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High-Affinity Monoclonal Antibodies for Detection of the Microbial Metabolite, 2-Methylisoborneol

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The production of 2-methylisoborneol (MIB) by certain fungi and algae can contribute musty off-flavors to foods and water supplies if uncontrolled. The goal of this research was to develop a nonsensory simple method for the detection of MIB. Anti-MIB monoclonal antibodies were produced by immunizing mice with borneol-conjugated protein and selecting positive clones with an MIB−protein conjugate. An indirect competitive immunoassay developed using this antibody had a detection limit of 0.6 μg L^{-1} and an I_{50} value of 5 μg L^{-1}. Detection was relatively specific for MIB and showed 20% cross-reactivity with borneol or isoborneol and 4−5% cross-reactivity with camphor. No cross-reactivity to geosmin was observed.

KEYWORDS: 2-Methylisoborneol; borneol; monoclonal antibodies; ELISA; immunoassay

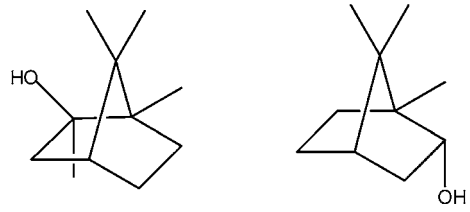
INTRODUCTION

2-Methylisoborneol (MIB, **Figure 1**) is produced by blue green algae (cyanobacteria), filamentous bacteria (actinomycetes), and fungi (*1*−*4*). MIB imparts musty flavor characteristics to potable water, mold-contaminated foods and some cultured or wild-caught fish (*5*−*8*) and has been an ongoing problem worldwide (*9*−*19*).

Sensory evaluation is a sensitive method of analysis currently available for detecting musty flavors in fish and is widely used by the catfish industry (*20*). For water, the odor threshold for humans has been reported as 30−40 ng L^{-1} (*21*, *22*). In fish, reported threshold values are 70−700 ng kg^{-1}, depending on the species tested (*23*). Sensory analysis is subjective, and for MIB, results can vary widely between tasters and replicate samples (*24*).

Gas chromatography−mass spectrometry (GC-MS) is considered the most useful nonsubjective method for MIB analysis and is used for MIB analysis in research settings (*11*, *25*, *26*). Ochiai et al. (*27*) reported a stir bar sorption−thermal desorption GC-MS for the extraction and analysis of water samples with a detection limit of approximately 0.1 ng L^{-1}. For the extraction and analysis of catfish samples, the limit of quantitation reported for microwave−distillation−solid phase microextraction GC-MS analysis is 100 ng kg^{-1} (*26*). The equipment and training requirements have limited the adoption of GC analysis by industry. There is a need for more rapid, simple, sensitive, and nonsubjective methods for routine quantitation of off-flavor compounds in water and foods.

Several authors have reported the development of antibodies for the detection of MIB, but these all resulted in detection limits that were significantly higher than the olfactory threshold levels. Chung et al. (*28*) and Chung (*29*) prepared polyclonal antibodies to a camphor−bovine serum albumin (BSA) conjugate. The resulting immunoassay gave a detection limit of only 1 mg L^{-1} and had 100% cross-reactivity to both camphor and the MIB dehydration product 2-methyl-2-borene. Miyamoto et al. (*30*) produced monoclonal antibodies to camphor−BSA and also obtained a detection limit for MIB of 1 mg L^{-1}. Onda et al. (*31*) describe the development of antiisoborneol monoclonal antibodies; the resulting immunoassay had a detection limit of 100 μg L^{-1} and showed 100% cross-reactivity with camphor and isoborneol.



a **b**

Figure 1. Structures of (**a**) the analyte of interest, MIB, and (**b**) the hapten used for synthesis of immunogen, (–)-borneol.

This paper describes the development of monoclonal antibodies with higher affinity to MIB than previous reports. Monoclonal antibodies were produced using a protein−borneol conjugate for immunization, selected for MIB affinity and used in a competitive indirect enzyme-linked immunosorbent assay (ELISA) to detect μg L^{-1} (ppb) levels of MIB.

