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Anthraquinone in Indonesian infusion tea: analysis by HPLC–UV and risk assessment

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Abstract

Background: Detection of anthraquinone in tea samples marketed in Europe is raising a concern due to the possible carcinogenicity of this compound. The European Union has set a very low maximum residue level (MRL) for anthraquinone residue in tea (0.02 mg/kg). The available method analyses for anthraquinone are mostly by gas chromatography (GC) coupled by mass spectrophotometer (MS) which are relatively more expensive instruments and not always available to moderately equipped laboratories. This study was aimed to analyze anthraquinone in infusion tea of Indonesian commercial tea using liquid chromatography–ultraviolet detector (HPLC–UV) and then assessed the risk associated with consuming infusion tea with anthraquinone residue. The analysis was conducted by low-volume liquid–liquid extraction followed by quantification by HPLC–UV, while the risk assessment was analyzed by calculating the risk quotient (HQ) and carcinogenic risk (*R*) based on the highest anthraquinone level detected in the sample.

Results: The low-volume liquid–liquid extraction followed by HPLC quantification was able to analyze anthraquinone in infusion tea with linearity of 0.9990 at 8.33 to 83.33 µg/L; precision of 2.25%; and recovery of 96.19 to 102.98%. The anthraquinone levels in infusion tea of Indonesian commercial tea varied from not detected to 0.44 µg/L. The calculated HQ is lower than 1 (0.033), implying that consuming Indonesian infusion tea with the detected anthraquinone residue is unlikely to cause non-carcinogenic effect. The *R* is 2.63×10^{-6} , but no parametric values have been regulated for anthraquinone; thus, its carcinogenic effect cannot be further assessed.

Conclusion: Low-volume liquid–liquid extraction followed by quantification by HPLC–UV was able to analyze anthraquinone in infusion tea of Indonesian commercial tea samples. Risk assessment showed no prove of adverse effect related to consuming infusion tea as high as the highest concentration detected in infusion tea samples. However, risk associated at higher concentration and/or long-term consumption may not be neglected.

Keywords: Anthraquinone, Tea infusion, Green analytical chemistry, Risk assessment

Background

The occurrence of anthraquinone in tea, which was previously neglected, is recently gaining concerns. It was first detected by laboratories in Europe in 2011 [1] for imported black tea sourcing from China, India, and Sri Lanka [2]. Due to the anthraquinone detection in tea, European Union has set a maximum residue limit of 0.02 mg/kg for tea Europe [3]. This limit is a two order

of magnitude lower compared to other pesticide residues in tea, and, thus, impacts the tea trade in Europe seriously.

Anthraquinone is a possibly carcinogen to humans. Research has shown that this compound caused tumors in kidney, liver, urinary bladder, and thyroid gland of mammalian [4]. International agency for research on cancers (IARC) has classified anthraquinone in group 2B, a possibly carcinogenic to humans, due to the sufficient evidence of carcinogenicity in animal but limited evidence in humans [5]. Chemical and physical properties of anthraquinone are given in Table 1.

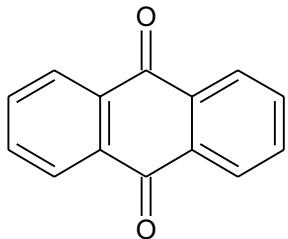
Anthraquinone occurrence in the environment is due to several factors. Anthraquinone and its derivatives are

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Table 1 Chemical and physical properties of anthraquinone

Chemical structure	
Chemical name	Anthraquinone; 9,10-anthraquinone
Molecular formula	C ₁₄ H ₈ O ₂
CAS No	84-65-1
Molecular weight	208.21
Boiling point	377 °C at 760 mmHg
Melting point	286 °C
Density/specific gravity	1.42–1.44 at 20 °C/4 °C
Octanol/water partition coefficient	log Kow = 3.39
Solubilities	0.44 g/100 g alcohol at 25 °C; 2.25 g/100 g boiling alcohol; 0.11 g/100 g ether at 25 °C; 0.61 g/100 g chloroform at 20 °C; 0.26 g/100 g benzene at 20 °C; 0.30 g/100 g toluene at 25 °C; soluble in concentrated sulfuric acid; soluble in acetone; 1.353 mg/L at 25 °C water

All properties are taken from Hazardous Substance Data Bank [6]

naturally found in many plants [6, 7] with several serve as natural pigments [6] or pose anti-insecticidal activity [2]. It is known as a bird repellent since 1940s [8] and used in paint, textile, and paper industries [6]. In addition, it was also known as air contaminant [9–12] sourced from diesel automobiles [9] or waste incinerator [13, 14].

