

Convenient Method to Determine Free Fatty Acid of Rice Using Thin-Layer Chromatography and Flame-Ionization Detection System¹

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ABSTRACT

Cereal Chem. 77(2):223–229

A convenient method which possessed simplicity and high sensitivity was designed to investigate the changes in free fatty acid (FFA) of rice during storage using a thin-layer chromatography and flame-ionization detection (TLC/FID) system. In this method, two different solvent systems for TLC were used according to the purpose of experiments. Solvent system A (hexane and diethyl ether and acetic acid 80:20:1) was suitable to obtain a chromatogram showing the overall state of rice lipid degradation. Using solvent system A, the degradation of triglyceride or the increase in FFA during storage was clearly visualized as changes in the

chromatogram. Solvent system B (hexane and acetic acid 100:1) was used to improve the low reproducibility of the TLC/FID method. When methyl stearate was used as an internal standard with solvent system B, high reproducibility of the FFA value was obtained, and very small changes were detectable in stored white milled rice. This method has small sample size and simple operation and is more sensitive than the standard titration method. Therefore, this seems to be an especially convenient method for small-scale storage tests or for experiments using many samples.

The degradation of rice lipids is closely related to quality deterioration of rice during storage (Yasumatsu and Moritaka 1964, Mitsuda et al 1972, Champagne et al 1992). The greater part of the rice lipids is triglycerides accumulated in the spherosomes that exist in the aleurone layer and the embryo of rice seeds. When rice seed is exposed to long-time storage, poor storage conditions, or physical damage, these neutral lipids leak out of the spherosomes with the collapse of the membrane and are degraded to free fatty acids (FFA) through the lipase reaction (Aibara et al 1986, Takano 1989, Ohta et al 1990). In addition, it is suggested that these released lipids are responsible for the quality changes in stored rice such as the generation of stale flavors (Yasumatsu et al 1966a,b; Shibuya et al 1974). Therefore, the measured value of rice FFA is widely used as one of the important indices of quality deterioration associated with storage.

The FFA content is generally determined by an alkaline titration method and expressed as fat acidity (mg of KOH necessary to neutralize FFA contained in 100 g of rice flour). This method is used as a conventional method by many investigators because of its simplicity and low cost. However, this method has several shortcomings. First, the titration endpoint may vary depending on the operator because the endpoint is judged by the naked eye. Second, the sensitivity is low and a lot of sample is required for accurate determination (10 g of brown rice). Analysis of low lipid materials such as white milled rice requires an especially large amount of sample and a careful determination.

Recently, a semiautomatic thin layer chromatography/flame ionization detection (TLC/FID) system was developed and many reports using this system have been published (Ackman et al 1990). This relatively new apparatus is briefly characterized as the combination of the simple operation of TLC and the sensitive detection of FID. The sample mixture is spotted on the edge of a quartz rod coated with silica gel and developed with a solvent system that is similar to the conventional planar TLC. After development, each component separated from the sample mixture is burned by passing the rod through a hydrogen flame and detected by FID. In this method, plural rods are usually developed at once and a batch

analysis is possible. Compared with other chromatographic methods such as HPLC or gas chromatography (GC), TLC/FID has advantages in simplicity and rapidity (Rao et al 1985, Zeman et al 1986). Although TLC/FID has been used to analyze lipids (Parrish and Ackman 1985, Schrijver and Vermeulen 1991, Sebedio and Juaneda 1991), to our knowledge there are no reports on the application of TLC/FID to cereal grains.

Judging from the characteristics mentioned above, TLC/FID seems to overcome the shortcomings of the standard titration method such as the low sensitivity or the individual variation caused by endpoint judgment with naked eye and is therefore best-fitted for routine determination of FFA content of rice during storage. However, TLC/FID has poor reproducibility of the peak area. It has been reported that the response of FID depended on the rod, and the reproducibility of TLC/FID was influenced by each rod's character (Ackman et al 1990). Therefore, some devices for improving accuracy are necessary for the application of this TLC/FID method to the quantitative and sensitive determination of rice FFA.

In this study, we attempted to establish suitable analytical conditions for the TLC/FID method with sufficient reproducibility for rice samples and to develop a simple and sensitive method for the routine analysis of the FFA content of rice during storage.

MATERIALS AND METHODS

Solvent Systems for TLC Development

Two different solvent systems were used in the experiments. Solvent system A (hexane and diethyl ether and acetic acid 80:20:1) was used to obtain the chromatogram revealing the overall state of lipid decomposition. Solvent system B (hexane and acetic acid 100:1) was used for accurate determination of the FFA content. For solvent system B, a methyl stearate solution in *n*-hexane was added to the sample extracts as an internal standard (at 1.25 mg/mL) before spotting.

