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# An optical DNA-based biosensor for the analysis of bioactive constituents with application in drug and herbal drug screening

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**Abstract**

The efficient and rapid detection of bioactive compounds in complex matrices of different origins (natural or synthetic) is a key step in the discovery of molecules with potential application in therapy. Among them, molecules able to interact with nucleic acids can represent important targets.

In this study, an optical DNA biosensor, based on surface plasmon resonance (SPR) transduction, has been studied in its potential application as new analytical device for drug screening. This device was applied to the analysis of pure synthetic or natural molecules and also to some fractions obtained by chromatographic separation of an extract of *Chelidonium majus* L. (great celandine), a plant containing benzo[*c*]phenanthridinium alkaloids having intercalating properties.

The ability of these molecules to interact with the double stranded nucleic acid (dsDNA) immobilised on the sensor surface has been investigated. The optical sensing relies on the SPR-based bench instrument Biacore X™ and represents an example of multiuse sensor. The results obtained demonstrate the potential application of this device for the rapid screening of bioeffective compounds. The characteristics of the biosensor offer the possibility to be coupled to chemical analysis as in hyphenated technologies.


*Keywords:* Biosensors; DNA intercalation; Synthetic and natural bioactive constituents; Drug discovery; Plant extracts; Bioactivity-guided isolation

## 1. Introduction

In the search for bioactive constituents both from synthetic or natural sources, the biological and pharmacological screening, at both primary and secondary levels, must deal with a large numbers of samples, asking essentially "yes/no" questions in automated analysis protocols. When dealing with extracts and fractions obtained from plants, the approach is generally based on bioactive-guided fractionation in order to obtain the active constituents. Thus, the preliminary identification of these compounds, at the earliest stage of separation, is a strategic element for guiding selective isolation procedure.

The chemical screening and characterization of extracts is well established by complex hyphenated techniques, which generate a huge amount of important information and, generally, are coupled to bioassays in order to obtain a bioactivity-guided fractionation procedure to isolate bioactive constituents. Thus, for the chemical screening, liquid chromatography (LC) or capillary electrophoresis (CE) techniques coupled with different detectors such as LC–UV, LC–MS, CE–UV, CE–MS and LC–NMR, led to a rapid initial screening of crude plant extracts, providing a great deal of preliminary information about the content and nature of constituents.

However, biological, pharmacological and toxicological off-line tests are needed [1]. These tests represent a limiting step of the rapid discovery of bioactive constituents, since they increase enormously the time for the bioactivity-guided fractionation. In fact, the isolation of bioactive compounds

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requires a preliminary fractionation, and then, the analysis of each fraction obtained, in order to find those having the investigated activity. The selected compounds are normally tested by in vitro assays, which are time consuming (the analysis time is in the range from hours to few days), generally expensive and not always reliable. In addition, false-positive and false-negative responses can cause difficulties in the interpretation of the results.

In order to rationalize this approach, it would be important to couple the purification–fractionation assay with on-line simple tests, which could give fast and reliable information about the bioactivity of the compound of interest. This step could be applied to both the analysis of new synthetic derivatives, or to natural products from plant sources.

In this regard, a new interesting approach could be, to combine a biosensor assay with classical fractionating approaches and with chemical screening methodologies in order to obtain a bioactivity-guided fractionation. And simultaneously, analysis of interesting compounds in terms of chemical characteristics, and eventually, the desired biological activity. A scheme of the proposed screening is illustrated in Fig. 1.

Some important advantages of this technology are: the short analysis time (in the order of few minutes), the absence of any labelling to reveal the bio-specific interaction, and also the easy of use. Information on "bioactive" substances, where the term bioactive stands for a compound able to interact with the target biomolecule immobilised on the sensor, can be obtained by off- or on-line coupling of the device with the fractionating step. Two interesting reviews report the application of an optical biosensor to drug screening and to the study of molecular interaction in pharmacology [2,3]. No tracers are required, and matrix effects are minimised or eliminated, since interfering and/or non-binding substances are removed in the first step. In particular, DNA-biosensor technology, on which this work is focused, can be used for a rapid and low-cost detection of anti-cancer, anti-viral drugs and other DNA-targeted molecules. In fact, immobilising DNA molecules on the transducer and looking at its interaction with the sample of interest, the ability of a molecule to bind to DNA, i.e. intercalator, can be shown in few seconds.

