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Development of an Immunoassay for the Residues of the Herbicide Bensulfuron-Methyl

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To develop a competitive indirect enzyme-linked immunosorbent assay based on polyclonal antibodies for the detection of the sulfonylurea herbicide bensulfuron-methyl, seven structurally related haptens were synthesized. Four of them mimicking the target analyte were conjugated to keyhole limpet hemocyanin by the *N*-hydroxysuccinimide activated ester method to use as immunogens, and all of them were conjugated to bovine serum albumin to use as plate-coating antigens. Polyclonal antibodies raised in rabbits and the coating antigens were screened and selected for the assay in simple homologous and heterologous ELISA formats. Three sensitive heterologous ELISAs were selected and optimized, showing the average IC_{50} values of bensulfuron-methyl as low as 0.17, 0.09, and 0.09 ng/mL, the detection ranges of 0.04−0.60, 0.01−0.60, and 0.04−0.25 ng/mL, and the lowest detection limits of 0.03, 0.002, and 0.03 ng/mL, respectively. The cross-reactivities of other sulfonylurea herbicides and metabolites of bensulfuron-methyl to the antibodies were less than 15% in the two assays. Recoveries from the analyte-fortified water samples in assay **I** were in the range of 81−125% by simple dilution. The correlation between the ELISA and HPLC was 0.999 ($n = 15$) with a slope of 1.37 in the analysis of groundwater samples fortified with bensulfuron-methyl. The results obtained strongly indicate that the ELISA can be a highly sensitive and convenient tool for detecting bensulfuron-methyl residues in agricultural and environmental samples.

KEYWORDS: Bensulfuron-methyl; sulfonylurea herbicide; ELISA; polyclonal antibodies; monitoring

INTRODUCTION

Since their development in the 1970s, sulfonylurea herbicides have been known to have many innovative properties. Because of their low mammalian toxicity and very high herbicidal activity, they have been in wide use to control broad-leaved weeds and some grasses in a variety of crops (*1*). Their extremely low application rate (as low as 2 g/ha), as well as their chemical and thermal instability, requires a highly sensitive analytical method for detecting their residues in environmental and agricultural samples. The early analytical approaches utilized normal-phase high-performance liquid chromatography (HPLC) with photoconductivity detection (*2*). Because of their extremely low volatility and thermal instability, they should be derivatized (*3*−*6*), or hydrolyzed (*7*), prior to gas chromatography (GC). The residues of some sulfonylurea herbicides in water (*8*−*9*) and soil (*10*) were determined by capillary electrophoresis (CE). Sensitive and selective immunoassays for some sulfonylurea herbicides in soil and water were relatively recently described for use with other complementary analytical techniques (*11*−*17*). Bioassays using some species, such as sugar beet, lentil, or green alga, susceptible to sulfonylurea herbicides were also reported (*18*−*19*).

Bensulfuron-methyl, methyl 2-[[[[[(4,6-dimethoxypyrimidin-2-yl)amino]carbonyl] amino]sulfonyl]methyl]benzoate, also belongs to the class of systemic sulfonylurea herbicides inhibiting acetolactate synthase, a key enzyme in the biosynthesis of the branched-chain amino acids of target plants. It is used for the pre- and post-emergence control of annual and perennial broad-leaved and sedge weeds in rice (*1*). The most common method for the detection of bensulfuron-methyl involves HPLC with either UV (*20*−*21*) or photoconductivity detection (*22*).

The instrumental methods such as HPLC, GC, and CE are accurate, but they are expensive and time-consuming and need much effort for sample preparation before analysis. Bioassay is a sensitive tool for the sulfonylurea herbicides but may lack selectivity, as it could encounter cross-reactivities by other herbicidally active compounds that are chemically similar to the target analyte. On the contrary, immunoassays generally are rapid, sensitive, selective, and cost-effective. Recently, Kawada et al. (*15*) reported on an ELISA for bensulfuron-methyl based on monoclonal antibodies. Yuan et al. (*17*) also worked on a fluoroimmunoassay for bensulfuron-methyl using a monoclonal antibody.

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The purpose of this investigation is to develop a competitive indirect enzyme-linked immunosorbent assay (ciELISA) for monitoring and efficiently quantifying bensulfuron-methyl residues in agricultural and environmental samples, using polyclonal antibodies produced against various immunogens mimicking the analyte.

MATERIALS AND METHODS

Chemicals. Bensulfuron-methyl of analytical and working grade was a gift from Du Pont. Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat anti-rabbit IgG peroxidase conjugate as the second antibody, and Freund's complete and incomplete adjuvants were all obtained from Sigma Chemical Co. (St. Louis, MO). All reagents for hapten synthesis were of analytical reagent grade and were used as supplied. For the cross-reactivity study, nicosulfuron was supplied by Ishihara Sangyo, azimsulfuron and thifensulfuron-methyl were supplied by Du Pont, ethoxysulfuron was provided by Aventis, cyclosulfamuron was from BASF, cinosulfuron was provided by Novartis, imazosulfuron was supplied by Takeda, and pyrazosulfuron-ethyl was provided by Nissan.

Instrument. ^{1}H nuclear magnetic resonance (NMR) spectra of synthesized compounds were obtained on a 300 MHz NMR spectrometer, DPX300 (Bruker, Germany) using tetramethylsilane as an internal standard. Infrared (IR) spectra were obtained on an FT−IR spectrometer, IFS-66/FRA106S (Bruker, Germany). Fast atom bombardment mass spectra (FABMS) by using 3-nitrobenzyl alcohol as a matrix were obtained on a JEOL four sector tandem mass spectrometer, JMS-HX/HX110A (JEOL, Japan), in the Korea Basic Science Institute. Melting points were determined on a Gallenhamp melting point apparatus. R_f values refer to thin-layer chromatography (TLC) on silica gel 60 F_{254}, precoated plates (Merck, Germany) with visualization under exposure to either ultraviolet light (254 nm) or iodine vapor. Ultraviolet−visible (UV/Vis) spectra were obtained on a U-2000 spectrometer (Hitachi, Japan). HPLC analysis was carried out by using a Hewlett-Packard 1100 series HPLC equipped with a diode array detector. ELISA was performed on 96-well microtiter plates (Nunc-Immuno plate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader, Bio-Rad Model 550 (Hercules, CA).

Hapten Synthesis and Verification. *Hapten-**1**, 2-{[({[(4,6-dimethoxypyrimidin-2-yl)amino]carbonyl}amino)sulfonyl]methyl}-benzoic acid*, *bensulfuron.* Hydrolysis of bensulfuron-methyl was conducted to synthesize hapten-***1***. That is, bensulfuron-methyl (1000 mg, 2.44 mmol) dissolved in 30 mL of 1 N NaOH solution was stirred at 65 °C until TLC showed no bensulfuron-methyl and the Na form of bensulfuron with a low R_f value, compound ***1***. After 90 min the reaction mixture was cooled and poured into 50 mL of water. The aqueous solution was washed with 50 mL of ethyl acetate to remove some impurities, acidified to pH 2 with 6 N hydrochloric acid, and then extracted with ethyl acetate (2 × 50 mL). The combined organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness under reduced pressure to give 850 mg of hapten-***1*** (bensulfuron) as a white solid: TLC (methanol/methylene chloride/acetic acid=1:9:0.1, v/v/v) R_f, 0.56; mp 186 °C. ^{1}H NMR (DMSO-d_6): δ 7.81 (d, 1 H, J = 7.5 Hz, Ar), 7.48−7.35 (m, 3H, Ar), 5.92 (s, 1H, pyrimidine H), 5.32 (s, 2H, CH_2SO$_2$), 3.73 (s, 6H, OCH_3). IR (ν_{max}, KBr) 3580−3210, 1718, 1654, 1457, 1364, 1160. Low FAB (+)-MS, m/z 397 [M + H]$^+$.

*Hapten-**2**, 4-[(2-{[({[(4,6-dimethoxypyrimidin-2-yl)amino]carbonyl}-amino)sulfonyl] methyl}benzoyl)oxy]butanoic acid.* To a cool, stirred solution of 2-(chloromethyl)benzoyl chloride, ***3*** (2400 mg, 12.70 mmol) in 20 mL of methylene chloride on an ice bath was added sodium 4-hydroxybutanoate (2160 mg, 15.22 mmol), together with triethylamine (1540 mg, 15.22 mmol). Thereafter, 4-(dimethylamino)pyridine (DMAP, 7 mg, 0.64 mmol) was added at room temperature. The mixture was stirred for 2 h, acidified with 20 mL of 5% HCl, and extracted with methylene chloride (2 × 30 mL). The combined organic layer was dried over anhydrous magnesium sulfate, and concentrated on a rotary evaporator under reduced pressure. The residue was purified by silica gel flash chromatography using ethyl acetate/*n*-hexane mixture (1:1, v/v) as an eluent and by preparative TLC using ethyl acetate as a developing solvent to give 383 mg of compound ***4*** as a white solid: TLC (ethyl acetate) R_f, 0.4 (tailing). ^{1}H NMR (CDCl$_3$): δ 7.95 (d, 1H, J = 7.5 Hz, Ar), 7.54−7.37 (m, 3H, Ar), 5.03 (s, 2H, CH$_2$Cl), 4.34 (m, 2H, OCH_2CH$_2$CH$_2$COOH), 2.55 (m, 2H, OCH$_2$CH$_2$CH_2COOH), 2.49 (m, 2H, OCH$_2$CH_2CH$_2$COOH).

To a solution of the compound ***4*** (383 mg, 1.49 mmol) in 5 mL of methanol was added thiourea (233 mg, 1.64 mmol). The mixture was refluxed for 18 h, and concentrated on a rotary evaporator under reduced pressure. The residue was purified by preparative TLC using ethyl acetate as a developing solvent to give 383 mg of compound ***5*** as a white solid. A suspension of the compound ***5*** (383 mg, 1.49 mmol) in 14 mL of methylene chloride/water mixture (1:1, v/v) was bubbled with excess chlorine gas for 1 h at 0 °C. The resulting yellow mixture was diluted with 30 mL of methylene chloride and then washed with water (2 × 30 mL). The organic layer was dried over anhydrous magnesium sulfate. The solvent was removed on a rotary evaporator under reduced pressure to give 512 mg of compound ***6*** as a white solid: ^{1}H NMR (CDCl$_3$): δ 8.02 (d, 1H, J = 7.5 Hz, Ar), 7.63−7.49 (m, 3H, Ar), 5.62 (s, 2H, CH$_2$SO$_2$), 4.39 (m, 2H, OCH_2CH$_2$CH$_2$COOH), 2.52 (m, 2H, OCH$_2$CH$_2$CH_2COOH), 2.10 (m, 2H, OCH$_2$CH_2CH$_2$-COOH).

