INTRODUCTION

Numerous reports\(^1\)\(^-\)\(^5\) have made it clear that the toxicity of oxidized edible oil is due mainly to oxidation products from unsaturated fatty acids. The ingredient standard for commercially processed foods, Japan, established the chemical properties of oil and fat at AV \(^3\) and POV \(^30\). The legislation seems to be based on the understanding that the safety of the edible oil and fat is ensured by setting limits on these two properties. In some other countries, only an AV cutoff point is set for oil and fat. Gotoh et al.\(^7\) have argued that AV does not reflect the risks due to oxidation of oil and fat at all. It is obvious that AV is relatively stable and that the cutoff point is hardly exceeded during usual food processing. We\(^8\) screened the AV of oils contained in batter coatings of various commercially deep-fried foods, such as cutlets, tempura, and croquettes, and found that only 4% of screened foods contained oil with AV>3 in spite of unpleasant appearance and odor.

Soriguer et al.\(^9\) found after extensive epidemiological studies that the risk of hypertension was positively and independently associated with the intake of cooking oil polar compounds. Sanchez-Muniz et al.\(^10\) reported that the triacylglycerol oligomer content in used frying oil gave more precise information about the alteration of the oil and its potential toxicity than PC did.

The present legislation on oil and fat described above may not be a problem, as no food poisoning due to deteriorated oil or fat has been reported officially in Japan in years. But consumption of deep-fried foods is huge nowadays\(^11\),\(^12\) and metabolic syndrome is common in the younger generation as well as in middle-aged people. Thus, it is essential to thoroughly address the effects of oxidized oil on human health from the point of lifestyle-related diseases. For several years, we have been studying\(^13\)\(^-\)\(^16\) the effects of oil thermally oxidized to such a degree that it causes neither diarrhea nor stomachache. Wistar rats were fed \(\text{ad libitum}\) a powdered diet (AIN93G; no fat) containing 7 wt% of fresh oil (control) or one of the frying oils described above. The rats were subjected to anthropometric measurements, hematological analyses, and observations of the liver and kidneys. All of the rats grew well, and no gross symptoms attributable to the experimental oils were observed. However, the rats fed a diet containing the heated oil developed apparent liver damage to different degrees regardless of the chemical properties of the ingested oils. Thus, it was suggested that the chemical properties evaluated here had little to do with the cytotoxicity of heated oil, although the properties express quality of oil. Volatile compounds seem to be major candidates for the toxic agents in heated oil because oils with rancid and deteriorated odor show strong toxicity.

Abstract: Heated frying oils with different chemical properties in terms of AV (acid value), POV (peroxide value), COV (carbonyl value), and contents of polar compounds (PC) and triacylglycerol (TG), as well as color and odor, were obtained. Male Wistar rats were fed \(\text{ad libitum}\) for 12 weeks a powdered diet (AIN93G; no fat) containing 7 wt% of fresh oil (control) or one of the frying oils described above. The rats were subjected to anthropometric measurements, hematological analyses, and observations of the liver and kidneys. All of the rats grew well, and no gross symptoms attributable to the experimental oils were observed. However, the rats fed a diet containing the heated oil developed apparent liver damage to different degrees regardless of the chemical properties of the ingested oils. Thus, it was suggested that the chemical properties evaluated here had little to do with the cytotoxicity of heated oil, although the properties express quality of oil. Volatile compounds seem to be major candidates for the toxic agents in heated oil because oils with rancid and deteriorated odor show strong toxicity.

Key words: chemical properties, acid value, peroxide value, polar compounds, thermally oxidized oil, volatile compounds, cytotoxicity, cell damage

1 INTRODUCTION

Numerous reports\(^1\)\(^-\)\(^5\) have made it clear that the toxicity of oxidized edible oil is due mainly to oxidation products from unsaturated fatty acids. The ingredient standard for commercially processed foods, Japan, established the chemical properties of oil and fat at AV \(\leq 3\) and POV \(\leq 30\). The legislation seems to be based on the understanding that the safety of the edible oil and fat is ensured by setting limits on these two properties. In some other countries, only an AV cutoff point is set for oil and fat. Gotoh et al.\(^7\) have argued that AV does not reflect the risks due to oxidation of oil and fat at all. It is obvious that AV is relatively stable and that the cutoff point is hardly exceeded during usual food processing. We\(^8\) screened the AV of oils contained in batter coatings of various commercially deep-fried foods, such as cutlets, tempura, and croquettes, and found that only 4% of screened foods contained oil with AV>3 in spite of unpleasant appearance and odor.

