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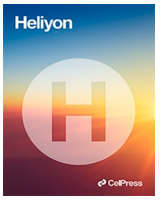

Research article

Discrimination of the geographical origin of gluten-free teff grains from northwestern parts of Ethiopia by fatty acid analysis

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ABSTRACT

Teff (*Eragrostis tef*) is a gluten-free cereal, and the consumer also prefers teff due to its nutritional composition. Determining the geographical origin of teff is important to select the right product for consumers. The quality and consumer preference of teff varies based on their production origin; consequently, their prices differ significantly. This work studied the profile of fatty acids in seventy-two teff samples by using gas chromatography coupled with mass spectrometry (GC-MS) and identifying the markers to discriminate the geographical origin of teff depending on their production region. Principal component analysis (PCA) and linear discriminat Analysis (LDA) were used to visualize data trends, and construct classification models for teff samples according to their geographical origins. Thirty different fatty acids were detected in all of the collected teff samples. The total mean concentration of fatty acids ranged from 739.85 to 938.06 mg/100g across the six districts in the three zones (East Gojjam,Awi, and West Gojjam). Stearic acid,*trans*-vaccenic acid, linoleic acid, azelaic acid, and capric acid were the most discriminating fatty acids of teff grains between East Gojjam and West Gojjam zones, while palmitic, palmitoleic, and oleic acid discriminated Awi zone teff samples from the other zones. The recognition and prediction abilities of the LDA model for the classification of the production zones were 98.6 % and 94.4 %, respectively. Hence, the fatty acid profiles combined with multivariate data analysis too can be used in the determination of the geographical origin of teff grains.

1. Introduction

Teff (*Eragrostis tef*) is a gluten-free cereal that originated from Ethiopia, between 4000 and 1000 BC [1]. Compared to most other cereals, it can tolerate harsh environments and grow in wider ecological conditions [2]. Moreover, it is a grain –free of gluten, which may be a good option to incorporate in the diet of people who suffer from disease caused as the results of gluten intolerance [1–5].

Teff is the most important cereal grain in Ethiopia, with very small seeds, colored from white to red and dark brown, milled into a whole meal, and is baked in the form of flat and thin fermented bread named injera [6,7]. The portion of bran and germ in whole meals containes nutritionally important compounds, such as health-conscious magnesium, calcium, and iron, able to cover the recommended nutritional allowances [8–10].

The main characteristics of cereal crops is the range of fatty acids they contain [11]. The main fatty acids present in most cereal

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crops, excluding *trans* fatty acids (TFAs), are stearic acid (C18:0), palmitic acid (C16:0), linoleic acid (C18:2), and oleic acid (C18:1) [12]. Majority of cereal grain are composed of nonpolar lipids, mostly stored as phospholipids, glycolipids, and acylglycerols [13]. This increases the value of cereal grains lipids for human nutrition, since essential fatty acids play key role in the metabolic processes like regulation of blood lipid levels [14].

Gas chromatography coupled with a mass spectrometer has been routinely used to profile the fatty acids in various commodities such as rice, maize, sorghum, wheat, millet cereal grains, fish, vegetables, and others [15–18]. In most of these studies oleic acid, linoleic acid, palmitic acid, α-linolenic acid, and stearic acid have been frequently reported as the most abundant fatty acids. However, these fatty acids have been reported in Teff samples collected from other regions of the country, while other trace fatty acids were not reported [19].

Fatty acids are important in assessing the quality and geographical originof food grains. According to Ref. [20], Gas chromatography-mass spectrometry is used for profiling fatty acids in green coffee (*Coffea arabica* L.) beans combined with multivariate data analysis for tracing geographical origins. The grain fatty acid composition is affected by the growing environment such as rainfall, temperature, altitude, and soil type. Therefore, fatty acids profiling can be used as a mechanisim for differentiating the origins of cereal grains [10,16].

Ethiopia's Amhara region is one of the country's leading producers of teff. The Gojjam zones (East Gojjam and West Gojjam), and Awizones are well-known for producing a large amount of teff and accountingfor the majority of the region's teff production. For example, the Bichena district in the East Gojjam zone produces the most preferable teff and is also the most expensive compared to a similar variety of teff cultivated in other zones of the region. However, low-quality teff is mislabeled and sold under the name Bichena teff. As a result, a simple method for distinguishing the growing zone of teff is in high demand to defend against market fraud and mislabeling of inferior teffs. It should be highlighted that mislabeling has a negative impact on the customer, farmer, and legitimate merchant. In this regard, it is critical to develop methods for identifying the geographical origin of teff to protect consumers and producers [21,22]. Furthermore, teff's fatty acid compositionhas not been studied in depth so far.

Thus, the main objective of this study was to determine the fatty acid profile of teff grains grown in the Amhara Regionusing GC-MS, and then apply multivariate chemometric models and evaluate if it the fatty acids could be linked to the cultivation area of the grains. The findings of this researchmay help the consumers to select the right teff product from the market based on their geographical origins.

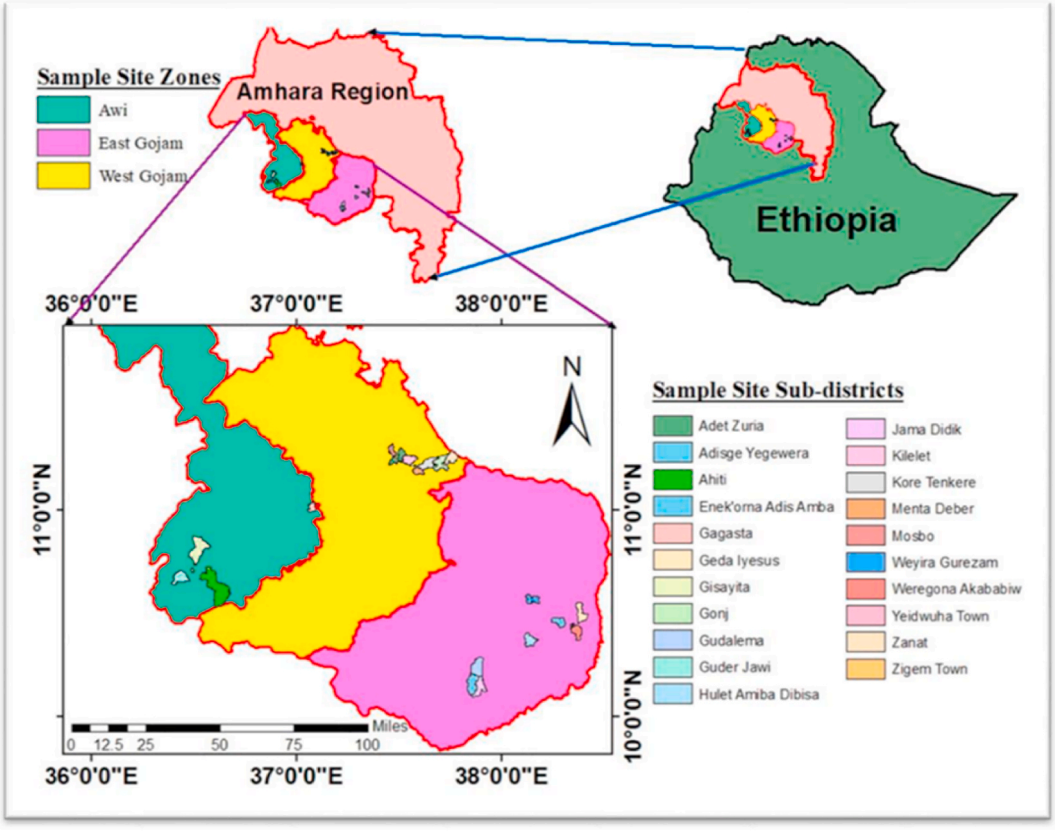

Fig. 1. Map of the sampling zones, districts and sub-districts. Source Reta et al. [23].

