Journal of Chromatography, 331 (1985) 141–148
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 833

EVALUATION OF A RAPID METHOD FOR PREPARATION OF FATTY ACID METHYL ESTERS FOR ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY

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(Received April 18th, 1985)

SUMMARY

The major limitation to fatty acid analysis by gas–liquid chromatography is associated with preparation of fatty acid methyl esters (FAME). In the present study, FAME preparations were made from plant oils (corn, olive, sunflower), sunflower oil margarine, lard and various animal tissue fats by a rapid transesterification involving tetramethylammonium hydroxide in methanol, and also by a longer conventional saponification–esterification method. Fats from animal (beef, mutton, pork) adipose tissues were extracted by a simpler modified procedure and also by the Folch method prior to the rapid and the conventional FAME preparations, respectively. FAME analysis on a gas–liquid chromatograph equipped with a Silar 10C glass capillary column indicated similar fatty acid composition of a given fat or oil, whether FAME was prepared by the rapid or the longer conventional method. The data obtained by both methods were very highly correlated for all the fats (r = 0.9895 – 0.9999). However, the rapid method showed a tendency for enhanced recoveries of lower chain fatty acids (*e.g.* 14:0), and also of unsaturated C_{18} isomers. Possibly, losses of fatty acids that occurred during the lengthy fat extraction, fatty acid esterification or ether-evaporation FAME concentration steps (conventional method) were minimised by the single transesterification step (rapid method). This rapid transesterification method appears to be an attractive alternative to FAME preparation from a wide variety of different fats for gas–liquid chromatographic analysis.

INTRODUCTION

The fatty acid composition of lipids is routinely determined by gas–liquid chromatography (GLC). Several methods are available for preparation of fatty acid methyl esters (FAME) for injection into the gas–liquid chromatograph[1–5].

In this laboratory, conventional FAME preparation[6] is a modification of an existing method[7], which involves saponification followed by esterification. This

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0021-9673/85/$03.30

method is accurate and reliable, but is tedious and requires large amounts of costly reagents. Metcalfe and Wang[8] described an alternative rapid FAME preparation method based on a single transesterification reaction, and found it suitable for fatty acid analysis by GLC on a packed stainless steel column.

The objective of this study was to extend the use of this rapid method to FAME preparation from a wide variety of fats, including those from animal tissues. Data reported herein were accumulated following comparative FAME analyses on a chromatograph equipped with a glass capillary column.

EXPERIMENTAL

Reagents and salt solution for lipid extraction

Reagent grade solvents and chemicals were obtained from suppliers in Canada (BDH, Toronto) or the U.S.A. (Aldrich, Milwaukee, WI; Fisher, Fairlawn, NJ). The oils (corn, olive and sunflower), margarine (100% sunflower oil), lard and tissue fat samples (beef, mutton and pork) were purchased from local suppliers.

A washing solvent was prepared by mixing chloroform, methanol and water in proportions of 3:48:47 by volume. Using this solvent, a salt solution was prepared to contain 0.02% calcium chloride, 0.017% magnesium chloride and 0.29% sodium chloride.

Tissue fat extraction

Fat was extracted[9] from individual portions of tissues randomly selected. Glass containers were used in all extractions and sample storage.

Extraction of tissue fat prior to conventional FAME preparation involved homogenising 5–10 g of tissue (1 g ≡ 1 ml) for 3 min at 50 rpm (manual homogeniser) in a 20-fold final dilution of chloroform–methanol (2:1). The fat extract was then decanted into a 100 × 13 mm test tube and the residual tissue discarded. The crude extract was mixed thoroughly with the salt solution (20% v/v), and the resulting mixture allowed to separate into two distinct phases (without interfacial fluff) by standing at room temperature for 10 min. Following careful withdrawal of the upper phase with a pasteur pipette, the remaining lower phase (containing the lipid extract) was washed twice with a small aliquot of the prepared solvent. The resulting extract was ready for methylation and could be diluted to any desired volume by the addition of chloroform–methanol, 2:1 (v/v). This extract was stored under gaseous nitrogen at 5°C in a closed screw-cap glass vial until required for FAME preparation.

