Journal of Chromatography, 143 (1977) 335—344
Biomedical Applications

CHROMBIO. 045

RAPID MICRODETERMINATION OF FATTY ACIDS IN BIOLOGICAL MATERIALS BY GAS—LIQUID CHROMATOGRAPHY*

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(Received November 3rd, 1976)

SUMMARY

Total and free fatty acids in general ranging from lauric to nervonic acid were separated and quantitated based on an internal standard method as methyl esters by "on column" methylation with trimethyl-(α,α,α-trifluoro-*m*-tolyl) ammonium hydroxide (TMTFTH) in a gas chromatographic system. This study represents an application of a method published by MacGee and Allen and a change to an internal standard technique. For the determination of the total fatty acids the samples were saponified with KOH—CH_3OH, acidified with H_3PO_4, and then the fatty acids were extracted into hexane. An aliquot of the hexane extract was then extracted with TMTFTH and chromatographed. For determination of free fatty acids the sample was acidified with H_3PO_4, immediately extracted with hexane and processed as described earlier. The relative standard deviation of 1.4 to 4.2% illustrates the precision of the method and the recovery of the fatty acids ranged from 88.5 to 100.5%. This method was applied to the determination of fecal fatty acids in conjunction with an interdepartmental study on "High protein diet in colon cancer" at the University of Missouri. In addition, the applicability of the analytical procedure (with small modifications) was shown for a wide variety of biological materials (serum, milk, skin tissue, fungal spores, food homogenates, beef tissues, and tumor cell cultures). The analyses were performed on different gas chromatographs by different analysts.

INTRODUCTION

A wide variety of analytical techniques have been developed and used for the separation and quantitation of fatty acids in biological materials [1, 2]. These techniques have usually produced reliable data. In conjunction with a nutritional study at the University of Missouri, concerned with the effect of high protein (beef) diet on anaerobic and aerobic flora and chemical components of human feces, a large number of fecal specimens and food homogenates were

*Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 7686. Approved by the Director.

analyzed for total and free fatty acids. A summary of the results is published by Hentges et al. [3] and this study is described in further detail by Flynn et al. [4].

A method that allowed for the rapid reliable determination of fatty acids from a large number of samples by "on column" methylation in a gas chromatographic system was published by MacGee and Allen [5]. After saponification of the sample with KOH—CH_3OH, acidification with H_3PO_4, and extraction of saponifiable fatty acids into hexane, the acids were extracted from hexane with a small amount of trimethyl-(α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH) as their quaternary ammonium salts. The salt solutions were injected together with a mixture of methyl propionate—methanol into a gas chromatograph at an injection port temperature of ca. 260°. This procedure was adapted for the analysis of fatty acids in fecal specimens, food homogenates, and modified to an internal standard method (heptadecanoic acid was used as internal standard). In addition, a variety of other biological materials were analyzed including serum (human, dog, and rat), tumor cell culture media, milk (shrew and cow), rat skin, and fungal spores.

EXPERIMENTAL

Apparatus

During the course of fatty acid analyses the following gas chromatographs, equipped with hydrogen flame detectors, were used: Barber-Colman 5000 (Barber-Colman, Rockford, Ill., U.S.A.); Tracor 222 (Tracor, Austin, Texas, U.S.A.), and Packard 7300 (Packard Instrument, Downers Grove, Ill., U.S.A.). The electrometers of these instruments were interfaced with a Hewlett-Packard 3352B laboratory data system (Hewlett-Packard, Avondale, Pa., U.S.A.) for identification and quantitation of the fatty acids.

The samples were analyzed on a column packed with 15% HI-EFF-1BP on 100—120 mesh Chromosorb W (AW), glass, 2 m × 2 mm I.D., or 2.5 m × 2 mm I.D. The injection port temperature was maintained, in general, at 260°, the detector temperature at 245°. The column oven temperature was usually kept at 155°, and varied slightly (140—155°) depending on the instrument used and the type of analysis performed. In general, a linear carrier gas flow (Nitrogen) of 20 cm/sec was used. To determine the average linear carrier gas flow, methane was injected into the column. Methane gas traveled practically with the same speed as the carrier nitrogen gas. The velocity was calculated by dividing the length of the column (cm) by the time (sec) measured with a stop watch from injection to detection.

