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Microimmunoanalysis on Standard Compact Discs To Determine Low Abundant Compounds

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High-density competitive indirect microimmunoassays were performed in both sides of compact discs by direct absorption of immunoreagents on polycarbonate surface, using gold- or enzyme-labeled immunoglobulins as tracers for displaying the immunoreaction. The operational principle is based on the use of a low-reflectivity compact disc as analytical platform that allows the reflection/transmission (30/70%) of the CD reader laser beam (λ 780 nm). The reflected light is used to scan the disc track keeping it in movement. The transmitted light is detected by a planar photodiode integrated on the CD drive. The variation of the optical transmission of the light caused by the immunoreaction products is related to the sample concentration. As a proof of concept, low abundant compounds, commonly used as pesticides, were detected in a 60-min total assay time, with a limit of detection ranging from 0.02 to 0.62 μg/L for 2,4,5-TP, chlorpyriphos, and metolachlor. The obtained results show the enormous prospective of compact discs in combination with CD players for multiresidue and drug discovery applications.

The scope of microarrays has expanded impressively in recent years. As a rapidly maturing technology, microarrays pave the way for high-throughput analysis.[1] Conventional microimmunoassay array fabrication involves the immobilization of either coating conjugates (indirect format) or antibodies (direct format) on a solid support depending on the analytical needs. As microarray elements become smaller for larger numbers of simultaneous tests, alternative platforms with high optical quality, chemically derivatizable, and easy manipulation are of huge interest for analytical purposes.

Different supports such as glass, silicon, and organic polymers have been used to develop high-density microarray assays. Among them, plastic discs have the advantage of large surface using the centrifugal force for fluid propulsion, performing different steps of the analytical process.

The current analytical applications using plastic discs as supports can be classified in two groups. One employs discs about 2-mm thickness and 12-cm diameter to accomplish the development of microfluidic-based assays for proteins and nucleic acids, in which, essentially, different steps including sample treatment are involved.[2−5] The analytical results are mining through conventional detectors such as densitometers, photometers, fluorometers, etc., adapted to the circular geometry, including modified CD drives as laser scanning microscope.[6] The other group is based on the use of compact discs audio−video technology for management and reading the chemical results.

Compact discs (CDs) are made from a 1.2-mm-thick disc of polycarbonate coated with a reflective layer of aluminum, silver, or gold protected by a lacquer resin with outstanding physical properties such as impact resistance, heat stability, large surface (94 cm^2), and good protein adsorption efficiency. The high optical quality of the polymeric materials used for CD and DVD audio−video disc manufacturing makes them a promising platform for microarraying purposes and molecular screening.[7]

Regarding the immobilization of probes (proteins, nucleic acids, etc.) on polycarbonate, adsorption of the molecules through hydrophobic interactions is the major driving force.[8] In a seminal paper, working with standard compact discs, Kido et al.[9] explored the principle of CD-based immunoassay by the adsorption of antibodies onto the polycarbonate side for the quantitative analysis of pesticide residues in water. High-throughput screening assays based on the passive adsorption of avidin on compact disc surfaces have also been reported, discriminating different types of plum pox virus by SNP analysis.[10] In relation to the use and application of optical compact disc technology for analysis of biological and chemical samples, several patents have been published.[11−13]

As far as detection is concerned, the use of a CD player would facilitate the automation and simplification of the analysis. This optical reader incorporates a sophisticated set of servo systems

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that make the laser beam accurately focus on the surface and track across the CD when it rotates. Being a mass-produced electronic consumer device with a market price running from 50 to 300 euros, CD players compare very favorably to microarray scanners (standard scanning area 18.75 cm^2) whose cost runs in the 30 000−60 000 euros. Additionally, the CD drive should increase the portability and frequency for real-time testing at the point of need.

Quantitative measurements using compact disc technology have been hardly approached. The contributions show different attempts regarding the use of discs and detectors (reader). Briefly, La Clair and Burkart[14] described the construction of a digital and biological layer on a standard compact disc and used error determination in an optical disc drive for direct detection of streptavidin with a response limited by the need of more than 50 μL of 6.5 pM protein solution in PBS. This innovative approximation shows as limitation that each drive manufacturer has its own proprietary firmware for error correction displaying a different sensitivity and thus different ability for analysis. Also, they use a PTFE mask to perform the assays in wells on the disc surface.

Nolte, Regnier, et al.[15] developed phase-contrast BioCDs that required a specific optical system for direct detection of immunoglobulins by spinning-disc interferometry using borosilicate glass or silicon discs of 10-cm diameter, coated with dielectric stacks that serve as a mirror with a center wavelength at 635 nm.

Within the CD approaches outside the label-free domain, Alexandre et al.[16] described an approximation based on the use of sectorial discs with limited analytical area, two lasers, and an additional steeping motor to move the detection head mounting the specific detector. In all cases, the disc, the detector, or both were not the standard audio−video elements.

On the other hand, approaches that do not employ the CD driver as a detector have also been published. This is the case of the work reported by Lange et al.[17] for the indirect detection of C-reactive protein using the pick-up head of a CD drive mounted on the stage of an optical microscope. This interesting approach leaves the simplicity and high reading speed of the CD drive system unused.