Some interaction studies based on the ability of different compounds to interact directly with DNA and employing the biosensor technology have been already reported by some authors using different transducers, mainly electrochemical and optical. In particular, electrochemical transducers have already been applied to environmental analysis [4,5], while optical systems like refractometric interference spectroscopy [6] and surface plasmon resonance (SPR) [7,8], were employed mostly for applications to drug analysis. The study of the binding of a homologous series of anthraquinones to DNA using an SPR-based instrumentation has been recently published [9]. Some optical sensors are well suited also for evaluating the kinetic parameters of the interaction, such as the affinity constant ($K_A$), from the binding profiles without the need of any label.

In the present work, the possibility of applying the biosensor approach, both to test synthetic molecules and in the search for bioactive constituents from plants has been investigated. In particular, an optical sensing method relying on the SPR-based bench instrument, Biacore X$^{TM}$ was used.

Different samples from pure compounds or purified fractions from plant extracts were analyzed with this DNA-based sensor. In particular, doxorubicin a well-known intercalator [10] was used as standard compound. Furthermore, some natural derivatives expected as DNA-interacting molecules, such as podophyllotoxin [11–13], emodine [14,15], chrysophanol [16] and sanguinarine [17–22] were investigated to evidence their mode of action ("yes/no" DNA binding ability). In addition, in order to evidence the suitability of biosensors in the search of active constituents in plant complex matrices, some fractions obtained from an ethanolic extract of the fresh plant material of *Chelidonium majus* L. (great celandine) were tested. This plant was chosen for its well-known content in benzo[*c*]phenanthridinium alkaloids, having multiple biological and pharmaceutical effects such as anti-inflammatory and anti-microbial activity, inhibition of SH-enzymes and microtubule assembly [23,24] and, in particular, because these alkaloids posses the ability to interact with DNA [25].

The presence of the benzo[*c*]phenanthridinium alkaloids in the investigated fractions obtained from the EtOH extract of *C. majus* L. was evaluated, in parallel, by conventional HPLC–DAD–MS analysis.

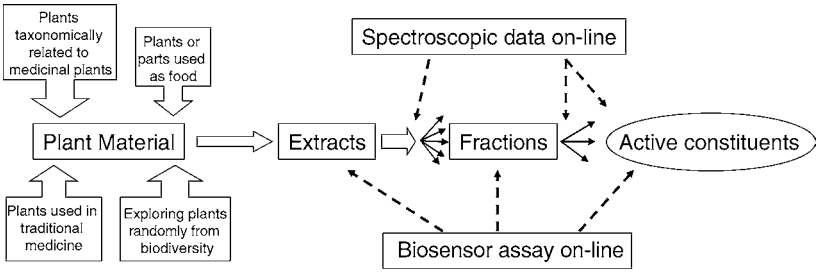



Fig. 1. Proposed analysis scheme: the biosensor analysis could be applied theoretically to plant material of different origins. The analysis can be performed on purified compounds or during fractionation on purified fractions and eventually to pure extracts.

## 2. Materials and methods

### 2.1. Reagents

11-Mercaptoundecanol was from Aldrich (Milan, Italy). Dextran was purchased from Pharmacia Biotech (Uppsala, Sweden); epichlorohydrin and *N*-hydroxysuccinimide (NHS) from Fluka (Milan, Italy). Ethanol and all the reagents for the buffers were purchased from Merck (Milan, Italy).

Acetonitrile and MeOH (HPLC grade), 85% orthophosphoric acid, hexane, diethyl ether and EtOH (analytical grade) were from Merck (Milan, Italy); Carlo Erba (Milan, Italy) provided 85% formic acid. Water was purified by a Milli-$Q_{plus}$ system from Millipore (Milford, MA, USA). $H_2SO_4$ (96%) was purchased from Aldrich (Milan, Italy).