Extraction of tissue fat for rapid FAME preparation involved solubilization. Portions of tissue (100–200 mg) were allowed to stand in 3 ml of diethyl ether in a glass test tube at room temperature for 4 h. A blunted glass rod was then used as a pestle to exude fat from the tissue. The residual tissue was discarded and the extracted fat sample treated as for oil.

FAME preparation

Extracted tissue fats, oils or melted fat were used for FAME preparation by each of the two methods.

Liquid fats (10 drops of lipid extract or one drop of oil, melted margarine or lard) were saponified in a 50-ml round-bottomed flask with a ground-glass top, as

follows. A 10-ml volume of 4% potassium hydroxide in methanol was added to the flask, fitted to a 60-cm glass column with a ground-glass joint and heated for 45 min to a soft rolling boil on a Variheat hot plate (Precision Scientific, Chicago, IL, U.S.A.). After cooling, the mixture was esterified by the addition of three drops of 0.1% bromophenol blue (in methanol), followed by 10 ml of 4% acetyl chloride (in methanol). The resulting mixture was refluxed for 45 min. After cooling, anhydrous sodium carbonate was added gradually to the flask with swirling until the blue colour reappeared. At this point the samples were transferred to individual 50-ml volumetric flasks, and 10 ml of petroleum ether (b.p. 30–60°C) were added. The sample was swirled gently and water added slowly to bring the petroleum ether layer up to the neck of the flask. After clearing, the upper petroleum ether layer was transferred to a calibrated test tube using a pasteur pipette. The petroleum ether was then evaporated under a stream of gaseous nitrogen with mild heating in a fume hood cupboard. Hexane (0.5 ml) was added to dissolve the residual FAME mixture.

Rapid transesterification involved dissolving 10 drops (100–200 mg) of a liquid sample (extracted tissue fat, oil or melted fat) in 3 ml of diethyl ether in a test tube to which 0.2 ml of 20% tetramethylammonium hydroxide (TMAH) was then added. The test tube was covered and shaken for 2 min, and the layers were allowed to separate. One drop of thymol blue indicator was added, followed by 0.5 *N* hydrochloric acid in methanol dropwise to turn the indicator from blue to yellow. Methanol (0.5 ml) was added to make the sample homogeneous.

FAME mixtures prepared from the various samples by both methods were kept under gaseous nitrogen in capped glass vials and stored at 4°C until required for injection into the chromatograph.

Chromatograpic methods

A Perkin-Elmer Sigma 2000 gas–liquid chromatograph (Norwalk, CT, U.S.A.) with a Hewlett-Packard 3390A integrator (Chicago, IL, U.S.A.) was used in this study. The chromatograph was equipped with a flame ionisation detector and a 40 m × 0.25 mm I.D. WCOT 12 glass column packed with polycyanopropylsiloxane, CS-10, Silar 10C, 0.4 μm (Terochem Labs., Edmonton, Canada). Nitrogen was the carrier gas at a flow-rate of 2.2 ml min^{-1}. The detector was optimised for linearity[10,11] and operated with air and hydrogen gas at a pressure of 14 kPa. The temperature programme and operating conditions are listed in Table I. Selection of in-

TABLE I

TEMPERATURE PROGRAMME FOR GLC AND MAXIMUM SET OPERATING CONDITIONS FOR FAME ANALYSIS

*Parameter**	*1*	*2*	*3*
Temperature (°C)**	150	200	220
Time (min)	4	5	3
Rate (°C min^{-1})	3	20	0

* Set operating conditions were: maximum temperature, 225°C; equilibration time, 8 min; range, 1 cm min^{-1}; attenuation, 2.

** Injection and detection temperatures were: 240 and 250°C *vs.* 290 and 270°C for conventional *vs.* rapid methods, respectively.

jection and detection temperatures for analysis of FAME prepared by both methods was based on data from preliminary runs. For all FAME samples analysed, the integrator run parameters were: zero, 5; attenuation, 0; chart speed, 0.1 cm min^{-1}; peak width, 0.16 min, threshold, 0; and area of rejection, 0.

A sample size of 0.5–1.0 μl was used for injection with a 36:1 split ratio. Fatty acids were identified using reference standards obtained from Sigma (St. Louis, MO, U.S.A.) and Terochem. Optimal conditions for GLC were estasblished using a mixture of NHI reference standards (H-104, Applied Scientific Labs., State College, PA, U.S.A.) containing alkyl chain lengths of 14, 16, 18, 20, 22 and 24 carbon atoms. For a given fat, 10–13 injections were made of FAME mixtures individually prepared.

