

# Development of a Selective Enzyme-Linked Immunosorbent Assay for 1-Naphthol—the Major Metabolite of Carbaryl (1-Naphthyl N-Methylcarbamate)

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A selective enzyme-linked immunosorbent assay for 1-naphthol was developed. Eight rabbits were immunized with either [(5-hydroxy-2-naphthyl)oxy]acetic acid (2b)-BSA, [(5-hydroxy-1-naphthyl)oxy]acetic acid (4)-BSA, or 5-hydroxy-2-naphthoic acid (7)-KLH, and all sera were screened against 14 different coating antigens. From this screening procedure, five assays could be developed, using two 7-KLH antisera, 3907 and 3909. The assay with the least interference and the best reliability (7-KLH antiserum 3907, 1:9000, and coating antigen 2b-BSA, 1.5  $\mu$ g/mL) was further optimized and characterized. It showed an  $I_{50}$  value of about 72  $\mu$ g/L, with a range of detection from 10 to 1000  $\mu$ g/L. There was no cross-reactivity with carbaryl, which makes this assay very suitable for the selective detection of 1-naphthol, for example, in undiluted human urine and in soil extract. The assay was tested in organic solvents and showed a tolerance of at least 10% solvent (acetone, acetonitrile, methanol).

## INTRODUCTION

1-Naphthol (MW 144.17) is the major hydrolysis product of carbaryl (SEVIN; 1-naphthyl *N*-methylcarbamate). Carbaryl is a broad-spectrum insecticide, which is widely applied on fruits, food crops, forests, and livestock against a wide variety of insects (over 100 species, Extension Toxicology Network, 1989). After it was invented in 1953, it became a great success, and by the year 1971 there was an annual production of 55 million pounds in the United States alone (Marshall, 1985). Carbaryl is a cholinesterase inhibitor. It is commonly judged to have moderate to low mammalian toxicity, because carbamylated esterases reactivate with a very short half-time of the inhibitor-enzyme complex (Hayes, 1982; Kaloyanova and El Batawi, 1991). On the other hand, more recent data show that, for example, in green algae and cyanobacteria a combination of carbaryl and 1-naphthol can show more toxicological effects than either substance alone (Megharaj et al., 1990).

Different methods are applied for the analysis of carbaryl and 1-naphthol, with high-pressure liquid chromatography (HPLC), gas chromatography (GC), spectrophotometry, and spectrofluorometry being the most common (Shafik et al., 1971; Nagasawa et al., 1977; DeBerardinis and Wargin, 1982; Hargreaves and Melksham, 1983; Nakamoto and Page, 1986; Garcia Sánchez and Cruces Blanco, 1987; Sastry et al., 1987; Ward et al., 1987; Yanez-Sedeno et al., 1988; Sancenón et al., 1989; Strait et al., 1991). In general, these methods are quite time-consuming and expensive, and sometimes derivatization is required. For the determination by GC, for example, Nagasawa et al. (1977) used the heptafluorobutyryl derivatives of carbaryl and 1-naphthol. The monitoring of large sample numbers for exposure studies of workers and for environmental and food control, though, asks for more cost-effective screening methods with the least amount of sample pretreatment. Immunochemical methods offer such a good screening method,

because they are very sensitive, often need little or no sample cleanup or preparation, and can be applied to numerous samples simultaneously (Hammock and Mumma, 1980; Cheung et al., 1988; Haberer and Krämer, 1988; Jung et al., 1989; Hammock et al., 1990). Because of the metabolic and environmental instability of carbaryl, an analytical method for carbaryl should incorporate the determination of 1-naphthol. After the development of a sensitive immunoassay against carbaryl, which does not interfere with 1-naphthol (Marco et al., 1993a), the need for an assay for 1-naphthol became apparent. The synthetic strategy, the subsequent development, and the main characteristics of the assay for 1-naphthol are shown in this paper.

## MATERIALS AND METHODS

**Chemicals.** Carbaryl and naphthaleneacetamide were purchased from Chem Service (Media, PA). Carbonates 12, 13, and 15, thiocarbonate 14, and ethylcarbamate 16 derivative, used for cross-reactivity studies, were synthesized and described by Huang et al. (1993). The synthesis of compounds 26 [*N*-(1-naphthoyl)-6-aminohexanoic acid], 27 [*N*-(2-naphthoyl)-6-aminohexanoic acid], 28 [*N*-(2-naphthylsulfonyl)-6-aminohexanoic acid], 29 [*N*-(1-naphthylacetyl)-6-aminohexanoic acid], and 30 [1-(5-carboxypentyl)-3-(1-naphthyl)urea], used during screening of coating antigens, is described by Marco et al. (1993a). Immunochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were from Aldrich Chemical Co. (Milwaukee, WI). Analytical stock solutions were prepared in either methanol, acetone, dimethyl sulfoxide, or dimethylformamide (DMF) and stored at -20 °C.

**Instruments.** Thin-layer chromatography (TLC) was performed on 0.25-mm precoated silica gel 60 F254 aluminum sheets from Merck (Gibbstown, NJ). <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectra were obtained with a QE-300 spectrometer (General Electric, 300 MHz for <sup>1</sup>H and 75 MHz for <sup>13</sup>C nuclei). Chemical shifts ( $\delta$ ) are given relative to tetramethylsilane (TMS) as an internal reference. Electron impact mass spectra (EIMS) were recorded on a Trio-2 (VG Masslab, Altrincham, U.K.) apparatus at 70 eV, and data are reported as *m/z* (relative intensity). A Hewlett-Packard 5890 gas chromatograph equipped with 15 m × 0.25 mm (i.d.) DB-5 column was interfaced to this mass spectrometer for GC-MS analyses. Fast atom bombardment mass spectra (FAB-MS) were obtained on a ZAB-HS-2F instrument (VG Analytical, Wythenshawe, U.K.). Melting points were determined with a Hoover capillary melting point apparatus (A. H. Thomas Co., PA) and are uncorrected. UV spectra were

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obtained with an UV-vis spectrophotometer 8450 A (Hewlett-Packard) and recorded with a Hewlett-Packard 7225 B plotter. Clear sera were obtained by centrifugation either in a microcentrifuge (Eppendorf 5415C) for small volumes ( $\leq 1\text{ mL}$ ) or in a Beckman centrifuge (GPR or J2-21M/E) for larger volumes (2–10 mL). Soil was extracted in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific Co., Inc., Edison, NJ). Polystyrene microtiter plates were purchased from Nunc (Maxisorb, Roskilde, Denmark). The absorbances were read with a  $V_{\text{max}}$  microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450 or 405–650 nm). The inhibition curves were analyzed with a commercial software package (Softmax, Molecular Devices) using a four-parameter logistic equation. Unless otherwise mentioned, all data presented from ELISA experiments correspond to the average of at least three well replicates on one plate.

**Hapten Synthesis.** Compounds 2a, 2b, 4, and 7 were synthesized as follows and were used for the preparation of immunogens or coating antigens.

