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Evaluation of susceptibility to oxidation of linoleyl derivatives by thin-layer chromatography with flame ionization detection

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Abstract

A simple and rapid method for the evaluation of susceptibility to oxidation in microsamples was developed using a thin-layer chromatographic–flame ionization detection (TLC–FID) system. The procedure was applied to linoleic acid, methyl linoleate, trilinolein and sucrose octalinoleate. Samples of 15 μg were spotted on Chromarods, subjected to different temperatures for various times and analysed by TLC–FID. Oxidized products from linoleyl derivatives were determined from the amounts of unoxidized samples, determined in turn by two approaches, *i.e.*, using calibration graphs or adding squalane an an internal standard.

1. Introduction

Although many analytical methods are available for the measurement of lipid oxidation [1,2], it is difficult to determine the extent of oxidation and its evolution owing to the complex nature and variety of lipid oxidation products formed [3]. Moreover, published studies vary widely in their experimental conditions and analytical methodology, hence there are difficulties in drawing general conclusions [4–9]. An indirect analytical approach consists in measuring oxidative stability by means of accelerated methods using elevated temperatures, such as the Rancimat test [10], but a possible drawback with this method is the requirement for 2–3 g of lipid sample. It must also be considered that oxidation occurring in accelerated heating tests involve mechanisms that can be different from those encountered in practical situations, *e.g.*, storage conditions. Therefore, the necessity to develop and standardize accelerated tests under ambient conditions, essentially based on the exposure of oil to atmospheric oxygen at very high surface-to-volume ratio, has been reported [11].

The thin-layer chromatography with flame ionization detection (TLC–FID) has been used for the determination of polar components and oxidation products in edible oils subjected to different oxidation tests [12–16]. However, there is little information relating to its direct application to determine susceptibility to oxidation [17,18]. One of the chief advantages of TLC–FID over the other separation methods is the possibility of simultaneously developing and analysing ten samples so that direct comparison among samples under identical conditions can be made. The rapidity of the analysis and the small amount of sample required are of great convenience when a large number of samples need

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to be prepared or when only minimum amounts are available. Manipulation of the sample prior to analysis is usually not necessary as multiple solvent systems for development may be tried depending on the compounds to be evaluated. Additionally, reference standards may be incorporated in the sample to evaluate response factors [19].

In this paper, we describe a new possibility for determining susceptibility to oxidation in microsamples using TLC–FID. The method was applied to linoleic acid, methyl linoleate, trilinolein and sucrose octalinoleate.

2. Experimental

2.1. Materials

Linoleic acid (LA) and methyl linoleate (ML) were purchased form Nu-Check-Prep (Elysian, MN, USA). Squalane was purchased from Sigma (St. Louis, MO, USA). Trilinolein (LLL) was obtained by esterification of linoleic acid and glycerol, using *p*-toluenesulphonic acid as a catalyst [20]. Sucrose octalinoleate (SOL) was prepared starting from sucrose and excess of linoleic chloride to form the complete ester [21]. Isolation and purification of trilinolein and sucrose octalinoleate were carried out by means of silica gel column chromatography as described elsewhere [22].

2.2. Sample oxidation

Samples were dissolved in hexane (15 mg/ml) and 1 μl (15 μg) was spotted on Chromarods S-III quartz rods with a coating of silica gel (Iatron Labs., Tokyo, Japan). In another set of experiments, samples were dissolved in hexane containing 10 mg/ml of squalane, used as an internal standard, so that 10 μg of squalane plus 15 μg of sample were applied to each rod. All analyses were performed in triplicate. In order to study surface oxidation at different temperatures, rods were maintained at room temperature or heated in an oven at 60 or 100°C. Samples were analysed at various intervals for up to 10 h at room temperature, 6 h at 60°C and 30 min at 100°C.

2.3. Determinations

After the oxidation phase the Chromarods were developed in light petroleum (b.p. 60–70°C–diethyl ether–acetic acid (90:10:2) for 35 min and scanned in an Iatroscan MK-5 TLC–FID analyser (Iatron Laboratories) equipped with a flame ionization detector. The Iatroscan was operated under the following conditions: flow-rate of hydrogen, 150 ml/min; flow-rate of air, 1500 ml/min; and scanning speed, 0.33 cm/s. Unoxidized substrate was analysed for LA, ML, LLL and SOL using calibration graphs of peak area *versus* amount. Five determinations for each of five concentrations (1, 2.5, 5, 10 and 15 μg/μl) were made. Alternatively, squalane was used as an internal standard. The percentage of total oxidized compound was calculated by subtracting the percentage of unaltered sample from 100. The induction time period was determined by the Rancimat method at 60 and 100°C [10] using 0.5-g samples.

2.4. Statistical analysis

Each reported value represents the mean of three determinations and the standard error of the mean (S.E.M.). Student's *t*-test was applied to determine the significance of differences between means ($P < 0.05$).