2. Materials and methods

2.1. Apparatus

PlatformShaker (Benchtop Shaker, ZHWY-304/334/344), centrifuge (portal centrifuge, Japan), Plastics Bag, GC-MS (Agilent Technologies 7890B-5977A, China), Beaker, Electrical Girder (FW-100, High-Speed Universal Disintegrator girders), Universal hot airoven (New Delhi-110,020, India, GST corporation ltd.), Balance (RADWAG:ps360/c/1), Spatula, Vials, Incubator (constant temperature and humidity incubator), Test Tube, Micro Pipit (China), Refrigerator (digital inverter technology, Samsung), syringe, membrane filter, and Crimper (crimper tool 11 mm hand crimper,QTY:1) were used for laboratory analysis.

2.2. Chemicals

Standard offatty acids, methanol (Mumbai400053, India), chloroform (99.8 %, 40005-India), toluene (99.99 %,121001-Blulux, laboratory),Chromatographicgrade n-hexane (99.9 %, France), acetone (99.5 %,20020-ARESE (Ml)), sulfuric acid (98 %,40005-India), anhydrous sodium sulfate (99 %,133001-India), sodiumchloride (99.5 %,121001-Blulux laboratory) and pentadecanoic acid (99 %, 16,823–0048, USA)as internalstandard were used for the analysis.

2.3. Sample collection and samplepreparation

In Ethiopia, there are different teffs named by their local varietal names. 'Kuncho', 'Tseday', 'Sergegna', 'Key-teff', etc are some of the local varietal names of the teff samples. Kuncho and tseday teff varieties are white and difficult to differentiate by the naked eye. The sergegna variety is mixed in color, while the key-tef is red. Kucho teff is cultivated in the East and West Gojjam zones, while Tseday and sergegna teff varieties are cultivated in the Awi zone of the Amhara region. These common local varieties were taken for this experiment.

During the 2020 crop years, 72 teff samples were gathered from farmers in the three zones (East Gojjam, West Gojjam, and Awi zones) of Ethiopia's Amhara region. As per the evidence from the Amhara region Bureau of Agriculture, the aforementioned three zones are the country's most important teff-producing areas, contributing a large amount of teff grains to the local and export market. Samples were taken from main teff-producing districts and subdistricts in each zone (Fig. 1). Three districts (Aneded, Shebelberenta, and, Enemay) and ninesub-districts (Jamadidik, Gudalema,Adisgeyegewera, Yeidwuhatown, Weregonaakababiw, Gedaiyesus, Weyiragurazam, Huletamibadibisa, and Enekornaadisamba) were considered from the East Gojjam zone. Wheareas, two districts (Goinjkolela and Adet/Yilmanadensa) and seven sub-districts (Zanat, Gonj,Koretenkere, Mentadeber, Kilelet, Adetzuria, and Mosbo) were considered from the West Gojjam zone of the region. Similarly, five sub-districts (Gisayita, Gangana, Ahiti, Zigemtown, and Gudarjawi) were selected from the Zigem district of the Awi zone.

From each sampling site, 500 g of teff grains were collected. In order to eliminate the adsorbed dust and particulate matter, all samples were washed with tap water and rinsed with distilled and deionized water respectively. The moisture was removed by oven-dried for 12 h until the sample obtained constant mass. The dried grains were ground using an electric grinder and kept in airtight plastic bottles until extraction, derivatization and analysis by GC-MS.

2.4. Crude fat extraction

Crude fat from teff samples was extracted and determined according to Ref. [24] with some modifications. About2.0g of powdered teff samples were extracted by Soxhletextraction apparatus set (sxt-06) by using hexane solvent for 6 h. Then the mass of crude fat was determined by drying the flask in an oven at 105 °C for 2 h until the constant mass of crude fat was obtained. Lastly, the mass of crude fat was calculated by using the formula [24]:

$$\text{Mass of crude fat } (\%) = \frac{\boldsymbol{W2 - W1}}{\boldsymbol{s}} \times \mathbf{100} \quad (1)$$

where, W_2 = mass of the flask with crude oil, $W_{1=}$ mass of empty flaskand, s = mass of sample used.

2.5. Sample preparation for GC-MS analysis

2.5.1. Extraction of lipids and derivation of the fatty acids into fatty acid methyl ester (FAME)

Lipids from teff samples were extracted following the procedure reported by Folch [25] with a slight modification. After proper optimization of the mass of the teff sample and the volume of extracting solvent, 1.0 g of teff sample was taken in a test tube and mixed with 15 mL of chloroform/methanol (2:1 ratio). The mixture was extracted for 36 h on a platform shaker at 300 rpm. Then, the extract was centrifuged, and the filtrate was taken. The lipid phase was separated with the aid of 2 mL of 0.73 % aqueous sodium chloride, then the upper phase was removed by using a micropipette, and the lower phase (chloroform) layer containing the lipid was taken andthe solvent was removed by letting the extract in a hood for two days, and the residue was reconstituted in 5.0 mL of toluene. Then a 2 mL portion of the lipid extract in toluene and an internal standard of 50 μL of 3.48 mg/mL pentadecanoic acid were taken and mixed with two mililitre of methanolic solution containing 1 % (v/v) sulfuric acid and then allowed to react for about 12 h in an incubator set at

50 °C. Then, 5 mL of 5 % brine solution was added to the resulting solution to create phase separation between the FAMEs and the other polar constituents, including unreacted sulfuric acid. Finally, the upper layer containing the FAMEswas extracted with hexane (2 × 3 mL). The two successive hexane extracts were combined) and dried over anhydrous sodium sulfate, filtered with an acrodisc syringe filter, transferred into the vial and analyzed and submitted to GC-MS analysis. Relative peak area wascalculated and a high relative peak area was obtained at 2 mL extract of 1g with 10 mL (2:1) chloroform/methanol and this optimization was applied for all seventy-two (72) teff samples [14,26]. Besides the internal standard to confirm the identity of the fatty acids in teff samples 19 mixed standards (heptanoic acid, nonanoic acid, undecanoic-acid,tridecanoic acid, pentadecanoic acid, 9-hexadecenoic acid, hecadecanoic acid, heptadecanoic acid, 9*cis*,12-*cis*--octadecadienoic acid, 9-octadecenoic acid, nonadecanoic acid, octadecanoic acid, 5,8,11,14-eicosatetraenoic acid, adipic acid, heneicosanoic acid, 4, 7, 10, 13, 16, 19-docosahexaenoic acid,13-docosenoic acid, adipic acid decyl 2,4-dimethylpent-3-methyl ester and tricosanoic acid) compounds were analyzed. Out of nineteen mixed standard solutions eight were identified in teff grain samples. The identified mixed standards match with teff samples are heptanoic acid, nonanoic acid, 15-methyl hexadic-9-enoic acid,9-hexadecenoic acid, 9-*cis*,12-*cis*,-octadecadienoic acid, tricosanoic acid, heptadecanoic acid, 9-*trans*--octadecanoic acid to confirm the identity of the fatty acids (Table 1) and Fig. 2.

2.5.2. Gas chromatography-mass spectrometry analysis (GC-MS)

An Agilent Technologies 8790A gas chromatographic system equipped with an autosampler, a split spitless injector, and a mass spectrometer (Agilent Technologies 7890B-5977A. China) was used for the analysis of the FAMEs. For the gas chromatographic conditions set were as follows: A split less mode and 1 μL sample injection volume, injector temperature of 280 °C, a fused silica capillary column with a stationary phase of DB-5 MS and column dimensions (30 m × 250 μm x 0.25 μm; Agilent Technologies China) were used. Temperature programming was set as follows. The column temperature was initially set at 60 °C and held for 3 min. Then it was ramped at 5 °C min^{-1} to 230 °C. This final temperature was held for 20 min. Helium gas with a flow rate of 1.68 mL/min was used as a mobile phase. For the mass spectrometer section, transfer line temperature (300 °C), a full scan mode (*m/z* 60–400), electron ionization (70 eV), and electron multiplier voltage (3000 V) were used.