*[(6-Hydroxy-1-naphthyl)oxy]acetic Acid Methyl Ester (1a) and [(5-Hydroxy-2-naphthyl)oxy]acetic Acid Methyl Ester (1b).* Methyl bromoacetate (1.91 g, 12.5 mmol) was added dropwise to a solution of 1,6-dihydroxynaphthalene (2.00 g, 12.5 mmol) in anhydrous acetone (100 mL) containing  $\text{K}_2\text{CO}_3$  (4.15 g, 30.0 mmol, 1.2 equiv). After refluxing for 4 h [TLC analysis  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (98/2)], the reaction mixture was poured into 1 N HCl (40 mL) and extracted with ethyl ether ( $3 \times 60\text{ mL}$ ). The combined organic fractions were washed with saturated NaCl solution ( $2 \times 60\text{ mL}$ ), dried with  $\text{MgSO}_4$ , filtered, and evaporated to give 2.5 g of a mixture that was repeatedly chromatographed in a silica gel column [ $\text{CHCl}_3$  to  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (98/2)] to isolate three pure compounds in this order. *[(6-[(Methoxycarbonyl)-methoxy]-1-naphthyl)oxy]acetic acid methyl ester (1c)* (1.2 g, 32%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.8 (s, 6H,  $\text{OCH}_3$ ), 4.72 (s, 2H,  $\text{CH}_2\text{COOCH}_3$ ), 4.77 (s, 2H,  $\text{CH}_2\text{COOCH}_3$ ), 6.57 (dd,  $J = 9.0$  and 1.7 Hz, 1H, H-2), 7.02 (d,  $J = 1.2$  Hz, 1H, H-5), 7.22–7.31 (cs, 3H, H-3, H-4, H-7), 8.27 (d,  $J = 8.6$  Hz, 1H, H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  51.41, 65.1, 65.4, 103.5, 106.0, 107.5, 118.4, 121.2, 123.1, 125.2, 127.2, 135.6, 153.7, 156.3, 169.1, 169.2; EIMS ( $m/z$ ) 304, 273, 231, 203, 180. *1b* (430 mg, 16%);  $^1\text{H}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  3.8 (s, 3H,  $\text{OCH}_3$ ), 4.72 (s, 2H,  $\text{CH}_2\text{COOCH}_3$ ), 6.70 (dd,  $J = 8.5$  and 1.8 Hz, 1H, H-2), 7.01 (d,  $J = 1.8$  Hz, 1H, H-5), 7.15–7.23 (cs, 3H, H-3, H-4, H-7), 8.15 (d,  $J = 9.2$  Hz, 1H, H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  52.2, 65.2, 106.8, 116.9, 118.5, 124.1, 126.9, 135.7, 152.6, 155.9, 169.6; FAB-MS ( $m/z$ ) 232, 154, 136. *1a* (580 mg, 20%); [structural differentiation from *1b* was accomplished by X-ray crystallographic analysis of the corresponding carboxylic acid (see below)]  $^1\text{H}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  3.78 (s, 3H,  $\text{OCH}_3$ ), 4.77 (s, 2H,  $\text{CH}_2\text{COOCH}_3$ ), 6.47 (dd,  $J = 9.0$  and 1.9 Hz, 1H, H-2), 7.12 (cs, 2H, H-5, H-4), 7.22 (cs, 2H, H-3, H-7), 8.17 (dd,  $J = 8.8$  and 1.9 Hz, 1H, H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  51.5, 64.7, 101.8, 108.4, 117.4, 119.3, 122.9, 123.3, 125.5, 135.7, 153.2, 155.4, 168.7; FAB-MS ( $m/z$ ) 232, 167, 155, 138.

*[(6-Hydroxy-1-naphthyl)oxy]acetic Acid (2a).* A mixture of *1a* (580 mg, 2.5 mmol) and 0.5 N NaOH (15 mL, 1.5 mmol, 3 equiv) in THF (20 mL) was stirred overnight at room temperature (RT). The day after, the reaction mixture was poured over 1 N HCl (10 mL), extracted with ethyl ether, dried over  $\text{MgSO}_4$ , filtered, and evaporated to obtain *2a* as brown solid (510 mg, 92%) which was crystallized from  $\text{CHCl}_3-\text{CH}_3\text{OH}$ . The crystals were used for X-ray structural analysis: compound *2a* crystallized in the monoclinic space group  $P2_1/n$ . The crystal data at 130 K are as follows:  $a = 10.055(3)$  Å,  $b = 11.264(3)$  Å,  $c = 12.831(4)$  Å,  $\beta = 102.96(3)$ °;  $d_{\text{calc}}$  1.38 mg/m<sup>3</sup>;  $Z = 4$ ; Siemens R3m/V diffractometer; Mo K $\alpha$  radiation;  $\lambda = 0.717073$  Å; graphite monochromator,  $2\theta$  (max) = 50°; 2012 observed reflections with  $F > 4.0\sigma$  ( $F'$ ) and 181 parameters used in refinement; crystallographic program SHELLXTL PLUS (version 4.2).  $^1\text{H}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  4.64 (s, 2H,  $\text{CH}_2\text{COOH}$ ), 6.42 (dd,  $J = 8.8$  and 1.2 Hz, 1H, H-2), 7.00 (s, 1H, H-5), 7.02–7.13 (cs, 3H, H-3, H-7, H-4), 8.06 (d,  $J = 9.0$  Hz, 1H, H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  63.8, 101.0, 107.8, 116.6, 118.2, 122.3, 122.8, 125.1, 135.0, 152.5, 154.7, 169.4; FAB-MS ( $m/z$ ) 218, 173, 149.

*[(5-Hydroxy-2-naphthyl)oxy]acetic Acid (2b).* As described in the preceding paragraph, from the methyl ester *1b* (480 mg, 2 mmol) the carboxylic acid *2b* was obtained as a brown

oil (370 mg, 82%);  $^1\text{H}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  4.7 (s, 2H,  $\text{CH}_2\text{COOH}$ ), 6.74 (dd,  $J = 8.5$  and 1.9 Hz, 1H, H-2), 7.02 (d,  $J = 1.8$  Hz, 1H, H-5), 7.14–7.22 (cs, 3H, H-3, H-4, H-7), 8.14 (d,  $J = 8.6$  Hz, 1H, H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  64.3, 106.2, 116.4, 117.7, 120.0, 123.5, 123.9, 126.6, 135.2, 152.5, 155.5, 170.6; FAB-MS ( $m/z$ ) 218, 167, 149.

*[(5-Hydroxy-1-naphthyl)oxy]acetic Acid Methyl Ester (3a).* Similarly, as described before for the preparation of *1a* and *1b*, 570 mg of a mixture of *3a* and *[(5-[(methoxycarbonyl)-methoxy]-1-naphthyl)oxy]acetic acid methyl ester (3b)* was obtained by reacting 1,5-dihydroxynaphthalene (400 mg, 2.5 mmol) with methyl bromoacetate (382.4 mg, 2.5 mmol) in anhydrous acetone (60 mL) containing  $\text{K}_2\text{CO}_3$  (829 mg, 6 mmol, 1.2 equiv). Separation of *3a* as a brown oil (220 mg, 38%) from the dialkylated *3b* as a beige solid (350 mg, 46%) was accomplished by chromatography of the mixture in a silica gel column [50 g, eluent  $\text{CHCl}_3$  to  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (98/2)]. *3a*:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.83 (s, 3H,  $\text{OCH}_3$ ), 4.81 (s, 2H,  $\text{CH}_2\text{COOCH}_3$ ), 6.72 (d,  $J = 8.6$  Hz, 1H, H-6), 6.84 (d,  $J = 8.7$  Hz, 1H, H-2), 7.18–7.36 (cs, 2H, H-3 and H-7), 7.79 (d,  $J = 8.7$  Hz, 1H, H-4 or H-8), 7.90 (d,  $J = 8.5$  Hz, 1H, H-4 or H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{CD}_3\text{OD}$ )  $\delta$  52.2, 65.4, 105.2, 108.7, 112.9, 115.6, 123.9, 124.2, 125.4, 152.4, 153.2, 169.6; FAB-MS ( $m/z$ ) 232, 113. *3b*:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.83 (s, 6H,  $\text{OCH}_3$ ), 4.82 (s, 4H,  $\text{CH}_2\text{COOCH}_3$ ), 6.75 (d,  $J = 9.2$  Hz, 2H, H-2 and H-6), 7.37 (t,  $J = 8.9$  Hz, 2H, H-3 and H-7), 7.98 (d,  $J = 9.1$  Hz, H-4 and H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{CD}_3\text{OD}$ )  $\delta$  52.9, 65.4, 114.6, 116.1, 124.2, 125.8, 153.1, 169.4; EIMS ( $m/z$ ) 304, 256, 216.

*[(5-Hydroxy-1-naphthyl)oxy]acetic Acid (4).* As described before for the preparation of *2a*, from the methyl ester *3a* (220 mg, 0.9 mmol) the carboxylic acid *4* was obtained as brown solid (190 mg, 92%);  $^1\text{H}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  4.68 (s, 2H,  $\text{CH}_2\text{COOH}$ ), 6.60 (d,  $J = 8.2$  Hz, 1H, H-2), 6.79 (d,  $J = 8.4$  Hz, 1H, H-6), 7.16 (t,  $J = 8.0$  Hz, 2H, H-3 and H-7), 7.77 (d,  $J = 8.1$  Hz, 2H, H-4 and H-8);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{DMSO}-d_6$ )  $\delta$  66.2, 104.92, 108.6, 112.1, 113.6, 115.6, 124.6, 125.4, 126.2, 152.0, 152.8, 170.6; FAB-MS ( $m/z$ ) 218, 173, 160, 131.

