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Article

Solid-Phase Purification of Deoxyguanosine-benzo[a]pyrene Diol Epoxide Adducts from Genomic DNA Adduct Synthesis

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Hidrocarbonetos polinucleares aromáticos são compostos largamente presentes no meio ambiente e são reconhecidamente considerados compostos carcinogênicos e/ou mutagênicos. Tais substâncias quando presentes em organismos são metabolizados e podem se ligar ao DNA formando adutos. Tais adutos podem induzir erros de replicação os quais podem causar um tumor carcinogênico ou uma mutação genética. Conseqüentemente, a determinação de tais adutos é útil na avaliação do risco de desenvolvimento de câncer. Neste estudo, a síntese do aduto de DNA formado da reação (±)-*anti*-7,8,9,10-tetrahidrobenzo[a]pireno-7,8-diol 9,10-epóxido (BPDE) com DNA de timo de bezerro foi conduzida. O maior produto formado nesta reação é a ligação do BPDE coma a base guanosina. Uma vez que a reação foi conduzida utilizando DNA genômico, um protocolo de purificação dos adutos foi necessário para isolar o principal aduto. Um método usando extração em fase sólida foi desenvolvido para isolamento e purificação do aduto. Os resultados mostraram que um gradiente seqüencial de água/metanol foi necessário para eluir o aduto. Eletroforese capilar de zona com detecção por fluorescência induzida a laser foi usada para monitorar a purificação a cada etapa do processo de desenvolvimento e o aduto de grau analítico foi caracterizado por espectroscopia UV/Vis e fluorescência e espectrometria de massas com ionização *electrospray*.

Polycyclic aromatic hydrocarbons are compounds widely present in the environment and well known to have carcinogenic and/or mutagenic properties. These substances when present in an organism are metabolized and can bind to DNA forming an adduct. Such adduct can induce replication errors that may cause carcinogenic tumor or genetic mutation. As a consequence, the determination of such adducts can be helpful in determining the risk for cancer development. In the present study, a synthesis of DNA adduct formed from the reaction of (±)-*anti*-7,8,9,10-tetrahydrobenzo[a]pyrene-7,8-diol 9,10-epoxide (BPDE) with calf thymus DNA has been carried out. The major product formed in this reaction is the binding of BPDE with the guanosine base. Since the reaction was conducted utilizing genomic DNA, a purification protocol for the adducts was necessary to isolate the main adduct. Solid phase extraction method was developed for isolation and purification of the adduct. The results have shown that a step gradient of water/methanol was needed to elute the adduct. Capillary zone electrophoresis with laser-induced fluorescence detection was used for monitoring the step-by-step purification procedure development and the analytical-grade adduct was characterized by UV/Vis and fluorescence spectroscopy, and electrospray ionization mass spectrometry.

Keywords: BPDE, biomarkers, carcinogens, capillary electrophoresis, mass spectrometry

Introduction

In recent years several studies have related the binding of chemicals to DNA since such reactions are thought to be crucial to the initiation of mutational and carcinogenic processes.[1] Polycyclic aromatic hydrocarbons (PAHs) have been identified as a major source of carcinogenic risk[2,3] and it has been found in large amounts in the environment. These substances are emitted to the environment by incomplete combustion processes of organic materials and can be introduced in an organism by inhalation, ingestion or absorption through the skin. Once in the organism these compounds are primarily activated through an oxidative metabolic pathway to electrophilic intermediates capable of covalently binding to DNA, thus forming DNA adducts, as summarized in Figure 1.[4]

Several studies have correlated the development of cancer and behavioral effects in a population from a highly polluted area, as well as in iron foundry workers, attributed to the presence of PAHs.[5] Benzo[a]pyrene (BP) is the most

