Analysis of Lipids from Cooked Beef by Thin-Layer Chromatography with Flame-Ionization Detection

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Lipids were extracted from cooked ground beef with methylene chloride/methanol (2:1). The lipids were separated on a silicic acid column, into neutral and polar fractions by elution with methylene chloride, followed by methanol. These fractions were analyzed by Iatroscan thin-layer chromatography with flame-ionization detection instrumentation on Chromarods S-III (silica gel-coated quartz rods). Comparison of cooked beef stored for 0, 4 and 7 d at 4°C indicated that storage caused a decrease in total lipids, an increase in neutral lipids and a decrease in polar lipids, specifically in phosphatidylcholine. These changes in the lipid fraction were associated with meat flavor deterioration and an increase in lipid oxidation.

KEY WORDS: Cooked beef lipids, Iatroscan, lipid extraction, lipid oxidation, phospholipids, TLC/FID.

Uncured cooked meat stored at 4°C for several days will develop off-flavors. These off-flavors also occur in raw meat. The result of this process is known as warmed-over flavor (WOF), as first described by Tims and Watts (1). Recently, WOF has been referred to as meat flavor deterioration (MFD) because desirable flavor notes decrease, whereas the off-flavor notes increase (2–5). A comprehensive explanation of the rational for adapting the more appropriate acronym, MFD, for describing the processes that occur during refrigerated storage was provided by Spanier (6). Henceforth, in this report, the phenomenon referred to as WOF will be alluded to as MFD.

Lipids in mammalian tissues are classified as either intermuscular (adipose or depot fat) or intramuscular fat (7,8). Reports from several laboratories (8–10) have confirmed that intramuscular fats, comprised of polar lipids that contain polyunsaturated fatty acids, are more susceptible to oxidation than adipose fat and thus are major contributors to off-flavor development. Igene and Pearson (11) investigated the role of phospholipids (PL) and triglycerides in MFD with model systems. They extracted and separated beef and chicken lipids into triglyceride and PL fractions, and then added them and commercial sources of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) back to lipid-extracted muscle fibers. They found that total added phospholipids decreased during cooking and storage (48 h, 4°C) of beef, and that the PLs, and particularly PE, were major contributors of MFD in cooked meat. PC did not influence MFD in the model system. Although total PLs decreased with cooking, PE did not decrease significantly, whereas PC decreased by 45%. They reported no appreciable change in the triglyceride fraction and no influence of the triglyceride fraction on MFD.

The present investigation was begun in order to develop a method to identify and quantify the extent of lipid degradation in cooked ground beef patties during storage at 4°C with Iatroscan thin-layer chromatography/flame-ionization detection (TLC/FID) instrumentation (Iatron Labs., Inc., Tokyo, Japan), a relatively new method for rapid quantitative analysis of organic substances, particularly lipids. A second objective was to investigate the differences among endogenous PLs extracted from the beef patties as lipid oxidation increased during the development of MFD. Hopefully, this investigation of the PLs in beef will increase our knowledge toward understanding the mechanism that produces MFD.

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EXPERIMENTAL PROCEDURES

Materials. PL standards, PE, PC, phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), lysophosphatidylglycerol (LPG), phosphatidic acid (PA) and sphingomyelin (Sp) were purchased from Sigma Chemical Co., (St. Louis, MO). Silicic acid (200 mesh) for column chromatography was obtained from J. T. Baker, Inc. (Phillipsburg, NJ). All other chemicals and organic solvents were of analytical grade.

Sample preparation. Top round steaks (semimembranosus muscle), choice cut, were purchased from a local supermarket (New Orleans, LA) the morning of sample preparation. After removal of visible fat, the lean meat was ground twice (1.0-cm plate followed by 0.8-cm plate) and separated into 85-g patties, which were cooked on a Farberware grill for 7 min/side. The fat content of patties prepared in this manner is generally from 4 to 5% (12,13). Several patties were immediately frozen after cooking and were placed in covered, glass petri plates. These patties were used as standards. Other cooked patties were placed in covered petri plates and stored at 4°C for up to 7 d to develop MFD. Experimental samples were 0-day patties (assayed immediately after cooking), and patties stored for 4 and 7 d. All standards and experimental samples were assayed by chemical, instrumental and sensory methods, except for the 7-d samples, which were not assayed by sensory methods due to the increased rancidity.