Determination of FFA Content Using TLC/FID

In this study, a TLC/FID analyzer, (Iatroskan MK-5, Iatron Co., Japan) was used in combination with silica gel coated quartz rods (Chromarod-S III, Iatron). This model has the capacity to analyze up to 10 rods at one time. The rods can be used repeatedly and are handled within a rod-holding rack throughout the whole operation. The detector was operated at a flow rate of 160 mL/min for H₂ gas and 2.0 L/min for air. The scanning speed of FID over the rod was 30 sec/rod.

Before use, the rods were cleaned and activated by passing through the hydrogen flame of the FID (blank scan). The developing tank (DT-150, Iatron) was lined with filter paper, and the solvent mixture

¹ Study supported in part by a grant-in-aid for a research program "Integrated Studies of New Rice Production Technology by Breeding Superior Varieties for the Next Millennium" provided by the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan.

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(70 mL) was poured into the tank, wetting the filter paper entirely. Sample extract (2 μ L) was spotted on each rod using a micro-dispenser (model 105, Drummond Scientific Co.), and the spotted rods were placed in a desiccator for 10 min. The rods were selected randomly for the spotting to minimize the influence of each rod's tendency. The filter paper was wetted again to saturate the tank with the solvent vapor thoroughly, and the rods were transferred into the developing tank immediately. After the development (40 min), the rods were dried using a rod drier (120°C, 3 min, TK-8, Iatron) and scanned with FID in the analyzer. Because the rods were cleaned and activated again during the scanning of the sample, the next spotting was carried out without a break.

Recording and peak area calculation were made using an Iatro-corder TC-21 integrator (Iatron), and the FFA content was calculated as linoleic acid expressed as mg/g of sample flour.

Calibration Curve for Linoleic Acid

For the calibration curve (solvent system B), various concentrations of linoleic acid solution in *n*-hexane containing methyl stearate (1.25 mg/mL) were analyzed. The result was expressed as means \pm standard deviations of four repetitions.

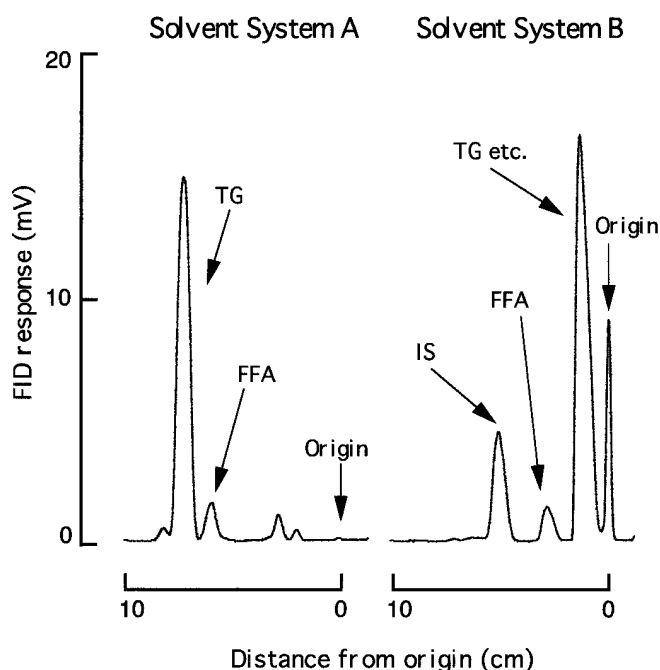


Fig. 1. Typical chromatograms of rice lipid extracts obtained by thin-layer chromatography and flame-ionization detection (TLC/FID) (white milled Yumehikari rice). Solvent system A: hexane and diethyl ether and acetic acid (80:20:1); solvent system B: hexane and acetic acid (100:1). Developing direction is from right to left, and scanning direction of FID is from left to right. FFA, free fatty acid; IS, internal standard (methyl stearate); TG, triglyceride.

Rice Samples

Rice cultivars used in this study (Hinohikari, Koganebare, Koshihikari, Nipponbare, and Yumehikari) were harvested at Kyushu National Agricultural Experiment Station (Department of Lowland Farming, Chikugo, Fukuoka) from the end of September to the beginning of November in 1996 and 1997. The brown rice of these cultivars, kept at room temperature, was transferred to the cold room (4°C) the following March and stored in a zippered polyethylene bag which was further contained in a sealed steel can until the test.

Preparation of Sample Extract for TLC/FID

Rice flour was prepared by grinding milled rice grains in an experimental blender (SCM-40A, Sibata Scientific Technology Ltd., Japan) for 1 min. The *n*-hexane (5 mL) was added to the sample flour (2.0 g of milled rice and 0.1 g of bran) and shaken vigorously. The supernatant obtained by centrifugation (2,000 rpm, 5 min, room temperature, Himac CF 7D2, Hitachi Koki Co., Ltd., Japan) was collected, and the same extraction was repeated three times. The extracts were combined, evaporated to dryness under reduced pressure at 40°C, and the resulting residue was dissolved in 1 mL of *n*-hexane. To remove the insoluble material, the centrifugation was done again (3,000 rpm, 10 min) and the supernatant was applied to TLC/FID analysis. For solvent system B, an equal volume of methyl stearate solution in *n*-hexane (2.5 mg/mL) was added to the sample extracts as an internal standard (1.25 mg/mL at final concentration). For solvent system A, an equal volume of *n*-hexane was added to the sample extracts to dilute the samples similarly to solvent system B in this study.

