Chemical Analysis of Ozonized Theobroma Fat

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ABSTRACT: Ozonized theobroma fat is used as raw material in the manufacture of pessaries and cosmetic creams. Ozonization of theobroma fat with water was carried out at different applied ozone dosages, and the resultant PV, acid value, iodine value, total hydroperoxide content, and FA content were determined. PV and total hydroperoxide content showed a notable increase with applied ozone dosage up to 35.7 mg/g. Acid value varied slightly from 4.1 to 9.9 mg KOH/g, and the iodine value fell to zero. PV and total hydroperoxide content increased slightly with a higher applied ozone dosage. The comparison of total hydroperoxide measurement using ferrous oxidation in xylenol orange assay and traditional iodometric assay for PV determination showed a significant linear correlation. Small amounts of oleic acid were found in ozonized theobroma fat samples with iodine value equaling zero, which demonstrated that iodine value determination is an inexact assay. During ozonization of theobroma fat, an increase in acid value of 18.9-fold with respect to the initial value was observed owing to decomposition of peroxide.

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KEY WORDS: Acid value, fatty acids, gas–liquid chromatography, hydroperoxides, iodine value, oleic acid, ozonized theobroma fat, peroxide value.

Theobroma fat is expressed from the seeds of *Theobroma cacao*. It is used in pharmaceutical, cosmetic, and food industries. Its m.p. (33–35°C) is very close to that of the human body temperature, which makes it especially suitable for preparation of vaginal suppositories (1). Theobroma fat is composed principally of TAG. The predominant FA in theobroma fat are stearic, oleic, and palmitic. However, these acyl groups are not distributed randomly across the glycerol positions. The saturated acyl groups are almost exclusively in the 1 and 3 positions and unsaturated acyl groups are in the 2 position (2,3).

The reaction of ozone with vegetable oils occurs almost exclusively at the carbon–carbon double bonds present in unsaturated FA (4,5). The ozonization of unsaturated FA gives 1,2,3-trioxolane, which decomposes rapidly to give a carbonyl oxide and an aldehyde. These two species recombine to give ozonides, hydroxyhydroperoxides, hydrogen peroxides, and carbonyl compounds (6). In participating solvents (such as water), the carbonyl oxide recombines with other carbonyl compound to form hydroperoxides, hydrogen peroxide, and aldehyde (7). We have suggested that peroxide compounds in conjunction with ozonides are involved in the germicidal effects of ozonized vegetable oil (8). Different ozonized oils are being studied at the Ozone Research Center of Cuba. Some of them, such as ozonized sunflower oil, present a remarkable germicidal effect, according to the research carried out in our Center (9,10). The efficacy of this product against fungi, bacteria, and viruses has been verified (11). The antimicrobial activity of ozonized theobroma fat against *Candida albicans* has been demonstrated, and its application for vaginal candidiasis has been recommended (12).

Assays for fats and oils presently in use include PV, acid value, and iodine value. These assays have been used in different ozonized vegetable oils (13). Recently, an assay based on ferrous oxidation in xylenol orange (FOX) was developed to measure hydroperoxides in edible and ozonized sunflower oils (14,15). The most frequent analysis of FA profiles in TAG is based on GLC (16).

In this study theobroma fat was ozonized at different ozone dosages and then its chemical characteristics were determined.

EXPERIMENTAL PROCEDURES

Solvents and reagents. Glacial acetic acid, chloroform, potassium iodide, sodium thiosulfate, starch, ethanol, diethyl ether, potassium hydroxide, phenolphthalein, iodine bromide, toluene, and dimethoxypropane were from Merck (Darmstadt, Germany). BHT and xylenol orange [*o*-cresolsulfonephthalein-3,3-bis(methyliminodiacetic acid sodium salt)] were obtained from Sigma Chemical (St. Louis, MO). Ammonium ferrous sulfate, sulfuric acid, propan-1-ol, and HPLC-grade methanol were purchased from BDH (Poole, United Kingdom). "Rubén David Suarez Abella" company (Baracoa, Cuba) supplied the theobroma fat.

General ozonization procedure. Ozone was generated by passing oxygen through a Trailigaz Labo model 12-02 ozone generator at a fixed voltage (170 V) and a constant flow rate of 42 L/h. The initial ozone concentration of the resulting stream (71.3 mg/L) was determined by Anseros Ozomat equipment. This stream was introduced into a mixture of 150 g theobroma fat and 15 mL of water in a bubbling reactor (17) at 45°C. The ozonization was allowed to continue at different applied ozone dosages (9.6, 11.8, 19.0, 26.1, 29.7, 33.1, 35.7, 69.6, 76.7, 87.4,

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95.0, and 146.2 mg/g). Three replicates were carried out for each ozone dosage. Replicates were pooled for each ozone dosage. Samples were stored at 2–8°C for 24 h before analysis.

