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Short communication

Rapid reversed-phase high-performance liquid chromatographic determination of the regiospecificity of lipoxygenase products on linoleic acid

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Abstract

A new reversed-phase high-performance liquid chromatography method for the separation of regioisomeric products from lipoxygenase acting on linoleic acid was studied. The addition of salts to the mobile phase improved the retention and separation behaviour of 13-hydroperoxy-9,11-octadecadienoic acid and 9-hydroperoxy-10,12-octadecadienoic acid with respect to the results obtained with other mobile phases reported in the literature. The effect of the pH and ionic strength of the buffer on the retention times, capacity factor and separation factor of these lipoxygenase products were also studied. The pH optimum coincided with the pK_a of linoleic acid (close to 7 depending on the fatty acid concentration). Phosphate concentrations close to 100 m*M* considerably reduced the retention times and led to better separation of the mixture of both products. Finally, this method was applied to the identification and separation of two linoleic acid hydroxides (13-hydroxy-9,11-octadecadienoic acid and 9-hydroxy-10,12-octadecadienoic acid) obtained by the reduction of their corresponding hydroperoxides.

Keywords: Regioisomers; Lipoxygenases; Linoleic acid; Enzymes; Fatty acids

1. Introduction

Lipoxygenases (LOXs; linoleate: oxygen oxidoreductase, EC 1.13.11.12) are a group of enzymes that catalyse the dioxygenation of polyunsaturated fatty acids (PUFAs) containing one or more 1,4-*cis*,*cis*-pentadiene systems, such as linoleic (LA), linolenic (LnA), or arachidonic acid (AA) to conjugated hydroperoxy fatty acids [1].

Most lipoxygenases have been shown to catalyse the dioxygenation reaction regio- and stereospecifically, which means that, depending on the source of the enzyme [2] and on the reaction conditions [3], the incubation of LA with a particular LOX may yield 13*S*-/13*R*-hydroperoxy-9,11-octadecadienoic acid (13-HPOD), 9*S*-/9*R*-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) or a mixture of 13- and 9-HPOD. These primary products of LOXs contribute to the organoleptic properties of fruits and vegetables. The hydroperoxides formed by LOXs from LA and LnA are converted by hydroperoxide lyases into C-6 or C-9 aldehydes [4]. The cleavage of 9-hydroperoxides produces C-9 aldehydes

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(cucumber flavour) [5]; 13-HPOD or 13-HPOT (13-hydroperoxy-9,11,15-octadecatrienoic acid) are precursors of C-6 compounds (the major contributors to the characteristic flavour of tomato) [6]. For this reason, a method to analyse the regiospecificity of LOX products is important in the flavouring industry.

During recent years, different methods to identify the products of the LOX reaction have been proposed [7–9]. Wu et al. [10] separated different HPODs and hydroxides [13-hydroxy-9,11-octadecadienoic acid (13-HOD) and 9-hydroxy-10,12-octadecadienoic acid (9-HOD)] with a good resolution using normal-phase high-performance liquid chromatography (NP-HPLC). However, each HPLC run takes approximately 50 to 60 min. Reversed-phase (RP) HPLC, the other type of HPLC used for HPOD and HOD analysis [11], is often used for the analysis of AA hydroperoxides but does not give good resolution in the separation of LOX products on LA. Moreover, the mobile phases described to identify the products of reaction catalysed by LOXs have not resolved a mixture of products of different regiospecificity [12–14]. Although Chan and Levet [15] reported a good method for examining peroxidation regiochemistry using RP-HPLC, the samples needed a previous treatment prior to HPLC analysis.

In this paper we describe a method which permits the identification and separation of regioisomers LOX products using RP-HPLC. Hydroperoxides produced by the action of soybean LOX-1 and potato 5-LOX of known regiospecificity (13-HPOD and 9-HPOD, respectively) were used as standards in this work.

2. Experimental

2.1. Solvent and reagents

LA was purchased from Cayman (Paris, France). Soybean LOX-1 type IV and $NaBH_4$ were from Sigma (Madrid, Spain). Potato LOX was purified according to the method of Mulliez et al. [16] and had a specific activity of 27 μmol O_2/min/mg protein. Bakerbond spe C_{18} (J.T. Baker octadecyl 7020-01, 1 ml, 100 mg) solid-phase extraction columns were used to extract the hydroperoxides. The solvents methanol (Lab-Scan, Dublin, Ireland), tetrahydrofuran (THF) (Merck, Darmstadt, Germany) and acetonitrile (Romil, Loughborough, UK) were of HPLC-grade. All the other chemicals used were of analytical grade.