2.6. Quantification of fatty acids in teff samples

The concentrations of 30 fatty acidswere identifiedwith relative percentages higher than 0.01 %, and the 23 fatty acids were detected with relative percentages higher than 0.1 % of the total fatty acidsand were quantified accurately and used for geographical origin classification of the teff samples as indicated in Fig. 2. These fatty acids are pelargonic acid, mystric acid, methyl palmitoleic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, arachidic acid, methyl eleostearioic acid, behenic acid,

Table 1
The chemical name, common name, molecular formula, means of identification and retention times of the fatty acids in the teff samples.

No.	Chemical name	Common Name	Molecular Formula	Means of Identification	Retention time
1.	hexanoic acid	Methyl caproic acid	$C_6H_{12}O_2$	NIST-MS	6.8
2.	heptanoic acid	Methyl enanthate	$C_7H_{14}O_2$	Standard	11
3.	2- heptenoic acid	–	$C_7H_{12}O_2$	NIST-MS	12.7
4.	octanoic acid	Caprylic acid	$C8H_{16}O_2$	NIST-MS	14.7
5.	2,4-heptadienoic acid	–	$C_7H_{10}O_2$	NIST-MS	15.8
6.	2-octenoic acid	–	$C_8H_{14}O_2$	NIST-MS	16.3
7.	nonanoic acid	Pelargonic acid	$C_9H_{18}O_2$	Standard	18
8.	hexanedioic acid	Adipic acid	$C_6H_{10}O_4$	NIST-MS	18.6
9.	decanoic acid	Capric acid	$C_{10}H_{20}O_2$	NIST-MS	20.9
10.	heptanedioic acid	Pimelic acid	$C_7H_{12}O_4$	NIST-MS	21.5
11.	octanedioic acid	Suberic acid	$C_8H_{14}O_4$	NIST-MS	24.2
12.	nanonedioic acid	Azelaic acid	$C_9H_{16}O_4$	NIST-MS	26.8
13.	decanedioic acid	Sebacic acid	$C_{10}H_{18}O_4$	NIST-MS	29.1
14.	tetradecanoic acid	Mystric acid	$C_{14}H_{28}O_2$	NIST-MS	30.7
15.	15-methylhexadec-9-enoic acid	Methyl palmitoleic acid	$C_{17}H_{32}O_2$	Standard	34.4
16.	hexadec-9-enoic acid	Palmitoleic acid	$C_{16}H_{30}O_2$	NIST-MS	34.5
17.	hexadecanoic acid	Palmitic acid	$C_{16}H_{32}O_2$	Standard	35
18.	heptadecanoic acid	Margaric acid	$C_{17}H_{34}O_2$	Standard	36.8
19.	9-*cis,12-cis*-octadecadieoic acid	Linoleic acid	$C_{18}H_{32}O_2$	Standard	38.1
20.	9-*trans*-octadecenoic acid	Oleic acid	$C_{18}H_{34}O_2$	Standard	38.2
21.	11-*trans*-octadecenoic acid	*trans*-vaccenic acid	$C_{18}H_{34}O_2$	NIST-MS	38.3
22.	octadecanoic acid	Stearic acid	$C_{18}H_{36}O_2$	NIST-MS	38.7
23.	9-*trans,11-trans*-octadecadienoic acid	Isolinoleic acid	$C_{18}H_{32}O_2$	NIST-MS	40.6
24.	11-eicosenoic acid	Gondoic acid	$C_{20}H_{38}O_2$	NIST-MS	41.7
25.	ecosanoicacid	Arachidic acid	$C_{20}H_{40}O_2$	NIST-MS	42.3
26.	5,11,14- eicosatrienoic acid	Sciadonic acid	$C_{20}H_{34}O_2$	NIST-MS	45
27.	9-*trans,*11-*cis,*13-*cis*-octadecatrienoic acid	Eleostearic acid	$C_{18}H_{30}O_2$	NIST-MS	45.5
28.	docosanoic acid	Behenic acid	$C_{22}H_{44}O_2$	NIST-MS	47.5
29.	tricosanoic acid	Tricosylic acid	$C_{23}H_{46}O_2$	Standard	51.2
30.	tetracosanoic acid	Lignoceric acid	$C_{24}H_{48}O_2$	NIST-MS	56.3

NIST- MS = National Institute of Standards and Technology-Mass Spectrometry.

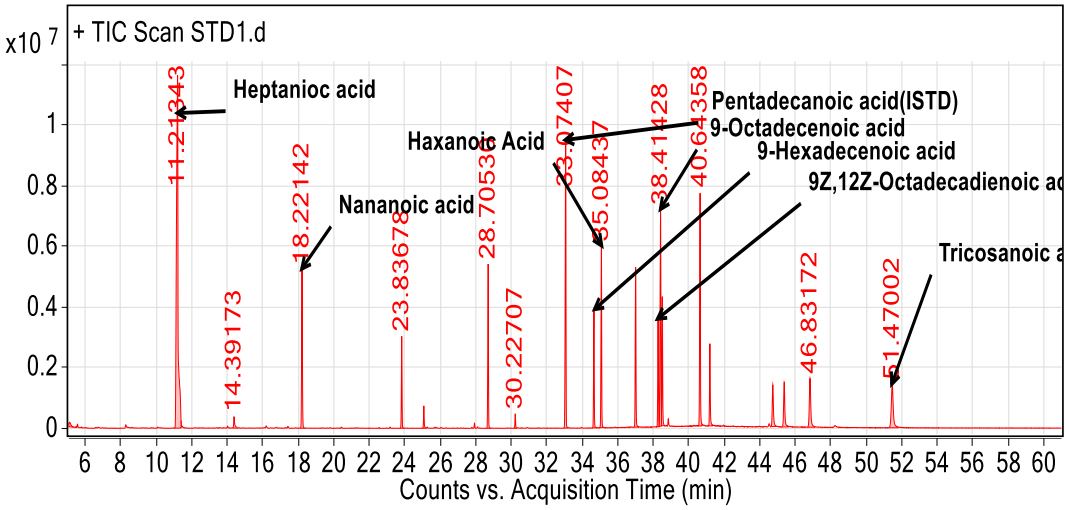

Fig. 2. Chromatogram of the mixed standards of nineteen mixed standards in which eight identifiedare heptanoic acid, nonanoic acid, 15-methyl hexadic-9-enoic acid, 9- hexadecenoic acid, 9-*cis*,12- *cis*, octadecadienoic acid, tricosanoic acid, heptadecanoic acid, 9- *trans*-- octadecanoic acid to confirm the identity of the fatty acids.

tricosylicacid, lignoceric acid, andfatty acids detected with relative percentages of less than 0.1 % were methyl enanthaoic acid, caprylic acid, hepta 2–4 dienoic acid, 2-octanoic acid, adipic acid, capric acid, and pimelic acidwere determined relative to the internal standard by using equation (2) [20,27,28]:

$$\frac{W}{W}\left(\frac{mg}{100g}\right) = \frac{A_{FA\ x\ m\mathrm{IS}}}{A_{IS}x\ m\ C} \tag{2}$$

where A_{FA} and A_{IS} are the peak area of the fatty acid and the peak area of the internal standard respectively, and m_{IS} is the mass of the internal standard, m_C is the mass of teff samples used for the analysis.

2.7. Statistical analysis

In this study, various statistical packages were used to analyzed the data. One-way Analysis of variance (ANOVA) was performed with SPSS software, while Principal component analysis (PCA), and linear discriminant Analysis, were performed with software StataSE 14, and SIMCA 13 (Umetrics, Sweden) software. One-way ANOVA was used to test the presence or absence of significant differences in the mean concentration of fatty acids among samples. Duncan's multiple comparisonswerecarried out to compare mean values of fatty acids among teff samples of three sampling zones. A 95 % probability level was taken to evaluate the statistical significance of the differences in the fatty acids among the sampling zones or districts. The natural grouping of the teff samples based on their fatty acid constituents was visualized using rincipal component analysis (PCA). The PCA was also used to reduce data dimensionality, visualize sample trends and identify the most discriminating fatty acids among samples [22,29]. Linear discriminant Analysis was used to classify the teff samples based on their sampling zones and districts. In addition, the geographical origin of teff samples were predicated on the trained LDA model.

