Application of High-Performance Liquid Chromatography for the Estimation of Peroxidized Phospholipids in Spray-Dried Egg and Muscle Foods

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High-performance liquid chromatography (HPLC) was applied to phospholipids extracted from raw egg and spray-dried egg, fresh and cooked ground beef, and raw, cooked, and salted-dried mackerel (*Cololabis saira*). On reversed-phase HPLC, stored spray-dried egg, fresh ground beef, and raw fish gave peaks corresponding to standard phospholipid hydroperoxides and were tentatively determined to contain hydroperoxides at the weight percentage in total phospholipids of around 2.4%, 0.2%, and 0.4%, respectively. No detectable level of phospholipid hydroperoxides was found in raw egg. Normal-phase HPLC provided an index of the oxidation levels for phosphatidylcholine and phosphatidylethanolamine by detecting at different wavelengths (203, 235 nm). Both HPLC analyses and the concomitant assays of thiobarbituric acid value and carbonyl value indicated that processing and cooking of such foods accelerate the formation of phospholipid hydroperoxides which, however, hardly accumulate during cooking.

Lipid peroxidation in food causes the appearance of unpleasant odor and toxic substances as well as nutritional damage. Lipid hydroperoxides, the primary products of peroxidation, have been suggested to possess a carcinogenic property (Cutler and Schneider, 1973, 1974). Therefore, it is of importance to quantify lipid hydroperoxides accumulated in food and foodstuff. Sensitive methods using fluorescence (Cathcart et al., 1983), chemiluminescence (Iwaoka and Tabata, 1984; Yamamoto et al, 1985), peroxidase (Heath and Tappel, 1976; Yamaguchi, 1980) or peroxidase activity of hemoglobin (Ohishi et al., 1985; Kikugawa et al., 1985), and cyclooxygenase (Marshall et al., 1985) were recently proposed for the quantification of lipid hydroperoxides in food or biological tissue.

We previously developed a method for detecting phospholipid hydroperoxides in vivo that combined reversed-phase high-performance liquid chromatography (HPLC) and normal-phase HPLC (Terao et al., 1984). The HPLC technique seems to be one of the most direct and specific methods for detecting lipid peroxides. The purpose of this work was to determine whether or not HPLC analysis is suitable for determining the phospholipid hydroperoxides in raw and spray-dried eggs, in fresh raw and cooked ground beef, and in raw, cooked, and salted-dried fish, all of which contain appreciable amounts of phospholipids (Weihrauch and Son, 1983). The results suggest that the HPLC technique can be applied to evaluate peroxidized phospholipids in these foods and foodstuffs.

MATERIALS AND METHODS

Materials. Spray-dried egg powder is a commercial product from whole egg. This was stored at room temperature for 9 months and then kept in the dark at 4 °C for 4 months prior to the experiment. A part of the spray-dried egg was incubated for another 3 months at 37 °C with adjustment of water activity (A_w = 0.80). Raw egg, fresh ground beef, and raw mackerel (*Cololabis saira*) were obtained from a local market. The round of beef was ground just before the experiment, and a part of the patties was stored at 4 °C for 2 days before and after cooking. Surface temperature of the patties was between 70 and 170 °C and the internal temperatures reached 70–100 °C. Mackerel was broiled in an oven at 300 °C for 4 min after removal of the head, gills, and intestines. Commercial salted-dried fish was obtained from the same source. Hydroperoxides of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from photosensitized oxidation products of respective phospholipids extracted from spray-dried egg and fresh ground beef by the procedure described previously (Terao et al., 1985a).

Preparation of the Phospholipid Fraction. The total lipids were extracted from the raw egg and spray-dried egg, fresh and cooked ground beef, and raw, cooked, and salted-dried mackerel by the method of Bligh and Dyer (1959). To obtain the phospholipid fraction, 5 mL of cold acetone and 100 μL of methanol containing 10% $MgCl_2 \cdot 6H_2O$ were added to each total lipid extract (ca. 100 mg), and the resultant mixture was cooled in an ice bath for 1 h (Kates, 1972). The mixture was centrifuged at 3000 rpm for 5 min. The precipitate was further washed with 2 mL of cold acetone and evaporated in vacuo. Residue was dissolved in methanol and chloroform (1:1, v/v) and then stored in the freezer (−20 °C) as the phospholipid fraction.

