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Species-Specific Identification of Seven Vegetable Oils Based on Suspension Bead Array

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ABSTRACT: Species adulteration of vegetable oils has become a main form of adulteration in vegetable oils, severely violating consumer rights and causing disorder in the market. A reliable method of species authentication of vegetable oils is desirable. This paper reports a novel method for identification of seven species of vegetable oils based on suspension bead array. One pair of universal primers and seven species-specific probes were designed targeting *rbcl* gene of the chloroplast. Each probe was coupled to a unique color-coded microsphere. Biotinylated PCR amplicons of seven oils were hybridized to the complementary probes on microsphere sets. Bound amplicons were detected fluorometrically using a reporter dye, streptavidin−R-phycoeryt hrin (SA-PE). A sample could be analyzed less than 1 h after PCR amplification. With the exception of olive probe, all probes showed no cross-reactivity with other species. Absolute detection limit of the seven probes ranged from 0.01 ng/μL to 0.0001 ng/μL. Detection limit in DNA mixture was from 10% to 5%. Detection of vegetable oils validated the effectiveness of the method. The suspension bead array as a rapid, sensitive, and high-throughput technology has potential to identify more species of vegetable oils with increased species of probes.

KEYWORDS: *suspension bead array, vegetable oils, PCR, adulteration*

INTRODUCTION

In the market of vegetable oils, adulteration of premium products with cheap species is commonly conducted by illegal operators for profiteering. In order to protect the interests of consumers and maintain market fairness, it is necessary to establish a sensitive and high-throughput method to identify complex adulteration of commercial oils. Some techniques have been reported for this purpose such as gas chromatography,[1,2] liquid chromatography,[3] mass spectrometry,[4] and spectroscopy.[5−7] Since analytes in these methods were usually chemical ingredients and their amount and structure could be affected by planting, processing, and storage, a DNA based method which could unambiguously determine biological identification of oil raw materials would be a better choice. For a DNA based method, real-time PCR is the most frequently used,[8−10] however it is limited to no more than five targets per reaction. We have reported a PCR-CE-SSCP to detect cheap oil blended in olive oil.[11] In the method, various species were detected simultaneously by two pairs of universal primers. The flaw was that the identity of fraud species needs to be confirmed by sequencing. Suspension bead array as a high-throughput detection technology has potential to simultaneously identify multiple fraudulent species of vegetable oils. According to a report, 45 human papillomaviruses (HPV) were simultaneously typed by use of a suspension system.[12] Currently, this technology has been used in a variety of applications such as SNP,[13,14] gene expression,[15] infectious disease,[16] HLA,[17] and microRNA.[18]

The suspension bead array system incorporates 5.6 μm polystyrene microspheres that are internally dyed with two spectrally distinct fluorochromes (red and infrared). Using precise amounts of each of these fluorochromes, an array is created consisting of 100 different microsphere sets with specific spectral addresses. A rapid flow cytometer is used to allow multiple color-coded microbeads to be detected in solution of a single tube or a single well of a 96-well microplate.[19] Species-specific probes are coupled with uniquely color-coded microbeads. Biotinylated PCR amplicons hybridized to the complementary probes on bead sets were detected fluorometrically using a reporter dye, streptavidin−R-phycoerythrin (SA-PE) (Figure 1). In our work, seven kinds of species-specific

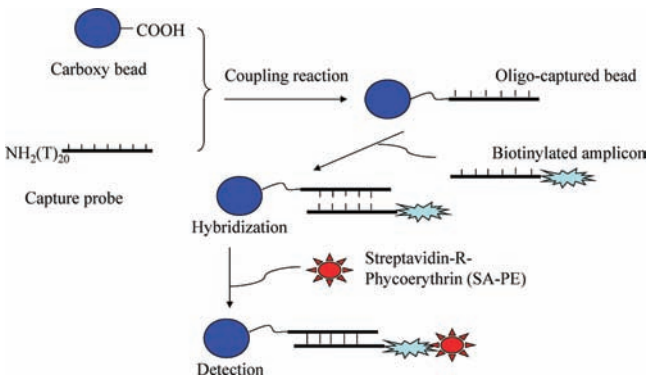

Figure 1. Schematic summary of the suspension bead array method.

