Development of an ELISA Using Polyclonal Antibodies Specific for 2-Methylisoborneol

Si-Yin Chung,*,† Peter B. Johnsen,† and Phillip H. Klesius‡

Southern Regional Research Center, U.S. Department of Agriculture—Agricultural Research Service, 1100 Robert E. Lee Boulevard, P.O. Box 19687, New Orleans, Louisiana 70179, and Animal Parasite Research Laboratory, U.S. Department of Agriculture—Agricultural Research Service, P.O. Box 952, Wire Road at Simms Drive, Auburn, Alabama 36830

An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of 2-methylisoborneol (MIB), a common cause for earthy/musty off-flavor. The antibody used in the assay was obtained from antisera raised against a conjugate of camphor linked to bovine serum albumin (BSA). The sensitivity and specificity of the antibody were tested in a microtiter plate coated with a camphor–ovalbumin conjugate. An antigoat IgG–horseradish peroxidase conjugate was used as the detecting system. Compounds such as MIB, camphor, geosmin, 2-methoxy-3-isopropylpyrazine, and seven other MIB structurally related analogues were tested. The relative percent cross-reactivity for each of these compounds was as follows: MIB, 100%; camphor, 100%; isoborneol, 100%; borneol, 100%; camphorquinone, 68%; norcamphor, 0%; *endo*-norborneol, 0%; norbornane, 0%; geosmin, 0%; 2-methoxy-3-isopropylpyrazine, 0%; 2-methyl-2-bornene (a MIB dehydration product), 100%. The assay has a sensitivity of 1 μg/mL. The methyl groups in camphor and MIB molecules were essential in antibody recognition.

Two metabolites of actinomycetes and algae, geosmin (1,10-dimethyl-9-decalol) and 2-methylisoborneol (MIB), impart earthy/musty off-flavors to their aquatic environment. Water and fish exposed to these compounds may become unacceptable to consumers. To monitor the levels of these compounds for quality control and abatement purposes, analytical techniques have been developed. The currently used methods, closed-loop stripping analysis (Krasner et al., 1983) and solvent extraction (Johnsen and Kuan, 1987), are both costly in terms of equipment and time. Therefore, a rapid, inexpensive analytical method such as ELISA (enzyme-linked immunosorbent assay) is needed. Similar systems have been used to detect low molecular weight compounds such as T-2 toxin (Wei et al., 1988), drugs (Hinds et al., 1986), enterotoxin (Lapeyre et al., 1988), and vitamins (Hansen and Holm, 1988).

This paper reports the development of an ELISA for MIB using a camphor–bovine serum albumin (BSA) conjugate as an immunogen for the production of antibodies. The feasibility of using camphor as a surrogate hapten for MIB lies in the fact that the two compounds are structurally similar (Figure 1). For use as an immunogen, the camphor was converted to a (carboxymethyl)oxime, followed by conjugation to the protein BSA. We believed that antibodies produced in this way might have equal affinity for both camphor and MIB.

MATERIALS AND METHODS

Materials. Camphor, camphorquinone, norcamphor, norbornane, norborneol, borneol, isoborneol, and *O*-carboxymethoxylamine hemihydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ovalbumin, BSA, rabbit anti-goat IgG peroxidase conjugate, *o*-phenylenediamine dihydrochloride, phosphate-buffered saline (PBS), 2,4,6-trinitrobenzenesulfonic acid, and isobutyl chloroformate were obtained from Sigma Chemical Co. (St. Louis, MO). Gelatin, Tween 20, and protein assay reagents were purchased from BioRad Laboratories (Rockville Center, NY). Triethylamine and tetrahydrofuran were from Fisher Scientific Co. (New Orleans, LA). Protein-G Sepharose was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Microtiter plates of Immulon II and plate reader M700 were purchased from Dynatech Laboratories, Inc. (Chantilly, VA). Geosmin was obtained from Givaudan Co. (Paramus, NJ). 2-Methoxy-3-isopropylpyrazine was purchased from Pyrazine Specialties (Atlanta, GA). MIB was synthesized in our laboratory by the method of Wood and Snoeyink (1977).