HPLC. HPLC was performed with a Shimadzu LC-4A equipped with a Rheodyne 7135 manual loop injector (200 μL), Shimadzu SPD-2A variable-wavelength detector (AUFS = 0.01), and Shimadzu CRI-B data processor. Reversed-phase HPLC was carried out with a YMC-packed ODS column (6 × 150 mm, Yamamura Kagaku, Co.). A mixture of methanol containing of 20 mM choline chloride/water/acetonitrile (90.5:7:0.5, v/v/v) was used as the eluent at a flow rate of 2 mL/min (Patton et al., 1982). Normal-phase YMC-packed SIL column (6 × 150 mm) was used for normal-phase HPLC. Acetonitrile was eluted isocratically for the first 5 min and then eluted with a linear gradient from 0 to 20% water in acetonitrile for 15 min. After 20 min, 20% water in acetonitrile was held constant for 20 min. The flow rate was 1.2 mL/min. The effluent was monitored at 203 and/or 235 nm. Sample solution (200 μL) was injected three times (each time with 800 μg of phospholipids for reversed-phase HPLC and 240 μg of phospholipids for normal-phase HPLC), and the average value was obtained.

Other Analytical Methods. TBA assay was carried out according to the method of Matsushita (1981). Total carbonyl compounds were determined by the procedure

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Table I. Fatty Acid Composition of Phospholipid Fraction Obtained from Raw Egg, Spray-Dried Egg, Raw Ground Beef, and Raw, Broiled, and Salted-Dried Mackerel

fatty acid	weight percentage					
	egg			mackerel		
	raw	spray-dried	raw ground beef	raw	broiled	salted-dried
14:0	*a*	0.4 ± 0.2	1.5 ± 0.4	1.9 ± 0.1	2.3 ± 0.1	2.3 ± 0.4
16:0	30.6 ± 0.7	30.5 ± 0.5	18.5 ± 0.3	19.4 ± 0.2	16.3 ± 1.0	20.7 ± 1.0
16:1	2.1 ± 0.3	2.2 ± 0.1	4.5 ± 0.6	0.9 ± 0.1	0.8	1.0 ± 0.1
18:0	14.0 ± 0.3	14.1	10.8 ± 0.2	9.7 ± 0.4	9.4 ± 0.7	8.8 ± 0.6
18:1	31.3 ± 0.5	32.0 ± 0.6	28.7 ± 0.5	3.7 ± 0.2	3.8 ± 0.2	4.5 ± 0.4
18:2	12.2 ± 0.1	12.5 ± 0.2	15.2 ± 0.3	0.5	0.5 ± 0.1	0.7 ± 0.1
18:3	0.4	0.6	0.6 ± 0.1	2.0	1.8 ± 0.3	3.6 ± 1.3
20:3	*a*	*a*	4.1 ± 0.2	*a*	*a*	*a*
20:4	4.6	3.9 ± 0.2	11.9 ± 0.3	2.3	2.2	4.2 ± 2.2
20:5	*a*	*a*	0.6 ± 0.1	3.9 ± 0.2	4.2 ± 0.5	3.8 ± 0.3
22:1	*a*	*a*	*a*	0.6	0.5	0.6 ± 0.1
22:4	*a*	*a*	1.8	*a*	*a*	*a*
22:5	*a*	*a*	*a*	1.4 ± 0.1	1.7 ± 0.1	1.3
22:6	4.9 ± 0.4	4.1 ± 0.4	1.4 ± 0.2	53.3 ± 0.6	55.6 ± 1.9	47.6 ± 3.9

[a] Less than 0.1%.

of Keller and Kinsella (1973). Fatty acid composition of the phospholipid fraction was determined by gas chromatographic analysis after methanolysis by the method described previously (Terao et al., 1985b). Contents of PC and PE in phospholipid fraction were determined with an Iatroscan TH-10 TLC/FID analyzer (Newman-Howells Associates, Ltd., Wolvesey Palace, Winchester, U.K.) (Tanaka et al., 1977). Three samples were prepared for all experiments involving HPLC analysis, and the results are expressed as the mean value with standard deviation. A significant difference was obtained by Student's t-test ($p < 0.05$).