The samples were stored in a Revco Ultra-low-temperature freezer at −70° (Revco Freezer from Scientific Products, St. Louis, Mo., U.S.A.).

Reagents

All fatty acids (of highest purity available), the pre-tested phase HI-EFF-1BP (diethylene glycol succinate) and Chromosorb W AW were obtained from

Applied Science Labs. (State College, Pa., U.S.A.). Potassium hydroxide (ACS grade), methanol (ACS grade), and silver oxide (purified) were purchased from Fisher Scientific (St. Louis, Mo., U.S.A.). Iodomethane (99% purity) and 3-aminobenzotrifluoride, 99+% (α,α,α-trifluoro-*m*-toluidine) were purchased from Aldrich (Milwaukee, Wis., U.S.A.). Methyl propionate was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). Phosphoric acid (85%, analytical reagent) was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Hexane (high purity) was purchased from Phillips Petroleum (Borger, Texas, U.S.A.). TMTFTH was prepared as described by MacGee and Allen [5].

Procedure

Analysis of saponifiable fecal fatty acids. All specimens were stored at -70°. For the analysis, samples were thawed, homogenized, ca. 100 mg weighed, the exact weight recorded, and then placed in a 12-ml centrifuge tube with a PTFE-lined cap. The original sample was immediately refrozen at -70°. The internal standard (I.S.), heptadecanoic acid (50 μg) in hexane, was then added and the hexane completely evaporated using a stream of pure nitrogen gas at room temperature. To the sample were added 2 ml of KOH—CH_3OH solution (15 g KOH in 100 ml CH_3OH). After purging with nitrogen gas, the centrifuge tube was capped tightly, sonicated briefly and saponified in a dry-bath at 65° for 1 h. During the saponification the contents of the tubes were mixed briefly on a Vortex-Genie every 10 min.

After 1 h the centrifuge tubes were cooled to room temperature and 3 ml of 1 *M* H_3PO_4 were added. The fatty acids were then extracted three times with 5-ml portions of hexane. For each extraction the centrifuge tube was shaken vigorously for 1 min by hand and centrifuged for 10 min. The upper hexane layer was drawn off with a Pasteur pipet and the extracts were pooled in a ca. 20-ml culture tube, and mixed well. A 5.0-ml aliquot was transferred into a 5-ml glass-stoppered centrifuge tube (Pyrex No. 8061), and 10 μl of TMTFTH (0.5 *M*) were added. The centrifuge tube was then shaken vigorously for 1 min and centrifuged at approximately 600 *g* for 1 min. For chromatography 1 μl of the TMTFTH extract was taken from the bottom of the centrifuge tube with a 10-μl syringe. The 1-μl TMTFTH extract was sandwiched between a methyl propionate—methanol mixture (1:2) and injected into a gas chromatograph at an inlet temperature of 260°.

Analysis of free fecal fatty acids. The samples were prepared as described for the determination of total or saponifiable acids with the exception that the KOH—CH_3OH saponification step was omitted, then the sample was acidified with 3 ml of 1 *M* H_3PO_4. The centrifuge tube was closed tightly and carefully mixed on a Vortex-Genie, and sonicated briefly. Distilled water (2 ml) was added to the sample, mixed again, and then extracted with hexane and processed as described previously.

Total fatty acids in food homogenate. The 100-mg samples of food homogenates were analyzed for saponifiable fatty acids in the same manner as described for saponifiable fecal fatty acids.

Saponifiable fatty acids in spores of fungus Helminthosporium. A mixture of 100 mg of spores and 100 μg heptadecanoic acid (I.S.) was saponified with 2

ml KOH—CH_3OH at 75° for 2 h and then treated as described for saponifiable fatty acids.

Total fatty acids in shrew milk. Shrew milk (100 mg) was processed as described for saponifiable fatty acids.

Total fatty acids in rat serum and rat skin. To 100 µl rat serum or 100 mg rat skin and 50 µg heptadecanoic acid (I.S.) 0.5 ml KOH—CH_3OH (15 g KOH in 100 ml CH_3OH) were added. The centrifuge tube was purged with pure nitrogen gas, capped tightly, and saponified at 65° for 30 min. After cooling, 0.7 ml of 1 *M* H_3PO_4 were added, and the assay was carried out as described for saponifiable fecal fatty acids.

