JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Quantitative Bioluminometric Method for DNA-Based Species/Varietal Identification in Food Authenticity Assessment

Ioannis A. Trantakis,[†] Theodore K. Christopoulos,*[,†,●] Stelios Spaniolas,[§,⊥] Panagiotis Kalaitzis,[§] Penelope C. Ioannou,[#] and Gregory A. Tucker[⊗]

[†]Department of Chemistry, University of Patras, Patras, Greece 26504

[§]Department of Horticultural Genetics and Biotechnology, Mediterranean Agronomic Institute, Chania, Greece 73100

[#]Department of Chemistry, University of Athens, Athens, Greece 15771

[⊗]Division of Nutritional Sciences, University of Nottingham, Nottingham, United Kingdom LE12 5RD

[●]Foundation for Research and Technology Hellas, Institute of Chemical Engineering and High Temperature Chemical Processes (FORTH/ICE-HT), Patras 26504, Greece

Supporting Information

ABSTRACT: A method is reported for species quantification by exploiting single-nucleotide polymorphisms (SNPs). These single-base changes in DNA are particularly useful because they enable discrimination of closely related species and/or varieties. As a model, quantitative authentication studies were performed on coffee. These involved the determination of the percentage of Arabica and Robusta species based on a SNP in the chloroplastic trnL(UAA)-trnF(GAA) intraspacer region. Following polymerase chain reaction (PCR), the Robusta-specific and Arabica-specific fragments were subjected to 15 min extension reactions by DNA polymerase using species-specific primers carrying oligo(dA) tags. Biotin was incorporated into the extended strands. The products were captured in streptavidin-coated microtiter wells and quantified by using oligo(dT)-conjugated photoprotein aequorin. Aequorin was measured within 3 s via its characteristic flash-type bioluminescent reaction that was triggered by the addition of Ca^{2+}. Because of the close resemblance between the two DNA fragments, during PCR one species serves as an internal standard for the other. The percentage of the total luminescence signal obtained from a certain species was linearly related to the percent content of the sample with respect to this species. The method is accurate and reproducible. The microtiter well-based assay configuration allows high sample throughput and facilitates greatly the automation.

KEYWORDS: *food authentication, quantitative, bioluminescence, photoprotein aequorin, authenticity, coffee*

INTRODUCTION

Protection of the consumer from accidental or deliberate substitution of valuable food constituents with other lower value components necessitates the development of convenient and robust analytical methods that are suitable for routine authenticity assessment. In particular, identification of the species of origin has become a major issue in food authentication. Species identification can be performed through the determination of characteristic small molecules, or protein-based tests (immunochemical methods), or DNA-based assays. DNA, however, is the preferred analyte, mainly due to its higher stability in a variety of food-processing conditions but also because it is present in nearly all of the cells of an organism, thereby facilitating sampling. Most DNA assays involve exponential amplification of the target sequence by the polymerase chain reaction (PCR).[1−4]

Quantification is an important aspect of food authenticity testing, which is recently gaining increasing attention. Quantification is accomplished either by real-time PCR or by quantitative competitive PCR.[3] In real-time PCR the amount of amplification product is measured continuously in the reaction mixture by a homogeneous fluorometric assay. However, real-time PCR requires costly equipment along with expensive reagents. In quantitative competitive PCR, the target DNA is coamplified with a synthetic DNA competitor (internal standard) that resembles, closely, the target and uses the same primers. The advantage of competitive PCR with common primers lies in the fact that any variation of the amplification efficiency affects equally the amplification of target and competitor and, consequently, the ratio of the products reflects the ratio of the initial amounts of the two sequences in the sample. Detection of the products is performed by capillary electrophoresis or by hybridization assays. Capillary electrophoresis employing lab-on-a-chip devices offers simplicity, low cost, and higher resolving power than standard gel electrophoresis.[5−7] However, there are problems associated with the presence of heteroduplexes when using restriction enzyme digestion. Indeed, heteroduplexes comprise one strand from the target DNA and one strand from the competitor and therefore resist enzyme digestion. Hybridization assays, especially bio(chemi)-luminometric methods performed in microtiter wells[8−10] or fluorometric methods performed on spectrally encoded microspheres,[11] are automatable and enable confirmation of the target sequence by using specific probes.

