Determination of Carbaryl in Well Water Samples by ELISA

This protocol describes the determination of the pesticide 1-naphthyl-*N*-methylcarbamate (carbaryl) in well water samples using an enzyme-linked immunosorbent assay (ELISA). Approximately 500 µL of well water sample are necessary to perform the analysis. The immunoassay described here uses a polyclonal antibody (R2114) developed against a carbaryl mimic coupled to keyhole limpet hemocyanin (KLH). The ELISA is carried out in microtiter plates using an indirect competitive ELISA format. The buffered samples are incubated with antibody solution and added to antigen-coated wells. The carbaryl present in the samples competes with the immobilized antigen for a fixed amount of antibody. After a 1-hr incubation at room temperature, a secondary antibody reagent (horseradish peroxidase–conjugated goat anti-rabbit IgG; GAR-HRP) is added and incubated for 1 hr. Finally, the presence of carbaryl in the samples is determined by adding a solution containing an appropriate enzyme substrate that will yield a colored product, and the absorbance of the colored product is measured at 450 nm. The absorbance is inversely proportional to the concentration of carbaryl in the sample. When analyzing well water samples, the working range of this immunoassay is 0.1 to 10 µg/L, and the limit of detection is 0.06 µg/L. The assay does not show cross-reactivity for 1-naphthol, the major degradation product of carbaryl. Cross-reaction for other carbamates is <5%. The concentration of antibody may need to be determined in a separate titration experiment. This procedure is presented in the Support Protocol.

QUALITY CONTROL

To verify method performance, negative and positive controls should be included on each microtiter plate. Two types of controls should be prepared. One set of controls should be prepared in assay buffer to indicate whether the immunoassay is under control. The other set of controls should be prepared in the sample matrix to indicate the relative magnitude of the matrix effect (see Critical Parameters).

If the negative control prepared in sample matrix produces a positive response in the ELISA, a matrix effect or prior contamination with analyte is presumed. This negative control value should be subtracted from the positive control prepared in sample matrix. If the resulting spiked recovery is within the quality control criteria, then the method of standard addition should be used to quantitate samples. If the values fall outside the quality control criteria, the method is out of control and the cause should be determined. Typically, the sample should be diluted or cleaned up to bring the method back into control. A solid-phase extraction method, such as that described by Marco et al. (1995), can be used.

Positive controls consist of assay buffer or sample matrix spiked with carbaryl at 0.1, 0.7, and 10 ng/mL. Recovery of carbaryl from the positive controls prepared in assay buffer should not deviate >15% from the expected value. Recovery of carbaryl from the positive controls prepared in sample matrix should be between 50% and 150%, with a relative percent standard deviation (RSD) of <30%. If these criteria are met, method performance is considered acceptable and sample analysis may begin.

Contributed by M.-Pilar Marco, Shirley J. Gee, and Bruce D. Hammock
Current Protocols in Field Analytical Chemistry (1998) 2C.8.1-2C.8.15

BASIC PROTOCOL

DETERMINATION OF CARBARYL IN WELL WATER

Materials

1 mg/mL coating antigen (5-CONA; see Reagents and Standards)
Coating buffer (see Reagents and Standards)
Anti-carbaryl polyclonal antibody (R2114; see Reagents and Standards)
1× and 10× PBST (see Reagents and Standards)
Well water samples for analysis
Carbaryl standard solutions (see Reagents and Standards)
Quality control samples (see Reagents and Standards)
GAR-HRP (see Reagents and Standards)
1% (v/v) H_2O_2 (store up to 1 month in a plastic container at 4°C)
6 mg/mL TMB (see Reagents and Standards)
Substrate buffer (see Reagents and Standards)
2 M sulfuric acid

Single-channel pipets (0.5- to 10-µL, 10- to 100-µL, 50- to 250-µL, 200- to 1000-µL, and 1- to 10-mL; e.g., Eppendorf) and appropriate tips
Multichannel (8- or 12-channel) pipettor capable of delivering 50 to 300 µL, with reservoirs and appropriate tips
96-well high-binding microtiter plates for enzyme-linked immunosorbant assays (ELISA plates; e.g., Nunc Maxisorb)
Refrigerator
Adhesive acetate plate sealers
Plate washer: 12-channel manual washer or an automatic washer
Absorbent paper
96-well polystyrene microtiter plate (mixing plate)
Microplate reader with a 450-nm filter
Computer and software to analyze the data using a four-parameter logistic curve-fitting equation (e.g., Genesis from Titertek, GraphPrism)

Coat ELISA plate

1. Prepare a 0.1 µg/mL coating antigen solution by diluting (for one microtiter plate) 1.1 µL of 1 mg/mL 5-CONA in 11 mL coating buffer.

   For optimal performance, the antigen concentration may need to be determined by titration (see Support Protocol).

2. Add 100 µL of 0.1 µg/mL coating antigen solution to each well of an ELISA plate using a multichannel pipettor.

3. Cover and seal the plate with an adhesive acetate plate sealer, and incubate overnight at 4°C.

   If the coated plates are to be used immediately, the preparation of samples (steps 4 to 7) can be conducted on the same day as the coating step and allowed to incubate over the same night.

Preincubate controls and samples with antibody

4. Prepare a 1:8000 anti-carbaryl antibody solution by diluting (for one microtiter plate) 1.25 µL R2114 in 10 mL of 1× PBST.

   For optimal performance, the antibody concentration may need to be determined by titration (see Support Protocol).

5. Mix 450 µL of each well water sample with 50 µL of 10× PBST.

   In the absence of a matrix effect, samples can be buffered and then used directly for analysis (see Quality Control and Support Protocol for information on determination of matrix effects).

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	S_A	S_A	QC-0	QC-0	QC-20	QC-20	QC-50	QC-50	QC-80	QC-80	
B	blank	S_B	S_B	U1	U1	U7	U7	U13	U13	U19	U19	
C	blank	S_C	S_C	U2	U2	U8	U8	U14	U14	U20	U20	
D	blank	S_D	S_D	U3	U3	U9	U9	U15	U15	U21	U21	
E	0	S_E	S_E	U4	U4	U10	U10	U16	U16	U22	U22	
F	0	S_F	S_F	U5	U5	U11	U11	U17	U17	U23	U23	
G	0	S_G	S_G	U6	U6	U12	U12	U18	U18	U24	U24	
H	0	S_H	S_H	QC-0	QC-0	QC-20	QC-20	QC-50	QC-50	QC-80	QC-80	

Figure 2C.8.1 Typical distribution on an ELISA plate. Column 1 contains quadruplicates of no-antibody blanks and zero-analyte standards (0). Remaining standards (S_A through S_H) are in columns 2 and 3. Quality control (QC) samples are shown as duplicates in both rows A and H (one row for QC samples prepared in blank matrix, one for QC samples prepared in assay buffer). Twenty four well water samples (U1 through U24) are in columns 4 through 11.

