ELSEVIER

Journal of Chromatography B, 707 (1998) 51–57

JOURNAL OF CHROMATOGRAPHY B

Determination of malondialdehyde in rat brain by capillary zone electrophoresis

Joanna Olsson[a], Bo Karlberg[a], Krister Kristensson[b], Fredrik Åberg[b,*]

[a]*Department of Analytical Chemistry, Stockholm University, 106 91 Stockholm, Sweden*
[b]*Department of Neuroscience, Karolinska Institute, Doktorsringen 17A, 171 77 Stockholm, Sweden*

Received 2 September 1997; received in revised form 18 November 1997; accepted 28 November 1997

Abstract

A method for determination of malondialdehyde with capillary electrophoresis using UV detection at 267 nm has been developed. The buffer system consisted of 10 m*M* borax and 0.5 m*M* CTAB at pH 9.3. Malondialdehyde migrated as the first peak in the electropherogram at 2.6 min. Limit of detection was 1.2 μ*M* corresponding to 7.8 pg. Malondialdehyde was determined before and after stimulating lipid peroxidation with the addition of ferrous ammonium sulphate to homogenates of rat brain tissue. Proteins were precipitated by boiling and removed from the brain homogenates with centrifugation. No further pretreatment was made before injecting the homogenates on the CE system. Non-precipitated homogenates could also be analyzed, but this required washing of the capillary with 0.1 *M* NaOH before introduction of the next sample. © 1998 Elsevier Science B.V.

Keywords: Brain; Malondialdehyde

1. Introduction

A number of neurodegenerative diseases have been suggested to be associated with oxidative stress [1]. The brain is especially vulnerable to oxidative stress due to its high oxygen consumption, generating prooxidants (reactive oxygen species, ROS), and the abundance of easily oxidizable substrates, for example unsaturated lipids. ROS activity on lipids results in drastic changes in the structure of the lipid, producing lipid hydroperoxides. When lipid hydroperoxides break down in biological systems aldehydes are formed [2]. Malondialdehyde (MDA) is one aldehyde traditionally used as a marker for the lipid peroxidation process [3,4]. It exists as a conjugated structure above pH 4.46 and has an absorption maximum at 267 nm [5]. A large body of the literature deals with the measurement of MDA by various techniques. A common analytical method involves mixing the sample with thiobarbituric acid (TBA) under acidic conditions to form an adduct with MDA, the absorbance of which is measured in the visible spectrum [6]. This method, commonly referred as the TBARS (thio barbitur acid reactive substances) is simple but lacks specificity. Various substances, besides MDA, also react with TBA [7]. Enhanced selectivity can be achieved by using high-performance liquid chromatography (HPLC), where the extracted TBA–MDA adduct is separated and detected by absorbance or fluorescence [8–13]. The fluorescent characteristics of the TBA–MDA adduct has lead to the development of methods with very

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00600-2

high sensitivity, resulting in a further decrease in the limit of detection (LOD) as compared with the absorbance detection [11]. These systems often use reversed-phase columns, but since the TBA–MDA complex elutes early this results in poor resolution. In addition, other substances may coelute with the TBA–MDA peak. These methods also require extensive washing procedures, frequent replacements of the guard column, and repacking or replacement of the column after some 300 determinations [12]. Alternative HPLC techniques to detect MDA have been described [5,15–17]. Usually these systems also require column regeneration since it looses efficiency and resolution with time, especially when crude biological samples are introduced. All reported and used HPLC systems are sensitive, with pmol amounts in submicromolar concentrations being measured when absorbance detection is employed. In view of the great interest in the investigation of the role of oxidative stress in neurodegeneration, it is important to develop new specific and sensitive methods to measure lipid peroxidation.

This paper describes a capillary zone electrophoresis (CZE) method utilising UV detection for determination of MDA in brain homogenates. The method is simple and enables the measurement of lipid peroxidation directly in small amounts of sample without additional substances, such as TBA, for complex binding of MDA. To evaluate the method we analyzed brain homogenates where lipid peroxidation had been stimulated by iron. This source of peroxidation is of particular interest in studies of the nervous tissue, since certain neurodegenerative diseases are associated with an imbalance in the iron homeostasis in the brain [18–26].