3. Results and discussion

Calibration graphs for LA, ML, LLL and SOL using TLC–FID were plotted as amount in micrograms (Q) *versus* peak area (A), according to the following equations ($n = 25$ in each instance): LA, $Q = 0.13 + 0.217 \cdot 10^{-3}\ A$, $r = 0.9974$; ML, $Q = 0.24 + 0.267 \cdot 10^{-3}\ A$, $r = 0.9989$; LLL, $Q = 0.70 + 0.189 \cdot 10^{-3}\ A$, $r = 0.9993$; and SOL, $Q = 0.76 + 0.191 \cdot 10^{-3}\ A$, $r = 0.9997$.

All the compounds showed fairly good linearity in the concentration range employed. The

relative response of ML, LLL and SOL with respect to LA were 0.81, 1.15 and 1.14, respectively. Such variations were expected according to previous results [23] and the specific characteristics of this technique [19]. The coefficient of variation of the area measurement ranged from 0.90% at 10-μg load levels of ML to 4.31% at 1-μg load levels of SOL, which indicated a high reproducibility. Unoxidized compounds remaining after the oxidation phase were determined using these calibration graphs or else by the internal standard method. Squalane was selected as the internal standard particularly for its resistance to alteration under the experimental conditions, provided that it fulfilled the basic requirements for use as an internal standard. In preliminary assays, five samples of 10 μg of squalane were spotted on Chromarods, heated in an oven at 100°C for 5 h and simultaneously analysed with five original samples. No significant differences were found between the treated and original squalane samples. The relative responses of squalane with respect to LA, ML and LLL were 1.13, 1.37 and 0.97, respectively. Total oxidized products, or higher polarity than that of the original lipid, were determined by difference. We used this approach because the polar fraction is a complex mixture of oxidation products and its composition depends on the heat treatment and could therefore change drastically from one sample to another. These differences would be expected to induce changes in the FID response factors and thereby considerable errors may result from direct analysis of the polar fraction. Moreover, volatile compounds originating from the breakdown of peroxides and hydroperoxides may be lost prior to detection.

Fig. 1 presents typical TLC–FID traces for initial samples and those obtained after oxidation at 100°C for 15 min. As can be observed, the selected solvent system permitted for all samples a good separation of the unoxidized compound from the more polar oxidized products. The procedure allowed the evaluation of susceptibility to oxidation in 15-μg samples at various temperatures and time periods, in a short analysis time and with the possibility of revealing the whole lipid pattern of the sample, thus adding to the reliability of the determination. Special care was taken with regard to sample purity and also cleanliness of the rods and evenness of the temperature within the oven, which were essential factors for good reproducibility.

Table 1 gives selected results for total oxidation products from all the compounds deter-

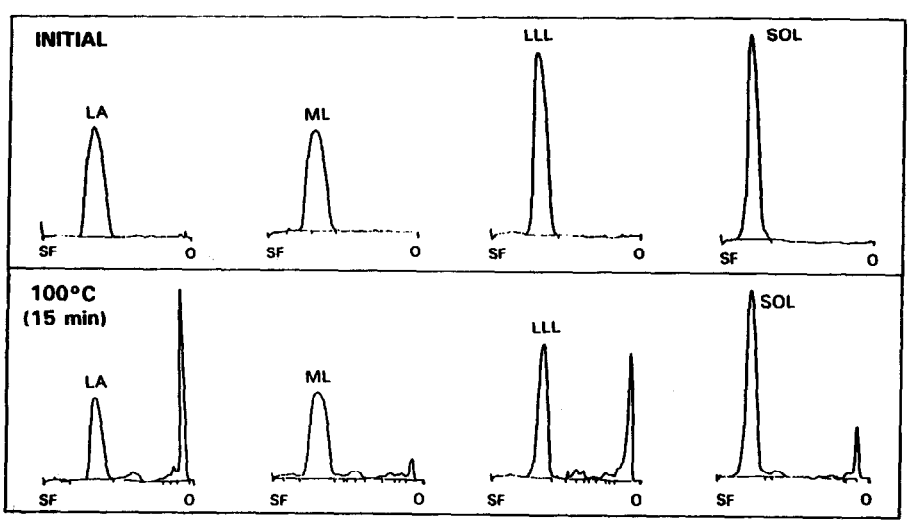

Fig. 1. Iatroscan TLC–FID traces for linoleic acid (LA), methyl linoleate (ML), trilinolein (LLL) and sucrose octalinoleate (SOL) showing the effect of oxidation on Chromarods after 15 min at 100°C. O = Origin; SF = solvent front.

Table 1
Total oxidation products of linoleic acid (LA), methyl linoleate (ML), trilinolein (LLL) and sucrose octalinoleate (SOL) on Chromarods S-III at 25 and 100°C (% of total compounds).

