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Evaluation of deactivated reversed phases for the analysis of an N,N,N-trimethylethanaminium analogue of α-tocopherol

Application to its purity control and determination in biological samples

J. Verne-Mismer*, M. Lamard and J. Wagner

Department of Analytical Chemistry, Marion Merrell Dow Research Institute, 16 Rue d'Ankara, B.P. 067, 67046 Strasbourg Cedex (France)

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ABSTRACT

Several non-deactivated and deactivated reversed-phase columns were evaluated for the determination of the quaternary ammonium analogue of α-tocopherol, 3,4-dihydro-6-hydroxy-N,N,N,2,5,7,8-heptamethyl-2*H*-1-benzopyran-2-ethanaminium 4-methylbenzenesulphonate, which is being developed as a cardioselective antioxidant. The retention characteristics of the compound, its corresponding quinone and their tertiary amine analogues were studied using UV detection at 280 nm. The effect of pH, ionic strength of the sodium phosphate buffer and nature of the organic solvent (methanol, acetonitrile) on the retention times and peak symmetry of the different compounds studied was evaluated. The effect of the addition of a basic modifier, N,N-dimethyloctylamine, was also investigated. The best results were obtained with deactivated C_8 and C_{18} phases and with a mixture of methanol and sodium phosphate buffer. The method was applied to the purity control of the title compound and is compatible with electrochemical detection, which allows the determination of this α-tocopherol analogue and its corresponding amine in the sub-picomolar range.

INTRODUCTION

The α-tocopherol analogue 3,4-dihydro-6-hydroxy-N,N,N,2,5,7,8-heptamethyl-2*H*-1-benzopyran-2-ethanaminium 4-methylbenzenesulphonate [chromanol, code No. MDL 73404; **1** (Fig. 1)] has been shown to be a potent cardioselective antioxidant [1]. The aim of this study was the development of HPLC procedures for the determination of this quaternary ammonium derivative of α-tocopherol. The method

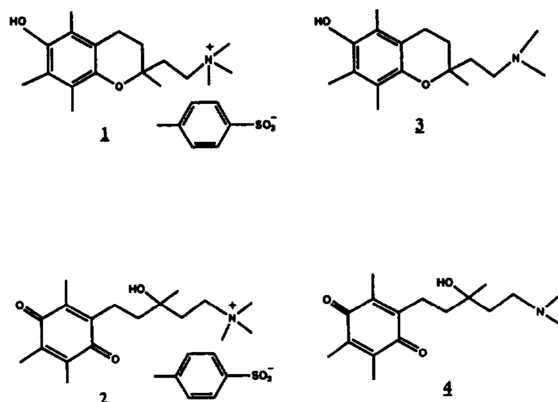

Fig. 1. Structures of the compounds studied.

* Corresponding author.

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should allow the separation of **1** from its quinone **2**, which occurs as an oxidation product, and from the corresponding tertiary amines **3** and **4**, which may arise as potential by-products of the synthesis or decomposition. In recent years, numerous HPLC procedures have been developed for the determination of α-tocopherol and model compounds in biological samples, foods and pharmaceuticals [2–6] and for the study of their oxidation pathways [7–11]. These methods are based on either normal- or reversed-phase HPLC, depending on the lipophilicity and charges of the compounds to be analysed. The present compounds are quaternary ammonium and tertiary amine analogues of 2,2,5,7,8-pentamethyl-6-chromanol, **1** and **3**, and their corresponding quinones, **2** and **4**. Tertiary amine and quarternary ammonium groups are well known to interact with residual silanol functions present in most reversed-phase packings, giving strongly tailing peaks [12–15]. These peak broadening problems are usually overcome by the addition of tertiary alkylamines to the eluent [12,16,17] or by masking the residual silanol groups by chemical derivatization and coating. Recently, so-called deactivated reversed-phase packings have been developed and have been shown to give excellent results with strongly basic analytes [14,18,19].

