ABSTRACT: Anthraquinone (AQ) levels in some Indonesian dried tea leaves samples from different plantation areas and their brewed tea samples were determined by gas chromatography–tandem mass spectrometry methods. The mean lower bound, middle bound, and upper bound of AQ levels in 59 dried tea leaves samples were 82.2, 82.8, and 83.4 μg/kg, respectively, while their 95th percentile values were identical at 190.3 μg/kg (0.1903 mg/kg). In a transfer rate study, the mean and 95th AQ levels in 30 dried tea leaves samples with AQ level ≥ LOQ (limit of quantification) were 128.6 and 194.5 μg/kg (0.1945 mg/kg), while those of their corresponding brewed tea samples were 2.1 and 3.4 μg/kg, respectively. The mean and 95th transfer rates of AQ into brewed tea samples were 51.99 and 88.17%. Using these data and taking into account daily tea consumption, calculated cancer potency slope factor, benchmark dose of 10% effect at lower bound 95% confidence interval of AQ, and average body weight, the risk characterization due to exposure to this compound from tea consumption was calculated and stated as incremental lifetime cancer risk (ILCR) and margin of exposure (MOE). The overall results revealed that AQ levels in dried tea leaves up to the highest level found in the samples lead to an ILCR of not more than 10^{-6} and an MOE of not less than 10^4 and hence was predicted to give sufficient consumer protection.

INTRODUCTION

Anthraquinone (AQ), a polycyclic aromatic hydrocarbon (PAH) compound, is found ubiquitously in the environment as a product of direct combustion process or the degradation of PAHs by atmospheric oxidants, residue of bird repellent, or waste of pulp and dye industries. However, a study conducted by Wei et al. stated that AQ may cause oxidative DNA damage, and therefore it is potentially carcinogenic, which was later strengthened by the International Agency for Research on Cancer (IARC), which classifies AQ as possibly carcinogenic to humans (2B). In line with the IARC, Commission Regulation (EU) 2017/776 classifies this compound as carcinogenic 1B with the hazard statement Code(s) of H350 (may cause cancer). With the entry into force of Commission Regulation (EU) No. 1146/2014 on 18 May 2015, the Annex II, III, IV, and V to Regulation (EC) No. 396/2005 has been amended. Consequently, the maximum residue limit (MRL) of AQ in tea has been added and set in Annex V to be 0.02 mg/kg. As the considerations, it was stated that the use of AQ is no longer authorized within the Union and also the third countries, so that it is appropriate to set the MRL at the specific limit of determination or at the default MRL in accordance with Article 18(1) (b) of Regulation (EC) No. 396/2005. Before the entry into force of the Commission Regulation No. 1146/2014, in the reasoned opinion on the review of the existing MRLs for AQ according to Article 12 of Regulation (EC) No. 396/2005, the European Food Safety Authority (EFSA) stated that residues of AQ are not expected to occur in any plant commodity or in any animal product because the pesticide containing AQ is no longer authorized within the EU, no Codex maximum residue limits (CXLs) are available for this active substance and no uses authorized in third countries were notified to the rapporteur member state. A risk assessment is therefore in principle not required. In Indonesia, AQ is also not listed as permitted pesticide for agriculture and forestry. From these points of views, the residues of AQ as pesticide residue are not expected to occur in any plant commodity.
RESULT AND DISCUSSION

In accordance with the risk analysis guidance, the presence of anthraquinone (AQ) in tea should be assessed as a non-threshold substance due to its carcinogenic properties. In this case, hazard characterization of AQ could be expressed as potential human carcinogenic risks in terms of an increased probability of developing cancer during a person’s lifetime. For example, $10^{-6}$ increased cancer risk represents an increased lifetime risk of 1 in 1,000,000 for developing cancer.

