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Analysis of 2-Alkylcyclobutanones with Accelerated Solvent Extraction To Detect Irradiated Meat and Fish

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A new analytical procedure has been developed to analyze 2-alkylcyclobutanones to detect γ-ray-irradiated fat-containing foodstuffs. Samples were extracted with an accelerated solvent extraction system via hot and pressurized ethyl acetate in cells. A large amount of fat in the extract was precipitated and removed with filtration by standing at −20 °C after the addition of acetonitrile. The extract was further cleaned with a 1 g silica gel mini column, and the radiolytic compounds of 2-docecylcyclobutanone (2-DCB) and 2-tetradecylcyclobutanone (2-TCB) were determined with gas chromatography with mass spectrometry (GC/MS). Sample preparation time before GC/MS was 7−8 h. At first, the procedure was evaluated with a recovery test in eight samples spiked with 2-DCB and 2-TCB at 20 ng/g, resulting in 70−105% recoveries with mostly less than 10% relative standard deviations. The procedure was further evaluated with beef, pork, chicken, and salmon samples irradiated with γ-rays from 0.7 to 7.0 kGy at −19 °C. Both 2-DCB and 2-TCB in most samples were detected with good dose−response relations at all doses, while salmon was detected more than 2 kGy irradiation. The amounts of 2-alkylcyclobutanones produced reflected precursor fatty acids levels in samples, especially for the combination of 2-TCB and stearic acid. The results indicated that the production rate of 2-TCB to stearic acid was more obvious than that of 2-DCB to palmitic acid in frozen samples with γ-ray irradiation.

KEYWORDS: 2-dodecylcyclobutanone; 2-tetradecylcyclobutane; β (gamma)-ray; irradiation; food; accelerated solvent extraction; CG>MS; beef; pork; chicken; salmon; fatty acid

INTRODUCTION

Irradiation of foodstuff by ionizing radiation to control microorganisms and extend product shelf life has become a popular preservation technique worldwide (*1*, *2*). Reliable and routine methods to confirm the irradiation history of foods would encourage consumer's acceptance of food irradiation and may help in the enforcement of labeling regulations. Various methods have been proposed to detect irradiated foodstuffs (*3*−*5*). Of these methods, analyses of 2-alkylcyclobutanones and volatile hydrocarbons during irradiation in fat-containing foods have been accepted by European Committee for Standardization (*6*, *7*). Radiolytic products of fatty acids, such as 2-dodecylcyclobutanone (2-DCB) from palmitic acid and 2-tetradecylcyclobutanone (2-TCB) from stearic acid, have been recommended as markers for irradiation of lipid-containing foods because 2-alkylcyclobutanones, including 2-DCB and 2-TCB, are considered as unique radiolytic products in irradiated lipids (*8*−*11*). 2-Alkylcyclobutanones were detected in irradiated seeds of mango and papaya (*12*), peanuts (*13*), salmon (*12*), and dried shrimp (*14*), as well as meats and eggs. European countries have adopted analysis of 2-alkylcyclobutanones to detect irradiated foods with fat such as chicken (*7*).

Concerning a toxic aspect of 2-alkylcyclobutanones, possibilities of genotoxin and cancer promoter were indicated (*15*, *16*). However, other groups claimed that genotoxic effects of 2-alkylcyclobutanones would be suspicious (*17*, *18*). A bacterial mutation test with *Escherichia coli* (*17*) and *Salmonella typhimurium* (*18*, *19*) showed that 2-DCB was not mutagenic with and without metabolic activation.

The analytical method EN 1785 that has been adopted in European countries as an official method requires a long time for sample preparation (*7*). EN 1785 sample preparation consists of two parts, fat extraction with the Soxhlet method for 6 h and Florisil column chromatography for cleanup, which requires a long time and large amounts of solvents. To reduce the extraction time and encourage selective extraction for 2-alkylcyclobutanones, supercritical fluid extraction (SFE) has been adopted instead of Soxhlet extraction and has succeeded in a greatly reduced extraction time to about 30−60 min (*13*, *20*−*22*). However, an SFE instrument with a fully automated system operation is not commercially available in Japan and legal permission is required for each instrument because of the extremely high gas pressure for operation; thus, it is not convenient to introduce SFE to monitor irradiated foodstuffs in Japan.

