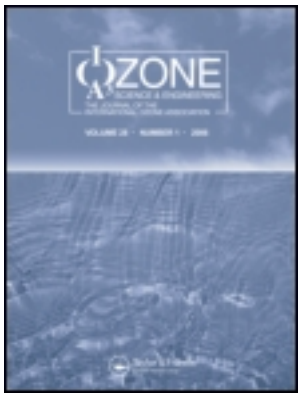

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Stability Studies of Ozonized Sunflower Oil and Enriched Cosmetics with a Dedicated Peroxide Value Determination

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Ozonized oils have interesting applications in the cosmetic industry and several patents on enriched products were developed in the last years. Ozonides are known for their high reactivity and stability data are required for enriched cosmetic products during storage. In this paper a dedicated determination of Peroxide Value (PV) was performed on ozonized sunflower oil (Neozone 4000) and on some enriched cosmetics, in order to control their stability during storage. The optimal conditions of the PV method were determined, following the decomposition reaction of ozonides with KI utilizing ^{1}H-NMR and GC/MS techniques.

Keywords Ozone, Ozonized Sunflower Oil, Cosmetic Product, Peroxide Value (PV), ^{1}H-NMR, GC-MS, Density

INTRODUCTION

Ozone is a powerful oxidant, principally applied as a disinfectant of drinking and waste water, but it can be used also in several medical applications, such as dermatology, gynecology, angiology, cardiology and also cosmetology (Bocci 2005). Recently, ozone has also been used to produce several forms of ozonized vegetable oils, applied in a large numbers of medical indications, because of their capability to damage bacterial nucleic acids and then to destroy the dangerous microorganisms. In fact, ozonized sunflower oil was applied with good results in the treatment of onychomycosis (Menéndez et al. 2010), while ozonized olive oil was tested on guinea pig for the acute cutaneous wound healing (Kim et al. 2009).

Ozone easily reacts with carbon-carbon double bonds of unsaturated fatty acids present in triglycerides of vegetable oils. This reaction gives rise to several oxygenated products, such as ozonides, hydroperoxides, aldehydes, peroxides, diperoxides and polyperoxides (Diaz et al. 2005). These oxygenated compounds confer to the oils many favorable characteristics, such as antibacterial, fungicidal and antiviral properties with consequently interesting applications in the cosmetic and pharmaceutical industries (Hernandez et al. 2009; Sadowska et al. 2008; Valacchi et al. 2005).

The raw material for the ozonization reaction has to present high levels of unsaturated fatty acids. Sunflower oil, very rich in linoleic acid and in oleic acid is the more utilized ozonized vegetable oil in the pharmaceutical field (Diaz et al. 2006). Also sesame oil (Zanardi et al. 2008) and soybean oil (Sadowska et al. 2008) are good substrates for the ozonization reaction, while olive oil, due to its lower level of polyunsaturated fatty acids, gives lower yields of ozonized products (Diaz et al. 2006). Ozonized oils have already been characterized under several points of view by many researchers.

In 2001, Rosado et al. reported the GC/MS characterization of the volatile fraction of ozonized sunflower oil, demonstrating that it was mainly composed of degradation compounds of fatty acid ozonides, such as saturated and unsaturated aldehydes (hexanal, nonanal, 3-nonenal and malonaldehyde), and carboxylic acids. In 2003, Soriano et al. monitored the ozonization of sunflower oil methyl esters by FT-IR and ^{1}H- and ^{13}C-NMR spectroscopy, identifying reaction products according to the Criegee mechanism. NMR is a very useful tool to follow the ozone reaction with unsaturated fatty acids of sunflower oil. In fact, the intensities of fatty acid olefinic proton signals decrease with increasing of ozone concentrations, and characteristic signals of ozonides appear in the spectra (Diaz et al. 2006; Gomez and Sazatornil 2005). Moreover, other measurements such as the determination of density, acidity, iodine values and peroxides value are useful to evaluate the ozonization degree.