6. Place 200 µL of each carbaryl standard, quality control sample, and buffered well water sample into a separate well in a 96-well mixing plate. Add 50 µL antibody solution to each well. For a blank, add 250 µL of 1× PBST with no antibody solution. For a zero-analyte standard, mix 200 µL of 1× PBST with 50 µL antibody solution.

   This amount of sample is enough for two well replicates in the ELISA plate. It is recommended that the zero-analyte standard and no-antibody blank be doubled and assayed in quadruplicate wells. To reduce errors when transferring, the same layout should be used for both the mixing plate and the ELISA plate, although the mixing plate will require half as many wells. See Figure 2C.8.1 for a typical distribution of samples, controls, and standards on an ELISA plate.

   The calibration curve is comprised of eight carbaryl concentrations (S_A through S_H, ranging from 1 µg/mL to 0.013 ng/mL), the zero-analyte standard, and the no-antibody blank.

   Quality control (QC) samples are used to test the accuracy of the assay and the presence or absence of matrix effects.

7. Cover and seal the mixing plate with an adhesive acetate plate sealer, and incubate overnight at 4°C.

Begin competition

8. After incubation, wash the coated ELISA plate five times with a plate washer by adding ~300 µL of 1× PBST to each well and aspirating the liquid. Tap the plate dry on absorbent paper.

   This procedure removes unbound reagents from the wells.

   Dry, coated ELISA plates are ready for immediate use. Although it is preferable to use freshly coated plates, the plates can be stored several days at −20°C with no significant change in assay characteristics. However, longer coating times result in an increase in well-to-well variability.

9. Transfer 100 µL from each well of the mixing plate to each of the corresponding duplicate wells of the ELISA plate (Fig. 2C.8.1).

10. Cover and seal the plate with an adhesive acetate plate sealer, and incubate 1 hr at room temperature.

Add secondary antibody

11. Prepare the working secondary antibody solution by diluting (for one microtiter plate) 2 µL GAR-HRP stock solution in 12 mL of 1× PBST (1:6000 dilution).

12. Wash the plate as described in step 8.

13. Add 100 µL GAR-HRP working solution to each well.

14. Cover and seal the plate with adhesive acetate plate sealer, and incubate 1 hr at room temperature.

Perform color detection

15. Prepare substrate solution for one assay plate by adding 44 µL of 1% (v/v) H_2O_2 (substrate) and 176 µL of 6 mg/mL TMB (chromogen) to 11 mL substrate buffer.

16. Wash the plate as described in step 8.

17. Add 100 µL substrate solution to each well and cover with a plate sealer. Incubate 30 min at room temperature.

18. Add 50 µL of 2 M sulfuric acid to each well.

19. Measure the absorbance at 450 nm for each well using a microplate reader.

Calculate results

20. Prepare a calibration curve for carbaryl by fitting the data for the standards using a four-parameter logistic equation:

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D$$

where y is absorbance, x is analyte concentration, A and D are the upper and lower asymptotes of the sigmoid curve (respectively), B is the slope, and C is the IC_{50} or the analyte concentration defining the midpoint of the linear portion of the curve.

21. Determine that the parameters of the four-parameter logistic equation (slope, IC_{50}, maximum and minimum absorbance, and the coefficient of correlation) are within expected values, and that the curve is well defined by the standards used.

22. Use the calibration curve to calculate the concentration of carbaryl present in well water samples and quality control samples.

If the calculated concentrations of the quality control samples deviate from the expected value by >15%, the method is out of control. See Quality Control and Commentary sections for strategies to bring the method back into control.

23. To determine if the carbaryl concentrations for well water samples and quality control samples are accurate, normalize the measured absorbances by dividing the absorbance value for each well water and quality control sample (B) by the mean of the absorbance at zero analyte concentration (B_0).

24. Calculate the absorbance value corresponding to 90%, 80%, and 20% of the B_0. From these absorbances use the calibration curve parameters determined in step 20 to calculate the corresponding concentrations of carbaryl.

The carbaryl concentration at 90% of B_0 (D-90) is the limit of detection. The carbaryl concentrations at 80% and 20% of B_0 (D-80 and D-20) are the working range of the assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	S_A	S_A	QC-0	QC-0	QC-20	QC-20	QC-50	QC-50	QC-80	QC-80	
B	blank	S_B	S_B	U1	U1	U7	U7	U13	U13	U19	U19	
C	blank	S_C	S_C	U2	U2	U8	U8	U14	U14	U20	U20	
D	blank	S_D	S_D	U3	U3	U9	U9	U15	U15	U21	U21	
E	0	S_E	S_E	U4	U4	U10	U10	U16	U16	U22	U22	
F	0	S_F	S_F	U5	U5	U11	U11	U17	U17	U23	U23	
G	0	S_G	S_G	U6	U6	U12	U12	U18	U18	U24	U24	
H	0	S_H	S_H	QC-0	QC-0	QC-20	QC-20	QC-50	QC-50	QC-80	QC-80	

Figure 2C.8.1 Typical distribution on an ELISA plate. Column 1 contains quadruplicates of no-antibody blanks and zero-analyte standards (0). Remaining standards (S_A through S_H) are in columns 2 and 3. Quality control (QC) samples are shown as duplicates in both rows A and H (one row for QC samples prepared in blank matrix, one for QC samples prepared in assay buffer). Twenty four well water samples (U1 through U24) are in columns 4 through 11.

6. Place 200 µL of each carbaryl standard, quality control sample, and buffered well water sample into a separate well in a 96-well mixing plate. Add 50 µL antibody solution to each well. For a blank, add 250 µL of 1× PBST with no antibody solution. For a zero-analyte standard, mix 200 µL of 1× PBST with 50 µL antibody solution.

   This amount of sample is enough for two well replicates in the ELISA plate. It is recommended that the zero-analyte standard and no-antibody blank be doubled and assayed in quadruplicate wells. To reduce errors when transferring, the same layout should be used for both the mixing plate and the ELISA plate, although the mixing plate will require half as many wells. See Figure 2C.8.1 for a typical distribution of samples, controls, and standards on an ELISA plate.

   The calibration curve is comprised of eight carbaryl concentrations (S_A through S_H, ranging from 1 µg/mL to 0.013 ng/mL), the zero-analyte standard, and the no-antibody blank.

   Quality control (QC) samples are used to test the accuracy of the assay and the presence or absence of matrix effects.