Total fatty acids in cell culture medium. A 2.00-ml sample containing 50 µg heptadecanoic acid (I.S.) was lyophilized to dryness, then 1 ml of KOH—CH_3OH (ca. 2 *N*) was added. The 12-ml centrifuge tube was purged with pure nitrogen gas, capped tightly, and heated at 65° in a dry-bath for 30 min. The samples were then treated as described for saponifiable fecal fatty acids.

RESULTS AND DISCUSSION

Calculations

The quantitation of the fatty acids (FA) was based on an internal standard method using heptadecanoic acid as internal standard.

The amount of each fatty acid in a sample was calculated as follows:

$$\mu g \text{ FA per sample} = \frac{\text{Area}_{FA} \times \mu g_{IS}}{\text{Area}_{IS} \times \text{RWR}_{FA/IS}} \quad \text{(samples)}$$

where:

$$\text{RWR}_{FA/IS} = \frac{\text{Area}_{FA} \times \mu g_{IS}}{\mu g_{FA} \times \text{Area}_{IS}} \quad \text{(standard)}$$

The relative weight response (RWR) values of each fatty acid from C12:0 to C20:4 were determined by repeated analyses of calibration standards of the combined fatty acids.

A computer interfaced with the GC instrumentation determined the peak area of the identified fatty acids and was programmed to calculate the amount of each fatty acid per sample.

Precision and recovery

The reliability of the method was continuously monitored during the course of analysis of the different biological materials. In Table I the relative standard deviations (R.S.D.) are listed from independent analyses of five types of biological samples: food homogenates, fecal specimens, shrew milk, rat serum, and tumor cell media.

The last column in Table I shows the average of five R.S.D. values. These

TABLE I

PRECISION OF FATTY ACID ANALYSIS IN DIFFERENT BIOLOGICALS

In most samples C20:1, C20:2, C20:3, and C20:4 appeared in trace to undetectable amounts. NA = Not analyzed, ND = not detected, TR = trace amount.

Fatty acid	R.S.D. (%)					
	Food homogenate	Fecal specimen	Shrew milk	Rat serum	Cell culture	Mean*
C12:0	2.9	6.4	0.7	NA	NA	3.3
C14:0	2.0	4.1	1.6	NA	NA	2.6
C16:0	3.2	2.2	1.1	1.5	1.1	1.8
C16:1	8.3	TR	1.3	5.2	1.8	4.2
C18:0	7.2	2.4	1.6	2.3	0.3	2.7
C18:1	6.0	1.2	1.4	1.0	2.2	2.4
C18:2	0.5	5.4	1.4	0.5	2.8	2.1
C18:3	2.8	ND	0.0	ND	ND	1.4
C20:1	ND	ND	TR	ND	ND	—
C20:2	ND	ND	0.0	ND	ND	—
C20:3	ND	ND	NA	ND	TR	—
C20:4	ND	ND	2.8	0.9	7.0	3.5

*Represents the average of relative standard deviations of five different biological samples. The R.S.D. for each individual biological is calculated from at least three independent analyses. The different biologicals were analyzed on different gas chromatographs with different columns (same type of packing) on different days by different analysts.

R.S.D. values ranged from 1.4 to 4.2%. The values of the precision study were obtained from at least three independent analyses of each different biological sample performed on different gas chromatographs with different types of columns (same type of packing) by different analysts on different days.

The percent recovery for each fatty acid added to five different biologicals is listed as the average in Table II which ranged from 88.5 to 100.5%. Columns three and four in this table show the standard deviation of the averaged recovery values and R.S.D. ranging from 3.3 to 7.5%.

Chromatography and analytical results

A good separation of all fatty acids of interest was achieved on a pre-tested HI-EFF-1BP (diethylene glycol succinate) liquid phase as illustrated in Figs. 1–4. The applicability of the analytical procedure was shown for a wide variety of biological materials with different matrices in Table III.

Fig. 2 shows a representative chromatogram of a standard mixture with fatty acids ranging from capric to nervonic. Shrew milk was analyzed over a wide range from capric to nervonic acids. The fatty acids of longer chain-length than arachidonic appeared usually in trace to undetectable amounts and are not listed in Tables I, II and III.