Analyses. PV, expressed as mmol-equiv of active oxygen in 1 kg of the substance (18); acid value, expressed as mg of KOH required to neutralize the free acids in 1 g of the substance (19); and iodine value, representing the double bonds contained in 1 kg of the substance (20), are described by the 2000 *British Pharmacopoeia*.

Total hydroperoxides were measured using the FOX assay (21). Stock FOX reagent contained 1 mM xylenol orange and 2.5 mM ammonium ferrous sulfate in 250 mM of sulfuric acid. One volume of stock solution was added to 9 vol of HPLC-grade methanol containing 4.4 mM BHT to make the working reagent. A drop of an ozonized theobroma fat sample was dissolved in propan-1-ol. An aliquot (100 μL) of this solution was brought to 1 mL by addition of FOX reagent for samples obtained with an applied ozone dosage from 9.55 to 76.72 mg/g. For samples obtained with a higher ozone dosage, 100 μL of fat/propan-1-ol solution was brought to 2 mL by addition of FOX reagent. Assay mixtures were incubated at room temperature for 30 min and then centrifuged at 2,700 × *g* for 30 min. The absorbance of the supernatant at 560 nm was used to determine total hydroperoxide content (THP) in accordance with the Lambert-Beer law (21).

Virgin theobroma fat and ozonized theobroma fat samples prepared using ozone dosages of 26.1, 29.7, 35.7, and 87.4 mg/g were transesterified for GLC analysis as described by Garcés *et al*. (22). Samples were stored at 20°C for 48 h before GLC analysis using a DB-17HT capillary column (15 m × 0.25 mm i.d., film thickness 0.15 μm) with an FID at 280°C. The carrier gas was hydrogen at 1 mL/min and a pressure of 50 kPa. The column temperature was programmed from 100 to 200°C at 8°C/min. Injection volume was 1 μL. A model 5890 Hewlett-Packard chromatographic system was used for the analysis. External FA standards (Sigma-Aldrich Chemical Company, St. Louis, MO) were used to identify components.

Statistical analysis. Analyses were in triplicate and data were expressed as mean ± SD. Linear regression analysis was performed using STATGRAPHICS (Herndon, VA), version 5.0 Statistical Program.

RESULTS AND DISCUSSION

During ozonization, PV, acid value, and total hydroperoxide content increased with ozone dosage, whereas iodine value decreased (Table 1). The increase of PV and total hydroperoxide content was due to formation of peroxidic substances when ozone reacts with unsaturated compounds through the known Criegee mechanism (23).

The trends with different applied ozone dosage showed two distinct phases. In the first, corresponding to an applied ozone dosage of 9.6–35.7 mg/g, there was an almost linear increase in PV and total hydroperoxide content with increased ozone dosage. This culminated in 1,495 and 1,038 mmol-equiv/kg of peroxide and hydroperoxide, respectively, at 35.7 mg/g. In the second phase, corresponding to 69.6–95.0 mg/g ozone dosage, PV increased more slowly from 1,523 to 1,564 mmol-equiv/kg. Thus, in this phase, PV was almost constant. When the applied ozone dosage was further increased from 95.0 to 146.2 mg/g, PV again showed an important increase. Hydroperoxide content showed a similar behavior, with a linear increase for ozone dosage from 9.6 to 35.7 mg/g, followed by an almost constant hydroperoxide content for ozone dosages of 35.7 to 76.7 mg/g and then a major increase in hydroperoxide content at or above 95.0 mg/g.