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In this study, we introduced an automated extraction instrument in which a sample is extracted with a hot and pressurized solvent, called accelerated solvent extraction (ASE) or pressurized liquid extraction, and developed original cleanup procedures with defatting under freezing temperatures and a disposal silica gel minicolumn to reduce operation time and solvent use.

The ASE uses organic solvents at high pressures and temperatures above boiling point; thus, solvents solubilize targeted compounds and penetrate sample matrices better than at atmospheric conditions and room temperature (*23*, *24*). Richter reported that environmental contaminants such as PCB and polycyclic aromatic hydrocarbons were rapidly and effectively extracted at temperatures above 100 °C (*24*). ASE was also effective in pesticides residue extraction (*25*−*27*) and was comparable with SFE in residues analysis in soils (*28*) and also dioxin analysis (*29*). Under ASE conditions, generally at above 100 °C, we expected that 2-alkylcyclobutanones in the samples would be extracted more easily and effectively with fats than the other extraction systems under room temperature.

The objectives of this study were (i) to evaluate the extraction power of ASE and the efficiency of the original cleanup procedure in various spiked and irradiated samples, (ii) to assess the availability of the proposed method in the detection of irradiated foods, and (iii) to show the relationship among the produced 2-alkylcyclobutanones and their precursor compounds in foods before irradiation.

MATERIALS AND METHODS

Reagents. 2-DCB and 2-TCB were purchased from Fluka (Switzerland). 2-Cyclohexylcyclohexanone, as an internal standard for gas chromatography/mass spectrometry (GC/MS) determination, was purchased from Wako Pure Chemical (Osaka, Japan). Each compound was dissolved in acetone to make a 1000 μg/mL stock solution. The 2-alkylcyclobutanone solutions were equally mixed and diluted for the running standards and spiking solution in recovery tests. Solvents and anhydrous Na_2SO_4 were pesticide analysis grade, and other reagents were analytical grade.

Materials. Particles of diatomaceous earth (Extrelut for refilling; particle size, 160−800 μm; Merck; Germany) were purchased from Merck (Germany) and used without any purification.

Silica Gel. Mega Bond Elut SI, 1 g/6 mL (Varian, CA). The sorbent was rinsed with 10 mL of 5% diethyl ether in *n*-hexane, followed by 10 mL of *n*-hexane for conditioning just before use.

Food Commodities. Meat and fish samples were purchased at a local market in Osaka. About a 500 g sample was chopped in a conventional food processor (MK-K3, Matsushita, Japan) for 5 min to obtain homogeneous fat distribution in each sample. About 30 g of the homogenized sample was placed into a small polyethylene bag and frozen at −20 °C for irradiation.

Irradiation. Frozen samples were irradiated with γ-rays from a ^{60}Co source (15 kGy/h) in the irradiation pool at the Research Institute for Advanced Science and Technology, Osaka Prefecture University, at doses from 0.7 to 7.0 kGy at −19 °C. The irradiation source was put on the bottom of the pool with 5 m depth of water to protect an operator. The dose was determined with Radiachromic dye film (FWT-60-1P, Fat West Technology, Inc., CA), which was put on each sample's plastic bag. The irradiated doses were compensated with a calibration curb for frozen temperature, since the film was less sensitive at cool temperature. A temperature of −19 °C was achieved using a NaCl−ice mixture as a refrigerant, and samples were stored at −20 °C after irradiation until analysis of 2-alkylcyclobutanones. Nonirradiated control samples were also stored under the same conditions. Possible irradiated meat and fish in Japan would be imported since irradiation of such foods is not permitted in Japan. A large amount of meats and fishes are imported frozen, so samples were irradiated under frozen conditions.

Sample Preparation for ASE. A 10 g aliquot sample and 10 g of diatomaceous earth particles, as a drying agent, were ground in a mortar (12 cm i.d.) with a pestle until the mixture became homogeneous to facilitate solvent penetration into sample matrices. The mixture was placed in stainless steel cells (33 mL; 11 cm × 1.9 cm i.d.). In the fortification study, 0.5 mL of 2-alkylcyclobutanone mixture at 0.4 μg/mL was added to the sample mixture in the cells and left for 30 min before extraction.

ASE. Extraction was performed with a Dionex AS 200 (Dionex, United States). The preset default conditions were as follows: extraction temperature, 100 °C; extraction pressure, 1500 psi; preheating period, 5 min; static extraction period, 5 min; solvent flash, 9.9 mL; nitrogen purge, 60 s; collection, 60 mL glass vials with Teflon-coated rubber caps (I-CHEM, United States). In the operation, ethyl acetate was used as an extracting solvent and both static extraction and solvent flash were performed twice for each sample.