*5-Hydroxy-2-naphthoic Acid (7).* The dibromoester *5* (Marco et al., 1993b) (100 mg, 0.26 mmol) in anhydrous THF (4 mL) was added, under  $\text{N}_2$  atmosphere, to a suspension of  $\text{NaOCH}_3$  (210 mg, 3.9 mmol) in the same solvent (1 mL) and stirred overnight at 4 °C. The day after, the reaction mixture was added to an aqueous solution of  $\text{H}_2\text{SO}_4$  (5%), stirred for 30 min at RT, and extracted with ethyl acetate ( $3 \times 30$  mL). The joined organic fractions were washed with saturated NaCl solution, dried over  $\text{MgSO}_4$ , filtered, and evaporated to dryness to obtain *7* as a brown solid (35 mg, 74%) after purification by preparative TLC [ $\text{CHCl}_3-\text{CH}_3\text{OH}$  (95/5)]; mp 162–165 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3-\text{CD}_3\text{OD}$ )  $\delta$  6.95 (dd,  $J = 8.2$  and 0.9 Hz, 1H, H-6), 7.36 (t,  $J = 7.8$  Hz, 1H, H-7), 7.49 (d,  $J = 6.8$  Hz, 1H, H-4), 8.04 (dd,  $J = 8.2$  and 0.6 Hz, 1H, H-8), 8.28 (d,  $J = 9$  Hz, 1H, H-3), 8.59 (s, 1H, H-1);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3-\text{CD}_3\text{OD}$ )  $\delta$  110.5, 120.8, 122.5, 124.2, 126.8, 126.9, 130.9, 131.3, 133.7, 152.5, 169.7; FAB-S ( $m/z$ ) 188.

**Preparation of Hapten-Protein Conjugates.** Haptens *2a*, *2b*, and *4* were conjugated to bovine serum albumin (BSA), conalbumin (CONA), ovalbumin (OVA), and keyhole limpet hemocyanin (KLH). Hapten *7* was conjugated to KLH only. Compounds *22–25* and 1- and 2-naphthyl acetic acid were conjugated to CONA and OVA. All haptens were conjugated via the mixed anhydride method [Rajkowska et al., 1977, modified after Erlanger et al. (1957, 1959)]. Here, the carboxylic acid haptens (0.08 mmol) were dissolved in anhydrous dimethylformamide, and tri-*n*-butylamine (0.0968 mmol) was added followed by isobutyl isochloroformate (0.088 mmol). The mixture was stirred for 30 min at RT and then added slowly to the corresponding protein solution (50 mg) in 6 mL of borate buffer (0.2 M borate–boric, pH 8.7). This was stirred overnight at 4 °C. The following day the solutions were transferred into dialysis bags (Spectra/Por, MW cutoff 12 000–14 000, Spectrum Medical Industries, TN) and dialyzed extensively against 0.01 M phosphate-buffered saline (pH 7.5), with a last exchange in water, lyophilized, and stored dry at –70 °C. Stock solutions of 1 mg/mL were prepared in 0.5 M carbonate buffer, pH 9.6, and stored at 4 or –20 °C.

**Determination of Hapten Density of Conjugates.** The conjugation of the most important hapten–protein conjugates used for this study was proved spectrophotometrically by

Table 1. Test of Coating Antigens and Screening for Inhibition for 1-Naphthol

		recognition/inhibition <sup>a</sup> for coating antigen											
immunogen serum	2b-CONA	2a-CONA	4-CONA	22-CONA	23-CONA	CONA	24-CONA	25-CONA	26-OVA	27-CONA	28-BSA	29-CONA	30-CONA
2b-BSA	3897	++/-	++/-	++/+-	++/+-	++	++	++	++	++	++	++	++
	3898	+++/--	++/--	++/+-	++/+-	++	++	++	++	++	++	++	++
4-BSA	3904	++/-	++/-	++/+-	++/+-	++	++	++	++	++	++	++	++
	3905	++/--	++/--	++/+-	++/+-	++	++	++	++	++	++	++	++
	3906	++/--	++/--	++/+-	++/+-	+	+	+	+	+	+	+	+
7-KLH	3907	++++/●●●	++/●	(++++/●●●) <sup>b</sup>	++/●	+++/nd <sup>c</sup>	+++/--	+++/rd	++	++/	++/	++/	++/
	3908	++++/●●●	++/●	+++/--	+++/--	+++/nd <sup>c</sup>	+++/--	+++/nd	++	++/	++/	++/	++/
	3909	++/●●●	(+/●●●) <sup>b</sup>	++/●●●	++/●●●	++/●●●	++/●●●	++/●●●	++/●●●	++/●●●	++/●●●	++/●●●	++/●●●

<sup>a</sup> Recognition—optical density (OD) values were obtained with antiserum 1:500 and coating antigen 10 µg/mL: ≤0.5 OD, +; 0.5–1.5 OD, ++; ≥1.5 OD, +++; underlined, on the higher range of the category (up to 3.5 OD). Inhibition—tests were carried out with optimized assay conditions:  $I_{50} \geq 1000 \mu\text{g/L}$ , ●; 100–1000 µg/L, ●●; ≤100 µg/L, ●●●; no inhibition, -; 26, N-(1-Naphthoyl)-6-aminohexanoic acid; 27, N-(2-naphthoyl)-6-aminoheptanoic acid; 28, N-(2-naphthylsulfonyl)-6-aminohexanoic acid; 29, N-(1-naphthylacetyl)-6-aminoheptanoic acid; 30, 1-(5-carboxypentyl)-3-(1-naphthyl)urea (Marco et al., 1993a). <sup>b</sup> Inhibition was in the lower ppb range, but standard curves had a very high background and/or the assay was not reliable, therefore no further testing was done. <sup>c</sup> nd, not determined. <sup>d</sup> Immunogen for these antisera was a BSA conjugate.

measuring absorbance of haptens and haptens–protein conjugates at wavelengths where the protein did not absorb. Haptens (10 µg/mL) were diluted in 0.5 M carbonate buffer, pH 9.6 (1% methanol), and haptens–protein conjugates (100 µg/mL) and proteins in the same buffer, respectively. The haptens density was determined via Lambert Beer's law, assuming that  $\epsilon$  for the unconjugated and  $\epsilon$  for the conjugated haptens are the same.

**Immunization of Rabbits.** Rabbits (female New Zealand, white, 3–5 kg) 3897 and 3898 were immunized with 2b-BSA, rabbits 3904, 3905, and 3906 were immunized with 4-BSA, and rabbits 3907, 3908, and 3909 were immunized with 7-KLH. The immunization was done as follows. Rabbits were injected intradermally (5–20 sites per animal) with 100 µg of the corresponding haptens–protein conjugate. For the initial injection, the corresponding haptens–protein conjugates (immunogens) were dissolved in 1.7% NaCl (w/v) and mixed 1:2 with Freund's complete adjuvants (Sigma) to a final volume of 900–1000 µL/injection. For all boost immunizations the immunogens were prepared in the same way but with Freund's incomplete adjuvants (Sigma). The boosting was started 1 month after the initial immunization and was repeated every 4 weeks (6 times); animals were bled 10 days after each boost (2–5 mL) from the marginal ear vein under light anesthesia. Ten days after the sixth boost the rabbits were exsanguinated under deep anesthesia by heart puncture. All procedures, except the preparation of the immunogens, were carried out by LAHC (Laboratory Animal Health Clinic, UCD) personnel. The blood sat in tubes overnight at 4 °C to allow it to clot. After removal of the clot and centrifugation of the serum (1000 rpm, 10 min), the clear serum was carefully drawn out with a Pasteur pipet, aliquotted, and stored in cryovials at -70 °C.

