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Simple chromatographic systems permitting both DNA purification and separation of 2′-deoxyribonucleoside 3′-monophosphates as substrates for ^{32}P-postlabelling studies☆

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ABSTRACT

The ^{32}P-postlabelling method has recently been applied to the measurement of oxidative DNA damage. The asssay requires the isolation of 2′-deoxyribonucleoside 3′-monophosphates subsequent to the extraction of DNA followed by its enzymatic digestion. As an alternative to the use of toxic and oxidizing solvents such as phenol, a simple purification method is proposed, based mainly on size-exclusion chromatography carried out either with ready-made columns (NAP-10, SEC-2000) or, more conveniently, with stainless-steel laboratory-packed columns (Fractogel HW 65 F). This method was applied to the purification of the DNA extracted from seeds of *Lactuca sativa*. After enzymatic digestion of DNA, the 2′-deoxyribonucleoside 3′-monophosphates may be further separated in less than 30 min by high-performance liquid chromatography on a Hypersil octadecylsilylsilica gel column in the ion-suppression mode by using either ammonium formate (0.05 *M*, pH 6.5) or sodium succinate (0.02 *M*, pH 6.0). The use of these eluent systems is compatible with straightforward ^{32}P-labelling of the 2′-deoxyribonucleoside 3′-monophosphates without any concentration and desalting steps.

INTRODUCTION

Evidence is accumulating that oxidative DNA base damage is responsible for the induction of mutations, carcinogenic processes and cell death [1]. Hence considerable efforts have been devoted recently to the development of sensitive methods for monitoring oxidative base modifications in DNA [2]. HPLC with appropriate systems allows both qualitative and quantitative determinations of mutagenic DNA adducts [3,4].

In a recent study dealing with an HPLC ^{32}P-postlabelling assay, Mouret *et al.* [5] adopted the following strategy: (1) chemical synthesis and structural characterization of a modified 2′-deoxyribonucleoside 3′-monophosphate; (2) chromatographic separation of the pool of the DNA enzymatic hydrolysate; and (3) polynucleotide kinase labelling of the modified 2′-deoxyribonucleoside 3′-monophosphate [6,7].

A simple general extraction and purification method for DNA is proposed which associates the classical lysis method with size-exclusion chromatography without phenol deproteinization. Isocratic reversed-phase HPLC with octadecylsilylsilica gel columns provides a rapid and efficient means of resolving the pool of deoxyribonucleoside 3′-phosphates. This was achieved with neutral buffers in the ion-suppression mode, an approach compatible with the use of polynucleotide kinase in the chromatographic eluate.

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EXPERIMENTAL

Chemicals and solvents

Stock buffer solutions of 0.5 *M* were prepared at room temperature and refrigerated until use and pH equilibration. The different salts used to prepare the solvents were of analytical-reagent grade and dissolved in water obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Ammonium formate, calcium formate, succinic acid and sodium chloride were purchased from Merck (Darmstadt, Germany). 2′-Deoxyribonucleoside 3′-monophosphates were obtained from Sigma (St. Louis, MO, USA) and used as received. Triethylamine, ethanol and 2-propanol were purchased from Prolabo (Paris, France) and 2-butanol from Sigma.

Biopolymers

The following nucleic acids were used as received unless stated otherwise: *E. coli*, strain B, fraction VIII, purchased from Sigma; calf thymus DNA and lambda DNA, obtained from Boehringer (Mannheim, Germany); and plant DNA, extracted from seeds of *Lactuca sativa* (Perlane variety, Rhonalpagri, Voreppe, France).

Enzymes

Proteinase K (EC 3.4.21.14) and RNase A (EC 3.1.27.5) were purchased from Boehringer. The enzymatic digestion into 2′-deoxyribonucleoside 3′-monophosphates was carried out with a cocktail of micrococcal nuclease (EC 3.1.4.7) and spleen phosphodiesterase (EC 3.1.4.8) obtained from Boehringer and Sigma, respectively. Dephosphorylation was achieved with alkaline phosphatase (EC 3.1.3.1) of molecular biology grade, purchased from Boehringer, according to the manufacturer's recommendations.

Extraction of DNA

The following protocol was used for the extraction of DNA from the seeds of *Lactuca sativa*. Before grinding, the mortar and pestle (porcelain) were precooled in liquid nitrogen. The seeds (250 mg) were ground to a fine powder until complete evaporation of liquid nitrogen. The operation was repeated four times in a total time of 15 min. The lysis buffer (5 ml), obtained from Applied Biosystems (Foster, CA, USA), was poured on the fine grey powder and rapidly solidified.

