Identification of 2-Alkylcyclobutanones in Nutmeg (*Myristica fragrans*)

Susu Chen [a, c], Tomoaki Tsutsumi [b], Satoshi Takatsuki [b], Rieko Matsuda [b], Hiromi Kameya [a], Mitsutoshi Nakajima [c], Masakazu Furuta [d], Setsuko Todoriki [a,*]

[a] Food Safety Division, National Food Research Institute, 2-1-12, Kannondai, Tsukuba, Ibaraki 305-8642, Japan

[b] Division of foods, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya, Tokyo 158-8501, Japan

[c] Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1, Tennodai, Tsukuba, Ibaraki 305-8572, Japan

[d] Laboratory of Quantum-beam Chemical Biology, Radiation Research Center, Osaka Prefecture University, 1-2, Gakuencho, Nakaku, Sakai, Osaka 599-8570, Japan

[*] Correspondence to: Setsuko Todoriki

Address: Food Safety Division, National Food Research Institute

2-1-12, Kannondai, Tsukuba, Ibaraki 305-8642, Japan

Telephone: +81- 29-838-8047

Fax: +81-29-839-1552

E-mail: setsuko@affrc.go.jp

Abstract

The natural existence of the irradiation markers, namely, 2-decylcyclobutanone (2-DCB) and 2-dodecylcyclobutanone (2-dDCB), in nutmeg (*Myristica fragrans*) has recently been reported. In this study, 2-DCB and 2-dDCB were extracted from nutmeg of 5 different origins using supercritical fluid extraction (SFE). A 50-kGy irradiated sample was used to optimize the parameters of SFE and solid-phase extraction. The irradiated samples were analysed by gas chromatography-mass spectrometry, whereas the non-irradiated samples were analysed with high-resolution gas chromatography-mass spectrometry. Both 2-DCB and 2-dDCB were detected and identified in the irradiated samples at 5 kGy or greater. However, neither was present in the non-irradiated samples. Moreover, although the concentrations of 2-DCB and 2-dDCB were significantly reduced, a positive identification was obtained in irradiated nutmeg even after 30 weeks of storage.

Keywords: irradiation, 2-alkylcyclobutanone, nutmeg, detection method, GC-HRMS

1. Introduction

The final microbial content of spices is determined by the natural content of microorganisms in plants and by the harvesting, drying, transporting, and packaging processes (Gould, 1996). Three major methods of bacterial reduction are used to ensure hygienic quality in the spice industry. These are fumigation with ethylene oxide, thermal treatment with steam, and irradiation with gamma rays or high-energy electrons (Leistritz, 1997). The irradiation treatment has developed greatly in the past 20 years and is used throughout much of the world (Kume, Furuta, Todoriki, Uenoyama, & Kobayashi, 2009). Although the popularity of this method is increasing in both developing and industrialized countries, consumers should be able to choose between irradiated and non-irradiated products. Thus, adequate product labelling is required.

To check for compliance with existing regulations, suitable methods must be available for the reliable authentication of irradiated products. Regarding the detection of irradiated spices, the international standard and the European standard include the photostimulated luminescence (PSL) (EN 13751) and thermoluminescence (TL) (EN 1788) methods. The PSL is a screening method that is widely used for detection of irradiation in nearly all foodstuffs. In contrast, the TL is the most promising detection method and was recognized as the standard in Japan in 2007.

Nutmeg (*Myristica fragrans*) is a popular spice used in sweet and savoury cooking. Like other spices, nutmeg is prone to contamination by microorganisms and insects from the time of harvest to sale. However, unlike other spices, nutmeg has a small quantity of silicate mineral because its edible part is the inner core of the seed. Previous research has shown that it is almost impossible to use the TL method for nutmeg

(Oduko & Spyrou, 1990). Therefore, another promising detection method is required.

When fat-containing foods are irradiated, free fatty acids and triacylglycerides in the foods are decomposed to 2-alkylcyclobutanones (2-ACBs). The 2-ACBs have the same number of carbon atoms as the parent fatty acids from which they are formed, with an alkyl group located in ring position 2. These compounds are cyclic compounds formed by the loss of an electron from the oxygen on the carbonyl of fatty acids or triacylglycerides, followed by rearrangement to produce 2-ACBs specific to the parent fatty acids (Letellie. Pr & Nawar, 1972). In addition, 2-ACBs originate during irradiation but are not formed during the cooking, decay, or oxidation processes, indicating that they could function as unique radiolytic products (URPs) and be used as a detection marker for fat-containing foods and also to estimate the absorbed dose (Stevenson, Crone, & Hamilton, 1990). Therefore, this 2-ACB method was adopted as a global standard by the European Committee for Normalization in 1996 (EN 1785) and declared a standard method in Japan in 2010. Thus far, this detection method has been successfully used a wide range of irradiated fat-containing foods, such as meat-based products (Boyd, Crone, Hamilton, Hand, Stevenson, & Stevenson, 1991; Zanardi, Battaglia, Ghidini, Conter, Badiani, & Ianieri, 2007), cheeses (Schreiber, Helle, Schulzki, Spiegelberg, Linke, Wagner, *et al.*, 1993; Rahman, Matabudall, Haque, & Sumar, 1996), eggs (Crone, Hand, Hamilton, Sharma, Boyd, & Stevenson, 1993), exotic fruits (D. W. Sin, Y. C. Wong, & M. Y. Y. Yao, 2006; Stewart, Moore, Graham, McRoberts, & Hamilton, 2000), and seafood (Tanabe & Goto, 2003).

A large number of extensive studies have shown that nutmeg is also characterized by high fatty acid content (mainly myristic and palmitic acid) (Niyas, Variyar, Gholap, & Sharma, 2003; Spricigo, Pinto, Bolzan, & Novais, 1999). Therefore, 2-ACB method

was expected to be applicable to irradiated nutmeg. However, the natural existence of 2-ACBs in non-irradiated nutmeg and cashew nut samples of India was reported, disproving the hypothesis that 2-ACBs are URPs (Variyar, Chatterjee, Sajilata, Singhal, & Sharma, 2008). Therefore, the purpose of the present study was to investigate whether 2-ACBs exist naturally in non-irradiated nutmeg and to determine whether the 2-ACB detection method can be used for routine analysis of irradiated nutmeg samples.

