® plus one part of deionized water) was used for the dilution. A 8 mL aliquot of each of the standard solution was used for fluoride analysis to construct a standard curve for quantification of fluoride in samples.

Quantification of Fluoride:

The 5 mL test medium that was treated with NaOH and H_2SO_4 from each of the 15 mL polypropylene tubes was thawed at room temperature and was centrifuged. Four milliliter aliquot from each of the tubes was transferred to a 50 mL polypropylene tube that contained 4 mL of TISABII® solution. After mixing, the medium was analyzed for fluoride using a 710 A Plus pH/ISE meter (Thermo Orion, serial # 066814) and a Ionplus fluoride selective electrode (Thermo Orion, model 96-09, lot # GX1). After filling the reference chamber with reference electrode filling solution (Thermo Orion), the electrode was inserted into each of the sample medium and fluoride standard solution and the conductivity in millivolts was recorded after 5 min of incubation. Each sample was measured twice. A standard curve was generated by plotting the standard fluoride concentration in LOG scale versus millivolts (Figure A-1) for quantification of fluoride.

4.0 RESULTS AND DISCUSSION

4.1 Under the test conditions, 8-2 TBA is not readily biodegradable (Figure 1, Table 1, and Table A-1).

- Spike recovery of 8-2 TBA from the test medium averaged 106 ± 5% at day 0, 91 ± 3% at day 7, 94 ± 2% at day 14, and 74 ± 2% at day 28 (Table A-2), indicating that MTBE extraction method obtained a good recovery of 8-2 TBA from the test medium, except for day 28 samples.
- At day 7, the loss of 8-2 TBA in test vessels (***Treatment 1***) was ~8% more (p <0.05) compared with abiotic control vessels (***Treatment 2***).
- At day 14, the loss of 8-2 TBA in test vessels (***Treatment 1***) was ~13% more (p <0.01) compared with abiotic control vessels (***Treatment 2***).
- At day 28, the concentrations of 8-2 TBA were not distinguishable between the test system and the abiotic controls. Most likely, the loss of 8-2 TBA during this 28-day test was due to abiotic removal mechanisms – volatility and/or adsorption rather than biotransformation.
- In the abiotic controls, the concentration of 8-2 TBA decreased continuously. The loss is possibly caused by volatilization during the incubation or by incomplete

extraction due to adsorption to the glass walls of the test vessels and/or the sludge inoculum.

4.2 Under the test conditions, no defluorination of 8-2 TBA occurred during the 28-d test.

- The fluoride concentration was the same in the test medium during the test period, averaged 17.9 ± 2.6 µg/L at day 0, 17.1 ± 0.8 µg/L at day 7, 17.4 ± 1.4 µg/L at day 14, and 17.5 ± 1.0 µg/L at day 28 (Table A-3).
- Under the test conditions, perfluorooctanoic acid (CAS# 335-67-1) is unlikely to be a major metabolite because defluorination was not observed during the test. On a molar basis, for one mole of 8-2 TBA to be converted to one mole of perfluorooctanoic acid, two moles of fluoride would be released. No increase in fluoride concentration was observed during the test. As a result, the absence of fluoride concentration increase during the test indicated that perfluorooctanoic acid is unlikely a major metabolite (>10% of total mass present) of 8-2 TBA biotransformation under these test conditions.

5.0 CONCLUSION

- Under the test conditions at room temperature (~22°C), 8-2 TBA is not readily biodegradable.
- Confirmed expectations that abiotic removal mechanisms – volatility and/or adsorption – will be factors that need consideration when designing future studies with objective of achieving mass balance.