The thawing of the ground seeds was achieved at room temperature in the absence of strong incident light for at least 3 h. A viscous brown liquid was obtained that was kept at 8°C overnight. After rinsing the mortar and pestle with 2 ml of lysis buffer, the disrupted biological material was centrifuged at a low speed (1000 *g*) at room temperature (15 min) in order to eliminate most of the rough cellular debris, then the lysate (0.7 ml) was placed in twelve Eppendorf tubes (2 ml), each containing 0.3 ml of 7.5 *M* ammonium acetate solution.

The salting out of DNA and RNA was carried out by adding 2-propanol (1 ml). A dark brown precipitate appeared when the tubes were mixed manually by inversion. The tubes were kept at −20°C for at least 1 h. Then precipitates were collected by centrifugation (Sigma MK 2 centrifuge, 1 h, 4°C, 12 000 *g*) and rinsed with 70% ethanol (1 ml). Once dried (10 min), the nucleic acids and other water-soluble products were extracted with fresh sterile Milli-Q-purified water (0.4 ml per tube) and subsequently vortex-mixed for 30 min at room temperature. The light brown aqueous solution obtained was concentrated with 2-butanol according to Maniatis *et al.* [8]. Phase separation was accelerated by centrifugation at room temperature for 10 min at 1000 *g*, giving a yellowish precipitate.

Purification of DNA by size-exclusion chromatography

DNA was purified by size-exclusion chromatography using NAP-10 desalting columns (Sephadex G-25, DNA grade) obtained from Pharmacia (Bromma, Sweden) and equilibrated with water. UV absorbance detection (260 nm) was used for monitoring the chromatographic eluates. The nucleic acids collected in the void volume were concentrated with either a Speed-Vac evaporator obtained from Savant Instruments (Farmingdale, NY, USA), or more conveniently with 2-butanol.

Traces of 2-butanol were removed by filtering the eluate (1 ml) through a NAP-10 column previously equilibrated with water. The resulting filtrate (1.5 ml) was divided into two Eppendorf tubes (0.75 ml each). RNase digestion (1 h, 37°C) was carried out with 10 μl of an aqueous solution of RNase (10 mg/ml) in a standard sodium citrate buffer while deproteinization (1 h, 55°C) was carried out after the destruction of RNA, adding to each tube 10 μl of an aqueous solution of proteinase K (20 mg/ml).

The crude DNA mixed with the RNA hydrolysate was submitted again to a size-exclusion chromatographic purification on a semi-preparative column.

Column preparation

Preliminary assays were carried out with an SEC-2000 column (300 mm × 7.5 mm I.D.; molecular mass operating range 1000–250 000) purchased from Beckman Instruments (Fullerton, CA, USA) with water as the eluent at a flow-rate of 0.5 ml/min.

All other chromatographic separations were carried out with classical stainless-steel semi-preparative laboratory-packed columns (300 mm × 7.5 mm I.D.) filled by gravity by using a slurry of Fractogel HW 65 F in water (molecular mass operating range $5 \cdot 10^4$–$5 \cdot 10^6$) from Merck. However, the packing was more conveniently achieved according to Coq *et al.* [9] with a Touzart et Matignon (Paris, France) pump for 1 min with Milli-Q-purified water.

Digestion of DNA into 2'-deoxyribonucleoside 3'-monophosphates

An aqueous solution of DNA (0.750 ml, 15 μg) was enzymatically digested (2 h, 37°C) with 1 μl (15 U) of micrococcal nuclease, 20 μl (0.4 U) of spleen phosphodiesterase in succinate buffer (20 m*M*, pH 6) with calcium chloride (10 m*M*) in a final volume of 0.840 ml.

High-performance liquid chromatography

The chromatographic solvents were prepared by using Milli-Q-purified water and subsequently degassed by filtration on a Millipore filter (HAWP, 0.45 μm). Samples were injected via a Rheodyne Model 7125 injector equipped with a 0.5-ml loop. Delivery of the solvents was achieved by using either a Merck L 6200 Intelligent pump or a Waters Model 501 pump system. The chromatographic eluates were monitored at 260 nm. The injector was rinsed prior to each analysis with 0.5 ml of 0.3 *M* NaH_2PO_4 solution (pH 4).