Preparation of Camphor *O*-(Carboxymethyl)oxime (Camphor-CMO). Camphor-CMO was prepared with a slight modification of the method of Langone and VanVunakis (1976). Briefly, a solution of camphor (1 g, 6.5 mmol) and *O*-carboxymethoxylamine hemihydrochloride (2.84 g, 13 mmol) in 14.0 mL of absolute ethanol and 16.5 mL of 2 N NaOH (33 mmol) was refluxed for 6 h. After the mixture was allowed to stand at 25 °C overnight, a 20-μL aliquot was drawn for thin-layer chromatography (TLC) analysis. Water (200 mL) was added to the remainder of the solution, the pH adjusted to 9.5 with 2 N NaOH, and the solution extracted three times with 100 mL of ethyl acetate. The aqueous layer was acidified to pH 3 with 1 N HCl and stored at 0 °C overnight to yield the oxime as a white precipitate. The precipitate was collected by centrifugation, washed with 500 mL of water, and dried in vacuo over anhydrous calcium sulfate. The camphor-CMO was subsequently characterized by infrared spectroscopy (IR), gas chromatography–mass spectrometry (GC–MS), elemental analysis, melting point, and TLC in ethyl acetate–methanol–ammonium hydroxide (65:35:11).

Preparation of Immunogen and Coating Antigen. Camphor was conjugated to BSA for use as the immunogen and to ovalbumin as a solid-phase antigen in the ELISA. The conjugates were prepared by the mixed-anhydride method (Dean et al., 1972). Briefly, a mixture of triethylamine (12 μL, 85 μmol) and camphor-CMO (18.9 mg, 84 μmol) in tetrahydrofuran (1 mL) was cooled to −5 °C. Isobutyl chloroformate (12 μL, 85 μmol) was added to the mixture and the resultant solution stirred for 30 min at −5 °C. The reaction mixture was then added dropwise to 5 mL of a cold solution of BSA (20 mg/mL, 1.5 μmol) in water–dioxane (7:3), pH 9. Sodium hydroxide was added to maintain the pH. The mixture was then stirred overnight at 4 °C, dialyzed against water, and stored below 0 °C. The molar ratio (moles of camphor bound per mole of protein) was deter-

† Southern Regional Research Center.
‡ Animal Parasite Research Laboratory.

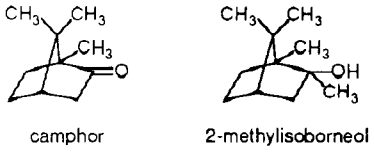


Figure 1. Structures of camphor and 2-methylisoborneol.

mined by the 2,4,6-trinitrobenzenesulfonic acid method (Fields, 1971).

For the preparation of camphor–ovalbumin conjugate, 5 mL of ovalbumin (5 mg/mL, 0.56 μmol), 12.5 mg of camphor-CMO (56 μmol), and 8 μL of triethylamine and isobutyl chloroformate (60 μmol each) were used, respectively. The resulting camphor–ovalbumin conjugate was then dialyzed before use as the coating antigen in the microtiter plate.

Immunization. Four goats were each injected subcutaneously with 2 mL of an emulsion consisting of 0.5 mg of camphor–BSA conjugate in a 1:2 mixture of PBS and Freund's complete adjuvant. A booster injection prepared in Freund's incomplete adjuvant was performed 3 weeks after the first injection. Two weeks after the second injection, the animals received intravenous injections of antigen alone, containing 2 mg for the next 3 weeks at weekly intervals. The goats were bled at different times during the immunization and the sera tested for the presence of antibodies by ELISA.

Determination of Optimal Coating Antigen Concentration by ELISA. Camphor–ovalbumin conjugate in 0.05 M carbonate buffer, pH 9.6, at concentrations of 20, 10, 5, 2.5, 1.25, and 0.63 μg/mL were added to wells of microtiter plate (100 μL/well). The plate was incubated at 4 °C overnight, washed with PBS–Tween 20 (0.05%), and then blocked with 0.5% gelatin in PBS–Tween (200 μL/well) at 37 °C for 3 h. After the plate was washed, a dilution (1:1000) of antiserum in PBS–Tween–ovalbumin (0.5%) was added, followed by incubation at 25 °C for 30 min. The plate was again washed and an anti-goat IgG–peroxidase conjugate (1:1000) in PBS–Tween–ovalbumin added. After incubation at 25 °C for 30 min, the plate was washed and a substrate solution containing *o*-phenylenediamine (0.5 mg/mL) in citrate–phosphate buffer, pH 5, and 0.01% hydrogen peroxide was added to the wells. After incubation in the dark at 25 °C for 15 min, the reaction was terminated by adding 50 μL of 4 N sulfuric acid. Absorbance at 490 nm was determined in a Dynatech MR700 plate reader.