Methods. The Iatroscan TH-10 TLC/FID Analyzer was interfaced with a Hewlett-Packard (Palo Alto, CA) 3390 Integrator. The FID was operated with a hydrogen gas flow of 160 mL/min. Air was supplied *via* an electrical air pump. The air flow was 2 L/min. Chart speed on the integrator was 24 cm/min. The Iatroscan and integrator were started simultaneously. Separations were performed on silica-coated Chromarods S-III (Iatron Labs., Inc.). Until initial use, the rods were stored in 60% sulfuric acid. Rods were thoroughly washed with distilled water immediately prior to use, and scanned twice to burn off impurities that may have remained on the rod. After spotting rods with extracts from experimental samples and scanning with the Iatroscan TLC/FID Analyzer, rods were routinely burned again and then stored in a 100% humidity chamber until needed for the next analysis.

Direct gas chromatography was performed with a Tracor 222 Gas Chromatograph, interfaced with an

external closed inlet device (Scientific Instruments Service, River Ridge, LA), according to the method of Dupuy *et al.* (14). 2-Thiobarbituric acid-reactive substances (TBA), measured at 532 nm with a Hewlett-Packard 8450-A diode array spectrophotometer, were determined by the method of Tarladgis *et al.* (15).

Sensory profiles were performed by the method of Meilgaard *et al.* (16). Evaluations were accomplished by a trained panel of twelve with the spectrum-universal intensity scale, 0–15, as described by Meilgaard *et al.* (16). Desirable beefy notes were: cooked beef/brothy, beefy/meaty (BM), brothy (BRO), browned/caramel (BRC) and sweet (SWT). Undesirable (off-flavor) notes were: Painty (PTY), cardboardy (CBD), sour (SUR) and bitter (BTR) (13).

Lipid extraction and separation. A modified Folch procedure was used for extraction of total lipids (17). The modified extraction solvent was methylene chloride/methanol (2:1, vol/vol). The sample-to-solvent ratio was 1:10. The samples plus solvent were placed in an Omni-Mixer cup (Dupont Co., Newtown, CT) in an ice-water bath and mixed three times for 1-min intervals with 30 s pauses between intervals. Extracts were filtered into a graduated cylinder. Residues were returned to the mixer cup, and the extraction procedure was repeated twice for a total of 30 mL/g sample. After the three extractions were completed, the total volume was measured, and the extract was transferred to a separatory funnel. Distilled water, equal to two-tenths the volume of the extract, was added, and the mixture was shaken vigorously for several minutes. Solutions were allowed to stand overnight (16 h) at room temperature. The organic phase, containing the lipid extract, was collected into a tared, round-bottom flask, and the solvent was evaporated under nitrogen until a constant weight was obtained. Residual lipids were dissolved in methylene chloride/methanol (2:1, vol/vol). Final concentration of lipid was 75–100 μg/μL.

The total lipid fraction was further fractionated by chromatography on a mini-column of silicic acid, which had been dried in an oven at 100°C for 18 h. The silicic acid was cooled in a dessicator, and a slurry was made with methylene chloride. A Pyrex straight-bore Teflon plug, 6 mm i.d., stopcock (Corning 7282-6) was used as the mini-column. Two grams of silicic acid in methylene chloride were poured onto a glass-wool plug, which was inserted just above the Teflon fitting. The mini-column was washed with five volumes of methylene chloride, followed by five volumes of methanol, and then again with five volumes of methylene chloride. The total lipid fraction, 40–50 mg, was applied to the mini-column. Neutral lipids were eluted with 50 mL methylene chloride, followed by the elution of polar (predominately PLs) lipids with 50 mL methanol. Both fractions were placed in round-bottom flasks and brought to dryness on a roto-evaporator under a stream of nitrogen. The dry residual lipids were dissolved in methylene chloride/methanol (2:1, vol/vol) and analyzed by Iatroscan TLC/FID. Neutral and polar fractions obtained were chromatographically pure as judged by Iatroscan TLC/FID.

For comparison purposes, total lipids were extracted from egg yolk, which contains PE and PC as its main PL components. This polar extract was used as a PL standard or as a marker for PE and PC in addition to commercial standards.