2.2. Preparation of HPODs and HODs

13-HPOD was prepared by incubating 0.1 m*M* LA with 15 μl of soybean LOX-1 (1 mg/ml) in 30 ml of borate buffer pH 9.0 at 4°C and under constant aeration. The reaction was followed at 25°C in a spectrophotometer Kontron Uvikon 940 equipped with thermostable cells and magnetic stirrer. The reaction, which was exhausted in 30 min, was stopped by acidification to pH 3.0. The solution was applied to an octadecyl solid-phase extraction column previously conditioned with two volumes of methanol and two volumes of water. The column was washed with four volumes of water and the products were eluted with one volume of methanol. 9-HPOD was obtained by incubation of 0.1 m*M* LA with 500 μl of 5-LOX potato (4.58 mg/ml) in 30 ml of 0.1 *M* phosphate buffer, pH 6.3 using the same procedure described for 13-HPOD. The concentration of the hydroperoxy fatty acids was determined from the absorbance at 234 nm (ϵ_{234}=25 000 M^{-1} cm^{-1} [16]).

9-HOD and 13-HOD were synthesised by reducing their corresponding hydroperoxides with $NaBH_4$ at 0°C under N_2. Then, water was added and the mixture was acidified with HCl to pH 3.0. The HOD was purified by solid-phase extraction using the procedure described for HPOD. HPODs and HODs were stored in methanol under N_2 at −20°C.

2.3. Analysis by RP-HPLC of HPODs and HODs

RP-HPLC analysis of the products was carried out on a Inertsil-2-ODS column (GL Science, 5 μm particles, 150×4.6 mm). The product samples were eluted isocratically at a flow-rate of 1 ml/min and detected at 234 nm using a Kontron 430 UV detector. A Reodyne 7125 loop injector was used to inject the sample solution (20 μl) into the column. In each sample a similar concentration (≈3 m*M*) of 13-HPOD and 9-HPOD was used.

