

# Differences in chemical characteristics due to different roasting of robusta coffee beans

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## ABSTRACT

Roasting is a process that contributes to the formation of compounds and flavors in coffee beans. Temperature and length of time are the main factors in the roasting process. Generally, the range of temperatures and roasting times varies significantly for different varieties of coffee beans. This study aims to determine the effect of roasting on changes in the chemical characteristics of robusta coffee beans (*Coffea canephora*) from Sidomulyo Village. This study used different brewing methods to brew roasted robusta coffee beans with the best chemical characteristics used a factorial randomized block design with two factors: roasting temperature, which consisted of three levels (185, 190, and 195 °C), and roasting time, which consisted of three levels (10, 13, and 16 minutes). The roasted coffee beans were analyzed for water content, ash content, caffeine content, total phenol, and pH. The results of this study obtained a water content value of  $3.523 \pm 0.129\%$  to  $1.939 \pm 0.025\%$ , ash content of  $8.119 \pm 0.115\%$  to  $4.315 \pm 0.260\%$ , a caffeine content of  $2.494 \pm 0.015\%$  to  $2.176 \pm 0.021\%$ ; total phenol of  $6.251 \pm 0.101\%$  to  $4.334 \pm 0.117\%$ ; and a pH value of  $6.675 \pm 0.126$  to  $4.075 \pm 0.171$ . At this stage, the best treatment (Zeleny method) is produced by robusta coffee beans roasted at 185°C for 10 minutes. Robusta coffee beans with the best treatment have a moisture content of  $3.523 \pm 0.129\%$ ; ash content of  $4.315 \pm 0.260\%$ , the caffeine content of  $2.494 \pm 0.015\%$ ; total phenol of  $6.251 \pm 0.101\%$ ; and a pH value of  $4.075 \pm 0.171$ .

**Key words:** *Coffea canephora*; roasting; caffeine; phenol.

## 1 INTRODUCTION

Coffee drinks are quite popular with the people of Indonesia and the world. Coffee is a commodity that grows a lot in Indonesia. Coffee production in Indonesia ranks as the third largest coffee producing country in the world after Brazil and India (International Coffee Organization, 2022). The quality of coffee is greatly influenced by its handling during harvest and post-harvest. Coffee is a complex product. According to Sunarharum, Williams and Smyth (2014), coffee *flavor* is influenced by many factors, including soil quality, seed maturity, geographical conditions of planting, production process, processing, roasting, and brewing.

The roasting process has a role in the formation of compounds and *flavors* with heat treatment and time. Roasting is also a very important process for developing the specific sensory properties (aroma, taste and color) that underlie coffee quality. The taste and aroma of roasted coffee is largely determined by the temperature and duration of roasting which affect changes in color, moisture content, bean size and bean shape (Sutarsi; Rhosida; Taruna, 2016). The quality of coffee beans can be improved if the roasting process is carried out at the right temperature and roasting time to get the water content and acidity level according to national standards. The aim of this study was to obtain the best combination of temperature and roasting time for robusta coffee beans according to national standards.

In the roasting process, there are several influences caused by temperature and length of roasting time, as in the

research of Fikri, Nuriman and Yushardi (2022), where the roasting temperature continues to increase but the density decreases; This shows an inverse relationship between temperature and density of robusta coffee beans. The higher the temperature used, the greater the value of the ash content of the coffee beans, the longer the roasting takes place, and the higher the ash content of the coffee beans (Edvan; Edison; Same, 2016). Several factors, including coffee quality, cause differences in coffee ash content.

## 2 MATERIAL AND METHODS

The main raw material used in this study was red pick robusta coffee beans obtained from Sidomulyo Village, Jember Regency with *fullwash post-harvest treatment* and then sorted. While the materials used for analysis included solid caffeine, chloroform, CaCO<sub>3</sub>, and 50% Folin-Ciocalteu reagent.

This research method used a randomized block design (RBD) which was arranged in a factorial 3 x 3, totaling 9 treatments and each combination was repeated four times. The treatments were obtained by combining temperature and drying time (roasting time). The first factor is the roasting temperature which consists of three levels (185, 190, and 195 °C). The second factor is the roasting time which consists of three levels (7, 10, and 13 minutes). The process of chemical analysis on roasted Robusta coffee beans includes: moisture content (Association of Official Analytical Chemistry - AOAC, 2016), ash content (AOAC, 2016), caffeine content (Maramis; Citraningtyas; Wehantouw, 2013), total phenols (Wungkana;

Suryanto; Momuat, 2013), and pH (AOAC, 2016). In addition, a volatile content test was also carried out.

Determination of the best treatment for roasting robusta coffee beans is determined by the *Multiple Attribute methods* (Zeleny, 1987). The parameters used in determining the best treatment are the lowest water content (%), highest ash content (%), lowest caffeine content (%), highest total phenol (%), and lowest pH.

