Journal of Chromatography, 593 (1992) 133–138
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2458

Reversed-phase high-performance liquid chromatography–thermospray mass spectrometry of radiation-induced decomposition products of thymine and thymidine

M. Berger and J. Cadet*

Laboratoire "Lésions des Acides Nucléiques", DRFMC/SESM, Centre d'Études Nucléaires, 85X, F-38041 Grenoble (France)

R. Berube

Laboratoire de Spectrométrie de Masse, Faculté de Médecine, Université de Sherbrooke, Québec J1H 5N4 (Canada)

R. Langlois and J. E. van Lier

Groupe CRM en Sciences des Radiations, Faculté de Médecine, Université de Sherbrooke, Québec J1H 5N4 (Canada)

ABSTRACT

High-performance liquid chromatography–thermospray mass spectrometry was applied to the analysis of various radiation-induced decomposition products of thymidine including N-(2-deoxy-β-D-*erythro*-pentofuranosyl)formamide and the various diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine, 5-hydroxy-5,6-dihydrothymidine and 5,6-dihydrothymidine. This method combines high sensitivity and product resolution, rendering it particularly useful for monitoring the formation of radiation-induced base damage within DNA.

INTRODUCTION

Measurement of individual base damage within DNA on exposure to radical agents such as ionizing radiation and hydroxyl radicals still remains a challenging analytical problem. Various methods involving either high-performance liquid chromatography (HPLC) or gas chromatography (GC) have been developed for the separation of the complex mixture of modified DNA components (for recent reviews, see refs. 1–3). Sensitive detection of DNA lesions can be achieved by using various methods, including amperometry [4,5] as well as colorimetric [6], fluorescent [7] and radioactive [8] post-labellings. Mass spectrometry (MS) is mostly used in combination with GC analysis [9,10]. On the other hand, this accurate method of detection has not often been associated with HPLC, only two examples of the application of HPLC–MS to measuring radiation-induced DNA base damage being available in the literature [11,12].

The main objective of this study was to explore the possibility of using the on-line HPLC–MS technique for monitoring the formation of radiation-mediated decomposition products of the base moieties of nucleic acids. We report here the HPLC–thermospray (TSP) MS analysis of the main radiation-induced decomposition products of thymine and its 2'-deoxyribonucleoside obtained in both aerated and oxygen-free aqueous solutions. The choice of both base and nucleoside decomposition products of the same DNA component (thymine) was dictated by the fact that these compounds may be obtained either by mild acidic hydrolysis [13] or by enzymic digestion of modified DNA [1]. This allows a comparative study of the thermospray mass

0021-9673/92/$05.00

spectrometric features of the two classes of compounds with emphasis on the sensitivity of detection.

EXPERIMENTAL

Chemicals

Thymine and 5,6-dihydrothymine were obtained from Sigma (St. Louis, MO, USA) Thymidine was purchased from Genofit (Geneva, Switzerland) and was used without further purification.

5-Hydroxy-5,6-dihydrothymine was synthesized according to Nofre *et al.* [14]. The four *cis* and *trans* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine were prepared by mild alkaline hydrolysis of *trans*-(5*R*,6*R*)- and -(5*S*,6*S*)-5-bromo-6-hydroxy-5,6-dihydrothymidine [15]. The 5*R* and 5*S* diastereomers of 5,6-dihydrothymidine and 5-hydroxy-5,6-dihydrothymidine were obtained by gamma radiolysis of thymidine in oxygen-free aqueous solutions containing cysteine [16]. N-(2-Deoxy-β-D-*erythro*-pentofuranosyl)formamide was prepared by menadione photooxidation of thymidine and purified by HPLC [17].

High-performance liquid chromatography

The HPLC separations of the various radiation-induced decomposition products of thymine and thymidine were performed using an HP 1090 system (Hewlett-Packard). Octadecylsilyl silica gel ODS 1 (100 mm × 4.6 mm I.D.) and/or ODS-2 (50 × 4.6 mm I.D.) reversed-phase analytical columns were packed with 3- and 5-μm particles, respectively (Whatman, Hillsboro, OR, USA). Samples were introduced using a Rheodyne (Berkeley, CA, USA) Model 7125 injection valve equipped with a 100-μl loop. The isocratic mobile phase was 0.1 *M* ammonium acetate (pH 6) at a flow-rate of 0.8 ml/min.