Received: September 3, 2011
Revised: December 29, 2011
Accepted: December 30, 2011
Published: December 30, 2011

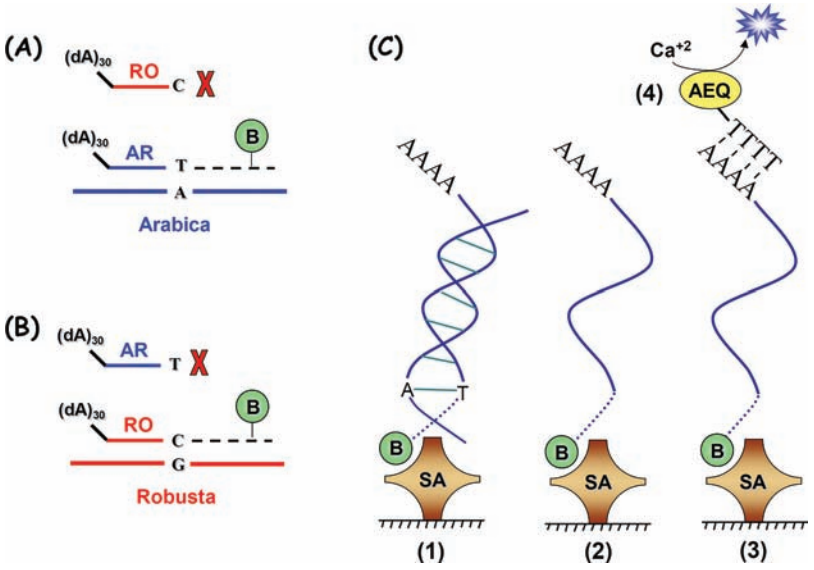

Figure 1. Differentiation of the Arabica PCR amplification product (A) from the Robusta PCR amplification product (B) by the primer extension assay. The Arabica-specific primer is extended by DNA polymerase only if it is hybridized to the Arabica DNA fragment. Similarly, the Robusta-specific primer is extended only if it is hybridized to the Robusta DNA fragment. Biotin is incorporated in the extended fragment. (C) Principle of the bioluminometric assay for quantification of each species: (1) The extended product is captured in a streptavidin-coated microtiter well. (2) The template strand is removed by NaOH treatment. (3) The immobilized strand hybridizes with the conjugate of the photoprotein aequorin (AEQ) with oligo(dT). (4) The bioluminescence of bound aequorin is measured by adding Ca^{2+} and integrating the signal for 3 s. B, biotin; SA, streptavidin.

Single-nucleotide polymorphisms (SNPs) (single-base changes in DNA) are particularly useful in authenticity testing because they enable discrimination of closely related species and/or varieties. Authentication studies have been reported on coffee, an important food commodity worldwide. Commercially, the most important coffee species are *Coffea arabica* and *Coffea robusta*. Arabica provides superior coffee, due to the finer flavor and better quality, and is sold at ∼2−3 times the price of Robusta coffee. A DNA-based species identification method for coffee was reported,[12] exploiting a SNP in the chloroplastic trnL(UAA)-trnF(GAA) intraspacer region that leads to the presence of a *Psu*I restriction site in Robusta but not in Arabica. The method involves PCR, restriction enzyme digestion, and fragment analysis by lab-on-a-chip capillary electrophoresis.

The aim of the present work is to develop a high-throughput method for the relative quantification of the two coffee species. Following PCR with common trnL(UAA)-trnF(GAA) primers,[12] the products are subjected to SNP-specific extension reactions using oligo(dA)-tagged primers designed to differentiate Robusta and Arabica products. Biotin is incorporated into the extended strands. The products are captured in streptavidin-coated wells and quantified by using oligo(dT)-conjugated photoprotein aequorin. Aequorin is measured within 3 s via its characteristic flash-type bioluminescent reaction that is triggered by the addition of Ca^{2+}. It is worth noting that, because of the close resemblance between the two DNA fragments, during PCR one species serves as an "internal standard" for the other.

MATERIALS AND METHODS

Instrumentation. The milling machine was purchased from Glen Creston (Stanmore, U.K.). PCR and primer extension reactions were performed in a MJ Research PTC-0150 thermocycler (Watertown, MA). Agarose gel electrophoresis was performed in Subcell GT, Bio-Rad (New York, NY). The digital camera, Dynax 5D, was obtained from Konica Minolta (Ramsey, NJ). The Gel Analyzer software for DNA documentation was purchased from Kodak (New York, NY). Hybridization assays were performed using an SH26, Ingenieurburo CAT M. shaker/incubator from Zipperer (Staufen, Germany). The microtiter plate washer Nunc Immuno Wash 8 was purchased from Nalge Nunc International (Roskilde, Denmark). Bioluminescence was measured using the PhL microplate luminometer from Mediators (Vienna, Austria).