Oligonucleotides were purchased from Amersham Biosciences (Uppsala, Sweden). The base sequences of the 5′-biotinylated probe (25-mer) and of the complementary oligonucleotide (25-mer) were as follows:

probe: 5′-biotin-GGCCATCGTTGAAGATGCCTC-TGCC-3′

target: 5′-GGCAGAGGCATCTTCAACGATGGCC-3′

### 2.2. Samples

#### 2.2.1. Pure compounds

Doxorubicin hydrochloride and podophyllotoxin were from Sigma (Milan, Italy); emodine was from Fluka (Milan, Italy); chrysophanol from Extrasynthese (Genay Cedex, France); sanguinarine nitrate was from City Chemical Corporation (New York). DMSO solution (1 mg ml$^{-1}$) of each sample was prepared.

#### 2.2.2. Plant extract and fractions

*C. majus* L. (great celandine) was collected in Castelnuovo di Garfagnana (Lucca, Italy) on July 2001 by one of the authors (ARB). A voucher plant sample is maintained in the Department of Pharmaceutical Sciences, University of Florence.

A crude EtOH extract (1000 ml) was obtained from whole fresh plant material of great celandine (150 g) by percolation. A portion of the extract was evaporated to dryness and the residue (2 g) was suspended in $CHCl_3$ (5 ml). The solution was then filtered to remove the insoluble part.

The chloroform solution was placed on the top of the column and fractionated by gravity column chromatography. Mixtures of $CHCl_3$–MeOH (from 100:1 to 1:100) were eluted (flow, 3 ml min$^{-1}$) to obtain 100 fractions of 20 ml. Fractions 1–35 were obtained from $CHCl_3$ 100%; fractions 36–40 were obtained by $CHCl_3$–MeOH 96:4; fractions 41–60 from $CHCl_3$–MeOH 9:1; fractions 61–73 from $CHCl_3$–MeOH 4:1; fractions 74–84 from $CHCl_3$–MeOH 70:30; fractions 85–96 from the mixture of $CHCl_3$–MeOH 1:1 and the last three fractions from 100% methanol. Fractions were examined by TLC as reported in the methods and those containing alkaloids were collected in eight main fractions (1–8). These solutions were evaporated to dryness and submitted to HPLC–DAD–MS and sensor analysis.

### 2.3. Biosensors instrumentation

The nucleic acid, double stranded DNA (dsDNA), was immobilised, on the sensing surface of the sensor chip of the commercially available optical sensor Biacore X$^{TM}$.

The affinity constants ($K_A$) of the interaction between the immobilised DNA and pure compounds were estimated by the use of the dedicated BIAevaluation software (Biacore AB, Uppsala, Sweden).

The instrument Biacore X$^{TM}$, and the Sensor Chips (Sensor surface, Au) used, were from Biacore AB (Uppsala, Sweden).

The detection principle of the biosensor is illustrated in Fig. 2. A DNA double helix is immobilised on the sensing surface and the interactant (intercalating agent) is in solution, flowing on the surface (Fig. 2a). If the molecule binds the DNA, then a signal is generated in real-time and is displaced on the sensorgram (resonance units versus time) (Fig. 2b).

### 2.4. Immobilisation of DNA

#### 2.4.1. Immobilisation of small DNA oligonucleotides

The gold sensor chip was modified with 11-mercaptoundecanol, carboxylated dextran and streptavidin following a published procedure [26]. The sensor chip was placed in the instruments and the biotinylated oligonucleotide (probe, 1 μM) was immobilised on streptavidin [27]. The instrument provides two flow cells: the probe was immobilised only on one of the two flowcells and the other was kept with streptavidin and was considered as a control cell. The DNA probe is single stranded and to obtain the double strand, a solution of target (1 μM) was injected only on the flowcell with the probe and incubated for 10 min.

### 2.5. Biosensor measurement procedure

All binding measurements were performed in a buffer composed of 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20 and pH 7.4. Binding interactions were monitored at a constant flow rate of 5 μl min$^{-1}$ at a temperature of 25 °C.