MATERIALS AND METHODS

Supplies. (1*S*)-*endo*-(−)-Borneol and (±)-isoborneol were purchased from Aldrich (Milwaukee, WI). MIB ([1*R*-*exo*]-1,2,7,7-tetramethylbicyclo-[2.2.1]heptan-2-ol), succinic anhydride, 4-(dimethyl amino) pyridine,

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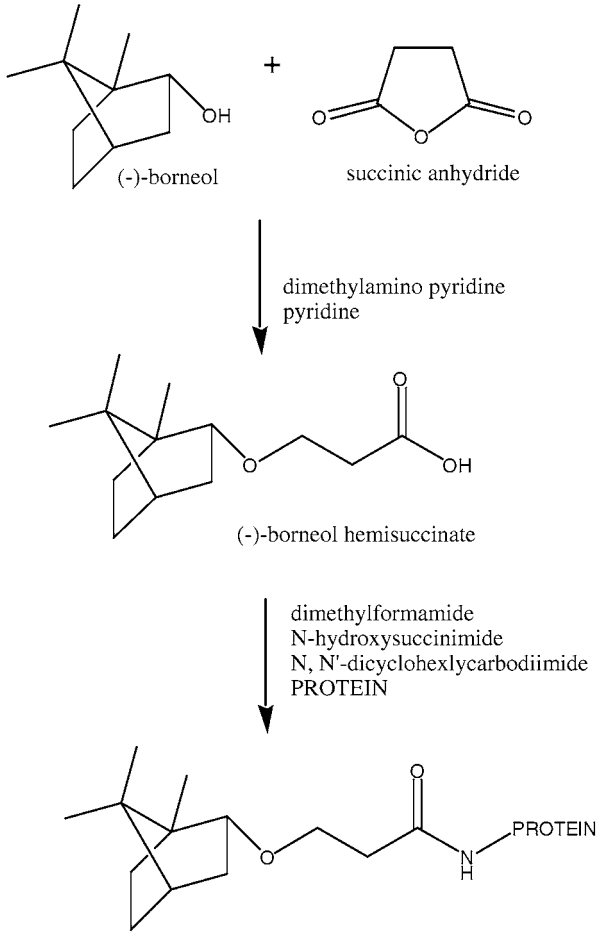
Scheme 1. Conjugation of (–)-Borneol to Protein by Hemisuccination and Active Ester Condensation



anisaldehyde, *N*-hydroxy succinimide, 1,3-dicyclohexylcarbodiimide, 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS), $(NH_4)_2CO_3$, urea, P_2O_5, BSA, *Limulus polyphemus* hemocyanin (LPH), tween-20, ISOstrip Mouse Isotyping Kit, and goat anti-mouse Ig G-peroxidase conjugate were obtained from Sigma (St. Louis, MO). Sterile poly(ethylene glycol) (PEG 1500) in 75 μM HEPES buffer was from Boehringer Mannheim (Indianapolis, IN). Ribi Adjuvant system (for mice) was from Ribi Immunochem Research (Hamilton, MA). Microtiter plates were Immulon 2 (Dynex Technologies, Chantilly, VA). Phosphate-buffered saline (PBS) solution was made as 0.9% NaCl in 0.01 M phosphate buffer, pH 7.3. To prepare PBST, tween 20 (0.05%, v/v) was added before the pH was adjusted.

Serum-free RPMI consisted of Rosewell Park Memorial Institute 1640 with added L-glutamine (2 μM), Na pyruvate (1 μM), penicillin (100 units/mL), and streptomycin (100 μg/mL) (all from Sigma). Complete RPMI was serum-free RPMI plus 20% (v/v) heat-inactivated fetal bovine serum (Sigma). Heat inactivation was achieved at 56 °C for 30 min. HT medium consisted of complete RPMI with 20% fetal bovine serum, sodium hypoxanthine (100 μM), and thymidine (16 μM). Hypoxanthine and thymidine were purchased sterile as 100× supplements from Gibco BRL (Gaithersburg, MD). HAT consisted of HT medium plus aminopterin (0.4 μM) (100× sterile, Gibco BRL).

Synthesis of Conjugates. Borneol−LPH and MIB−LPH were prepared as immunogens. MIB−BSA and borneol−BSA were prepared as solid phase protein conjugates. **Scheme 1** shows the conjugation of borneol to protein; the same reaction was used for the synthesis of all conjugates.

