Preliminary study for 9,10-anthraquinone residue analysis in tea-based functional beverage: GC-ECD optimization and method development

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Abstract. 9,10-Anthraquinone, a possible carcinogenic compound, is recently detected above the regulated limit in tea samples sourced from Asian countries. Its occurrence is previously neglected. The European Union has set a limit of 0.02 mg/kg for anthraquinone residue in tea. The available analysis methods for anthraquinone are mostly by gas chromatography (GC) coupled with mass spectrophotometer (MS). This study was aimed to analyse anthraquinone in an infusion of tea-based functional beverage based on low volume liquid-liquid extraction followed by a GC-µECD quantification. The experiment included optimisation of GC-µECD for anthraquinone quantification. The result showed that GC-µECD was able to detect anthraquinone at 1 µg/L with good repeatability showed by %RSD of 6.68. The calibration curve at the range of 1 to 10 µg/L showed good linearity (r=0.9973). The low volume liquid-liquid extraction sample preparation was able to extract anthraquinone from the infusion samples as low as 0.67 µg/L.

1. Introduction
Tea is the most consumed beverage in the world after water. Food and Agriculture Organization of the United Nations [1] reported that in 2013 the world tea consumption was increasing to 4.8 million tonnes. The popularity of tea products has opened a way to the production of a variety of tea-based beverages. Moreover, advances in food technology have allowed the addition of functional ingredients to tea-based products to produce tea-based functional beverages such as slimming tea, breast milk enhancing tea, anti-diabetic tea, anti-hypertensive tea, or antioxidant tea.

However, as tea products, the tea-based functional beverages may pose a risk associated with the contaminants present in the tea. Pesticide residues are one of the major class contaminants in tea [2] and consequently to tea-based functional beverages. Moreover, polycyclic aromatic hydrocarbons [3] such as anthraquinone are also among the contaminants reported for tea.

Anthraquinone, classified by IARC (International Agency for Research on Cancer) as possibly carcinogenic to humans (2B) [4], is recently gaining concern since its detection above the 0.02 mg/kg level set by European Union [5] in tea imported to Europe in 2012 [6]. The contamination source was still arguable [6], although the use of tea bags as well as the application of pesticide during plantation [7], and the contamination of the atmosphere of the plantation area [6,8] were suspected contributing to the tea contamination.
Anthraquinone has a formula of $\text{C}_{14}\text{H}_8\text{O}_2$. It was also known as 9,10-anthraquinone to differentiate it from the anthraquinones class, a phenolic compounds group. It is one of polycyclic aromatic hydrocarbons and commonly used in the paint, textile, paper and bird repellent productions [4].

Analysis of anthraquinone is usually performed in relation to plant extracts with bioactivity since several of anthraquinones pose anti-cancer or anti-bacterial properties [9,10]. It was known as a contaminant in air particulate in the 1980s to early 2000s [11-15] but recently was also becoming a tea contaminant [6].

Formerly, anthraquinone analysis as anthraquinones group involved solid-liquid extraction, maceration, and Soxhlet extraction [9,10,16]. However, these methods used a high volume of samples and solvents that will produce more chemical waste and also need more time. In addition, some of the sample preparation techniques use toxic solvents such as dichloromethane [16]. Additional instruments such as accelerated solvent extraction [9], ultrasound-assisted extraction and microwave-assisted extraction [17] are also being used although these are uncommon since not all laboratories have access to those instruments. Recently, anthraquinone analysis in tea has been done but still utilized Soxhlet [8] or liquid-liquid extraction [6].

Several instruments have been used for identification and quantification of anthraquinones. Instruments coupled with traditional detectors such as high-performance liquid chromatography (HPLC) with photodiode array detector (PAD) [16] and HPLC with diode array detector (DAD) [17] were commonly used especially in relation to the analysis of plant extracts which did not require a low level of detection. For low level analysis, mass spectrophotometer (MS) is normally used as a detector in combination with liquid chromatography (LC) [8] and gas chromatography (GC) [6] due to its sensitivity. However, the mass spectrophotometer is relatively costly and needs high maintenance cost in addition to not available in moderately equipped laboratories [18]. Therefore alternative detector with relatively low cost is needed for anthraquinone analysis.

This study reports the preliminary study for 9,10-anthraquinone residue analysis in tea-based functional beverage where micro electron capture detector (ECD) was optimized as a detector for gas chromatography separation.

2. Experimental

2.1. Chemicals and reagents

Anthraquinone was sourced from Chem Service, USA. Solvents and other chemicals were sourced from Merck unless otherwise stated. The stock solution was prepared in acetonitrile at 39 mg/L. Standard solutions were prepared by diluting the stock solution in acetonitrile.

2.2. Apparatus

GC-ECD analysis was carried out with an Agilent 7890B coupled with a micro ECD. The tea samples were collected from the market around Bandung area, West Java, Indonesia.

2.3. GC-ECD optimization and evaluation

The GC-ECD was optimized in an HP-5 (30 m x 0.320 mm x 0.25) Agilent column with helium and nitrogen gas as carrier gas and make-up gas, respectively. The initial program was set as in Paramasivam and Chandrasekaran [19] with some modification on the detector temperature. In brief: injector temperature was 250°C, detector temperature was 350°C, autosampler split ratio 10:1, oven program 160°C for 1 min then ramped up at 15°C/min to 200°C and held for 2 min and ramped up at 10°C/min to 280°C and held for 8 min. The program was then modified based on the resulted chromatogram to obtain the condition for good separation. The optimum condition was determined by comparing the chromatogram at different conditions. The optimum condition was then evaluated by instrument detection limit, the linearity of standard and precision.
2.4. Analysis of spiked tea samples
The optimum condition of the GC-ECD was also used to analyse tea samples that have been spiked with anthraquinone and prepared by low volume liquid-liquid extraction. Tea sample for the infusion tea was a commercial black tea obtained from supermarkets in Bandung area. The commercial tea was packaged into tea bags.