RESULTS

Phospholipids corresponding the 18.5 ± 2.0%, 0.5%, and 0.7% of total samples were extracted from stored spray-dried egg, fresh ground beef, and raw mackerel, respectively. Their fatty acid compositions are listed in Table I. Spray-dried egg showed almost the same fatty acid composition as raw egg in which oleic acid (18:1) and linoleic acid (18:2) were the major unsaturated fatty acids. The level of arachidonic acid (20:4) was higher in fresh ground beef phospholipids than in spray-dried egg. Little change was observed in the fatty acid composition in spray-dried egg and ground beef before and after additional storage for 3 months and before and after cooking (data not shown here). Docosahexaenoic acid (22:6) constituted more than half of the total fatty acids in raw mackerel phospholipids. Salted-dried material showed a slightly lower content of 22:6, although this difference was not significant ($p < 0.25$). There were no changes in the fatty acid composition of the raw fish and broiled fish.

Figure 1 shows the typical elution patterns of unoxidized PC and PE and their hydroperoxides prepared from photosensitized oxidation products of spray-dried egg and fresh ground beef on reversed-phase HPLC. Each hydroperoxide could be clearly distinguished from its respective unoxidized species. However, hydroperoxides of PC and PE appeared with almost the same retention time in the range from 13 to 30 min. Standard curves for the determination of phospholipid hydroperoxides on reversed-phase HPLC were developed from the amounts ranging from 1 to 10 or 10 to 40 μg for PC and PE, respectively. A linear relationship was observed between the total peak areas due to phospholipid hydroperoxides and the amounts of hydroperoxides (μg) injected to HPLC (correlation coefficient >0.99) with similar regression lines (Spray-dried egg: PC, $y = 1.02x - 0.14$; PE, $y = 1.10x - 1.75$. Ground beef: PC, $y = 0.92x - 0.33$; PE, $y = 1.00x - 0.62$). The lowest detectable level for each injection was estimated to be 0.5 and 2.0 μg for PC-hydroperoxides and PE-hydroperoxides from the two sources, respectively.

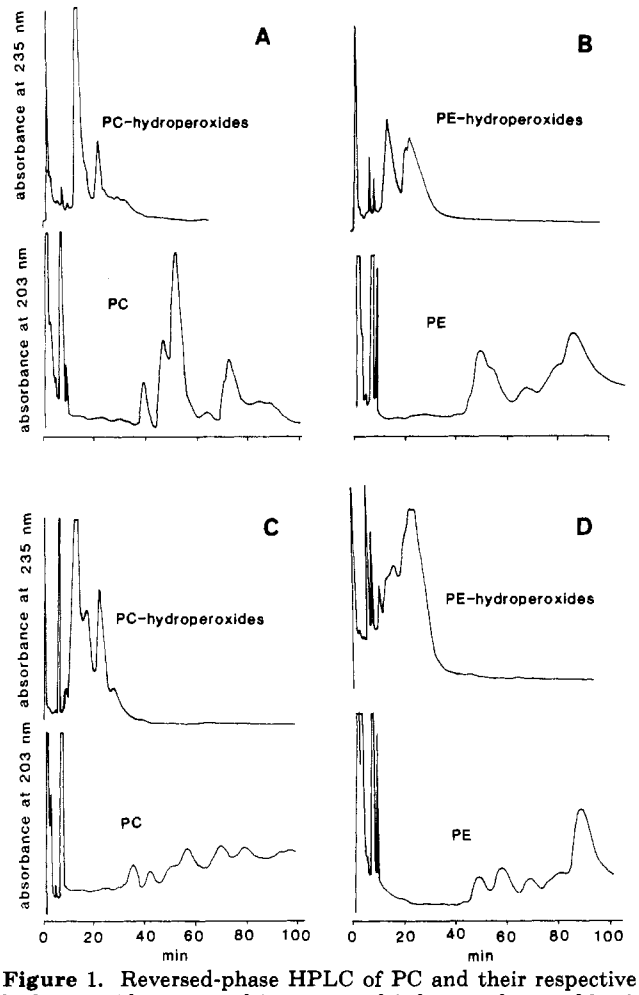

Figure 1. Reversed-phase HPLC of PC and their respective hydroperoxides prepared from spray-dried egg and ground beef: (A) PC and PC-hydroperoxides from spray-dried egg; (B) PE and PE-hydroperoxides from spray-dried egg; (C) PC and PC-hydroperoxides from ground beef; (D) PE and PE-hydroperoxides from ground beef.