Determination of Antibody Titers. Serial dilutions of antiserum or preimmune serum in PBS–Tween–ovalbumin were added to wells of the microtiter plate coated with the optimal antigen concentration as determined above. For control, ovalbumin was used. Assay procedure was the same as described above. Antibody titer was defined as the antiserum dilution that gave an absorbance reading of 0.3 unit above the blank.

Purification of Antibody by Protein-G Sepharose. Before being applied to a Protein-G Sepharose (3-mL) column, the antiserum (3 mL) was centrifuged, filtered through a Millipore membrane (Millex Gv, 22 μm), and diluted 1:1 with 0.02 M sodium phosphate buffer, pH 7. IgG was isolated by passing the diluted antiserum through the column and eluting with 0.1 M glycine hydrochloride, pH 2.7. The eluted IgG fractions (1 mL each) were immediately neutralized by adding 1 M Tris, pH 9 (80 μL/mL fraction), concentrated in an Amicon cell (YM 30 membrane), and stored at −20 °C. The protein concentration was determined from an extinction coefficient $\epsilon^{0.1\%}$ at 280 nm (1.4). The purity of the antibody was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reduced condition. Bands corresponding to the IgG heavy (50 000 Da) and light chains (25 000 Da) were observed.

Competitive ELISA. A competitive ELISA between free antigen and antigen absorbed to the plate for the antibody was used. To determine a standard curve, camphor or MIB in the concentration range 0.1–1000 μg/mL in 10% ethanol–PBS–Tween was used. Fifty microliters of the camphor or MIB solution was incubated with 50 μL of primary antibody (1:10 000) in PBS–Tween–ovalbumin at 25 °C for 30 min. The mixture was added to wells coated with camphor–ovalbumin or ovalbumin (control), followed by incubation for 15 min. The plate

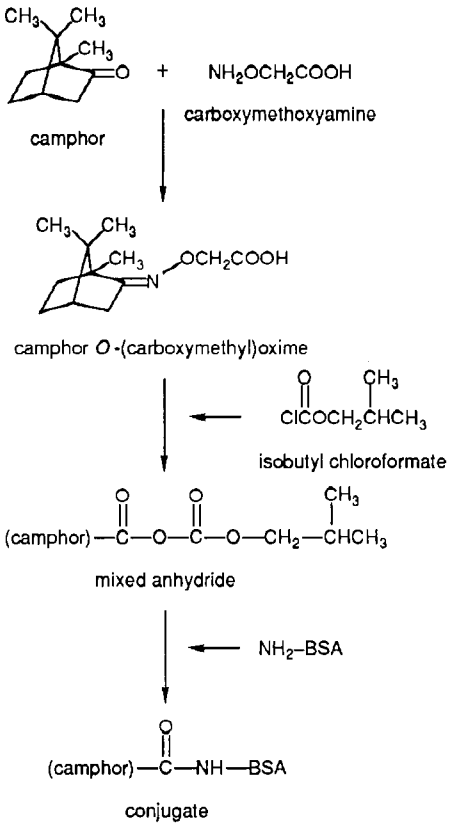


Figure 2. Steps in preparation of camphor–BSA conjugate.

was then washed with PBS–Tween. Peroxidase conjugate and substrate solutions were added as described above except that the incubation time for color development was 30 min. A standard curve was prepared by plotting the percent of maximum absorbance versus the concentration of camphor on semilog scale. The percent of maximum absorbance is defined as $(A_s/A_0) \times 100$, where A_s and A_0 are the absorbance obtained at each standard concentration and the absorbance of the blank in the absence of camphor, respectively.