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studied PAH due its high carcinogenicity; it is metabolized to *anti*-7,8,9,10-tetrahydrobenzo[a]pyrene-7,8-diol 9,10-epoxide, commonly known as BPDE, which can further react with DNA either *in vivo* or *in vitro*.[6] The adduct formation involves the reaction of the exocyclic amino group of guanosine with the benzylic carbon of the epoxide. Other PAH-DNA adducts are also formed but in much lower quantities.[7]

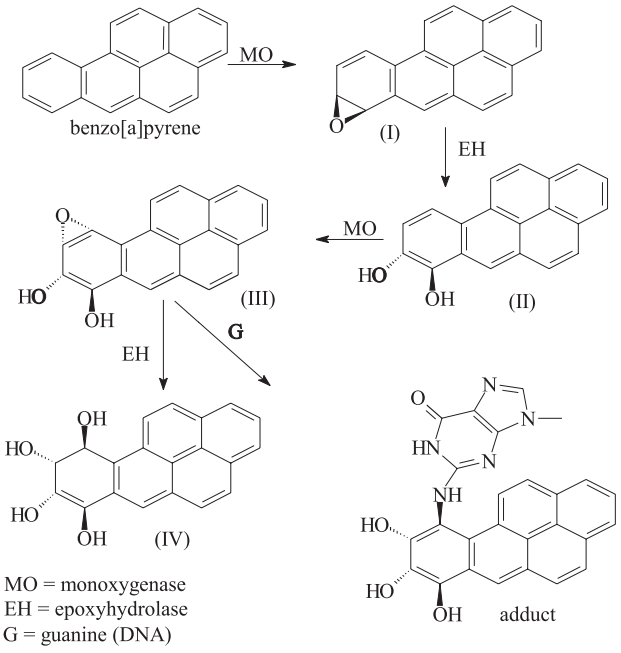

Figure 1. *In vivo* oxidative metabolic pathway of benzo[a]pyrene *via* hydrophilic intermediates (I-IV) and formation of DNA adducts with guanine base.

Determination of DNA adducts is important because they can be considered as biomarkers. Their levels reflect the personal exposure to a determined chemical, therefore determining the risk for cancer development. The average level of adduct formation is 1 modification in 10^6 to 10^9 unmodified base pairs and for this reason, any analytical method used for adduct detection requires very high sensitivity. Several analytical methods have been demonstrated to be useful for such purposes including ^{32}P-postlabeling,[8] immunological assays,[9] and fluorescence techniques.[10] ^{32}P-postlabeling is a technique that has been largely used for analysis of adducts due to its high sensitivity. It is capable of detecting DNA adducts present at levels as low as 1 adduct/10^9–10^{10} nucleotides.[11] However, ^{32}P-postlabeling technique presents some disadvantages such as the use of radiolabeled compounds that increases the cost of analysis and poses health risks due to the radioactivity.

Immunosorbent assays have also demonstrated high sensitivity but are limited to a specific chemical constituent and consequently, elevating the cost of the analysis.[12] Fluorescence detection methods (especially laser induced fluorescence detection) are known to be very sensitive and when this detection mode is used with capillary electrophoresis (CE) low adduct levels can be detected.[13] The advantages of CE are that this technique provides fast analysis time with superior separation efficiencies, ease of use and low cost. The use of laser as an excitation source yields an increase in the fluorescence intensity and then, laser induced fluorescence method can increase the sensitivity of fluorescence detection methods by several orders of magnitude.

The purification of the DNA adduct samples is also an important subject since these samples are digested with enzymes for cleavage of the DNA to oligonucleotides and then to nucleotides. DNA adducts are found either in organisms or *in vitro* synthesis in low quantities. The use of solid-phase extraction (SPE) can be applied for simultaneous pre-concentration of adducts and removal of non-modified bases, proteins, and other secondary products such as salts and buffers.[14] In this work BPDE-DNA adduct was synthesized with calf thymus DNA and the major adduct, formed with the amino group of guanosine, was isolated and purified. Purification of this digestate was conducted utilizing an SPE procedure similar to that described by Melikian *et al.*[15] and Barry *et al.*[16] However, our results have shown that more studies were needed for the complete purification of the adducts and a detailed SPE protocol is now presented. Capillary electrophoresis was used with laser-induced fluorescence detection for monitoring the method development. The purified DNA adducts were characterized by UV/Vis, fluorescence and mass spectrometry. The data was compared with that obtained from the literature.