After the immobilisation of small oligonucleotides, the interaction between the immobilised DNA and the different compounds was monitored with an association time of 6 min followed by 5 min of dissociation. Solutions of each compound were injected in the system and association and dissociation phase of the interaction were recorded (Fig. 2b). The flowcell without DNA was used as control cell to verify the absence of non-specific adsorption of the compounds on the sensor chip.

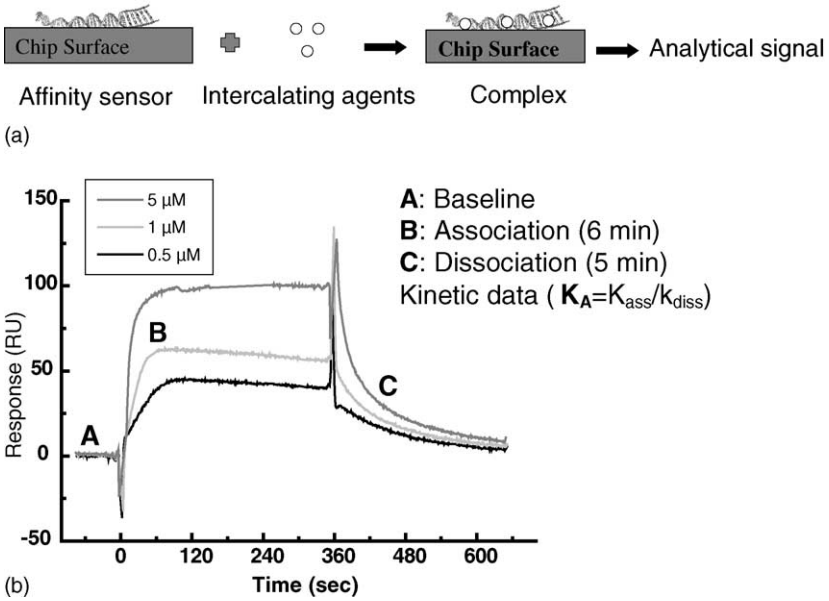



Fig. 2. (a) Biosensor scheme: the analyte in solution binds the biological receptor immobilised on the transduction surface. From the interaction a signal is generated and recorded. (b) Biosensor principle: interaction between immobilised DNA and the molecule in solution able to bind it, i.e. an intercalating substance. Here, reported a sensorgram obtained with the BiacoreX$^{TM}$; the binding is revealed by the increase in the resonance units (RU) after the injection of the analyte. The recorded analytical signal is the increase in RU vs. the base line (C–A). The intercalating agent was tested at concentrations: 0.5–1–5 μM.

The recorded signal is shown as a sensorgram, where the profile of the interaction is displaced over time. Each sensorgram was corrected subtracting the one recorded with the corresponding blank solution to delete any effects due to changes in the refractive index of the bulk solution. Most of the tested compounds were soluble in ethanol or DMSO and blank solutions composed of these solvents were used to verify the absence of interaction between the solvent and DNA.

The fractions in DMSO were diluted in buffer (1:10, v/v) and blank solutions composed of DMSO diluted in buffer (1:10, v/v) were used to verify the absence of interaction between the solvent and DNA.

After one measurement cycle, the DNA surface is regenerated by a treatment with HCl 1 mM. This solution removes both the interactant and the target DNA strand, complementary to the probe immobilised on the surface. With a new hybridisation step a fresh layer of double-stranded oligonucleotides can be obtained, ready for a new interaction meaurement. Equilibrium binding (affinity) constants ($K_A$, $M^{-1}$) were evaluated from the association and dissociation phases of the sensorgrams using BIAevaluation Software (Biacore AB, Uppsala, Sweden).

### *2.6. Chromatographic analysis of purified fractions from great celandine*

#### *2.6.1. Thin layer chromatography (TLC)*

The analytical TLC was performed on Silica gel 60 $F_{254}$ aluminium sheets (20 cm × 20 cm) (Merck, Darmstadt, Germany). Crude extract and fractions were analyzed by TLC eluting with $CHCl_3$–MeOH (9:1 and 1:1) using a light at 365 nm as detector.

#### *2.6.2. HPLC–DAD analysis*

The HPLC system consisted of a HP 1090L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Helwett & Packard, Palo Alto, CA, USA).