2. Materials and methods

2.1. Chemicals and food samples

2-Cyclohexylcyclohexanone (2-CHCH) as an internal standard for gas chromatography-mass spectrometry (GC-MS) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in *n*-hexane to produce a 0.1 μg/mL stock solution. The solution was preserved at −20°C. The 2-ACB standards, including 2-DCB and 2-dDCB, which were synthesized by Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan) were equally mixed and diluted with *n*-hexane in a range of 0.02–20.00 μg/mL for the running standards and spiking solution in recovery tests. Sodium sulphate anhydrous (Na_2SO_4) was heated for 5 h at 600°C before use.

Nutmeg samples—1 lot from Sri Lanka (A), 3 lots from different farms in Indonesia (B, C, and D), and 1 lot from India (E)—were kind gifts from Japanese spice makers. They were excised homogeneously with a food cutter and preserved at −80°C before use. For the room temperature storage test, ground nutmeg samples were protected from light in aluminium-sealed polyethylene bags at a temperature of 8 ± 2°C.

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114 *2.2. Irradiation system*

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116 The ground nutmeg samples in aluminium-sealed polyethylene bags were irradiated
117 with gamma rays from a cobalt 60 source (Gammacell 220; MDS Nordion International
118 Co. Ltd., Ottawa, Ontario, Canada) at the National Food Research Institute of Japan.
119 The dose rate was 6 kGy/h. The samples were irradiated at doses of 5 and 10 kGy at
120 room temperature and were stored for 0, 1, 5, 15, and 30 weeks at 8 ± 2°C. To
121 investigate the efficiency of 2-ACB extraction from the matrix, we prepared and used
122 nutmeg at a dose of 50 kGy for method development and stored it at −80°C until
123 analysis. An alanine pellet dosimeter (Bruker Biospin Ltd., Rheinstetten, Germany) was
124 attached to the surface of each sample, and the absorbed dose was determined using an
125 electron spin paramagnetic spectrophotometer (Bruker EMX; Bruker Biospin Ltd.).
126 Non-irradiated nutmeg was used as a control and stored under the same conditions.

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128 *2.3. Extraction of 2-ACBs*

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130 The extraction of 2-ACBs from the nutmeg samples for GC-MS measurement was
131 performed using a supercritical fluid extraction (SFE) system (Model SFX1220;
132 Teledyne Isco, Inc., Lincoln, NE, USA) with carbon dioxide. A 1.5-g sample of ground
133 nutmeg was homogenized with wet support (ratio of 1:1) in a mortar. A 5-mL SFE
134 cartridge (i.d., 15 mm; length, 56 mm; Teledyne Isco, Inc.) was loaded with sand (about
135 2 g), and then the nutmeg-wet support mixture was placed in the extractor. The sand
136 protected the seal of the extraction cartridge. Four measurement samples were extracted

for each lot. The blank control (with only wet support and sand), non-irradiated nutmeg samples, and irradiated nutmeg samples were prepared and analysed in the same way.

Extraction was carried out under the following conditions: pressure, 150 atm; temperature, 80°C; 5 min static and 60 min dynamic with a CO_2 flow rate of 2 mL/min. The 2-ACBs were trapped with 6 mL *n*-hexane in a test tube (i.d., 13 mm; length, 125 mm). In this extraction process, *n*-hexane was added frequently to maintain the appropriate *n*-hexane amount. Finally, after dried with $NaSO_4$ the nutmeg extract was concentrated to 5 mL under a stream of nitrogen at 40°C for cleanup.

2.4. Cleanup of the extraction and preparation of samples

About 0.25 mL of the nutmeg extract was added to a silica solid-phase extraction (SPE) cartridge column (5000 mg/25 mL; Alltech Associates Inc., Grace Co., USA), whose packing bed was rinsed with 20 mL *n*-hexane for conditioning. A 10-mL aliquot of *n*-hexane was eluted and discarded. Then, a 55-mL aliquot of 2% diethyl ether/*n*-hexane (2:98, v/v) was eluted, and a 25–55 mL fraction was collected as the 2-ACB fraction. The flow rate of this column was about 1.00 mL/min. After concentrating to 1 mL, the extract was further subjected to a supelclean sulfoxide SPE cartridge column (3000 mg/6 mL; Supeluco, Bellefonte, PA, USA), which was conditioned with 10 mL acetone to remove residual moisture and equilibrated with 20 mL *n*-hexane, after which a 4–14 mL fraction was collected with *n*-hexane. The flow rate was about 0.72 mL/min. Then, the eluted *n*-hexane was carefully concentrated to a volume of 1 mL in a rotary vacuum evaporator, further concentrated to near dryness under a stream of nitrogen at 40°C, and then added to 0.2 mL 2-CHCH (0.05 μg/mL) as

an internal standard. Finally, the mixture was transferred into a glass vial insert for GC-MS and high-resolution gas chromatography-mass spectrometry (GC-HRMS) analysis. A single sample was processed within 200–240 min.

2.5. GC-MS and GC-HRMS analysis of 2-ACBs

Samples were analysed using a QP-2010 Plus model GC-MS system (Shimadzu, Kyoto, Japan) for irradiated samples and a JMS-700 MStation GC-HRMS system (JEOL Ltd., Tokyo, Japan) equipped with GC HP-6890 Plus (Agilent, Toronto, Ontario, Canada) for blank controls and non-irradiated samples. Data acquisition and control were performed using GC-MS-Solution Ver. 2.53 SU3 software and DioK software (JEOL Ltd., Tokyo, Japan), respectively.

The GC conditions of the QP-2010 Plus model GC-MS system were as follows: column, DB-5MS (Agilent Technologies J & W Scientific, USA) 60 m × 0.25 mm i.d., and 0.25-μm film; column temperature program, 55°C (2 min), 55–175°C at 20°C/min, 175–250°C at 2°C/min, 250–270°C at 10°C/min, 270°C (5 min), 270–280°C at 10°C/min, and 280°C (10 min); carrier gas, helium 1.00 mL/min; injection temperature, 250°C; injection single taper inlet liner (SGE Analytical Science, Australia); injection mode, splitless; and injection volume, 1 μL. The GC conditions of the GC-HRMS were almost identical, but the injection volume was 2 μL.