The peroxide value (PV), in particular, is the quantity of the peroxides reported as milliequivalents of active oxygen in 1000 g of oil sample. Its determination requires a reaction

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of the peroxides of oil with potassium iodide in an acidic medium. After reaction, the iodine formed is titrated with sodium thiosulphate and the value is reported as PV. The variables that can influence the results are: the weight of sample used, the volume of solvents, the amount of potassium iodide added, the reaction time and the temperature. Some researchers (Diaz et al. 2006; Sadowska et al. 2008) determined PV of ozonized oils using the official method reported in the British Pharmacopoeia (2000, 2008), others (Tellez et al. 2006; Zanardi et al. 2008) modified this method in reason of the very high peroxide values usually showed by ozonized oils. Moreover, there is still the need of improving the PV procedures to better measure the peroxide title of ozonized oils and of enriched cosmetics as skin creams and massage oils that undergo some degradation of the added active oil during storage, and have to be checked to know the effective stability of the cosmetic products in the time.

To correctly evaluate the quality and the stability in the time of the ozonized sunflower oil, alone and in cosmetics, we further checked the PV method, by studying the behavior of the reaction between ozonides and potassium iodide under different experimental conditions in order to find the best conditions to determine the oxidation properties of a high value ozonized sunflower oil (Neozone 4000) and those of the enriched cosmetics.

High resolution NMR spectroscopy was used to evaluate the content of the ozonide functions and to monitor the reaction with potassium iodide during the PV determination, in order to find the end point of the reaction and the time necessary to reach it. The main volatile products formed during the reaction with KI were recovered and identified by GC/MS analysis. The preservation of ozonized sunflower oil and several derivatives was also checked by measuring the peroxides value under different storage periods and conditions.

EXPERIMENTAL PROCEDURE

Materials

Chemicals were purchased from Sigma-Aldrich (Milan, Italy) and used without further purifications. All the ozonized samples were supplied from a cosmetic factory (Cosmoproject s.p.a., Parma, Italy).

Ozonized sunflower oil samples

Ozone was produced by an O_3 generator (LA/003, Ozonline International, Brandizzi, Italy) using medical-grade oxygen. Ozone formation was achieved using the electrical corona discharge method, applying a voltage of 6400 V, for a generator of 5 L of capacity, while in the case of a generator of 30 L of capacity a voltage of 9500 V was used. The generated ozone was led to a reaction vessel where ozone was bubbled through the oil. 60 samples of ozonized sunflower oil were analyzed and the amount of ozone absorbed was controlled both by determination of PV value and by density measurements.

Cosmetics containing ozonated sunflower oil

The samples used for the stability experiments are listed in Table 1. In order to study the stability of the sunflower oil and the enriched cosmetics during storage, three groups of samples were prepared: one stored at 4 °C, a second kept at 25 °C and a third maintained at 40 °C. The control analyses were done in triplicate on the fresh products (time zero), and then on all the samples after 30, 60, 90, 180 and 360 days. All the collected samples, oils and cosmetics, were placed and maintained in standard plastic containers in order to avoid eventual differences caused by a different storage materials.

Density Measurement

The density measurements were carried out on 60 oil samples using a Mettler Toledo 30PX densitometer (Columbus, OH, USA), at room temperature (25°C). The samples utilized for this analysis were oils obtained from the same starting sunflower oil, treated with increasing amounts of ozone.

Peroxides Value Determination

The peroxide value (PV) or peroxide index (PI) is a number that expresses in milliequivalents of active oxygen the quantity of peroxides contained in 1000g of the substance (British Pharmacopoeia 2008). In this work, PV was determined introducing changes to the official method. In particular, 0.1 g of ozonized sunflower oil or 0.3–0.6 g of ozonized cosmetic products were weighed in a 100 mL conical flask and added of 20 mL of methylene chloride/glacial acetic acid (2/3 v/v). When the sample was completely dissolved, 1 mL of a saturated potassium iodide solution was added. Then, the solution was stored for 16 h at 25 °C in the dark. After this time, the solution was added of 20 mL of distilled water and then titrated with sodium thiosulphate solution (0.05 M), adding 1 mL of starch solution. Peroxide value was calculated by the following equation:

$$PV = (V^*c^*1000)/w$$

where "PV" is the peroxide value expressed in meq kg^{-1}, "V" is the volume measured (mL) of sodium thiosulphate used for the sample titration corrected for the volume used for the blank titration, "c" is the normality of sodium thiosulphate and "w" represents the grams of sample used.

