Improved Enzyme-Linked Immunosorbent Assay To Detect Didecyldimethylammonium Chloride, a Quaternary Ammonium Compound

Jennifer P. Bull, Alessio N. Serreqi, Hector R. Gamboa, and Colette Breuil*

Department of Wood Science, Faculty of Forestry, University of British Columbia, 4041-2424 Main Mall, Vancouver, British Columbia, Canada V6T 1Z4

Didecyldimethylammonium chloride (DDAC) is a commonly used wood protectant in Canada and must be monitored due to environmental concerns. Three new DDAC-like immunogens were synthesized and tested for their ability to produce antibodies that complex DDAC. The polyclonal antisera from rabbits immunized with the new hapten conjugates were compared by hapten homologous indirect competitive enzyme-linked immunosorbent assay (CELISA). The resulting antisera recognized DDAC to different degrees. CELISA based on anti-DDAC 3, 2, and 1 antisera had IC_{50}s of 0.05, 16.0, and 17.2 μg/mL, respectively. All the antisera were more sensitive to DDAC than a previously reported polyclonal antiserum. The most sensitive and selective antiserum was produced from the hapten that most closely mimicked DDAC. The selectivity of the three immunoassays for compounds representing DDAC's different epitopes was tested. Each immunoassay exhibited the highest selectivity with compounds that most closely resembled the hapten. The effect of solvents and detergents was determined for the most sensitive immunoassay: CELISA based on anti-DDAC 3 antiserum. Recovery experiments were also carried out with this assay. This immunoassay (IC_{50} 50 ppb) holds promise for the analysis of environmental samples since the regulatory discharge limit is 700 ppb.

Keywords: *Didecyldimethylammonium chloride; quaternary ammonium compounds; ELISA; immunoassay*

INTRODUCTION

Quaternary ammonium compounds (QACs) have long been recognized as an important class of cationic surfactants with both industrial and commercial uses. One important class, the dialkyldimethylammonium chlorides (DADACs) are commonly used in fabric softeners and oil-based drilling muds (Boethling, 1984). The DADAC, didecyldimethylammonium chloride (DDAC; Figure 1) is also effective in controlling sapstain in freshly cut lumber and is the most common active ingredient in antisapstain products used in Canada today. While DDAC is less toxic to mammals than its predecessors, the chlorophenols, it is not free from environmental concerns. DDAC is toxic to aquatic life, with LC_{50}s of 1 mg/L to invertebrates and fish (Cooper, 1988) and may pose a threat when entering waterways from sawmill sites via stormwater runoff.

To ensure that optimum treatment levels are applied to wood, it is necessary to monitor DDAC working solutions in sawmills. Environment Canada also requires monitoring of DDAC at antisapstain facilities to ensure protection of worker health and the environment. Currently, DDAC has a stormwater discharge limit of 700 μg/L (ppb) (Konasewich and St. Quintin, 1994). DDAC is, however, difficult to analyze because it has a low volatility, lacks a chromophore, and is difficult to derivatize (Suortti and Sirviö, 1990). Despite this, methods have been developed to detect DDAC, which include titration (Lonza Inc., 1988), gas chromatography (GC) (Metcalfe, 1962; Suzuki et al., 1986; Konasewich and St. Quintin, 1994), liquid chromatography (LC) (Larson and Pfeiffer, 1983), and high-performance liquid chromatography (HPLC) (Suortti and Sirviö, 1990; Daniels, 1992; AWPA, 1993). Indirect LC and HPLC methods employ photometric detection (Larson and Pfeiffer, 1983), UV detection (AWPA, 1993), and refractive index detection (Suortti and Sirviö, 1990). However, a direct detection method for HPLC was developed utilizing an evaporative light-scattering detector (ELSD) (Daniels, 1992). The methods reported have relatively high detection limits, which may not be suitable for environmental analysis of DDAC. The detection limit for various QACs using an LC method was 0.6−0.8 μg/mL (ppm) (Larson and Pfeiffer, 1983). While Suortti and Sirviö (1990) developed an HPLC method with a detection limit of approximately 0.3 μg/mL for DDAC. The HPLC method described by Daniels (1992) was developed to detect DDAC within the range of 30−250 ppm, after extraction from wood surfaces. Generally, HPLC and GC are time-consuming, expensive, and have low sample throughput capabilities (Wittmann and Hock, 1990; Van Emon and Gerlach, 1992). In addition, these techniques are not easily adapted to on-site applications.

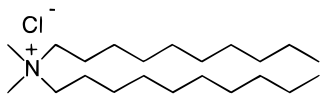

Figure 1. Didecyldimethylammonium chloride (DDAC).

Immunoassays can provide an alternative to or complement conventional analyses such as HPLC and GC. They are sensitive, easy to use, fast, cost-efficient, and

* Author to whom correspondence should be addressed (fax, 604-822-9104; email, breuil@unixg.ubc.ca).