6.0 LIMITATIONS OF THE TEST

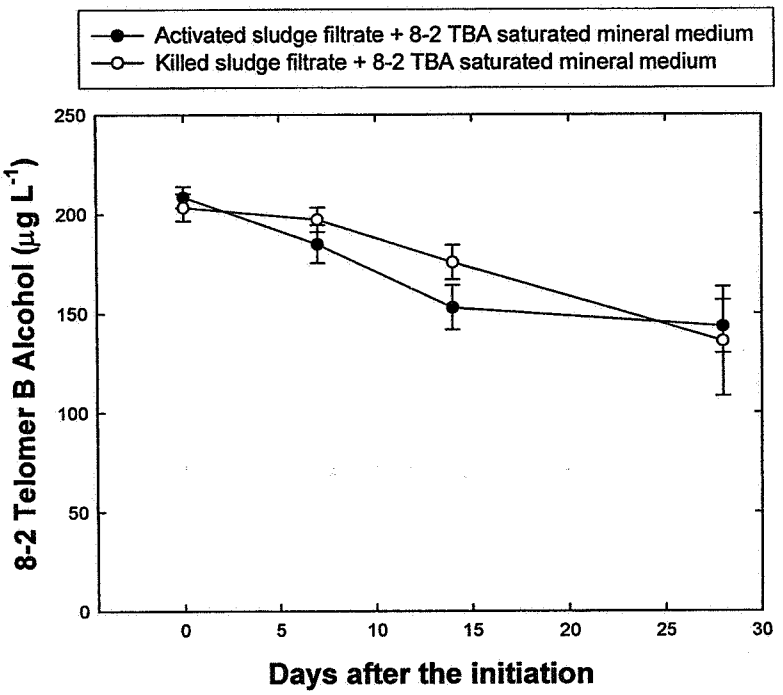
- Due to the low water solubility, volatility, and strong surface adsorption properties of 8-2 TBA, it is difficult to conduct a test with a good mass balance (90%) for the abiotic control, whereas, the mass balance achieved in this study was only 67%. During the test, the concentration of 8-2 TBA continued to decrease in the abiotic controls, complicating the experimental data interpretation.

Taken together, the conclusion drawn from this test is indicative, not conclusive. Further studies will be conducted to determine the potential major biotransformation products of 8-2 TBA.

7.0 REFERENCE

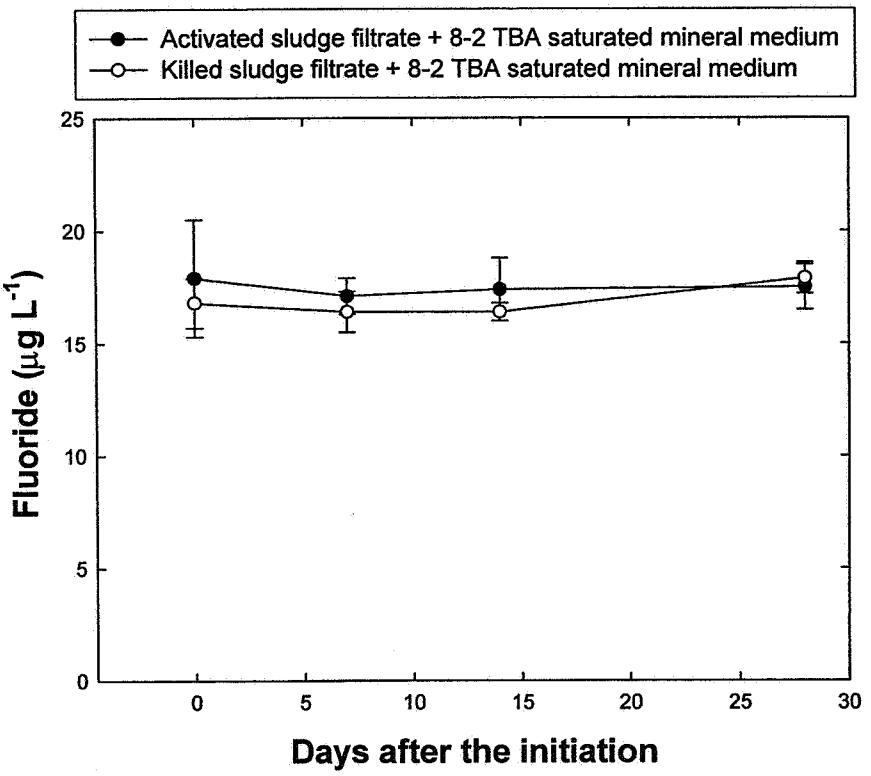
7.1 OECD Guideline for Testing of Chemicals, Section 3: Ready Biodegradability: 301 D Closed Bottle Test. (1992).

FIGURE 1
8-2 TELOMER B ALCOHOL (8-2 TBA) CONCENTRATION DURING THE 28-DAY TEST*



* The test was initiated on 31 December 2002 and finished on 28 January 2003. The graph is derived from the original data of Table A-1.

FIGURE 2
FLUORIDE CONCENTRATION DURING THE 28-DAY TEST*



* The test was initiated on 31 December 2002 and finished on 28 January 2003. The graph is derived from the original data of Table A-3.