7. Cover and seal the mixing plate with an adhesive acetate plate sealer, and incubate overnight at 4°C.

Begin competition

8. After incubation, wash the coated ELISA plate five times with a plate washer by adding ~300 µL of 1× PBST to each well and aspirating the liquid. Tap the plate dry on absorbent paper.

   This procedure removes unbound reagents from the wells.

   Dry, coated ELISA plates are ready for immediate use. Although it is preferable to use freshly coated plates, the plates can be stored several days at −20°C with no significant change in assay characteristics. However, longer coating times result in an increase in well-to-well variability.

9. Transfer 100 µL from each well of the mixing plate to each of the corresponding duplicate wells of the ELISA plate (Fig. 2C.8.1).

10. Cover and seal the plate with an adhesive acetate plate sealer, and incubate 1 hr at room temperature.

Add secondary antibody

11. Prepare the working secondary antibody solution by diluting (for one microtiter plate) 2 µL GAR-HRP stock solution in 12 mL of 1× PBST (1:6000 dilution).

12. Wash the plate as described in step 8.

13. Add 100 µL GAR-HRP working solution to each well.

14. Cover and seal the plate with adhesive acetate plate sealer, and incubate 1 hr at room temperature.

Perform color detection

15. Prepare substrate solution for one assay plate by adding 44 µL of 1% (v/v) H_2O_2 (substrate) and 176 µL of 6 mg/mL TMB (chromogen) to 11 mL substrate buffer.

16. Wash the plate as described in step 8.

17. Add 100 µL substrate solution to each well and cover with a plate sealer. Incubate 30 min at room temperature.

18. Add 50 µL of 2 M sulfuric acid to each well.

19. Measure the absorbance at 450 nm for each well using a microplate reader.

Calculate results

20. Prepare a calibration curve for carbaryl by fitting the data for the standards using a four-parameter logistic equation:

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D$$

where y is absorbance, x is analyte concentration, A and D are the upper and lower asymptotes of the sigmoid curve (respectively), B is the slope, and C is the IC_{50} or the analyte concentration defining the midpoint of the linear portion of the curve.

21. Determine that the parameters of the four-parameter logistic equation (slope, IC_{50}, maximum and minimum absorbance, and the coefficient of correlation) are within expected values, and that the curve is well defined by the standards used.

22. Use the calibration curve to calculate the concentration of carbaryl present in well water samples and quality control samples.

If the calculated concentrations of the quality control samples deviate from the expected value by >15%, the method is out of control. See Quality Control and Commentary sections for strategies to bring the method back into control.

23. To determine if the carbaryl concentrations for well water samples and quality control samples are accurate, normalize the measured absorbances by dividing the absorbance value for each well water and quality control sample (B) by the mean of the absorbance at zero analyte concentration (B_0).

24. Calculate the absorbance value corresponding to 90%, 80%, and 20% of the B_0. From these absorbances use the calibration curve parameters determined in step 20 to calculate the corresponding concentrations of carbaryl.

The carbaryl concentration at 90% of B_0 (D-90) is the limit of detection. The carbaryl concentrations at 80% and 20% of B_0 (D-80 and D-20) are the working range of the assay.

25. If well water samples give absorbances <20% of B_0, dilute and reanalyze them to obtain a more accurate carbaryl concentration value.

26. If well water give absorbances >80% of B_0 and a lower limit of detection is desired, concentrate samples by solid-phase extraction so that resulting absorbances fall into the working range (i.e., 20% to 80% of B_0).

27. Calculate the concentration of carbaryl in the well water samples using values that fall within the working range.

TWO-DIMENSIONAL TITRATION OF ANTIGEN AND ANTIBODY

SUPPORT PROTOCOL

The concentrations of coating antigen (5-CONA) and antibody (R2114) solutions may need to be adjusted for the assay to meet performance specifications. Optimizing reagent concentrations is particularly important when new immunoreagents are prepared, when assay conditions (e.g., incubation period or temperature, washing procedure) are modified, or when changes in the assay parameters (e.g., IC_{50}, maximal absorbance, slope, background noise) are observed. In this experiment, varying dilutions of the antiserum are tested for avidity to varying amounts of coating antigen in a two-dimensional titration paradigm. The titration reaction is the same as that for the ELISA assay itself (see Basic Protocol) and uses the same materials.

Coat ELISA plate with antigen dilutions

1. Prepare 2 mL of a 6 µg/mL coating antigen solution by diluting 12 µL of 1 mg/mL 5-CONA in 1988 µL coating buffer.

2. Add 100 µL coating buffer to all wells of an ELISA plate except for column 1 (wells A1 to H1).

3. Add 200 µL of 6 µg/mL coating antigen solution (prepared in step 1) to each well of column 1.

4. Transfer 100 µL from well A1 to well A2 (final 3 µg/mL coating antigen). Transfer 100 µL from well A2 to well A3 (final 1.5 µg/mL), and continue 2-fold serial dilutions in this manner to well A11 (final 750, 375, 188, 94, 47, 23, 12, and 6 ng/mL). Discard 100 µL from well A11. Repeat the serial dilution procedure for rows B through H.

   Wells in column 12 are zero–coating antigen controls and contain coating buffer only.

   The serial dilutions can be facilitated by the use of an 8-channel multichannel pipettor. With this device, 100 µL from each well in column 1 can be transferred simultaneously to wells in column 2, and so on.

5. Cover and seal the plate with an adhesive acetate plate sealer and incubate overnight at 4°C.

   If conditions such as assay incubation time or temperature are to be modified for the Basic Protocol, use these modified conditions for titration as well.

Prepare antibody dilutions

6. Prepare 4 mL of a 1:1000 anti-carbaryl antibody solution by diluting 4 µL R2114 in 4 mL of 1× PBST, and place it in the first channel of an 8-channel reagent reservoir.

7. Add 2 mL of 1× PBST to the remaining channels of the reservoir.

8. Transfer 2 mL from channel 1 to channel 2 in the reservoir (final 1:2000). Transfer 2 mL from channel 2 to channel 3 (final 1:4000), and continue 2-fold serial dilutions in this manner to channel 7 (final 1:64,000). Discard 2 mL from channel 7.

   Channel 8 is a zero-antibody control and contains 1× PBST only.

Perform immunoreaction

9. Wash the plate as described (see Basic Protocol, step 8).

10. With a 12-channel pipettor, transfer 100 µL from the first channel of the reagent reservoir (1:1000 R2114) to each well of the first row of the ELISA plate (wells A1 to A12). Transfer 1:2000 R2114 from the second channel of the reagent reservoir to the second row of the ELISA plate (wells B1 to B12). Continue so that each row of the ELISA plate contains a different dilution of the antiserum.