To a cooled solution of the compound ***6*** (512 mg, 1.60 mmol) in 20 mL of ethyl acetate on an ice bath was added an aqueous solution of 25% ammonium hydroxide (0.25 mL). The reaction mixture was stirred for 20 min at 0 °C. Thereafter, an excess of ammonium hydroxide solution (0.2 mL) was again added. The mixture was stirred for 2 h and then washed with 20 mL of 5% hydrochloric acid. The organic layer was dried over anhydrous magnesium sulfate. The solvent was removed by evaporation to give 31 mg of compound ***8*** as a white solid: ^{1}H NMR (CDCl$_3$): δ 7.97 (d, 1H, J = 7.5 Hz, Ar), 7.57−7.50 (m, 3H, Ar), 4.92 (s, 2H, CH$_2$SO$_2$), 4.77 (br, 2H, NH$_2$), 4.41 (m, 2H, OCH_2CH$_2$CH$_2$COOH), 2.55 (m, 2H, OCH$_2$CH$_2$CH_2COOH), 2.12 (m, 2H, OCH$_2$CH_2CH$_2$COOH).

To a stirred solution of the compound ***8*** (31 mg, 0.11 mmol) in 3 mL of acetonitrile were added phenyl (4,6-dimethoxypyrimidin-2-yl)-carbamate (32 mg, 0.11 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 34 mg, 0.22 mmol). The reaction mixture was stirred at room temperature for 30 min, diluted with ethyl acetate (30 mL), and washed with 30 mL of 5% HCl. After the organic layer was dried over anhydrous magnesium sulfate, the solvent was removed by a rotary evaporator under reduced pressure. The residue was recrystallized from diethyl ether/*n*-hexane mixture (1:1, v/v) to give 27 mg of pure hapten-***2*** as a white solid: TLC (methanol/methylene chloride/acetic acid, 1:9:0.1, v/v/v) R_f, 0.56. ^{1}H NMR (CDCl$_3$): δ 7.91 (m, 1H, J = 7.5 Hz, Ar), 7.9 (br s, 1H, NHPy), 7.52−7.49 (m, 3H, Ar), 5.72 (s, 1H, pyrimidine H), 5.39 (s, 2H, CH$_2$SO$_2$), 4.40 (m, 2H, OCH_2CH$_2$CH$_2$-COOH), 3.78 (s, 6H, OCH$_3$), 2.53 (m, 2H, OCH$_2$CH$_2$CH_2COOH), 2.03 (m, 2H, OCH$_2$CH_2CH$_2$COOH). Low FAB (+)-MS, m/z 483.8 [M + H]$^+$.

*Hapten-**3**, 6-[(2-{[({[(4,6-dimethoxypyrimidin-2-yl)amino]carbonyl}-amino)sulfonyl] methyl}benzoyl)oxy]hexanoic acid.* For the synthesis of an ester ***10***, the compound ***3*** (1000 mg, 5.29 mmol) was added to 5 mL of methanol. The solution was refluxed for 3 h, and the solvent was removed on a rotary evaporator under reduced pressure to give 1000 mg of methyl 2-(chloromethyl)benzoate, ***10***, as an oily product: TLC (methylene chloride) R_f, 0.77.

Thiourea (413 mg, 5.42 mmol) was added to a solution of the compound ***10*** (1000 mg, 5.42 mmol) in 10 mL of methanol and the mixture was refluxed for 18 h. The resulting solution was concentrated under reduced pressure and the residue was recrystallized twice from ethyl acetate to give 1450 mg of compound ***11*** as a malodorous solid: TLC (ethyl acetate) R_f, 0.08.

The suspension of the compound ***11*** (1450 mg) in 30 mL of methylene chloride/water mixture (1:1, v/v) was bubbled with an excess of chlorine gas at 0 °C for 1 h. The resulting mixture was diluted with 30 mL of methylene chloride and then washed twice with 20 mL of water. The organic layer was dried over anhydrous magnesium sulfate and concentrated. The residue was recrystallized from ethyl acetate and then purified by filtration through a short pad of silica gel using ethyl acetate/*n*-hexane mixture (3:7, v/v) as an eluent to give 1390 mg of compound ***12*** as a white solid: TLC (ethyl acetate) R_f, 0.93. ^{1}H NMR

($CDCl_3$): δ 7.81 (d, 1H, $J = 7.5$ Hz, Ar), 7.53−7.37 (m, 3H, Ar), 5.64 (s, 2H, CH_2SO_2), 3.89 (s, 3H, $COOCH_3$).

The compound ***12*** (695 mg, 2.80 mmol) was dissolved in 30 mL of ethyl acetate, and the solution was cooled on an ice bath. *Tert*-butylamine (409 mg, 5.60 mmol) was added slowly to the solution. The resulting white suspension was stirred at 0 °C for 1 h, and the stirring was continued at room temperature for 2 h. The mixture was washed with 30 mL of 5% hydrochloric acid. The organic layer was dried over anhydrous magnesium sulfate and evaporated under reduced pressure to give 440 mg of compound ***13*** as a white solid: TLC (ethyl acetate/*n*-hexane, 1:1, v/v) R_f, 0.73.

The hydrolysis of compound ***13*** (440 mg, 1.62 mmol) was conducted in 20 mL of tetrahydrofuran (THF)/1 N NaOH mixture (1:1, v/v) at 65 °C for 5 h. THF was removed on a rotary evaporator under reduced pressure. The alkaline solution was acidified with 20 mL of 5% hydrochloric acid, and then extracted with ethyl acetate (2 × 30 mL). The combined organic layer was dried over anhydrous magnesium sulfate and concentrated on a rotary evaporator to give 270 mg of compound ***14*** as a white solid: TLC (ethyl acetate/*n*-hexane, 1:1, v/v) R_f, 0.22 (tailing). ^{1}H NMR ($CDCl_3$): δ 13.1 (br s, 1H, COOH), 7.81 (d, 1H, $J = 7.5$ Hz, Ar), 7.53−7.37 (m, 3H, Ar), 4.88 (s, 2H, CH_2SO_2), 1.23 (s, 9H, NHC$(CH_3)_3$).

To a stirred solution of the compound ***14*** (1000 mg, 3.7 mmol) in 20 mL of acetonitrile were added 6-bromohexanoic acid (1008 mg, 5.6 mmol) and DBU (1127 mg, 7.4 mmol). After the reaction mixture was heated at 50 °C for 1 h, it was concentrated on a rotary evaporator, acidified with 30 mL of 5% hydrochloric acid, and extracted with ethyl acetate (2 × 30 mL). The combined organic layer was dried over anhydrous magnesium sulfate, and the solvent was removed on a rotary evaporator. The residue was purified by using preparative TLC on silica gel twice with methylene chloride/isopropyl alcohol mixture (9.5:0.5, v/v) as a developing solvent to give 180 mg of compound ***15*** as a white solid: TLC (methylene chloride/isopropyl alcohol, 9.5:0.5, v/v) R_f, 0.4. ^{1}H NMR ($CDCl_3$): δ 7.91 (d, 1H, $J = 7.5$ Hz, Ar), 7.60−7.30 (m, 3H, Ar), 4.88 (s, 2H, CH_2SO_2), 4.30 (m, 2H, O$CH_2$$CH_2CH_2CH_2$-$CH_2$COOH), 2.61 (br s, 1H, CH_2SO_2NH), 2.36 (m, 2H, OCH_2CH_2-$CH_2CH_2CH_2$COOH), 1.78 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2$COOH), 1.67 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2$COOH), 1.48 (m, 2H, $OCH_2CH_2CH_2$-CH_2CH_2COOH), 1.25 (s, 9H, NHC$(CH_3)_3$).

To 1 mL of trifluoroacetic acid was added the compound ***15*** (150 mg, 0.49 mmol). The mixture was stirred at room temperature for 2 h and then evaporated to dryness. Water (30 mL) was added to the residue, and the mixture was extracted with ethyl acetate (2 × 30 mL). The combined organic layer was dried over anhydrous magnesium sulfate and concentrated on a rotary evaporator. The residue was purified by using a chromatotron using ethyl acetate/*n*-hexane mixture (1:1, v/v) as an eluent to give 113 mg of a transparent oily compound ***16***: TLC (methylene chloride/methanol, 1:9, v/v) R_f, 0.8. ^{1}H NMR ($CDCl_3$) δ 7.91 (d, 1H, $J = 7.5$ Hz, Ar), 7.52 (m, 2H, Ar), 7.42 (m, 1H, Ar), 4.88 (s, 2H, CH_2SO_2), 4.85 (s, 2H, $CH_2SO_2NH_2$), 4.32 (t, 2H, $J = 7$ Hz, O$CH_2CH_2CH_2CH_2CH_2$COOH), 2.36 (m, 2H, $OCH_2CH_2CH_2CH_2$-COOH), 1.78 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2$COOH), 1.67 (m, 2H, $OCH_2CH_2CH_2$ CH_2CH_2COOH), 1.48 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2$-COOH).

To a stirred solution of the compound ***16*** (113 mg, 0.37 mmol) in 4 mL of acetonitrile were added phenyl (4,6-dimethoxypyrimidin-2-yl)carbamate (101 mg, 0.37 mmol) and DBU (113 mg, 0.74 mmol). The reaction mixture was stirred at room temperature for 1 h, diluted with 30 mL of ethyl acetate, and washed with 30 mL of water. The organic layer was discarded, and the aqueous layer was acidified to pH 3 with 5% hydrochloric acid, and then extracted with ethyl acetate (2 × 30 mL). The combined organic layer was dried over anhydrous magnesium sulfate, the solvent was removed by evaporation under reduced pressure, and the residue was recrystallized from ethyl acetate/*n*-hexane mixture to give 97 mg of compound ***17*** (hapten-***3***) as a white solid: TLC (methanol/methylene chloride/acetic acid, 1:9:0.1, v/v/v) R_f, 0.56. ^{1}H NMR (DMSO-d_6): δ 7.81 (d, 1H, $J = 7.5$ Hz, Ar), 7.60−7.40 (m, 3H, Ar), 5.92 (s, 1H, pyrimidine H), 5.29 (s, 2H, CH_2SO_2), 4.18 (m, 2H, O$CH_2CH_2CH_2CH_2CH_2$COOH), 3.66 (s, 6H, OCH_3), 2.21 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2$COOH), 1.65 (m, 2H, $OCH_2CH_2CH_2$-CH_2CH_2COOH), 1.52 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2$COOH), 1.37 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2$COOH). Low FAB (+)-MS, *m*/*z* 511.14 $[M + H]^+$.