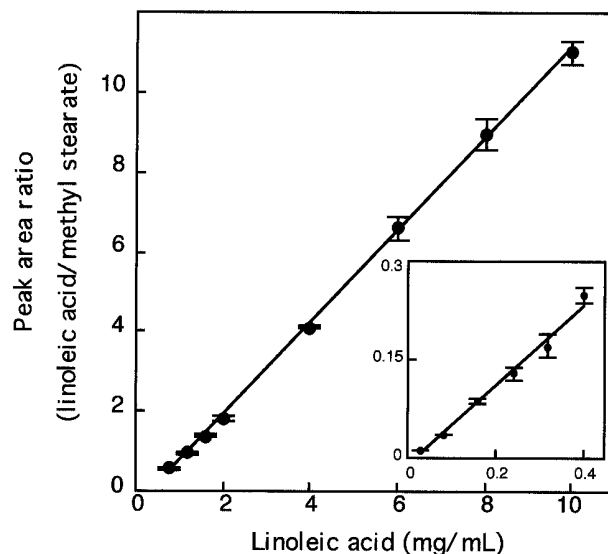


Fig. 2. Calibration curve for linoleic acid obtained by thin-layer chromatography and flame-ionization detection (TLC/FID) using solvent system B, hexane and acetic acid (100:1).

TABLE I
Reproducibility of Measured Value with Solvent Systems A and B

Development	Solvent System A Peak Area (linoleic acid) ^a		Solvent System B Peak Area (linoleic acid) ^b		Solvent System B Peak Area Ratio (linoleic acid/methyl stearate) ^b	
	Average (10 rods)	CV ^c (%)	Average (10 rods)	CV (%)	Average (10 rods)	CV (%)
1st	95,362	9.82	106,963	8.34	1.0697	3.38
2nd	87,667	11.92	100,395	12.63	1.0978	3.93
3rd	101,134	9.84	110,324	8.97	1.0910	3.38
4th	95,580	11.71	101,447	8.31	1.0833	3.36
5th	100,999	9.02	113,498	9.69	1.0746	3.34

^a Two μ L of linoleic acid solution (1.25 mg/mL) was spotted.

^b Two μ L of linoleic acid solution (1.25 mg/mL) containing methyl stearate (1.25 mg/mL) was spotted.

^c Coefficient of variation.

Storage Test of Milled Rice and Rice Bran

The storage test of milled rice and rice bran using TLC/FID was conducted using five cultivars (Hinohikari, Koganebare, Koshihikari, Nipponbare, and Yumehikari) harvested in 1997. The storage test started July 22, 1998. On the first day, the brown rice that had been stored in the cold room was returned to room temperature in the sealed container, and the white milled rice (91% milled) and bran were obtained by milling the brown rice with a National KG-15 mill (Matsushita Electric Industrial Co., Ltd., Japan). The samples of milled rice (100 g) and rice bran (12 g) were contained in a wide-mouth glass bottle without a cap (100 mL for milled rice and 50 mL for bran) and subjected to the storage test in the same 25°C incubator. The relative humidity in the incubator had a range of 61–74% during the storage period of 69 days, and the sampling was made five times (0, 7, 20, 36, 69 days) for the milled rice. For the bran, the storage period was 64 days, and the sampling was made four times (0, 13, 33, 64 days). On each sampling day, ≈8 g of milled rice or ≈0.5 g of bran were taken from the incubator and sample extracts were prepared as described above and analyzed by TLC/FID. For the determination of FFA content using solvent system B, three sample extracts were prepared from the milled rice flour or the bran of each cultivar, and TLC/FID analysis was performed once for each sample extract. In addition, the changes of the chromatograms were also investigated using solvent system A. For solvent system A, one sample extract was selected randomly from the three sample extracts of solvent system B. On the first day of the storage test, the grinding of milled rice grains and the preparation of sample extracts (milled rice and bran) were conducted within 5–6 hr after milling.