However, the source of anthraquinone in tea is still uncertain. Despite its application as pesticide had never been registered in China [2], it was detected in exported tea sourced from China, thus, raised questions on how it contaminated the tea. Several sources were suspected contributing to the anthraquinone detection. They were the use of tea bags [15], application of pesticides during plantation [15], and contamination of atmosphere in the plantation [2]. Nevertheless, none is scientifically proven yet.

Analysis of anthraquinone was mostly done as plant extract of anthraquinones [16–18] where the conventional methods such as solid–liquid extraction, maceration, or Soxhlet extraction were performed using high volume of solvents and samples before being analyzed by HPLC–UV. However, as plant extract, anthraquinone analysis was not up to a low value of method detection limit. This is not the case for tea residue analysis where the low detection limit was important and mostly impaired by the complicated matrix of tea. For anthraquinone analysis in tea, the report is limited [2, 19] and all the analysis was performed on gas chromatography coupled with tandem mass spectrophotometer (MS/

MS). However, MS, both for single and tandem MS, is relatively costly and usually not available in moderately equipped laboratories [20], and thus, alternative method utilizing relatively cheaper instruments such as HPLC–UV is still needed.

Meanwhile, tea is usually consumed as tea infusion. If anthraquinone is present in tea, the channel for anthraquinone to enter the human body is through tea infusion. This study was aimed to analyze anthraquinone in tea infusion of commercial Indonesian tea by utilizing low-volume liquid–liquid extraction followed by quantification using HPLC–UV. The anthraquinone concentration in tea infusion data was then used to assess the risk accompanying drinking tea infusion with anthraquinone residue.

Materials and methods

Chemicals and reagents

Anthraquinone with the purity of 99.4% was sourced from Chem Service, West Chester, USA. Acetonitrile, *n*-hexane, and acetone were sourced from Merck. Stock solution of anthraquinone was prepared at 39 mg/L in acetonitrile. Standard solutions were prepared from stock solution by dilution.

Tea samples

Tea samples are Indonesian commercial tea that was purchased from local supermarkets. The samples were in the package of tea bags or loose leaf. All products were the

common tea brand produced and sold in Indonesia and were accepted as products indicated in their labels.

Apparatus

HPLC–UV analysis was performed on an HPLC 7000 series from Hitachi coupled with a UV–visible detector. A μ -BondapakTM C₁₈ (10 μ m; 125 Å; 3.9 \times 300 mm) from waters was used for separation. The temperature of the column oven was set at 30 °C. The mobile phase was methanol and water with the ratio of 70:30, and was set in an isocratic mode with the flow rate of 0.8 mL/min for 15 min. The UV–Vis detector was set at 260 nm. The volume of each injection was 20 μ L.

Analytical procedures and analysis of tea samples

Performance of the method was validated through spike experiment in which the tea infusion was spiked with anthraquinone. The no-spike experiment was also conducted in parallel with the spike experiment as a control. All the spike and no-spike experiments were extracted according to Fig. 1.

For the real tea sample analysis, tea infusion was prepared by infusing 2 g of tea samples in 200 mL of boiling water. The tea infusion was then filtered before being used for the extraction. For the extraction, a 30 mL of the tea infusion aliquot was used to extract with 3 mL of 1% acetone in *n*-hexane. The extraction was performed on a rotary agitator for 15 min at low speed (22.5 rpm). More details on the extraction procedures are shown in Fig. 1.

Mass spectrometry confirmation

Confirmation of anthraquinone was done on a Shimadzu GCMS-TQ8030, a gas chromatography coupled with triple quadrupole mass spectrometer (GC–MS/MS). The system was operated by GCMS solution software. The chromatographic separation was performed on an Rxi[®]-1 ms (30 m \times 0.25 mm \times 0.25 μ m) from Restek. Each sample was injected 1 μ L at splitless mode at 270 °C. The oven temperature was programmed at 150 °C for 2 min and increased at 5 mL/min to 300 °C and then was held for 2 min. Helium was used as the carrier gas at 2.5 mL/min. The ion source temperature and interface temperature were 250 °C and 280 °C, respectively. The solvent cut time was 3.5 min. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode at electron energy of 70 eV.