**Screening of Antisera with Two-Dimensional Titration.** For the determination of the initial titers, for each serum one microtiter plate (Nunc) was divided into three equal parts (one part: 4 columns, 8 rows) and coated with 100 µL/well of three different coating antigens (2a-, 2b-, and 4-CONA), respectively, at 8 concentrations (0.08–10 µg/mL) in 0.5 M carbonate-bicarbonate buffer (pH 9.6). Plates were sealed with adhesive plate sealers and incubated overnight at 4 °C. The following day, the coated plates were washed [10 times, 0.2 M phosphate-buffered saline with 0.5% Tween 20 (PBST)] and 100 µL/well of diluted serum was incubated for 2 h at RT. The sera were checked in a two-dimensional titration with four different dilutions (4 columns: 1:500, 1:2000, 1:4000, and 1:10000 in 0.2 M PBST, pH 7.5) against the coating antigens. After another washing step, 100 µL/well of goat anti-rabbit IgG, conjugated with alkaline phosphatase (Sigma), diluted 1:4000 in 0.2 M PBST, pH 7.5, was added and incubated for 2 h at RT. Then plates were washed again, and the substrate *p*-nitrophenyl phosphate (tablets, 1 mg/mL, Sigma) for the enzyme reaction was added (100 µL/well) in 10% diethanolamine buffer, pH 9.8. After incubation for 30 min at RT, the absorbance values were read at 405/650 nm, and the data were collected with the PC-EIA program (Softmax).

For more extensive screening of additional coating antigens (see Table 1) all sera were checked again in two-dimensional titrations.

**ELISA—Coating Antigen Format. Competitive Inhibition.** To shorten incubation times, the ELISA for the competitive inhibition was slightly varied as follows. Microtiter plates were coated with the optimized concentration (1–2 µg/mL; 100 µL/well) of the corresponding haptens–protein conjugate overnight at 4 °C. The next day, plates were washed (10 times, 0.2 M PBST, pH 7.5), and the analyte (0.001–100,000 µg/L in 0.2 M phosphate-buffered saline (PBS), pH 7.5; 50 µL/well) and the antiserum (optimized dilution from 1:4000 to 1:12000, depending upon the assay, in 0.2 M PBST, pH 7.5) were added and incubated for 35 min at RT. After another washing step, 100 µL/well HRP-labeled goat anti-rabbit IgG was added (1:10000 in 0.2 M PBST, pH 7.5; Sigma) and incubated for 90 min at RT. Plates were washed again, 100 µL of substrate/well was added (tetramethylbenzidine (TMB)/1% H<sub>2</sub>O<sub>2</sub>; 6 mg of TMB/mL DMSO; 400 µL of TMB, and 100 µL of 1% H<sub>2</sub>O<sub>2</sub> in 25 mL of 0.1 M sodium acetate, pH 5.5), and, after 10–20 min, the enzyme reaction was stopped with 50 µL/well 4 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 450/650 nm. With this assay format the color development

is inversely proportional to the amount of analyte. For better comparison of standard curves, the absorbance values ( $A$ ) were converted to percent control values, according to the formula

$$\% \text{ control} = \frac{A - A_{\text{background}}}{A_{\text{max}} - A_{\text{background}}} \times 100$$

where  $A_{\text{background}}$  is the absorbance without addition of antiserum and  $A_{\text{max}}$  is the absorbance without analyte.

**pH Effect.** The influence of pH on the assay was studied with 0.2 M phosphate buffer ( $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  in various proportions) in the pH range from pH 5 to pH 8 in steps of 0.5 pH units (for example, for pH 7, 41 mL of 0.2 M  $\text{KH}_2\text{PO}_4$ –59 mL of 0.2 M  $\text{Na}_2\text{HPO}_4$ ). For this study, standards were determined in quadruplicate, set up either in 0.2 M phosphate buffer at one pH or in the usual buffer (0.2 M PBS, pH 7.5).

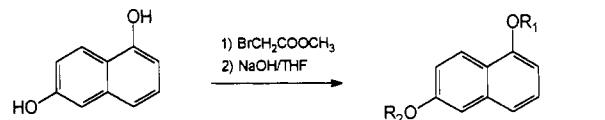
**Solvent Tolerance.** The most commonly used solvents for conventional LC methods were tested in the assay: acetone, acetonitrile, and methanol (0–50% or 100%, respectively, in 0.2 M PBS). Standards of 1-naphthol in solvent were determined in triplicate and compared to standards in 0.2 M PBS (duplicate determination) on the same plate. Diluted antiserum (50  $\mu\text{L}$ /well) was added in 0.2 M PBST, pH 7.5. The assay was performed as described under ELISA Coating Antigen Format. Competitive Inhibition.

**Matrix Effects in Human Urine and in Soil Extract.** For the effect of different matrices in the assay, the standard curves were run in one spiked human urine sample of different dilutions (0%, 1%, 2%, 5%, 10%, 25%, 50%, and 100%, diluted in water). The antiserum was added in 0.4 M PBST, pH 7.5, and the assay was performed as described formerly for the competitive inhibition format.

Yolo silt loam (fine-silty, mixed, nonacid thermic, typic xerorthent, 1.3% organic carbon) was used as one example to investigate the possible matrix effect of soil. Two grams of soil in 100 mL of a chloroform–methanol mixture (90:10) was shaken for 20 h at RT. The extract was filtered through Whatman filter paper No. 1 and then evaporated. The residue was redissolved in 200  $\mu\text{L}$  of methanol and diluted with 0.2 M PBS to a final volume of 2 mL. This solution was spiked with 1-naphthol (100 000  $\mu\text{g}/\text{L}$ , serially diluted to 0.001  $\mu\text{g}/\text{L}$ ) and compared to the standard curve in 0.2 M PBS. The assay was performed as described earlier.

## RESULTS AND DISCUSSION

**Hapten Synthesis.** Since hapten antibody recognition is higher towards the portion of the hapten molecule which is furthest from the carrier protein, the selected synthetic strategy was directed toward the introduction of a carboxylic group on the opposite side of the hydroxyl group on the 1-naphthol molecule. We introduced a carboxylic acid terminated handle by formation of an ether prepared from the phenoxylate anion, generated with potassium carbonate, and methyl bromoacetate (see Figure 1). When 1,6-diol was the starting material, a mixture of the dialkylated compound 1c and the two monoalkylated compounds 1a and 1b was obtained. For 1,5-diol, only two compounds are possible due to the symmetry of the molecule, the monoalkylated 3a and the dialkylated 3b. Unfortunately, dialkylated compounds 1c and 3b were the main reaction products for 1,6- and 1,5-diol, respectively. Repeated purification by silica gel column chromatography resulted in the isolation of the methyl esters 1a, 1b, and 3a. Subsequently, the methyl esters were hydrolyzed with aqueous NaOH to give the corresponding carboxylic acids 2a, 2b, and 4. Structural differentiation of positional isomers 2a and 2b was accomplished by spectroscopic data and X-ray structural analysis of the crystals obtained for 2b. Carboxylic acid derivatives 2b and 4 were meant to produce antibodies recognizing preferentially 1-naphthol, whereas 2a was designed as a possible coating antigen.



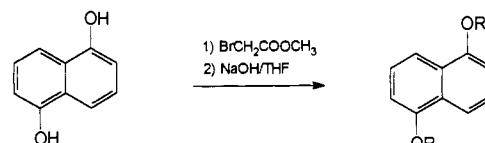
1a  $\text{R}_1=\text{CH}_2\text{COOCH}_3$ ;  $\text{R}_2=\text{H}$

1b  $\text{R}_1=\text{H}$ ;  $\text{R}_2=\text{CH}_2\text{COOCH}_3$

1c  $\text{R}_1=\text{R}_2=\text{CH}_2\text{COOCH}_3$

2a  $\text{R}_1=\text{CH}_2\text{COOH}$ ;  $\text{R}_2=\text{H}$

2b  $\text{R}_1=\text{H}$ ;  $\text{R}_2=\text{CH}_2\text{COOH}$



3a  $\text{R}_1=\text{CH}_2\text{COOCH}_3$ ;  $\text{R}_2=\text{H}$

3b  $\text{R}_1=\text{R}_2=\text{CH}_2\text{COOCH}_3$

4  $\text{R}_1=\text{CH}_2\text{COOH}$ ;  $\text{R}_2=\text{H}$

Figure 1. Synthesis of haptens used for immunogens and for coating antigens.