Finally, the proposal of Potyrailo et al.[18] utilized a standard CD/DVD reader to analyze Ca^{2+} in the milligram per liter range by attaching macroscopic films (3 × 4 mm) onto standard DVDs. This attempt is beyond the reach of high-density bioassays, which are more sophisticated and demand a microgram per liter level or even better sensitivity.

Our hypothesis concerns the use of standard compact discs and transmission/reflection of the CD reader laser light to develop high-density microimmunoassays. However, it is necessary to resolve some drawbacks regarding the disc and detector management. First, the analytical platform should be a standard compact disc to be read by also using standard drivers. At the same time, the disc has to allow transmission of the incident laser radiation, so that the variation of the transmittance would be related to the sample concentration. Because of that, the use of partially transparent discs is the key to achieve both goals, since with this detection principle, the reflectivity of the analytical platform ensures that the laser beam of the CD drive follows the spiral track of the disc, a circumstance that it is essential to simultaneously rotate the disc and detect thousands of samples.

A broad spectrum of solid reaction products that absorb the light of the CD reader laser beam modifying the optical properties of the surface can be compatible with our detection mode. Thus, enzyme-mediated methods such as those using horseradish peroxidase and other enzymes such as alkaline phosphatase, β-galactosidase, and glucose oxidase produce insoluble reaction products that can modify the transmitted light profile of the disc. Also, colloidal gold markers can be well-suited to act as a nucleation point around which a solid reaction product is formed after reaction with silver salts.[19] On the other hand, it would be interesting that the laser intensity hitting the samples was the maximum, so that the sensitivity of the assay should be the highest. In this sense, no data in the literature have been found regarding this issue.

Another aspect to be taken into account is the focal diameter of the laser beam that hits the disc. While at the down side of the disc (polycarbonate) the laser beam has a diameter of 728 μm, in the top side it is focused down to 1.7 μm. Working with narrow beams, higher optical resolution can be achieved, and therefore, the possibility to work with arrays of higher density will increase CD working capacity and probably sensitivity.

Regarding the type of detector for reading CDs, two different strategies have been proposed. Recently, the pickup head as detection unit either outside or inside the optical disc drive has been used as a laser scanner detector, but until now, minimum information using this interesting strategy has been published in relation to high-density microassay arrays. So much more research should be done regarding optical alignment issues demonstrating its potential. Nevertheless, the use of the CD drive as detector without modification presents an optical limitation for performing microassay arrays at the polycarbonate surface of the disc. Because the diameter of the laser beam at the reading surface of the CD (polycarbonate) is 728 μm, spots with smaller track pitch cannot be precisely resolved. Sensing on the top side of the discs will overcome this issue.

The second strategy consists of mounting an extra detector apart from that of the CD drive to sense the changes of the signal intensity. In this case, Gordon[20] proposed either the use of two detectors or an additional source of visible radiation coupled to an absorptiometric detector. Both proposals made difficult the use of these detectors to read standard CDs. Up to now, nobody has reported the use of the laser from the standard CD drive as the unique light source to generate the analytical signal while tracking gradually across the CD surface, developing the chemical assays on the top side of the discs.

Herein presented research addresses with the use of low-reflectivity compact discs, transmission of the laser light directly detected by a photodiode integrated into the CD reader unit,

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taking a comprehensive approach to perform high-density chemical assays. The variation of the transmittance caused by the immunoreaction products (absorption, scattering, reflection, etc.) is related to the sample concentration, allowing the quantification of the extension of the immunoreaction. Therefore, the goal of this research is twofold: first, the use of standard compact discs as an analytical platform for developing microassays and, second, making a CD player work as a sensitive microarray detector.

We demonstrate the proof-of-concept developing microimmunoassay with microgram per liter sensitivity for chlorpyriphos, metolachlor, and 2,4,5-TP on both sides of standard CDs, exploiting the full potential of the CD technology: simplicity, sensitivity, versatility, high capacity of analysis, low-cost, and portability.

EXPERIMENTAL SECTION

Chemicals. Buffers [coating buffer, CB, 50 mM sodium carbonate buffer, pH 9.6; PBS-T, 10 mM sodium phosphate buffer, 150 mM NaCl, 0.05% Tween 20, pH 7.4; printing buffer, PB, PBS-T with 5% (v/v) glycerol] and washing solutions were filtered through a 0.22-μm-pore size disc before use. Previously characterized polyclonal antibodies (C2-II, R48, and R58) and coating conjugates (OVA-C5, OVA-metolachlor, and OVA-2,4-D) were used as immunoreagents for the determination of chlorpyriphos,[21] metolachlor,[22] and 2,4,5-TP,[23] respectively. Chlorpyriphos, metolachlor, and 2,4,5-TP standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Nanogold-labeled goat anti-rabbit immunoglobulins (GAR-Au) and gold enhance kit were from Nanoprobes, Inc. (Yaphank, NY). Silver enhancer solution, 3,3′-diaminobenzidine (DAB), 3,3′,5,5′-tetramethylbenzidine liquid substrate (TMB), 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt with nitro blue tetrazolium chloride purple liquid substrate (BCIP/NBT), and HRP-labeled goat anti-rabbit immunoglobulins (GAR-HRP) were from Sigma-Aldrich (Madrid, Spain). Alkaline phosphatase-labeled goat anti-rabbit immunoglobulins were from EMD Biosciences, Inc. (San Diego, CA). Caution: Pesticides, silver enhancer solutions, and enzymatic substrates are toxic chemicals. Working in a well-ventilated fume hood and handling them carefully is recommended. Wastes should be discarded following the security recommendations for hazardous reagents.