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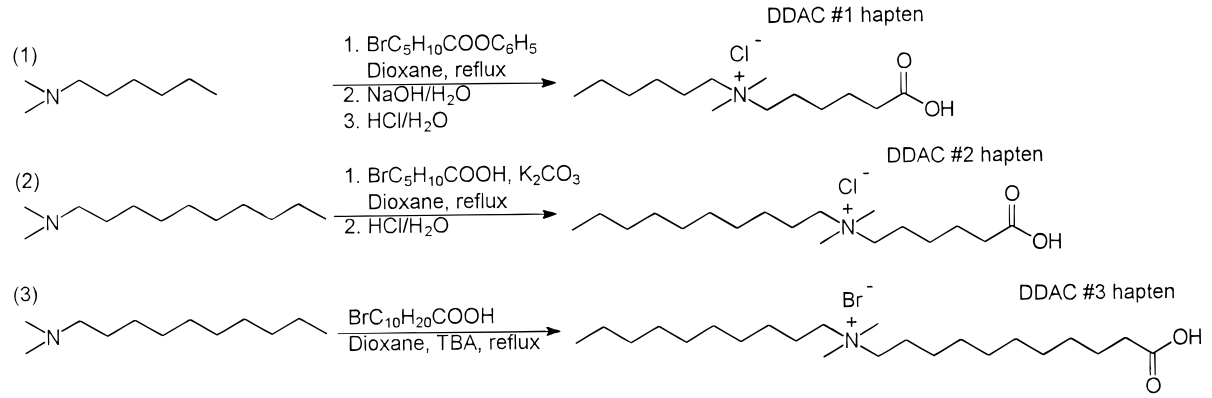


Figure 2. Syntheses of the DDAC 1 (1), 2 (2), and 3 (3) haptens. TBA is tributylamine.

have high sample throughput capabilities (Wittmann and Hock, 1990; Van Emon and Gerlach, 1992; Mountfort et al., 1994). Immunoassays are also gaining increasing acceptance as monitoring tools for pesticide residues and other toxic residues in agricultural products and the environment (Van Emon and Lopez-Avila, 1992; Bekheit et al., 1993; Richman et al., 1996; Stanker et al., 1996). An indirect competitive enzyme-linked immunosorbent assay (CELISA), based on rabbit polyclonal antibodies, was developed for detecting DDAC (Chen et al., 1995), but the sensitivity was inadequate for an environmental discharge limit of 700 ppb. The IC_{50} and detection limit for DDAC were 29 and 8 ppm respectively (Chen et al., 1995). The immunoassay also was selective for other compounds, such as fatty acids and alcohols, by as much as 54%. This is undesirable as these compounds may be present in environmental samples. These results suggested that raising antibodies against DDAC may be difficult because of its homogeneous structure and lack of functionality.

The objective of this work was to develop an improved immunoassay for DDAC detection. Three new DDAC haptens and immunogens were synthesized to generate more sensitive and specific antibodies. Hapten homologous CELISAs were developed for each polyclonal antiserum and the sensitivities and specificities for DDAC were determined. These results were compared to those reported by Chen et al. (1995). The effects of other surfactants and solvents were tested on the most sensitive immunoassay. In addition, recoveries of DDAC spikes in milk samples were carried out.

MATERIALS AND METHODS

Reagents. DDAC (~98%) was donated by Forintek Canada Corporation (Vancouver, BC). Didodecyldimethylammonium chloride ($DD_{12}AC$), *N*,*N*-dimethyldecylamine (*N*,*N*-$DMD_{10}A$, >95%), *N*-methyldidecylamine (*N*-$MDD_{10}A$, >95%), and *N*-methyldioctylamine (*N*-MDO_8A, >95%) were obtained from Lonza Inc. (Annandale, NJ). Bovine serum albumin (BSA), carbonate−bicarbonate buffer capsules, Freund's complete and incomplete adjuvants, *o*-phenylenediamine dihydrochloride (OPD) tablets (toxic), and rabbit serum albumin (RSA) were purchased from Sigma (St. Louis, MO). Didecyldimethylammonium bromide (DDAB, 98%), dihexylamine, *N*,*N*-dimethylbutylamine (*N*,*N*-DMB_4A), *N*,*N*-dimethylhexylamine (*N*,*N*-DMH_6A), *N*,*N*-dimethyloctylamine (*N*,*N*-DMO_8A, 95%), *N*-methyldibutylamine (*N*-MDB_4A), and tetramethylammonium chloride (TMAC) were purchased from Aldrich (Milwaukee, WI). The anti-rabbit IgG-horseradish peroxidase was obtained from Caltag Laboratories (San Francisco, CA). Bacto dehydrated skim milk powder was obtained from Difco Laboratories (Detroit, MI). All solvents were of HPLC grade, and the deionized water was purified by the NANOpure ultrapure water system (Barnstead-Thermolyne). Dioxane should be handled with care since it is a flammable liquid, potentially explosive if contaminated with peroxides, and a cancer suspect agent. Many quaternary ammonium compounds are toxic irritants.

Equipment and Data Analysis. Microtitration plates (Immulon 4, Dynatech Laboratories, Inc., Chantilly, VA) were used for ELISAs. ELISA plates were assessed using a THERMOmax microplate reader and standard curves were fitted to the four-parameter logistic equation using SOFTmax version 2.01 software (Molecular Devices Corp., Menlo Park, CA). When necessary, some standard curves were fitted to semilog equations. Mathcad PLUS 6.0 software, employing the Dixon test and the *G*-statistic test, was used to detect outliers in data points containing at least four replicates. ^1H NMRs were obtained on a Bruker 200 MHz spectrometer. IRs were obtained on a Perkin-Elmer 1600 series FTIR with a diffuse reflectance accessory.

