

# LIPID AUTOXIDATION OF FISH, LARD, CORN AND LINSEED OILS BY ISOTHERMAL CALORIMETRY

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## ABSTRACT

This work proposes an approach to characterize lipid oxidation of oils based on the measurement of isothermal calorimetry data, including information on the duration of the monomolecular, bimolecular and termination periods. The approach has been exploited with fish and lard oil samples at temperatures from 40 to 80°C and with corn and linseed oils at 80°C. The length of the monomolecular period was the most sensitive to the variation of temperature. Accordingly, the monomolecular period was used as index of oxidative stability of oils. Thus, the highest oxidative stability was observed for corn oil samples at 80°C ( $t_{\text{mono}} = 2060$  min), followed by linseed ( $t_{\text{mono}} = 390$  min), fish ( $t_{\text{mono}} = 40$  min) and lard oils ( $t_{\text{mono}} = 30$  min). The different stability of the samples was discussed with their fatty acids profile and antioxidant activity. The results confirmed that the content in natural antioxidants is the key responsible for the final oxidative stability of the samples.

*Keywords:* autoxidation periods, fish oil, edible oils, isothermal calorimetry, oxidative stability

## 1. INTRODUCTION

Lipid autoxidation is a radical chain mechanism that involves a reaction between unsaturated fatty acids and oxygen (JOHNSON and DECKER, 2015; TUORILA and CARDELLO, 2002). This reaction is of great importance for the food industry, as its occurrence is often associated with quality defects, like rancidity or nutritional loss. The mechanism of reaction is generally described in three main stages: an “*initial phase*”, comprising the monomolecular phase of hydroperoxide formation. This is followed by the “*propagation phase*”, where radicals are formed with autocatalytic, monomolecular and bimolecular or branching reactions, resulting in the exponential increase of the rate of lipid autoxidation. Then, the process ends with a “*termination phase*”, where the rate of lipid reaction slow down and increase the formation of secondary oxidation products (GHNIMI, 2017). Between the first and second phase, the abrupt change in the rate of lipid autoxidation is often defined as the induction period (IP). This value is widespread used by food industry as index to characterize the oxidative stability of an oil sample (FRANKEL, 2014).

The measurement of the induction period is generally obtained by differential scan calorimetry (DSC) (SHAHIDI and ZHONG, 2015; LITWINIENKO *et al.*, 1999) or differential thermal analysis (DTA) (BACHA *et al.*, 2015). However, the IP value obtained with these techniques is often difficult to interpret when the samples have a complex composition. One drawback of DSC is that, at low temperatures, the exothermic peak of oxidation is flat, and the IP value cannot be determined unambiguously. Moreover, when DSC is run at high temperatures, the oxidative behavior of foods become dependent on the temperature gradient used and also on the geometry of the samples (FOX and STACHOWIAK, 2007; KOUSKSOU *et al.*, 2011). Furthermore, the analysis of oil samples that contain antioxidants at high temperatures may lead to misleading results when the antioxidants are volatile and can leave the sample before they can exert their protective action (HAMAMA and NAWAR, 1991).

An alternative approach for the analysis of lipid autoxidation is offered by isothermal calorimetry (HAMAN *et al.*, 2017a). This technique has some advantages respect DSC and DTA, as it can be used to monitor in real-time the rate of autoxidation of unsaturated fatty acids under mild temperatures. Its theoretical background is relatively simple and well-described by Willson (WILLSON *et al.*, 1995) and Hansen (HANSEN *et al.*, 1990). Recent applications of isothermal calorimetry include the study of oxidation of lipid oxidation (DRIDI *et al.*, 2016), the study of the radical scavenging activity of lipophilic antioxidants (HAMAN *et al.*, 2017b), antioxidant and pro-oxidant activity of spent coffee extracts (HAMAN *et al.*, 2018) and the study of shelf-life of foods (RIVA *et al.*, 2001). However, in these studies, the calorimetric trace was mainly used to provide the cumulative enthalpy evolution of the sample, or the induction time. In such way, only a small fraction of the information contained in the calorimetric trace is used.