Confirmation of matched rods. Two sets of ten Chromarods each were used to obtain a matched set of ten rods. Each of the twenty rods was spotted with 3.0 μg of PC. After application of the sample, the rods were air-dried and then developed in a two-step solvent system. The first phase was comprised of benzene/chloroform/formic acid (50:20:1.5, vol/vol/vol), the development time was for 30 min, air-drying time was 5 min. This phase was used to remove any neutral lipid impurities that may be present. After drying, the rods were developed in the second solvent system, chloroform/methanol/29.3% ammonium hydroxide (50:50:5, vol/vol/vol) for 15 min. The rods were allowed to dry for 5 min and then scanned in the Iatroscan TLC/FID Analyzer. This procedure was repeated seven times on each rod. From the twenty rods tested, a set of ten were selected statistically to form a matched set (Fig. 1).

Calibration of standard curves. Standard curves were run under the two-step system described previously. Commercial standards of PG, PE, PC, LPG, LPE, LPC and Sp, in amounts from 1–5 μg of each PL were used. Regression analyses were done for the following linear and quadratic functions, respectively: $y = ax + b$ and $y = ax^2 + bx + c$, where y is area counts and x is concentration in μg. From these equations, correlation coefficients (r^2 values) were calculated. Equations of the standard curves are shown in Table 1.

Egg yolk PLs as standards. In the present study, hen eggs were purchased from a local supermarket and the yolks were physically separated from the whites. Total lipids were extracted (n = 11) from the yolks with methylene chloride/methanol (2:1, vol/vol) as the extracting solvent. The final concentration of the extract was 222 μg/μL. The rods were spotted with 0.2 μL of this extract and then developed in the two-solvent development system to obtain markers for PLs.

Analysis of beef lipids. A two-step solvent system was used to separate lipid components extracted from cooked ground beef patties stored for 0, 4 and 7 d at 4°C. Experimental samples were applied at the point of origin on the rods. The rods were air-dried and then developed in benzene/chloroform/formic acid (50:20:1.5, vol/vol/vol) for 30 min, then air-dried for 5 min. The neutral lipids

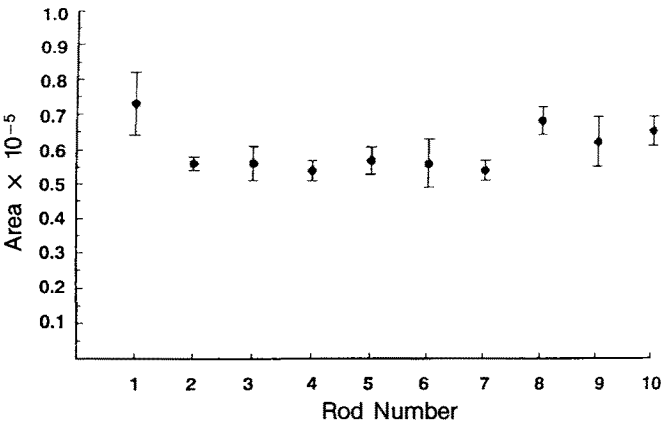

FIG. 1. Calibration of chromarods: mean peak areas × 10^{-5} for 3.0 μg phosphatidylcholine; each mean value represents an n = 7; error bars represent standard error of the mean.

TABLE 1

Linear and Quadratic Regression Constants and Correlation Coefficients for Phospholipid Calibration Curves

	Linear (ax + b)			Quadratic (ax^2 + bx + c)			
Lipid[a]	a	b	r^2	a	b	c	r^2
PG	0.3756	−0.2333	0.9433	0.0590	0.0617	0.0444	0.9883
PE	0.4507	−0.3244	0.9293	0.0531	0.1806	−0.0879	0.9589
PC	0.2922	−0.2125	0.9351	0.0284	0.1413	−0.0786	0.9514
Sp	0.2934	−0.1839	0.9473	0.0456	0.0862	−0.0256	0.9772
LPG	0.1867	−0.0718	0.9662	0.0231	0.0714	0.0051	0.9976
LPE	0.0849	−0.0401	0.9407	0.0106	0.0320	−0.0049	0.9719
LPC	0.3511	−0.1354	0.9610	−0.0094	0.1621	0.0378	0.9847

[a]Abbreviations: PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sp, sphingomyelin; LPG, lysophosphatidylglycerol; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine.

migrated during this development phase, polar lipids remained at the point of origin. The rods were not scanned at this point. After drying, the rods were developed in the second solvent system, chloroform/methanol/ 29.3% ammonium hydroxide (50:50:5, vol/vol/vol) for 15 min. The polar lipids were separated during this phase of the development. Upon completion of the two-stage development process and the drying of the rods, the rods were scanned in the Iatroscan TLC/FID Analyzer. This procedure was repeated three times for each sample.