Reaction of Ozonized Sunflower Oil and Potassium Iodide

The reaction between ozonides of the oil and KI was investigated at room temperature (25°C) following the procedure described for the peroxide value determination. The oil samples were titrated with sodium thiosulphate solution (0.05 M) after 0.5, 2, 6 and 16 h of reaction with KI. Titrated samples were extracted with 20 mL of methylene chloride and evaporated under vacuum at room temperature. The residues were dissolved in 0.5 mL of $CDCl_3$ and analyzed by ^{1}H-NMR

TABLE 1. Peroxides Values (PV, mEq/1000g) in Ozonized Oil and Enriched Cosmetics Determined at Time Zero and after 30, 60, 90, 180 and 360 Days of Storage at Three Different Temperatures (4, 25 and 40 °C)

Sample	Ozonized oil (Neozone) percentage	Initial PV	Storage temperature (°C)	PV 30 days	PV 60 days	PV 90 days	PV 180 days	PV 360 days
Skin cream 1	10	507	4	465	522	518	453	454
			25	413	533	542	382	410
			40	413	375	362	173	157
Skin cream 2	10	536	4	514	544	531	469	438
			25	577	525	555	408	418
			40	449	377	540	174	173
Massage oil 1	10	560	4	647	703	608	426	439
			25	611	638	542	414	426
			40	529	448	504	320	254
Massage oil 2	20	1034	4	1059	1096	1057	741	737
			25	947	885	1035	637	604
			40	937	786	828	439	380
Ointment 1	10	480	4	511	500	513	368	369
			25	515	500	507	386	346
			40	421	474	517	250	152
Ointment 2	20	833	4	841	828	823	683	673
			25	813	896	848	620	633
			40	762	737	767	458	320
Ozonized sunflower oil (Neozone 4000)	100	4167	4	4235	4062	4051	3524	3524
			25	4263	4045	4062	3425	3354
			40	3853	3434	3433	1415	1317

Standard deviations were lower than 5%.

(600 MHz). The residue of the sample titrated after 16 h was further analyzed by GC-MS.

NMR Spectroscopy

NMR spectra were recorded on a VARIAN INOVA-600MHz spectrometer (Palo Alto, CA, USA), operating at 14.1 T, equipped with a 5-mm triple resonance inverse probe. Data were collected at 298K, with 32K complex points, using a 45° pulse length. 32 scans were acquired with an acquisition time of 1.896 and a recycle delay of 1 s. The NMR spectra were processed by MestreC software (Mestrelab Research, Santiago de Compestela, Spain): spectra were Fourier transformed with FT size of 32 k and 0.3 Hz line-broadening factor, phased and baseline corrected, and referenced to the chloroform signal (7.26 ppm).

GC/MS Analysis

First, 50 mg of residue of ozonized oil treated with KI, and titrated with thiosulphate after 16 h were dissolved in 2 mL of acid methanol (5% HCl) and heated for 30 min at 60 °C in a closed vial to obtain methyl esters of fatty acids. The sample was then evaporated at room temperature under a nitrogen flow and recovered with dichloromethane (1 mL). Then, 1 μL of the solution was injected into an Agilent Technologies 6890N gas-chromatograph (Santa Clara, CA, USA) coupled to an Agilent Technologies 5973 mass spectrometer with split/splitless injector and analyzed on a CW capillary column (MEGAWAX, 25 m × 0.25 mm, f.t. 0.25 μm). Helium was used as carrier gas. The injector temperature was 250 °C and the injection mode was split. Oven temperature increased from 40 °C to 180 °C, at 10 °C per min after an initial hold at 40 °C for 2 min. Final temperature was maintained for 10 min. The detector temperature was 250 °C and the acquisition mode was full scan (from 40 m/z to 500 m/z).

RESULTS

Study of the Reaction between Ozonized Sunflower Oil and Potassium Iodide

The problem of the determination of peroxide value on oils with high peroxide content is well known and many authors proposed modified methods, enhancing the reaction time, the temperature or the KI concentration (Tellez et al. 2006; Zanardi et al. 2008). In this paper we directly follow the evolution of the reaction of ozonides with KI by analyzing ozonized oil samples (Neozone 4000) prepared as described