Determining Specificity of the Antibody. To determine the antibody specificity, MIB, seven structurally related camphor compounds and two other earthy/musty compounds were tested in the competitive ELISA. In addition, MIB dehydration products reported to have earthy/musty odors in fish tissue (Martin et al., 1988) were prepared (described below) and tested. The relative percent cross-reactivity was then determined and defined as the concentration (i.e., molar IC_{50}) of camphor causing 50% reduction in maximum absorbance divided by the concentration of test compound causing 50% reduction in maximum absorbance.

Preparation of MIB Dehydration Products for Test in ELISA. MIB dehydration products, 2-methyl-2-bornene and/or 2-methylenebornane, were prepared to determine whether the antibodies against MIB recognize these products. Briefly, a solution of MIB (45 mg) in 10 mL of ethanol was added dropwise to a mixture of concentrated sulfuric acid and distilled water (1:1) (100 mL) at 0 °C. The final mixture was stirred for 1 h at 0 °C before being extracted three times with hexane (Photrex) (10 mL each). The hexane layers were combined, washed with methanol three times (10 mL each), and concentrated with nitrogen to a final volume of 400 μL. Identification of dehydration products was made with Hewlett-Packard 5970 GC–MS system.

For the competitive ELISA, 50 μL of the hexane concentrate was used. This concentrate was blown dry with nitrogen, dissolved in ethanol (100 μL), and mixed with PBS–Tween (900 μL). Serial dilutions (10-fold) with 10% ethanol–PBS–Tween were made, and 50 μL of the diluted sample was applied in the competitive ELISA as described above.

Table I. Chemical Properties of Camphor, Camphor-CMO, and Carboxymethoxyamine Hydrochloride (CMA·HCl)

	camphor	camphor-CMO	CMA·HCl
mp, °C	178–180	95.6–97	156
MW	152.24	225[a]	218.6
UV: λ, nm	202, 287	197–200	194
acid group	NIL	–COOH[b]	–COOH
R_f (TLC)	0.76	0.31	0.078

[a] Confirmed by GC/MS and elemental analysis. [b] Confirmed by IR.

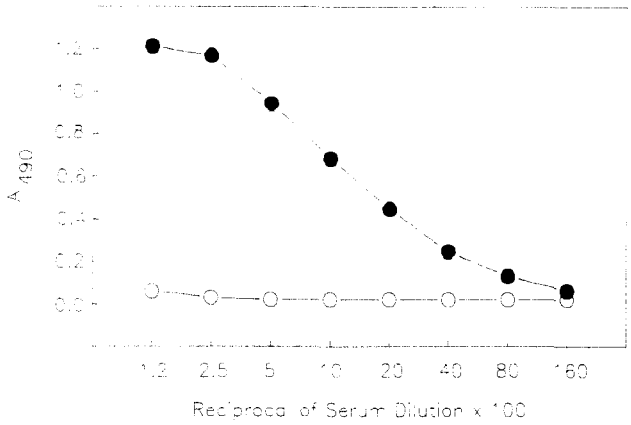


Figure 3. Titration of goat antiserum in plate coated with camphor–ovalbumin (5 μg/mL). Preimmune serum (○) and antiserum (●) were obtained from bleeds collected before and 3 weeks after the first injection of camphor–BSA. An antigoat IgG–peroxidase conjugate (1:1000) was used as the detecting system. Incubation time with substrate was 15 min.

RESULTS AND DISCUSSION

Characterization of the Camphor Derivative and Conjugate. Camphor was coupled to BSA as outlined in Figure 2. Camphor has one carbonyl group available for introduction of reactive group. By derivatizing with carboxymethoxylamine, camphor was converted to an oxime with a free carboxyl group available for coupling to BSA. The characteristics of this camphor derivative compared to the parent molecule are shown in Table I. The derivative (i.e., camphor-CMO) has a melting point of 95.6–97 °C compared to 178–180 °C of camphor and a molecular weight of 225 as confirmed by GC–MS and elemental analysis. The latter showed that the camphor derivative contains the following. Anal. Calcd for $C_{12}H_{19}NO_3$: C, 64; H, 8.44; N, 6.22; O, 21.33. Found: C, 63.75; H, 8.65; N, 6.24; O, 20.94. Analysis by TLC gave one spot with R_f 0.31 (0.76 for camphor). The presence of the free –COOH group in the derivative was confirmed by IR spectrometry.