The observed data were statistically analyzed using the Minitab 17 application with the Analysis of Variance (ANOVA) method with a 95% confidence interval. If the factors studied were significantly different ( $P < 0.05$ ), Tukey's follow-up test was carried out with a 95% confidence interval.

## 2.1 Water content

Analysis of water content using the thermogravimetric method was carried out by weighing 2 g of sample in a cup of known weight and then drying it in an oven at 105 °C for 4 hours. After that, it was cooled in a desiccator and weighed until a constant weight was obtained. The weight is considered constant when the difference in weighing is not more than 0.2 mg. Calculation of the water content is obtained by comparing the sample weight before drying and the sample weight lost after drying multiplied by 100%.

## 2.2 Ash content

Accurately weigh 2 g of the sample in a porcelain cup of known weight. Charcoal samples above the burner flame, then ashes in an electric furnace. At a maximum temperature of 550 °C until complete ashing (occasionally open the furnace door slightly so that oxygen can enter). Cool in a desiccator, then weigh with a fixed weight.

## 2.3 Caffeine content

Caffeine analysis was carried out using a spectrophotometric method which involved adding the sample to hot water, then stirring and filtering it to produce a liquid which was then added to  $\text{CaCO}_3$  (calcium carbonate). The coffee solution was placed in a separatory funnel and extracted four times, with chloroform added each time. A bottom layer will form, which can be removed with the extract (chloroform phase), then placed in a rotary evaporator until all the chloroform has evaporated. Solvent-free caffeine extract was precipitated in a measuring flask, diluted with distilled water, and homogenized. Caffeine levels were measured using a UV-VIS spectrophotometer at a wavelength of 275 nm. From the results obtained, the standard curve that represents a standard solution of caffeine as an equation is  $y = ax + b$  and produces a correlation coefficient value in each equation.

## 2.4 Total phenols

The fraction was determined for the total phenolic content using the Folin-Ciocalteu method. As much as 0.1 mL

of each 1000 halm fraction was put in a test tube, then 0.1 mL of 50% Folin-Ciocalteu reagent was added. The mixture was vortexed for 2 minutes, then 2 mL of 2% sodium carbonate solution was added. Then the mixture was incubated in a dark room for 30 minutes. The absorbance was read at  $\lambda$  750 nm with a spectrophotometer. The total phenolic content was expressed as gallic acid equivalent to mg/kg of extract.

## 2.5 pH

pH levels are measured using a pH meter; the first step is to turn on the pH meter by pressing the ON button, then calibrate it first with a buffer solution of pH 4.0 and pH 7.0. After calibration, the sample is put into a tube or glass, then the pH meter is inserted into the sample, and the results will appear on the pH meter screen and then be recorded.

# 3 RESULTS

## 3.1 Water content

The results of the analysis of water content can be seen in Table 1. Based on the results of the ANOVA analysis, the treatment of temperature and roasting time had a significant effect ( $\alpha = 0.05$ ) on the decrease in water content in robusta coffee beans.

**Table 1:** Water content of roasted robusta coffee.

Treatments	Temperature(°C)	Time (minutes)	Water content (%)
1		10	3.523 ± 0.129a
2	185	13	2.287 ± 0.025cd
3		16	2.085 ± 0.023d
4		10	3.018 ± 0.341b
5	190	13	2.212 ± 0.026d
6		16	2.048 ± 0.027d
7		10	2.745 ± 0.474bc
8	195	13	2.104 ± 0.078d
9		16	1.939 ± 0.025d

It can be seen that roasting at 185 °C for 10 minutes produces the highest water content of 3.523 ± 0.129%, while roasting at 195 °C for 16 minutes produces the lowest moisture content of 1.939 ± 0.025%, the results of the analysis show the effect of temperature and the roasting time where the higher the temperature and the longer the roasting time, the lower the water content contained in the Robusta coffee beans. The water content contained in Robusta coffee beans in all treatments meets national standards where the maximum water content contained in coffee is 5% (Standar Nasional Indonesia, 2021).

### 3.2 Ash Content

The results of the ash content analysis can be seen in Table 2. Based on the results of the ANOVA analysis, the treatment of temperature and roasting time had a significant effect ( $\alpha=0.05$ ) on the ash content in Robusta coffee beans.

**Table 2:** Ash content of roasted robusta coffee

Treatments	Temperature (°C)	Time (minutes)	Ash Content (%)
1		10	4.315 ± 0.260e
2	185	13	5.622 ± 0.146cd
3		16	6.887 ± 0.116b
4		10	4.646 ± 0.188e
5	190	13	5.653 ± 0.175cd
6		16	7.752 ± 0.185ab
7		10	5.362 ± 0.314d
8	195	13	5.975 ± 0.1047c
9		16	8.119 ± 0.115a

It can be seen that roasting at 195 °C for 16 minutes produces the highest ash content of 8.119 ± 0.115%, while roasting at 185 °C for 10 minutes produces the lowest ash content of 4.315 ± 0.260%, the results of the analysis show that the higher the temperature and the longer the roasting time, the higher the ash content contained in Robusta coffee beans. In this experiment, only the roasting treatment at 185 °C, 190 °C and 195 °C with a time of 10 and 13 minutes had the ash content in accordance with the national standard where the maximum ash content was 6% (Standar Nasional Indonesia, 2021).