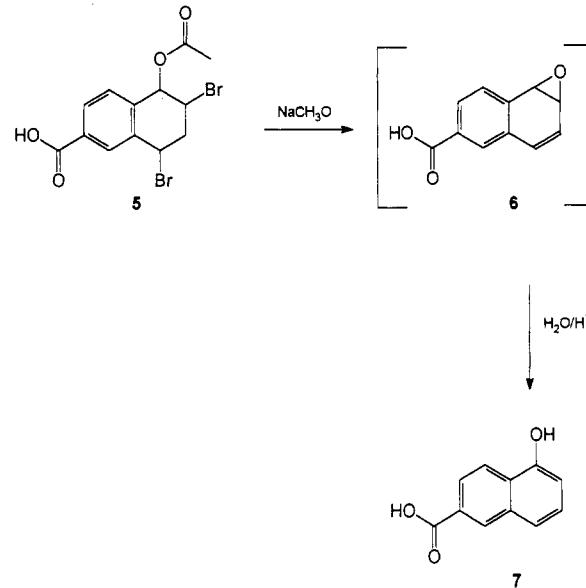
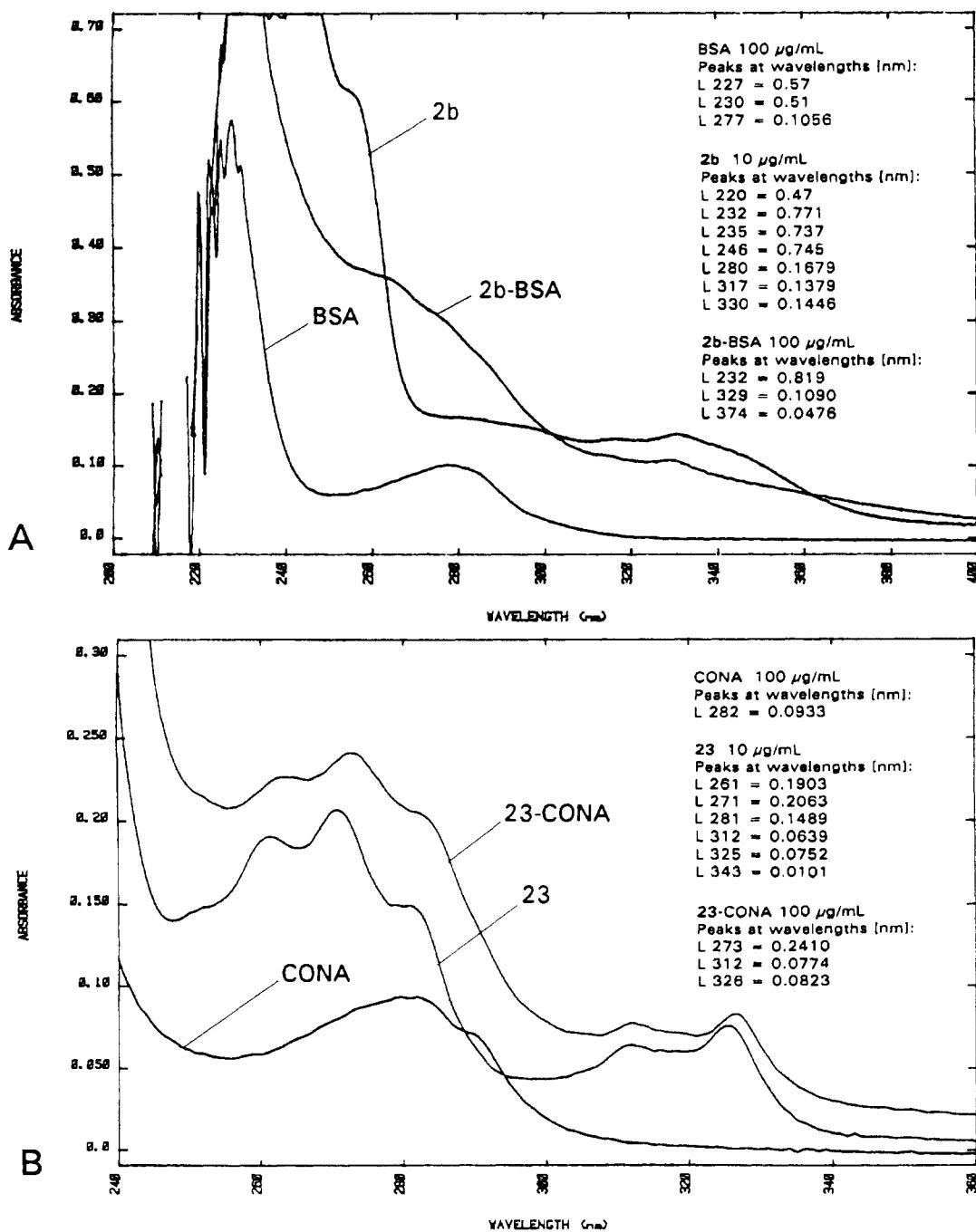


Figure 2. Synthesis of hapten for conjugation to KLH and for subsequent use as an immunogen.

In addition, the hapten 5-hydroxy-2-naphthoic acid (7) was considered to be an interesting hapten for immunization, because of the short “handle”. As starting material 5-acetyl-6-bromo-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid (5) was used (Figure 2), because this compound was available from a previous study (Marco et al., 1993b).

**Conjugation of Haptens to Proteins.** Compounds 2b, 4, and 7 were used to raise antibodies against 1-naphthol after covalent coupling to carrier proteins. 2b- and 4-BSA-, and 7-KLH-protein conjugates were used for the immunization protocol. Additionally, CONA and OVA conjugates were prepared with these haptens for subsequent use as coating antigens. Covalent coupling to the amino groups of the protein was accomplished by formation of a mixed anhydride. Competition of the free naphthoxylate generated on the basic conditions with the amino groups of the lysine residues of the protein could be produced during the coupling reaction. Higher  $\text{pK}_a$  values



**Figure 3.** Confirmation of the conjugation via UV spectroscopy with the example of two conjugates used in the assay development for 1-naphthol. Haptens were used in a concentration of 10 µg/mL, whereas the hapten-protein conjugate and the protein concentrations were 100 µg/mL, respectively. (A) 2b-BSA; absorbance at 329/330 nm was used to determine the hapten density. (B) 23-CONA; absorbance at 325/326 nm was used to determine hapten density.

**Table 2. Different Combinations and Standard Curve Characteristics for Different Assays for 1-Naphthol**

antiserum	dilution	coating antigen	N <sup>a</sup>	$I_{50}$ (C) (µg/L)	SD (µg/L)	A	B	D	corr coeff
3909	1:4000	23-CONA, 2 µg/mL	6	288	144	$0.323 \pm 0.028$	$0.478 \pm 0.079$	$0.0274 \pm 0.013$	$0.982 \pm 0.008$
	1:4000	22-CONA, 1 µg/mL	6	96.7	12.5	$0.285 \pm 0.016$	$1.017 \pm 0.120$	$0.0320 \pm 0.006$	$0.969 \pm 0.022$
	1:5000	4-BSA, 1.5 µg/mL	5	46.3	16.3	$0.197 \pm 0.033$	$0.723 \pm 0.343$	$0.0110 \pm 0.023$	$0.917 \pm 0.076$
3907	1:12000	23-CONA, 2 µg/mL	11	53.8	27.5	$0.185 \pm 0.041$	$1.037 \pm 0.333$	$0.0483 \pm 0.019$	$0.886 \pm 0.104$
	1:9000	2b-BSA, 1.5 µg/mL	6	66.9	5.0	$0.256 \pm 0.018$	$0.910 \pm 0.141$	$0.0103 \pm 0.008$	$0.979 \pm 0.019$

<sup>a</sup> N, number of plates that were taken for the determination of the average and standard deviation values. On each plate the standard curve was run with standards from 0.001 to 100 000 µg/L; each standard was run at least in duplicates. Maximal absorbance (A), slope (B),  $I_{50}$  (C), minimal absorbance (D), and correlation coefficient (corr coeff) are values from the four-parameter curve fit given by the equation  $y = (A - D)/[1 + (x/C)^B] + D$  (SOFTmax).

indicate weak acid and stronger base conjugates. Since stronger bases are better nucleophiles, a certain preference for the reactivity of the lysine residues vs the phenolic

moieties can be predicted according to the  $pK_a$  values ( $pK_a$  1-naphthol = 9.35,  $pK_a$  2-naphthol = 9.51,  $pK_a$  lysine = 10.53).