The MS conditions of the QP-2010 Plus model GC-MS system were as follows: ionization mode, electron ionization (EI); ion detection, selected ion monitoring (SIM); event time, 0.20 s; detector voltage, 0.84 kV; ion source temperature, 200°C; and interface temperature, 280°C. The monitored ions were *m/z* 98 and 112, and *m/z* 98 was

selected for determination. The MS conditions of the GC-HRMS system were similar to those of the GC-MS system: ionization mode, EI; ion detection, SIM; resolution performance, 10000; ionization voltage, 70 eV; ionization current, 500 μA; ion source temperature, 230°C; interface temperature, 280°C; and event time, 2 cycle/s. The monitored ions of *m/z* 98.073 and 112.088 were chosen, and 2-ACBs were identified with the area ratio of these two peaks according to EN 1785. Each sample was repeated at least 4 times.

2.6. Statistical analysis

Statistical tests were performed using Microsoft Office Standard 2007 Excel software (Microsoft Corporation, Redmond, WA, USA). Results were expressed as means ± standard deviation for each determination. Data were analysed using Welch's *t*-test (SSRI Co., Ltd., Tokyo, Japan). $p > 0.05$ between groups was accepted as not significantly different.

3. Results and discussion

3.1. Method development

To date, extraction methods developed to detect 2-ACBs from irradiated samples have been based mainly on soxhlet extraction (EN 1785 method), accelerated solvent extraction (Obana, Furuta, & Tanaka, 2005), direct solvent extraction (Hijaz, Kumar, & Smith, 2010; Tewfik, 2008), or SFE (Horvatovich, Miesch, Hasselmann, & Marchioni,

2000; Lembke, Bornert, & Engelhardt, 1995; Rahman, Matabudall, Haque, & Sumar, 1996; Stewart, McRoberts, Hamilton, & Graham, 2001). The use of solid phase microextraction to isolate 2-ACBs from irradiated meat was also reported recently (Blanch, Caja, Flores, & del Castillo, 2009; Caja, del Castillo, & Blanch, 2008). Among these methods, SFE is the most effective, and selective extraction method and was even able to extract a small amount of 2-ACBs from cowpeas irradiated at 50 Gy and rice irradiated at 100 Gy (Horvatovich, Miesch, Hasselmann, & Marchioni, 2002). Because nutmeg contains a lot of essential oil, all extractions were carried out using SFE in this study. To optimize the parameters of SFE, we irradiated a sample of nutmeg at a very high dose (50 kGy) in order to obtain a high concentration of 2-ACBs. As previously mentioned, because of the fatty acid profile of lipids in nutmeg, 2-DCB and 2-dDCB were chosen as the main targets of analysis in this study.

Fig. 1 shows the extraction rates of 2-DCB and 2-dDCB under the 3 different extraction pressures at 80°C. To define the total abundance of 2-ACBs, we extracted the samples at 150, 200, and 250 atm with 200-mL CO_2. In the case of 150 atm, when the volume of CO_2 increased to 120 mL, both 2-DCB and 2-dDCB reached maximum values, which means that henceforth those compounds were not obtained in volume. In the case of 200 and 250 atm, when the volume of CO_2 increased to 80 mL, both 2-DCB and 2-dDCB reached maximum values, and higher amounts could no longer be extracted (Fig. 1). The concentrations of 2-DCB and 2-dDCB at 150 atm was 9.59 ± 0.85 and 1.58 ± 0.07 μg/g fresh weight (FW) and at 250 atm was 8.68 ± 0.51 and 1.42 ± 0.02 μg/g FW, respectively. Further, there was no significant difference in the extracted 2-ACB amounts between these 2 groups ($p > 0.05$). However, the chromatogram obtained at 150 atm showed lesser impurity peaks close to the retention time of 2-dDCB

than chromatograms obtained at 200 or 250 atm (data not shown). Horvatovich *et al.* advised increasing the temperature to reduce lipids (Horvatovich, Miesch, Hasselmann, & Marchioni, 2000). Thus, the samples were also extracted at 150 atm and 90°C. The chromatograms at 80°C were much clearer and more intuitive than those at 90°C (data not shown). Therefore, the conditions of SFE extraction were fixed at 150 atm, 80°C, and 120 mL CO_2 volume.

For the cleanup procedure, Horvatovich *et al.* reported that a silica trap should be used for purification instead of the existing large-scale Florisil columns as in the EN 1785 method, because of its ability to retain 2-ACBs from the SFE extract substance (Horvatovich, Miesch, Hasselmann, & Marchioni, 2000). In our study, we first attempted to use a 5-g silica SPE column; however, as the components of nutmeg are quite complex, a large number of impurities still remained after this cleanup procedure. In addition, Chen *et al.* reported recently that a sulfoxide SPE column was successfully used to separate 2-ACBs (Chen, Morita, Saito, Kameya, Nakajima, & Todoriki, 2011). Therefore, a sulfoxide SPE column was tried after the silica SPE column. The impurity peaks that were not removed with the silica SPE column were almost completely removed with the sulfoxide SPE column (data not shown).

The signals to n-hexane background noise ratio above 3 were consistently obtained for both m/z 98 and 112 ions for 0.008 μg/mL 2-ACBs. This protocol was evaluated with recovery tests in which 2-DCB and 2-dDCB were spiked at 0.125 μg/g into control nutmeg samples and analysed with GC-MS (n = 4). The 2-ACBs were not detected in the control samples. Both 2-DCB and 2-dDCB were recovered at a rate of 90% ± 4% and 138% ± 5%, respectively, with mostly less than 5% relative standard deviation. The chromatograms and mass spectra of GC-MS analysis were clear enough to identify the