### 3.3 Caffeine Levels

Based on the results of ANOVA analysis, temperature treatment and roasting time had a significant effect ( $\alpha=0.05$ ) on the caffeine content in Robusta coffee beans. Table 3 shows an increase in caffeine content in Robusta coffee beans with the highest caffeine content of 2.494 ± 0.015% at 195 °C roasting for 16 minutes, while the lowest caffeine content was 2.176 ± 0.021% at 185 °C roasting for 10 minutes.

From these data it can be seen that the higher the temperature and the longer the time in the roasting process, the higher the caffeine content in coffee.

### 3.4 Total Phenol

Based on the results of the ANOVA analysis, temperature treatment and roasting time had a significant effect ( $\alpha=0.05$ ) on total phenol in robusta coffee beans. Table 4 shows a decrease in total phenol in Robusta coffee beans with the highest phenolic content of 6.521 ± 0.101% at roasting with a temperature of 185 °C and 10 minutes, while

the lowest phenolic content was 4.334 ± 0.117% at roasting with a temperature of 195 °C and 16 minutes time.

**Table 3:** Caffeine levels of roasted robusta coffee.

Treatments	Temperature (°C)	Time (minutes)	Caffeine Content (%)
1		10	2.176 ± 0.021c
2	185	13	2.405 ± 0.029ab
3		16	2.481 ± 0.021a
4		10	2.310 ± 0.087b
5	190	13	2.431 ± 0.021a
6		16	2.488 ± 0.091a
7		10	2.386 ± 0.024ab
8	195	13	2.462 ± 0.024a
9		16	2.494 ± 0.015a

**Table 4:** Total phenol of roasted robusta coffee.

Treatments	Temperature (°C)	Time (minutes)	Total Phenol (%)
1		10	6.251 ± 0.101a
2	185	13	5.628 ± 0.166cd
3		16	5.070 ± 0.070e
4		10	5.960 ± 0.053b
5	190	13	5.513 ± 0.073d
6		16	4.549 ± 0.102f
7		10	5.794 ± 0.031bc
8	195	13	5.213 ± 0.066e
9		16	4.334 ± 0.117g

### 3.5 pH

The results of the pH analysis can be seen in Table 5. Based on the results of the ANOVA analysis, the treatment of temperature and roasting time had a significant effect ( $\alpha=0.05$ ) on the decrease in pH in robusta coffee beans.

**Table 5:** The pH value of roasted robusta coffee beans.

Treatments	Temperature(°C)	Time (minutes)	pH value
1		10	4.075 ± 0.171d
2	185	13	5.850 ± 0.191c
3		16	6.325 ± 0.222ab
4		10	4.475 ± 0.150d
5	190	13	6.125 ± 0.171bc
6		16	6.350 ± 0.208ab
7		10	5.850 ± 0.173c
8	195	13	6.175 ± 0.150bc
9		16	6.675 ± 0.126a

It can be seen that roasting with a temperature of 195 °C for 16 minutes produced the highest pH value of  $6.675 \pm 0.126$ , while roasting with a temperature of 185 for 10 minutes produced the lowest pH value of  $4.075 \pm 0.171$ , the results of the analysis showed that the higher the temperature and the longer the roasting time, the higher the pH value of Robusta coffee beans.

### 3.6 Volatile Content

The samples used as samples for further analysis using GC-MS to estimate the volatile content in roasted Robusta coffee beans used samples treated at 185 °C for 10, 13, and 16

minutes, as well as two other treatments, namely 190 °C, and 190 °C. 195 °C for 10 minutes. The use of this treatment is because this test is only to add information and determine the volatile content in Robusta coffee at differences in roasting temperature and roasting time so that treatment is chosen at the same temperature and different roasting time; Besides that, it also uses a treatment with a different temperature and sam time of roasting. The chromatogram of roasted Robusta coffee beans can be seen in Figure 1, Figure 2, Figure 3, Figure 4 and Figure 5, while the components detected in roasted coffee beans can be seen in Table 6, Table 7, Table 8, Table 9, and Table 10.