Hapten Syntheses. *DDAC 1*: (*6-Carboxylic hexanyl*)*dimethylhexylammoniun Chloride.* A mixture of 6-bromohexanoic acid phenyl ester (0.43 g, 1.6 mmol) and *N*,*N*-dimethylhexylamine (0.32 g, 2.5 mmol) in 10 mL of dried dioxane was refluxed for 18 h. Removal of dioxane by rotary evaporation and drying by vacuum pump yielded the phenyl ester intermediate as a yellow oil (0.6 g, 1.5 mmol, 94% yield): ^1H NMR ($CDCl_3$, 200 MHz) δ 0.82 (t, 3H), 1.10−1.85 (m, 14H), 2.58 (t, 2H), 3.30 (s, 6H), 3.35−3.66 (m, 4H), 6.95−7.40 (m, 5H). To a solution of this compound (0.35 g, 0.88 mmol) in dioxane (10 mL) was added 1 N NaOH (2 mL). The reaction mixture was stirred for 5 h and then acidified to pH 1.0 with 1 N HCl. Dioxane was removed by rotary evaporation, and the aqueous layer was washed with ether (15 mL), frozen, and then freeze-dried. The product (Figure 2, part 1) was redissolved in 1:1 dioxane/methanol (20 mL), filtered, concentrated by rotary evaporation, and vacuum pump dried to give a yellow residue (0.24 g, 0.86 mmol, 98% yield): ^1H NMR ($CDCl_3$, 200 MHz) δ 0.85 (t, 3H), 1.30 (m, 8H), 1.70 (m, 6H), 2.40 (t, 2H), 3.08 (s, 6H), 3.25 (m, 4H); IR (ν_{max} KCl cm^{-1}) 2930 (s), 2345 (m), 1730 (s), 1465 (m).

DDAC 2: (*6-Carboxylic hexanyl*)*decyldimethylammoniun Chloride.* *N*,*N*-Dimethyldecylamine (0.50 g, 2.7 mmol) and anhydrous potassium carbonate (0.37 g, 2.7 mmol) were added to a solution of 6-bromohexanoic acid (0.55 g, 2.8 mmol) in dry dioxane (5 mL), and refluxed for 18 h. The residue obtained after filtration and removal of dioxane by rotary evaporation was dissolved in 1:1 H_2O/ethanol (30 mL). The solution was acidified to pH 2.0 with 2 N HCl. The organic layer was removed by rotary evaporation, and the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined extracts were dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. Placing the quaternary ammonium product in a desiccator in the presence of P_2O_5 gave a white amorphous solid (0.24 g, 0.72 mmol, 27% yield): ^1H NMR ($CDCl_3$, 200 MHz) δ 0.81 (t, 3H), 1.20 (m, 16H), 1.6 (m, 2H), 1.77 (m, 4H), 2.30 (t, 2H), 2.70 (m, 6H), 2.90 (m, 4H); IR (ν_{max} KCl cm^{-1})

Table 1. Quantities of Reagents Used in the Syntheses of the DDAC 1, 2, and 3 Immunogens and Coating Antigens

	hapten					
	DDAC 1		DDAC 2		DDAC 3	
reagent	immunogen	coating antigen	immunogen	coating antigen	immunogen	coating antigen
IBCF (μmol)	130	160	70	70	46	70
TBA (μmol)	130	160	70	70	42	67
hapten (μmol)	110	140	60	60	33	42
DMF (mL)	3.0	3.0	1.0[a]	1.0[a]	2.0	1.0
protein (mg)	109	112	40	40	25	48
buffer (mL)	4.0[b]	6.0[c]	5.0	5.0	3.0	5.0
product (mg)	85	90	41	36	18	44

[a] Dioxane was used instead of *N*,*N*-dimethylformamide (DMF). [b] Buffer is a 3:1 solution of $NaHCO_3$ buffer (50 mM, pH 9.6)−DMF. [c] Buffer is a 5:1 solution of $NaHCO_3$ buffer (50 mM, pH 9.6)−DMF.

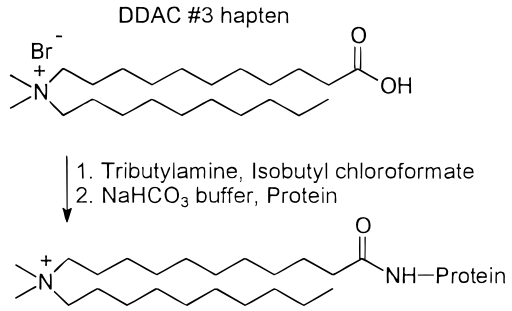


Figure 3. Synthesis of the DDAC 3 immunogen and coating antigen, starting from the DDAC 3 hapten. The carrier proteins were bovine serum albumin for the immunogen and rabbit serum albumin for the coating antigen.

2940 (s), 2510 (br, s), 1730 (s), 1455 (m). The hapten is shown in Figure 2, part 2.

DDAC 3: (11-Carboxylic undecanyl)decyldimethylammoniun Chloride. *N*,*N*-Dimethyldecylamine (0.20 g, 1.1 mmol) and tributylamine (0.34 mL, 1.4 mmol) were added to a solution of 11-bromoundecanoic acid (0.31 g, 1.2 mmol) in dry dioxane (3 mL), and refluxed for 18 h. Ethyl acetate was added to the reaction mixture, and a precipitate formed which was filtered and washed with *N*,*N*-dimethylformamide (DMF). Drying in a desiccator in the presence of P_2O_5 gave the quaternary ammonium product as a white amorphous solid (0.30 g, 0.67 mmol, 61% yield): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.83 (t, 3H), 1.30 (m, 26H), 1.70 (m, 6H), 2.33 (m, 2H), 3.34 (s, 6H), 3.50 (m, 4H); IR (ν_{max} KCl cm^{-1}) 2913 (br, s), 2350(w), 1730 (s), 1475 (m). The hapten is shown in Figure 2, part 3.

Syntheses of the Immunogens and Coating Antigens. The three immunogens and three coating antigens were all synthesized by the same method, except with different volumes, which are reported in Table 1. Isobutyl chloroformate (IBCF) and tributylamine (TBA) were added to a solution of the hapten in DMF (or dioxane) which was cooled in a water bath, and stirred for 30 min to 1 h. The reaction mixture was then added to either bovine serum albumin (BSA) for the immunogen, or rabbit serum albumin (RSA) for the coating antigen, dissolved in $NaHCO_3$ buffer (50 mM, pH 9.6), and stirred at room temperature. The DDAC 1, 2, and 3 immunogens and coating antigens were stirred for 6, 5, and 18 h, respectively. The conjugates were then dialyzed against deionized water for 24 h with four changes of water (2 L) and lyophilized to give white powders. The synthesis scheme for the DDAC 3 immunogen and antigen is shown, as an example, in Figure 3.