11. Cover and seal the plate with an acetate plate sealer and incubate 1 hr at room temperature.

If this incubation time or temperature are to be modified for the Basic Protocol, use these modified conditions for titration as well.

Label bound antibody

12. Perform secondary antibody reaction, color development, and measurement of A_{450} as described (see Basic Protocol, steps 11 to 19).

Calculate results

13. Plot absorbance versus coating antigen concentration for each antiserum dilution (Fig. 2C.8.2). Choose the coating antigen concentration at which the absorbance is beginning to plateau, at an antibody dilution that gives a maximal absorbance of 0.7 to 1.5 units.

These criteria ensure that the coating antigen molecules will cover the plastic surface approximating a monolayer, and that the maximal absorbance is large enough to give a reasonable range of response.

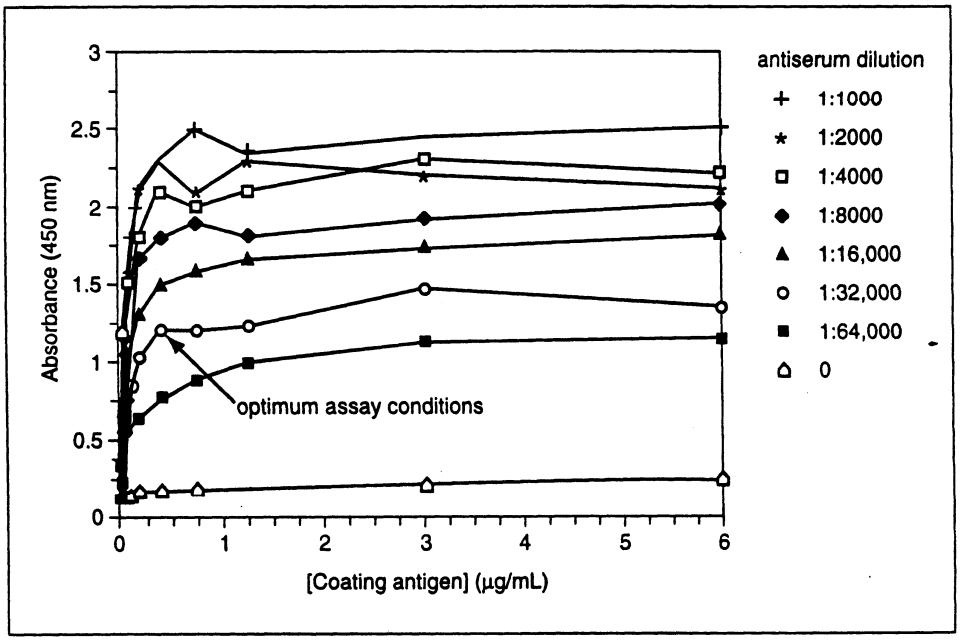

Figure 2C.8.2 An example of a family of titration curves obtained by plotting absorbance versus coating antigen concentration for each antiserum dilution. The arrow indicates the optimum conditions chosen to ensure a monolayer of coating antigen and an acceptable signal. Coating antigen or antiserum concentrations that are too high may lead to a decrease in assay sensitivity and to higher well-to-well variation.

REAGENTS AND STANDARDS

NOTE: Prepare all buffers with Milli-Q-purified water. Although 0.02% (w/v) sodium azide or 0.005% (w/v) sodium ethylmercurithiosalicylate can be added as preservatives, their use can be eliminated when buffers are used within 1 to 2 weeks and stored at 4°C. Because sodium azide inhibits horseradish peroxidase activity, better assay performance may be obtained by eliminating the preservative from the final wash step. Preservative should never be added to substrate buffer.

Anti-carbaryl polyclonal antibody (R2114)

Upon receipt, divide antiserum (available upon request from B.D. Hammock, University of California, Davis) into 50-µL aliquots and store up to 2 years at –20°C. Store thawed aliquots up to 2 weeks at 4°C.

This antiserum was developed in New Zealand white rabbits immunized with 8-KLH (Fig. 2C.8.3), a conjugate of 1-(5-carboxypentyl)-3-(1-naphthyl)urea and keyhole limpet hemocyanin (KLH). The description and derivation of this reagent are reported in Marco et al. (1993).

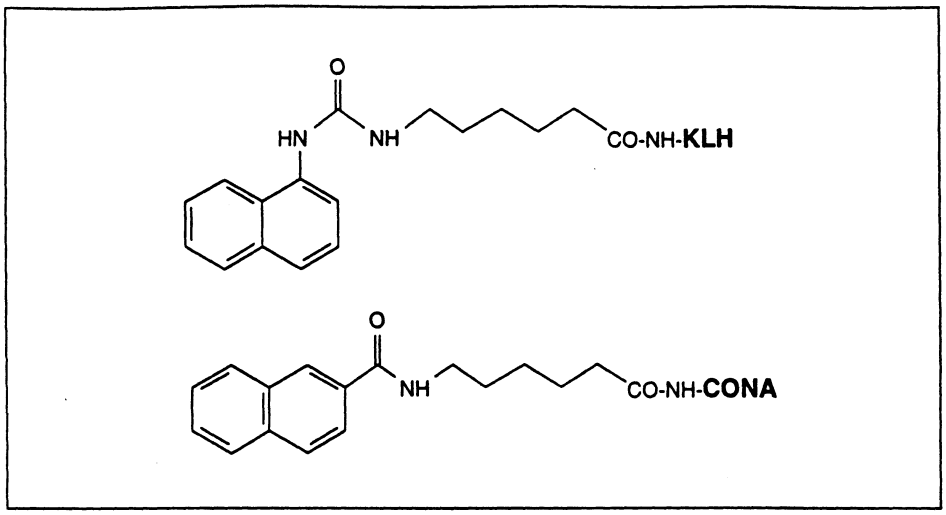

Figure 2C.8.3 Chemical structures of 1-(5-carboxypentyl)-3-(1-naphthyl)urea (hapten 8, top) and *N*-(2-naphthoyl)-6-aminohexanoic acid (hapten 5, bottom). Hapten 8 was covalently coupled to keyhole limpet hemocyanin (KLH) to produce 8-KLH, which was used as the immunogen for producing the anti-carbaryl antibody, R2114. Hapten 5 was covalently coupled to conalbumin (CONA) to produce 5-CONA, which is used as the coating antigen in the indirect ELISA.

Carbaryl standard solutions

Stock solution: Prepare a 2 mg/mL carbaryl stock solution by dissolving 20 mg analytical grade carbaryl in 10 mL dimethyl sulfoxide (DMSO). Store up to 2 weeks at room temperature.

