Journal of Chromatography, 385 (1987) 109–117
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1025

QUANTITATIVE DETERMINATION OF VITAMIN E AND OXIDIZED AND REDUCED COENZYME Q BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH IN-LINE ULTRAVIOLET AND ELECTROCHEMICAL DETECTION

JOHANNA K. LANG* and LESTER PACKER
Membrane Bioenergetics Group, Lawrence Berkeley Laboratory and the Department of Physiology/Anatomy, University of California, Berkeley, CA 94720 (U.S.A.)

SUMMARY

We describe a sensitive quantitative high-performance liquid chromatographic (HPLC) assay for the simultaneous determination of vitamin E isomers (α-, γ- and δ-tocopherol), oxidized coenzyme Q species (ubiquinone 9, ubiquinone 10) and reduced coenzyme Q homologues (ubiquinol 9, ubiquinol 10) in various tissues, including blood and plasma. The compounds of interest are quantitatively extracted with a fast one-step lipid extraction procedure and subjected to HPLC without further purification. The extract is separated on a reversed-phase column and the eluted compounds are monitored by sequential UV and electrochemical detection. Ubiquinones are detected at their 275 nm absorbance maximum, by the UV detector, whereas tocopherols and ubiquinols are monitored by the electrochemical detector with high sensitivity and selectivity. The method can detect as little as 1 pmol of the individual ubiquinones. Detection limits for tocopherols and ubiquinols are at least two orders of magnitude lower.

INTRODUCTION

Since lipid peroxidation has been recognized as a possible factor in human pathology, there has been an increased interest in the determination of endogenous prooxidant and antioxidant compounds in tissue lipids. Vitamin E, which encompasses the various tocopherol isomers and is ubiquitous in biomembranes, is considered as the major lipid antioxidant[1]. Coenzyme Q, which is preferably located in the mitochondria and functions as an electron carrier in the electron transport chain[2], comprises the different ubiquinone homologues and their reduced forms, the ubiquinols. Due to their antioxidant properties[3], ubiquinols can be regarded as another class of endogenous antioxidants, whereas the ubiquinones are potential prooxidants[4]. A number of analytical techniques have been employed for the determination of vitamin E or coenzyme Q from plant, bacterial, and mammalian sources, but so far no procedure for the simultaneous determination of individual vitamin E and coenzyme Q species has been described. Older procedures rely on spectrophotometry,

0021-9673/87/$03.50

fluorimetry or polarography and require lengthy isolation and purification steps to remove interfering compounds. (For reviews see ref. 5, and refs. 6 and 7 for vitamin E and coenzyme Q, respectively). More recent procedures for tocopherols are based on gas chromatography[8] and high-performance liquid chromatography (HPLC) with UV[9] and fluorescence detection[10,11]. Individual coenzyme Q homologues were analyzed by HPLC with UV and electrochemical detection[12].

The analytical procedure described here is a simultaneous assay of individual vitamin E isomers and oxidized and reduced coenzyme Q species in tissues. The method is based on the procedure of Ikenoya *et al.*[12] and includes HPLC on a reeversed-phase column with in-line UV and electrochemical detection. The compounds of interest are quantitatively extracted by a one-step procedure, recently described by Burton *et al.*[13]. This procedure does not require saponification or repeated extraction, thereby saving time and minimizing sample decomposition and artifact formation.

EXPERIMENTAL

Reagents and standards

All solvents were of HPLC grade. Reagent alcohol (Aldrich, Milwaukee, WI, U.S.A.) consisted of ethanol–2-propanol (95:5, v/v).