3. Results and discussion

3.1. Crude fat in teff grain samples

The mean crude fat in the studied teff samples collected from six districts was found in the range of 2.51–3.51 % as indicated in (Table 2). The teff sample collected from Enemay was the highest in crude fat content, followedby Shebelberenta and Adet districts. One-way ANOVA ($p < 0.05$) revealed that there is a significant difference in crude fat between samples from the East Gojjam zone and the other two zones (Table 4). On the other hand, the difference in crude fat between West Gojjam and Awi Zones was insignificant ($p > 0.05$). The existence of significant differencesin crude fat between the sampling zones might be differences in genetic composition, the growing environmental conditions such as soil chemistry and climatic conditions of the sampling districts [30–32].

3.2. Characterization of fatty acids

A total of 30 different fatty acids were identified in all of the teff samples collected from three zones of the Amhara region (Fig. 3). The identities of the detected fatty acids were determined by using standard fatty acids and NIST-MS spectral library as a reference

Table 2
The Average concentrations (mg/100g) of fatty acids and Crude fat (%) found in teff from different districts of Amhara region, Ethiopia.

Number of fatty	Chemical name	Districts and number of samples					
		Common name	Aneded (n = 9)	Shebelberenta (n = 12	Enemay (n = 9)	Adet (n = 9)	Goinjikolela (n = 12)	Zigem (n = 21)
			mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
1.	hexanoic acid	Methyl caproic acid	2.14 ± 0.77	0.67 ± 0.44	0.41 ± 0.19	3.26 ± 1.41	1.06 ± 0.73	2.14 ± 1.47
2.	heptanoic acid	Methyl enanthate	0.40 ± 0.22	0.72 ± 0.35	0.67 ± 0.49	0.55 ± 0.35	0.31 ± 0.22	0.33 ± 0.16
3.	2- heptenoic acid	–	0.08 ± 0.04	2.03 ± 1.33	0.61 ± 0.57	16.3 ± 24.7	9.90 ± 9.51	0.11 ± 0.01
4.	octanoic acid	Caprylic acid	0.80 ± 0.37	0.04 ± 0.00	0.15 ± 0.08	1.22 ± 0.91	0.25 ± 0.19	0.71 ± 0.40
5.	hepta 2–4 dienoic acid	–	1.54 ± 1.29	0.81 ± 0.49	0.12 ± 0.10	0.41 ± 0.34	0.66 ± 0.61	0.22 ± 0.01
6.	2-octanoic acid	–	0.44 ± 0.25	0.08 ± 0.07	0.05 ± 0.01	0.43 ± 0.14	0.17 ± 0.11	0.32 ± 0.19
7.	nonanoic acid	Pelargonic acid	2.16 ± 1.75	0.81 ± 0.38	0.40 ± 0.14	7.35 ± 5.02	4.04 ± 3.30	1.07 ± 0.59
8.	hexanedioic acid	Adipic acid	0.29 ± 0.17	0.70 ± 0.67	0.06 ± 0.03	0.37 ± 0.52	0.69 ± 2.05	0.12 ± 0.05
9.	decanoic acid	capric acid	0.20 ± 0.08	0.08 ± 0.02	0.07 ± 0.02	0.30 ± 0.18	0.13 ± 0.05	0.15 ± 0.06
10.	heptanedioic acid	Pimelic acid	0.84 ± 0.53	0.08 ± 0.06	0.21 ± 0.23	0.81 ± 0.57	0.30 ± 0.23	0.97 ± 0.59
11.	octanedioic acid	Suberic acid	8.54 ± 3.88	0.35 ± 0.15	1.02 ± 0.83	7.67 ± 2.19	2.31 ± 1.89	4.35 ± 4.46
12.	nanonedioic acid	Azelaic acid	41.37 ± 16.0	1.66 ± 0.72	2.74 ± 2.27	34.3 ± 9.43	11.2 ± 10.3	21.60 ± 8.92
13.	decanedioic acid	Sebacic acid	2.24 ± 0.88	0.32 ± 0.21	0.70 ± 0.33	2.11 ± 0.59	1.35 ± 0.77	1.46 ± 0.65
14.	tetradecanoic acid	Myristic acid	1.45 ± 0.12	1.58 ± 0.31	0.98 ± 0.41	1.43 ± 0.49	1.11 ± 0.09	1.09 ± 0.24
15.	15-methylhexadec-9-enoic acid	Methyl palmitoleic acid	1.27 ± 0.99	1.21 ± 0.37	1.30 ± 0.49	1.70 ± 1.01	1.03 ± 0.35	1.09 ± 0.48
16.	hexadec-9-enoic acid	Palmitoleic acid	1.29 ± 0.38	1.79 ± 0.86	1.96 ± 0.67	1.28 ± 0.64	1.27 ± 0.29	2.23 ± 0.71
17.	hexadecanoic acid	Palmitic acid	288.15 ± 4.67	249.67 ± 37.3	187.51 ± 47.9	262.39 ± 23.0	234.47 ± 33.6	212.32 ± 27.3
18.	heptadecanoic acid	Margaric acid	3.84 ± 1.31	3.14 ± 0.63	15.49 ± 20.9	3.86 ± 0.98	2.82 ± 1.20	2.34 ± 0.43
19.	9-*cis*,12-*cis*-octadecadieoic acid	Linoleic acid	85.53 ± 34.8	267.79 ± 32.1	252.93 ± 56.6	70.82 ± 12.7	149.87 ± 56.5	181.72 ± 45.0
20.	9-*trans*-octadecenoic acid	Oleic acid	110.36 ± 57.7	202.82 ± 34.5	262.39 ± 56.8	173.01 ± 61.9	150.61 ± 26.3	251.96 ± 31.5
21.	11-*trans*-Octadecenoic acid	*Trans*-vaccenic acid	3.08 ± 2.28	43.19 ± 40.3	54.94 ± 20.1	4.68 ± 1.76	24.62 ± 23.9	5.42 ± 3.71
22.	octadecanoic acid	Stearic acid	19.11 ± 10.5	17.73 ± 8.97	12.34 ± 5.31	98.92 ± 9.95	71.0 ± 37.8	68.37 ± 14.9
23.	9-*trans*,11-*trans*--Octadecadienoic acid	Isolinoleic acid	22.60 ± 4.39	7.21 ± 2.83	2.70 ± 0.77	20.61 ± 8.25	7.26 ± 4.20	12.63 ± 6.83
24.	11-eicosanoic acid	Gondoic acid	4.72 ± 2.93	5.46 ± 3.38	6.38 ± 5.55	5.23 ± 2.06	4.35 ± 2.36	6.40 ± 3.84
25.	ecosanoicacid	Arachidic acid	20.91 ± 3.63	15.1 ± 11.3	9.33 ± 5.33	21.53 ± 2.06	17.58 ± 3.50	16.72 ± 5.66
26.	5,11,14- eicosatrienoic acid	Sciadonic Acid	85.20 ± 36.1	81.14 ± 48.6	2.71 ± 1.43	29.47 ± 32.7	60.49 ± 12.4	3.65 ± 1.44
27.	9-*trans*,11-*cis*,13-*cis*--octadecatrienoic acid	Eleostearic acid	14.06 ± 11.7	18.28 ± 7.79	1.77 ± 1.61	4.12 ± 4.02	8.59 ± 4.01	1.66 ± 1.46
28.	docosanoic acid	Behenic acid	8.51 ± 0.77	6.28 ± 2.75	4.77 ± 1.55	7.96 ± 0.92	6.66 ± 1.14	6.13 ± 1.28
29.	tricosanoic acid	Tricosylic acid	3.05 ± 0.38	2.50 ± 0.58	1.23 ± 0.36	2.25 ± 0.85	2.63 ± 1.09	1.44 ± 0.25
30.	tetracosanoic acid	Lignoceric acid	5.54 ± 0.51	4.59 ± 1.62	2.60 ± 0.86	4.79 ± 0.48	3.98 ± 0.73	3.71 ± 0.75
		Total	739.85 ± 199.75	938.06 ± 239.56	828.69 ± 232.23	789.33 ± 210.49	780.92 ± 0.73	812.58 ± 163.62

(*continued on next page*)

Table 2 (*continued*)

Number of fatty	Chemical name	Districts and number of samples					
		Common name	Aneded (n = 9)	Shebelberenta (n = 12	Enemay (n = 9)	Adet (n = 9)	Goinjikolela (n = 12)	Zigem (n = 21)
			mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
		Crude fat (%)	2.23 ± 0.19	3.26 ± 0.34	3.511 ± 0.17	2.51 ± 0.10	2.63 ± 0.22	2.60 ± 0.27

NB n = number of in each district.