*Hapten-**4**,4-({[2-(methoxycarbonyl)benzyl]sulfonyl}amino)-4-oxobutanoic acid.* To 1 mL of trifluoroacetic acid was added the compound ***13*** (300 mg, 1.05 mmol). The mixture was stirred at room temperature for 3 h and then evaporated to dryness. To the residue was added 30 mL of distilled water, and the mixture was extracted with ethyl acetate (2 × 30 mL). The combined organic layer was dried over anhydrous magnesium sulfate and concentrated on a rotary evaporator under reduced pressure. The residue was recrystallized from ethyl acetate to give 255 mg of compound ***18*** as a white solid: TLC (methylene chloride/methanol, 1:9, v/v) R_f, 0.8. ^{1}H NMR ($CDCl_3$): δ 8.01 (d, 1H, $J = 7.5$ Hz, Ar), 7.61−7.40 (m, 3H, Ar), 4.90 (s, 2H, CH_2SO_2), 4.56 (s, 2H, SO_2NH_2), 3.94 (s, 3H, OCH_3).

Hapten-***4*** was synthesized according to a method introduced by Schlaeppi et al. (*12*). To 5 mL of acetonitrile was added 200 mg (0.87 mmol) of methyl-(aminosulfonyl)-*o*-toluate, compound ***18***, together with 87 mg (0.87 mmol) of succinic anhydride. Then, 265.5 mg (1.74 mmol) of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was added dropwise for 10 min under slightly cooled conditions (22 °C). After this was stirred for 2 h, the resulting solution was poured into 50 mL of distilled water and washed with 50 mL of ethyl acetate to remove some impurities. The organic layer was discarded, and the aqueous phase was acidified to pH 2 with hydrochloric acid, and then extracted with ethyl acetate (2 × 50 mL). The combined organic layer was dried over anhydrous magnesium sulfate, the solvent was removed by evaporation, and the residue was recrystallized from ethyl acetate to give 100 mg of hapten-***4*** as a white solid: TLC (methanol/methylene chloride/acetic acid, 1:9:0.1, v/v/v) R_f, 0.47. ^{1}H NMR (CD_3OD): δ 7.91 (d, 1H, $J = 7.5$ Hz, Ar), 7.52−7.42 (m, 3H, Ar), 4.88 (s, 2H, CH_2SO_2), 3.85 (s, 3H, OCH_3), 2.48 (m, 2H, COCH_2CH_2COOH), 2.43 (m, 2H, $COCH_2CH_2$COOH). Low FAB (+)-MS, *m*/*z* 329.9 $[M + H]^+$.

*Hapten-**5**, ({({({[(4,6-dimethoxypyrimidin-2-yl)amino]carbonyl}-amino)sulfonyl} methyl)benzoyl}amino)acetic acid.* To a solution of bensulfuron, the compound ***2*** (398 mg, 1 mmol) in dry *N*, *N*-dimethylformamide (DMF) was added 1,1′-carbonyldiimidazole (CDI) (172 mg, 1.06 mmol) at 0 °C. After the initial vigorous evolution of gas, the reaction mixture (the temperature of which was allowed to increase to room temperature) was stirred until no more gas evolved. Solid ethyl glycinate hydrochloride (142 mg, 1.02 mmol) was added, and the mixture was stirred until a clear solution resulted. On adding water dropwise to this solution, a precipitate formed. The precipitate was filtered out and the filtrate was concentrated under reduced pressure to afford a pale-yellow solid. It was column chromatographed on silica gel using ethyl acetate/*n*-hexane mixture (1:2, v/v) as an eluent to afford compound ***20*** (213 mg, 74.9%): mp 165 °C. ^{1}H NMR ($CDCl_3$): δ 9.30 (br s, 1 H), 7.17 (br s, 1 H), 5.70 (s, 1 H), 4.26−4.18 (m, 4 H), 3.94 (s, 6 H), 1.08 (t, $J = 7.10$ Hz, 3 H). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 179.39, 168.21, 154.75, 151.23, 67.34, 54.34, 51.20, 37.91, 14.68. IR (ν_{max}, KBr) 1723, 1610, 1454.

A solution of the compound ***20*** (103 mg, 0.29 mmol) in THF (5 mL) was cooled to 5 °C. To this solution was slowly added lithium hydroxide monohydrate (36 mg, 0.86 mmol) in water (0.9 mL). The resulting solution was stirred at 5−10 °C for 1.5 h and then diluted with 1 N hydrochloric acid (0.86 mL). The mixture was concentrated under reduced pressure to give the crude product. It was chromatographed on silica gel using methanol/chloroform mixture (1:3, v/v) as an eluent to afford hapten-***5*** (32 mg, 35%) as a pale-yellow crystalline solid: mp 220 °C. ^{1}H NMR (DMSO-d_6): δ 9.30 (br s, 1H), 7.17 (br s, 1H), 5.70 (s, 1H), 4.26−4.19 (m, 2H), 3.94 (s, 6H). ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 171.87, 168.12, 154.75, 151.23, 69.73, 56.43, 38.76. IR (ν_{max}, KBr) 3400−2460, 1714, 1456. Low FAB (+)-MS, *m*/*z* 257 $[M + H]^+$.

Hapten-***6***, *4-(3-(4,6-dimethoxypyrimidin-2-yl)-5-{[2-(methoxycarbonyl)benzyl] sulfonyl}-4-oxo-1,3,5-triazinan-1-yl)butanoic acid and Hapten-**7**, 6-(3-(4,6-dimethoxypyrimidin-2-yl)-5-{[2-{methoxycarbonyl}-benzyl]sulfonyl}-4-oxo-1,3,5-triazinan-1-yl)hexanoic acid.* A mixture of bensulfuron-methyl (2.1 g, 5.0 mmol), 4-aminobutyric acid (0.52 g, 5.0 mmol) for the synthesis of hapten-***6*** or 6-aminohexanoic acid (0.66 g, 5.0 mmol) for synthesis of hapten-***7***, and formaldehyde (35%, 5.6

g, 30.0 mmol) was stirred at 40 °C for 30 min in a three-necked round-bottomed flask equipped with an internal thermometer and a Dean−Stark trap. A solution of *N*-methylmorpholine (1.1 mL, 10.0 mmol), 1,4-dioxane (1 mL), and toluene (40 mL) was added, and the temperature was raised to 85 °C, whereupon the solution became homogeneous. Over a 90-min period, 35 mL of distillate was collected in the trap, and toluene (15 mL) was added to the reaction mixture as the internal temperature rose to 90−110 °C. The reaction mixture was cooled and concentrated to a semisolid residue. Chromatography on silica gel using methanol/chloroform mixture (1:10, v/v) as an eluent afforded hapten-***6*** as a pale-yellow crystalline solid (1.57 g, 58.4%): mp 275 °C. ^{1}H NMR (DMSO-d_6): δ 12.02 (br s, 1H), 7.86 (d, J = 7.42 Hz, 1H), 7.50 (dd, J = 7.43 Hz, J = 1.87 Hz, 1H), 7.45 (dd, J = 7.36 Hz, J = 1.87 Hz, 1H), 7.35 (d, J = 7.36 Hz, 1H), 5.91 (s, 1H), 5.32 (s, 2H), 4.71 (s, 4H), 3.91 (s, 3H), 3.83 (s, 6H), 2.44−2.03 (m, 4H), 1.39−1.49 (m, 2H). ^{13}C NMR (75.47 MHz, DMSO-d_6): 180.04, 179.99, 168.61, 166.74, 158.78, 141.49, 136.25, 133.67, 126.40, 77.92, 58.11, 57.51, 57.28, 57.62, 54.43, 51.60, 30.75, 23.35. IR (ν_{max}, KBr): 3300−2400, 1713, 1459. Low FAB (+)-MS, *m*/*z* 538 $[M + H]^+$.

According to the method mentioned above, hapten-***7*** was also obtained as the same colored crystalline solid (1.52 g, 53.7%): mp 250 °C; ^{1}H NMR (DMSO-d_6) δ 8.55 (br s, 1H), 7.69−7.58 (m, 1H), 7.51−7.33 (m, 3H), 5.83 (s, 1H), 5.30 (s, 2H), 4.72 (s, 4H), 3.92 (s, 3H), 3.86 (s, 6H), 2.46−2.05 (m, 4H), 1.47−1.36 (m, 6H); ^{13}C NMR (75.47 MHz, DMSO-d_6) δ 183.08, 180.01, 168.16, 165.58, 158.77, 141.61, 136.56, 131.34, 129.05, 77.25, 58.56, 57.49, 57.28, 56.70, 54.69, 51.10, 41.68, 35.41, 27.56, 23.45; IR (ν_{max}, KBr) 3300−2400, 1714, 1463; Low FAB (+)-MS, *m*/*z* 566 $[M+H]^+$.

Conjugation of Carboxylic Acid Haptens to Carrier Proteins. The haptens were coupled covalently to the lysine groups of the carrier proteins such as keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) according to the activated ester method (*23*). That is, each hapten (0.04 mmol) was dissolved in 0.2 mL of dry DMF with equimolar *N*-hydroxysuccinimide (NHS) and a 10% molar excess of dicyclohexylcarbodiimide. After stirring at 22 °C for 5 h, the precipitated dicyclohexylurea was removed by filtration, and about 0.2 mL of the active ester was added slowly to the protein solution (10 mg of protein in 1 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was stirred gently at 4 °C for 24 h to complete the conjugation and then dialyzed exhaustively against normal strength phosphate-buffered saline (1 × PBS; 8 g of NaCl, 0.2 g of KH_2PO_4, 1.2 g of Na_2HPO_4, and 0.2 g of KCl), which was changed twice a day for 5 days. Finally, the conjugates were dispensed into the 2-mL cryogenic vial and stored at −80 °C.

To calculate the amount of the hapten−protein conjugate to be used as immunogens or coating antigens as a protein equivalent, its protein content was determined by the Bio-Rad protein assay based on the method of Bradford (*24*). The extent of coupling of each hapten to the carrier protein was determined according to the UV spectrophotometric (*25*) or the trinitrobenzenesulfonic acid (TNBSA) method (*26*).