257 presence of both 2-DCB and 2-dDCB according to the conditions of EN 1785 (data not
258 shown).
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260 *3.2. Detection of 2-ACBs from irradiated nutmeg and room temperature storage*
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262 Because previous studies used doses of 5–10 kGy to decontaminate spices
263 (Ananthakumar, Variyar, & Sharma, 2006; Kume, Furuta, Todoriki, Uenoyama, &
264 Kobayashi, 2009; Pauli, 1995), each of the 5 lots of nutmeg was irradiated at 5 or 10
265 kGy. 2-ACBs were measured using GC-MS. Peaks corresponding to 2-DCB and
266 2-dDCB in the chromatogram were identified according to the EN 1785 method.
267 In the selected ion monitoring mode of GC-MS, the *m/z* 98 ion was used to quantify
268 2-ACBs with the *m/z* 112 ion used as a monitor ion. 2-DCB extracted from the
269 irradiated nutmeg samples produced peaks for ions *m/z* 98 and 112 in a ratio of
270 3.63–4.01:1, whereas for 2-dDCB the corresponding ratio was 4.04–4.25:1. Both of
271 these ratios were similar to those obtained for the standard solutions of 3.75–4.04:1 and
272 4.23–4.44:1, respectively.
273 Quantification of 2-DCB and 2-dDCB in each lot irradiated at 5 kGy was carried out
274 after 1 day of storage. Lots C and D showed the greatest content of 2-DCB. In addition,
275 C and D had similar amounts of 2-DCB and 2-dDCB: 2.14 ± 0.17 μg/g FW and 0.33 ±
276 0.01 μg/g FW, respectively, for C; and 2.19 ± 0.12 μg/g FW and 0.33 ± 0.01 μg/g FW,
277 respectively, for D. This may have been because they came from the same area.
278 Furthermore, E showed the largest content of 2-dDCB at 0.55 ± 0.04 μg/g FW. However,
279 A showed the smallest content of 2-DCB or 2-dDCB at 1.52 ± 0.18 μg/g FW and 0.21 ±
280 0.02 μg/g FW, respectively. For this reason, A was chosen for the stability study, which

281 involved storing samples 0, 1, 5, 15, and 30 weeks after irradiation at 5 and 10 kGy.

282 2-ACB formation is dependent on various environmental factors such as atmosphere,
283 water activity, pH, humidity, irradiation temperature, and storage temperature (Hilmy,
284 Chosdu, & Matsuyama, 1995; Ndiaye, Jamet, Miesch, Hasselmann, & Marchioni, 1999;
285 Stevenson, Crone, Hamilton, & McMurray, 1993). In this study, all nutmeg samples
286 were irradiated at room temperature (about 10°C) and stored at 8 ± 2°C, and the
287 humidity was controlled at 20 ± 5%, taking the condition of market circulation into
288 consideration .

289 Fig. 2 shows the GC-MS chromatograms at *m/z* 98 of 5 kGy-irradiated A after 30
290 weeks of storage (a). There were clear peaks in the retention times of 2-DCB (20.72 ±
291 0.01 min) and 2-dDCB (27.69 ± 0.01 min), which were the same as the pure standards
292 (d); however, neither peak was observed in the control sample (c). In addition, (b)
293 shows non-irradiated nutmeg spiked with 0.125 μg/g each of 2-DCB and 2-dDCB in the
294 recovery tests. The signal-to-noise (S/N) ratio of each of these detected ions was greater
295 than 3:1.

296 In the scan mode of GC-MS, the molecular ion *m/z* 210 of 2-DCB was observed in 5
297 kGy-irradiated A after storage for 30 weeks, but the concentration of 2-dDCB (ion *m/z*
298 238) was too low to be observed. However, in both 2-DCB and 2-dDCB, the ion *m/z* 98
299 was the base ion peak, and the relative intensities of ions *m/z* 98 to 112 were compared
300 with 2-DCB and 2-dDCB standards with MS spectra that were practically the same in
301 the detected range of *m/z* 50 to 250 (data not shown).

302 Thus, using GC-MS analysis, we confirmed the qualitative and quantitative presence
303 of 2-DCB and 2-dDCB in 5 kGy-irradiated nutmeg after storage for 30 weeks. For this
304 reason, 2-DCB and 2-dDCB were considered present in the other samples irradiated at

305 the higher dose of 10 kGy and for shorter times.

306 Table 1 provides a summary of the temporal change in both 2-DCB and 2-dDCB in
307 nutmeg samples irradiated at 5 and 10 kGy. Neither 2-DCB nor 2-dDCB remained
308 unchanged ($p > 0.05$) in either 5- or 10 kGy-irradiated samples until 5 weeks of storage.
309 However, there was a sluggish but significant diminution after 5 weeks of storage: both
310 2-DCB and 2-dDCB decreased by about 65% to 75% after 30 weeks of storage. These
311 losses were less than those of 2-ACBs in irradiated tropical fruits such as papayas and
312 mangos (Stewart, Moore, Graham, McRoberts, & Hamilton, 2000). Furthermore, this
313 result is different from a previous study (D. W. M. Sin, Y. C. Wong, & W. Y. Yao, 2006)
314 that showed that more than 85% of 2-ACB content in black melon seed samples
315 decomposed after 120 days of storage. The reason for the slow degradation of 2-ACBs
316 in nutmeg might be the large amount of anti-oxidative compounds such as argenteane,
317 meso-dihydroguaiaretic acid and erythro-austrobailignan-6 (C. A. Calliste, D.
318 Kozlowski, J. L. Duroux, Y. Champavier, A. J. Chulia, & P. Trouillas, 2010), although
319 further study is necessary to elucidate this hypothesis. Further, another reason might be
320 the low temperature (around 8°C) during the storage period.

321 Both 2-DCB and 2-dDCB were absent in the entire control sample as referred to
322 above. However, 2-DCB and 2-dDCB may exist naturally in commercial non-irradiated
323 nutmeg (Variyar, Chatterjee, Sajilata, Singhal, & Sharma, 2008). Therefore, the control
324 samples were analysed using GC-HRMS, which is more sensitive than GC-MS and is
325 able to detect smaller concentrations of 2-ACBs.

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327 *3.3. Detection of 2-ACBs in non-irradiated nutmeg*

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To provide accurate and reliable information on the existence of 2-ACBs in non-irradiated samples, we subjected 5 lots of nutmeg to GC-HRMS. The limit of detection of GC-HRMS was examined using the solvent standard solution (*n*-hexane). The signals to *n*-hexane background noise ratio above 3 were consistently obtained for both *m/z* 98.073 and 112.088 ions for 0.002 μg/mL 2-ACBs (2-DCB and 2-dDCB). We supposed that 2-ACB concentrations of at least 3 to 6 times more could be detected in the matrix sample solution with GC-HRMS. Therefore, 2-DCB and 2-dDCB were spiked into the 5 lots of nutmeg at concentrations of 0.016 and 0.032 μg/g FW nutmegs, and the expected final concentrations were 0.006 and 0.012 μg/mL, respectively. Then, these spiked samples and control samples were subjected to analysis simultaneously.

