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Metabolite Formation of Pesticide Isoprocarb in Coffee Beans During Short-Term Storage and Condition

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Abstract

Isoprocarb (IPC) is one of the most important carbamate pesticides for white flea control in coffee plants. The prevalence of isoprocarb pesticide residues in coffee, i.e., green coffee beans, is a cause for concern. Green coffee beans were intentionally contaminated with isoprocarb at concentrations >0.01 mg/kg, which was investigated in this research. Quantitative analysis of isoprocarb with the QuEChERS method and qualitative analysis for metabolite formation was characterized using UV-Vis, FTIR, ^{1}H-NMR, GC-MS, and LC-QTOF-MS. Based on the data, the metabolite formed is *o*-cumenol through the hydrolysis reaction of *o*-aryl carbamate with the enzyme carboxylesterase. *o*-cumenol or 2- isopropylphenol as a phenol derivative. To verify the existence of metabolite analytes that are believed to be there in coffee tainted with isoprocarb, more reliable analytical techniques utilizing analytical standards must be developed. Isoprocarb concentrations decreased along the storage time, especially in unsterilized conditions. As a toxic compound, a quantitative structural activity relationship study (QSAR) was initially carried out through a software application for estimating toxicity (TEST) provided by the US Environmental Protection Agency (EPA). For additional information, based on the LC_{50} and LD_{50} data confirmed from the TEST application, it was concluded that isoprocarb is more toxic than *o*-cumenol.

1. Introduction

Pesticides are indispensable in agricultural production to control weeds and insects [1]. Pesticides are classified into five main classifications based on their chemical composition: organochlorines, organophosphates, carbamates, pyrethrins, and pyrethroids [2]. One of the classifications of pesticides is carbamate, which is widely used because it has several advantages, including degradation easily in the natural environment, leading to less contamination, lower toxicity, and more widespread use than organophosphate pesticides [3, 4, 5]. Although carbamate pesticides are less potentially hazardous than organophosphate pesticides, they must be used cautiously because excessive pesticide use will still impact the environment. Regarding this lack of caution, at the end of 2021, the international coffee trade became concerned due to the presence of isoprocarb pesticide residues in coffee export commodities to Japan [6]. Isoprocarb is a pesticide in the carbamate class of insecticides. Figure 2(a) shows the chemical structure of isoprocarb.

One popular beverage that also has Indonesian origins is coffee. Based on the International Coffee Organization (ICO) data, global coffee consumption reached 166.35 million 60-kilogram bags in 2020/2021. This number increased by 1.3% compared to the previous period, 164.2 million bags measuring 60 kilograms [7]. The BPS-Statistics Indonesia (Badan Pusat Statistik, BPS) has officially reported that the anticipated coffee production in Indonesia for 2022 is projected to reach 794,800 tonnes. The amount has increased by 1.10% compared to the previous year, which amounted to 786,191 tonnes [8]. In Indonesia, the government has approved using pesticides containing isoprocarb to

control mealybugs in coffee plants, especially green coffee beans. One of the consequences of the unwise use of pesticides is the contamination of coffee with isoprocarb, which started with unscrupulous farmers spraying insecticide containing the active ingredient isoprocarb on coffee plants to repel ants during harvest.

Liu *et al.* [9] examined metabolite mapping and pollutant monitoring in chicory plants subjected in vivo to several carbamate herbicides. Based on this research, it can be assumed that the probable metabolite found in the isoprocarb-contamined coffee beans is *o*-cumenol. Therefore, there is a probability that the isoprocarb that contaminates coffee beans may likewise break down into its metabolites. According to this reasoning, the isoprocarb contaminating coffee beans may also decrease its metabolites during storage. To enhance coffee commodity-based food safety, it is necessary to determine whether the isoprocarb that contaminates coffee beans converts into other metabolites that are also hazardous to human health. This research presents several instrumentation approaches for identifying the presence of isoprocarb metabolites in coffee beans contaminated with isoprocarb in this research.