Instrumentation. Low-reflectivity compact discs (L-CD) were from Media Corp. (Tau-Yuan Shien, China). The disc surface was gold metallized to reflect at 780 nm 30% of the light, allowing the rest to be transmitted through the disc.

The compact disc arrayer from V & P Scientific, Inc. (San Diego, CA) matched with four rows of five pins (20 in total); each one carries 6 nL and leave a 3-nL drop on the CD, producing 500-μm-diameter dots. This size allows arraying 320 spots with a horizontal and vertical pitch of 1,125 μm, and eight arrays fit on a disc (2560 spots/disc).

Contact angles were measured using OCA 20 video-based contact angle meter from DataPhysics Instruments GmbH (Filderstadt, Germany).

Protocol of Microimmunoassays on Disc. The microimmunoassays were based on an indirect competitive format. Briefly, the CD was first conditioned by gently washing with 96% ethanol followed by rinsing with deionized water and were dried by slight centrifugation (840 rpm). Afterward, the coating conjugates for chlorpyriphos, metolachlor, and 2,4,5-TP were passively adsorbed on the polycarbonate side of the disc, dispensing 30 μL in CB evenly distributed using a glass coverslip (22 × 22 mm). Then, the coated disc was set into a CD box for 16 h at 4 °C. After, the disc surface was thoroughly washed by immersing in deionized water for 1 min and dried as before. Antibody solutions in PB with or without analyte were dispensed in a 384-well plate (20 μL/well) and incubated for 15 min. Then, the solutions were transferred onto the disc using a CD arrayer. Each pin delivered a different solution that was stamped four times generating a 4 × 20 array (80 spots). The stamping of such an array size took less than 1 min. After 10-min incubation, it was washed with PBS-T buffer for 1 min and rinsed with deionized water. Next, 17 μL of GAR-Au solution (1:100 in PBS-T) or 17 μL of GAR-HRP (1:100 in PBS-T) for an enzymatically based reaction was dispensed onto sample areas and 22 × 22 mm glass coverslips were used to make sure of an even distribution. After 10 min at room temperature, the disc was washed and dried as described before.

For displaying the immunoreaction, each detection area was incubated with 17 μL of the silver enhancer solution. For enzymatic tracers, 17 μL of TMB solution was used. A 22 × 22 mm glass coverslip was used to distribute the solution along the detection area. Silver or dark blue deposits selectively occurred at the spots in ~5 min for gold or enzymatic tracers, respectively. After stopping the reaction by washing with deionized water and drying as described, the disc was read by the CD player.

Detection System. A conventional optical disc drive (Premium, Plextor America, Fremont, CA) was used as detector. The drive has an optical system with a laser (λ 780 nm) to read standard CDs and uses the servo focus/tracking system to center and focus the beam on the spiral data track across the whole disc surface. Our detector system takes advantage of the CD driver optical system to illuminate accurately the down side of the disc by the laser. The controlled parameters of the optical disc drive include positioning of the laser toward the disc, scanning the whole disc surface while controlling the spatial resolution and the linear rotation velocity of the disc.

A planar photodiode (SLSD-71N6, Silonex, Montreal, Canada), 25.4 mm long, and 5.04 mm width with a spectral sensitivity of 0.55 A/W at 940 nm, spectral range between 400 and 1100 nm, and acceptance half-angle of 60°, was used to detect the transmitted laser light and convert it into an analog electrical signal. When scanning, in the absence of biochemical interaction (no immunoreaction product), the laser beam of the CD drive is transmitted through L-CD and the intensity detected by the photodiode is the background signal. In contrast, as the laser scanned across the disc, the positive spots attenuate the intensity of the laser beam that reaches the photodiode, resulting in a distinctive signal from the background level. In this process, the variation of the light intensity is related to sample concentration.