Working solutions (Fig. 2C.8.4): Dilute 100 µL of 2 mg/mL stock solution in 900 µL DMSO to make a 0.2 mg/mL working solution. Dilute 5 µL of the 0.2 mg/mL working solution in 995 µL of 1× PBST to make a 1 µg/mL standard solution, the highest standard concentration to be tested. Place 300 µL of the 1 µg/mL standard solution into the well of a mixing plate (a standard 96-well microtiter plate), and add 240 µL of 1× PBST to seven additional wells. Transfer 60 µL of 1 µg/mL

continued

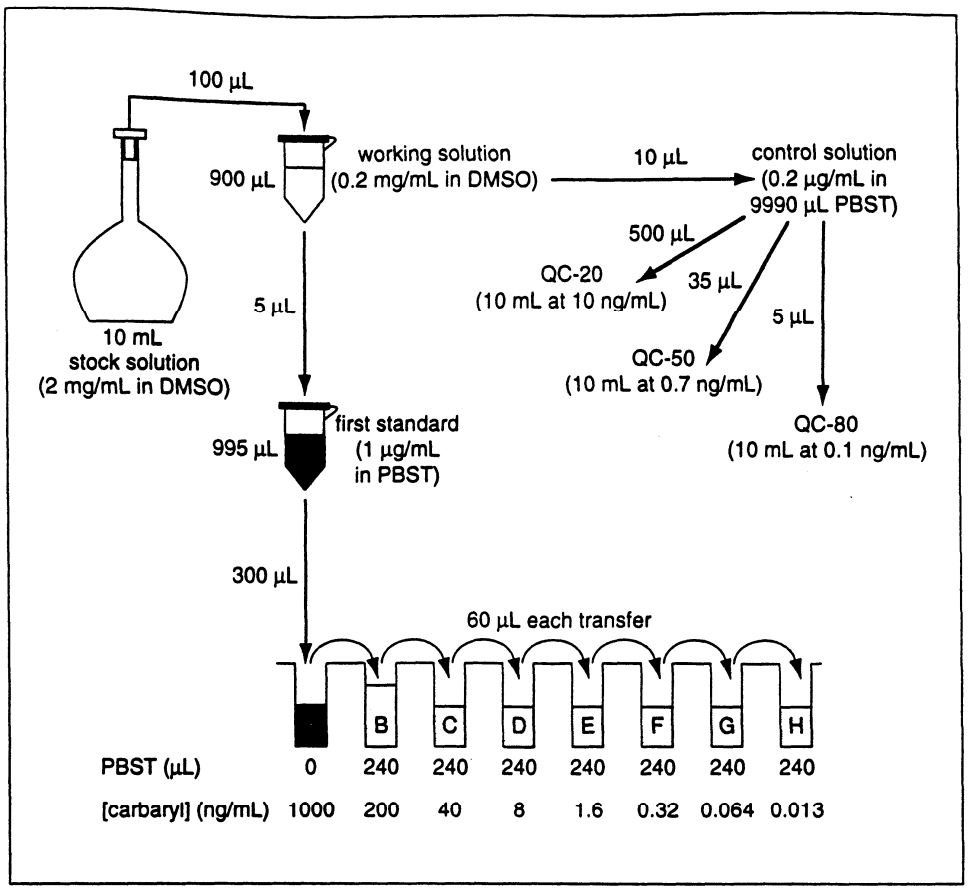

Figure 2C.8.4 Schematic representation of the steps involved in preparation of carbaryl standards (bottom) and quality control samples (right) for the carbaryl ELISA. Quality control samples are prepared both in blank matrix and assay buffer (1× PBST).

solution to the first PBST well (final 200 ng/mL). Continue to prepare 1/5 serial dilutions in the remaining wells by transferring 60 µL from each well to the next (final 40, 8, 1.6, 0.32, 0.064, and 0.013 ng/mL). Prepare working standards fresh before each assay.

This procedure utilizes an amount of analytical standard that can be weighed with reasonable accuracy. An analytical balance with precision of 0.1 mg should be used. DMSO is a very polar, water-miscible solvent of low volatility. The final concentration of organic solvent in the well containing the 1 µg/mL standard is only 5%. Carbaryl slowly hydrolyzes in water, producing 1-naphthol. Therefore, a fresh standard should be prepared and a new standard curve measured with each assay. The use of cluster tubes in a 96-well microtiter plate format to prepare the standard curve simplifies further steps of the Basic Protocol.

Coating antigen (5-CONA), 1 mg/mL

Prepare a 1 mg/mL working solution of 5-CONA (available upon request from B.D. Hammock, University of California, Davis) in 1× PBS (see below). Divide into 50-µL aliquots and store up to 2 years at –20°C. Store thawed aliquots at 4°C for up to 2 weeks.

The coating antigen is a conjugate of N-(2-naphthoyl)-6-aminohexanoic acid (Fig. 2C.8.3) covalently coupled to conalbumin (CONA). It should be stored freeze-dried or frozen in aliquots as indicated, and should show no loss of performance under these conditions. The description and derivation of this reagent are reported in Marco et al. (1993).

Coating buffer

To ~350 mL H_2O add:
0.795 g Na_2CO_3
1.465 g $NaHCO_3$
Adjust to pH 9.5
Adjust to 500 mL with H_2O
Store up to 1 month at 4°C

Goat anti-rabbit IgG, horseradish peroxidase–conjugate (GAR-HRP)

To make a stock solution, reconstitute horseradish peroxidase–conjugated goat anti–rabbit IgG (Sigma, cat. no. R-2004) with 1 mL 0.135 M NaCl. Divide into 50-µL aliquots and store up to 6 months at –20°C. Store thawed aliquots at 4°C for 1 to 2 weeks.

Phosphate-buffered saline (PBS), 10× and 1×

For 10× solution, add the following to ~700 mL H_2O:
80 g NaCl
2 g KCl
2 g KH_2PO_4
11.5 g Na_2HPO_4
Adjust pH to 7.5
Adjust to 1 liter with H_2O
Store up to 4 weeks at room temperature

Add Na_2HPO_4 slowly to prevent clumping of salts.

For 1× solution: Dilute 50 mL of 10× PBS to a final volume of 500 mL with water (final 0.2 M). Adjust pH to 7.5 if necessary and store up to 2 weeks at 4°C.

PBS-Tween (PBST), 10× and 1×

For 1× solution: Mix 50 mL of 10× PBS (see above) and 5 mL of 5% (v/v) Tween 20. Adjust to 500 mL with H_2O. Store up to 2 weeks at room temperature.