Synthesis of Dihexyldimethylammonium Bromide (*DH_6AB*). Bromohexane (95 mg, 0.58 mmol) and potassium carbonate (88 mg, 0.64 mmol) were added to a solution of *N*,*N*-dimethylhexylamine (74 mg, 0.59 mmol) in THF (3 mL), and refluxed for 18 h. The reaction mixture was filtered, concentrated by rotary evaporation, and dried under vacuum to yield a brown oil (0.15 g, 0.51 mmol, 86% yield): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.82 (t, 6H), 1.22 (m, 12H), 1.62 (m, 4H), 3.30 (s, 6H), 3.40 (m, 4H); IR (ν_{max} KCl cm^{-1}) 2925 (s), 2860(s), 1465 (m).

Synthesis of Dimethyldioctylammonium Chloride (*DO_8AC*). Iodomethane (0.27 mL, 4.34 mmol) and potassium carbonate (75 mg, 0.54 mmol) were added to a solution of *N*-methyldioctylamine (0.20 g, 0.78 mmol) in dioxane (3 mL), and refluxed for 18 h. After filtration and removal of solvent, the residue was dissolved in water (10 mL) and acidified to pH 1.0 with 2 N HCl. The solution was extracted with chloroform (3 × 10 mL), and the combined extracts were dried over anhydrous magnesium sulfate, filtered, concentrated by rotary evaporation, and dried under vacuum to give a brown oil (0.24 g, 0.78 mmol, 100% yield): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.82 (t, 6H), 1.22 (m, 20H), 1.65 (m, 4H), 3.30 (s, 6H), 3.42 (m, 4H); IR (ν_{max} KCl cm^{-1}) 2925 (br, s), 2855(m), 1465 (m).

Producing and Screening the Polyclonal Antibodies. For each immunogen, two New Zealand white female rabbits weighing between 2 and 4 kg were immunized subcutaneously with 1 mL of the following emulsions, each containing approximately 3 mg of an immunogen. The DDAC 1 and 3 immunogens were added to 0.8 mL of PBS, 0.4 mL of dimethyl sulfoxide (DMSO), and 1.2 mL of complete Freund's adjuvant; while the DDAC 2 was added to 1.2 mL of PBS and 1.2 mL of complete Freund's adjuvant. The rabbits were boosted intramuscularly three times, with two week resting periods, using 1 mL from emulsions consisting of approximately 2.5 mg of an immunogen in 1.2 mL of PBS and 1.2 mL of incomplete Freund's adjuvant. The antibody titers of each sera were determined by hapten homologous indirect ELISA, using 100 ng/well coating antigen concentration, after the first, second, and third boosts. When a high titer was reached, the rabbits were bled, and the serum was separated by centrifugation and stored at −70 °C.

Indirect ELISA and CELISA. In indirect ELISAs, microtitration plates were coated with either DDAC 1, 2, or 3-RSA (100 ng/100 μL per well) in carbonate−bicarbonate buffer (pH 9.6) and dried overnight at 37 °C. The plates were washed four times with PBS buffer (pH 7.4) and blocked with a 2% solution of milk powder in PBS (200 μL/well) for 1 h at 37 °C, and then washed again four times with PBS. The respective anti-BSA−hapten polyclonal sera were serially diluted in a 0.1% solution of milk powder in PBS (100 μL/well), added to the plate, and incubated for 2 h at 37 °C. The plate was washed four times with PBS, and a secondary antibody−enzyme conjugate, anti-rabbit IgG-horseradish peroxidase, diluted 1:3000 in a 0.1% solution of milk powder in PBS (100 μL/well), was added and incubated for 1 h at 37 °C. The enzymatic reaction was carried out at room temperature, in the dark, using *o*-phenylenediamine dihydrochloride (OPD) dissolved in 0.05 M phosphate−citrate buffer with sodium perborate as the substrate (100 μL/well). The reaction was stopped after 30 min with 2.5 M H_2SO_4 (50 μL/well), and the absorbance or optical density (OD) was measured at 490 nm.

CELISAs were used to assess the sensitivity and selectivity of the immunoassays for DDAC and recovery of DDAC from spiked milk samples. Stock solutions of 1, 0.4 and 0.1 mg/mL of DDAC and related compounds were prepared in Nanopure water, methanol, or acetonitrile, depending on the solubility of the compound. Appropriate concentrations of the chemical, based on the working linear ranges or the IC_{50}s of each antiserum, were mixed with the polyclonal sera and diluted