Due to carcinogenicity of a substance, the probability of an individual developing cancer over a lifetime is estimated by multiplying the cancer slope factor (mg/kg/day)$^{-1}$ for the substance by the chronic (70 year average) daily intake (mg/kg/day). More precisely, the United States Environmental Protection Agency (US EPA) uses the concept of chemical-specific excess lifetime cancer risk,$^{12,13}$ which is calculated using the following equations:

$$\text{Risk} = \text{CDI} \times \text{CSF}$$  

(1)

where Risk, is the unitless probability of an individual developing cancer as the result of exposure to chemical $i$, CDI, is the chronic daily intake of chemical $i$ averaged over 70 years (mg/kg/day), and CSF, is the US EPA cancer slope factor (CSF) for chemical $i$ (mg/kg/day)$^{-1}$.

The US EPA recommends tolerable cumulative risks ranging from $10^{-4}$ [1 in 10,000] to $10^{-6}$ [1 in 1 million] incremental individual lifetime cancer risk.$^{14}$

Similarly, Health Canada uses incremental lifetime cancer risk (ILCR) to calculate the cancer risk as follows:$^{15}$

$$\text{ILCR} = \text{lifetime average daily dose (µg/kg/BW/day)} \times \text{cancer slope factor (µg/kg/BW/day)}^{-1}$$  

(2)

Accordingly, risk characterization of carcinogens is based on 100% of the acceptable risk value of $1 \times 10^{-5}$ since the ILCR is independent of background sources. The total cancer risk will be deemed to be “essentially negligible” where the total ILCR estimated is $\leq 1$ in 100,000 ($1 \times 10^{-5}$).$^{14}$

To date, no cancer slope factor (CSF) data is available for AQ. However, based on the data of liver tumor development in male mice as presented in the NTP carcinogenicity study, the New York State Department of Health (NYSDOH) calculated a cancer potency slope factor of 0.043 (mg/kg/day)$^{-1}$ for AQ.$^{16}$ In addition, by applying benchmark dose software, benchmark dose (BMD) and potency of AQ have been calculated using a linear risk model, which gave potency factor and BMD of 0.04 (mg/kg/day)$^{-1}$ and 2.63 mg/kg/day, respectively.$^{17}$ Furthermore, by applying carcinogenic studies data provided by the NTP, the US EPA has calculated that the lowest benchmark dose corresponds to 10% effect at lower bound 95% confidence interval (BMDL10) of AQ, which is published in the Provisional Peer-Reviewed Toxicity Values (PPRTV) for 9,10-anthraquinone.$^{18}$ Accordingly, the BMDL10 for AQ is 2.61 mg/kg/BW/day, which is based on the increase in hepatocellular adenoma, carcinoma, or hepatoblastoma in male mice. This BMDL10 value represents a point of departure (POD) for margin of exposure (MOE) calculation. MOE is the ratio between a defined point on the dose—response curve for the adverse effect and the human intake.$^{19}$ MOE approach has been used to assess potentially genotoxic and carcinogenic compounds.$^{19-22}$ Using BMDL10 as the POD, MOE can be calculated as follows:

$$\text{MOE} = \frac{\text{BMDL10}}{\text{BMDL10 for AQ}} = \frac{2.61 \text{ mg/kgBW/day}}{2.63 \text{ mg/kgBW/day}} = 0.99$$

From this point of view, AQ could also be treated in an analogue way with those of process contaminants, i.e., the establishment of a maximum level (ML) based on the risk analysis approach.