Generally, the samples were chromatographed isothermally at 140 or 155°. To determine the fatty acids in shrew milk ranging from capric (C10:0) to nervonic acids (C24:1), chromatography was started at 65°. The temperature was then raised by 7°/min to 175° to obtain well shaped peaks especially for

TABLE II

RECOVERY OF FATTY ACIDS ADDED TO DIFFERENT BIOLOGICALS

Fatty acid	Mean recovery (%)*	Standard deviation	R.S.D. (%)
C12:0	88.5	2.9	3.3
C14:0	89.8	6.3	7.0
C16:0	95.0	5.2	5.4
C16:1	95.0	5.5	5.8
C18:0	97.3	6.2	6.3
C18:1	95.6	5.1	5.4
C18:2	97.7	6.9	7.1
C18:3	100.5	6.4	6.4
C20:1	98.8	6.0	6.1
C20:2	99.3	7.4	7.5
C20:3	96.9	5.8	6.0
C20:4	97.4	3.2	3.3
AV.	96.0	5.6	5.8

*Represents at least two independent runs for each of five different biological samples performed on different gas chromatographs with different columns (same type of packing), on different days by different analysts.

the long chain acids in a reasonable time. To avoid excessive column bleeding and shifting of the baseline the column temperature was not increased higher than 175°.

A good example for the efficiency of the method by MacGee and Allen [5] is shown in Fig. 1 depicting the fatty acid composition of a fecal specimen. Practically no interference from background was observed. The extraction of the fatty acids from the acidic methanol—water phase into the hexane phase and the back-extraction of the acids from the supernatant hexane into the aqueous system containing Oakes and Willis base TMTFTH [6] functions as a very efficient cleanup step. A further advantage inherent in this method is the concentrating effect [5] by extracting the fatty acids out of 5 ml hexane into 10 μl TMTFTH. This is illustrated in Fig. 4, where low levels of fatty acids were determined in cell culture media. To ensure complete saponification of fungal spores the samples were heated at 75° for 2 h. The completeness of saponification of the pellet was determined by resaponifying and analyzing. No fatty acids were found. For the determination of the fatty acids in rat serum, rat skin tissue, and all culture media samples were saponified for 30 minutes which was sufficient.

Fig. 3 shows the chromatogram of the fatty acid pattern in rat serum. Only small samples were necessary for the determination. The samples were obtained from Dr. Boyd L. O'Dell who is conducting nutritional fatty acid deficiency studies; note in Fig. 3, the low level of 8,11,14-eicosatrienoic acid (C20:3ω6) and the high level of abnormal 5,8,11-eicosatrienoic acid (C20:3ω9) which appears when essential fatty acid deficiency in certain mammals is induced (for the explanation of the abbreviated fatty acid formulae see legend to Fig. 3 and Press et al. [7]. Since 5,8,11-eicosatrienoic acid was not available as a reference compound we had to deduce the presence of this abnormal acid

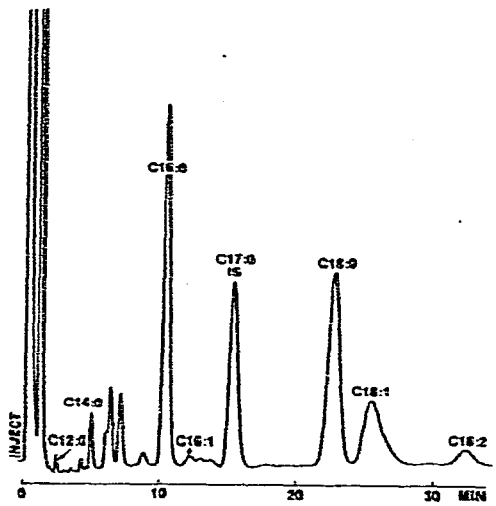

Fig. 1. Gas—liquid chromatographic analysis of saponifiable fecal (human) fatty acids as methyl esters. Sample, 100 mg (amount of sample injected into instrument, ca. 3 mg). Column: 15% HI-EFF-1BP on 100—120 mesh Chromosorb W AW, 2.5 m × 2 mm I.D., glass; volume injected, 1 μl of TMTFTH extract sandwiched between a mixture of methyl propionate—methanol (1:2); column temperature, 140° (isothermal); detector temperature, 210°; and injection temperature, 240°. Gas chromatograph: Barber-Colman 5000.