A reflective photosensor (EE-SY125, Omron) used to detect the analytical areas includes an infrared led of 950 nm and a phototransistor, with a sensing distance ranging from 0.5 to 2 mm. The operational principle of the photosensor is based on the detection of the different reflectivity between the sensing object

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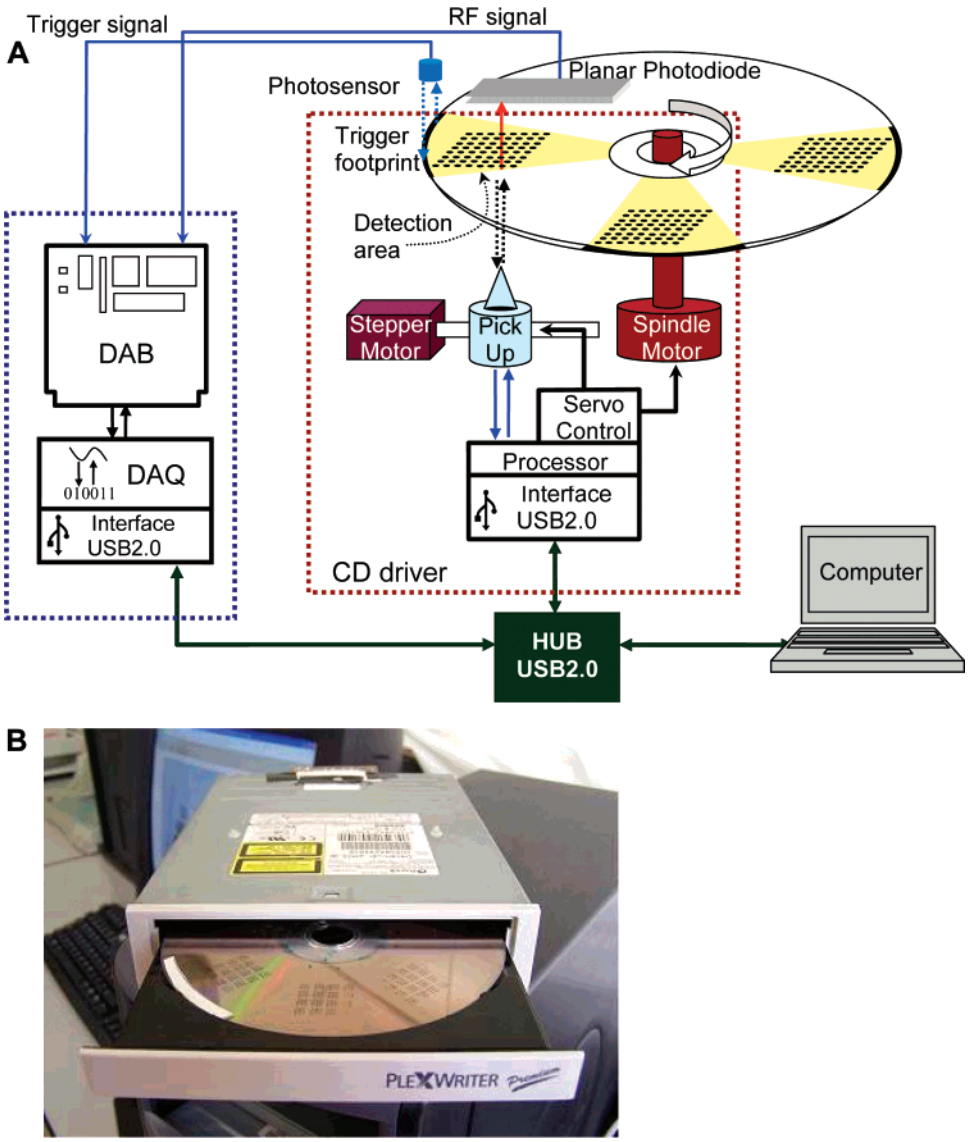

Figure 1. (A) Schematic representation of the detection system. The set of servo systems (spindle and stepper motors) of the CD drive keeps the laser beam focused on the spiral track, allowing disc rotation and laser scanning. The transmitted light through the disc is transformed by the photodiode into an analog electrical signal (rf signal). At the same time, the photosensor detects the trigger footprints, starting the data collection on disc. The DAB is integrated into the CD drive unit and contains the planar photodiode and the photosensor. DAB brings the rf and trigger signals to the DAQ. The DAQ digitizes the analog signals and transfers them to the computer for processing. The CD drive is controlled by software and connected to the computer by USB2.0 interface. On the disc, three different detection areas are represented, simulating the arrays corresponding to each studied analyte. (B) Picture of the CD player used in this work.

and the disc. For that, the analytical areas are marked in the outer rim of the disc by low-reflectivity trigger footprints of 3.5 cm. Because the unmarked perimeter presents higher reflectivity, the photosensor detects the marked areas providing a trigger signal to the data acquisition board in order to start capturing data exclusively from those zones.

A custom-built electronic board (DAB) incorporates the planar photodiode and the photosensor. The function of the board is twofold: first, the amplification of the analog signal and, second, the detection of analytical areas. Both functions are carried out at the same time the CD drive performs its original function of reading and writing data. In this way, during the data acquisition process only the signals from the detection areas are digitized by the data acquisition board (DAQ), stored in the computer, and deconvoluted into an image to further quantification. A scheme of the developed detection system is shown in Figure 1A. Furthermore, the detector system is controlled by software running on a Windows-based computer connected to the PC through a universal serial bus interface (USB2.0) to become portable (see Figure 1B).