Comparison Between TLC/FID and Titration Methods

To investigate the correlation of the measured value of FFA between TLC/FID (solvent system B) and the standard titration method, white milled rice stored at accelerated conditions was subjected to both methods. On the first day of storage (October 26, 1998), the milled rice of four cultivars (Koganebare, Koshihikari, Nipponbare, and Yumehikari) harvested in 1996 were prepared as for the storage test and stored at 40°C, 75% rh. The storage period was 30 days, and the sampling frequency was seven times (0, 4, 9, 15, 21, 25, 30 days). On each sampling day, a portion of the milled rice sample was transferred to the freezer (–80°C) and stored until the test. After the storage period, the milled rice flour was prepared by grinding milled rice grains and subjecting them to both TLC/FID (solvent system B) and the titration method (single experiment). The standard titration method was performed (Ohtsubo et al 1987). A suspension of 10 g of sample flour in 50 mL of toluene (with a screw cap) was kept at 30°C for 30 min, with 1 min of shaking at 10 min intervals. The extract (25 mL) from the paper filtration (Advantec No. 2, Toyo Roshi Kaisha, Ltd.) was combined with 0.04% phenolphthalein and 95% ethanol (25 mL) and titrated with 0.1N KOH. The same volume of toluene (25 mL) was titrated similarly as a blank. The results of the titration method were expressed as fat acidity (mg of KOH necessary to neutralize the FFA contained in 100 g of rice flour).

RESULTS AND DISCUSSION

Analytical Conditions of TLC/FID

The analytical conditions for TLC/FID of rice samples were determined as a result of preliminary experiments. The procedure was designed to be as simple as possible. Typical chromatograms with the two solvent systems are shown in Fig. 1. FFA was detected at a position separated from triglyceride in both of the solvent systems. The major fatty acids of the rice lipids, palmitic acid, oleic acid, and linoleic acid were not separated on the rods under these conditions. Therefore, a measured value of FFA is obtained as a total fatty acid content. In solvent system A, the distances of each component from the origin were larger than those of solvent

system B, and the separation of each component was also superior. On the other hand, the components except FFA moved slightly from the origin in solvent system B and separation was not good. The triglyceride and FFA positions were reversed and the peak of TG appears to have combined with the other minor peaks. However, methyl stearate was in a good position as an internal standard in solvent system B.

Simplicity and rapidity are required for routine analysis and a small sample size is preferable. In TLC/FID method, the recommended sample amount is 2 g for milled rice, 0.5 g for brown rice, and 0.1 g for bran. This small sample size is used because of the high sensitivity of FID. Time required is ≈70–80 min to complete one cycle of this method (except for the sample extraction), and analysis of 20–30 samples in a cycle using several developing tanks is possible. In our laboratory, ≈10 samples can be analyzed in the same period using the standard titration method. Therefore, TLC/FID seems to be a rapid method in comparison with the standard manually operated titration method or other chromatographic methods like HPLC or GC.

In the chromatogram with solvent system A, the peak of FFA or triglyceride was satisfactorily separated from the other peaks (Fig. 1). Therefore, the information on the overall lipid decomposition, such as the degree of remaining triglycerides or the ratio of FFA and triglycerides, was easily obtained using solvent system A. However, the peak area reproducibility was not good. When linoleic acid (1.25 mg/mL) was analyzed with 10 rods, the coefficient of variation (CV) in the set was 9.02–11.92% (Table I). We tried to find a suitable internal standard for solvent system A because low

TABLE II
Measured Value of Each Fatty Acid Species Obtained by TLC/FID^a
Method Using Solvent System B

Fatty Acid Species (5 μmol/mL)	Peak Area Ratio ^b (fatty acid/methyl stearate)
Palmitic acid	1.0132 ± 0.01674
Oleic acid	1.1619 ± 0.02378
Linoleic acid	1.1168 ± 0.02550

^a Thin-layer chromatography and flame-ionization detection.

^b Two μL of each fatty acid solution containing methyl stearate (1.25 mg/mL) was spotted. Results expressed as mean ± standard deviations of five rods.

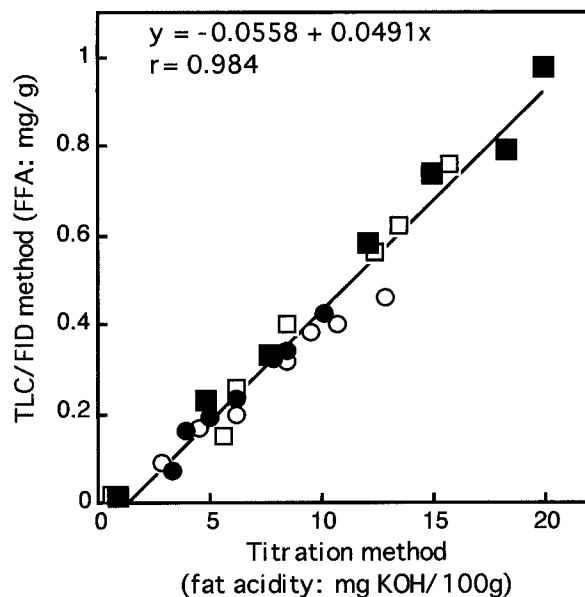


Fig. 3. Correlation of the measured value of free fatty acid (FFA) between the titration method and thin-layer chromatography and flame-ionization detection (TLC/FID) using solvent system B, hexane and acetic acid (100:1). Koganebare (○); Koshihikari (□); Nipponbare (●); Yumehikari (■).

reproducibility of TLC/FID was speculated to be caused by the differences in each rod's sensitivity. However, we could not measure these differences so we examined another solvent system and internal standard, and established the combination of solvent system B and methyl stearate.