Experimental

Chemicals

(±)-*Anti*-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) was purchased from the NCI Chemical Carcinogen Reference Standard Repositories at Midwest Research Institute (Kansas City, MO). Calf thymus DNA, DNase I, snake venom phosphodiesterase I and tetrahydrofuran (THF) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). THF was purified for total removal of water utilizing an alumina column (10 g). Solvents, such as methanol, ethylacetate, and diethylether, were obtained from

Mallinckrodt (Phillipsburg, NJ) while Tris/TAPS buffers were obtained from Sigma (Saint Louis, MO).

Caution. It is well established that BPDE is a potent mutagenic and/or carcinogenic agent and should be handled with care.

Apparatus

Capillary electrophoresis Beckman P/ACE 5000 system (Fullerton, CA) with laser-induced fluorescence (LIF) detection was used for DNA adducts analysis during method development. An Omnichrome He/Cd laser (Melles Griot, Carlsbad, CA) was used for LIF excitation at 325 nm and a lab-made edge filter at 375 nm (70% of transmittance) was used for selection of the fluorescent light. All separations were carried out using a poly(vinylalcohol) (PVA) coated fused-silica capillary column with suppressed electroosmotic flow (EOF) with 75 μm i.d. and 47 cm of total length (40 cm from injection to detection point). Hydrodynamic injections varied from 1 to 15 s at 0.5 psi and the separations were conducted applying –20 kV at the injection end (reverse polarity). Separation was carried out in a medium with 50 mmol L^{-1} Tris-TAPS pH 8.3 buffer.

Spectrometric characterization of the dG-BPDE adduct was carried out utilizing a Micromass Platform LC mass spectrometer (Manchester, UK) with direct injection of 10 μL of the adduct fractions in acetonitrile/water (50/50 v/v) mobile phase at a flow rate of 10 μL min^{-1}. The ionization mode utilized was electrospray with negative ion detection; no modifiers were added to enhance ion formation. The electrospray needle voltage was maintained at -3 kV, the source temperature was 90 °C, and the cone voltage was set at 35 V for recording the $[M–H]^-$ spectra. Data acquisition was performed in the scan mode from *m/z* 200 to *m/z* 700 at 1 Hz. Characterization of the purified adduct was also carried out utilizing a Hitachi U-3501 spectrophotometer and a Hitachi F-4500 spectrofluorimeter.

Synthesis of DNA adducts

Calf thymus DNA (5.0 mg) was mixed with 5 mL of 10 mmol L^{-1} Tris-HCl buffer (pH 7). Separately, 500 μg of (±)-anti-BPDE was dissolved in 500 μL of purified tetrahydrofuran (THF). These two solutions were mixed and were incubated at 37 °C overnight. The BPDE that did not react with DNA (BPDE easily hydrolyzes to tetraols analogue in aqueous medium) was then removed from solution by three extractions with ethyl acetate and one extraction with diethyl ether.[17] The DNA was enzymatically hydrolyzed to deoxyribonucleotides utilizing DNase I and snake venom phosphodiesterase I enzymes.[18]

