Racemic Lactones from Butterfat: An Advanced Approach That Includes Stereodifferentiation

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A new method was developed to obtain lactones from butterfat. The application of lipases and capillary gas-liquid chromatography with chiral phases for the separation of γ-lactone enantiomers resulted in elucidation of the racemic composition of γ-lactones from butterfat. The seasonal distribution of lactones in butterfat was determined.

KEY WORDS: Butterfat, lactones, lipase, organic solvents, stereodifferentiation.

Since the early 1960's lactones and their hydroxy fatty acid glyceride precursors have been known to be flavor constituents of milk and milk products (1,2). Jurriens and Oelle (1,2) detected γ-lactones by means of gas-liquid chromatography-mass spectrometry (GLC-MS) and infrared spectroscopy after laborious isolation from butterfat with liquid chromatography. Ellis and Wong (3) isolated γ- and δ-lactones from margarine and butterfat by evaporation from heating samples up to 150°C. Distribution of lactones in other natural foods has been reviewed by Maga (4). Lactones are intramolecular esters of 4- (γ-) and 5-(δ-) hydyroxy fatty acids, which contain a chiral center. The use of enantiomerically pure lactones as synthons for pheromones (5–8) and pharmaceuticals (9,10) made them a topic for organic chemical synthesis (11,15). In recent years, enzymes also have been used for the preparation of enantiomerically enriched lactones (16,17). In the present study, a new method was developed to obtain lactones from butterfat with a technique that also may be applied to other fats. To our knowledge, we report here for the first time on totally racemic γ-lactones in a natural food.

MATERIAL AND METHODS

Butter samples (500 g each) were kindly collected by Nordbutter (Hohenwested, Germany) every 4–6 wk during a period in 1989–1990 and stored at −20°C until experiments were performed. The samples were from: May 16, 1989; June 12, 1989; July 4, 1989; August 8, 1989; September 21, 1989; October 26, 1989; December 29, 1989; January 23, 1990; and February 21, 1990 (hereafter designated by month and day, *i.e.*, Feb/21).

Determination of physical and chemical parameters. Estimation of iodine value was performed according to IDF Standard 8 (18), and melting curves were recorded on a calibrated differential scanning calorimeter (Heraeus TA 500, WSK, Rodenbach, Germany), equipped with a liquid nitrogen cooling device (19,20). We used sealed aluminum capsules as sample holders. After each experiment, the capsule was weighed to assure that no material had evaporated. The temperature interval investigated was −100°C to + 100°C, and the applied heating rate was 5°C/min. The accuracy of the instrument was ± 4% for enthalpy determinations and ± 0.5°C for temperature measurements.

Preparation and oxidation of fat fractions. Fat fractions were prepared by dissolving butter samples in hexane at 40°C, drying with anhydrous Na_2SO_4 and removing precipitates by centrifugation. The soluble fraction was concentrated by evaporation at room temperature and used as fat fraction. Fat fractions diluted in dry hexane were used for further experiments. One fat fraction was diluted with dry hexane and oxidized with a continuous stream of air at 30°C for 24 h. Volatile substances were trapped by a cooling device at the outlet and added to the treated fat fraction. A second fat fraction was diluted with dry hexane, treated with H_2O_2 (30%, 10 mL) and vigorously stirred for 24 h.

Conditions for transesterification. For transesterification of glycerol-bound hydroxy fatty acids, fat fractions were diluted in dry hexane and incubated with lipase powder (1 g) or ion exchange material (Dowex 50, acid form, Fluka, Buchs, Switzerland) for 24 h at 30°C under low stirring. The reaction was stopped by removing the lipase powder or ion exchange material by filtration.

Purification of lactones from fat fractions. After each experiment, lactones were stripped from solution by a continuous stream of nitrogen at 60°C and collected in a trap cooled by liquid nitrogen. Fractions obtained were purified by liquid column chromatography on prepacked Silica gel-60 columns (Merck Lobar, Darmstadt, Germany) with hexane/ethyl acetate (95:5, vol/vol) as eluent. Fractions of 15 mL were collected and analyzed by thin-layer chromatography (TLC) on silica gel plates (Merck). TLC plates were developed with hexane/ethyl acetate (7:3, vol/vol). Lactones were detected by spraying with ceric sulfate reagent. The stain reagent consisting of ceric sulfate (10 g, Merck), molybdatophosphoric acid (25 g, Merck), conc. sulfuric acid (60 mL) and water (940 mL).