Compound	25°C			100°C		Induction period at 100°C
	3 h	5 h	10 h	15 min	30 min	(h)
LA	n.d.	8.3 ± 2.9^{a}	28.4 ± 3.3^{a}	52.9 ± 3.6^{a}	69.7 ± 6.1^{a}	0.6
ML	n.d.	n.d.	11.9 ± 3.0^{b}	18.1 ± 2.9^{b}	30.5 ± 3.0^{b}	0.9
LLL	n.d.	19.6 ± 3.1^{b}	52.9 ± 3.1^{c}	48.1 ± 3.1^{a}	62.7 ± 3.2^{a}	1.1
SOL	n.d.	5.1 ± 2.9^{a}	16.3 ± 2.8^{b}	8.3 ± 1.8^{c}	21.7 ± 2.8^{b}	1.3

Values are means ± S.E.M. of three determinations. Values in a column with different superscript letters are significantly different ($P < 0.05$). n.d. = Not detectable.

mined following oxidation at room temperature (*ca.* 25°C) and 100°C. Unoxidized substrate was determined using calibration graphs. The last column shows the induction periods at 100°C using the Rancimat apparatus. From the results obtained, comparisons between sample behaviours were possible. The oxidation rate of compounds was dependent on the temperature tested. Thus, the rise in oxidized substrate was fastest and most extensive for LA at 25°C whereas both LA and LLL showed the highest values at 100°C. Several papers have been published on the comparison of the oxidation rates of various types of unsaturated fatty acid esters [24–27]. It has been shown that oxidation of the fatty acid was more rapid than that of the methyl or ethyl ester, probably owing to the participation of the carboxyl groups in the decomposition of peroxides [25,28]. Also, higher oxidation rates have been found for triacylglycerol than for the fatty acid ester, although the kinetics of triacylglycerol autoxidation did not seem to follow the usual rate law [26]. It is important to note that among linoleic esters, LLL and SOL, in contrast to ML, may undergo oxidation in up to three or eight fatty acyls, respectively. Hence greater total oxidized substrates, as determined by this method, could be expected for LLL and SOL than for ME, substantiated by the fact that a considerable proportion of oxidized molecules contain unoxidized fatty acyl groups [29]. This could explain in part that higher oxidized amounts resulted from LLL than for ML. However, SOL showed unexpected lower percentages of oxidized products than LLL at any temperature tested, and generally similar values to those for ML. Further, it should be stressed that similar values of total oxidation products for SOL and ML would still indicate lower levels of oxidized fatty acyls for SOL (eight fatty acyls per molecule) than those for ML (1 fatty acyl per molecule). These data were consistent with the induction period at 100°C, which was longer for SOL than for ML and LLL. The differences between LLL and SOL agreed with our previous results [22], which indicated that the oxidation of sucrose octaesters occurred more slowly than that of the triglyceride with a similar fatty acid composition when starting from pure compounds. In contrast, it has also been reported that the autoxidation rates of sucrose esters were higher than those of triacylglycerols and methyl esters of safflower oil [27]. However, whereas triacylglycerols and methyl esters were purified, sucrose esters were not completely acylated as the average number of acyl groups per molecule was six, which means the presence of compounds with a wide range of polarity.

Table 2 summarizes the results obtained at 60°C when the internal standard method was used for quantification. The last column lists the induction periods as measured with the Rancimat apparatus. SOL was not included as it overlapped with squalane with the elution system used. Clearly, the use of an internal standard was advantageous in that the reproducibility and

Table 2
Total oxidation products of linoleic acid (LA), methyl linoleate (ML) and trilinolein (LLL) on Chromarods S-III at 60°C (% of total compounds)

Compound	1 h	2 h	4 h	6 h	Induction period at 60°C (h)
LA	n.d.	11.0 ± 2.1	84.6 ± 0.8[a]	85.2 ± 0.4[a]	2.5
ML	n.d.	n.d.	9.4 ± 0.7[b]	18.6 ± 1.8[b]	12.2
LLL	n.d.	n.d.	21.1 ± 0.9[c]	20.8 ± 1.6[c]	14.9

Values are means ± S.E.M. of three determinations. Values in a column with different superscript letters are significantly different ($P < 0.05$). n.d. = Not detectable.

accuracy of the determination were not affected by possible variations in the amount of sample applied to the rod. In general, the results showed the same alteration order as that observed at 100°C. LA oxidized more rapidly than LLL and ML and these findings were in good agreement with the induction periods. Differences between the Rancimat and TLC–FID techniques, such as the surface-to-volume ratio, detection system and the possible influence of silanol groups accessible on the surface, did not contribute to modifying the alteration order of the samples, which was found to be consistent with both methods.

Overall, the results obtained in this study with linoleyl derivatives give evidence of the important influence of the compound structure on the oxidation rate. The approach suggested here for evaluating oxidative stability by TLC–FID can be widely applied to compare different lipid samples that are available only in milligram amounts. Another valuable application of the proposed technique could be as a rapid and simple method to determine the potential influence of minor compounds and the protective effect of antioxidants on oil and fat stability. Experiments along these lines are in progress.

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5. References

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