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The present legislation on oil and fat described above may not be a problem, as no food poisoning due to deteriorated oil or fat has been reported officially in Japan in years. But consumption of deep-fried foods is huge nowadays\(^11\),\(^12\) and metabolic syndrome is common in the younger generation as well as in middle-aged people. Thus, it is essential to thoroughly address the effects of oxidized oil on human health from the point of lifestyle-related diseases. For several years, we have been studying\(^13\)\(^-\)\(^16\) the effects of oil thermally oxidized to such a degree that it causes neither diarrhea nor stomachache. Wistar rats were fed \(\text{ad libitum}\) a standard diet containing 7 wt% of the oil for 8-12 weeks. The animals developed histological damage in the liver and kidneys and hematological changes without gross symptoms attributable to the experimental oils. In the present study, the relation between chemical properties of thermally oxidized frying oil and cytotoxicity
was investigated when Wistar rats were fed a diet containing the oil for a long period.

2 EXPERIMENTAL

2.1 Materials

2.1.1 Oil

The fresh oil 1 (used for animal experiment 1) was made of fresh soybean and rapeseed oils. One liter of this oil was heated with and without 1% gluten (Nacalai Tesque, Inc., Kyoto) at 180°C for 10 h in a 2-L four-necked flask, respectively. Oil heated with gluten was filtered over filter paper under a reduced pressure.

The fresh oil 2 (used for animal experiment 2) was also made of fresh soybean and rapeseed oils. To 1 L of this oil each of the following four different nutrient groups was added, and the mixture was heated at 180°C for 20 h in a 2-L four-necked flask under a stream of air at 3 L/min. The four groups were a mixture of amino acids, Gln, Gly, Ala, Tyr, Arg, Pro, Thr, Asp (Nacalai Tesque, Inc., Kyoto), 200 ppm each; 1% gluten (Nacalai Tesque, Inc., Kyoto) from wheat; 1% sucrose (Wako Pure Chemical Industries Ltd., Osaka); and 1% wheat flour (Nacalai Tesque, Inc., Kyoto). Experimental oils, thus obtained, were allowed to stand at a room temperature for a day to precipitate solid materials, and each supernatant was employed for an animal experiment. “B grade recovered vegetable oil” (recovered oil) was obtained from Miyoshi Oil & Fat Co., Ltd. The oil is composed mainly of soybean and rapeseed oils.

2.1.2 Diets

A commercial pelleted diet (Labo MR Stock, Nihon Nosan Kohgyo, Japan) and a powdered AIN93G diet without fat (Japan Clea, Tokyo) were purchased. Using a blender, the latter was mixed uniformly with 7 wt% experimental oils and fresh oil, respectively. The 9 kinds of diets, thus prepared, were handled and provided as described in our previous papers.

2.1.3 Chemical analyses

Methods for chemical analyses of oil were the same as in our previous papers. The fatty acid compositions of the fresh oils 1 and 2 analyzed as previously were as follows: myristic acid 0.1% and 0.1%; palmitic acid 9.4% and 8.4%; palmitoleic acid 0.1% and 0.1%; stearic acid 3.6% and 3.3%; oleic acid 32.6% and 38.2%; linoleic acid 44.1% and 39.2%; linolenic acid 6.3% and 6.5%; eicosenoic acid 0.5%; and α-linolenic acid 6.3% and 6.5%; eicosenoic acid 0.5% and 0.6%; others 3.3% and 3.6%, respectively.