Custom software was written in Visual C++ (BioDisk). The software provides control to the CD/DVD driver to scan the surface of the disc, to modulate the disc linear rotation velocity to a specified spatial resolution, and to write data in the CD or DVD. Also, BioDisk software provides control to the data acquisition board to configure the sampling frequency according to the disc rotation speed and desired angular resolution. To scan completely the surface of the L-CD, the software simulates the writing process of a 700 MByte size file to a controlled disc rotation speed. The scan begins from the inner tracks of the disc, following the continuous spiral toward the outer tracks. The captured data of each detection area are represented within a sector that is

formed by a set of arcs centered over a radial direction, starting from the inner toward the outer radius. The software collects the data of each area and arranges them in a well-ordered rectangular shape. Also, it stores them in independent files in uncompressed binary format and displays them onto a graphical image.

Due to the spatial difference between samples taken horizontally (each 13 μm) and vertically (each 1.6 μm), a graphical adjustment is done to display a proportional $X-Y$ image. The software also allows exporting the image in a gray scale code to a compressed tif format or bitmap for further quantification by the graphical software.

The images were processed with Photoshop 7.0 (Adobe Systems Inc., San Jose, CA) to map the lightest and darkest pixels into black and white before quantifying with GenePix software (Axon Inst., Union City, CA). Signal intensities of each spot were calculated by background subtraction. Inhibition curves were mathematically analyzed by fitting experimental results to a sigmoidal four-parameter logistic equation.

RESULTS AND DISCUSSION

CD Microarraying Conditions. Attaching probes on polymeric surfaces might be carried out directly by physical adsorption, covalently or indirectly through a bridge system such as avidin/biotin. In this work, the coating conjugates were adsorbed on the disc surface, as a simple way for the development of microimmunoassay arrays. The use of disc surfaces [polycarbonate, poly(methyl methacrylate)] as high-throughput screening platforms where probes are attached to the surface by adsorption has been reported.[9,10] Different concentrations of coating conjugates ranging from 10 μg/L to 10 mg/L were used to test the coating efficiency on the polycarbonate side of the disc and concentrations of 0.25, 1.0, and 4.0 mg/L were the best in terms of sensitivity for chlorpyriphos, metolachlor, and 2,4,5-TP microimmunoassays, respectively.

Contact angle measurements were randomly carried out on different parts of the disc to test the coating uniformity along the disc surface (CV 4.1%, $n = 12$) showing a mean value of 65.2°, which was lower than that of bare disc surface (82.1°). Contact angle studies indicated a change of the hydrophobicity of the surface due to the passive immobilization of the coating protein.

According to our experience, glycerol was selected to be added to printing buffers, preventing the instantaneous evaporation of the nanoliter droplets and reducing the air bubble formation during the transfer process.[10,24] A set of experiments were carried out to evaluate the influence of glycerol and surfactant concentrations on spot homogeneity. Glycerol content in printing buffer ranged from 0.5 to 50% and Tween from 0.01 to 0.5%. The presence of Tween 20 in the printing buffer reduced the nonspecific signal and resulted in better spot homogeneity. Both assay sensitivity and signal-to-noise ratio improved as Tween concentration decreased to 0.05%. As far as reproducibility and quality of the spots are concerned, neither higher nor lower glycerol content in the printing buffer improved the results obtained with PBS-T, 5% glycerol. For this reason, and in order to protect against the potential protein denaturating effects and control spot morphology, the composition of the printing buffer was amended to a final glycerol content of 5% and 0.05% Tween.

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Regarding the spotting area, two sizes of pins were compared. The diameter variability of the spots stamped with 0.45-mm-diameter pins was lower than that obtained with 0.29-mm-diameter pins. In addition, the signal-to-noise ratio (SNR) was higher printing with pins of 0.45 mm, but the sensitivity of the microimmunoassays was similar. For further experiments, 0.45-mm-diameter pins were used.

Detector setup. A commercial CD reader, commonly used for data storage and retrieval, was thought of as an optical detector. The intensity of transmitted light through the L-CD was detected by a planar photodiode that was integrated in the amplification/detection board (DAB). The photodiode was statically fixed 2 mm above the top side of the disc covering the radial displacement of the pickup head. Thus, during the reading, a disc surface of 65 cm^2 was detected by the photodiode, enough area to perform simultaneously eight microarray assays, each one of 320 spots. In particular, the triggers make the BioDisk software to collect data from ∼6.1 cm^2, producing a total of 120 Mb raw data information. The photosensor provided control to the data acquisition process, being the data exclusively extracted from the detection marked areas. The DAB brought the detected signal to the DAQ for digitization.

Sampling rate F_s and lineal velocity (V_L) of the disc limit the maximum spatial resolution in the angular movement (S), which is given by the following equation:

$$S = 2V_L/F_s$$

On the other hand, the radial resolution is constant along the disc surface as determined by the track pitch (1.6 μm for CDs and 0.74 μm for DVDs).