For this reason, this work aims to propose a methodological approach that can extend the information normally achievable with calorimetric data, providing information about the duration of the monomolecular, bimolecular and termination phase of the lipid autoxidation process. For this purpose, fish and lard oil samples were analyzed by isothermal calorimetry at different temperatures (from 40 to 80°C), and the results compared with those obtained with linseed and corn oils at 80°C. The relevance and utility of this approach was discussed considering the fatty acid composition and the antioxidant capacity of the oils.

## 2. MATERIAL AND METHODS

### 2.1. Materials

Refined corn oil, fish oil, lard oil, cyclohexane (purity  $\geq 99\%$ ), sodium thiosulphate (purity  $\geq 99\%$ ) and potassium iodide (purity  $\geq 99\%$ ) were purchased from Sigma-Aldrich (Milan, Italy). Refined linseed oil (cold pressed) was from Sabo Italia s.r.l. (Firenze, Italy). Chloroform (stabilized with ethanol) was purchased from PanReac AppliChem (Milan, Italy). Acetic acid (purity  $\geq 99.7\%$ ) was purchased from Fluka (Milan, Italy).

### 2.2. Fatty acid profile by near infrared spectroscopy

Fatty acids were determined by near infrared (NIR) spectrometer with a Multipurpose Analyzer (MPA) (Bruker Optik GmbH, Germany). Acquisition software was OPUS 7.5 (Bruker Optik GmbH, Germany). The background spectrum consisted of empty cell at the same temperature of analysis. OPUS/QUANT package was used to quantify the composition of fatty acids (palmitic, stearic, oleic, linoleic and linolenic acid) in fish, lard, linseed and corn oils. The analysis was performed at the same conditions of the calibration kit (calibrations based on 8084 spectra) in the range of 12500-4000  $\text{cm}^{-1}$  (resolution, 16  $\text{cm}^{-1}$ ; sample, 64 scans) at 40°C.

### 2.3. Isothermal calorimetry

A micro-calorimeter (Thermal Activity Monitor, Model 421 TAM III, TA Instruments), equipped with 24 channels, was used to measure the heat rate. Each channel of the instrument is a twin calorimeter where the two units are positioned above each other. In isothermal mode, the oil in the thermostat was maintained at a constant temperature with an absolute accuracy of  $\pm 0.0005$  °C (manufacturer's data). The micro-calorimeters were equipped with built-in metal reference specimens having a heat capacity approximately equal to that of a vial.

The heat generated or absorbed was continuously measured over time. Following the manufacturer's instructions, each channel was calibrated prior to measurement using a gain calibration procedure with electric impulses. Ampoules were first lowered into the thermal equilibration position and left there for 15 min. Then, the ampoules were lowered into the measurement position and the heat flow rates recorded for up to 5 and 8 days at 10-second intervals. The auto-oxidation reaction was monitored by calorimetry with close glass ampoules containing  $100 \pm 3$  mg of oil in isothermal conditions in the presence of air in the head space.

### 2.4. DPPH assays for the determination of the antioxidant activity

The antioxidant activity of the oils was evaluated using 1,1-diphenyl-2-picrylhydrazil (DPPH) as described by Brand Williams (BRAND-WILLIAMS *et al.*, 1995) with small modifications. DPPH is a free radical that can react directly with most of the antioxidants and be captured by them (KOLEVA *et al.*, 2002). The reduction of DPPH was measured by the decrease in absorbance at a characteristic wavelength (517 nm) and a determined time during the reaction (minimum 60 min). In the radical form (DPPH $\cdot$ ) presented a violet color at 517 nm. With the addition of a reduced antioxidant (AH), its color changed from violet to yellow and consequently the absorption decreased as a result of the decrease of the DPPH concentration. Briefly, 10 mg of DPPH were dissolved in 250 mL of ethanol and sonicated for 1 min. A defined amount of oil was dissolved in 1 mL ethanol and sonicated

(DU-06, China) for 5 min. To perform the measurements, 1.9 mL of DPPH solution were transferred into the cuvettes and 100  $\mu$ L of the extracts solutions were added. The samples were kept in dark for 1 h at room temperature (ca. 25 °C) and subsequently the absorbance was recorded at 517 nm with a spectrophotometer (Cary 100 Series UV-Vis Spectrophotometer, Agilent Technologies, Italy). The antioxidant capacity of the oils was determined as the IC<sub>50</sub> - the concentration of antioxidant, which reduces the radical form (DPPH•) of 50%. A triplicate assay was performed for each sample.