Coupling of the camphor-CMO to BSA was achieved by the mixed-anhydride method. This method has the advantage over the carbodiimide procedure in that protein–protein cross-linkages can be prevented (Samokhin and Filimonov, 1985). The resulting coupling gave a molar ratio of 10:1 (camphor to BSA). A similar ratio was also reported in the study of antibody against aflatoxin (Chu and Ueno, 1977).

Determination of Antibody Titers. Antibody titer was determined by titration of serial dilutions of goat antisera in wells of a microtiter plate coated with camphor–ovalbumin at a concentration of 5 μg/mL. Results for a typical titration of antibody against camphor are shown in Figure 3. The significant difference in the titration curves of both preimmune serum and antiserum indicated that antibodies against camphor were present in the antiserum. Among the four goats tested, two gave the lowest background in ELISA. Antibody titers produced by these goats over a period of 13 weeks were shown in Figure 4. These results indicated that antibodies could be detected as early as 3 weeks after immunization of the animals. The titers continued to rise after two boosters and then declined 10 weeks after boosting. Goat A gave the best response. Its antiserum at week 10 was collected, purified, and used throughout the study.

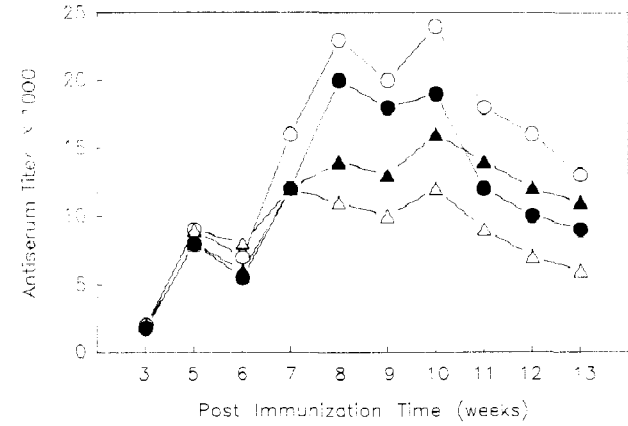


Figure 4. Production of antibodies in goats. Bleeds were collected at indicated intervals following immunization of goats A (○), B (●), C (▲), and D (△).

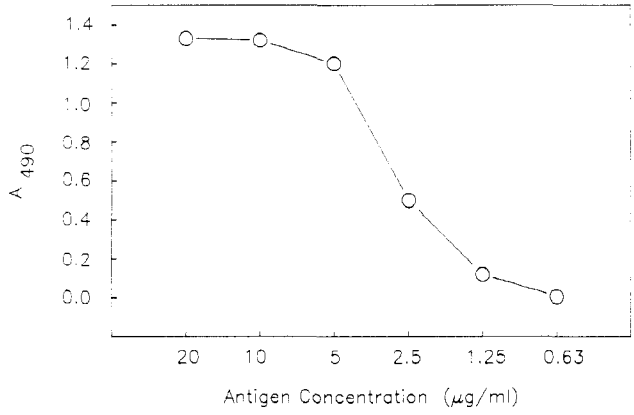


Figure 5. Titration of coating antigen. Plate was coated with camphor–ovalbumin at concentrations shown and was titrated with an antiserum. Incubation time with substrate was 15 min.

Optimization of Assay Conditions in ELISA. The assay conditions were optimized by determining the optimum concentration of the coating antigen (i.e., camphor–ovalbumin) and the dilution of antibodies. Figure 5 shows that the optimal concentration of coating antigen for the assay was 5 μg/mL. Although it has been reported (Hassen et al., 1988) that using a concentration lower than the optimum might enhance the sensitivity of the competitive ELISA, no improvement in sensitivity was found (data not shown) when a concentration ranging from 2.5 to 0.62 μg/mL was used in this study.

With use of plates coated with camphor–ovalbumin at the optimal concentration of 5 μg/mL, standard curves of camphor were prepared to establish the best purified antibody dilution to use. Dilutions of 1:10 000, 1:20 000, and 1:40 000 were tested in the competitive ELISA (Figure 6). The rate of color development at the latter two dilutions was slow, and sensitivity was not improved. Therefore, a dilution of 1:10 000 was selected for use throughout the study.