The sampling frequency is configured in the DAQ by BioDisk software according to the rotation speed and desired angular resolution. A lineal velocity of 13 m/s (10×) was the highest reading speed, taking the CD player 7 min to scan the full disc. The captured data are transferred to the computer through the USB2.0 interface for its quantification. In the setup, the USB2.0 data acquisition board (model DT9832A-02-OEM from Data Translation) provides two 16-bit ADC, an input voltage range of ±10 V, and a sample rate up 2 megasamples/s reaching a maximum angular resolution of 13 μm at the metal surface. So, given a spot of 500-μm diameter and a scan speed of 13 m/s, the number of the data samples taken in the angular direction were up to 76 while those in the radial direction were 312, with a total of ∼18 720 samples allowing an accurate identification of the spot. A lower linear velocity would increase the angular resolution and identify smaller spots. However, the maximum spatial resolution is limited on CDs to ∼1.7 μm at the metal surface, the lower focal diameter reached by the CD drive optical system. The optical resolution at the polycarbonate side of the disc is 728 μm, given by the focal diameter of the laser beam. Because the spot pitch of the CD arrayer is 1,125 μm, the CD drive is able to accurately identify the printed spots at the down side of the disc.

Staining Strategies. Immunodetection was attempted by two different staining strategies. This study was performed with the chlorpyriphos microarray as a model assay in a noncompetitive format. For these experiments, the coating conjugate concentration (OVA-C5) was 0.25 mg/L and the antibody dilution ranged from 1/250 to 1/4000.

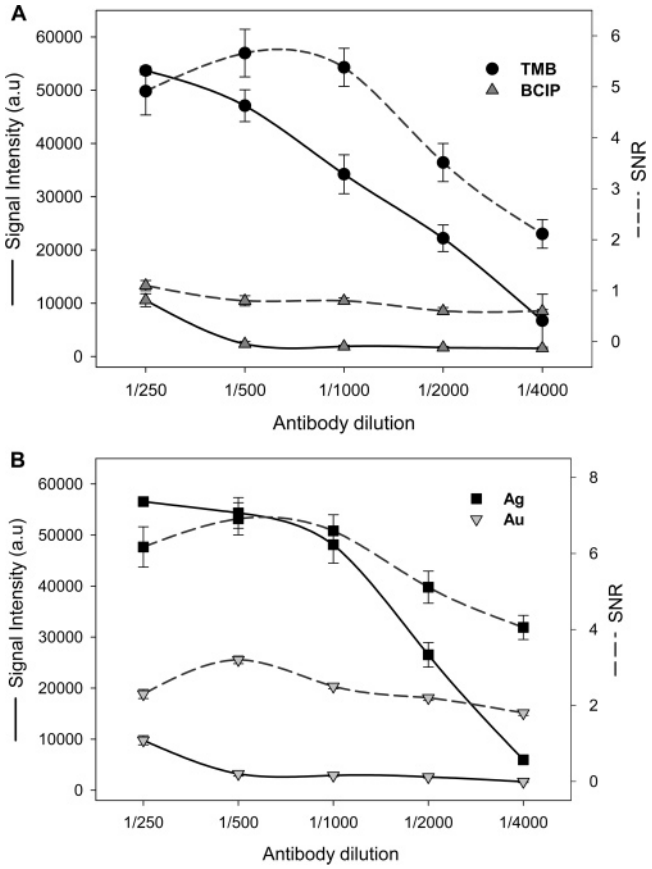

Figure 2. Signal intensity (solid lines) and SNR (dash lines) obtained for different antibody dilutions using different markers and staining strategies for the chlorpyriphos microimmunoassay. The plotted results are those obtained using (A) the enzymatic substrates TMB (●) and BCIP (▲) and (B) silver (■) and gold (▼) developing approaches.

The first approach was based on the use of substrates enzymatically transformable in nonsoluble products, which absorb the light emitted by the laser of the CD reader. The enzymatic conversion of an organic substrate to an insoluble reaction product is widely used as a detection method in immunohistochemistry.[25] The enzymatic substrates TMB, DAB, and BCIP/NBT were tested. The best results in terms of SNR and capacity to absorb the laser light were obtained with TMB. The four different formulations tested using DAB (black, blue, gray, or brown precipitates) did not generate detectable products. In contrast, as shown in Figure 2A, the use of TMB allowed the detection of immunoreaction products using high antibody dilution (1/1000 and 1/2000). The signal intensity detected, which is directly proportional to the absorbed light, was at least 8-fold higher than that obtained with BCIP/NBT. Also, higher SNRs were obtained by using TMB as the developing system with coefficients of variation ranging from 9.1 to 11.2%.

In the presence of an appropriate metal source and activating agents, horseradish peroxidase-conjugated probes can selectively deposit metal to give a black spot.[26] Indeed, enzyme metallography has proven to be highly effective as a developing method for in situ hybridization.[27] However, this approach did not succeed in detecting the immunoreaction when silver was used as the metal source. The signal intensity was as low as that obtained using BCIP/NBT (data not shown).