Immunization. Female New Zealand white rabbits weighing 3 kg were used for raising polyclonal antibodies. Routinely, 100 μg (protein equivalent) of each immunogen (hapten−KLH conjugate) in 0.5 mL of 0.85% saline was thoroughly emulsified with an equal volume of Freund's adjuvant. The emulsion was subcutaneously injected at five different sites on the neck and back of a rabbit. Complete Freund's adjuvant was used in the first injection, and Freund's incomplete adjuvant was used for the subsequent boost injections. Boosts were given every three weeks in the same manner. On the seventh day after each boost, about 10 mL of blood sample was drawn from the jugular vein of the ear to check the titer of the polyclonal antibody. The blood sample was allowed to coagulate for about 2 h at room temperature, and then it was left to stand overnight in a refrigerator. The serum was decanted and centrifuged (800*g*), and then the supernatant was stored in conveniently sized aliquots at −80 °C. Boosts were given six times.

Checkerboard Titration. A checkerboard titration (*27*) was performed with each of the antisera collected from each rabbit. The checkerboard assay selected the combination of antiserum dilution and coating antigen concentration (hapten−BSA conjugate) that would provide the greatest sensitivity in ELISA. The optimized ELISA for bensulfuron-methyl used a coating antigen concentration between 0.01 and 1 μg/mL and an antiserum dilution between 16000 and 256000. Titers of the antisera produced by twelve rabbits were evaluated. Rabbits A−C were immunized against hapten-***1***−KLH, D−F were immunized against hapten-***2***−KLH, G−I were immunized against hapten-***3***−KLH, and J−L were immunized against hapten-***4***−KLH. To check the titers of the antisera by the homologous indirect ELISA, each antiserum was diluted 256000-fold.

Indirect ELISA, Competitive Inhibition ELISA, and Cross-Reactivities. Indirect ELISA and competitive indirect ELISA were performed according to the method of Voller et al. (*28*) as modified by Harrison et al. (*29*). For the checkerboard titration, an indirect ELISA was conducted. That is, 96-well microtiter plates were coated with 100 μL/well of the hapten−BSA conjugate in a carbonate buffer and allowed to stand overnight at 4 °C. On the following day, the plates were washed five times with one-tenth strength PBST (0.1 × PBS with 0.05% Tween 20) and thoroughly tapped dry. Sites not coated with the conjugate were blocked with 200 μL/well of 3% (w/v) skim milk with 1 × PBS. After incubation at 37 °C for 1 h, the plates were washed as described above. To the wells was added 100 μL of an anti-bensulfuron-methyl antiserum diluted with 1 × PBS, and the plates were incubated at room temperature for 1 h. After washing the plate, 100 μL of a secondary antibody conjugated to the anti-rabbit IgG-horseradish peroxidase diluted 1:10000 with 1 × PBST was added, and the plates were incubated for 1 h at room temperature. The plate was washed, and 100 μL of a substrate solution (0.1 mL of 1% hydrogen peroxide and 0.4 mL of 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide (DMSO) added to 25 mL of citrate-acetate buffer, pH 5.5) was added to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μL of 4 N sulfuric acid. The yellow-colored plate was read spectrophotometrically in a dual wavelength mode at 450 nm using a reference wavelength of 655 nm. The amount of the enzyme bound, as indicated by the change of colorless substrate to blue product, is directly related to the amount of the rabbit anti-hapten antibody bound to the plate-coating antigen.

A competitive inhibition ELISA was used to assess the specificity of the antibody to free bensulfuron-methyl and the cross-reactivities of structurally related compounds to the antibody. For competition, 50 μL of standards in assay buffer or diluted samples were placed in the wells, and 50 μL of the anti-bensulfuron-methyl antiserum diluted with assay buffer was added to it. After these mixed for 30 s and incubated at room temperature for 1 h, the plate was washed. The following procedure was followed as described for the indirect ELISA. With the inhibition ELISA format, analytes that do not react with the antibody would produce absorbances near 100%; conversely, analytes that do react with the antibody would decrease in percentage of absorbance. Standard curves were calculated by mathematically fitting experimental points to a four-parameter logistic equation (*30*) using a commercial software package (Origin, Microcal).

Because the cross-reactivity is defined as the ability of structurally related compounds of the target analyte to bind to the specific antibody raised against the analyte, some other sulfonylurea herbicides, haptens, and major metabolites of bensulfuron-methyl were tested for selectivity of the ELISA by determining their respective IC_{50} values in the competitive assays. Cross-reactivity values were calculated as the ratio of the IC_{50} of the bensulfuron-methyl standard to the IC_{50} of the test compounds and expressed as a percentage.

Water Samples. All water samples were collected from agricultural areas where bensulfuron-methyl was not applied. Surface water samples were collected from a pond, a stream, and a rice paddy.A groundwater sample was from an underground well, and a soil leachate sample was from a 1-m-deep lysimeter. The water samples were collected in brown glass bottles and stored in a dark, cool place until the analyses. They are characterized by pH, electric conductivity (EC), color, and smell, as presented in **Table 1**.

ELISA and HPLC Analysis of Water Samples Fortified with Bensulfuron-Methyl. *ELISA.* The samples were filtered through a 0.45-μm filter to remove suspended solids. To test matrix effects, the water samples were diluted 10 and 50 times with ultrapure water at pH 8. For the recovery test, five concentrations (0, 2.5, 5, 10, and 25 ng/mL) of bensulfuron-methyl in water samples were prepared with the bensulfuron-methyl stock solution in ethyl acetate. For the ELISA of

Table 1. Characteristics of Water Samples Collected from the Agricultural Areas near Cheongju

water source	pH	EC[a]	color	smell
pond	7.8	0.1	pale yellow	odorless
stream	6.7	0.2	pale yellow	slightly rotten odor
rice paddy	8.4	0.3	pale yellow	odorless
soil leachate	7.9	0.4	pale yellow	odorless
underground	7.2	0.3	colorless	odorless
1 × PBS	8.0	13.2	colorless	odorless

[a] Electric conductivity, reported as mS/cm.

bensulfuron-methyl, the aliquots of each sample were diluted 50 times with ultrapure water of pH 8. Each analysis was done in triplicate.

HPLC. One liter of each groundwater sample fortified with the analyte by the above procedures was transferred into a 2-L separatory funnel, and 50 mL of a saturated NaCl solution was added to it. The solution was acidified to pH 3 with 4 N hydrochloric acid and then extracted twice with 80 mL of methylene chloride. The combined organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. After the residue was redissolved in 10 mL of distilled water, the pH of the sample was adjusted to 2−3 with 4 N hydrochloric acid. A 6-mL cartridge packed with 0.5-g C18 (Alltech Associates, Inc.) was preconditioned by passing 5 mL of methanol through it, followed by 5 mL of water. A low vacuum and a flow rate of 5−10 mL/min were used. The acidified sample and then 5 mL of distilled water were passed through the cartridge. The eluate was discarded and the cartridge was dried by suction for 15 min. Finally, the cartridge was eluted with 5 mL of 0.1% (v/v) acetic acid in ethyl acetate and the eluate was evaporated to dryness under reduced pressure. The residue was redissolved in 2.5 mL of acetonitrile, and bensulfuron-methyl was determined by HPLC (Hewlett-Packard HP 1100 series) equipped with a diode array detector on a LiChrosorb R-18 (250 mm × 4.6 mm i.d.). Operating conditions were as follows: injection volume, 20 μL; flow rate, 1.0 mL/min; detection wavelength, 254 nm; mobile phase, acetonitrile/water (pH 2.3 adjusted with H_3PO_4) (50:50, v/v).

RESULTS AND DISCUSSION

Synthesis of Haptens and Their Conjugation to Carrier Proteins. The herbicide bensulfuron-methyl in itself cannot be used as an immunogen because of its low molecular weight (MW 410.4). Therefore, haptens mimicking the analyte herbicide and containing reactive groups for conjugation to carrier proteins should be synthesized to develop an ELISA for it. The hapten is also a small molecule with a great diversity of reactive groups such as carboxyl, amino, or hydroxyl group for conjugation to proteins, and cannot, by itself, elicit an antibody response. In the present study, seven analogues of the target analyte were synthesized for use as immunogens and plate-coating antigens. For the production of antibodies capable of recognizing bensulfuron-methyl, the immunizing haptens were designed as almost perfect mimics of the structure of the target molecule in terms of electronic and hydrophobic properties. The immunizing hapten-***1*** was synthesized as bensulfuron with a carboxyl group by hydrolyzing the aromatic methyl ester group in the target molecule. Because the spacer arms with different lengths to connect the target analyte to the carrier protein have an effect on the formation of the specific and sensitive antibody (*31*−*32*), two other immunizing haptens, hapten-***2*** and hapten-***3***, were synthesized by replacing the methyl group in the ester of the

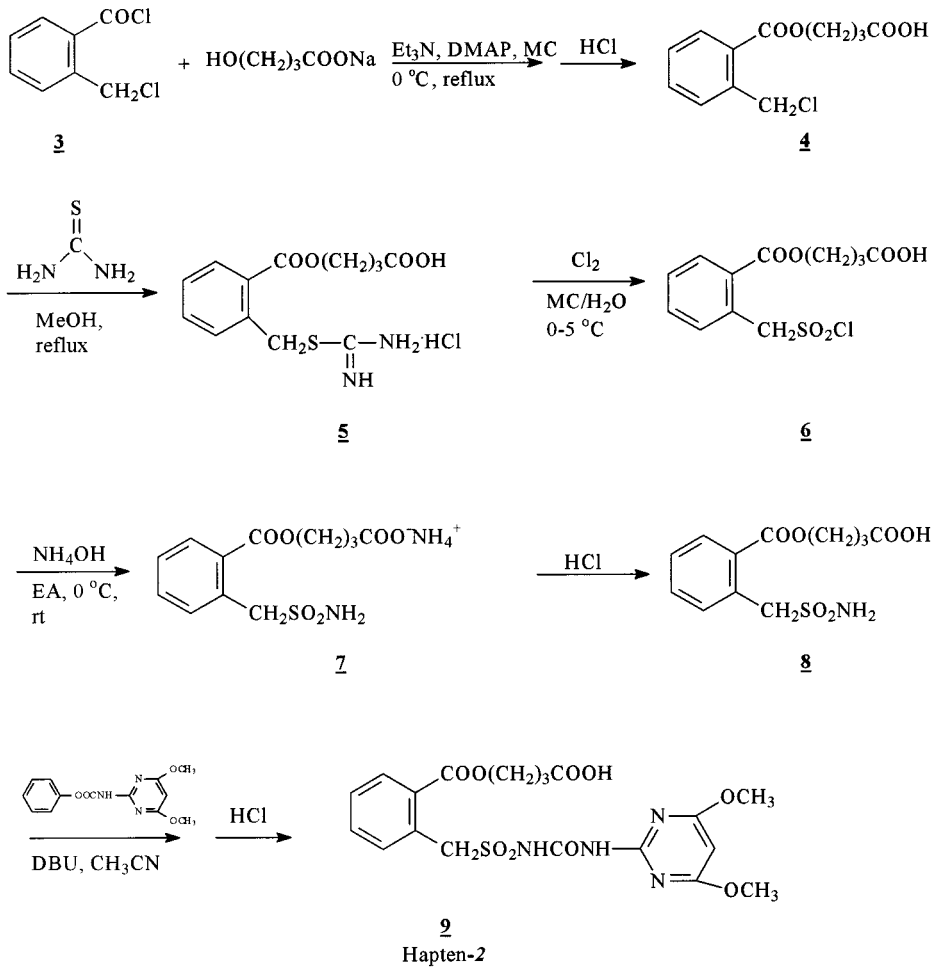


Figure 1. Synthetic route of hapten-***2***.