Water was purified in a Sybron/Barnstead Nanopure system (Barnstead Sybron, Boston, MA, U.S.A.). Lithium perchlorate, ACS grade, was from Aesar (Johnson Matthey, Seabrook, NH, U.S.A.). Sodium dodecylsulfate (SDS) (from Sigma, St. Louis, MO, U.S.A.) was purified as follows: 10 g was suspended in 100 ml of HPLC-grade hexane, sonicated for 30 s, and filtered. The residue was then collected and dried under reduced pressure. Purified sodium dithionite was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.), γ-tocopherol was from Eastman Kodak (Rochester, NY, U.S.A.), δ-tocopherol was a gift from G. W. Burton (National Research Council of Canada, Ottawa, Canada). 2,6-Di-*tert.*-butyl-*p*-cresol (BHT), ascorbic acid, α-tocopherol, ubiquinone 9 and ubiquinone 10 were Sigma products. Ubiquinol 9 and 10 were prepared from the quinones as follows: ubiquinone (0.2–0.3 mg) was dissolved in 1 ml of ethanol. After addition of 3 ml of water and approximately 100 mg of sodium dithionite and brief sonication, the mixture was vortex-mixed for 3 min and then incubated in the dark for 30 min. The ubiquinol was extracted into 4 ml of hexane, and the upper (organic) phase was washed once with 4 ml of water. The hexane layer was carefully removed, dried under nitrogen and resuspended in 3–5 ml of ethanol to yield the quinol stock solution, the concentration of which was determined spectrophotometrically, as described previously[6]. This procedure leads to pure solutions of quinols as confirmed by UV spectra and HPLC analysis. However, on prolonged standing (> 15–30 min, 25°C) the quinol is reoxidized and decomposes. Stock solutions of α-tocopherol, γ-tocopherol, δ-tocopherol, ubiquinone 9 and ubiquinone 10 were prepared by dissolving the pure compounds in the reagent alcohol to yield final concentrations of approximately 50–100 μM. The accurate concentration of each standard solution was determined spectrophotometrically using molar extinction coefficients as published previously[5,6]. A working standard for HPLC calibration was prepared by combining individual stock solutions to give final concentrations of approximately 2 μM for each of the quinones, 3 μM for toco-

pherols, and 10 μM for quinols in reagent alcohol–methanol (1:1, v/v). To account for concentration changes after mixing of the individual components, their final concentrations in the HPLC working standard were determined by comparison of the measured HPLC peak heights to those of the pure appropriately diluted stock solutions. The working standard is stable for several weeks at −20°C.

Apparatus

The modular HPLC system consisted of a Beckman 114 M HPLC pump (Beckman Instruments, Altex Division, San Ramon, CA, U.S.A.), a Rheodyne injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop, an Altex Ultrasphere ODS or Octyl column, 25 cm × 4.6 mm I.D., 5 μm particle size (Beckman Instruments) a Beckman 165 variable-wavelength detector (Beckman Instruments) and a Bioanalytical Systems LC 4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). Alternatively an Isco V4 variable-wavelength detector with a standard flow-cell (Isco, Lincoln, NE, U.S.A.) was used. The two detectors were set up in line, the column effluent first passing through the UV detector. This arrangement resulted in a delay of 3 s in retention times between the UV and the electrochemical detector.

Lipid extraction

Approximately 100 mg freeze clamped* tissue was accurately weighed in the frozen state and subsequently homogenized with 1 ml of water in a Potter-Elvehjem homogenizer with a motor-driven pestle. A volume of 50 μl of a solution of BHT in reagent alcohol (10 mg/ml) was added to each sample to prevent autoxidation. After addition of 1 ml of 0.1 M SDS and brief mixing by homogenization, the sample was transferred to a 10-ml test tube fitted with a PTFE-lined screw cap, 2 ml of reagent alcohol was added, and the mixture was vortex-mixed for 30 s. Then 2 ml of hexane was added and the tightly capped test tube was vigorously vortex-mixed for 2 min. It was then centrifuged for 5 min at 1000 g and 1 ml of the hexane layer was transferred to a small vial. The solvent was evaporated under nitrogen and the residue redissolved in methanol–reagent alcohol (1:1, v/v). Final volumes varied from 0.2 to 1.0 ml, depending on analyte concentrations in the tissue. Extracts from liver and adipose tissue could not be fully redissolved in the methanol–ethanol mixture, but after brief sonification (10 s) and subsequent centrifugation, the compounds of interest were quantitatively dissolved in the clear supernatant. Samples were injected shortly after preparation, as the ubiquinols are rapidly oxidized and decompose. Even if kept in a dry state under nitrogen at −20°C, the extracts cannot be stored for more than 2 h. Sample vials were wrapped with alumina foil during all the preparation steps to prevent photodegradation.

Heparinized blood plasma was diluted five-fold with water and 1 ml was mixed with 1 ml of 0.02 M SDS. The further extraction procedure was essentially as described above for tissue samples. The final resuspension volume was 0.2 ml.

For whole blood, aliquots of 0.2 ml were mixed with 0.8 ml of 0.1 M SDS.

---

* Freeze clamping is a rapid freezing technique for tissues. It is used to minimize changes in metabolite composition once the tissue has been disected. Freeze clamped tissues are stored in liquid nitrogen for later analysis.