In solvent system B, the reproducibility of the measured value was improved remarkably (CV 3.34–3.93%, Table I). Although the peak area reproducibility was similar to that of solvent system A, the peak area ratio (linoleic acid to methyl stearate) was stable and therefore showed that the use of methyl stearate as an internal standard would be effective. The calibration curve for linoleic acid with solvent system B showed a linear relationship between concentration and measured value covering a wide range (Fig. 2). In addition, the FFA content of milled rice stored for various periods was determined using both the universal titration method and TLC/FID (solvent system B), and the relationship between these methods was investigated (Fig. 3). The regression curve was $y = -0.0558 + 0.0491x$, and $r = 0.984$. One unit of fat acidity (1 mg KOH/100 g) corresponds to 0.04998 mg/g of FFA content (as linoleic acid) theoretically, so the slope of the experimental regression curve was generally equal to the theoretical value. On the other hand, the intercept of y-axis was below the origin, which suggests that the standard titration method had a tendency to estimate FFA content slightly higher than the TLC/FID method. Some other acidic compounds may have been extracted from the rice samples and influenced the FFA value in the titration method. As a good correlation was observed between these two methods, it seems possible

that TLC/FID possessed the continuity relative to the past data obtained by the titration method. However, this problem should be considered when comparing the data from these two methods.

Because the individual fatty acid species were not separated under this condition, it was suspected that the measured value of FFA might be influenced by the fatty acid composition and the differences in FID response to each fatty acid. Actually, some differences were noticed in the response of FID to each fatty acid species in this method (Table II). It was reported previously that the cultivar difference in the fatty acid composition of rice lipids was 13.7–21.1% (palmitic acid), 36.6–51.6% (oleic acid), and 28.6–39.2% (linoleic acid) (Taira et al 1988). Judging from these data and the values listed in Table II, the maximum calculated error caused by differences of fatty acid composition seems to be $\approx 1.3\%$. Therefore, it should be noted that TLC/FID is not suitable for detecting a small difference in samples, even when solvent system B is used.

TABLE III
Analysis of Variance of Free Fatty Acid (FFA) Content
in Storage Test of Rice^a

Factor	Degrees of Freedom	Sum of Square	Variance	F-Value
Cultivar	4	0.07189	0.01797	213.2** ^b
Error	10	0.0008431	0.00008431	

^a Milled rice stored for 69 days.

^b Significant at 1% level.