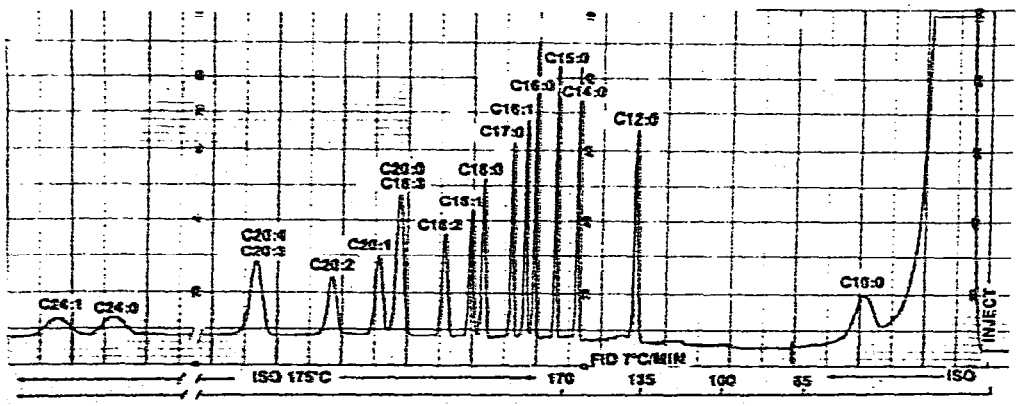

Fig. 2. Gas—liquid chromatography of fatty acids as methyl esters in a standard mixture, ranging from caprie to nervonic acid. Volume injected, 1 μl of TMTFTH extract, 200 ng of each fatty acid. Column and sandwiching as in Fig. 1. Initial column temperature 65°, isothermal for 28 min, programmed 7°/min, final temperature 175°. Injector temperature 250°, detector temperature 210°. Instrument Tracor 222.

TABLE III

SAPONIFIABLE FATTY ACID COMPOSITION IN BIOLOGICAL SAMPLES

ND = Not detected, NA = not analyzed.

Fatty acid	Food homogenates, dry wt. (mg/g)	Fecal specimen, dry wt. (mg/g)	Spores *Helminthosporium*, dry wt. (mg/g)	Shrew milk, wet wt. (mg/g)	Rat serum (mg/100 ml)	Rat skin, wet wt. (mg/100 g)	Tumor cell culture (μg/ml)
Lauric C12:0	1.08	0.29	ND	8.31	NA	NA	NA
Myristic C14:0	4.25	1.15	0.06	10.11	NA	NA	NA
Palmitic C16:0	20.66	11.79	6.11	13.47	46.6	248.9	31.3
Palmitoleic C16:1	1.35	0.34	0.26	1.29	18.8	91.7	6.9
Stearic C18:0	12.77	11.92	1.20	2.00	23.1	91.1	22.9
Oleic C18:1	82.99	12.39	6.31	8.47	86.8	459.3	40.3
Linoleic C18:2	10.32	1.21	12.74	8.90	8.2	486.5	55.8
Linolenic C18:3	1.36	0.17	1.22	0.73	ND	8.3	7.5
11-Eicosenoic C20:1	ND	ND	ND	0.31	ND	9.0	0.7
11,14-Eicosadienoic C20:2	ND	ND	ND	0.38	ND	17.4	ND
8,11,14-Eicosatrienoic C20:3	ND	ND	ND	ND	38.0*	5.4	3.1
Arachidonic C20:4	ND	ND	ND	0.32	11.5	81.7	7.8

*5,8,11-eicosatrienoic acid.