2. Experimental

2.1. Chemicals

Ferrous ammonium sulphate, sodium hydroxide, Borax and cetyl trimethyl ammonium bromide (CTAB) were purchased from Merck (Darmstadt, Germany). Butylated hydroxy toluene (BHT), 1,1,3,3-tetramethoxypropane was obtained from Sigma (St. Louis, MO, USA). All chemicals and solvents used were of analytical grade. Water was purified with an Elgastat UHQ II (ELGA, UK). The electrophoresis buffer consisted of CTAB corresponding to 0.5 m*M* added to a 10 m*M* Borax solution (pH 9.3). Before use it was degassed and filtered through a 0.25-μm filter.

Malondialdehyde standard was prepared as described by Esterbauer and Cheeseman [27]. Briefly, a 10 m*M* stock solution was prepared by mixing 1,1,3,3-tetramethoxypropane in 1% sulphuric acid at room temperature for 2 h. This solution was then further diluted with distilled water to prepare various working standard solutions. The absorbance spectrum of the standard solution was confirmed by using a Pharmacia UV-Vis spectrophotometer, Ultraspec Plus (Uppsala, Sweden) operated in the wavelength scanning mode.

2.2. Animals

Male Sprague–Dawley rats (B&K, Sollentuna, Sweden), 180–200 g in mass, were used throughout the study. The rats were kept at the animal facility with a 12 h light and dark cycle and fed ad libitum on ordinary chow.

2.3. Sample preparation

Rats were killed by CO_2 and decapitated. The collected heads were stored at −70°C until further use. The brains were quickly excised and put on ice. Tissue homogenates (20%) were then prepared in distilled water using an ultraturrax at 800 rpm. The homogenisation procedure was performed on ice.

Lipid peroxidation was stimulated by the addition of ferrous ammonium sulphate to a final concentration of 100 μ*M*. After 1 h incubation under continuous shaking at room temperature, the reaction was terminated with the addition of 0.005% BHT. The homogenates were precipitated by putting them in capped tubes in a boiling water bath for 5 min. After boiling, the homogenates were chilled on ice and centrifuged at 15 000 *g* for 10 min. This boiling and subsequent chilling procedure was repeated on the recovered supernatants, which were finally frozen and stored at −20°C. BHT (0.005%) was added to the control brain tissue directly after dissection of the brains and homogenisation was performed as described above, but without the addition of ferrous

ammonium sulphate. This control homogenate was also used when the linearity of the method was examined. Standard malondialdehyde was added to different samples in different concentrations ranging from 5 to 100 μ*M*. A homogenate without the precipitating procedure described above was also prepared. The crude 20% homogenate were centrifuged in order to remove particular matter. Prior to analysis, the supernatants were thawed and centrifuged.

2.4. Capillary zone electrophoresis system

Untreated fused-silica capillaries, 70 cm (55 cm to the detector)×75 μm I.D.×375 μm O.D., were obtained from Polymicro Technologies (Phoenix, AZ, USA). Before use, the capillaries were washed with 0.1 *M* NaOH for 20 min, with water for 10 min and, finally, with buffer for 20 min. After every 10th run the capillary was washed with 0.1 *M* NaOH for 3 min, with water for 2 min and, finally, with buffer for 3 min. The input of the high-voltage power supply, ±0–30 kV, (Brandenburg, Thornton Heath, UK), together with the injection end of the capillary, were placed in a Plexiglas box with an interlock on the access door for protection. Rinsing of the capillary was accomplished by applying vacuum on the detection buffer. Injections of samples were made hydrodynamically by elevating the sample beaker 1.5 cm for 30 s, corresponding to an injection plug length of 0.5 cm (90 nl injected). A CV4 UV detector (ISCO, Lincoln, NE, USA) at 267 nm was employed for on-column detection. Electropherograms were recorded with a sampling frequency of 20 Hz using an ELDS 900 laboratory data system (Chromatography Data Systems, Kungshög, Sweden).

