



## Content of Organic Acid and Essential Oil in Natural Sweet Chestnuts (*Castanea Sativa* Mill) Growing in Giresun/TURKEY

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### ABSTRACT

This study investigated organic acid and essential oil components in sweet chestnut (*Castanea Sativa* Mill) fruits collected from naturally growing chestnut trees within the borders of Giresun province. For this purpose, chestnut samples were collected from trees in 10 different areas in Giresun. The organic acid composition of chestnuts was determined by high-performance liquid chromatography (HPLC). Essential oil components were identified with gas chromatography-mass spectrometry

(GC-MS). Five different organic acids, including oxalic, quinic, maleic, citric and succinic acid, were investigated in chestnut samples, and the highest concentrations in chestnut samples were oxalic and citric acid. In the GC-MS analysis of chestnut samples, 18 essential oils were detected and terpinolene (TPO) was the most abundant. Another vital component found in chestnut samples is limonene.

Keywords: Chestnut, organic acid, essential oil, HPLC, GC-MS, monoterpenes.

## 1. Introduction

Chestnut is a tree from the beech family with upright trunk, reddish bark and hard leaves. Thirteen chestnut species are found in the northern hemisphere. The most common type of chestnut is "*Castanea sativa* Mill", which is delicious and consumable (Burnham et al. 1986; Soyulu 2004; Serdar et al. 2014). It is the most common chestnut species in Turkey, which is the reason for selecting it for research. Sweet chestnut (*Castanea sativa* Mill.) forests represent an important landscape component in European forest ecosystems, covering more than 2.5 million hectares. Sweet chestnut has a long history in Europe and its distribution area has expanded since the IV century BC (Silva et al. 2002; Silva et al. 2004a; Conedera et al. 2004; Conedera et al. 2016).

Chestnuts play an important role in human nutrition due to containing vitamins, minerals, amino acids, and antioxidant compounds (Alasalvar & Shahidi 2008). Chestnut has important health effects linked to its nutritional composition. It is cholesterol-free, rich in vitamin C, high in fiber and gluten-free. Its importance in human nutrition increases for cases of coronary heart disease, cancer, and celiac disease (Gonçalves et al. 2010). Although chestnuts contain less fat than other types of nuts, they are a rich source of essential oil, especially linoleic acid, which is essential for the human body. The omega-3 content in chestnuts slows down the growth of cancerous cells, increases the effectiveness of chemotherapy and reduces side effects of chemotherapy (Hardman 2002).

There are studies in the literature that determined the organic acid content (Vaughan & Geissler 1997; Silva et al. 2002; Silva et al. 2004a) and essential oil content in foods (Ferreira-Cardoso et al. 1999; Benatti et al. 2004). Organic acids are compounds that have acidic properties, are classified according to the number of carboxylic functions, and have a protective effect against various diseases due to their antioxidant activities. They are involved as intermediate or final products in many important metabolism and catabolism mechanisms in biological processes, animals and plants (Vaughan & Geissler 1997; Silva et al. 2002; Silva et al. 2004a; Silva et al. 2004b; Chahardoli 2020). The nutritional value of chestnuts is affected by the nature and concentration of organic acids. Some organic acids, such as citric, succinic, fumaric and malic acids, have important roles in human metabolism (Delgado et al. 2018). Essential oils have important effects in regulating plasma lipid levels, cardiovascular diseases and many chronic diseases relating to the immune system, insulin levels, neuron development and visual function (Ferreira-Cardoso et al. 1999; Benatti et al. 2004).

The aim of this study was to examine the fruits of natural chestnut trees growing in Giresun province in terms of organic acid and essential oil components. There is no research about organic acids and essential oil, which have significant effects on human health, in chestnuts grown in the Giresun region. In addition, the number of studies about this subject worldwide is quite low. It is thought that the results obtained will contribute to the scientific literature and future research.

## 2. Material and Methods

### 2.1. Chestnut samples

Chestnut samples were taken from chestnut trees in 10 different locations close to each other in Giresun province. Approximately 2 kg of chestnut samples were collected from each sampling area. The locations of the sampling areas are given in Figure 1 and their coordinates are given in Table 1. The shells and pellicles of the chestnuts were peeled by hand. The peeled samples were kept in a refrigerator at +4 °C in sterile bags and the analysis was started the next day.