Subsequently 1.0 ml of 5 m*M* ascorbate in 5 m*M* phosphate buffer (pH 7.4) was added, and the whole mixture was vortex-mixed for 1 min. Addition of reagent alcohol and hexane extraction were performed, as described for tissue samples. The dry lipid extract was redissolved in 0.2 ml methanol–reagent alcohol (1:1, v/v).

High-performance liquid chromatography

Mixtures of methanol–reagent alcohol containing 20 m*M* lithium perchlorate were used as mobile phases at a flow-rate of 1 ml/min. Typical detector settings were 275 nm, 0.005–0.01 a.u.f.s. for the UV detector and +0.5–0.7 V, 5–50 nA for full recorder scale (nAFS) for the EC detector. Unless otherwise stated, analyses were performed on the ODS column. To obtain maximum speed of analysis without loss of resolution, different eluent compositions were chosen for different tissues. Blood, plasma, muscle, brain and adipose tissue extracts can be analyzed with an eluent composition of methanol–reagent alcohol (1:9, v/v) containing 20 m*M* lithium perchlorate, in a total analysis time of 12 min. Rat liver preparations, which yield more complex chromatograms require an eluent composition of methanol–reagent alcohol (3:7, v/v) containing 20 m*M* lithium perchlorate and an overall elution time of 24 min. Some liver samples were analyzed on an Octyl column with and a more polar eluent (methanol–reagent alcohol (7:3, v/v) containing 20 m*M* lithium perchlorate. In these cases, the chromatograms were similar to those from the ODS column. Concentrations of analytes were calculated fom peak heights by external standardization. The quantitative working standard, containing tocopherols, ubiquinols 9 and 10 and ubiquinones 9 and 10, was injected frequently between samples to account for changes of detector sensitivity.

RESULTS AND DISCUSSION

The prevailing endogenous tocopherols (α-tocopherol, γ-tocopherol), ubiquinols (ubiquinol 9, ubiquinol 10), and ubiquinones (ubiquinone 9, ubiquinone 10) were well separated by reversed-phase HPLC (Fig. 1). δ-Tocopherol was well separated from γ-tocopherol but β-tocopherol, a positional isomer of γ-tocopherol coelutes with the latter. Neither δ-tocopherol nor β-tocopherol occur in animal tissues in bigger than trace amounts. Ubiquinones were detected with maximal sensitivity at their UV maximum at 275 nm. Tocopherols and ubiquinols, which absorb poorly at 275 nm were detected with high sensitivity and specificity by the electrochemical detector. The selectivity of the electrochemical detector allowed accurate determination of tocopherols and ubiquinols in whole lipid extracts, which yielded complex chromatograms using UV detection, in which tocopherol and ubquinol peaks were small or not separated from interfering compounds (Figs. 1–3). Ubiquinone 9 and 10 were well resolved in all tissues examined except for rat liver and some guinea pig livers, where an unidentified peak was not separated from ubiquinone 9 (Fig. 1c).

Responses from UV and electrochemical detectors were linear within the concentration range of interest (0.1–10 μM for tocopherols and ubiquinols, 0.1–20 μM for ubiquinones). Final concentrations of analytes in the different samples could be kept in a narrow range by adjusting the final volume.

Voltage *vs.* current responses (hydrodynamic voltammograms) were measured on the electrochemical detector for α-tocopherol, ubiquinol 9 and ubiquinol 10. Hy-