Defatting. ASE extracts in a collecting vial were suspended with water, and a slight water layer, which was also coextracted with solvent from the sample, was separated at the bottom of the vial. The extract was transferred to a 50 mL volumetric cylinder with a cap. The collecting vial was washed with 5 mL of ethyl acetate, which was added to the extract. The extract was made up to 50 mL with ethyl acetate and vigorously shaken to make the suspension homogeneous. An aliquot of 25 mL of suspended extract for less fatty samples such as liver and thigh was placed in a conical flask, followed by addition of 20 mL of acetonitrile. For fat rich samples such as beef intestine and minced meat, 10 mL of extract was taken and 10 mL of acetonitrile was added. The mixture was kept at −20 °C for 30 min to precipitate fat. The precipitated fat was removed by immediate filtration with coarse filter paper. Both the flask and the filter were washed with 5 mL of acetonitrile, which was also cooled at −20 °C and collected as a filtrate. If the filtrate was suspended after acetonitrile washing, the precipitating procedure was repeated at −20 °C. The filtrate in a 100 mL round bottom flask was carefully evaporated almost to dryness, and then, evaporation was repeated after the addition of 5 mL of acetone and 5 mL of *n*-hexane twice to remove water and polar solvents for cleanup with silica gel.

Fat Content. The remaining 25 mL of suspended extract was passed through about 10 g of anhydrous Na_2SO_4 on the filter paper and evaporated to almost to dryness. The fat residue was weighed after overnight standing.

Cleanup. Defatted extract was dissolved in *n*-hexane (about 1 mL) and was added to a silica gel cartridge, which was connected to a glass syringe (10 mL) as a reservoir. A 10 mL amount of *n*-hexane was first eluted and discarded. Then, 10 mL of 2% diethyl ether in *n*-hexane was further eluted and collected as a 2-alkylcyclobutanones fraction. The elution was concentrated to 2 mL with a nitrogen stream under warming conditions, after addition of 2-cyclohexylcyclohexanone at a final concentration of 0.1 μg/mL.

GC/MS Determination. An Auto Mass 120 (JEOL, Tokyo, Japan) was connected to an HP5890 gas chromatograph (Hewlett-Packard, CA). The GC conditions were as follows: column, RTX-5ms (Restek Bellefonte, PA) 30 m × 0.25 mm i.d., 0.25 μm thickness; column temperature program, 60 °C (1 min), 60−300 °C at 8 °C/min, 300 °C (5 min); carrier gas, He; injection temperature, 250 °C; injection volume, 2 μL with an HP7673 autosampler (Hewlett-Packard); injection mode, splitless. The MS conditions were as follows: ionization mode, electron ionization; ion detection, selected ion monitoring; ionization voltage, 70 V; filament current, 0.3 mA; detector voltage, −0.7 kV; scan time, 100 ms; ion source temperature, 200 °C; transfer line temperature, 250 °C. The monitored ions were *m*/*z* 98 and 112, and *m*/*z* 98 was selected for determination.

Fatty Acids. Fatty acids in the crude fat were determined as described by Methods of Analysis in Health Science (*30*). In brief, 20 mg of fat was dissolved in 1 mL of benzene, followed by the addition of sodium methoxide and shaking, for hydrolysis of triglycerides and methylation of fatty acids. Methylated fatty acids were extracted with *n*-hexane after the addition of acetic acid. Fatty acids were determined with GC/MS.

Statistics. Corelation of produced 2-alkylcyclobutanones by irradiation and fatty acids in the sample was statistically evaluated with Microsoft Excel 2004 for Macintosh.