## 2.5. Determination of peroxide value

The peroxide value (PV) of the samples was measured according to the AOAC Official Method 965.33 (HORWITZ, 2002). The method is based on an iodometric titration, which measures the iodine produced from the potassium iodide by the peroxides present in the oil. The measurements were performed in triplicate for each sample and PV values were expressed as meqO<sub>2</sub> per Kg of oil.

## 2.6. Statistical analysis

Calorimetric results were reported as the mean of six independent experiments. All other measurements (NIR and DPPH) were performed in triplicate (n = 3). All the results are reported as mean, together with the relative standard deviation. To compare the means values, data were subjected to the analysis of variance (ANOVA) using Fisher's least significance difference method with a 95 % confidence level.

# 3. RESULTS AND DISCUSSION

## 3.1 Oxidative stability based on monomolecular and bimolecular periods

Figure 1 shows the heat-flow signal of a fish oil sample recorded at 40°C. At any point, the intensity of the heat-flow signal reflects the rate of the autoxidation process that is occurring to the sample, accordingly to the following general relationship (eq. 1) (WILLSON *et al.*, 1995):

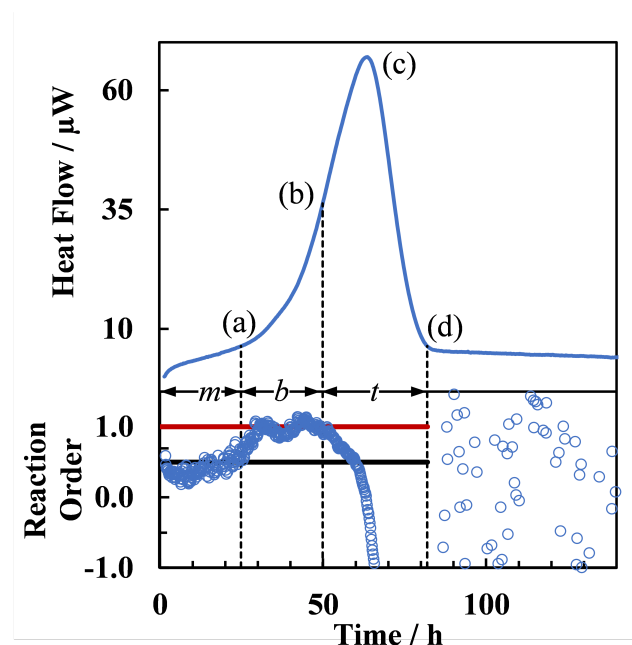
$$\text{heat flow} = \text{rate} \times \text{enthalpy} \quad (1)$$

At the beginning of the experiment, the signal is negligible (from the beginning to the point (a), Fig. 1). This corresponds to a slow autoxidation process. At about 25 h (point (a) of Fig. 1), the rate of heat production suddenly increases, at point (b), reaching a maximum heat flow value at point (c). After that, the signal fades down to a minimum steady state, at point (d). The final heat-flow signal has an intensity close to that recorded at the beginning of the experiment.

Figure 1 also shows the changes in the apparent reaction order as a function of time. The apparent reaction order was determined at any time following an approach previously described by Haman (HAMAN *et al.*, 2017a). Briefly, for a reaction  $A \rightarrow B$ , where A represents unsaturated fatty acids and B is their oxidation products, the heat-flow signal ( $dq/dt$ ) is equal to  $k \cdot H \cdot x^n$ , where  $k$  is the rate constant,  $H$  is the reaction enthalpy,  $x$  is the number of moles of product formed at time  $t$  and  $n$  is the reaction order. Since  $x$  can be obtained, at any time, as the ratio between the heat ( $q$ ) and the enthalpy ( $H$ ), then this equation becomes (eq. 2):

$$\frac{dq}{dt} = k \cdot H \cdot \left(\frac{q}{H}\right)^n = k \cdot H^{1-n} \cdot q^n$$