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Table 1. Information of Seven Species-Specific Hybridization Probes Used in This Method

target species	specificity	microsphere no.[a]	probe sequence (5′−3′)[b]	GenBank Accession No.
olive	*Olea europaea*	28	CCATATTGAGCCCGTTCCTG	DQ673304.1
peanut	*Arachis hypogaea*	35	CCGGTTGCTGGCGAAGAAAA	U74247.1
rapeseed	*Braccica rapa*	25	CCAGGAGAAGAAACTCAATT	AY167977.1
sesame	*Sesamum indium*	42	CCGGTTCCTGGAGAAACAGA	L14408.1
soybean	*Glycine max*	50	CCTGTTGCTGGGGAAGAAAA	Z95552.1
sunflower	*Helianthus annuus*	43	TGGACTTGAGCCTGTTCCTG	L13929.1
maize	*Zea mays*	45	CCCGTTCCTGGGGACCCAGA	Z11973.1

[a]The probes were coupled to seven types of microspheres coded with different colors, and the number corresponding to each type of microsphere was generated automatically by the system. [b]The 5′ end of the probe was linked with 20 T which was modified with an amino group to conjugate with carboxyl group on the surface of microspheres.

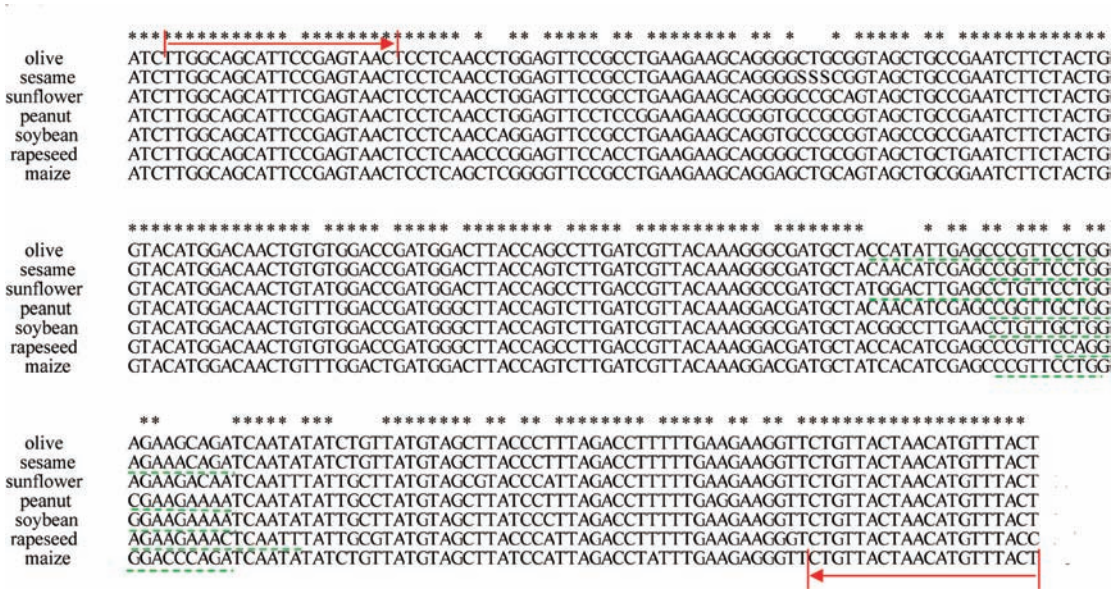

Figure 2. Results of *rbcl* gene sequences alignment of seven vegetable oils. The universal primer pairs are marked with arrows. Species-specific probes were synthesized according to sequence of variable regions underlined.

probes were fixed on uniquely color-coded beads to detect corresponding DNA fragments in the sample. The throughput could be improved because up to 100 probes could be coupled with 100 kinds of beads.