For 10× solution: Mix 450 mL of 10× PBS (see above) and 50 mL of 5% (v/v) Tween 20. Store up to 2 weeks at room temperature.

Quality control (QC) samples

Prepare one set of negative (zero-analyte; QC-0) and positive controls (analyte concentrations giving 20%, 50%, and 80% maximal absorbance; QC-20, QC-50, QC-80) in carbaryl-free well water of the source to be analyzed (blank matrix). Prepare a second set of similar controls with 1× PBST. Prepare QC samples fresh before each assay (see Critical Parameters).

For this purpose, prepare a 0.2 µg/mL control solution of carbaryl by diluting 10 µL of 0.2 mg/mL working carbaryl standard solution (see above) in 9990 µL of 1× PBST (Fig. 2C.8.4). From this solution prepare both sets of quality control samples as follows:

QC-0 (zero analyte): blank matrix or PBST only
QC-20 (10 ng/mL carbaryl): 500 µL control solution in 9500 µL blank matrix or PBST
QC-50 (0.7 ng/mL carbaryl): 35 µL control solution in 9965 µL blank matrix or PBST
QC-80 (0.1 ng/mL carbaryl): 5 µL control solution in 9995 µL blank matrix or PBST

Substrate buffer

Add 6.81 g sodium citrate to ~350 mL H_2O. Adjust pH to 5.5 with acetic acid and bring to 500 mL with H_2O. Store up to 2 weeks at 4°C.

3,3′,5,5′-Tetramethylbenzidine (TMB), 6 mg/mL

Store 3,3′,5,5′-tetramethylbenzidine (TMB; >95% purity) dry at 2° to 8°C. Prepare a 6 mg/mL working solution in dimethyl sulfoxide (DMSO) and store at room temperature protected from light (stable at least 3 weeks under these conditions).

The solution should be colorless, and should not be used if it is yellow or brown. Use of low-purity TMB will result in a colored solution even when freshly prepared in DMSO.

COMMENTARY

Background Information

Immunochemical techniques are based on the ability of the immune system to produce an almost unlimited amount of molecules with different specificities. Immunoassays use a specific antibody as detector of the target analyte. The reaction of the analyte (or antigen) and the antibody follows the law of mass action. There are many kinds of immunoassay techniques, but the most commonly used in environmental analysis has been the enzyme-linked immunosorbent assay (ELISA). The ELISA is a heterogeneous immunoassay that uses enzymes as labels to measure the extent of the immune reaction. These enzymes catalyze the formation of a colorimetric substrate. Immunoassays can be designed as laboratory or field-portable methods, and as semiquantitative or quantitative procedures. The main advantages of immunoassays are that they are rapid, sensitive, selective, and cost effective for large sample loads. Immunoassays are therefore exceptional screening tools.

Safety assurance for human and environmental health requires analytical support that employs rapid and simple analytical procedures in order to establish efficient testing programs. However interferences from the matrix, or from other analytes likely to be present in the sample, should be evaluated (see Critical Parameters, evaluation of matrix effects). Since immunoassays are essentially carried out in aqueous media, water samples are the ideal matrices to be analyzed by this method. Application of clean-up procedures are often not necessary and samples can be directly measured. Therefore the ELISA protocol described in this unit is an appropriate option to detect carbaryl in well water samples.

Monoclonal antibodies for carbaryl were developed by Abad and Montoya (1994) using a hapten that preserves the carbamate function. With this antibody, a very sensitive indirect ELISA was developed (Abad and Montoya, 1997; Abad et al., 1997). The assay detected carbaryl down to 0.022 µg/L (D-90), with an IC_{50} of 0.058 µg/L. Only the hydroxylated degradation products of carbaryl interfered with the assay, showing cross-reactivity values from 20% to 40%. The immunoassay was validated using well water samples. The average coefficient of variation found with fortified samples was 10.7%, and the average recovery was 112%. The anti-carbaryl polyclonal antibody, R2114, and the coating antigen, 5-CONA, are available for demonstration or collaborative projects in limited amounts.

Additionally, an ELISA kit for carbaryl determination is commercially available from Strategic Diagnostics. The assay is based on the use of antibody-coated paramagnetic beads (Itak et al., 1993).

Definitions

NOTE: See UNIT 2C.1 for additional definitions.

Antigen a substance that binds the antibody in an immune reaction.

Antiserum serum from a mammal containing, among other components, the fraction of immunoglobulins.

Blank matrix sample that does not contain the analyte of interest.

Coating process of passively adsorbing an immunoreactive material on a solid phase, usually through noncovalent interactions.

Coating antigen antigen used to coat the microtiter plate in a indirect ELISA.

Competitive ELISA the analyte competes for the specific antibody with another immunoreactive reagent (i.e., the coating antigen in an indirect ELISA).

Conjugation procedure of covalently binding a hapten to a carrier protein or enzyme label.

Direct ELISA format immunoassay method consisting of a single step. The primary antibody or an analyte derivative are labeled with the enzyme to allow measurement of their binding to an antigen- or antibody-coated solid surface, respectively.

Hapten a molecule that cannot by itself induce an immune response, but can bind to the antibody.

IgG the most common subclass of immunoglobulins present in the serum.

Immunogen antigen employed to raise specific antibodies.

Indirect ELISA format immunoassay method in which the amount of a primary antibody is detected by using labeled anti-IgG antibodies.

Matrix that which encloses the target of the analysis.

Microtiter plate polystyrene plates arranged in a 96-well (8 × 12 array) format.

Serum a clear, pale yellow liquid that separates from the clot on coagulation of the blood. This fluid contains the immunoglobulin fraction.

Safety

NOTE: *Review all relevant Material Safety Data Sheets (MSDSs) prior to the analysis and take appropriate safety precautions. MSDSs are available from reagent manufacturers and on the World Wide Web.*

Read the Material Safety Data Sheet (MSDS) provided by the manufacturer for any compound with which you are not familiar. If using sodium azide to prepare buffers, avoid breathing vapors or skin contact. Carbaryl has only moderate mammalian toxicity; however, samples and particularly the concentrated stock solutions should be handled with care. DMSO is known to increase skin absorption.

Critical Parameters

General considerations

Samples and analyte. Because carbaryl hydrolyzes in water, samples should be stored at 4°C and analyzed within 4 or 5 days after collection. Similarly, a fresh calibration standard should be prepared each time an assay is run. Carbaryl is highly susceptible to chemical hydrolysis and biodegradation, leading to 1-naphthol as the main degradation product. Therefore, when previous contamination with carbaryl is to be assessed, measurement of 1-naphthol is recommended (*UNIT 2C.9*).