Reversed-phase HPLC was applied to the phospholipid fraction extracted from raw egg and spray-dried egg, fresh and cooked beef, and raw, broiled, and salted-dried fish. Peaks whose retention times correspond to those of respective standard phospholipid hydroperoxides appeared

Table II. Determination of Peroxidized Phospholipids in Raw Egg, Spray-Dried Egg, Ground Beef, and Mackerel with and without Treatments

treatment	hydroperoxides, mg/g phospholipids	peak ratio (235 nm/203 nm) PE ($\times 10^{-2}$)	peak ratio (235 nm/203 nm) PC ($\times 10^{-2}$)
raw egg	nd[d]	1.6 ± 0.2[a]	2.7 ± 0.1[a]
spray-dried egg			
0-day storage	24 ± 1	2.9 ± 0.2	4.4 ± 0.4
1-month storage	37 ± 11	3.2 ± 0.7	4.7 ± 1.2
3-months storage	64 ± 10[a]	3.6 ± 0.7	4.5 ± 0.8
ground beef			
fresh	1.8 ± 0.4	1.5	2.1 ± 0.2
fresh-stored[e]	2.1 ± 0.8	1.9 ± 0.4	1.9 ± 0.1
cooked	1.1 ± 0.2[b]	1.3 ± 0.1	1.6 ± 0.1[b]
cooked-stored[e]	1.2 ± 0.2[b]	1.4 ± 0.1	1.5 ± 0.1[b]
mackerel			
raw	3.9 ± 0.7	2.9 ± 0.4	1.4 ± 0.2
broiled	6.4 ± 0.6	5.2 ± 1.2	2.1 ± 0.3
salted-dried	13.2 ± 3.7[c]	6.0 ± 1.5[c]	3.7 ± 0.3[c]

[a] Significantly different from spray-dried eggs before additional storage. [b] Significantly different from fresh ground beef. [c] Significantly different from raw mackerel. [d] Not detected. [e] Stored at 4 °C for 2 days.

for samples prepared from spray-dried egg and fresh and cooked beef. Such peaks were assumed to be phospholipid hydroperoxides, and their contents were calculated by matching the areas of all these peaks to the standard curve of phospholipid hydroperoxides. The contents of phospholipid hydroperoxides were obtained by using the respective standard curve as follows: phospholipid hydroperoxides = PC-hydroperoxides + PE-hydroperoxides; PC-hydroperoxides = (weight ratio of PC in phospholipids) × (the amounts determined by the standard curve for PC-hydroperoxides); PE-hydroperoxides = (weight ratio of PE in phospholipids) × (the amounts determined by the standard curve for PE-hydroperoxides). Weight percentages of PC and PE in total phospholipids were determined to be 75 ± 1% and 23 ± 1% for spray-dried egg and 58 ± 3% and 40 ± 1% for raw ground beef, respectively, and neither cooking nor additional storage changed this ratio significantly (data not shown here). Weight percentages of PC and PE in the phospholipid fraction of raw mackerel were determined to be 53 ± 2% and 35 ± 1%, respectively. In raw, broiled, and salted-dried fish, peaks appeared at the retention time corresponding to the hydroperoxides prepared from ground beef. Phospholipid hydroperoxides in fish were tentatively determined from the standard curves of phospholipid hydroperoxides of ground beef.

Weight percentages of hydroperoxides in total phospholipids were determined to be around 2.4%, 0.2%, and 0.4% for spray-dried egg, fresh ground beef, and raw fish, respectively (Table II). No detectable level of phospholipid hydroperoxides was found in raw egg. In ground beef, this level was decreased by cooking although not changed by storage in a refrigerator for 2 days. Clearly, salted-dried fish had a higher level of hydroperoxides than raw or broiled fish.

Next, the phospholipid fraction was applied to normal-phase HPLC, typical examples of which are shown in Figure 2. All classes of phospholipids were found to be completely separated within 40 min. Our previous work (Terao et al., 1984) indicated that the ratio of the peak area of each phospholipid detected by the absorption due to conjugated diene and that detected by the nonspecific end absorption can be used as an index of peroxidation level of each phospholipid (235 nm/203 nm). Table II shows the ratio of the peaks area of PC and PE from each phospholipid fraction. Raw egg gave lower ratios than spray-dried egg for both phospholipids. For ground beef, the ratios were lower after cooking, whereas the ratios for salted-dried fish were apparently higher than those for raw fish.