Other coating antigens with different handle structure and position such as compound **2a**, 1- (22) and 2-naphthoxyacetic acid (23), 1- and 2-naphthylacetic acid, and 1- (24) and 2-napthoic acid (25), conjugated to CONA and OVA, respectively, were successfully prepared by the same method. All of these coating antigens were well recognized by the 7-KLH antisera, but only the coating antigens using compounds **22** and **23** could be used in the inhibition assay against 1-naphthol. Compounds **22**–**25** were subsequently tested also as competitors in the optimized assay (antisera 3907 1:9000 and coating antigen **2b**-BSA 1.5  $\mu$ g/mL, Table 3) but did not show any substantial cross-reactivity ( $\leq 0.3\%$  CR).

**Confirmation of Conjugation.** The determination of the hapten density of the main hapten–protein conjugates for the assay development was based on UV spectroscopy (Figure 3). The molecular weights of the proteins used for these determinations were 68 000 for BSA, 76 000 for CONA, and 45 000 for OVA. Hapten **2b** showed one characteristic peak at 329/330 nm, that was observed in both, the hapten–protein-conjugate and the free hapten, but not in the protein (BSA) alone (Figure 3A). The hapten density was determined by using the absorbances at this wavelength for both compound **2b** and the **2b**-BSA conjugate, respectively. The hapten density for the **2b**-BSA conjugate was determined as 24 haptens/BSA. Hapten **4** showed distinct absorbance maxima at 227, 284, 298, 313, 327, and 337 nm (spectrum not shown). After conjugation to BSA or CONA, respectively, the main peak was broader at 230–233 nm, where the maxima of the other hapten peaks were at 285, 298/9, 310, and 323/4 nm, respectively. Because the main peak at 227 nm interfered with the main protein peak at 226 nm, the determination of hapten density was performed at 327 nm for the hapten and at 323, 324 nm for the 4-BSA conjugate and 4-CONA conjugate, respectively. The density was 30 for the 4-BSA conjugate and 47 for the 4-CONA conjugate. The hapten 2-naphthoxyacetic acid (**23**) showed distinct absorbances at 261, 271, 281, 312, and 325 nm that were seen also in the corresponding hapten–protein conjugates. Only absorbance maxima at 312 and 325 nm did not show interference with the protein absorbance after conjugation; therefore, 325 nm was chosen for hapten density determination (Figure 3B). Hapten density was 41 for the 23-CONA conjugate and 24 for the 23-OVA conjugate. The latter estimate of hapten load for the OVA conjugate seems too high, because recent studies of the sequence of OVA show only 20 free lysines.

**Screening of the Sera against 1-Naphthol.** To obtain the most sensitive and reliable assay that also showed the least interference with other related structures, all sera were tested against 14 different coating antigens (Table 1). Although almost all antisera showed good recognition for numerous coating antigens (Table 1), only the 7-KLH sera could be inhibited by 1-naphthol. In general, all coating antigens using CONA as the protein (Table 1) gave higher absorbances but were less sensitive when used in the assays for inhibition.

From the screening procedure, five assays that showed standard curves for 1-naphthol (Table 2) could be made out. In addition, there were two more assays, using antisera 3907 (1:6000) with coating antigen 4-BSA (1  $\mu$ g/mL) and antisera 3909 (1:4000) with coating antigen **2a**-BSA (2  $\mu$ g/mL), which showed inhibition curves for 1-naphthol with  $I_{50}$  values in the lower micrograms per liter range (Table 1). They were not taken into further consideration, because they either showed a too high

background (about 40%) or were less specific for 1-naphthol (about 25% cross-reactivity for 2-naphthol).

The ELISA (competitive inhibition) with the most reliable data (lowest standard deviation) and with the least interference with other cross-reactants used the 7-KLH antiserum 3907 and the coating antigen **2b**-BSA (Tables 2 and 3). For these reasons it was taken for further characterizations, although it was not the most sensitive assay.

**Assay Characteristics.** Under optimized conditions (7-KLH antiserum 3907 dilution 1:9000 end dilution in the well, **2b**-BSA 1.5  $\mu$ g/mL), the ELISA for 1-naphthol showed a standard curve that allowed the measurement of the analyte from 10 to 1000  $\mu$ g/L with an average  $I_{50}$  value of  $72 \pm 22 \mu$ g/L (Table 3; Figure 4). The analyte concentrations in this study are the original concentrations pipetted into the wells of the microtitration plates. This was preferred because of better comparison with conventional methods, which also refer to the original concentration in the sample. Other ELISA studies, though, give often the final concentration in the well, which means that the values shown here would have to be divided by 2 (50  $\mu$ L of antiserum and 50  $\mu$ L of analyte), for example, for the comparison with the ELISA for carbaryl (Marco et al., 1993a).

**Cross-Reactivities.** A study of nearly 30 compounds as cross-reactants in this ELISA system showed that the 7-KLH antiserum 3907 recognized mainly compounds that have substitutes in the 1- and/or 6-position on the naphthalene ring system. The best recognized compound tested for cross-reactivity was [(5-hydroxy-2-naphthyl-*enyl*)oxy]acetic acid methyl ester (**1b**,  $I_{50}$  is about 7  $\mu$ g/L), which is the methyl ester of the hapten **2b** used as coating antigen (conjugated to BSA) in this assay (Figure 1). Compound **2b** itself showed also a very high cross-reactivity (96%). Compounds **1b** and **2b** will probably not interfere with the analysis of 1-naphthol in environmental samples, because they are not very likely to appear. The only compound that might occur in the matrix, together with 1-naphthol, is 1,6-dihydroxynaphthalene, which shows a cross-reactivity of 66%. 5-Hydroxy-2-naphthoic acid (**7**), which was the hapten for the immunogen, showed about 25% cross-reactivity. All other tested combinations of substitutions did not show crucial interferences with this assay. 1-Naphthaleneacetamide, 1-naphthoic acid, naphthalene, and pentachlorophenol did not show cross-reactivity up to 100 mg/L. 1- and 2-naphthoxyacetic acid also did not cross-react, although these compounds (**22**, **23**) were used in the screening procedure (Table 1) as haptens for coating antigens and could be used for the development of three assays, respectively (Table 2). A similar result was obtained with 1- and 2-napthoic acid (**24**, **25**). Both compounds gave negligible inhibition in the assay, but they were recognized when they were conjugated to a protein and used for screening of the sera (Table 1). They were not useful, though, for the development of an assay. Both results show that the hydroxyl group in the 1-position is essential for the inhibition in this assay. By varying the structure of the coating antigen, it is likely that this library of antisera will yield selective assays for a variety of phenolic compounds.

A very important feature is that this assay did not show cross-reactivity with carbaryl (Table 3), from which 1-naphthol is most likely derived. In a very brief study this was already investigated, when carbaryl standards were stored for more than 1 month. Up to 25% cross-reactivity with these “aged” carbaryl standards was observed in the 1-naphthol assay (freshly prepared standards:  $\leq 0.5\%$ ). The same tendency was observed