Human risk assessment

The anthraquinone concentrations in infusion tea samples obtained in this study were then used to assess the

carcinogenic and non-carcinogenic risks of anthraquinone as a result of consuming infusion tea from Indonesian market. The hazard quotient (HQ) and carcinogenic risk (*R*) were estimated to analyze the non-carcinogenic and carcinogenic risks of anthraquinone using Eq. (1) [21], Eq. (2) [22], and Eq. (3) [23, 24]. The HQ was calculated based on the following:

$$HQ = \frac{CDI}{RfD}, \quad (1)$$

where chronic daily intake (CDI) represented the amount of ingested anthraquinone per kilogram body weight; RfD was the oral reference dose of the anthraquinone (mg/kg day). Because reference dose (RfD) for anthraquinone was another unknown, the provisional reference dose (P-RfD) for anthraquinone (0.002 mg/kg day) proposed by USEPA [25] was used. The CDI was calculated by the following:

$$CDI = \frac{C \times IR \times EF \times ED}{BW \times AT}, \quad (2)$$

where *C* represented the measured concentration (mg/L) of anthraquinone; IR was the infusion tea ingested rate of an Indonesian person that was accounted for 0.38 kg/year or 190 cups/year (1 cup = 200 mL) [26]; EF was the exposure frequency (365 days/year); ED was the exposure duration (70 years for adult); BW was the body weight of an adult (70 kg); and AT was the average lifespan (25,550 for adult). For the carcinogenic risk (*R*), the formulation below was used by the following:

$$R = CDI \times SF \times ADAF, \quad (3)$$

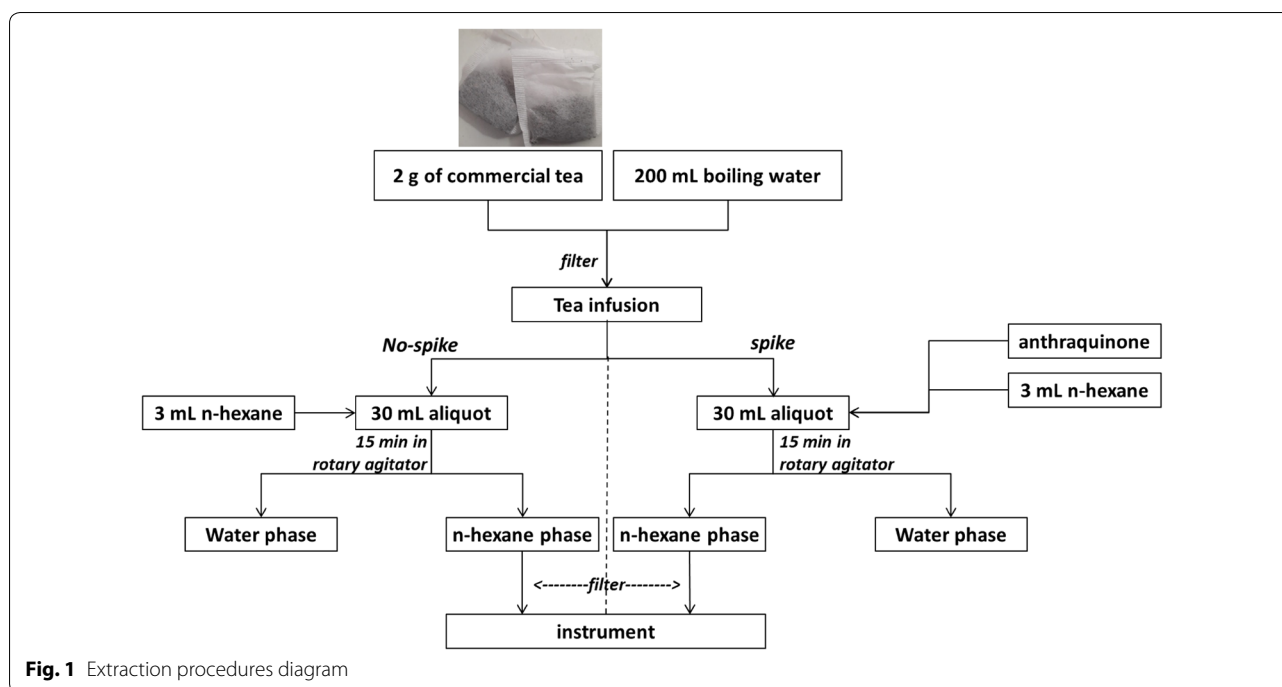
where SF was the cancer slope factor (mg/kg day). Similar to RfD, because of cancer slope factor for anthraquinone which was another unknown, the provisional oral slope factor (P-OSF) for anthraquinone (0.04 mg/kg day) proposed by USEPA [25] was used. ADAF was the age-dependent adjustment factor (1 for above 16 years old).