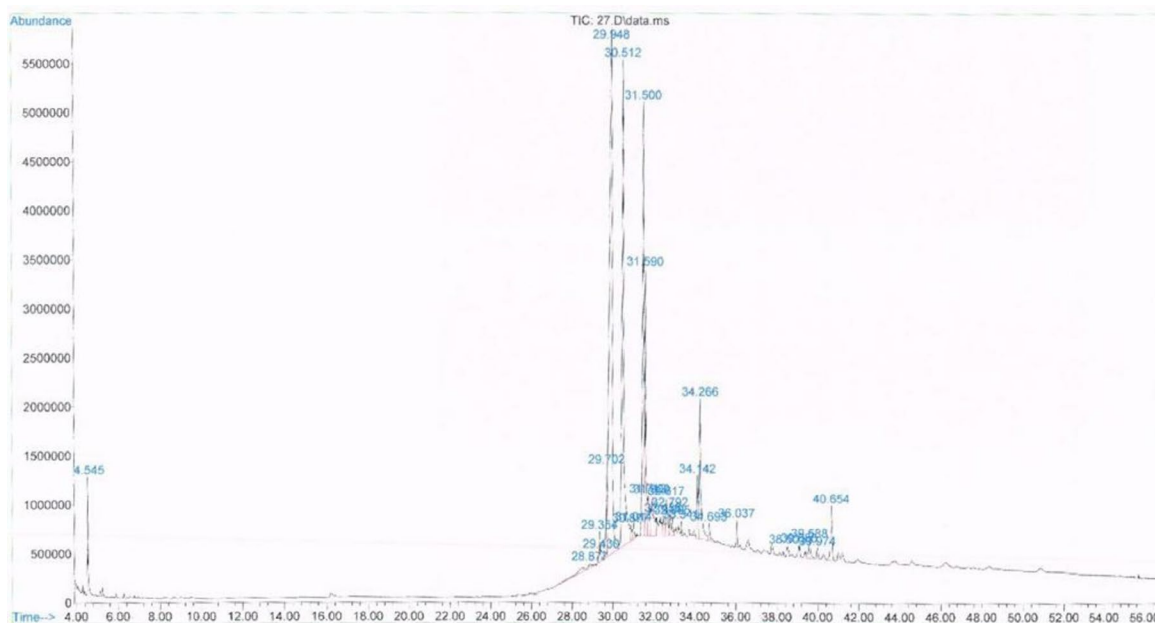


Figure 1: Chromatogram of robusta coffee beans roasted at 185 °C for 10 minutes.

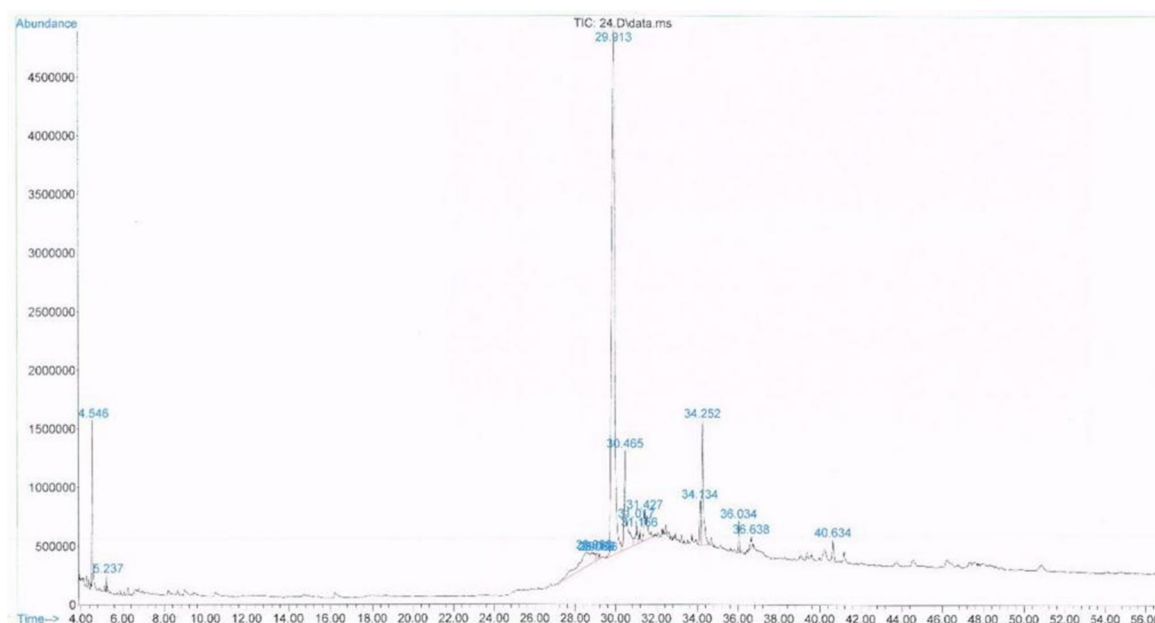


Figure 2: Chromatogram of robusta coffee beans roasted at 185 °C for 13 minutes.