Thermospray mass spectrometry

The HPLC–TSP-MS system consisted of an HP 5988A quadrupole mass analyser (Hewlett-Packard) and a Vestec (Houston, TX, USA) thermospray interface equipped with a CC 100 Cryocool immersion cooler. Acquisition and treatment of the spectrometric data were achieved with an HP 9000 Model 216 computer. The conditions used for the TSP-MS analysis were as follows: filament, "mode on"; stem temperature, 140°C with a tip temperature of 220°C; electron multiplier voltage, 2500; temperature of the source, 300°C; and calibration of the spectra was achieved by using polypropylene glycol.

RESULTS AND DISCUSSION

The thymidine decomposition products which were analysed include the 5*R* and 5*S* diastereomers of 5,6-dihydrothymidine and 5-hydroxy-5,6-dihydrothymidine, the four *cis* and *trans* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine and N-(2-deoxy-β-D-*erythro*-pentofuranosyl)formamide. In addition, 5,6-dihydrothymine, 5-hydroxy-5,6-dihydrothymine and *cis*-5,6-dihydroxy-5,6-dihydrothymine, the corresponding racemic 5,6-saturated thymine derivatives and thymidine were also included. It is worth noting that N-(2-deoxy-β-D-*erythro*-pentofuranosyl)formamide and the four diastereoisomeric thymidine glycols are two of the three main classes of decomposition products of thymidine arising from both hydroxyl radical reactions [18–20] and electron transfer processes [21,22]. The latter oxidation reaction, which may be initiated by both high-intensity laser pulses and photosensitization [22], involves the transient formation of a pyrimidine radical cation [21,22]. It is also interesting that the 5*R* and 5*S* diastereomers of 5,6-dihydrothymidine and 5-hydroxy-5,6-dihydrothymidine were found to be the main radiation-induced decomposition products of thymidine in deaerated aqueous solutions when cysteine, a known radioprotector agent, was present [14].

HPLC–TSP-MS of thymidine

HPLC–TSP-MS was performed in either the positive or negative-ion mode (Figs. 1 and 2). It is interesting to note an almost complete lack of fragmentation in the negative-ion spectrum with a predominant quasi-molecular ion at *m/z* 301 $(M + CH_3COO)^-$ and a relatively minor fragment at *m/z* 125 $(\text{thymine} - H)^-$. In the positive-ion mode the fragment corresponding to the cleavage of the N-glycosidic bond at *m/z* 127 $(\text{thymine} + H)^+$ is the base peak. However, a significant quasi-molecular ion at *m/z* 243 $(M + H)^+$ is also observed.

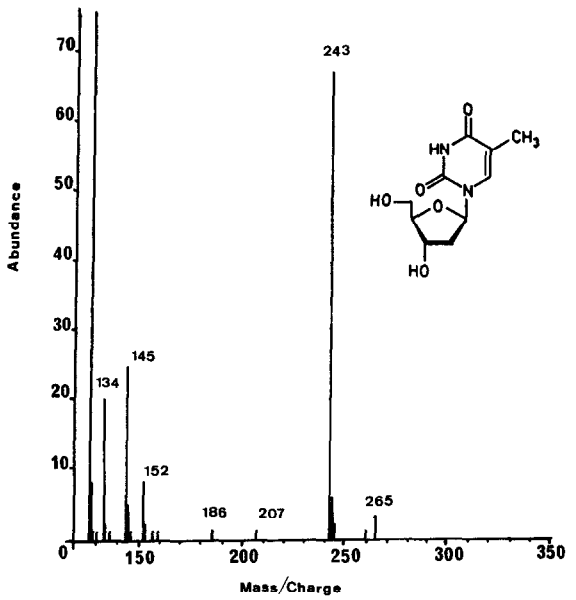

Fig. 1. Scale corrected thermospray mass spectrum in the positive-ion mode of thymidine. HPLC and TSP conditions as described under Experimental.