All the fractions obtained by the column chromatography were analyzed to obtain the amount of alkaloids. Commercial sanguinarine was used as an external standard. Calibration graphs for HPLC were recorded with sample amounts ranging from 0.10 to 2.5 μg ($r > 0.99$). Linearity range of responses was determined on five concentration levels with three injections for each level.

The column was a 201 TP 54 RP-18 (254 mm × 4.6 mm, 5 μm, 300 Å, Vydac Separation Group Hesperia, CA, USA) maintained at 26 °C. The mobile phase was a step linear isocratic method slightly modified from literature in [28]. The solvent was a mixture of $CH_3OH/CH_3CN/H_2O$ with $H_3PO_4$ (pH 3.2), respectively, 21:55:24 during a 60 min period at a flow rate of 1.0 ml $min^{-1}$. Samples were dissolved in MeOH–$H_2O$–AcCN and were filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane before HPLC analysis. The volume of sample injected was 25 μl. UV–vis spectra were recorded in the range of 190–450 nm, and chromatograms were acquired at 225, 254, 280 and 320 nm.

#### *2.6.3. HPLC–MS analysis*

The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Hewlett & Packard, Palo Alto, CA, USA).

Similar analytical conditions to HPLC–DAD analysis were used to evaluate the peak purity and molecular weights

of the peaks corresponding to the alkaloids. Mass spectrometer conditions were optimized in order to achieve the maximum sensitivity of ESI values. The same column, time period and flow rate were used. Since, phosphoric acid was not suitable for HPLC–MS operations, separation was performed using aqueous formic acid (pH 3.0), without appreciable variations in the chromatographic profile. Mass spectrometry operating conditions were: gas temperature 350 °C at a flow rate of 10 μL $min^{-1}$; nebuliser pressure 30 psi; quadrupole temperature 30 °C; and capillary voltage 3500 V. Full scan spectra from *m*/*z* 100 to *m*/*z* 800 in the positive ion mode were obtained (scan time, 1 s).

## 3. Results and discussion

All the pure compounds and the fractions obtained by the extract of great celandine were tested with the optical DNA-based sensor. The presence of alkaloids in each fraction was evaluated by TLC and HPLC/DAD/MS analysis.

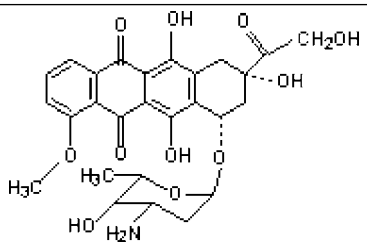
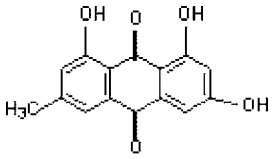
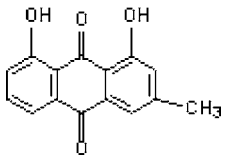
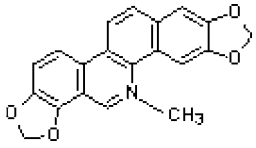
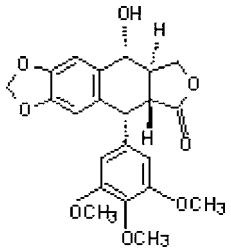
### 3.1. Analysis of pure compounds with the optical DNA-based sensor

Doxorubicin was used as positive control for DNA binding. In Table 1, the results obtained with the optical sensor are shown. In table, we report the name, structure and molecular weight of the tested compound with the calculated affinity constant ($K_A$).

Among the different tested compounds, emodine, chrysophanol and podophyllotoxin did not show the ability to bind the DNA double strand ((–) in Table 1).

For kinetic analysis of the interaction between the pure compounds and DNA, they have been tested at the range of 10–20–50 ppm. The affinity interaction values of doxorubicin and sanguinarine were calculated ($9 \times 10^5$). These two

Table 1
Pure compounds tested with the biosensor

| Name | Structure | MW | $K_A$ ($M^{-1}$) |
|---|---|---|---|
| Doxorubicin |  | 579.99 | (9±4) × $10^5$ |
| Emodine |  | 270.2 | – |
| Chrysophanol |  | 254 | – |
| Sanguinarine |  | 394.22 | (9±3) × $10^5$ |
| Podophyllotoxin |  | 414.41 | – |

$K_A$ ($M^{-1}$) is the estimated affinity constant for the interaction.