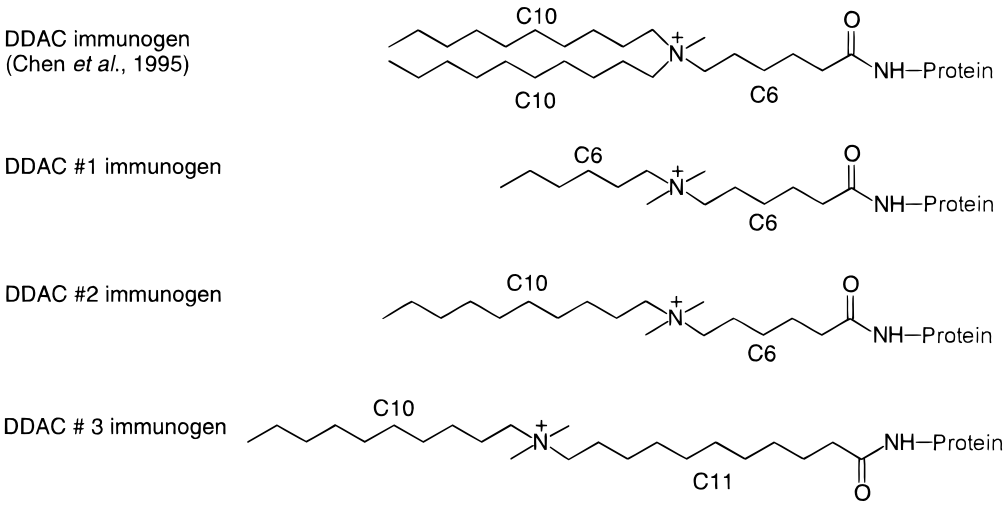


Figure 4. Structures of the immunogens representing DDAC 1, 2, and 3, and that synthesized by Chen et al. (1995).

by one-half to the desired final concentrations in a 0.1% solution of milk powder in PBS, and added to the plate wells in quadruplicate. The rest of the ELISA was carried out as described above. Semilog equations were used to analyze inhibition curves for the anti-DDAC 1 and 2 antisera, while the four-parameter logistic equation was used to analyze inhibition curves for the anti-DDAC 3 antiserum. The IC_{50}s, IC_{20}s, IC_{70}s, detection limits, and unknown concentrations were also calculated from each plate's DDAC inhibition curve.

RESULTS AND DISCUSSION

Hapten Designs. The choice and design of the hapten, linker arm (if used), protein, and assembly of the complex are important for the production of highly selective antibodies (Harrison et al., 1991; Carter and Friedman, 1992; Schneider and Hammock, 1992). The purpose of a linker arm is to maximize exposure of the analyte for antibody production and increase assay sensitivity. Because DDAC and related QACs have no functional group suitable for conjugation with carrier proteins, new haptens were synthesized. In Chen et al. (1995), didecylmethylamine was coupled to a linker arm, 6-bromohexanoic acid, to give the hapten. A protein was then conjugated through the carboxylic acid group to give the immunogen and coating antigen, which retained the didecyl chain structure of the DDAC molecule (Figure 4). However, the desired sensitivity and selectivity for DDAC were not achieved with this immunogen design. To produce antibodies with higher sensitivities and selectivities for DDAC, new DDAC immunogens were synthesized (Figure 4). The DDAC 1 hapten was designed with a C_6 chain and dimethylammonium center, while the DDAC 2 and DDAC 3 haptens contained a C_{10} chain and dimethylammonium center, except with differences in the length of the linker arm. The new immunogens retained the important structural features of DDAC, namely the dimethyl quaternary ammonium structure and dialkyl chains of at least six carbons.

Characterizing the Polyclonal Antibodies. *Antibody Production.* Each DDAC hapten was conjugated to two proteins: BSA and RSA. BSA−hapten conjugates were used as immunogens and RSA−hapten conjugates were used as coating antigens. The titer response and sensitivity for DDAC of the test and final bleeds were determined using hapten homologous indirect ELISAs and CELISAs, with coating antigen concentrations of 100 ng/well. The most sensitive

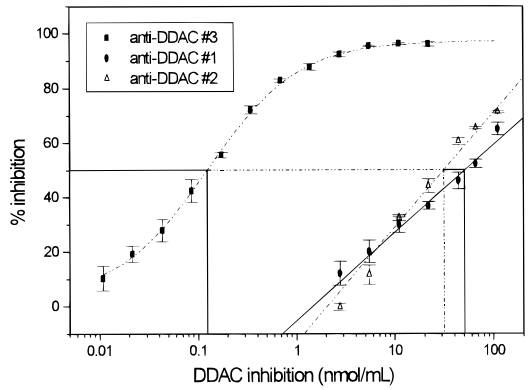


Figure 5. Standard inhibition curves for the anti-DDAC 1, 2, and 3 polyclonal antibodies, against free DDAC. The indirect CELISAs were done using antiserum dilutions of 1:70000, 1:6000, and 1:4000, respectively, with coating antigen concentrations of 100 ng/well. The logistic correlation coefficient for the anti-DDAC 3 antiserum was 0.99. The linear correlation coefficients for the anti-DDAC 1 and 2 antisera were also 0.99. Inhibition curves were repeated twice per serum and in quadruplicate. Error bars represent standard deviations.

antiserum against DDAC in each case was used for further CELISA experiments. Absorbance or optical density (OD) readings of approximately 1.1 were obtained for antiserum dilutions of 1:70000, 1:6000, and 1:4000 for the anti-DDAC 1, 2, and 3 antisera, respectively. For DDAC 1 and 2, the antisera obtained after the second test bleeds were used, while the final bleed antiserum was used for DDAC 3 experiments.

Sensitivity of the Polyclonal Antisera for DDAC. The sensitivities of the antisera for free DDAC, which can be characterized by both the IC_{50} and the detection limit, were determined by hapten homologous indirect CELISA. Percent inhibition was calculated as $100[1 - (A/A_0)]$, where A_0 was the absorbance with no DDAC present (blank response) and A was the absorbance in the presence of DDAC. The detection limit was defined from the logistic or semilog curve fit, as the concentration of DDAC that gave an OD of $A_0 - 3{\cdot}SD_0$, where SD_0 was the standard deviation of the absorbance for the blank response (Fleeker, 1987). The A_0 value was a mean of $n = 12$. Figure 5 shows typical inhibition curves for DDAC using the anti-DDAC 1, 2, and 3 polyclonal antisera. Concentrations of DDAC ranged from 0 to 110.5 nmol/mL (0 to 40 μg/mL) for the DDAC 1 and 2 curves, and from 0 to 22 nmol/mL (0 to 8 μg/