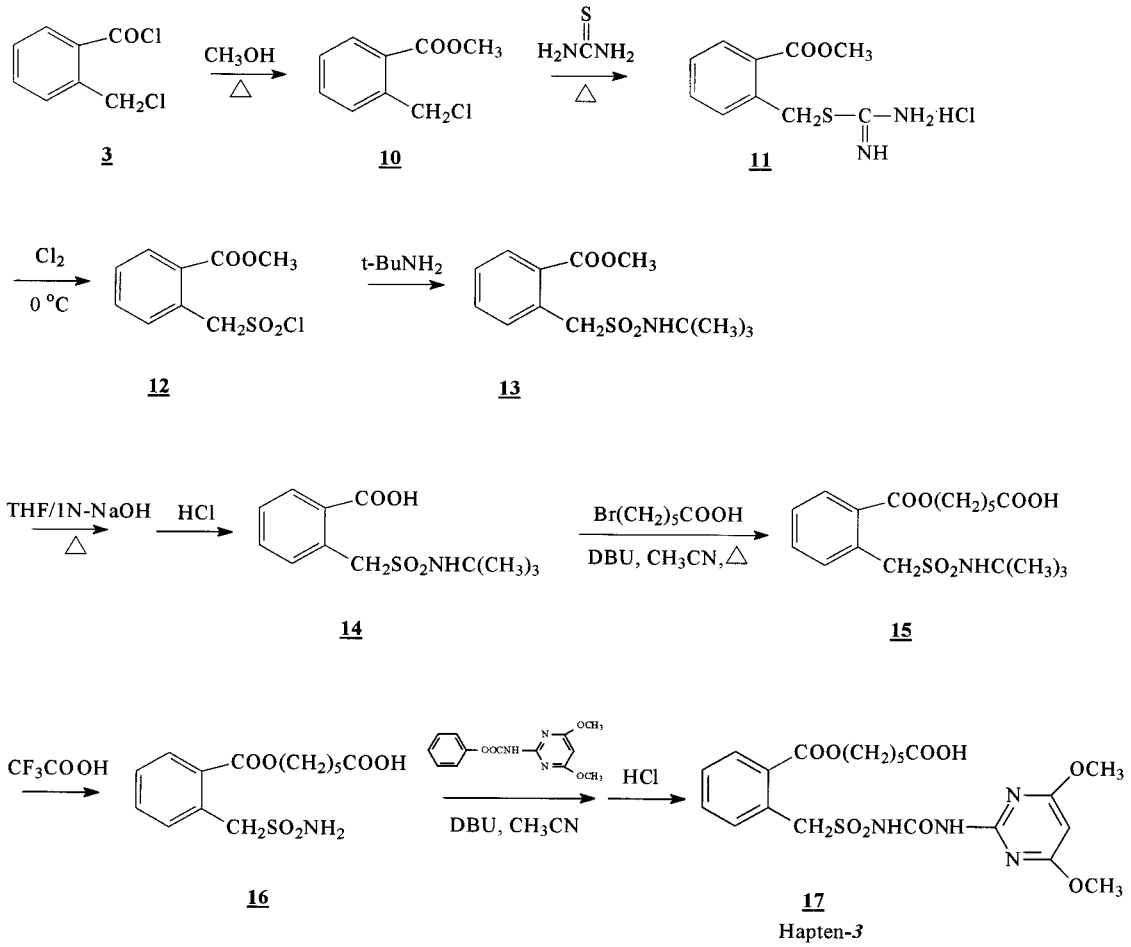


Figure 2. Synthetic route of hapten-***3***.

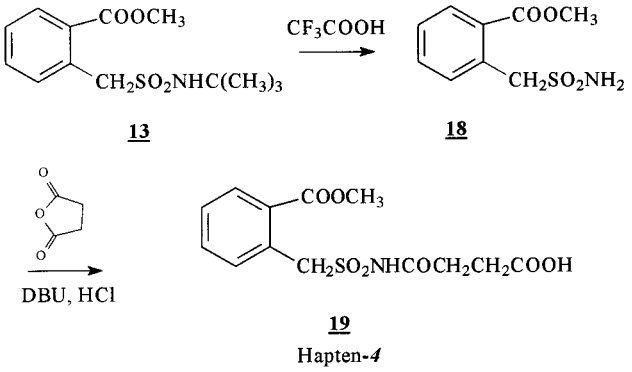


Figure 3. Synthetic route of hapten-***4***.

target molecule with four- and six-carbon spacer arms with a carboxyl group at the end for conjugation (**Figures 1** and **2**). In contrast to the above three haptens, hapten-***4*** was a mimic of only the aryl sulfonamide moiety of the analyte molecule, the other part of which was replaced by a three-carbon spacer arm. It was synthesized by the reaction of succinic anhydride as a spacer arm with the methylaminosulfonyl-*o*-toluate moiety of the analyte (**Figure 3**). Hapten-***4*** was designed adopting the methods by Schlaeppi et al. (*12*) and Simon et al. (*14*). Using haptens that were the sulfonamide moieties of triasulfuron and metsulfuron-methyl containing succinic acid as a spacer arm, they produced antibodies with a higher affinity for the parent herbicides than a hapten imitating the complete analyte molecule.

The four haptens were conjugated to KLH for use as immunogens for the antibody production. The molecular models of the immunizing haptens were identical to the target molecule in structure and geometry (data not shown).

Heterology is commonly used to eliminate problems coming from no or poor inhibition by the target compound, associated with the strong affinity of the antibodies for the bridging groups. It usually results in somewhat weaker recognition of plate-coating antigens than recognition of the target analyte. Thus, lower analyte concentrations can compete with these reagents under equilibrium conditions, which results in better assay sensitivity than homologous system (*33*−*34*). For the heterologous bensulfuron-methyl ELISA in this study, heterology included hapten heterology using different hapten structures, site heterology, linker heterology using spacer arms with different lengths (*35*−*36*), and carrier heterology. Additional haptens for plate-coating were synthesized (**Figures 4** and **5**): one coating hapten (hapten-***5***) contained a partial structure of the target molecule and two other coating haptens (hapten-***6*** and -***7***) contained a cyclization of the sulfonylurea bridge in the target molecule. All of the seven haptens containing spacer arms of different lengths and partial or cyclized structures of the sulfonylurea bridge of the target molecule were conjugated to BSA for use as coating antigens to compare homologous and heterologous ELISA formats.

The protein contents of the hapten−protein conjugates were in the range of 3.8−6.9 mg/mL. By the UV spectrophotometric method, the conjugation molar ratios of each hapten to BSA were determined as 26/1, 21/1, 59/1, 34/1, and 79/1 for hapten-***1***, -***2***, -***3***, -***5***, and -***7***, respectively, and those of each hapten to KLH were 1677/1, 606/1, and 2918/1 for hapten-***1***, -***2***, and -***3***, respectively. On the other hand, the main peak of hapten-***4*** or

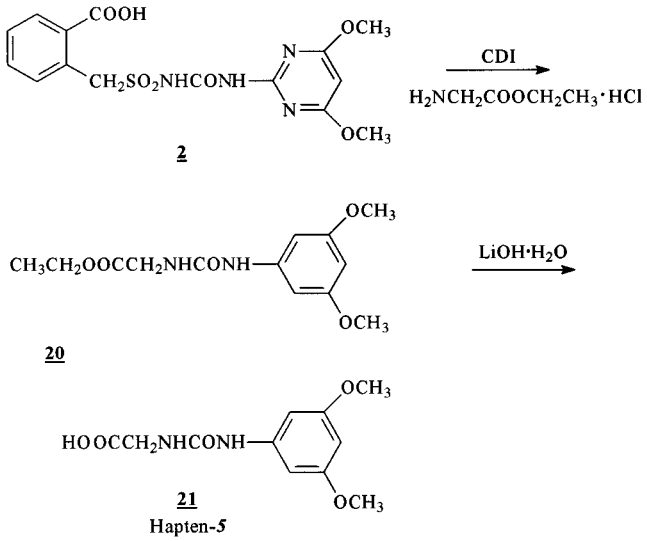


Figure 4. Synthetic route of hapten-***5***.

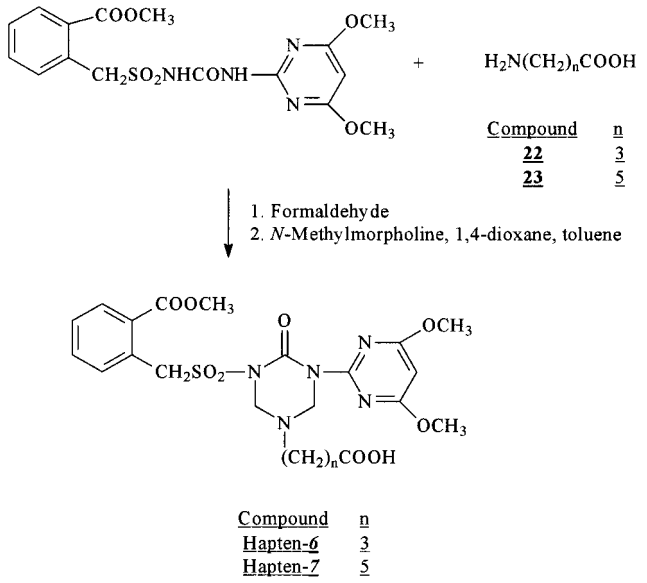


Figure 5. Synthetic routes of hapten-***6*** and hapten-***7***.

hapten-***6*** was overlapped with that of BSA or KLH. Therefore, the conjugation ratios of the hapten-***4***-protein conjugates and the hapten-***6***−BSA conjugate were determined by the TNBSA method. The coupling density was estimated by comparing the absorbance with the corresponding values of hapten-free proteins. From the available amino groups, 38% of hapten-***4*** and 64% of hapten-***6*** were conjugated in the hapten−BSA conjugates and 47% of hapten-***4*** was conjugated in the hapten−KLH conjugate.