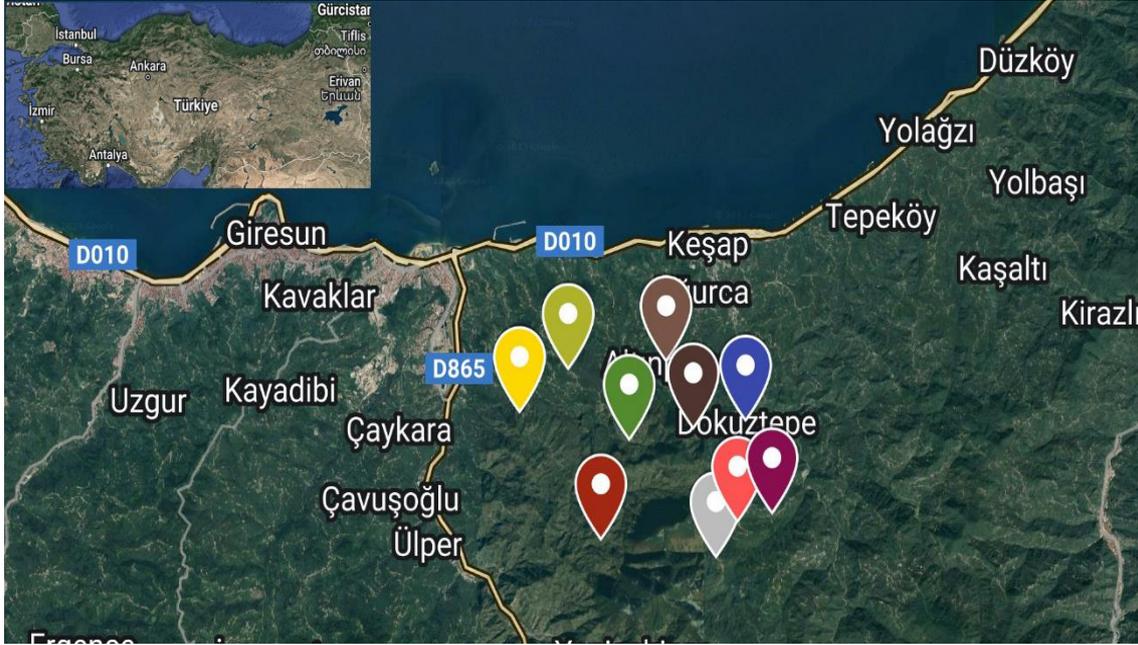


Figure 1-  Location points represent Güneyköy, Kayabaşı, Güveç, Sarvan, Alataş, Demirci, Altınpınar, Bayrambey, Sayca, and Dokuztepe, respectively

Table 1- Sampling areas and coordinates

Sample No	Location name	Latitude (°N)	Longitude (°E)
1	Güneyköy	40° 53' 34"	38° 28' 29"
2	Kayabaşı	40° 52' 32"	38° 29' 11"
3	Güveç	40° 52' 20"	38° 28' 53"
4	Sarvan	40° 52' 41"	38° 27' 49"
5	Altınpınar	40° 53' 37"	38° 30' 45"
6	Sayca	40° 52' 52"	38° 30' 32"
7	Dokuztepe	40° 52' 57"	38° 31' 24"
8	Alataş	40° 51' 55"	38° 31' 46"
9	Demirci	40° 51' 51"	38° 31' 16"
10	Bayrambey	40° 51' 24"	38° 30' 53"

### 2.2. Reagent and solutions

Oxalic acid, quinic acid, maleic acid, citric acid, and succinic acid with analytical standard (500 mg; Supelco) were purchased from Sigma-Aldrich Chemie GmbH. Ultrapure deionized water ( $\approx 18.2$  M $\Omega$ .cm) used in sample preparation and analysis was obtained from Sartorius Arium Model Ultra-Pure Water Systems. All other reagents and chemicals used were of analytical grade and solvents were HPLC gradient grade and obtained from a variety of commercial vendors (Merck and Sigma-Aldrich). An ISOLUTE C18 solid-phase extraction column (SPE; average particle size 50  $\mu$ m, pore diameter 60 Å, sorbent type non-polar, 10g /70mL) was used for the extraction of the chestnut samples.