The peak shapes (as measured by the asymmetry factor) and separation efficiencies of compounds **1–4** were studied using conventional and deactivated C_8 and C_{18} reversed-phase columns. The following parameters were studied: effect of addition of a lipophilic amine (N,N-dimethyloctylamine), effect of the nature and strength of the buffer salt, effect of pH and the influence of the nature of the organic solvent. It was found that the best results were obtained with deactivated phases, which allowed an efficient separation of **1** from its potential by-products **2–4**. The method, using UV detection, was applied to the purity control of **1** and can be used for stability studies. The use of an electrochemical detector gives a marked increase in sensitivity for the oxidizable compound, *i.e.*, **1** and **3**.

EXPERIMENTAL

Chemicals

3,4-Dihydro-6-hydroxy-N,N,N,2,5,7,8-heptamethyl-2*H*-1-benzopyran-2-ethanaminium 4-methylbenzenesulphonate (**1**) and the amine 3,4-dihydro-2-[2-(dimethylamino)ethyl]-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (**3**) were synthesized as described previously [1]. The quinones 3-hydroxy-N,N,N,3-tetramethyl-5-(2,4,5-trimethyl-3,6-dioxo-1,4-cyclohexadien-1-yl)pentanaminium, 4-methylbenzene sulfonate (**2**) and 1-(3-hydroxy-3-methyl-5-dimethylaminopentyl)-2,4,5-trimethyl-1,4-cyclohexadien-3,6-dione (**4**) were obtained by established procedures [20]. N,N-Dimethyloctylamine (DMOA) was obtained from ICN Pharmaceuticals (Plainview, NY, USA), ammonium acetate from Prolabo (Paris, France) and trifluoroacetic acid from Janssen (Beerse, Belgium). Acetonitrile (LiChrosolv), methanol (Uvasol) and all other chemicals (analytical-reagent grade) were purchased from Merck (Darmstadt, Germany).

Liquid chromatographic system

A modular HPLC system was used, consisting of a Model 9010 solvent delivery system from Varian (Palo Alto, CA, USA), a WISP 715 automatic injector with thermostating from Waters (Milford, MA, USA) and a Model 486 UV detector from Waters. An HP 1049A electrochemical detector from Hewlett-Packard (Palo Alto, CA, USA) with a glassy carbon electrode whose potential was maintained at 0.6 V *versus* a silver–silver chloride reference electrode was used for electrochemical detection. The chromatograms were recorded and integrated with a Nelson 6000 data acquisition system. The columns were thermostated at 40°C with a Croco-cil from Interchim (Montluçon, France). The flow-rate was usually 1 ml/min.

Columns

The following columns were used: LiChroCART Superspher 60 RP-8e and 100 RP-18e (4 μm) (250 × 4.0 mm I.D.) from Merck, Ultrabase

C_8 and C_{18} (5 μm) (250 × 4.6 mm I.D.) from Shandon-SFCC (Eragny, France), Zorbax Rx-C_8 (5 μm) (250 × 4.6 mm I.D.) from Rockland Technologies (supplied by Shandon-SFCC) and Nucleosil 100-5 C_{18}AB (5 μm) (250 × 4.0 mm I.D.) from Macherey–Nagel (Düren, Germany).

Mobile phases

Different mobile phases were used under isocratic conditions. For the Superspher RP-8e and RP-18e columns, the following eluents were prepared: 0.15 *M* NaH_2PO_4 buffer–methanol (65:35, v/v) with apparent pH 5.2; and the same eluent but with addition of 0.02% (v/v) of DMOA and with the pH adjusted to 5.2 with 1 *M* H_3PO_4. For the studies with the other columns, the mobile phases were 0.12 *M* NaH_2PO_4–acetonitrile (80:20, v/v) or 0.15 *M* NaH_2PO_4–methanol (65:35, v/v). The pH was adjusted to lower values, *e.g.*, 3 and 4, by addition of H_3PO_4 and to higher values, *e.g.*, 6, 7 and 8, with 4 *M* NaOH. For LC–MS studies, the mobile phase was 0.15 *M* ammonium acetate buffer–methanol (65:35, v/v) with an apparent pH adjusted to 5.2 with concentrated acetic acid. For semi-preparative HPLC, the eluent was water–methanol–concentrated trifluoroacetic acid (65:35:1, v/v/v) with an apparent pH of 2.00.