Toxicity studies of *o*-cumenol as metabolite formation of isoprocarb continue to develop. To achieve the objectives of this study, a method for analyzing carbaryl residues in green coffee beans using extraction methods used in quality assurance was employed. The testing results on coffee beans that still contain isoprocarb pesticide residue strengthen the case for this research to be carried out as an input for handling and describing the quality of coffee beans before they are shipped to the destination country. Furthermore, QSARs have been identified using the TEST application provided by the US EPA to determine the toxicity of isoprocarb metabolite products formed during storage. The data generated by the TEST application will be used to guide future treatments.

2. Experimental

The research started by identifying isoprocarb-contaminated coffee stored in the laboratory and subsequently observing a degradation in isoprocarb concentration in contaminated coffee after 90 days of storage. Then, it concluded by identifying metabolite formation in various analytical instruments.

2.1. Materials and Instruments

The materials used in this study were contaminated green coffee beans. Isoprocarb with analytical standard grade and magnesium sulfate anhydrous were purchased from Sigma-Aldrich. Acetonitrile, acetic acid, and sodium acetate were purchased from Merck. Primary-secondary amine bulk, C-18 bulk, and graphitized carbon black bulk were purchased from Agilent. The equipment used in this study was disposable plasticware for QuEChERS extraction, micropipette 10–100 µL and 100–1000 µL, precisa XT 220A analytical balance, and refrigerated centrifuge. UV-Vis Spectrophotometer (SHIMADZU UV- 2600) FTIR (SHIMADZU IRPrestige-21), ^{1}H-NMR (Bruker Avance Neo 500 MHz with Cyro), GC-MS/MS (SHIMADZU TQ-8040NX), and LC-QTOF-MS (Waters, Xevo G2-S QTof) were used for characterization.

2.2. Storage of Isoprocarb-Contaminated Coffee in the Laboratory

Coffee spiked with isoprocarb pesticide was stored in a container in the laboratory for 90 days at a temperature of 25.2°C and 54% humidity. Control samples were prepared by sterilizing coffee samples stored for 20 days using a UV lamp. Control samples were stored in a container that had been sterilized as well and given silica gel to regulate the humidity. Both samples were stored at 25.2°C and 54% humidity.

Table 1. GC-MS/MS conditions for isoprocarb analysis

Parameter	Setting
Interface temperature	290°C
Pressure	76.2 kPa
Total flow	14.4 mL/min
Column flow	1.04 mL/min
Linear velocity	37.9 cm/s
Purge flow	3.0 mL/min
Slit ratio	-1.0
Injection mode	Splitless
Carrier gas	Helium
Column	Rtx5-MS
Ion source temperature	100°C
m/z Isoprocarb 1	136.00 > 121.00
m/z Isoprocarb 2	136.00 > 103.00

Table 2. LC-QTOF-MS condition for metabolite screening

Parameter	Setting
Chromatographic Separation	
LC system	Ultra Performance Liquid Chromatography (UPLC)
Column	C18 (1.8 µm 2.1 × 100 mm) HSS
Temperature	50°C (column), 25°C (room)
Mobile phase	(A) Water + 5 mM ammonium formic and (B) Acetonitrile + 0.05 % formic acid
Injection volume	5 µL
Mass Spectrometry	
System	ESI (electrospray ionization)
Mode	Positive
Mass analysis range	50 – 1200 m/z
Source temperature	100°C
Desolvation temperature	350°C
Cone gas flow	0 L/hr
Desolvation gas flow	793 L/hr
Collision energy	4 V (low energy)
Rampt collision energy	25 – 60 V (high energy)

2.3. Identification of Degradation for Isoprocarb Concentration in Contaminated Coffee after Storing for 90 Days

The concentration of isoprocarb in coffee was tested during short-term storage time (90 days), according to the test method that was also carried out for carbaryl pesticide residues in coffee [10]. Isoprocarb concentration was calculated using the external standard method. The sample was calculated with an isoprocarb calibration curve, which was prepared with a spiking standard isoprocarb analytical standard. Coffee was extracted using the QuEChERS (Quick Easy Cheap Effective Rugged Safe) extraction method using acetonitrile solvent. This step served to guarantee that the concentration of the coffee sample that was to be identified dropped while it was being stored. Isoprocarb concentration in the contaminated coffee samples was calculated using GC- MS/MS. The method and condition for analysis in GC-MS/MS are shown in Table 1.