Fig. 3 shows the *m/z* 98.073 and 112.088 GC-HRMS chromatograms of the control and spiked samples of lot E. The typical chromatogram of 2-DCB in standard solution was illustrated with peaks at 17.24 min (c), and 2-dDCB was illustrated at 23.29 min (f). A peak was observed around the retention time in the sample spiked with 0.016 μg/g of 2-DCB (b), but no significant peaks were found at the control sample retention time of 2-DCB (a). A peak was found in a sample spiked with 0.032 μg/g of 2-dDCB (e). However, there was an explicit peak around the retention time of 2-dDCB (d). Similar trends were observed in other lots (data not shown). Therefore, the relationship of ion intensities between m/z 98.073 and 112.088 was checked for the identification of 2-ACBs according to the EN 1785 method. When the peak area ratio of *m/z* 112.088 to 98.073 was within ±20% of that of 0.01 μg/mL standard solution and the retention times were within ±0.02 min, the peaks would be identified as 2-DCB or 2-dDCB.

Table 2 summarizes the results of 2-DCB peak identification with GC-HRMS. No serious peaks were observed in any of the control samples; however, all samples that

were spiked with 0.016 μg/g 2-DCB gave a positive identification, because the S/N ratio was greater than 3:1, the retention time was ±0.02 min, and the relative ion intensities of *m/z* 98.073 and 112.088 were within 20% of those of the standard solution.

Table 3 shows the results for 2-dDCB. Unlike 2-DCB, a little peak of S/N ratio greater than 3:1 was observed in non-irradiated samples near the standard retention time (±0.02 min). However, all of the relative ion intensities of *m/z* 98.073 and 112.088 were greater than 20%, leading to a negative identification. In contrast, in all of the samples spiked with 0.032 μg/g 2-dDCB, the retention time, S/N ratio, and area conformed to the conditions for identification stated previously. Moreover, those spiked samples were not detected with GC-MS analysis (data not shown).

These results indicated that both 2-DCB and 2-dDCB were less than detectable levels in all of the non-irradiated nutmeg samples in the present study, and 2-DCB and 2-dDCB were detected in samples spiked at 0.016 and 0.032 μg/g in the same analytical conditions. In contrast, Variyar *et.al* reported the natural occurrence of 2-DCB and 2-dDCB at concentrations of 2.67 ± 0.21 and 0.58 ± 0.19 μg/g, respectively, in commercial non-irradiated nutmeg samples (Variyar, Chatterjee, Sajilata, Singhal, & Sharma, 2008). However, the lowest detectable levels found in the present study were quite lower than the natural levels in the previous report, and these results strongly support that 2-DCB and 2-dDCB could be employed as markers for irradiated nutmeg samples.

4. Conclusions

2-ACBs were found only in irradiated nutmeg samples, and their amount increased

proportionally with an increase in the irradiation dose. However, neither 2-DCB nor 2-dDCB was observed in any of the non-irradiated nutmeg samples. Although after up to 30 weeks of storage the concentrations of 2-ACBs had decreased to 65–75% compared with those after 1 day, both 2-DCB and 2-dDCB could be detected in 5 kGy-irradiated samples. Therefore, this 2-ACBs method can be used to check irradiated spices when the TL method is difficult to apply. The positive identification and detection of 2-ACBs in irradiated nutmeg samples after a post-irradiation storage period of 30 weeks confirms that 2-ACB analysis with GC-MS is an accurate and reliable chemical technique for determining irradiation status. Moreover, the proposed protocol saves a large amount of time and solvent compared to the EN 1785 method.

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390

Abbreviations

2-ACB, 2-Alkylcyclobutanone; 2-CHCH, 2-Cyclohexylcyclohexanone; 2-DCB, 2-decylcyclobutanone; 2-dDCB, 2-dodecylcyclobutanone; EI, electron ionization; EN, European Norm; FW, fresh weight; GC, Gas chromatograph; GC-MS, gas chromatography-mass spectrometry; GC-HRMS, high resolution gas chromatography mass spectrometer; IS, internal standard; LOD, limit of detection; SFE, Supercritical Fluid Extraction; SIM, selected-ion monitoring; SPE, solid-phase extraction; S/N, signal to noise; TL, Thermoluminescence; URP, unique radiolytic product

References

Ananthakumar, A., Variyar, P. S., & Sharma, A. (2006). Estimation of aroma glycosides of nutmeg and their changes during radiation processing. *Journal of Chromatography A, 1108*(2), 252-257.

Blanch, G. P., Caja, M. D., Flores, G., & del Castillo, M. L. R. (2009). Identification of 2-dodecylcyclobutanone and linear-alkanes as irradiation markers in sliced dry-cured ham. *Food Chemistry, 113*(2), 616-620.

Boyd, D. R., Crone, A. V. J., Hamilton, J. T. G., Hand, M. V., Stevenson, M. H., & Stevenson, P. J. (1991). Synthesis, characterization, and potential use of 2-dodecylcyclobutanone as a marker for irradiated chicken. *Journal of Agricultural and Food Chemistry, 39*(4), 789-792.

Caja, M. M., del Castillo, M. L. R., & Blanch, G. P. (2008). Solid phase microextraction as a methodology in the detection of irradiation markers in ground beef. *Food Chemistry, 110*(2), 531-537.

Calliste, C. A., Kozlowski, D., Duroux, J. L., Champavier, Y., Chulia, A. J., & Trouillas, P. (2010). A new antioxidant form wild nutmeg. *Food Chemistry, 118*, 489-496.

Chen, S. S., Morita, Y., Saito, K., Kameya, H., Nakajima, M., & Todoriki, S. (2011). Identification of Irradiated prawn (Penaeus monodon) using thermoluminescence and 2-alkylcyclobutanone analyses. *Journal of Agricultural and Food Chemistry, 59*(1), 78-84.