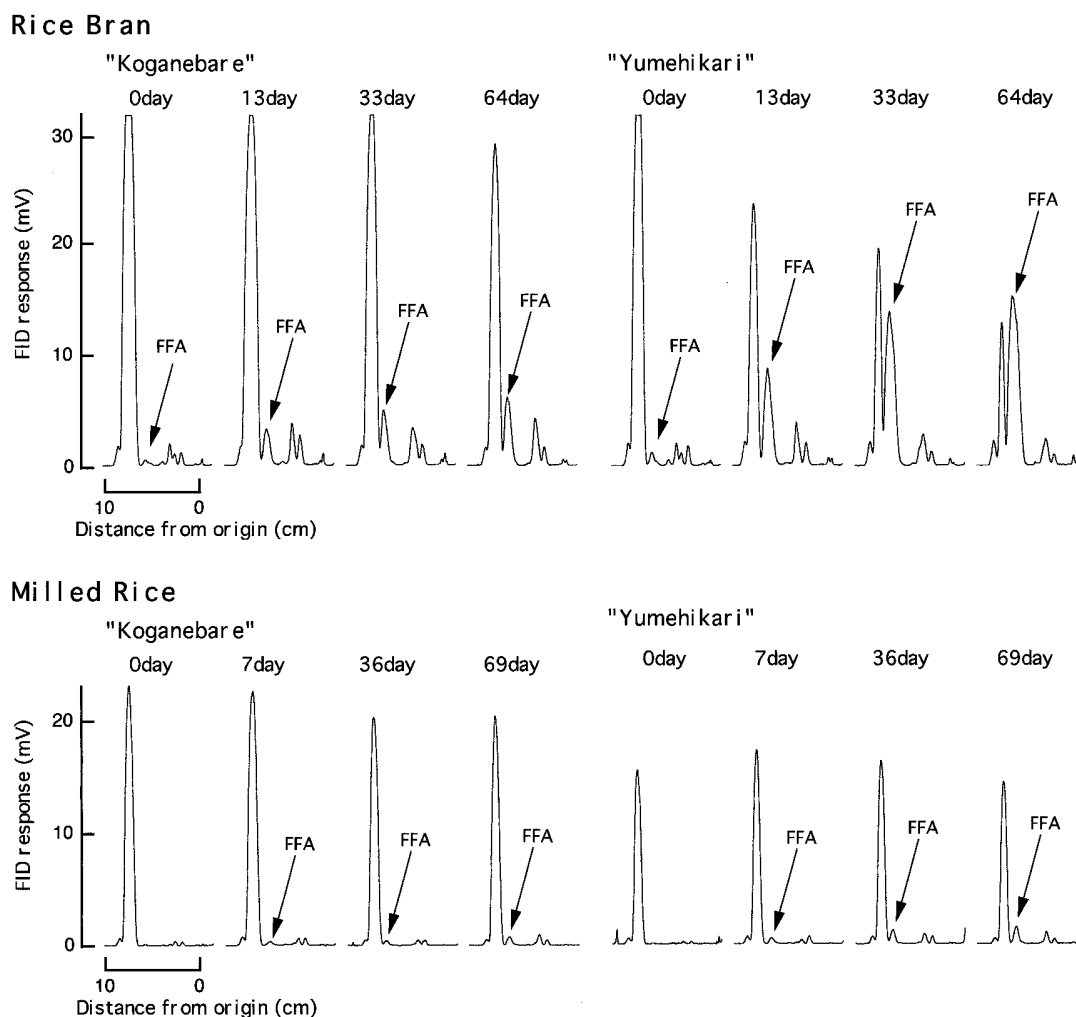


Fig. 4. Changes in the chromatograms of rice bran and milled rice during storage obtained by thin-layer chromatography and flame-ionization detection (TLC/FID). Koganebare and Yumehikari are slow- and fast-degrading cultivars, respectively. Solvent system A, hexane and diethyl ether and acetic acid (80:20:1). Developing direction is from right to left, and scanning direction of FID is from left to right.

Consequently, analytical conditions for TLC/FID with improved reproducibility were established. Solvent system B can be used for routine determination of FFA content as well as rough estimation of the overall lipid decomposition. If an investigator needs more exact information on the overall lipid decomposition, solvent system A is preferred because the separation of each component is better. The primary advantages of TLC/FID (simplicity and high sensitivity) seem to be exhibited sufficiently under these conditions, and beneficial data will be obtained using these two solvent systems according to the purpose of experiments.

Application of TLC/FID to Storage Testing

The applicability of TLC/FID to the storage test of white milled rice was investigated. Stored rice bran was also examined. Chromatogram changes during the storage are shown in Fig. 4 (solvent system A, Koganebare and Yumehikari). Degradation of rice lipids is accelerated by the milling of brown rice, and the quality deterioration of rice is faster in stored milled rice (Shibuya et al 1974). In this study, the overall state of lipid decomposition was clearly visualized using solvent system A. In a fast-degrading cultivar like Yumehikari, a rapid degradation of lipid was observed in the rice bran, and the greater part of the triglycerides was converted to FFA in about two months. However, in stored milled rice, only a small portion of the lipids was degraded in the same period. Although the lipid degradation should be accelerated in the milled rice, an unexpectedly large amount of triglycerides remained intact as compared with the rice bran.

Chromatogram changes of solvent system B are also shown in Fig. 5. Although the separation of each component was inferior to that of solvent system A, the rough information about the overall lipid degradation could be obtained from solvent system B. On the other hand, it was observed that the response of the internal standard (1.25 mg/mL of methyl stearate) was unstable in the chromatograms of solvent system B. The instability of the peak area in TLC/FID was recognizable in this figure, and it is also suggested that the suitable internal standard is necessary for the accurate determination.

The study using solvent system B showed that there was a considerable cultivar difference in the changes in FFA content during the storage (Fig. 6). Analysis of variance of FFA content was done for each point of the storage test. The result of milled rice (69 days) is shown in Table III as an example. As a result, the *F* values for each point were: rice bran, 165.5** (0 day), 132.0** (13 days), 287.4** (33 days), and 153.9** (64 days); milled rice, 62.09** (0 day), 70.35** (7 days), 38.18** (20 days), 41.65** (36 days), and 213.2** (69 days) ($P < 0.01$).

At 0 day, Koshihikari was isolated from the other cultivars (rice bran and milled rice), however the cultivar differences were still significant even excluding the value of Koshihikari. Therefore, the differences in FFA content with cultivar were statistically significant at each point, and the TLC/FID was able to investigate the cultivar differences of rice FFA content from the early stages of storage.