Another approach to develop the extension of immunoreaction was based on the use of Nanogold-labeled immunoglobulins. Two different procedures known as gold and silver enhancement[28] were evaluated. Under the tested conditions, the gold procedure did not produce detectable deposits. In contrast, as can be seen in Figure 2B, the signal intensities obtained with the silver enhancement procedure were in the range 6000−56000 (arbitrary units) from 1/4000 to 1/250 antibody dilutions, respectively. The coefficients of variation ranged from 1.6 to 13.6%. In comparison with the results obtained with TMB, the silver enhancement achieved a slightly higher sensitivity with good SNR values. Besides, silver precipitates are stable for months, which keep results for long time and block light at a broad spectrum of wavelengths,[29] allowing the use of a variety of laser and photodetectors.

Competitive Microimmunoassay. According to a previous characterization study by ELISA,[21−23] polyclonal antibodies C2-II, R48, and R58 and coating conjugates OVA-C5, OVA-metolachlor, and BSA-2,4-D were used to setup high sensitivity methods to determine chlorpyriphos, metolachlor, and 2,4,5-TP, respectively. In this work, the microimmunoanalysis was performed on both sides (down and top) of L-CD.

(A) Down Side. Sensing on the polycarbonate face of low-reflectivity discs, different competition times (5, 10, 15, 30, 45, and 60 min) were tested to study the effect on absolute signal intensity and sensitivity. It was observed that long competition times reduced assay sensitivity (higher IC_{50} values) while the absolute signal intensity was similar for all incubation tested times. The dose−response curve obtained after 10-min incubation is shown in Figure 3, displaying the mean of 32 curves performed in four discs (two curves per array and four arrays per disc). Each analyte concentration was run in four replicates. In total, 128 spots per concentration of analyte were averaged. As analyte concentration increased, the gray scale intensity changed from black (100% normalized signal corresponding to 0 μg/L) to white (2.5% at 1000 μg/L) (see panels in Figure 3). The resulting silver deposit from the highest analyte concentration was minimal (<5%), indicating a small degree of nonspecific response. The limit of detection (IC_{10}) was 0.39, 0.62, and 0.03 μg/L and the sensitivity (IC_{50}) 2.51, 5.72, and 0.78 μg/L for chlorpyriphos, metolachlor, and 2,4,5-TP, respectively.

As it is shown in Table 1, the coefficient of variations (CV) intradiscs ranged from 9.6 (disc I, metolachlor assay) to 18.8% (disc III, 2,4,5-TP assay). Similar precision is achieved by ELISA plate format using the same immunoreagents. Assays performed on four independent discs (eight calibration curves per disc) showed an average variation ranging from 13.7 to 17.2%, indicating a suitable reproducibility between microarrays in different discs.

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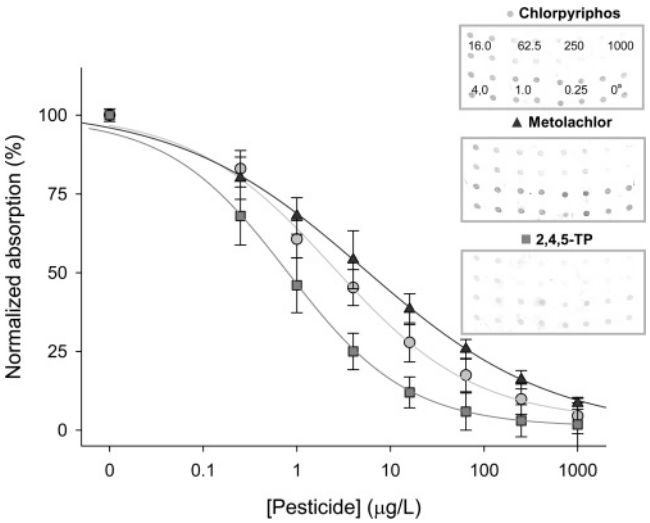

Figure 3. Standard calibration curves and representative gray scale images of competitive microimmunoassays at the down side of L-CD for chlorpyriphos (●), metolachlor (▲), and 2,4,5-TP (■). For the three analytes, the numbers inside the chlorpyriphos panel correspond to pesticide concentrations in μg/L.

Table 1. Intra- and Interdisc Reproducibility of the Microimmunoassays Developed on the Down and Top Side of the Discs[a]

analyte	disc I	disc II	disc III	disc IV	mean interdisk
			Down Side		
chlorpyriphos	14.6[b]	12.8	15.5	12.4	13.8
metolachlor	9.6	14.9	16.8	13.3	13.7
2,4,5-TP	16.6	15.8	18.8	17.4	17.2
			Top Side		
chlorpyriphos	12.2	11.8	13.4	14.3	13.0
metolachlor	10.6	9.9	12.6	11.1	11.0
2,4,5-TP	14.4	13.5	12.9	16.8	14.4

[a] Data expressed as CV (%). [b] In each disc, eight calibration curves were run with four replicates per concentration of analyte. In total, 128 spots per concentration were averaged.

The dose−response curves and the limits of detection were similar and independent of the processed disc.

(B) Top Side. To sense on this side of the disc, a 0.6-mm-thick polycarbonate film was assembled onto the low-reflectivity compact disc to carry out the assays. The microimmunoassay protocol was the same as described above. At this working side, the original incident diameter of the laser beam (~728 μm entering the polycarbonate) is focused to 13 μm, the resolution being limited by the scan speed and the rf bandwidth. The resolution could be easily improved up to 1.7 μm by reading the disc at 1×, but it would imply a decrease on the scan speed to 1.3 m/s taking 60 min to scan the whole disc.