Sample identification measurements were performed by using a Hewlett-Packard 3D CE system (Palo Alto, CA, USA).

3. Results and discussion

3.1. Choice of separation buffer

Different separation buffers were evaluated in order to find an appropriate buffer for separation of MDA from the sample matrix (Fig. 1a–c). With 10 m*M* borax, pH 9.3, and a separation voltage of 25 kV the migration time for MDA was 8.2 min. With reversed flow, employing buffers containing 0.5 m*M* CTAB, and −25 kV, MDA migrated among the first peaks. The migration times were 4.2 min with 10

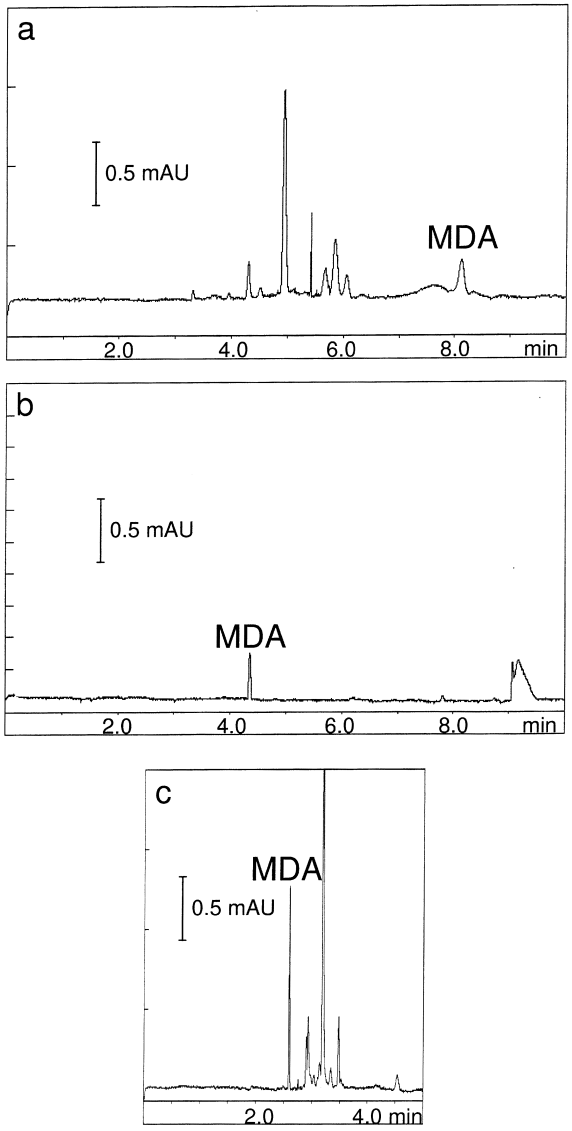

Fig. 1. Electropherogram of a precipitated brain homogenate. (a) Buffer 10 m*M* Borax pH 9.3. (b) Buffer: 10 m*M* phosphate pH 7.0–0.5 m*M* CTAB. (c) Buffer: 10 m*M* borax (pH 9.3)–0.5 m*M* CTAB. Applied voltage 25 kV. Hydrodynamic injection: 90 nl; λ=267 nm.

m*M* phosphate, pH 7.0, and 2.6 min with 10 m*M* borax 10, pH 9.3 (Fig. 1b,c). This latter buffer was employed for all subsequent analyses, since it provided the shortest migration time for MDA, and also the lowest LOD. With this buffer it was possible to inject as much as 90 nl (corresponding to 0.5% of the capillary) of the sample without affecting peak width or migration time of the MDA peak.

3.2. Sample identification

Sample identification was performed in the biological sample by spiking with MDA standard (Fig. 2a,b). Furthermore, diode array spectroscopy measurements made on standard and sample peaks confirmed the identity of MDA (Fig. 3). A statistical evaluation [28] of the peak purity of the MDA peak gave a peak purity factor of 993 in the sample compared to 994 in the standard. This suggests that the peak is pure.