Table 1. Results of Anthraquinone Determinations in Different Samples

| no. | samples         | sample code     | anthraquinone (mg/kg) | testing laboratory |
|-----|----------------|-----------------|-----------------------|-------------------|
| 1   | soil (from tea plantation area) | 00619-Pn 1246 | <0.01$\text{a}$ | Ministry of Trade, Republic of Indonesia |
| 2   | fresh Leaf      | 00619-Pn 1247 | <0.01$\text{a}$ | |
| 3   | silver oak (protecting tree) | 00619-Pn 1248 | <0.01$\text{a}$ | |
| 4   | withered Leaf (protecting tree) | 00619-Pn 1249 | <0.01$\text{a}$ | |
| 5   | green tea (Pan- ning) | 0555-Pn 1037 | <0.01$\text{a}$ | |
| 6   | green tea (Java Preanger) | 388-2014-00090865 | <0.01$\text{a}$ | Dr. Specht Laboratorium (Eurofins) |
| 7   | white tea (Java Preanger) | 388-2014-00090863 | <0.01$\text{a}$ | |

$\text{a}$Reporting limit (RL) = LOQ = 0.01 mg/kg.
MOE = POD/intake \hspace{1cm} (3)

In addition, BMDL10 can be used to derive the provisional oral slope factor (p-OSF) according to the following equation:\textsuperscript{23}

\[ p\text{-OSF} = 0.1 / \text{BMDL10} = 0.1 / 2.61 \text{ mg/kg/day} = 0.0385 \text{ (mg/kg/day)}^{-1} \] \hspace{1cm} (4)

p-OSF seems similar with the calculated cancer potency slope factor or potency factor as described before. Since the three calculations gave nearly identical results, these values will also give a similar unit risk of AQ. For our purposes, a calculated cancer potency slope factor of 0.043 (mg/kg/day)\textsuperscript{-1} as the most sensitive one will be selected for unit risk calculation.

Chronic daily intake or ingestion data of AQ due to daily tea consumption is also needed for risk calculation. Hence, daily tea consumption was estimated from annual tea consumption per capita in several countries with the highest tea consumption. In 2015, four European countries with the highest tea consumption per capita included: Ireland with 2.20 kg, UK 1.90 kg, Poland 1.00 kg, and The Netherlands 0.80 kg, while in 2016, the tea consumption per capita in those countries were respectively 4.83, 4.28, 2.20, and 1.72 lb or equivalent to 2.19, 1.94, 1.00, and 0.78 kg.\textsuperscript{23,24} These annual data were then converted to daily consumption and showed that in 2015 the daily tea consumption in Ireland, UK, Poland, and The Netherlands were 0.0060, 0.0052, 0.0027, and 0.0022 kg, while in 2016 were 0.0060, 0.0053, 0.0027, and 0.0021 kg, respectively. Later, the highest consumption was chosen for risk characterization calculation. If it is assumed that tea contains AQ at the EU MRL level (0.02 mg/kg), then the total daily consumption in Ireland, UK, Poland, and The Netherlands, respectively, would be 0.006 kg, 0.0053 kg, 0.0027 kg, and 0.0021 kg. In 2016, the tea consumption per capita in those countries were respectively 4.83, 4.28, 2.20, and 1.72 lb or equivalent to 2.19, 1.94, 1.00, and 0.78 kg, respectively. The highest tea consumption per capita included: Ireland with 2.20 kg, UK 1.90 kg, Poland 1.00 kg, and The Netherlands 0.80 kg, while in 2016, the tea consumption per capita in those countries were respectively 4.83, 4.28, 2.20, and 1.72 lb or equivalent to 2.19, 1.94, 1.00, and 0.78 kg, respectively. Later, the highest consumption was chosen for risk characterization calculation. If it is assumed that tea contains AQ at the EU MRL level (0.02 mg/kg), then the total daily consumption in Ireland, UK, Poland, and The Netherlands, respectively, would be 0.006 kg, 0.0053 kg, 0.0027 kg, and 0.0021 kg.

To support the exposure assessment of AQ from tea in Indonesia, we have validated a GC-MS/MS analytical method for quantification of AQ in dried tea leaves and tea infusions/brewed tea samples. The validation results confirm that all EU performance criteria are fulfilled, and hence the method can be applied for the determination of AQ in both dry tea samples and their infusions. Table 2 shows the summary results of AQ determinations in 59 dried tea leaves samples from different tea plantation in Java and Sumatra.