All the eluents were filtered under vacuum with a 0.45-μm Millipore HV filter.

Solutions

Standard solutions of compounds **1–4** were prepared by dissolving 0.5–3 mg (standard 1) or 0.2–0.8 mg (standard 2) of the different compounds in 10 ml of water. Solutions were stored at 0–5°C under nitrogen to prevent oxidation. Aliquots of 50 μl of the standard solutions were usually injected, corresponding to 3–9 μg (10–35 nmol) or 1–3 μg (2–12 nmol), respectively, of the different compounds. The automatic injector was thermostated at 4°C to limit thermal degradation of the compounds.

Calculations

The following factors were used for the column evaluation. The capacity factor was measured using the relationship $(t_r - t_0)/t_0$, where t_r is the retention time of the compound and t_0 that of a non-retained compound measured by using the solvent disturbance peak [21]. The peak asymmetry factor B/A was measured at 10% of the peak height using the ratio of the widths of the rear and front sides of the peak [21,22]. The Foley–Dorsey method [22] was used to measure the column efficiency as skewed peaks were often involved. The method uses the equation

$$N = \frac{41.7(t_R/w_{0.1})^2}{B/A + 1.25}$$

were N is the efficiency (plates per column) and $w_{0.1}$ the peak width at 10% height.

RESULTS AND DISCUSSION

Use of conventional reversed-phase columns and effect of amine modifier

The first studies with the quaternary ammonium compound **1** and its analogues **2–4** were done by using the classical end-capped Superspher RP-18e and RP-8e stationary phases. With mixtures of 0.15 *M* sodium phosphate buffer and methanol at an apparent pH of 5.2, separation of the four compounds was obtained with badly tailing peaks, the separation being only partial for **2** and **3** with the RP-8e column, as illustrated in Fig. 2a and c. The addition of a small amount of DMOA, *i.e.*, 0.02% (v/v), to the mobile phase at the same pH greatly improved the peak shapes, with a slight decrease in retention times. Satisfactory separation of the four compounds was obtained with the RP-18e column, whereas with the RP-8e column **2** and **3** co-eluted (see Fig. 2b and d). Under these conditions, **1** was always eluted first, followed by **3**, **2** and **4**. This is in agreement with the differences in lipophilicity, log *P* being −0.60 for **1** and 1.15 for **3** [1]. The chromatographic characteristics for **1** are summarized in Table I. For the injection of smaller amounts, *eg.*, 1.6 μg (5 nmol) of **1**, no change in k', a marked

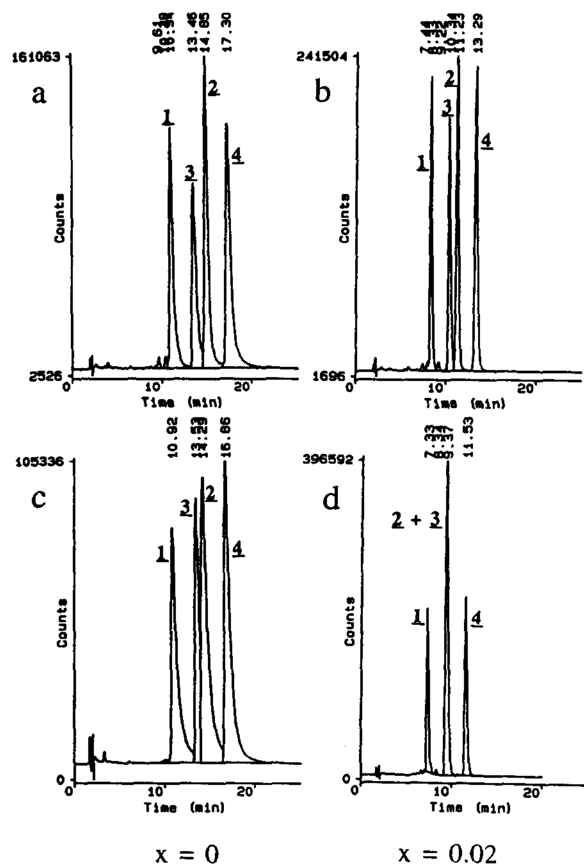

Fig. 2. Effect of DMOA on the separation of compounds **1–4**. Column: (a,b) Superspher RP-18e; (c,d) Superspher RP-8e. Conditions as defined in Table I. x = % of DMOA.

decrease in the asymmetry factor, *e.g.*, 1.6 for the RP-18e column with DMOA instead of 2.6, and nearly a doubling of the plate number, *e.g.*, 6700 for the RP-18e column with DMOA instead of 3500, were observed.