Statistical analyses

Significant differences between corresponding fatty acid means were established by the paired or unpaired student-t test. Correlation coefficients were computed using the mean values of those fatty acids recovered following FAME preparation by the conventional *versus* the rapid method[12].

RESULTS AND DISCUSSION

The method of fat extraction (*i.e.* ether solubilisation or Folch procedure[9]) prior to rapid FAME preparation[8] had no effect on the fatty acid composition of a given animal tissue fat (Misir *et al.*, unpublished data). Therefore, it was decided to use the simple ether solubilisation in preference to the more elaborate Folch procedure for extraction of the animal tissue fats. This preferred method might be particularly useful in certain studies where sample size is limited.

FAME analysed were those for which standards were available in the laboratory. Recoveries were in excess of 95% by weight of the total fatty acid content for all fats, including mutton fat (Fig. 1).

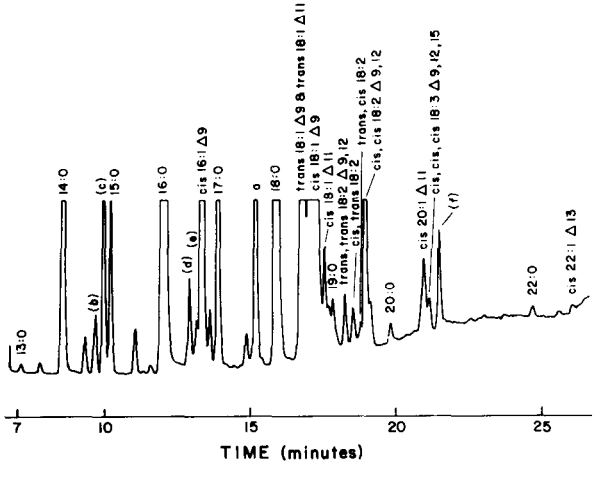

Fig. 1. A typical chromatogram to illustrate a split sample injection for mutton fat FAME prepared by the rapid method. Split ratio, 36:1. Unidentified peaks are designated by letters a–f.

The fatty acid composition of FAME mixtures prepared by both methods were similar (Tables II–V). Correlation coefficients for the two FAME preparation methods were highly significant ($P < 0.001$) for the eight fats (Table VI), indicating that rapid transesterification using TMAH (in methanol)[8] was equally as effective as the conventional saponification–esterification[6]. However, there were some differences between the mean values for some fatty acids. Lower concentrations of shorter chain fatty acids (14:0, 15:0) were detected when the conventional in contrast to the rapid method was used. For 14:0, the values (%) were 0.04 *vs.* 0.06 ($P < 0.05$), sunflower oil; 3.43 *vs.* 3.84 ($P < 0.05$), beef fat; 2.68 *vs.* 3.13 ($P < 0.01$), mutton fat; and 1.13 *vs.* 1.21 ($P < 0.01$), pork fat; whereas for 15:0, the values were 0.05 *vs.* 0.07 for pork fat. These data indicate that some loss of short chain fatty acids occurred probably during the ether evaporation–FAME concentration step of the conventional esterification–saponification[13,14]. Furthermore, conventional FAME preparation resulted in lower levels of unsaturated C_{18} isomers, *e.g.* values for *cis,cis*-18:2$\Delta^{9,12}$ (%) were 60.71 *vs.* 62.38 ($P < 0.01$), corn oil; 41.47 *vs.* 43.66 ($P < 0.05$), sunflower oil margarine; 12.34 *vs.* 13.28 ($P < 0.01$), pork fat; 7.37 *vs.* 7.56 ($P < 0.05$), lard; and 2.62 *vs.* 2.98 ($P < 0.01$), mutton fat. In the case of beef fat, lower values for certain unsaturated C_{18} isomers (*e.g.* 36.79 *vs.* 40.20% for *cis,cis*-18:2 $\Delta^{9,12}$) accompanied increases in the recovery of the corresponding saturated C_{18} isomer (*i.e.* 18.67 *vs.* 13.45% for 18:0). These results suggest that increased hydrogenation or loss of fatty acids probably occurred during sample heating (saponification) and refluxing (esterification) by the conventional method. These processes would likely be minimised under the milder conditions (*e.g.* room temperature, 20–22°C) of the rapid transesterification method.