Purification

The separation of unmodified nucleotides from modified nucleotides (adducts) was carried out on SPE cartridges containing 100 mg of C_{18} phase (Amersham Life Sciences, Buckinghamshire, UK). Initially, this purification was conducted adding 500 μL of the adduct solution in the cartridge and then 2 mL of water for elution of the unmodified nucleotides and 2 mL of methanol for elution of the modified nucleotides according to Barry *et al.*[16] In a second experiment for purification of total DNA adduct synthesis 5 mL of water and 5 mL of methanol were eluted sequentially in the C_{18} cartridge in 1 mL aliquots. After the conclusion of the second clean up, a third experiment was carried out utilizing different proportions of water and methanol, simulating a step-gradient elution. In all experiments, the methanol from fraction was totally evaporated and 100 μL of 5 mmol L^{-1} Tris-TAPS pH 8.3 buffer was added to each fraction. After purification, both fractions of 1 mL water/methanol were pooled together and an aliquot of 500 μL was diluted with 1 mL of water for spectroscopic characterization and quantitation.

Results and Discussion

Genomic DNA adduct synthesis and SPE purification

DNA adducts can be formed when electrophilic compounds bind covalently to DNA. BPDE can bind to DNA mainly via deoxyguanosine base, forming an adduct. This binding can induce to DNA replication errors and even induce the formation of a carcinogenic tumor.[19] Adducts with other DNA bases can also be formed but at lower quantities. However, according Ibanez *et al.*,[20] only 5 to 10% of the BPDE that is added to a solution containing calf thymus DNA bind covalently to DNA. Therefore, the major portion of BPDE is hydrolyzed to a tetraol analogue.

The presence of high quantities of unmodified nucleotides can limit the amount of sample that may be introduced in the capillary column during electrophoretic injection of the sample, thus decreasing the sensitivity of the analysis; purification of the adduct sample must be performed. As such, an SPE procedure was employed to remove the unmodified nucleotides and some degradation products. In the first purification experiment, the modified deoxyribonucleotides were separated from unmodified deoxyribonucleotides using a C_{18} SPE cartridge based on their hydrophilic/hydrophobic properties.

An aliquot of 500 μL of DNA adducts was added to the cartridge and eluted with a 2 mL aliquot of water and a 2 mL aliquot of methanol. Figure 2 shows the

electropherograms of the purified products from the synthesis of BPDE with calf thymus DNA (Figure 2a) and its control reaction and purification (Figure 2b). The electropherogram obtained from the adduct synthesis shows an intense peak at *ca.* 19 min that can be attributed to deoxyguanosine-BPDE adduct, the major adduct formed. It can also be observed the presence of several other smaller peaks migrating between 5 and 18 min. All peaks could be attributed to BPDE bound to the other DNA bases and/or adducts from BPDE degradation products. A blank reaction for control of the adduct synthesis was also prepared and the electropherogram obtained (Figure 2b) shows that no fluorescent compounds were detected; therefore, all detected fluorescence signals originated from BPDE-derived compounds. For all CE analysis a PVA coated capillary was used to avoid the electroosmotic flow, therefore all the separations were anionic only. Since the adduct molecules have a permanent negative charge due to the phosphate group, the separation of these adducts was carried out basically by their size difference, therefore neutral compounds such as tetraols could be neither separated nor detected.

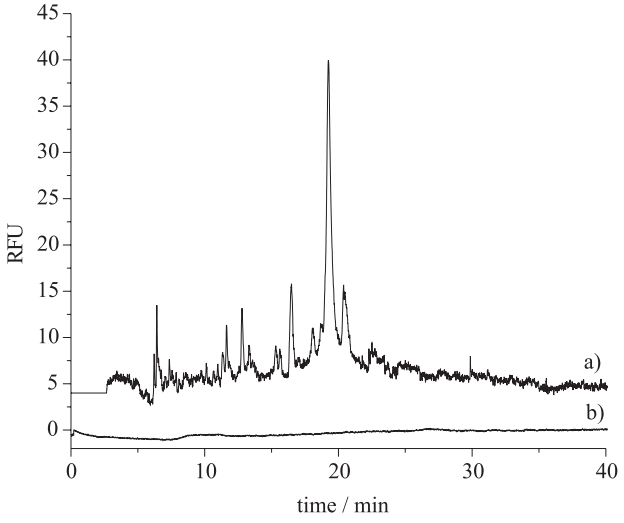

Figure 2. Electropherograms of a) first DNA adduct synthesis and purification, and b) blank reaction. Conditions of analysis: injection time was 15 s and the voltage applied was –20 kV. Running buffer was 50 mmol L^{-1} Tris-TAPS pH 8.3 in a PVA coated capillary. Data were stacked for clarity.