Titers of the Antisera. The four hapten−KLH conjugates were injected seven times into each rabbit as immunogens, respectively. The antisera collected after each boost were subjected to titration by the homologous indirect ELISA. All of the antisera showed higher titers after the fourth, fifth, or sixth boosting than those of the others. These results indicate that specific antibodies in the rabbit antiserum were produced against each immunogen. The final antisera were used for the subsequent screening in search of antibodies specific to the target compound bensulfuron-methyl. All antisera did not show any significant affinity for BSA itself as a coating antigen.

Screening and Selection of Coating Antigens and Antisera. To establish a sensitive ELISA, all combinations between coating antigens and antisera were screened via the inhibition by two different concentrations (10 and 1000 ng/mL) of the analyte dissolved in the assay buffer, using the homologous or heterologous competitive indirect ELISA system. The inhibition ratio was expressed as a percentage of the difference between the absorbance of the analyte-free buffer and that of the analyte-containing buffer, divided by the former. The antibodies raised against hapten-***2***, -***3***, and -***4*** with a spacer arm of a four- or six-carbon chain were more specific to the target analyte in the heterologous ELISAs than those raised against hapten-***1*** without a bridge group. These results prove that a spacer arm in a hapten is essential for the production of antibodies specific to the analyte.

There was almost no or very low inhibition by the analyte in homologous ELISAs using the same hapten for a coating antigen and an immunogen. Whereas, there were very high inhibitions in heterologous ELISAs using the combinations of hapten-***1***, -***5***, -***6***, and -***7*** as coating antigens with the antisera raised against the hapten-***2***−KLH and hapten-***3***−KLH immunogens, and the combinations of hapten-***1***, -***2***, -***3***, -***5***, -***6***, and -***7*** as coating antigens with the antisera against the hapten-***4***−KLH immunogen. It is interesting that rabbits injected with hapten-***2*** and hapten-***3*** closely related to the analyte bensulfuron-methyl produced antisera exhibiting a high inhibition, whereas rabbits injected with hapten-***4*** mimicking only a portion of the analyte structure also produced antisera showing a similar inhibition.

The nine heterologous ELISAs using the combinations of the coating hapten-***1***, -***2***, -***3***, -***5***, and -***7*** and the antiserum D, F, and K, which showed higher inhibition ratios, were conducted at 10 concentrations of bensulfuron-methyl. The IC_{50} values of the rabbit antiserum D and F raised against the hapten-***2***−KLH immunogen were, respectively, 0.47 and 0.70 ng/mL on a plate coated with hapten-***1***−BSA, 0.68 and 4.98 ng/mL with hapten-***5***−BSA, and 23 and 0.61 ng/mL with hapten-***7***−BSA, and those of the antiserum K against the hapten-***4***−KLH were 0.16 ng/mL with the hapten-***1***−BSA, 0.18 ng/mL with the hapten-***2***−BSA, and 0.51 ng/mL with the hapten-***3***−BSA. For the development of the bensulfuron-methyl ELISA, we optimized three sensitive heterologous ciELISAs using three combinations of the coating antigens with the antisera: assays **I**, **II**, and **III** were the combinations of the coating hapten-***1*** with the antiserum D, of the coating hapten-***2*** with the antiserum K, and of the coating hapten-***7*** with the antiserum F, respectively. The assay **I** was used to optimize blocking conditions of the coated plate and the buffer for the assay, because the antiserum D had the highest titer.

Optimization of the ELISA. To determine the bensulfuron-methyl residues in the agricultural and environmental samples, it is essential to develop an ELISA with optimum sensitivity. For that purpose, the effects of blocking agents on the plate coated with a coating antigen, and the assay buffer-related factors such as solvent, detergent, ionic strength, and pH were evaluated.

Effect of Blocking Agents. To minimize the background and maximize signal-to-noise ratios in the assay, blocking agents were evaluated to hinder nonspecific sorption of an antibody onto the less-coated plate. Because there is no universal blocking buffer for all immunoassays, various blocking agents for each immunoassay need to be tested. As shown in **Figure 6**, all the tested blockers enhanced the assay sensitivity compared to no blocking. Skim milk as a blocker showed a lower background signal, a higher ratio of maximum absorbance (*A*) to minimum absorbance (*D*), and a higher sensitivity for the ELISA than other blockers. In particular, blocking with 3% skim milk at 37

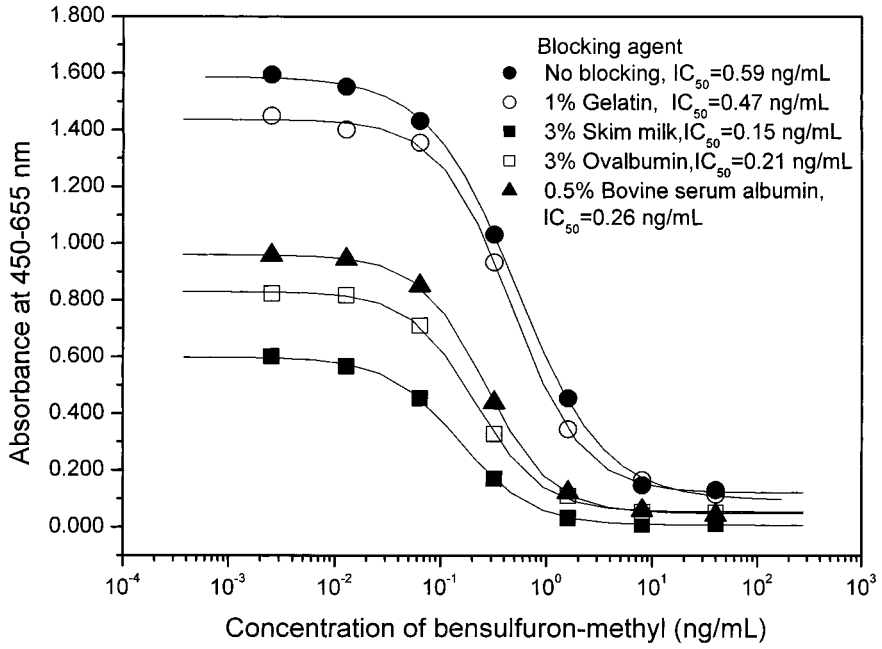


Figure 6. Effect of the blocking agents on the sensitivity of the ELISA.

Table 2. Effect of the Assay Buffer-Related Factors on the Sensitivity of the ELISA[a]

factor	A_{max} (*A*)	slope (*B*)	IC_{50} (ng/mL) (*C*)	A_{min} (*D*)	*A/D*
blocking temperature					
rt	1.342	1.34	0.31	0.048	27.96
37 °C	1.055	1.46	0.17	0.030	35.17
organic solvent in the assay buffer[b]					
methanol (%)					
0	0.994	1.36	0.20	0.024	
2	0.996	1.05	0.30	0.004	
4	0.939	0.83	0.34	–0.011	
10	0.778	0.96	0.63	0.020	
acetone (%)					
0	0.945	0.85	0.12	–0.008	
2	0.992	0.72	0.12	–0.019	
4	0.955	0.77	0.20	–0.037	
10	0.957	0.63	0.32	–0.069	
20	0.994	0.90	0.47	–0.017	
concentration of the assay buffer (ionic strength)[c]					
0.5	1.055	1.46	0.17	0.030	
1	0.668	1.35	0.14	0.015	
1.5	0.377	1.00	0.15	0.003	
dilution of the antibody					
1:32000	1.080	1.41	0.22	0.022	
1:48000	1.054	1.46	0.17	0.030	
1:64000	0.941	1.23	0.10	0.006	

[a] Assay conditions: coating antigen, hapten-***1***–BSA (1 μg/mL); blocking with 3% skim milk in 1 × PBS at 37 °C; antiserum, rabbit D (1:48000) in 1 × PBS; standard series of bensulfuron-methyl were dissolved in ultrapure water; goat anti-rabbit IgG-HRP (1:10000). Data are the means of quadruplicate samples. [b] Antiserum, rabbit D (1:32000). [c] The ionic strength is the mean of the buffers containing the analyte and the antiserum.

°C resulted in lower nonspecific binding of an antibody to the solid phase, and hence the sensitivity almost doubled that at room temperature (**Table 2**).

Effect of Organic Solvent and Detergent. The effects of the organic solvent (methanol or acetone) and detergent on the ELISA were evaluated. As shown in **Table 2**, the assay sensitivity was higher in the buffer containing no or a small amount of organic solvents. The IC_{50} value in the assay buffer containing 10% methanol or acetone was about three times higher than that without the organic solvents, indicating that an increase in the amount of organic solvent decreases the assay sensitivity in the bensulfuron-methyl ELISA. The amounts of the organic solvents that are less than 4% in the assay buffer had little effect on the sensitivity of the ELISA. It will be necessary to use a small amount of organic solvent in the assay buffer to reduce assay variability by decreasing interference of lipid micelles with complex matrixes and by reducing binding of analyte to surfaces of the plate (*37*). As water solubility of bensulfuron-methyl at pH 8 is 1200 mg/L (*38*), the use of large amounts of organic solvents will not be necessary for the ELISA.

Meanwhile, Tween 20 is a nonionic detergent and has been used in immunoassays to reduce nonspecific binding and improve sensitivity (*39*). However, in this study, as shown in **Figure 7**, the IC_{50} value in the buffer to which 0.05% (v/v) Tween 20 was added was about twice higher than that without the detergent, indicating that the addition of Tween 20 to the buffer did not enhance the sensitivity of the ELISA for the detection of bensulfuron-methyl. This result is in agreement with the studies of the esfenvalerate ELISA (*40*) and the polychlorinated dibenzo-*p*-dioxin ELISA (*41*) that showed Tween 20 decreased the sensitivity due to nonspecific hydrophobic interactions between the detergent and nonpolar small analytes in an aqueous environment, thereby interfering with the specific analyte−antibody interaction. Manclús and Montoya (*42*) also reported that Tween 20 had no effect on the sensitivity of an ELISA for a highly polar compound such as TCP (3,5,6-trichloro-2-pyridinol). The buffer without a detergent in the ELISAs for the polar sulfonylurea herbicides, metsulfuron-methyl and chlorsulfuron, might be also used for this reason (*14, 43*).