2.4. Identification of Metabolite Formation on Various Analytical Instruments

Residual extracts of coffee samples in acetonitrile solvent were analyzed using various instrumentation, including UV-Vis spectrophotometer, FTIR, ^{1}H-NMR, GC-MS, and LC-QTOF-MS spectrometers. These various instruments were used to identify the formation of metabolite products derived from isoprocarb before specific quantitative analysis methods were developed. The method and condition for analysis in LC-QTOF-MS are shown in Table 2. Before conducting the ^{1}H-NMR analysis, phytochemical screening was carried out using TLC analysis to prove the presence of phenol in the sample extract [11].

2.5. QSAR Study on Isoprocarb and Metabolite Products

A QSAR study was conducted using the TEST application provided by the US EPA to determine the toxicity of isoprocarb metabolite products formed during storage. This study is essential for evaluating the toxicity level of isoprocarb and metabolites that may be formed during storage.

3. Results and Discussion

The preliminary identification of coffee extract was successfully carried out by determining the concentration of isoprocarb in coffee stored for 90 days (Table 3). The coffee was extracted with acetonitrile using the QuEChERS extraction method. As reported by Liu *et al.* [9], the contamination of isoprocarb in mustard plants resulted in the formation of a new metabolite, 2- isopropylphenol or *o*-cumenol. Figure 2(b) shows the chemical structure of *o*-cumenol. Table 3 shows the concentration of isoprocarb in coffee decreased after storage for 90 days using GC-MS/MS. Based on this data, a preliminary identification of the formation of metabolite products from contaminated isoprocarb coffee beans on various suitable analytical instrumentation was carried out.

Table 3. Isoprocarb concentration during 90 days of storage using GC-MS/MS

No.	Day	Concentration (ppb)
1	0	2991.98
2	7	2611.74
3	20	2355.47
4	90	525.37

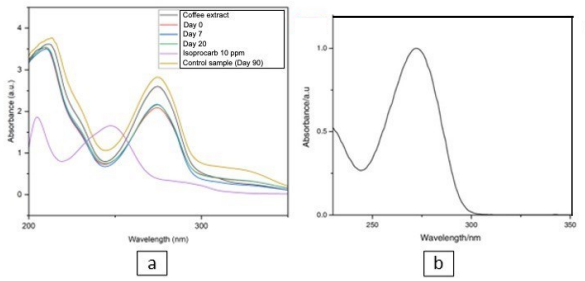


Figure 1. (a) UV-Vis spectra of coffee extract during storage in acetonitrile solvent at various storage times, and (b) UV-Vis spectra of caffeine from literature

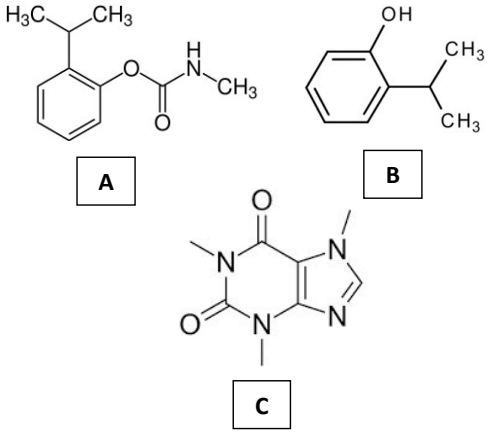


Figure 2. (a) Chemical structure of isoprocarb, (b) *o*- cumenol, and (c) caffeine