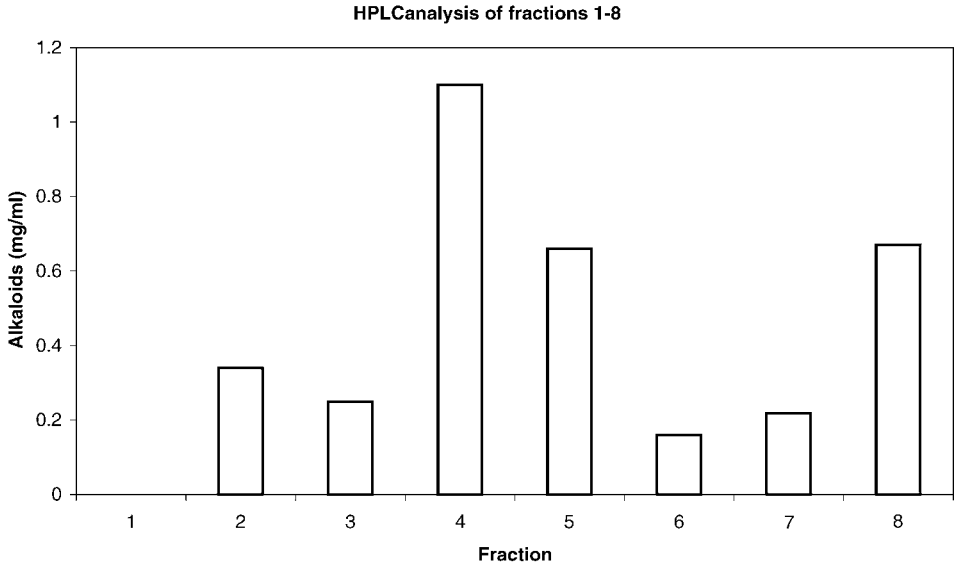



Fig. 3. HPLC/DAD/MS analyses conducted on the obtained eight fractions by column chromatography purification and combined by TLCs. The chemical profile in terms alkaloids content is reported.

compounds have already been used in other studies based on DNA-based biosensors employing different transduction principles. Where available, these literature data were compared with our findings and a very good agreement has been found for all the comparisons ($8 \times 10^4$) for doxorubicin [6] and $2 \times 10^5$ to $2 \times 10^6$ for sanguinarine [19]). The system is then very specific for molecules with DNA affinity.

The compounds giving non-detectable analytical binding signal (i.e. emodine, chrysophanol and podophyllotoxin) were initially classified as intercalating agent in literature of [29], but very recently a different mechanism for their DNA breaking action has been reported [30,31]. They inhibit the enzymes involved in DNA repair (SOS) causing an increase in the DNA breakage initially explained as a consequence of an intercalating activity. This can clearly explain the absence of binding with our sensor.

Anyhow, it is here important to underline that in the analyzed literature relative to the compounds emodine, chrysophanol and podophyllotoxin there is a gap of almost a decade to clarify the mechanism of action for these control compounds but we could affirm that we were able to rapidly classify (about 15 min) these compounds as molecules that do not bind directly DNA.

### *3.2. Analysis of great celandine fractions by HPLC/DAD/MS*

All fractions obtained by column chromatography purification and combined by TLC were tested by HPLC/DAD/MS analyses in order to evaluate the chemical profile in terms alkaloids content (Fig. 3). In the figure we report the alkaloids content (mg $ml^{-1}$) in each of the eight fraction isolated as explained in Section 2.2.2.

### *3.3. Analysis of great celandine fractions with the optical biosensor*

The optical sensor was applied to the analysis of the fractions obtained by column chromatography.