TABLE 1
CONCENTRATION OF 8-2 TBA AT DAY 0 (31-DEC-2002), DAY 7 (7-JAN-2003), DAY 14 (14-JAN-2003), AND DAY 28 (28-JAN-2003)*

Type of Treatments	Time	Date	8-2 TBA Concentration	Test Substance remaining At day 7, day 14, and day 28 Compared to Day 0‡
	Day		*μg L⁻¹*	*%*
Treatment 1 - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	0	31-Dec-2002	209 ± 5†	100
	7	7-Jan-2003	185 ± 10	89
	14	14-Jan-2003	153 ± 11	73
	28	28-Jan-2003	144 ± 13	69
Treatment 2 - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	0	31-Dec-2002	204 ± 7	100
	7	7-Jan-2003	197 ± 6	97
	14	14-Jan-2003	176 ± 9	86
	28	28-Jan-2003	136 ± 28	67

* This table is derived from the original data of Table A-1.

† Mean ± standard deviation; n = 4.

‡ Test substance remaining at Days 7, 14, and 28 compared to Day 0, % = $(C_x/C_0) \times 100$, where:
C_0 = 8-2 TBA concentration at Day 0,
C_x = 8-2 TBA concentration at Days 7, 14, and 28.

TABLE 2
DAILY TEMPERATURE READINGS WITH A CALIBRATED DIGITAL THERMOMETER INSIDE A CHEMICAL HOOD WHERE THE TEST VESSELS WERE INCUBATED

Time	Date	Temperature
Day		°C
0	31-Dec-2002	21.6
1	1-Jan-2003	22.8†
2	2-Jan-2003	21.8
3	3-Jan-2003	21.1
4	4-Jan-2003	23.5†
5	5-Jan-2003	23.5†
6	6-Jan-2003	22.4
7	7-Jan-2003	22.3
8	8-Jan-2003	21.9
9	9-Jan-2003	22.1
10	10-Jan-2003	22.0
11	11-Jan-2003	23.8†
12	12-Jan-2003	23.8†
13	13-Jan-2003	22.0
14	14-Jan-2003	22.2
15	15-Jan-2003	22.0
16	16-Jan-2003	22.3
17	17-Jan-2003	21.9
18	18-Jan-2003	23.0†
19	19-Jan-2003	23.0†
20	20-Jan-2003	21.9
21	21-Jan-2003	22.1
22	22-Jan-2003	22.1
23	23-Jan-2003	22.3
24	24-Jan-2003	21.9
25	25-Jan-2003	25.0†
26	26-Jan-2003	25.3†
27	27-Jan-2003	21.7
28	28-Jan-2003	22.0
Average, n = 20		**22.0**
Standard Deviation		**0.3**

† Temperature reading in the test lab recorded by a calibrated Dickson recorder (Model THDx, serial number 01118-247). The temperature readings by the recorder were not used for calculation of the daily average temperature.

APPENDIX A:

TABLE A-1.
ANALYTICAL RESULTS OF 8-2 TBA CONCENTRATION AT DAY 0 (31-DEC-2002), DAY 7 (7-JAN-2003), DAY 14 (14-JAN-2003), AND DAY 28 (28-JAN-2003)

No	Type of Treatment	Time	Rep	8-2 TBA Analytical concentration	8-2 TBA Analytical Average	8-2 TBA final concentration†
		day		*μg L⁻¹*	*μg L⁻¹*	*μg L⁻¹*
E93384 EJ-1	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	0	1	107	106	212
			1	104		
E93384 EJ-2		0	2	101	102	204
			2	103		
E93384 EJ-3		0	3	102	102	204
			3	101		
E93384 EJ-4		0	4	107	107	214
			4	106		
Average ± Standard Deviation‡						**209 ± 5**
E93384 EJ-5	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	0	1	102	101	202
			1	99.4		
E93384 EJ-6		0	2	97.4	97.8	196
			2	98.2		
E93384 EJ-7		0	3	106	102	204
			3	97.2		
E93384 EJ-8		0	4	107	106	212
			4	104		
Average ± Standard Deviation						**204 ± 7**
E93384 EJ-15	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	7	1	89.9	90.5	191
			1	91.0		
E93384 EJ-16		7	2	92.8	93.3	187
			2	93.8		
E93384 EJ-17		7	3	89.9	90.5	191
			3	91.0		
E93384 EJ-18		7	4	85.6	85.4	171
			4	85.2		
Average ± Standard Deviation						**185 ± 10**
E93384 EJ-19	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	7	1	98.4	98.2	196
			1	97.9		
E93384 EJ-20		7	2	96.6	96.8	194
			2	97.0		
E93384 EJ-21		7	3	103	103	206
			3	103		
E93384 EJ-22		7	4	97.4	96.3	193
			4	95.1		
Average ± Standard Deviation						**197 ± 6**