The results in Figure 2a have shown that the purification was not efficient, since several compounds were present. A second purification was then carried out using 5 mL of water and 5 mL of methanol with fractionation of 1 mL aliquots. The results obtained with this second clean up can be seen in Figure 3. Observe that few compounds were eluted in the first 1 mL extraction with water (Figure 3a) but most of fluorescent compounds were still present in the first 1 mL methanol extraction (Figure 3b). All other fractions with 1 mL of either water or methanol did not present any fluorescent compounds (results not shown). The substances that were eluted with the first 1 mL of water are probably highly hydrophilic BPDE degradation-product adducts, since the unmodified nucleotides have not presented any fluorescence signal in the control reaction. For the electropherogram obtained from the elution with the first 1 mL methanol fraction, various compounds were eluted showing that even though 5 ml of water was used before the methanol, the solvent was not strong enough to elute other compounds. In this case products of degradation with hydrophobic properties could have been formed. Barry *et al.*[16] reported that in a purified fraction, collected from an *in vitro* DNA reaction, there was more than one hydrolysis product and that these compounds also contained the BPDE structure, what was confirmed here in this work with the selective fluorescence detection. Therefore we have decided to use different proportions of water and methanol to obtain a fraction containing only the major adduct and no other fluorescent compounds.

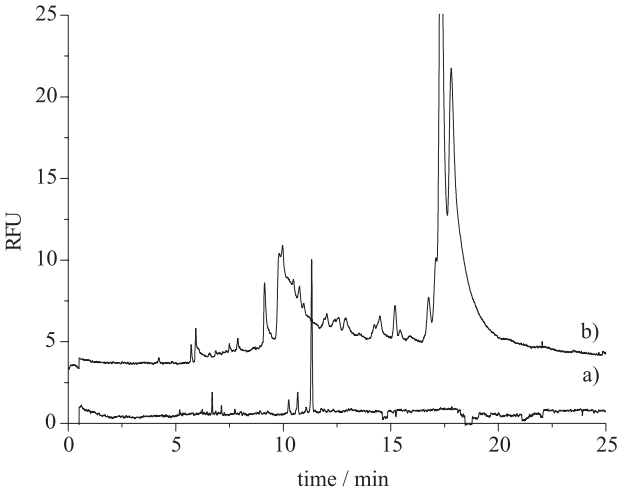

Figure 3. Electropherograms of the fractions obtained from the second adduct purification protocol. a) First 1 mL fraction of water and b) first 1 mL fraction of methanol. Conditions of analysis: injection time was 5 s and the voltage applied was –20 kV. Running buffer was 50 mmol L^{-1} Tris-TAPS, pH 8.3 in a PVA coated capillary.

The use of a gradient elution for the adduct purification is useful because the mixture of two solvents could increase gradually the solvent strength for appropriate elution of the compound of interest. In Figure 4, extractions obtained with different proportions of water and methanol is presented. It can be observed that both the first (Figure 4a) and the second fraction (Figure 4b) containing 1 mL of water presented some peaks that could be due to the elution of some hydrophilic degradation products. It can also be

observed that pure DNA adducts were eluted from the C_{18} cartridge with 1 mL of water/methanol (6:4 v/v) (Figure 4d) while no other peaks were detected when pure methanol was eluted from the cartridge (Figure 4e).