THP was linearly related to PV with correlation (99.4%) and determination (98.8%) coefficients, over the ozone dosage interval from 9.6 to 76.7 mg/g. The equation obtained by linear regression was

$$\text{THP} = 0.60\ \text{PV} - 125.63 \quad [1]$$

TABLE 1
Physicochemical Parameters of Ozonized Theobroma Fat[a]

AOD (mg/g)	PV (mmol-equiv/kg)	AV (mg KOH/g)	IV (g iodine/100 g)	THP (mmol-equiv/kg)
First phase				
9.6	227 ± 4	4.1 ± 0.1	27.9 ± 0.3	236 ± 26
11.8	338 ± 10	4.2 ± 0.1	25.8 ± 0.2	379 ± 1
19.0	554 ± 6	4.2 ± 0.1	21.7 ± 0.7	476 ± 14
26.1	877 ± 11	5.0 ± 0.1	18.4 ± 0.6	617 ± 15
29.7	1,037 ± 7	5.8 ± 0.2	10.9 ± 0.3	751 ± 8
33.1	1,303 ± 2	6.9 ± 0.1	3.8 ± 0.1	858 ± 2
35.7	1,495 ± 10	9.9 ± 0.1	0 ± 0	1,038 ± 8
Second phase				
69.6	1,523 ± 5	20.3 ± 0.4	0 ± 0	1,071 ± 49
76.7	1,527 ± 1	28.4 ± 0.3	0 ± 0	1,075 ± 14
87.4	1,544 ± 8	34.1 ± 0.2	0 ± 0	1,304 ± 50
95.0	1,564 ± 9	39.5 ± 1.2	0 ± 0	1,420 ± 28
146.2	1,766 ± 8	77.4 ± 0.6	0 ± 0	1,538 ± 15

[a]Values represent mean ± SD of three determinations. Abbreviations: AOD, applied ozone dosage; AV, acid value; IV, iodine value; THP, total hydroperoxide content.

TABLE 2
Content (%) of Oleic and Linoleic Acid in Virgin and Ozonized Theobroma Fat[a]

	AOD (mg/g)				
	0[b]	26.1	29.7	35.7	87.4
Oleic acid	35.6 ± 0.1	16.4 ± 0.03	8.7 ± 0.002	6.1 ± 0.002	3.3 ± 0.001
Linoleic acid	2.8 ± 0.1	1.5 ± 0.001	0 ± 0	0 ± 0	0 ± 0

[a]Values represent mean ± SD of three determinations. For abbreviation see Table 1.
[b]Represents virgin theobroma fat

which supported the use of the FOX assay for determining hydroperoxides in ozonized theobroma fat. This result was demonstrated previously using ozonized sunflower oil (15), but this was the first application of the FOX assay to ozonized theobroma fat.

For an applied ozone dosage greater than 76.7 mg/g this linear relationship was lost, because total hydroperoxides are formed in greater proportion than other peroxidic species. Traces of water promote the formation of hydroperoxides at the expense of ozonides by acid-catalyzed hydrolysis (24), which should favor the formation of total hydroperoxides.

Acid value also increased this ozone dosage (Table 1). Acids are formed directly during ozonization, TAG hydrolysis, and peroxide decomposition (25). In the first phase, where PV increased rapidly up to 35.7 mg/g of applied ozone dosage, acid values showed a slight increase from 4.1 to 9.9 mg KOH/g. Acid value continued to increase in the second phase, up to 77.4 mg KOH/g, which represented an increase of 18.9-fold with respect to initial acid value. In this latter phase, the increase of acid value occurred principally due to peroxide decomposition. These results agree with those obtained by ozonizing sunflower oil (26), where a high PV (2,500 mmol-equiv/kg), coincided with high acid value (86.9 mg KOH/g).

Iodine value decreased with an increase of applied ozone dosage (Table 1). The iodine value is a measure of double bond content in oils (18), principally oleic and linoleic acids in theobroma oil. Ozone attacked the double bonds of FA (6), and thus iodine value decreased with ozone dosage. Contents of oleic and linoleic FA in virgin theobroma fat, obtained with GLC, are shown in Table 2. These results were within the reported ranges from 29 to 37% for oleic acid and from 2 to 4% for linoleic acid. (2,3). During ozonization, these two unsaturated FA decreased (Table 2). In contrast to theobroma fat, sunflower oil has a high content of linoleic acid, from 48 to 74% (1); therefore, theobroma fat has comparatively fewer double bonds for reaction with ozone.

GC analysis of ozonized theobroma fat samples showed that oleic acid was still present even at an applied ozone dosage of 87.4 mg/g (Table 2). Therefore, double bonds continue to be present in the system for ozone reaction. In contrast, the results obtained using iodine value determination (Table 1) indicate that double bonds are not detected at an applied ozone dosage of 35.7 mg/g or higher. This demonstrates that iodine value determination is not sufficiently sensitive under these conditions. When applied ozone dosage is elevated, polymerizations occur due to condensation of peroxides that have been formed *via* ozonization (13) and the resultant high viscosity obstructs the access to the double bonds of the iodine bromide.

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