TABLE A-1 (CONTINUED)

No	Type of Treatment	Time	Rep	8-2 TBA Analytical concentration	8-2 TBA Analytical Average	8-2 TBA final concentration†
		day		*μg L⁻¹*	*μg L⁻¹*	*μg L⁻¹*
E93384 EJ-29	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	14	1	78.5	81.1	162
			1	83.7		
E93384 EJ-30		14	2	78.3	78	156
			2	77.7		
E93384 EJ-31		14	3	68.6	68.3	137
			3	68		
E93384 EJ-32		14	4	77.6	78.3	157
			4	78.9		
Average ± Standard Deviation						**153 ± 11**
E93384 EJ-33	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	14	1	90.5	90.6	181
			1	90.7		
E93384 EJ-34		14	2	83.1	81.6	163
			2	80.1		
E93384 EJ-35		14	3	88.4	88.0	176
			3	87.6		
E93384 EJ-36		14	4	91.0	91.1	182
			4	91.1		
Average ± Standard Deviation						**176 ± 9**
E93384 EJ-57	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	28	1	75	79.5	159
			1	84		
E93384 EJ-58		28	2	74.4	74.8	150
			2	75.1		
E93384 EJ-59		28	3	64.4	65.4	131
			3	66.4		
E93384 EJ-60		28	4	61.6	66.8	134
			4	72.0		
Average ± Standard Deviation						**144 ± 13**
E93384 EJ-61	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	28	1	66.2	64.2	128
			1	62.1		
E93384 EJ-62		28	2	91.9	86.8	174
			2	81.7		
E93384 EJ-63		28	3	58.1	53.9	108
			3	49.6		
E93384 EJ-64		28	4	63.4	66.9	134
			4	70.4		
Average ± Standard Deviation						**136 ± 28**

† Final concentration of 8-2 TBA, $C_f = C_a \times (V_{MTBE}/V_t)$ where:
C_a = 8-2 TBA analytical average value,
V_{MTBE} = 50 mL (MTBE used to extract the test medium),
V_t = 25 mL (Test medium used fro extraction).
‡ Standard Deviation was calculated using the final concentration of 8-2 TBA; n = 4.

TABLE A-2.
ANALYTICAL RESULTS OF SPIKE RECOVERY OF 8-2 TBA FROM THE SAMPLE MATRIX (TREATMENT 3, ACTIVATED SLUDGE FILTRATE PLUS MINERAL MEDIUM) AT DAY 0 (31-DEC-2002), DAY 7 (7-JAN-2003), DAY 14 (14-JAN-2003), AND DAY 28 (28-JAN-2003)

No	Time	Rep	8-2 TBA Analytical Concentration	8-2 TBA Analytical Average	Final 8-2 TBA Concentration†	% of Spike Recovery‡
	day		*µg L^{-1}*	*µg L^{-1}*	*µg L^{-1}*	
E93384 EJ-9	0	1	223	227	454	101
		1	230			
E93384 EJ-10	0	2	249	252	504	113
		2	254			
E93384 EJ-11	0	3	232	233	466	104
		3	234			
E93384 EJ-12	0	4	240	239	478	107
		4	238			
Average ± Standard Deviation						**106 ± 5**
E93384 EJ-23	7	1	205	204	408	91
		1	202			
E93384 EJ-24	7	2	202	202	404	90
		2	202			
E93384 EJ-25	7	3	196	196	392	88
		3	195			
E93384 EJ-26	7	4	213	212	424	95
		4	210			
Average ± Standard Deviation						**91 ± 3**
E93384 EJ-37	14	1	211	212	424	95
		1	212			
E93384 EJ-38	14	2	212	212	424	95
		2	212			
E93384 EJ-39	14	3	201	206	412	92
		3	211			
E93384 EJ-40	14	4	213	212	424	95
		4	211			
Average ± Standard Deviation						**94 ± 2**

TABLE A-2 (CONTINUED)