As the oil heated with gluten showed almost no toxicity in the experiment 2, low boiling compounds in three kinds of oils employed in the animal experiment 1 were analyzed preliminarily by head-space GC (Head-space sampler, 7694 Hewlett-Packard Company; oven temp., 80°C; vial heating time, 30 min; loop temp., 150°C; transfer line temp., 200°C) and GC-MS (6890/5973 Hewlett-Packard Company; Column, DB-WAX, (Agilent Technologies) Φ 0.25 mm × 60 m; split (1:4); Column temp. 40°C for 1 min, then raised at 5°C/min to 200°C; carrier gas He 1 mL/min; electron impact ionization; ionization voltage 70 eV).

2.2 Animals

Male Wistar rats aged 9 weeks were obtained from Japan SLC, Inc., Shizuoka, Japan, and were housed separately in wire cages at 24 ± 2°C and humidity 50 ± 10%, with light from 7:00 to 19:00 at Japan SLC, Inc., Animal Experiment Center, Shizuoka, Japan. Animal care and handling were in accordance with the Ethical Agreement Concerning Care and Use of Laboratory Animals for Research and Education, Kobe-Gakuin University.

2.3 Procedure

2.3.1 Animal experiment 1

Twenty-four animals were maintained on radio-sterilized Labo MR Stock for 1 week of adaptation; animals were then divided into three groups (8 rats/group). Two groups were fed a diet containing respectively 7-wt% oils heated with/without gluten, and the third group was fed a diet containing 7-wt% fresh oil 1. All animals were allowed feed and water ad libitum throughout the experiment. Autoxidation of oil in the diet was avoided by supplying a fresh diet daily as described in our previous paper. After 12 weeks, a fasting period of 18 h was imposed prior to the administration of anesthesia. Serum was obtained from blood drawn from the abdominal aorta. Livers and kidneys were excised, weighed, and examined.

2.3.2 Animal experiment 2

Forty-eight animals (6 groups) were subjected to the animal experiment as in 2.3.1; Five groups (amino acid group, gluten group, sugar group, wheat starch group and recovered oil group) were fed a diet containing 7-wt% experimental oils, respectively. The last group was fed a diet containing 7-wt% fresh oil 2.

2.4 Hematological analyses

Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined as in our previous papers.

2.5 Statistical analysis

All the values obtained from animals are revealed as mean ± SD. Data from 8 animals each for experimental and control groups were analyzed using Student’s t-test for unpaired observations and results were considered significant at p<0.05.

3 RESULTS

3.1 Chemical properties of the oils used
As shown in Table 1, the six laboratory-heated oils had about 20% polar compounds, 83–91% TG, COV values of about 30, and AV values of 0.1–0.3. Oil heated with/without gluten had low POV, probably because the decomposition of peroxides to carbonyl compounds was active in the first 10 h of heating. Recovered oil showed PC and TG concentrations similar to those of other experimental oils, but with low COV and high AV. The odor of the recovered oil was the worst of all and that of oil heated with gluten was mild and acceptable.

### Table 1  Chemical Properties of Frying Oil.

<table>
<thead>
<tr>
<th></th>
<th>Oil heated at 180°C for 10 h with</th>
<th>Fresh oil 1</th>
<th>Recovered oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gluten</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>PC (%)</td>
<td>21.6</td>
<td>23.9</td>
<td>4.2</td>
</tr>
<tr>
<td>TG (%)</td>
<td>83.5</td>
<td>85.0</td>
<td>98.6</td>
</tr>
<tr>
<td>COV</td>
<td>30.6</td>
<td>35.4</td>
<td>2.7</td>
</tr>
<tr>
<td>POV (mEq/kg)</td>
<td>1.4</td>
<td>3.6</td>
<td>0.9</td>
</tr>
<tr>
<td>AV</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Color (Gardner)</td>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Smell</td>
<td>mild</td>
<td>rancid</td>
<td>fresh</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Oil heated at 180°C for 20 h with</th>
<th>Wheat starch</th>
<th>Fresh oil 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acids</td>
<td>Gluten</td>
<td>Sucrose</td>
</tr>
<tr>
<td>PC (%)</td>
<td>21.6</td>
<td>21.6</td>
<td>17.8</td>
</tr>
<tr>
<td>TG (%)</td>
<td>83.8</td>
<td>82.6</td>
<td>90.0</td>
</tr>
<tr>
<td>COV</td>
<td>31.9</td>
<td>34.1</td>
<td>39.1</td>
</tr>
<tr>
<td>POV (mEq/kg)</td>
<td>62.7</td>
<td>63.4</td>
<td>76.9</td>
</tr>
<tr>
<td>AV</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Color (Gardner)</td>
<td>11</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Smell</td>
<td>rancid</td>
<td>mild</td>
<td>rancid</td>
</tr>
</tbody>
</table>