Comparative HPLC–TSP-MS analysis of the 5R and 5S diastereomers of 5-hydroxy-5,6-dihydrothymidine and 5,6-dihydrothymidine

The positive-ion thermospray mass spectra of the 5*R* and 5*S* diastereomers of 5-hydroxy-5,6-dihydrothymidine are presented in Figs. 3 and 4. The two spectra are almost identical, indicating that the stereochemistry at C-5 does not exert any significant influence on the fragmentation pattern. Both molecules show little fragmentation with characteristic quasi-molecular ions at *m/z* 261 $(M + H)^+$ (base peak) and *m/z* 278 $(M + NH_4)^+$.

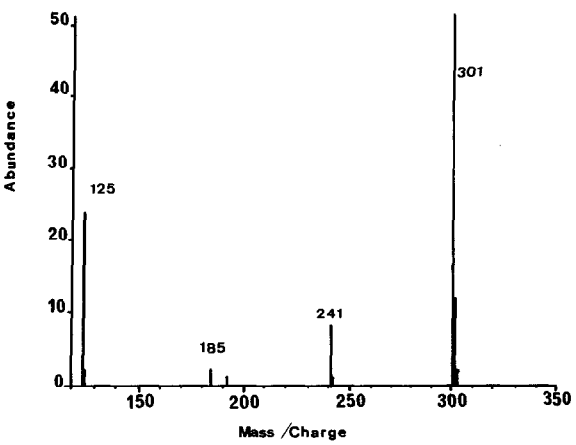

Fig. 2. Scale corrected thermospray mass spectrum in the negative-ion mode of thymidine.

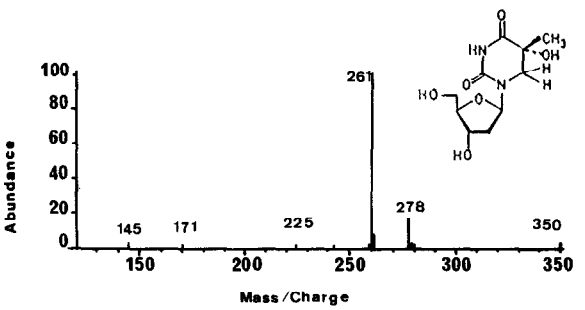

Fig. 3. Scale thermospray mass spectrum (positive-ion mode) obtained from (5*R*)-5-hydroxy-5,6-dihydrothymidine.

It is of interest that the positive- and negative-ion spectra of the 5*R* and 5*S* diastereomers of 5,6-dihydrothymidine exibit a predominant quasi-molecular ion at *m/z* 245 $(M + H)^+$ and *m/z* 303 $(M + CH_3COO)^-$, respectively (data not shown). No fragmentation of the pyrimidine ring is observed. In addition, we note a complete lack of any fragment corresponding to the release of the free base subsequent to the cleavage of the N–glycosidic bond. The only additional significant peaks which were detected are the ions at *m/z* 262 $(M + NH_4)^+$ and *m/z* 243 $(M - H)^-$, which exhibit relative intensities of 17–25% and 5–8% in the positive- and negative-ion spectra, respectively. Again, the mass spectra of the 5*R* and 5*S* diastereomers are identical.

The thermospray mass spectra of the racemic 5,6-dihydrothymine and 5-hydroxy-5,6-dihydrothymine exhibit two major peaks, which correspond to the quasi-molecular ions $(M + H)^+$ and $(M +$

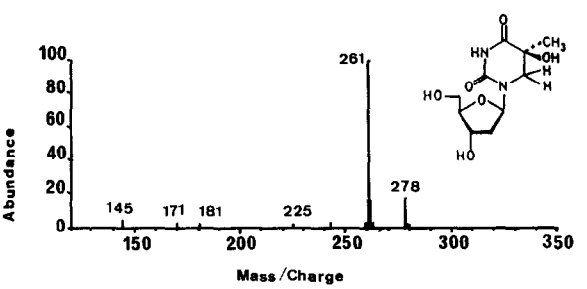

Fig. 4. Scale thermospray mass spectrum (positive-ion mode) obtained from (5*S*)-5-hydroxy-5,6-dihydrothymidine.