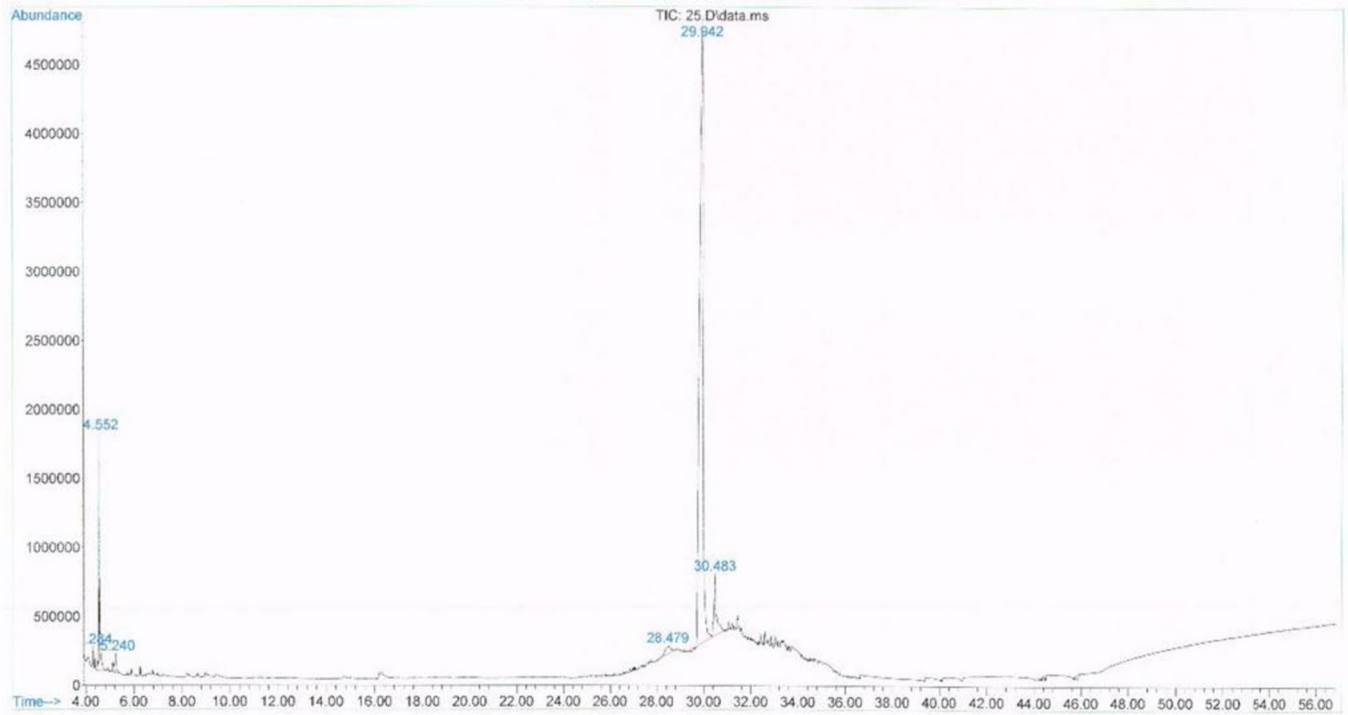


Figure 3: Chromatogram of robusta coffee beans roasted at 185 °C for 16 minutes.