MATERIALS AND METHODS

Sample Collection. Seeds of maize (*Zea mays*), rapeseed (Braccica rapa), soybean (*Glycine max*), sesame (Sesaum indium), sunflower (*Helianthus annuus*), and peanut (*Arachis hypogaea*) and leaves of olive (*Olea europaea*) were provided by Institute of Oil Plant, Chinese Academy of Agricultural Science. The other biological materials, including rice, wheat, cottonseed, cashew, pine nut, ordinary sunflower, mustard, celery, camellia, sheep, and pig, were obtained from a local market in Beijing. Oils were prepared in our lab from seeds of peanut, soybean, olive, and sunflower. Leaves of olive were stored at −80 °C. Oils were stored in the dark at room temperature. Other samples were stored at 4 °C.

DNA Extraction. The Wizard Magnetic DNA Purification System for Food (Promega, USA) was used for extraction of DNA from all samples. DNA extraction of vegetable oils followed a modified kit method. 20 mL of oil was halved in two tubes. 2 mL of lysis solution A, 1 mL of lysis solution B and 10 mL of *n*-hexane were added to every tube and centrifuged for 1 h at 7000*g*. The lower liquid was transferred into a new 50 mL tube. 5 mL of chloroform was added and centrifuged at 12000*g* for 15 min. The upper phase was transferred into a new 50 mL tube. 0.8 vol of the transferred solution of isopropanol was added for nucleic acid precipitation. After incubation at room temperature for 1 h, the sample was centrifuged at 12000*g* for 15 min. The pellet was washed with 1 mL of 70% (v/v) ethanol and dissolved in 200 μL of ddH_2O. MagneSil Paramagnetic Particles (PMPs) were used to purify DNA following the corresponding protocol of the kit. Magnetic beads were added only once. Finally, 50 μL of ddH_2O was used to dissolved DNA.. The DNA was quantified with a DU640 nucleic acid and protein analyzers (Backman, USA). DNA extraction of other samples was followed the protocol of the kit.

PCR Amplification. Universal primers targeting the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (*rbcl*) were used. The forward primer was 5′-TTGGCAGCATTCCGAGTAAC-3′; the reverse primer was 5′-AGTAAACATGTTAGTAACAG-3′. The reverse primer was biotinylated at the 5′ end to bind to streptavidin−R-phycoerythrin (SA-PE). The PCR reactions were set up in a total volume of 25 μL containing 1×Multi HotStart Buffer,1.5 mmol/L $MgCl_2$, 1 U of Multi HotStart DNA Polymerase (Qiagen, Germany), 200 nmol/L dNTP mixture (TaKaRa, Japan), 200 nmol/L of each primer (synthesized by Invitrogen company, China), 5 μL of template DNA, and 12.8 μL of sterilized ddH_2O.The blank control used water instead of template DNA. The PCR was performed in a thermal cycler (Eppendorf, Germany), according to the following protocol: 95 °C for 10 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; a final elongation at 72 °C for 5 min, followed by cooling at 4 °C. PCR products were analyzed by electrophoresis on 2% (w/v) agarose gels and stained with ethidium

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bromide (EB). Gels were photographed with a Bio Imaging system (Gene Genius, Germany).

Probe Design. Selection of hybridization probe was based on comprehensive *rbcl* sequence alignment of seven species listed in Table 1 by ClustalW (Figure 2). Targeting a region which had intraspecies consensus and interspecies diversity, we designed seven species-specific probes. Each probe had an optimal length of 20 nucleotides, and their G + C content were around 55%. This facilitated multiplex hybridization under uniform conditions. All probes were tagged with the 5′ end amino-modified 20 T (thymidine) as spacers. The reactive primary amino group facilitated the coupling to the carboxylated polystyrene microspheres.