Crone, A. V. J., Hand, M. V., Hamilton, J. T. G., Sharma, N. D., Boyd, D. R., & Stevenson, M. H. (1993). Synthesis, characterization, and use of 2-tetradecylcyclobutanone together with other cyclobutanones as markers for

422 irradiated liquid whole egg. *Journal of the Science of Food and Agriculture, 62*(4), 361-367.
424 Gould, G. W. (1996). Methods for preservation and extension of shelf life. International Journal of Food Microbiology, 33, 51-64.
426 Hijaz, F., Kumar, A., & Smith, J. S. (2010). A rapid direct solvent extraction method for the extraction of 2-dodecylcyclobutanone from irradiated ground beef patties using acetonitrile. *Journal of Food Science, 75*(6), T118-T122.
429 Hilmy, N., Chosdu, R., & Matsuyama, A. (1995). The effect of humidity after gamma-irradiation on aflatoxin b-1 production of aspergillus-flavus in ground nutmeg and peanut. *Radiation Physics and Chemistry, 46*(4-6), 705-711.
432 Horvatovich, P., Miesch, M., Hasselmann, C., & Marchioni, E. (2000). Supercritical fluid extraction of hydrocarbons and 2-alkylcyclobutanones for the detection of irradiated foodstuffs. *Journal of Chromatography A, 897*(1-2), 259-268.
435 Horvatovich, P., Miesch, M., Hasselmann, C., & Marchioni, E. (2002). Supercritical fluid extraction for the detection of 2-dodecylcyclobutanone in low dose irradiated plant foods. *Journal of Chromatography A, 968*(1-2), 251-255.
438 Kume, T., Furuta, M., Todoriki, S., Uenoyama, N., & Kobayashi, Y. (2009). Status of food irradiation in the world. *Radiation Physics and Chemistry, 78*(3), 222-226.
440 Leistritz, W. (1997). Methods of bacterial reduction in spices. *Spices, 660,* 7-10.
441 Lembke, P., Bornert, J., & Engelhardt, H. (1995). Characterization of irradiated food by SFE and GC-MSD. *Journal of Agricultural and Food Chemistry, 43*(1), 38-45.
443 Ndiaye, B., Jamet, G., Miesch, M., Hasselmann, C., & Marchioni, E. (1999). 2-Alkylcyclobutanones as markers for irradiated foodstuffs II. The CEN

445 (European Committee for Standardization) method: field of application and limit
446 of utilization. *Radiation Physics and Chemistry, 55*(4), 437-445.
447 Niyas, Z., Variyar, P. S., Gholap, A. S., & Sharma, A. (2003). Effect of
448 gamma-irradiation on the lipid profile of nutmeg (Myristica fragrans Houtt.).
449 *Journal of Agricultural and Food Chemistry, 51*(22), 6502-6504.
450 Obana, H., Furuta, M., & Tanaka, Y. (2005). Analysis of 2-alkylcyclobutanones with
451 accelerated solvent extraction to detect irradiated meat and fish. *Journal of*
452 *Agricultural and Food Chemistry, 53*(17), 6603-6608.
453 Oduko, J. M., & Spyrou, N. M. (1990). Thermoluminescence of irradiated foodstuffs.
454 *International Journal of Radiation Applications and Instrumentation. Part C.*
455 *Radiation Physics and Chemistry, 36*(5), 603-607.
456 Pauli, G. H. (1995). Evaluating the safety of irradiated foods. *Radiation and Public*
457 *Perception, 243*, 89-101.
458 Rahman, R., Matabudall, D., Haque, A. K., & Sumar, S. (1996). A rapid method
459 (SFE-TLC) for the identification of irradiated chicken. *Food Research*
460 *International, 29*(3-4), 301-307.
461 Schreiber, G. A., Helle, N., Schulzki, G., Spiegelberg, A., Linke, B., Wagner, U., & Bogl,
462 K. W. (1993). Intercomparisons to evaluate the suitability of
463 gas-chromatographic, electron-spin-resonance spectrometric and
464 thermoluminescence methods to detect irradiated foods in routine control.
465 *Radiation Physics and Chemistry, 42*(1-3), 391-396.
466 Sin, D. W., Wong, Y. C., & Yao, M. Y. Y. (2006). Application of pentafluorophenyl
467 hydrazine on detecting 2-dodecylcyclobutanone and 2-tetradecylcyclobutanone

in irradiated chicken, pork and mangoes by gas chromatography-mass spectrometry. *European Food Research and Technology, 222*(5-6), 674-680.

Sin, D. W. M., Wong, Y. C., & Yao, W. Y. (2006). Analysis of gamma-irradiated melon, pumpkin, and sunflower seeds by electron paramagnetic resonance spectroscopy and gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry, 54*(19), 7159-7166.

Spricigo, C. B., Pinto, L. T., Bolzan, A., & Novais, A. F. (1999). Extraction of essential oil and lipids from nutmeg by liquid carbon dioxide. *Journal of Supercritical Fluids, 15*(3), 253-259.

Stevenson, M. H., Crone, A. V. J., & Hamilton, J. T. G. (1990). Irradiation detection. *Nature, 344*(6263), 202-203.

Stevenson, M. H., Crone, A. V. J., Hamilton, J. T. G., & McMurray, C. H. (1993). The use of 2-dodecylcyobutanone for the identification of irradiated chicken meat and eggs. *Radiation Physics and Chemistry, 42*(1-3), 363-366.

Stewart, E. M., McRoberts, W. C., Hamilton, J. T. G., & Graham, W. D. (2001). Isolation of lipid and 2-alkylcyclobutanones from irradiated foods by supercritical fluid extraction. *Journal of Aoac International, 84*(3), 976-986.

Stewart, E. M., Moore, S., Graham, W. D., McRoberts, W. C., & Hamilton, J. T. G. (2000). 2-Alkylcyclobutanones as markers for the detection of irradiated mango, papaya, Camembert cheese and salmon meat. *Journal of the Science of Food and Agriculture, 80*(1), 121-130.