Analysis of lactones. Fractions containing lactones were pooled and analyzed on a Hewlett-Packard GLC-MS equipped with a Gerstel (Mühlheim, Ruhr, Germany) two-channel GLC system. Helium was the carrier gas at a flow rate of 2 mL/min. In the first channel, samples were separated on a 10-m Carbowax capillary column (Chrompack, Middleburg, The Netherland), in the second channel, γ-lactone enantiomers were separated on a Lipodex D capillary column (20 m) (Macherey and Nagel, Düren, Germany). Mass spectra were recorded with the Hewlett-Packard Quadrupole MS. An equimolar mixture of racemic γ-lactones with 6, 7, 8, 9, 10 and 12 C-atoms was used as standard. γ-Lactones were identified by means of the retention time and by the typical fragment with the mass 85 corresponding to the furanone ring structure of γ-lactonens.

Enzymes and chemicals. Lipases used for transesterification were purchased from Merck (*Porcine pancreas*), Gist Brocades (Charlotte, NC) (*Mucor miehei*), Serva (Heidelberg, Germany) (*Porcine pancreas*) and Amano (Troy, VA) (*Penicillium* sp.). Dowex 50 (acid form) was from Fluka, solvents for chromatography and Na_2SO_4 were of analytical grade from Merck.

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TABLE 1

Iodine Values from Butter Samples Before and After Treatment with Oxygen or Lipase

Sample	Iodine value (mean of three determinations)	Melting curve[a]
May/16 fat fraction	30.3	a
After oxygen treatment	30.4	b
After transesterification	29.2	c
Aug/08 fat fraction	31.9	
After transesterification	32.2	

[a]As displayed in Figure 1.

RESULTS AND DISCUSSION

Butterfat triglycerides sometimes contain hydroxy fatty acids in addition to the normal saturated and unsaturated fatty acids, as well as small amounts of free lactones, as shown by Jurriens and Oelle (1,2). Lipases can be used for the hydrolysis of glycerides in a system that contains distinct amounts of water as co-substrate. γ-Lactones are intramolecular esters of 4-hydroxy fatty acids. We used several different lipases and an acidic ion exchange material in an anhydrous environment to perform a transesterification of the glycerol hydroxy fatty acid esters to lactones and to avoid hydrolysis of glycerol-bound saturated and unsaturated fatty acids. Under anhydrous conditions, transesterification, but no hydrolysis, should occur when appropriate catalysts are added.

Effects of fat fraction pretreatment. Because oxidation of unsaturated fatty acids was assumed to be a method for converting them to hydroxy fatty acids, one sample of fat fraction was treated with oxygen and another with H_2O_2. With these experiments we wished to determine whether lactones could be formed spontaneously from unsaturated fatty acids. Iodine values and melting curves of one oxidized sample are compared to one enzyme-treated sample in Tables 1 and 2, as well as in Figure 1.

As Tables 1 and 2 and Figure 1 show, no significant changes or effects can be deduced. The shape of the melting curves suggests the occurrence of 4–5 melting fractions. Mild oxidation or enzyme treatment had no significant influence upon these characteristic parameters. It can be concluded that under the above conditions, oxidation of unsaturated fatty acids did not occur, because the