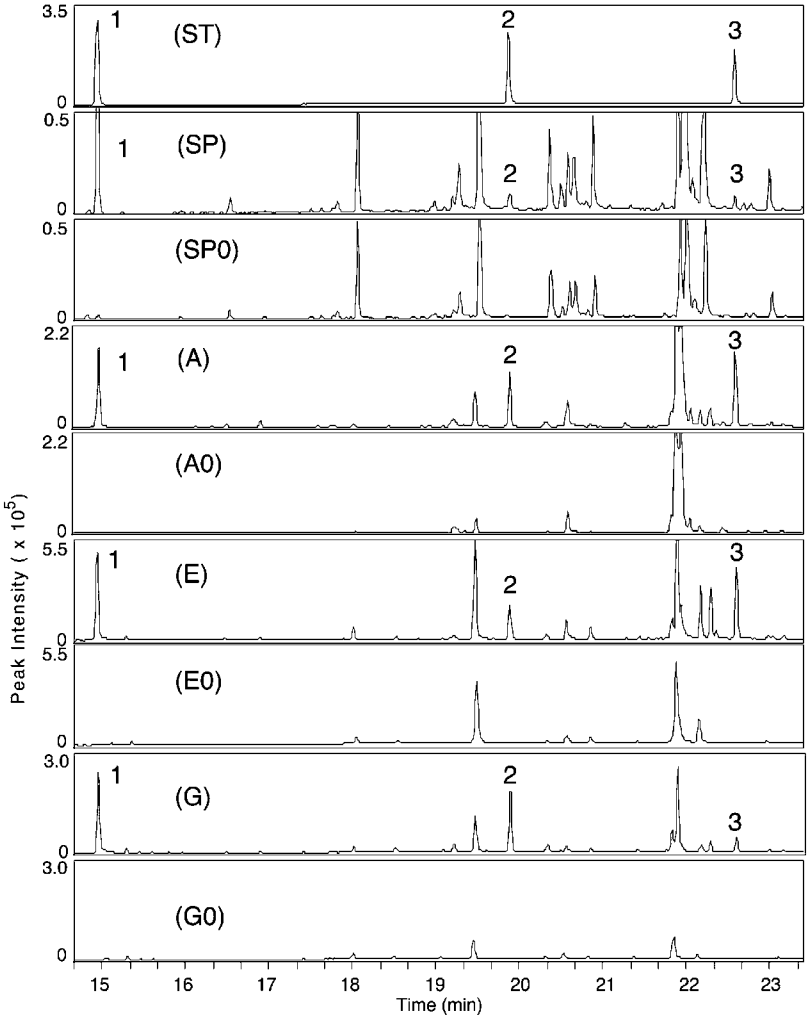

Figure 1. Typical chromatograms of 2-DCB and 2-TCB monitored at *m/z* 98. ST, 2-DCB and 2-TCB at 100 ng/mL; SP, minced beef spiked at 20 ng/g; SP0, blank beef; A, 4.7 kGy irradiated beef intestine; A0, nonirradiated beef intestine; E, 4.6 kGy irradiated minced pork; E0, nonirradiated minced pork; G, 4.3 kGy irradiated chicken thigh; and G0, nonirradiated chicken thigh. Peak labels: 1, 2-cyclohexylcyclohexanone; 2, 2-DCB; and 3, 2-TCB.

RESULTS AND DISCUSSION

Method Development. With ASE, a number of samples in the cell are automatically and consecutively extracted and the extracts are also collected in the sample vials. For these two reasons, ethyl acetate was selected for extraction. Ethyl acetate is generally a good solvent for extraction and showed better extraction power than other solvent systems when crude fat extraction was an indicator in a preliminary study (data not shown). The second reason is that ethyl acetate solution is suitable to remove fat in an extract with the addition of acetonitrile. Ethyl acetate is freely miscible with acetonitrile, which practically does not dissolve triglycerides, the main component of the crude fat in meat and fish. Using this property, much of the fat could be removed from the primal ethyl acetate extract. Fat in ethyl acetate solution could be precipitated if it was stored in a freezer; however, this would take a long time and success is not guaranteed. By adding a large amount of acetonitrile to the ethyl acetate extract, fat is instantly precipitated; however, a rapid reaction might cause loss of 2-alkylcyclobutanones by coprecipitation with fat. Thus, about an equal amount of extract was added so that the fat was still dissolved, and it was precipitated gradually and obviously when the mixed solution was cooled at −20 °C for 30 min. The next filtration should be done rapidly to avoid melting the solid fat. This operation required about 40 min, including standing time, was sometimes doubled by a repeat operation in fat rich samples, and removed about 80−90% fat from the crude extract by residual weight basis (data not shown). With this easy operation, more selected extract was charged to a 1 g silica gel column, which could accept equivalent to 0.5 g of crude fat in the primal extract. The EN1785 method allowed to charge 0.2 g of crude fat to about 40 g of Florisil for defatting and cleanup (*7*).