No	Time	Rep	8-2 TBA Analytical Concentration	8-2 TBA Analytical Average	Final 8-2 TBA Concentration†	% of Spike Recovery‡
	day		*μg L^{-1}*	*μg L^{-1}*	*μg L^{-1}*	
E93384 EJ-65	28	1	163	162	324	72
		1	160			
E93384 EJ-66	28	2	168	168	336	75
		2	168			
E93384 EJ-67	28	3	164	171	342	76
		3	177			
E93384 EJ-68	28	4	161	163	326	73
		4	164			
Average ± Standard Deviation						**74 ± 2**

† Final 8-2 TBA concentration, $C_f = C_a \times (V_{MTBE}/V_t)$ where:

C_a = 8-2 TBA analytical average value,

V_{MTBE} = 50 mL (MTBE used to extract the test medium),

V_t = 25 mL (Test medium used fro extraction).

‡ % of spike recovery, $R = (C_f/C_s) \times 100$, where:

C_f = final 8-2 TBA concentration,

C_s = Concentration of 8-2 TBA spiked into the sample bottles (448 μg/L).

TABLE A-3.
ANALYTICAL RESULTS OF FLUORIDE CONCENTRATION AT DAY 0 (31-DEC-2002), DAY 7 (7-JAN-2003), DAY 14 (14-JAN-2003), AND DAY 28 (28-JAN-2003)

No	Type of Treatment	Time	Rep	Fluoride Analytical concentration	Fluoride Analytical Average	Fluoride final concentration†
		day		*µg L⁻¹*	*µg L⁻¹*	*µg L⁻¹*
E93384 EJ-1	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	0	1	10.7	10.6	21.2
			1	10.4		
E93384 EJ-2		0	2	9.3	9.4	18.8
			2	9.4		
E93384 EJ-3		0	3	7.4	7.9	15.8
			3	8.3		
E93384 EJ-4		0	4	7.6	7.9	15.8
			4	8.1		
Average ± Standard Deviation‡						**17.9 ± 2.6**
E93384 EJ-5	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	0	1	9.8	9.1	18.2
			1	8.3		
E93384 EJ-6		0	2	8.9	8.6	17.2
			2	8.2		
E93384 EJ-7		0	3	8.4	8.1	16.2
			3	7.7		
E93384 EJ-8		0	4	8.1	7.8	15.6
			4	7.4		
Average ± Standard Deviation						**16.8± 1.1**
E93384 EJ-15	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	7	1	7.8	8.4	16.8
			1	9.0		
E93384 EJ-16		7	2	7.6	8.1	16.2
			2	8.6		
E93384 EJ-17		7	3	7.1	8.5	17.0
			3	9.8		
E93384 EJ-18		7	4	9.3	9.1	18.2
			4	8.8		
Average ± Standard Deviation						**17.1 ± 0.8**
E93384 EJ-19	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	7	1	10.0	8.8	17.6
			1	7.5		
E93384 EJ-20		7	2	8.8	8.0	16.0
			2	7.2		
E93384 EJ-21		7	3	8.6	8.1	16.2
			3	7.5		
E93384 EJ-22		7	4	8.3	7.8	15.6
			4	7.3		
Average ± Standard Deviation						**16.4 ± 0.9**

TABLE A-3 (CONTINUED)

No	Type of Treatment	Time	Rep	Fluoride Analytical concentration	Fluoride Analytical Average	Fluoride final concentration†
		day		*µg L⁻¹*	*µg L⁻¹*	*µg L⁻¹*
E93384 EJ-29	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	14	1	8.9	8.4	16.8
			1	7.9		
E93384 EJ-30		14	2	8.3	8.1	16.2
			2	7.9		
E93384 EJ-31		14	3	8.4	8.5	17.0
			3	8.6		
E93384 EJ-32		14	4	11.1	9.7	19.4
			4	8.3		
Average ± Standard Deviation						**17.4 ± 1.4**
E93384 EJ-33	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	14	1	8.3	8.5	17.0
			1	8.6		
E93384 EJ-34		14	2	7.8	8.0	16.0
			2	8.2		
E93384 EJ-35		14	3	8.0	8.1	16.2
			3	8.1		
E93384 EJ-36		14	4	8.1	8.1	16.2
			4	8.0		
Average ± Standard Deviation						**16.4 ± 0.4**
E93384 EJ-57	***Treatment 1*** - Activated sludge filtrate plus 8-2 TBA saturated mineral medium	28	1	9.3	9.3	18.6
			1	9.3		
E93384 EJ-58		28	2	8.6	9.0	18.0
			2	9.3		
E93384 EJ-59		28	3	8.0	8.2	16.4
			3	8.3		
E93384 EJ-60		28	4	8.3	8.5	17.0
			4	8.6		
Average ± Standard Deviation						**17.5 ± 1.0**
E93384 EJ-61	***Treatment 2*** - Killed sludge filtrate plus 8-2 TBA saturated mineral medium	28	1	8.5	9.0	18.0
			1	9.4		
E93384 EJ-62		28	2	8.8	9.4	18.8
			2	10.0		
E93384 EJ-63		28	3	8.5	8.7	17.4
			3	8.9		
E93384 EJ-64		28	4	7.9	8.6	17.2
			4	9.3		
Average ± Standard Deviation						**17.9 ± 0.7**