HPLC separations were carried out on a Hypersil (5 μm) silica gel column (250 mm × 4.5 mm I.D.) obtained from Shandon (Runcorn, UK).

RESULTS AND DISCUSSION

DNA extraction

The DNA extraction procedure was improved in order to shorten the precipitation and drying steps which lower the overall yield of isolated nucleic acids. The main DNA extraction steps from seeds of *Lactuca sativa* may by summarized as follows: (1) mechanical grinding in the cold (liquid nitrogen) for 15 min; (2) lysis for 4 h (room temperature) then overnight (4°C); (3) low-speed centrifugation (1000 *g*, 15 min); (4) salting out with ammonium acetate and 2-propanol; (5) precipitation step (1 h, −20°C) + centrifugation (1 h, 12 000 *g*); (6) solubilization of DNA + RNA + proteins (water, 30 min); (7) concentration of the aqueous solution to 1 ml with 2-butanol; (7) prepurification on a NAP-10 column; (8) RNase (1 h, 37°C) and proteinase K (1 h, 55°C) treatments; (9) size-exclusion chromatography of DNA by using either a NAP-10 column or a semi-preparative HPLC column packed with Fractogel HW 65 F with water as eluent.

After concentration of the chromatographic eluate to a convenient volume, the purified DNA can be enzymatically hydrolysed with an appropriate buffer and metallic cofactors if necessary.

The procedure may be applied to any DNA obtained from various biological materials when speed, suppression of toxic organic solvents and handling of several samples are required.

The final purification steps were performed using size-exclusion chromatography at low pres-

sure. Any ready-made size-exclusion chromatographic column could be used for this purpose provided that the nucleic fraction is eluted first before any undesirable lower-molecular-mass compounds [10]. This was the case with the two HPLC columns used in this work; the nucleic acids were present in the void volume and lower molecular mass products were eluted with a capacity factor $k' = 0.9$.

Once collected and concentrated to a convenient volume, the RNAs were eliminated with a concomitant improvement in the separation of two groups of products: DNA in the void volume and the ribonucleotides which are more retained ($k' = 0.9$). An identical behaviour was observed after the destruction of the residual proteins with proteinase K (Fig. 1).

In the last step, a relatively pure DNA was obtained as inferred from the 260 nm/280 nm absorbance ratio of 1.80 (35 μg for 250 mg of seeds of *Lactuca sativa*). This was also confirmed by the electrophoretic pattern on agarose gel (data not shown) [11].

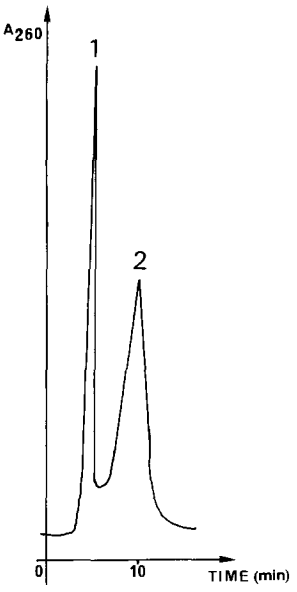

Fig. 1. Purification of *Lactuca sativa* DNA by size-exclusion chromatography. Column, laboratory packed semi-preparative stainless-steel column filled with Fractogel HW 65 F (30 cm × 0.75 cm I.D.); eluent, Milli-Q-purified; flow-rate, 1.0 ml/min. Peaks: 1 = DNA; 2 = hydrolysis products.

On reviewing the abundant literature on methods for the extraction of DNA from different biological sources, it appeared that each of the steps possessed its own importance. Grinding the biological material in liquid nitrogen is a convenient means of converting it into a finely divided powder, while the released nucleases were inhibited by the low temperature used [12].

Polyphenols are present in various amounts in plant tissues. They are important in seeds as natural protective agents against viral infections or physical stress, *e.g.*, low temperature or mechanical shock [13]. Polyphenols, in the presence of oxygen, undergo a polymerization reaction mediated by polyphenol oxidases, giving rise to complex polymeric products [14]. The net consequences of these spontaneous reactions resulted in low DNA yields and co-extraction of undesirable products that inhibit all operations generally associated with genomic studies such as enzymatic digestions by restriction enzymes [15]. These difficulties are generally circumvented by adding a reducing agent to the lysis buffer when the nucleic acids are released from the plant material [16]. However, the transition metals, even chelated by EDTA, trigger the Fenton reaction [17,18]:

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + OH^{\cdot} + OH^{-}$$

It was found very convenient to use the extraction medium without adding any other compounds, generally employed to eliminate the polyphenols such as polyvinylpyrrolidone [19]. Polyphenols and citrate buffers have opposite effects on the background formation of 8-hydroxy-2′-deoxyguanosine [20,21], while phenol-based DNA purification has been demonstrated to increase twenty-fold the level of this oxidative damage [22].