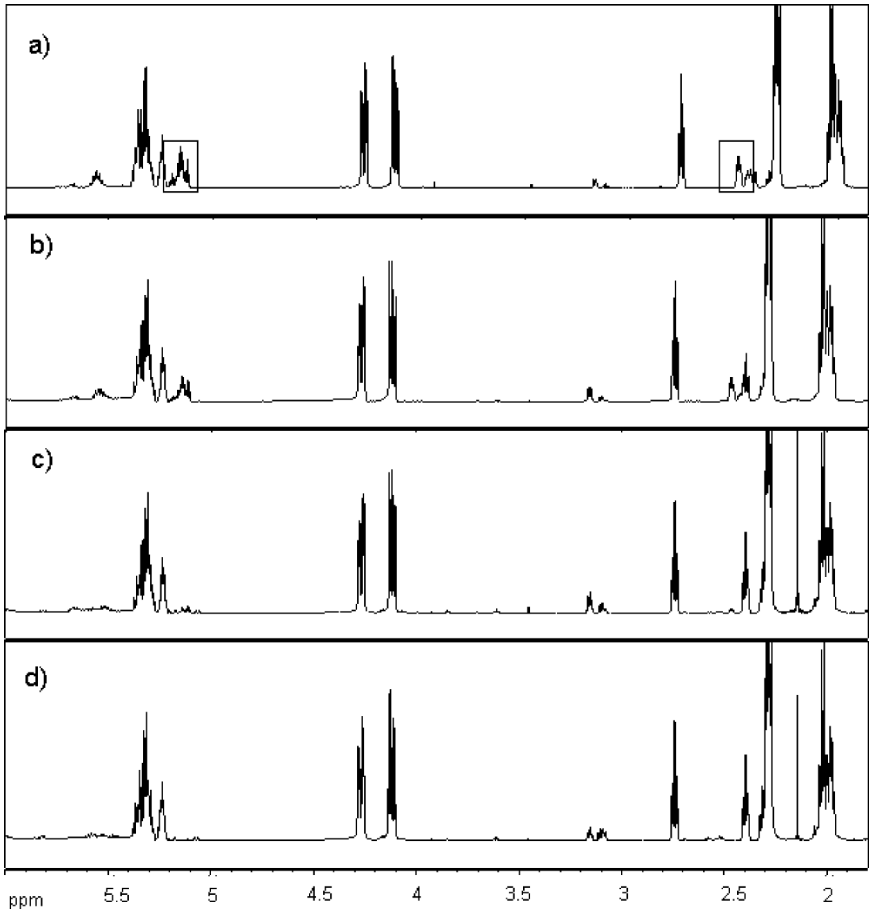


FIGURE 1. ^{1}H-NMR spectrum of ozonized sunflower oil with KI for 2 (b), 6 (c) and 16 (d) hours, compared with ^{1}H-NMR spectrum of ozonized sunflower oil not treated (a).

in paragraph 2.4, by NMR spectroscopy after 0.5, 2, 6 and 16 h of reaction with KI.

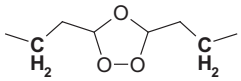
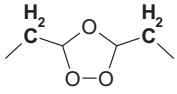
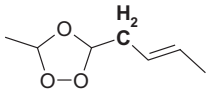
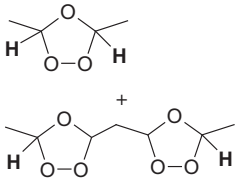
The ^{1}H-NMR spectra (Figure 1) showed that several regions are characteristic of the ozonide signals, in particular those centered at 1.41, 1.70, 2.48 and 5.15 ppm, reported in Table 2. Comparing the NMR spectra registered at different reaction times, it is possible to observe that the integrals of the signals characteristic of the ozonide functions gradually decreased during the reaction time: after 16 hours the reaction of KI can be considered complete as the ozonide signals are disappeared. For this reason, a reaction time of 16 h at 25 °C has been chosen for PV determination.

Moreover, the determination of PV values of ozonized oil was determined on sample after 0.5, 2, 6 and 16 h of reaction with KI, obtaining respectively 1041, 2291, 3163, 3877 $mEqO_2/kg$. To further confirm the degradation of ozonides, the products obtained from the reaction between ozonides and potassium iodide after a reaction time of 16 h was identified by GC/MS technique. The gas-chromatogram (Figure 2) showed that, as already described by other authors (Diaz et al. 2005) saturated and unsaturated carboxylic acids and aldehydes are the main products found, but it is also possible to observe other minor cyclic compounds as furyl derivatives, 1-iodooctane, octanol and octenol. The list of the degradation compounds recorded by GC-MS is reported in Table 3.