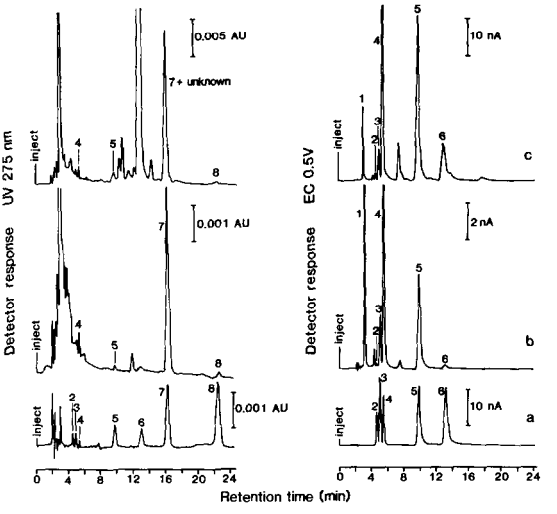

Fig. 1. Chromatograms obtained with UV and electrochemical (EC) detection of a standard mixture (a), rat muscle (b), and rat liver (c). A standard mixture, rat muscle and rat liver were separated on an ODS column with methanol–reagent alcohol (3:7, v/v) containing 20 m*M* lithium perchlorate as eluent. The standard mixture contained 4.9 μM δ-tocopherol (2) 5.2 μM γ-tocopherol (3), 2.4 μM α-tocopherol (4), 9.1 μM ubiquinol 9 (5), 11.0 μM ubiquinol 10 (6), 2.2 μM ubiquinone 9 (7) and 2.4 μM ubiquinone 10 (8). Peak 1 is BHT. Rat muscle was prepared as described from 101.2 mg tissue, rat liver from 121.0 mg tissue, final volume was 0.2 ml each.

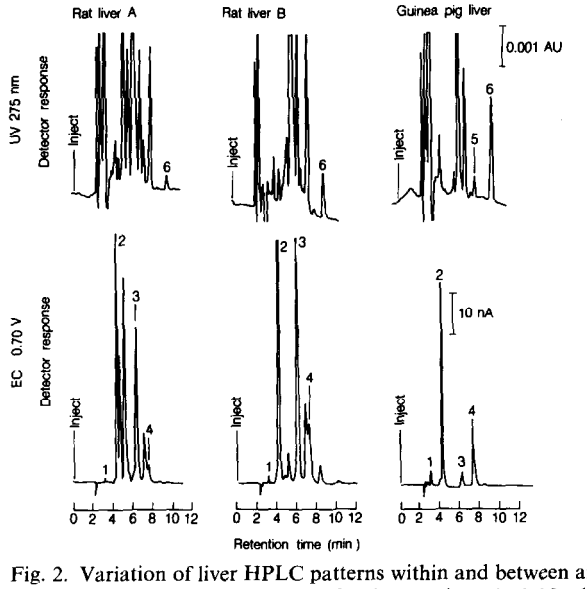

Fig. 2. Variation of liver HPLC patterns within and between animal species. Rat liver A: 104.8 mg tissue in final volume of 0.5 ml. Rat liver B: 93.0 mg tissue in 0.25 ml. Guinea pig liver: 100.8 mg in 0.25 ml. In this case, HPLC was performed on an Altex Ultrasphere C_8, 5-μm particle size column with an eluent composition of methanol–reagent alcohol (7:3, v/v) containing 20 m*M* lithium perchlorate. Peaks: 1 = BHT, 2 = α-tocopherol, 3 = ubiquinol 9, 4 = ubiquinol 10, 5 = ubiquinone 9, 6 = ubiquinone 10. Under these separation conditions β- and γ-tocopherol are not clearly separated from α-tocopherol but appear as tiny shoulder at the onset of the large α-tocopherol peak.

TABLE I

TOCOPHEROLS AND OXIDIZED AND REDUCED COENZYME Q IN VARIOUS TISSUES

Values are mean ± standard error of the mean, in nmol/g wet weight (solid tissues) or nmol/ml (blood, plasma). n.s. = not separated.

Tissue	*Source*	*β+γ-Tocopherol*	*α-Tocopherol*	*Ubiquinol 9*	*Ubiquinol 10*	*Ubiquinone 9*	*Ubiquinone 10*	*n*
Liver	Rat (A)*	<0.5	68.5 ± 4.5	102.7 ± 16.8	11.7 ± 4.0	n.s.	1.7 ± 0.4	8
Liver	Rat (B)*	<0.5	45.0 ± 5.2	77.1 ± 9.4	8.7 ± 1.0	n.s.	2.7 ± 0.5	10
Liver	Guinea pig	<0.5	17.0 ± 4.2	4.7 ± 0.8	23.9 ± 3.3	n.s.	20.4 ± 1.6	6
Muscle	Rat	3.1 ± 0.2	42.4 ± 0.8	4.4 ± 1.0	0.1 ± 0.1	41.2 ± 1.7	3.1 ± 0.3	9
Muscle	Guinea pig	<0.3	11.1 ± 1.3	0.5 ± 0.1	5.4 ± 0.1	0.9 ± 0.1	12.5 ± 1.5	6
Muscle	Human	8.3 ± 0.5**	33.4 ± 4.4**	<0.5	6.5 ± 3.2**	1.1 ± 0.3**	15.8 ± 5.6**	2
Brown adipose tissue	Rat	<1.0	187.6 ± 8.8	49.4 ± 5.0	7.0 ± 1.3	37.4 ± 3.0	6.2 ± 1.1	7
Brown adipose tissue	Guinea pig	<1.0	126.6 ± 24.8	17.2 ± 1.2	293.0 ± 23.6	10.9 ± 2.1	155.2 ± 35.7	6
Heart	Rabbit	1.2	6.1	<0.2	1.2	4.9	119.7	1
Spinal cord	Rabbit	0.5	9.1	<1.5	<1.5	4.1	8.2	1
Plasma	Rat	<0.5	28.8 ± 1.8	<1.0	<1.0	<0.5	<0.5	3
Plasma	Human	2.5 ± 0.4	23.5 ± 1.4	<0.1	<0.1	<0.1	<0.1	10
Blood	Human	1.3 ± 0.2	13.1 ± 0.7	<0.1	<0.1	<0.1	<0.1	10