The log-log plot of the heat flow with the heat leads a slope, which corresponds to the reaction order ( $n$ ). Thus, by following the changes of  $n$  as a function of time, it is possible to identify three distinct phases of the autoxidation process. For fish oil, the first phase is characterized by a reaction order equals to  $\sim 0.5$ . This order corresponds to the *monomolecular period*, during which the reaction is catalyzed by monomolecular decomposition of the hydroperoxides ( $\text{LOOH} + \text{LH} \rightarrow \text{LO}^* + \text{L}^* + \text{H}_2\text{O}$ ) (LABUZA *et al.*, 1969; GHIMNI *et al.*, 2017). The duration of such phase lasts in about  $25 \pm 0.3$  h. Interestingly, this phase ends with the onset point of the calorimetric trace (also called induction point). This point is very informative since can be interpreted as the moment when a food become unacceptable for human consumption (LABUZA and DUGAN Jr, 1971). After this point, the reaction order becomes close to 1. It is during this period that the rate of lipid autoxidation rises exponentially. In this exponential stage, the bimolecular decomposition of hydroperoxides is the dominant mechanism ( $\text{LOOH} + \text{LOOH} \rightarrow \text{LO}^* + \text{LOO}^* + \text{H}_2\text{O}$ ). This process is known to follow the *bimolecular period* (LABUZA and DUGAN Jr, 1971). The length of such phase lasts in about  $25 \pm 0.3$  h (from point (a) to point (b) of Fig. 1).



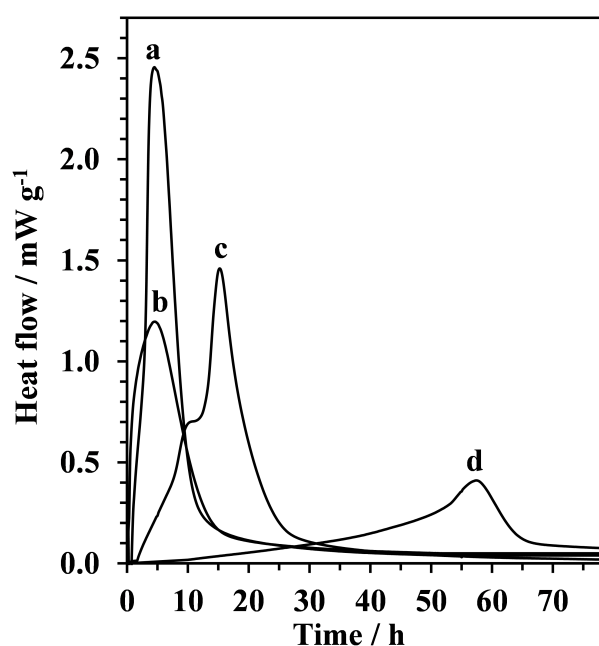
**Figure 1.** Calorimetric trace of fish oil in isothermal conditions at  $40^\circ\text{C}$ . Legend: (a) end of the monomolecular phase; (b) end of the bimolecular phase; (c) the maximum heat flow and (d) the end of the termination phase; Legend:  $m$  stands for monomolecular phase;  $b$  stands for bimolecular phase and  $t$  stands for termination phase.

After this point, the reaction order, suddenly, declines towards negative values. Negative reaction orders correspond to the so-called "*termination phase*", where the rate of the reaction is either inhibited by the high concentration of the reactants (i.e. radicals species) or by the absence of oxygen in the ampoule. In this stage, radical-radical combinations occur, and hydroperoxides start to exponentially decompose to secondary oxidation products, including aldehydes and ketones (GHINMI *et al.*, 2017). In the experimental

setup used here, the termination phase occurred when the concentration of oxygen in the ampoule was limiting. This was verified in a control experiment, where, successively to the analysis of a fish oil sample, the ampoule was opened without removing the sample, fresh air was left to enter into the headspace of the ampoule, and the recording of the calorimetric signal re-started. In such way, we observed that a new exothermic peak could be observed. This result confirms that the termination phase was limited by oxygen and that further monomolecular and bimolecular process could occur provided that oxygen is available. Accordingly, the termination period of the observed autoxidation process in the ampoule was defined as the period occurring from point (b), where the reaction order starts decreasing, to point (d), where the reaction is likely terminated because of oxygen depletion in the headspace of the ampoule. At times above 80 h, the reaction was completed. The measure of the reaction order become unpredictable because of the heat flow values were nearly constant, with slopes equals nearly to zero.