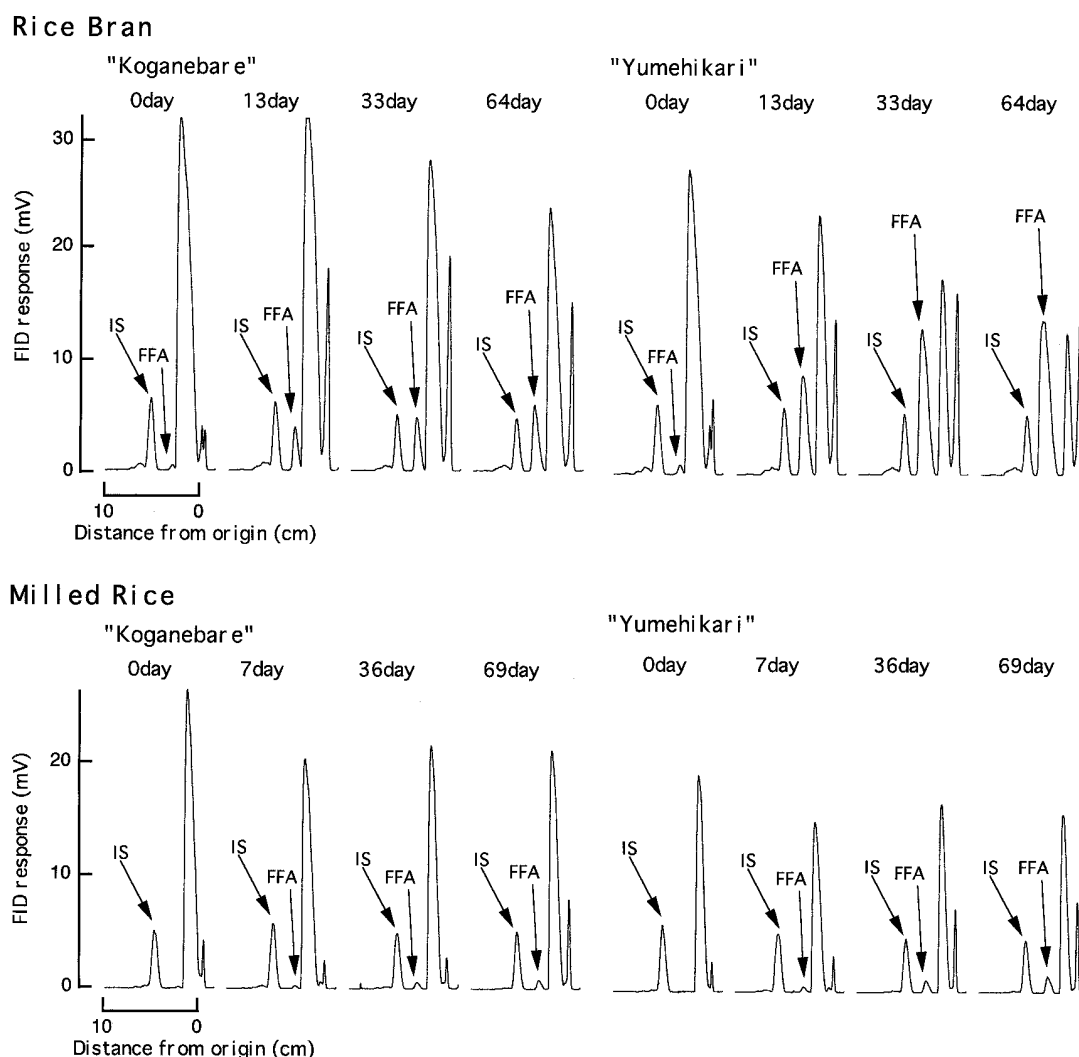


Fig. 5. Changes in the chromatograms of rice bran and milled rice during storage obtained by thin-layer chromatography and flame-ionization detection (TLC/FID). Koganebare and Yumehikari are slow- and fast-degrading cultivars, respectively. Solvent system B, hexane and acetic acid (100:1). Developing direction is from right to left, and scanning direction of FID is from left to right.

The detected amount of FFA and the increasing rate was extremely different between rice bran and milled rice. After about two months of storage, the ratio of the FFA content (rice bran and milled rice, 64 and 69 days, respectively) reached 201.6 (37.27 and 0.1849, Koganebare), 256.2 (67.34 and 0.2628, Koshihikari), 278.5 (58.76 and 0.2110, Nipponbare), 322.7 (95.26 and 0.2952, Hinohikari), and 323.8 (123.7 and 0.3822, Yumehikari). Therefore, it was suggested that the difference between rice bran and milled rice had a tendency to become larger in the fast-degrading cultivars.

On the other hand, the pattern of cultivar differences was quite similar between milled rice and rice bran, in spite of the large gap in the degradation rate (Fig. 6). Therefore, the rate of lipid degradation in the milled rice fundamentally reflected the properties of rice bran. The quality of the cooked rice, such as the generation of stale flavors, may be affected by a very small quantity of the bran that sticks to the surface of the milled rice (Shibuya et al 1974, Tsugita et al 1980). Thus, the relationship between milled rice and rice bran observed in this study is interesting from the standpoint of the influence of rice bran on the quality of cooked rice.