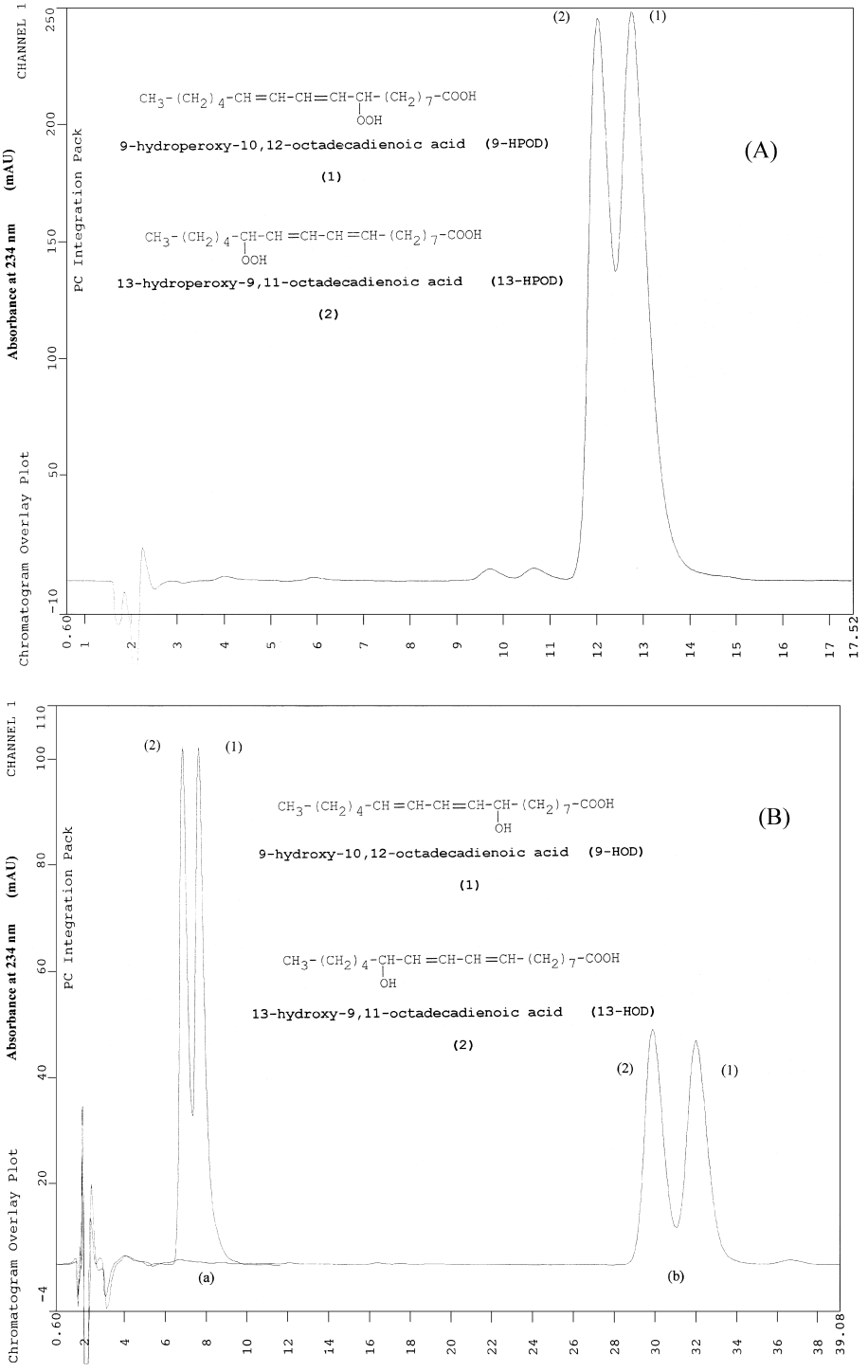

Fig. 1. (A) RP-HPLC analysis of 13- and 9-HPOD using: THF–MeOH–0.1 *M* phosphate buffer, pH 7.0–HAc (25:30:45:0.1) as mobile phase. (B) Reversed phase chromatograms of 13- and 9-HOD using (a) buffered mobile phase: THF–MeOH–0.1 *M* phosphate buffer, pH 7.0–HAc (25:30:45:0.1) and (b) THF–MeOH–water–HAc (25:30:45:0.1).

3. Results and discussion

3.1. Choice of the mobile phase

The addition of buffers to the mobile phase is a useful strategy frequently used in RP-HPLC to modify the resolution [17] although, to our knowledge, it has never been applied to the analysis of LOX products. As shown in Fig. 1A, the addition of 0.1 *M* phosphate buffer, pH 7.0 as a component of the THF–MeOH–buffer–HAc (25:30:45:0.1) mobile phase leads to a substantial decrease in retention times with respect to those reported by other authors (i.e., from the 40 min needed by Van Aarle et al. [14] to approximately 12 min). Furthermore, as will be later discussed, the resolution is also improved. This method facilitates the analysis of PUFA oxidation by LOXs as a large number of samples can be processed in a short period of time.

The next step was to study the different characteristics of the buffer that may influence the separation.

3.2. Effect of pH

The role played by the pH of the mobile phase in the identification and separation of 9-HPOD and 13-HPOD has been studied. In the acid and neutral pH region, the retention times are around 40 min and are not affected by the pH (data not shown). However, a dramatic decrease in the retention time is observed between pH 5.5 and pH 7.4, when HPODs elute at 10 min. The variation of the retention time of a sample with pH describes a sigmoid curve [18], the midpoint of this curve occurring at the pK_a of the sample. In the case of 13- and 9-HPOD, the midpoint of this curve is around 6.5 (data not shown), which is close to the pK_a of their corresponding fatty acid [19]. In addition, a decrease in the separation factor (α) is obtained when the pH is increased from 5.5 to 7.5 (data not shown). In conclusion, when the pH of the mobile phase is close to the pK_a of the fatty acid hydroperoxide, the separation of 9- and 13-HPOD is improved.

3.3. Effect of ionic strength

We studied the effect of another chemical–physical factor, the ionic strength of the buffer, on the separation of the primary products of LOXs by RP-HPLC. An increase in the ionic strength of the buffer leads to a considerable displacement of the retention times (data not shown). Thus, when no phosphate is used or when the phosphate concentration is low (22 m*M*), the retention times are over 30 min, while a 100 m*M* concentration of phosphate buffer shortens the retention time from 42 min (in the absence of phosphate) to 12 min. The presence of 100 m*M* of phosphate buffer produces a substantial decrease in the ln k' (capacity factor) of about 1.25 units (2.75 to 1.5) (data not shown). Furthermore, the separation factor (α) between 13-HPOD and 9-HPOD improves with increasing phosphate concentrations.

3.4. Use of the buffered mobile phase for the separation of 9-HOD and 13-HOD

Finally, we applied this method to the identification and separation of the 9-HOD and 13-HOD obtained by the reduction of the respective HPODs. The result is shown in Fig. 1B. As can be seen, the use of the THF–MeOH–buffer–HAc (25:30:45:0.1) mobile phase improves the sensitivity of the analysis and substantially reduces the retention times with respect to those obtained with THF–MeOH–water–HAc (25:30:45:0.1) (from 35 to 10 min), in the same way as was demonstrated for the primary reaction products of LOXs.

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