### 2.3. Extraction of organic acids

Extraction of organic acids from chestnut samples was performed according to the method used by Silva et al. (2004b) and modified by Ribeiro et al. (2007) (Silva et al. 2004b; Ribeiro et al. 2007). Five grams of each chestnut sample was weighed and extracted by grinding with 50 mL of methanol at 40 °C with a mixer. The methanolic extract was filtered and the supernatant was re-extracted with methanol. The collected filtrates were passed through an ISOLUTE C18 column (solid phase extraction column) preconditioned with 30 ml of methanol and 70 mL of HCl (pH 2). Non-polar compounds remained in the column, polar ones such as organic acids remained in aqueous solution. The aqueous solution was evaporated, redissolved in 0.01 N sulfuric acid (1 mL) and analyzed by HPLC. The analysis results were calculated on a dry matter basis from the moisture content of chestnut samples determined by drying them in an oven at 105 °C.

### 2.4. HPLC/UV analysis of organic acids

HPLC/UV analyses of organic acids were carried out with an Agilent 1260 Infinity series with UV detection at 210 nm, using phosphate buffer ( $\text{KH}_2\text{PO}_4 - \text{H}_3\text{PO}_4$ ) with pH 2.70 as the mobile phase, at a flow rate of 0.2 mL/min with an injection volume of 5  $\mu\text{L}$ . The analytical column was Zorbax Eclipse XDB-C8 (150 mm x 4.6 mm, 5  $\mu\text{m}$ ) and column temperature was 30 °C.

### 2.5. Essential oil extraction with Clevenger apparatus and GC-MS analysis

Twenty five grams of chestnut sample was placed in a 500 mL glass bottle belonging to the Clevenger apparatus and boiled with 250 mL distilled water for 2.5 hours to obtain essential oils under reflux. The oil in the capillary, where the essential oil was collected, was sent to the separatory funnel and 5 mL of n-hexane and about 1 g of NaCl were added, and then a sufficient amount of  $\text{Na}_2\text{SO}_4$  was added to retain the remaining water.

The extracts were transferred to vials and analyzed with GC-MS (Agilent 7694 model with FID detector and 5975C series MS) instrument. Device parameters were HP-5MS column (30 m length, 0.25 mm inner diameter, 0.25  $\mu\text{m}$  film thickness), injection temperature 250 °C; carrier gas helium; flow rate: 1 mL/min; electron energy: 70 eV; and detector temperature: 280 °C. The oven temperature program had initial temperature of 50 °C for 4 minutes, increasing at 10 °C/min to 200 °C, left for 1 minute, and finally increasing with 20 °C/min to 230 °C and left for 1 minute. The data were identified using MS Library NIST and Wiley Library (Sevindik et al. 2019).