For the preparation of borneol−hemisuccinate, 31.1 mg of 4-dimethyl amino pyridine was added to 9.1 mL of pyridine prior to the addition of succinic anhydride (487.7 mg). Borneol (168.4 mg) was added, and the reaction mixture was heated under reflux, with stirring for 4 days, 5 h at 58 °C. The reaction was stopped by adding 20 mL of H_2O and was extracted three times with 25 mL of methylene chloride, and the combined organic layers (lower) were rotoevaporated and then dried under a vacuum over P_2O_5.

To prepare MIB hemisuccinate, the above procedure was followed but using 38.4 mg of 4-dimethylamino pyridine, 8.9 mL of pyridine, 445.7 mg of succinic anhydride, and 82 mg of MIB. The mixture was heated under reflux at 74 °C. After 3 days of reaction, most of the MIB had reacted, yielding MIB−hemisuccinate. The reaction product was extracted with methylene chloride and dried (154 mg).

To prepare borneol−LPH conjugate, all of the borneol−hemisuccinate produced, as described above, was dissolved in 4 mL of anhydrous dimethyl formamide (DMF); *N*,*N*′-dicyclohexyl-carbodiimide (87.9 mg) and *N*-hydroxysuccinimide (107 mg) were added; and the mixture was stirred for 12 h at 4 °C to form the active ester. LPH (25.1 mg) was dissolved in 4 mL of PBS, pH 7.4. Half of the active ester reaction mixture was filtered through glass wool into the LPH/PBS solution. The mixture was stirred for 22 h at 4 °C and then dialyzed for 2 h against 8 M urea (0.9 L) and then 1 h against 50 mM ammonium carbonate (2 L) and finally overnight against 25 mM ammonium carbonate (4 L). The final dialyzed product was lyophilized to 23.1 mg.

MIB−hemisuccinate (62 mg) was dissolved in 2 mL of anhydrous DMF, and then, *N*-hydroxysuccinimide (35 mg) and *N*,*N*′-dicyclohexyl-carbodiimide (51 mg) were added. The reaction mixture was stirred for 24 h at 4 °C and then filtered through glass wool into a BSA solution (127.3 mg in 3 mL of PBS). The mixture was stirred for 2 days at 4 °C, dialyzed as described above, and lyophilized (102.5 mg). To obtain only soluble conjugate, the dialysis product (80.2 mg) was dissolved in 15 mL of ammonium carbonate and centrifuged at 800*g* for 15 min. The supernatant was lyophilized and yielded 27.6 mg of soluble MIB−BSA conjugate.

Borneol−BSA was prepared using 109.4 mg of borneol−hemisuccinate, 117.3 mg of *N*-hydroxysuccinimide, and 133.9 mg of dicyclohexylcarbodimide in 4 mL of DMF. After 20 h at 4 °C, the product was added to a BSA solution (199.6 mg of BSA in 10 mL of PBS). After 2 days at 4 °C, the conjugate was dialyzed and lyophilized (196.8 mg).

Hemisuccination reactions were monitored by thin-layer chromatography (TLC): silica gel 60 F264 (0.2 mm thick) aluminum sheets from E. Merck, (Darmstadt, Germany) with a mobile phase of ethyl acetate, methanol, and 1% aqueous ammonia (80:20:10). Visualization was accomplished by spraying with anisaldehyde, glacial acetic acid, and concentrated sulfuric acid (0.5:50:1).

Matrix-Assisted Laser Desorption/Ionization (MALDI)-MS. The MW of the conjugates was determined using MALDI time-of-flight mass spectrometry (Bruker Proflex II MALDI-TOF, LSU Department of Chemistry MS Facility) with 2,5-dihydroxybenzoic acid (2,5-DHB) matrix solution and BSA reference. The analyte−matrix mixture was applied to the sample probe and air-dried. A linear, time lag focusing TOF MS was used to collect all mass spectra with 290 (350) laser shots. MIB−BSA was found to contain 10 mol MIB per BSA, isoborneol−BSA contained 15 isoborneol per BSA, and borneol−BSA contained 13 borneol per BSA.