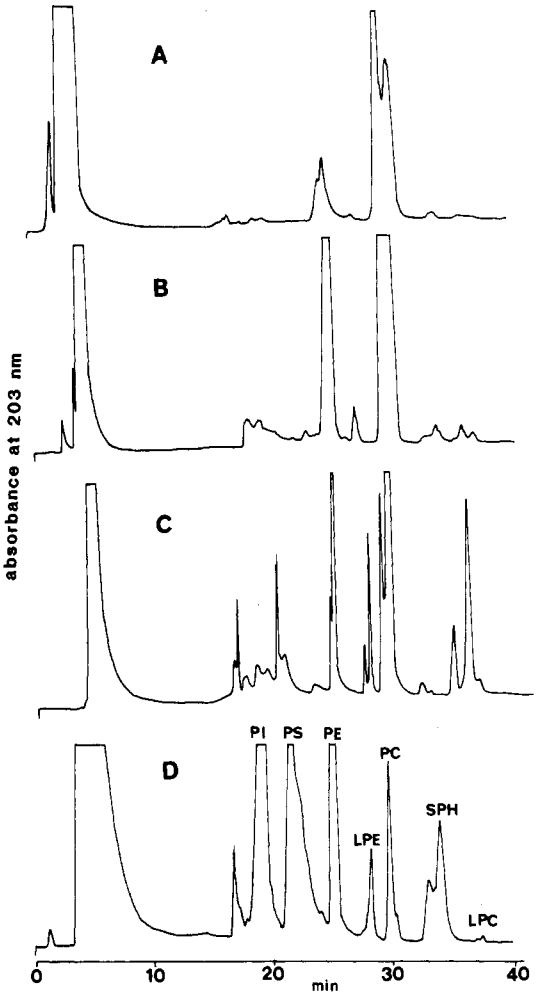

Figure 2. Normal-phase HPLC analysis of phospholipid fraction extracted from (A) spray-dried egg, (B) fresh ground beef, and (C) raw mackerel and that of a mixture of standard phospholipids (D). Key: PI, phosphatidylinositol; PS, phosphatidylserine; LPE, lysophosphatidylethanolamine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

Table III. Contents of TBA-Reactive Materials and Total Carbonyl Compounds in Raw Egg, Spray-Dried Egg, Ground Beef, and Mackerel with and without Treatment

treatment	TBA no., mg malondialdehyde/kg	total carbonyls, μmol/g
raw egg	0.5 ± 0.1[a]	*d*
spray-dried egg		
0-day storage	5.7 ± 0.2	0.20 ± 0.01
1-month storage	4.2 ± 0.8	0.79 ± 0.01[a]
3-months storage	6.0 ± 0.3	0.74 ± 0.01[a]
ground beef		
fresh	1.2 ± 0.1	0.10 ± 0.01
fresh-stored	0.9 ± 0.1	0.12 ● 0.01
cooked	3.7 ± 0.5[b]	0.21 ± 0.01[b]
cooked-stored	2.5 ± 0.5[b]	0.25 ± 0.05[b]
mackerel		
raw	23 ± 6	0.48 ± 0.16
broiled	31 ± 1	2.00 ± 0.47[c]
salted-dried	48 ± 9[c]	0.57 ± 0.15

[a] Significantly different from spray-dried egg before additional storage. [b] Significantly different from fresh ground beef. [c] Significantly different from raw mackerel. [d] Not determined. [e] Stored at 4 °C for 2 days.

The amounts of TBA-reactive materials and carbonyl compounds in whole tissue are shown in Table III. Mackerel gave a much higher level of TBA-reactive materials than spray-dried egg and ground beef regardless of treatments. The level of TBA-reactive materials in raw egg is lower than that for spray-dried egg. Cooking of ground beef increased TBA-reactive materials and carbonyl compounds. A higher level of carbonyl compounds was found in the fish after broiling.