* (A) and (B) denote different diets.

** Values are mean ± range.

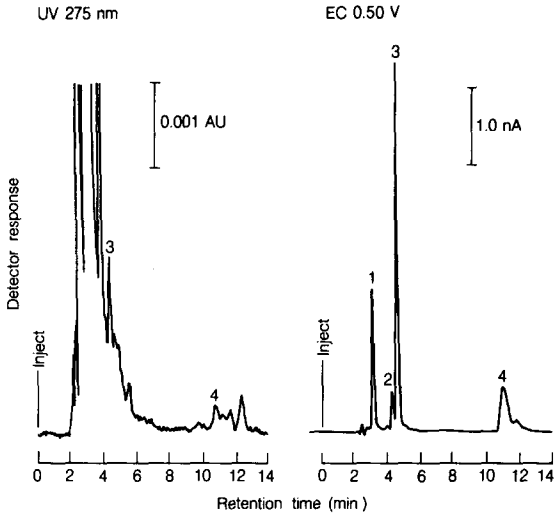

Fig. 3. HPLC profiles of human blood obtained with UV and electrochemical detection. A lipid extract of 0.2 ml human blood was obtained according to the described procedure and chromatographed on an Altex ODS column with methanol–reagent alcohol (1:9, v/v) containing 20 m*M* lithium perchlorate. Peaks: 1 = BHT, 2 = $\beta+\gamma$-tocopherol, 3 = α-tocopherol, 4 = unknown.

drodynamic voltammograms for the two ubiquinols were identical, while the α-tocopherol response was very similar and only slightly (0.02 V) shifted towards higher potentials. The lowest measurable signals were obtained at + 0.3 V, and the plateau was reached at + 0.9 V. Due to the high sensitivity of the electrochemical detector —standard concentrations in Fig. 1 are about three orders of magnitude above the detection limit— it is possible to operate at low potentials (+ 0.5–0.7 V), thereby achieving enhanced selectivity for the easily oxidizable tocopherols and ubiquinols. The sensitivity of the method is limited to the lowest amount of analyte detectable by the UV detector, which is about 1 pmol of ubiquinone.

Recovery of the compounds of interest by the lipid extraction procedure was determined by standard addition to a reagent blank and a liver homogenate. For these two samples, respective recoveries were 100 and 99% for α-tocopherol, 95 and 98% for ubiquinol 9, 89 and 95% for ubiquinol 10, 93% for ubiquinone 9 (peak was not completely resolved in rat liver and therefore not calculated), and 111 and 118% for ubiquinone 10. These results indicate that all compounds are quantitatively extracted and that a small amount of ubiquinol is oxidized to ubiquinone during sample preparation. Blood, but not plasma samples, required addition of ascorbate to obtain good recoveries, especially of ubiquinols. Without ascorbate, recovery of an added ubiquinol standard from blood was only 39 and 33%.

As the sample preparation procedure extracts all analytes quantitatively, their tissue concentrations can be determined by external standardization, *i.e.* by comparing sample peak heights with those of a standard mixture of defined composition (working standard, as described in Experimental). The amount of ubiquinol oxidation, likely to occur during sample preparation, was considered negligible. We had once considered to use a long chain ubiquinol analog as internal standard in order

to account for any ubiquinol oxidation during the sample preparation. Internal standard procedures had been used for tocopherols and ubiquinones[14], but they did not seem feasible for ubiquinols, because the ubiquinol analogs are too unstable.