Result and discussion

Chromatographic performance

The absorption spectra of anthraquinone were studied at wavelength of 254–270 nm (Fig. 2) with methanol and water as the mobile phase. The compound showed the maximum absorbance at 260 nm, so, for the next experiment, the HPLC analysis was performed at 260 nm.

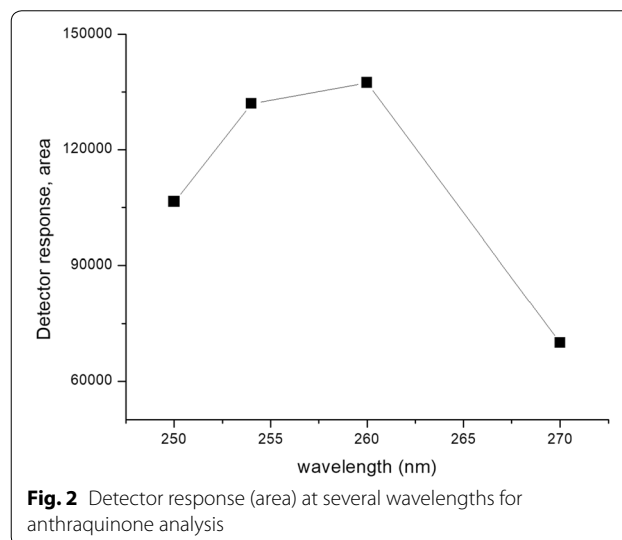
Figure 3 shows the chromatograms of standard solution of anthraquinone, and tea sample extract with and without anthraquinone. It can be seen that anthraquinone was eluted at around 10.24 min. Meanwhile, for tea infusion matrix, the interference peaks came early and did not interfere with the target compound anthraquinone. This benefited the extraction, since no



further clean-up is needed. For residue analysis in tea samples that performed on MS or MS/MS detectors, this usually was not the case. Tea was known of its complicated matrix, and clean up using primary secondary amine (PSA), florisil, and graphitized carbon black (GCB) are usually applied during sample preparation/extraction to remove interferences [2, 19, 27–29]. For anthraquinone analysis in tea, PSA combined with GCB [19] and florisil [2] were normally used to clean up interferences. However, in our case, since the interferences in tea that usually observed during GC–MS quantification were not detected due to their inactivity to the UV detector, the clean-up step was not required and, thus, simplified the extraction method. Without clean-up step that normally is applied for tea-based analysis, the time for the extraction is shortened and budget for clean-up chemicals is minimized.

Calibration curve for standard solutions of anthraquinone was constructed at 13 concentration levels from 6.24 to 156 µg/L and then plotted the peak area against each concentration. Concentration of 6.24 µg/L was the lowest concentration that can be detected, and, therefore, was appointed as the instrument detection limit (IDL). The detector response for all the standard solutions was fitted to linear regression and gave a coefficient correlation of 0.9999 (Table 2), indicating good linearity of the standard solutions.