Effect of pH and Ionic Strength. To determine the effect of pH on the assay, the phosphate buffer was used in the range of pH 6.5 to 9.5, and results were compared with one another. As shown in **Figure 8**, in the range tested, the pH value affected the assay. The sensitivity of the ELISA was a little higher under slightly alkaline conditions than under slightly acidic and neutral conditions, as shown by the IC_{50} values. This means that the interaction between the antibody and the target analyte was most favored under this alkaline condition of pH 8.5. Because

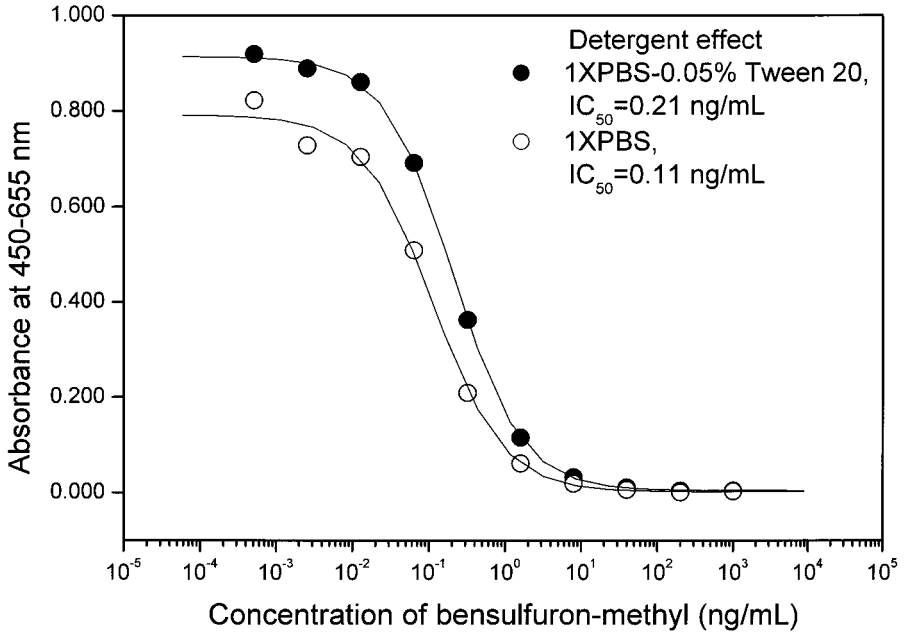


Figure 7. Effect of the detergent in the assay buffer on the sensitivity of the ELISA.

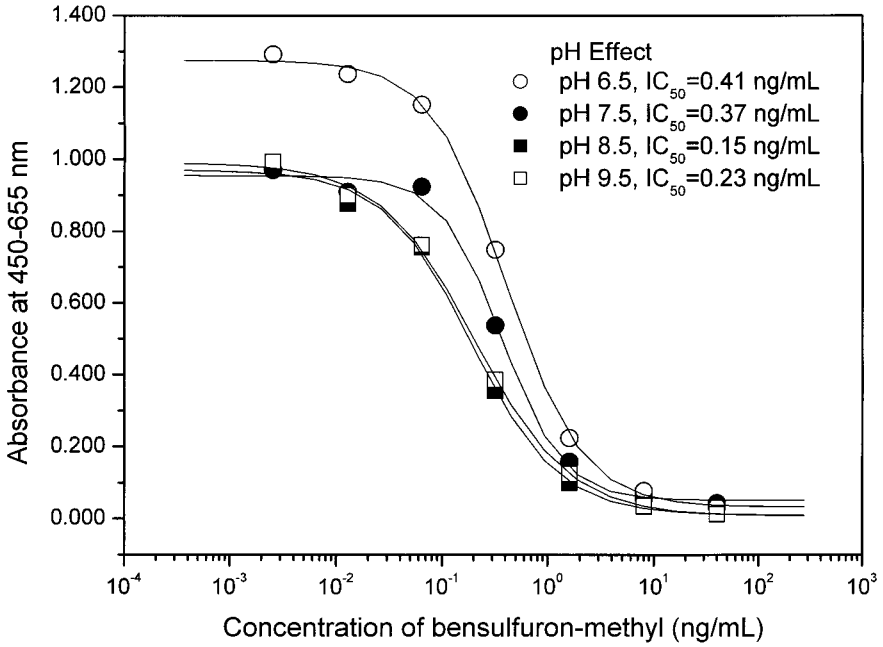


Figure 8. Effect of pH on the sensitivity of the ELISA, as shown by the IC_{50} value.

bensulfuron-methyl is most stable under a slightly alkaline condition (pH 8), the optimum pH range of the assay buffer was set at 8−8.5 for the ELISA.

As shown in **Table 2**, the binding of the antibody to the coating antigen decreased with the increasing salt concentration of the assay buffer. The optimum ion strength of the assay phosphate buffer for a maximum binding was that of 0.5 × PBS.

Dilution of the Antisera. The concentrations of immunoreagents such as the antisera and the coating antigens selected for the ELISA were determined in the checkerboard titration. In addition, an intensive investigation for a more sensitive assay was also carried out at different concentrations of the antiserum on the same amount of a coating antigen. As shown in **Table 2**, the higher the antibody dilution is, the lower the IC_{50} value is. The ELISA becomes more sensitive by reducing the amount of the antibody. It is possible to obtain a more sensitive ELISA for bensulfuron-methyl by decreasing the concentration of the antibody.

On the basis of these results, the optimal conditions for the bensulfuron-methyl ELISA are summarized as follows (**Table 3**): A quantity of 1 μg/mL of the hapten-***1***−BSA conjugate as a coating antigen was coated onto the plate and stored at 4 °C overnight, and then the plate was blocked with 3% skim milk at 37 °C for 1 h. The antiserum D raised against the hapten-***2***−KLH conjugate as an immunogen was diluted 64000-fold in the assay buffer at pH 8 without detergent or organic solvent and competed with the target analyte dissolved in pH 8 ultrapure water in the blocked plate. With the heterologous assay **I** optimized for bensulfuron-methyl under these conditions, the IC_{50} value of the analyte as low as 0.17 ± 0.04 ng/mL was obtained, showing the detection range of 0.04−0.60 ng/mL, and

Table 3. Optimized Conditions for the ELISAs

assay	immunogen	coating antigen	blocking agent	antiserum (dilution)	assay buffer (pH 8)
I	hapten-***2***–KLH	1 μg/mL of hapten-***1***–BSA at 4 °C overnight	3% skim milk in 1 × PBS at 37 °C	rabbit D (1:64000)	0.5 × PBS
II	hapten-***4***–KLH	0.5 μg/mL of hapten-***2***–BSA at 37 °C for 2 h	3% skim milk in 1 × PBS at 37 °C	rabbit K (1:30000)	1 × PBS
III	hapten-***2***–KLH	1 μg/mL of hapten-***7***–BSA at 37 °C for 2 h	3% skim milk in 1 × PBS at 37 °C	rabbit F (1:24000)	1 × PBS

the lowest detection limit (LOD) of 0.02 ng/mL (**Figure 9**). Whereas, as shown in **Table 3** and **Figure 9**, under the optimized conditions of the assays **II** and **III**, the IC_{50} values of 0.09 ± 0.02, and 0.09 ± 0.03 ng/mL were obtained by using hapten-***2***−BSA and hapten-***7***−BSA, respectively, as plate-coating antigens and hapten-***4***−KLH and hapten-***2***−KLH conjugates as immunogens, showing the detection ranges of 0.01−0.60 and 0.04−0.25 and LODs of 0.002 and 0.03 ng/mL, respectively.

Kawada et al. (*15*) reported on the monoclonal antibody-based ELISAs for bensulfuron-methyl, using haptens with a spacer arm of various carbon chains. The detection ranges were reported to be 0.2−2 and 0.5−5 ng/mL in the competitive indirect and direct inhibition ELISAs, respectively. Whereas in our ELISA, the detection range was 0.04−0.60 ng/mL in the assay **I**, which is far lower than their detection range. Besides, the haptenic synthesis was quite different from theirs. Meanwhile, the monoclonal antibody-based fluoroimmunoassay for bensulfuron-methyl developed by Yuan et al. (*17*) showed the detection limit of 0.024 ng/mL. Even if they used a tetradentate β-diketonate europium fluorescent chelate to label a bensulfuron-methyl BSA conjugate for this assay, the detection limit was almost the same as ours, which was 0.03 ng/mL in the assay **I**.

Cross-Reactivities. In assay **I** that is the heterologous combination of hapten-***2***−KLH (immunogen) and hapten-***1***−BSA (coating antigen), the antiserum D recognized some structurally related compounds, but not the two possible metabolites. On the basis of the cross-reactivity (**Table 4**), the antigenic determinants in each compound to which the rabbit D antibody binds specifically would be a phenyl group connected to the sulfonylurea moiety via methylene, oxygen,