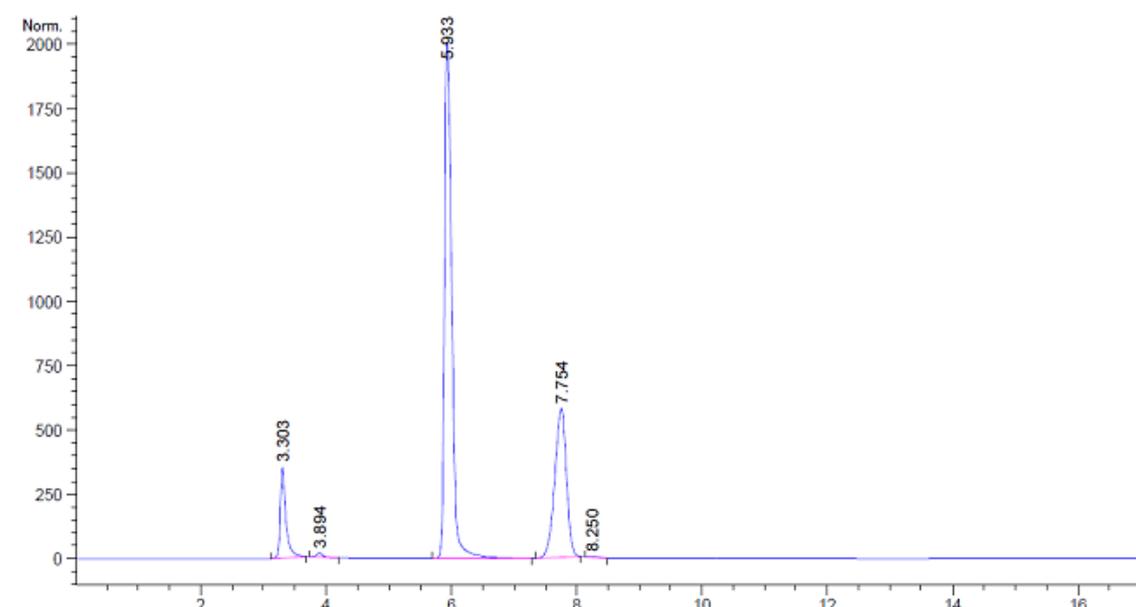
### 2.6. Linearity

The calibration curve values, limits of detection (LOD) and limits of quantification (LOQ) of chestnut samples confirming the HPLC-MS/MS analytical method are given in Table 2. The calibration curve method was used to calculate LOD and LOQ (Şengül 2016). Stock organic acid standard solutions of 5.00 mg/mL were prepared with ultrapure water and 5-point calibration curves in the range of 0.100-0.500 mg/mL were plotted for quantitative analysis of organic acids in chestnut samples. Good linearity was observed for all of them. Each sample was studied with three parallel repeats.

## 3. Results and Discussion

The peaks detected with HPLC analysis for organic acid standards are given in Figure 2 and good separation was observed.

The oxalic acid peak was observed first, followed by quinic acid, maleic acid, citric acid, and succinic acid peaks. In Table 2, the arrival times of the peaks, linear correlation coefficient ( $R^2$ ) values for the calibration curves and LOD and LOQ values are given. The  $R^2$  values were  $>0.999$ .



**Figure 2-** HPLC chromatogram for standard organic acid mix solution (according to retention times 3.303-oxalic acid, 3.894-quinic acid, 5.933-maleic acid, 7.754-citric acid, 8.250-succinic acid)

**Table 2-** Calibration curve, LOD and LOQ values for chestnut sample analyses

<i>Organic acids</i>	<i>R<sup>2</sup>*</i>	<i>Retention time (min)</i>	<i>LOD* (mg/mL)</i>	<i>LOQ* (mg/mL)</i>
Oxalic acid	0.9994	3.486	0.0013	0.0156
Quinic acid	0.9995	3.784	0.0009	0.0108
Maleic acid	0.9995	5.895	0.0009	0.0108
Citric acid	0.9998	7.765	0.0007	0.0084
Succinic acid	0.9995	8.241	0.0010	0.0116

\*R<sup>2</sup>: Linear correlation coefficient; LOD: Detection limit; LOQ: Quantification limit

The structure and amounts of organic acids are important factors affecting the flavor of fruits and vegetables. The presence or absence and amount of each organic acid is important for taxonomic studies. In addition, organic acids may have protective roles against various diseases due to their antioxidant activity; therefore, the qualitative and quantitative availability and analysis of organic acids in chestnuts are very important (Ribeiro et al. 2007). Organic compound concentrations in chestnut samples are given in Table 3 and Table 4.