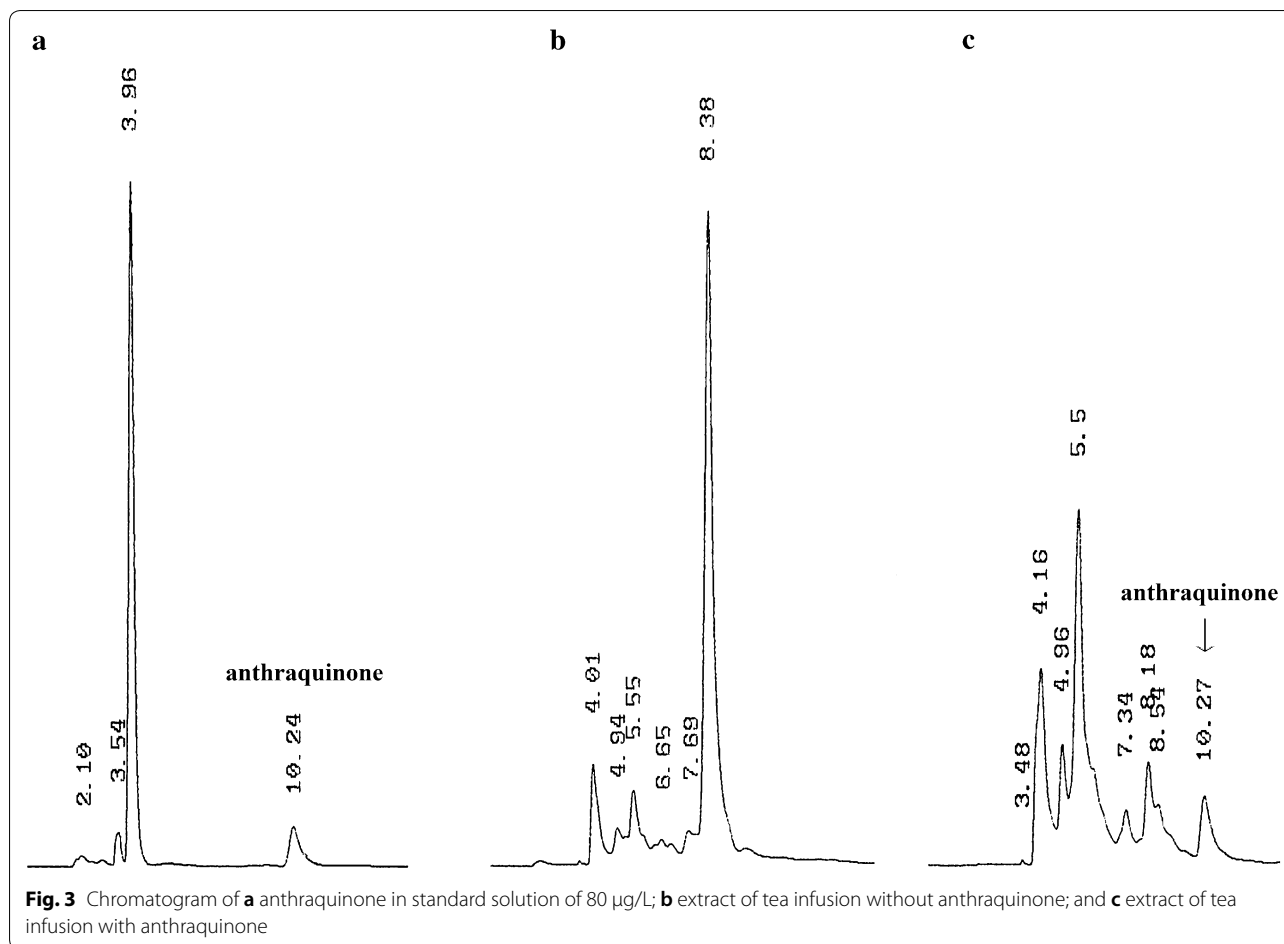
The precision of chromatographic system was evaluated by analyzing anthraquinone standard solution at



6.24 µg/L by seven independent injections. The percent relative standard deviation (RSD) was acceptable with a value below of the 2/3 Horwitz coefficient of variation (CV_{Horwitz}) value, as shown in Table 2.

Analytical performance

The linearity of the method for the spike experiment was evaluated at one order of magnitude concentration, ranging from 8.33 to 83.33 µg/L. Linear relationship between the corresponding peak area and the concentration of



analytes was observed, showed by a close to 1 r value accounted for 0.9990 (Fig. 4).

The precision of the method was assessed by applying the extraction procedures to the tea infusion with anthraquinone and the extraction was done in seven replicates for the same tea infusion. As shown in Table 3, the precision of the method was acceptable with a lower % RSD (2.25%) compared to the two-thirds of calculated CV_{Horwitz} (21.96%). Similarly, the % RSD also lower compared to AOAC guideline [30]. This implies that the extraction method is precise.

The accuracy of the analytical method was evaluated by the recovery data. The tea infusion was spiked with anthraquinone at concentration levels of 8.33 to 83.33 µg/L and then was extracted by the method. Prior to analyte fortification, the background level of [2] tea infusion was also determined for the recovery calculation. The recoveries obtained in this study were in between 96.19 and 102.98% with an average of 99.52%. These values are in the acceptable range as in the AOAC guidelines (80–110%) [30] and European Union guideline (70–120%) [31].