The infusion tea was prepared by infusing 5 g of tea into 150 mL of boiling water for 5 min [19]. The infusion tea was then filtered before being used. For the tea extraction, 30 mL of infusion tea was spiked with anthraquinone at 0.01 mg/L (or without spiking for control experiment) and extracted by low volume liquid-liquid extraction with 3 mL of n-hexane in a rotary agitator. The organic phase was then collected and the extraction was repeated with the fresh n-hexane. The n-hexane fraction was then filtered and analysed by GC-ECD. The spike experiment was also conducted for the infusion tea at anthraquinone concentration of 0.67 μg/L. This concentration was calculated based on the MRL of anthraquinone in tea [5], assuming all the anthraquinone in the black tea dissolved in the infusion tea.

3. Result and discussion
3.1. Instrument optimization
The running time for the starting condition was 21.7 minutes and the anthraquinone was detected at 7.039 minutes. To shorten the running time, the initial temperature of the oven and the ramping was changed (condition ii and iii) while the injection split ratio was changed to 1:1 (condition iv) to increase the area. The GC-ECD program for each condition and the resulting chromatogram is given in Figure 1.

![Figure 1](image)

Figure 1. The GC-ECD program for condition i to iv and the resulting chromatograms.

Figure 1 shows that condition iii and iv give similar time analysis (13.0 minutes) as programmed. These two conditions also gave a lower retention time compared to the other two conditions (3.526 and 3.519) due to the fact that the oven temperature for conditions iii and iv was higher than i and ii thus anthraquinone came out earlier. Condition iii and iv were the same, except for the split ratio which was 1:10 for condition iii and 1:1 for condition iv. Since a lower split ratio may result in a lower detection limit, condition iv was then preferably chosen for further analysis of anthraquinone.

3.2. Instrument evaluation
3.2.1. Instrument detection limit. The instrument detection limit (IDL) was measured by analysing series of anthraquinone standard solutions until the detector could not detect anthraquinone. The IDL was measured as the concentration above the concentration that could not be detected by the detector.
The IDL of the GC-ECD in this experiment was 0.5 µg/L while the minimum regulated limit (MRL) of anthraquinone in tea for European Union was set to 0.02 mg/kg [5]. This suggests that the GC-ECD can be applied to detect anthraquinone at concentration level above the MRL.

### Figure 2. Chromatogram of anthraquinone.

### Figure 3. Linearity of standard solutions.

3.2.2. *Linearity*. Good linearity (r=0.9973) of standard solutions at 1 to 10 µg/L for GC-ECD quantification was obtained in this study as shown in Figure 3.

3.2.3. *Precision*. The precision of the retention time and area was evaluated by injecting a standard solution (1 µg/L) for seven times and calculating the relative standard deviation (RSD, Table 1). It was shown that the RSD (%) was all below the AOAC guideline (30% at 1 ppb) [20] or the 2/3 CV Horwitz (10.67%) suggesting good repeatability of the analysis.
Table 1. Precision of the retention time and the area at 1 μg/L.

| Replicate No. | Retention time (minutes) | Area  |
|---------------|--------------------------|-------|
| 1             | 3.511                    | 7.268 |
| 2             | 3.511                    | 8.163 |
| 3             | 3.511                    | 7.672 |
| 4             | 3.510                    | 8.222 |
| 5             | 3.510                    | 7.484 |
| 6             | 3.510                    | 8.364 |
| 7             | 3.510                    | 8.770 |

Average 3.510 7.992
Standard deviation 0.0005 0.534
CV Horwitz (%) 16 16
Relative standard deviation (%) 0.015 6.681

Figure 4. Chromatogram for the spiked sample (bottom) at 0.01 mg/L and unspiked sample (top).

3.3. Analysis of tea samples
Analysis of tea samples that had been spiked with anthraquinone and prepared by low volume liquid-liquid extraction was also done. It was shown that the method was able to detect anthraquinone at 0.01 mg/L in the infusion tea (Figure 4) since there was anthraquinone peak observed at the chromatogram of the spiked sample but not for the control (unspiked sample). Similarly, when the concentration of anthraquinone was lowered to 0.67 μg/L (Figure 5), anthraquinone peak was also observed in the chromatogram of the spiked sample. This suggests that this method can be applied as low as European Union MRL level for anthraquinone [5], assuming that all anthraquinone in tea is dissolved in the infusion tea. However, since many unknown peaks were also observed in the chromatogram, further investigation for sample preparation is still needed to eliminate the interferences from the samples.
4. Conclusion
GC-ECD may become an alternative way for anthraquinone analysis in tea samples. The optimization gives an IDL of 0.05 μg/L, the repeatability was good (0.015% for retention time and 6.681% for area) and the linearity was also good (r=9.9973). Analysis of spiked sample with low volume liquid-liquid extraction was able to extract anthraquinone as low as 0.67 μg/L. This study suggests some improvement to eliminate interferences in the tea samples and to validate the method.

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