Reproducibility of the procedure, as expressed by coefficients of variation was checked by three parallel extractions of a rat liver and four parallel extractions of a rat muscle homogenate. Values were 2.4 and 2.9% for α-tocopherol, 9.5 anmd 8.3% for ubiquinol 9, 9.3% for ubiquinol 10, 9.9% for ubiquinone 9 and 16.9 and 16.7% for ubiquinone 10. The large variation for ubiquinone 10 is most probably due to the low levels of ubiquinone 10 in these rat tissues.

Different species show characteristic qualitative and quantitative differences in their HPLC–UV and HPLC–electrochemical detection patterns (Fig. 2). Within species, the UV and EC chromatograms strongly reflect different diets (Fig. 2).

Table I summarizes tissues levels of tocopherols, ubiquinols, and ubiquinones, as obtained by the described procedure. As far as comparable data are available, the values agree well with those published previously[12,15].

The chromatograms obtained with electrochemical detection of most tissues show additional, well reproducible, yet unidentified peaks (Figs. 1–3). The fact that they are extractable with a lipid extraction procedure and generate a signal on the electrochemical detector defines them as redox-active lipids. They could be expected to have antioxidant properties similar to tocopherols and ubiquinols. Retention times and electrochemical behavior rule out retinyl esters or β-carotene.

CONCLUSIONS

The described procedure allows quantitative determination of endogenous tocopherols, ubiquinols, and ubiquinones from tissue lipid extracts by a single HPLC analysis. The lipid extraction procedure is fast, highly versatile, and quantitative for the compounds of interest. As a standard procedure we extract 50–100 mg tissue and redissolve the lipid extract in a final volume of 0.2–1.0 ml. However, the amount of tissue required can easily be scaled down by a factor of ten without loss of sensitivity, as the final volume can be decreased by the same factor (as little as 30 μl are sufficient for HPLC). This feature makes the procedure applicable to the analysis of human biopsy tissue.

ACKNOWLEDGEMENTS

We wish to thank Laurie Rothfuss for excellent technical assistance. This work was supported by the National Institutes of Health (AG 04818), the National Foundation for Cancer Research, the Office of Naval Research and Hoffmann-La Roche. J. K. Lang was supported by a Schroedinger fellowship from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung.

REFERENCES

1 G. W. Burton, K. H. Cheeseman, T. Doba, K. U. Ingold and T. F. Slater, in *Biology of Vitamin E (Ciba Foundation Symposium 101)*, Pitman Books, London, 1983, pp. 4–18.
2 P. Mitchell, *FEBS lett.*, 56 (1975) 1–6.
3 H. Bindoli, L. Cavallini and P. Jocelyn, *Biochim. Biophys. Acta*, 681 (1082) 496–503).

4 H. Kappus and H. Sies, *Experientia*, 37 (1981) 1233–1241.
5 M. Kofler, P. F. Sommer, H. R. Bolliger, B. Schmidli and M. Vecchi, *Vitam. Horm. (N.Y.)*, 20 (1962) 407–439.
6 Y. Hatefi, *Adv. Enzymol.*, 25 (1963) 275–328.
7 F. L. Crane and R. A. Dilley, in D. Glick (Editor), *Methods of Biochemical Analysis*, Vol. XI, Wiley-Interscience, New York, 1963, p. 279–306.
8 S.-H. Lin and E. C. Horning, *J. Chromatogr.*, 112 (1975) 465–482.
9 A. P. De Leenheer, V. O. R. C. De Bevere, M. G. M. De Ruyter and A. E. Claeys, *J. Chromatogr.*, 162 (1979) 408–413.
10 C. H. McMurray and W. J. Blanchflower, *J. Chromatogr.*, 178 (1979) 525–531.
11 L. J. Hatam and H. J. Kayden, *J. Lipid Res.*, 20 (1979) 639–645.
12 S. Ikenoya, M. Takada, K. Yuzurika, K. Abe and K. Katayama, *Chem. Pharm. Bull.*, 29 (1981) 158–164.
13 G. W. Burton, A. Webb and K. A. Ingold, *Lipids*, 20 (1985) 29–39.
14 S. Ikenoya, K. Abe, T. Tsuda, Y. Yamano, O. Hiroshima, M. Ohmae and K. Kawabe, *Chem. Pharm. Bull.*, 27 (1979) 1237–1244.
15 G. W. Burton, A. Joyce and K. A. Ingold, *Arch. Biochem. Biophys.*, 221 (1983) 281–290.