Determination of Peroxide Values and Density on Ozonized Sunflower Oils

Applying the previously optimized method, both peroxides values and density were determined on 60 samples of ozonized sunflower oil. Data, reported in Figure 3, show the correlation between peroxide values and density of the oil. This correlation can be explained considering the saturation of carbon-carbon double bonds in the oils treated with ozone, which determines an increase of oil specific gravity. The determination of density can therefore be used to control the ozonization reaction during the industrial process.

TABLE 2. 1H NMR Assignment of the Main Ozonides Signals and Corresponding Integrals in Untreated Ozonized Oil and after 2, 6 and 16 h of Reaction with KI

		Integrals*				
δ (ppm)	Assignment	Untreated	after 0,5 h	after 2 h	after 6 h	after 16 h
1.41		0.89	0.66	0.44	0.11	0.00
1.704		0.87	0.60	0.36	0.07	0.00
2.488		0.21	0.18	0.10	0.02	0.00
5.159		0.41	0.15	0.19	0.03	0.00

*Referred to glycerol.

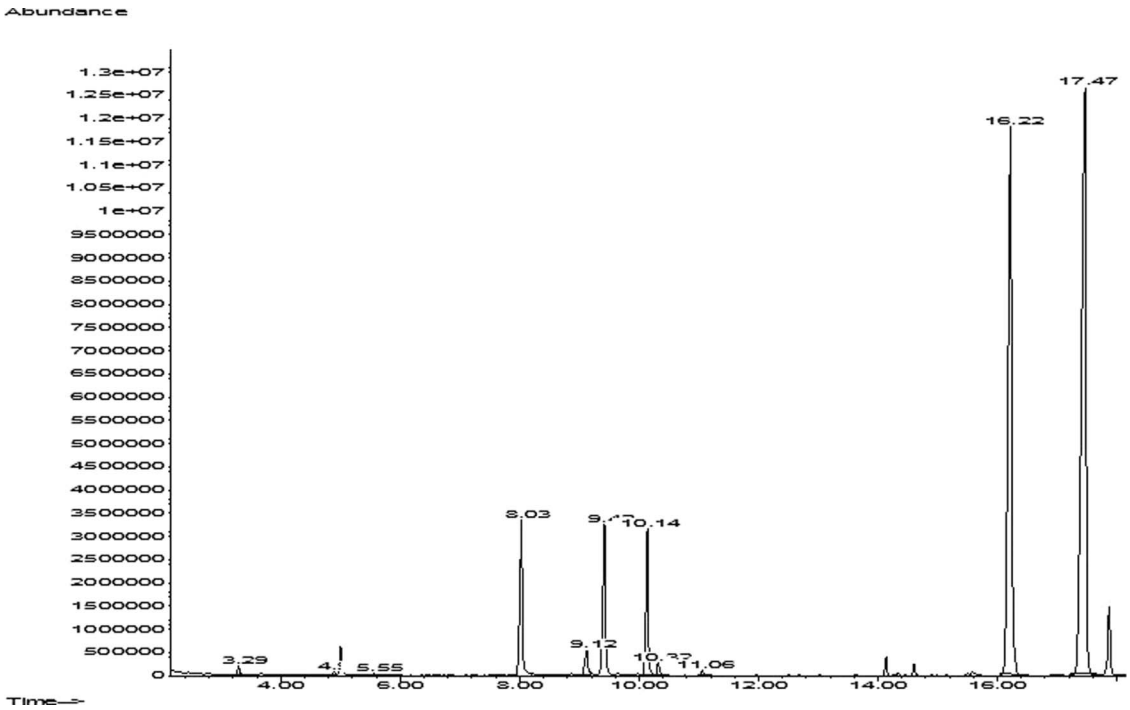


FIGURE 2. GC/MS chromatogram of short-chain compounds detected after reaction between ozonides of sunflower oil and KI (Reaction time = 16 h).

Determination of Peroxides Value in the Time at Different Temperatures

To evaluate the shelf-life and the stability of ozonized sunflower oils and variously enriched cosmetic products (Table 1), a series of samples were stored at different temperatures: 4 °C (fridge), 25 °C (room temperature), and 40 °C (laboratory oven), and controlled for their peroxide content at different times (0, 30, 60, 90, 180 and 360 days).