3.3. Calibration strategy

The sample matrix can affect the area and the migration time of the MDA peak of the samples, compared to those obtained when pure standards are run in the CE system. Therefore, for the quantification of MDA, a standard curve was created by spiking the control brain tissue with increasing amounts of MDA standard. This curve was also used for studying the linearity and the LOD of the method. The control brain tissue was found to contain 1.2 μ*M* of MDA, see Fig. 2c. After correcting for this value the linear calibration curve obtained was, $y=16.1x+27.3\cdot10^{-6}$ ($R=0.9975$), where y is the area of the peak (AU s) and x is the concentration of MDA (M). Standard deviation for the slope was 0.44 AU s M^{-1} and for the intercept $21.6\cdot10^{-6}$ AU s. To calculate the unknown concentration of MDA in a sample the three data points closest to the sample were used.

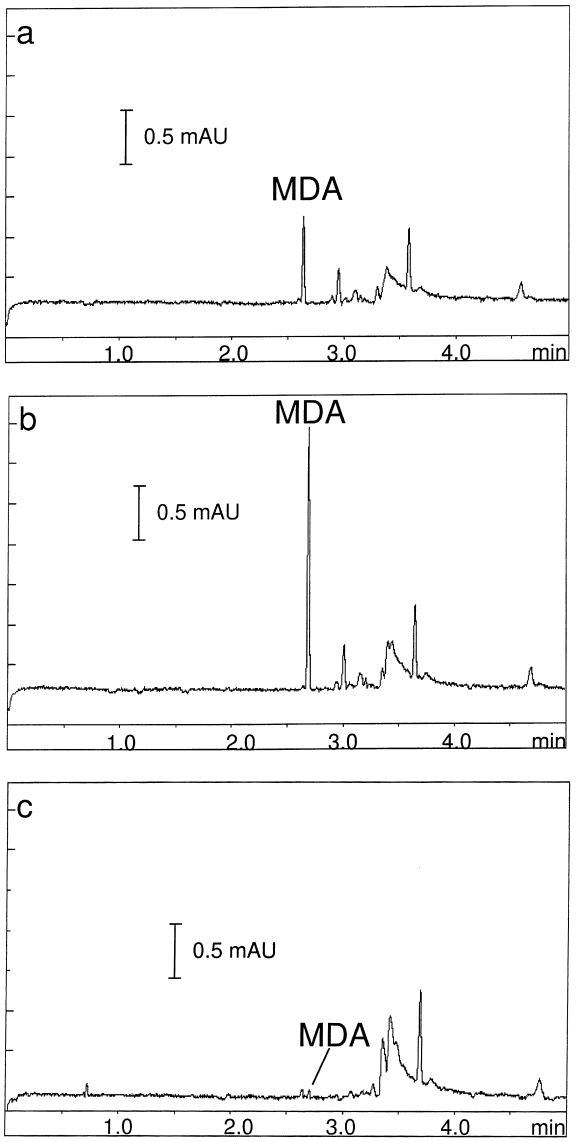

Fig. 2. Electropherograms of brain tissue. (a) Peroxidized tissue. (b) Peroxidized tissue spiked with MDA standard as described in Section 2.3. (c) Control brain tissue. Sample treatment and separation conditions as in Fig. 1c.