Plant polyphenols are known to have both beneficial [23,24] and hazardous effects [25–27] on biological macromolecules. Reddy *et al.* [28], using the ^{32}P-postlabelling method, demonstrated the formation of phenolic adducts to DNA. It can be noted that in the methodology proposed, the classical phenolic deproteinization was avoid-

ed in order to prevent the covalent attachment of phenolic adducts to DNA.

In order to facilitate the separation of nucleic acid material from other undesirable products, a long lysis period was preferred to the inhibition of the spontaneous polymerization of polyphenols. The complex mixture was in fact composed of two chemically different groups of products: the compounds of interest (nucleic acids) are hydrophilic polyanions whereas the polyphenols are hydrophobic, thus rendering easier the extraction of the former products by using water [29]. The water extraction step was accompanied by a noticeable increase in the dilution of the nucleic acids. This dilution may be considered as an advantage when the concentration of the aqueous solution is carried out with 2-butanol, making the elimination of insoluble polyphenols easier.

The concentration of the aqueous solution of nucleic acids made with 2-butanol was followed by a centrifugation step. This facilitates the clearing of the solution before size-exclusion chromatography of DNA. The plant polyphenols were extracted in a similar way with 2-butoxyethanol [30].

Ammonium acetate has often been used to salt out macromolecules from an aqueous solution with the addition of 2-propanol [31,32]. Performing this reaction at the beginning of our procedure is a convenient means to obtain both DNA and RNA in only one precipitate, from which DNA may be subsequently separated [33].

It is now well established that phenol may produce DNA damage by autoxidation processes. Taking this drawback into account, the deproteinization step was carried out by using proteinase K. The sequential treatment of the bulk of nucleic acids initiated with RNase followed by proteinase K shortens the procedure and avoids further handling. The deproteinization reaction carried out for at least 1 h at 55°C is a convenient means to destroy the constitutive proteins [34].

From a practical point of view, commercially available HPLC columns can be used to purify DNA. It should be mentioned that the Beckman SEC-2000 column may conveniently be replaced by laboratory-made stainless-steel columns packed with two kinds of size-exclusion chromatographic gels, either Fractogel HW 65 F (present data) or HW 55 F [10]. Under these conditions, the nucleic acids are eluted in the void volume.

The latter system is very cheap and the high-performance stainless-steel columns may be packed by gravity even in the absence of any disposable filling system.

HPLC of 2′-deoxyribonucleoside 3′-monophosphates

In order to shorten and simplify the ^{32}P-postlabelling of the 2′-deoxyribonucleoside 3′-monophosphates arising from enzymatic digestion of DNA, we searched for simple chromatographic systems allowing a straightforward use of the chromatographic eluate in the labelling reaction. As reviewed by Singhal *et al.* [35], deoxyribonucleosides and other nucleic acid components may be efficiently separated by HPLC techniques. This involved in most instances the use of non-volatile phosphate-buffered solutions often associated with organic modifiers (acetonitrile and/or methanol). However, these solvents cannot be used for the ^{32}P-postlabelling of 2-deoxyribonucleoside 3′-monophosphates, as the phosphate anion is an inhibitor of the T4 polynucleotide kinase [36].

Isocratic versus gradient solvent systems. The use of reversed-phase HPLC constitutes the simplest analytical approach. This is particularly true when volatile solvents are used for structural studies, thus avoiding tedious desalting operations.

Relatively good correlations were established between the structure of modified nucleosides and the capacity factors (k') when chromatographic separations were carried out on reversed-phase silica gel columns [37]. The elution is controlled by the apparent charge of the molecule [38]. This was the reason why the negative charge of the ionized phosphate group was suppressed by adding inorganic counter ions for the separation of nucleotides in the ion-suppression mode [39]. Gloor and Johnson [40] suggested the pref-

erential use of ion-pair chromatography with large organic counter ions when the samples to be analysed contain both ionic and non-ionic components.