DISCUSSION

It has already been demonstrated that PC-hydroperoxides can be eluted separately from unoxidized PC on reversed-phase HPLC (Porter et al., 1980; Crawford et al., 1980; Daolio et al., 1983; Ursini et al., 1983). We previously succeeded in the separation of PC- and PE-hydroperoxides from respective phospholipids of rat liver (Terao et al., 1984). However, the separation was definitely improved in this work by using the mixture of methanol, water, and acetonitrile. This eluting solvent was originally used to separate phospholipid molecular species (Patton et al., 1982). Thus, different molecular species of phospholipid hydroperoxides might produce broad peaks on the chromatogram as shown in Figure 1. A linear relationship between the total peak areas and the amounts of standard hydroperoxides confirms that this analytical condition is suitable for quantification of phospholipid hydroperoxides. In addition, it was found that normal-phase HPLC for the separation of the classes of phospholipids could be used to measure the oxidation level of each phospholipid in foods by using the ratio of the peak areas at the two different wavelengths. It should be noted that this ratio is a relative value and the absorption at 203 nm is influenced by the polar head group and fatty acid composition (Guerts Van Kessel et al., 1977). Recovery of hydroperoxides by extraction may be a problem when HPLC is applied to quantify phospholipid hydroperoxides. Phospholipid hydroperoxides would be recovered incompletely by the acetone precipitation method. However, the method of preparation of phospholipid fraction in this experiment is basically the same as that used in our previous work from which good recovery of phospholipid hydroperoxides was obtained (Terao et al., 1984). Accordingly, our HPLC method described here is considered to measure satisfactorily peroxidized phospholipids in food and foodstuff. Moreover, this technique facilitates the characterization of lipid hydroperoxides, and the analytical values seem to be scarcely influenced by other food components, although standard materials for the calibration curve should be derived from actual food materials studied when HPLC is applied to the quantification of lipid hydroperoxides in each food.

Tsai and Hudson (1984, 1985) and our group (Sugino et al., 1986) found that cholesterol epoxides accumulated in egg products as a result of the free-radical oxidation of cholesterol during processing or storage. Peroxidation of phospholipids seems to be easily induced in the spray-dried egg because polyunsaturated fatty acids (PUFA) in the phospholipids (18:2, 20:4, 22:6) are more sensitive to free-radical oxidation than cholesterol (Terao et al., 1985b). The spray-dried egg used in this work was stored for more than 1 year before the start of the experiment. Thus, the high level of peroxidized phospholipids estimated by HPLC analyses may have resulted from lipid peroxidation during long storage rather than processing. On the other hand, fresh ground beef and raw fish had much lower levels of phospholipid hydroperoxides than stored spray-dried egg, in spite of the high content of PUFA in their phospholipid fractions. Decreases in peroxidized phospholipid levels shown by HPLC analyses and increases in TBA reactive materials and carbonyl compounds after cooking of ground beef may indicate decomposition of hydroperoxides by heating resulting in secondary products. Moreover, muscle food contains ferric heme pigments, which have been suggested as major prooxidants (Love and Pearson, 1971) and are known to decompose PUFA hydroperoxides (Hamberg, 1975). Thus, hydroperoxides would hardly accumulate in cooked muscle foods. The high level of peroxidized phospholipids in salted-dried mackerel supports the idea that the drying process accelerates formation of lipid peroxides in fish (Shimizu and Kaneda, 1969).

To fully understand how lipid peroxidation occurs in foods, lipids other than phospholipids, such as triacylglycerol and free fatty acids, should be analyzed (Pizzocaro et al., 1985). However, phospholipids in general are the primary species responsible for lipid peroxidation in muscle foods (Younathan and Watts, 1960; Igene and Pearson, 1979; Pikul et al., 1984). Therefore, the results obtained from the HPLC analysis of phospholipid fractions may at least reflect the level of total lipid peroxides in such foods.

In conclusion, HPLC can be satisfactorily used for estimating peroxidized phospholipids in spray-dried egg and raw and cooked muscle foods. Furthermore, this technique seems to be helpful in indicating the level of lipid peroxidation in foods and foodstuffs.