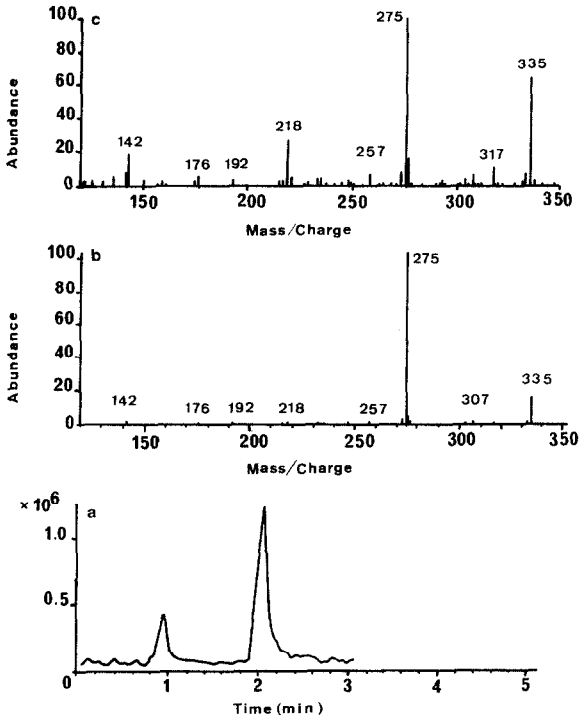

Fig. 5. (a) Total ion chromatogram (TIC) profile of (−)-*trans*-(5*S*,6*S*)- and (+)-*cis*-(5*S*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine. (b) Scale corrected thermospray mass spectrum (negative-ion mode) of (+)-*cis*-(5*R*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine. (c) Scale corrected thermospray mass spectrum (negative-ion mode; of (−)-*trans*-(5*S*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine.

$NH_4)^+$ in the positive-ion mode and $(M - H)^-$ and $(M + CH_3COO)^-$ in the negative-ion mode.

HPLC–TSP-MS analysis of the cis and trans diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine

Fig. 5a shows the total ion chromatogram obtained from the HPLC–TSP-MS analysis of a mixture of the (−)-*trans*-(5*S*,6*S*)-and (+)-*cis*-(5*S*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine in the negative-ion mode. The two modified nucleosides are well separated ($\alpha = 2.9$), the *trans* diastereomer being eluted faster than the corresponding C-6 epimer [6]. The fragmentation pattern of each of the two diastereomers is similar. We note in particular the presence of the two characteristic pseudo-molecular ions at *m/z* 275 $(M - H)^-$ and *m/z* 335 $(M + CH_3COO)^-$ (Fig. 5b and c). The main difference between the two spectra concerns the intensity of the fragment *m/z* 218, which corresponds to the cleavage of the N–glycosidic bond (base − H + $CH_3COO)^-$. Under these conditions, the *trans*-(6*S*)-diol appears to be more thermally unstable than the *cis*-(6*R*)-diol. Similarly, the dehydration process (*m/z* 257) which is likely to involve the loss of the hydroxyl group at C-6 [23] is higher for the *trans*-diol. Intense pseudo-molecular ions at *m/z* 277 $(M + H)^+$ and *m/z* 294 $(M + NH_4)^+$ were observed when the two thymidine glycols were analysed in the positive-ion mode (data not shown).