Tewfik, I. (2008). A rapid direct solvent extraction method for the extraction of cyclobutanones from irradiated chicken and liquid whole egg. *International*

491 *Journal of Food Science and Technology, 43*(1), 108-113.
492 Variyar, P. S., Chatterjee, S., Sajilata, M. G., Singhal, R. S., & Sharma, A. (2008).
493 Natural existence of 2-alkylcyclobutanones. *Journal of Agricultural and Food*
494 *Chemistry, 56*(24), 11817-11823.
495 Zanardi, E., Battaglia, A., Ghidini, S., Conter, M., Badiani, A., & Ianieri, A. (2007).
496 Evaluation of 2-alkylcyclobutanones in irradiated cured pork products during
497 vacuum-packed storage. *Journal of Agricultural and Food Chemistry, 55*(10),
498 4264-4270.
499

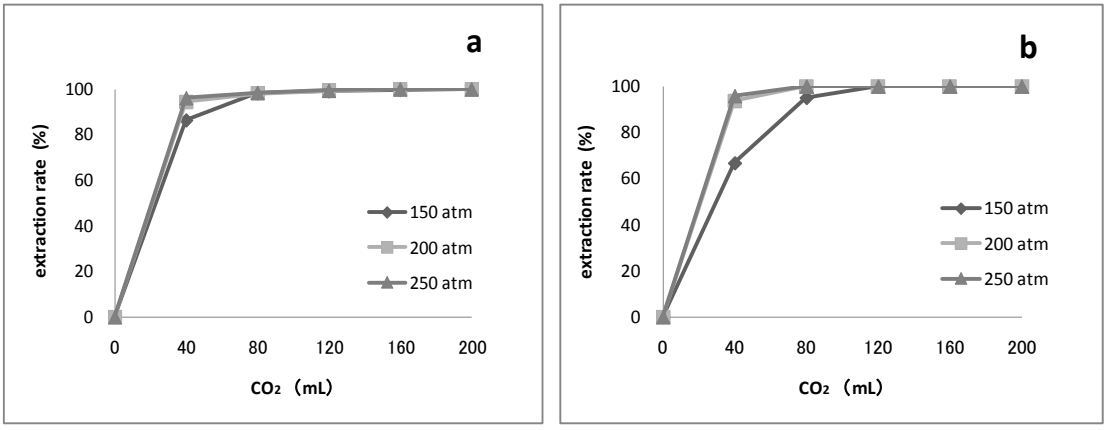
Figure captions

Fig. 1. The extraction rate of 2-ACBs under different extraction pressures at 80°C. (a), 2-DCB; (b), 2-dDCB.

Fig. 2. GC-MS chromatograms of non-irradiated and 5 kGy-irradiated lot A nutmeg at *m/z* 98. (a), 5 kGy-irradiated A after 30 weeks of storage; (b), non-irradiated nutmeg spiked with 0.125 μg/g each of 2-DCB and 2-dDCB; (c), non-irradiated A sample (control); (d), 0.08 μg/mL standard solutions for 2-DCB and 2-dDCB. Peak labels: 1, IS; 2, 2-DCB; 3, 2-dDCB.

Fig. 3. GC-HRMS chromatograms of non-irradiated nutmeg confirming the absence of 2-DCB and 2-dDCB at *m/z* 98.073 and 112.088. (a), non-irradiated nutmeg sample (control); (b), non-irradiated nutmeg spiked with 0.016 μg/g 2-DCB; (c), 0.006-μg/mL standard solution of 2-DCB; (d), non-irradiated nutmeg sample (control); (e), non-irradiated nutmeg spiked with 0.032 μg/g 2-dDCB; (f), 0.012-μg/mL standard solution of 2-dDCB. Chromatograms: *m/z* 98.073, —; *m/z* 112.088, ---.

514



515 Fig. 1

516

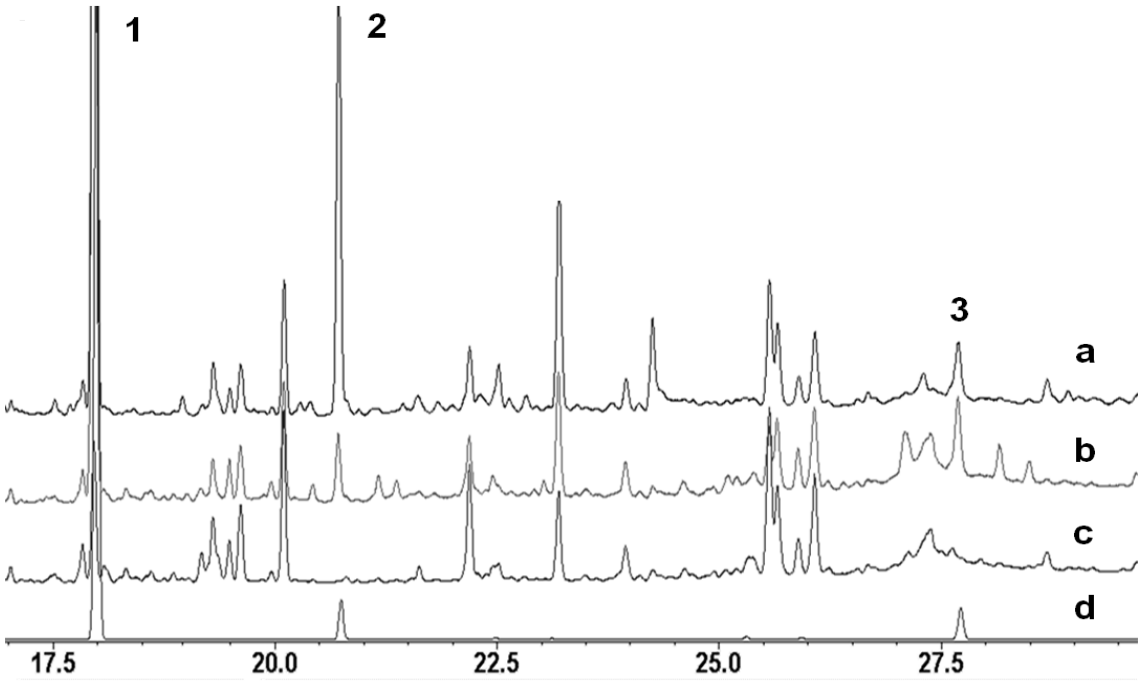


Fig. 2.