3.4. Method characteristics

Calibration data for MDA including migration time, peak area, separation efficiency and relative standard deviations for the oxidized biological sample, are presented in Table 1. Manually performed hydrodynamic injection proved to be acceptable in terms of repeatability. The relative standard deviation for the peak area of MDA is 3.54% when relative areas are calculated, therefore, with the use of an automatic injector it should be possible to improve the repeatability. Variations in migration times, were sometimes observed, probably due to

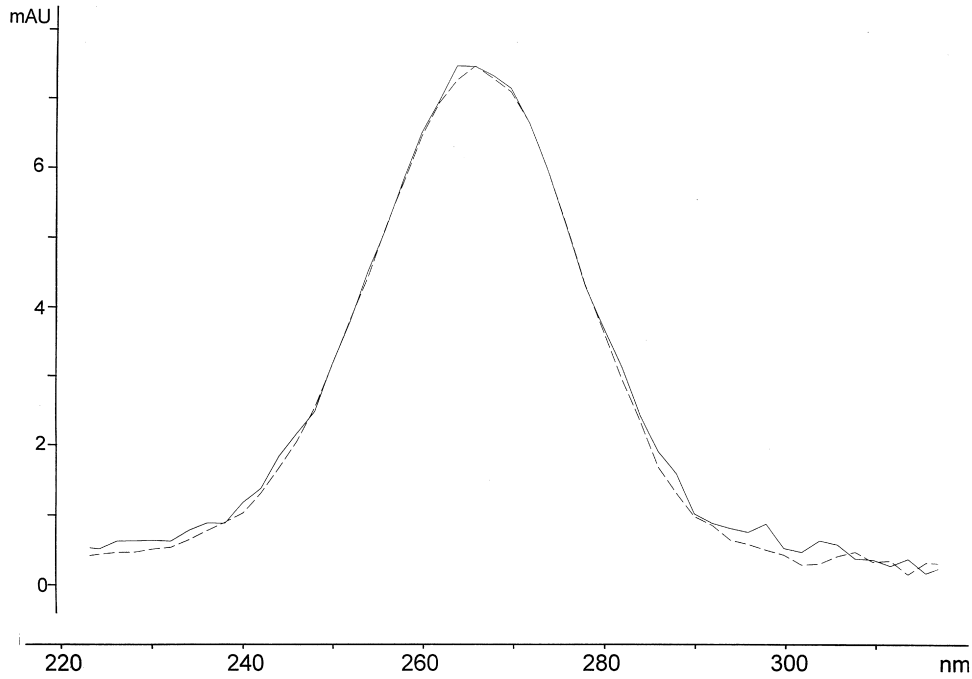

Fig. 3. Spectrum of the MDA standard peak (dotted line) and the MDA peak in a sample.

differences in the sample composition. However, these did not affect the MDA assay, since MDA was easily identified as it eluted as the first peak in the electropherogram. The LOD (3 times noise) was found to be 1.2 μ*M*.

3.5. Sample pretreatment – influence on the analytical results

A unprecipitated brain homogenate, merely subjected to a centrifugation step to remove particular matter, and thereby avoiding clogging of the capillary, was analyzed, see Fig. 4a. It was essential, however, with this type of sample, to wash the capillary with NaOH between each run in order to maintain the electroosmotic flow. Failure to do this increased the migration time, presumably due to adsorption of proteins on the capillary wall. An electropherogram of the corresponding precipitated homogenate is shown in Fig. 4b. This applied sample preparation procedure allowed adequate removal of protein and particular material from the sample. Rinsing of the capillary was only needed every 10th run, and therefore these homogenates were applied for MDA determinations. No loss in the performance of the capillary was observed.

3.6. Determination of MDA in rat brain tissue

Control brain tissue was found to contain small but detectable amounts of MDA, 1.2 μ*M*, corre-

Table 1
Migration data for MDA

Migration time t_m (min)	Area	Plate number N	R.S.D.$_{time}$ (%)[a]	R.S.D.$_{area}$ (%)[a]	R.S.D.$_{relative\ area}$ (%)[a]
2.57	20 467	217 000	1.12	6.37	3.54

[a] n=8.