Use of deactivated C_8 and C_{18} phases

In order to improve the separation between the amine **3** and the quinone **2**, which corresponds to the first and major oxidation product formed, and to apply the HPLC method to coupled LC–MS or semi-preparative chromatography using simplified mobile phases without addition of an amine modifier, several deactivated C_8 and C_{18} phases were tested (a complete stability study with isolation and characterization of the major oxidation compounds formed has been carried out and will be described elsewhere). These deactivated supports have been shown to improve greatly the separation and peak shapes of basic analytes without addition of basic modifiers such as triethylamine or DMOA [14,18,19].

Method development: effect of ionic strength, DMOA addition, pH and nature of the organic solvent. The optimization of the mobile phase conditions was done with the Ultrabase C_8 column. In fact, the conditions giving the best results with the Superspher columns without DMOA addition, *i.e.*, 0.15 *M* NaH_2PO_4 buffer–methanol (65:35, v/v) at an apparent pH of 5.2, gave a very good separation of **1–4** with the Ultrabase C_8 column.

As expected, an increase of the ionic strength from 0.038 to 0.15 *M* NaH_2PO_4 clearly improved the efficiency of the column and the asymmetry factors of the peaks. For an amount of *ca.* 9 μg

TABLE I

EFFECT OF DMOA ON THE CHROMATOGRAPHIC CHARACTERISTICS OF COMPOUND 1 WITH THE SUPERSPHER RP-8E AND RP-18E COLUMNS

Conditions: mobile phase, 0.15 *M* NaH_2PO_4–methanol (65:35, v/v) with or without 0.02% of DMOA, apparent pH = 5.2; flow-rate, 1 ml/min; temperature, 40°C; detection, UV at 280 nm; sample, 50 μl of standard 1 corresponding to amounts of *ca.* 9 μg of **1** and **3** and *ca.* 3 μg of **2** and **4**; k', B/A and N as defined in the text.

Eluent	k'		B/A		N	
	RP-8e	RP-18e	RP-8e	RP-18e	RP-8e	RP-18e
Without DMOA	4.20	3.68	6.37	4.82	250	850
With DMOA	2.49	2.56	2.45	2.58	2400	3500

(30 nmol) of **1** injected, the plate number N increased from 1300 to 2800 and the asymmetry factor decreased from 3.2 to 2.7. Addition of 0.02% (v/v) of DMOA to the eluent containing 0.15 M NaH_2PO_4 buffer further improved the plate number and asymmetry factor to 6000 and 1.9, respectively. Further addition of up to 0.1% of DMOA led only to a slight additional improvement. A typical chromatogram obtained for **1–4** on the Ultrabase C_8 column under the above-described conditions, without addition of DMOA, is given in Fig. 3a.

Variation of the pH between 3 and 6 with the 0.15 M NaH_2PO_4 buffer–methanol (65:35, v/v) eluent had no effect on the capacity factors for the four compounds. Above pH 6 the capacity factors of the amines **3** and **4** rapidly increased, as expected, owing to the deprotonation of the amino function. An apparent pH of 5.2 was adopted for all further studies. No systematic study of the effect of temperature was made, but a temperature of 40°C was found to provide a good compromise between reduced pressure and column lifetime.