Generation of a Standard Curve by Competitive ELISA. The antibody used to generate the standard curve for MIB was purified through a Protein-G Sepharose

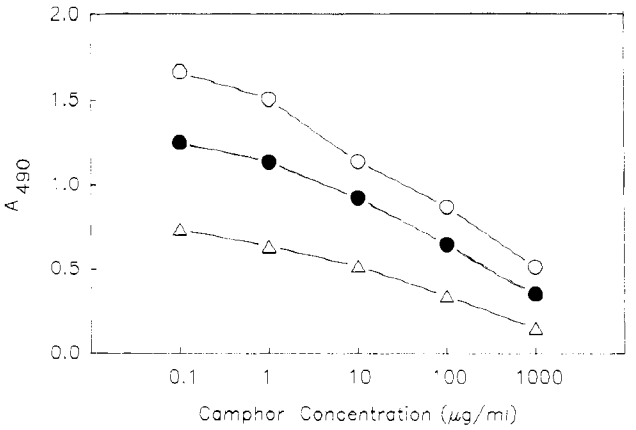


Figure 6. Effect of different antibody dilutions on the development of standard curve for camphor. Purified IgG at dilutions of 1:10 000 (○), 1:20 000 (●), or 1:40 000 (△) was incubated with an equal volume of camphor solution for 30 min at 25 °C. The mixture was then added to wells coated with camphor–ovalbumin (5 μg/mL). Assay was performed as described in text with a peroxidase conjugate (1:1000). Incubation time with substrate was 30 min.

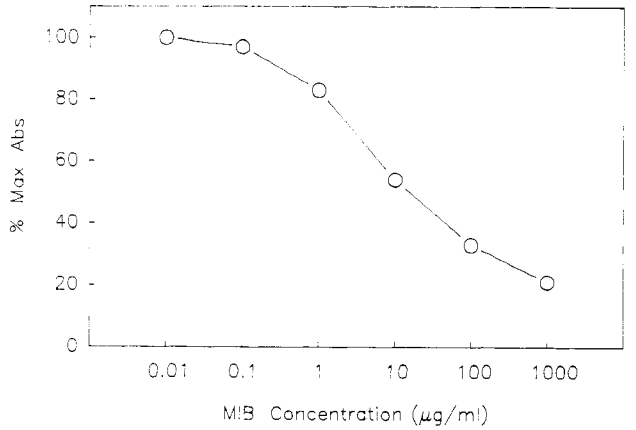


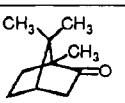
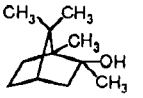
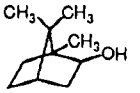
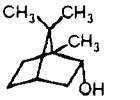
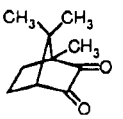
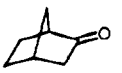
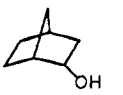
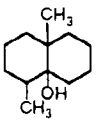
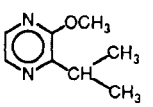
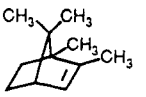
Figure 7. Standard curve for MIB. MIB at a concentration was incubated with an equal volume of purified IgG (1:10 000) for 30 min at 25 °C. The mixture was then added to wells coated with camphor–ovalbumin, and assay was performed as described in Figure 6.

column. Protein-G has been known to bind to IgG from a wide range of species, including goat, sheep, cow, mouse, human, rat, rabbit, and others (McGuire, 1988). Protein-G is preferred in this study to Protein-A because the latter has a poor affinity for goat IgG (McGuire, 1988).

The standard curve for MIB is shown in Figure 7. Although the purity of the antibody is an important factor in the sensitivity of an assay, our results show that a detection limit of only 1 μg/mL could be obtained. It is thought that the sensitivity may be dependent upon the titer of the antibody rather than its purity. Indeed, in most cases (Martlbauer et al., 1988; Zhang et al., 1986; Newsome, 1985) antibodies either were partially or were not purified at all and still exhibited good sensitivity. These antibodies were obtained from bleeds collected at periods ranging from 6 to 17 months. In an attempt to improve the sensitivity, bleeds beyond 13 weeks were collected and tested. However, the titer of the bleeds and sensitivity did not seem to improve.