**Table 3-** Organic acid content in chestnut samples (n=10, mg/g)

<i>Organic acids</i>	<i>Mean</i>	<i>Median</i>	<i>±SD*</i>	<i>Min.</i>	<i>Max.</i>
Oxalic acid	0.988	1.038	0.593	0.304	1.834
Quinic acid	0.362	0.364	0.237	0.123	0.597
Maleic acid	0.004	0.003	0.004	0.001	0.014
Citric acid	0.498	0.584	0.168	0.199	0.640
Succinic acid	0.254	0.157	0.220	0.059	0.748

\* SD: Standard deviation

Among the organic acids, oxalic acid had the highest value followed by citric, quinic, succinic and maleic acid in all samples. Oxalic acid is found in many plants, and in addition to its antioxidant properties, consumption of plants with high oxalic acid content can have a negative effect on metabolism as oxalates bind calcium and other minerals and form calcium oxalate urinary stones (Kayashima & Katayama 2002). The concentration of oxalic acid in chestnut samples was between 0.304-1.834 mg/g, with an average concentration of 0.988 mg/g. The organic acid amounts in two Portuguese chestnut cultivars were studied by Ribeiro et al. (2007) and the oxalic acid concentrations were found in the range of 0.0013-0.0019 mg/g in Judia cultivar and 0.0014-0.0017 mg/g in Longal cultivar (Ribeiro et al. 2007). These values are quite different from our oxalic acid results. The differences between these results may be due to differences in chestnut variety, soil conditions, cultural practices, climate, and analysis method (Gölükcü & Tokgöz 2018; Santos et al. 2022). In the study conducted by Suárez et al. (2012), oxalic acid was in the range of 0.073-1.667 mg/g in chestnuts from Spain, with an average of 0.525 mg/g (Suárez et al. 2012). In their study, Carochó et al. (2013) determined the oxalic acid concentration in Portuguese chestnuts was 0.7 mg/g (Carochó et al. 2013). The results of these last two studies are consistent with our findings and support our results. If the organic acid values of chestnuts

are compared with vegetables containing high concentrations of oxalic acid, values were; spinach (3.64-11.45 mg/g), rhubarb (5.11-9.84 mg/g), beets (0.369-7.94 mg/g) (Attalla & Monga 2014), the tropical vegetables of taro (*Colocasia esculenta*) (2.78-5.74 mg/g FW), sweet potato (*Ipomoea batatas*) (4.70 mg/g FW) and yam (*Dioscorea alata*) (4.86-7.81 mg/g) (Noonan & Savage 1999). The organic acid content of chestnuts is slightly below these values. Oxalic acid was found in significant amounts in chestnuts.

Quinic acid is a natural compound with antioxidant and anti-inflammatory properties found abundantly in plants. It increases the production of tryptophan and nicotinamide in the gut, which in turn increases the concentrations of all lipoproteins that can improve metabolic health and are effective in the treatment of diabetes (Heikkilä et al. 2019; Dong et al. 2022). Quinic acid was detected in only three of the ten chestnut samples and quinic acid concentrations had an average of 0.362 mg/g, ranging between 0.123-0.597 mg/g. In similar studies, Ribeiro et al. (2007) found malic acid+quinic acid concentrations in the range of 0.036-0.051 mg/g (Judia) and 0.055-0.113 mg/g (Longal) in two different chestnut species (Ribeiro et al. 2007). Carocho et al. (2013) found the quinic acid concentration in chestnuts was 13.000 mg/g (Carocho et al. 2013). Our results are close to the study by Ribeiro et al. but quite different from Carocho et al. Quinic acid (1, 3, 4, 5-tetrahydroxy cyclohexane carboxylic acid) is mostly found in coffee beans, cinchona bark, potatoes, apples, and peaches (Ercan & Doğru 2022). Moldoveanu (2012) determined quinic acid concentrations in 23 tobacco samples and found they ranged from 0.750-11.450 mg/g (Moldoveanu 2012). Since there is no study in the literature about the content of quinic acid in foods, it is only possible to state that the amount of quinic acid in chestnuts has lower concentration than in tobacco.