† Final fluoride concentration, $C_f = C_a \times [(V_i+V_t)/V_t]$ where:
C_a = Fluoride analytical average value,
V_i = 4 mL (TISABII® buffer added to the test medium for fluoride measurement),
V_t = 4 mL (Test medium used for fluoride measurement).
‡Standard Deviation was calculated using the final fluoride concentration; n = 4.

TABLE A-4.
PREPARATION OF THE MINERAL MEDIUM FOR THE TEST

Prepare the following stock solutions, using analytical grade reagents (From OECD 301 D Test Guidelines)

(a) Potassium dihydrogen orthophosphate, $KH_2 PO_4$	8.50 g
Dipotassium hydrogen orthophosphate, $K_2 HPO_4$	21.75 g
Disodium hydrogen orthophosphate, Na_2HPO_4	28.39g
Ammonium chloride, NH_4Cl	0.50 g

Dissolve in water and make up to 1 liter. The pH of the solution should be 7.4.

(b) Calcium chloride, anhydrous, $CaCl_2$	27.50 g
or Calcium chloride dihydrate, $CaCl_2.2H2\ 0$	36.40 g

Dissolve in water and make up to 1 liter.

(c) Magnesium sulfate heptahydrate, $MgS0_4.7H_20$	22.50 g

Dissolve in water and make up to 1 liter.

(d) Iron (III) chloride hexahydrate, $FeCl_3.6H_20$	0.25 g

Dissolve in water and make up to 1 liter.

(e) Filter each stock solution through a sterilized filtration unit with 0.2 µm size pore.

Note: In order to avoid having to prepare iron (III) chloride stock solution immediately before use, add one drop of concentrated HCl per liter. If a precipitate forms in a stock solution, replace it with a fresh-made solution.