In order to isolate 2′-dexoyribonucleoside 3′-phosphates as substrates for postlabelling, the ion-suppression mode was preferred, provided that the counter ion used in the chromatographic solvent does not inhibit the polynucleotide kinase. Gradient systems involving organic modifiers such as methanol and acetonitrile were chosen for their good separation properties. However, their use required a drying step before the polynucleotide kinase labelling reaction [4,5].

From these considerations, low ionic strength and volatility constitute the main characteristics of a suitable HPLC solvent for direct labelling of a 2′-deoxyribonucleoside 3′-monophosphate. The octadecylsilylsilica gel column combined with a volatile solvent in the isocratic mode appears to be a convenient system for the separation of 2′-deoxyribonucleoside 3′-monophosphates and subsequent direct ^{32}P-postlabelling of oxidative DNA base lesions [41].

Counter-ion effect. Ammonium formate appears to be one of the best candidates for this purpose. Its use is straightforward as the neutral pH of the solution (pH 6.5), ensured ionization of all nucleotides when considering their pK_a values [42] (Table I).

A change in the nature of the inorganic counter ion (*i.e.*, calcium instead of ammonium) does not modify the order of elution of the 2′-deoxyribonucleoside 3′-monophosphates: dCMP > dGMP > dTMP > dAMP, which was similar to that described for the deoxyribonucleoside 5′-phosphates [43].

When calcium formate is used to separate the 2′-deoxyribonucleosides, its influence is comparable to that of triethylammonium acetate in terms of the measured capacity factors (Table I). Calcium is a divalent cation used by Lin *et al.* [44]. Its interesting chelating properties allow the formation of complexes with the hydroxyl groups of carbohydrates [45,46] and also with phosphate groups in the hydroxyapatite chromatography of DNA [47]. This cation is also involved in the formation of a ternary complex between thymidine 3′,5′-biphosphate and the micrococcal nuclease [48].

The well known interaction of triethylamine with the free silanol groups is evidenced in Table I and Fig. 2a but calcium exerted the same effect [49]. As surface derivatization is never total, one

TABLE I

CAPACITY FACTORS (k') OF 2′-DEOXYRIBONUCLEOSIDE 3′-MONOPHOSPHATES AND THEIR CORRESPONDING 2′-DEOXYRIBONUCLEOSIDES

Compound[a]	Ammonium (formate) (0.05 *M*)	Calcium (formate) (0.05 *M*)	Triethylammonium (acetate) (0.05 *M*)	Sodium (succinate) (0.002 *M*)
3′-dAMP	4.70	7.50	21.60	3.30
3′-dCMP	0.60	0.70	1.55	0.25
3′-dGMP	1.40	2.10	7.30	0.80
3′-dTMP	2.30	3.40	9.40	1.50
2′-dAdo	45.80	45.10	21.90	21.80
2′-dCyd	4.00	3.60	0.30	4.30
2′-dGuo	16.15	15.00	8.10	15.70
2′-dThd	19.30	18.10	9.25	18.10

[a] 3′-dAMP = deoxyadenosine 3′-monophosphate; 3′-dCMP = deoxycytidine 3′-monophosphate; 3′-dGMP = deoxyguanosine 3′-monophosphate; 3′-dTMP = thymidine 3′-monophosphate; 2′-dAdo = 2′-deoxyadenosine; 2′-dCyd = 2′-deoxycytidine; 2′-dGuo = 2′-deoxyguanosine; 2′-dThd = thymidine.