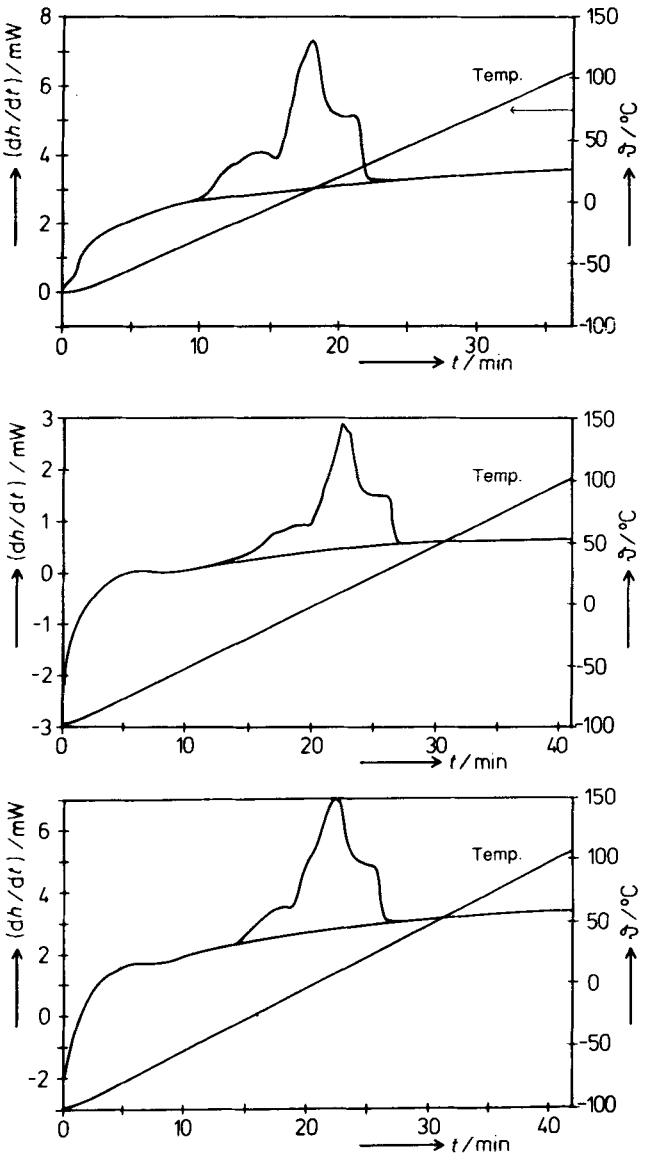

FIG. 1. Melting curves of butter samples, recorded between −100°C and +100°C. The enthalpy and actual temperature are displayed on the left and on the right y-axis, respectively. Inside the diagram, the temperature curve is marked with Temp. A displays the melting behavior of May/16 before treatment; B after oxygen treatment; and C after additional enzymatic treatment.

TABLE 2

Temperature Range of Melting and Melting Enthalpy of Butter Samples Before and After Treatment with Oxygen or Lipase

Sample	Amount used (mg)	Melting interval	Melting enthalpy (J/g)
May/16 fat fraction	18.640	−40°C + 34°C	71.1
After oxygen treatment	9.265	−39°C + 36°C	71.5
After transesterification	19.450	−36°C + 35°C	67.8
Aug/08 fat fraction	16.975	−53°C + 38°C	67.5
After transesterification	10.810	−48°C + 41°C	63.5

TABLE 3

Butter Samples, Weights of Recovered Material After Chromatography of Cold-Trap Fractions, Weights of Fat Fractions

Sample	Weight of extract (mg)	Weight of fat fraction (g)
May/16	26	360
Jun/12/a	9	370
Jun/12/b	18	—
Jul/04	74	383
Aug/08	40	410
Sep/21	48	400
Oct/26	10	410
Dec/29	21	380
Jan/23	34	385
Feb/21	17	400

TABLE 4

Distribution of Lactones in Butterfat During the Year 1989/90

	Lactone[a]						
Sample	γ-7	γ-8	γ-9	γ-10	γ-11	γ-12	δ-10
May/16	+	+	+	+	+	+	
Jun/12	+	+	+	+	+	+	
Jul/04	−	−	−	+	+	+	
Aug/08	+	+	+	+	+	−	
Sep/21	+	+	+	+	−	−	
Oct/26	−	−	−	−	−	−	
Dec/29	−	−	−	+	+	+	+
Jan/23	−	−	−	+	+	−	
Feb/21	−	−	−	−	−	−	

[a]Abbreviations: γ-7, γ-Heptalactone; γ-8, γ-octalactone; γ-9, γ-nonalactone; γ-10, γ-decalactone; γ-11, γ-undecalactone; γ-12, γ-dodecalactone; and δ-10, δ-decalactone.

iodine value did not decrease significantly after treatment with oxygen. Glycerides also were not hydrolyzed under these conditions, because the melting curves of enzymatically treated samples did not change.