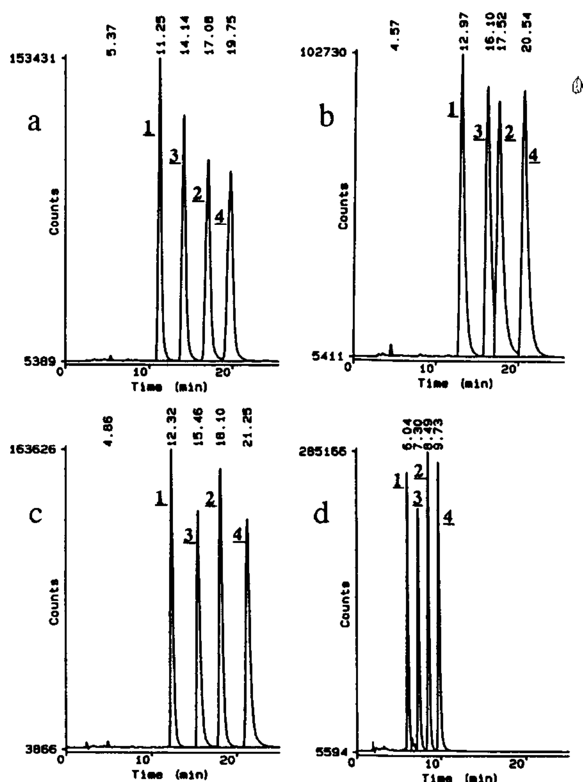

Fig. 3. Separation of compounds **1–4**. Column: (a) Ultrabase C_8; (b) Zorbax Rx-C_8; (c) Ultrabase C_{18}; (d) Nucleosil C_{18} AB. Conditions as defined in Table II.

The use of acetonitrile instead of methanol as an organic modifier, *i.e.*, 0.12 M NaH_2PO_4–acetonitrile mixture (80:20, v/v) at an apparent pH of 5.2, which gave similar capacity factors, led to large and unresolved peaks for **1** and its analogues **2–4**. The addition of 0.02% (v/v) of DMOA restored satisfactory peak shapes, but with incomplete resolution of the four compounds. In particular, the quinone **2** was eluted very close to **1**.

This is a further example of a specific solvent interaction with analytes and reversed-phase packings, especially with quaternary ammonium and tertiary amine analogues [12,13–15]. It is noteworthy that the four compounds here have significantly lower solubilities in acetonitrile than in methanol.

These different trials led us to adopt 0.15 M sodium phosphate buffer–methanol (65:35, v/v) at an apparent pH of 5.2 for the remainder of the experiments.

Comparison of different deactivated phases. Considering the remarkable peak-shape improvements observed with the Ultrabase C_8 column without addition of DMOA, it was of obvious interest to study some other commercially available deactivated phases. Three of them were tested, Zorbax Rx-C_8, Ultrabase C_{18} and Nucleosil C_{18} AB.

The same amount of the standard solution of the four compounds was injected, using the chromatographic conditions that gave the best separation with the Ultrabase C_8 column, *i.e.*, 0.15 M NaH_2PO_4 buffer–methanol (65:35, v/v) at an apparent pH of 5.2 at 40°C and a flow-rate of 1 ml/min. The general elution patterns shown in Fig. 3b–d are similar although the separation of **2** and **3** with the Zorbax Rx-C_8 column is clearly less effective. The retention times for the C_8 and C_{18} columns, except for the Nucleosil C_{18} phase, are nearly the same.

The chromatographic characteristics of **1** (k', B/A and N) are summarized in Table II. The best peak shapes and efficiencies were obtained with the Ultrabase C_8 and C_{18} and the Nucleosil

TABLE II

CHROMATOGRAPHIC CHARACTERISTICS FOR COMPOUND 1 WITH THE DIFFERENT DEACTIVATED COLUMNS

Conditions: mobile phase, 0.15 *M* NaH_2PO_4–methanol (65:35, v/v), apparent pH = 5.2; other conditions as in Table I: *k'*, *B/A* and *N* as defined in the text.

Column	*k'*	*B/A*	*N*
Superspher RP-8e	4.20	6.37	250
Superspher RP-18e	3.68	4.82	850
Ultrabase C_8	2.97	2.67	3500
Ultrabase C_{18}	3.79	2.79	3400
Zorbax Rx-C_8	3.67	4.23	800
Nucleosil C_{18}AB	1.88	2.54	2450

C_{18} columns. The results are similar to those obtained with the RP-8e and RP-18e columns but in the presence of DMOA. These results show that the Ultrabase and Nucleosil phases tested present less residual silanol activity than the Superspher phases and that the end-capping as done with the Superspher phases is not sufficient to minimize silanol interactions with these quaternary ammonium and tertiary amine analogues.