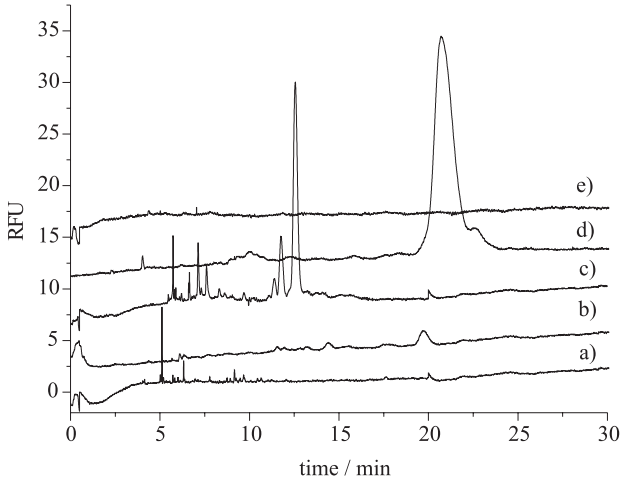

Figure 4. Electropherograms of the fractions obtained from the third adduct purification protocol: a) first 1 mL of water; b) second 1 mL of water; c) 1 mL of water/methanol 8:2 (v/v); d) 1 mL of water/methanol 6:4 (v/v), and e) first 1 mL of methanol. Conditions of analysis: injection time was 5 s and the voltage applied was –20 kV. Running buffer was 50 mmol L-1 Tris-TAPS, pH 8.3 in a PVA coated capillary.

Electrophoretic analysis of BPDE DNA adducts

Earlier work from our group has shown the versatility of CE methods in separating DNA adducts and it was found that the use of coated capillaries was better suited for this work.[21] Since a PVA coated capillary was used and the BPDE DNA adducts have a permanent negative charge from the phosphate group, the CE analysis was carried out in the anionic mode, *i.e.*, with reverse polarity, in which only anions were separated and detected. Because no EOF is present, anions with high electrophoretic mobility are detected first and anions with lower electrophoretic mobility are detected later. If it is assumed that deoxynucleotide BPDE adducts carry only one negative charge differences in mobility are mainly attributed to differences in molecular size. From Figures 2-4 it can be noticed that the earlier migrating smaller peaks are possibly attributed either to singly charged smaller BPDE adducts such as deoxycytidine and deoxythymidine adducts, or BPDE degradation-product adducts, or even charged BPDE degradation products only (not an adduct). The presence of a second major peak migrating after the peak attributed to the dGMP-BPDE adduct can be due to the formation of an adduct with the adenosine base, the second major adduct formed between BPDE and DNA.[22]

Spectrometric characterization of the purified dG-BPDE adducts

The experiments for adduct characterization by mass spectrometry (MS) were conducted for all fractions obtained in the purification of adducts from the total-DNA synthesis. The samples were directly introduced in the mass spectrometer with electrospray negative ionization mode thus producing pseudo-molecular ions $(M - H)^-$. After the injection of the first fraction eluted with 1 mL of water, ions with *m/z* 346 were detected, which corresponded to the deoxyguanosine (molecular mass of 347 Da). The injection of the second fraction of 1 mL of water still presented a small quantity of dGMP.

The analysis of the fraction eluted with 1mL of water/methanol 6:4 (v/v) has shown ions with *m/z* 648, which corresponded to the pseudo-molecular ion of the dG-BPDE adduct with a molecular mass of the 649 Da. In the second fraction with 1 mL of water/methanol 6:4 (v/v), the adduct was not detected anymore, showing that all adduct was eluted from the cartridge with 1 mL of water/methanol 6:4 (v/v).