Table 2. Comparison of the IC_{50} and Detection Limits for Anti-DDAC 1, 2, 3, and Chen et al. (1995) Polyclonal Antisera

hapten	IC_{50} (μg/mL)	detection limit (μg/mL)
Chen et al. (1995)	29	8
DDAC 1	17.2 ± 1.6^b	15.2 ± 0.2^d
DDAC 2	16.0 ± 4.8^a	2.6 ± 0.3^d
DDAC 3	0.050 ± 0.008^e	0.0080 ± 0.0005^c

[a–e] Uncertainties are ± standard deviations with means of (*a*) $n = 6$, (*b*) $n = 5$, (*c*) $n = 3$, (*d*) $n = 2$, and (*e*) $n = 14$.

mL) for DDAC 3. Results from the inhibition curves are summarized in Table 2. The anti-DDAC 3 antiserum showed the best sensitivity for DDAC, with an IC_{50} of 0.05 μg/mL (50 ppb) and a detection limit of 0.008 μg/mL (8 ppb). These antibodies showed a 600-fold improvement in the IC_{50} over the antiserum produced by Chen et al. (1995), which had an IC_{50} of 29 μg/mL (ppm) and a detection limit of 8 μg/mL (ppm) for DDAC. The IC_{50}s were similar for the anti-DDAC 1 and 2 antisera, at 17.2 and 16.0 μg/mL, respectively. DDAC became insoluble in the PBS buffer−milk system at concentrations ≥ 50 μg/mL. Complete sigmoidal curves could not be produced for the anti-DDAC 1 and 2 antisera because of their high IC_{50}s relative to DDACs solubility limit. Consequently, these results were best fitted with semilog rather than four-parameter curves.

Selectivity of the Polyclonal Antisera. Polyclonal antibodies produced against a given immunogen can recognize one or several epitopes of the antigen, with varying affinities for the different epitopes. The ability of the antibodies to complex with other compounds is usually quantified by determining the cross-reactivities (CR). Determination of CRs requires a substantial amount of experimental work when many samples need to be tested. For each compound a standard curve must be generated to determine its IC_{50} and these experiments are usually performed in triplicate. Therefore, a different approach was used for the preliminary characterization of selectivity of the antisera. Compounds were tested for their ability to inhibit the antisera at molar concentrations equivalent to the respective IC_{50}s for DDAC (Table 3) i.e., at 47.5, 44.2, and 0.14 nmol/mL for the anti-DDAC 1, 2, and 3 antisera, respectively. The result for each compound (*x*) was reported as a selectivity factor (SF) and calculated as 100[% inhibition(*x*)/% inhibition (DDAC)], where (*x*) and (DDAC) are at the molar DDAC IC_{50} concentration for the antiserum tested. This approach enables many compounds to be rapidly tested since they are tested only at one concentration.

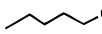
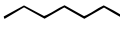
The chemical structure of DDAC offers two types of epitopes: the long aliphatic C_{10} chain(s) and the dimethylammonium center. The anti-DDAC antisera were tested for their selectivity toward compounds representing DDACs different epitopes to assess the antigenic determinants of each immunogen. Table 3 shows that the three antisera reacted with fatty acids and fatty alcohols to different degrees. The anti-DDAC 2 antiserum was most selective for these compounds, followed by the anti-DDAC 1 and 3 antisera. Reactivity of the antisera with simple fatty acids and alcohols can be a problem since these compounds may cause interference in various environmental matrices and because fatty acids may denature antibodies (Hefle, 1995). The selectivity factor ranged from 0 to 69%, from 0 to 158%, and from 0 to 16% for DDAC 1, 2, and 3 haptens, respectively. The selectivity factor of the DDAC 1 and 2 antisera for fatty acids and fatty alcohols were similar to and higher than those reported by Chen et al. (1995). Because of the significant reactivity of these compounds, the immunogens that produced these antisera were not ideal, even though there were slight improvements in the IC_{50}s for DDAC.

There was concern that tertiary amines may be present in environmental samples as degradation products of quaternary ammounium compounds. Therefore, the selectivity of the anti-DDAC antisera for several tertiary amines was determined (Table 3). The anti-DDAC 1 antiserum was most selective for amines having C_6 chains, the same chain length as the hapten. Conversely, both the anti-DDAC 2 and 3 antisera were very selective for amines having C_{10} chains, the same as their haptens. The anti-DDAC 2 and 3 antisera were most selective for *N*,*N*-dimethyldecylamine (132% and 106% respectively), and they were also very selective for *N*-methydidecylamine (92% and 57%), *N*,*N*-dimethyloctylamine (83% and 60%), and *N*-methyldioctylamine (77% and 83%). These results support the observation that antibody specificity is generally highest for the part of the molecule that is furthest from the carrier protein, which in this case is the amine structure (Hammock and Mumma, 1980; Sherry, 1992).

Other dialkyldimethylammonium chlorides were also tested to determine whether the antisera would recognize closely related compounds (Table 4). Reactivity with related dialkyldimethylammonium chlorides are a concern because they may cause interference in environmental samples. The anti-DDAC 1 antiserum was most selective for dihexyldimethylammonium chloride (205% SF) and also showed a high selectivity for dihexylamine (125% SF). This suggests that the antibodies recognized the C_6 chains combined with the ammonium center, a structure similar to the hapten. However, the serum was more selective for dimethyldioctylammonium chloride (181% SF) than dihexylamine, suggesting that the charged nitrogen was also important in the binding reaction. Conversely, the anti-DDAC 2 and 3 antisera showed the highest selectivity for DDAC. This, along with the amine results, suggested that the C_{10} chain(s) combined with the dimethylammonium center were important for antibody recognition of the haptens for both sera. Chen et al. tested their anti-DDAC antiserum against several small QACs and observed the antiserum had little affinity for these compounds. However, QACs that closely mimicked DDAC were not tested. The results of this work indicated that the antiserum most selective for DDAC was anti-DDAC antiserum. It had the lowest affinity for other similar compounds. The antiserum was obtained from the hapten that most closely mimicked DDAC. These results are consistent with those reported by Harrison et al. (1991), who showed that chemical structures similar to the hapten will generally have the highest affinity for the antibodies raised.