Another factor that may play a role in the sensitivity of the assay is the coating antigen. Proteins such as hemocyanin and fibrinogens were said to give higher sensitivity than both BSA and ovalbumin when used as protein carriers (Gee et al., 1988). A poly(amino acid) such as poly-L-lysine also has been used (Fan and Chu, 1984). In addition, the nature of the hapten linkage may be critical. In some cases (Schubring and Chu, 1987), the linking group was recognized by the antibodies, thus decreasing the sensitivity of the assay. However, we found no improvement in the sensitivity using another linking group, hemisuccinate of isoborneol, or a different carrier, poly-L-lysine. Using an alkaline phosphatase instead of IgG peroxidase conjugate with *p*-nitrophenyl phosphate as the substrate did not seem to improve the sensitivity. Additionally, increasing reaction and color development times from the standard 30 min to 60 and 120 min had no positive effects.

Table II. Specificity of Antibodies toward Related Compounds

compound	structure	% cross-reactivity[a]	IC_{50},[b] M
camphor		100	1.44×10^{-4}
2-methylisoborneol		100	1.42×10^{-4}
isoborneol		100	1.38×10^{-4}
borneol		100	1.38×10^{-4}
camphorquinone		68	2.10×10^{-4}
norcamphor		0	nil
endo-norborneol		0	nil
norbornane		0	nil
geosmin		0	nil
2-methoxy-3-isopropylpyrazine		0	nil
2-methyl-2-bornene		100	1.40×10^{-4}

[a,b] See text for explanation.

Determination of Specificity of Antibody. The specificity of the antibody was tested by using structurally related compounds as competitive antigens in the ELISA. The structures of these compounds, the percent cross-reactivity, and the molar IC_{50} are listed in Table II. It is noted that the antibody was most specific for camphor, MIB, isoborneol, and borneol. Geosmin and 2-methoxy-3-isopropylpyrazine were not recognized by the antibody, confirming that they have a structure significantly different from that of camphor or MIB.

The importance of structure in antibody recognition is also demonstrated (Table II). While compounds such as norcamphor and norborneol are similar to camphor but lack the methyl groups, they were poorly recognized by the antibody, indicating that the methyl groups in camphor or MIB played an important role in antibody recognition. Both norcamphor and norborneol had the same cross-reactivity (i.e., 0%) as norbornane, indicating that the hydroxyl or carbonyl group at the 2-position on the molecule has no effect on the binding of antibody. Tests with camphorquinone, which has an additional carbonyl group as compared to camphor, showed some cross-reactivity with the antibody. This decrease in cross-reactivity compared to camphor may indicate the specificity of the antibody.

Further evidence for the specificity of the antibody came from the study of MIB dehydration products. Four major peaks were found in the GC–MS total ion chromatogram profile of the dehydration products. The relative ratio of the four products was 26:22:7:45. The 26% peak was identified as 2-methyl-2-bornene, a compound similar to MIB in structure (Table II). In contrast to the findings of Martin et al. (1988) that MIB was dehydrated to both 2-methyl-2-bornene and 2-methyleneborнane, the latter compound was not found in this study to be among the four major peaks. The other three peaks were not identified and did not contain fragments indicating structural similarity to MIB. When tested in the competitive ELISA, the dehydration product mixture showed approximately 20% cross-reactivity based on the total concentration of all four products. Because 2-methyl-2-bornene was the only probable reaction product, and its relative abundance was close to the 20% value, the cross-reactivity of this compound was interpreted to be 100%.

Summary. An indirect competitive ELISA for MIB was developed. A camphor–BSA conjugate was used as the immunogen. We have demonstrated in this study that an antibody directed against camphor was specific for MIB and did not react with the similar smelling odorants geosmin and 2-methoxy-3-isopropylpyrazine. The antibody had no cross-reactivity with norcamphor and norborneol, indicating that only the methyl groups in the camphor or MIB molecule were essential in antibody recognition. Additional side groups (e.g. two carbonyl groups in camphorquinone as compared to one carbonyl group in camphor) may also have an effect on antibody recognition. The importance of side chain groups was stressed in the case of antibodies against trichothecene mycotoxin (Hack et al., 1988) and sulfonamide antibiotics (Singh et al., 1989).