TABLE II

EFFECT OF FAME PREPARATION METHOD ON FATTY ACID COMPOSITION OF CORN AND OLIVE OILS

Values are percent of total fatty acids.

Fatty acids	*Corn oil**		*Olive oil**	
	Conventional	*Rapid*	*Conventional*	*Rapid*
16:0	10.94 ± 0.628	10.59 ± 0.714	12.44 ± 0.253	12.45 ± 0.454
cis-16:1 Δ^9	0.11 ± 0.032	0.08 ± 0.031 (11)	0.99 ± 0.023 (9)	1.02 ± 0.045
17:0	0.06 ± 0.010	0.07 ± 0.015 (7)	0.07 ± 0.005 (9)	0.06 ± 0.002
18:0	1.86 ± 0.108	1.77 ± 0.150	2.63 ± 0.375	2.37 ± 0.034
trans-18:1 Δ^9, and Δ^{11}	0	0	0.04 ± 0.014 (9)	0.02 ± 0.011** (9)
cis-18:1 Δ^9	24.01 ± 0.997	23.28 ± 0.844	68.15 ± 4.775	71.61 ± 1.010
cis,cis-18:2 $\Delta^{9,12}$	60.71 ± 2.421	62.38 ± 0.530**	11.84 ± 1.301	11.24 ± 0.143 (10)
20:0	0.43 ± 0.089	0.37 ± 0.056	0.43 ± 0.016 (9)	0.39 ± 0.020***
cis-20:1 Δ^{11}	0.30 ± 0.030 (4)	0.27 ± 0.030 (9)	0.22 ± 0.015 (8)	0.28 ± 0.020
cis,cis,cis-18:3 $\Delta^{9,12,15}$	0.76 ± 0.133 (4)	0.77 ± 0.017 (9)	0.59 ± 0.017 (7)	0.58 ± 0.024
22:0	0.15 ± 0.068	0.14 ± 0.107 (8)	0.19 ± 0.060 (9)	0.14 ± 0.049 (11)
cis-22:1 Δ^{13}	0.04 ± 0.041 (6)	0.07 ± 0.029 (3)	0.11 ± 0.077 (9)	0.14 ± 0.074 (7)

* Mean ± standard deviation for different sample analyses: corn oil, 11 (conventional) or 12 (rapid); olive oil, 10 (conventional) or 12 (rapid), unless otherwise indicated ( ).

** $P < 0.05$.

*** $P < 0.01$.

TABLE III

EFFECT OF FAME PREPARATION METHOD ON THE FATTY ACID COMPOSITION OF SUNFLOWER OIL AND MARGARINE

Values are percent of total fatty acids.

Fatty acid	*Sunflower oil**		*Sunflower oil margarine**	
	Conventional	*Rapid*	*Conventional*	*Rapid*
14:0	0.04 ± 0.010 (8)	0.06 ± 0.005 (12)**	0.07 ± 0.025 (7)	0.07 ± 0.005 (9)
16:0	6.73 ± 0.216	6.55 ± 0.142	7.44 ± 1.063	6.83 ± 0.240
cis-16:1 Δ^9	0.07 ± 0.014 (9)	0.07 ± 0.011 (12)	0.07 ± 0.023 (7)	0.07 ± 0.021 (8)
17:0	0.05 ± 0.011 (6)	0.04 ± 0.005 (7)***	0.06 ± 0.016 (6)	0.05 ± 0.007 (6)
18:0	5.25 ± 0.180	4.96 ± 0.506	9.20 ± 0.725	9.31 ± 0.360
trans-18:1 Δ^9, and Δ^{11}	0	0	12.17 ± 0.582	12.38 ± 0.382
cis-18:1 Δ^9	14.00 ± 0.583	14.93 ± 1.934	23.82 ± 1.006	23.52 ± 0.245
cis-18:1 Δ^{11}	0	0	0.23 ± 0.024 (9)	0.24 ± 0.036
trans,trans-18:2 $\Delta^{9,12}$	0	0	0.10 ± 0.009 (8)	0.10 ± 0.004 (6)
cis,cis-18:2 $\Delta^{9,12}$	70.56 ± 1.770	71.96 ± 0.616	41.47 ± 2.816	43.66 ± 1.077**
20:0	0.34 ± 0.016	0.32 ± 0.057	0.42 ± 0.080 (10)	0.40 ± 0.056
cis-20:1 Δ^{11}	0.18 ± 0.064	0.16 ± 0.013 (12)**	0.57 ± 0.328 (10)	0.38 ± 0.097
cis,cis,cis-18:3 $\Delta^{9,12,15}$	0.23 ± 0.103	0.17 ± 0.014 (12)**	0.54 ± 0.197 (9)	0.38 ± 0.079
22:0	0.91 ± 0.072 (9)	1.00 ± 0.342	0.88 ± 0.099 (9)	0.82 ± 0.168
cis-22:1 Δ^{13}	0	0	0.10 ± 0.037 (7)	0.09 ± 0.020 (10)