After much of the fat was removed, the extract could be cleaned up with a silica gel cartridge so that the elution solvents and operation time were substantially reduced to 20 mL and about 40 min as compared with a conventional Florisil cleanup, which requires time-consuming activation and further deactivation of Florisil and about 300 mL elution of solvents.

Evaluation of the Method. The proposed method was primarily evaluated with recovery tests in which 2-DCB and 2-TCB were spiked at 20 ng/g to beef (large intestine, minced meat, thigh, and liver), pork (minced meat and thigh), chicken thigh with skin, and salmon. Recovery studies were conducted with five experiments in each food sample, and the results are summarized in **Table 1**. Both 2-DCB and 2-TCB were recovered from 70 to 105%, with mostly less than 10% relative standard deviation (RSD). There was no significant difference in recoveries among the two target 2-alkylcyclobutanones and sample types. These results indicated that the ASE method at 100 °C was effective and did not affect the stabilities of 2-DCB or

Table 1. Recoveries of 2-DCB and 2-TCB in Meat and Fish Spiked at 20 ng/g

		2-DCB		2-TCB	
sample	part	average[a] (%)	RSD[b] (%)	average[a] (%)	RSD[b] (%)
beef	minced	83	6	96	4
	large intestine	91	9	78	11
	thigh	104	6	87	3
	liver	105	4	100	4
pork	minced	79	10	80	6
	thigh	94	8	100	14
chicken	thigh	70	2	74	5
salmon		102	5	87	5

[a] Average of five experiments. [b] Relative standard deviation of five experiments.

2-TCB. Chromatograms were clear enough to identify and determine each compound with detection of a primary ion (*m*/*z* 98). Blank control samples did not show serious interference peaks around two 2-alkylcyclobutanone peaks on GC/MS chromatograms. Typical chromatograms of spiked 2-DCB and 2-TCB at 20 ng/g to minced beef and nonspiked blank sample are illustrated in **Figure 1**.

The operation time for sample preparation with five samples and one blank sample was around 7−8 h. The SFE method does not require a defatting process; however, a cleanup would be necessary to determine low levels of 2-alkylcyclobutanones (*31*), and fat extraction was also necessary when 2-alkylcyclobutanone concentrations were expressed on a fat basis (*31*), and the further overnight lyophilization of the samples might be necessary to remove water before SFE extraction (*21*); thus, the total operation time and labor intensity with the proposed method are comparable with SFE or better. Two days are required for the sample preparation with EN1785 method, which consists of 6 h for the Soxhlet extraction, drying extract to obtain crude fat, large scale Florisil column chromatography with unstable fractionation of 2-alkylcyclobutanones because of considerable deactivation of Florisil with water (*7*). We think that the two-step cleanup such as defatting and application of the cartridge column would be the key point, which makes the entire method simple and stable in this study.

Eight frozen samples, as above, were irradiated at four dose levels, ranged from 0.7−7.0 kGy, and produced 2-DCB and 2-TCB concentrations and are illustrated in **Figure 2**. 2-DCB and 2-TCB were detected in most samples, and their production increased as the irradiated dose increased. 2-TCB levels were dominant or slightly higher than 2-DCB levels in beef and pork, while the ratio was the opposite in chicken and salmon at all doses. High fat samples such as beef intestine, minced beef, minced pork, and chicken thigh showed high concentrations of two compounds, while less fatty samples such as beef thigh, beef liver, and pork thigh showed low levels of 2-DCB and 2-TCB, about 1/10 to 1/5 the levels of the high fat samples. Fat concentrations are listed in **Table 2**. No 2-DCB or 2-TCB was detected in all of the nonirradiated control samples.

2-DCB and 2-TCB concentrations are expressed on a sample weight basis in this study, even though it has been expressed on a fat basis as in other studies. If 2-alkylcyclobutanone concentrations were expressed on a fat basis, the levels would be relatively higher than those on a weight basis since the levels were compensated by fat concentrations. This will make it difficult to distinguish between high fat samples with much production of 2-alkylcyclobutanones and low fat samples with slight production of them.