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Comparison of Methods for Determining Myrosinase Activity

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Four assays for plant myrosinase (EC 3.2.3.1) were compared for linearity, sensitivity, reproducibility, and suitability for routine analyses. Methods: (i) pH-stat assay (pHSA); (ii) spectrophotometric coupled enzyme assay (SCEA); (iii) direct spectrophotometric assay (DSA); (iv) a new polarographic coupled assay (PCA) involving glucose oxidase and catalase, which measures the rate of glucose release as O_2 uptake during substrate hydrolysis. PCA and pHSA showed comparable activities and were linear with increasing amounts of purified enzyme up to 10 μg. As originally proposed, SCEA showed complete nonlinearity due both to the presence of ascorbate as myrosinase activator and to the low concentrations of Mg^{2+} and hexokinase–glucose 6-phosphate dehydrogenase. None of these methods appear suitable for routine work for different reasons. On the contrary, although DSA gave the expected lower activity compared to pHSA due to the suboptimum substrate concentration, it appears the most suitable for routine analyses given its simplicity and reliability.

Meals of rapeseed and other cruciferous seeds have high protein content and a well-balanced amino acid composition (Van Etten et al., 1965). However, the presence of large amounts of glucosinolates (thioglucosides) limits their use as feed, especially for meals produced with varieties not improved for this characteristic.

Glucosinolates are not deleterious in themselves; however, in the presence of myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) they degrade rapidly to give glucose, bisulfate, and an aglucon, which can undergo a spontaneous Lössen rearrangement to produce goitrogenic isothiocyanates or, via a protonation mechanism at low pH, toxic nitriles (Gil and MacLeod, 1980) as outlined in Scheme I.

The enzyme myrosinase seems to occur in all plants that contain glucosinolates (Ettlinger and Kjaer, 1968) even though enzymes with myrosinase activity have also been detected in some microorganisms, insects, and mammals (Björkman, 1976). Myrosinase appears to be an important enzyme, mostly for the biological and technological implications in food and feed quality and the safety of cruciferous material. For studies in this field it is evident that a dependable, rapid, and inexpensive assay to measure myrosinase activity is essential.

Over the last decade, numerous publications have described indirect and direct techniques for assaying myrosinase from several sources. The direct methods, viz. titration of released acid with alkali using pH-stat apparatus (Tookey and Wolff, 1970; Björkman and Lönnerdal, 1973), spectrophotometric measurements of the decrease in absorbance at 227 nm during sinigrin disappearance (Schwimmer, 1961; Gil and MacLeod, 1980; Palmieri et al., 1982), and recently a spectrophotometric coupled assay that measures the released glucose via hexokinase–glucose 6-phosphate dehydrogenase (Wilkinson et al., 1984a,b)

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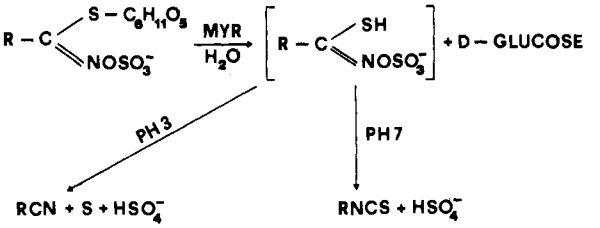
Scheme I



appear to be better than the indirect ones and are in theory all equally efficient, simple to use, and suitable for kinetic analyses as they allow one to determine myrosinase activity continuously. However, to our knowledge the relative advantages and disadvantages of the cited methods have never been extensively and objectively examined, especially in view of their utilization in breeding programs and in comparative studies of different plant tissues and cruciferous species.

This paper is a continuation of our work on cruciferous oil-bearing seeds (Palmieri et al., 1982, 1986; Iori et al., 1983). These studies prompted us to find other convenient methods for routine myrosinase determination that overcome the potential differences between enzyme sources and/or isoenzymes that could affect activity measurement. Therefore, the purpose of this study was to find a simple, dependable, and inexpensive assay that can rapidly analyze large numbers of crude or partially purified myrosinase samples. To this aim, an additional new polarographic assay, not previously used for this purpose, was tested. The present paper is a comparative study of four methods and also examines the effect of ascorbic acid.

MATERIALS AND METHODS

Materials. Commercial varieties of rapeseeds, *Brassica napus* L. cv. Jet Neuf (low erucic acid) and cv. Jade (low erucic acid and glucosinolate contents), were obtained from Ringot (Lille, France) and Norddeutsche Pflanzenzücht (Hohenliet, W. Germany) respectively. White mustard

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