Materials. The GeneSpin DNA extraction kit was purchased from Genescan Analytics (Freiburg, Germany). Agarose and deoxynucleoside triphosphates (dNTPs) were obtained from HT Biotechnology (Cambridge, U.K.). Biotin-21-dUTP was purchased from Clontech Laboratories (Palo Alto, CA). Ethidium bromide was purchased from Research Organics (Cleveland, OH). Phusion high-fidelity DNA polymerase was obtained from Finnzymes (Espoo, Finland). DNA molecular weight markers (PhiX *Hae*III digest) and Taq DNA polymerase Vent(exo-) were from New England Biolabs (Beverly, MA). Opaque Microlite 2 polystyrene microtiter wells were obtained from Thermo Labsystems (Franklin, MA). Streptavidin from *Streptomyces avidinii* was purchased from Roche Diagnostics (Mannheim, Germany). The aequorin−$(dT)_{30}$ conjugate was prepared as described previously.[13,14] The primers and probes used in this study were synthesized by VBC-Genomics (Vienna, Austria). The 20-mer 5′-GGTTCAAGTCCCTCTATCCC-3′ was used as the upstream primer (Ucoffee), and the 25-mer 5′-AGCATCCTCATTTTATGAGAAAAGG-3′ was used as the downstream primer (Coffea1-R) for amplification of a segment of the chloroplastic trnL(UAA)-trnF(GAA) intraspacer region. Primers used for differentiation of Arabica and Robusta coffee were as follows: The 50-mer $(dA)_{30}$-TTCTAGTACCTAGATAAAAT was used as an Arabica-specific primer (Usnp4arab), and the 50-mer $(dA)_{30}$-TTCTAGTACCTAGATAAAAC was used as a Robusta-specific primer (Usnp4rob). All other common reagents were from Sigma (St. Louis, MO).

Coffee Samples. The coffee material used in this study comprised Arabica and Robusta green beans from several geographical origins kindly provided by Mercanta "The Coffee Hunters" (London, U.K.).

DNA Extraction. Green beans (20 g) were ground in a milling machine using a mesh of 2.0 mm. For the analysis of admixtures, green coffee bean powders containing Robusta and Arabica were mixed at various ratios. DNA was extracted from 150 mg of sample of the above powder using the GeneSpin DNA extraction kit according to the manufacturer's instructions.

dx.doi.org/10.1021/jf203531h | J. Agric.Food Chem. 2012, 60, 912−916

Quantitative Authenticity Test. *Polymerase Chain Reaction.* PCR was performed in a total volume of 25 μL containing 0.5 U of Phusion high-fidelity DNA polymerase, 1× Phusion HF reaction buffer, 2 mM $MgCl_2$, 250 μM of each dNTP, 0.25 μM of each primer for the amplification of a 362 bp section of the chloroplastic trnL(UAA)-trnF(GAA) intraspacer region, and 0.5 μL of DNA. The thermal cycling parameters were as follows: initial denaturation at 98 °C for 40 s and 35 cycles of 98 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and finally incubation at 72 °C for 7 min. The concentration of the PCR product was determined by agarose gel electrophoresis (2% agarose gel) and ethidium bromide staining followed by densitometric analysis of the picture of the gel taken by a digital camera.

Primer Extension Reaction. PCR-amplified DNA was subjected to two separate extension reactions, one with the Arabica-specific primer and the other with the Robusta-specific primer. All primer extension reactions were carried out in a total volume of 20 μL, containing 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 1 mL/L Triton X-100, 1 mM $MgSO_4$, 0.25 U of Vent (exo-) DNA polymerase, 0.1 pmol of amplified DNA, 1 pmol of the appropriate primer (Arabica- or Robusta-specific), 2.5 μM each of dATP, dCTP, and dGTP, 1.875 μM dTTP, and 0.625 μM biotin-21-dUTP. The thermal cycling conditions for the extension reactions were an initial denaturation step at 95 °C for 5 min followed by three cycles of denaturation at 95 °C for 15 s, primer annealing for 10 s at 65 °C, and primer extension at 72 °C for 15 s.