RESULTS AND DISCUSSION

A set of ten chromarods were used to evaluate inter- and intrarod precision. The peak mean area for all ten rods was 59,661 counts ± 1,788 (Fig. 1). There were no statistical differences ($P > 0.05$) between the responses of each rod. Thus, the interrod precision indicated that the 10 rods were a matched set, which is important to obtain accurate results without an internal standard (18), and it eliminated the need for response factor calculations/rod.

The matched set of 10 Chromarods were used to investigate the linearity and precision of the FID response at microquantities of PLs. There was essentially no significant difference between the correlation coefficients of the linear and quadratic functions for each PL (Table 1). However, upon plotting the data, the quadratic functions deviated slightly from linearity when compared to that of the linear function (Fig. 2). Therefore, the linear functions were used to simplify calculations.

To quantitate individual PLs in the extracts of ground beef patties, standard solutions of seven PLs, ranging from 1 to 5 μg, were analyzed by Iatroscan TLC/FID. The developing method was the same as that used in calibrating the rods. Figure 3 represents the PLs; Figure 4, the lysophospholipids. The plots represent a linear relationship up to the 5 μg quantities, although the y axis intercept was below "zero." This phenomenon was also observed by others (19-20). From 5 to 20 μg, the standard curves were slightly better fitted to a quadratic equation than to a linear equation. These results were similar to those of a plot for the lower concentrations, shown in

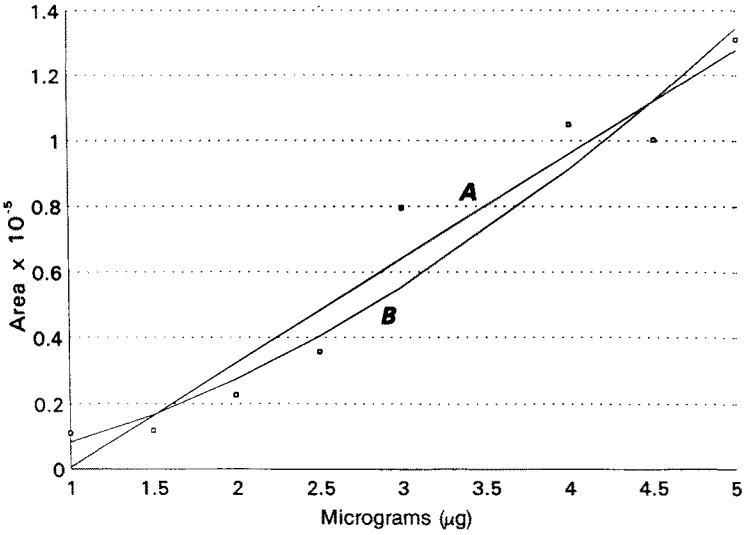

FIG. 2. Computer-drawn response curves in the range of 1 to 5 μg of phosphatidylcholine; A, linear regression response; B, quadratic regression response; n = 3.

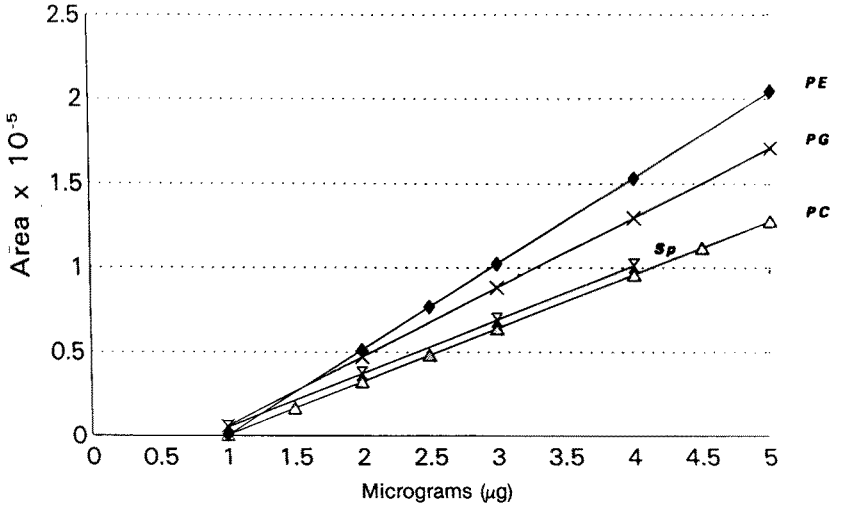

FIG. 3. Calibration curves of Iatroscan thin-layer chromatography thin-layer chromatography/flame-ionization detector (Iatron Labs., Inc., Tokyo, Japan) peak areas *vs.* weights of phospholipid standards spotted on Chromarods (Iatron Labs., Inc.); phosphatidylethanolamine (PE); phosphatidylglycerol (PG); phosphatidylcholine (PC); sphingomylin (Sp); n = 3.