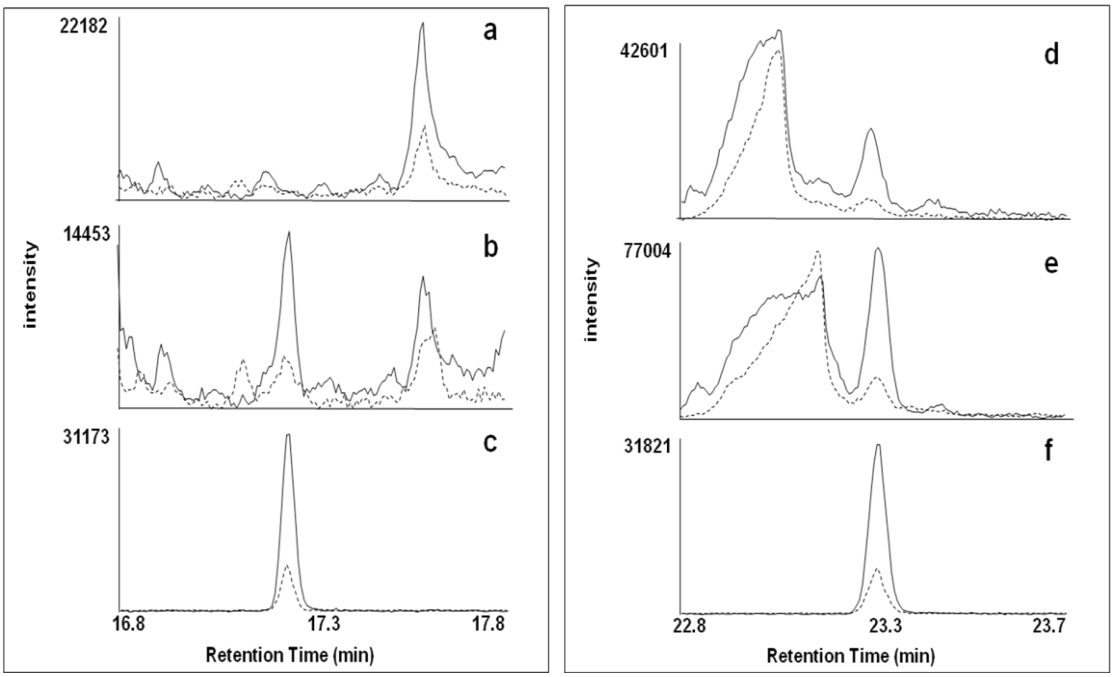

22182
a
14453
b
31173
c
intensity
16.8
17.3
17.8
Retention Time (min)
42601
d
77004
e
31821
f
intensity
22.8
23.3
23.7
Retention Time (min)

Fig. 3.

524 **Table 1.** Concentrations of radiation-induced 2-DCB and 2-dDCB during room

525 temperature storage (μg/g FW sample)

2-ACBs	Storage (Weeks)	Irradiation dose (kGy)[1]		
		0	5	10
2-DCB	0	ND[2]	1.52 ± 0.18[a]	2.52 ± 0.25[a]
	1	ND	1.30 ± 0.16[a]	2.57 ± 0.16[a]
	5	ND	1.51 ± 0.30[a]	2.05 ± 0.19[b]
	15	ND	1.16 ± 0.21[b]	1.96 ± 0.12[b]
	30	ND	0.99 ± 0.20[b]	1.64 ± 0.13[c]
2-dDCB	0	ND	0.21 ± 0.02[a]	0.41 ± 0.04[a]
	1	ND	0.19 ± 0.01[a]	0.42 ± 0.02[a]
	5	ND	0.20 ± 0.03[a]	0.31 ± 0.02[b]
	15	ND	0.18 ± 0.00[b]	0.29 ± 0.05[b]
	30	ND	0.17 ± 0.01[b]	0.27 ± 0.03[b]

526 For each compound, the concentrations of 2-DCB and 2-dDCB were compared using

527 Welch's *t*-test ($p > 0.05$). [a-c] Means in the same column of the same compound with the

528 same superscript are not significantly different ($p > 0.05$). [1] Mean of 4 replications

529 (standard deviation). [2] Not detected.

Table 2. Confirmation of the detection peaks of 2-DCB from non-irradiated nutmeg and samples spiked with 0.016 μg/g 2-DCB by GC-HRMS

	lot	RT[1] (min)	difference with std(min)	98.073 area	98.073 S/N	112.088 area	112.088 S/N	AR[2] (%)	difference with std (%)[3]	determination
STD[4]		17.24	-	1246	184	241	91	19	-7	**detected**
	A	-	-	-	-	-	-	-	-	ND*
	B	-	-	-	-	-	-	-	-	ND
0 kGy	C	-	-	-	-	-	-	-	-	ND
	D	-	-	-	-	-	-	-	-	ND
	E	-	-	-	-	-	-	-	-	ND
	A	17.22	-0.02	763	9	138	4	18	-12	**detected**
	B	17.23	-0.01	1055	11	184	5	17	-16	**detected**
spiked	C	17.24	0.00	995	14	175	5	18	-15	**detected**
	D	17.23	-0.01	761	8	126	3	17	-20	**detected**
	E	17.24	0.00	335	7	63	3	19	-9	**detected**

1, RT, retention time (min); 2, AR, area ratio of 112.088/98.073 (%); 3, difference with std (%), the difference with the area ratio of 0.10-μg/mL standard solution (21%); 4, STD, standard of 0.006 μg/mL; * ND, not detected.

535 **Table 3**. Confirmation of the peaks of 2-dDCB from non-irradiated nutmeg and samples

536 spiked with 0.032 μg/g 2-dDCB by GC-HRMS

	lot	RT[1]	difference	98.073		112.088		AR[2]	difference	determination
		(min)	with std(min)	area	S/N	area	S/N	(%)	with std (%)[3]	
STD[4]		23.29	-	1569	151	332	126	21	-4	**detected**
	A	23.28	-0.01	549	14	168	6	31	39	ND*
	B	23.27	-0.02	1248	7	-	-	-	-	ND
0 kGy	C	23.27	-0.02	2068	8	605	4	29	33	ND
	D	23.27	-0.02	706	5	359	4	51	131	ND
	E	23.28	-0.01	857	9	301	6	35	60	ND
	A	23.29	0.00	3729	27	933	12	25	14	**detected**
	B	23.31	0.02	3220	18	838	8	26	18	**detected**
spiked	C	23.28	-0.01	5025	12	1156	8	23	5	**detected**
	D	23.29	0.00	4242	26	1058	9	25	13	**detected**
	E	23.29	0.00	3981	25	844	6	21	-4	**detected**

537 1, RT, retention time (min); 2, AR, area ratio of 112.088/98.073 (%); 3, difference with

538 std (%), the difference with the area ratio of 0.10-μg/mL standard solution (22%); 4,

539 STD, standard of 0.012 μg/mL; * ND, not detected.

540