The documented bacterial breakdown processes of various carbamates, including oxidoreductases and hydrolases, are essential to point out to share metabolic pathways, i.e., ring hydroxylation, the hydrolysis products by amidases or esterases in the case of aryl carbamates, the production of related dihydroxy chemicals, and ring-cleavage that ensues to produce aliphatic intermediates that are directed toward the core metabolism [12]. This scientifically clarifies the reason why the concentration of isoprocarb in contaminated coffee beans decreases over time. In this case, this study compared sterile and nonsterile conditions at room temperature storage. The presence of bacteria in unsterilized storage conditions accelerates the decomposition of isoprocarb. Table 3 shows that isoprocarb concentrations decrease with storage time.

3.1. UV-Vis Spectra

Raheem *et al.* [13] constructed a method for testing pesticide residues in raw milk using a UV-Vis spectrophotometer. The optimum wavelengths of identification using UV-Vis for the initial coffee extract containing isoprocarb, standard isoprocarb solution, and coffee extract after 90 days of storage are compared in Figure 1. Due to the interference of caffeine in the wavelength range of 250–300 nm, these identification results have not been able to shed light on the phenomena that occur in coffee samples when compared to the absorbance of caffeine (Figure 2(c) shows the chemical structure of caffeine) from the literature, which is 274 nm [14]. The analyzed QuEChERS sample extract still contains much caffeine, so the reading with the UV-Vis spectrophotometer is disturbed. Moreover, the reliability of identification through UV-Vis is compromised due to the prevalence of caffeine compounds in the sample extract. Consequently, desired analytes, such as isoprocarb and the possible metabolite product, *o*- cumenol, are not properly observed.

3.2. FTIR Spectra

Further identification to determine the possibility of metabolites formed is using FTIR. FTIR has been widely used to detect the presence of pesticide residues for various practical reasons that can support the analysis [15, 16, 17]. Figure 3 presents a comparative image of the FTIR spectra between samples stored for 90 days and those stored for a shorter duration. The FTIR spectrum from sample stored for 20 days reveals the formation of compounds other than isoprocarb. These compounds were identified by the presence of a broadened peak within the wavenumbers of 3600–3000 cm^{-1} [18]. This indicates that phenolic compounds were formed in the sample, as shown by the vibration of the O–H stretching band (3584–3700 cm^{-1}). The results of this analysis need to be reinforced with the results of ^{1}H-NMR readings.

3.3. ^{1}H-NMR Spectra

Preliminary studies to determine phenol in the sample extract using TLC proved the presence of phenol in the sample extract, indicated by a purple spot [11]. Figure 4 shows TLC results for phenol presence. Identification of phenol compounds can also be done using NIR, FTIR, and LC-MS instrumentation [19, 20]. The ^{1}H-NMR characterization strengthened the evidence for forming *o*-cumenol (Figure 5). In contrast, the *o*- cumenol structure is shown in Figure 2(b). ^{1}H-NMR spectra were used to analyze more complex samples containing residue pesticide [21, 22, 23]. ^{1}H-NMR identification confirmed the formation of a new compound, indicating the formation of new or higher peaks around the chemical shift (δ) 1–3 ppm. For phenols, the ^{1}H-NMR chemical changes are not very noticeable. On the other hand, one anticipates the aromatic protons to be discovered at 7–8 ppm and the -OH signal to be in the 4– 7 ppm region [24]. It can also be seen that chemical shifts of 1-5 ppm can indicate phenol compounds [25].