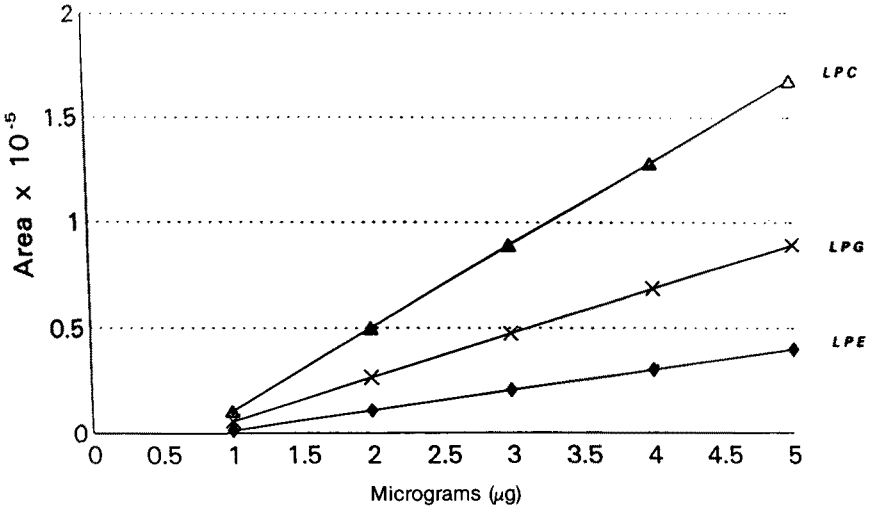

FIG. 4. Calibration curves of Iatroscan thin-layer chromatography/flame-ionization detector peak areas *vs.* weights of phospholipid standards spotted on Chromarods; lysophosphatidylethanololamine (LPE); lysophosphatidylglycerol (LPG); lysophosphatidylcholine (LPC); n = 3. See Figure 3 for company sources.

Table 1. As observed, the r^2 values from the quadratic equation are slightly greater than those from the linear regression plots. Moreover, the r^2 values for both the linear and quadratic regressions were similar to that previously reported by Ackman and associates (19–20).

Egg yolk, a biological material that is rich in PLs, has been used in the large-scale preparation of PLs (21,22). The Iatroscan TLC/FID profile of egg yolk total lipid extract is shown in Figure 5. The peak areas of PE and PC, the most abundant PL of egg yolk, were applied to their respective standard curves. The percentages of PE and PC were 17.93 and 82.06 ± 5.17%, respectively, based on the total PL content. These values are in agreement with those reported by Parkinson (23). Retention times for the two markers were also noted. Consequently, Iatroscan profiles of the two egg yolk PLs were used as markers in addition to those of standards, to evaluate PE and PC, the two main PLs found in beef.

MFD is a highly complex process that involves a loss of desirable flavor notes, such as BM, BRO, BRC and SWT, accompanied by an increase in off-flavor notes, such as, PTY, CBD, SUR and BTR. Other markers of MFD are an increase in TBA numbers and an increase in the intensities of hexanal and total volatiles (13). The intensities of these sensory and chemical markers for the cooked beef patties are shown in Table 2. The 0-day controls had high intensities for the desirable sensory markers, BM, BRO and BRC, low intensities for the off-flavor markers, PTY,

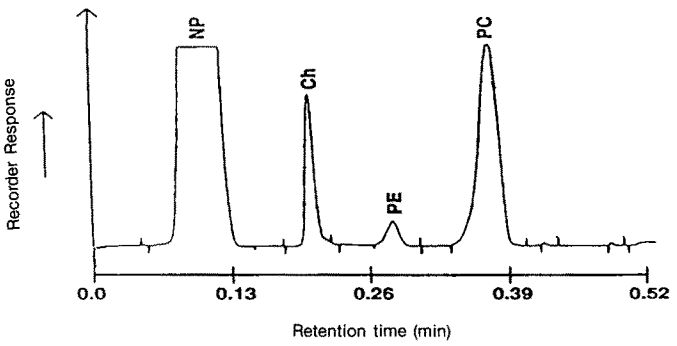

FIG. 5. Typical Iatroscan thin-layer chromatography/flame-ionization detector chromatogram of fresh egg yolk lipid extract; NP, nonpolar lipids; Ch, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine. See Figure 3 for company source.