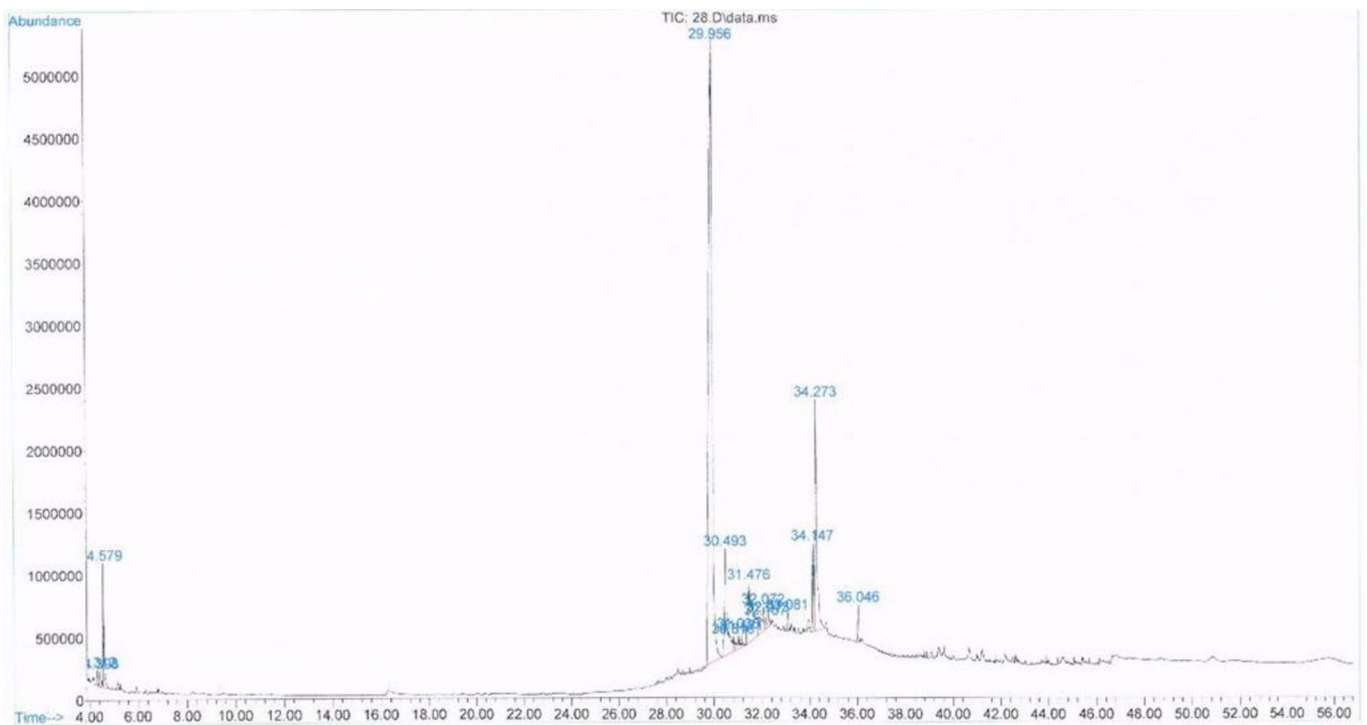
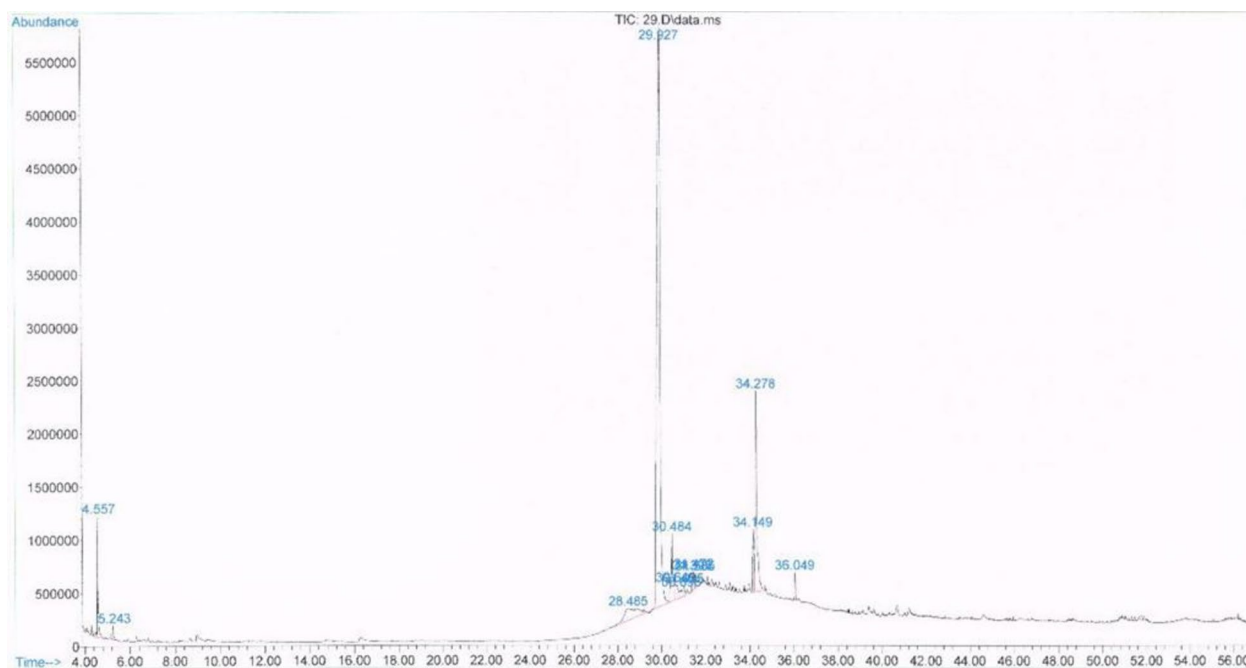


Figure 4: Chromatogram of robusta coffee beans roasted at 190 °C for 10 minutes.



**Figure 5:** Chromatogram of robusta coffee beans roasted at 195 °C for 10 minutes.

**Table 6:** Identified chemical components in robusta coffee beans roasted at a temperature of 185 °C with a time of 10 minutes.

Compounds	Retention Times (minutes)	Areas (%)
2-furanmethanol	4.542	1.96
1-methyloctahydro-2(1h)-naphthalenone	28.875	1.06
caffeine	29.951	34.09
hexadecanoic acid	30.51	20.18
9,12-octadecadienoic acid	31.503	13.5
octadecanoic acid	31.592	5.29
eicosanoic acid	32.62	1.17
5-pregnen-3.beta.-ol-20-one, trifluoacetat	34.144	1.53
7,9-dimethoxy-8-isoprophyl-4-methyl-1h-phenalen-1-one	34.268	5.29
stigmastan-3,5-diene	40.653	2.1

**Table 7:** Identified chemical components in robusta coffee beans roasted at a temperature of 185 °C with a time of 13 minutes.