The assay has a detection limit of 1 μg/mL. Similar sensitivity was also reported with monoclonal antibodies directed against an insecticide (Brimfield et al., 1985) and mycotoxin (Casale et al., 1988). The fact that high sensitivity was not achieved may be due to the low affinity of the antibody for the hapten (Tuzi et al., 1988). While the human odor detection threshold is near 1 ng/L in water, the high specificity of the antibody described here could be useful in the rapid and simple routine screening for MIB in some circumstances.

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Registry No. MIB, 2371-42-8; camphor, 76-22-2; camphor-CMO, 124155-79-9.

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Determination of Bis(2-ethylhexyl) Phthalate in Cow's Milk and Infant Formula by High-Performance Liquid Chromatography

John A. Giust,† C. Thomas Seipelt, Brian K. Anderson, David A. Deis,* and John D. Hinders

Ross Laboratories, 625 Cleveland Avenue, Columbus, Ohio 43215

A method for the rapid extraction and analysis of milk or infant formula for bis(2-ethylhexyl) phthalate (DEHP) by high-performance liquid chromatography (HPLC) is reported. The method eliminates separatory funnel extraction and does not require a residue cleanup by gel permeation chromatography (GPC). Samples are solvent-extracted after being mixed with 10% deactivated silica. The extract is concentrated by Kuderna–Danish evaporation and the resulting residue analyzed directly by HPLC with UV detection. A method detection limit of 100 ppb was calculated. The overall time required for a complete determination is improved over methods using separatory funnel extraction and GC analysis by approximately 3-fold. Spike recovery of DEHP from infant formula and four different milk types (raw, whole, low-fat, and skim milk) ranged from 65% to 80% recovery at the 10 mg/L spike level.

Plasticizers are nonvolatile solvents compounded with plastic resins to increase their workability, flexibility, or extensibility. Bis(2-ethylhexyl) phthalate (DEHP) is the most common plasticizer, with an estimated annual production rate of 2 billion pounds worldwide (National Research Council, 1986).

DEHP has been found in soils, human and animal tissues, air, foods, and marine life (Peakall, 1975). Two studies have estimated the total daily human consumption of DEHP from all sources of exposure at 5.8 mg in the United States (U.S. Department of Health and Human Services, 1985) and 2.1 mg in Japan (Nakamura et al., 1979). Due to the apparent ubiquity of DEHP in the environment, the effects of human exposure to DEHP have been extensively researched during the past two decades (Department of Health, Education and Welfare, 1972; National Toxicology Program, 1981; Thomas et al., 1978).

Foods become contaminated with DEHP by contacting containers, wrappings, and food-processing equipment made of plastic or using plastic parts containing DEHP. For example, various types of plastic tubing are commonly used for conveying milk, and PVC food wrap is widely used in the United Kingdom (Castle et al., 1987). Since a plasticizer is not permanently bound to the plastic resin, it will migrate into foods containing lipophilic materials such as fats and oils. Because of this, the Food and Drug Administration (FDA) limits the DEHP content in materials contacting food to 3% (w/w) (*Code of Federal Regulations*, 1987).

Analytical methods commonly used for determining DEHP in milk or infant formula have been residue methods based upon a separatory funnel extraction and gas chromatographic analysis with flame ionization, electron capture, or mass detectors (Cocchieri, 1986; Thuren, 1986; Ferrario et al., 1985; Kamps et al., 1985; Petitjean-Jacquet and Vergnaud , 1983; Suzuki et al., 1979; Giam et al., 1975; Anderson and Lam, 1979). These analytical methods have excellent sensitivity; however, they often have very tedious extraction procedures. Also, milk extracts generally require a rigorous cleanup step, usually by gel permeation chromatography (GPC), to remove coextracted fats and oils before GC analysis.

In some instances, impurities can be intolerable since they limit the size of injections and require long, high-temperature oven hold times to completely desorb the chromatographic column. In these cases, a second cleanup step, either column chromatography or additional GPC, has been used (Burns et al., 1981). This laboratory initially employed a pesticide method developed by Kamps et al. (1985). The method consisted of a separatory funnel extraction, centrifugation of the resulting emulsion, and GPC cleanup of the residue, followed by GC/MS analysis. The method was effective, but very laborious. To overcome this disadvantage, a new method was devel-

† Present address: Hewlett-Packard, 675 Brooksedge Blvd., Westerville, OH 43081.

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