Bioluminometric Assay. Opaque polystyrene microtiter wells were coated with 50 μL of 1.4 mg/L streptavidin in phosphate-buffered saline, pH 7.4, and incubated overnight at 4 °C. The wells were then washed three times with wash solution (50 mM Tris-HCl, 0.15 M NaCl, 2 mM EDTA, 0.2 mL/L Tween 20, pH 7.5). A 10 μL aliquot of the extension products along with 40 μL of assay buffer (0.15 M NaCl, 15 mM sodium citrate, 10 g/L BSA, and 2 mM EDTA, pH 7.0) was added into each well and incubated for 30 min at ambient temperature under gentle shaking to allow binding of biotinylated extension products to streptavidin. The wells were washed three times, and 50 μL of 0.1 M NaOH was added. After 5 min of incubation, the nonbiotinylated DNA strand was removed by washing the wells as above. Then, 50 μL of 22 nmol/L aequorin-$(dT)_{30}$ conjugate, previously diluted in reconstitution buffer (0.15 M NaCl, 15 mM sodium citrate, 10 g/L BSA, 2 mM EDTA, and 0.5 mL/L Tween 20, pH 7.0), was added into the wells and incubated for 30 min (ambient temperature with gentle shaking) to allow hybridization with the immobilized single-stranded extension product carrying a $(dA)_{30}$ tail. The wells were washed, and the activity of bound aequorin was measured in the luminometer by injecting 50 μL of light-triggering solution (25 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5) and integrating the luminescence signal for 3 s.

RESULTS AND DISCUSSION

The principle of the proposed method is illustrated in Figure 1. A 362 bp section of the chloroplast *trn*L(UAA)-*trn*F(GAA) intraspacer region was amplified by PCR using DNA extracted from Arabica and Robusta green beans as well as from admixtures of coffee powder. A SNP in this chloroplast region was used as a target for the discrimination of the Arabica and Robusta species. The PCR product was subjected to two separate primer extension reactions using species-specific primers. The primers were identical except for a single nucleotide at their 3′ end. Each primer comprised a sequence adjacent to the polymorphic site, with the 3′-end nucleotide complementary to the variant one and a $(dA)_{30}$ tag at the 5′-end. Due to the high accuracy of nucleotide incorporation by Vent (exo-) DNA polymerase, which lacks 5′→3′ or 3′→5′ exonuclease activity, extension occurs only if the primer matches perfectly the target sequence. Biotin−dUTP is incorporated into the extended primer along with the other dNTPs. Biotin serves for affinity capture of the extension products onto microtitration wells coated with streptavidin. The $(dA)_{30}$ segment at the 5′-end of both species-specific primers enables hybridization of immobilized extension products with the $(dT)_{30}$−AEQ conjugate. The luminescence of bound AEQ is measured within seconds by simply adding Ca^{2+}. If primer extension has not taken place, then no signal is observed because biotin is not incorporated.

Figure S1 (Supporting Information) presents the dependence of the luminescence signal on the amount of Arabica-amplified DNA product (femtomoles) introduced into the primer extension reaction. A linear relationship between the luminescence (RLU) and the amount of Arabica DNA (femtomoles) is observed. The linear regression equation is RLU = −757 + 335(fmol), with a correlation coefficient of R = 0.991. Similarly, a linear relationship was obtained between the amount of Robusta-amplified DNA (femtomoles) and the luminescence signal (Figure S2, Supporting Information). The regression equation is RLU = 2208 + 583(fmol), with R = 0.990.

The calibration curves for the determination of each species (percent content) were constructed simply by mixing PCR products from the Robusta and Arabica DNA at various ratios, covering the entire range from 0 to 100%. Each mixture (calibrator) was subjected to two extension reactions using the species-specific primers. The extension products were then quantified by the bioluminometric hybridization assay. The calibration graph for the Arabica species is the plot of the ratio $AR = 100L_A/(L_A + L_R)$ versus the percent of Arabica DNA in the mixture. (Figure S3, Supporting Information). L_A and L_R are the luminescence signals obtained from the extension reactions performed with the Arabica- and Robusta-specific primers, respectively. The linear regression equation is AR = 8.92 + 0.88(% Arabica), with a correlation coefficient of R = 0.987. In a similar manner, the calibration graph for the Robusta species is the plot of the ratio $RO = 100L_R/(L_A + L_R)$ versus the percent Robusta in the DNA mixture (Figure S4, Supporting Information). The linear regression equation is RO = 2.95 + 0.88(% Robusta), with a correlation coefficient of R = 0.987. Each of the AR and RO ratios represents the percentage of the total signal that is due to the Arabica and Robusta species, respectively.

Reproducibility (%CV, coefficients of variation expressed as percentage) at the levels of 25, 50, and 90% Arabica was 8.1, 1.6, and 1.2, respectively (n = 3 separate determinations).