Maleic acid (MA) is a very important chemical intermediate widely used in industrial chemistry. It is used as a raw material in the production of lubricant additives, unsaturated polyester resins, surface coatings, plasticizers, copolymers, and agrochemicals (Wojcieszak et al. 2015). Maleic acid is found at very low amounts in plants and is produced commercially by synthesizing from maleic acid anhydride, 5-hydroxymethylfurfural (HMF), or furfural (Thiyagarajan et al. 2020). Organic acids are known as phytoremediation agents and exogenous preservatives, but this property of MA has not been investigated yet (Mahmud et al. 2017). Among the organic acids in our chestnut samples, the organic acid with lowest concentration was maleic acid. It was found in the concentration range of 0.001-0.014 mg/g in 8 of 10 chestnut samples. Its average concentration was 0.004 mg/g. In studies about the organic acid content of plants, maleic acid has been investigated very little and no study was found examining the maleic acid content in chestnuts. Related to maleic acid in the literature, the organic acid content of honey was investigated, and it was found in the range of 0.0002-0.0005 mg/g in chestnut honey (Suárez-Luque et al. 2002). Maleic acid concentrations are low in both chestnuts and chestnut honey.

Citric acid is an important organic acid for metabolism. The citric acid cycle is the most important metabolic pathway that provides energy to the body, which living cells use to completely oxidize biofuels to carbon dioxide and water. It also provides many intermediate products required for the synthesis of amino acids, glucose, heme, etc. (Akram 2014). Citric acid is found mostly in citrus fruits and the concentration of citric acid in lemon is around 48 mg/g (Penniston et al. 2008). In our sweet chestnut samples, the highest concentration was observed for citric acid after oxalic acid among organic acids. The average citric acid concentration was 0.498 mg/g and the concentration range was 0.199 to 0.640 mg/g. Citric acid concentrations in organic acid research conducted in Portugal on sweet chestnuts: were 5.220 mg/g according to Delgado et al. (2018) for the Longal variety and 9.550 mg/g for the Judia variety (Delgado et al. 2018). Riberio et al. (2007) found values were between 0.040-0.107 mg/g for the Longal variety and 0.050-0.090 mg/g for the Judia variety (Ribeiro et al. 2007). Gonçalves et al. (2010) detected citric acid in the range of 1.460–8.790 mg/g in ten Portuguese cultivars (Gonçalves et al. 2010). Vekiari et al. (2006) found citric acid concentrations between 1.27 and 4.30 mg/g in fifteen European chestnut varieties collected from different European countries (Vekiari et al. 2006). The organic acid contents were identified in 21 chestnuts obtained from Tenerife (Spain) by Suárez et al. (2012) and the average citric acid concentrations were between 0.261 and 1.135 mg/g (Suárez et al. 2012). Differences in the organic acid content of plants are affected by the type of chestnut, soil conditions, climate and even the time of collection (Gölükçü & Tokgöz 2018). Organic acid levels vary between species, cultivars, and even individual tissues of a plant grown under the same conditions (López-Bucio et al. 2001). For these reasons, there are differences in the citric acid values in chestnuts between these studies and our study.

Succinic acid is a good antioxidant and immune system stimulant, stabilizing the structure and functional activity of mitochondria (Lieschova et al. 2020). In nine of the ten chestnut samples examined, an average of 0.254 mg/g succinic acid was found, and the concentration range was 0.059-0.748 mg/g. There is no study in the literature that determined the succinic acid concentration in chestnuts. Succinic acid is found more in foods such as broccoli, rhubarb and sugar beet. Succinic acid was identified in dry rhubarb stems and its concentration was found to be 2.600 and 8.990 mg/g in two different varieties (Golubkina et al. 2022). The concentration of succinic acid in tomatoes was examined by Marconi and Montanari, and the range was 0.088-0.190 mg/g in 5 of the 7 tomato juice samples (Marconi et al. 2007). Succinic acid concentrations in chestnuts and tomatoes are close to each other.