Compounds	Retention Times (minutes)	Areas (%)
2-furanmethanol	4.549	4.75
6-methoxy-8-thiabicyclo [3.2.1] octan-3-ol	28.938	8
caffeine	29.91	59.3
hexadecanoic acid	30.468	8.34
tricyclo [5.1.0.0 (3,5) octane-2,6-dione, 1,3,5,7-tetramethyl-	31.02	1.59
7-pentadecyne	31.427	3.6
methanone, (5-hydroxy-3-benzofuryl) (2,5-dimethoxyphenyl)	34.137	1.69
7,9-dimethoxy-8-isoprophyl-4-methyl-1h-phenalen-1-one	34.254	7.65
squalen	36.033	1.01
stigmastan-3,5-diene	40.632	1.43

**Table 8:** Identified chemical components in robusta coffee beans roasted at a temperature of 185 °C with a time of 16 minutes.

Compounds	Retention Times (minutes)	Areas (%)
2-furanmethanol	4.549	7.89
6-methoxy-8-thiabicyclo [3.2.1] octan-3-ol	28.482	1.56
caffeine	29.944	84.07
hexadecanoic acid	30.482	5.48

**Table 9:** Identified chemical components in robusta coffee beans roasted at a temperature of 190 °C with a time of 10 minutes.

Compounds	Retention Times (minutes)	Areas (%)
2-furanmethanol	4.576	3.13
caffeine	29.958	60.75
hexadecanoic acid	30.496	6.57
9,12-octadecadecioic acid	31.475	7.78
s-[2-[n,n-dimethylamino]ethyl]n,n-dimethylcarbamoyl thiocarbohydroximate	32.075	2.35
1h-cyclopropa[3,4]benz[1,2-f]azulene-5,8-dione, 1a,3a,4,4a,6,7,7a,9b-octahydro-1,1,3a,6,9-pentamethyl-, [1as-(1a.alpha., 3a.alpha., 6.alpha., 9b.alpha.)]-	34.15	2.99
7,9-dimethoxy-8-isopropyl-4-methyl-1h-phenalen-1-one	34.274	12.04

**Table 10:** Identified chemical components in robusta coffee beans roasted at a temperature of 195 °C with a time of 10 minutes.

Compounds	Retention Times (minutes)	Areas (%)
2-furanmethanol	4.556	3.69
6-methoxy-8-thiabicyclo [3.2.1] octan-3-ol	28.482	5.64
caffeine	29.93	65.31
hexadecanoic acid	30.482	5.6
1h-cyclopropa[3,4]benz[1,2-f]azulene-5,8-dione, 1a,3a,4,4a,6,7,7a,9b-octahydro-1,1,3a,6,9-pentamethyl-, [1as-(1a.alpha., 3a.alpha., 6.alpha., 9b.alpha.)]-	34.150	2.58
7,9-dimethoxy-8-isopropyl-4-methyl-1h-phenalen-1-one	34.275	12.45

There were 3 components detected in the five samples tested as shown in Table 11 and Table 12 below:

**Table 11:** Component area volatile based on roast time differences on robusta coffee.

Temperature	Time	Areas (%)		
		2-furanmethanol	caffeine	hexadecanoic acid
185 °C	10 minutes	1.96	34.09	20.18
	13 minutes	4.75	59.3	8.34
	16 minutes	7.89	84.07	5.48

**Table 12:** Component area volati based on temperature difference roasted robusta coffee.

Time	Temperature	Areas (%)		
		2-furanmethanol	caffeine	hexadecanoic acid
10 minutes	185°C	1.96	34.09	20.18
	190°C	3.13	60.75	6.57
	195°C	3.69	65.31	5.6

## 4 DISCUSSION

### 4.1 Water content

Water is an important component in a food product whose existence can affect the appearance, texture, taste of food, determine the acceptability, freshness and durability of food ingredients in the storage process. According to Saloko et al. (2019), the decrease in coffee water content can be caused by heat transfer that occurs in the roaster into the coffee during the roasting process so that the phase contained in the coffee beans changes from the liquid phase to vapor. This phase change causes the water content contained in coffee to decrease. Musa, Charnia and Salma (2019) added, the greater the temperature difference between the heating medium and the food, the faster the transfer of heat to the food and the faster the evaporation of water. So that the hotter the temperature used, the more water in the coffee evaporates and the more water content in the coffee is reduced.

### 4.2 Ash Content

Ash is an inorganic residue after going through the process of burning organic matter. The content and composition

of ash depends on the type of material and how it is used. The mineral content in coffee is calcium, potassium, magnesium, and non-metallic compounds such as phosphorus and sulfur (Janda et al., 2020). Siregar et al. (2021) states that the higher the temperature and the longer the time used for roasting, the higher the ash content of a material. The increase in the ash content produced in Robusta ground coffee can be caused by the temperature treatment and roasting time which has an impact on reducing the water content and other compounds such as antioxidants (Yohanes; Kompiang; Agus, 2022). Research conducted by Edvan, Edison and Same (2016) and (Saloko et al., 2019) also shows that differences in temperature and roasting time have a significant effect on increasing the ash content of roasted coffee beans. Differences in the origin of raw materials and environmental factors are external factors that affect the ash content in coffee beans. (Hameed; Nuriman; Yushardi, 2018).