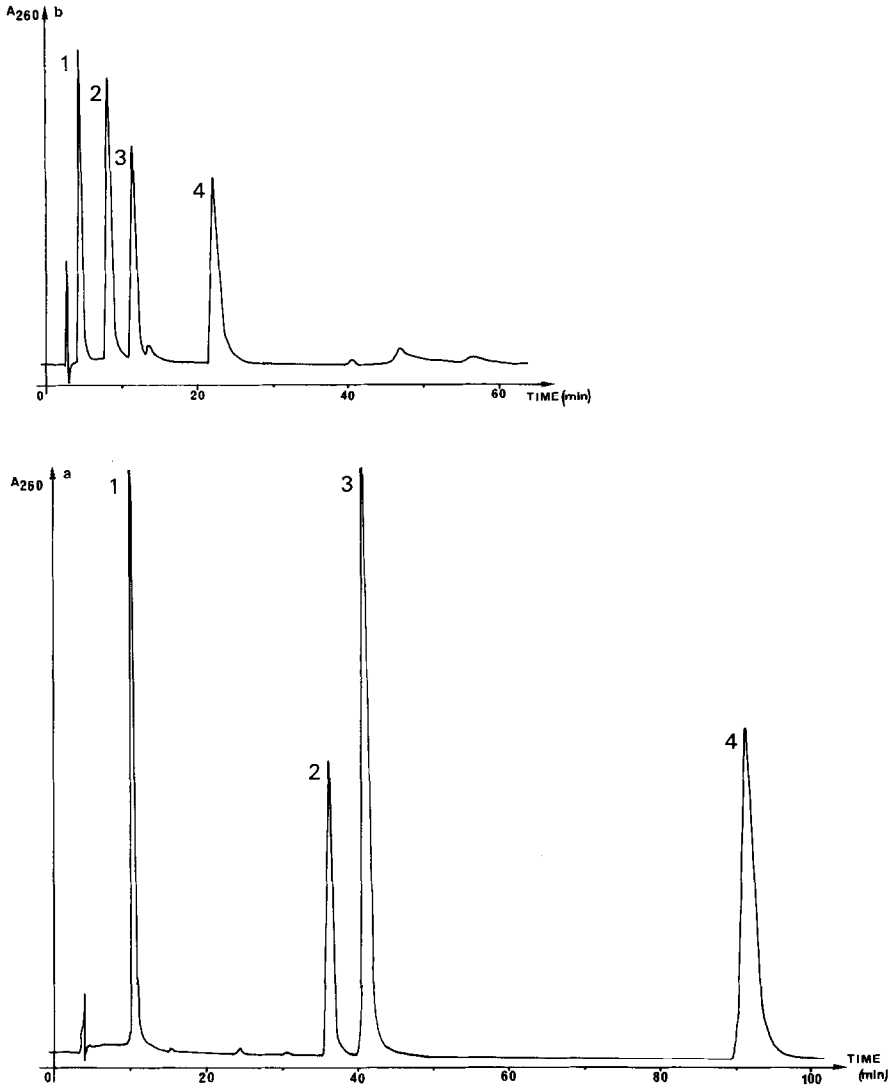

Fig. 2. HPLC separation of 2′-deoxyribonucleoside 3′-phosphates after enzymatic hydrolysis of a DNA extracted from *Lactuca sativa*. Column, Hypersil C_{18}, particle size 5 μm (25 cm × 0.46 cm I.D.); eluents, (a) 0.05 *M* triethylammonium acetate (pH 6.75) and (b) 0.05 *M* calcium formate (pH 6.0); flow-rate, 1.0 ml/min; detection, 260 nm. Peaks: 1 = 3′-dCMP; 2 = 3′-dGMP; 3 = 3′-dTMP; 4 = 3′-dAMP.

can consider that calcium interacts both with one free silanol group and with one charge of the 3′-phosphate group [50].

The succinate buffer used for the enzymatic digestion of DNA buffer and recently for the separation of dideoxyribonucleosides [51] was as efficient as ammonium formate in the resolution of the mixture of 2′-deoxyribonucleoside 3′-phosphates (Table I). Once separated with sodium succinate, the 2′-deoxyribonucleoside 3′-monophosphates can be directly phosphorylated in the chromatographic eluate, thus permitting any modification of the calcium chloride concentration to optimize the subsequent ^{32}P-labelling reaction [52].

CONCLUSION

When applying the ^{32}P-postlabelling method for the measurement of DNA oxidative base lesions arising from different biological sources, it is important to rationalize and standardize the DNA extraction. A simple extraction method for DNA was presented here that includes the following main steps: destruction of the biological structures in the cold, lysis in a standardized buffer without reducing agents and phenol and subsequent size-exclusion chromatography.

Size-exclusion chromatography with commercially available HPLC columns (Beckman SEC-2000) or, more conveniently, with laboratory-made columns (Merck Fractogel HW 65 F) represents an inexpensive way to apply semi-preparative high-performance size-exclusion chromatography, which permits the purification of DNA and also UV monitoring of the completeness of the hydrolysis of DNA into 2′-deoxyribonucleoside 3′-monophosphates, which is recognized as one of the main critical steps in ^{32}P-postlabelling [53].

The use of volatile solvents (ammonium formate) in the isocratic mode without any organic modifier represents a good approach for automation, avoiding the risk of baseline drift which occurs with gradient systems. In addition, the system could be suitable for eventual coupling with a mass spectrometer [54].

Calcium can be considered as a new counter ion for the chromatographic separation of the 2′-deoxyribonucleoside 3′-monophosphates.

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