The analytical method applied for the determination of tea samples has an LOQ of 10 μg/kg. Among 59 tea samples, there were 7 samples (11.9%) with AQ level < LOQ (left censored data), which were then treated applying the substitution method either as lower bound (LB), middle bound (MB), or upper bound (UB). Accordingly, the left censored data considered as 0, LOQ/2, or LOQ, respectively. From Table 2, it is clear that the AQ level, neither at average (82.2–83.4 μg/kg) nor at the 95th percentile (190.3 μg/kg), showed significant difference when the left censored data were calculated as LB, MB, or UB.

### Table 2. Summary Results of Anthraquinone Determinations in Dried Tea Leaves Samples\textsuperscript{a}

| concentration (μg/kg) | LB | MB | UB |
|----------------------|----|----|----|
| sample count         | 59 | 59 | 59 |
| min.                 | 0.0| 0.0| 10.0|
| max.                 | 199.0| 199.0| 199.0|
| mean                 | 82.2| 82.8| 83.4|
| SD                   | 61.5| 60.7| 60.0|
| 95th percentile      | 190.3| 190.3| 190.3|

\textsuperscript{a}Limit of quantification (LOQ) = 10 μg/kg. LB = lower bound, non-quantified samples = 0 μg/kg. MB = middle bound, non-quantified samples = LOQ/2 = 5 μg/kg. UB = upper bound, non-quantified samples = LOQ = 10 μg/kg. SD = standard of deviation.

In addition, 30 dried tea leaves samples with AQ level ≥ LOQ were further evaluated for transfer rate experiment. The summary results of the experiment are displayed in Table 3.

### Table 3. Summary Results of Anthraquinone Determinations and Transfer Rate Calculations

| concentration | dried tea leaves (μg/kg)
|---------------|--------------------------|
|               | brewed tea samples (μg/L)
|               | transfer rate % |
| sample count  | 60                        | 60                      | 30          |
| min.          | 62                        | 0.5                     | 14.32       |
| max.          | 202                       | 3.5                     | 91.62       |
| mean          | 128.6                     | 2.1                     | 51.99       |
| standard dev. | 36                        | 0.7                     | 20.03       |
| 95th percentile | 194.5                   | 3.4                     | 88.17       |

\textsuperscript{a}Samples weight: 5 g. 30 samples, each sample was doubly determined. \textsuperscript{b}Infusion volume: 150 mL, 30 samples, each sample was doubly determined. \textsuperscript{c}Taking into account the total amount of anthraquinone transferred into tea infusion, the average result of double determination was used for transfer rate calculation of each sample.

The concentration of AQ in both dried tea leaf samples and their infusions vary significantly. The mean concentrations of AQ in dried tea leaves samples and their infusions are respectively 128.6 μg/kg and 2.11 μg/L, while their 95th percentile levels are 194.50 μg/kg and 3.37 μg/L. The transfer rates of AQ also vary in the range of 14.32–91.62% with the mean of 52.18% and the 95th percentile of 88.17%.

The results of these laboratory tests and tea consumption data can be applied for calculation/estimation of exposure to AQ. For this purpose, the average daily tea consumption of Ireland in 2015 and 2016, which reflects the highest consumption (0.006 kg/person), and the AQ level at the 95th percentile (194.50 μg/kg = 0.1945 mg/kg) are chosen. The daily AQ intake will be 0.006 kg/person × 0.1945 mg/kg = 0.001167 mg/kg/person/day. Assuming the body weight of 70 kg, the intake will be equivalent to 1.71 × 10⁻⁶ mg/kgBW/day. By using a CSF of 0.043 (mg/kg/day)⁻¹, the risk or ILCR will be (1.71 × 10⁻⁶ mg/kgBW/day) × 0.043 (mg/kg/day)⁻¹ = 7.37 × 10⁻⁸, which is much lower than tolerable cumulative risks set by the US EPA and Health Canada.