Immunization. Two 4 month old BALB/c inbred female mice (Charles River Laboratories, Wilmington, MA) were housed in the LSU Life Sciences animal facility under an approved institutional animal care and use protocol. Preimmune blood samples (200 μL) were obtained (retro-orbital), and mice were immunized with 0.3 mg of borneol−LPH conjugate in 0.6 mL of sterile PBS/Ribi adjuvant system. Two 0.1 mL injections (sc and ip) were administered to each mouse. Subsequent injection boosts were made on days 21, 46, and 67. Blood samples were collected (retro-orbital) 7 days after each immunization. Blood samples were clotted at 37 °C for 30 min, stored at 4 °C overnight, centrifuged at 15 000*g*, and tested for antibody titer.

Cell Fusion, Selection, and Cloning. Myeloma cells (NS-1, ATCC No. TIB 18) were grown in sterile tissue culture flasks using complete RPMI with 20% fetal bovine serum at 37 °C with 5% CO_2 and 90−100% humidity. Cells were subcultured every 2−4 days to maintain cell density from about 10^5 to 10^6 cells per mL. On the day of fusion, actively dividing myeloma cells were washed twice with 15 mL of serum-free RPMI.

Three days after final injection, the selected mouse was asphyxiated using CO_2 and its spleen was removed under sterile conditions. Splenocytes were flushed from the spleen using two sterile needles (21 gauge) and syringes, transferred to a 15 mL conical centrifuge tube, and centrifuged at 250*g* for 5 min. The cells were washed twice with 10 mL of serum-free RPMI. Red blood cells were lysed by suspending the cells in 4 mL of 0.8% aqueous NH_4Cl for 1.5 min. The remaining intact splenocytes were centrifuged at 250*g* for 10 min, resuspended in 10 mL of serum-free RPMI, counted, and immediately used for fusion.

Splenocytes and myeloma cells were combined in a ratio of 13:1. The combined cells were gently mixed and centrifuged at 800*g* for 5 min. One milliliter of 1% PEG1500 in HEPES buffer was added to the cells with gentle mixing over 2 min. Serum-free RPMI (1 mL) was slowly added over the next 1 min with an additional 9 mL added over the next 3 min. The cell suspension was centrifuged in 8 mL of HAT medium, resuspended in 50 mL, and plated as 100 μL cell suspension per well in 96 well sterile plates. Control myeloma cells were added to eight wells. The cells were cultured in HAT medium for the first 10 days. For the next 10 days, cells were subcultured in HT medium.

After 5 days, supernatant (20 μL) from each well was screened for anti-MIB−BSA antibodies using a noncompetitive ELISA. The relative antibody (Ab) activity was expressed as the absorbance at 405 nm, measured after 30 min at room temperature.

Cells that showed relative Ab activities of greater than 1.0 were subcultured in 24 well plates and cloned using limiting dilution. Cells were diluted to be 10, 1, and 0.1 cells per 200 μL of conditioned medium in 96 well plates (30 wells of each concentration). Conditioned medium was 3 day old myeloma cell supernatant (RPMI containing 20% fetal bovine serum) that was centrifuged at 250*g* for 10 min, filtered (0.2 μm), and fortified with 20% fetal bovine serum and 2 μM glutamine. Macroscopic colonies, as seen by looking at the under surface of the plate, became visible after 1 week. Supernatants were assayed for anti-MIB Ab, and positive clones were transferred to 24 well plates. Selection was based on Ab production/affinity, sensitivity in competitive EI, and cell growth. The cloning procedure was repeated three times for the selected clone (f6b4 g7b4).

Determination of Antibody Titer. Ab titers of mouse sera or cell culture supernatants were tested using a checkerboard noncompetitve ELISA. A stock solution (100 μg mL^{-1}) of the solid phase MIB−BSA conjugate was serially diluted three times with PBS to obtain 100, 10, 1, 0.1, and 0 μg mL^{-1}. Each row of a microtiter plate was filled with 100 μL of one of the above concentrations, and the plates were stored overnight at 4 °C. The solution was removed from the plate, and each well was blocked with 200 μL of 1% BSA in PBS for 30 min at 37 °C and then washed three times with 200 μL of PBST for 5 min at room temperature.

Dilutions of mouse serum (1/500, 1/1000, 1/5000, 1/10 000, 1/100 000) or cell culture supernatant (1/125, 1/250, 1/500) were prepared in 1% BSA in PBS or culture media, respectively, and were added to plate wells (50 μL per well) so that each column contained a different serum concentration. After incubating for 30 min at 37 °C, wells were washed three times with 200 μL of PBST. The next steps, incubation with goat anti-mouse IgG−peroxidase conjugate, washing, incubation with substrate solution, and determination of absorbance, were as described for Indirect ELISA, below.