HPLC–TSP-MS analysis of N-(2-deoxy-2-β-D-erythro-pentofuranosyl)formamide

The positive-ion mass spectrum of N(2-deoxy-β-D-*erythro*-pentofuranosyl)formamide is shown in Fig. 6. Very little fragmentation occurs, the relative intensity of the ion at *m/z* 134 (1-amino-2-D-*erythro*-pentose) being only 10%. The two main frag-

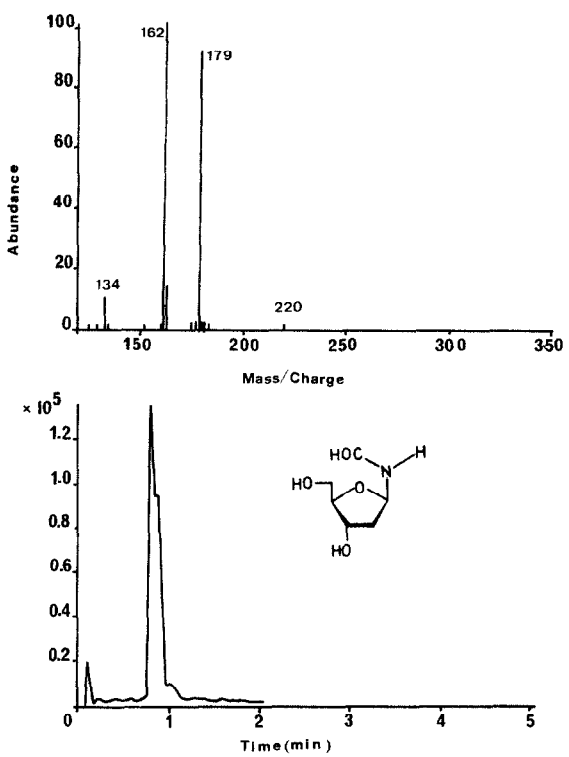

Fig. 6. Scaled thermospray mass spectrum (positive-ion mode) and total ion chromatogram (TIC) profile of N-(2-deoxy-β-D-*erythro*-pentofuranosyl)formamide.

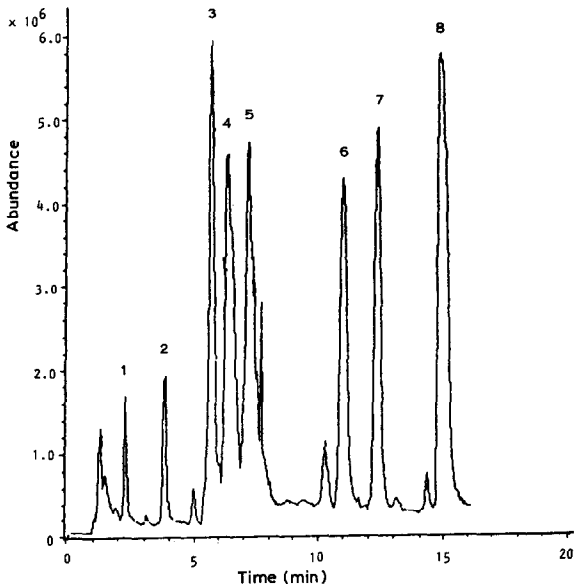

Fig. 7. Total ion chromatogram (TIC) profile of a mixture of nucleobases and nucleosides: (1) 5-hydroxy-5,6-dihydrothymine, (2) (5*R*)-5-hydroxy-5,6-dihydrothymidine, (3) (5*S*)-5-hydroxy-5,6-dihydrothymidine, (4) 5,6-dihydrothymine, (5) thymine, (6) (5*R*)-5,6-dihydrothymidine, (7) (5*S*)-5,6-dihydrothymidine and (8) thymidine.

ments correspond to the characteristic quasi-molecular ions at *m/z* 162 $(M + H)^+$ and *m/z* 179 $(M + NH_4)^+$. It is also interesting that the base peak in the negative-ion spectrum is the quasi-molecular ion at *m/z* 220 $(M + CH_3COO)^-$ (data not shown).