### 4.3 Caffeine Levels

Natural caffeine is found in coffee beans, tea leaves, cocoa beans, guarana berries and yerba maté leaves (van Dam et al., 2020). The increase in the amount of caffeine can be caused by the evaporation of water content and acids such as chlorogenic acid during the roasting process, and it is suspected that the temperature and roasting time used have not been able to evaporate the caffeine content during the roasting process (Agustina et al., 2019). Thus the percentage of non-volatile substances such as caffeine, fat and minerals will increase. The increase in the amount of caffeine in roasted coffee is thought to be caused by the decomposition of liquid and acidic substances during the roasting process, so that the amount of non-liquid content such as the percentage of caffeine, fat and minerals increases (Pilipczuk et al., 2015). The chemical properties of caffeine can melt at 236 °C and boil at 178 °C (Virhananda et al., 2022).

### 4.4 Total Phenol

Phenolic compounds are a group of molecules and their function in growth and development with defense mechanisms in plants (Abdul Gani, 2021). Basically the high temperature and heating time will cause the total phenol content to be higher as well (Krisnawan et al., 2022). However, in this study the total phenol content decreased, this was due to the roasting temperature used, which was 185-195 °C. During roasting there are several active ingredients that are damaged at high temperatures such as phenolic compounds which have an optimal temperature range of 0-90 °C. (Dewata et al., 2017). This causes the total phenol content of roasted robusta coffee beans to decrease.

### 4.5 pH

An increase in the pH value occurs because high temperatures can reduce a number of volatile acids such as

acetic acid, so that the coffee beans are roasted to decrease the acidity level (Fuller; Rao, 2017). The degree of acidity (pH) of coffee drinks greatly affects the taste. The sour taste detected in brewed coffee comes from simple aliphatic acid compounds such as acetic acid, citric acid, malic acid and pyruvic acid (Abubakar et al., 2021). Cuong et al. (2014) stated that the higher the temperature and the longer the roasting time, the higher the pH due to the degradation of various important compounds in coffee, including protein, polysaccharides, trigonellin, and chlorogenic acid. This is in accordance with the statement Ifmalinda et al. (2018) which states that coffee beans naturally contain various types of volatile compounds such as aldehydes, furfural, ketones, alcohols, esters, formic acid, and acetic acid. Roasting coffee beans will speed up the process of evaporation of volatile compounds which will be directly proportional to the increase in the pH value to near neutral.

### 4.6 Volatile Content

There was a change in the composition of the volatile content in roasted Robusta coffee which was influenced by differences in temperature and roasting time. In the difference in the length of roasting time, the longer the roasting time, the less volatile content is detected. At different roasting temperatures with the same roasting time, there was a change in the composition of the detected volatile content, where the higher the roasting temperature, the more acid content was detected. In Table 11 and Table 12, it can be seen that the longer the roasting time and temperature, the higher the area value of *2-Furanmethanol* and caffeine. Meanwhile, the area of *hexadecanoic acid* in coffee decreases with increasing temperature and time of roasting.

According to Caporaso et al. (2018), the most volatile content in coffee that can affect the aroma are *2-Furanmethanol*, acetic acid and *2-methyl pyrazine*. Each volatile component in coffee can produce a different aroma. *2-Furanmethanol* produces a smoky, burning odor (Toledo et al., 2016). Agustina et al. (2019) stated that the higher the temperature used for the roasting process, the stronger the aroma produced by roasted coffee beans. This is caused by the ripening of the roasted coffee beans. Caffeine and *hexadecanoic acid* have an influence on the taste of coffee. According to (Poole; Tordoff, 2017), caffeine is a prototypical poor bitter taste stimulus because it acts on bitter taste receptor-independent pathways, and caffeinated products most likely stimulate 'taste' receptors in nongustatory cells. Nugraha et al. (2020) also added that *hexadecanoic acid* has the ability to lower cholesterol in the blood, this compound can also be used as a flavoring, fragrance, lubricant, and cosmetic additives.

### 4.6 Best Treatment Determination

The best treatment for roasting Robusta coffee beans was obtained from roasting Robusta coffee beans at 185 °C



with a roasting time of 10 minutes. The parameter values of this best treatment already meet national standards where the maximum moisture content is 5%, the maximum ash content is 6%, and the caffeine content is 0.9-2.5% (Standar Nasional Indonesia, 2021).

## 5 CONCLUSIONS

The roasting method using a temperature of 185°C with a time of 10 minutes is the best roasting method for Sidomulyo robusta coffee beans.

## 6 AUTHORS' CONTRIBUTIONS

AFR wrote the manuscript and performed the experiment, FCH supervised the experiment and co-worked the manuscript, Y supervised the experiment and co-worked the manuscript, AFR conducted all statistical analyses, FCH and Y reviewed and approved the final version of the work.

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