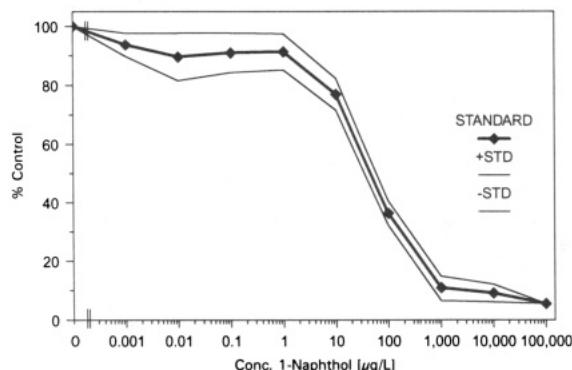
Table 3. Cross-Reactivities for the ELISA for 1-Naphthol<sup>b</sup>

compound	no.	chemical structure	av (μg/L)	SD (μg/L)	CR (%)
1-naphthol			72 <sup>a</sup>	22 <sup>a</sup>	100
[(5-hydroxy-2-naphthyl)oxy]acetic acid methyl ester	1b		6.5 <sup>b</sup>	0.8 <sup>b</sup>	1103
[(5-hydroxy-2-naphthyl)oxy]acetic acid	2b		75 <sup>c</sup>	20 <sup>c</sup>	96
1,6-dihydroxynaphthalene			109 <sup>d</sup>	29 <sup>d</sup>	66
5-hydroxy-2-naphthoic acid	7		291 <sup>c</sup>	113 <sup>c</sup>	25
[(6-hydroxy-1-naphthyl)oxy]acetic acid	2a		13910 <sup>e</sup>	11036 <sup>e</sup>	0.5*
2-naphthol			6225 <sup>f</sup>	1895 <sup>f</sup>	1.1
2-naphthyl ethyl carbonate	13		12900 <sup>f</sup>	1700 <sup>f</sup>	0.6*
O-2-naphthyl S-ethyl carbonate	14		18100 <sup>f</sup>		0.4*
2-naphthoxyacetic acid	23		37700 <sup>e1</sup>		0.2*
2-naphthoic acid	25		25200 <sup>f</sup>	8061	0.3*
1,5-dihydroxynaphthalene			2205 <sup>d</sup>	690 <sup>d</sup>	3.2
[(5-hydroxy-1-naphthyl)oxy]acetic acid	4		12100 <sup>f</sup>	3500 <sup>f</sup>	0.6*
1-naphthaleneacetamide	11				nc
1-naphthyl ethyl carbonate	12		8815 <sup>f</sup>	4385 <sup>f</sup>	0.8
1-naphthyl methyl carbonate	15		1128 <sup>f</sup>	383 <sup>f</sup>	6.3
1-naphthyl N-ethylcarbamate	16		8565 <sup>f</sup>	755 <sup>f</sup>	0.8*
carbaryl (1-naphthyl N-methylcarbamate)	17		13563 <sup>d</sup>	5303 <sup>d</sup>	0.5
1-naphthoxyacetic acid	22		23100 <sup>e1</sup>		0.3*
1-naphthoic acid	24		23700 <sup>f1</sup>		0.3*
naphthalene					nc
3-hydroxycarbaryl	8		2793 <sup>d</sup>	785 <sup>d</sup>	2.6
4-hydroxycarbaryl	9		16100 <sup>d</sup>		0.4
5-hydroxycarbaryl	10		4515 <sup>f</sup>	1345 <sup>f</sup>	1.6
phenol	18		15200 <sup>d</sup>		0.5*
4-nitrophenol	19		10600 <sup>d</sup>		0.7*
isopropyl N-(3-chlorophenyl)carbamate	21		4050 <sup>d</sup>		1.8*

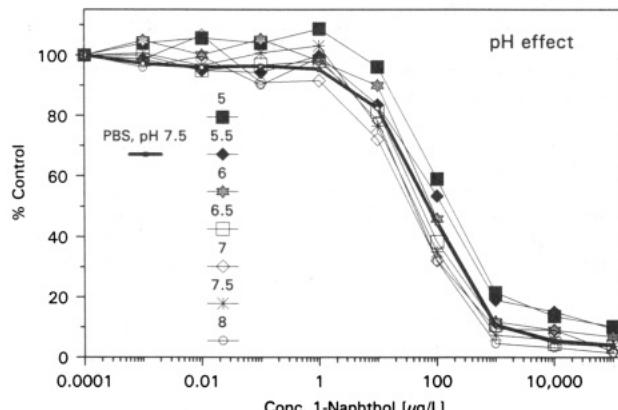
Table 3 (Continued)

compound	no.	chemical structure	av (μg/L)	SD (μg/L)	CR (%)
pentachlorophenol	20				nc

<sup>a</sup> Average and standard deviation from 27 plates, determined with 5 independent experiments over a period of 3 months; values on one plate were either an average of double or quadruplicate determination. Values were the average and standard deviation of <sup>b</sup>5 plates, <sup>c</sup>7 plates, <sup>d</sup>6 plates, <sup>e</sup>3 plates, <sup>f</sup>2 plates, triplicate determination on each plate. <sup>g</sup> Value was determined on one plate (triplicate determination). <sup>h</sup> 3 plates were determined, but only one plate showed  $I_{50}$  value, basically nc. <sup>i</sup> 2 plates were determined, but only one plate showed  $I_{50}$  value, basically nc. nc, no cross-reactivity. <sup>j</sup> Microtiter plates were coated with 1.5 μg/mL 2b-BSA. The serum from rabbit 3907 was used with a 1:9000 dilution. CR (%), % cross-reactivity; values were obtained by four-parameter curve fit of standard curves with the equation  $y = (A - D)/[1 + (x/C)^B] + D$  (SOFTmax); \* standard curves were only obtained with log-logit curve fit.



**Figure 4.** Standard curve ( $\pm$  standard deviation) for 1-naphthol using antiserum 3907 (diluted 1:9000, final dilution in the well) and coating antigen 2b-BSA (1.5 μg/mL). The absorbance (450–650 nm) was converted to percent control values (see Materials and Methods). The standard curve was linear from 10 to 1000 μg/L with an  $I_{50}$  of about 70 μg/L. This standard curve represents an average of 12 plates. On each plate every standard (0.001–100 000 μg/L, serial dilution) was determined in duplicate.



**Figure 5.** Effect of pH on the ELISA for 1-naphthol. Phosphate buffer (0.2 M) was used from pH 5 to pH 8 and compared to the usually used buffer (0.2 M PBS, pH 7.5). Each standard was determined in quadruplicate. The standard curve for PBS represents an average of seven plates, with quadruplicate determination on each plate. All absorbances with the 0.2 M phosphate buffers were about 30% lower than with PBS, which might have been caused by the different salt concentrations of the two buffer systems. In addition, the absorbance of the assay with 0.2 M phosphate buffer dropped about 40% from pH 8 to pH 5.

with 1-naphthyl *N*-ethylcarbamate (Schneider et al., 1994). For future studies, the selectivity of the assay will most likely allow its usage for the monitoring of the degradation of carbaryl or for the monitoring of 1-naphthol from other sources.

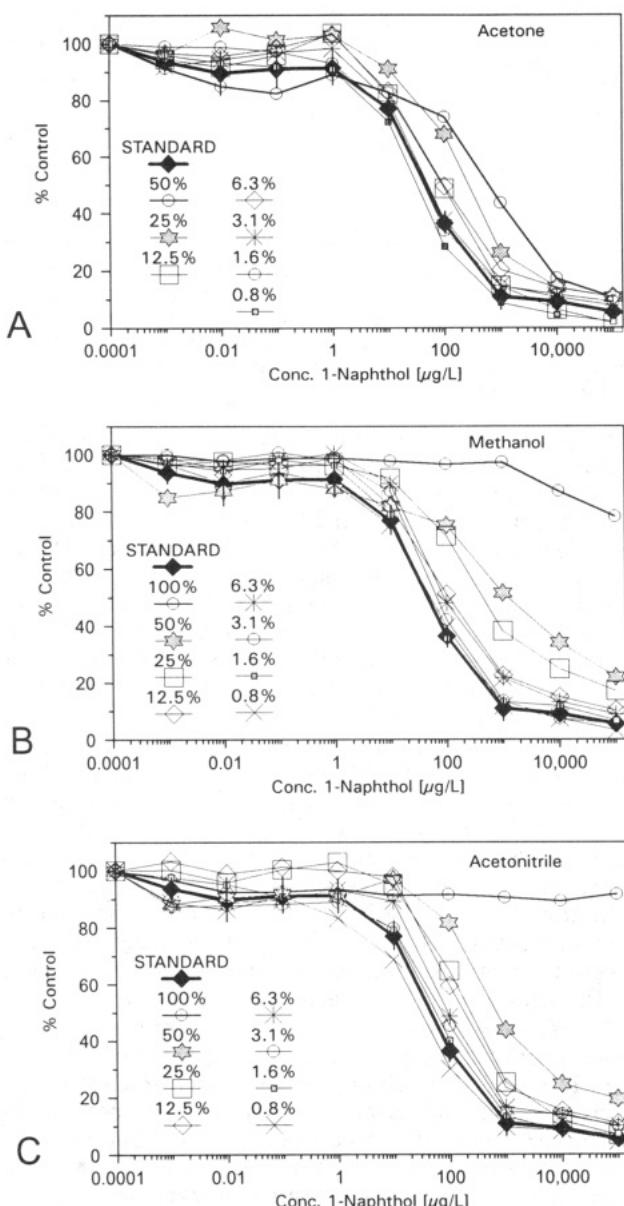
**pH Effect Studies.** To determine the potential influence of pH, 0.2 M phosphate buffer was used in the range from pH 5 to pH 8 and compared with 0.2 M PBS,