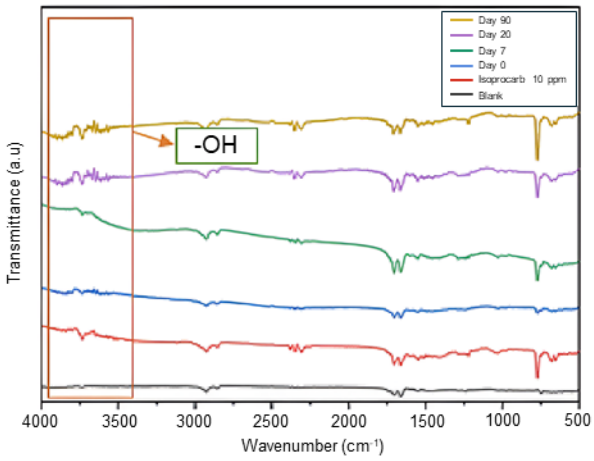
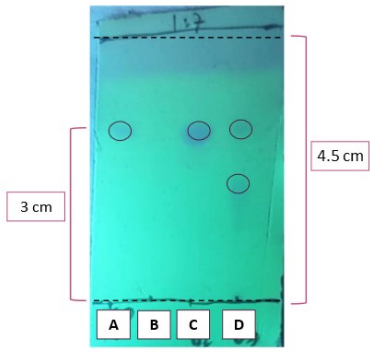


Figure 3. FTIR spectra of coffee samples containing isoprocarb at different storage times and standard isoprocarb in acetonitrile as a solvent



Description:
A: Spot for *o*-cumenol standard in methanol solvent
B: Spot for freshly contaminated green coffee bean extract in methanol solvent
C: Spot for green coffee bean extract after 90 days of storage in methanol extract
D: Spot for green coffee bean extract after 90 days of storage in 2-propanol extract

Figure 4. TLC screening for phenol identification on a 254 nm UV lamp

The ^{1}H-NMR data of the initial sample and the ^{1}H- NMR data after being stored for 90 days were compared, revealing the chemical shift of isoprocarb removed from the sample in both the initial and after it was stored. Based on Figure 5, the ^{1}H-NMR spectra for the initial sample are δ -3.42 (4H, s), 3.59 (4H, s), 4.00 (4H, s), 7.27 (2H, s), and 7.52 (1H, s). Meanwhile, for ^{1}H-NMR spectra after 90 days of storage are δ -0.88 (0H, s), 1.25 (3H, s), 3.41 (4H, s), 3.59 (4H, s), 4.00 (4H, d, J = 0.35 Hz), 7.27 (0H, s), and 7.52 (1H, d, J = 0.43 Hz).

Chemical shifts in the range of 0.5–3 ppm indicate other chemical contents in the coffee extract that need further analysis. Based on Figure 5, there is a peak at chemical shift around 1.26 ppm, which indicates the presence of the -CH_3 group. The most obvious change is the chemical shift around 7 ppm, where the presence of chemical content in the coffee extract changes the multiplicity of isoprocarb or *o*-cumenol standards to be different from the initial multiplicity. This phenomenon shows that for complex matrices such as coffee, NMR analysis still requires preliminary separation to produce a more precise analysis, and the QuEChERS method Figure 6 shows the comparison of the spectra of coffee extract after storage with the standards of isoprocarb and *o*- cumenol in pure solvent.

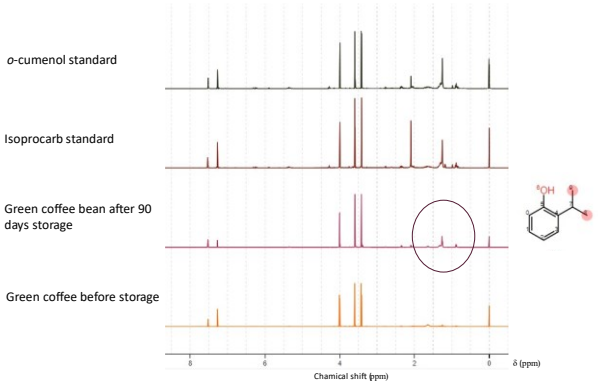


Figure 5. ^{1}H-NMR spectra for coffee extract in storage compared with standards in coffee extract diluted with $CDCl_3$ solvent