CBD, SUR and BTR, and low intensities for the lipid oxidation chemical markers, TBA, hexanal and total volatiles. These results are characteristic of those found in meat that has not undergone the MFD process and has retained the desirable beefy taste, as judged by a trained taste panel using descriptive analysis (13). On the other hand, the opposite was observed in the MFD sample that was stored for 4 d. The desirable sensory notes were lower than those in the 0-day controls, whereas the off-flavor notes and the chemical markers were higher than those of the control samples. The 7-day samples were not tasted by the sensory panel, but the chemical indicators were determined. Results indicated that lipid oxidation, and thus MFD, in the 7-day samples were greater than in the samples stored for 4 d.

Typical Iatroscan TLC/FID chromatograms of total lipid extracted from cooked ground beef stored at 4°C for 0, 4 and 7 d are shown in Figure 5. Changing profiles in the chromatograms demonstrate the complexity of the lipids and the changes they undergo under refrigerated storage conditions. All but two of the major peaks were identified. The first was near the origin (the area where the sample was applied to the rod) at a scanning retention time of 0.46 min, and the second appeared at a retention time of *ca.* 21 min. The peak in the polar area of the chromatogram could be either PA, PS or PI, all of which co-eluted in this solvent system. As observed in Figure 6, the changes that occurred during storage appeared to be very slight. However, by compilation of the differences in the peak areas from replicates, the slight changes were more noticeable (Table 3).

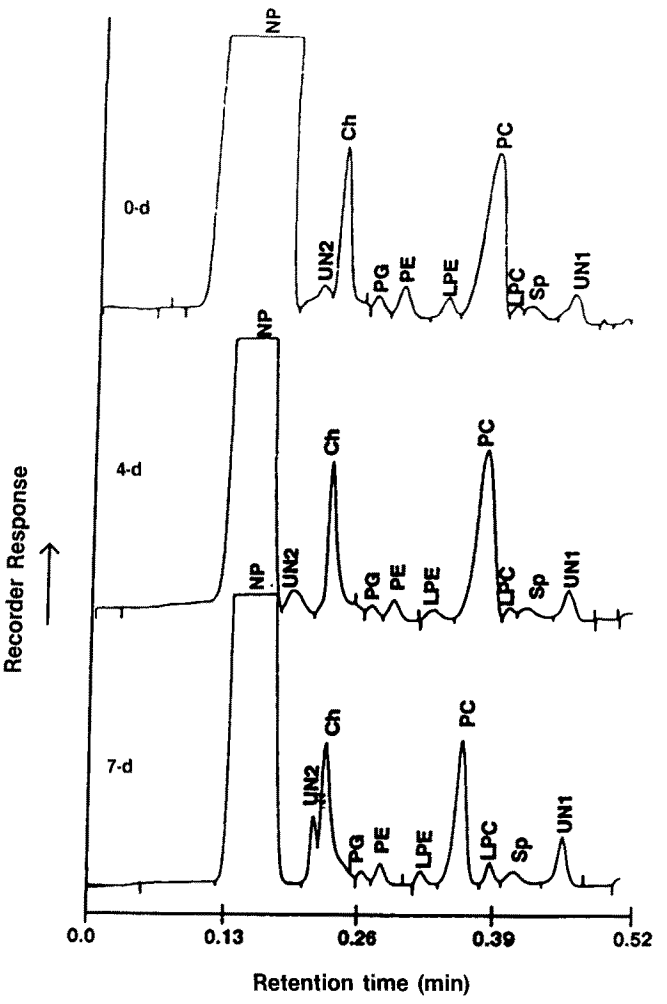

FIG. 6. Iatroscan thein-layer chromatography/flame-ionization detector chromatograms of total lipids from cooked beef patties stored at 4°C; abbreviations as in Figures 3, 4 and 5. UN, unknown. See Figure 3 for company source.