pH 7.5. In the range tested, the pH had only a minor influence on the assay. At pH 5 and 5.5, the assay lost sensitivity, and the standard curve shifted from an average  $I_{50}$  value of 75 μg/L (0.2 M PBS, pH 7.5) to an  $I_{50}$  value of 107 μg/L (pH 5; Figure 5). In the range from pH 6 to pH 8 the assay gained in sensitivity, compared to the standard PBS buffer, which might be an effect of the lower salt concentration of the 0.2 M phosphate buffer. The  $I_{50}$  values (averages of quadruplicate determination) were 69 μg/L (pH 6), 55 μg/L (pH 6.5), 30 μg/L (pH 7), 41 μg/L (pH 7.5), and 45 μg/L (pH 8).

**Matrix Effects.** (a) *Solvent Tolerance.* A solvent content up to about 10% was tolerated with all three solvents tested: acetone, methanol, and acetonitrile (Figure 6). These solvents were chosen because they are most commonly used in conventional LC methods for 1-naphthol. Both acetonitrile and methanol showed no inhibition curves at 100% solvent. The assay can tolerate 50% of solvent, but the sensitivity of the assay decreases (acetone, about 10-fold; acetonitrile, about 14-fold; and methanol, about 20-fold decrease in sensitivity).

(b) *Human Urine.* 1-Naphthol was determined as a metabolite of carbaryl in urine in a study done by Shafik et al. (1971). The method (electron capture GC) required derivatization and cleanup steps for the increase in sensitivity (limit of detection 0.02 ppm). Shafik et al. (1971) measured concentrations of 1-naphthol ranging from 6.2 to 78.8 ppm in the urine of workers who worked as formulators of technical carbaryl at a manufacturing plant. In the urine of agricultural workers who used carbaryl for pest control, they measured 1-naphthol in the range from 0.07 to 1.7 ppm. In our preliminary study, we spiked undiluted and diluted human urine and ran it in the ELISA. The urine used did not show a significant matrix effect (Figure 7A). This indicates that the assay could be used in future studies as a screening method to measure 1-naphthol in human urine directly without derivatization or sample cleanup. At analyte concentrations above 1000 μg/L, urine would have to be diluted to measure in the linear range of the standard curve.

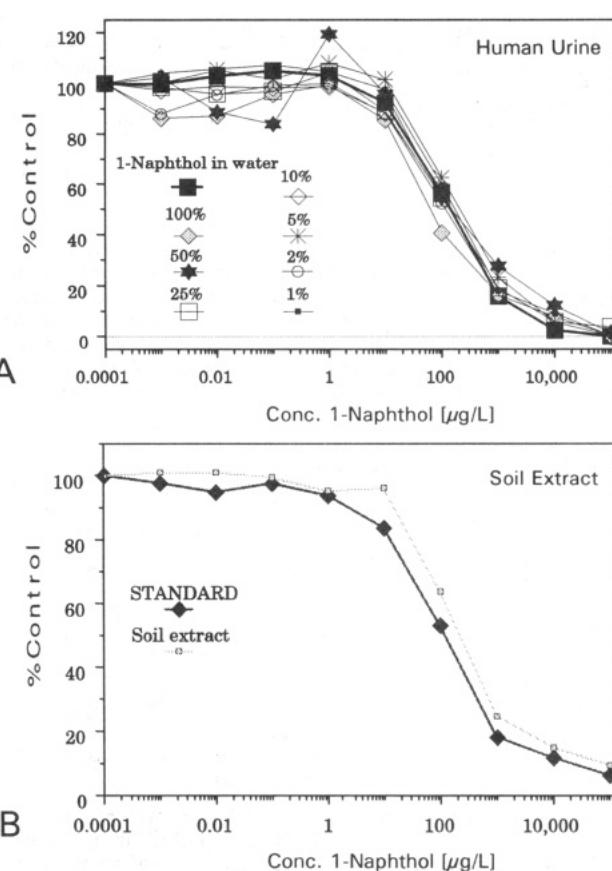
(c) *Soil.* In a recent study, Dikshith et al. (1990) analyzed 1-naphthol in environmental samples taken in and around Bhopal, India, where carbaryl was produced for more than a decade. Among other matrices, they collected soil samples from ponds. The data showed, as they pointed out, "alarming levels", ranging between 0.153 and 0.656 ppm. On the basis of this background information, preliminary studies of the potential behavior of the ELISA for 1-naphthol in soil extracts were carried out, using Yolo silt loam as an example. The  $I_{50}$  value of the standard curve in the soil extract shifted to the right compared to the one in 0.2 M PBS (95 μg/L in PBS vs 158 μg/L in soil extract, Figure 7B), and the absorbance values in the assay with soil extract were about 20% lower than with PBS.



**Figure 6.** Solvent tolerance of the ELISA for 1-naphthol. (A) Acetone. The absorbance started to drop slowly (5%) with about 6% acetone in the assay. With 50% acetone, the absorbance dropped to 50% that of the standard assay. (B) Methanol. The absorbance of the assay increased slowly compared to the performance with 0.2 M PBS, pH 7.5 (about 20% increase in absorbance with 50% methanol). (C) Acetonitrile. The absorbance dropped only about 25% with 50% acetonitrile; with all other solvent contents the absorbance remained the same as with 0.2 M PBS, pH 7.5.

Besides these matrix effects, the assay could be performed in this soil extract without any further cleanup steps. For future studies, similar to the one carried out in Bhopal, this assay might provide a fast, easy, and therefore very useful tool for the screening of residues of 1-naphthol.

**Conclusion.** The moderate to low mammalian toxicity of carbaryl (U.S. EPA, 1984) together with its effectiveness against many agricultural pests has led to an extensive use of this insecticide on a wide variety of crops. The specific monitoring, however, reveals difficulties, because of the fast hydrolysis of carbaryl to 1-naphthol, especially under basic conditions ( $\text{pH} > 9$ ). Therefore, the development of this selective ELISA for 1-naphthol might provide a good screening tool for the monitoring of the degradation of carbaryl or for the monitoring of 1-naphthol, deriving from other sources. On the basis of the solvent tolerance



**Figure 7.** Preliminary studies of matrix effects on the ELISA for 1-naphthol. (A) Human urine. The absorbance of the assay decreased with increasing urine concentration (data not shown), but the matrix urine has no effect on the performance of the assay, when data are expressed as percent control values. To obtain quantitative results, the standards and the unknowns should be run in the same concentration of urine. Antiserum dilution (3907) for this experiment was 1:9000; coating antigen (2b-BSA) concentration was 1  $\mu\text{g}/\text{mL}$ . Each data point represents the average of a triplicate determination on one plate. Standard curve (in water) represents the average of four plates, with duplicate determination of each standard/plate. (B) Soil extract. An extract of Yolo silt loam was used to test the potential performance of the ELISA in the matrix soil. The standard curve in the soil extract shifted to the right, but the assay could be performed without further cleanup steps. Each data point represents the average of a triplicate determination on the same plate.

of the assay, the ELISA will be suitable to use as a tool for possible integrations with other analytical methods, for example, as a detector for the HPLC (Krämer et al., 1994). For potential future studies, preliminary tests were carried out in one sample of human urine and in one soil extract, which indicate that this assay might be utilized for human exposure studies and for environmental control, respectively.

#### ABBREVIATIONS USED

BSA, bovine serum albumin; CONA, conalbumin A; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase;  $I_{50}$ , concentration of the analyte, where 50% of the antibody is inhibited by the analyte, midpoint of the assay; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.5% Tween 20 (polyoxyethylene sorbitan monolaurate); RT, room temperature (21–23 °C); TLC, thin-layer chromatography.

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