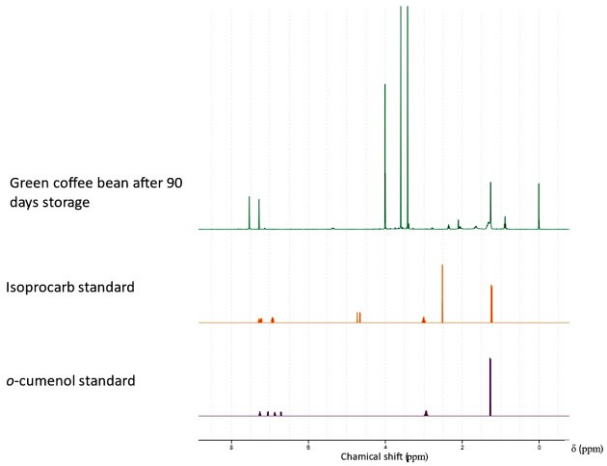


Figure 6. ^{1}H-NMR spectra for coffee extract in storage compared with standards in $CDCl_3$ solvent

Due to the limitations of interpretation of ^{1}H-NMR related to the complexity of the matrix, identification with other instrumentation was added to strengthen the suspicion of compounds formed in coffee bean samples contaminated with isoprocarb pesticides.

3.4. GC-MS Spectra

The most commonly used instrument for analyzing volatile pesticide compounds is GC-MS. Gas chromatography-tandem MS/MS is a powerful instrument for various pesticide residue analysis purposes [26, 27, 28, 29]. GC-MS identification is based on a peak representing a product metabolite formed from isoprocarb. The chromatogram is compared to the GC-MS library, which is provided by WILEY7 LIB for this instrument (Figure 7). The library discovered that a phenol compound had been formed in contaminated coffee beans that had been stored.

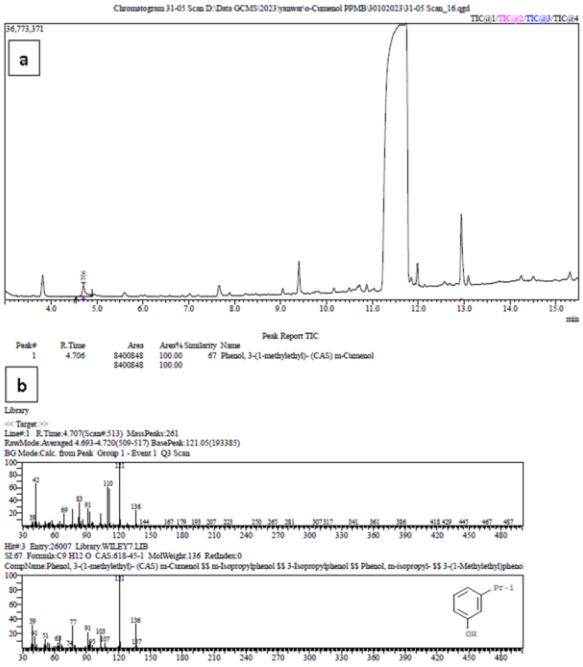


Figure 7. Scan results using GC-MS instrumentation after the sample was stored for 20 days of (a) a sample full-scan chromatogram and (b) spectrogram of the matching peak at a retention time of 4.7 in the library

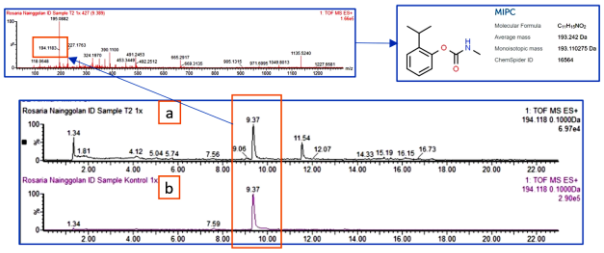


Figure 8. LC-QTOF-MS Isoprocarb spectra of a) sample T2, and b) control sample after 90 days storage