**Table 4- Organic acid content (mg/g) of chestnut samples from each location (n=3)\***

<i>Sample No</i>	<i>Oxalic acid</i>	<i>Quinic acid</i>	<i>Maleic acid</i>	<i>Citric acid</i>	<i>Succinic Acid</i>
1	0.304±0.023	0.364±0.132	0.004±0.003	0.200±0.063	0.441±0.140
2	1.427±0.178	<LOD	0.014±0.002	0.640±0.020	0.332±0.107
3	1.329±0.407	<LOD	<LOD	0.600±0.140	0.146±0.104
4	1.834±0.117	<LOD	0.004±0.003	0.603±0.087	0.113±0.102
5	1.433±0.293	<LOD	0.007±0.003	0.438±0.070	0.059±0.016
6	1.594±0.215	<LOD	0.006±0.003	0.602±0.035	<LOD
7	0.308±0.015	<LOD	0.002±0.001	0.622±0.029	0.160±0.069
8	0.746±0.474	0.597±0.020	0.002±0.001	0.508±0.046	0.748±0.082
9	0.541±0.182	<LOD	<LOD	0.568±0.070	0.157±0.134
10	0.365±0.097	0.123±0.121	0.001±0.001	0.199±0.019	0.128±0.036

\*: mean±SD

The oil obtained from chestnut samples with the Clevenger apparatus was analyzed for essential oil components by GC-MS and the chromatogram is shown in Figure 3. When the analysis output was evaluated, a total of thirty-three peaks were found, as seen in Figure 3. These peaks were evaluated using the Wiley7 and NIST05 libraries on the device. Eighteen of these peaks were clearly identified and are shown in Table 5. Essential oils, characterized by their strong odor, are aromatic hydrophobic volatile liquids obtained from different parts of plants, such as flowers, roots, bark, leaves, seeds, bark and fruits. (O'Bryan et al. 2015). Essential oils are an important group of plant secondary metabolites and include a variety of volatile terpenes, aldehydes, alcohols, ketones and simple phenolics (Bunse et al. 2022). Terpenes, and especially monoterpenes, are the most common components in essential oils. Among the eighteen compounds identified with GC-MS analysis of chestnut samples, the most prominent ones are terpinolene (TPO) and limonene. Among the compounds identified, terpinolene appears to be the most abundant compound (retention time: 19.929). Terpinolene and limonene are monoterpenes. Terpinolene is a bioactive compound with important pharmacological activities. This compound is commonly found in the chemical composition of aromatic plants, especially plants of Asian origin such as black currant and saffron (*Curcuma longa*). It has pharmacological activity such as antifungal, antioxidant and insecticide features (Menezes et al. 2021). TPO has a wide range of biological activities including anticancer, antioxidant, antifungal and antiviral effects. Limonene (retention time: 14.764 and 18.668) was another important component among the 18 components in chestnut samples. Limonene is a monoterpene with a citrus odor at room temperature, commonly found in plants of the Rutaceae family. It is abundant in citrus plants such as lemon, grapefruit, and orange and in grapes (Amini et al. 2020; AlSaffar et al. 2022). Monoterpenes are known to have anticancer properties (Sobral et al. 2014). D-limonene, the most common isomer of limonene, was classified as having a wide range of biological activities, including antioxidant, anti-inflammatory, antibacterial, antiviral, anticancer, anti-fibrotic, vasodilator, and anti-hypertensive effects (Sun 2007, AlSaffar et al. 2022). Studies show that the anti-tumorigenic properties of d-limonene were evaluated for various types of cancer including breast, stomach, prostate, bladder and colon cancer (Anandakumar et al. 2021). In addition to its medicinal potential, limonene also has a wide range of uses in cosmetics. It is a commercially important molecule given its use in soft drinks, many sweetening products, and other household uses (Erasto & Viljoen 2008). The fact that chestnuts contain these two important components increases their importance as a nutritional substance.