Indirect ELISA. Optimum concentrations (or ranges) of antibody and solid phase protein conjugate were predetermined using the checkerboard ELISA, above. Microplate wells were coated with solid phase protein conjugate (100 μL) and stored overnight at 4 °C. The next day, the solution was removed and the wells were blocked with 200 μL of 1% BSA in PBS for 1 h at room temperature. The wells were then washed three times with 200 μL of PBST for 5 min. MIB solutions (10-fold dilutions in 10% methanol) were added (50 μL per well) followed by prediluted anti-borneol Ab (50 μL per well). After they were incubated for 2 h at room temperature on an orbital shaker, the wells were washed three times with 200 μL of PBST. Prediluted goat anti-mouse IgG−peroxidase conjugate (1/2000) was added to the wells and incubated at room temperature for 2 h. The wells were then washed as before. Peroxidase substrate solution, consisting of ABTS (0.5 mg ml^{-1}) with 0.01% hydrogen peroxide in 0.1 M citrate buffer (pH 3.8), was added (100 μL per well). After the wells were incubated for 30 min at room temperature, the absorbance at 405 nm was measured using a plate reader (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA). Standard curve equations were determined using SOFTmax Pro 2.2 (Molecular Devices) as four parameter curves. % Cross-reactivity was calculated as (I_{50} value for MIB)/(I_{50} value for the comparison compound) × 100%.

Table 1. Comparison of A_0, I_{50}, and r^2 Values When Various Concentrations of MIB–BSA and Borneol–BSA Solid Phase Conjugates Were Used in Competitive Indirect ELISA[a]

solid phase conjugate	Mab supernatant dilution	[solid phase] (μg mL^{-1})	A_0	I_{50} (μg L^{-1})	r^2
borneol–BSA	1/250	1	3.454	251	0.926
		0.5	3.490	347	0.999
		0.25	3.522	95	0.999
		0.125	2.899	19	0.976
	1/500	1	3.140	153	0.998
		0.5	3.245	42	0.995
		0.25	3.051	17	0.993
		0.125	1.753	17	0.932
MIB–BSA	1/250	1	3.092	27	0.998
		0.5	2.886	3	0.995
		0.25	1.923	0.09	0.991
		0.125	0.593	0.45	0.955
		1	2.412	3	0.984
	1/500	0.5	1.243	1	0.992
		0.25	0.460	0.41	0.991
		0.125	0.082	1	0.917

[a] Mab was f6b4g7b4 culture supernatant diluted 1/250 or 1/500. Each value is the average of three curves determined on three microtiter plates.

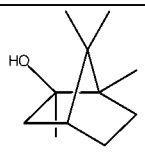
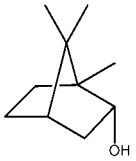
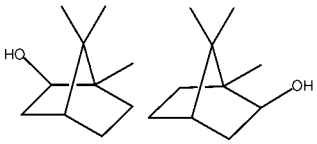
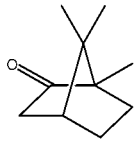
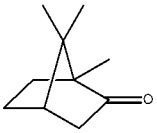
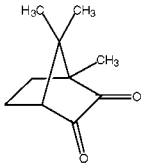
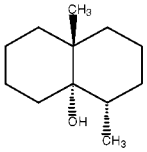
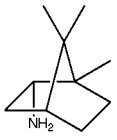
RESULTS AND DISCUSSION

Our approach for preparing conjugates for the production of anti-MIB antibodies was to use (−)-borneol, which has structural similarity to MIB but lacks methyl substitution at C2 and is both a stereoisomer and an epimeric isomer (**Figure 1**). After two immunizations, both mice showed the presence of antibodies that recognized MIB−BSA solid phase in noncompetitive ELISA. Mouse titers were approximately 1/10 000 (signal >3× background) using 1 μg mL^{-1} MIB−BSA solid phase. Competitive ELISA confirmed that serum antibodies also recognized unconjugated MIB in 10% methanol. The I_{50} values were approximately 1 mg L^{-1} for both polyclonal sera (diluted 1/5000) when 1 μg mL^{-1} MIB−BSA was used for the assay solid phase.