Coupling of Capture Probes to Carboxylated Microspheres. A total of 6.25×10^5 (50 μL) polystyrene microspheres (Bio-Rad, USA) were centrifuged at 10000*g* in a 1.5 mL centrifuge tube. The supernatant was removed, and the microspheres were resuspended in 50 μL of 100 nmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 4.5). 0.05 mol of probe and 2.5 μL of 10 mg/mL fresh *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC, Sigma) were added and incubated in the dark for 30 min at room temperature with constant shaking. The EDC (freshly prepared) treatment and incubation steps were then repeated once. Coupled microspheres were washed with 1 mL 0.02% Tween20 followed by a second wash with 1 mL of 0.1% (w/v) SDS (sodium dodecyl sulfate). Coupled microspheres were resuspended in 50 μL of TE buffer (10 mmol/L Tris-HCl,1 mmol/L EDTA, pH 8.0) and stored in the dark at 4 °C.

Hybridization Reaction and Fluorescence Intensity Measurement. Coupled microsphere sets were selected and resuspended by vortex and sonicated for approximately 20 s. The hybridization reaction was set up in a total volume of 50 μL containing 0.5 μL of each type of coupled microsphere (approximately 5000 microspheres per type), 33 μL of 1.5×TMAC (tetramethylammonium chloride) hybridization buffer (4.5 mol/L, 0.15% TMAC, Sarkosyl, 75 mmol/L Tris-HCl [pH 8.0], 6 mmol/L EDTA [pH8.0]), 1 μL of PCR amplicons and TE buffer. The reaction mixture was denatured at 95 °C for 5 min in a PCR thermocycler (Eppendorf, Germany), followed by 10 min of hybridization at 50 °C. In order to avoid cross-reactivity, hybridization buffer was drained soon after reaction. 50 μL of 10 ng/μL streptavidin−R-phycoerythrin (SA-PE, Invitrogen, China) was added to every reaction well, and the mixture was incubated for 5 min in the dark. Every reaction well was washed with TE once. Microspheres were resuspended with 125 μL of TE.

Resuspended microspheres were analyzed by Bio-Plex 200 suspension array system (Bio-Rad, USA). The detection platform automatically calculated the median fluorescence intensity (MFI) of 50 microspheres of each set. The MFI values presented were all subtracted from the background MFI determined by blank control of PCR. Signals were regarded as positive if the MFI of samples was at least 10 times higher than the background MFI.

RESULTS

Amplification by Universal Primers. PCR products from 18 samples were analyzed by 2% agarose gel electrophoresis (Figure 3). It showed that amplicons with predicted length (246 bp) were amplified from all plants.[1−16] No product was found in the lanes of pig,[17] sheep,[18] and blank (B). Therefore the universal primer pair is efficient and specific to amplify target fragment from plant materials.

Specificity. PCR products from the 18 samples were further tested by suspension bead array. As shown in Table 2, the MFI (mean fluorescence intensity) value of all samples was recorded. The cutoff value was set as 10 times higher than the background MFI, e.g. for probe 35, the cutoff value would be 50. Accordingly, probe 35 hybridized exclusively with peanut, probe 42 with sesame, probe 25 with rapeseed, probe 28 with maize (slightly higher than cutoff) and olive, probe 43 with sunflower (oil and ordinary sunflower), probe 50 with soybean, probe 45 with maize. The weak cross-reactivity between probe 28 and maize DNA would not compromise the specificity of the whole system because maize DNA was specifically identified by probe 45.

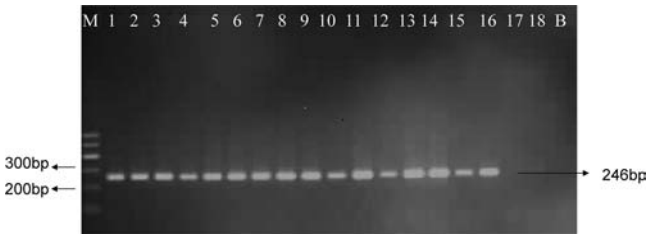

Figure 3. vAgarose gel electrophoresis of PCR amplification product of different samples: M, 100 bp DNA ladder marker; 1, maize; 2, rapeseed; 3, soybean; 4, olive; 5, peanut; 6, oil sunflower; 7, sesame; 8, ordinary sunflower; 9, cottonseed; 10, pine nut; 11, cashew; 12, wheat; 13, rice; 14, mustard; 15, celery; 16, camellia; 17, pig; 18, sheep; B, blank.