If the level of AQ is corrected by the transfer rate value (95th percentile), then the daily AQ intake will be 0.006 kg/person × 0.1945 mg/kg × 0.8817 = 0.001029 mg/person/day. Assuming the body weight of 70 kg, the intake will be...
exposure to this compound from other sources at the following if the AQ level increases to be more than 0.2713 mg/kg for also shows that tolerable risk unit/ILCR will be calculation results are summarized in Table 4. However, it is still in a tolerable range of 10−6 US EPA unit risk will be exceeded. First, if the daily consumption increases up to 0.010 kg/day, the 10−6 US EPA unit risk will be exceeded. However, it is still in a tolerable range of 10−6 to 10−5. Table 4 also shows that tolerable risk unit/ILCR will be first exceeded if the AQ level increases to be more than 0.2713 mg/kg for 100% transfer rate scenario and more than 0.3077 mg/kg for 88.17% transfer rate scenario (95th percentile of our results). It also means that exposure to AQ at our 95th percentile level due to tea consumption still provide possible tolerable exposure to this compound from other sources at the following daily exposure levels:

1. 100% transfer rate scenario:

\[
\frac{[(0.2713 - 0.1945) \text{mg/kg} \times 0.006 \text{kg}]/70 \text{kg}}{0.000000658 \text{mg/kgBW/day or 0.00658}} = 0.000000658 \text{mg/kgBW/day or 0.00658}
\]

\[
\mu g/kgBW/day
\]

2. 88.17% transfer rate scenario (95th percentile of our results):

\[
\frac{[(0.3077 - 0.1945) \text{mg/kg} \times 0.006 \text{kg}]/70 \text{kg}}{0.000000970 \text{mg/kgBW/day or 0.00970}} = 0.000000970 \text{mg/kgBW/day or 0.00970}
\]

\[
\mu g/kgBW/day
\]

In addition to unit risk/ILCR, risk of AQ can be characterized as MOE, which can be calculated using eq 3. MOEs for various intake scenarios are summarized in Table 5. Table 5 shows that, at any level of AQ in tea with both 88.17 or 100% transfer rate, the MOEs remain higher than 10,000. In the scientific opinion of the EFSA, an MOE of 10,000, if based on the BMDL10 from an animal study, would be of low concern from a public health point of view and might be considered as a low priority for risk management actions.19 MOE calculations results reveal that the risk of AQ due to daily intake from tea up 0.006 kg/day at AQ level of 0.2 mg/kg/tea, which is practically identical with the highest AQ level in the tea samples, could still be considered as low or tolerable. Up to now, no dietary exposure assessment of AQ has been conducted. Cassia gum, a food additive used as a gelling agent and thickener, can be considered as the main source of dietary intake of total AQs. Based on the Joint FAO/WHO Expert Committee on Food Additives (JEFCFA) specification for cassia gum (INS 427), the ML of total AQs in this food additive is 0.5 mg/kg.25 The EFSA referred to a petitioner’s proposed use levels of cassia gum as a food additive, and conservative assumption of daily exposure to cassia gum was accordingly
estimated to be 2.1 mg/kgBW/day at the mean and 4.9 mg/kgBW/day at the 90th percentile.26 In the safety evaluation of certain food additives, the JECFA concluded that the estimated 90th-percentile dietary exposure to cassia gum from the proposed uses would be less than 6 mg/kgBW/day.27 Meanwhile, it was reported that all naturally occurring AQs are derivatives of AQ only with a different chemical structure compared to AQ28 and hence cannot be considered as additional/dietary source of AQ.

The calculation results in Table 4 and Table 5 reveal that the unit risk/ILCR and MOE due to exposure to AQ are less than 10−6 (unit risk/ILCR) and higher than 10,000 (MOE), respectively. The overall results show that the AQ level up to 0.2 mg/kg tea still gives sufficient protection for consumers.