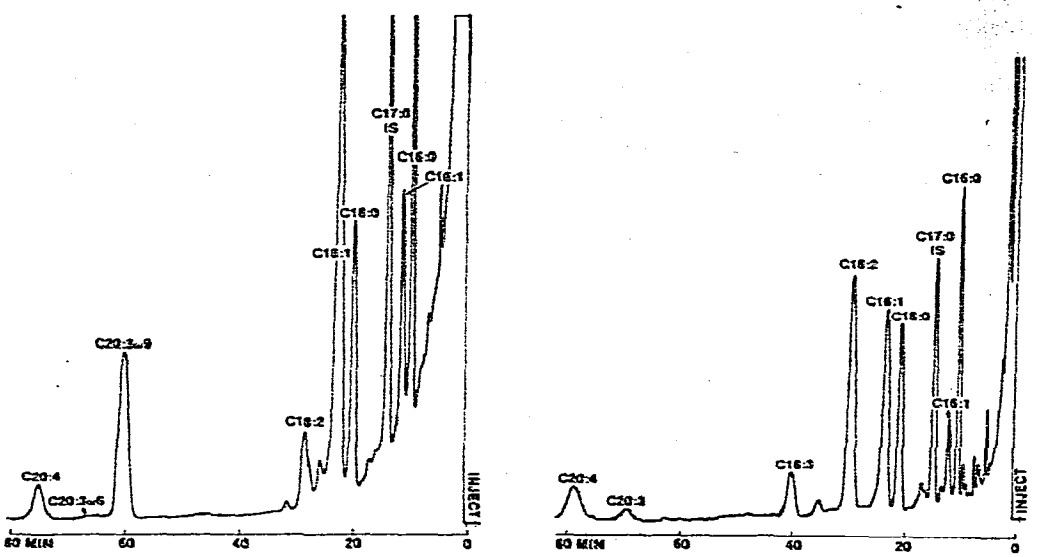

Fig. 3. Gas—liquid chromatography of saponifiable fatty acids as methyl esters in serum of rats with essential fatty acid deficiency manifested by the presence of abnormal 5,8,11-eicosatrienoic acid (C20:3ω9), the practical disappearance of 8,11,14-eicosatrienoic acid (C20:3ω6) and a rather low level of arachidonic acid (C20:4). Sample: 100 μl, sample size injected ca. 3 μl. Chromatographic conditions: volume injected, 1 μl TMTFTH extract; the 2 m × 2 mm I.D. glass column was packed with 15% HI-EFF-1BP on Chromosorb W AW (100—120 mesh); column temperature 155° (isothermal), detector temperature 240° and inlet temperature 260°. Gas chromatograph: Packard 7300. The abbreviated formula (C20:3ω9) indicates the number of carbon atoms and the numbers of double bonds. The position of the double bond nearest to the methyl terminus, counting CH_3 as 1, is indicated by the symbol ω.

Fig. 4. Gas—liquid chromatographic analysis of saponifiable fatty acids as methyl esters in a cell culture medium of prostate (human) cancer. Sample: 2.00 ml, sample size injected ca. 50 μl. Lyophilized to dryness; volume injected, 1 μl TMTFTH extract. Gas chromatograph: Packard 7300. See legend to Fig. 3 for further details.

Based on the elution pattern of a chromatogram published by Press et al. [7]. The 5,8,11-eicosatrienoic acid was quantitated on the assumption that its RWR factor would be similar to that of the isomer 8,11,14-eicosatrienoic acid. Based on our experience, this should be a reasonable approach.

Occasionally "memory" peaks were observed when samples of relative high fatty acid concentration were analyzed prior to those of lower concentration. Repeated injections of a mixture of TMTFTH and methyl propionate—methanol eliminated the "ghosting" effect.

When the hexane solution of the internal standard has been added to the sample it is very important to remove all hexane by use of a gentle stream of pure nitrogen gas to avoid incomplete saponification under the given experimental conditions.

CONCLUSION

The described internal standard method proved to be rapid and reliable for the determination of fatty acids at low levels in a variety of biologicals. Sample preparation and analysis requires approximately 1½ h. The TMTFTH extraction of the acids from the organic phase functions as a cleanup and concentration step. Good precision and accuracy of the method was demonstrated by use of different instrumentation and different analysts.

ACKNOWLEDGEMENTS

This research was performed in part under Contract No. NO1 CP 33335 from the Division of Cancer Cause and Prevention, National Cancer Institute. Samples were kindly provided by Professors D.J. Hentges, M.A. Flynn, R.R. Anderson, O.H. Calvert, B.L. O'Dell, and R.D. Williams. We thank Messrs. David P. Pozniak and Michael T. Patterson for technical assistance.

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