Table 2. MFI Values Resulting from Hybridization between Seven Probes and 18 DNA Samples

	capture probe no.[a]						
target	35	42	25	28	43	50	45
maize	4	3	−1	**102**	26	65	**1091**
rapeseed	5	7	**1573**	56	19	3	10
soybean	4	−1	5	8	18	**1095**	1
olive	5	4	5	**813**	24	4	4
peanut	**2301**[b]	2	5	8	14	3	4
oil sunflower	−5	6	4	14	**2894**	−1	5
sesame	5	**1323**	2	12	18	1	3
ordinary sunflower	8	5	1	13	**1818**	−2	8
cottonseed	1	−1	7	61	25	0	1
pinenut	5	4	9	17	20	2	2
walnut	3	−2	8	9	6	0	10
cashew	17	0	7	11	5	3	7
wheat	9	5	21	10	50	−1	8
rice	5	3	1	8	1	1	9
potato	1	2	1	9	−5	9	−2
mustard	4	4	50	11	8	−2	3
celery	6	−3	7	8	9	3	−2
sheep	3	−3	6	8	7	0	6
pig	0	1	−5	8	9	3	7
blank	5	10	10	8	9	10	6

[a]Capture probes 35, 42, 25, 28, 43, 50, and 45 were designed to specifically detect peanut, sesame, rapeseed, olive, sunflower, soybean and maize. All probes target plant *rbcl* gene. [b]MFI (median fluorescent intensity) was subtracted from MFI of the background. Cutoff value was set as 10 × MFI of the blank control of that probe set. Cutoff values of seven probes were 50, 100, 100, 80, 90, 100, 60. MFI higher than the cutoffs was a positive reaction.

Reproducibility. In order to assess reproducibility of each specific probe, three independent assays were performed in three days. In each assay, 5 ng of template DNA was amplified. Amplicons were hybridized to specific probes coupled to uniquely defined, color-coded microspheres. The measurement of MFI of each sample was performed in triplicate, and the mean MFI was calculated. Table 3 shows the results of reproducibility assays. The coefficient of variation (CV) of each

Table 3. Results of Reproducibility Assays

Target	mean MFI of each indep assay	mean MFI of three indep assays	CV (%) of three indep assays
olive	1977, 2071, 1952	1997	3.3
peanut	2645, 2665, 2500	2603	3.4
soybean	1074, 1084, 1075	1078	0.5
sunflower	4172, 4895, 4843	4637	8.7
maize	1400, 1312, 1512	1408	7.1
sesame	2010, 1989, 2210	2069	5.9
rapeseed	2465, 2578, 2598	2547	2.8

probe in three independent assays was below 10%, indicating the good reproducibility of this method.

Sensitivity. Plant DNA was serially diluted (10^{-5} to 10 ng/μL) to determine the absolute sensitivity of the system. Each sample was analyzed in 10 independent assays. Positive rate (frequency of positive results/10) was calculated for each dilution. As shown in Table 4, data from 10 independent assays indicated that MFI of soybean and sesame probes were above cutoff value when DNA concentration was as low as 0.0001 ng/μL; MFI of peanut, sunflower, rapeseed, and maize probe were above threshold when DNA was as low as 0.001 ng/μL; and for olive DNA, MFI of olive probe was above cutoff value when DNA was reduced to 0.01 ng/μL. Therefore the absolute sensitivity of the system would be 0.01 ng/μL to 0.0001 pg/μL.

To investigate relative sensitivity of the system, as shown in Table 5, four pairs of plant DNA were mixed at series ratios to be detected by corresponding probes. It was evident that the MFI value decreased with decreasing ratio of target DNA. Detection limit of olive, maize, soybean, peanut, and sesame probes was 5%. Detection limit of sunflower and rapeseed was 10%.