CONCLUSIONS

By applying our validated method, the AQ determination in 59 dried tea leaves samples gave mean LB, MB, and UB values of 82.2, 82.8, and 83.4 μg/kg, respectively, with the highest level of 199.0 μg/kg, while their 95th percentile values were identical, i.e., 190.3 μg/kg (0.1903 mg/kg). Furthermore, in a transfer rate study, the mean and 95th% AQ levels in 30 dried tea leaves samples with AQ level ≥ LOQ were 128.6 and 194.5 μg/kg (0.1945 mg/kg), while those of their corresponding brewed tea samples were 2.1 and 3.4 μg/kg, respectively. The mean and 95th% transfer rates of AQ into brewed tea samples were 51.99 and 88.17%.

Taking into account the calculated cancer potency slope factor and benchmark dose lower bound 95% confidence interval of 10% extra risk (BMDL10) of AQ as its hazard characteristics and dietary exposure to this compound and daily tea consumption, risk characteristic of this compound due to tea consumption can be quantitatively estimated. Applying the calculated cancer potency slope factor of 0.043 (mg/kgBW/day)−1 and BMDL10 of 2.61 mg/kgBW/day as the POD and a high tea daily intake of 0.006 kg/day (equivalent to 1.71 × 10−5 mg AQ/kgBW/day), the ILCR value due exposure to AQ was calculated to be less than 10−6 and its MOE value was greater than 104. Since the analysis results of some supporting samples, as indicated in Table 1, showed that AQ levels in these samples were no more than the reporting limit (RL) and no reported source of natural AQ, AQ in dried/processed tea can be considered as the main source of AQ from tea products. Based on all facts, AQ levels in dried/processed tea leaves up to the highest level found in the samples would still give sufficient and reasonable consumer protection in accordance with risk analysis principles.

MATERIALS AND METHODS

Materials. Black tea samples from two different processes, namely, CTC (Crush, Tear, and Curl) and Orthodox, in various grades were obtained from four big tea companies in Indonesia. The samples came from 26 different fields in Sumatra and Java islands, which are the main production areas in Indonesia.

Analytical grade standard of AQ was ordered from Supelco (Belleville, PA, USA). Anhydrous MgSO₄, anhydrous CH₃COONa, anhydrous NaCl, PSA, and Bond Elut Carbon/NH₄ cartridge were purchased from Agilent (Santa Clara, USA). Ethyl acetate, cyclohexane, formic acid, and acetic acid were supplied by Merck (Darmstadt, Germany).

Sample Homogenization. About 500 g of black tea sample was finely grounded and homogenized by using a grinder homogenizer to reduce sample preparation variability. Sample was stored in a tightly sealed container until the analysis is performed.

Sample Preparation of Dried Black Tea. About 5 g of homogenized sample was weighed and put into a 50 mL extraction tube. Then, the sample was spiked with AQ standard. An addition standard used for the calibration curve with the concentrations of 10, 20, 50, 100, and 150 μg/kg was prepared by spiking the sample with the AQ standard. This calibration curve was used for quantification of AQ in Indonesian dried tea leave samples. The samples were left to stand for 30 min to distribute the AQ standard evenly and given time to interact with the matrix before extraction. A 15 mL solution of cold water was added and homogenized using a vortex for 1 min. Samples were left for 30 min for soaking. For the extraction procedure, 15 mL of ethyl acetate (containing 1% of acetic acid) was added and vortexed for 3 min followed by extraction using an end-over-end shaker for 30 min. Thereafter, 4 g of anhydrous MgSO₄, 1 g of anhydrous CH₃COONa, and 1 g of anhydrous NaCl were added, and the tubes were capped immediately and briefly shaken by hand to prevent agglomeration of the salts. Then, the tubes were centrifuged at 7000 rpm and 14 °C for 5 min. The extracts were then cleaned up with Bond Elut Carbon/NH₄ cartridge after being conditioned with 10 mL of ethyl acetate (containing 1% formic acid). A 2 mL solution of extracts was passed through the SPE column and eluted with 10 mL of ethyl acetate (containing 1% formic acid). The eluent was evaporated to dryness by using a nitrogen evaporator. The residues were reconstituted with 1 mL of ethyl acetate and cyclohexane (9:1, v/v) and then filtered with a 0.2 μm PTFE syringe. After filtration, aliquots were transferred into the vials for analysis by gas chromatography–tandem mass spectrometry (GC-MS/MS).