The fusion of splenocytes from one mouse with myeloma cells gave rise to between 5 and 10 hybridoma per well, for a total of approximately 400 hybridoma. The hybridoma actively grew in the presence of HAT medium while control myeloma did not. Five days after fusion, screening revealed that three wells contained Ab that recognized MIB−BSA. Of these, cells from two of these wells retained the ability to make Ab and recognized both MIB−BSA and unconjugated MIB. One of these was cloned three times to yield the f6b4g7b4 cell line that was used as the Mab source in the following experiments. The isotype was found to be IgG1.

The ELISA absorbance values without added analyte (A_o) showed that borneol−BSA had a higher signal as compared to MIB−BSA when equal concentrations were used for coating (**Table 1**). This may be due either to difference in affinity of the Ab to the two conjugates, the greater hapten substitution rate obtained for the borneol−BSA (13 vs 10 mol per BSA, determined by MALDI), or by a difference in their ability to

Table 2. Comparison of I_{50} Values and % Cross-Reactivity for Various Analytes (Solutions Made in 10% Methanol)[a]

Compound	I_{50} values ($\mu g\ l^{-1}$)	% Cross reactivity	Structure
MIB	19	100	
(-)-borneol	94	19.9	
(+/-)-isoborneol	95	19.6	
(+)-camphor	396	4.7	
(-)-camphor	496	3.8	
camphorquinone	589	3.2	
geosmin	1,090	1.7	
(+)-bornylamine	301,000	0	

[a] MIB–BSA solid phase was used at 0.5 μg mL^{-1}, and Mab was f6b4g7b4 culture supernatant diluted 1/250. Each value is the average of two curves determined on two microtiter plates.

bind to the microtiter plate plastic. It was expected that the anti-borneol Ab would show less affinity to conjugated MIB because of the steric hindrance by the C-2 methyl group. Screening of hybridoma using the MIB−BSA conjugate may have selected for antibodies with greater relative affinity to MIB, even though the immunization was made using a borneol-containing immunogen.

For a comparison of assay detection level, however, I_{50} values for competitive curves were compared (**Table 1**). As expected, the level of detection was lower when the solid phase conjugates and Mab were limiting but correlation coefficients (r^2) also decreased. Interestingly, the I_{50} values were lower when MIB−BSA was used as the solid phase, indicating that the assay sensitivity was improved with hapten heterogeneity. Good r^2

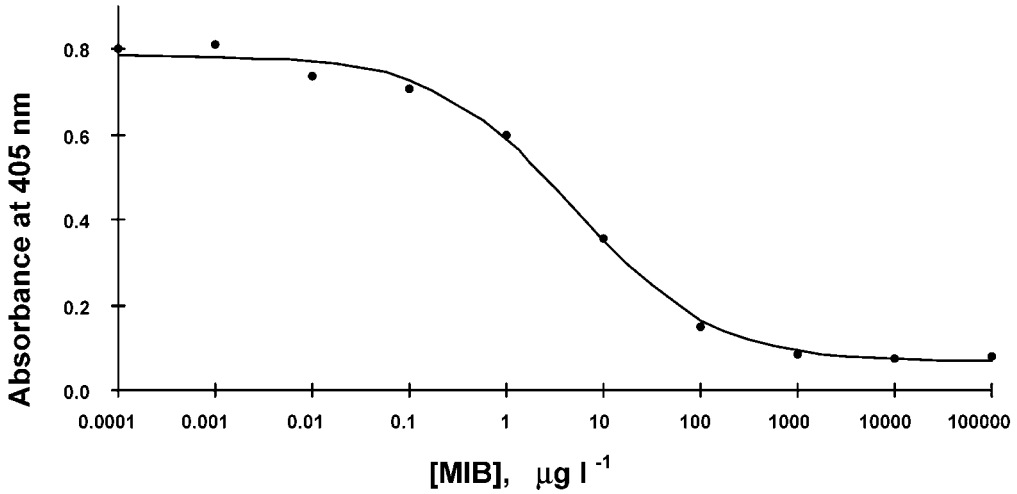


Figure 2. Representative standard curve for MIB in 10% methanol using competitve indirect ELISA with MIB–BSA (0.5 μg ml^{-1}) and antiborneol Mab (1/500 culture supernatant). Each point is the average of two determinations.

values were obtained for the four parameter curves for MIB−BSA concentrations of 0.5 or 1 μg mL^{-1} and Mab supernatant dilutions of 1/250 or 1/500. For the conditions used, the I_{50} values were from 1 to 27 μg L^{-1} for MIB−BSA.