The accuracy of the proposed method was assessed by analyzing coffee powder mixtures. Arabica and Robusta green coffee beans were powdered and mixed at various ratios. Each mixture was prepared in triplicate. DNA was extracted from each mixture followed by PCR amplification, primer extension, and hybridization assay. The percent content of each mixture with respect to Arabica and Robusta was determined by using the luminescence signals L_A and L_R and the calibration graphs. The experimentally determined percentages of each species along with the corresponding nominal values are presented in Table 1. The standard deviation for each triplicate determination is also shown in Table 1.

In addition, 14 coffee samples from various geographical origins were analyzed by using the proposed method (Table 2). The results are presented as scatter plots, that is, plots of the calculated percentage of the signals for each species versus the sample number (Figure 2). Eleven of the 14 samples were found to contain only Arabica, whereas the remaining 3 were found to contain only Robusta coffee. The results obtained

Table 1. Robusta and Arabica Contents, Expressed as Percentages, in Coffee Powder Mixtures[a]

% Robusta		% Arabica	
nominal	experimental	nominal	experimental
1	4 ± 1	99	96 ± 1
5	5 ± 2	95	95 ± 2
10	10 ± 2	90	90 ± 2
30	32 ± 3	70	68 ± 3
50	60 ± 2	50	40 ± 2

[a]Comparison between experimentally determined values and nominal values. The standard deviation for each triplicate determination is also shown.

Table 2. Green Coffee Samples

green coffee sample	origin	species[a]
1	Café de Cuba, Cuba	*C. arabica*
2	Mysore A, India	*C. arabica*
3	Sumatra Lintung	*C. arabica*
4	Jacaranda organic coffee, Brazil	*C. arabica*
5	El Salvador, El Carmen Estate (Icatu)	*C. arabica*
6	Robusta, Rwanda	*C. canephora*
7	Maragogype, Nicaragua, Finca el Platanillo	*C. arabica*
8	Mondo Novo, Natural Sertarzinho farm	*C. arabica*
9	Yellow bourbon, Brazil, Cachoeira farm	*C. arabica*
10	India Robusta Mysore	*C. canephora*
11	Coopronaranjo Bandola Costa Rica	*C. arabica*
12	Dominican Republic, Montana Verde	*C. arabica*
13	Accession 1S/2, Uganda	*C. canephora*
14	Kalosi Indonesia	*C. arabica*

[a]*C. canephora* is the scientific name of Robusta.

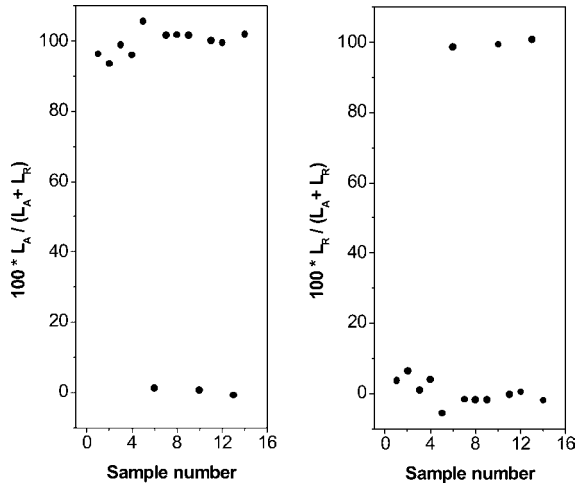

Figure 2. Analysis of a series of commercial coffee samples: (left) primer extension reaction was performed using the Arabica-specific primer; (right) Robusta-specific primer was employed in the primer extension reaction.

from the two species-specific primers are in complete concordance.