The weights of organs excised are shown in Fig. 2. No difference in the weight of kidneys was detected between groups. However, the recovered oil and sugar groups had significantly heavier livers than those of the control group.

### 3.4 Hematological analyses

In animal experiment 1, the heated oil group had the highest AST, followed by the group that received oil heated with gluten and the control group, with no significant difference from each other. ALT measurement did not show any difference among the three. In animal experiment 2, the magnitude of AST and ALT was in the order of recovered oil group, wheat starch group, amino acid group, gluten group, sugar group, and control.

There was a significant difference in AST and ALT between the recovered oil group and the control (Table 2). The occurrence of AST and ALT, both higher than the maximum AST (101 IU/L for Experiment 1 and 118 IU/L for Experiment 2) and ALT (69 IU/L for Experiment 1 and 97 IU/L for Experiment 2) of the control, was assessed for each group and listed in Table 3.

### 3.5 Visible changes in livers and kidneys

Examination of the livers and kidneys revealed dark-red patches due to dotted bleeding on the surface of the livers, particularly from the recovered oil, amino acids, sugar, and wheat starch groups, suggesting degeneration of inner tissues (Table 3). A control rat and a rat in the gluten group also had patches, but their numbers were
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3.6 Correlation of the chemical properties and toxicity of heated oil

Chemical properties (PC, TG, COV, POV) of the oil and damage, based on the number of rats with dark-red patches on the liver/8 rats, were plotted, respectively, to determine the relation between them (Fig. 3), but the chemical properties proved unrelated to the occurrence of damage.

4 DISCUSSION

All the experimental oils had high PC and COV and low TG (Table 1). POVs of the oils used for animal experiments 1 and 2 were low and high, respectively. The reason for high POV seems to be attributable to the long heating time under air supply and leaving the oil overnight for sedimentation of solid materials. AVs of all the oils except the recovered oil were low. The color of the experimental oils ranged from yellow to brown. The experimental oils smelled rancid except for the oil heated with gluten, which had PC, COV, TG, POV, and AV values often found in heated oil, but had a mild odor with very slight rancidity. In order to investigate the effect of gluten on the alteration of oil odor during heating, the odor of three oils used in animal experiment 1 was analyzed by head-space GC (Fig. 1).

Several peaks probably attributable to the rancid odor became obviously smaller by the addition of gluten, while the peak at RT 8.0 min increased. Because peak size is determined by the summed intensity of fragments generated from the compound by electron impact, quantification of each compound is not possible; peaks with the same RT can be compared because all the GC-MS conditions for each run were identical. In addition, odor is not related to the size of the peak, so a small peak can be attributable to a rancid odor. The mechanism of the reactions between oil and gluten under heating is not currently known.

In both animal experiments, all the rats grew well and appeared normal. As shown in Fig. 2, big differences were not found in the weights of the liver and kidneys. It was confirmed that frying oil heated for 10-20 h and recovered oil apparently did not impair the health condition of the animals. The AST value reveals cell damage in the liver and kidneys, but the average AST of each group did not show a significant difference from that of the control except in the recovered oil group. As described in 3-5, the percentage of rats with dark-red patches was employed as the index of cytotoxicity and used for the evaluation of chemical properties.

Studies of the toxicity of oxidized oil have been focused on its acute toxicity, such as in food poisoning. The present study on chronic symptoms caused by the ingestion of oxidized oil indicated that the chemical properties evaluated here did not correspond to the degree of cytotoxicity (Fig. 3), and that low-molecular-weight volatile compounds...
Fig. 2  Organ Weights of Rats Fed a Diet Containing Oil Heated with a Nutrient at 180°C for 10 h* or 20 h**.