In Fig. 4, the results relative to the analysis with BiacoreX$^{TM}$ are reported. With this instrument the biointeraction between the ligand and the immobilised DNA, can be followed in real-time, and without the use of any label. For this reason, the association and dissociation phases of the interaction can be clearly distinguished, as showed in the sensorgram reported in Fig. 2. In the association phase the ligand interacts with the DNA, forming a complex, in the dissociation phase, the complex resulting from the interaction, starts to dissociate, while the chip is washed with flowing buffer (phase C). The results are reported as resonance shift (RU), which is the difference between the final value of resonance units at the end of the association or dissociation phase and the initial value with buffer.

In the results relative to the dissociation phase, it is shown that some compounds in the fractions can very quickly be washed off by the flowing buffer, while other remain bound to DNA. This is indicative somehow of the “strength” of the binding between the active compounds and immobilised DNA.

Comparing the results of the optical biosensor with the chromatographic analysis of the tested fractions, it could be seen how the fraction showing the highest ability in DNA binding (fraction 4) is the one with the highest alkaloid contents as resulted from HPLC/MS analysis.

Moreover, a good agreement can be found between the general behaviour of the eight fractions on the biosen-

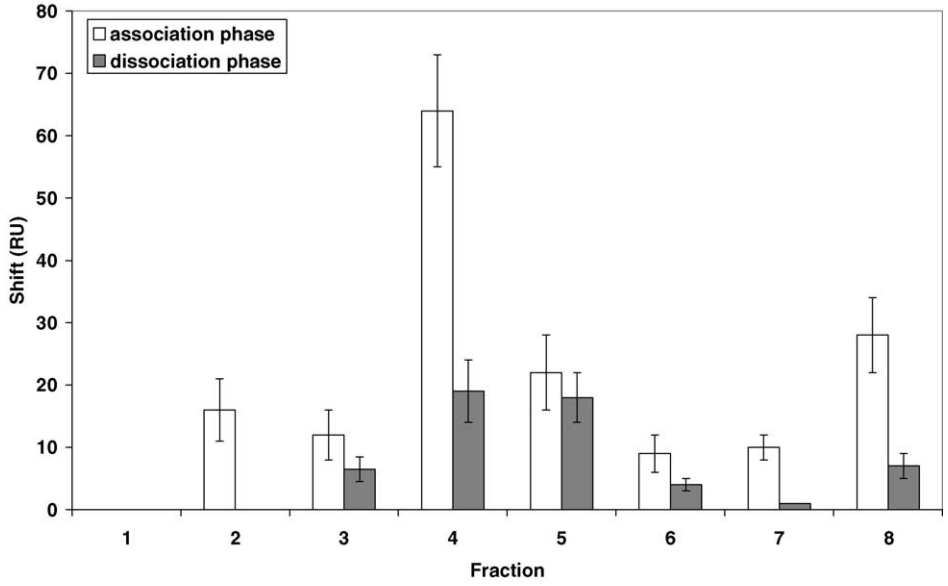



Fig. 4. BiacoreX$^{TM}$ analysis conducted on the purified eight fractions is reported. The bio-interaction between the ligand (intercalating agent) and the immobilised DNA is distinguished in the association and dissociation phase.

sor, and the relative presence of alkaloids, estimated by HPLC/MS.

The results obtained with this biosensor, demonstrate the potentialities of this device in the drug screening and in bioassay-guided fractionation of active constituents from plant.

## 4. Conclusions

The biosensor approach demonstrates the high potentialities of these devices in affinity studies regarding, in particular, interaction with nucleic acids, to be used for fast screening of natural compounds in fractions from plant extracts.

The biosensor has been successfully employed in the analysis of real samples and the obtained results were compared well with traditional analysis by HPLC.

Conventional eluents employed in separation methods are compatible with the biosensor analysis within a certain range. The sample could be diluted many times in water solution to allow biosensor analysis.

The analysis can be easily performed in short time, representing then a competitive technology with respect to traditional approaches. Moreover, the optical sensor can be used to evaluate the detailed kinetic effects of identified positive compounds.

Thus, the impact of biosensors in drug discovery is enormous because they are a label-free screening system, which imparts flexibility to the process of assay design and facilitates successful integration with other technologies thank to the increasing number of commercially available instruments having novel sensor surfaces, immobilisation techniques and attachment chemistries.

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