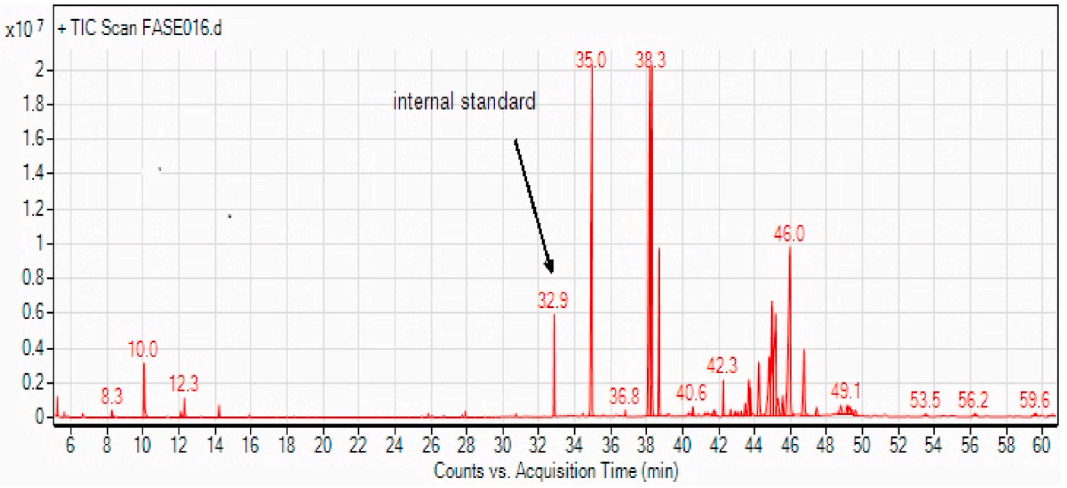

Fig. 3. Typical GC-MS chromatogram of teff grain extract, indicating the 30 identified fatty acids.

(Table 1).

3.3. Fatty acid profiles of teff samples

The total mean concentration of fatty acidsin the teff samples ranged from 739.85 ± 199.75 to 938.06 ± 239.56 mg/100 g across the six districts (Table 2). The most abundant fatty acids in the teff samples were palmitic acid, oleic acid, linoleic acid, stearic acid, and sciadonic acid. The highest mean concentration of palmitic acid (288.15 ± 4.67 mg/100g) was found in the sample from the Aneded district (262.39 ± 56.8 mg/100g) followed bya sample from the Adet district (262.39 ± 23.0 mg/100g) and the lowest in the samples collected from Enemay district (187.51 ± 47.9 mg/100g).

The other major fatty acid constituent was stearic acid, which was the highest concentration of fatty acids in samples from districts of Adet, Goinjikolela, and Zigem, with 98.92 ± 9.95, 71.0 ± 37.8, and 68.37 ± 14.9 mg/100g,respectively.These concentration difference is due to the existence of different soil type, climate conditions, and geographical locationsbetween the districts [33].

Palmitic acid constituted 22.90–36.22 % of the total fatty acid in the teff samples (Table 3). Teff samples collected from Aneded were the highest inpalmitic acid (36.22 %)followedby samples from Goinjkolela (34.34 %). Compared with the other cereal grains, the

Table 3
The average percentage compositions of individual fatty acids from the total fatty acid found in teff samples across districts.

Fatty acid	Aneded (n = 9)	Shebelberenta (n = 12)	Enemay (n = 9)	Adet (n = 9)	Goinjikolela (n = 12)	Zigem (n = 21)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
azelaic acid	4.79 ± 2.73	2.17 ± 0.07	0.34 ± 0.38	1.47 ± 1.38	4.48 ± 1.20	2.68 ± 1.31
palmitic acid	36.22 ± 2.21	26.31 ± 3.22	22.90 ± 22.6	29.09 ± 4.76	34.34 ± 3.53	26.24 ± 2.84
linoleic acid	15.12 ± 5.07	28.45 ± 4.49	31.70 ± 31.4	18.11 ± 5.18	14.21 ± 2.17	20.87 ± 6.54
oleic acid	13.80 ± 7.25	20.22 ± 5.34	25.20 ± 4.58	18.46 ± 1.77	22.46 ± 7.61	27.69 ± 3.56
trans--vaccenic acid	2.30 ± 0.25	3.84 ± 4.24	6.71 ± 6.79	4.69 ± 7.06	6.86 ± 224	1.06 ± 1.71
stearic acid	8.46 ± 5.92	3.40 ± 3.75	4.10 ± 1.27.	8.74 ± 5.57	9.83 ± 5.63	8.27 ± 2.79
isolinoleic acid	2.84 ± 0.58	0.76 ± 0.28	0.33 ± 0.38	0.93 ± 0.57	2.69 ± 1.10	1.59 ± 0.85
arachidic acid	2.52 ± 0.73	1.58 ± 1.18	1.18 ± 1.16	2.18 ± 0.46	2.82 ± 0.35	2.14 ± 0.93
sciadonic acid	10.86 ± 4.89	8.60 ± 5.10	5.45 ± 0.36	7.52 ± 1.63	4.29 ± 3.85	3.81 ± 0.19
eleostearioc acid	1.71 ± 1.35	2.79 ± 3.12	0.26 ± 0.24	1.07 ± 0.53	0.55 ± 0.55	0.21 ± 0.17

percentage composition of palmitic acid in the fatty acid of teff grains was higher than rice while its Oleic acid and linoleic acid compositions were lower than that reported for rice grains [34]. According to Zhang et al. [15] linoleic acid (66.68 %) in foxtail millets was higher than linoleic acid in teffgrains (present study). A report by Amare et al. [16] indicated that different varieties of teff contained palmitic acid within the range of 16.40–18.90 % of the total fatty acid, which is lower than this study.

Oleic acid was the second most abundant fatty acid followed by linoleic acid, representing 27.69–31.82 % and 15.12–31.70 % of the total fatty acids, respectively. This range was obtained from samples collectedfrom Zigem and Enemay districts. According to Amare et al. [16] the levels of Oleic acid ranged from 20.99 % to 26.25 % and linoleic acid ranged from 29.42 % to 43.33 % of the total fatty acids for teff samples. This variation may be due to soil type, ways of farming, geographical locations, and rainfall [21].

3.4. Fatty acid variation among different zones

The concentration of fatty acids in teff samples from the three sampling zones was in decreasing order of palmitic acid > oleic acid > linoleic acid > stearic acid > sciadonic acid > azelaic acid > *trans*-vaccenicacid > capric acid (Table 4). The concentrationsof palmitic acid, oleic acid, linoleic acid, stearic acid, sciadonic acid, and azelaic acidfound in this study were in agreement witha previous study by Amare et al. [16].

Statistical analysis with one-way ANOVA ($\alpha = 0.05$) was used to test the presence or absence of significant differences in the average concentration of the individual fattyacids of teff from the three administrative zones, a one way ANOVA ($\alpha = 0.05$) was used. The differences were considered significant when $P < 0.05$; otherwise, the differences were considered insignificant. Results are presented in Table 4. Samples from East Gojjam and West Gojjam zones contained significantly higher mean concentrations of Methyl enanthoic acid, palmitic acid, sciadonic acid, and eleostearic acidthan teff samples from the Awi zone. On the other hand insignificant differences in the mentioned fatty acids in teff samples collected from two Gojjam zones (East and West) were observed. Significantly higher levels of palmitoleic acid and oleic acid in samples from the Awi zone than in West Gojjam and East Gojjam teff samples were noted. In addition, the mean concentration of palmitoleic acid and oleic acidin Awizone's teff samples was significantly different from the teff samples from East Gojjam and West Gojjam teff samples. East Gojjamteff samples were significantly higher in the concentration of*trans*-vaccenic acidand significantly lower in the mean concentration of azelaic acid in samples from Awi and West Gojjam teff samples.