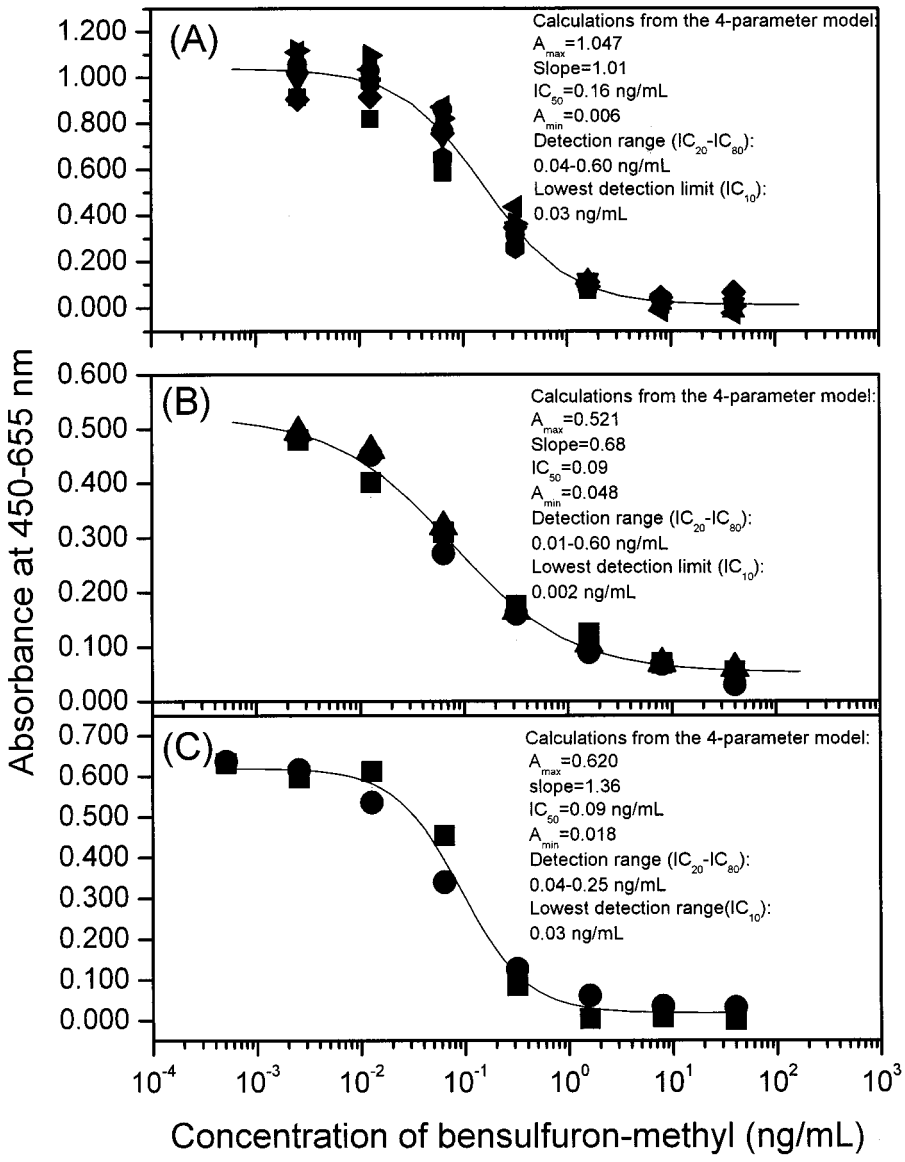
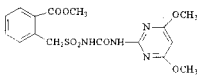
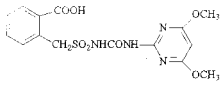
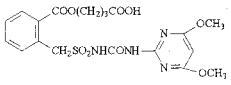
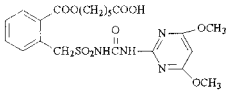
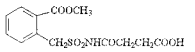
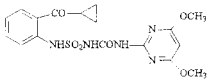
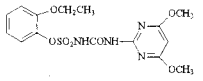
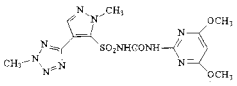
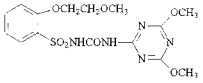
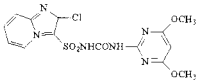
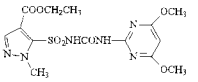
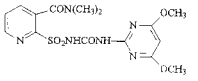
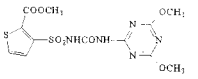
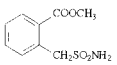
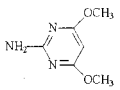


Figure 9. Standard curves obtained under the optimized conditions in the ELISAs.

Table 4. Cross-Reactivity of Some Structurally Related Compounds to the Rabbit D and K Antisera in the ELISAs[a]

compound	chemical structure	assay I		assay II	
		IC_{50} (ng/mL)	CR[b] (%)	IC_{50} (ng/mL)	CR (%)
bensulfuron-methyl		0.16	100	0.09	100
hapten-*1*, bensulfuron		1.63	9.8	>1000	<0.1
hapten-*2*		1.05	15.2	>1000	<0.1
hapten-*3*		1.58	10.1	>1000	<0.1
hapten-*4*		NI[c]	0	0.08	112.5
cyclosulfamuron		2.13	7.5	5.29	1.7
ethoxysulfuron		1.30	12.3	3.21	2.8
azimsulfuron		43.2	0.4	>1000	<0.1
cinosulfuron		37.2	0.4	>1000	<0.1
imazosulfuron		32.0	0.5	>1000	<0.1
pyrazosulfuron-ethyl		53.3	0.3	>1000	<0.1
nicosulfuron		50.4	0.3	>1000	<0.1
thifensulfuron-methyl		>1000	<0.1	>1000	<0.1
metabolite-*1*, methyl 2-(aminosulfonyl-methyl)benzoate		NI	0	5.45	1.65
metabolite-*2*, 2-amino-4,6-dimethoxypyrimidine		NI	0	NI	0

[a]Rabbit D and K antisera were raised against the immunogens hapten-***2***–KLH conjugate (assay **I**) and hapten-***4***–KLH conjugate (assay **II**), respectively. [b] % Cross-reactivity (CR) = (IC_{50} of bensulfuron-methyl/IC_{50} of other compounds) × 100. [c] NI, no inhibition.

or NH moieties. The fact that the covalent radii of carbon, oxygen, and nitrogen atoms are 0.771, 0.74, and 0.74 Å, respectively, indicates that the steric fitness is very important in the antigen−antibody interaction. This argument is based on

the result that the sulfonylureas such as azimsulfuron, imazosulfuron, pyrazosulfuron-ethyl, and nicosulfuron that contain 4,6-dimethoxypyrimidine and sulfonylurea moieties without methylene group showed very low cross-reactivities. The fact that metabolite-1 and metabolite-2 containing no complete sulfonylurea moiety showed no cross-reactivity supports the above reasoning.

Schlaeppi et al. (*12*) reported an ELISA for the sulfonylurea herbicide triasulfuron based on monoclonal antibodies (MAbs). They generated MAbs with high affinity for the analyte by a simple hapten corresponding only to the chloroethoxy sulfonamide moiety of triasulfuron with an additional succinic acid spacer. They indicated that the urea function of the bridge structure was a prerequisite for the binding of the MAb. In addition, they concluded that the chloroethoxy group interacted with the MAb. Similarly, Kelley et al. (*11*) worked on another sulfonylurea herbicide, chlorsulfuron. They suggested that the bridge and heterocycle structure is related to the antigen−antibody binding, based on the cross-reactivity changes as affected by the modification of the above structures. In the case of assay **II**, in which the combination of hapten-***4***−KLH (immunogen) and hapten-***2***−BSA (coating antigen) was used, only ethoxysulfuron exhibited a low cross-reactivity (2.8%) except for hapten-***4*** that was used as the immunogen. The cross-reactivities of the two compounds would be based on the fact that ethoxysulfuron is very similar to bensulfuron-methyl in structure in that the lengths of the $-COOCH_3$ in bensulfuron-methyl and $-OCH_2CH_3$ group in ethoxysulfuron are both 5.14 Å, in addition to the similar sizes of the $-CH_2-$ group in the former and the oxygen atom in the latter. Meanwhile, in assay **III** (not presented), where the combination of hapten-***2***−KLH (immunogen) and hapten-***7***−BSA (coating antigen) was used, quite a few sulfonylurea compounds showed relatively high cross-reactivities. This result could be due to the fact that the structure of coating antigen is quite different from that of the immunogen, rendering high affinity between the antibody and the test compound.

Matrix Effect. Because of the matrixes such as inorganic and organic compounds contained in water samples, the test samples used in ELISA should be diluted or purified by a solid-phase extraction or other methods prior to the determination of bensulfuron-methyl residues. In assay **I**, the water samples collected from nearby agricultural areas were diluted 10 and 50 times with ultrapure water, after filtration and pH adjustment to 8, to prevent the interference by matrixes. Standard curves of the water samples were prepared with each diluted water sample, and compared to the calibration curve prepared with ultrapure as a control. The matrix effects of water samples by the 50-fold dilution fairly diminished, showing that their standard curves were almost close to the control curve (data not shown). These results indicate that the ELISA can determine bensulfuron-methyl residues in water samples at the ppb-level only by simple dilution.

Recovery of Bensulfuron-Methyl Residues from the Fortified Water Samples. Five water samples fortified with four different levels of the analyte were analyzed by the optimized assay **I**. The recovery of bensulfuron-methyl from all the water samples was very good, being in the range of 81 to 125% (**Table 5**). Although assay **I** showed a little lower sensitivity than assay **II**, it had a slope value of about 1 in an equation obtained from the bensulfuron-methyl standard curve, whereas the value in the assay **II** was about 0.7. In addition, the recovery of the analyte fortified to the water samples was in the range of 110 to 180% in assay **II** (data not shown). Therefore, the assay **I** was adopted for the better quantitation of the analyte in various water samples.

Table 5. Recovery of the Analyte Bensulfuron-Methyl Fortified to Some Water Samples by the ELISA

water sample	fortified (ng/mL)	50-fold diluted (ng/mL)	detected (ng/mL)	mean recovery (%, $n = 3$)	coefficient of variation (%)[a]
rice paddy	0	0	<LOD[b]	-	0.4
	2.5	0.05	0.042	84	4
	5	0.10	0.108	108	8
	10	0.20	0.166	83	5
	25	0.50	0.470	94	5
pond	0	0	<LOD	-	2
	2.5	0.05	0.054	108	4
	5	0.10	0.086	86	10
	10	0.20	0.162	81	3
	25	0.50	0.530	106	7
stream	0	0	<LOD	-	2
	2.5	0.05	0.046	92	1
	5	0.10	0.090	90	5
	10	0.20	0.200	100	8
	25	0.50	0.612	122	10
underground	0	0	<LOD	-	1
	2.5	0.05	0.046	92	2
	5	0.10	0.094	94	6
	10	0.20	0.234	117	6
	25	0.50	0.600	120	9
soil leachate	0	0	<LOD	-	5
	2.5	0.05	0.058	116	2
	5	0.10	0.082	82	1
	10	0.20	0.250	125	3
	25	0.50	0.482	96	8

[a] Coefficient of variation is defined as the standard deviation divided by the mean, expressed as a percentage. [b] LOD means the lowest detection limit (0.03 ng/mL) in assay I.

To validate the ELISA, the same fortified groundwater samples were subjected to HPLC after a cleanup step. The equation of the linear regression correlation between the ELISA (Y) and HPLC (X) results was $Y = 1.38X - 1.78$ ($r = 0.999$, $n = 15$). The ELISA is comparable to HPLC in the analysis of bensulfuron-methyl residues in water samples. Therefore, this ELISA could be used as an alternative to the conventional instrumental methods for monitoring bensulfuron-methyl residues in agricultural and environmental samples.

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