According to The Metabolomics Innovation Centre (TMIC), through the BioTransformer 3.0 application as an *in-silico* metabolism prediction tool (https://biotransformer.ca), several metabolite products may be produced by isoprocarb. The possible pathways for forming isoprocarb metabolites are transformations that occur in the human gut and abiotic transformations. Based on the GC-MS library confirmed that there is a phenolic compound inside contaminated green coffee beans, as shown in Figure 7. Regrettably, GC-MS cannot distinguish the exact location of the functional groups. This identification can only read the relative molecular abundance of the compound.

3.5. LC-QTOF-MS Spectra

Coffee beans are contaminated with isoprocarb pesticide residues, which create metabolite products. This has led to additional investigation of coffee bean extracts using LC-QTOF-MS devices. LC-QTOF-MS is used to identify another possibility of product metabolites from contaminated coffee isoprocarb. Figure 8 shows the result of isoprocarb concentration identification using LC- QTOF-MS. The control samples are contaminated coffee beans stored under sterile containers and sterilized with UV light before storing at room temperature. In contrast, T2 samples are contaminated coffee samples not stored in sterile conditions and at the same temperature and humidity (25.2°C and 54% or room temperature).

The data presented in Figure 9 indicates that the concentration of the control sample is higher than that of sample T2. This is supported by the relative abundance of m/z isoprocarb (194.118) in the chromatogram of the control sample, which is 2.90×10^5) higher than that of the T2 sample, which is 6.97×10^4. It concludes that the concentration of isoprocarb in sample T2 has decreased, so it can be assumed that there has been a change in isoprocarb into another compound.

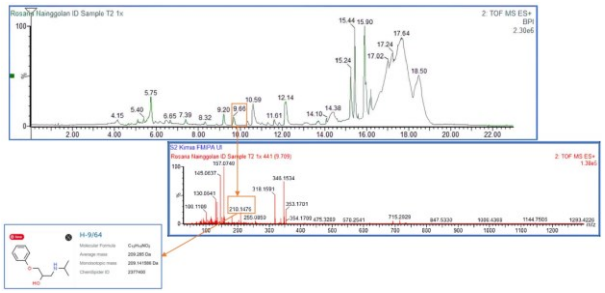


Figure 9. LC-QTOF-MS chromatogram and spectrogram of a sample of possible metabolites formed at a retention time of 9.65 with m/z 210.5476

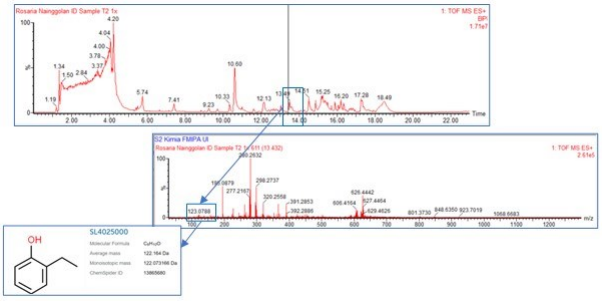


Figure 10. LC-QTOF-MS chromatogram and spectrogram of a sample of possible metabolites formed at a retention time of 13.49 with m/z 123.0788

Analysis using a Q-TOF tandem MS detector is useful in determining the possibility of metabolites forming from the original compound [30, 31, 32]. Figure 9 is one of the possible metabolites, m/z 210.1476, and Figure 10 is another possible metabolite with m/z 123.0788. This information was obtained from MassLynx software from Waters™. After obtaining the mass fragmentation of the compound, the ChemSpider website was used to obtain molecular formulas that may be similar to the target compound (compared with the corresponding literature).

According to The Metabolomics Innovation Centre (TMIC), through the BioTransformer 3.0 application (https://biotransformer.ca), several metabolite products may be produced by isoprocarb. Isoprocarb in coffee can yield several metabolite products (8 metabolites) via several different processes, such as abiotic transformation and the o-aryl carbamate hydrolysis process.