Concerning the other major fatty acids (FAs), such as linoleic acid and stearic acid, there was nosignificant difference in their average concentration of fatty acid among the two zones. The noted significant variation in the fatty acids content in teff samples of the two zones might be due to the differences in one or more factors such as climatic conditions, soil composition, farming practices and genetic make of the teff samples [30–32]. The concentrations of oleic acid found in Awi zone's teff samples (average 251.96 mg/100g) are significantly higher than those present in teff samples from the other zones ($P < 0.05$). The box plot constructed from the concentrations of stearic acid separates these teff samples from those of the other two zones (Fig. 4) and is used to show overall patterns of response. It provides a useful way to visualize the range and other characteristics of responses for three zones. The box plot of stearic acid in the East Gojjam zone was comparatively short. The box plot was comparatively tall at West Gojjam and Awi zone. This could suggest a significantdifferencein the concentration of stearic acid between the three zones. To have comprehensive data on teff's chemical composition, further study may be needed on the fatty acid composition of teff samples from other regions of Ethiopia.

3.5. Origin discrimination model

3.5.1. Principal component analysis

Principal component analysis (PCA) was performed to evaluate sample trends and identify the most discriminating fatty acids between teff samples. Before PCA the data was standardized with Pareto scaling. The PCA extracted 6 PCs, each with eigenvalue> 1,

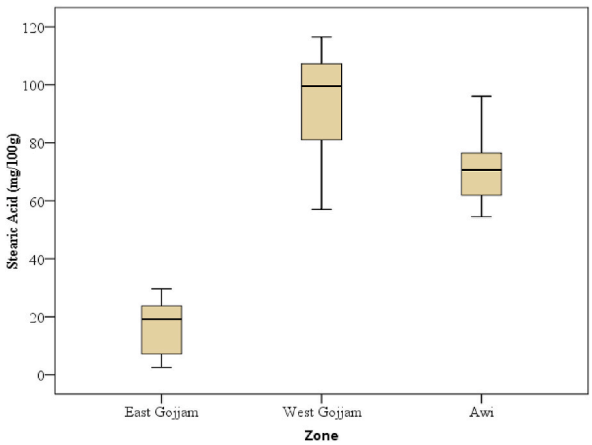

Fig. 4. Box plot showing the allocation of stearic acid in samples grown in three zones of Amhara Region, Ethiopia.

that explained 93 % of the information in the data set. Of these, the first principal component (PC) accounted for 44 % while the second 22 % of the variation in the data. The distribution of the samples in the space created by these two PCs is displayed in Fig. 5. The samples tend to form separate groupings, especially samples from Awi and West Gojjam tend to form different categories separated by the first PC.

Contributions of each fatty acid to the first and second PCs are visualized with the loadings plot in Fig. 6. Fatty acids with a loading value ≥ 0.2 were considered the most discriminating fatty acids among samples (Table 5). These fatty acids also showed significant differences ($p < 0.05$) in their average concentrations among samples from the different production areas.

3.5.2. Linear discriminant analysis of fatty acids in districts and zones

Based on the fatty acid contents of the 30 observed fatty acids, an attempt was made to establish chemometric methodologies effective for the verification of the geographical origin of the teff samples. Teff samples from various zones and districts have varying fatty acid concentrations, and LDA is assessing the contributions of many causes to these variances. The study's hypothesis was that the various teff growing zones might differ in one or more of the following: soil type, agronomic practices, local climate (rainfall, temperature, humidity, and solar radiation), and so on. These variations could lead to notable differences in the fatty acid contents of teff grains grown in various locations. Thus, an attempt was undertaken to assess the suitability of the overall differences in fatty acid concentrations for use as markers in chemometric models. An LDA was employed to develop classification models for teff grains cultivated in the three studied zones, as depicted in Fig. 7. In light of this, two discriminant functions were calculated, and 98.6 % of the samples were accurately placed in the appropriate production zone. Only one East Gojjam sample was mistakenly identified as being from Awi.

3.6. Model validation for classification of teff samples at zonal level

3.6.1. Leave-one-out cross-validation

Leave-one-out cross-validation provided 90.3 % of samples correctly classified into their respective zones. Only 7 out of the 72 samples were misclassified (3 East as Awi, 3 West 2 as Awi and 1 as East, 1 Awi as East).

3.6.2. Prediction ability

To assess the prediction ability of the LDA model, 18 samples were selected randomly and used as the prediction set, while 54 samples as the training set. The Analysis provided 100 % recognition ability, where all of the 54 samples in the training set were correctly classified into their respective cultivation zone. On the other hand, the analysis provided a 94.4 % prediction success rate,

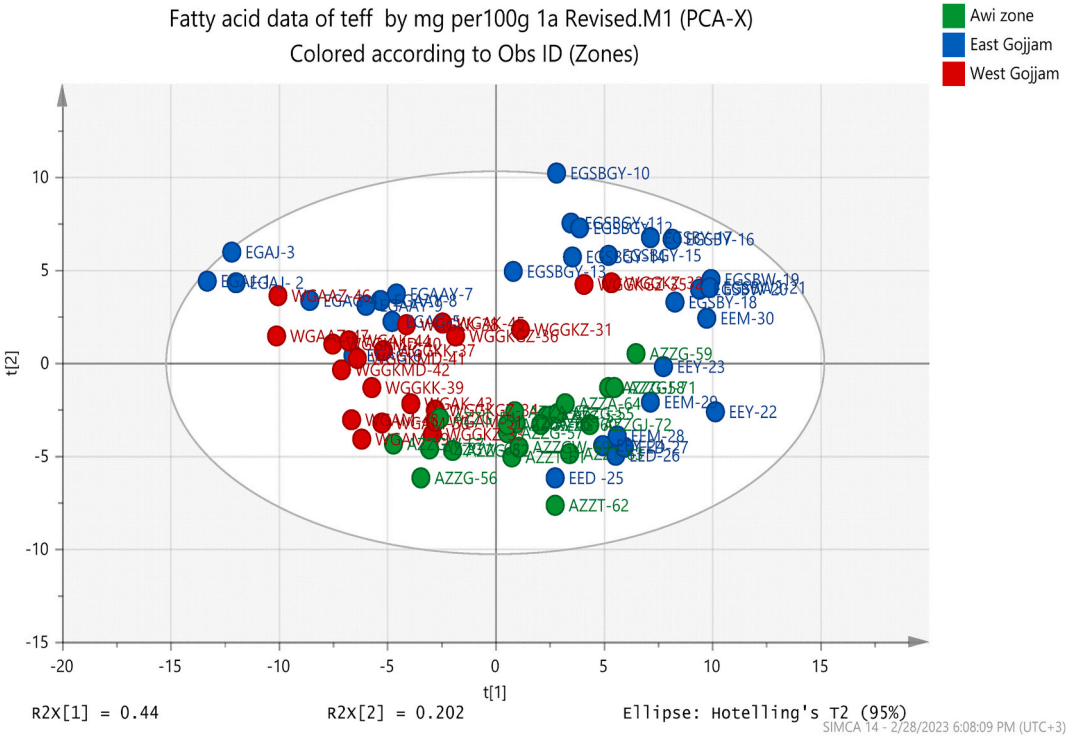

Fig. 5. PCA scores plot of PC1 *vs.* PC2 based on GC-MS data, indicating the natural grouping of teff samples into three sampling zones based on their fatty acid composition.