Analysis of a complex mixture of radiation-induced decomposition products of thymine and thymidine by thermospray mass spectrometry in line with reversed-phase HPLC

Fig. 7 shows the total ion chromatogram obtained by reversed-phase HPLC–TSP-MS analysis of a complex mixture of eight radiation-induced decomposition products of thymine and thymidine. A complete separation of the modified bases and nucleosides was achieved on the ODS-3 column [24] in less than 16 min by using 0.1 *M* ammonium acetate as the mobile phase. The following compounds were separated in decreasing order of elution: 5-hydroxy-5,6-dihydrothymine (1) > (5*R*)-5-hydroxy-5,6-dihydrothymidine (2) > (5*S*)-5-hydroxy-5,6-dihydrothymidine (3) > 5,6-dihydrothymine (4) > thymine (5) > (5*R*)-5,6-dihydrothymidine (6) > (5*S*)-5,6-dihydrothymidine (7) > thymidine (8).

TABLE I

DETECTION LIMITS OF BASES AND NUCLEOSIDES IN THE POSITIVE- AND NEGATIVE-ION MODES

Compound	Amount (ng)
Thymidine	0.1–0.3
5,6-Dihydroxy-5,6-dihydrothymidines	5
5-Hydroxy-5,6-dihydrothymidines	0.1
5,6-Dihydrothymidine	0.2
Thymine	0.1

Quantitative aspects and sensitivity: selective ion monitoring measurements

The sensitivity of detection of the modified bases and nucleosides analysed by the HPLC–TSP-MS method is significantly increased by using the selective ion monitoring (SIM) technique under optimized conditions of measurement (source temperature varying from 275 to 300°C according to the compounds analysed, mode "on"). The detection limits of several compounds were determined by measuring the predominant pseudo-molecular ion by using the SIM technique and are listed in Table I. The detector response was linear with the amount of the compounds being analysed, as illustrated for the 5*R* diastereomer of 5-hydroxy-5,6-dihydrothy-

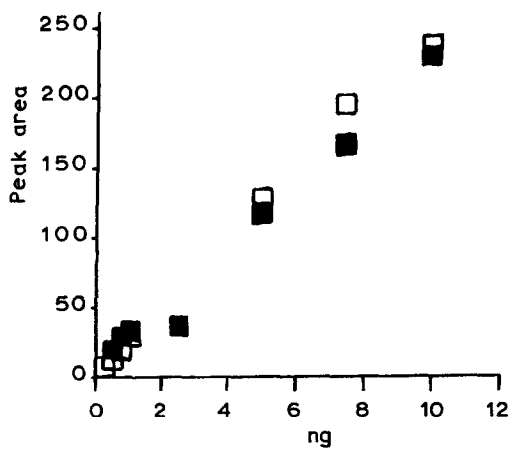

Fig. 8. Relationship between peak area on the SIM chromatogram and amount of (5*R*)-5-hydroxy-5,6-dihydrothymidine in the (□) positive- and (■) negative-ion modes.

midine (Fig. 8). Similar behaviour was observed for the other radiation-induced decomposition products of thymine and thymidine.

CONCLUSIONS

Reversed-phase HPLC–TSP-MS is a powerful technique, combining efficient product separation with detection sensitivity, for the accurate detection of radiation-induced decomposition products of thymine and thymidine. The method is an interesting alternative (and probably a complementary tool) to the GC–MS method [7], which requires a derivatization step prior to the analysis. It is likely that a similar range of sensitivity is provided by both techniques, depending, however, on the nature of the compounds to be analysed. In this respect, it is worth noting that the sensitivity of detection of the thymine glycol is relatively poor, irrespective of the HPLC–MS or GC–MS approach used. On the other hand, HPLC–TSP-MS has the potential to detect one 5,6-dihydrothymine or 5-hydroxy-5,6-dihydrothymine residue per 10^5 nucleobases within 20–30 μg of DNA, which is similar to the sensitivity offered by the HPLC–amperometric assay [4,5]. It should be mentioned that accurate determination of modified nucleobases and nucleosides would require, for both methods, the use of internal isotopically enriched standards.

Interesting and useful applications of the HPLC–TSP-MS assay involve DNA repair studies (determination of substrate specificity for repair enzymes) and the search for modified bases within cellular DNA. This complements the array of existing methods, including the specific HPLC–electrochemical assay [4,5] and the HPLC–^{32}P post-labelling method [8].