* Mean ± standard deviation for different sample analyses: sunflower oil, 10 (conventional) or 13 (rapid); sunflower oil margarine, 11 (conventional) or 12 (rapid), unless otherwise indicated ( ).

** $P < 0.05$.

*** $P < 0.01$.

TABLE IV

EFFECT OF FAME PREPARATION METHOD ON FATTY ACID COMPOSITION OF PORK FAT AND LARD

Values are percent of total fatty acids.

Fatty acid	*Pork fat**		*Lard**	
	Conventional	*Rapid*	*Conventional*	*Rapid*
14:0	1.13 ± 0.044	1.21 ± 0.057 ***	1.46 ± 0.036	1.47 ± 0.090
15:0	0.05 ± 0.004	0.07 ± 0.013***	0.11 ± 0.039 (9)	0.09 ± 0.009
16:0	22.59 ± 0.295	22.02 ± 0.752	26.71 ± 0.305	26.74 ± 0.399
cis-16:1 Δ^9	2.27 ± 0.088	2.51 ± 0.124***	2.54 ± 0.050	2.57 ± 0.100
17:0	0.30 ± 0.009	0.35 ± 0.090**	0.52 ± 0.031	0.50 ± 0.015
18:0	11.30 ± 0.438	10.76 ± 0.722	15.69 ± 0.210	15.31 ± 0.570
cis-18:1 Δ^9	44.63 ± 0.601	43.80 ± 1.753	40.33 ± 0.689	41.45 ± 0.585***
cis-18:1 Δ^{11}	0.05 ± 0.079	0.06 ± 0.029 (8)	0.04 ± 0.022 (5)	0.06 ± 0.005
cis,cis-18:2 $\Delta^{9,12}$	12.34 ± 0.291	13.28 ± 0.321***	7.37 ± 0.166	7.56 ± 0.189**
20:0	0.24 ± 0.016	0.23 ± 0.048	0.18 ± 0.061	0.22 ± 0.044
cis-20:1 Δ^{11}	0.03 ± 0.015 (9)	0.03 ± 0.027 (8)	0.08 ± 0.097 (2)	0.03 ± 0.039
cis,cis,cis-18:3 $\Delta^{9,12,15}$	1.98 ± 0.075	2.05 ± 0.075	1.44 ± 0.135	1.41 ± 0.039
22:0	0.72 ± 0.302	0.87 ± 0.310	0.63 ± 0.350	0.38 ± 0.086
cis-22:1 Δ^{13}	0.29 ± 0.024	0.43 ± 0.150***	0.43 ± 0.369	0.38 ± 0.046

* Mean ± standard deviation for ten different sample analyses, unless otherwise indicated ( ).

** $P < 0.05$.

*** $P < 0.01$.

TABLE V

EFFECT OF FAME PREPARATION METHOD ON FATTY ACID COMPOSITION OF BEEF AND MUTTON FATS

Values are percent of total fatty acids.