It is extremely important that measurements take place in the linear range of the sigmoid curve. Therefore, the working range (80% to 20% maximal absorbance) should be determined on each plate. It will also speed sample analysis to prepare and analyze several dilutions of the sample, ensuring that one is within the working range.

Incubation and storage. Allow all reagents and buffers to reach room temperature before use. Careful control of incubation times is also advisable to ensure reproducibility. Because temperature and time cannot be stringently controlled when multiple plates are run simultaneously or when conducting analysis in the field, each ELISA plate should contain a calibration curve. Since this technique is based on the use of biological reagents, it is important to follow the storage instructions given by the supplier.

Solvent. Whenever a solid-phase extraction technique is used to clean up or concentrate a sample, be sure that the amount of organic solvent employed does not interfere with the assay, or prepare the standard curve in the presence of the same amount of solvent as contained in the sample to be analyzed (see evaluation of matrix effects below). Of the solvents studied with this method (methanol, acetone, and acetonitrile), methanol is the most tolerated by the assay. Concentrations of methanol below 10% do not affect assay performance. As little as 2% acetone or acetonitrile dramatically changes the calibration curve parameters (Marco et al., 1993).

Evaluation of matrix effects

When a series of analyses of a particular kind of sample are to be performed, the effect of the matrix on the ELISA must be determined. Matrix effects of water samples are usually a consequence of pH or of the presence of trace metals, dissolved organic matter, or excessive salt concentration. Matrix effects are most often manifested as an inhibition of color in a sample that should not contain analyte (QC-0; see Quality Control), although in some cases an increase in the absorbance can be observed.

Matrix effects can be determined by ELISA using several methods. Whenever a "blank" sample matrix (i.e., sample matrix that is free of the analyte of interest) is available, a standard calibration curve can be prepared in this matrix. By simultaneously analyzing a curve prepared from blank matrix standards and one prepared with assay buffer, it is possible to observe whether the parameters of the two curves are significantly different (see Fig. 2C.8.5 for as-

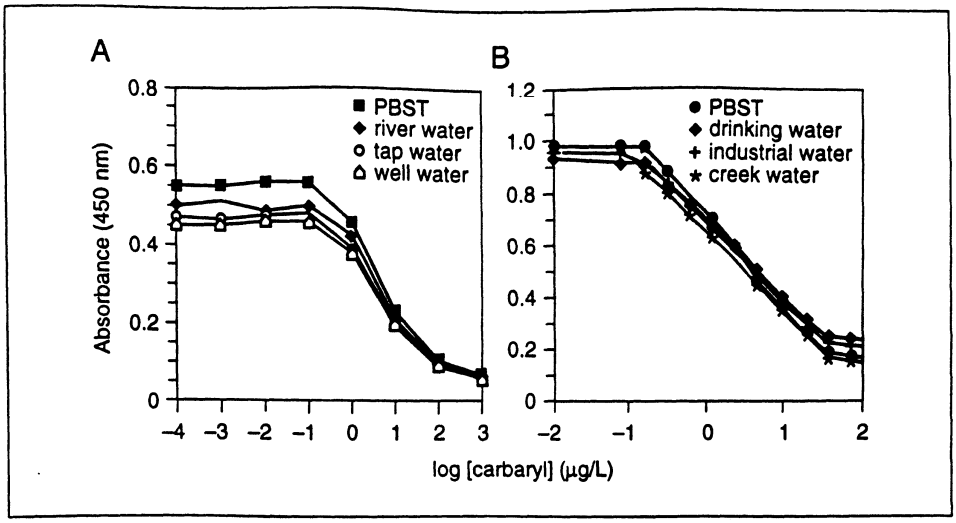

Figure 2C.8.5 Parallelism between standard curves prepared in assay buffer and those prepared in different matrices. Water samples were used without any dilution or clean-up step.

sessment of the effect of various matrices on the carbaryl ELISA). The regular use of quality control samples prepared with blank sample matrix verifies the integrity of the data.

If a blank sample matrix is not available, a sample (preferably with a low analyte level) can be used and the method of standard additions applied (Miller and Miller, 1984). The method consists of splitting the sample into two fractions, one of which is fortified. The concentration of both fractions is then measured by ELISA, and the concentration determined for the sample is subtracted from the one that was fortified. The difference should correspond to the fortification level; otherwise a matrix effect should be assumed.

Another way to verify the results when a blank sample matrix is not available is to analyze the sample at several dilutions. The dilutions chosen should yield absorbances on the linear portion of the standard curve. In the absence of a matrix effect, a plot of the absorbances obtained from each dilution should be parallel to the slope of the calibration curve prepared in the assay buffer.

When a matrix effect is evidenced, several approaches can remedy the effects. (1) A clean-up method can be used on the sample before analysis by ELISA. Several procedures have been described for water samples containing carbaryl. Many of them are based on solid-phase extraction techniques (e.g., Marco et al., 1995). (2) The sample can be diluted, which takes advantage of the assay sensitivity, as many interferences are simply "diluted away." The dilution factor can be determined in a simple experiment: the matrix is serially diluted with the assay buffer and each dilution is fortified with a known amount of the target analyte. By measuring these samples in the ELISA, it is possible to see the minimal dilution factor that will give 100% recovery of the fortified amount. The dilution factor then becomes a factor in reporting the assay's detection limit. (3) The standard curve can be prepared in the matrix of interest. In this way the interferences are normalized across the standard curve.

Troubleshooting

The reader is directed to other publications for general information regarding guides to troubleshooting an immunoassay (e.g., Schneider et al., 1995; Gee et al., 1996). For carbaryl, a decrease in the assay sensitivity may be attributed to the hydrolysis of the standard stock solution. When carbaryl standards were stored in aqueous media at 4°C for 1 month, as much as 25% of the carbaryl was converted to 1-naphthol (Krämer et al., 1994). Additional information on the environmental persistence of carbaryl can be found in Ripley and Chau (1982).

Method Performance

Accuracy

The routine use of quality control samples serves to evaluate method accuracy at three different levels within the linear range. In a program that monitored the input of agrochemicals into the ground water of Almería (Andalucia, Spain; Marco et al., 1995), the method performance for well water samples

was evaluated. Samples were spiked at different levels, as shown in Table 2C.8.1. As an indication of the method accuracy, all recoveries were high, especially when samples had been spiked at low concentration levels. Examination of the standard deviation revealed that precision was higher if measurements were within the working range of the assay. Table 2C.8.2 shows the intraplate and interplate (day-to-day) variation observed during this study, as well as the observed shift in calibration curve parameters. As a demonstration of method performance, the ELISA protocol for carbaryl analysis was compared with EPA Method 531 (EPA, 1991) using on-line solid-phase extraction liquid chromatography and postcolumn derivatization with fluorescence detection (on-line SPE-LC-PCR-FD; Marco et al. 1995). Ten blank well water samples were spiked with carbaryl and split prior to analysis by both methods. Linear regression analysis of the correlation between the two methods yielded a slope of 1.08 and an intercept of 0.02, with a correlation of 0.998.