Extension to other buffers. Most of the studies were done with the Ultrabase C_8 column, although the results obtained with the Ultrabase C_{18} and Nucleosil C_{18} are nearly equivalent in terms of asymmetry factor and separation efficiency for the four compounds of interest. Sodium phosphate can be replaced with ammonium acetate for coupled LC–MS studies. The separation obtained with the Ultrabase C_8 column with ammonium acetate buffer–methanol (65:35, v/v) is shown in Fig. 4a.

The use of 0.1% trifluoroacetic acid also gives a satisfactory separation (see Fig. 4b) and allows an easy scale-up to the semi-preparative HPLC separation of **1** from its decomposition products.

Applications

Purity control of compound 1. The method using the Ultrabase C_8 column and 0.15 *M* NaH_2PO_4–methanol (65:35, v/v) at an apparent pH of 5.2 has been routinely applied for the purity control of **1**. Addition of DMOA to the mobile phase to improve the peak shape of the compounds was not used for the final purity control conditions. Indeed, **1** is determined as a *p*-toluenesulphonate salt and with DMOA addition the retention time of the *p*-toluenesulphonate group is increased, *e.g.*, addition of 0.02% (v/v) of DMOA increases its retention time from 5 to 7 min, and it will interfere with one of the oxidation products of **1**. A typical chromatogram

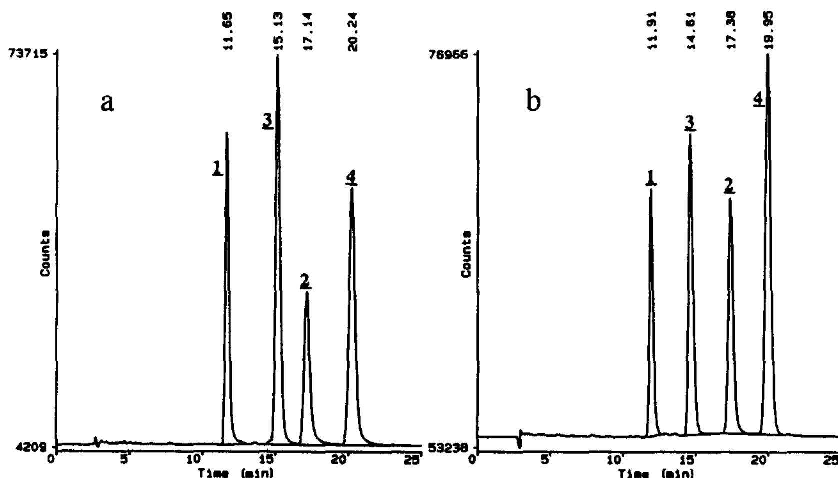

Fig. 4. Separation of compounds **1–4** with the Ultrabase C_8 column. Conditions: mobile phase, (a) 0.15 *M* ammonium acetate–methanol (65:35, v/v), apparent pH = 5.2 and (b) water–methanol–concentrated trifluoroacetic acid (65:35:1, v/v/v), apparent pH = 2.00; sample, 50 μl of standard 2 corresponding to amounts of *ca.* 3 μg of **1** and **3** and *ca.* 1 μg of **2** and **4**; other conditions as defined in Table I.