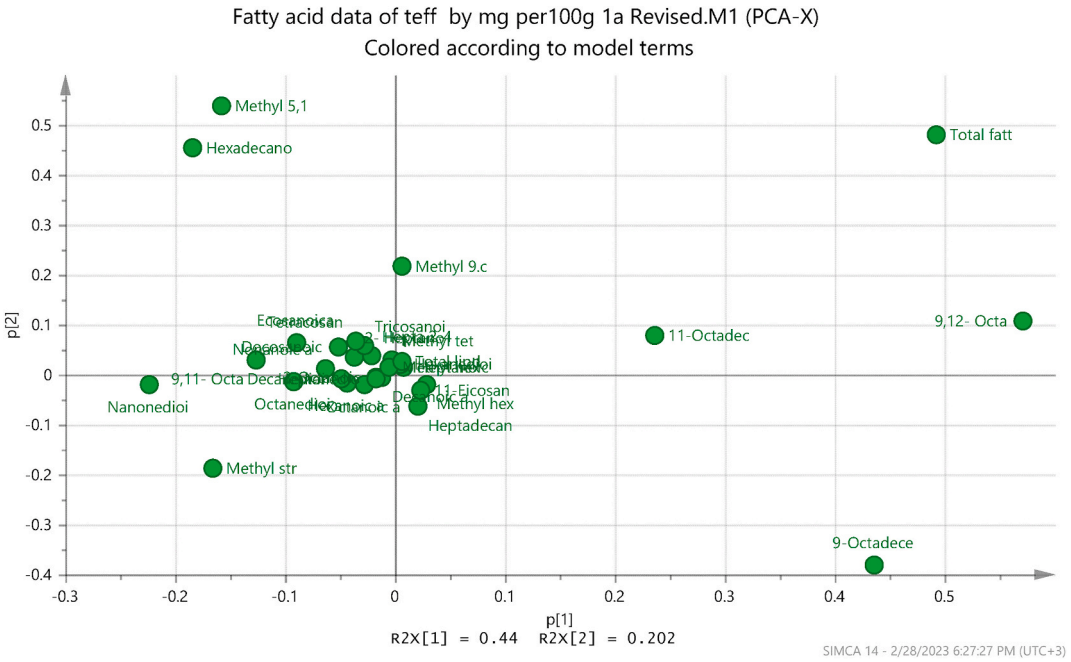

Fig. 6. Loading plots showing the contribution of fatty acids to the first two PCs obtained from principal component analysis of teff grains from the different production areas of Amhara region.

where only one out of the 18 samples was incorrectly classified.

3.7. Forward stepwise variable selection

Forward stepwise variable selection was used to identify the most important fatty acids that best discriminate among production zones. Four out of the thirty fatty acids were found to be discriminant markers of the zones (Table 6). Stearic acid, in particular, was responsible for the discrimination of teff grains grown in West Gojjam (average 82.9 mg/100g) from East Gojjam (16.5 mg/100g). The box and whiskers plot (Fig. 4) show the differentiation of East Gojjam and West Gojjam teff grains based on their stearic acid contents. On the other hand, the higher oleic acid in teff grains from the Awi zone (average 251.9 mg/100g) enabled distinguishing the grains from the other zones (average 160–193 mg/100g).

The application of LDA provided 98.6 % of samples were correctly classified into the six production districts. Only one sample from Adet was misclassified as Goinjikolela. Two districts' teff samples are a good cluster and are evident from the LDA scores plot of teff grains from the six districts (Fig. 8). These are teff samples coming from Aneded and Shebelberenta districts.5,11,14- Eicosatrienoic acid was in particular fatty acid that, was responsible for the discrimination of teff grains grown in Shebelberenta and Aneded within an average of 81.14 and 85.20 mg/100g concentration fatty acids in teff samples.

3.8. Model validation for classification of teff samples at district level

Leave-one-out cross-validation.

Leave-one-out cross-validation provided 86 % correct classification of samples into their respective districts.

***Prediction* ability:** The Analysis provided a 78 % prediction success rate.

4. Conclusion

A total of 30 different fatty acids were detected in allof the72 teff samples and their concentration were according to the decreasing order of palmitic acid > oleic acid > linoleic acid > stearic acid > sciadonic acid > azelaic acid > *trans*-vaccenicacid > capric acid. One-way ANOVA revealed a significant difference ($p < 0.05$) in the average concentrations of fatty acids from different production zones and districts. Samples from East Gojjam and West Gojjam zones contained significantly higher mean concentrations of2-heptanoic acid, palmitic acid, sciadonic acid, and eleostearic acid than teff samples from the Awi zone. Significantly higher levels of palmitoleic acid andoleic acid were in samples from the Awi zone than in West Gojjam and East Gojjam teff samples. Using linear discriminant analysis 98.6 % of samples were correctly classified into their respective production zones. Stearic acid, linoleicacid, oleic acid, and, palmitic acids were found to be important in discriminating teff grains according to their production zones. Of these fatty acids, the concentration of Stearic acidwas found to be a suitable discriminant marker for West Gojjam teff grains. Hence, fatty acid

Table 4
The mean, standard error (SE), minimum (Min), and maximum (Max) concentrations (mg/100g) of fatty acids and crude fat (%) in samples from East Gojjam, West Gojjam, and Awi zone.