During this evaluation the method did not produce any false negative results. Table 2C.8.3 summarizes the results obtained during a four-month monitoring period of five wells of the Aquifer Campo de Nijar in Almería (Andalucia, Spain). During the period of pesticide application (May and June), the carbaryl level in ground water clearly exceeded the upper limit of 0.1 μg/L established for drinking waters by the European Community (Commission of the European Communities, 1980). The results determined by the ELISA protocol described in this unit are in close approximation to those

Table 2C.8.1 Recoveries Obtained by On-line SPE-LC-PCR-FD and ELISA Measuring Well Water Samples Spiked with Different Levels of Carbaryl[a]

Spike level (μg/L)	SPE-LC-PCR-FD (% recovery)	ELISA (% recovery)	ELISA (% RSD)
0.02	200	200	30
0.05	115	120	45
0.1	110	130	12
0.25	88	44	8
0.8	97.5	71.2	11
1	120	135	9
2	98	98	12
5	99.6	64	15
50	99.6	99	8
100	100	110	41

[a]Average and percent relative standard deviation (% RSD) of recoveries found using two plates measured on different days, each using three replicates. Data published by Marco et al. (1995).

Table 2C.8.2 ELISA Parameters[a]

Parameter	Average value	Standard deviation	*n*
IC_{50} (μg/L)	0.71	0.25	20
Slope	0.77	0.19	20
Working range (μg/L)			
Upper limit	10.6	8.05	20
Lower limit	0.11	0.08	20
Coefficient of variation (%)			
Intraplate	2.2	1.36	9
Day-to-day	21	8	6

[a]IC_{50} and slope values are averages of parameters extracted from four-parameter logistic equations used to fit the standard curve (see Basic Protocol). Working range defines the linear portion of the curve, with upper and lower limits equal to concentrations giving 20% and 80% B_0, respectively. Coefficient of variation refers to the IC_{50} value. Data published by Marco et al. (1995).

Table 2C.8.3 Results from a 4-Month Dual-Method Well-Water Monitoring Program for Carbaryl[a]

Sample	May		June		July		August	
	ELISA	EPA	ELISA	EPA	ELISA	EPA	ELISA	EPA
Well 1	0.11 ± 0.2	0.12	1.37 ± 0.2	1.03	ND	ND	ND	ND
Well 2	0.09 ± 0.00	0.04	0.64 ± 0.22	0.71	ND	ND	ND	ND
Well 3	0.12 ± 0.1	0.1	0.41 ± 0.23	0.68	ND	ND	ND	ND
Well 4	0.08 ± 0.01	0.05	0.56 ± 0.19	0.89	ND	ND	ND	ND
Well 5	0.17 ± 0.01	0.14	0.34 ± 0.18	0.103	NT	NT	NT	NT

[a]Samples were collected monthly in 1994 from five wells of the aquifer Campo de Nijar, Almería (Andalucia, Spain). Samples were split and analyzed by ELISA and on-line SPE-LC-PCR-FD (EPA Method 531.1). ELISA data is the mean and standard deviation from at least three experiments run on three different days, utilizing three replicates each. Values expressed in µg/L. ND, not detected (detection limit = 0.06 µg/L); NT, not tested.

obtained by on-line SPE-LC-PCR-FD (Marco et al., 1995).

Cross-reactivity

The potential interference produced in the assay by other compounds is evaluated in cross-reactivity studies. Cross-reactivity may occur when interfering compounds share common chemical or structural features with the target analyte and/or the immunizing hapten used to produce the antiserum. Several methods to calculate cross-reactivity have been reported. A simple way is to prepare a standard curve for the interfering compound as described for the target analyte, and to analyze the compound on the ELISA plate simultaneously with carbaryl. Cross-reactivity values can then be calculated as follows: [IC_{50} (carbaryl)/IC_{50} (cross-reactant)] × 100. The ELISA protocol described in this unit is very specific for carbaryl. Carbaryl degradation products, other carbamate insecticides, phenylurea herbicides, and naphthalene derivatives have been evaluated and their interference with the assay is negligible (Table 2C.8.4). Only the herbicide naphthaleneacetamide shows significant cross-reactivity; however, this compound is rarely used, and is thus less likely to be found in environmental samples.

Time Considerations

Set up for the ELISA should begin a day in advance. Coating the plates takes ~30 min plus an overnight incubation, and although coated ELISA plates can be stored frozen for several days, it is preferable to coat fresh plates for each experiment. Additionally, the sensitivity of the assay is improved by preincubating the samples (and standards) with the antibody overnight at 4°C. Preparing standards and quality control samples takes ~1 hour, and setting up samples and controls for preincubation with antibody takes ~1 hour plus the overnight incubation. On the day of the assay, processing ELISA plates takes ~3 hr.

An advantage of using ELISA to screen environmental samples is that multiple samples can be run on the same ELISA plate. Additionally, several plates can be processed simultaneously, increasing the sample load per assay day. For example, an average analyst can easily prepare and process 10 plates per day. From Figure 2C.8.1, 24 samples can be placed on a single plate, thus as many as 240 samples can be processed in ~2 days (from coating the plates to final report). In contrast it takes 85 min for a single sample run using on-line SPE-LC-PCR-FD.

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Table 2C.8.4 Cross-reactivites of Compounds Structurally Related to Carbaryl and to Hapten 8[a]

Compound	Cross-reactivity
Aldicarb	4.2
Carbaryl	100
Carbofuran	3
3,7-Dihydroxycarbofuran	<0.001
Diuron	0.14
Fenuron	0.009
3-Hydroxycarbaryl	3.7
4-Hydroxycarbaryl	3.6
5-Hydroxycarbaryl	4
7-Hydroxy-3-oxocarbofuran	<0.001
Monuron	0.02
Naphthalene	<0.001
1-Naphthaleneacetamide	84
1-Naphthol	<0.001
Naptalam	0.05
3-Oxocarbofuran	<0.001
Propham	0.001
Propoxur	4.2

[a]Hapten 8 is the hapten used for raising the anti-carbaryl polyclonal antibody R2114 (see Fig. 2C.8.3). Data for 3-oxocarbofuran, 7-hydroxy-3-oxocarbofuran, and 3,7-dihydroxycarbofuran from Marco et al. (1995); all other data extracted from Marco et al. (1993).

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