3.6. QSAR Results for Isoprocarb and Metabolite Products

Based on preliminary identification obtained from several different instrumentations, it was found that the potentially formed metabolites are phenol compounds. *o*- Cumenol or 2-isopropylphenol are most likely the chemicals found in tainted green coffee beans, based on the pathways of potential metabolite production in isoprocarb. The possible reactions based on The Metabolomics Innovation Center (TMIC) through the BioTransformer 3.0 application are explained in Figure 11. This reaction, referred to as hydrolysis of *o*- arylcarbamate (Figure 12), occurs with the presence of the enzyme carboxylesterase.

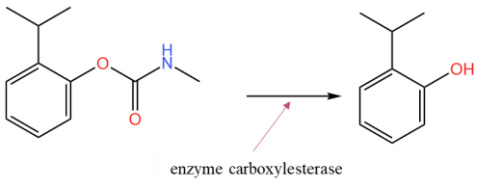


Figure 11. *o*-Cumenol formation reaction (TMIC, through the BioTransformer 3.0 application)

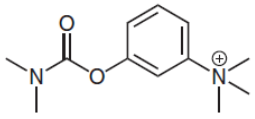


Figure 12. *o*-Arylcarbamate

Table 4. TEST result for isoprocarb and *o*-cumenol

Data compared	Isoprocarb	*o*-Cumenol
Fathead minnow LC_{50} (96 hr) (mg/L)	7.34	19.55
Daphnia magna LC_{50} (48 hr) (mg/L)	1.81	5.78
T. Pyriformis IGC_{50} (48 hr) (mg/L)	101.52	75.27
Oral rat LD_{50} (mg/kg)	288.81	551.08

The toxicity level of metabolite products in this research is also calculated using a QSARs study through the TEST application [33] provided by the US EPA. The results (Table 4) showed that the metabolite *o*-cumenol formed from isoprocarb is less toxic than isoprocarb. This is known from the LC_{50} and LD_{50} data values of isoprocarb, which are smaller than *o*-cumenol (a possible metabolite product).

Based on TEST data, it is determined that the metabolite product formed, *o*-cumenol, has lower toxicity compared to the original compound isoprocarb, as indicated by the values of Fathead minnow LC_{50} (96 hr) (mg/L), *Daphnia magna* LC_{50} (48 hr) (mg/L), and Oral rat LD_{50} (mg/kg) for isoprocarb. In terms of toxic effects, both of these compounds have the potential to become environmental toxins, but *o*-cumenol does not. According to Sharma *et al.* [23] research, increasing functional groups causes increased toxicity. Idroxyl-group molecules are more responsive, which is an important identification throughout the process. Sharma *et al.* [23] identified hydrolysis as a dangerous insecticide.

4. Conclusion

Based on the different identifications performed, it was determined that more advanced instruments are needed to analyze samples with complex matrices to lessen the matrix's impact on the target analytes. In conclusion, there's a chance that metabolite chemicals made from isoprocarb-contaminated coffee beans exist. In Chinese mustard samples, particularly from GC-MS scanning, several identification and characterization results result in synthesizing phenol compounds, which is the same as converting isoprocarb to its metabolites. Phenol compounds theoretically produced from isoprocarb metabolism are 2-isopropylphenol, also known as *o*-cumenol. Developing more rigorous analytical methods using analytical standards is needed to confirm the presence of metabolite analytes suspected in isoprocarb-contaminated coffee to determine how quickly isoprocarb changes its metabolites (*o*-cumenol) during storage. Chromatographic methods with high precision and accuracy may be a reference for analytical methods on samples with complex matrices. As additional information, the QSARs study concludes that the possible metabolite, *o*-cumenol, formed from isoprocarb, is less toxic than isoprocarb. However, it needs to be studied further for safety, whether it accumulates in the body or causes the same danger as isoprocarb.

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