In Figure 5 is presented the mass spectrum of dG-BPDE adduct purified from the total-DNA synthesis. Observe that the base peak ion is the pseudo-molecular adduct ion (*m/z* 648) and a second abundant ion is the dGMP (*m/z* 346). The presence of the dGMP may be either due to a small contamination of the adduct solution with the deoxynucleotide (which could not be detected by CE-LIF since it does not fluoresce) or to a fragmentation of the adduct molecule, such as described by Barry *et al.*[16] The fragmentation between the guanosine base and the benzo[a]pyrene triol may occur. If the negative charge stays with the triol, the ion observed could have *m/z* 302, however, if the negative charge stays with the guanosine base, then one should have a fragment with *m/z* 346. In this experiment, however, we cannot conclude which one was the source of the *m/z* 346 ion because the sample injection was done directly in the spectrometer without chromatographic or electrophoretic separation and no MS/MS capability.

For further spectroscopic characterization of the purified dG-BPDE adduct obtained from the total-DNA synthesis, Figure 5 also shows the absorbance and fluorescence spectra. The absorbance spectrum of the covalent adduct is characterized by three maxima at 315, 330 and 345 nm.[23] The concentration of the solution could be calculated measuring the maximum absorption in 345 nm and utilizing the molar absorption coefficient of the 2.9×10^4 L mol^{-1} cm^{-1}, obtained from the literature.[24] The fluorescence spectrum presented also three maxima at 380, 400 and 420 nm, which were in fully agreement with the published data.[25]

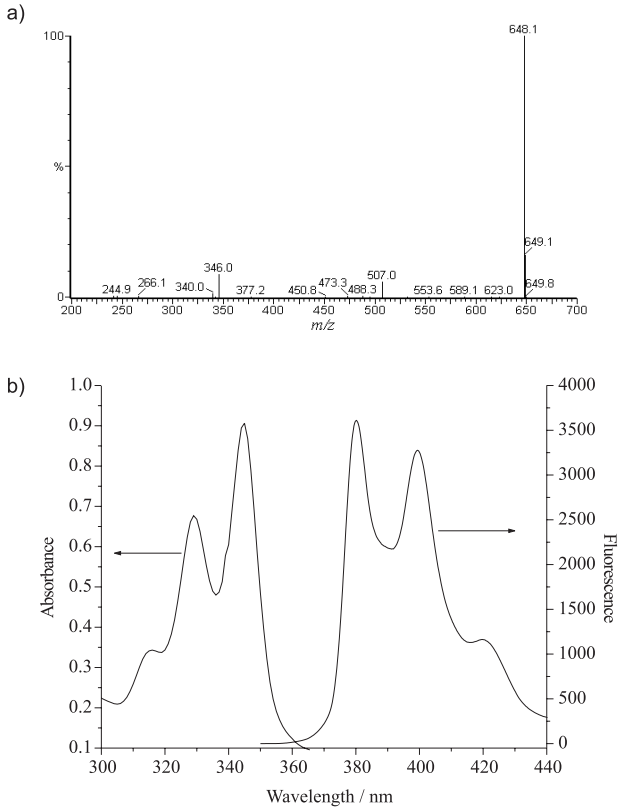

Figure 5. a) Negative-ion electrospray mass spectrum of dG-BPDE adduct obtained from total-DNA synthesis and isolated by the SPE purification procedure. b) Absorbance and fluorescence spectrum of the purified adduct. Excitation wavelength for the fluorescence spectrum was 325 nm (same wavelength used in the CE-LIF system).

Conclusions

The BPDE-DNA adduct synthesis from genomic DNA was successful as well as the purification methodology for isolation of its major adduct, dG-BPDE. Spectrometric characterization of the purified adduct, corroborated with data from literature, supplied appropriate evidences of its positive isolation and identification. The development of such methodology was fundamental for quantitative determination of dG-BPDE adducts, as biomarkers, in order to evaluate the exposure to PAHs of rural workers in the sugar cane harvesting.[26] Capillary electrophoresis analysis with LIF detection provided excellent results for step-by-step monitoring of the synthesis and the purification yielding shorter analysis time, smaller sample volume, lower detection levels, and no solvent consumption when compared to HPLC analysis.

Acknowledgments

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