Table 2 Evaluation of HPLC–UV performance

Parameters	Anthraquinone
Linear regression equation	$y = 117.26x + 95.449$
Coefficient correlation (r)	0.9999
Linear range	6.24–156 µg/L
Instrument detection limit (IDL)	6.24 µg/L
Relative standard deviation (RSD) at 6.24 µg/L ($n = 7$)	15.68%
CV_{Horwitz}	34.15%

This method was able to quantify anthraquinone in infusion tea as low as 8.33 µg/L. If we assume that all anthraquinone in tea sample (2 g) is transferred to the infusion tea (200 mL), then the LOQ of this method is equivalent to 0.833 mg/kg which is around 42-fold of the MRL regulated by European Union [3]. However, the previous report showed that anthraquinone has low solubility with only 1.6 to 13.7% brew to the infusion tea [2], and thus, the equivalent LOQ of this method will be ranging from 0.013 to 0.114 mg/kg. It can be seen that the lowest

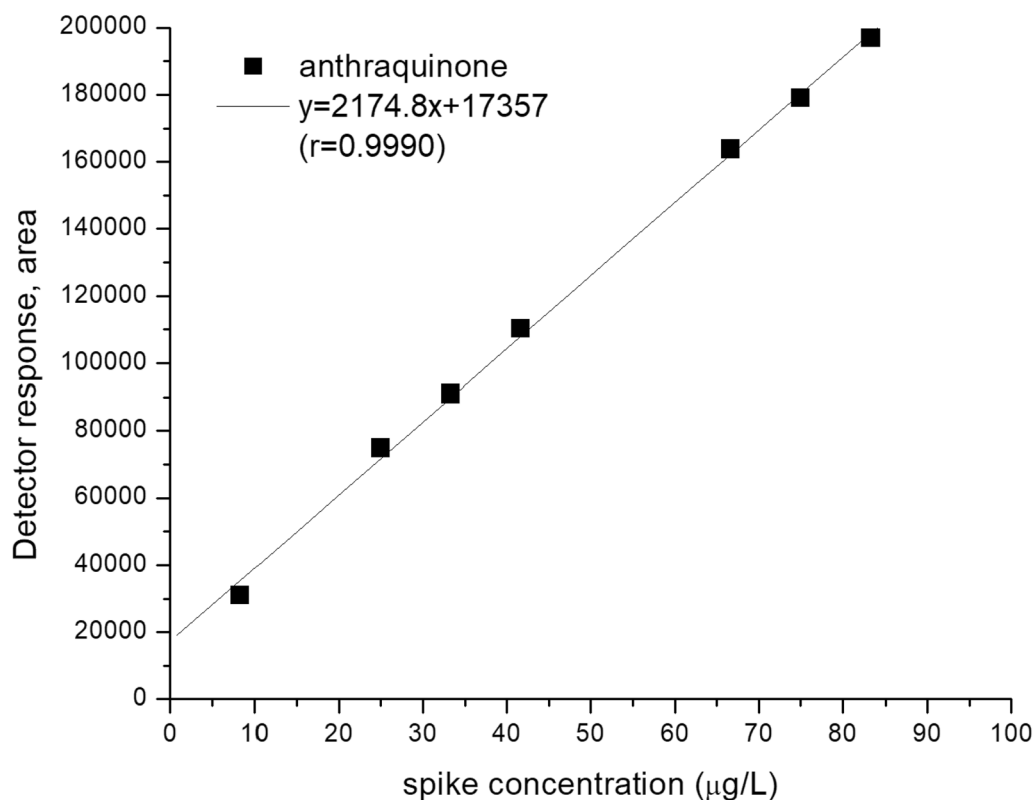


Fig. 4 Linearity of the method at 8.33–83.33 µg/L

value of the equivalent LOQ (0.013 mg/kg) is lower than the MRL, while the highest value is higher than the MRL. The transfer behavior of anthraquinone from dried tea to infusion tea is likely to be temperature- and concentration-dependent [2], and thus, the equivalent LOQ values obtained in our study are likely to be affected by temperature when preparing the infusion tea and the anthraquinone concentration in the dried tea used to prepare the infusion tea. Therefore, this method is not suggested to be used to determine the anthraquinone level in tea (dried) to meet the requirement set by the European Union and can only be used to determine the anthraquinone level in infusion tea as low as 8.33 µg/L. In the case of anthraquinone determination in dried tea (as required by European Union), method which utilizes gas chromatography with tandem mass spectrophotometer (GC–MS/MS) is still more sensitive and can achieve LOQ as low as 0.01 mg/kg [2] which is lower than the MRL. Similarly, utilization of GC–MS/MS for anthraquinone analysis in infusion tea can achieve a lower LOQ (0.4 µg/L) [2] compared to our method. However, both applied clean-up step which was not use in our method.

