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Note

Direct high-performance liquid chromatographic separation of hydroperoxide isomers of linoleic acid

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Lipid peroxidation has important effects *in vivo* because the free radicals that are formed by the decomposition of lipid hydroperoxides can lead to cellular damage possibly responsible for ageing[1]. Polyunsaturated fatty acids are easily oxidizable and many workers have studied the autoxidation of linoleic acid and have identified four hydroperoxide isomers: 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid (13-*c*-*t*), 13-hydroperoxy-9-*trans*-11-*trans*-octadecadienoic acid (13-*t*-*t*), 9-hydroperoxy-10-*trans*-12-*cis*-octadecadienoic acid (9-*t*-*c*) and 9-hydroperoxy-10-*trans*-12-*trans*-octadecadienoic acid (9-*t*-*t*). These isomers have been separated by high-performance liquid chromatography (HPLC) after hydroxylation and methylation[2,3] or only after hydroxylation[4].

The purpose of this paper is to describe a method for the separation of these isomers directly without hydroxylation and/or methylation, which may cause isomerization of the unstable hydroperoxides.

EXPERIMENTAL

Reagents and chemicals

Linoleic acid ($\geq$99% pure) was purchased from Koch-Light, Tween 20 from Merck and disodium phosphate from Prolabo. *n*-Heptane (Chromasol) was supplied by S.D.S., ethanol by Merck and glacial acetic acid by Prolabo.

Instrumentation

An LDC high-performance liquid chromatograph was purchased from Sopares-France and equipped with a Constametric III pump, a Valco 7000 p.s.i. injector and a Spectromonitor III UV detector set at 234–239 nm.

Chromatography

Adsorption chromatography was carried out on a stainless-steel column (20 × 0.47 cm I.D.) of Spherisorb Si60 (particle size 3–4 μm). To improve the separation of the hydroperoxide isomers, we used solvents composed of *n*-heptane, ethanol and acetic acid in different proportions.

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TABLE I

COMPOSITION OF SOLVENT SYSTEMS USED FOR THE SEPARATION OF HYDROPEROXIDE ISOMERS AND WAVELENGTH USED FOR DETECTION

Solvent	*Component (v/v)*			*λ (nm)*
	n-Heptane	*Ethanol*	*Acetic acid*	
I	100	0.5	0.1	234
II	100	0.3	0.1	234
III	100	0.3	0.2	234
IV	100	0	1.7	238
V	100	0	2.3	239

Linoleic acid autoxidation

Linoleic acid was dispersed at a concentration of $2.5 \cdot 10^{-3}$ *M* with 0.5% Tween 20 in phosphate-buffered aqueous solution (0.025 *M*) at pH 9 under a nitrogen atmosphere.

The aqueous micellar solution of linoleic acid was adjusted to pH 6.9, placed in a glass tube and left in the dark, exposed to air, at room temperature for about 6 days.

Extraction of hydroperoxides

Hydroperoxides were extracted from the aqueous media with chloroform–methanol (1:1). After evaporation to dryness under reduced pressure at low temperature (30°C), the dry residue was dissolved in diethyl ether.

Chromatography of hydroperoxides

Different solvents were tested in order to achieve the most efficient separation of hydroperoxide isomers (Table I).

Hydroperoxides have maximum absorption at about 234 nm. Solvents I, II and III allowed hydroperoxides to be detected at this maximum because they contained a low percentage of acetic acid: Solvents IV and V, which contained a higher percentage of acetic acid, required detection at 238 and 239 nm, respectively. The flow-rate was 2 ml min^{-1}.

TABLE II

RETENTION TIMES (min) OF HYDROPEROXIDE ISOMERS ELUTED BY THE DIFFERENT SOLVENTS

Solvent	*13-c-t*	*13-t-t*	*9-t-c*	*9-t-t*
I	46	56	69	71
II	55	67	86	88
III	56	66	81	83
IV	64	70	77	80
V	36	39	46	48

RESULTS AND DISCUSSION

The retention times varied with the composition of the solvent (Table II).