As a result of applying this method to the storage test of milled rice, an extremely small change in the FFA, which occurs in the early stage of the storage, was detectable and cultivar differences could also be investigated. Although the sample size was small, sufficient reproducibility was obtained. Therefore, this method is applicable to low-lipid materials such as white milled rice, and the increase in sample number and the required sample size is an improvement over the conventional method. In addition, operating costs to perform this method were low. Only H₂ gas and organic solvents for the extraction and TLC development were used in this method. No expensive reagents or toxic substances were required. Judging

from these results, TLC/FID appears to be a convenient method for a routine analysis of FFA content in stored rice.

There were some notable points about the operation. For example, sharp spikes were observed on the chromatograms occasionally, and the area calculation was disturbed. These spikes were results of small dust particles that stuck on the rods. Because of the high sensitivity of FID, the slight pollution or the dust on the rod was reflected clearly on the chromatograms, therefore a dusty environment should be avoided for TLC/FID method. Furthermore, the reproducibility of TLC is usually affected by environmental factors such as temperature or humidity. Therefore, it is desirable to perform this method at fixed conditions.

CONCLUSIONS

A convenient method for the routine analysis of rice FFA was established using TLC/FID. An effective investigation was achieved employing two solvent systems. TLC/FID has three major advantages. First, it is a sensitive and stable method compared with the standard titration method. This method is applicable to the time course of the study of low lipid samples such as white milled rice, and only a small amount of sample is required. Second, it is possible to obtain a chromatogram showing the overall state of rice lipid degradation. Using this method, the degree of remaining triglycerides or the ratio of FFA and triglycerides are clearly visualized as changes in the chromatogram. Third, the determination method is simple with low operating costs. This method requires no expensive reagents or toxic substances. Only H₂ gas and the organic solvents for the extraction and TLC development are used. Therefore, it seems to be a suitable method for routine determination of rice FFA content.

On the other hand, as in standard planar TLC, some factors should be taken into account for accurate determination. For example, it is desirable to perform this method at a temperature or humidity as fixed as possible because the reproducibility of TLC is affected by the changes in these parameters. Furthermore, a dusty environment should be avoided. Dust on the rods causes sharp spikes on the chromatogram that disturb the area calculation. By addressing these problems, TLC/FID can become a remarkably convenient method for investigating rice lipid degradation during storage, especially for small-scale storage tests or experiments using many samples.

ACKNOWLEDGMENTS

We are grateful to the Laboratory of Rice Breeding (Kyushu National Agricultural Experiment Station, Japan) for providing the rice samples used in this study.

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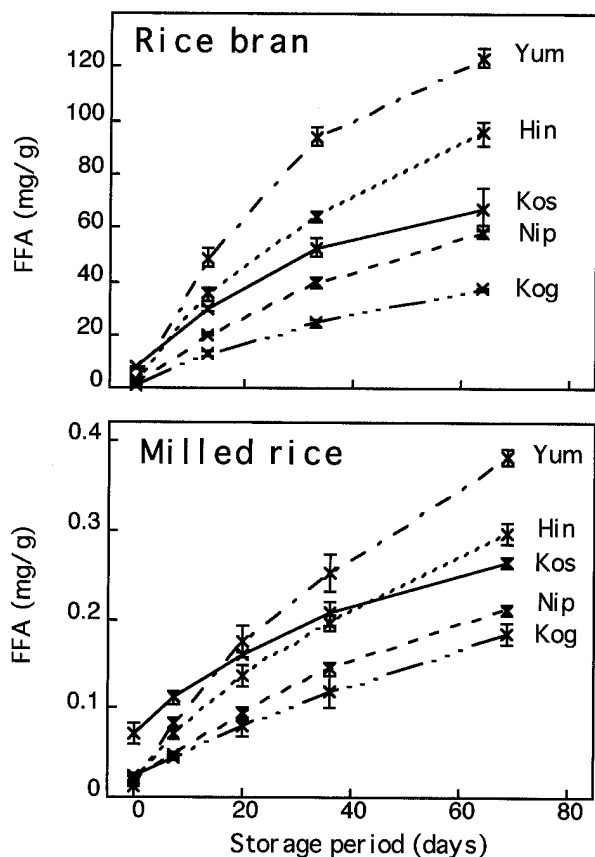


Fig. 6. Changes in the free fatty acid (FFA) content of rice bran and milled rice during storage obtained by thin-layer chromatography and flame-ionization detection (TLC/FID) using solvent system B, hexane and acetic acid (100:1). Cultivars: Hinohikari (Hin); Koganebare (Kog); Koshihikari (Kos); Nipponbare (Nip); Yumehikari (Yum). Experimental error expressed as error bars (means \pm standard deviations of three determinations).

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[Received July 1, 1999. Accepted December 16, 1999.]