The total lipid extracted from the ground beef was 4.28% (Table 3), which was in the range previously reported (12,13). The total polar lipid of the original 2-g sample was 0.99%, which is in agreement with that of Hornstein *et al.* (24) and Dugan (25). The neutral lipid content was 3.19%, which is also in the range reported by Hornstein *et al.* (24). As storage time caused MFD development, there was a slight decrease in total lipids, an

TABLE 2

Sensory and Chemical Markers for Cooked/Stored Ground Beef Patties (n = 3)[a]

	TBA	HXL	TV	BM	BRO	BRC	SWT	PTY	CBD	SUR	BTR
0-day	5.49	24.0	278.9	3.47	2.08	2.31	0.85	0.22	0.23	0.53	0.26
± SEM	0.74	2.6	40.1	0.06	0.12	0.02	0.04	0.02	0.01	0.05	0.02
4-day	13.47	66.6	355.3	3.14	1.96	2.00	0.86	0.55	0.51	0.62	0.35
± SEM	2.18	13.9	58.6	0.05	0.04	0.04	0.08	0.03	0.03	0.02	0.03
7-day	16.93	76.7	516.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
± SEM	1.02	3.1	45.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

[a]Abbreviations: TBA, 2-thiobarbituric acid reactive substances; HXL, hexanal; TV, total volatiles; BM, beefy/meaty; BRO, brothy; BRC, browned/caramel; SWT, sweet; PTY, painty; CBD, cardboardy; SUR, sour; BTR, bitter; n.a., not applicable.

TABLE 3

Lipid Composition (%) of Stored Cooked Ground Beef (n = 3)

	Total lipids	Neutral lipids	Polar lipids
0-day	4.28	3.19	0.99
± SEM	0.91	0.74	0.05
4-day	4.18	3.23	0.79
± SEM	0.49	0.44	0.01
7-day	4.08	3.36	0.73
± SEM	0.62	0.61	0.07

increase in neutral lipids and a decrease in polar lipids. However, the changes that were observed in the total lipid and neutral fractions were not statistically significant.

Typical Iatroscan TLC/FID chromatograms (not included) of neutral and polar lipids extracted from cooked ground beef stored at 4°C for 0, 4 and 7 d and separated on silicic acid columns were highly comparable to those obtained from the chromatogram of the total lipid profile (Fig. 6).

As expected during storage, there was an increase in lipid oxidation, as indicated by elevated TBA, hexanal and total volatile values (Table 2). The increase in neutral lipids has been previously reported by Willemot *et al.* (26) on the refrigerated storage of pork. Likewise, the decrease in PLs during refrigerated storage of cooked pork was reported by Willemot *et al.* (26), and of cooked beef by Igene and Pearson (11). Both groups reported that the PLs were the main source of lipid oxidation during the MFD process. Furthermore, as lipid oxidation increased, PL content decreased (27). Owing to the reports that triglyceride fats are not involved in the development of MFD (11,28), no further experiments were conducted on the neutral lipid fractions.

The changes in PLs during storage were further investigated (Table 4). The data represent evaluation of three samples of each of the three storage periods, 0, 4 and 7 d, analyzed from 4 to 6 times each. The data also show the PL composition for each of the polar lipid fractions shown in Table 3. PC, by far the most abundant PL found in cooked beef, underwent the largest change and was the only PL that was found to decrease upon storage. All other measured PL were found to increase slightly. Igene and Pearson (11) found that all PL decreased after storage for two days and that PC decreased the most (54%).

TABLE 4

Phospholipid (% ± SEM) Content in Stored Cooked Ground Beef (n = 3)[a]

Phospholipid	0-day	4-day	7-day
PG	12.07 ± 1.35	12.74 ± 0.36	14.02 ± 0.86
PE	13.98 ± 1.05	14.95 ± 0.61	17.94 ± 0.76
LPE	16.88 ± 1.09	16.51 ± 1.08	18.72 ± 0.15
PC	48.41 ± 3.30	43.22 ± 0.23	35.73 ± 2.52
LPC	6.56 ± 0.75	6.73 ± 0.24	8.39 ± 0.17
Sp	9.39 ± 0.87	10.37 ± 0.54	11.93 ± 0.37

[a]Abbreviations as in Table 1.

However, unlike our reference point, freshly cooked beef patties, their reference point was fresh (uncooked) ground beef. Also, our studies were conducted on cooked ground beef stored for four and seven days. Their data were from a storage time of only two days.

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