* p < 0.05, significantly different from the value of control 2 (unpaired t-test).

Fig. 3  Relation between Chemical Properties and Liver Damage in Rats Fed a Diet Containing the Oil.
<table>
<thead>
<tr>
<th></th>
<th>Gluten*</th>
<th>None*</th>
<th>Fresh oil 1</th>
<th>Recovered oil</th>
<th>Amino acids**</th>
<th>Gluten**</th>
<th>Sucrose**</th>
<th>Wheat starch**</th>
<th>Fresh oil 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>95.4 ± 34.1</td>
<td>100.1 ± 33.0</td>
<td>87.6 ± 17.9</td>
<td>181.6 ± 161.9†</td>
<td>105.4 ± 50.6</td>
<td>100.8 ± 60.4</td>
<td>96.1 ± 38.6</td>
<td>136.1 ± 121.4</td>
<td>73.1 ± 16.5</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>52.2 ± 11.1</td>
<td>53.3 ± 9.1</td>
<td>54.4 ± 23.9</td>
<td>114.8 ± 104.2†</td>
<td>71.4 ± 46.8</td>
<td>63.0 ± 29.2</td>
<td>55.3 ± 18.5</td>
<td>100.9 ± 105.1</td>
<td>48.9 ± 11.4</td>
</tr>
</tbody>
</table>

Values are means ± SD for eight animals.

†p < 0.05, significantly different from the value of control 2 (unpaired t-test).

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Occurrence of AST higher than maximum AST of control/8 rats</td>
<td>1/8</td>
<td>2/8</td>
<td>—</td>
<td>4/8</td>
<td>4/8</td>
<td>1/8</td>
<td>3/8</td>
<td>3/8</td>
<td>—</td>
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<tr>
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<td>0/8</td>
<td>0/8</td>
<td>—</td>
<td>4/8</td>
<td>3/8</td>
<td>2/8</td>
<td>2/8</td>
<td>3/8</td>
<td>—</td>
</tr>
</tbody>
</table>
Toxicity of Oxidized Oil

could be candidates for the cause of cytotoxicity. Our previous paper reported that most of the generated carbonyl compounds vaporized from oil during frying: frying operators could be exposed to a large quantity of carbonyl compounds vaporizing with steam generated from water during the frying of foodstuffs.

When COV started to drastically increase after reaching the maximum POV in the autooxidation of methyl linoleate, low-molecular-weight compounds, secondary decomposition products of peroxides, contributed to the toxicity of the oxidized oil. Among the low-molecular-weight compounds, 4-hydroperoxy-2-en-1-al with 5-9 carbons was reported to have the strongest toxicity. Gabriel et al. gave Wistar rats fresh olive oil, and distillable fractions of fresh and thermally oxidized olive oil, respectively, and found out that only the rats that received the distillable fraction of thermally oxidized olive oil showed overt symptoms of heated fat toxicity. This was reflected in the histological scores of these animals, with the liver sustaining the most numerous and severe lesions. These reports support our speculation that cytotoxicity of thermally oxidized oil was attributable to low-molecular-weight decomposition products.

Leung et al. tested food samples for acrylamide by an LC-MS method and found high levels in all kinds of crisps. But we did not detect it in used frying oil. Velasco et al. investigated the formation of monoepoxy fatty acids arising from oleic and linoleic acids in olive oil and sunflower oil. Their results showed that the monoepoxides constituted a major group among the oxidized fatty acid monomers formed at a high temperature. The content of monoepoxides in used frying oils from restaurants and fried-food outlets in Spain was found to range from 3.37 to 14.42 mg/g of oil. These authors, however, did not evaluate the characteristic cytotoxicity of monoepoxides.

ACKNOWLEDGMENTS

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References

22. Gabriel, H.G.; Alexander, J.C.; Valli, V.E. Nutritional and metabolic studies of distillable fractions from fresh and