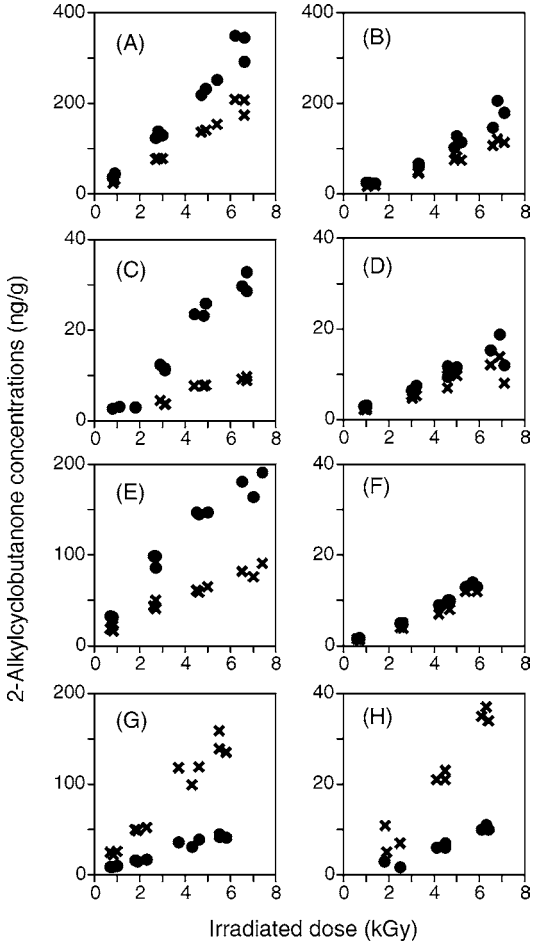

Figure 2. Production of 2-alkylcyclobutanons with γ-ray irradiation in meat and fish samples. (**A**) Beef intestine, (**B**) minced beef, (**C**) beef liver, (**D**) beef thigh, (**E**) minced pork, (**F**) pork thigh, (**G**) chicken thigh, and (**H**) salmon. 2-DCB, ×; 2-TCB, ●. Experiments were carried out with three trials at each irradiated dose.

Typical chromatograms of 2-DCB and 2-TCB in irradiated and nonirradiated samples are illustrated in **Figure 1**. The two compounds and 2-cyclohexylcyclohexanone (internal standard) were detected without serious interference in all of the samples. The abundances of contaminant peaks were reduced as compared with SFE extraction and EN1785 method (*7*, *20*−*22*). Detected 2-DCB and 2-TCB, more than 30 ng/g, were confirmed by mass spectra under scan mode. The relative intensities of major fragment ions such as 98, 84, and 112 were compared with 2-DCB and 2-TCB standards whose MS spectra were practically the same in the detected *m*/*z* range from 80 to 270.

Relative peak areas of 2-DCB and 2-TCB standards against the area of the internal standard showed a linear calibration from 2.5 to 500 ng/mL with correlation coefficients of >0.999.

The limit of detection (LOD) was calculated from the peak intensity at 20 ng/g and blank noise levels in recovery tests. LOD was defined as S/N > 5 so that it is in the linear range of the standard calibration. The LODs of 2-DCB and 2-TCB were 2.5 ng/mL in the test solution, which would be 3 ng/g weight in high fat samples and would be 1−2 ng/g weight in low fat and less interfered samples. LOD levels of 2-DCB and 2-TCB were about 0.1 μg/g fat when a concentration was expressed on a fat basis. On the basis of the chromatograms in **Figure 1** and LOD, the lowest detectable levels of irradiation doses would be 0.5 kGy or less in high fat samples such as minced beef, beef intestine, minced pork, and chicken thigh, 1 kGy for less

Table 2. Relationship of Cyclobutanones and Precursor Fatty Acids

sample	part	irradiated dose (kGy)	2-DCB[a] (ng/g)	palmitic acid (mg/g)	2-TCB[a] (ng/g)	stearic acid (mg/g)	ratio 2-DCB/2-TCB	ratio P/S[b]	crude fat[a] (%)
beef	minced	3.9	77	34	115	19	0.7	1.8	16.7
	large intestine	3.9	144	46	234	40	0.6	1.2	19.5
	thigh	3.7	8.8	4	11	2	0.8	2.1	1.9
	liver	3.6	8	1	24	1	0.3	1.2	1.4
pork	minced	3.6	62	58	146	28	0.4	2	23.2
	thigh	3.5	8	3	10	1	0.8	2.4	1.6
chicken	thigh	3.2	112	27	35	5	3.2	5.6	9.5
salmon		3.4	22	8	6	2	3.7	4.2	5.3

[a] Average of three irradiated samples. [b] Palmitic acid to stearic acid.

fatty samples such as beef thigh, beef liver, and pork thigh, and 2 kGy for salmon. Those levels were similar to values described in EN1785 method (*7*), except for salmon. Because the fatty acids composition of salmon was different from animal meats, cleanup was not enough for intensive concentration like other less fatty samples.