Sample Preparation for Tea Infusion. Brewing Procedure. About 5 g of dried black tea leaves as accurately weighed and put into a 250 mL Erlenmeyer flask. Then, 150 mL of distilled boiling water was added into the flask. After 5 min of brewing, the sample was swirled for 30 s by using an electromagnetic stirrer. The total of brewing time was 10 min. Immediately after the brewing, the tea infusion samples were cooled to 5 °C for 30 min by immersing the flask into cold water. Then, the tea infusion samples were ready for analysis using the QuEChERS method for AQ determination. The method has been described for pesticide determination.

Sample Preparation of Brewed Tea. A 10 mL solution of brewed tea samples were transferred into a 50 mL polypropylene extraction tube. For the fortification experiment (recovery study), the sample was spiked with AQ standard solution. A six-level calibration curve in concentrations of 0.5, 2.5, 10, 20, 30, and 50 μg/L was also prepared by spiking the blank samples with AQ standard solution. This calibration curve was used for quantification of AQ in brewed tea. After leaving the samples for 30 min for equilibration, 15 mL of ethyl acetate (containing 1% of acetic acid) was added and vortexed for 3 min followed by extraction using an end-over-end shaker for 30 min. Thereafter, 4 g of anhydrous MgSO₄, 1 g of anhydrous CH₃COONa, and 1 g of anhydrous NaCl were added, and the tubes were capped immediately and briefly shaken by hand to prevent agglomeration of the salts. Then, the tubes were centrifuged at 7000 rpm and 14 °C for 5 min.
Supernatants were then transferred into the 15 mL centrifuge tube containing 950 mg of anhydrous MgSO₄, 150 mg of C₁₅₇ and 300 mg of PSA. The tubes were capped and mixed using a vortex for 3 min and centrifuged the tubes at 7000 rpm and 14 °C for 3 min. A 2.5 mL solution of supernatants was transferred into 5 mL glass tubes and then evaporated to dryness using a nitrogen evaporator. The residues were reconstituted with 1 mL ethyl acetate + cyclohexane (9:1, v/v), filtered with a 0.2 μm PTFE syringe, and transferred into the vials for analysis by GC-MS/MS.

**AQ Determination.** The determination of AQ in dried tea and its infusion samples were performed in the Laboratory for Quality Testing of Goods, Ministry of Trade, Jakarta, Indonesia, by gas chromatography—triple quadrupole mass spectrometry (GC-MS/MS). A Shimadzu GC-MS-TQ8030 was operated in multiple reaction monitoring (MRM) mode with an ionization voltage of 70 eV. The transitions of 180 > 152 and 208 > 152 were used as quantifier and qualifier MRM, respectively. A GC Column SH-Rxi-1MS 30 m × 0.25 m × 0.25 μm was used to separate AQ. A 1 μL solution of the final clean extract of the sample was injected in splitless mode at 270 °C. The ion source and interface temperatures were 250 and 280 °C, respectively. Helium was used as a carrier gas. The initial oven temperature was programmed at 90 °C and increased to 150 °C (30 °C/min). After being held for 1 min, the temperature was increased to 300 °C (15 °C/min) and held for 2 min. Figure 1 shows the GC chromatogram of AQ for a 10 μg/kg spike in the black tea sample and a 0.5 μg/L spike in the brewed tea sample. GC systems has been able to detect up to 0.5 ppb levels in brewed tea because the matrix effect was much lower than in dried tea. For the quantification of AQ, the peak areas of the samples were plotted into the calibration curve described in the sample preparation procedure. Calibration curves in both dried tea and brewed tea were prepared through standard additions to compensate matrix effects and improve accuracy of the test results.