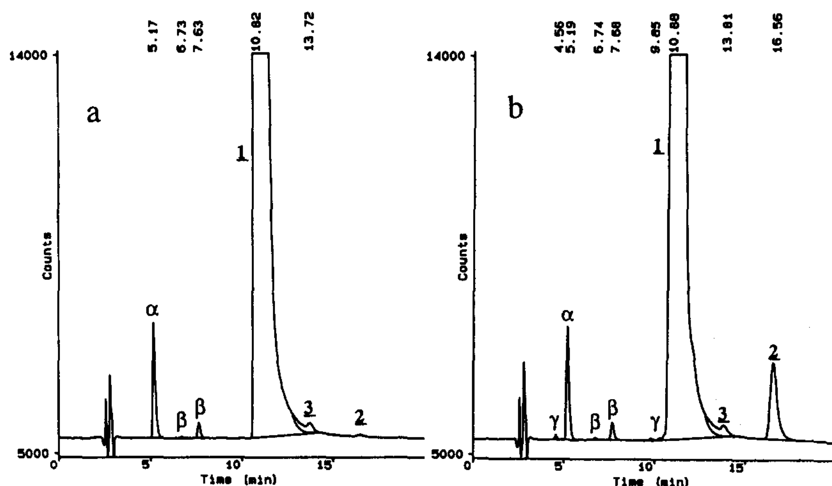

Fig. 5. Purity control of compound **1**. Conditions: column, Ultrabase C_8; mobile phase, 0.15 *M* NaH_2PO_4–methanol (65:35, v/v), apparent pH = 5.2; flow-rate, 1 ml/min; temperature, 40°C; detection, UV at 280 nm; sample, 50 μl of solution of **1** corresponding to 15 μg. (a) At time zero; (b) after 1 day. α = *p*-Toluenesulphonate; β = impurities; γ = oxidation compounds.

obtained with UV detection at 280 nm for the injection of 50 μl of a 5 mg per 10 ml aqueous solution of **1** is given in Fig. 5a. No detectable quinone **2** at a level of 0.05% by area was observed. The sensitivity of the procedure is illustrated by the chromatogram in Fig. 5b, which corresponds to the same solution of **1** left in presence of air for 1 day. The quinone **2** is now clearly observed and corresponds to 0.67% by area or 0.16 mol%.

Use of electrochemical detection. Vitamin E analogues are easily oxidizable compounds and therefore electrochemical detection has been a method of choice for the determination of these analogues in various media [4–6]. Using an electrochemical detector, the sensitivity of detection was markedly increased for **1** and **3**. Compounds **2** and **4** were no longer detected. The chromatogram obtained with UV detection at 280 nm with injection of amounts ranging from

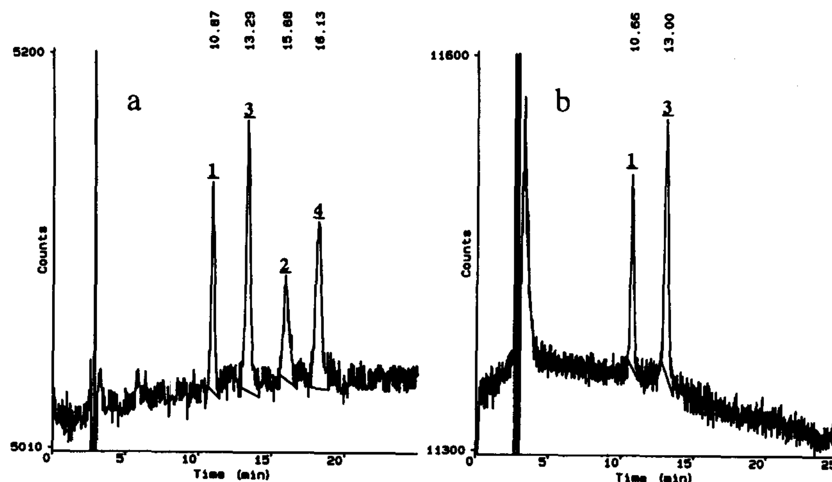

Fig. 6. Detection limits obtained for compounds **1–4** with the Ultrabase C_8 column, with conditions as defined in Fig. 5. (a) UV detection at 280 nm; amounts injected, *ca.* 5 ng of **1** and **3** and 1.5 ng of **2** and **4**. (b) Electrochemical detection; electrode potential, 0.6 V *versus* Ag/AgCl reference electrode; amounts injected, *ca.* 100 pg of **1** and **3** and 30 pg of **2** and **4**.