### 3.2. Oxidative stability of corn and linseed oil

The same approach used before with fish oil was next applied to characterize the oxidative stability of different oil samples, including fish, lard, linseed and corn oils. All the sample showed similar initial peroxide values. The resulting heat flows were plotted as a function of time (Fig. 2).



**Figure 2.** Heat flow *vs.* time curve plot of a) fish oil, b) lard oil c) linseed oil and d) corn oil, placed isothermally at 80°C into closed glass ampoule.

Table 1 reports the duration of the monomolecular, bimolecular and termination step. By comparing the duration of the monomolecular period at 80°C, the highest oxidative stability was observed for the corn oil sample (2060 min), followed by linseed (390 min), fish (50 min) and lard (30 min) oil samples.

**Table 1.** Kinetic and thermodynamic parameters of fish, lard, corn and linseed oil.

Oil	Temperature (°C)	Phase duration			$\Delta H$ (kJ/mol O <sub>2</sub> )
		Monomolecular (min)	Bimolecular (min)	Termination (min)	
Fish	40	2220±20	802±10	1780±35	43.1±0.1
Fish	50	600±8	653±11	1102±24	59.3±0.4
Fish	60	220±2	450±4	802±8	71.9±0.5
Fish	70	90±4	311±2	648±6	84.0±0.6
Fish	80	40±3	224±4	470±9	97.8±0.3
Lard	40	2490±35	1519±25	5161±71	36.4±0.3
Lard	50	550±2	190±3	3120±59	47.8±0.7
Lard	60	200±3	121±3	2402±53	66.2±0.9
Lard	70	600±3	60±2	1570±31	74.7±0.8
Lard	80	30±4	104±18	1143±21	67.7±0.5
Linseed	80	390±7	1102±18	1681±31	77.3±0.2
Corn	80	2060±25	1121±11	1160±13	46.2±0.1

Furthermore, Table 1 shows the changes of the monomolecular, bimolecular and termination durations as a function of temperature. For fish and lard oil samples, higher temperatures corresponded with faster reaction rates and, consequently, shorter duration of these periods. Moreover, the relationship between the duration of such phases follows the so-called Arrhenius function, where the logarithm of the duration of each phase leads a linear relationship when plotted as a function of the inverse of temperature ( $\ln(t)$  vs  $1/T$ ). For fish oil samples, the slopes of such linear relationships were  $1.1 \cdot 10^3$  K,  $3.6 \cdot 10^3$  K and  $3.5 \cdot 10^3$  K, respectively, for the monomolecular, bimolecular and termination phase. Similar results were obtained for lard oil samples. The slopes were in this case equal to  $1.2 \cdot 10^3$  K,  $3.7 \cdot 10^3$  K and  $4.1 \cdot 10^3$  K, respectively, for the monomolecular, bimolecular and termination phase of lard oil samples. These values suggest that the most sensitive phase to the changes in temperature is the monomolecular phase. This information is important for food manufacturers, since it provides an evidence that the antioxidants needed to stabilize such oils are those with the highest capacity to retard or stabilize the monomolecular phase.

Last experiments aimed to understand the reasons of the different oxidative stability observed between the oil samples. The ranking observed could be explained considering either the quality of fatty acids composition (i.e. presence of mono- or poly-unsaturated fatty acids) or the antioxidant content of the samples. Table 2 reports the fatty acids profile and the antioxidant content of the oil samples. What is striking is that the ranking of oxidative stability of the oil samples obtained according to the duration of the monomolecular phase cannot be predicted simply by looking at the polyunsaturated fatty acid composition of the samples. Instead, the oxidative stability of the samples matches perfectly with the antioxidant capacity determined by the DPPH assay on the methanolic extracts of the samples. In practice, the oil samples with the highest oxidative stability (i.e. longer duration of the monomolecular phase, as determined by calorimetry) are also those with the highest antioxidant capacity (i.e. shorter value of the DPPH assay).