Fatty acid	*Beef fat**		*Mutton fat**	
	Conventional	*Rapid*	*Conventional*	*Rapid*
14:0	3.43 ± 0.446	3.84 ± 0.343**	2.68 ± 0.174	3.13 ± 0.370***
15:0	0.71 ± 0.072	0.79 ± 0.141	0.87 ± 0.026	0.88 ± 0.199
16:0	27.74 ± 0.841	27.56 ± 1.443	26.00 ± 0.483	25.57 ± 1.840
cis-16:1 Δ^9	3.37 ± 0.695	4.95 ± 0.524***	2.00 ± 0.047	2.24 ± 0.184***
17:0	1.86 ± 0.342	1.35 ± 0.149***	2.55 ± 0.156	2.25 ± 0.200**
17:1	1.04 ± 0.061	1.13 ± 0.286	0	0
18:0	18.67 ± 2.204	13.45 ± 2.430***	23.47 ± 1.517	22.08 ± 2.131**
trans-18:1 Δ^9, and Δ^{11}	1.77 ± 0.279 (9)	1.72 ± 0.459	1.25 ± 0.212 (6)	1.71 ± 0.559
cis-18:1 Δ^9	36.79 ± 1.945	40.20 ± 1.223***	32.83 ± 2.155	33.53 ± 3.398
trans,trans-18:2 $\Delta^{9,12}$	0.10 ± 0.061	0.19 ± 0.053***	0.25 ± 0.041	0.24 ± 0.056
cis,cis-18:2 $\Delta^{9,12}$	1.36 ± 0.578	1.22 ± 0.060	2.62 ± 0.243	2.98 ± 0.185***
20:0	0.14 ± 0.015	0.11 ± 0.031***	0.18 ± 0.019	0.18 ± 0.047
cis-20:1 Δ^{11}	0.45 ± 0.082 (6)	0.35 ± 0.065	0.11 ± − (1)	0.11 ± 0.021 (12)
cis,cis,cis-18:3 $\Delta^{9,12,15}$	0	0	0.40 ± − (1)	0.54 ± 0.102
22:0	0.13 ± 0.160 (4)	0.07 ± 0.110 (10)	0.07 ± 0.011	0.09 ± 0.017 (9)
cis-22:1 Δ^{13}	0.04 ± 0.070 (4)	0.02 ± 0.006 (9)	0.16 ± 0.037	0.20 ± 0.024 (9)

* Mean ± standard deviation for different sample analyses: beef fat, 12 (conventional), 13 (rapid); or mutton fat, 10 (conventional) or 13 (rapid), unless otherwise indicated ( ).

** $P < 0.05$.

*** $P < 0.01$.

TABLE VI

CORRELATION COEFFICIENTS FOR FAME PREPARED FROM VARIOUS LIPIDS BY THE CONVENTIONAL *VS.* THE RAPID METHOD

Lipid	*r value**
Corn oil	0.9997
Olive oil	0.9998
Sunflower oil	0.9999
Margarine (sunflower oil)	0.9994
Pork fat	0.9996
Lard	0.9998
Beef fat	0.9895
Mutton fat	0.9991

* $P < 0.001$.

For a given fat, the number of fatty acid replicates detected by GLC following rapid transesterification was generally greater than those for conventional saponification–esterification (Tables II–V). For certain fatty acids, *e.g.* *cis*-20:1 Δ^{11} and *cis,cis,cis*-18:3 $\Delta^{9,12,15}$ of mutton fat, nearly all the expected values (12–13) were detected, in contrast to only one value for each of these acids following conventional

FAME preparation (Table IV). Therefore, the rapid method seemed to promote clearer separation of FAME mixtures during GLC analysis.

The major limitation in GLC analyses of fatty acids is associated with FAME preparation. Perhaps the greatest advantage of the current rapid transesterification method[8] is elimination of the refluxing and FAME concentration steps[6], during which major losses of FAME are likely to occur. For certain fats analysed the rapid method also seemed to facilitate clearer separation of FAME, including unsaturated isomers.

In conclusion, the present study shows that the rapid transesterification method seems suitable for FAME preparation from a wide variety of animal and plant fats. Satisfactory FAME separation may be achieved using a glass capillary column. In view of the minimal sample degradation, simplicity and low cost, this method is an attractive alternative to conventional saponification–methylation procedures.

ACKNOWLEDGEMENTS

This study was supported by the Saskatchewan Farmlab Research Program. The authors express their appreciation to Ms. D. L. Thierry and Mr. M. J. Farmer for technical assistance.

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