To conclude, the proposed method provides excellent discrimination of the two species and simultaneously gives the percentage of each species in the sample. The specificity of the entire assay arises from (a) the specificity of the primers used for PCR amplification, (b) the specificity of the Arabica and Robusta primers used for the extension reaction, and (c) the specificity of the bioluminometric assay. With regard to equipment, the method requires a conventional thermal cycler and a luminometer. The results are obtained in <90 min after PCR. The microtiter well-based assay configuration allows parallel analysis of many samples (high sample throughput) and facilitates greatly the automation of the method. Heteroduplex formation during PCR is not a source of error for this method because only single-stranded DNA is measured. No purification of the PCR products is required for the primer extension reaction. Also, the extension products are introduced directly into the wells without prior treatment. Aequorin is an excellent bioluminescent reporter molecule because it can be detected down to 10^{-18} mol by simply adding excess Ca^{2+}, and the reaction is complete within 3 s. This is a significant advantage over enzyme reporters that require long incubation times. Because of the high sensitivity of the bioluminescent reaction of aequorin, only three cycles of primer extension reaction (15 min) are sufficient for detection. The throughput can be further enhanced by exploiting a dual label bioluminometric assay[9] that enables simultaneous determination of the two species in a single well. More bioluminescent labels could be utilized if quantification of more than two species is needed.[9] The method is general and enables relative quantification of species (percent content) based on DNA fragments differing by as little as a single nucleotide. At its present format, the assay determines the content of Arabica and Robusta in the sample. Quantification of more species in the sample could be accomplished by designing appropriate primers for the extension reaction.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (+30)2610 962951. E-mail: tchrist@upatras.gr.

Present Address

⊥Department of Laboratory Tests, Hellenic Food Authority − Regional Division of Northern Aegean, 12 Kavetsou. 81100 Mytilini, Greece.

■ REFERENCES

(1) Woolfe, M.; Primrose, S. Food forensics: using DNA technology to combat misdescription and fraud. *Trends Biotechnol.* **2004**, *22*, 222−226.

(2) Sforza, S.; Corradini, R.; Tedeschi, T.; Marchelli, R. Food analysis and food authentication by peptide nucleic acid (PNA)-based technologies. *Chem. Soc. Rev.* **2011**, *40*, 221−232.

(3) Mafra, I.; Ferreira, I.; Oliveira, M. Food authentication by PCR-based methods. *Eur. Food Res. Technol.* **2008**, *227*, 649−665.

(4) Lockley, A. K.; Bardsley, R. G. DNA-based methods for food authentication. *Trends Food Sci. Technol.* **2000**, *11*, 67−77.

(5) Garciá-Cañas, V.; González, R.; Cifuentes, A. The combined use of molecular techniques and capillary electrophoresis in food analysis. *Trends Anal. Chem.* **2004**, *23*, 637−643.

(6) García-Cañas, V.; Cifuentes, A. Simultaneous confirmatory analysis of different transgenic maize (*Zea mays*) lines using multiplex polymerase chain reaction-restriction analysis and capillary gel electrophoresis with laser induced fluorescence detection. *J. Agric. Food Chem.* **2008**, *56*, 8280−8286.

(7) Scott, M.; Knight, A. Quantitative PCR analysis for fruit juice authentication using PCR and laboratory-on-a-chip capillary electrophoresis according to the Hardy-Weinberg law. *J. Agric. Food Chem.* **2009**, *57*, 4545−4551.
(8) Mavropoulou, A. K.; Koraki, T.; Ioannou, P. C.; Christopoulos, T. K. High-throughput double quantitative competitive polymerase chain reaction for determination of genetically modified organisms. *Anal. Chem.* **2005**, *77*, 4785−4791.
(9) Elenis, D. S.; Ioannou, P. C.; Christopoulos, T. K. Quadruple-analyte chemiluminometric hybridization assay. Application to double quantitative competitive polymerase chain reaction. *Anal. Chem.* **2007**, *79*, 9433−9440.
(10) Elenis, D. S.; Kalogianni, D. P.; Glynou, K.; Ioannou, P. C.; Christopoulos, T. K. Advances in molecular techniques for detection and quantification of genetically modified organisms. *Anal. Bioanal. Chem.* **2008**, *392*, 347−354.
(11) Kalogianni, D. P.; Elenis, D. S.; Christopoulos, T. K.; Ioannou, P. C. Multiplex quantitative competitive polymerase chain reaction (MQC-PCR) based on a multianalyte hybridization assay performed on spectrally encoded microspheres. *Anal. Chem.* **2007**, *79*, 6655−6661.
(12) Spaniolas, S.; May, S. T.; Bennett, M. J.; Tucker, G. A. Authentication of coffee by means of PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis. *J. Agric. Food Chem.* **2006**, *54*, 7466−7470.
(13) Glynou, K.; Ioannou, P. C.; Christopoulos, T. K. One-step purification of recombinant photoprotein aequorin by immobilized metal-ion affinity chromatography. *Protein Express. Purif.* **2003**, *27*, 384−390.
(14) Glynou, K.; Ioannou, P. C.; Christopoulos, T. K. Affinity capture-facilitated preparation of aequorin-oligonucleotide conjugates for rapid hybridization assays. *Bioconjugate Chem.* **2003**, *14*, 1024−1029.