The limit of detection (IC_{10}) was 0.33, 0.54, and 0.02 μg/L and the sensitivity (IC_{50}) was 1.81, 2.72, and 0.63 μg/L for chlorpyriphos, metolachlor, and 2,4,5-TP, respectively. The sensitivity of the assays was not improved by reading the disc at lower speed; however, a better precision of the readings was achieved, increasing the reproducibility of the signals. Also, the sensitivity was similar to that obtained on the down side of the disc.

The CV intradiscs ranged from 9.9 (disc II, metolachlor assay) to 16.8% (disc IV, 2,4,5-TP assay), while the interdisc variation went from 11.0 (metolachlor assay) to 14.4% (2,4,5-TP assay) as shown in Table 1.

The good results obtained by sensing on the top side of L-CD open the way (work in progress) to performing assays by spin coating transparent organic polymers for further covalent and oriented immobilization of biomolecules (proteins, DNA, carbohydrates, etc.).

High-Throughput Disc Microassay Performances. Under the current working conditions, each detection area fits up to 320 spots distributed in 72 samples and 8 standards (four replicates) and 8 areas per disc (576 samples) taking the CD drive 7 min to detect them. Also, taking into account the system resolution (13 μm), the good signal-to-noise ratio and sensitivity, the large surface of the disc (94 cm^2), and high-density printing performances (spot diameter and distance between spots), thousands of microspots are detectable by the CD drive.

In terms of reagent consumption, an assay performed on disc needs less than 2% coating conjugate of that required on plate (considering 100 μL/well). Also, working with L-CDs, less than 0.05% specific antibody and 2.64% labeled secondary antibody of that used per assay on plate is required, so there is an important immunoreagent saving. Moreover, the assays can be developed with a well-established and inexpensive silver staining process. It is worth mentioning that an important time saving on critical steps compared with the optimized 96-well plate ELISA format is reached.

As far as sensitivity is concerned, experimental results obtained for 2,4,5-TP, chlorpyriphos, and metolachlor indicated that the sensitivity was in the range of that reached in plates using the same immunoreagents.[21−23] However, considering each particular system, sensitivity was slightly lower for metolachlor (IC_{50}: 0.70 μg/L in plate and 2.72 in CD), slightly better for 2,4,5-TP (IC_{50}: 0.80 μg/L in plate and 0.63 μg/L in CD), and less sensitive for chlorpyriphos (IC_{50}: 7×10^{-3} μg/L in plate and 1.81 μg/L in CD). These results, the same as when setting up ELISA assays, can be improved by using other immunoreagents and formats, and they are only indicative.

Considering the practical aspect of the disc developed method, target analytes down to the 1 μg/L level are quantified directly with our approach, because in the less favorable case (metolachlor), the limit of detection is 0.54 μg/L. This value is very impressive in view of the multiresidual capacity of the CDs, demonstrating the enormous prospective, especially for screening purposes.

In terms of reproducibility, similar results have been reported for microarrays in microscope glass slide format for the analysis of small molecules. Thus, an average variance between 11 and 14% was described,[30] showing sensitivity in the range between 1 ng/L and 1 mg/L. Other authors[31] reported comparable reproducibility (9−12%) and sensitivity in the quantification of pesticide residues by microarrays on slide supports. In both works, fluorescent detection was used.

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CONCLUSIONS

The use of a low-reflectivity compact disc and an adapted CD drive as the detector is a suitable methodology to sense low abundant molecules in a high-density format with microgram per liter level sensitivity. The detection setup fully uses all the servo systems of a CD drive, improving and implementing the former detection CD-based analytical methodologies. Sensing on the top side of standard compact discs resulted in a good imaging resolution to readily detect as small as 13-μm-diameter spots. The response capacity of the CD drive is huge. In the current configuration, up to 2560 spots of 500 μm in diameter in 7 min can be detected. Furthermore, considering that the active surface of a disc is a circular band of 3 cm width and a track pitch of 200 μm (center-to-center distance between spots), a total of 300 000 spots of 100-μm diameter could be deposited on the disc and detected by the CD reader in a few minutes. Also, the automation of the printing step (for instance, the use of inkjet CD printers, robot stampers adapted to the CD platform, etc.) would permit us to set up a quicker, less expensive, more precise, and higher throughput technique.

Among other advantages of the compact disc as an analytical tool, direct adsorption of probes, the use of centrifugal force for washing and drying steps, and universal and mass production surface at low cost are included. It is also worth mentioning that chemical derivatization of discs and the use of surface coating technologies will allow covalent and oriented immobilization for developing protein or DNA microarrays.

In summary, the herein developed methodology is sensitive, versatile, portable, low cost, and easy to operate and could become a very advantageous system for a simultaneous multiresidue and real-time high-throughput technique of general application in molecular recognition-based biochemical methods at the point of need for home health monitoring or in situ analysis.

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