Efficiency. One advantage of this method was that multiple ingredients could be detected in one reaction. As shown in Figure 4, DNA from peanut, soybean, sunflower, and sesame were mixed in equal proportions (v/v) and tested in a single well with mixed beads coupled with corresponding probes. The histogram revealed positive signals from the probes of all four target species. Therefore amplicons derived from different species could be detected simultaneously in one assay, which would contribute to improve efficiency in routine inspection.

Detection of Vegetable Oil Samples. To validate feasibility of the method in detecting oil samples, DNA was extracted from 4 oil samples and PCR with the universal primer pair was conducted. In suspension bead array microspheres coupled with 7 probes were mixed and loaded to one reaction well. As revealed in Table 6, for each sample, a positive signal was obtained only from the probe corresponding to the species from which the oil was originated. For instance, when the PCR product from sunflower oil was hybridized with mixed beads, the system detected a strong MFI signal (4779) as compared to the blank value (12) from bead coupled with sunflower specific probe, while the signals from the other beads were much lower than the cutoff value (120). This experiment demonstrated that the method applied as well to oil products as to raw materials in terms of specificity and signal intensity.

In conclusion, species adulteration of vegetable oils has become a main concern for government, the food industry, and consumers. Adulterated vegetable oils are often simultaneously adulterated with several other species of vegetable oils. Using the presently available methods, it is difficult to rapidly detect multiple fraud species in vegetable oils. A rapid and high-throughput detection method for species-specific identification of vegetable oils is desirable. In this paper we reported a highly efficient method applying suspension bead array to detect the origin of seven vegetable oils at one time. With the help of this highly efficient system, detection of 7 vegetable oils could be finished within 1 h after PCR. Each probe showed no cross-reactivity with other species except for weak reactivity between olive probe with maize amplicon which could be offset by strong specificity of maize probe. The absolute detection limit of the seven probes ranged from 0.01 ng/μL to 0.0001 ng/μL.

Plant chloroplast gene is often used to study plant species owing to its intraspecies consensus and interspecies variability. Vegetable oils are deeply processed food, and DNA exists in residual, rendering a great challenge for a DNA based method. Since chloroplast is present in multiple copies in a cell, a PCR method based on chloroplast gene would be more sensitive. In this work, a pair of universal primers targeting *rbcl* were designed to simultaneously amplify from seven species, and species-specific probes based on the variable region of the gene were designed to identify the species. Compared with a specific PCR method, a universal system is more efficient and easier for operation. Though so far only seven probes were included in the experiment, much more species-specific probes would be synthesized to include a wider range of oil species in the future. As the species of probes increase, the system has potential to detect more species composition in vegetable oils.

The universal primer pair and seven species-specific probes targeting seven vegetable oils are the core of the method. In case some advanced instruments, such as Bio-Plex 200 suspension array system, are not available in certain laboratories, other alternatives can be chosen to achieve the same detection goal.

Table 4. Results of Absolute Sensitivity Assay of Seven Probes

concn of target DNA (ng/μL)	positive rate of 10 indep assays						
	maize	rapeseed	soybean	olive	peanut	sesame	sunflower
10	10[a]/10[b]	10/10	10/10	10/10	10/10	10/10	10/10
1	10/10	10/10	10/10	10/10	10/10	10/10	10/10
0.1	10/10	10/10	10/10	10/10	10/10	10/10	10/10
0.01	10/10	10/10	10/10	10/10	10/10	10/10	10/10
0.001	10/10	10/10	10/10	0/10	10/10	10/10	10/10
0.0001	0/10	0/10	10/10	0/10	2/10	10/10	0/10

[a]The frequency of positive results in 10 independent assays. [b]10 independent assays.