2-Alkylcyclobutanones and Precursors. Because 2-alkylcyclobutanone concentrations with γ-ray irradiation are considered to be related to fatty acid concentrations in the sample, the relationshipa of 2-alkylcyclobutanone concentrations and their precursor compounds such as palmitic acid and stearic acid at similar irradiated doses were studied. It was reported that γ-ray irradiation would cause a reaction from palmitic acid to 2-DCB and stearic acid to 2-TCB (*12*, *32*).

2-Alkylcyclobutanone concentrations at similar irradiation doses and respective precursor fatty acid levels in the samples before irradiation are listed in **Table 2**. Palmitic acid concentrations were higher than stearic acids levels in all of the samples, although 2-DCB concentrations were lower than those of 2-TCB in six out of eight samples. The results became clear when they expressed the ratio of two compounds in either precursors or products. The ratios of precursors, palmitic acid to stearic acid, were greater than 1 in all of the samples, while those of the products, 2-DCB to 2-TCB, were less than 1 in six samples except for chicken and salmon. The results indicated that γ-ray irradiation of frozen samples was likely to produce more 2-TCB from stearic acid than 2-DCB from palmitic acid and the ratios of 2-DCB to 2-TCB were less than those of palmitic acid to stearic acid. Production of radiolytic compounds was clearly affected by the irradiated temperature of samples. Stevenson reported that ratio of 2-DCB to 2-TCB in frozen pork was reduced by 60% from that in fresh pork (*32*). Stewart reported that 2-DCB production was reduced in frozen salmon as compared with chilled salmon while 2-TCB production was not affected (*12*). Those reports indicated that the 2-DCB/2-TCB ratio did not directly reflect the ratios of precursor fatty acids under the frozen state. All of the samples in this study were irradiated under the frozen state. Further study will be necessary to confirm the relationship of the ratios of 2-DCB/2-TCB and those of precursor fatty acids under various irradiation temperatures.

The relationship of 2-alkylcyclobutanone and its precursor fatty acid was also figured and showed good correlative linearity ($\gamma^2 = 0.988$, $p < 0.01$) with 2-TCB and stearic acid (**Figure 3**), even though the samples resources were different kinds of foodstuffs. 2-DCB levels also seemed to depend on palmitic acid but did not show a clear correlation ($\gamma^2 = 0.577$, $p < 0.05$). The 2-DCB concentration in the minced pork was quite a lot lower than the expected level in 2-DCB and palmitic acid relationship. Stevenson pointed out that the position of palmitic acid and stearic acid in triglyceride in pork, in which palmitic acid bound to at position 2 of glycerol, while stearic acid was likely to bind to position 1, would affect yields of 2-DCB and 2-TCB with irradiation (*32*).

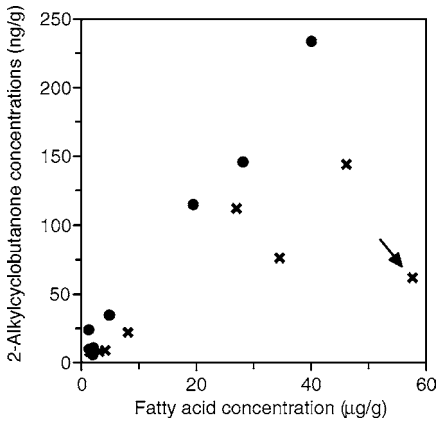

Figure 3. Relationship of 2-alkylcyclobutanones and precursor fatty acids before irradiation. 2-DCB and 2-TCB levels were the average of three experiments in eight samples in **Table 2**. 2-DCB vs methyl palmitate, ×; 2-TCB vs methyl stearate, ●. The arrow indicates minced pork.

The results indicate that a rapid analytical method for 2-DCB and 2-TCB with ASE has been developed, that both 2-DCB and 2-TCB are reliable indicators to detect irradiated foodstuffs, which contain fat. Both 2-alkylcyclobutanones should be monitored because an abundance of the two compounds is not expected, and they would diversify by precursor fatty acids in the samples and the irradiated temperature.

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