Treatment of fat fraction. Fat fractions, with and without pretreatment, were transesterified by different lipases or acidic ion exchange material in anhydrous organic solvent. Lactones initially contained in the sample (Jun/12/a) and those formed by the transesterification (Jun/12/b) were stripped from solution by a stream of nitrogen (21) and collected in a trap cooled by liquid nitrogen. This seemed to be a simple, but powerful, method as compared to other procedures investigated. We found triglycerides containing short-chain fatty acids and lactones in the cooling trap fraction accompanied by only small amounts of free fatty acids. Table 3 summarizes the amounts of isolated substances of the cooling-trap fractions after liquid chromatography. A significant amount of lactone-containing extracts could be isolated by stripping with nitrogen and collecting in a cooling trap. Samples Jun/12/a and Jun/12/b indicate that a large amount of lactones typically occurs as esterified hydroxy fatty acids in butterfat.

Results from the GLC-MS measurements. Samples were analyzed regarding their lactone composition and distribution of enantiomers *via* two-channel GLC-MS on a chiral phase (Lipodex D, Machery and Nagel). Figure 2 shows ion-stream chromatograms recorded at the outlet of the chiral column by a quadrupole mass-spectrometer. In Figure 2a an ion-stream chromatogram of a racemic γ-lactone mixture is shown. Column switching of the two-channel GLC is performed by changing the gas pressure. This procedure results in slight changes of retention times between different runs. Below each sample chromatogram a mass selective chromatogram is displayed. Only peaks containing mass 85, the mass of the γ-lactone furanone ring, are shown.

The ion-stream chromatograms showed that the samples contained γ-lactones and only one δ-lactone. Samples Oct/26 and Feb/21 contained no lactones. In all samples containing lactones, only racemic mixtures appeared. Neither enzymatic and chemical transesterification resulted in enantiomerically enriched lactones. The δ-lactone could not be separated into enantiomers by GLC on the chiral phase. Over the year, a change in γ-lactone composition occurred, resulting in a complete lack of lactones both in late autumn and in February. Samples collected during late spring and summer contained a broad spectrum of lactones, especially with short 4-alkyl chains, as compared to those collected during the winter.

According to results reported by Schulz *et al.* (22), who showed that a time course of the iodine value exists, a time-dependent "lactone course" over the year also seems to exist. Since the change in iodine value has been correlated with changes in climate and composition of fatty acids in hay for cow feed, it may be postulated that the changes in lactone composition are also due to different feed. Perhaps lactone composition could serve as a marker for a certain type of feed. Table 4 gives the results