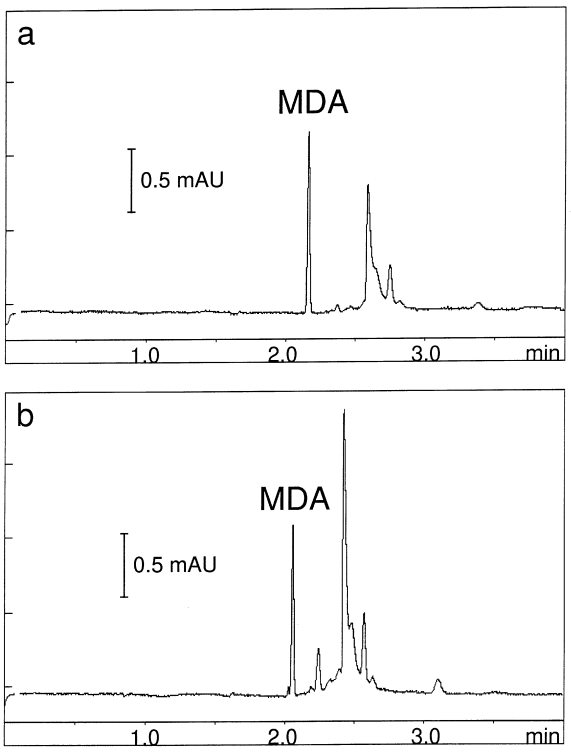

Fig. 4. Electropherogram of peroxidized brain tissue. (a) Supernatant from 20% homogenate centrifuged at 15 000 *g* for 10 min. (b) 20% homogenate precipitated as described in Section 2.3. Homogenates were boiled in capped tubes, chilled and centrifuged. Buffer: 10 m*M* borax–0.5 m*M* CTAB, pH 9.3; applied voltage: −25 kV; hydrodynamic injection: 90 nl; λ=267 nm.

sponding to 0.11 pmol in a 90 nl injection (Fig. 2c). In lipid peroxidation stimulated tissue homogenates this value was increased to 27 μ*M* in a 90 nl injection (Fig. 2a). The small amounts of MDA detected in the nonoxidized material can be due to the workup procedure, or, alternatively, has a biosynthetic origin, e.g., eicosanoid synthesis where MDA is also formed in small quantities. Our system using ferrous ammonium sulphate for inducing lipid peroxidation was not optimised in order to attain maximum lipid peroxidation, but only to create a positive control of oxidized sample. We are in the process of evaluating different approaches for the recovery of possible bound forms in brain tissue employing CZE for detection and separation.

3.7. Comparison with other methods

Several methods have been described for the determination of MDA [8–17]. The method described in this paper has several advantages over those currently used. MDA is negatively charged and UV absorbing at the pH used, and it can therefore be separated and detected directly, without the addition of substances for complex binding. The method is simple and easy to use, and can be readily automated. The short migration time of MDA, 2.6 min, makes it possible to run many samples in a short time; when MDA has migrated past the detector, other sample components can be flushed out from the capillary. Washing of the capillary is quick and easy to perform. Due to the high separation capacity (N=217 000), the appearance of interfering peaks, which comigrate with the MDA peak, is not probable. Furthermore, the method is advantageous in that very small amounts of samples, nl volumes, are required, leaving sample available for other types of analysis. Very small amounts of buffer are consumed. Crude biological samples can be analyzed. Many runs can be performed on the same capillary without any loss in performance. This contrasts HPLC where loss of column performance is a problem. Replacing the capillary is inexpensive compared to the cost of replacing a HPLC column. The usually low concentration sensitivity, typical for CZE separations with UV detection, is in this method closer to the HPLC methods than expected. Reports have described detection limits between 0.25–0.5 μ*M* using HPLC and UV detection [5,15,16]. In our CZE system the detection limit is 1.2 μ*M*. This low LOD was due to the large stacking effect produced, since the sample has a lower conductivity than the separation buffer, generating a narrow MDA peak. The method reported here thus represents a rapid and straightforward approach when MDA is considered as the index for lipid peroxidaton in a system where oxidative stress has been experimentally induced.

Acknowledgements

This work was supported by the Swedish Medical Research Council and the Osterman foundation. We

greatly appreciate the assistance of Ms. Britta Fängström and Ms. Josefin Lundgren.