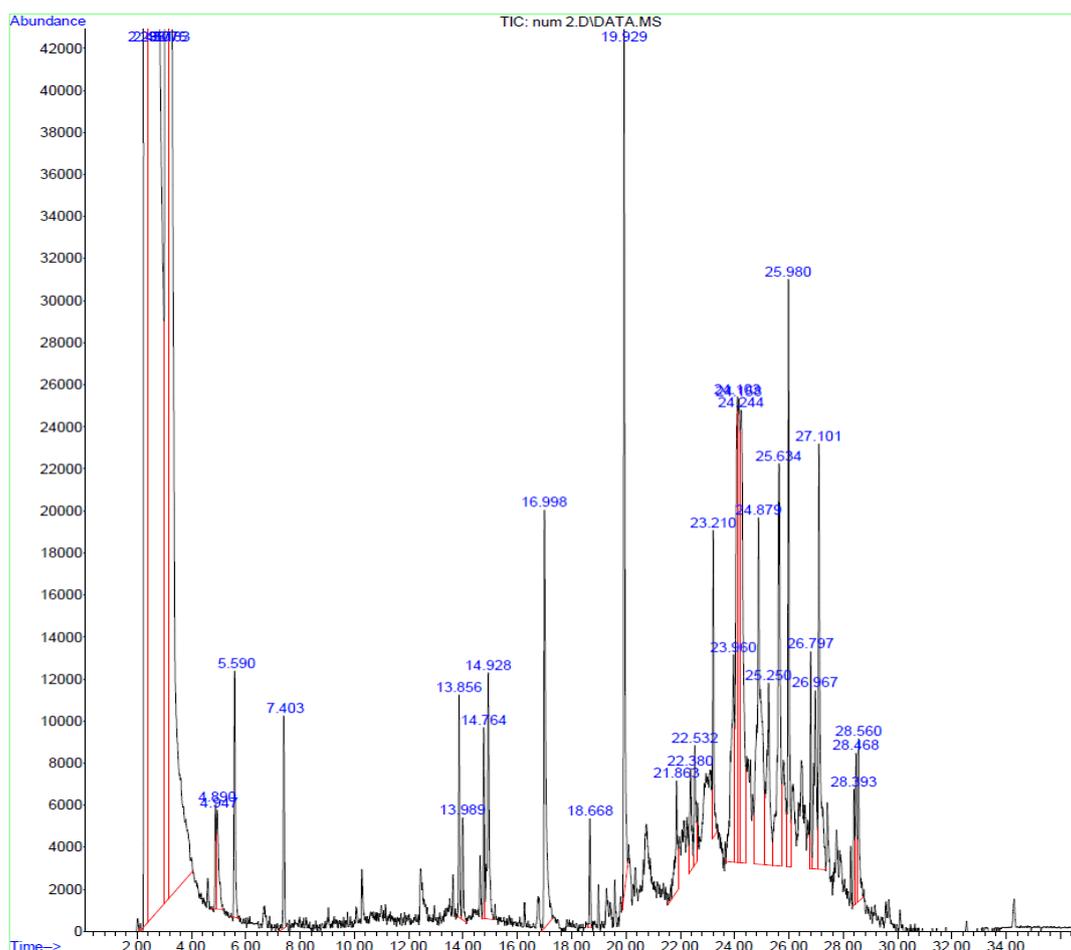


Figure 3- GC-MS chromatogram for chestnut essential oil

Table 5- Chestnut essential oil composition based on Wiley7 and NIST05 libraries

<i>Retention time</i>	<i>Component name</i>
4.890	3-methyl-1-butanol (isoamyl alcohol)
5.590	Toluene
7.403	Hexamethylcyclotrisiloxane
13.806	Octamethylcyclotetrasiloxane
14.764	Limonene
14.928	1,2-Dichlorobenzene
16.998	Benzoic acid, methyl ester (methyl benzoate)
18.668	Limonene
19.929	Terpinolene (3-Cyclohexene-1-methanol, $\alpha,\alpha$ 4-trimethyl-)
22.380	5-Eicosyne
23.210	1,1,6-Trimethyl-1,2-dihydronaphthalene
24.104	Longifolene, 1,4-Methanoazulene, decahydro-4,8,8-trimethyl-9-methylene-
25.634	Tritetracontane
26.797	Naphthalene
27.101	Pentatriacontane
28.393	1-Octanol, 2-butyl
28.468	Hexadecane, 2,6,11,15-tetramethyl (crocetane)

#### 4. Conclusions

In this study, organic acid and essential oil contents of chestnut fruits from trees growing spontaneously in forest areas within the borders of Giresun province were determined. There were significant differences in organic acid concentration between samples taken within the same provincial borders. Organic acids and essential oil are natural sources of antioxidants and are therefore very important for metabolism. Due to these properties, studies identifying the content of these components in edible plants need to be increased. According to the research results, chestnut is a useful source of organic acids and essential oils.

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## Conflict of Interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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