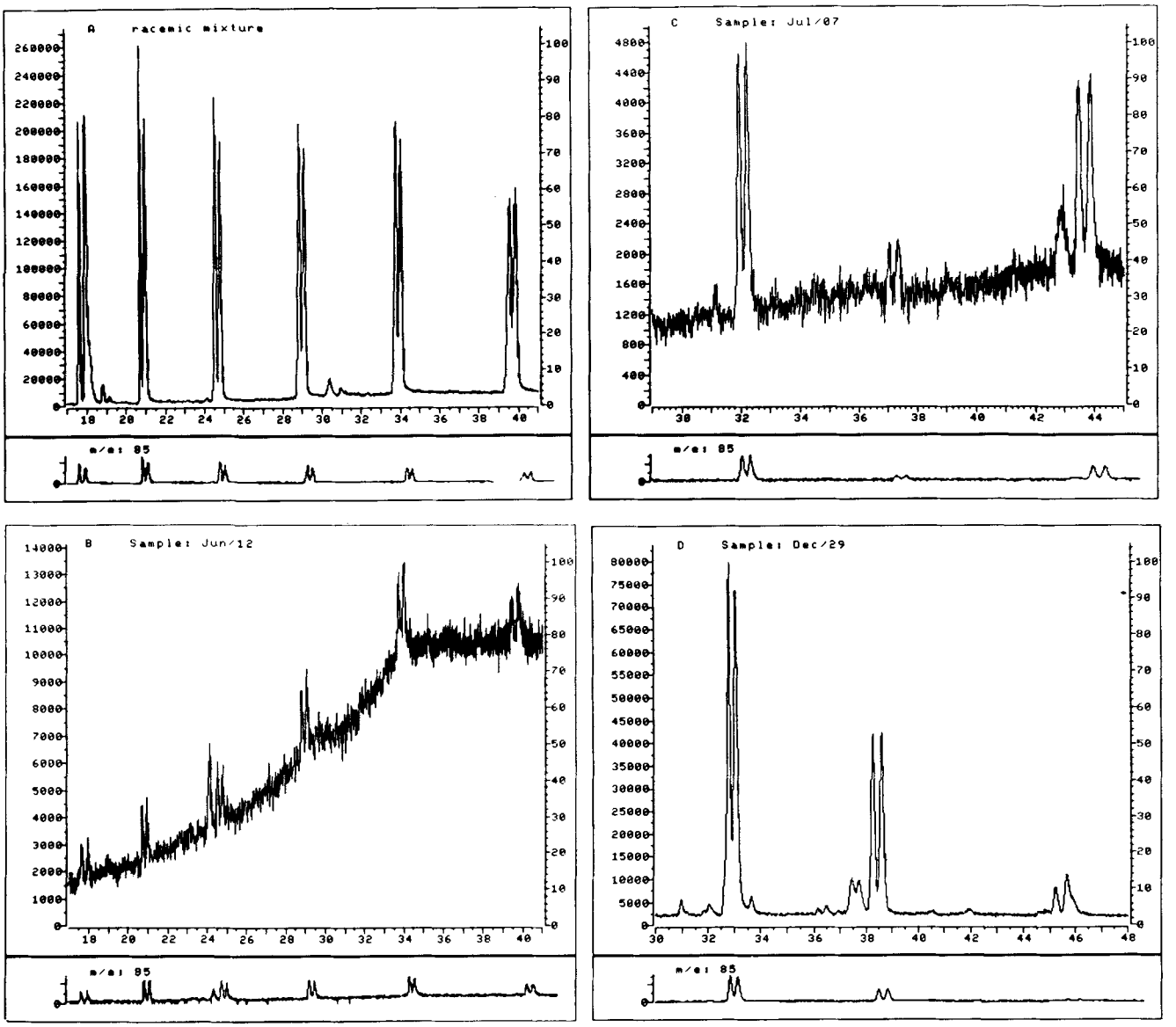

FIG. 2. Ion-stream chromatograms of investigated samples containing lactones. Below the chromatogram a mass-selective chromatogram is displayed showing peaks that contain the fragment with mass 85, the typical fragmentation particle of γ-lactones. A, GLC-MS ion-stream chromatogram of a racemic γ-lactone mixture from 7 to 12 carbon atoms, separated as described in Materials and Methods; B, Jun/12; C, Jul/04; and D, Dec/29; all separated under the same conditions.

from the ion-stream chromatograms in a qualitative form. It can be seen that the composition of γ-lactones changes with a certain trend over the year.

Lactones as constituents of natural fruit flavors have been analyzed in the last few years with respect to their enantiomeric composition (23,24). Enantiomerically enriched γ-lactones seem to be markers for a natural food product. Addition of chemically synthesized aroma constituents must be indicated on food packaes as "identical to nature". Only those directly obtained from natural sources are presently allowed to be titled "natural" in Germany. Mosandl *et al.* (25) detected racemic γ-lactones in different fruit juices and concluded that synthetic aroma constituents had been added. In his review on the use of capillary columns with cyclodextrin phases for GLC, König (26) demonstrated that cocoa contains δ-lactones with different chainlengths and different enantiomeric composition. Short-chain δ-lactones were enantiomerically enriched, and long-chain δ-lactones were nearly racemic.

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