TABLE 3. Retention Times and Relative % Content of the Short-Chain Compounds Detected by GC/MS after Reaction of Ozonides with KI (Reaction time = 16 hours)

Identified compound	Retention time (min)	Relative percentage (%)
Hexanal	3.29	0.69
Hexanoic acid, methyl ester	4.90	0.26
Furan, 2-pentyl-	5.55	0.17
Nonanal	8.033	15.6
Octane, 1-iodo-	9.12	2.86
Nonanoic acid, methyl ester	9.42	15.71
3-Nonenoic acid, methyl ester	10.14	12.07
1-Octanol	10.32	1.16
2-Octen-1-ol	11.06	0.47
Nonanoic acid, 9-oxo-, methyl ester	16.22	76.95
Nonanedioic acid, dimethyl ester	17.47	100.00

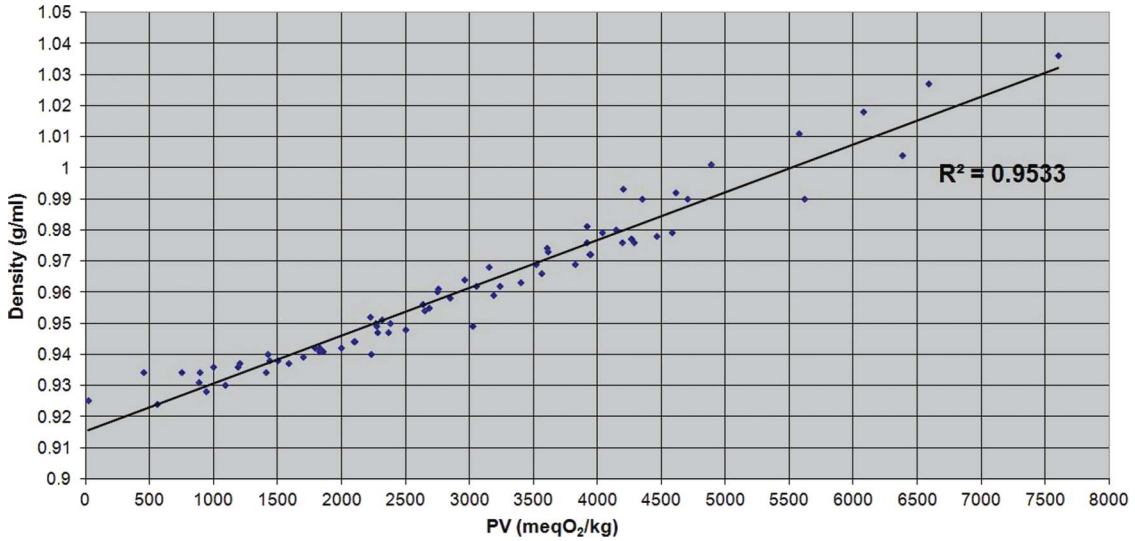


FIGURE 3. Peroxide values and density trend for ozonized sunflower oil samples (color figure available online).

The initial peroxides values (PV) show that some enriched cosmetics present PVs slightly higher (10–20%) than expected if considering the percentage of the ozonized oil added. The peroxide value can be affected by the cosmetic matrix than can interact with ozonides, and increases by reducing the sample weight for the analysis, that has to be standardized. Moreover, the peroxides indexes of samples determined at 4°C and at room temperature surprisingly slightly increase during the first months of shelf-life, while values of samples kept at 40°C decrease, as expected. After 90 days of staying, PVs decrease constantly even for refrigerated samples.

Comparing the peroxide values obtained at time zero with those registered after 360 days, it results that samples maintained at 4°C show a reduced activity of about 15 %, those kept at 25°C were reduced of about 20 % of peroxides content, and those heated at 40°C maintain only the 30% of the starting activity.

SUMMARY

In conclusion, the stability controls on ozonized sunflower oil and enriched cosmetics such as skin cream, massage oil and ointment, stored at different temperatures and times, evidence that all these products maintain a satisfying stability and good peroxide values for at least six months, if refrigerated or kept at room temperature, while a gradual but significant decrease of the ozonide content occurs on samples stored at higher temperatures (40 °C) or for more prolonged times.

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