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Centre National d'Études Spatiales (90/312), the France–Québec Exchange Programme and the Medical Research Council of Canada.

REFERENCES

1 P. Vigny and J. Cadet, in A. Favre, R. M. Tyrrell and J. Cadet (Editors) *From Photophysics to Photobiology*, Elsevier, Amsterdam, 1987, p. 123.
2 J. Cadet and P. Vigny, in H. Morrison (Editor), *Bioorganic Photochemistry*, Vol. 1, Wiley, New York, 1990, p. 1.
3 M. Dizdaroglu, *Free Rad. Biol. Med.*, 10 (1991) 222.
4 R. A. Floyd, J. J. Watson, P. K. Wong, D. H. Atmiller and R. C. Rickard, *Free Rad. Res. Commun.*, 1 (1986) 163.
5 M. Berger, C. Anselmino, J.-F. Mouret and J. Cadet, *J. Liq. Chromatogr.*, 13 (1990) 199.
6 J. R. Wagner, M. Berger, J. Cadet and J. E. van Lier, *J. Chromatogr.*, 504 (1990) 191.
7 M. Sharma, H. C. Box and D. J. Kelman, *Chem.-Biol. Interact.*, 74 (1990) 107.
8 J.-F. Mouret, F. Odin, M. Polverelli and J. Cadet, *Chem. Res. Toxicol.*, 3 (1990) 102.
9 A. F. Fuciarelli, B. J. Wegher, E. Gajewski, M. Dizdaroglu and W. F. Blakely, *Radiat. Res.*, 119 (1989) 219.
10 W. G. Stillwell, H.-X. Xu, J. A. Adkins, J. S. Wishnol and S. R. Tannenbaum, *Chem. Res. Toxicol.*, 2 (1989) 94.
11 A. J. Alexander, P. Kebarle, A. F. Fuciarelli and J. A. Raleigh, *Anal. Chem.*, 59 (1987) 2484.
12 J. Cadet, M. Berger, C. Decarroz, A. Shaw, J. R. Wagner, E. Keskinova and D. Angelov, in A. Y. Spasov (Editor), *Lasers and Applications*, World Scientific, Singapore, 1987, p. 508.
13 M. Polverelli, M. Berger, J.-F. Mouret, F. Odin and J. Cadet, *Nucleosides Nucleotides*, 9 (1990) 451.
14 C. Nofre, A. Cier, R. Chapurlat and J.-P. Mareschi, *Bull. Soc. Chim. Fr.*, (1965) 332.
15 J. Cadet, J. Ulrich and R. Téoule, *Tetrahedron*, 31 (1975) 2657.
16 J. Cadet, M. Berger, P. Demonchaux and J. Lhomme, *Radiat. Phys. Chem.*, 32 (1988) 197.
17 R. J. Wagner, J. E. van Lier, C. Decarroz, M. Berger and J. Cadet, *Methods Enzymol*, 186 (1990) 502.
18 R. Téoule, *Int. J. Radiat. Biol.*, 51 (1987) 573.
19 C. von Sonntag, *The Chemical Basis of Radiation Biology*, Taylor and Francis, London, 1987.
20 G. W. Teebor, R. J. Boorstein and J. Cadet, *Int. J. Radiat. Biol.*, 54 (1988) 131.
21 D. J. Deeble, M. N. Schuchmann, S. Steenken and C. von Sonntag, *J. Phys. Chem.*, 94 (1990) 8186.
22 J. Cadet, M. Berger, C. Decarroz, J.-F. Mouret, J. E. van Lier and R. J. Wagner, *J. Chim. Phys.*, 88 (1991) 1021.
23 J. R. Wagner, J. E. van Lier, C. Decarroz and J. Cadet, *Bioelectron. Bionerg.*, 18 (1987) 155.
24 J. Cadet, M. Berger and L. Voituriez, *J. Chromatogr.*, 238 (1982) 488.