FIGURE A-1
A FLUORIDE STANDARD CALIBRATION CURVE USED FOR FLUORIDE QUANTIFICATION OF SAMPLES E93384EJ-1 TO E93384EJ-64

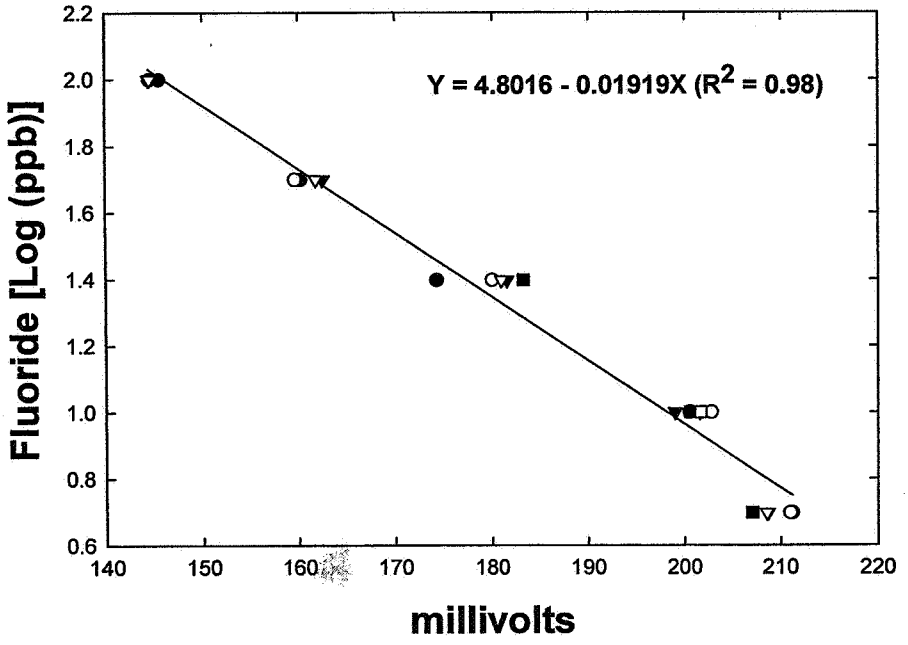
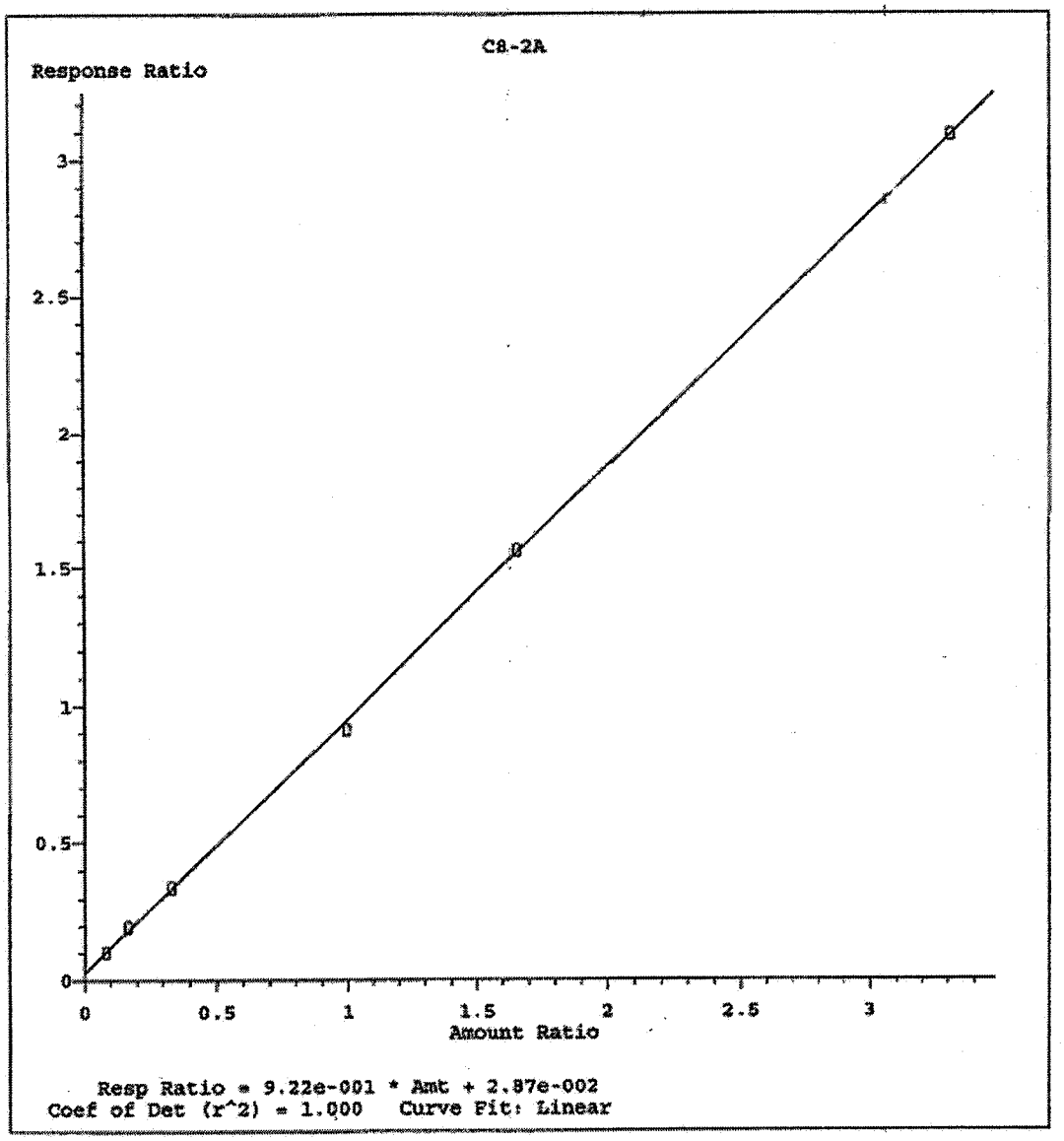
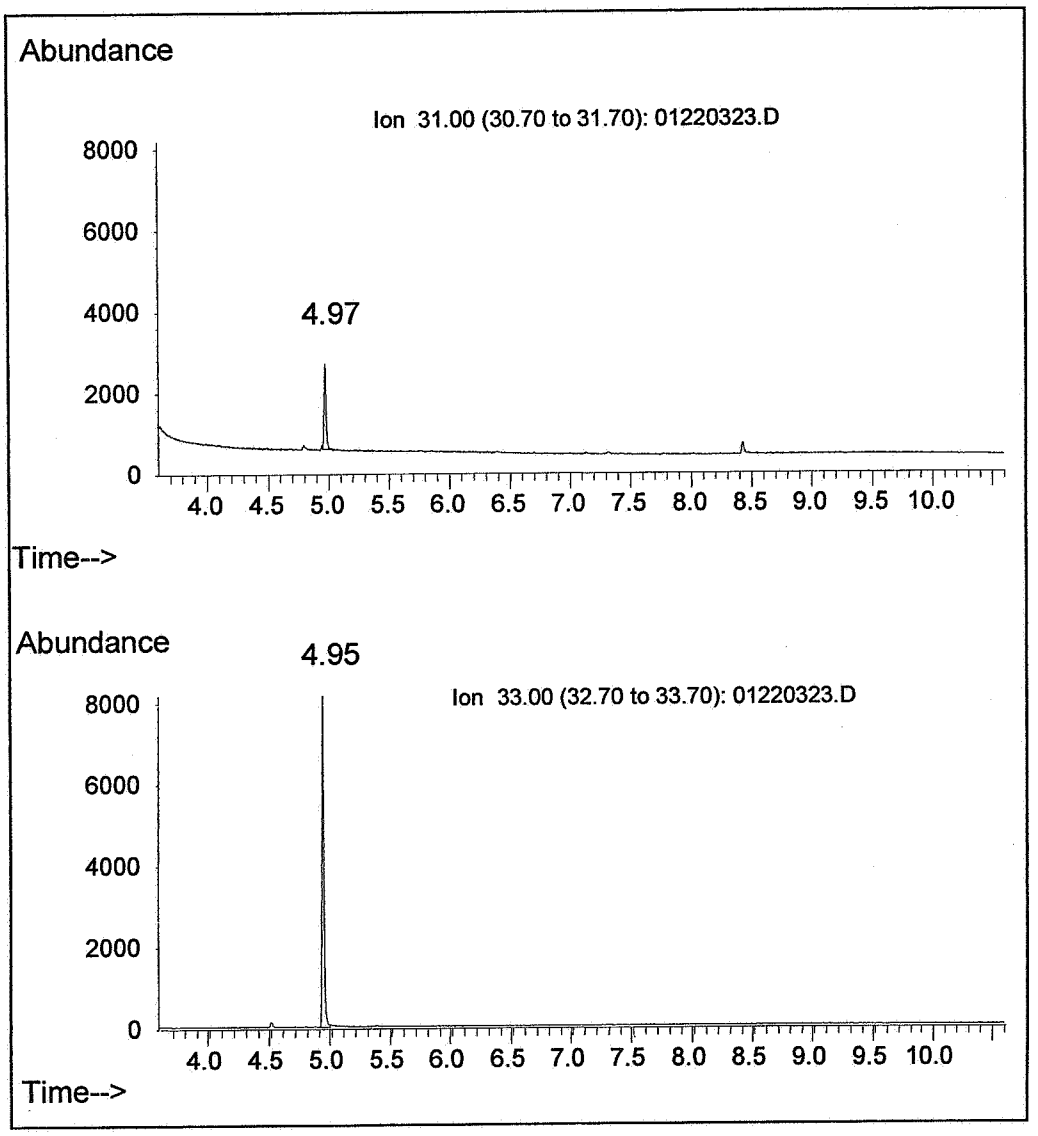


FIGURE A-2
A CALIBRATION CURVE USED FOR 8-2 TBA QUANTIFICATION OF SAMPLES E93384EJ-1 TO E93384EJ-28



A calibration curve used for samples E93384 EJ-1 to E93384 EJ-28. The concentration of the internal standard: 308 µg/L of D-8-2 TBA. The range of concentrations of 8-2 TBA: 25.5 - 1020 µg/L.

FIGURE A-3
A CHROMATOGRAPH OF SAMPLE E93384EJ-29 USED FOR 8-2 TBA ANALYSIS



Sample E93384 EJ-29; Ion 31, retention time 4.97 min = 8-2 TBA; Ion 33, RT 4.95 min = D-8-2 TBA (internal standard).