References

[1] N.A. Simonian, J.T. Coyle, Annu. Rev. Pharmacol. Toxicol. 36 (1996) 83.
[2] B. Halliwell and J.M.C. Gutteridge, Free Radicals in Biology and Medicine, Clarendon Press, Oxford, 1989.
[3] D.R. Janero, Free Rad. Biol. Med. 9 (1990) 515.
[4] H.H. Draper and M. Hadley, in L. Packer and A.N. Glazer (Editors), Methods in Enzymology, Vol. 186, Academic Press, San Diego, CA, 1990, p. 421.
[5] A.W. Bull, L.J. Marnett, Anal. Biochem. 149 (1985) 284.
[6] L. Ernster and K. Nordenbrand, in R.W. Estabrook and M.E. Pullman (Editors), Methods in Enzymology, Vol. 10, Academic Press, San Diego, CA, 1967, p. 574.
[7] J.M.C. Gutteridge, Adv. Biosci. 64 (1987) 1.
[8] R.P. Bird, S.S.O. Hung, M. Hadley, H.H. Draper, Anal. Biochem. 128 (1983) 240.
[9] V.L. Tatum, C. Changchit, C.K. Chow, Lipids 25 (1990) 226.
[10] M.J. Richard, P. Guiraud, J. Meo, A. Favier, J. Chromatogr. 577 (1992) 9.
[11] K. Fukunaga, T. Suzuki, K. Takama, J. Chromatogr. 621 (1993) 77.
[12] S. Chirico, in L. Packer (Editor), Methods in Enzymology, Vol. 233, Academic Press, San Diego, CA, 1994, p. 314.
[13] D. Londero, P.L. Greco, J. Chromatogr. A 729 (1996) 207.
[14] C.R. Wade, P.G. Jackson, A.M. Van Rij, Biochem. Med. 33 (1985) 291.
[15] C. Largillière, S.B. Mélancon, Anal. Biochim. 170 (1988) 123.
[16] W.A. Behrens, R. Madère, Lipids 26 (1991) 232.
[17] M. Cini, R.G. Fariello, A. Bianchetti, A. Moretti, Neurochem. Res. 19 (1994) 283.
[18] D.T. Dexter, C.J. Carter, F.R. Wells, F. Javoy-Agid, Y. Agid, A. Lees, P. Jenner, C.D. Marsden, J. Neurochem. 52 (1989) 381.
[19] K.V. Subbarao, J.S. Richardsson, L.C. Ang, J. Neurochem. 55 (1990) 342.
[20] I. Hajimohammadreza, M. Brammer, Neurosci. Lett. 112 (1990) 333.
[21] D.T. Dexter, A.E. Holley, W.D. Flitter, T.F. Slater, F.R. Wells, S.E. Daniel, A.J. Lees, P. Jenner, C.D. Marsden, Movement Disord. 9 (1994) 92.
[22] B. Hallgren, P. Sourander, J. Neurochem. 3 (1958) 41.
[23] P. Riederer, E. Sofic, W.-D. Rausch, B. Schmidt, G.P. Reynolds, K. Jellinger, M.B.H. Youdim, J. Neurochem. 52 (1989) 515.
[24] D.T. Dexter, A. Carayon, F. Javoy-Agid, Y. Agid, F.R. Wells, S.E. Daniel, A.J. Lees, P. Jenner, C.D. Marsden, Brain 114 (1991) 1953.
[25] J.R. Connor, B.S. Snyder, J.L. Beard, R.E. Fine, E.J. Mufson, J. Neurosci. Res. 31 (1992) 327.
[26] K. Jellinger, E. Kienzl, G. Rumpelmair, P. Riederer, H. Stachelberger, D. Ben-Schachar, M.B.H. Youdim, J. Neurochem. 59 (1992) 1168.
[27] H. Esterbauer and K. Cheeseman, in L. Packer (Editor), Methods in Enzymology, Vol. 186, Academic Press, San Diego, CA, 1996, p. 407.
[28] Hewlett-Packard ChemStation, Waldbronn, Germany, 1997.