Solvent systems composed of *n*-hexane with ethanol[3,5,6] or isopropanol[7] did not yield complete and direct separation of the hydroperoxide isomers without derivatization.

It was difficult to separate efficiently 9-*t*-*c* and 9-*t*-*t*. To improve the separation of these two isomers we first tested a solvent system composed of *n*-heptane (in place of *n*-hexane, which was less pure), ethanol and acetic acid in different proportions (solvents I, II and III). The results showed that an increase in the proportion of ethanol reduced the time required for chromatography but we obtained an incomplete separation of 9-*t*-*c* and 9-*t*-*t*.

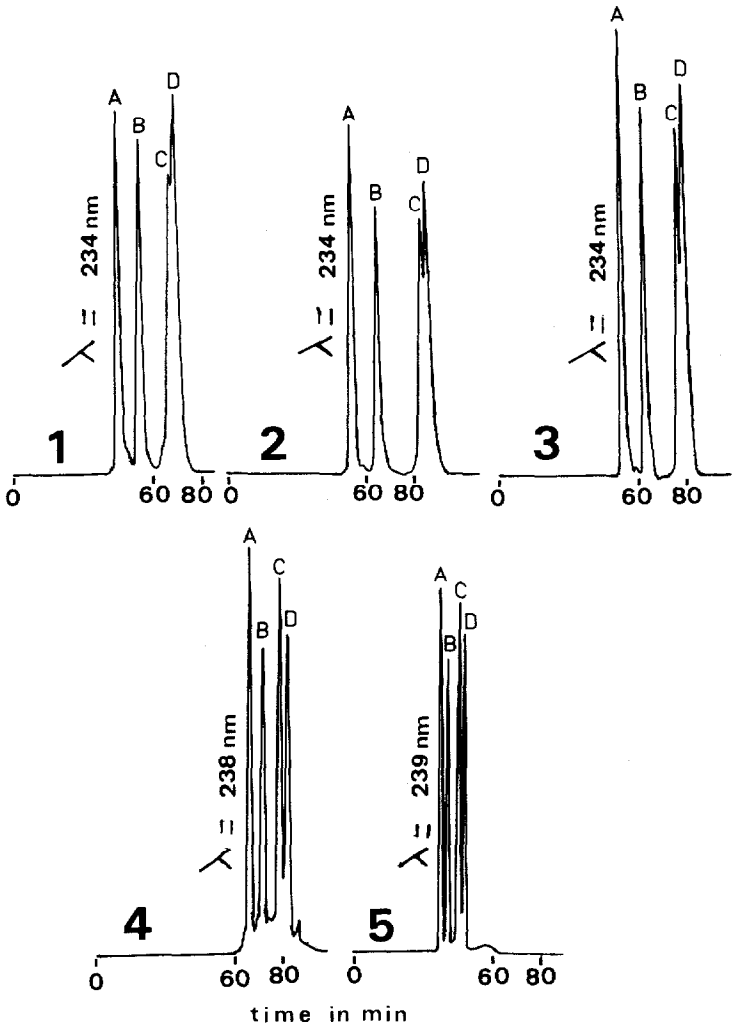

Fig. 1. HPLC separation of hydroperoxide isomers using different solvent systems. Solvents (Table I): 1, I; 2, II; 3, III; 4, IV; 5, V. Peaks: A = 13-*c*-*t*; B = 13-*t*-*t*; C = 9-*t*-*c*; D = 9-*t*-*t*.

An increase in the proportion of acetic acid improved the separation of these isomers as it reduced the time required for chromatography. The best result (complete separation and shortest time of chromatography) was obtained with 2.3% of acetic acid and without ethanol. The use of this percentage of acetic acid required detection of the isomers at 239 nm. This modification of the wavelength from that of maximum absorption of the hydroperoxides (234 nm) led to only a slight decrease in sensitivity (Fig. 1).

CONCLUSION

The method described allows the complete separation of the four hydroperoxides of linoleic acid without their hydroxylation and/or methylation.

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