Peak Number	Geographical origin and number of samples									
	Chemical name	Common name	East Gojjam zone (n = 30)			West Gojjam zone (n = 21)			Awi zone (n = 21)		
			Mean ± SE	Min	Max	Mean ± SE	min	Max	Mean ± SE	Min	Max
1.	hexanoic acid	Methyl caproic acid	1.03 ± 0.16[a]	0.2	3.12	2.00 ± 0.33 [b]	0.37	5.39	2.14 ± 0.32 [b]	0.11	4.63
2.	heptanoic acid	Methyl enanthate	0.61 ± 0.07[a]	0.03	1.62	0.41 ± 0.06[a]	0.1	1.17	0.33 ± 0.03 [b]	0.08	0.65
3.	2- heptanoic acid	–	1.02 ± 0.22[a]	0.05	3.94	12.4 ± 3.84 [b]	0.02	56.69	0.11 ± 0.02[a]	0.01	0.48
4.	octanoic acid	Caprylic acid	0.30 ± 0.07[a]	0.03	1.42	0.67 ± 0.16 [b]	0.03	2.71	0.71 ± 0.08 [b]	0.09	1.61
5.	hepta 2–4 dienoic acid	–	0.82 ± 0.17[a]	0.03	4.52	0.55 ± 0.11 [ba]	0.03	2.43	0.22 ± 0.08 [b]	0.02	1.85
6.	2-octanoic acid	–	0.18 ± 0.04 [b]	0.03	1.02	0.28 ± 0.03 [b]	0.04	0.56	0.32 ± 0.04 [b]	0.03	1.03
7.	nonanoic acid	Pelargonic acid	1.09 ± 0.21[a]	0.18	5.23	5.45 ± 0.94 [b]	0.3	15.05	1.07 ± 0.12[a]	0.13	2.37
8.	hexanedioic acid	Adipic acid	0.39 ± 0.09 [b]	0.01	2.31	0.55 ± 0.34 [b]	0.03	7.21	0.12 ± 0.01 [b]	0.04	0.23
9.	decanoic acid	Capric acid	0.11 ± 0.01 [b]	0.03	0.36	0.20 ± 0.03[a]	0.06	0.75	0.15 ± 0.01[a]	0.04	0.28
10.	heptanedioic acid	Pimelic acid	0.35 ± 0.08 [b]	0.04	1.94	0.52 ± 0.10 [b]	0.06	1.42	0.59 ± 0.21 [b]	0.05	4.68
11.	octanedioic acid	Suberic acid	3.01 ± 0.77 [b]	0.15	14.18	4.61 ± 0.73 [b]	0.19	10.43	4.35 ± 0.97 [b]	0.52	21.73
12.	nanonedioic acid	Azelaic acid	13.9 ± 3.68 [b]	0.07	63.43	21.1 ± 3.32[a]	1	47.43	21.6 ± 1.94[a]	5.43	40.14
13.	decanedioic acid	Sebacic acid	1.01 ± 0.17 [b]	0.06	3.46	1.68 ± 0.17[a]	0.27	2.84	1.46 ± 0.14 [ab]	0.43	3.03
14.	tetradecanoic acid	Myristic acid	1.36 ± 0.07[a]	0.52	2.34	1.25 ± 0.07 [ba]	0.9	2.66	1.09 ± 0.05[b]	0.6	1.72
15.	15-methylhexadec-9-enoic acid	Methyl palmitoleic acid	1.26 ± 0.11[a]	0.56	3.92	1.32 ± 0.16 [b]	0.27	3.73	1.09 ± 0.10 [ab]	0.19	2.65
16.	hexadec-9-enoic acid	Palmitoleic acid	1.69 ± 0.13[a]	0.29	3.55	1.27 ± 0.10[a]	0.49	2.45	2.23 ± 0.15 [b]	0.51	3.46
17.	hexadecanoic acid	Palmitic acid	242.57 ± 9.61[a]	140.87	294.64	246.42 ± 7.02[a]	168.6	297.31	212.39 ± 5.97 [b]	120.24	246.39
18.	heptadecanoic acid	Margaric acid	7.05 ± 2.26 [b]	0.24	53.92	3.26 ± 0.26 [b]	0.37	6.19	2.34 ± 0.09 [b]	1.19	2.95
19.	9-*cis,*12-*cis*-- octadecadieoic acid	Linoleic acid	208.65 ± 16.7[a]	43.62	322.03	115.99 ± 12.7 [b]	57.13	253.94	181.72 ± 9.82[a]	94.24	254.01
20.	9-*trans*-octadecenoic acid	Oleic acid	192.95 ± 14.0[a]	37.15	361.77	160.21 ± 9.86[a]	79.67	236.67	251.96 ± 6.88 [b]	198.25	300.8
21.	11-trans-octadecenoic acid	*Trans*-vaccenic acid	34.6 ± 6.31[a]	0.28	92.59	16.0 ± 4.46 [b]	0.41	59.50	5.42 ± 0.81 [b]	0.16	12.81
22.	octadecanoic acid	Stearic acid	16.5 ± 1.60 [b]	2.5	29.66	82.92 ± 6.99[a]	6.84	116.49	68.3 ± 3.25[a]	22.18	96.08
23.	9-*trans,*11-*trans*- octadecadienoicacid	Isolinoleic acid	10.4 ± 1.60 [b]	1.89	27.16	12.9 ± 1.98 [b]	1.26	33.30	12.6 ± 1.49 [b]	3.48	27.48
24.	11-eicosanoic acid	Gondoic acid	5.51 ± 0.72 [b]	0.96	18.95	4.73 ± 0.48 [b]	1.85	11.33	6.40 ± 0.83 [b]	1.49	18.47
25.	ecosanoic acid	Arachidic acid	15.1 ± 1.64 [b]	0.14	30.02	19.2 ± 0.78 [b]	10.55	26.30	16.7 ± 1.23 [b]	4.84	35.87
26.	5,11,14- eicosatrienoic acid	Sciadonic Acid	58.8 ± 9.41[a]	1.02	140.48	47.1 ± 6.02[a]	1.3	88.61	3.65 ± 0.31 [b]	1.52	6.31
27.	9-*trans,*11-*cis,*13-*cis*-octadecatrienoic acid	Eleostearic acid	12.0 ± 1.92[a]	0.05	32.12	6.68 ± 0.98[a]	0.23	14.98	1.66 ± 0.31 [b]	0.09	5.56
28.	docosanoic acid	Behenic acid	6.49 ± 0.44 [b]	0.78	9.93	7.22 ± 0.26 [b]	4.69	10.14	6.13 ± 0.28 [b]	2.82	9.77
29.	tricosanoic acid	Tricosylic acid	2.28 ± 0.15 [b]	0.9	3.74	2.47 ± 0.21[a]	1.02	4.79	1.44 ± 0.05 [b]	0.64	1.81
30.	tetracosanoic acid	Lignoceric acid	4.28 ± 0.29 [b]	0.43	7.04	4.33 ± 0.16 [b]	2.88	5.40	3.71 ± 0.16 [b]	1.68	4.87
		Total	845.79 ± 72.88	233.11	1540.41	784.30 ± 62.88	340.96	1327.89	812.19 ± 35.89	461.1	1113.72
		Crude fat (%)	3.45 ± 0.05	2.65	3.85	2.58 ± 0.04	2.26	2.9	2.60 ± 0.05	2.05	2.95

*Mean values with no common letter in a row are significantly different at $p < 0.05$).

Table 5
Fatty acids with higher discriminatory characteristics among teff grainsfrom Amhara region.

IUPAC Name	Common Name
5,11,14- eicosatrienoic acid	Sciadonic acid
hexadecanoic acid	Palmitic acid
9-*trans*,11-*cis*,13-*cis*-octadecatrienoic acid	Eleostearic acid
9-*cis*,12-*cis*-octadecadienoic acid	Linoleic acid
9-*trans*,11-*trans*-octadecadienoic acid	Isolinoleic acid
octadecanoic acid	Stearic acid
9-*trans*-octadecenoic acid	Oleic acid

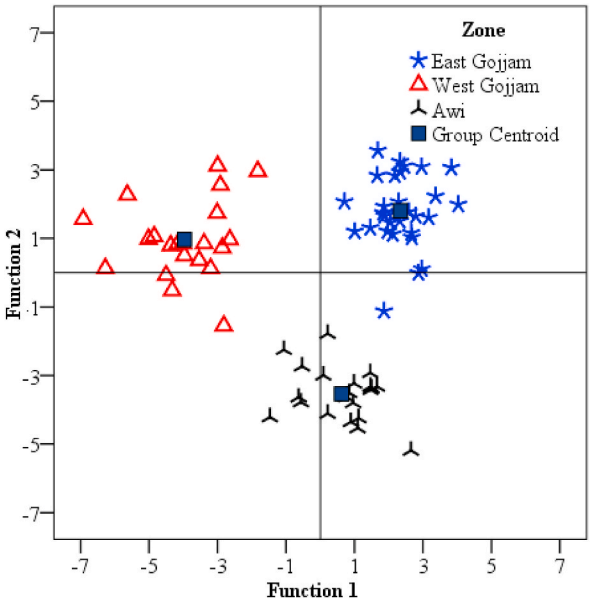

Fig. 7. Scores plot of the two discriminant functions obtained from the linear discriminant analysis of the three production zones based on the fatty acid contents of teff grains.

Table 6
Canonical discriminant function coefficients of the linear discriminant model constructed with the fatty acid concentration of teff cereal samples from three production zones of the Amhara Regional state of Ethiopia.

Fatty acid	Function	
	1	2
stearic acid	−0.856	0.039
linoleic acid	0.274	0.238
oleic acid	0.012	0.575
palmitic acid	0.050	−0.322

data combined with linear discriminant analysismodeling can be used to authenticate the geographical origin of teff from the Amhara region.

Data availability statement

Data will be made accessible on request.

CRediT authorship contribution statement

Chaltu Reta: Writing – original draft. **Minaleshewa Atlabachew:** Supervision, Conceptualization. **Bewketu Mehari:** Data curation. **Kidanemariam Teklay Hilawea:** Formal analysis. **Tihitinna Asmellash:** Supervision, Conceptualization.

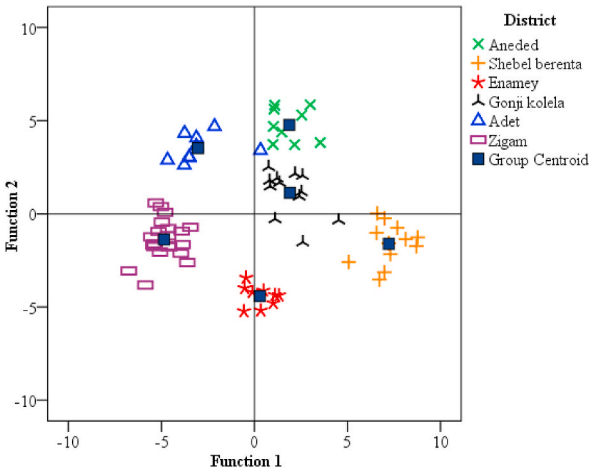

Fig. 8. Scores plot of the two discriminant functions obtained from the linear discriminant Analysis of teff grains based on their fatty acid contents.

Declaration of competing interest

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

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