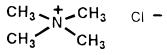
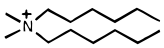
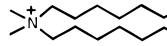
Further Characterization of the Anti-DDAC 3 Antiserum. *Effects of Solvents on the Anti-DDAC 3 Antiserum*. Solvents are often used in commercial antisapstain formulations and to extract analytes from environmental matrices such as soil and water. In addition, when the selectivities of some of the water-insoluble compounds were tested (Tables 3 and 4), methanol and acetonitrile were used as solvents. Solvents, however, have been shown to affect the response

Table 3. Selectivities of the Anti-DDAC Polyclonal Antisera with Compounds Representing DDAC's Different Epitopes[a]

Chemical*	Structure	Selectivity Factor (%) Anti #1	Anti #2	Anti #3
	Fatty Acids			
caproic acid (C6:0)		< 1	< 1	< 1
octanoic acid (C8:0)		< 1	< 1	< 1
decanoic acid (C10:0)		12 ± 11	77 ± 15	< 1
lauric acid (C12:0)		32 ± 4	158 ± 3	< 1
myristic acid (C14:0)		33 ± 11	134 ± 4	7 ± 9
palmitic acid (C16:0)		31 ± 8	63 ± 6	16 ± 5.
stearic acid (C18:0)		40 ± 5	94 ± 8	< 1
	Fatty Alcohols			
hexanol (C6:0)		15 ± 6	2 ± 14	< 1
octanol (C8:0)		22 ± 8	16 ± 14	< 1
decanol (C10:0)		38 ± 5	58 ± 15	< 1
lauryl alcohol (C12:0)		69 ± 6	104 ± 5	10 ± 18
	Amines			
N,N-DMB$_4$A		27 ± 10	2 ± 6	17 ± 14
N,N-DMH$_6$A		124 ± 7	45 ± 13	37 ± 11
N,N-DMO$_8$A		114 ± 8	83 ± 11	60 ± 8
N,N-DMD$_{10}$A		76 ± 5	132 ± 6	106 ± 4
N-MDB$_4$A		80 ± 4	25 ± 9	37 ± 3
dihexylamine		125 ± 5	85 ± 4	< 1
N-MDO$_8$A		110 ± 12	77 ± 13	83 ± 6
N-MDD$_{10}$A		11 ± 6	92 ± 7	57 ± 6

[a] N.B. Compounds were tested in quadruplicate and uncertainties are ± standard deviations. Percent selectivity factor (% SF) for each compound, *x*, was calculated as 100[% inhibition(*x*)/% inhibition (DDAC)], at the molar DDAC IC_{50} concentration for the hapten tested. *Chemical names of abbreviations are given in reagents section of Materials and Methods.

Table 4. Selectivities of the Anti-DDAC Polyclonal Antisera with Compounds Representing DDAC's Different Epitopes[a]

Chemical*	Structure	Selectivity Factor (%) Anti #1	Anti #2	Anti #3
	Quaternary ammonium compounds			
TMAC		< 1	< 1	< 1
DH$_6$AB		205 ± 1	< 1	10 ± 11
DO$_8$AC		181 ± 8	66 ± 6	59 ± 19
DDAC		100 ± 6	100 ± 5	100 ± 3
DD$_{12}$AB		5 ± 6	< 1	15 ± 8

[a] N.B. Compounds were tested in quadruplicate and uncertainties are ± standard deviations. Percent selectivity factor (% SF) for each compound, *x*, was calculated as 100[% inhibition(*x*)/% inhibition (DDAC)], at the molar DDAC IC_{50} concentration for the hapten tested. *Chemical names of abbreviations are given in reagents section of Materials and Methods.

of antibodies in noncompetitive ELISAs (Harrison et al., 1991; Bekheit et al., 1993; Wittmann and Hock, 1993). Solvents can also disrupt the hydrophobic binding forces between the antibody and analyte, which can result in a reduction in affinity and decrease in assay sensitivity (Carter and Friedman, 1992). The effect of different solvents such as acetone, acetonitrile, methanol, and propylene glycol on the response of the anti-DDAC 3 antiserum in a noncompetitive ELISA was determined (Table 5). The response of the antibodies decreased by 10% when solutions contained 4% acetone, 8% acetonitrile, 15% methanol, or 6% propylene glycol. Consequently, solvents were used only when necessary and only at concentrations low enough to have no significant effect on antibody response.

Effects of Surfactants on the Anti-DDAC 3 Antiserum. We were concerned that antibodies raised against surfactants such as DDAC may be susceptible to general surfactant effects rather than interacting specifically with the analyte. Surfactants can interact nonspecifically with proteins or may even denature the antibody's protein structure. Thus three surfactants, sodium

Table 5. Solvent Effect on the Response of the Anti-DDAC 3 Polyclonal Antibodies: Percent Residual Activity vs Solvent Concentration[a]

	solvent concentration							
solvent	0.5%	1%	2%	4%	6%	8%	10%	15%
acetone			96	91	84	72	56	31
acetonitrile			99	89	87	89	79	69
methanol		99	96	93	93	95	89	92
propylene glycol					94	85	85	74

[a] Numbers are reported when less than 100%.