To ensure the validity of the test results, all glassware were washed with a specific detergent, rinsed with distilled water and acetone, and dried before use. To ensure that there is no contamination, a reagent blank and sample blank were checked before the determination of AQ in the samples. Reagent blanks are distilled water or other relevant solvents and all of the reagents used in the sample preparation procedure.

**Sample Preparation.** The determination of AQ in both dried and brewed tea was performed in accordance with the performance criteria required by EU document SANTE/11945/2015. Recovery, repeatability (RSDᵣ), and within-lab reproducibility (RSDᵢ) for 10 and 50 μg/kg spike levels ranged from 78−98%, 1.5−1.9%, and 3.1−4.0%, respectively. In brewed tea samples, recovery, repeatability (RSDᵣ), and within-lab reproducibility (RSDᵢ) for 0.5 and 5 μg/L spike levels ranged from 78−102%, 0.5−8.4%, and 10.2−11.7%, respectively. LOQs for AQ in dried tea and brewed tea were 10 and 0.5 μg/kg, respectively. The LOQ of AQ in brewed tea was much lower than in dried tea because the low concentration of tea extract in aqueous solutions did not significantly interfere with the response of the analyte in the GC system. Linearity (R²) values of AQ in dried tea and brewed tea were 0.9986 (calibration level: 10, 20, 50, 100, and 150 μg/kg) and 0.9961 (calibration level: 0.5, 2.5, 10, 20, 30, and 50 μg/L), respectively. As stated in the SANTE document, analytes below the reporting limit (RL) must be reported as <RL. In our article, the LOQs are equal to the RL.

**Method Validation.** The validation method for the determination of AQ in both dried and brewed tea was performed in accordance with the performance criteria required by EU document SANTE/11945/2015. Recovery, repeatability (RSDᵣ), and within-lab reproducibility (RSDᵢ) for 10 and 50 μg/kg spike levels ranged from 78−98%, 1.5−1.9%, and 3.1−4.0%, respectively. In brewed tea samples, recovery, repeatability (RSDᵣ), and within-lab reproducibility (RSDᵢ) for 0.5 and 5 μg/L spike levels ranged from 78−102%, 0.5−8.4%, and 10.2−11.7%, respectively. LOQs for AQ in dried tea and brewed tea were 10 and 0.5 μg/kg, respectively. The LOQ of AQ in brewed tea was much lower than in dried tea because the low concentration of tea extract in aqueous solutions did not significantly interfere with the response of the analyte in the GC system. Linearity (R²) values of AQ in dried tea and brewed tea were 0.9986 (calibration level: 10, 20, 50, 100, and 150 μg/kg) and 0.9961 (calibration level: 0.5, 2.5, 10, 20, 30, and 50 μg/L), respectively. As stated in the SANTE document, analytes below the reporting limit (RL) must be reported as <RL. In our article, the LOQs are equal to the RL.

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Figure 1. GC chromatogram of AQ.
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ABBREVIATIONS USED
AQ, anthraquinone; BMD, benchmark dose; BNN, Bundesverband Naturkost Naturwaren; CSF, cancer slope factor; CXL, oxidative DNA damages in humans; MB, middle bound; ML, maximum level; MOE, margin of exposure; MRL, maximum residue limit; p-OSF, provisional oral slope factor; PPRTV, Provisional Peer-Reviewed Toxicity Values; POD, point of departure; UB, upper bound; US EPA, United States Environmental Protection Agency

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