Anthraquinone was confirmed by GC–MS/MS at conditions explained in the methods. The transition of

Table 3 Evaluation of the method performance (precision, recovery, and uncertainty measurement)

Parameters	Results
Spiked concentration	8.33 µg/L
Extracted concentration (average)	8.29 µg/L
Percent recovery	96.19–102.98% (average 99.52%)
<i>N</i>	7
RSD, %	2.25
CV_{Horwitz}	32.94
$2/3 CV_{\text{Horwitz}}$	21.96
Uncertainty	8.29 ± 0.42 µg/L

m/z 180 to m/z 152 was used as quantifier while transition of m/z 208 to m/z 152 was used for anthraquinone confirmation. These transitions are corresponding to the removal of one molecule of CO (180–152) or two molecules of CO (208–152) as proposed in the previous research of anthraquinone analysis by GC–MS/MS [2].

Real sample analysis and risk assessment

The performance of HPLC–UV and analytical method described above was applied for anthraquinone analysis in tea infusion for the real samples of tea bags and loose leaf produced and marketed in Indonesia. Tea bags and loose leaf tea were chosen, since they are the most consumed tea products in Indonesia [32]. Moreover, since tea bags and loose leaf tea were consumed as tea infusion, thus, anthraquinone, if present in tea, will be exposed to humans through tea infusion. Therefore, tea infusion was chosen as the matrix for the analysis.

For the analysis, tea samples were extracted according to the method (Fig. 1). The extraction was performed in duplicate for each sample and the results are summarized in Table 4. As shown in the table, there is a high degree of variability for the anthraquinone content in commercial tea marketed in Indonesia, as low as not detected to 44 µg/L. To our knowledge, there was no report on anthraquinone concentration on tea infusion for real samples. Report on anthraquinone concentration on tea infusion was limited to anthraquinone fortification on tea samples (anthraquinone has been applied in field trial) to see the infusion behavior of anthraquinone from tea to tea brew [2]. Since anthraquinone pose a low solubility in water (1.353 mg/L at 25 °C) [6] with only 1.6–13.7% of it brews into tea infusion [2], thus, it can be predicted that anthraquinone concentrations in tea bag or loose tea samples are higher than what are detected in tea infusion as investigated in this study.

However, as mentioned earlier, since tea bags and loose leaf tea are consumed as tea infusion, the risk of anthraquinone in tea bags and loose tea that are not leached into tea infusion (or anthraquinone in spent tea) is not important and can be neglected. Nevertheless, anthraquinone detection on tea infusion is a concern due to its carcinogenicity, although daily permitted intake of anthraquinone for human has not yet been determined or evaluated.

Assuming that anthraquinone daily intake is only from tea infusion, the risk for consuming infusion tea with anthraquinone residue with concentration level as detected in this study is predicted. The non-carcinogenic risk was estimated based on the hazard quotient or HQ (Eq. 1) and the result is given in Table 5. If the Indonesian tea consumption per capita is 0.38 kg/years [26] and the intake of anthraquinone is the highest anthraquinone detected in this study (44 µg/L), then HQ for an adult with a 70-kg body weight is 0.033. This value (HQ < 1) implies that there is non-carcinogenic effect resulting from consuming Indonesian infusion tea.

The carcinogenic risk (*R*) value was calculated using Eqs. (2 and 3). The calculated *R* value is 2.63×10^{-6} (Table 5). Usually, the *R* value is compared to parametric

Table 4 Anthraquinone and recovery in Indonesian commercial tea (*n* = 2)

Real samples	Anthraquinone (µg/L)	Recovery (%)
SP-01	ND	95.78
SP-02	ND	101.73
SP-03	ND	97.92
SP-04	8.17	105.07
SP-05	ND	88.87
SP-06	0.57	106.75
SP-07	16.24	86.74
SP-08	7.06	97.53
SP-09	20.41	94.14
SP-10	ND	82.35
SP-11	22.80	117.53
SP-12	ND	105.39
SP-13	6.32	99.54
SP-14	6.12	89.52
SP-15	26.26	77.76
SP-16	44.23	84.18
SP-17	32.82	112.96

ND non detected

value recommended by regulators. However, since anthraquinone carcinogenic study is limited and parametric value recommended by regulators such as EU, USEPA, or WHO is absent, that obtained *R* value is meaningless and cannot be used to assess the carcinogenic risk of anthraquinone.