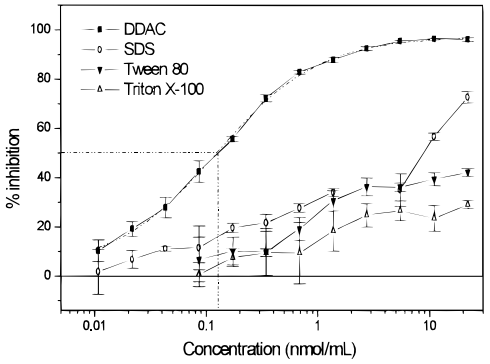


Figure 6. Inhibition of the anti-DDAC 3 antibodies with DDAC, Triton X-100, Tween 80, and SDS at equivalent molar concentrations and in quadruplicate. Error bars represent standard deviations.

Table 6. Precision and Reproducibility of the Standard Curve Working Parameters Using the Anti-DDAC 3 Antiserum

working parameter	mean (ng/mL)[a]	CV (%)
IC_{50} (ng/mL)	50.3 ± 8.3	16.4
slope	0.86 ± 0.11	13.0
IC_{20} (ng/mL)	9.2 ± 3.0	32.8
IC_{70} (ng/mL)	149.4 ± 27.5	18.4

[a] Uncertainties are ± standard deviations with means of n = 14.

dodecyl sulfate (SDS), Triton X-100, and Tween 80, were tested at molar concentrations equivalent to those used in DDAC inhibition curves for the anti-DDAC 3 antiserum. Compared to the inhibition curve for DDAC, these surfactants did not greatly affect the response of the antiserum and did not lead to sigmoidal response curves typical for compounds recognized by antibodies (Figure 6). As well, at the molar equivalent of the IC_{50} for DDAC (0.14 nmol/mL), SDS, Tween 80, and Triton X-100 affected the response of the antibodies by 13.6%, 8.9%, and 0% respectively. In particular, SDS, which most resembles DDAC, with a C_{12} carbon chain linked to a sulfate, did not associate significantly with the antibodies. Thus, the antiserum specifically recognized DDAC and not detergents in general.

Precision and Reproducibility. The CELISA for DDAC using the anti-DDAC 3 antiserum was precise and reproducible. Variation in the standard curve working parameters is shown in Table 6. The coefficients of variation (% CV) and thus interassay variation for all parameters ranged from 13% to 33% over 14 experiments. However, reproducibility of the IC_{50} and slope were good, with CVs of 16.4% and 13.0% respectively. This variation was within acceptable limits for the purposes of this work.

Recovery of DDAC from Spiked Samples. Working solutions of DDAC from mills were analyzed by CELISA and HPLC. The HPLC analysis was carried out by Forintek Canada while the immunoassays were done in our lab. The correlation between the two methods was modest. It appears that compounds present in the DDAC working solutions either originating from the wood or as part of the fungicide formulation interfered with the CELISA. Therefore, more research is needed to develop an appropriate sample pretreatment, such as a solid-phase extraction, to remove these interfering components from complex samples.

Table 7. Recoveries of Spiked Parts per Billion Concentrations of DDAC from Milk Using the Anti-DDAC 3 Antiserum: Percent Recovery by ELISA Based on Spiked Concentrations

DDAC spike (ng/mL)	DDAC measured by CELISA (ng/mL)	CV (%)	recovery by CELISA (%)
25	25.7 ± 6.5[a]	25.3	102.8
50	55.8 ± 11.6[a]	20.7	111.7
100	99.7 ± 0.115[b]	30.0	99.7

[a,b] Values expressed as ± standard deviation with means of (*a*) n = 12 and (*b*) n = 8.

Recoveries from laboratory samples, however, were well within acceptable parameters. Milk solutions (0.1%) were spiked with pure DDAC at concentrations of 25, 50, and 100 ng/mL (ppb). The CELISA included a DDAC standard curve and samples spiked near the IC_{50} to take advantage of the precision in this part of the standard curve (Stanker et al., 1996). Table 7 shows that the recovery of DDAC by CELISA ranged from 99.7% to 111.7%, with coefficients of variation ranging from 21% to 30%. These results indicate that analysis of DDAC in environmental samples by CELISA should be possible provided the samples are in the appropriate solution matrix.

CONCLUSIONS

Polyclonal antisera were successfully raised against three DDAC immunogens that were different from the immunogen used by Chen et al. (1995). The antisera recognized DDAC to different degrees, with the anti-DDAC 3 antiserum showing the greatest sensitivity, with an IC_{50} of 50 ppb. Of the three haptens synthesized, the one that most closely mimicked DDAC produced the most sensitive antiserum to DDAC. The differences in selectivities for DDAC and other compounds observed between the three antisera demonstrates the importance of hapten design in producing selective antibodies. The anti-DDAC 3 antiserum was not selective for long-chain fatty acids and alcohols, which was a marked improvement over the previous antiserum (Chen et al., 1995). The anti-DDAC 3 antiserum could be used to monitor DDAC in stormwater since the sensitivity of the assay is well below the discharge limit of 700 ppb. These antibodies show great promise for further development into an immunoassay kit.

ABBREVIATIONS USED

BSA, bovine serum albumin; CELISA, competitive enzyme-linked immunosorbent assay; CR, % cross-reactivity; CV, coefficient of variation; DDAC, didecyldimethylammonium chloride; ELISA, enzyme-linked immunosorbent assay; IC_{50}, analyte concentration causing 50% inhibition of antibody binding; IC_{20}, 20% antibody inhibition concentration, representing the upper ELISA limit; IC_{70}, 70% antibody inhibition concen-

tration, representing the lower ELISA limit; OD, optical density; QAC, quaternary ammonium chloride; RSA, rabbit serum albumin; SD, standard deviation; PBS, phosphate-buffered saline.

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