1.5 to 5 ng (from 4 to 24 pmol) of **1–4** is shown in Fig. 6a; the detection limit corresponds to *ca.* 2 ng (7 pmol) injected for **1** and **3** and *ca.* 1 ng (3.5 pmol) for the corresponding quinones **2** and **4** at a signal-to-noise ratio of 3. Fig. 6b illustrates the gain in sensitivity obtained for **1** and **3** with the electrochemical detector by applying a working potential of 0.6 V. The detection limits are *ca.* 40 pg (0.15 pmol) for both compounds, which corresponds to a gain in sensitivity by a factor of 50 compared with UV detection at 280 nm. This method is now routinely applied to the determination of **1** and analogues in biological samples such as brain and heart homogenates of mice and rats [23].

CONCLUSIONS

The best results for the separation of **1** from its potential impurities **2–4** were obtained on deactivated phases, *i.e.*, Ultrabase C_8 and C_{18} and the Nucleosil C_{18}AB columns, with addition of DMOA to the eluent. The purity control of **1** was developed on an Ultrabase C_8 column with sodium phosphate buffer–methanol as eluent, but without DMOA addition. These conditions gave the best compromise between good peak shapes and separation of all the potential impurities considered. The method was easily adapted to coupled LC–MS studies and to semipreparative chromatography by replacing sodium phosphate with ammonium acetate or trifluoroacetic acid. The method was also applied to the determination of **1** in biological samples by using electrochemical detection, allowing a great enhancement of sensitivity.

REFERENCES

1 J.M. Grisar, M.A. Petty, F.N. Bolkenius, J. Dow, J. Wagner, E.R. Wagner, K.D. Haegele and W. De Jong, *J. Med. Chem.*, 34 (1991) 257.
2 A. Deschuytere and H. Deelstra, *Fresenius' Z. Anal. Chem.*, 324 (1986) 1; and references cited therein.
3 M. Balz, E. Schulte and H.P. Thier, *Fett Wiss. Technol.*, 94 (1992) 209.
4 J.K. Lang, K. Gohil and L. Packer, *Anal. Biochem.*, 157 (1986) 106.
5 M.E. Murphy and J.P. Kehrer, *J. Chromatogr.*, 421 (1987) 71.
6 G.A. Pascoe, C.T. Duda and D.J. Reed, *J. Chromatogr.*, 414 (1987) 440.
7 M. Matsuo, S. Matsumoto, Y. Iiataka and E. Niki, *J. Am. Chem. Soc.*, 111 (1989) 7179.
8 D.C. Liebler, P.F. Baker and K.L. Kaysen, *J. Am. Chem. Soc.*, 112 (1990) 6995.
9 D.C. Liebler and J.A. Burr, *Biochemistry*, 31 (1992) 8278.
10 M. Baca, C. Suarna and P.T. Southwell-Keely, *J. Liq. Chromatogr.*, 14 (1991) 1957.
11 I. Molnar and S. Koswig, *J. Chromatogr.*, 605 (1992) 49.
12 E. Bayer and A. Paulus, *J. Chromatogr.*, 400 (1987) 1.
13 M.A. Stadalius, J.S. Berus and L.R. Snyder, *LC·GC*, 6 (1988) 494.
14 R.J.M. Vervoort, F.A. Maris and H. Hindriks, *J. Chromatogr.*, 623 (1992) 207.
15 B. Buszewski, J. Schmid, K. Albert and E. Bayer, *J. Chromatogr.*, 552 (1991) 415.
16 J.S. Kiel, S.L. Morgan and R.K. Abramson, *J. Chromatogr.*, 320 (1985) 313.
17 K.-G. Wahlund and A. Sokolowski, *J. Chromatogr.*, 151 (1978) 299.
18 T.L. Ascah and B. Feibush, *J. Chromatogr.*, 506 (1990) 357.
19 J. Coene, M. Ghijs, E. Van Den Eeckhout, W. Van Den Bossche and P. Sandra, *J. Chromatogr.*, 553 (1991) 285.
20 J.M. Grisar, unpublished work.
21 L.R. Snyder and J.J. Kirkland (Editors), *Introdution to Modern Liquid Chromatography*, Wiley, New York, 1979, 2nd ed., Ch. 5.
22 J.P. Foley and J.G. Dorsey, *Anal. Chem.*, 55 (1983) 730.
23 F. Bolkenius and J. Verne, unpublished work.