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Table 5. Test of DNA Mixture of Two Different Species in Different Proportion in a Single Well

DNA mixture (A and B)	mix proportion (A:B, v/v)	specific probe, MFI	detection result (species)
olive and maize	95:5	olive, 1637; maize, 481	olive, maize
	90:10	olive, 1549; maize, 954	olive, maize
	70:30	olive, 915; maize, 1291	olive, maize
	50:50	olive, 589; maize, 1316	olive, maize
	30:70	olive, 424; maize, 1445	olive, maize
	10:90	olive, 383; maize, 1474	olive, maize
	5:95	olive, 365; maize, 1568	olive, maize
sunflower and soybean	95:5	sunflower, 4840; soybean, 204	sunflower, soybean
	90:10	sunflower, 4869; soybean, 379	sunflower, soybean
	70:30	sunflower, 4221; soybean, 567	sunflower, soybean
	50:50	sunflower, 2864; soybean, 858	sunflower, soybean
	30:70	sunflower, 1099; soybean, 1069	sunflower, soybean
	10:90	sunflower, 432; soybean, 1295	sunflower, soybean
	5:95	sunflower, 40; soybean, 1338	soybean
peanut and soybean	95:5	peanut, 1956; soybean, 624	peanut, soybean
	90:10	peanut, 1677; soybean, 778	peanut, soybean
	70:30	peanut, 1059; soybean, 854	peanut, soybean
	50:50	peanut, 1021; soybean, 1020	peanut, soybean
	30:70	peanut, 551; soybean, 1200	peanut, soybean
	10:90	peanut, 123; soybean, 1307	peanut, soybean
	5:95	peanut, 93; soybean, 1274	peanut, soybean
sesame and rapeseed	95:5	sesame, 1943; rapeseed, 48	sesame
	90:10	sesame, 1965; rapeseed, 102	sesame, rapeseed
	70:30	sesame, 1900; rapeseed, 424	sesame, rapeseed
	50:50	sesame, 1842; rapeseed, 779	sesame, rapeseed
	30:70	sesame, 1720; rapeseed, 1021	sesame, rapeseed
	10:90	sesame, 1512; rapeseed, 1523	sesame, rapeseed
	5:95	sesame, 434; rapeseed, 2003	sesame, rapeseed

For instance, probes can be fixed on the surface of a biochip. SA-PE could be substituted with anti-biotin horseradish peroxidase conjugate and a precipitable horseradish peroxidase substrate. Biotinylated amplicons perfectly matched with the probes can be visualized as a color change on the biochip surface. Other chromogenic enzymatic reactions also are likely to be used to indicate whether amplicons are hybridized to probes. However, the detection throughput of biochip technology may be less high than that of suspension bead array.

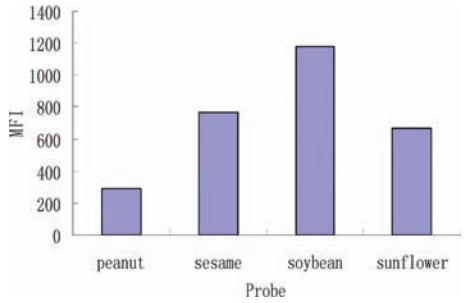

Figure 4. Test of DNA mixture. DNA from peanut, soybean, sunflower, and sesame were mixed in equal proportions (v/v) and tested in a single well with mixed beads coupled with corresponding probes.

Table 6. Detection Results of Four Vegetable Oil Samples

	MFI value of capture probe						
oil samples	peanut	sesame	rapeseed	olive	sunflower	soybean	maize
sunflower	4	5	3	79	**4779**	3	1
peanut	**1789**	12	48	47	36	22	7
olive	5	6	10	**1680**	12	15	9
soybean	3	6	3	2	12	**1359**	8
blank	13	12	8	11	12	11	7

Considering experimental purpose and instrumental conditions, the method can be modified flexibly.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

EB, ethidium bromide; SA-PE, streptavidin–R-phycoerythrin; *rbcl*, ribulose 1,5-bisphosphate carboxylase/oxygenase; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; TMAC, tetramethylammonium chloride

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