Further assessment was then conducted based on the previous reports on anthraquinone carcinogenicity for mammals as comparison. Again, assuming that anthraquinone daily intake is only from tea infusion, for an adult with 70-kg body weight who drinks two cups of tea infusion (200 mL) daily with 44 µg/L of anthraquinone, the anthraquinone daily intake will be a total of 0.0176 mg or 2.5×10^{-4} mg anthraquinone per kg of body weight. The previous research showed that daily intake of 125.3 mg/kg day (human equivalent dose) of anthraquinone in male mice for 2 years, which is 6 order of magnitude greater than the simulated concentration in this study, has bring negative effect such as increasing the incident of tumors (hepatocellular adenoma, carcinoma, and hepatoblastoma) up to 98% [25]. Moreover, daily intake of 50 mg/kg body weight of female rats for 105 weeks resulted in incidence of papilloma that was significantly different from the control [6]. Unfortunately, there was no information on lower concentration of daily intake of anthraquinone in the last report [6]. In addition, excretion of anthraquinone was quick in animal, with almost 96% was excreted in urine and feces within 2 days [6].

Table 5 Human risk assessment

Measured concentration	44.23 µg/L
Infusion tea ingested rate of Indonesian (IR)	0.38 kg/year (or 190 cups/year or 0.38 L/year)
Exposure frequency (EF)	365 days/year
Exposure duration (ED)	70 years (adult)
Body weight (BW)	70 kg
Average lifespan	25,550 day (adult)
Chronic daily intake (CDI)	6.57824×10^{-5} mg/kg day
Oral reference dose	0.002 mg/kg day ^a
HAZARD quotient (HQ)	0.033
Provisional oral slope factor (P-OSF)	0.04 mg/kg day ^a
Age-dependent adjustment fact (ADAF)	1 (> 16 years old)
Carcinogenic risk (R)	2.63×10^{-6}

^a USEPA [25]

From the above-mentioned reports, there was no proof for the adverse effects of anthraquinone if it was taken as tea infusion contaminant at the level of concentration detected in this study for short period of exposure. However, for daily intake at higher concentration and or at prolonged exposure of anthraquinone, the adverse effect may not be neglected. Nevertheless, this prediction is needed to be scientifically proven.

The accuracy of the method in term of recovery was also evaluated for all the samples. Anthraquinone was spiked to all samples and the area was subtracted from the no-spike experiment. It was found that the recovery for all samples was in the range suggested by European Union [31] for pesticide residue analysis, and 15 of 17 samples were in the range suggested by AOAC [30] (Table 4).

Conclusion

A simple extraction procedure including low-volume liquid–liquid extraction without a clean-up step, followed by HPLC–UV quantification, was able to determination anthraquinone in infusion tea of commercial Indonesian tea and no adverse effect resulting from consuming Indonesian infusion tea observed in this study.

Abbreviations

ADAF: age-dependent adjustment factor; AT: average lifespan; BW: body weight; C: concentration; CDI: chronic daily intake; CV_{Horwitz} : Horwitz coefficient of variation; ED: exposure duration; EF: exposure frequency; GC: gas chromatography; GCB: graphitized carbon black; HPLC: high-performance liquid chromatography; HQ: hazard quotient; IDL: instrument detection limit; IR: ingested rate; MRL: maximum residue level; MS: mass spectrophotometer; MS/MS: tandem mass spectrophotometer; P-OSF: provisional oral slope factor; P-RfD: provisional reference dose; PSA: primary secondary amine; R: carcinogenic risk; RfD: oral reference dose; RSD: relative standard deviation; SF: slope factor; UV: ultra violet; UV–Vis: ultraviolet–visible.

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Authors' contributions

RY planned the experimental design, conducted the research including validate the method for the analysis, and interpreted the data; MMP (corresponding author) prepared the manuscript; MA prepared the risk assessment and critically reviewed the manuscript. TAK provided the guidance for method validation; HM provided the guidance for tea and helped with the tea samples. All authors read and approved the final manuscript.

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Availability of data and materials

Additional data may be available on request to the authors; please contact the corresponding author.

Ethics approval and consent to participate

The authors declare that this study does not involve human subjects, human material, and human data. This manuscript is an original article and has not published in other journals. The authors agree to comply with the copyright rule.

Consent for publication

The authors agreed to the publication of the manuscript in this journal. The authors grant the accessibility of the scientific article in agreement of the springer policy.

Competing interests

The authors declare that they have no competing interests.

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