**Table 2.** Fatty acid composition, peroxide value and antioxidant capacity of fish, lard, corn and linseed oil.

	Corn oil	Linseed oil	Fish oil	Lard oil
<i>Fatty acids (% w/w)</i>				
Palmitic acid (16:0)	14.7±0.6 <sup>b</sup>	6.3±0.5 <sup>c</sup>	15.6±1.4 <sup>b</sup>	26.6±1.6 <sup>a</sup>
Stearic acid (18:0)	2.9±0.3 <sup>b</sup>	4.2±0.6 <sup>b</sup>	3.5±0.8 <sup>b</sup>	10.7±0.9 <sup>a</sup>
Oleic acid (18:1)	30.8±1.8 <sup>b</sup>	18.8±1.6 <sup>c</sup>	11.1±0.9 <sup>d</sup>	46.8±2.8 <sup>a</sup>
Linoleic acid (18:2)	48.4±1.7 <sup>a</sup>	17.9±1.3 <sup>b</sup>	2.5±0.5 <sup>d</sup>	9.3±0.7 <sup>c</sup>
Linolenic acid (18:3)	1.1±0.2 <sup>b</sup>	50.4±3.1 <sup>a</sup>	1.5±0.3 <sup>b</sup>	1.8±0.2 <sup>b</sup>
Total SFA	17.6±0.5 <sup>b</sup>	11.1±0.7 <sup>b</sup>	23.1±1.1 <sup>b</sup>	37.3±1.5 <sup>a</sup>
Total PUFA	49.5±2.3 <sup>b</sup>	68.3±3.9 <sup>a</sup>	51.7±2.3 <sup>b</sup>	11.1±0.6 <sup>c</sup>
Total MUFA	30.8±1.4 <sup>b</sup>	19.2±1.8 <sup>d</sup>	22.6±2.1 <sup>c</sup>	49.6±2.4 <sup>a</sup>
Others	2.1±0.2 <sup>a</sup>	1.4±0.4 <sup>a</sup>	2.6±0.6 <sup>a</sup>	1.9±0.2 <sup>a</sup>
<i>Peroxide value (m<sub>eq</sub>O<sub>2</sub>/kg oil)</i>	2.9±1.2 <sup>b</sup>	1.6±0.5 <sup>c</sup>	4.2±0.3 <sup>a</sup>	5.4±1.7 <sup>a</sup>
<i>DPPH (IC<sub>50</sub>)</i>	70.3±1.5 <sup>d</sup>	83.9±2.7 <sup>c</sup>	251.8±6.4 <sup>a</sup>	98.9±3.4 <sup>b</sup>

Legend: SFA is saturated fatty acids; PUFA is polyunsaturated fatty acids; MUFA is monounsaturated fatty acids.

Values with different letters in the same row are significantly different ( $p < 0.05$ ).

Values are presented as mean±standard deviation ( $n = 3$ ).

#### 4. CONCLUSIONS

The results of the present study show the potential of isothermal calorimetry to monitor the oxidation processes of edible oils and exemplify a procedure to determine the monomolecular, bimolecular and termination periods according to the changes in the apparent reaction order of the overall process. In addition, we observed that the monomolecular phase was the most sensitive to the changes in temperature. This implied that the duration of the monomolecular phase is very informative to explain the oxidative stability of an oil sample. According to corn, linseed, lard and fish oil samples, their oxidative stability was not correlated with their qualitative composition in fatty acids. Instead, their oxidative stability was explained with their antioxidant capacity. On a more general perspective, this work demonstrated the potential of isothermal calorimetry to predict the oxidative stability of oil samples and characterizing the duration of the monomolecular, bimolecular and termination phase of the autoxidation process.

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