Because the assay depends on the competition of the antibody binding to either immobilized conjugate or soluble MIB in the analyte solution, the sensitivity will be greatest if the antibody affinity for the soluble MIB is enhanced relative to the immobilized conjugate. Our choice of haptens for preparation of the immunogen and solid phase conjugates introduced both hapten and site heterology and may have lead to the low level of detection that could be obtained in the immunoassay.

In a separate experiment, the specificity of the MAb was determined by comparing the cross-reactivity toward several compounds with structural similarity to MIB in ELISA using 0.5 μg mL^{-1} and Mab diluted 1/500 (**Table 2**). MIB had a cross-reactivity of 100%, by definition, and showed the highest cross-reactivity as compared to all of the compounds tested. MAb were made using (−)-borneol−LPH immunogen, which is both a stereoisomer and an epimeric isomer of MIB. (−)-Borneol, however, showed only 19.9% cross-reactivity as compared to MIB, similar to the cross-reactivity of (±)-isoborneol (19.6%). The position of the hydroxyl group therefore had minimal influence on binding affinity (regardless of the epimer), but the presence of the C-2 methyl group in MIB seemed to be more important. Lower antibody recognition of borneol and isoborneol, as compared to MIB, is desirable since these compounds may be present in the environment but are not known to contribute to musty off-odors.

Camphor showed low cross-reactivity, approximately 4%. This may be attributed to the presence of its ketone, which would influence the bond angles and lengths in the ring structure. Camphorquinone, with an additional carbonyl group as compared to camphor, showed even lower cross-reactivity. The polyclonal antibody produced by Chung et al. (*28*) and Chung (*29*), using a camphor−BSA immunogen, showed a relatively low affinity to MIB. Therefore, for structures containing rings, hapten heterogeneity may only be useful if the immunogen and solid phase haptens are only slightly different in structure. Again, low cross-reactivity to camphorlike structures will improve the assay specificity for MIB.

Geosmin, while not close to MIB structurally, was evaluated for cross-reactivity since it can also cause musty flavor, has similar origins (fungal, algal) as MIB, and can be present in similar types of samples (*11*, *24*). The Mab developed in this study will not detect geosmin (**Table 2**). This would need to be considered if one was interested in the evaluation of musty flavor of samples, rather than specifically MIB.

2-Methyleneborane and 2-methyl-2-borene are dehydration products of MIB, do not cause off-flavor, but can be present in chronically off-flavored catfish flesh (*32*−*34*). These two dehydration products were not evaluated for cross-reactivity in this study, but this is expected to be low since, as with camphor, the presence of double bonds would greatly affect the ring structure bond angles. Further studies will be needed to determine if they could yield false positives.

A typical standard curve is shown for the ELISA using antiborneol Mab diluted 1/500 times and 0.5 μg/mL of MIB−BSA solid phase conjugate (**Figure 2**). MIB was diluted in 10% methanol from 100 mg/L to 0.1 ng/L, and each concentration was run in duplicate. For this standard curve, the I_{50} value was 5 μg L^{-1}, the r^2 was 0.997, and the detection limit was 0.6 μg L^{-1}.

CONCLUSIONS

It was demonstrated that monoclonal antibodies and an immunoassay are able be produced with sufficiently high MIB sensitivity to detect this compound at levels that are approximately 100-fold lower than previously reported ELISA methods. Although the immunogen was a protein conjugate of (−)-borneol, selection of positive clones using an MIB−BSA conjugate enabled us to obtain a monoclonal antibody with greater relative affinity to unconjugated MIB vs unconjugated borneol. We suggest that the choice of heterologous haptens for immunization and the screening assay was necessary for this purpose.

This immunochemical method, if adapted and validated for the analysis of MIB in potable water and foods, may prove to be an advantage over chromatographic analysis, especially for field testing. Rapid tests may be developed that will provide on-site analysis without the need for sample purification or specialized equipment. A U.S. Patent has been issued for this technology (No. U.S. 6,444,433 B1).

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